Modulatory properties of dietary fiber and role of intestinal mucus on Enterotoxigenic Escherichia coli (ETEC) pathogenicity

Thomas Sauvaitre

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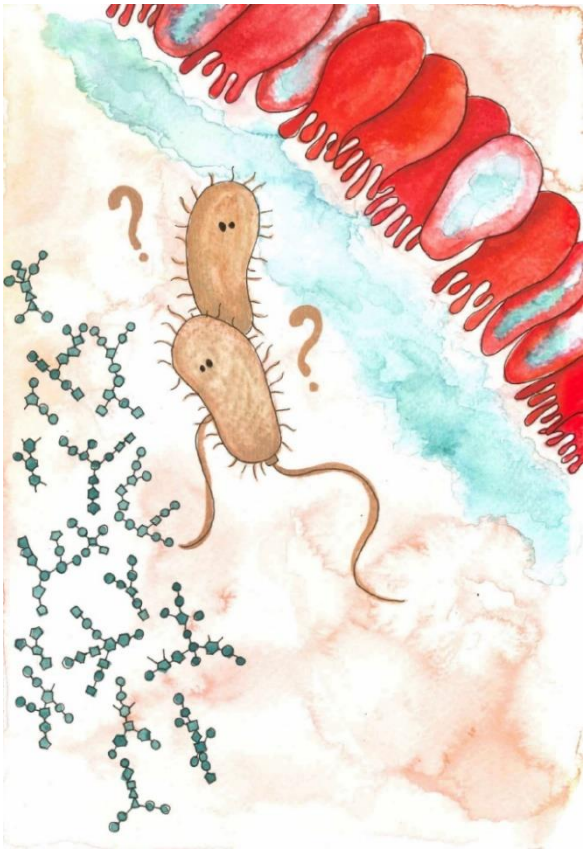

Thomas Sauvaître

Defended on the 3rd of June 2022

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Modulatory properties of dietary fiber and role of intestinal mucus in Enterotoxigenic *Escherichia coli* (ETEC) pathogenicity

Thomas Sauvaître

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences at Ghent University (Belgium) and Biology and Health at Université Clermont Auvergne (France).

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Scientific Curriculum Vitae

SCIENTIFIC DEGREES AND TRAININGS

- **2018 – 2022: PhD student Microbiology and Biotechnology**
  Joint Phd between Université Clermont Auvergne (France) and Ghent University (Belgium)

Thesis project: *Modulatory properties of dietary fiber and role of intestinal mucus on Enterotoxigenic Escherichia coli (ETEC) pathogenicity*

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Host laboratories : joint PhD UMR MEDIS (France) / CMET (Belgium)
- UMR 454 MEDIS, Université Clermont Auvergne - INRAE, Clermont-Fd, (France)
- Center for Microbial Ecology and Toxicology, Ghent (CMET), Ghent University, Ghent (Belgium)

- **2017 – 2018: Master 2 in Biology, Genetics, Physiology, Pathologies and Health**
  Université Clermont Auvergne, Clermont-Ferrand (France) – Graduation with honors

Training project: *Study on the impact of titanium dioxide in the form of food additives (E171) on the colic carcinogenesis and on the intestinal microbiota in murin model APC$^{min/+}$*

Co-Advisors: Dr Boucher Delphine
Dr Bonnet Mathilde

Host Laboratory: Microbes Intestine Inflammation and Susceptibility of the Host (UMR1071 Inserm/USC INRA 2018), Université Clermont Auvergne, Clermont-Ferrand, (France).

- **2016 – 2017: Master 1 in Genetics and Physiology**
  Université Clermont Auvergne, Clermont-Ferrand (France) – Graduation with honors

Training project: *Investigation of the role of the dilp8 in Lactobacillus plantarum supplementation beneficial effect on the growth retardation of Drosophilia larvae in a poor environment*

Advisor: Dr Ramos Cathy

Host Laboratory: Functional Genomics Institute of Lyon – Functional Genomic Team of host / bacteria interactions, UMR5242 ENS/CNRS/INRA/University Lyon 1, Lyon (France).

TECHNICAL SKILLS

Microbiology: identification, culture and numeration of microorganisms (aerobic and anaerobic), flow cytometry

Molecular biology: *RNA and DNA analysis*: DNA purification, PCR amplification, RNA purification and mRNA quantification, qPCR, qRT-PCR; *Protein analysis:* ELISA technique.

Analytical chemistry: Metabolite extraction, gas chromatography, HPLC.

Cellular Biology: cell culture of intestinal epithelial cells (Caco-2, HT29-MTX, T84), cell infection with pathogenic microorganisms (Bio-safety level 3) and adhesion assays.

Biotechnology: *In vitro* digestion in artificial model of the upper GI tract (TIM1, TNO gastro Intestinal tract Model), fecal batch fermentation experiments.

Histology: tissue preparation and staining (H&E, Alcian blue).

Bioinformatics and statistics: biological databases (e.g. UniProt, BLAST), statistical softwares (R-studio, Graph-Pad, QIIME 2), treatment of *16S RNA* gene amplicon sequencing data.

Animal experimentation: gavage, injection, blood and organ collection on wild type and transgenic mice, training in animal experimentation, conception level, rodent specialty (Level 1).

TEACHING AND MANAGEMENT ACTIVITIES

2018-2020 Teaching assistant - Practical courses of Microbiology for undergraduate students (1st year of technological school, IUT Clermont Auvergne), practical classes in Medical Microbiology
Université Clermont Auvergne, Clermont-Ferrand, FRANCE

2021 Teaching assistant - Practical courses of Food Microbiology for undergraduate students (1st year of technological school, IUT Clermont Auvergne)
Université Clermont Auvergne, Clermont-Ferrand, FRANCE

2019-2020 Supervision of Claude Durif (laboratory technician)

LIST OF PUBLICATIONS

Articles published

1. Sauvaitre, T., F. Van Herreweghen, K. Delbaere, C. Durif, J. Van Landuyt, K. Fadhlaoui, S. Huile, F. Chaucheyras-Durand, L. Etienne-Mesmin, S. Blanquet-Diot and T. Van de Wiele (**2022**). Lentils and yeast fibers: a new strategy to mitigate Enterotoxigenic *Escherichia coli* (ETEC) strain H10407 virulence?

2. Sauvaitre, T., C. Durif, A. Sivignon, S. Chalancon. T. Van de Wiele, L. Etienne-Mesmin and S. Blanquet-Diot (**2021**). *In vitro* evaluation of dietary fiber anti-Infectious properties against food-borne enterotoxigenic *Escherichia coli*. *Nutrients* 13(9):3188.

3. Sauvaitre, T., L. Etienne-Mesmin, A. Sivignon, P. Mosoni, C. M. Courtin, T. Van de Wiele and S. Blanquet-Diot (**2021**). Tripartite relationship between gut microbiota, intestinal mucus and dietary fibers: towards preventive strategies against enteric infections. *FEMS Microbiol Rev* 45(2):fuaa052.

4. Etienne-Mesmin, L., B. Chassaing, M. Desvaux, K. De Paepe, R. Gresse, T. Sauvaitre, E. Forano, T. V. de Wiele, S. Schuller, N. Juge and S. Blanquet-Diot (**2019**). Experimental models to study intestinal microbes-mucus interactions in health and disease. *FEMS Microbiol Rev* **43**(5): 457-489.

Articles in preparation

5. Sauvaitre, T., J. Van Landuyt, C. Durif, C. Roussel, A. Sivignon, S. Chalancon, O. Uriot, F. Van Herreweghen, T. Van de Wiele, L. Etienne-Mesmin and S. Blanquet-Diot. Role of mucus-bacteria interactions in Enterotoxigenic *Escherichia coli* (ETEC) strain H10407 virulence.

PARTICIPATION AT NATIONAL AND INTERNATIONAL CONFERENCES

Oral presentations

- International Conference on probiotics, prebiotics, gut microbiota and health, June 2022, Bratislva, Slovakia

Use of a Fiber-based Strategy to Mitigate Virulence of Enterotoxigenic Escherichia coli Food-borne Pathogen Responsible for Travelers' Diarrhea
Sauvaitre, T., Van Landuyt, J., Durif, C., Delbaere, K., Huile, S., Chaucheyras-Durand, F., Van Herreweghen, F., Van de Wiele, T., Blanquet-Diot, S., Etienne-Mesmin, L.

- Groupe Régional de Recherche en Microbiologie des Interactions, May 2022, Lyon, France (online)

Sauvaitre, T., Van de Wiele, T., Etienne-Mesmin, L., Blanquet-Diot, S. Role of mucus compartment in Enterotoxigenic *Escherichia coli* (ETEC) pathogenicity?

- Rencontre des Microbiologistes du Pôle Clermontois, April 2021, Clermont-Ferrand, France (online)

Sauvaitre, T., Etienne-Mesmin, L., Roussel, C., Chalancon, S., Durif, C., Uriot, O., Van de Wiele, T., Blanquet-Diot, S. Deciphering the role of intestinal mucus in enterotoxigenic *Escherichia coli* infection : toward a fiber-based preventive strategy ?

- 24ème journée de l'Ecole Doctorale SVSAE, October 2020, Clermont-Ferrand, France

Sauvaitre, T., Etienne-Mesmin, L., Durif, C., Sivignon, A., Fadhlaoui, K., Van de Wiele, T., Blanquet-Diot, S. Towards a fiber-based strategy to prevent bacteria-mucus interactions in Enterotoxigenic *Escherichia coli* infections ?

Poster presentations

- Beneficial Microbes meeting, March 2021, Amsterdam, The Netherlands (online)

Sauvaitre, T., Etienne-Mesmin, L., Roussel, C., Chalancon, S., Durif, C., Uriot, O., Van de Wiele, T., Blanquet-Diot, S. Use of a fiber-based strategy to prevent bacteria-mucus interactions in enterotoxigenic *Escherichia coli* infections?

- 12th international symposium on Gut Microbiology, October 2021, Clermont-Ferrand, France (online)

Sauvaitre, T., Roussel, C., Sivignon, A., Chalancon, S., Durif, C., Huille, S., Chaucheyras-Durand, F., Van de Wiele, T., Etienne-Mesmin, L., Blanquet-Diot, S. Use of a fiber-based strategy to prevent enterotoxigenic *Escherichia coli* infections: *in vitro* investigation on their antagonistic effects

- Digestive Disease Week, May 2019, San Diego, United States

Brugiroux, S., Sauvaitre, T.; Roche, G., Ledieu, S., Chevarin, C., Godfraind, C., Massard, C., Awitor, O., Barnich, N., Bonnet, M., Boucher, D. Impacts of additive food E171 (titanium dioxide) on the gut microbiota and colorectal carcinogenesis in $APC^{min/+}$ murine model.

AWARDS

- Award for best oral communication during 24ème journée de l'Ecole Doctorale SVSAE, Université Clermont Auvergne, October 2020, Clermont-Ferrand, France

- Recipient of 1st prize from audience and 2nd prize from jury at the finale of My Thesis in 180 seconds (MT180).

Abstract

The human digestive tract is a key player at the boundary between the external environment and the host. At the interface between the digestive lumen and the intestinal epithelium, the mucus layer, a complex viscoelastic adherent secretion, acts as a major modulator of human health. In order to reach the intestinal cells and/or colonize, several enteric pathogens have to interact with and get through this physical, chemical and biological line of defense. Enterotoxigenic *Escherichia coli* (ETEC), the main pathogenic agent of travelers' diarrhea, does not escape this rule. To fulfil its infection cycle, ETEC is equipped with an arsenal of adhesins and mucinases allowing cellular adhesion and mucus degradation, respectively. These colonization mechanisms facilitate the production and release of heat labile (LT) and/or heat-stable (ST) enterotoxins, ultimately responsible for cholera-like watery diarrhea. To date the treatment of ETEC infection remains mainly symptomatic with a frequent use of antibiotics. Given the global burden of antibiotic resistance and its negative impact on human health, it is urgent to find new preventive strategies against these infections. Among the candidates, dietary fibers have been recently investigated for their antagonistic properties against enteric pathogens. A low number of studies has suggested that they may act through various means: (i) direct antagonism (bacteriostatic effect, inhibition of cell adhesion and toxin production) or (ii) indirect antagonism *via* modulation of gut microbiota composition/activity or decoy of resident gut microbes from mucus layer consumption. In this context, this joint doctoral research work between Ghent University (Belgium) and Université Clermont Auvergne (France) aimed to (1) unravel how the mucus compartment can modulate the prototypical ETEC strain H10407 survival and virulence, and (2) decipher if dietary fiber-containing products could present ETEC anti-infectious properties, notably by preventing ETEC-mucus interactions.

In the first axis, we confirmed the prototypical ETEC strain H10407 adhesion propensity for the intestinal mucus by using different simple *in vitro* approaches. The introduction of mucin secretion and physical surface in the dynamic TIM-1 digestive model showed that mucus could favor ETEC survival during gastrointestinal passage without significantly affecting its virulence. However, when reaching the host intestinal cells simulated by mucus-secreting Caco-2/HT29-MTX co-culture, ETEC virulence gene expression was significantly induced confirming that the host is a key driver of pathogen's virulence. When simulating the complex microbial background of the human gut, mucin addition did not impact significantly ETEC survival, but we showed that the mucosal compartment was colonized by a specific microbiota particularly affected by ETEC. In the second axis, a screening program was first performed to select among 8 fiber candidates from cereals, legumes or microbes the two most relevant based on their anti-infectious properties against ETEC strain H10407, namely a lentil fiber extract and specific yeast cell walls from *Saccharomyces cerevisiae*. In-depth investigations indicated that the lentil extract reduced LT toxin concentration while the yeast product decreased ETEC adhesion to the mucus secreting co-culture model. Also, in cell assays, both lentils and yeast fiber products were able to modulate ETEC virulence gene expression and innate immune response induction. Mainly yeast cell walls were able to strengthen intestinal barrier function. Finally, in batch experiments with fecal microbiota, we reported that the yeast product supported the prevalence of some phylogroups as *Parabacteroides* or commensal *E. coli*, which could be of interest in traveler's diarrhea prevention.

To conclude, this PhD research provided meaningful *in vitro* insights on how the mucus compartment could shape ETEC virulence and brought solid evidences concerning dietary fiber-containing products antagonistic properties. In a next future, these promising results could be confirmed using more complex approaches such as dynamic digestive systems coupled with human cell lines or animal models. This work opens up avenues in the development of new relevant prophylactic anti-infectious strategies against ETEC based on the use of fibers.

Samenvatting

Het menselijke spijsverteringskanaal is een belangrijke speler op de grens tussen het externe milieu en de gastheer. Op het grensvlak tussen het spijsverteringslumen en het darmepitheel fungeert de mucuslaag, een complexe visco-elastische adherente secretie, als een belangrijke modulator van de menselijke gezondheid. Om de darmcellen te bereiken en/of zich te koloniseren, moeten verschillende enterische pathogenen met deze fysische, chemische en biologische verdedigingslinie interageren en er doorheen geraken. Enterotoxigene *Escherichia coli* (ETEC), het belangrijkste pathogene agens van reizigersdiarree, ontsnapt niet aan deze regel. Om zijn infectiecyclus te kunnen volbrengen, is ETEC uitgerust met een arsenaal van adhesines en mucinases die respectievelijk celadhesie en mucusafbraak mogelijk maken. Deze kolonisatiemechanismen vergemakkelijken de productie en het vrijkomen van hitte-labiele (LT) en/of hitte-stabiele (ST) enterotoxinen, die uiteindelijk verantwoordelijk zijn voor cholera-achtige waterige diarree. Tot op heden blijft de behandeling van ETEC-infectie voornamelijk symptomatisch met een frequent gebruik van antibiotica. Gezien de mondiale last van antibioticaresistentie en de negatieve invloed daarvan op de menselijke gezondheid, is het dringend noodzakelijk nieuwe preventieve strategieën tegen deze infecties te vinden. Onder de kandidaten zijn voedingsvezels recent onderzocht op hun antagonistische eigenschappen tegen enterische pathogenen. Een klein aantal studies heeft gesuggereerd dat zij op verschillende manieren kunnen werken: (i) direct antagonisme (bacteriostatisch effect, remming van celadhesie en toxineproductie) of (ii) indirect antagonisme *via* modulatie van de darmmicrobiota samenstelling/activiteit of decoy van residente darmmicroben door consumptie van de mucuslaag. In deze context had dit gezamenlijk doctoraatsonderzoek tussen de Universiteit Gent (België) en de Université Clermont Auvergne (Frankrijk) tot doel (1) te ontrafelen hoe het mucuscompartiment de overleving en virulentie van de prototypische ETEC-stam H10407 kan moduleren, en (2) te ontcijferen of voedingsvezelbevattende producten anti-infectieuze eigenschappen tegen ETEC kunnen hebben, met name door ETEC-mucusinteracties te voorkomen

In de eerste as, bevestigden wij de prototypische ETEC stam H10407 adhesiegeneigdheid voor het intestinale mucus door gebruik te maken van verschillende eenvoudige *in vitro* benaderingen. De introductie van mucine secretie en fysieke oppervlakte in het dynamische TIM-1 spijsverteringsmodel toonde aan dat mucus de overleving van ETEC tijdens de gastro-intestinale passage kon bevorderen zonder de virulentie significant te beïnvloeden. Echter, bij het bereiken van de darmcellen van de gastheer, gesimuleerd door mucus afscheidende Caco-2/HT29-MTX co-cultuur, werd ETEC virulentie genexpressie aanzienlijk geïnduceerd, wat bevestigt dat de gastheer een belangrijke drijfveer is voor de virulentie van de ziekteverwekker. Bij simulatie van de complexe microbiële achtergrond van de menselijke darm had de toevoeging van mucine geen significante invloed op de overleving van ETEC, maar we toonden aan dat het mucosale compartiment gekoloniseerd werd door een specifieke microbiota die in het bijzonder door ETEC werd aangetast. In de tweede as werd eerst een screening uitgevoerd om uit 8 vezelkandidaten van granen, peulvruchten of microben de twee meest relevante te selecteren op basis van hun anti-infectieuze eigenschappen tegen ETEC stam H10407, namelijk een linzenvezelextract en specifieke gistcelwanden van *Saccharomyces cerevisiae*. Diepgaand onderzoek wees uit dat het linzenextract de LT-toxineconcentratie verminderde, terwijl het gistproduct de ETEC-adhesie aan het mucusafscheidende co-cultuurmodel verminderde. Ook in celtests waren zowel linzen als gistvezelproducten in staat om ETEC virulentie genexpressie en aangeboren immuunrespons inductie te moduleren. Voornamelijk gistcelwanden waren in staat om de intestinale barrièrefunctie te versterken. Tenslotte, in batch experimenten met fecale microbiota, rapporteerden we dat het gistproduct de prevalentie van sommige fylogroepen zoals *Parabacteroides* of commensale *E. coli* ondersteunde, wat van belang zou kunnen zijn bij het voorkomen van reizigersdiarree.

Concluderend kan worden gesteld dat dit doctoraatsonderzoek zinvolle *in vitro* inzichten heeft opgeleverd over hoe het mucuscompartiment vorm kan geven aan de virulentie van ETEC en solide bewijzen heeft opgeleverd over de antagonistische eigenschappen van voedingsvezel-bevattende producten. In een volgende toekomst zouden deze veelbelovende resultaten kunnen worden bevestigd met behulp van meer complexe benaderingen zoals dynamische spijsverteringssystemen gekoppeld aan menselijke cellijnen of diermodellen. Dit werk opent perspectieven voor de ontwikkeling van nieuwe relevante profylactische anti-infectiestrategieën tegen ETEC gebaseerd op het gebruik van vezels.

Résumé

Contexte scientifique de la thèse

Les fibres alimentaires : présentation générale

Diverses définitions des fibres alimentaires ont été émises par des organismes scientifiques et réglementaires du monde entier. La définition qui prévaut le plus est toutefois celle du *Codex Alimentarius* émanant de l'Organisation des Nations unies pour l'alimentation et l'agriculture (FAO) et de l'Organisation Mondiale de la Santé (OMS). Les fibres alimentaires sont définies comme des polymères de carbohydrates de plus de trois résidus non hydrolysés par les enzymes endogènes de l'intestin humain. De ce fait, elles sont potentiellement consommées par les micro-organismes, en particulier les bactéries, constituant le microbiote intestinal humain. Les fibres alimentaires peuvent être divisées en sous-groupes selon leur origine, leur structure et leurs propriétés physicochimiques. Néanmoins, la plupart des fibres alimentaires consommées par les humains sont d'origine végétale et se retrouvent dans des proportions différentes dans les fruits, les légumes, les légumineuses, les céréales, les noix et les graines. Certaines d'entre elles peuvent également être dérivées d'animaux, de champignons ou de bactéries. C'est notamment le cas des oligosaccharides du lait humain, des mannanes de levures, de la chitine des champignons et des exopolysaccharides des bactéries retrouvés dans les aliments fermentés comme le pain, le fromage ou le yaourt. Les fibres alimentaires peuvent aussi être divisées en oligosaccharides (entre 3 et 10 unités monomériques) ou en polysaccharides (plus de 10 unités). Parmi ces derniers, il existe notamment différents types : amidons résistants, la cellulose, les hémicelluloses, les fructanes comme l'inuline et les pectines. Les fibres alimentaires comprennent également des oligosaccharides résistants à base de fructose (fructooligosaccharide, FOS), de galactose (galactooligosaccharide, GOS), de xylose (xylooligosaccharide, XOS), de mélanges d'arabinose et de xylose (arabinoxylooligosaccharide, AXOS) ou de sucres pectiques (oligosaccharide pectique, POS). En conséquence, il existe une énorme diversité de fibres alimentaires qui diffèrent par leur composition en résidus saccharidiques, le type de liaison entre les sucres, le degré de polymérisation ou de ramification. Ces caractéristiques structurelles confèrent aux fibres des propriétés diverses, notamment en termes de cristallinité, viscosité ou solubilité. Cette dernière est particulièrement pertinente pour une fermentation efficace et rapide par les micro-organismes présents dans le tractus digestif humain.

Les fibres alimentaires : les effets bénéfiques reconnus

L'apport en fibres alimentaires varie considérablement d'un pays à l'autre. Les régimes alimentaires « occidentalisés » des pays industrialisés sont appauvris en fibres au profit des protéines animales, des graisses, du sucre et de l'amidon, tandis que les populations rurales non industrialisées consomment davantage de fibres grâce à leurs régimes riches en végétaux. Des études sur les habitudes alimentaires ont révélé que les adultes consomment en moyenne entre 12 et 18 grammes, 14 grammes et 16 à 29 grammes de fibres par jour aux États-Unis, Royaume-

Uni et Europe, respectivement. Ces quantités sont globalement inférieures aux recommandations proposées par le département de l'Agriculture des États-Unis (USDA) de 25 grammes par jour pour les femmes et de 38 grammes par jour pour les hommes jusqu'à l'âge de 50 ans. Or, les effets bénéfiques des fibres alimentaires sur la santé sont maintenant largement reconnus. Les fibres présentent de nombreux avantages physiologiques directs tels que l'augmentation du volume des matières fécales, la diminution du temps de transit, la baisse de la glycémie, du taux de cholestérol, de l'adiposité et des paramètres associés au syndrome métabolique. Les populations qui consomment plus de fibres alimentaires présentent une incidence plus faible de dérégulation du système immunitaire, avec un risque plus faible de développer de l'asthme, des allergies, des maladies inflammatoires chroniques de l'intestin (MICI), un diabète de type 2 et un cancer colorectal. L'apport alimentaire insuffisant dans les pays industrialisés est de son côté très largement relié à une perturbation de la relation hôte-microbiote, communément appelée dysbiose, associée une incidence accrue de troubles digestifs et extra-digestifs.

Les fibres alimentaires et la préservation du microbiote intestinal

Plusieurs études convergent et tendent à montrer que l'effet bénéfique des fibres alimentaires sur la santé de l'homme passerait très certainement en grande partie par son impact sur le microbiote intestinal. Par définition, les fibres ne sont pas digérées dans la partie haute du tractus digestif mais fermentées dans l'intestin par les micro-organismes. Il en découle une production de métabolites comme des acides gras à chaine courte ou AGCC (principalement acétate, butyrate et propionate) et des gaz (dihydrogène H_2, dioxyde de carbone CO_2, méthane CH_4 et sulfure d'hydrogène H_2S). La vitesse de dégradation des fibres va dépendre de leur accessibilité par les micro-organismes, qui elle-même résulte des propriétés physicochimiques des fibres et des espèces microbiennes présentes. Les fibres solubles sont plus accessibles et digérées dans les parties hautes de l'intestin, alors que les fibres insolubles forment des particules solides, plus difficilement accessibles et digérés plus en aval. Comme les fibres présentent une incroyable diversité de structure, leur dégradation nécessite un arsenal d'enzymes. Plus de mille enzymes spécialisés dans la digestion des carbohydrates ont été recensés dans le métagénome du microbiote humain, contre entre 8 et 17 pour l'hôte. La dégradation des fibres est séquentielle et peut impliquer de nombreuses enzymes différentes d'origine bactérienne multiple. Certaines bactéries ont des capacités de dégradation très étendues, comme *Bacteroides thetaiotaomicron*, alors que d'autres sont plus spécialisées, comme *Ruminococcus bromii*, une bactérie capable de coloniser et dégrader l'amidon. Les bactéries intestinales utilisent les fibres comme source majoritaire de carbone. De nombreuses études ont démontré à différentes échelles (de la population à l'individu) qu'un régime appauvri en fibres alimentaires était associé à une diminution de la diversité microbienne. Certaines études conduites chez la souris ont même démontré une disparition progressive de la diversité microbienne lorsque les apports diminuaient, alors que le retour à un niveau basal de diversité demanderait plusieurs générations successives. Or, comme évoqué précédemment, un

déséquilibre persistant des communautés microbiennes intestinales a été associé à de nombreuses pathologies.

Les fibres alimentaires : des propriétés anti-infectieuses méconnues

Un effet moins connu des fibres est leur potentiel anti-infectieux. En effet, les fibres peuvent inhiber, ou du moins réduire, les infections microbiennes par différents mécanismes directs ou indirects. Concernant l'effet direct, le chitosan (dérivé de la chitine) a montré un effet bactériostatique direct en inhibant la croissance de divers pathogènes, et en particulier les *Escherichia coli* enterohémorragiques (EHEC). Des fibres alimentaires de différentes sources (en particulier d'origine végétale) ont aussi prouvé leur efficacité dans la réduction de l'adhésion de pathogènes aux cellules épithéliales intestinales. Cet effet pourrait être expliqué par des motifs partagés entre les récepteurs présents au niveau du mucus intestinal et les fibres alimentaires, ces dernières agissant comme un leurre détournant les pathogènes de leur cible. Il a également été montré que certains oligosaccharides du lait humain ont un effet inhibiteur sur l'adhésion des toxines produites par le pathogène à leur récepteur. Concernant l'effet indirect, celui-ci peut faire intervenir l'hôte. En effet, certaines fibres alimentaires ont montré un effet immunomodulateur en réduisant l'activation de l'immunité innée de l'hôte. Par exemple, il a été montré que certains oligosaccharides du lait humain réduisent l'expression du récepteur CD14 limitant ainsi l'induction de l'inflammation. Outre l'effet *via* l'hôte, les fibres peuvent également moduler le microbiote intestinal, reconnu comme un obstacle important à la colonisation des pathogènes. Ce rôle protecteur est étayé par de nombreuses études montrant que certaines souches commensales du microbiote intestinal favorisent les mécanismes d'inhibition des pathogènes. Les effets inhibiteurs directs sont conférés par la production d'acides, la sécrétion de molécules inhibitrices comme des bactériocines ou la production de composés microbiens (inconnus pour la plupart) capables de réprimer les gènes de virulence. Par conséquent, la modulation du microbiote intestinal et le soutien de sa diversité avec des apports en fibres alimentaires pourraient être un moyen pertinent et efficace de prévenir les infections entériques. En ce sens, il a été montré à de nombreuses reprises que l'administration conjointe d'oligosaccharides et d'espèces probiotiques inhibait la colonisation par des pathogènes comme *Salmonella enterica* serovar Typhimurium, *Acinetobacter baumannii* et *Clostridioides difficile*. L'effet anti-infectieux peut être associé à l'augmentation d'un groupe microbien ou des modifications de l'activité métabolique bactérienne, comme des AGCC, produits majeurs de la fermentation des fibres. Récemment, un nouvel potentiel anti-infectieux des fibres a été mis en évidence *via* la préservation du mucus intestinal, comme décrit ci-dessous, offrant de nouvelles perspectives de recherche.

Le mucus intestinal : présentation générale

Le mucus intestinal est un gel viscoélastique et adhérent continuellement produit et sécrété par les cellules caliciformes. Le mucus est présent dans l'ensemble du tractus gastro-intestinal humain de l'estomac au gros intestin mais avec des variations en termes de structure et de composition. Dans le côlon, le mucus présente une structure à double couche, avec une

couche interne fermement attachée à l'épithélium et une couche externe superposée à la première. Le mucus subit une érosion mécanique et protéolytique constante due aux frottements des particules alimentaires et à l'action des enzymes digestives et des bactéries commensales. Composé d'eau, d'électrolytes, de lipides et de protéines, les principaux composants structurels du mucus (environ 5 %) sont des glycoprotéines de haut poids moléculaire appelées mucines. Les parties glycanes des mucines sont rattachées à la partie protéique par une liaison *O* à la sérine et à la thréonine, ou une liaison *N* à l'asparagine. La base des glycanes est formée par une combinaison de trois sucres, le galactose, la N-acétylgalactosamine et la N-acétylglucosamine auxquels différentes chaînes de saccharides peuvent être attachées. Le monosaccharide terminal est généralement le fucose ou l'acide sialique. Les chaînes d'oligosaccharides peuvent également subir des modifications comme la sulfatation, en particulier dans les régions coliques. À ce jour, plusieurs gènes codant des mucines ont été décrits chez l'homme et nommés en fonction de leur ordre de découverte. Parmi les mucines, certaines appartiennent à la famille des mucines gélifiantes sécrétées, tandis que d'autres sont classées dans la famille des mucines associées aux membranes. La glycoprotéine mucine 2 (MUC2) sécrétée par l'hôte est un constituant majeur du mucus de l'intestin grêle et du côlon humain, tandis que MUC1, MUC5AC et MUC6 sont prédominantes dans l'estomac. Le mucus intestinal a plusieurs fonctions. La première, la plus basique, est la lubrification de l'épithélium, permettant la progression du bol alimentaire le long du tractus digestif. Les autres (sources de nutriments, niche microbienne) sont détaillées plus amplement ci-dessous.

Le mucus intestinal : une seconde source de glucides en miroir des fibres

Les mucines étant des polymères protéiques glycosylés, elles peuvent constituer une source de carbone et d'énergie pour la croissance du microbiote intestinal, au même titre que les fibres alimentaires. Si l'apport en fibres peut être variable, le mucus est toujours qualitativement présent. Il constitue aussi une niche microbienne et sa colonisation est nécessaire pour que les micro-organismes résidant dans le tractus digestif puissent se maintenir. Des études ont démontré que les communautés microbiennes de la lumière digestive diffèrent en termes de composition et d'abondance par rapport à celles associées au mucus, notamment en raison de différences en termes de disponibilité en nutriments (e.g. oxygène et substrats glucidiques). Comme pour le microbiote luminal, ces communautés diffèrent selon le segment du tractus digestif considéré. En particulier, les communautés mucosales au niveau du côlon sont enrichies en certaines espèces comme *Bacteroides acidifaciens*, *Bacteroides fragilis*, *Akkermansia muciniphila* et en espèces appartenant à la famille des *Lachnospiraceae*. En comparaison avec les fibres, les glucides du mucus sont constitués d'un groupe plus restreint de résidus avec seulement six monomères possibles (galactose, N-acétylgalactosamine, N-acétylglucosamine, mannose, fucose et acide sialique). Toutefois, bien que les monomères et les liaisons osidiques soient différents, il existe des similitudes structurelles entre mucus et fibres. Comme pour les fibres, certaines liaisons osidiques du mucus sont spécifiquement dégradées par les bactéries du microbiote qui possèdent l'enzyme associée avec une dégradation

séquentielle. Certaines espèces sont spécialisées dans la dégradation du mucus, comme *Akkermansia muciniphila*, tandis que d'autres plus polyvalentes peuvent consommer à la fois fibres et mucus. Ces espèces polyvalentes peuvent toutefois présenter une orientation préférentielle pour le mucus comme *Bacteroides massiliensis* et *Bacteroides fragilis* ou pour les fibres comme *Bacteroides thetaiotaomicron*. Ainsi, lorsque les quantités de fibres alimentaires diminuent, les micro-organismes peuvent orienter leur activité vers la consommation des glycanes du mucus. Les similitudes entre oligosaccharides du lait maternel humain (HMOs, human milk oligosaccharides) et antigènes des groupes sanguins illustrent bien les analogies possibles entre fibres alimentaires et glycanes du mucus. Les HMOs sont composés d'unités de lactose ou de N-acétyl-lactosamine répétées et ramifiées, souvent décorées d'acides sialiques et de fucoses. Ces structures partagent des schémas communs avec les antigènes des groupes sanguins humains, retrouvés dans le mucus chez 80% des Nord-Américains et des Européens). Lors de la petite enfance, les oligosaccharides du lait maternel peuvent être considérés comme la seule source de fibres alimentaires, initiant le microbiote du nourrisson à la consommation des polysaccharides du mucus.

Le mucus intestinal : un rôle de barrière protégeant l'homéostasie intestinale

A côté de son rôle de niche microbienne et de substrats pour le microbiote résident, le mucus exerce une fonction barrière contre les agressions physicochimiques et les invasions microbiennes, montrant son rôle crucial dans le maintien de l'homéostasie intestinale, en étroite collaboration avec le système immunitaire. Le mucus est notamment une réserve de molécules antimicrobiennes (e.g. défensines α et β, IgA et IgM). De nombreuses études ont pu montrer que des défauts d'intégrité du mucus sont associés à des risques accrus de maladies, au même titre que des déficits en fibres alimentaires. Face à ses deux rôles antagonistes, l'organisation structurale du mucus doit être finement contrôlée. La colonisation microbienne du mucus selon l'axe transversal illustre cette dualité. La couche externe est densément colonisée par les micro-organismes et présente un taux de renouvellement rapide, alors que la couche interne, solidement attachée aux cellules épithéliales, a longtemps été considérée comme dépourvue de bactéries en raison de ses propriétés physicochimiques plus contraignantes.

Les pathogènes et l'enjeux de colonisation du mucus

Nombre de pathogènes intestinaux tels que les *Escherichia coli* ou les salmonelles ont besoin de coloniser l'épithélium intestinal pour réaliser efficacement leur cycle infectieux. Dans ce contexte, le mucus intestinal exerce son rôle de barrière physique et les pathogènes ont dû développer des stratégies pour s'y adapter. Ainsi, il a été montré que l'adhésion de *Salmonella enterica* serovar Typhimurium et des EHEC est plus élevée sur des modèles cellulaires producteurs de mucus que sur ceux n'en produisant pas. Comme pour les microorganismes commensaux, les agents pathogènes utilisent des appendices de surface (adhésines, fimbriae et flagelles) pour se lier au mucus et à ses motifs osidiques. Par exemple, *Helicobacter pylori* et *Campylobacter jejuni* possèdent plusieurs adhésines qui se lient aux antigènes des groupes

sanguins (exprimés dans le mucus intestinal) et à l'acide sialique. L'adhésine GbpA de *Vibrio cholerae* pour sa part se lie à la N-acétylglucosamine. Les sous-unités flagellaires de *Campylobacter jejuni*, des *Escherichia coli* entéropathogènes et de *Clostridioides difficile* sont toutes capables de se lier aux polysaccharides du mucus. Pour faire face aux propriétés de type « filet » du mucus, les agents pathogènes, comme leurs homologues commensaux, possèdent des protéases appelées mucinases. Ces dernières facilitent l'accès à la couche de mucus par protéolyse des protéines de mucines. Pour contrebalancer leur effet et maintenir sa structure en forme de filet retenant le microbiote, le mucus contient des inhibiteurs de protéases qui protègent le mucus d'une dégradation trop importante. Ces mucinases ont été particulièrement bien caractérisées chez les entérobactéries. Les pathovars d'*Escherichia coli* sont armés d'un arsenal diversifié de mucinases, telles que SslE, StcE, Hbp, YghJ et EatA. Ces mucinases participent à la colonisation intestinale, la pénétration des bactéries dans la couche de mucus, l'adressage des toxines et l'adhérence aux cellules épithéliales. A noter que les *Escherichia coli* pathogènes génèrent des quantités plus importantes de YghJ par rapport à leurs homologues commensaux, alors qu'il n'y a pas de différence dans leurs séquences d'acides aminés cataboliques putatives. Les pathogènes aussi peuvent utiliser les sucres du mucus comme sources de carbone et d'énergie. *Salmonella enterica* serovar Typhimurium peut par exemple libérer les glucides du mucus en utilisant une sialidase. Mais en général les pathogènes ne sont pas des dégradeurs dit primaires du mucus. Ils ont un arsenal limité de CAZymes et comptent souvent sur d'autres acteurs microbiens pour se nourrir du mucus.

Les fibres alimentaires : des appâts pour éviter la dégradation du mucus

Vu le rôle prépondérant du mucus comme barrière contre les pathogènes et l'adaptation évidente de ces derniers à cette barrière physique, il apparait intéressant de préserver cette barrière physique dans une stratégie anti-infectieuse. De par leurs similitudes structurelles avec les glycanes du mucus, les fibres pourraient jouer ce rôle *via* différents mécanismes. Tout d'abord, les motifs ou les récepteurs osidiques du mucus reconnus par les pathogènes peuvent être également retrouvés dans certaines fibres. Ces dernières pourraient alors être utilisées pour détourner les pathogènes de leur cible. Par exemple, la protéine GbpA de *Vibrio cholerae* permet l'adhésion du pathogène à la fois au mucus et à la chitine. Les fimbriae F17 produits par les souches d'*Escherichia coli* entérotoxinogènes (ETEC) reconnaissent à la fois les récepteurs présentant des N-acétylglucosamine sur l'épithélium intestinal et des oligomères de N-acétylglucosamine sur le mucus. Comme mentionné précédemment, des apports en fibres importants peuvent aussi empêcher la dégradation du mucus. La capacité du microbiote intestinal résident à orienter son métabolisme vers la consommation des glycanes du mucus lorsque l'apport en fibres est faible est une découverte relativement nouvelle et l'extension de cette idée aux pathogènes entériques est encore plus novatrice. Dans un modèle de souris gnotobiotique, Desai et son équipe ont montré qu'un régime pauvre en fibres conduisait à l'enrichissement du mucus en bactéries capables de le dégrader. L'expression des gènes dégradant le mucus était aussi favorisée conduisant à son érosion et à une plus grande susceptibilité à l'infection par le pathogène murin *Citrobacter rodentium*. Ces résultats

suggèrent qu'un apport adéquat en fibres alimentaires devrait permettre d'éviter la dégradation des polysaccharides du mucus et donc permettre le maintien d'une barrière efficace contre la colonisation par des agents pathogènes. Empêcher la dégradation du mucus par la partie versatile du microbiote résident permettrait aussi de réduire les sources de carbones et d'énergie disponibles pour les pathogènes.

Les *Escherichia coli* entérotoxinogènes (ETEC)

Les ETEC représentent l'agent pathogène le plus couramment identifié dans la diarrhée du voyageur (près d'un voyageur sur six soit 10 millions de voyageurs). Les infections à ETEC représentent également une cause émergente de diarrhée infectieuse dans les pays industrialisés. Même si chez les adultes les infections à ETEC restent de gravité modérée, des études récentes ont montré qu'elles étaient associées à un plus grand risque de développer un syndrome d'intestin irritable post-infectieux (SII-PI) ou des troubles musculo-squelettiques. Au-delà de l'impact sanitaire, la prise en charge de ces infections génère d'importantes pertes économiques au niveau mondial. Chez l'adulte, une fois ingérés à une dose comprise entre 10^6 et 10^{10} bactéries, les ETEC poursuivent une stratégie des plus sophistiquées pour résister aux conditions drastiques de l'environnement digestif humain et atteindre leur site d'infection, probablement situé dans la partie distale de l'intestin grêle. Le pathogène est capable d'adhérer à un large panel de récepteurs, grâce à une myriade d'adhésines parmi lesquelles se trouvent des facteurs de colonisation fimbriaux (CFA/I, FimH) et des protéines de la membrane externe (Tia, TibA, EtpA). Les facteurs de colonisation CFA/I, CS2, CS5 et CS6 et l'adhésine EtpA seraient capables de reconnaitre spécifiquement des motifs osidiques du mucus. Les ETEC peuvent aussi dégrader le mucus pour accéder plus facilement à l'épithélium sous-jacent, grâce à deux mucinases, YghJ and EatA, très fréquemment détectées chez les souches d'ETEC (90% et de 55-70% des souches, respectivement). Malgré toutes ces interactions entre ETEC et mucus, il n'existe quasiment aucune étude évaluant la modulation de la virulence des ETEC par le compartiment mucus. Les ETEC sécrètent deux toxines, une thermolabile (LT) et/ou une thermostable (ST). La toxine LT, partageant 80% d'homologie avec la toxine cholérique (*Vibrio cholerae*), est sécrétée par le système de sécrétion de type 2 impliquant notamment la protéine LeoA, tandis que la toxine ST est sécrétée par le système de pompes à efflux TolC. La production de ces toxines au niveau de l'épithélium intestinal permet l'activation d'une cascade de signalisation, à l'origine de diarrhées aqueuses abondantes de type cholériforme. A noter que les deux mucinases YghJ et EatA favorisent l'action de la toxine LT. L'infection à ETEC est aussi associée à une inflammation intestinale modérée, caractérisée par la présence dans les selles de leucocytes, de lactoferrine et de myélopéroxydase. Les cytokines et chimiokines de l'inflammation sont aussi induites, comme les interleukines (IL) IL-8, IL-1β, IL-17A et l'interféron γ (IFN-γ) et retrouvées dans les selles ou le sang. L'activation de l'immunité innée semble positivement liée à la sévérité de l'infection. Enfin, même si les données divergent, il semble que les ETEC induisent des modifications de l'activité et de la composition du microbiote fécal des patients infectés.

Quelle stratégie thérapeutique ou préventive contre les ETEC ?

A ce jour, aucun traitement reconnu et spécifique contre les ETEC n'est commercialisé dans le monde. La prise en charge des infections reste essentiellement symptomatique et suit les recommandations générales associées à tout épisode diarrhéique, c'est à dire une réhydratation orale, l'usage d'anti-diarrhéiques ou de ralentisseurs de transit. Le recours à l'antibiothérapie a également été fréquemment rapporté, contribuant au phénomène d'antibiorésistance et à son impact négatif sur la santé humaine. Il apparait donc important de développer de nouvelles stratégies anti-infectieuses préventives. Parmi les pistes envisagées, un vaccin commercialisé contre *Vibrio cholerae* a prouvé un effet bénéfique à court contre les ETEC. Le vaccin candidat le plus avancé en essai clinique de phase I/II est l'Etvax®, qui utilise quatre souches d'*E. coli* inactivées sur-exprimant certains facteurs de colonisation et une sous-unité de la toxine LT. L'usage de probiotiques pour la prévention des infections est aussi une autre piste prometteuse. Certaines études ont montré l'efficacité de probiotiques comme *Lactobacillus rhamnosus, Pediococcus pentosaceus* ou *Saccharomyces cerevisiae.* Ces microorganismes exercent un effet anti-infectieux en inhibant la croissance du pathogène, la production de toxine, l'adhésion aux cellules intestinales ou encore la réponse inflammatoire. A ce jour, aucun probiotique n'est actuellement spécifiquement prescrit en traitement préventif ou curatif des infections à ETEC.

Les fibres, une stratégie anti-ETEC à valoriser ?

Etant donné les interactions démontrées entre ETEC et mucus intestinal (adhesines, mucinases), l'utilisation d'une stratégie de prévention basée sur l'utilisation de fibres alimentaires semble intéressante à évaluer. Pourtant, les études portant sur la capacité de fibres à prévenir une infection à ETEC chez l'Homme sont rares et un nombre limité de fibres a été étudié *in vitro*. Il a été montré que les oligosaccharides du lait et les fibres solubles de plantain à des concentrations respectives de 1 $g.L^{-1}$ et 5 $g. L^{-1}$ réduisent l'adhésion des ETEC aux cellules épithéliales intestinales Caco-2. Diverses études ont étudié la capacité de fibres alimentaires à empêcher la liaison de la toxine d'ETEC à son récepteur. Ces études ont toutes été menées avec des oligosaccharides du lait humain, en raison des similitudes de structure avec les récepteurs de toxines. En effet, le récepteur GM1 de la toxine LT est un motif osidique également retrouvé dans le lait humain. En modèle d'anse iléale chez le lapin, il a été montré que l'oligosaccharide GM1 mais également le siallylactose sont capables d'inhiber la liaison de la toxine LT à son récepteur endogène, limitant ainsi les diarrhées aqueuses. Il a aussi été montré que la fraction fucosylée des oligosaccharides du lait maternel augmentait la survie des souris après ingestion de la toxine ST. Face à ces résultats, Paton et ses collègues ont développé des versions génétiquement modifiées d'*E. coli* exprimant à leur surface un récepteur GM2 et d'autres oligosaccharides, permettant d'inhiber la liaison des toxines à leur récepteur et réduisant les pertes en eau en modèle d'anse iléale.

Contexte organisationnel et objectifs de la cotutelle de thèse

Dans ce contexte, les trois années de cotutelles européenne de thèse se sont déroulées entre deux laboratoires universitaires, l'Unité Mixte de Recherche Microbiologie, Environnement Digestif et Santé (UMR MEDIS 454, Université Clermont-Auvergne, INRAE, Clermont-Ferrand, France) et le Center for Microbial Ecology and Technology (CMET, Université de Gand, Gand, Belgique). Ces deux laboratoires bénéficient de plus de 20 ans d'expertises technique et scientifique dans les domaines de la digestion et fermentation artificielles. L'intérêt majeur de cette collaboration était d'associer l'expertise de l'UMR MEDIS dans la simulation de la partie haute du tractus digestif et celle du CMET dans les modèles de fermentations simulant la partie distale du tractus digestif, mais aussi de profiter de leur expertise commune dans les modèles cellulaires reproduisant l'épithélium intestinal. Le travail de thèse avait pour objectifs de : **(i)** mieux comprendre les interactions entre le mucus intestinal et la virulence d'une souche ETEC de référence infectant l'homme et **(ii)** évaluer le potentiel anti-infectieux de produits contenant des fibres. La première partie de la thèse s'est déroulée en France. Dans le cadre de l'axe **1** (rôle du mucus de la physiopathologie des ETEC), des expériences ont été effectuées sur le modèle TIM-1 (TNO gastro Intestinal Model-1) reproduisant l'estomac et l'intestin grêle auquel un compartiment mucus a été ajouté pour évaluer son impact sur la virulence et la survie des ETEC. Des expériences de culture cellulaires comparant un modèle de co-culture Caco-2/ HT29-MTX secrétant du mucus à un modèle de monoculture Caco-2 ont permis de mesurer l'impact de la présence de cellules sécrétrices de mucus sur l'adhésion et la virulence des ETEC. Concernant l'axe **2** (potentiel des fibres comme stratégie anti-infectieuse), des expériences en culture simple, sur des modèles d'adhésion aux billes de mucine et sur le modèle cellulaire Caco-2/ HT29-MTX ont permis de **(i)** sélectionner 2 produits contenant des fibres parmi 8 candidats pour leur potentiel anti-infectieux et **(ii)** de confirmer l'effet anti-infectieux de ces deux produits sur la production de toxine par la bactérie, son adhésion aux cellules et son induction de la réponse inflammatoire. En raison de la crise sanitaire liée à la pandémie de Covid-19, le séjour d'un an et demi initialement prévu au CMET à Gand, en Belgique, a dû être réduit à une durée de 9 mois. Ce séjour a été maintenu grâce à une prolongation de 6 mois du contrat doctoral sur des fonds FEDER. Les objectifs des travaux effectués en Belgique ont dû être revus et simplifiés : les expériences sur le modèle M-SHIME n'ont pu être réalisées et ont été remplacées par des expériences de fermentation fécale en batch, moins chronophages et aux capacités de screening étendues. L'objectif était d'évaluer l'effet du mucus et des produits contenant des fibres sur la virulence et la survie des ETEC en présence d'un microbiote complexe. Au-delà de ce partenariat académique, ce travail de thèse a fait l'objet d'un partenariat avec différents industriels de la région Auvergne-Rhône-Alpes. Parmi ces groupes industriels, Lallemand SAS est un leader mondial dans le développement, la production et la mise en marché de levures, bactéries et ingrédients dérivés. Limagrain est un groupe coopératif agricole français spécialisé dans les semences de grandes cultures, les semences potagères et les produits céréaliers. PiLeJe fabrique et distribue des solutions de micronutrition, phytonutrition et des souches microbiotiques. HARi&CO est une start-up

lyonnaise qui a pour mission valoriser les légumineuses dans l'alimentation humaine. Ces sociétés ont notamment fourni la majeure partie des produits contenant des fibres testées pour leur propriétés anti-infectieuses. Le projet de thèse a été entièrement financé par un pack Ambition Recherche de la région Auvergne-Rhône-Alpes/FEDER appelé DYSFIBRE et coordonné par l'UMR MEDIS.

Descriptif du contenu du manuscrit de thèse

Le manuscrit de thèse comprend tout d'abord une revue de la littérature (section I) répartie en 5 grands chapitres. Le chapitre 1 est dédié à la présentation de la physiologie digestive humaine et des principaux paramètres digestifs abiotiques et microbiens. Le chapitre 2 aborde les interactions entre fibres et mucus mais aussi leur relation étroite avec le microbiote intestinal. Le chapitre 3 présente les interactions des pathogènes intestinaux avec le mucus intestinal puis introduit comment les fibres alimentaires pourraient limiter ces infections, notamment en inhibant les interactions pathogènes-mucus. Le chapitre 4 présente le pathogène sujet de cette thèse, l'ETEC et notamment la souche de référence H10407, isolée chez un adulte au Bangladesh en 1977. Une attention particulière a été portée sur les interactions du pathogène avec le mucus intestinal, les fibres, le microbiote intestinal et l'immunité innée. Enfin, le chapitre 5 fait la liaison avec le travail expérimental en décrivant les modèles *in vitro* qui pourraient être utilisés pour élucider les interactions entre ETEC, fibres et mucus. La partie expérimentale de ce travail de thèse (section II) s'articule autour de trois grands chapitres. Le chapitre 1 s'intéresse au rôle du mucus dans les infections à ETEC grâce à diverses approches *in vitro* complémentaires. Le chapitre 2 se focalise sur le programme de screening conduit afin de sélectionner 2 produits contenant des fibres (parmi 8 testés) pour leurs propriétés anti-infectieuses contre la souche ETEC H10407. Enfin, le chapitre 3 s'attache à démontrer plus amplement les propriétés anti-infectieuses des deux produits sélectionnés en utilisant un large panel de modèles *in vitro*. Dans la section III, les principaux résultats expérimentaux, les méthodes employées pour les obtenir et leurs perspectives sont discutés.

Principaux résultats et éléments de discussion des chapitres expérimentaux

Les principaux résultats expérimentaux, les méthodes employées pour les obtenir et éléments de discussion décrits dans les différents chapitres sont scindés en deux principales parties pour répondre aux deux objectifs de la thèse mentionnés ci-dessus.

Axe 1 : Impact du mucus intestinal sur la physiopathologie des ETEC

1.1 Spécificité d'adhésion de la souche ETEC H10407 pour le mucus

Différents modèles ont permis de confirmer l'affinité de la souche H10407 pour le compartiment mucus. L'affinité de la bactérie pour la mucine/agar est plus élevée que celle du contrôle (agar seul) avec 60% d'adhésion contre 10%. Ce modèle très simple ne prend en compte l'impact des paramètres physicochimiques de l'environnement digestif sur l'adhérence d'une bactérie. Afin de pallier cette limite, des tests d'adhésion complémentaires ont été réalisés après un processus simple de digestion gastro-intestinale, en présence de billes d'alginate contenant ou non de la mucine. Ces billes étaient constamment agitées pour éviter toute sédimentation. Même dans ces conditions (pH gastrique acide, présence de sels de biliaires, agitation), la souche ETEC H10407 présente un tropisme pour les billes avec mucine. Pour intégrer d'avantage les interactions avec l'hôte, des expériences d'adhésion sur cellules intestinales humaines ont été réalisées. La souche ETEC H10407 adhère davantage (environ 1 log de différence) à la co-culture Caco-2/HT29-MTX (ratio70:30) qu'à la monoculture de cellules Caco-2. La lignée Caco-2 se différenciant en entérocytes et la lignée HT29-MTX en cellules caliciformes sécrétrices de mucus, nous montrons ici que le pathogène possède un tropisme pour le mucus ou, du moins, pour des récepteurs présents sur les cellules caliciformes. Il est impossible de conclure à ce stade si le mucus secrété par les cellules HT29-MTX est seul responsable d'une plus grande adhésion des ETEC. Des expériences d'adhésion complémentaires comparant cellules HT29-MTX et HT29 (non sécrétrices de mucus) et impliquant de l'imagerie électronique pourraient apporter des précisions supplémentaires.

1.2 Rôle du mucus dans la survie de la souche H10407 dans la partie haute du tractus digestif

L'utilisation du modèle TIM-1, un simulateur de la partie haute du tractus digestif, a permis d'étudier l'impact du mucus sur la survie de la souche ETEC H10407 de son ingestion jusqu'à l'intestin grêle distal, site présumé d'action de la bactérie. Le TIM-1 consiste en quatre compartiments successifs simulant l'estomac humain et les trois parties de l'intestin grêle (duodénum, jéjunum et iléon), reproduisant les principaux paramètres physico-chimiques de la digestion humaine (température, pH, enzymes, péristaltisme, absorption). Pour simuler la présence du compartiment muqueux, une sécrétion de mucine a été ajoutée pour apporter de la mucine dans le duodénum à une concentration finale de 3 $g.L^{-1}$. Deux poches en polyester contenant 40 billes de billes de mucine-alginate ont également été placées dans chaque compartiment digestif pour fournir une matrice d'adhésion. Dans l'estomac et le duodénum, le compartiment mucus n'impacte que modérément la perte de viabilité des ETEC associée aux conditions drastiques de ces compartiments (acidité gastrique, sels biliaires), avec une augmentation de survie dans le duodénum à 30 minutes. A la fin de la digestion, le pourcentage de bactéries associé aux billes est particulièrement élevé dans ces deux compartiments (90% dans l'estomac et 60% dans le duodénum). Ainsi, le compartiment mucus pourrait constituer une niche pour permettre au pathogène de se maintenir dans les conditions digestives les plus néfastes. Dans le jéjunum et l'iléon, la souche ETEC H10407 reprend sa croissance de façon exponentielle, ce qui reflète une amélioration des conditions physicochimiques, mais également

l'utilisation potentielle de la mucine comme source de carbone et d'énergie. Confortant ces résultats, des tests de croissance en milieu minimal (M9) ont montré que la souche ETEC H10407 est capable de se diviser en présence de mucine. Des expériences complémentaires dans un modèle *in vitro* comme le SHIME permettraient de savoir si la présence d'un microbiote complexe influence les résultats obtenus dans le TIM-1 qui lui en est dépourvu.

1.3 Rôle du mucus dans l'expression des gènes de virulence de la souche H10407

Afin de mener à bien son cycle infectieux, le pathogène doit pouvoir réguler ses facteurs de virulence en fonction des différentes niches qu'il rencontre dans le tractus intestinal. Nos propres travaux dans le système TIM-1 ont montré que l'adhésion aux billes de mucines n'est pas associée à des changements profonds dans le profil d'expression des gènes bactériens (comparativement à la lumière digestive), que ce soit dans l'estomac ou l'iléon. Ceci est peut-être dû à la présence de mucine à la fois dans la lumière (sécrétion à 3 $g.L^{-1}$) et dans les billes. Une autre explication est simplement que la présence de mucine a moins d'impact sur l'expression des gènes de virulence que les variations physicochimiques observées le long du tractus digestif. Globalement, les gènes sont réprimés dans l'iléon, à l'exception des deux gènes codant pour les mucinases *YghJ* et *eatA*. Cette dernière observation pourrait indiquer une activation séquentielle des gènes de virulence dans la partie distale de l'intestin grêle. Une autre hypothèse est que l'activation des autres gènes nécessite la proximité ces cellules intestinales, un paramètre de la physiologie digestive non reproduit dans le TIM-1. Ainsi, des expériences de suivi de l'expression des gènes d'ETEC sur modèles cellulaires ont également été conduites sur modèles Caco-2/HT29-MTX et Caco-2. Quel que soit le modèle, les gènes de virulence étaient globalement activés par l'adhésion aux cellules, confirmant que la partie hôte pourrait être le paramètre décisif permettant à la souche H10407 l'induction de ses gènes de virulence. De plus, l'activation des gènes de virulence est plus forte avec le modèle de co-culture, suggérant que le mucus ou des composants présentés par les cellules caliciformes, augmentent la virulence de la bactérie. Ces résultats vont dans le sens de la seule autre étude qui a suivi la modulation des gènes de virulence d'une ETEC suite à l'adhésion à un modèle cellulaire, où le gène *eltB* de la souche H10407 était augmenté par l'adhérence aux cellules Caco-2. En revanche, l'utilisation d'une autre souche (E24377A) avait montré des effets opposés avec une inhibition des gènes de virulence. Ces données suggèrent de réitérer les expériences de culture cellulaire avec d'autres souches d'ETEC.

1.4 Impact des cellules sécrétrices de mucus sur l'induction de l'inflammation

Comme évoqué dans le contexte bibliographique de cette thèse, les infections à ETEC sont accompagnées d'une élévation des marqueurs inflammatoires intestinaux et sérologiques. Le mucus est présenté comme une barrière aux pathogènes, empêchant l'accès à l'épithélium sous-jacent et protégeant l'hôte de leurs agressions. Pour autant, nous avons montré en suivant la production d'interleukine-8 (IL-8), que la bactérie était capable d'induire une réponse inflammatoire dans les modèles de co-culture HT29-MTX/Caco-2 et de monoculture Caco-2. Il semblerait donc que le mucus secrété par le modèle Caco-2/HT29-MTX ne soit pas suffisant

pour inhiber l'induction de l'inflammation par la souche H10407. Toutefois, les niveaux basaux (non infectés) de production d'IL-8 sont plus élevés dans le modèle de co-culture (170 fois plus d'IL-8 intracellulaire), probablement dû aux deux lignées cellulaires réagissant à leur présence mutuelle. Ces derniers résultats limitent nos conclusions quant à l'impact du mucus sur l'induction de la réponse immunitaire innée. Afin de répondre à cette limite, nous préconisons de réitérer les expériences en comparant le modèle de monoculture HT29-MTX secrétant du mucus au modèle HT29 n'en sécrétant pas. Supportant cette perspective, la littérature rapporte que les cellules HT29-MTX en monoculture secrètent une couche de mucus uniforme, plus proche de la physiologie intestinale, que celle obtenue avec le mélange Caco-2/HT29-MTX.

1.5 Effet du mucus sur la croissance de la souche H10407 en présence du microbiote fécal

Le site d'action des ETEC est généralement considéré comme étant la partie distale de l'intestin grêle. Cependant, l'excrétion fécale des ETEC après l'infection est particulièrement importante et pourrait refléter la stratégie de contamination l'environnement par la bactérie. Il est donc apparu pertinent d'étudier les interactions de la bactérie avec le microbiote résident humain dans des expériences de fermentation du microbiote fécal en batch. Certains auteurs ont déjà évalué la survie des ETEC dans des expériences de fermentation avec des souches infectant l'homme, que ce soit en batch ou dans le modèle continu M-SHIME. Ces travaux ont été réalisés avec des microcosmes recouverts de mucines pour simuler le compartiment mucus. Toutefois, aucune de ces études ne traite spécifiquement de l'impact du compartiment mucus sur la survie des ETEC en le comparant à une condition de contrôle appropriée. Nos travaux montrent que comparativement à des billes contrôles d'alginate, l'utilisation de billes de mucine-alginate limite la colonisation par les ETEC. Nous proposons qu'une colonisation des billes de mucines par un microbiote spécifique (discuté dans les paragraphes suivants) protège de l'établissement excessif des ETEC. Toutefois, nos observations restent à confirmer car le manque de renouvellement du milieu du système batch limite la pertinence du modèle. Des systèmes de fermentation continue plus complexes auraient permis d'étudier le rôle du mucus comme niche écologique permettant le maintien du pathogène dans l'intestin.

1.6 Effet du compartiment mucus sur la modulation de la composition microbienne par les ETEC

Des études précédentes supportent une modulation de la composition et de l'activité du microbiote intestinal humain par les ETEC. Comme les altérations du microbiote peuvent favoriser les infections de différentes manières, il est crucial de caractériser ces modifications. A notre connaissance, ce travail est le premier à étudier spécifiquement l'impact du compartiment mucus sur la modulation du microbiote humain par les ETEC. Comme déjà rapporté par d'autres études, ces travaux de thèse mettent en évidence une colonisation par un microbiote spécifique des billes de mucines, notamment enrichi en *Clostridium* et en *Lactobacillaceae*. Ces groupes, qui pourraient avoir un rôle dans la santé humaine, sont particulièrement impactées par l'infection à ETEC. Les lactobacilles ont notamment démontré

des effets anti-infectieux contre les ETEC. Ce travail met donc un peu plus en exergue l'importance d'intégrer et de considérer le microbiote mucosal dans l'étude des infections à ETEC que soit *in vitro* ou *in* vi*vo*. Ces résultats demandent néanmoins à être confirmés dans des systèmes plus proches de la physiologie humaine.

1.7 Effet du compartiment mucus sur la modulation de l'activité microbienne par les ETEC

Dans ce travail, nous avons montré que l'utilisation de billes de mucine, un substrat nutritif riche, entraîne une production accrue de produits de fermentation (gaz et AGCC). La modulation des métabolites microbiens par les ETEC est moins évidente à expliquer, puisqu'en effet l'inoculation de l'agent pathogène tend à augmenter la plupart des paramètres de fermentation étudiés (gaz et AGCC tels que le butyrate), mais semble limiter la diminution du pH. Une hypothèse envisageable pour expliquer cette dernière observation pourrait provenir des systèmes de résistance à l'acidité d'*E. coli*. Dans des conditions anaérobies, les systèmes de résistance à l'acidité d'*E. coli* consomment notamment des protons H^+ et produisent de l'eau (H_2O), du dihydrogène (H_2) et du dioxyde de carbone (CO_2). Il semblerait également que l'inoculation de l'ETEC augmente la dégradation du compartiment mucus représentés par les billes de mucines. Cela pourrait être dû aux mucinases du pathogène. D'autres travaux sur les modulations de l'activité microbienne par les ETEC, à la fois *in vitro* et *in vivo*, sont nécessaires.

Axe2 : Propriétés anti-infectieuses de produits contenant des fibres sur la physiopathologie des ETEC

2.1 Screening de produits contenant des fibres pour leurs propriétés anti-infectieuses vis-à-vis de la souche H10407

Afin d'évaluer les propriétés antagonistes de produits contenant des fibres vis-à-vis des ETEC, les partenaires industriels associés au projet ont fourni divers produits contenant des fibres. Parmi eux, des extraits d'amidon de blé, d'avoine, de son d'avoine, d'haricots rouges, de lentilles et de parois cellulaires de la levure *Saccharomyces cerevisiae*. Certains de ces extraits ont été fournis directement par les partenaires, tandis que d'autres ont dû être extraits au laboratoire ou achetés auprès de distributeurs (gommes de guar et de locuste). Ces derniers produits ont été sélectionnés pour contenir des galactomananes ; des polysaccharides ayant déjà montré des propriétés anti-infectieuses contre d'autres pathotypes d'*E. coli* comme les EHEC, notamment des capacités d'inhibition d'adhésion. Ces 8 produits représentaient des sources de fibres d'origines variées et contenant d'après la littérature des fibres à la structure diversifiée (amidon résistant, hémicellulose, cellulose, oligosaccharides, xylanes, mannanes, galactanes). L'ensemble des expérimentations ont été réalisées à la concentration physiologique en fibres de 2 $g.L^{-1}$, une concentration physiologique de l'intestin. Lors de tests de croissance, aucun des produits n'a montré une capacité à réduire la croissance de la souche H10407 en milieu riche (medium LB) alors que tous ces produits permettent la croissance de la bactérie en milieu minimal (M9). Cette utilisation des produits comme substrat par les ETEC ne devrait néanmoins

pas poser de problème *in vivo* lorsque le pathogène sera en compétition avec l'hôte et le microbiote pour les nutriments simples contenus dans les produits. Cette hypothèse pourrait être vérifiée lors de futures expériences dans des systèmes *in vitro* incluant le microbiote ou *in vivo*. Nous avons ensuite montré que l'extrait de lentilles diminuait les niveaux de la toxine LT dans le surnageant de culture de la souche H10407. Enfin, comme l'un des mécanismes majeurs d'inhibition des pathogènes par les fibres passe par l'inhibition de l'adhésion, des tests d'adhésion ont été conduits. Lors de tests d'adhésions sur billes de mucines (intégrant une agitation constante), l'extrait de lentilles et les parois de levures ont montré des propriétés intéressantes en réduisant de plus de 4 fois l'adhésion de la souche ETEC H10407 aux billes. Ces résultats ont été confirmés dans des tests d'adhésion cellulaire avec le modèle Caco-2/HT29-MTX, dans lesquels d'autres produits tels que la gomme de guar et l'extrait d'amidon résistant de blé ont aussi montré des propriétés antiadhésives intéressantes. Ce travail est pionnier dans la mise en évidence des propriétés anti-infectieuse des produits contenant des fibres contre un souche d'ETEC infectant l'homme et dans l'intégration de la composante mucus.

2.2 Inhibition de la détection de la toxine LT par l'extrait de lentilles

Le programme de screening ci-dessus a mis en évidence un effet inhibiteur dose-dépendant de l'extrait de lentille à 2 $g.L^{-1}$ de fibres sur la détection de la toxine LT dans le surnageant de culture de la souche H10407. Ces tests ont été conduits dans le milieu CAYE (Casamino Acids-Yeast Extract) connu pour induire la production de toxines des ETEC. Afin de déterminer si l'effet passait par une modulation de la virulence de la bactérie ou était dû à un effet direct sur la détection de la toxine, des tests ont été menés sur la toxine purifiée. Les résultats ont confirmé que l'extrait de lentilles impactait directement la détection de la toxine. Cette observation est donc au moins partiellement responsable de la diminution de détection de la toxine observée dans le surnageant de culture bactérienne. Des tests complémentaires sur cellules T-84 ont été conduits. En effet, ces cellules expriment le récepteur à la toxine LT et induisent en cascade la synthèse d'AMP cyclique (AMPc) intracellulaire. De façon surprenante, ces tests n'ont pas mis en évidence d'augmentation significative des taux d'AMPc intracellulaire ni par le surnageant de la souche H10407, ni par la toxine cholérique (proche parent de la LT), ni par la bactérie elle-même. Ces données ont empêché toute conclusion quant au mécanisme d'action de l'extrait de lentille sur la toxine LT.

2.3 Inhibition d'adhésion de la souche H10407 par les parois de *Saccharomyces cerevisiae*

De nouvelles expériences ont confirmé que les parois de levures limitaient l'adhésion des ETEC sur cellules Caco-2/HT29-MTX. Afin de confirmer que cet effet pouvait être dû à une affinité de la bactérie pour des composants des parois agissant tels des leurres, des expériences d'adhésion supplémentaires ont été entreprises. En particulier, il a été montré que la souche H10407 a une affinité plus importante (environ 1 log de différence) pour les billes d'alginate contenant des parois de levures à $2g.L^{-1}$ que pour les billes contenant de la mucine à

la même concentration. Ce résultat confirme ainsi l'affinité forte de la souche ETEC pour le produit testé. Il a été déjà été montré dans la littérature que certaines fibres, telles que des oligosaccharides du lait humain ou les fibres de plantain, inhibaient l'adhésion de souches humaines d'ETEC aux cellules Caco-2. Dans cette étude, cette notion est étendue à des fibres provenant de microorganismes. Des études précédentes conduites au laboratoire ont montré qu'une souche probiotique de levure avait la capacité de réduire l'adhésion de la souche H10407 aux cellules Caco-2, et que des résidus mannose étaient impliqués. En opposition avec ces résultats, nous montrons ici que l'ajout dans le milieu de culture cellulaire de mannose à 10 $g.L^{-1}$ n'a pas réduit significativement l'adhésion de la souche H10407 sur les billes de levures. Il semblerait que d'autres motifs moléculaires soient probablement impliqués.

2.4 Modulation de l'expression des gènes de virulence de la souche H10407

Nous avons aussi étudié les propriétés des produits sur l'expression des gènes de virulence du pathogène. Ces expériences conduites sur les cellules Caco-2/HT29-MTX ont montré que les gènes de virulence de la souche H10407 sont modulés par les produits contenant des fibres, que la bactérie soit sous forme planctonique ou adhérée. Sous forme planctonique, les parois de levures augmentent l'expression des gènes tandis que les modulations engendrées par l'extrait de lentilles dépendent du gène considéré. Globalement, les gènes impliqués dans la sécrétion de toxines sont inhibés, tandis que les gènes impliqués dans la dégradation du mucus et l'adhésion sont activés. Concernant les bactéries adhérées, pour lesquelles les variations sont davantage susceptibles d'impacter la physiopathologie, les effets des deux produits sont concordants. Il est observé une inhibition des gènes impliqués dans la sécrétion de toxines, tandis que les gènes codant des mucinases ou adhésines sont surexprimés. Ce travail est le premier à faire la démonstration d'une modulation des gènes de virulence d'une souche d'ETEC par des produits comprenant des fibres.

2.5 Inhibition de l'activation de l'immunité innée

Sur le modèle cellulaire Caco-2/HT29-MTX, l'effet des produits contenant des fibres sur l'activation de gènes impliqués dans la réponse immunitaire innée a été testé. Globalement, la souche H10407 active l'expression de tous les gènes codant pour ces cytokines, indépendamment de leur statut pro- ou anti-inflammatoire. De manière intéressante, les produits contenant des fibres ont un effet immuno-modulateur. Les lentilles, en particulier, réduisent l'activation de l'IL-1β, l'IL-6, IL-8 de l'ordre de 50%. De même concernant les gènes impliqués dans la synthèse de mucus, l'expression de ces derniers est significativement augmentée en présence de la souche ETEC, tandis que l'extrait de lentilles limite cette augmentation et l'effet des parois de levures est plus modeste. Le niveau d'expression des protéines de jonctions serrées assurant notamment la perméabilité de l'épithélium a également été étudié. Le niveau d'expression de ces protéines s'est révélé peu affecté par la souche H10407, seul l'expression du gène codant pour la claudine-1 est induit. Nous avons pu montrer que les parois de levures sont capables de réduire l'expression du gène codant la claudine-1. En conclusion, l'ETEC induit une activation globale de l'expression des gènes de l'immunité innée, avec une limitation

de cette activation par les lentilles et un effet plus modeste des parois de levures. Au niveau protéique, nous avons montré que l'ETEC induit la production d'IL-8 intracellulaire. Les 2 produits contenant des fibres ont montré leur capacité à réduire la production induite par l'ETEC, cette induction étant diminuée de 50% par les parois de levures et totalement inhibée par les lentilles. Plusieurs mécanismes peuvent expliquer ces résultats. Il semble probable que l'effet bénéfique soit issu d'une combinaison d'effets directs sur la bactérie (diminution de l'adhésion, modulation des gènes de virulence) et immunomodulateurs (effet basal des produits sur l'immunité sans même que la bactérie soit impliquée). A notre connaissance, ce travail est le premier à mesurer l'effet bénéfique de produits contenant des fibres sur l'induction de l'immunité innée par un pathogène avec une telle ampleur, notamment en intégrant des gènes de la réponse effectrice à l'étude.

2.6 Effet des produits sur la perméabilité cellulaire

Face à l'effet mitigé des produits à base de fibres sur l'expression des protéines des jonctions serrées, des expériences complémentaires ont été conduites. L'addition des produits du côté apical de cellules Caco-2/HT29-MTX cultivées en inserts augmente la résistance trans-épithéliale (30% d'augmentation de résistance au bout de 3 heures). De plus, les parois de levures diminuent de 20% la perméabilité trans-cellulaire évaluée par le suivi de l'absorption de la caféine. La perméabilité para-cellulaire, évaluée par le suivi de l'absorption de l'aténolol, est diminuée de 70% en présence des 2 produits. Ces résultats montrent que les produits pourraient même participer au renforcement de la barrière intestinale contre les ETEC. À court terme, des expériences cellulaires complémentaires permettant de s'affranchir du phénomène de sédimentation des produits sont nécessaires.

2.7 Effet des produits sur la croissance de la souche H10407 en présence du microbiote

En étant dégradées en fragments osidiques par le microbiote intestinal endogène, les fibres alimentaires peuvent fournir un substrat aux agents pathogènes se comportant en dégradeurs secondaires. Ainsi dans nos expériences de batch en présence du microbiote fécal, nous n'avons pas mis en évidence d'augmentation de la croissance des ETEC en présence des produits contenant des fibres alimentaires. Au contraire, les parois de levure tendent à diminuer l'expansion des ETEC que ce soit en présence du microbiote luminal ou du microbiote mucosal. Plusieurs études indépendantes ont montré que les fibres alimentaires peuvent limiter la croissance des agents pathogènes en présence du microbiote. Les fibres peuvent notamment exercer un rôle de prébiotique sur des espèces commensales probiotiques. Dans notre expérience, les produits contenant des fibres ont permis l'expansion de phylogroupes comme les *Escherichia/Shigella* dans les conditions non-infectées. Ce groupe pourrait jouer un rôle dans le contrôle de l'infection en occupant les mêmes niches écologiques que les ETEC, comme cela a déjà été observé avec d'autres agents pathogènes. Dans l'objectif de mettre en évidence de plus amples bénéfices, à terme, l'utilisation de modèles intégrant à la fois la partie hôte et fermentation microbienne devrait être envisagée.

2.8 Effet du compartiment mucus sur la modulation par les fibres de la composition microbienne par les ETEC

A notre connaissance, ce travail est le premier à étudier le potentiel de produits contenant des fibres pour moduler les effets d'une infection à ETEC sur le microbiote humain. Nous rapportons que les produits utilisés tendent à prévenir de la disparition partielle des *Clostridia* consécutive à l'administration des ETEC. L'effet des parois de levures sur la composition du microbiote est significatif, en induisant certaines modifications de composition et ce de façon indépendante de l'infection. Le produit augmente notamment l'abondance des *Parabacteroides*, un genre bactérien associé notamment à des propriétés anti-inflammatoires dans différents syndromes. Ainsi, si nos résultats montrent un impact mineur des produits à base de fibres sur la composition du microbiote, cet effet ne semble pas néfaste et pourrait même avoir son utilité dans une stratégie anti-infectieuse. Afin de vérifier cette observation, il pourrait être envisagé de déposer des échantillons fermentaires sur des modèles cellulaires complexes intégrant les échanges avec le microbiote, comme des intestins sur puce ou « Gut-chips ».

2.9 Modulation de l'activité microbienne

L'ajout des produits contenant des fibres ne semble pas être en mesure de limiter l'impact des ETEC sur l'activité du microbiote. Cette addition favorise même certains paramètres de fermentation associés comme la production de gaz qui pourrait être dû à une augmentation de l'activité fermentaire du microbiote et à une plus grande activité des systèmes de résistance à l'acidité des ETEC. Pour répondre à la question, des expériences de transcriptomique ou des knock-out de gènes de résistance à l'acidité des ETEC pourraient être envisagés.

Discussion générale

Cette section discute de manière plus globale les chapitres expérimentaux et repositionne les travaux de recherche dans un contexte plus global notamment vis-à-vis de la littérature existante dans ce domaine.

La spécificité des résultats au niveau de la souche, une limite aux deux axes

Force est de constater que les deux axes de ce travail de thèse sont concernés par une limite commune. En effet, une seule souche d'ETEC a été testée. De ce fait, les modulations observées un niveau de la physiopathologie des ETEC que ce soit par le mucus ou les produits contenant des fibres ne peuvent être étendues à l'ensemble du pathotype ETEC. Ce constat est une limite de ce travail, puisque certaines études ont démontré que la réponse transcriptomique des ETEC aux contacts mucosaux est dépendante de la souche. De nouvelles expériences avec d'autres souches humaines seront nécessaires pour appuyer nos observations.

Utilisation des modèles *in vitro* pour décoder l'impact du mucus et des fibres sur la physiopathologie ETEC

Tout d'abord, le choix des modèles *in vitro* plutôt que d'autres approches est une première source de questionnement. Les approches *in vivo* chez l'homme représentent évidemment l'approche idéale pour étudier les interactions entre fibres alimentaires, microbiote intestinal et agents pathogènes entériques. Cependant, plusieurs limites entravent leur utilisation, parmi lesquelles la variabilité interindividuelle, l'accessibilité au site d'action de l'ETEC et les risques liés à l'administration d'un agent pathogène chez l'homme, même si de nombreuses études sur volontaires ont été conduites dans le cas des ETEC. Une alternative aux études cliniques est l'utilisation de modèles animaux. Néanmoins, une attention croissante est accordée à la réduction des études animales du fait d'une pression éthique et sociétale grandissante. Il convient également de faire preuve d'une grande prudence lors de la transposition à l'homme de données obtenues dans des modèles animaux. Concernant les ETEC, il n'existe pas de modèles animaux pertinents permettant de reproduire toute la complexité de la physiopathologie infectieuse de cette bactérie. De nombreuses différences subsistent notamment en ce qui concernent les facteurs de virulence. Chez l'animal, les adhésines et les toxines reconnaissent des récepteurs différents des ETEC infectant l'homme, indiquant que les facteurs de virulence des souches d'ETEC sont spécifiques de l'espèce infectée. Ainsi, les modèles *in vitro* reproduisant l'environnement digestif humain représente une alternative de choix. Ces modèles présentent de nombreux avantages en terme de coût, de temps, de flexibilité (capacité de screening), de reproductibilité (contrôle précis des paramètres) mais aussi en terme d'échantillonnage dans l'espace et le temps. Ces modèles ne sont pas limités par des contraintes éthiques ou des problèmes de sécurité rencontrés notamment lors des études avec des agents pathogènes humains. Néanmoins, ces modèles *in vitro* présentent certaines limites comme l'absence des contrôles nerveux, endocriniens et immunitaires de l'hôte.

Rôle du mucus dans la physiopathologie des ETEC

L'étude des interactions entre le microbiote intestinal et le mucus n'est qu'à ses prémices, de même que la relation entre les agents pathogènes et la couche de mucus. Grâce à l'utilisation de modèles *in vitro* complémentaires de l'environnement digestif humain, l'objectif principal de l'axe 1 était de mieux comprendre les interactions d'ETEC H10407 avec le compartiment du mucus. Une étude conduite il y a une vingtaine d'années en modèle de culture cellulaire avait montré que la souche H10407 était co-localisée au niveau de la bordure en brosse des entérocytes, et non pas au niveau du mucus présent sur les cellules HT29-MTX. D'autres études conduites dans des systèmes *in vitro* simples ont montré que la mucine gastrique de porc favorisait l'expression des facteurs de colonisation de la souche ETEC 258909–3. Enfin, une autre étude a rapporté que l'adhésion de la souche H10407 sur un modèle cellulaire sans mucus (Caco-2) s'accompagnait d'une induction de l'expression de la toxine LT et de facteurs de colonisation. Nos expériences ont confirmé l'affinité d'adhésion des ETEC pour les modèles intégrant le mucus. L'utilisation du TIM-1 a montré pour la première fois que l'adaptation des ETEC infectant l'homme au mucus leur permettait probablement de survivre

plus efficacement aux conditions physico-chimiques rencontrées dans la lumière de l'environnement digestif. Les expériences de culture cellulaire ont montré que l'adhésion aux cellules et particulièrement celles productrices de mucus était un facteur clé dans l'activation de la cascade des gènes de virulence. Enfin, l'impact du compartiment mucus sur la physiopathologie de l'ETEC en présence du microbiote humain a été adressé spécifiquement pour la première fois, montrant que le microbiote colonisant spécifiquement le mucus pourrait être un élément clé dans l'étiologie des infections aux ETEC. Grâce aux nouveau mécanismes rapportés, ce projet pourrait renforcer la conscience de la communauté scientifique de l'importance de considérer le compartiment mucus lors des études menées sur la pathogénicité des ETEC. Il pourrait aussi permettre l'élaboration de nouvelles stratégies de prévention des infections à ETEC, qui cibleraient spécifiquement l'interaction avec le mucus.

Potentiel anti-infectieux de produits contenant des fibres vis-à-vis des ETEC

A ce jour, seules quelques études ont étudié les propriétés anti-infectieuses potentielles de fibres alimentaires vis-à-vis des infections humaines à ETEC. Plusieurs études ont montré que des oligosaccharides du lait humain ou des fibres de plantain pouvaient diminuer l'adhésion des ETEC et de leurs toxines aux cellules intestinales Caco-2. Une étude en modèle murin a rapporté que le chitosan limitait la colonisation intestinale par les ETEC. Le présent travail montre que des fibres alimentaires de diverses origines peuvent exercer des effets antagonistes multiples notamment en limitant l'adhésion, l'induction de la réponse inflammatoire et en modulant l'expression de gènes de virulence du pathogène. De plus, ce travail suggère pour la première fois que les produits contenant des fibres pourraient avoir un effet bénéfique sur les modulations du microbiote intestinal associées à l'infection. Ainsi, ce travail devrait attirer l'attention de la communauté scientifique sur le potentiel anti-infectieux sous exploité des produits contenants des fibres dans la lutte contre les infections à ETEC. Il pourrait aussi déboucher sur un futur développement produit et une confirmation de la résistance de l'effet des produits à digestion humaine, en partie dans la partie distale de l'intestin grêle est un prérequis. L'utilisation de modèles *in vitro* simulant la digestion humaine et intégrant à la fois le microbiote de l'intestin grêle et la partie hôte seront nécessaires. La caractérisation de composés actifs contenus dans les produits devra aussi être envisagée.

Conclusion

En utilisant des approches *in vitro* complémentaires, ce projet doctoral fournit de plus amples informations sur la manière dont le compartiment du mucus peut moduler la physiopathologie des infections à ETEC chez l'homme et a apporté des preuves solides concernant les propriétés anti-infectieuses de fibres alimentaires vis-à-vis de ce pathogène. Ces résultats nécessitent d'être confirmés et approfondis en modèles *in vitro* plus complexe ou *in vivo pour* notamment mieux décrire les mécanismes associés.

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This acknowledgement section will be written in both English and French (sorry for my poor improvements in Dutch, my Flemish friends) depending on the person concerned.

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Figure 0.1. The PhD candidate's family helping him relaxing
Despite the facial expressions, no imminent danger was present.

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Figure 0.2 The PhD candidate visting ghent with its advisors.
Supervision did not stop at the lab.

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Figure 0.3. The PhD candidate could count on the support of many friends.
Support has been shown on many occasions as hiking trips, modest parties or during the "Ma Thèse en 180 Secondes" contest.

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Figure 0.4 The PhD candidate had formidable colleagues.
The PhD colleagues were all very supportive, creating a good dynamic.

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Notation index

A	**AA**	Aggregative Adherence
	AAF	Aggregative Adherence Factor
	A/E	Attaching/Effacing
	AIEC	Adherent Invasive *E. coli*
	AMP	Antimicrobial Peptides
	ARCOL	Artificial CoLon
	ASV	Amplicon Sequence Variant
	AXOS	Arabinoxylanoligosaccharides
B	**BLAST**	Basic Local Alignment Search Tool
	BFP	Bundle Forming Pili
C	**cAMP**	cyclic AMP
	CAYE	Casamino-Acid Yeast Extract
	CAZymes	Carbohydrate-Active enzymes
	CBM	Carbohydrate-Binding Modules
	CCL20	Chemokine (C-C motif) ligand 20
	CD	Crohn's Disease
	CDC	Center for Disease Control
	CE	Carbohydrate Esterases
	CFA/I	Colonization Factor Antigen I
	CFs	Colonization Factors
	CFTR	Cystic Fibrosis Transmembrane Regulator
	CFU	Colony Forming Units
	cGMP	cyclic GMP
	CHERG	Child Health Epidemiology Reference Group
	CLDN	Claudin
	CMET	Center for Microbial Ecosystem and Technology
	CNCM	National Collection of Microorganism Cultures
	CoMiniGut	Copenhagen MiniGut
	CRP	c-AMP Receptor Protein
	CS	Coli Surface antigen
	CT	Cholera Toxin
	CXCL8	Interleukine-8
D	**DAEC**	Diffusely Adherent E. coli
	DAF	Decay-Accelerating Factor
	db-RDA	Distance-based redundancy analysis
	DC	Dendritic Cells
	DEC	Diarrheagenic *E. coli*
	DGM	Dynamic Gastric Model
	DLP	Dynamin-Like Protein
	DMEM	Dulbecco's Modified Eagle's medium
	DNA	Deoxyribonucleic Acid
	DsbA	Disulfide Bond protein A
E	**EAEC**	Enteroaggregative *E. coli*
	EAST1	EnteroAggregative heat-Stable enteroToxin 1
	EFSA	Food Safety Authority
	EHEC	Enterohemorrhagic *E. coli*
	EIEC	Enteroinvasive *E. coli*
	ELISA	Enzyme-Linked Immunosorbent Assay
	EPEC	Enteropathogenic *E. coli*
	ER	Endoplasmic Reticulum
	ESIN	Engineered Stomach and Small Intestine
	ETEC	Enterotoxigenic *E. coli*
	ETpA	ETEC two partner protein A
	ExPEC	Extraintestinal pathogenic *E. coli*
F	**FEDER**	Funding for Regional Developments
	FBS	Fœtal Bovine Serum
	(Flow-)FISH	Fluorescent In-situ Hybridization
	FITC	Fluorescein Isothiocyanate
	FNR	Fumarate and Nitrate Reduction
	FOS	fructooligosaccharides
G	**GAPDH**	Glyceraldehyde 3-Phosphate Dehydrogenase
	Gb3	Globotriaosylceramide
	GBD	Global Burden of Disease
	GDS	Gastric Digestion Simulator
	GC-C	Guanylyl Cyclase
	GH	Glycoside Hydrolases
	GIT	Gastro-Intestinal Tract
	GM	Monosialoganglioside
	GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
	GOS	Galactooligosaccharides
H	**HCl**	Hydrochloridric acid
	HCM	Human Colonic Model
	HCO_3^-	Bicarbonate
	HGS	Human Gastric Simulator
	HMI	Host Microbiota Interaction
	HMO	Human-Milk Oligosaccharides
	H-NS	Heat-stable Nucleoid Structural protein
	HuMix	Human Microbial Cross-talk
	HUS	Hemolytic Uremic Syndrome
I	**IBS**	Irritable Bowel Syndrome
	IBD	Inflammatory Bowel Diseases
	IECs	Intestinal Epithelial Cells
	IESC	Intestinal Epithelial Stem Cells
	Ig	Immunoglobulin
	IL	Interleukin
	IM	Inner Membrane
	INAF	Nutrition and Functional Foods Institute
	iViDiS	*In Vitro* Digestive System
	IVOC	*In Vitro* Organ Culture
K	**KLF4**	Kruppel-Like Factor 4
L	**LB**	Luria Bertani
	Lcn-2	Lipocalin-2
	LeoA	Labile enterotoxin output
	LPS	Lipopolysaccharide
	LPF	Long Polar Fimbriae
	LT	Heat-labile toxin
M	**MAMPs**	Microbe-Associated Molecular Patterns
	MAPK	Mitogen Activated Protein Kinase
	Mbp	Million base pairs
	M cells	Microfold cells
	MEDIS	Microbiology Digestive Environment and Health
	MOI	Multiplicity Of Infection
	MPO	Myeloperoxidase
	M-SHIME	Mucosal-Simulator of the Human Intestinal Microbial Ecosystem
	MUC	Mucin
	M2iSH	Microbes, Intestin, Inflammation et Susceptibilité de l'Hôte (UMR)
N	**NaCl**	Sodium Chloride
	NCBI	National Center for Biotechnology Information

	NF-κB	Nuclear Factor-kappa B
	NHE	Na^+ /H^+-exchanger
	NMDS	Non-Metric Multidimensional Scaling
O	**OCLN**	Occludin
	OM	Outer Membrane
	OMVs	Outer Membrane Vesicles
	OTU	Operational Taxonomic Units
P	**PAMPs**	Pathogen-Associated Molecular Patterns
	PBS	Phosphate Buffered Saline
	PCoA	Principle Coordinate Analysis
	(q)PCR	(quantitative) Polymerase Chain Reaction
	PDE3	Phosphodiesterase 3
	PERMANOVA	Permutational Multivariate Analysis Of Variance
	PG	Peptidoglycan
	PhD	Doctor of Philosophy
	PKA	Phosphokinase A
	PKGII	cGMP-dependent protein kinase II
	PPR	Pattern Recognition Receptors
	PolyfermS	Polyfermentor Intestinal Model
	POS	Pectin-oligosaccharides
	PL	Polysaccharide Lyases
R	**Reg-1b**	Lithostathine-1-beta
	RNA	Ribonucleic Acid
	rpm	Rotation per minute
	RS	Resistant Starch
	RT	Reverse Transcription
S	**SAS**	Société par Actions Simplifiée
	SCFA	Short Chain Fatty Acids
	SD	Standard Deviation
	Sec	Secretory machinery
	SEM	Standard Error of Means
	SGF	Simulated Gastric Fluid
	SIMGI	Multicompartmental Dynamic Model of the Gastrointestinal System
	SPATE	Serine Protease Autotransporters of the Enterobacteriaceae
	SsLE	Secreted and Surface associated Lipoprotein
	ST	Heat-stable toxin
	STEAC	Shiga-Toxin producing EnteroAggregative *E. coli*
	Stx	Shiga-toxin
	Sus	Starch-Utilization System
T	**TEER**	TransEpithelial Electrical Resistance
	TD	Travelers' diarrhea
	Tia	Enterotoxigenic invasion locus A
	TibA	Enterotoxigenic invasion locus B
	TFF3	Trefoil factor 3
	TIM-1	TNO gastrointestinal model 1
	TIM-2	TNO gastrointestinal model 2
	TIM-agc	TIM-advanced gastric compartment
	Tiny-TIM	Tiny-TIM model
	TJP	Tight Junction Protein
	TLR	Toll-Like Receptor
	TNF	Tumor Necrosis Factor
	TolC	TolC efflux protein
	TSI	The Smallest Intestine
	TTP	Thrombotic Thrombocytopenic Purpura
	T1SS	Type 1 Secretion System
	T2SS	Type 2 Secretion System
	T3SS	Type 3 Secretion System
U	**UCA**	Université Clermont Auvergne
	UPEC	Uropathogenic E. coli
	UMR	Unité Mixte de Recherche
	USDA	US Department of Agriculture
W	**WHO**	World Health Organization
X	**XOS**	Xylooligosaccharides
Z	**ZO**	Zonula occuldens

Section I - Literature review

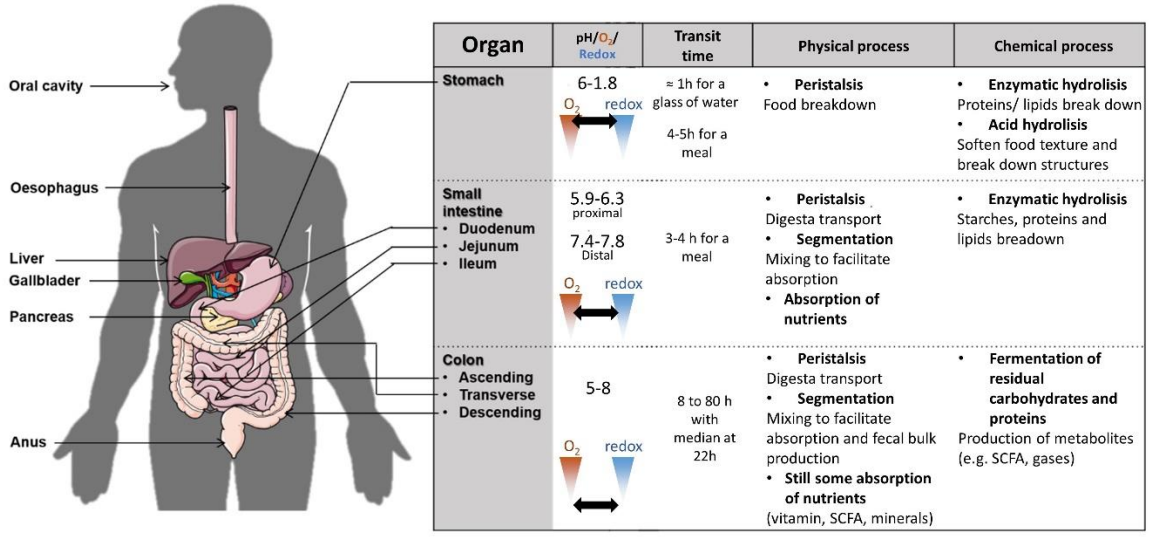
Table of contents

1. Gastrointestinal physiology and microbiota

The following sections are dedicated to the definition of the human gut physiology. Focus will be successively brought on gastric and intestine parts of the gastrointestinal tract (GIT), introducing their abiotic and microbial factors and epithelium characteristics. The mucus compartment residing in these gut compartments is willingly omitted in this first part, to be developed more extensively in the second part of this thesis introduction (Section 2) alongside with another polysaccharide containing compartment present in the gut, namely, dietary fiber.

1.1. Abiotic physiology of the human gut

The human digestion is a multi-step process including mechanical and chemical breakdown by which foods are converted into organic nutrients that can be absorbed and assimilated by the body. Thus, the digestive tract is formed by a group of hollow organs from the mouth to anus and ancillary solid organs (e.g. pancreas, liver and gallbladder) (**Fig. 1.1**). These organs are involved in the ingestion, digestion and elimination of foods (https://www.iffgd.org/, consulted on 03/2021). The abiotic factors of the GI digestion of a healthy adult, starting from the stomach to the large intestine (colon) will be detailed in the following section.



Organ	pH/O_2/ Redox	Transit time	Physical process	Chemical process
Stomach	6-1.8 O_2 redox	≈ 1h for a glass of water 4-5h for a meal	• **Peristalsis** Food breakdown	• **Enzymatic hydrolisis** Proteins/ lipids break down • **Acid hydrolisis** Soften food texture and break down structures
Small intestine • **Duodenum** • **Jejunum** • **Ileum**	5.9-6.3 proximal 7.4-7.8 Distal O_2 redox	3-4 h for a meal	• **Peristalsis** Digesta transport • **Segmentation** Mixing to facilitate absorption • **Absorption of nutrients**	• **Enzymatic hydrolisis** Starches, proteins and lipids breadown
Colon • **Ascending** • **Transverse** • **Descending**	5-8 O_2 redox	8 to 80 h with median at 22h	• **Peristalsis** Digesta transport • **Segmentation** Mixing to facilitate absorption and fecal bulk production • **Still some absorption of nutrients** (vitamin, SCFA, minerals)	• **Fermentation of residual carbohydrates and proteins** Production of metabolites (e.g. SCFA, gases)

Figure 1.1. Gastrointestinal tract of healthy adults: summary of the key physical and chemical processes.
SCFA: Short Chain Fatty Acids.
Modified from Bornhorst and Singh, 2014.

1.1.1 Oral phase

Often neglected, the first stage of digestion is the oral phase. In the mouth, chewing mixes the food with saliva and the mechanical process of digestion begins. Saliva contains digestive enzymes called amylase and lingual lipase, secreted by the salivary and serous glands on the tongue. These enzymes respectfully begin to degrade starch and lipids. These processes produce a bolus, which can be swallowed down through the esophagus to enter the stomach.

1.1.2. Stomach

1.1.2.1. Anatomy and physiological parameters

The stomach is divided in three main anatomical regions: the fundus, the body and the antrum (Boland 2016). Because the upper part of the stomach (fundus) is highly distensible, the stomach can hold about one liter of food (Ferrua, Kong and Singh 2011). Two sphincters keep the contents of the stomach contained; the lower esophageal sphincter namely cardia at the junction of the esophagus and stomach, and the pylorus at the junction of the stomach with the duodenum. The stomach is not depleted in oxygen and is thus considered as an aerobic compartment. As explained below, the gastric pH depends on the digestion phase. The oxydo-reduction (redox) potential, which reflect the affinity of electron transfer to or from a chemical species in solution, is around +200mV (Obrenovich *et al.* 2020). Its temperature is steadily maintained at 37°C as in all subsequent GI segments (Koziolek *et al.* 2015) and will thus be no longer mentioned.

1.1.2.2. Digestive function

Following ingestion of liquids, the average gastric emptying is around 13 minutes as evaluated by magnetic resonance (Mudie *et al.* 2014). On the contrary, after ingestion of a solid meal, the total gastric emptying lasts around 4 to 5 hours, with considerable variations between individuals and according to the composition of the food ingested (Boland 2016; Grimm *et al.* 2018). Solid components remain in the stomach until they are small enough to be slowly released into the small intestine (Ramsay and Carr 2011). Also, it should be noted that this heterogeneity of the gastric emptying is conserved within one meal in which particles larger than 1–2 mm are the last ones to reach the duodenum (Siegel *et al.* 1988). Such aspect is important because the stomach is the last process in the gastrotintestinal tract (GIT) that has a mechanical function to break down particles. The proximal stomach is thought to act as a food

reservoir, while the distal stomach is the main location of the physical breakdown of foods into particles resulting in chime formation. In fact, the peristaltic movements of the gastric wall act to crush and grind food particles (Schulze 2006).

The stomach secretes on a daily basis around 2-3 liters of gastric juice (e.g. mucus, acid, ions, enzymes, and intrinsic factors) (Johnson *et al.* 2012; Sherwood and Ectors 2015), actively participating in the digestion process. A hydrochlorhydric acid (HCl) secretion allows the gastric pH to stay acidic (Boland 2016). However, gastric pH is dependent of many parameters and especially the feeding status. In fasted individual, the gastric pH is considered to stay around 2, since food intake tends to elevate the pH, that in turn has to be counteracted by the HCl secretion (Barlow *et al.* 1994). Such acidic environment inhibits bacterial growth (Beasley *et al.* 2015) and allows the action of digestive proteases (Gore and Levine 2007). Among them, pepsin is an endopeptidase released in its inactive form pepsinogen and then converted into its active proteolytic form upon contact with HCl. Pepsin is then able of hydrolyzing peptide bonds (e.g. phenylalanine, tyrosine and leucine) of most proteins (Fruton 2002). The gastric lipase also takes part in the gastric digestion process and especially in lipids breakdown accounting for 10-30% of dietary triglyceride hydrolysis (Gallier and Singh 2012). As gastric acid pH would damage the stomach wall, mucus is constantly secreted by innumerable gastric glands to provide a slimy protective layer (Boland 2016).

1.1.2.3. Gastric cell lining

Different cell types participate in gastric secretions (Johnson *et al.* 2012; Sherwood and Ectors 2015). The faveolar cells found in the superficial phase of the gastric mucosa and the mucous neck cells in the crypt are the responsible for mucus secretion. Parietal cells secrete HCl (Boland 2016). The chief cells are involved in the enzymatic secretion of pepsinogen and gastric lipase (Gallier and Singh 2012). Finally, deeper in the crypts, G cells secrete hormones such as gastrin, ghrelin, somatostatin or cholecystokinin mediating the overall digestion process (Vasavid *et al.* 2014).

1.1.3 Small intestine and colon

1.1.3.1. Small intestine

1.1.3.1.1. Anatomy and physiological parameters

The human small intestine, separated into three sections (e.g. duodenum, jejunum and ileum), extends from the pyloric sphincter to the ileocaecal junction, representing 6-7 m long, the longest part of the digestive tract. The duodenum, jejunum and ileum segments are respectively around 1 m, 2.5 m and 3.5 m in length. The total transit time in the small intestine is around 3 to 4 hours (Yuen 2010; Boland 2016). Compared to the stomach, which is considered as an aerobic environment, small intestine is depleted in oxygen (30-50 mmHg O_2) (Zheng, Kelly and Colgan 2015). The redox potential becomes negative favoring anaerobic bacterial metabolism. Values are in ranges between −65 and −200 mV (Boland 2016). pH is comprised between 6 and 8 depending on the segment considered, with huge inter-individual variations (Johnson *et al.* 2012; Boland 2016).The proximal small intestine has been shown to be more acidic, with pH values between 5.9 and 6.3, while the distal small intestine appears a bit more alkaline with pH values ranging from 7.4 and 7.8 in fasted individuals (Koziolek *et al.* 2015).

1.1.3.1.2. Digestive function

In the small intestine, food bolus is propelled forward by contraction and relaxation movement, termed peristalsis and occurs thanks to longitudinal smooth muscle contractions. Meanwhile, segmentation movements by circular smooth muscle contraction/relaxation enable chyme mixing and increase intra-luminal contact time which aids in digestion, secretion, and absorption (Kumral and Zfass 2018).

The duodenum secretes bicarbonate to neutralize gastric acid and provides an appropriate pH for further enzymatic digestion to occur. It receives secretions of enzymes and bile from the pancreas and liver, respectively (Vasavid *et al.* 2014). Briefly, in response to food ingestion, the exocrine pancreas secretes a pancreatic juice containing fluids, bicarbonate and diverse enzymes as lipases, proteases and amylases. Some of these enzymes, as trypsin, are secreted in an inactive form and are activated once delivered into the duodenal lumen (Chandra and Liddle 2014). Bile is produced in the liver, stored in the gallbladder and released into the duodenum. It is a complex mixture of bile acids, cholesterol, pigments, lecithin and mineral salts. In the small intestine, bile acids assist in the emulsification and absorption of fatty acids.

They also stimulate lipolysis by facilitating binding of pancreatic lipase with its co-lipase (Chandra and Liddle 2014), which results in the formation of small particles of emulsified fats called micelles. Following action of digestive enzymes and bile acids, hydrolyzed nutrients (mono- and di-saccharides, amino acids, small oligopeptides and micelles) are ready for uptake. All together, the digestion in the luminal phase of the small-bowel is complete for the lipids only, while saccharides and peptides are not all hydrolyzed to single residues. Intestinal epithelial cells will achieve carbohydrates and proteins digestion. The majority of nutrient absorption occurs in the jejunum but is completed in the ileum. The distal ileum is the only site where bile salts are re-absorbed (Johnson *et al.* 2012).

1.1.3.2. Colon

1.1.3.1.1. Anatomy and physiological parameters

The colon, with a significant microbial mass, extends from the ileocecal junction to the anal canal, with a larger diameter, but shorter length (1.5 m) and is divided into four sections (ascending, transverse, descending and sigmoid colon). The colonic transit time showed a median of 21.6 hours (Rao *et al.* 2009), with variations according to dietary intakes and individuals. Hence, it fluctuates between 3 to 5 h in the ascending colon, 0.2 to 4 h in the transverse colon and 5 to 72 h in the descending and sigmoid colon (Wilson 2010). The colon is even more anaerobic environment than the small intestine (<10 mmHg O_2), (Zheng, Kelly and Colgan 2015) and the redox potential becomes further negative (-415 mV in the ascending, -400 mV in the transversal and -380 mV in the descending colon) (Wilson 2010; Boland 2016). These two parameters favor microbial growth and metabolism than in the small intestine (Obrenovich *et al.* 2020). Due to the huge microbial activity, pH should decrease in the colon, but a bicarbonate secretion in the ascending colon counterbalances (Boland 2016). pH electrodes implanted on the colon wall during colonoscopy in areas free of debris indicate that patients with a normal bowel have a more acidic right colon (pH 7.05± 0.32), followed by a more alkaline transverse colon (pH 7.42± 0.51), becoming more acid moving towards the rectum (p 7.15± 0.44). The lumen pH mirrors the changes of the wall, but remains more acidic (average value of 6.5) and with more inter-individual variayion going from 5 to 8 in the ascending colon and 6 to 8 in the transversal colon (McDougall *et al.* 1993; Wilson 2010; Koziolek *et al.* 2015).

1.1.3.1.2. Digestive function

Peristaltic movments occur in the colon to propel forward the excreta to reach the rectum and exit via defecation. Mixing of watery waste also occurs by segmentation (every 30 minutes) and mass movements (1 to 3 times per day) (Rao *et al.* 2009). The colon processes indigestible watery-food waste to produce fecal bulk, which in turn is an important determinant of bowel health (Johnson *et al.* 2012). In the colon, the colonic anaerobic microbes will enable fermentation of food particles partially digested or prebviously undigested. As only lipid digestion is completed in the luminal phase of the small intestine, these particles contain carbohydrates, but also proteins, particularly when such macronutrients are ingested in high quantities (Yao, Muir and Gibson 2016). The fermentation process of these compounds produces gaseous products including dihydrogen (H_2), Carbon dioxide (CO_2), for some individual's methane (CH_4), and short-chain fatty acids (SCFAs). The three most abundant SCFA, acetate, propionate, and butyrate are playing a primary role in host energy metabolism. The fermentation of proteins also results in the production of hydrogen sulfide (H_2S), ammonia, N-nitroso, amines, phenolic and indolic compounds (Yao, Muir and Gibson 2016). SCFA and water are continuously absorbed along the colon.

1.1.3.3. Small intestine and colonic cell lining

1.1.3.3.1. Intestinal epithelium structure and function

The intestinal epithelium is a complex layer representing the largest epithelium of the body's mucosal surfaces, with an average surface of 32 m^2, with approximately 2m^2 refering to the large intestine (Helander and Fändriks 2014). The intestinal epithelium is formed by a single layer of polarized cells organized into crypts and villi in the small intestine but not in the colon. Different cell types compose this layer, with two main functions, digestion/absorption and protection against invaders. The major cell types include enterocytes (termed colonocytes in the colon), goblet cells, Paneth cells and enteroendocrine cells (**Fig. 1.2**). Enterocytes are the only cells with digestive and absorptive functions. The three others cell types are specialized for maintaining the digestive or barrier functions of the epithelium. Intestinal epithelial cells are continuously replaced every 4-5 days, and new cells are produced by stem cells located in crypts. In addition, M cells are found in the ileum and are associated with the immune system (Peterson and Artis 2014).

The functions of each cell types are succinctly described:

(i) Enterocytes (colonocytes in the colon) – They are the most numerous cell type, covering 80% of the villi in the small intestine, where they express on their apical surface hydrolytic enzymes to perform terminal digestion of polysaccharides and peptides (e.g. enterokinase, aminopeptidase), in concertation with enzymes present in the luminal phase. These cells play an important role in nutrient absorption (e.g. ions, water, mono- and disaccharides, amino acids, oligopeptides, lipids, SCFA), and in secreting immunoglobulins.

(ii) Goblet cells – Cover around 10% of all intestinal epithelial cells (25% in the colon). They are specialized in the synthesis and continuous secretion of mucus (Birchenough *et al.* 2015). The mucus layer on itself has a very particular dual function in intestinal physiology. In accordance to the classification of goblet cells into protective cells, the mucus layer controls microbiota interactions with its host by acting as a barrier. However, the mucus layer also represents a nutrient niche favoring microbe maintenance and growth. Details about the structure and functions of the mucus layer in health and disease will be thus extensively provided in Section 2.

(iii) Paneth cells – Only found in the small intestine, especially in the ileum, and preferentially at the crypt. They synthesize and secrete antimicrobial peptides (AMP) and proteins such as defensins and lysozymes.

(iv) Enteroendocrine cells – Release intestinal hormones or peptides (e.g. glucagon, cholecystokinin) into the bloodstream. They are also known to act as chemoreceptors, detecting harmful substances and initiating protective responses (Van der Flier and Clevers 2009; Kong, Zhang and Zhang 2018).

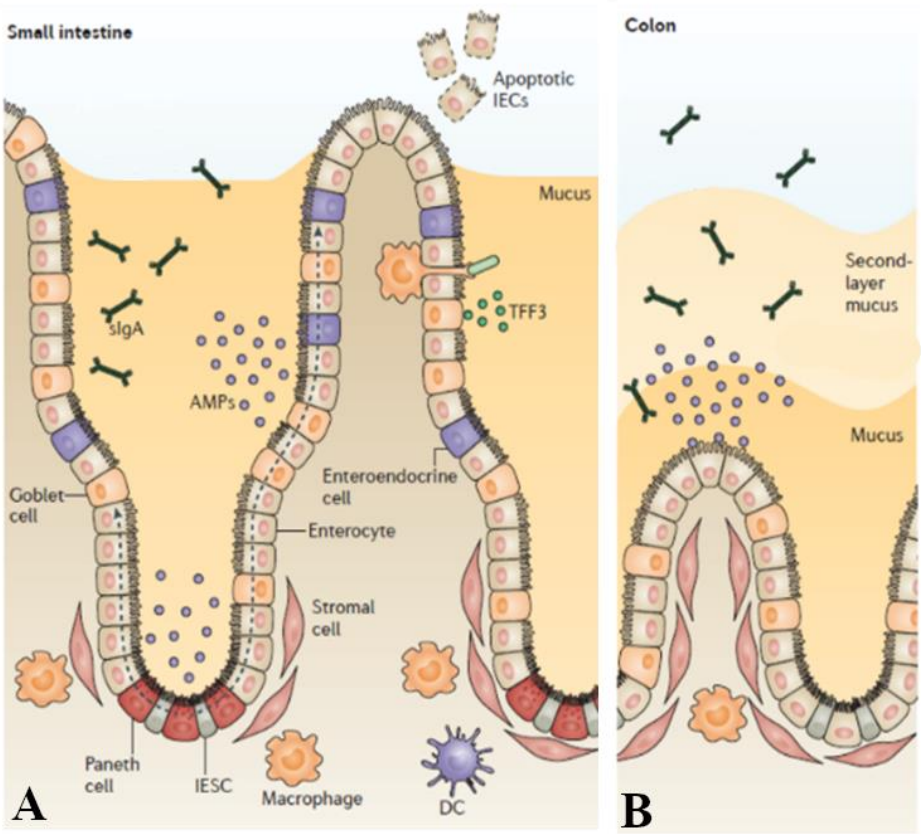


Figure 1.2. Cell lining of the small intestine (A) and colon (B).
Intestinal epithelial cells form a biochemical and physical barrier between the lumen and underlying mucosa. The intestinal epithelial stem cell (IESC) niche controls the continuous renewal of the epithelial cell layer by crypt-resident stem cells. Secretory goblet cells and Paneth cells secrete mucus and antimicrobial peptides (AMP). The transcytosis and luminal release of secretory Immunoglobulin A (SIgA) further contribute to this barrier function. The Trefoil factor 3 (TFF3) is a protein involved in the maintenance and repair of the intestinal mucosa.
AMPs: amtimicrobial peptides, DC: dendritic cells, IEC: Intestinal epithelial cells, IESC: Intestinal epithelial stem cells, IgA: immunoglobulin A, TFF3: Trefoil factor 3.
Modified from Peterson and Artis, 2014.

1.1.3.3.2. The tight control of intestinal permeability

As illustrated by the main functions of mucosal cells, the intestinal epithelium has two functions, which at first sight could seem contradictory. If the epithelium must act as an efficient barrier to protect the host, it must also allow exchanges of compounds like nutrients with the luminal environment. There are several pathways, tightly controlled, for luminal compounds to cross the intestinal epithelium. Such pathways should avoid exposing the host to armful component like antigens, endotoxins, pathogens, and other pro-inflammatory substances. Depending on the size, hydrophobicity, and other chemical characteristics of the compound different pathways can be considered. Small hydrophilic and lipophilic compounds can use the

transcellular route to cross the plasma membrane of the enterocytes/coloncytes. Transport of amino acids, vitamins, and sugars through enterocytes/colonocytes requires energy consuming epithelial transporters (active transport) (Said 2013). In the colon, SCFA can use either passive diffusion and active transport mechanisms which are related to various ion exchange transporters (Velázquez, Lederer and Rombeau 1997). Finally, ions, water, and larger hydrophilic compounds (from 400 Da to 10–20 kDa) use the paracellular route to cross enterocytes/colonocytes (Farré *et al.* 2020).

There, key players are the different kinds of intercellular junctions including tight junction, adherens junction, gap junction, desmosome, and hemidesmosome. Particularly, the tight junction proteins play leading roles in paracellular permeability and finely regulate this route. Tight junctions are dynamic cell-to-cell adhesion complexes that polarizes the intestinal epithelium. They are comprised of four unique groups of transmembrane proteins: claudins, occludins, zonula occludens protein and junctional adhesion molecules (Laukoetter, Bruewer and Nusrat 2006). These networks of transmembrane proteins interact between each other and link laterally adjacent cells near the apical surface of the epithelium (Zihni *et al.* 2016). Thus, the expression levels of these proteins are related to the intestinal barrier integrity (Zeissig *et al.* 2007; Ahmad *et al.* 2014; Chelakkot, Ghim and Ryu 2018). At least, tight junctions regulate two types of pores. The first one is a high capacity and charge selective pore, which is permeable to small ions and small-uncharged molecules (also known as “pore” pathway). The second one is a much larger pore with low capacity (also known as the “leak” pathway), which is permeable to large ions and molecules regardless of their charge. Mainly claudin proteins regulate the “pore pathway” and in contrast, occludin and zonula occludens proteins regulate the “leak pathway”. The permeability of both pathways can be measured using different methods. The transepithelial electrical resistance (TEER) measures the net movement of all ions (cations and anions) through the epithelium. TEER reflects not only the contribution of the paracellular resistance regulated by tight junctions, but also the transcellular and the sub-epithelial resistance. The permeability of the paracellular leak pathway can be specifically assessed by measuring the flux of large molecules from 4 up to 20 kDa across the epithelium. Alterations in the paracellular pathway are supposed to be relevant in the pathogenesis of several GI diseases as inflammatory bowel diseases (IBD) and metabolic diseases (Zeisel, Dhawan and Baumert 2019; Farré *et al.* 2020).

Bullet points, abiotic physiology of the human gut

- The digestive tract is formed by a group of hollow organs from mouth to anus passing through the GIT and also by ancillary solid organs, all involved in the ingestion, digestion and elimination of foods.
- Along the longitudinal axis, the GIT faces changes in anatomy and physicochemical parameters reflecting the segments specialisation in different steps of the digestion process.
- The small intestine and colon epithelia comprise four different cells types, specialised in digestion/absorption of nutrients or protection of the host. The ratio of these cells also change depending on the segment, reflecting its digestive function.
- Intercellular junctions, and in particular tight junctions, play a preponderant role in the balance between nutrients absorption and host protection by regulating the epithelium permeability.

1.2. Gastro-intestinal gut microbiota

Gut microbes live in close symbiosis with the human host and play a vital role in health. In terms of composition and abundance, the gut microbiota is characterized by pronounced regional differences (described in the section below), influenced notably by physicochemical, nutritional and immunological gradients (Donaldson, Lee and Mazmanian 2016). In addition to these dissimilarities, intra- and inter-individual variabilities are important (at least at family, genus and species levels), making the awareness of the gut microbiota even more complex, while the phylum level is generally more conserved. A non-exhaustive list of contributing factors can be draw up with a variation of diet, lifestyle, geographic and ethnical origin, health status, gender, menstrual cycle and age-range. Interestingly, within one individual, the microbial community (at phylum level) is fairly stable over time within adulthood (Donaldson, Lee and Mazmanian 2016). The largest shifts occur during the first 3 years of life, when the gut microbial community establishes and in elderly due to age-related changes in host physiology and diet (Hidalgo-Cantabrana *et al.* 2014; Maynard and Weinkove 2018). It is important to mention that in addition to bacteria, other key microorganisms are present in the gut including yeast, fungi, Archaea, viruses and phages, thus constituting the gut microbiota. The following sub-sections will describe only the human adult gut microbiota and will focus on the bacterial component.

1.2.1. Biogeography of the gut microbiota

The adult human gastrointestinal tract is estimated to harbor 10^{13} bacteria, which is equivalent to the number of human cells (Sender, Fuchs and Milo 2016). The stomach, which

was long be considered as a sterile organ, has been found to harbor quite abundant microbial communities dominated at the phylum level by Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria (Bik *et al.* 2006; Klymiuk *et al.* 2017; Zhang *et al.* 2019). The main genera found in stomach are *Streptococcus*, *Prevotella*, *Enterococcus*, *Lactobacillus*, *Veillonella* and *Rothia* (Bik *et al.* 2006; Engstrand and Lindberg 2013; Jandhyala 2015), the specie *Helicobacter pylori* is also prevalent (Bik *et al.* 2006). The stomach still shelters the lowest number of microbes in the GI tract ranging between 10 and 10^3 colony forming units (CFU) per gram of content (Hillman *et al.* 2017). In the duodenum and jejunum, the high levels of bile acids, antimicrobials and a short transit time limit bacterial growth to 10^3 to 10^6 CFU.mL^{-1} with huge interindividuals variations. Due to the mild oxygen levels, the microbial communities of these proximal parts of the small intestine are dominated by facultative anaerobic bacteria (Sundin *et al.* 2017). Few studies have investigated their composition. In a study conducted on five healthy volunteers that have ingested a meal, the duodenum was aspirated 90 min post-intake. Results showed the presence of two dominant phyla Firmicutes and Actinobacteria and four dominant genera with *Streptococcus*, *Actinomyces*, *Propionibacterium* and *Granulicatella* (Angelakis *et al.* 2015). In contrast, a recent study conducted on 20 adults has shown that the jejunum is dominated by Firmicutes, Proteobacteria and Bacteroidetes phyla. It also revealed a recurring core of abundant species in the jejunum belonging to *Streptococcus*, *Prevotella*, *Veillonella*, *Haemophilus* and *Escherichia* genera (Sundin *et al.* 2017). In the ileum, due to lower bile salts concentrations, lower level of oxygen, slower transit times and possible retrograde flow by the ileocecal valve from the colonic microbes, bacterial concentrations increase from 10^7 to 10^8 CFU.mL^{-1} of luminal content (Booijink *et al.* 2010; Quigley 2013). The ileal microbiota is dominated by the Firmicutes phylum and the following genera *Clostridium*, *Streptococcus*, *Escherichia* and *Veillonella* are the most commonly found. Contrary to the duodenum and jejunum, Bacteroidetes phylum is found in half of the volunteers, but remains still below the levels found in the colon (Booijink *et al.* 2010; Zoetendal *et al.* 2012; van den Bogert *et al.* 2013).

In contrast, colonic conditions exhibit lower cell turnover rate, lower redox potential and a longer transit time. The colon supports a dense and diverse community of bacteria with 10^{10} to 10^{12} CFU.mL^{-1} of colonic content according to the segment location, mainly anaerobes with the ability to utilize complex carbohydrates undigested from the small intestine. The colonic microbiota is dominated by members of Firmicutes and Bacteroidetes, followed by Proteobacteria and Actinobacteria. The three main dominant genus reported are *Bacteroides*, *Clostridium* and *Prevotella* (Miller *et al.* 2021). Due to sample accessibility, the numbers of

studies focusing on the human fecal bacterial communities is immeasurable compared to studies conducted directly in segments of the GI tract. The microbial load of fecal samples is estimated to be around 10^{10}-10^{11} CFU per gram of feces (Galazzo *et al.* 2020). Because of their immediate proximity, the fecal microbiota is thought to exhibit similarities with the colonic one. Analysis of fecal materials from healthy adults have shown that bacteria were predominantly members of the phyla Firmicutes and Bacteroidetes, followed by Proteobacteria and Actinobacteria. Moreover, about twenty genera belong to the aforementioned phyla such as *Bacteroides*, *Prevotella*, *Alistipes*, *Eubacterium*, *Ruminococcus*, *Roseburia*, *Faecalibacterium*, *Lactobacillus*, *Enterococcus*, *Blautia*, *Enterobacteriaceae* (family), *Fusobacteria* and hundreds to thousands of species belongs from these genera (Eckburg *et al.* 2005; Ley *et al.* 2006; Turnbaugh *et al.* 2007; Ruan *et al.* 2020). Among the most represent species, are *Bacteroides* spp. *Escherichia* spp and *Lactobacillus* spp. This core microbiota plays crucial roles in the gut, as explained in the next section. The next figure summarizes the bioregional composition of the gut microbiota along the longitudinal axis (**Fig. 1.3.**).

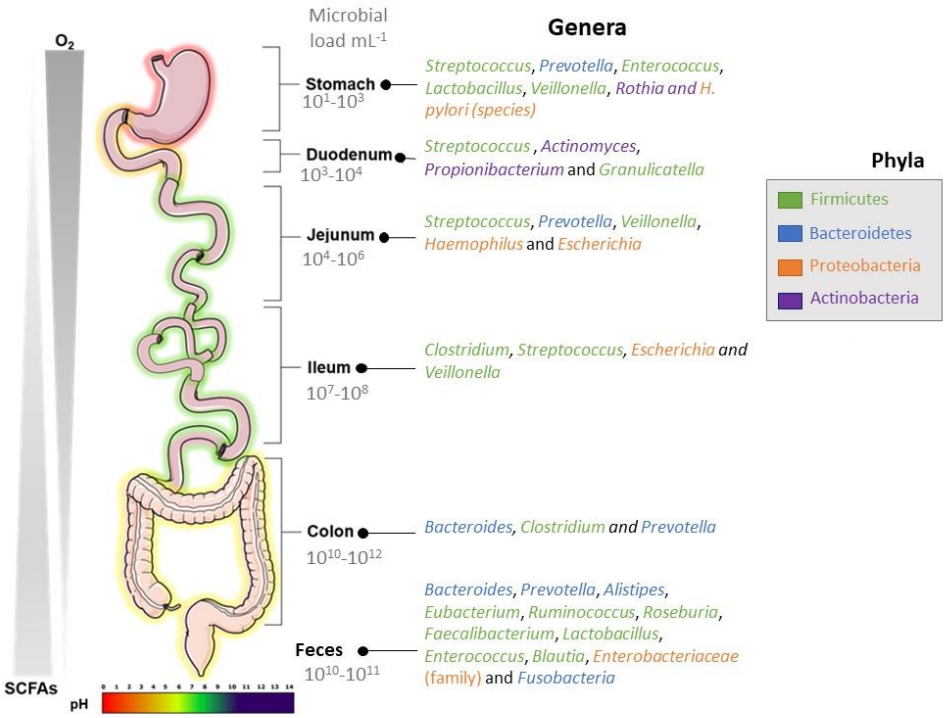


Figure 1.3. Biogeography of the luminal microbial composition of gastrointestinal tract in a healthy adult.
The most common genera in each gastrointestinal tract location are represented, belonging to a phylum represented in color (legend on the right). The gastrointestinal tract scheme is also colored according to the pH scale shown at the bottom.
SCFAs: Short Chain Fatty Acids.
Modified from Charlene Roussel's PhD manuscript.

1.2.2. Key functions of the gut microbiota

1.2.2.1 Functions in eubiosis

The gut microbiota plays important role in human health and metabolism, and huge efforts have been made over the last decade to explore the functional repertoire contributing to human physiology (Fan and Pedersen 2021). However, the associated mechanisms are complex and remain largely to be described. In healthy physiological state, named as eubiosis, the trillions of microbes colonising the human GIT contribute to various systemic processes. In particular, the human microbiota has a preponderant role in nutrient transformation and vitamin supply (Said 2013; Sonnenburg and Bäckhed 2016). Indeed, the human genome is very limited in term of variety of digestive enzymes (only 8 to 17 carbohydrate digestive enzymes in the human genome) and most human digestion occurs in the upper GIT (up to the ileum). Thus, the microbiota diversity serves as a reservoir of digestion capabilities (more than 1000 carbohydrate digestive enzymes) allowing the breakdown of the non-digested particles mostly in the colon, where most of the microbial mass prevails. The carbohydrate degrading function of the microbiota will be specifically addressed and detailed in Section 2. However, it is important to underline that dietary fibers are not the only nutrients processed by the microbiota. The later also participates in the degradation of proteins that escaped the human digestion process and polyphenols. The microbiota is also involved in vitamin B and K synthesis (O'Hara and Shanahan 2006). Concerning local health of the gut, the microbiota has important trophic effects on intestinal epithelia, by favoring the development of intestinal microvilli, epithelial cell differentiation and proliferation (Li *et al.* 2012). For example, without gut microbiota, the speed of cells renewal is diminished by 20% and mucosa thickness is also reduced in a mouse model (Alam, Midtvedt and Uribe 1994). Finally, and of particular interest to this PhD work, healthy intestinal microbiota contributes to host resistance to enteric infection through its involvement in the development of the host immune system and provision of colonisation resistance. The different colonisation resistance mechanisms can be gathered under microbe-microbe and microbe-host interactions (through stimulation of the host immune system (Wu and Wu 2012; Leshem, Liwinski and Elinav 2020). The microbe-microbe interactions include complex bacterial networks, competition for nutrients and inhibition by antimicrobial peptides.

1.2.2.1. Dysbiosis association with diseases

Given the multiple roles of the microbiota in human health, it is not surprising that shifts in composition/activity lead to a disease-promoting imbalance, which is often referred to as dysbiosis. Dysbiosis is defined by some authors as: "a compositional and functional alteration in the microbiota in individuals with disease compared with healthy subjects(Levy *et al.* 2017). Sometimes, the microbiota alterations associated with dysbiosis contributes to disease development and/or severity. Some authors considered the disease-promoting imbalance as part of the definition, while some others do not. In term of composition characteristics, authors often associate dysbiosis to a reduction of microbial diversity concomitant with an increase of Proteobacteria abundance (Weiss and Hennet 2017). Of note, it is important to underline that the dysbiosis concept could be criticized as years of microbiome data collection failed to define properly a well-balanced microbial community (Tiffany and Bäumler 2019; Shanahan, Ghosh and O'Toole 2021).

Growing number of diseases is associated with intestinal dysbiosis, which in some cases contributes to digestive and extra-digestive disease development or severity. Dysbiosis is a hallmark of IBD such as ulcerative colitis and Crohn's disease (Wlodarska, Kostic and Xavier 2015), colorectal cancer (Schwabe and Jobin 2013), metabolic disorders (Gérard 2016) and necrotizing enterocolitis (Neu and Walker 2011). Disrupted microbiota also promote the onset of enteric infections or at least increase their severity. Supporting this view, mice treated with antibiotics or bred in sterile environments (known as germ-free mice) are more susceptible to enteric pathogenic bacteria (Ferreira *et al.* 2011; Jump *et al.* 2014). Thus, it seems that, facing a long-time established microbiota, pathogens probably account on perturbations to colonise. Such perturbations could be induced by external factors or the pathogen itself (Ghosh *et al.* 2011; Willing *et al.* 2011). Finally, dysbiosis is also involved in extra-intestinal diseases, like autoimmune and neurological disorders (Knip and Siljander 2016; Tremlett *et al.* 2017).

Bullet points, gastro-intestinal human gut microbiota

- The adult human GIT harbors microbial communities known as gutmicrobiota whose composition varies along the longitudinal axis due to physicochemical parameters of the compartments.
- When correctly balanced (named as eubiosis), this gut microbiota helps its host in numerous biological process/functions as nutrient transformation, intestinal epithelium development and resistance to pathogens. Disturbance (termed dysbiosis) is associated to numerous intestinal and extra-intestinal diseases.

1.3. Intestinal immune response surveillance

Facing the importance of gut microbiota and especially pathogens on human health, it is not surprising that the human body have checkpoints related to microbiota activity *via* the immune system. Located at the interface between the lumen and the underlying host, the epithelial barrier acts as the first line of defense and monitor unfavorable activation of the host immune system. Disruption of this barrier leads to immune system activation. The immune system is composed of two parts. First, the innate immunity is acting as the first line of immunological defense present in the gut lumen and interacts with the microbiota *via* nonspecific innate immune receptors expressed on the different cell types in the mucosa (enterocytes, polynuclear cells, mast cells, macrophages and dendritic cells). Second, the adaptive immune system allows a highly targeted immune response of the host against the aggressors. The adaptive immune system interacts with gut microbiota *via* highly specific receptors expressed on lymphoid cells. This immunity has a memory of the signals triggering its activation and thus, will allow to respond more efficiently to future aggressions presenting the same signals (Thaiss *et al.* 2016; Allaire *et al.* 2018).

The activation of the innate immunity by microbial proximity occurs *via* the recognition of conserve microbe-associated molecular patterns (MAMPs). Of note, when these patterns originate from pathogens, they are termed pathogen-associated molecular patterns (PAMPs). MAMPs (and PAMPs) include diverse microbial components such as lipopolysaccharide (LPS), lipid A, flagelin, nucleic acids (Takeuchi and Akira 2010). Those MAMPs are recognised by pattern recognition receptors (PRR) such as toll like receptors (TLR), the C-type lectin receptors and NLRs (NOD-like receptors) of eukaryotic cells, triggering the host innate immunity and downstream signaling pathways (Ausubel 2005; Bailey et al. 2005).

2. Dietary fiber and mucus, the two glycan compartments of the gut

As presented in the previous section, the human gut microbiota is involved in many physiological processes. Disturbances of gut microbiota have been associated with negative health outcomes, and especially could promote the onset of enteric infections. To sustain its growth and persistence within the human digestive tract, the gut microbiota relies on two main glycan compartments, namely dietary fibers and mucus glycans. In this section, we will describe these two compartments along sides first by shedding light on similarities and differences

between dietary fibers and mucus structures and functions, then by providing an overview of their interactions with the third partner, namely the gut microbiota.

It is worth to note that this state of the art has been published in a review article in the FEMS Microbiol. Rev. journal (Impact Factor: 16.408) and redrafted / updated for the present section.

Review: SAUVAITRE T, ETIENNE-MESMIN L, SIVIGNON A, MOSONI P, COURTIN CM, VAN DE WIELE T, BLANQUET-DIOT S. Tripartite relationship between gut microbiota, intestinal mucus and dietary fibers: towards preventive strategies against enteric infections. FEMS Microbiol Rev. 2021 Mar 16;45(2): fuaa052. doi: 10.1093/femsre/fuaa052.

2.1. The analogy between dietary fibers and mucus glycans

2.1.1 Brief overview of dietary fibers and mucus: structure and properties

2.1.2.1. Dietary fibers

2.1.2.1.1. Definition and structure

Varieties of definitions for dietary fiber have been proposed by scientific and regulatory agencies worldwide. According to the Codex Alimentarius, dietary fibers are defined as carbohydrate polymers with 10 or more monomeric units, which are not hydrolysed by the endogenous enzymes in the small intestine of humans (Jones 2014; Porter and Martens 2017). The Codex Alimentarius also states that the dietary fiber definition could be extented to oligosaccharides containing between 3–9 monomeric units, depending on national authorities' recommendations (Jones 2014). This lack of consensus raises some issues as some of authors consider oligosaccharides as part of the dietary fiber group while some other do not. In this thesis manuscript, oligosaccharides that resist human digestion have been considered as part of dietary fibers. Dietary fibers include carbohydrate polymers naturally occurring in the food as consumed, as well as polymers obtained from raw food materials or chemically synthetized. Such dietary fibers have been shown to have a beneficial effect on health as demonstrated by scientific evidences from competent authorities (Jones 2014).

Dietary fibers can be divided into subgroups according to their origin, structure and physicochemical properties (**Fig. 2.1**) (Porter and Martens 2017; Deehan *et al.* 2018). Nevertheless, most dietary fibers consumed by humans are generally of plant origin and found in different proportions in fruits, vegetables, legumes, cereals, nuts and seeds. Some of them

are also derived from animals, fungi or bacteria. This is the case for human milk oligosaccharides (HMO), mannans from yeasts, chitin from fungi, and exopolysaccharides from bacteria, which are found in fermented foods such as bread, cheese or yogurt (Porter and Martens 2017). Depending of the numbers saccharides units the chain contains, dietary fibers can be divided into either oligosaccharide (between 3 and 10 monomeric units) or polysaccharides. Among the latter, there are different types (I to V) of resistant starches. They are called this way because their constitutive α(1→4) linked D-glucose polymer cannot be hydrolyzed by human amylases in the time between ingestion and reaching the large intestine (Fuentes-Zaragoza *et al.* 2010). Then, there are non-starch polysaccharides which comprise cellulose (polymer made of β(1→4) linked D-glucose units), hemicelluloses (set of branched polysaccharides based on xylose, mannose, arabinose, glucose), fructans like inulin (β(2→1) linked fructose units) and pectins (complex polysaccharides composed of mostly galacturonic acid, galactose, arabinose and rhamnose) (Deehan *et al.* 2018). Dietary fibers also comprise resistant oligosaccharides made of fructose (FOS), galactose (GOS), xylose (XOS), mixtures of arabinose and xylose (AXOS), or pectic sugars (POS) (Deehan *et al.* 2018). In consequence, there is a tremendous diversity of plant-derived dietary fibers that differ in their sugar composition, type of linkage between sugars, degree of polymerization, or branching. These structural characteristics impart dietary fiber with various physicochemical properties, notably solubility, viscosity and fermentability (Gill *et al.* 2021). Solubility refers to the ability of dietary fibres to be dissolve in water. Conrary to insoluble fibres that remain as discrete particles, soluble fibers have a high affinity for water. Viscosity is defined as the degree of resistance to a certain flow. It is generally associated with soluble dietary fibres (such as gums, pectins, β-glucans and psyllium) and relates to the ability of a fiber, when hydrated, to thicken in a concentration-dependent manner. Finally, fermentability refers the ability of an individual microbiome to degrade dietary fiber. Fementabilty is particularly corelated to solubility, as insoluble dietary fiber particles are less accessible to microorganisms degrading enzymes (Holscher 2017; Gill *et al.* 2021).

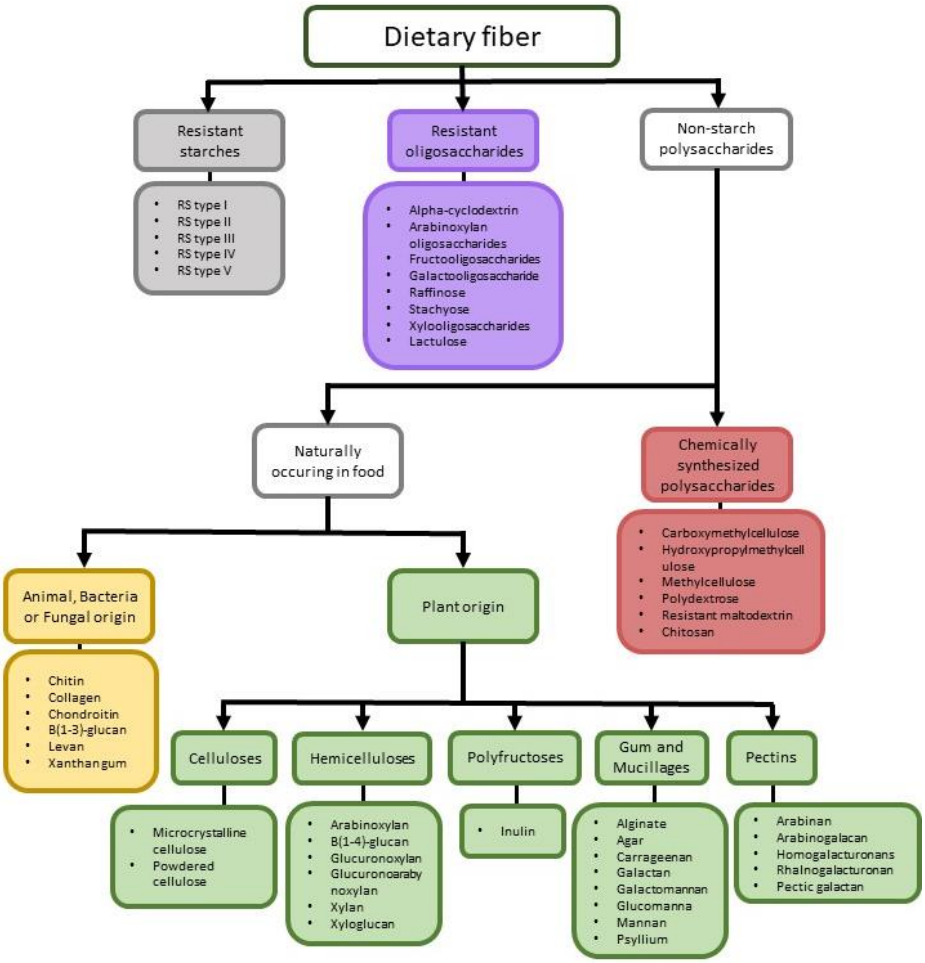


Figure 2.1. Classification of dietary fibers.
Dietary fibers are classified between resistant starches, non-starch polysaccharides and resistant oligossacharides. As presented for non-starch polysaccharides, resistant starches and resistant oligosaccharides originate from different sources. Resistant starches are either from plant origin or chemically synthesized (Type IV: starches that are chemically modified to obtain resistance to enzymatic digestion) (Cummings and Stephen 2007). Resistant oligosaccharides are either from plant, animal origin or chemically synthesized
RS: resistant starch.
Modified from Deehan *et al*. 2017.

2.1.2.1.1. Dietary fiber intake and health effects

Dietary fiber intake varies substantially among countries. Diets in industrialized countries are often depleted in fibers in favor of animal protein, fat, sugar and starch; while non-industrialized rural communities have greater fiber intake through fibrous plant-rich diets (De Filippo *et al.* 2010; Schnorr *et al.* 2014). Investigations into dietary habits revealed that on average adults consume between 12–18 grams, 14 grams and 16–29 grams of fibers per day in the United-States (US), Kingdom (UK) and Europe, respectively (EFSA Panel on Dietetic Products, King, Mainous and Lambourne 2012; Holscher 2017). These dietary amounts are below the US department of Agriculture (USDA) recommendation of 25 grams for women and 38 grams for men up to 50 years old (Jones 2014; Holscher 2017). The beneficial effects of

dietary fiber have been acknowledged by the European Food Safety Authority (EFSA) for two specific health claims : the decreased transit time and increase of fecal bulking (EFSA Panel on Dietetic Products, Nutrition, and Allergies (NDA) 2010). Dietary fiber intakes may also lower glycemia and cholesterol levels, decrease adiposity and the associated parameters of metabolic syndrome (Dhingra *et al.* 2012; Zou *et al.* 2018). Populations with high dietary fiber intake present a lower incidence of immune dysregulation, with a lower risk to develop asthma, allergies, IBD, diabetes and colorectal cancer (Burkitt, Walker and Painter 1972; Sonnenburg and Sonnenburg 2014). Insufficient dietary intake in industrialized countries has been associated with a disrupted host-microbiota relationship leading to an increased incidence of inflammatory-related disorders (Makki *et al.* 2018; Zou *et al.* 2018).

Bullet points, overview of dietary fiber structure and properties

- Dietary fibers are carbohydrate polymers (originating from plants, animals, fungi or bacteria), which are not hydrolysed by the endogenous enzymes in the small intestine of humans and are thus available for fermentation by microorganisms.
- Dietary fiber intakes vary substantially among countries and low intakes are widely recognized as detrimental for human health, even if the associated mechanisms are not yet fully described.

2.1.2.1. Intestinal mucus

2.1.2.1.1. Structure

The intestinal mucus is continuously produced and secreted by goblet cells. The goblet cell-to-enterocyte ratio changes along the intestinal tract, with an estimated percentage of goblet cells in the intestinal epithelium of approximately 4% in the duodenum, 6% in the jejunum, 12% in the ileum and 16% in the distal colon. The intestinal mucus also varies in terms of structure and composition according to the considered species (Hugenholtz and de Vos 2018; Etienne-Mesmin *et al.* 2019). In human, mucus is found throughout the entire GIT of human from stomach to large intestine, with its thickness and structure varying depending on the segment of the digestive tract considered, but also with cross-sectional differences (**Fig. 2.2**). In the colon, the mucus layer shows a double-layer structure, with an inner layer firmly attached to the epithelium, and an outer layer superimposed to the first one, constantly shed into the lumen and showing an expended volume due to proteolytic activities provided by the host but also by commensal bacteria (Atuma *et al.* 2001; Ijssennagger, van der Meer and van Mil 2016).

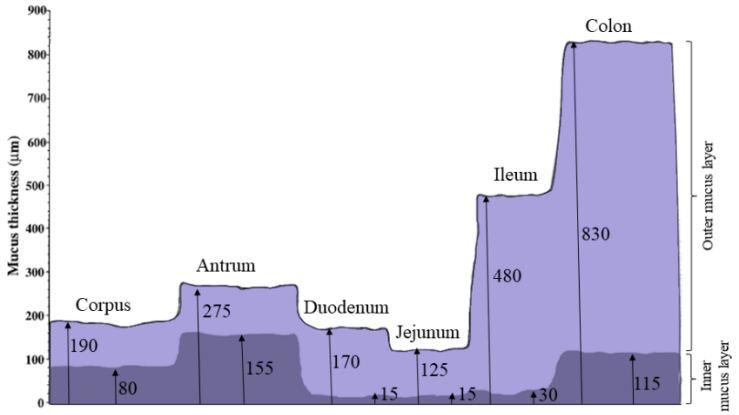


Figure 2.2. Mucus thickness along the human gastro intestinal tract.
Both outer and inner mucus layer thickness are represented all along the digestive tract.
Modified from Atuma et al. 2001.

Mucus is a complex viscoelastic adherent secretion composed of water, electrolytes, lipids and the main structural components (around 5 %) glycoproteins, which are called mucins. Mucin as polymer consists of a polypeptide backbone with glycan side chains. The core region of the glycans is formed by a combination of three sugars, galactose, N-acetylgalactosamine and N-acetylglucosamine, to which different chains of glycans can be attached. The terminal monosaccharide is usually fucose or sialic acid (Holmén Larsson *et al.* 2009; Juge 2012). Oligosaccharide chains can also be sulfated, especially in colonic regions (Rho *et al.* 2005). The glycan moieties are conjugated to proteins, mostly by O-link to serine and threonine but also by N-link to asparagine (Porter and Martens 2017). To date, several MUC genes have been described in human and named based on their order of discovery. Some of them belong to the secreted gel-forming mucin family, while others are classified in the membrane-associated family (**Table 2.1**). Mucins in the gastrointestinal tract). Host-secreted mucin 2 (MUC2) glycoprotein is a major constituent of human small intestinal and colonic mucus, while MUC1, MUC5AC, and MUC6 are predominant in the stomach (Sicard *et al.* 2017).

Table 2.1. Mucins in the human gastrointestinal tract.
Modified from Johansson *et al*. 2007.

Mucin glycoproteins	Types	Number of amino acids	Cell type expression	Functions
MUC1	Transmembrane	~1,250	Epithelial cells	Signaling, protection
MUC2	Gel-forming	~5,200	Goblet and paneth cells	Protection, lubrication
MUC3	Transmembrane	>2,550	Enterocytes	Apical surface protection
MUC4	Transmembrane	~5,300	Epithelial cells Goblet cells	Signaling, protection

MUC5AC	Gel-forming	>5,050	Mucous cells	Protection, lubrication
MUC5B	Gel-forming	~5,700	Mucous cells Goblet cells	Protection, lubrication
MUC6	Gel-forming	~2,400	Mucous cells	Protection, lubrication
MUC7	Gel-forming	377	Mucous cells	Protection
MUC12	Transmembrane	~5,500	Enterocytes	Apical surface protection
MUC13	Transmembrane	512	Enterocytes	Apical surface protection?
MUC16	Transmembrane	~22,000	Epithelial cells	Apical surface protection
MUC17	Transmembrane	~4,500	Enterocytes	Apical surface protection

2.1.1.2.2. Main functions

The mucus barrier has several functions, a primary one being the lubrication of the epithelium helping the passage of food material along the GIT. Mucin proteins are glycosylated polymers that constitute a carbon and energy source for the growth of resident gut microbiota (Tailford *et al.* 2015). Accumulating evidence demonstrates a crucial role of the mucus layer in maintaining gut homeostasis (Martens, Neumann and Desai 2018). Notably, it contains a large variety of host antimicrobial molecules (e.g. α and β defensins, IgA and IgM) that are retained within the net-like polymer structure of mucin glycoproteins. In close collaboration with the immune system and the gut microbiota, the mucus is the first line of defense against encroaching bacteria that can breach and persist on the epithelial surface (Johansson and Hansson 2016). In particular, bacteriophages are able to interact with mucus and studies in mice demonstrated that phage particles are 4-fold more concentrated in mucus layer compared to the lumen content (Barr *et al.* 2013). Recent studies showed that phages represent key players in limiting bacterial persistence close to the epithelium and may play an important role in the homeostasis of the gut microbiota (Almeida *et al.* 2019; Rasmussen *et al.* 2020; Sausset *et al.* 2020). The mucus layer therefore has a dual role. On the one hand, it lubricates the intestine and acts as a defensive barrier against harmful aggressors. On the other hand, it provides an ecological niche for bacteria by providing adhesion sites and nutrients, as described in section 2.2.1.

Bullet points, overview of intestinal mucus structure and properties

- Secreted by goblet cells, mucus covers the entire GIT of humans with longitudinal and cross-sectional differences.
- Mucus is a complex viscoelastic adherent secretion which main structural components are large glycoproteins called mucins.
- Mucus has a dual role. On one hand, it protects the epithelium from luminal biological, chemical and physical aggressions. On the other hand, it constitutes a niche for the resident gut microbiota, providing adhesion sites, carbon and energy sources.

2.1.2. Similarities and differences between dietary fiber and mucus as glycan compartments

2.1.2.1. Origin and metabolism

The first major distinction between dietary fibers and mucus carbohydrates is their origins. While dietary fibers are provided only from the external environment through diet, the glycans presented by the mucus layer originate from the host itself. Consequently, dietary fiber uptake is variable in quantity and composition throughout daytime, life and individual, while mucus carbohydrates are chemically more homogeneous (**Fig. 2.3**) and always present as an energy source for the microbial ecosystem. Nonetheless, dietary fibers and mucus carbohydrates are both non-digestible by host enzymes but can be metabolized in the intestine by the resident members of the gut microbiota and further fermented to yield gases (e.g. dihydrogen, carbon dioxide, methane) and SCFAs (Morrison and Preston 2016). SCFA are a subset of saturated fatty acids containing six or less carbon molecules that include acetate, propionate, butyrate, pentanoic (valeric) acid and hexanoic (caproic) acid (Tan *et al.* 2014). Dietary fiber fermentation results mainly in acetate, butyrate and propionate production (Morrison and Preston 2016). As dietary fibers, mucus carbohydrates can also be fermented in the digestive lumen due to constant shedding of the mucus layer (Johnson *et al.* 2012) and SCFA, especially butyrate, resulting from their metabolism provide an energy source directly usable by nearby colonocytes (Ouwerkerk, de Vos and Belzer 2013).

2.1.2.2. Structure

As a result of different linkages and more than twenty possible numerous monomeric units, the structure of fiber carbohydrates is amazingly diverse as illustrated by dietary fiber heterogeneity (Porter and Martens 2017). By comparison, the carbohydrates presented by the

mucus layer constitute a more restricted group with only six possible monomeric sugar units (galactose, N-acetylgalactosamine, N-acetylglucosamine, mannose, fucose and sialic acid) (Etienne-Mesmin *et al.* 2019) (**Fig. 2.3**). Still, mucus carbohydrate can offer structural similarities with dietary fibers (Porter and Martens 2017), in terms of polymerization, high cross-linkage, with linkages solely and specifically broken down by certain bacteria. Futhermore, similar patterns can be found between mucus carbohydrates and dietary fibers.

HMO from human breast milk and blood group antigens illustrate this tight line between dietary fibers and mucus carbohydrates structure. HMO are oligosaccharides composed of repeated and variably branched lactose or N-acetyl-lactosamine units often decorated with sialic acid and fucose monosaccharides (Kunz *et al.* 2000; Ninonuevo *et al.* 2006). Interestingly, HMO structures share common patterns with human blood group antigens (Porter and Martens 2017), known to be expressed on the surface of blood cells, but also, in most humans (e.g. 80% of North Americans and Europeans), expressed on mucin-O linked glycans in mucus. Such observation is related to *fut2* expression in these individuals called “secretors” (Kelly *et al.* 1995). As HMO can be considered as the sole source of dietary fibers at early age, some authors have postulated that their intake could initiate the use of mucus carbohydrates as a nutritive source by the infant gut microbiota (Koropatkin, Cameron and Martens 2012). In support, study showed that some *Bacteroides* induce the same bacterial genes for the consumption of HMO and mucus glycans (Marcobal *et al.* 2013).

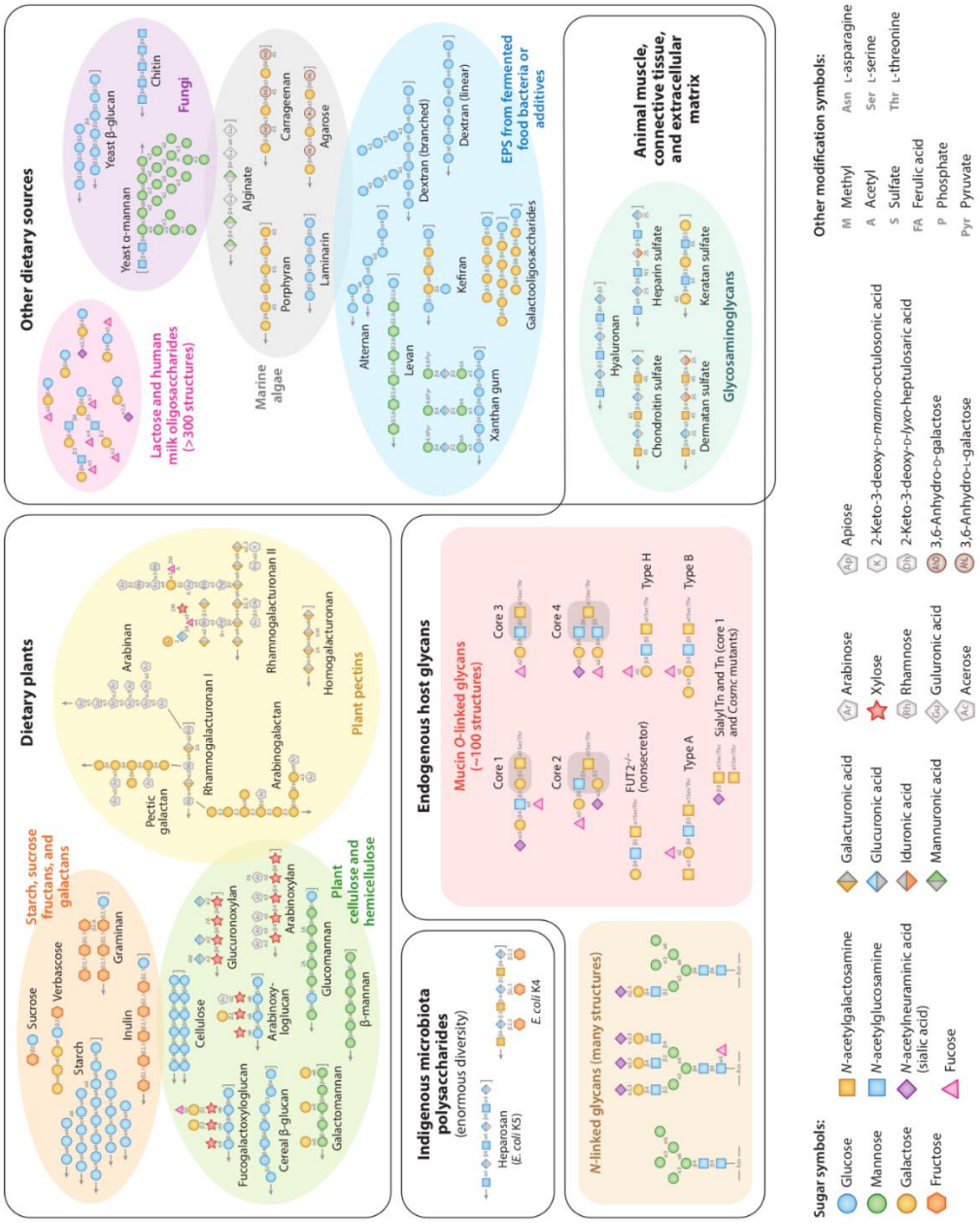


Figure 2.3. The diversity of sources and structures of non-digestible oligosaccharides and polysaccharides in the human gut.

Representative diversity of oligosaccharides/polysaccharides derived from various dietary, host, and microbial sources. Gray brackets indicate reducing ends of polysaccharides and gray arrows indicate the possibility of extended polymer length. Most of the oligosaccharides/polysaccharides shown are known to be degraded by human gut bacteria. The three major sources of polysaccharides (diet, endogenous host glycans, and microbially produced glycans indigenous to the microbiota) are shown. Endogenous microbiota polysaccharides, which are not mentioned in the main text to avoid confusion, are restricted here to two *Escherichia coli* (*E. coli*) capsule polysaccharide structures. Linkage types (α or β) between sugars are indicated, and where the donor sugar is linked via carbon 1 to another monosaccharide, this number is not indicated (e.g., β1,4 linkage between two sugars is written as β4).

Printed with permission from Porter and Martens 2017.

Bullet points, similarities and differences between dietary fibers and mucus glycan compartments

- Both dietary fibers and mucus glycans can serve as nutrients for microorganisms. However, if mucus is constantly produced, fiber intakes depend on human diet.
- Compared to mucus glycan, the structural diversity of dietary fibers is incredibly more important. Still, some structural similarities can be found between these two gut glycan compartments, as illustrated by HMO and blood antigens.

2.2. Interactions of dietary fibers and mucus-associated polysaccharides with human gut microbiota

2.2.1. Substrate accessibility and microbial niches

2.2.1.1. Dietary fiber as a particle niche for microbes

Substrate accessibility is the first determinant of microbial colonisation of dietary fibers and subsequent degradation and fermentation of their constituting carbohydrates. Restricted to the intestinal transit time, dietary fiber fermentation in the gut can take place in-between 18 hours up to 60 hours (De Paepe *et al.* 2020). For effective dietary fiber fermentation, poly- or oligosaccharide accessibility is therefore crucial. Soluble fiber, such as oligosaccharides (which are often soluble due to their low chain length), are free and easily accessible to microbes in the lumen (Koropatkin, Cameron and Martens 2012). They are thus easily metabolized in the proximal GIT (mainly ileum and proximal colon), especially in normal transit individuals (Koropatkin, Cameron and Martens 2012).

In contrast, insoluble fibers consist of a complex tridimensional network of different polysaccharides (for example, plant cell wall particles made of cellulose, hemicellulose and pectins) that render these carbohydrates less accessible to microorganisms. Hence, insoluble fiber degradation will take more time and is generally completed in the distal colon where the microbial bacteria richness is the most important (Koropatkin, Cameron and Martens 2012). By themselves, these insoluble dietary fiber particles can be considered as microbial niches since they face an ecological succession of microbial colonisers able to gradually degrade them along their progression through the GIT (De Paepe *et al.* 2020). The colonising microbial actors are dietary fiber specific (Leitch *et al.* 2007) and *in vitro* studies of these dynamic communities could be highly predictive of their fiber-degrading capacities (De Paepe *et al.* 2019). For instance, using anaerobic batch cultures of fecal microbiota, De Paepe and colleagues showed that colonisation of wheat bran particles by *Bacteroides ovatus*/*stercoris*, *Prevotella copri* and Firmicutes was associated with an increase in fermentation activity (De Paepe *et al.* 2019).

Similarly, Leitch and colleagues found that resistant starch particles were enriched in *Ruminococcus bromii*, a starch-colonising and degrading bacterium (Leitch *et al.* 2007; Ze *et al.* 2012; Vital *et al.* 2018). Some coloniser species, such as *Bacteroides thetaiotaomicron* and *Roseburia intestinalis* could even form biofilms at the surface of dietary fiber particles in the luminal digestive content (Mirande *et al.* 2010; Li *et al.* 2015). Insoluble dietary fibers have also a huge impact on microbial microbial diversity and functionality, not only in distal but also in proximal colon and probably even before in the GIT.

2.2.1.2. Mucus niche and mucus-associated microbiota

The mucus layer is considered a well-known microbial niche in the GIT where its colonisation is necessary for resident microorganisms to maintain their presence (Ouwerkerk, de Vos and Belzer 2013). Bacterial mucinases are one of the main actors of this colonisation. Described both in commensal bacteria and in pathogenic strains, these enzymes allow access to the mucus layer by proteolysis of the core of mucin proteins then enabling bacterial colonisation (Etienne-Mesmin *et al.* 2019). To counterbalance mucinase action and maintain its net-like structure that retains the gut microbiota, the mucus contains structural proteins including protease inhibitors that protect the mucus from extensive degradation (Bansil and Turner 2018). Not all microbial species are adapted to mucosal colonisation though and studies have demonstrated that microbial communities from the digestive lumen differ in terms of composition and abundance from the mucus-associated ones, driven by differences in nutrient availability and physicochemical gradients like oxygen availability (Chassaing and Gewirtz 2019). Gastric mucosal samples are more oriented towards Firmicutes and Proteobacteria (Sung *et al.* 2016). For its part, the mucosal jejunal microbiota displays great similarity with the luminal one (Dlugosz *et al.* 2015; Sundin *et al.* 2017). Only one work has studied the ileal mucosa, with an unhealthy volunteer, making difficult any conclusion (Patrascu *et al.* 2017). Then, most of the studies about differences in microbiota composition between the lumen and the mucus have been conducted in the colonic compartment. Compared to the luminal one, the human colonic mucus layer displays a markedly higher level of Firmicutes, Actinobacteria and Proteobacteria and a lower level of Bacteroidetes (Donaldson, Lee and Mazmanian 2016; Richard *et al.* 2018; Chassaing and Gewirtz 2019; Vuik *et al.* 2019). Especially, mucosal communities are highly enriched in *Bacteroides acidifaciens*, *Bacteroides fragilis*, the mucin-degrader *Akkermansia muciniphila* and in species belonging to the Lachnospiraceae taxa (Donaldson, Lee and Mazmanian 2016; Pereira and Berry 2017) (**Fig. 2.4**). **Figure 2.4**

summarizes the changes in microbiota composition between luminal and mucosal phases of the human gut.

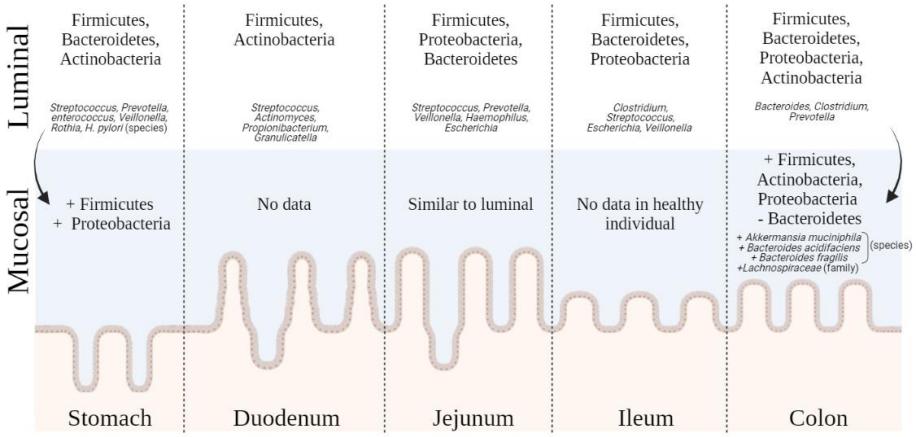


Figure 2.4. Changes in microbiota composition between luminal and mucosal compartments throughout the human gut.
The composition of the luminal microbiota (phyla and genera) presented in section 1.2.1 is represented alongside with changes observed in the mucosal phase of the human gut.
Built from personal source.

Difference in microbiota communities are also observed on the transversal axis inside the mucus layer. A gradial density of microbial colonisation is found from the outer mucus layer to the intestinal epithelium, mostly due to niche accessibility. The outer colonic layer is more densely colonised thanks to proteolytic activities loosening the net-like structure. The inner colonic mucus layer has for long been believed to be devoid of bacteria in accordance with its more constraining physical properties (Johansson, Sjövall and Hansson 2013). At tissue scale, single-cell imaging conducted in mouse model revealed the presence of bacteria in close proximity of the epithelium (Earle *et al.* 2015). Among them, Segmented Filamentous Bacteria have been identified in many vertebrate intestines (humans, rodents, chickens) as commensal strains able to invade this mucus layer without invading the host (Chen *et al.* 2018; Hedblom *et al.* 2018; Ladinsky *et al.* 2019).

Bullet points, substrate accessibility and microbial niche

- Accessibility of dietary fibers depends on their micro- and macrostructure. Insoluble fibers are less accessible and, in that sense, a important driver of colonic ecosystem diversity and functionality.
- These particles have been recently shown to constitute a niche on their own, facing an ecological succession of microbial colonisers able to degrade them gradually along their progression in the GIT.
- Bacterial enzymes called mucinases are able to disrupt the core of mucin proteins and enable colonisation by a mucus specific microbiota.
- The intestinal mucus is also a well described microbial niche, with, in the colon, a markedly higher level of Firmicutes, Actinobacteria and Proteobacteria compared to the digestive lumen.

2.2.2. Recognition and binding strategies

2.2.2.1. Dietary fibers

Among the fiber-degrading bacteria isolated from the human gut, the *Bacteroides* genus has been the most extensively studied. Several members of this genus (e.g. *Bacteroides thetaiotaomicron*, *Bacteroides xylanisolvens*, *Bacteroides intestinalis*, *Bacteroides ovatus*) are able to forage an important repertoire of glycans in the gut (Kaoutari *et al.* 2013). These bacteria produce cell-surface enzyme systems that allow them to convert dietary fibers into oligosaccharides that are then internalized into the cell and further hydrolyzed into simple sugars. All of these enzyme systems have the same cellular organization and operating mode as the Starch-Utilization System (Sus) of *Bacteroides thetaiotaomicron* in which substrate recognition is ensured by the cell-surface protein called SusD (Martens *et al.* 2009). Each enzyme system is dedicated to a specific polysaccharide and contains a SusD-like protein recognizing fructans (Sonnenburg *et al.* 2010), xylans (Rogowski *et al.* 2015; Despres *et al.* 2016a), xyloglucans (Larsbrink *et al.* 2014) and pectins (Martens *et al.* 2011; Despres *et al.* 2016b).

Among the Firmicutes, the fiber-degrading bacteria belonging to the *Ruminococcus* genus also rely on very complex enzyme complexes called cellulosomes (*Ruminococcus champanellensis*, *Ruminococcus flavefaciens*) or amylosomes (*Ruminococcus bromii*) for substrate recognition and binding (Ben David *et al.* 2015; Cann, Bernardi and Mackie 2016). *Ruminococcus albus* and *Ruminococcus flavefaciens* have also been shown to attach to cellulose *via* type IV pili (Rakotoarivonina *et al.* 2002; Vodovnik *et al.* 2013). Studies of the complex polysaccharide degrading apparatus in Firmicutes species (other than *Ruminococcus*) are just in their infancy. Recently, studies have shown that *Roseburia intestinalis* and *Monoglobus*

pectinilyticus belonging to the Firmicutes phylum display the appropriate gear to be mannan and pectin primary degraders, respectively (Kim *et al.* 2019; La Rosa *et al.* 2019). Sheridan and colleagues also reported that *Roseburia* spp. and *Eubacterium rectale* possess their own Gram-positive polysaccharide utilization loci allowing complex glycans degradation (Sheridan, Paul O. *et al.* 2016). Otherwise, Firmicutes species are known to rely on a diverse array of transporters (such as ABC transporters) to import smaller sugars for intracellular processing. In particular, ABC transporters own an extracellular substrate-binding site for sugar recognition (Chen 2013).

2.2.2.2. Mucus polysaccharides

Microorganisms have developed different binding strategies to mucin. As for dietary fibers, Bacteroides species recognise mucus carbohydrates *via* a SusD-like protein belonging to the enzyme system involved in mucin glycan degradation (Martens *et al.* 2009; Sonnenburg *et al.* 2010). Bacteria can also use specialized cell-surface adhesins or lectins. For instance, the well-known mucus-binding protein MUB, produced by *Lactobacillus reuteri* ATCC 53608, is able to interact with terminal sialic acid of mucin (Etzold *et al.* 2014). Another strategy is to employ appendages such as pili and flagella. *Lactobacillus rhamnosus* SpaC adhesins are positioned along the complete length of the bacterial pili. This is supposed to reinforce mucin-binding strength (Reunanen *et al.* 2012). As their surface counterparts, these pili adhesins also recognise precise carbohydrate patterns (Troge *et al.* 2012). Interestingly, some adhesins have been shown to recognise patterns encountered in both mucins and dietary fibers, likely due to structural similarities (Cooling 2015; Dotz and Wuhrer 2016; Taylor *et al.* 2018). Hence, in addition to binding to mucin, *Lactobacillus plantarum*, a mannose-specific adhesin is also able to bind glycan structures from yeast cell walls. Also, it has been demonstrated that *Bifidobacterium infantis* adhesins recognise HMO (Pretzer *et al.* 2005; Garrido *et al.* 2011).

Bullet points, recognition and binding strategies

- Different dietary fiber binding proteins coexist in the gut communities, differing according to their microbial origin and targeted-carbohydrates. Complex polysaccharides will generally be bound by complex enzyme apparatus, allowing binding, primary degradation and import of the resulting saccharides.
- Different mucus binding strategies coexist as *(i)* complex enzyme apparatus similar as the one used for complex dietary fibers, *(ii)* adhesins or lectins and *(iii)* appendages such as pili and flagella.
- Some adhesins have been shown to recognise both mucins and dietary fibers, likely due to structural similarities.

2.2.3. Carbohydrate metabolism by human gut microbiota

2.2.3.1. Specialized carbohydrate-active enzymes

Enzymes involved in carbohydrate metabolism are named CAZymes (for Carbohydrate-active enzymes) and represent 2.6 % of the total enzymes encoded by the human gut microbiota (Turnbaugh *et al.* 2008). Of note, carbohydrate metabolism is almost exclusively supported by the gut microbiota, with around 10,000 CAZymes found in the genome of 177 reference gut bacteria, compared to only 8 to 17 GHs in the human genome involved in carbohydrate digestion in the gut (Kaoutari *et al.* 2013; El Kaoutari *et al.* 2014). In the CAZyme super family, glycoside hydrolases (GHs) hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety, whereas polysaccharide lyases (PLs) cleave uronic-acid containing polysaccharides via a β-elimination mechanism and carbohydrate esterases (CEs) catalyze the de-O or de-N-acylation of substituted saccharides (Kaoutari *et al.* 2013). Based on their sequences, GHs are classified into 167 families, PLs into 40 families, and CEs in 17 families (see http://www.cazy.org/). One CAZyme is often associated with the degradation of one type of linkage (Snart *et al.* 2006; Chassard *et al.* 2010; Hamaker and Tuncil 2014). CAZymes do not only contain catalytic modules. Carbohydrate-binding modules (CBM) keep them to bind the substrate (Bolam *et al.* 1998; Boraston *et al.* 2004).

CAZymes families contain plant dietary fiber specialized CAZymes (e.g. GH5, GH6, GH9, GH10, GH11, GH12, GH28, GH44, GH45, GH74, GH88, GH105, PL1, PL2, PL3, PL4, PL9, PL10, PL11, PL15) while other contain mucus polysaccharide specialized ones (e.g. GH20, GH29, GH33, GH42, GH84, GH85, GH89, GH95, GH98, GH101, GH112, GH129) (Hamaker and Tuncil 2014). CAZymes relative to dietary fiber utilization are well characterized (White *et al.* 2014; Grondin *et al.* 2017). CAZymes involved in mucin metabolism have also been functionally characterized in resident members of the gut microbiota able to feed on mucins, including *Akkermansia muciniphila*, *Bacteroides thetaiotaomicron*, *Bacteroides fragilis*, *Bifidobacterium bifidum* and *Ruminococcus gnavus* (Tailford *et al.* 2015; Ndeh and Gilbert 2018). Of note, β-galactosidases from the GH2 family has been associated with the degradation of both mucus carbohydrates and dietary fibers (Turnbaugh *et al.* 2009). If most CBMs are involved in enzyme binding to dietary fiber polysaccharides, CBM in families 32, 40, 47 and 51 also recognise mucus carbohydrates.

2.2.3.2. Vertical distribution of carbohydrate degradation inside the ecological niches

According to the degree of dietary fiber complexity, several CAZymes are needed for their complete hydrolysis (Martens *et al.* 2011) and the length of time for their degradation in the human gut will vary (Sanchez *et al.* 2009). Such degradation process can be sequential and involves several different microorganisms. For example, *Bifidobacterium* spp. commonly need primary degradation of starch and xylan by species like *Ruminoccocus bromii* and *Bacteroides ovatus* to use the resulting malto- and xylo- oligosaccharides, respectively (Turroni *et al.* 2018). This relationship by which one microorganism allows another to feed is called cross-feeding (Falony *et al.* 2006). Cross-feeding is possible because GHs, PLs and CEs are typically secreted or cell surface-associated enzymes whose activity results in the availability of the released mono- or oligosaccharides for uptake by the hydrolase-producing organism itself but also by nearby bacteria. In the cross-feeding chain, microorganisms required to initiate the degradation are called primary degraders and are defined as "bacteria that are able to detect and degrade a complex carbohydrate owing to enzymatic equipment that is missing in other species" (Kaoutari *et al.* 2013). If a primary degrader outcompetes the other organisms by being the most efficient in degrading a particular polysaccharide, hence being essential for further degradation by the resident microbiota, it is called bacteria with keystone functions or keystone species (Ze *et al.* 2012). For example, *Ruminoccocus bromii* has been regularly described as a robust starch keystone species, and its absence within the ecosystem is associated with resistant starch indigestibility by the host (Ze *et al.* 2012; Vital *et al.* 2018).

Mucus glycans are also concerned by this cross-feeding strategy (Png *et al.* 2010; Marcobal *et al.* 2013; Egan *et al.* 2014), since a combination of enzymatic activities from several mucolytic bacteria is required to complete mucin degradation (Derrien *et al.* 2010; Marcobal *et al.* 2013). As the O-glycans are covalently attached to the mucin peptides, the peripheral residues are the first targets for GH enzymes. Removal of these peripheral residues composed of sialic acid, fucose and glycosulfate is necessary to gain access to and degrade the O-glycan chains (Corfield 2018). *Bacteroides thetaiotaomicron*, *Bacteroides ovatus*, *Prevotella* spp. strain RS2, *Bifidobacterium breve* UCC2003, or *Bacteroides fragilis* all possess mucin-sulfatases or glycosulfatases and are thus potential primary degraders (Salyers *et al.* 1977; Berteau *et al.* 2006; Benjdia *et al.* 2011; Egan *et al.* 2016; Praharaj *et al.* 2018). This high number of primary degraders with high level of redundancies in their CAZYmes arsenal targeting mucin carbohydrates probably reflects the huge amount of constantly renewed mucus

substrate. To date, no keystone species has been found for mucus glycan degradation. Still, *Akkermansia muciniphila* has been often highlighted as a species allowing higher mucus consumption in host intestinal tract (Cheng and Xie 2021), with a central role within the mucus degrading communities (Van Herreweghen *et al.* 2018, 2021). Once mucus glycans peripheral residues have been removed, the remainders of the O-glycan chains can be hydrolyzed. The released saccharides, such as N-acetylglucosamine, N-acetylgalactosamine, galactose, fucose and N-acetylneuraminic acid (sialic acid) can be used by the bacterial degraders themselves or by other resident bacteria (Bjursell, Martens and Gordon 2006; Martens, Chiang and Gordon 2008; Sonnenburg *et al.* 2010). Commensal *E. coli* and *Enterococcus* are examples of cross-feeders unable to feed on mucin without microbial pre-digestion (Sicard *et al.* 2017).

2.2.3.3 Horizontal distribution of carbohydrate degradation in and between the ecological niches

Inside the ecological niche, microorganisms can be classified as generalists or specialists based on their CAZyme equipment. Generalists can use a large number of different carbohydrate structures. When comparing the two main phyla inhabiting the human gut, *Bacteroidetes* are usually considered more generalist than Firmicutes (Kaoutari *et al.* 2013). With 308 CAZyme genes, *Bacteroidetes thetaiotaomicron* is a good example of a generalist species (Martens, Chiang and Gordon 2008). On the opposite, other bacteria using relatively few polysaccharides, such as *Ruminoccocus bromii* (starch degrader only) and *Roseburia inulinivorans* (inulin degrader), are termed as “specialists” (Koropatkin, Cameron and Martens 2012). Thanks to their CAZyme arsenal, generalist microorganisms can shift their metabolism depending on the diet and are thought to be highly adaptable to different conditions depending on dietary fiber availability (Koropatkin, Cameron and Martens 2012). When several carbon sources are available, generalists exhibit hierarchical polysaccharide preferences (Rogers *et al.* 2013). Generalists can even switch between the consumption of glycans from different ecological niches like dietary fiber and mucus glycans (Sonnenburg 2005). When both compartments are available, some species as *Bacteroides thetaiotaomicron* prioritizes dietary fiber over mucus glycans consumption (Kashyap *et al.* 2013), but this sense of priority is not shared by all microorganisms. *Bacteroides massiliensis* and *Bacteroides fragilis* are more oriented towards mucosa-associated glycans (Pudlo *et al.* 2015). Indeed, large differences can be observed between species of a same genus. As an example, *Bacteroides thetaiotaomicron* and *Bacteroides ovatus*, which have 96.5% identity in their 16S rRNA gene sequences have less than one-third of their *sus*-like systems genes in common (Martens *et al.* 2011). This

versatility in carbohydrate consumption implies that a fiber-depleted diet will drive the microbiota to use the pool of indigenous host glycans present in the mucus (Earle *et al.* 2015; Desai *et al.* 2016). Accordingly, low fiber diets increase the expression of microbiota O-glycan CAZymes (Sonnenburg 2005), as well as mucinases (Desai *et al.* 2016). This results in increased inner mucus layer permeability as illustrated in murine models (Schroeder *et al.* 2018; Khoshbin and Camilleri 2020), in which dietary fiber supplementation can reverse the loss of mucus integrity (Schroeder *et al.* 2018). Lastly, "versatile" species are often opposed to "mucus specialists" which rely on mucus glycans as sole carbon source (Cockburn and Koropatkin 2016). *Akkermansia muciniphila* is a good example of a mucus specialist (Derrien, Belzer and de Vos 2017).

Bullet points, carbohydrate metabolism by human gut microbiota

- The microbial enzymes involved in carbohydrate degradation are named CAZymes (for carbohydrate-active enzymes). Some of them target both mucus glycans and dietary fibers.
- The complete degradation of both dietary fibers and mucus glycans involves several enzymes carried by different microorganisms. Thus, their degradation is sequencial, involving primary degraders and cross-feeders.
- Some organisms can degrade both fibers and mucus glycans. They are called generalists as opposed to specialists. This substrate versatility implies that a fiber-deprived diet forces a part of the microbiota to switch to mucus consumption, imparing mucus integrity.

2.2.4. Effect of carbohydrates on gut microbiota composition and sources of variability

2.2.4.1. Well-known effect of dietary fibers on the gut microbiota

Large observational studies taught us that long-term dietary fiber consumption affects human gut microbiota composition by evolutionary means (Yatsunenko *et al.* 2012; Clemente *et al.* 2015; Smits *et al.* 2017). Globally, consumption of fiber-rich diet protects microbiota diversity and preserves (and selects) fiber-degrading species. Multiple independent studies in humans have demonstrated stark differences in terms of gut microbiota composition and activity between urbanized populations consuming low fiber diets and rural populations. Westernization is characterized by a lower bacterial diversity, a lower *Prevotella*/*Bacteroides* ratio and a loss of CAZymes genes (Yatsunenko *et al.* 2012; Clemente *et al.* 2015; Martínez *et al.* 2015; Smits *et al.* 2017; Makki *et al.* 2018), supporting the disappearance of bacterial species and their degrading functions over time. These observations have been confirmed in a mice model in which fiber-low diet results in a progressive loss of microbiota diversity over

generations. The authors showed that this loss can be reversed by fiber administration at the scale of one individual, but not after several generations (Sonnenburg *et al.* 2016). Besides, it seems that long-term cross-generational consumption of (previously) indigestible dietary fiber can select for new specific degrading capabilities of the microbiota in a specific population (Hehemann *et al.* 2012). In that sense, even rarely ingested dietary fibers (Kitahara *et al.* 2005; Hehemann *et al.* 2010, 2012) or long-considered unfermentable ones (De Filippo *et al.* 2010) can be catabolised by the gut microbiota of accustomed populations. Japanese consuming diets enriched in uncooked seaweed possess in their microbiota very rare genes (acquired from the environmental bacterium *Bacteroides plebeius)* encoding porphyranase and agarase enzymes enabling their digestion (Kitahara *et al.* 2005; Hehemann *et al.* 2010, 2012).

Compared to the variations observed in long-term studies, the effect of short-term interventions with dietary fiber appears much more modest, less permanent and with higher inter-subject variability, suggesting a day-to-day adaptation of the gut microbiota to the diet and dietary fiber in particular (Turnbaugh *et al.* 2009; Wu *et al.* 2011; ANR MicroObes consortium *et al.* 2013). Interestingly, most of these short-term studies have focused on the effect of a specific fiber rather than using a rich/low fiber diet. Thus, the reported effects vary widely depending on the type of fiber investigated (Martínez *et al.* 2010), its crystalline form (Tester, Karkalas and Qi 2004; Lesmes *et al.* 2008), the degree of polymerization (Hughes *et al.* 2008; Sanchez *et al.* 2009; Van den Abbeele *et al.* 2011; Zhu *et al.* 2017) and the dose (Bouhnik *et al.* 1999; Davis *et al.* 2011). There are different mechanisms by which a dietary fiber could influence microbiota composition on a short-term scale. For instance, dietary fiber fermentation generates SCFA leading to a lower colonic pH. High and rapid decrease of pH with highly fermentable fibers then will enrich gut microbiota in microbial groups resistant to low pH (Scott, Duncan and Flint 2008; Duncan *et al.* 2009). Dietary fibers are also able to trap bile salts (Story and Kritchevsky 1978), slow glucose absorption and modulate the immune system (Hooper, Littman and Macpherson 2012), mechanisms by which the microbiota composition is in turn affected. Then, as seen in long-term studies, dietary fiber can specifically enrich groups with corresponding dietary fiber degradation capabilities if these groups are already present. For instance, resistant starch supplementation has been found to increase *Ruminoccocus bromii* population, a well-known resistant starch degrader, in human feces (Salonen *et al.* 2014; Vital *et al.* 2018).

2.2.4.2. Impact of mucus and mucus-associated glycans on gut microbiota composition

Numerous *in vivo* studies have shown different composition between luminal and mucosal microbial communities (Section 2.2.1). Thanks to their flexibility, *in vitro* models may also bring complementary information. In the Simulator of the Human Intestinal Microbial Ecosystem model (SHIME) (detailed in section 5.3), incorporation of mucin-agar microscosms resulted in enrichement of this mucin layer in Firmicutes (with *Clostridium* cluster XIVa accounting for almost 60% of the mucin-adhered microbiota) and in species with known mucosal adherence capabilities as *Lactobacillus mucosae* as observed *in vivo* (Van den Abbeele *et al.* 2012, 2013). In the same model, Van Herreweghen and colleagues evaluated the effect of mucin at 4.g.L^{-1} on gut microbiota composition. Such dose significantly impacted microbial communities (26% of observed variations at the OTU level), with enrichment in *Akkermansia*, *Bacteroides* and *Ruminococcus* genera known to have mucin-degrading capabilities (Van Herreweghen *et al.* 2018).

Concerning the specific impact of mucus carbohydrates on gut microbiota composition, some pieces of evidence have been gathered *in vivo*. Mice deficient in core 1- derived O-glycans exhibit a dysbiotic faecal microbiota (Sommer *et al.* 2014) and mice deficient in core 1- and core 2- derived O-glycans develop microbiota-dependent colitis (Bergstrom *et al.* 2016). However, since modifications of mucin glycosylation patterns affect mucus barrier function, it appears challenging to decipher whether this dysbiotic microbiota results from direct modulation of microbial communities or from other induced phenomenon, such as inflammation. More interestingly, Wacklin and colleagues have shown that human ABO blood groups, expressed in mucus O-linked glycans, are also involved in differences in intestinal microbiota composition (Wacklin *et al.* 2011). Specifically, fecal microbiota from individuals harboring the B antigen on their mucosal surface (secretor B and AB) differed from the non-B antigen groups (Mäkivuokko *et al.* 2012). A study performed in mice confirmed these observations with differences in microbiota composition according to the presence or not of blood groups antigens, but also gave additional information on the effect of dietary fibers. Differences in blood group antigen microbiota were noticed only when mice diet was depleted in dietary fiber, suggesting the impact of mucus glycosylation on microbiota composition gains importance when mucus polysaccharides are the sole carbohydrate type left (Kashyap *et al.* 2013).

Bullet points, effect of carbohydrates on gut microbiota composition

- Dietary fibers and mucin are both known to affect gut microbiota composition, certainly through substrate availability. However, other indirect effects have been suggested especially for dietary fiber (e.g. bile salt trapping, inhibition of glucose absorption, modulation of the innate immune system)

- Some clues in favor of the specific impact of mucus carbohydrates are also available *in vivo*, essentially based on blood group antigen in mucus-O linked glycans.

To summarize this chapter, similarities and differences between dietary fibers and mucus-glycans are summarized bellow in **Table 2.2**.

Table 2.2. Similarities and differences between dietary fibers and mucus glycans.
Updated from Sauvaitre *et al.* 2021.
GIT: GastroIntestinal Tract, PUL: Polysaccharide Utilization Loci.

	Dietary fibers	Mucus glycans
1. General features		
Origin	Exogenous	Endogenous
Qualitative presence in the gut	Variable (dependent upon dietary intakes)	Constant (continuously produced/secreted by goblet cells)
Structure (polysaccharide composition)	More than 20 possible monosaccharide units	6 possible monosaccharide units, some in common with dietary fiber
Non-microbial factors influencing composition	Environmental factors (diet including cooking/preparation methods)	Environmental factors (mainly diet) Region of the GIT Genetic Ageing
Physiological functions / Health promotion properties	Faecal bulking / Transit time reduction Trapping of bile salts Reduction of glucose absorption Immune system modulation Microbiota maintenance	Lubrication of the epithelium Maintenance of the epithelial barrier Immune system modulation Microbiota maintenance
2. Feeding mechanisms		
Microbiota accessibility	Soluble fibers are easily accessible Insoluble fibers can be considered as a physical niche with reduced accessibility	Mucus shed in the digestive lumen is easily accessible Inner colonic layer is a physical niche nearly devoid of bacteria
Niche colonisation	The microbial communities colonising insoluble fiber particles are enriched in microorganisms with degrading functions	Mucus is colonised by microorganisms with more or less degrading functions, the presence of such microbes is dependent upon dietary fiber availability
Binding	Microorganisms are able to use carbohydrate-binding molecules, specific proteins from extracellular structure and lectins to bind to dietary fiber	Microorganisms are able to use carbohydrate adhesins to bind to mucus
Degradation	Degradation involves several enzymes: Glycoside Hydrolases, Polysaccharide Lyases and Carbohydrate Esterases	Degradation involves several enzymes: Sulfatases, Glycoside Hydrolases and Polysaccharide Lyases. Some enzymes are common with dietary fiber consumption
Fermentation	Once hydrolyzed, mucus and dietary fiber polysaccharides monomers are fermented by gut microbiota leading to the production of metabolites such as SCFA.	
3. Ecological characteristics		
Vertical / Cross-feeding relationships	By releasing or exposing simple polysaccharides, primary degrading-species allow cross-feeding species to feed themselves Primary degraders are considered to harbor complex dietary fiber degrading apparatus (cellulosome, PULs, …)	Primary degraders have to handle external residues and possess appropriate GHs (sulfatase, sialidases…)
Horizontal ecological relationships	Degradation by dietary fiber degrading species and versatile species	Degradation by mucin degrading specialists and versatile species
Impact on gut microbiota composition	Gut microbiota composition is highly dependent on the daily and long-term dietary fiber intakes and composition	The potential impact of mucus polysaccharides composition on gut microbiota composition gains in importance when the diet is depleted in dietary fiber

3. Interactions enteric pathogens / mucus / dietary fiber

Several evidences suggest that the three-way relationship between gut microbiota, dietary fiber and mucus layer could unravel the capacity of enteric pathogens to colonise the human digestive tract and ultimately lead to infection. In this section, we argue of the relevance of using dietary fiber interventions to prevent enteric infections with a focus on gut microbial imbalance and impaired-mucus integrity.

Some parts of this state of the art have been published in a review article in the FEMS Microbiol. Rev journal (Impact Factor: 16.408) and redrafted / updated for the present section.

Review: SAUVAITRE T, ETIENNE-MESMIN L, SIVIGNON A, MOSONI P, COURTIN CM, VAN DE WIELE T, BLANQUET-DIOT S. Tripartite relationship between gut microbiota, intestinal mucus and dietary fibers: towards preventive strategies against enteric infections. FEMS Microbiol Rev. 2021 Mar 16;45(2): fuaa052. doi: 10.1093/femsre/fuaa052.

3.1. Mucus role in pathogens virulence

3.1.1. Pathogens binding to mucus

Most of the enteric pathogens including *Enterobacteriaceae* have to reach the intestinal epithelium and invade the mucosal barrier to promote their colonisation or persistence. Binding to mucus is, therefore, the primary colonisation challenge for pathogens (Sicard *et al.* 2017) but it can also favor subsequent bacterial adhesion. *In vitro* adherence of *Salmonella enterica* serovar Typhimurium and Enterohemorrhagic *E. coli* (EHEC) is higher on high-mucus producing cells (e.g. Ht29-MTX or LS174T) than in non- or low-mucus producing cells (e.g. Caco-2 or HT29) (Gagnon *et al.* 2013; Hews *et al.* 2017). As for commensals, pathogens use surface-associated appendages (surface adhesins, fimbriae and flagella) to bind to mucus polysaccharides. For instance, *Helicobacter pylori* and *Campylobacter jejuni* possess several characterized adhesins that notably bind to blood group antigens and to sialic acid (Mahdavi 2002; Avril *et al.* 2006; Heikema *et al.* 2010; Moran, Gupta and Joshi 2011; Kenny *et al.* 2012; Rossez *et al.* 2014) while GbpA from *Vibrio cholerae* binds to N-acetylglucosamine (Bhowmick *et al.* 2008; Wong *et al.* 2012). Flagellar subunits of *Campylobacter jejuni* (Sicard *et al.* 2017), enteropathogenic *E. coli* (EPEC) (Erdem *et al.* 2007) and *Clostridioides difficile* are all able to bind mucus polysaccharides. *Enterobacteriaceae* can interact with mucus glycans via various appendanges like type 1 pili (Sokurenko *et al.* 1998; Schembri *et al.* 2001; Aprikian *et al.* 2007). Std fimbriae from *Salmonella enterica* serovar Typhimurium interacts with terminal fucose residues (Chessa *et al.* 2009) and mannose (Vimal *et al.* 2000).

Pathogen-mucus interactions are built on the recognition of specific saccharide motifs. As mucus polysaccharide composition changes all along the human GIT, it could be a strategy for precise site targeting in the gut (Owen *et al.* 2017). The pathogens also have to deal with host-related parameters known to induce variations in mucus structure and composition, such as genetics, diet, degradation by host microbiota and diseases. Illustrating this pathogen pattern dependency, *Helicobacter pylori* infections were found most prevalent in individuals from O than A group, suggesting a preferred attachment of the bacteria to O- blood group antigen present in the mucus (Kościelak 2012). Some pathogenic bacteria as *Shigella flexneri*, *Helicobacter pylori* and *Citrobacter rodentium* are even able to reshape mucin glycosylation patterns (Sperandio *et al.* 2013; Pham *et al.* 2014; Magalhães *et al.* 2015). These modifications may adjust the expression of bacterial receptors (Barnich *et al.* 2007; Corfield 2018) or impact the gut microbiota colonisation barrier (Pham *et al.* 2014). For instance, *Helicobacter pylori* infection affects host expression resulting in increased sialylation patterns that favor *Helicobacter pylori* SabA-mediated adhesion (Magalhães *et al.* 2015).

3.1.2. Mucus degradation by pathogens

To face the mucus net-like properties, pathogens possess proteases called mucinases. These mucinases are classified according to the functional group involved in catalysis (e.g. metallo, serine and aspartic proteases), their site of action (endo- or exo- proteases) and their evolutionary relationships related to their amino acid sequence (Carroll 2013). Even if some mucinases, as SslE, are known to exist in both secreted and surface-associated forms, most of the characterized mucinases are secreted in the external environment by pathogens, probably for a wider impact on the mucus structure (Etienne-Mesmin *et al.* 2019). Mucinases have been well characterized in *Enterobacteriaceae*, in particular in enterotoxigenic (ETEC) and enterohemoragic (EHEC) *E. coli*, with a diverse arsenal of mucinases, such as SslE, StcE, Hbp, YghJ and EatA (Kumar *et al.* 2014). In Adherent Invasive *E. coli* (AIEC), mucinase Vat-AIEC is over-expressed in the presence of bile salts and mucin, and contribute to bacteria penetration in the mucus layer to establish gut colonisation (Gibold *et al.* 2016). Mucinases have also been evidenced in *Vibrio cholerae* (Szabady *et al.* 2011), *Yersinia enterocolitica* (Mantle and Rombough 1993) and *Clostridioides difficile* (Janoir *et al.* 2007), suggesting the involvement of mucus depolymerisation during infection processes.

As already mentioned, some commensal bacteria also possess mucinases, highlighting the fine line between pathogenicity and commensalism in the GIT. As an example, SslE is found both in commensal *E. coli* and in ETEC and EHEC pathogenic strains (Etienne-Mesmin *et al.*

2019; Tapader, Basu and Pal 2019). Nevertheless, differences between commensal and pathogen mucinases reside at least in their expression levels. For example, pathogenic *E. coli* generate significant amounts of YghJ compared to their commensal counterparts, while there is no difference in the putative catabolic amino acid sequences (Luo *et al.* 2014). Lastly, as for CAZymes, mucinases seem to have substrate specificities. For example, StcE preferentially cleaves MUC2 compared to MUC5AC (Hews *et al.* 2017) and YghJ targets MUC2 and MUC3 (Luo *et al.* 2014).

By their degrading potential, CAZymes, and notably GHs, could be another way to cleave mucus but this activity has been poorly described in pathogens. To date, pathogen GHs, as commensal ones, have been preferentially studied as feeding tools rather than mucus-degrading enzymes. As a nice example, *Salmonella* contains 47 GHs that may degrade glycans. During infection in mice, specific deletion of *nanH* and *malS* genes impedes bacterial invasion, suggesting that these GHs may be considered as new virulence factors (Arabyan *et al.* 2016). *Bacteroides fragilis* has also been shown to require special polysaccharide utilization loci (containing GH along other CAZymes) for crypt colonisation, and mutants strains deficient for these loci failed to occupy crypts (Lee *et al.* 2013). However, it is not possible to decipher if these GH knock-out defects in colonisation can be attributed to mucus-degrading defect or to loss of feeding capabilities on other carbohydrate sources.

3.1.3. Mucus-based feeding of pathogens

3.1.3.1. Primary degraders or cross-feeding strategies

CAZymes are also used by some pathogens to release mucus-derived sugars for their own consumption (Mondal *et al.* 2014; Arabyan *et al.* 2016). *Salmonella enterica* serovar Typhimurium has the ability to release carbohydrates from mucus by using its sialidase (Hoyer *et al.* 1992). Interestingly, *Vibrio cholerae* uses its chitinase ChiA2 to feed on both chitin fibers found in the aquatic environment and mucins in the gut (Mondal *et al.* 2014), most probably because of their structural similarities (chain polymers of β-1,4 linked N-acetylglucosamine residues). In line with this observation, mutants for chitin utilization pathway display less capacity to penetrate mucus and are hypovirulent in a mouse model (Chourashi *et al.* 2016). Besides these examples, pathogens usually behave as non-primary degraders. They have limited CAZymes arsenal and often count on other mucin degraders to cross-feed. *E. coli* pathogens represent a good example of this strategy. Indeed, they colonise the mouse large intestine by growing in intestinal mucus, but as they do not secrete extracellular GHs, they cannot degrade

mucin-derived oligo- and polysaccharides and depend on other microbes which feed them with small saccharides and promote their own growth (Conway and Cohen 2015). In a gnotobiotic mouse model, EHEC colonise the mucus layer within the cooperation of local bacterial communities including *Bacteroides thetaiotaomicron* that cleaves host glycan-derived sugars and produces fucose (Pacheco *et al.* 2012). Similarly, *Bacteroides thetaiotaomicron* is also able to release free sialic acid from colon mucus glycans that can be further used by *Clostridioides difficile* and *Salmonella enterica* serovar Typhimurium to promote their own colonisation and persistence in the gnotobiotic mice gut (Ng *et al.* 2013). To date, more investigations are required to decipher if these cross-feed relationships also exist in the human gut.

3.1.3.2. Importance of microbial background

When gut microbiota is not disturbed, pathogens have to compete with commensal non-primary feeders to use mucus carbohydrates. Conway and Cohen (2015) showed that when gnotobiotic mice are pre-colonised with only three commensal *E. coli* strains, these strains use all the mucus monosaccharides uptake possibilities to outcompete the pathogenic EHEC strain, leading to pathogen elimination (Leatham *et al.* 2009). In response, EHEC can utilize a large panel of mucus-derived monosaccharides and thereby compete with commensal *E. coli* (Fabich *et al.* 2008). The metabolic flexibility of some pathogenic strains to use both glycolytic and gluconeogenic nutrients from the host may also represent a competitive advantage (Bertin *et al.* 2013). To outcompete the native microbiota, pathogens can benefit from gut disturbance that will let ecological niches free. For instance, in human, antibiotic use is one of the leading risk factors for enteric diseases associated with *Salmonella* and *Clostridioides difficile* infections (Pépin *et al.* 2005; Doorduyn *et al.* 2006; Dethlefsen *et al.* 2008). Of interest, antibiotic treatment is also one of the drivers modulating mucin carbohydrates availability. Studies in mice showed that antibiotic treatment induced a spike in mucus-derived monosaccharides such as sialic acid, and these high concentrations of free monosaccharides facilitated the expansion of *Salmonella enterica* serovar Typhimurium and *Clostridioides difficile* (Ng *et al.* 2013). As further evidence, colonisation of gnotobiotic mice with a sialidase-deficient mutant of *Bacteroides thetaiotaomicron* induced reduction of free sialic acid levels impairing expansion of *Clostridioides difficile*. These transient effects could be reversed by exogenous dietary administration of free sialic acid (Ng *et al.* 2013).

3.1.4. Pathogens and inflammation in a mucus-altered context

There is scarce but promising evidence that inflammation driven by mucus alterations may support pathogen infection. First, in mouse models, defects in mucus glycosylation are clearly associated with inflammation (An *et al.* 2007; Stone *et al.* 2009; Burger-van Paassen *et al.* 2011). This inflammation occurs only when gut microbiota is present, suggesting that the close proximity between microbes and the epithelial brush border drives the response (Bergstrom *et al.* 2016). Besides, mice with genetically impaired mucus layer are more susceptible to pathogens such as *Salmonella enterica* (Bergstrom *et al.* 2010; Zarepour *et al.* 2013; Hecht *et al.* 2017). Altogether, mucus defects appear to be involved both in inflammation and pathogen susceptibility. As mucus over-degradation triggers an inflammatory state, we may hypothesize that mucus-degrading microorganisms or microorganisms benefiting from mucus degradation would be more adapted to an inflammatory environment. In this sense, colitis induced with dextran sodium sulfate seemed to favor microorganisms expressing genes involved in mucus polysaccharide utilization (Schwab *et al.* 2014). In the same way, studies suggest that pathogens could also benefit from this pro-inflammatory state. In both human and mice, inflamed microbiota is characterized by a reduced abundance of obligate anaerobic bacteria and expansion of facultative anaerobic bacteria from Proteobacteria phylum, mostly members of the family *Enterobacteriaceae* (Seksik 2003; Gophna *et al.* 2006; Baumgart *et al.* 2007; Lupp *et al.* 2007; Walker *et al.* 2011; Gevers *et al.* 2014; Chiodini *et al.* 2015). *Enterobacteriaceae* may also support this inflammatory state to promote their own persistence in the gut (Garrett *et al.* 2010). Interestingly, inflammation could also impact the mucus layer itself to favor the pathogen. *Salmonella* have adapted their own metabolism and trigger inflammation-induced mucus fucosylation, allowing the pathogen to feed on fucose (Ansong *et al.* 2012; Bäumler and Sperandio 2016) in an inflammatory state.

3.1.5. Modulation of virulence genes by mucin and their degradation products

In addition to acting as binding sites or carbon sources for pathogens, mucin glycoproteins can influence the expression of different pathogen virulence genes, as shown by many *in vitro* studies (Vogt, Peña-Díaz and Finlay 2015). Many virulence genes of *Campylobacter jejuni* are upregulated *in vitro* in the presence of MUC2 glycoprotein (Tu, McGuckin and Mendz 2008) and fucose especially influences chemotaxis and biofilm formation that are important during gut infection (Dwivedi *et al.* 2016). In response to mucins,

Vibrio cholerae also downregulates polysaccharide synthesis pathways involved in biofilm formation, thus promoting its motility within the mucus (Liu *et al.* 2015). Released monosaccharides from mucin O-glycan degradation can also act as a chemical cue to help pathogens to sense their environment and adapt accordingly. As illustrated with EHEC, fucose represses EHEC LEE (Locus of Enterocyte Effacement) expression involved in the formation of attachment and effacement lesions (Pacheco *et al.* 2012). The study postulates that gene repression through fucose-sensing may prevent energy expense in EHEC during LEE production before reaching the epithelial surface, where free fucose is not present (Pacheco *et al.* 2012). N-acetylglucosamine and sialic acid have also a negative effect on LEE expression under aerobic conditions (Le Bihan *et al.* 2017) but stimulate the production of a LEE effector (EspB) under micro-aerobic conditions, which are those found at a close proximity of the intestinal epithelium (Carlson-Banning and Sperandio 2016). Therefore, the availability of free monosaccharides is not the sole determinant factor in pathogen virulence regulation, but other parameters associated to bacterial localization, such as oxygen conditions, must be considered.

Bullet points, mucus role in pathogens virulence

- Pathogens recognise/bind mucus polysaccharides by adhesins on the cell surface or associated to appendages (fimbriae and flagella), degrade mucin thanks to mucinases to facilitate access to the intestinal epithelium, and feed with CAZymes.
- To date, pathogenic bacteria are considered to behave as non-primary degraders with limited CAZymes arsenal, cross-feeding on simple carbohydrates released by other mucus specialists.
- Inflammation driven by mucus alterations may support pathogen infection as pathogens have adapted to benefit from such inflammation and its impact on mucus composition.

3.2. Dietary fiber modulation of enteric pathogen virulence

3.2.1. Direct antagonistic effect of dietary fibers on pathogens

3.2.1.1. Bacteriostatic effect

Some dietary fibers as chitosan (derived from chitin) have shown a direct bacteriostatic effect by inhibiting the growth of various pathogens (**Table 3.1**), especially EHEC (Chantarasataporn *et al.* 2014; Ma *et al.* 2016; Vardaka, Yehia and Savvaidis 2016; Garrido-Maestu *et al.* 2018). Chitosan antimicrobial activity probably results from the intracellular leakage via binding positively charged chitosan to negatively charged bacterial surface, leading membrane permeability alteration causing cell death (Jeon *et al.* 2014). Of interest, the broad

in vitro effect of chitosan is also conserved *in vivo* for ETEC, EHEC and others animal pathogens, by decreasing pathogen colonisation (Jeong *et al.* 2011; Xiao *et al.* 2014; Jeon *et al.* 2016; Liu *et al.* 2016a).

Table 3.1. Summary of studies investigating dietary fiber inhibition properties against pathogen growth.
Extraced from Sauvaitre *et al.* 2021.

References	Tested fiber(s)	Doses	Pathogens	Growth media	Observed effect
Liu *et al* 2000	Chitosan	1 $g.L^{-1}$	*Agrobacterium tumefaciens*, *Bacillus cereus*, *Corinebacterium michiganence*, *Erwinia sp.*, *Erwinia carotovora subsp*, *Escherichia coli*, *Klebsiella pneumoniae*, *Micrococcus luteus*, *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Xanthomonas campestris* (strains unspecified)	Acetic acid (2M)	Bacteria growth inhibition
Qi *et al* 2004	Chitonsan nanoparticules	Nanoparticles at 0.0125 mg.L-1 and raw chitosan at 64 $mg.L^{-1}$	*Escherichia coli* (strains K88 and ATCC 25922), *Salmonella choleraesuis* (strain ATCC 50020), *Salmonella typhimurium* (strain ATCC 50013) and *Staphylococcus aureus* (strain ATCC 25923)	Acetic acid (0.25%) in water at pH 5.0	100 % bacterial lethality
Chantarasata porn *et al* 2014	Chitosan derived oligosaccharid es	Up to 0.2 $g.L^{-1}$	EHEC O157 :H7 (strain DMST 12743), *Staphylococcus aureus* (strain ATCC 6538), *Listeria monocytogenes* (strain ATCC 19115), *Bacillus cereus* (strain C113) and *Salmonella enteritidis* (strain DMST 1706)	Trypticase Soy broth	Bactericidal activity
Jeon *et al* 2014	Chitosan microparticules	2 $g.L^{-1}$	EHEC O157:H7 EDL933 (strain ATCC48935), intra-uterine pathogenic *Escherichia coli* (strain unspecified), *Salmonella enterica* strain CDC3041-1, *Klebsiella pneumoniae* (strain unspecified), *Vibrio cholera* (strain 395 classical O1) and S*treptococcus uberis* (strain unspecified)	Luria Bertani medium Brain Heart Infusion broth (for *Streptococcus uberis*)	100 % bacterial lethality
Ma *et al* 2016	Chitosan microparticules	40 $mg.L^{-1}$	EHEC O157:H7, S*treptococcus uberis*, *Salmonella enterica*, *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Enterococcus*, *Vibrio cholerae* (O1 El Tor), *Vibrio cholerae* (non-O1), *Vibrio cholerae* (O395)	Mueller Hinton broth Simulated gastrointestinal fluids	100 % bacterial lethality
Garrido-Maestu *et al* 2018	Chitosan nanoparticules	2 $g.L^{-1}$	EHEC O157 H7 (strain unspecified)	Luria Bertani broth	100 % bacterial lethality

3.2.1.2. Inhibition of cell adhesion

Dietary fibers from different sources have proven efficiency in reducing pathogenic *E. coli* adhesion to intestinal epithelial cells. Many of these fibers have a plant origin (Rhoades *et al.* 2008; Roubos-van den Hil *et al.* 2009, 2010; Gonzales *et al.* 2013; Di *et al.* 2017). For example, soluble fiber extract from plantain bananas reduced adhesion of AIEC, ETEC and *Shigella* strains to intestinal epithelial cells (Martin *et al.* 2004; Roberts *et al.* 2010). β-

galactomannan from yeast are also able to decrease ETEC adhesion on Caco-2 cells (Badia *et al.* 2012). Yeasts harbor numerous oligomannosides on their cell wall able to interact with FimH adhesin of type 1 pili and represent an interesting anti-adherence strategy in reducing pathogenic *E. coli* adhesion (Sivignon *et al.* 2015; Roussel *et al.* 2018b). Bacterial exopolysaccharides from *Lactobacillus* spp. also inhibited EHEC adhesion on HT29 cells as well as biofilm formation (Kim, oh and Kim 2009; Liu *et al.* 2017). These exopolysaccharides do not necessarily contain mannose supporting other possible inhibitory effects (Liu *et al.* 2017). Lastly, adhesion of ETEC strains to intestinal Caco-2 cells was reduced by addition of human HMO (Idota and Kawakami 1995; Salcedo *et al.* 2013) and goat milk oligosaccharides were also proven to decrease adhesion of human enteric pathogen as *E. coli* and *Salmonella enterica* serovar Typhimurium in a Caco-2 cells model (Leong *et al.* 2019). Reduction of bacteria adhesion could be explained by shared patterns between mucin polysaccharides and dietary fibers, resulting in dietary fibers acting as a bait for bacteria which will be decoyed from the mucus compartment. **Table 3.2** summarizes the studies reporting dietary fiber inhibition properties against pathogen adhesion.

Table 3.2. Summary of studies investigating dietary fiber anti-adhesion effect against pathogens.

Updated from Sauvaitre *et al.* 2021.

AIEC: Adherent-invasive *E. coli*, CCL20: Chemokine (C-C motif) ligand 20, CXCL8: interleukine 8, EHEC: Enterohemorrhagic *E. coli*, EPEC: enteropathogenic *E. coli*, ETEC: enterotoxigenic *E. coli*, GM-CSF: Granulocyte-macrophage colony-stimulating factor, IL6: interleukin-6, M-SHIME: Mucosal Simulator of the Human Intestinal Microbial Ecosystem, TNFα: tumor necrosis factor.

References	Tested fiber(s)	Doses	Pathogens	Cell or adhesion test model	Observed effect
Cravioto *et al* 1991	Human milk oligosaccharides	3 $g.L^{-1}$	EPEC (strains O1163, O1736, 851/71, E2348)	Hep-2 cells (Human, carcinoma)	Up to 92.8 % adhesion inhibition with the pentasaccharides fraction against EPEC strain 01163
Stins *et al* 1994	NeuAc alpha 2,3-sialyl lactose	50 µM	S fimbriated *Escherichia coli* (strain GB101/13)	Bovine brain cortices endothelial cells	80 % adhesion inhibition
Idota and Kawakami 1995	Human milk oligosaccharides (G_{M1} and G_{M3})	1 $g.L^{-1}$	ETEC (strain Pb-176)	Caco-2 cells (Human, colorectal adenocarcinoma)	70 and 80 % adhesion inhibition for G_{M3} and G_{M1} respectively
Martín *et al* 2002	Bovine milk oligosaccharides	0.33 g.L-1	ETEC strains from calves (K99-12, F41-15, K99-4, CCB1, CCB22, CCB33, CCB37)	Hemagglutination of erythrocytes	Hemagglutination inhibition depending on the saccharides and tested ETEC strains
Ruiz-palacios *et al* 2003	Alpha1,2-Fucosyllactose	0.2 $g.L^{-1}$	*Campylobacter jejuni* (invasive strain 287i)	Hep-2 cells (Human, carcinoma)	54.8 % adhesion inhibition
Martin *et al* 2004	Soluble plaintain fibers	5 $g.L^{-1}$	AIEC (strains HM427 and HM545)	HM427 cells (isolated from Crohn's disease patients) and HM545 cells (from the tumor tissue of a colon cancer patient)	83 to 95 % adhesion inhibition for the AIEC strains HM545 and HM427, respectively
Coppa *et al* 2006	Human milk oligosaccharides	10 $g.L^{-1}$	EPEC O119, *Vibrio cholerae* (strain ATCC 14034), and *Salmonella fyris* (unspecified strain)	Caco-2 cells (Human, adenocarcinoma)	Up to 42.2 % adhesion inhibition against EPEC strain O119
Shoaf *et al* 2006	Galactooligosaccharides	16 $g.L^{-1}$	EPEC (strain E2348/69)	HEp-2 cells (Human, carcinoma) and Caco-2 cells (Human, colorectal adenocarcinoma)	65 to 70% adhesion inhibition on Hep-2 and Caco-2 cells, respectively
Rhoades *et al* 2008	Pectin derived oligosaccharides	2.5 $g.L^{-1}$	EPEC (strains O11:H27, 0O19H4,O128:H12), EHEC (strains 123900, 13127, 13128), *Desulfovibrio desulfuricans* (strain 12833)	HT-29 cells (Human, colorectal adenocarcinoma)	Up to 90 %, 85 %, and 99 % adhesion inhibition for EPEC, EHEC and *Desulfovibrio desulfuricans* strains respectively
Kim *et al* 2009	*Lactobacillus acidophilus* exopolysaccharides	1 $g.L^{-1}$	EHEC O157:H7, *Salmonella enteritidis*, *Salmonella typhimurium* (strain KCCM 11806), *Yersinia enterocolitica*, *Pseudomonas aeruginosa* KCCM 11321, *Listeria monocytogenes* ScottA, and *Bacillus cereus* (unspecified strain)	Biofilm test formation	Up to 95 % biofilm formation inhibition with *Listeria monocytogenes* ScottA
Roubos-van den Hil *et al* 2009	Soluble fermented soya beans extract	2.5 $g.L^{-1}$	ETEC K88 (strain ID1000)	Caco-2 cells (Human, colorectal adenocarcinoma)	40 % adhesion inhibition
Roberts *et al* 2010	Plaintain and broccoli soluble fibers	5 $g.L^{-1}$	AIEC (strains LF82, HM580, HM605, HM615)	Caco2-cl1 cells (Human, colorectal adenocarcinoma) and Raji B cells (Human, burkitt's lymphoma) = M cell model	45.3 to 82.6 % inhibition of translocation of AIEC strains across M-cells for broccoli and plantain soluble fibers, respectively

Roubos-van den Hil 2010	Soluble fermented soya beans extract	10 $g.L^{-1}$	ETEC K88 (strain ID1000)	*Ex vivo* adhesion test to pig intestinal brush borders	99 % adhesion inhibition
Wang *et al* 2010	Reuteran and levan	5 and 10 $g.L^{-1}$	ETEC K88 (strains ECL13086, ECL13795, ECL13998 and ECL14048)	Hemagglutination of erythrocytes	Inhibition of hemagglutination
Badia *et al* 2012, a and b	Beta-galactomannan	0.5 to 20 $mg.L^{-1}$	*Salmonella enterica* serovar Typhimurium	IPI-2I cells (porcine, small intestine epithelium)	Up to 70 % adhesion inhibition Decrease of inflammation marker expression and cytokines production (IL6, CXCL8)
Badia *et al* 2012	Beta-galactomannan	10 $g.L^{-1}$	ETEC K88 (strains 56190 and GN1034)	Caco-2 cells (Human, colorectal adenocarcinoma))	80 % adhesion inhibition 50 % reduction of TNFα, GM-CSF and CCL20 expression, from 4 to 10- and 1.4-fold reduction of IL-6 and CXCL8 secretion, respectively
Salcedo *et al* 2012	Human milk oligosaccharides motifs	0.004 to 0.8 $mg.L^{-1}$	ETEC (strain CECT 685), EPEC (strain CECT 729), *Listeria monocytogenes* (strain CECT 935)	Caco-2 cells (Human, colorectal adenocarcinoma)	Up to 28 % adhesion inhibition on EPEC with GM_1 at 0.004 $mg.L^{-1}$
Gonzalez-Ortiz *et al* 2013	Locust bean, wheat bran soluble extract, exopolysaccharides	1 and 10 $g.L^{-1}$	ETEC K88 (strains: O149:K91:H10 [K-88]/LT-I/STb and F4-, F6-, F18-, LT1- ST1-, ST2+ Stx2e-)	IPEC-J2 cells (porcine, jejunal epithelium)	Up to 80 % adhesion inhibition depending on the strains with 10 $g.L^{-1}$ locust bean extract
Quintero-Villegas *et al* 2013	Chito-oligosaccharide	0.5 to 16 $g.L^{-1}$	EPEC (strain E2348/69, O127:H6)	HEp-2 cells (Human, carcinoma)	Up to 95 % adhesion inhibition at the dose 16 $g.L^{-1}$
Roberts *et al* 2013	Soluble plaintain fibers	5 $g.L^{-1}$	*Salmonella enterica* serovar Typhimurium (strain LT2), *Shigella sonnei* (strain unspecified), ETEC (C410) and *Clostridium difficile* (strain 080042)	Co-culture of Caco-2 (Human, colorectal adenocarcinoma) and Raji B cells (Human, burkitt's lymphoma) = M cell model	46.6 to 85 % inhibition of adhesion and 46.4 to 80.2 % decrease of translocation depending on the strains
Sarabia-sainz *et al* 2013	Neoglycans composed of conjugated porcine albumin and galactooligosaccharides	1 $g.L^{-1}$	ETEC K88 (strain unspecified)	Porcin gastric mucin	Adhesion inhibition as measured by decreased optical density
Chen et al 2014	Reuteran and levan	10 $g.L^{-1}$	ETEC K88 (strains ECL13795 and ECL13998)	Hemagglutination of erythrocytes	Inhibition of hemagglutination
Gonzalez-Ortiz *et al* 2014	Locust bean, wheat bran soluble extract	10$g.L^{-1}$	ETEC K88 (strains: O149:K91:H10 [K-88]/LT-I/STb and F4-, F6-, F18-, LT1- ST1-, ST2+ Stx2e-)	Microtitration-based adhesion tests on ileal mucus from piglets	Up to 95 % adhesion inhibition with wheat bran extract
Cilieborg *et al* 2016	Lactose and alpha1,2-Fucosyllactose	1 and 5 $g.L^{-1}$	ETEC F18 (strain 9910297–2STM)	PSIc1 cells (porcine, jejunal epithelium)	Up to 70 % adhesion inhibition with α-1,2-fucosyllactose at 5 $g.L^{-1}$
Van den Abbeele *et al* 2016	Inulin and galacto-oligosaccharides	3 g per day added to a continuously renewed compartment	AIEC (strain LF82)	M-SHIME experiment with a mucus compartment comprising mucin-agar-covered microcosms	More than 1 log decrease of AIEC counts in the mucus (could result from microbiota modulation - notably increase of mucosal lactobacilli and bifidobacteria counts)
Di *et al* 2017	Pectin derived oligosaccharides	0.001 to 5 $g.L^{-1}$	EHEC (strain ATCC43895)	HT-29 cells (Human, colorectal adenocarcinoma)	Up to 90 % bacterial adhesion inhibition at the dose 0.005 $g.L^{-1}$
Kuda *et al* 2017	Alginate	1 $g.L^{-1}$	*Salmonella enterica* serovar Typhimurium (strain NBRC 13245T)	HT-29 Luc cells (Human, colorectal adenocarcinoma)	70 to 80 % adhesion/invasion inhibition depending on alginate molecular weight

Liu *et al* 2017	*Lactobacillus plantarum* WLPL04 exopolysaccharides	0.01 to 1 $g.L^{-1}$	EHEC O157 H7 (strain unspecified)	HT-29 cells (Human, colorectal adenocarcinoma)	Up to 30 % adhesion inhibition and 60 % anti biofilm activity at the highest dose
Zhu *et al* 2018	Exopolysaccharides produced during industrial fermentation of olives	10 $g.L^{-1}$	ETEC K88 (strains: O149:K91:H10 [K-88]/LT-I/STb and F4-, F6-, F18-, LT1- ST1-, ST2+ Stx2e-)	IPEC-J2 cells (porcine, jejunal epithelium)	Up to 50 % adhesion inhibition depending on the exopolysaccharides
Leong *et al* 2019	Goat milk oligosaccharides and galactooligosaccharides	20$g.L^{-1}$ for galactooligosaccharides and at concentration found in infant formula for goat milk oligosaccharides	EPEC (strain NCTC 10418) and *Salmonella enterica* serovar Typhimurium (strain unspecified)	Caco-2 cells (Human, colorectal adenocarcinoma)	30 % adhesion inhibition for EPEC and *Salmonella enterica* serovar *Typhimurium*

3.2.1.3. Inhibition of toxin binding and activity

Interestingly, dietary fibers from human milk have also a direct inhibitory effect on pathogen toxins. Notably, sialyl lactose contained in milk is able to inhibit Cholera toxin (from *V. cholerae*) binding to its receptor the monosialotetrahexosylganglioside 1 (GM1) (Idota *et al.* 1995). Most of the results concerning toxin binding inhibition by HMO concern ETEC toxins (**Table 3.3**), and they they will be further developed in Section 4.4.5.

Table 3.3. Summary of studies investigating dietary fiber inhibition of toxin effects.
EHEC: Enterohemorrhagic *E. coli*, ELISA: ELISA: Enzyme-Linked Immuno Sorbent Assay, ETEC: enterotoxigenic *E. coli*, Gb3: Globotriaosylceramide, GM1: monosialotetrahexosylganglioside, GM2: monosialicganglioside 2, LT: heat labile toxin, ST: heat stable toxin, Stx: Shiga toxin.
Updated from Sauvaitre *et al.* 2021.

References	**Tested fiber(s) / Microorganisms**	**Doses**	**Toxins**	***In vitro* and *in vivo* models**	**Observed effect**
Otnaess *et al* 1983	GM_1	Unspecified	Cholera toxin and LT toxin from ETEC	Toxin binding ELISA assay and rabbit ileal loop assays	Inhibition of toxin binding to receptor and fluid secretions in rabbits' intestinal loops
Newburg *et al* 1990	Fucosylated fraction of human milk oligosaccharides	Unspecified	ST toxin	Mice	Higher mice survival rate
Idota *et al* 1995	Sialyllactose	75 and 100 $mg.L^{-1}$	Cholera toxin	Toxin binding assay and rabbits	Inhibition of toxin binding to receptor and fluid secretions in rabbit intestinal loops
Paton *et al* 2000	Gb3 expressing *E. coli*	Unspecified	Shiga toxin	Toxin binding assay and mice	Inhibition of toxin binding and full protection against EHEC (strains B2F1 and 97MW1) in mice
Paton *et al* 2005	GM_2 and other oligosaccharides expressing *E. coli*	Unspecified	LT toxin from E*scherichia coli* C600:pEWD299 (cloned LT operon)	Toxin binding assay and rabbits	Inhibition of toxin binding and reduction of fluid secretion in rabbits
Rhoades *et al* 2008	Pectic oligosaccharides	From 0.01 to 100 $mg.L^{-1}$	Shiga toxin (Stx1 and Stx2)	HT-29 cells viability test	Decreased intestinal cell death whatever the dose tested
Di *et al* 2017	Pectic oligosaccharides	From 1 to 100 $mg.L^{-1}$	Shiga toxin (Stx2)	HT-29 rRNA depurination test	Up to 44 % reduction of rRNA depurination

3.2.2. Indirect effect of dietary fibers through gut microbiota modulation

3.2.2.1. Modulation of microbiota composition

The resident microbiota is now widely recognised as a significant barrier to pathogen colonisation. This protective role is supported by many studies showing that commensal strains from gut microbiota promote inhibition mechanisms towards pathogens. Direct inhibitory effects are mediated by acid production, secretion of inhibitory molecules like bacteriocin or production of (mostly) unknown compounds able to repress virulence genes (Corr, Gahan and Hill 2007; Schoster *et al.* 2013; Sikorska and Smoragiewicz 2013). Therefore, microbiota modulation with dietary fibers may be a relevant mean to prevent enteric infections (Conway and Cohen 2015). However, demonstrating a positive effect mediated by microbiota modulation is not easy. Even if a dietary fiber supplementation does modify the microbiota and has anti-infectious properties, how to prove that the beneficial effect results from the increase or decrease of specific microbial groups? Some clues can emerge from the simultaneous administration of probiotic strains and dietary fiber to specifically support the probiotic growth (resulting in a prebiotic effect for dietary fiber). In 2001, Asahara and colleagues showed that pre-colonisation of mice with probiotic *Bifidobacterium breve* inhibited *Salmonella enterica* serovar Typhimurium growth and translocation in others organs (Asahara *et al.* 2001). This effect was strengthened by co-administration of *Bifidobacterium breve* with prebiotic GOS, while GOS alone did not show any anti-infectious properties. However, the authors did not prove any change in *Bifidobacterium breve* proportion or activity by GOS administration (Asahara *et al.* 2001). The continuous oral administration of the probiotic *Bifodobacterium breve* strain Yakult® inhibited mice infection by a multidrug-resistant *Acinetobacter baumannii* and GOS markedly potentiated the probiotic effect without providing any protection alone (Asahara *et al.* 2016). Another mouse study showed that the second generation probiotic *Faecalibacterium prausnitzii* plus potato starch reduced *Clostridioides difficile* colonisation in mice model, the combined effect being slightly better than the individual one (Roychowdhury *et al.* 2018). Lastly, in a continuous anaerobic fermentation system inoculated with human feces, combination of *Lactobacillus plantarum* 0407 and *Bifidobacterium bifidum* Bb12 together with oligofructose and XOS reduced *Campylobacter jejuni* growth whatever the mode of administration (prophylaxis treatment or co-administration with the pathogen). The dietary fiber alone failed to reproduce the combined effect of dietary fiber and probiotics but the dietary fiber did increase *Bifidobacteria* counts, supporting a prebiotic effect (Fooks and Gibson 2003).

Taken together, these *in vivo* and *in vitro* studies support that prevention of enteric infections by dietary fiber supplementation may be achievable. Nevertheless, the beneficial effect firstly depends on the previous identification of a specific probiotic group that can act in synergy with dietary fibers, without obvious associated prebiotic effect. Some evidences of dietary fiber efficiency against enteric infections are also available in humans, with the well-known prebiotics FOS and GOS. A study on 281 healthy infants reported that supplementation with GOS and/or FOS resulted in fewer episodes of acute diarrhea. Another study on 342 infants reported a lower incidence of gastroenteritis in the supplemented group with GOS and FOS compared to controls and reduced antibiotic courses/year (Bruzzese *et al.* 2009). Nevertheless, interpretation of these results is impeded by the lack of pathogen identification and gut microbiota characterization.

3.2.2.2. Modulation of gut microbiota activity

Microbial metabolites resulting from dietary fiber fermentation, such as SCFA can also modulate pathogen virulence. Acetate at the concentration found in the human ileum stimulates the expression of Type III secretion System (T3SS) from *Salmonella enterica* serovar Typhimurium, while propionate added at the typical concentration of the human colon, represses T3SS expression (Lawhon *et al.* 2002). Contradictory results have been obtained for butyrate (at concentrations found in the human colon) with repression or overexpression of T3SS depending on the studies (Lawhon *et al.* 2002; Takao, Yen and Tobe 2014). Mice fed a diet rich in highly fermentable guar gum exhibited a 10- to 100-fold increase in EHEC colonisation and developed illness compared to the control group fed with cellulose, which is considered as non-fermentable fiber (Zumbrun *et al.* 2013). This increased pathogenicity was associated to a rise in globotriaosylceramide expression (Shiga-toxin receptor), upregulated due to increase in butyrate concentrations (Zumbrun *et al.* 2013). Acetate produced by *Bifidobacteria* seemed to protect mice from EHEC toxic effect by increasing intestinal epithelium barrier function (Fukuda *et al.* 2011). Lastly, an elegant gnotobiotic mouse study showed that a dietary fiber-rich diet could promote *Clostridioides difficile* colonisation in presence of succinate produced by *Bacteroides thetaiotaomicron* (Ferreyra *et al.* 2014). Of note, such a study must be interpreted cautiously since the experiments have been conducted in gnotobiotic mice lacking a competitive microbiota that would normally occupy the succinate-feeding niche. These examples illustrate the complexity in dietary fiber-microbiota-pathogens interactions and the need to investigate in depth pathogen specificities before assuming any dietary recommendation.

3.2.3. Dietary fiber inhibition of pathogen interactions with mucus

3.2.3.1. Binding to mucus: dietary fiber acting as a decoy

Mucus polysaccharide patterns represent potential binding sites for intestinal pathogens and this observation can be extended to all mucosa surface-associated carbohydrates. Interestingly, saccharide-binding patterns are also found in dietary fibers and the hypothesis here is that dietary fibers can lure pathogens from mucus polysaccharides associated patterns by presenting similar binding site. The chitin-binding protein GbpA of *Vibrio cholerae* has been described as a common adherence factor for both chitin and intestinal surface, including mucus polysaccharides (Kirn, Jude and Taylor 2005; Wong *et al.* 2012; Younes and Rinaudo 2015). F17 fimbriae produced by ETEC strains targeting animals recognises N-acetylglucosamine-presenting receptors on the mucosa and this binding is inhibited by N-acetylglucosamine as well as N-acetylglucosamine oligomers (Buts *et al.* 2004). Blood group antigens on soluble glycans such as mucins or HMO may serve as decoy receptors in pathogen defense (Pendu *et al.* 1983; Renkonen 2000; Yu *et al.* 2001). It was shown that HMO have the potential to inhibit many pathogens binding to mucus. These results are relevant for both pathogens with a tropism to ileum and colon since over 90% of ingested HMO survive transit through the gut (Chaturvedi *et al.* 2001). HMO supplementation inhibited *Campylobacter* colonisation of mice *in vivo* and human intestinal mucosa *ex vivo* (Ruiz-Palacios *et al.* 2003). Specifically, *Campylobacter jejuni* binds to fucosylated carbohydrates containing the H(O) blood group epitope and this binding is inhibited by HMO. First evidences of HMO relevance in human enteric infection prevention come from breastfed infants who are at a 6-fold to 10-fold lower risk of developing necrotising enterocolitis than formula-fed ones (Lucas and Cole 1990; Schanler 2005; Poindexter *et al.* 2009). The infant protection would depend on HMO composition of the milk (Autran *et al.* 2018).

3.2.3.2. Inhibition of mucus degradation by dietary fiber, a new anti-infectious mechanism

The gut microbiota ability to switch to mucus polysaccharide consumption when fiber intake is low is a relatively new discovery (Sonnenburg 2005). Desai and colleagues were pioneers in extending this notion to enteric pathogen (Desai *et al.* 2016). In a gnotobiotic mice model colonised by a synthetic human microbiota of 14 species, they showed that a low fiber diet led the microbiota to switch to mucus polysaccharide consumption, and to enrichment in mucus degrading bacteria and mucus erosion. This greater penetrability induced a lethal

susceptibility to the murine pathogen *Citrobacter rodentium* (Desai *et al.* 2016). These results were recently confirmed in mice colonized with a conventional gut microbiota in which the lack of dietary fiber also results in microbiome-mediated intestinal permeability contributing to lethal colitis induced by the mucosal pathogen *C. rodentium* (Neumann *et al.* 2021). Avoidance of mucus polysaccharides over degradation with adequate dietary fiber intake should allow a safe mucus-consuming microbiota to maintain, prevention of inflammatory reactions and therefore increased barrier to pathogen colonisation (Leatham *et al.* 2009). Furthermore, to maintain in the intestinal mucus layer, pathogens generally rely on cross-feeding (Pacheco *et al.* 2012; Ng *et al.* 2013). Distracting the versatile part of the microbiota from mucus degradation could prevent their adaptation to mucus consumption (Desai *et al.* 2016), thus avoiding them to feed pathogens in the mucus niche. On the contrary, other studies have reported that dietary fiber rich diet could promote pathogen colonisation by cross-feeding on fiber-derived metabolites from the lumen (Ferreyra *et al.* 2014). However, these studies have been conducted in antibiotic treated mice, and we can argue that in a more complex physiological situation, other commensal microorganisms could have outcompeted with pathogens for fiber metabolites (Ferreyra *et al.* 2014). Altogether, these results indicate that other investigations are needed to address the question of whether the enteric infection may benefit from dietary fiber intake or not. This would necessarily depend on fiber characteristics (fermentable or not), but also on the studied microbiota (e.g. selected strains or complex microbiota, antibiotic treatment, inflammation or not...) and type of models used. In any way, it should be interesting to evaluate dietary fiber anti-infectious properties under dysbiotic conditions (e.g. following antibiotic treatment, inflammation, metabolic disorders) to anticipate the effects due to the lack of competition by a diverse long-term resident microbiota.

Bullet points, dietary fiber inhibition of pathogen interactions with mucus

- By presenting carbohydrate patterns that can be recognized by pathogens, dietary fibers can decoy them from interacting with the mucus layer, thus limiting their infectious cycle.
- In the last decade, a new way for dietary fibers to exert their antifectious properties though mucus protection gains the scientific community attention. By offering an alternative nutrient source, dietary fibers can preserve the mucus from microbiota consumption, thereby reinforcing mucus barrier integrity against pathogens.

Figure 3.1 summarizes the potential role of dietary fibers in enteric infections seen in section 3.2, with an emphasis on mucus layer interactions.

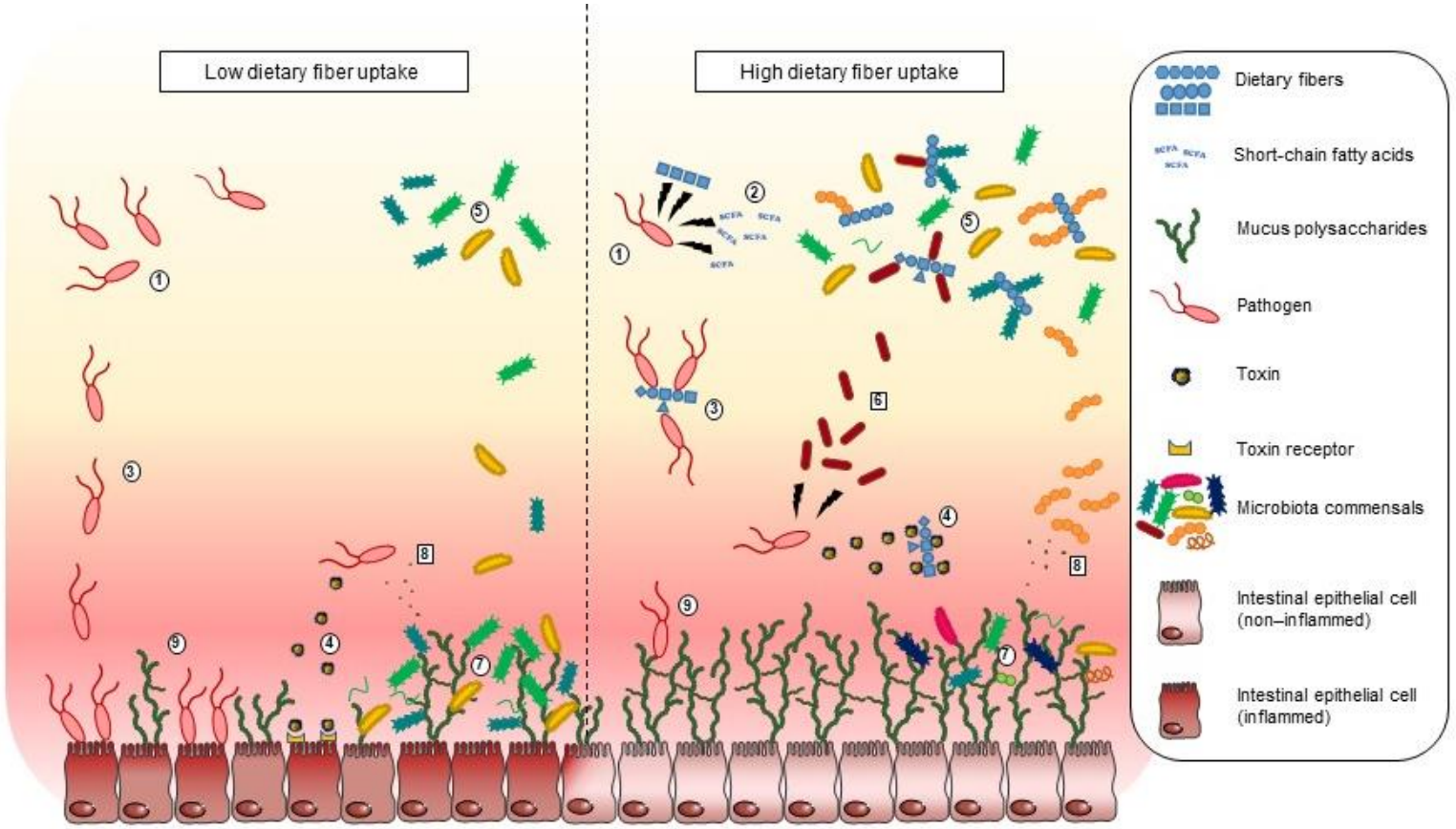


Fig. 3.1. Overview of the potential role of dietary fibers in preventing enteric infections. Each potential mechanism is illustrated by a comparison of the intestinal health with (right panel) and without dietary fiber (left panel). Reliable and converging data from scientific literature are represented with numbers in circles, while data more hypothetical needing further investigations are represented with numbers in square. ① Some dietary fiber exhibit direct bacteriostatic effects against pathogens. ② Dietary fiber degradation lead to SCFA production that can modulate pathogen's virulence. ③ By presenting structure similarities with receptors, some dietary fiber can prevent pathogen adhesin binding to their receptors. ④ By the same competition mechanism, dietary fiber can also prevent toxin binding to their receptors. ⑤ Dietary fibers are able to promote gut microbiota diversity. ⑥Dietary fiber may promote the growth of specific strains with probiotic properties and therefore exhibit anti-infectious properties. ⑦ Suitable dietary fiber intake prevents microbiota's switch to mucus consumption, limiting subsequent commensal microbiota encroachement and associated intestinal inflammation. ⑧ Dietary fiber may prevent pathogen cross-feeding on mucus by limiting mucus degradation and/or by preserving the diversity of competing bacterial species. ⑨ By preventing mucus over-degradation by switchers microbes, dietary fiber can hamper pathogen progression close to the epithelial brush border and further restrict subsequent inflammation.
Printed from Sauvaitre et al. 2021.

4. Enterotoxigenic *Escherichia coli* (ETEC)

This section provides an overview of the enteric pathogen Enterotoxigenic *E. coli* (ETEC) with a particular emphasis on its interactions with the themes aforementioned such as human intestinal physiology, innate immune response, mucus and dietary fiber.

4.1. *Escherichia coli*

4.1.1. General presentation of *Escherichia coli*

Physiologically, it is a non sporulated Gram-negative, rod-shaped, coliform bacillus, measuring about 1 µm long by 0.35 µm wide, although this can vary depending of the strain and its culture condition (**Fig. 4.1**). Besides, the bacterium is oxidase-negative and a facultative anaerobe, growing in presence or absence of oxygen. Phylogenetically, *E. coli* belongs to Proteobacteria phylum and is a member of *Enterobacteriaceae* family. It typically represents only 0.1 to 5% of the total microbial community in the human gut (Hacker and Blum-Oehler 2007; Blount 2015). Importantly, within one host, the *E. coli* community faces important variations, with multiple clones cohabiting over time and appearing/disappearing along lifespans (Martinson and Walk 2020).

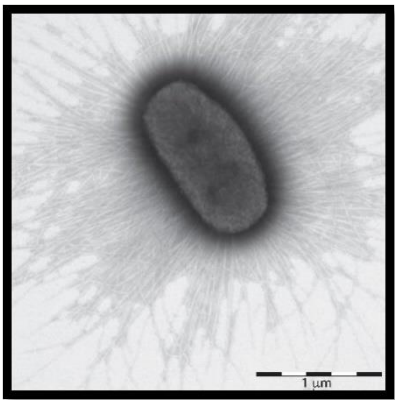


Figure 4.1. Transmission electron microscopy of *E. coli* isolate E873.
The picture of a phosphotungstic staining shows that *E. coli* is a heavily fimbriated Gram-negative bacterium
Reprinted with permission from Von Mentzer, 2017.

Notwithstanding that *E. coli* is a harmless intestinal inhabitant, horizontal gene transfer and pathogenicity islands play a major role in the evolution and gain of pathogenic properties in *E. coli* genome, contributing significantly to the burden of infectious diseases in human and animal (Messerer, Fischer and Schubert 2017). The versatile *E. coli* pathogen is estimated to cause millions of deaths annually through both intestinal and extra-intestinal infections in humans (Nataro and Kaper 1998; Clements *et al.* 2012; Khalil *et al.* 2019).

4.1.2. Classification

4.1.2.1. Non-phylogenic classification

For taxonomic and epidemiological purposes, serotyping of O-LPS antigens, H-flagellar antigens and K- capsular antigens has long being regarded as the gold standard in classification of commensal and pathogenic *E. coli* (Fratamico, DebRoy and Needleman 2016). However, *E. coli* serotypes are not immutable, and can change rapidly due to horizontal gene transfer mediated by mobile genetic elements, including plasmids, phages, and integrative and conjugating elements. Thus, this classification is not necessarily relevant in term of phylogeny and pathogenicity (Denamur *et al.* 2021).

Another model of classification based on pathotypes (also known as pathotypes) has been constructed. These pathotypes are identified using acronyms and have been proposed over time as specific discoveries have been made and are not unified in a meaningful way (Denamur *et al.* 2021). The definition of these pathotypes can be based on various criteria, such as the target organ, the infected hosts (human or others), the presence of specific genes (mainly virulence factors), the pathology caused by the strains and specific phenotypes (Denamur *et al.* 2021). This model contains at least ten well-recognised pathotypes of human pathogenic *E. coli* divided in two groups: (i) extraintestinal pathogenic *E. coli* (ExPEC), colonising various sites in the human body; and (ii) enteric or diarrheagenic *E. coli* (DEC) —although not all of the subtypes in this group necessarily cause diarrhea. The ExPEC group includes subtypes such as uropathogenic *E. coli* (UPEC), neonatal meningitis-associated *E. coli* (NMEC) and sepsis-associated *E. coli* (SEPEC) (Manges *et al.* 2019).

Each DEC pathotype represents a collection of strains that possess similar virulence factors to each other and cause similar diseases (**Table 4.1**). Collectively, DEC represent the most common bacterial pathogens worldwide and some of these pathotypes are a major cause of morbidity and mortality in low-income countries (Gomes *et al.* 2016). Of note, the determining factors of the ETEC pathotype, aim of this PhD, is the production of two toxins: heat-stable enterotoxin (ST) and/or heat-labile enterotoxin (LT).

Table 4.1. Main characteristics of DEC pathotypes.

The determining factors sustaining the pathotype definition are indicated in bold.

AAF: Aggregative Adherence Factor, A/E: Attaching/Effacing, BFP: Bundle Forming Pili, DAF: Decay-Accelerating Factor, EAST1: Enteroaggregative heat-stable enterotoxin 1, HUS: Hemolytic Uremic Syndrome, IECs: Intestinal Epithelial Cells, LEE: locus of enterocyte effacement, LPF: Long Polar Fimbriae, LT: Heat-Labile Toxins, ST: Heat-Stable Toxins, Stx: Shiga-Toxin, TTP: Thrombotic Thrombocytopenic Purpura.

Built from personal source.

Pathotypes	Virulence factors			Action site	Associated mechanisms	Associated disease
	Adhesion factors	Toxins	Other			
ETEC (Enterotoxigenic *E. coli*)	**Multiple colonisations factors (CFA/I, FimH…)**	**Enterotoxins (LT/ST)**	**Mucinases YghJ and eatA**	Distal small intestine	• Attachment to the surface of IECs thanks to colonisation factors (>20 CF). • Mucus degradation by mucinases. • Secretions of at least one enterotoxin leading the release of ions and water in the intestinal lumen	Acute watery diarrhea Traveler's diarrhea Infant diarrhea (<5 years)
EPEC (Enteropathogenic *E. coli*)	Intimin, BFP, **A/E**	**Several enterotoxins**	LEE chromosomal island	Terminal ileum Colon	• Attachment to IECs (type IV pilus) and colonisation factor intimin (attaching and effacing" (A/E) lesions on microvilli). •20 secretory toxins are injected in the enterocyte by a type III injectisome. • Increased permeability of tight junctions, alterations in water absorption and electrolyte secretion in the small intestine.	Infant diarrhea (<6 months)
EHEC/STEC (Enterohemorrhagic *E. coli*)	**A/E**	**Shiga-toxins A and B**	Type 3 secretion system	Terminal ileum Colon	• Inhibition of protein synthesis by shiga toxins (by targeting eukaryotic ribosomes) resulting in cell death and subsequent inflammatory colitis. • Toxins can also cause systemic damages (small intestine, kidneys, brain).	Aqueous diarrhea and hemorrhagic colitis, complications: HUS, TTP
AIEC (Adherent invasive *E. coli*)	Several adhesin as the type 1 pili (FimH), LPF		Mucinase (Vat-AIEC)	Ileum Colon	**Adhesion to and invasion of IECs and replication within macrophages**	Crohn's disease
EAEC (Enteroaggregative *E. coli*)	AAF	EAST1, Sat, ShET1	Type 4 secretion system	Colon	Formation an **aggregative adherence (AA) pattern**, characterized by adherent bacteria in a stacked-brick arrangement on the surface of IECs.	Acute watery diarrhea with blood and mucus, infant diarrhea, Traveler's diarrhea
EIEC/Shigella (Enteroinvasive *E. coli*)		Toxins ShET1/2	Type 3 secretion system (pINV)	Colon	The plasmid **pINV** encodes a T3SS system and a number of effectors that allow Shigellae/EIEC to penetrate epithelial cells, move within these cells and invade neighboring cells	Shigellosis: blood, mucus and leucocytes in stool
DAEC (Diffusely adherent *E. coli*)	Afimbrial (Afa) or fimbrial (Dr) adhesins, DAF (adhesins involved in diffuse adherence)			Unclear	**Distinctive pattern of adherence to tissue culture cells**.	Acute watery diarrhea (children <5 years) Urinary tract infections

Classification of *E. coli* based on pathotypes have proved being valuable in identifying and tracking outbreaks, and for prognostication in individual cases of infection. However, we should mention that this classification also faced some of its own limits. Notably, due to *E. coli* genetic recombination capabilities, some hybrids isolates that do not comply with the standard classification have been reported (Frank *et al.* 2011; Boisen *et al.* 2015). This is perfectly illustrated by the EHEC–EAEC hybrid strain, combining EHEC Shiga toxin production and EAEC adherence phenotype, which caused a major outbreak in Germany in 2011 (Frank *et al.* 2011; Boisen *et al.* 2015). Furthermore, this classification does not relate of *E. coli* phylogeny.

4.1.2.2. *Escherichia coli* phylogeny classification

All *E. coli* strains are phylogenetically assigned to 9 groups, i.e., A, B1, B2, C, D, E , F, G and H (Denamur *et al.* 2021). *E. coli* pathotypes do not group together in these groups, demonstrating their phylogenetic disparate nature (Croxen *et al.* 2013). In fact, all human *E. coli* pathotypes show a highly diverse genomic background with many lineages inside a pathotype (Denamur *et al.* 2021). Concerning human ETEC in particular, ETEC lineages appeared several times in different phylogroups (von Mentzer *et al.* 2014). Whole genome sequencing studies have identified 22 robust lineages belonging to phylogroups A, B1, C and E (Karnisova *et al.* 2018; Denamur *et al.* 2021) (**Fig 4.2**). Within the phylogroup A, no phylogenetic differences between commensal *E. coli* and ETEC are displayed, based on the 16S rRNA gene sequence (Croxen *et al.* 2013). If the *E. coli* pathotypes are not relevant phylogenetically, at least, some phylogroups are more associated to virulence than others, indicating that the genetic background has a major role in the emergence of the virulence (**Fig 4.2**) (Denamur *et al.* 2021).

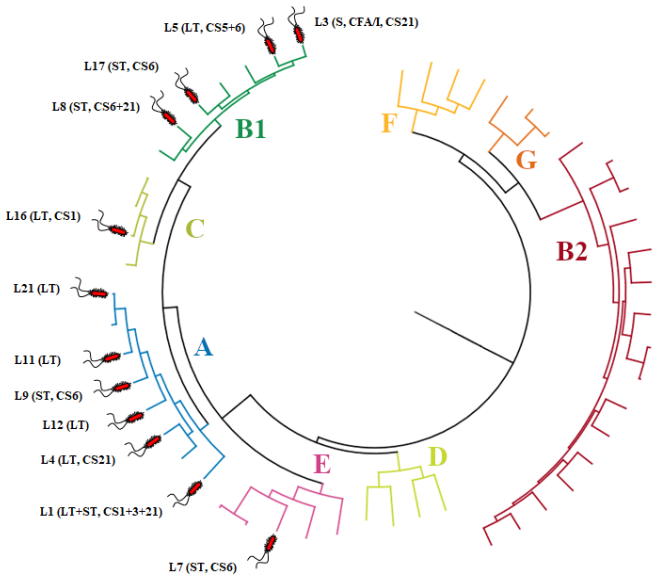


Figure 4.2. The disparity of ETEC lineages in the *E. coli* phylogenetic tree and its phylogroups. ETEC main lineages (groups of organisms that consist of a common ancestor and all its lineal descendants) are distributed within the *E. coli* phylogeny and its phylogroups (A, B1, B2, C, D, E, F and G. *E. coli* phylogroup H is excluded). ETEC lineages are represented by a red bacteria. The identified virulence factors of these lineages are indicated in brackets.
Modified from Denamur *et al.* 2021.

Bullet points, *Escherichia coli* and its pathotypes

- *E. coli* is a bacterial species belonging to Proteobacteria phylum and *Enterobacteriaceae* family, commonly found in the lower part of the intestine of human and warm-blooded animals.
- Different *E. coli* classifications coexist inside the *E. coli* species. Several limits are associated to the phylogroup classification. ETEC, the pathotype subject of this PhD thesis, is no exception with at least 21 lineages in different phylogroups and crossing-border strains like LT-toxin producing EPEC and Shiga-producing ETEC.
- The determining factor of the ETEC pathotype is the production of two toxins: heat- stable enterotoxin (ST) and/or heat- labile enterotoxin (LT).

4.2. Epidemiology of ETEC

4.2.1. History of ETEC

The history of one of the leading causes of diarrhea in the world, called ETEC, begins in 1956 in Calcutta, India. The bacterium was discovered in the course of clinical investigation of children and adult patients with *Vibrio cholerae* culture-negative stools, presenting a cholera-like syndrome, characterized by acute onset of watery diarrhea and severe dehydration. In the late 1960s, a subsequent study by a team of cholera investigators from Johns Hopkins

University in Calcutta led to definitive identification of ETEC, notably by detecting the production of a heat-labile filterable enterotoxin from the bacteria (Carpenter *et al.* 1965; Sack, R. B. *et al.* 1971). Then, the ETEC strain H10407 originally isolated in Dhaka (Bangladesh) from an adult patient with acute diarrhea became the prototypical or reference strain (Evans *et al.* 1977). Concomitantly, similar studies were being conducted with animals that also demonstrated ETEC strain to be responsible for diarrheal disease in several animal species.

4.2.2. ETEC burden in the world: epidemiological data and clinical features

I-4

ETEC pathogens represent a major health concern for both humans and farm animals. The pathogen is transmitted between humans and/or animals through the fecal-oral route, by ingestion of contaminated food and water exposed to animal and/or human sewage (Qadri *et al.* 2005). In the frame of this research project, this section will focus on providing a complete and detailed description of the epidemiology and clinics in the human population only.

In 2016, diarrhea caused more than 1.6 million human deaths worldwide (Khalil *et al.* 2018). Among the 13 recognised etiological agents (e.g. bacteria, parasites, viruses) for diarrheal diseases across all geographies, ETEC alone annually accounts for hundreds of millions of diarrheal episodes over the world (Khalil *et al.* 2018). ETEC is detected by the presence of enterotoxins LT and/or ST in stool samples thanks to molecular techniques.

Unsurprisingly, ETEC mortality rates are higher in low-incomes countries (endemic countries) such as Africa, South America and South Asia (Khalil *et al.* 2018) (**Fig. 4.3**). In 2016, ETEC was the eighth leading cause of diarrhea mortality, accounting for an estimated 51,000 deaths. About 3.2% of all diarrhea-associated deaths were attributable to ETEC. However, between 1990 and 2016, the diarrhea mortality rate attributable to ETEC decreased from about 60% (Khalil *et al.* 2018). A systematic review of the literature across 35 countries in the world found that approximately 45% of ETEC isolates expressed ST toxin only, 25% expressed LT toxin only, and 30% expressed both LT and ST toxins (Isidean *et al.* 2011). ST is recognised to be more frequently associated with diarrhea (Troeger *et al.* 2017).

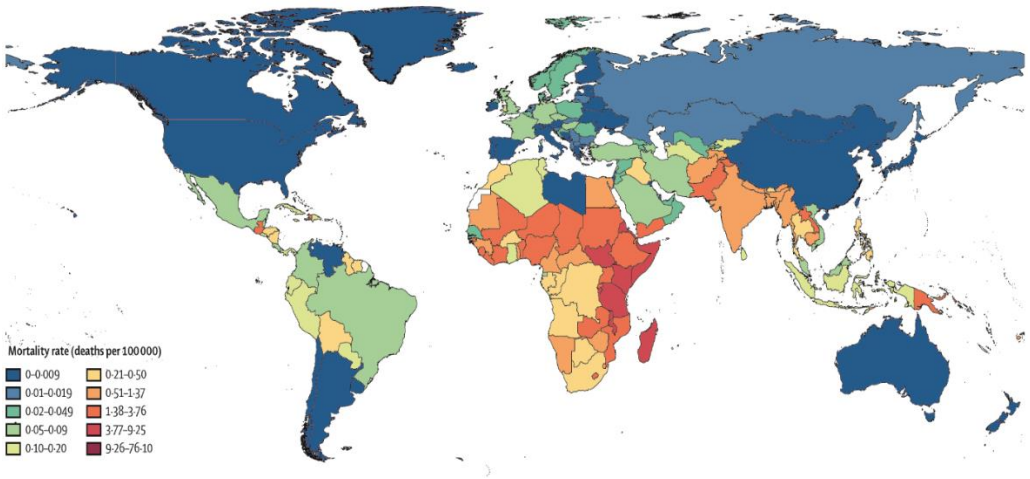


Figure 4.3. Enterotoxigenic *E. coli* diarrhea mortality rate per 100,000 persons in 2016 for all ages.
Modified from Khalil *et al*., 2018.

In term of age, any age groups is susceptible to be ETEC-infected, however the most vulnerable group remains children below five years of age, who may suffer from multiple diarrheal episodes each year (Qadri *et al.* 2005). Moreover, the epidemiology of ETEC infection attests important disparities according to the socio-economic status and living conditions. Thus, globally, two main at-risk groups for ETEC infections have been recognised with (i) infants living in low and/or middle-income countries and, (ii) adults traveling and/or working occasionally in endemic countries. These two at-risk populations will be presented in the following sections. Beyond this classification, we should mention that other human populations are at significant risk, even if often forgotten by the literature. According to the Global Burden of Disease (GBD) study in 2016 around 18,152 deaths from ETEC occurred among adults older than 70 years worldwide (Khalil *et al.* 2018). In the last decade, awareness has also risen concerning ETEC domestically acquired infection in high-income countries (Medus *et al.* 2016; Buuck *et al.* 2020) (Boxall *et al.* 2020). Finally, many ETEC outbreaks have occurred during natural disasters such as the floods in Bangladesh in 2004, provoking 17,000 cases of acute diarrhea (Qadri *et al.* 2005).

4.2.2.1. Infant diarrhea in low and middle-income countries

ETEC is responsible for an estimated 75 million cases that accounted for 19,000 deaths among children below 5 years old, which represent about 4% of all diarrhea deaths in this age group in 2016 (Khalil *et al.* 2018). Remarkably, even if still considerable, the number of deaths

due to ETEC has strongly declined year by year (WHO, 2006, Walker *et al.* 2017; Khalil *et al.* 2018) (**Fig. 4.4**).

Of note, high-income zones like North America, Asia-Pacific or Western Europe report nearly no death of infant related to ETEC infection (Khalil *et al.* 2018), illustrating the worldwide discrepancies in term of hygiene capacities and access to health care facilities. Globally, ETEC children infection mostly occurs in south Asia, Africa and Latin America. The GBD 2016 meta-analysis has shown that ETEC is responsible for more than 8 deaths per 100,000 infants in Eastern and Western sub-Saharan Africa. Madagascar was the country with the highest number of deaths per children (23.6 per 100,000) (Khalil *et al.* 2018).

ETEC particularly impact children from poor communities (Liu *et al.* 2016b; Kotloff 2017) and malnutrition is strongly associated with diarrhea severity (Liu *et al.* 2016b). ETEC infection is often the first bacterial illness that these infants and young children experienced in low-income countries with a median of 3.2 diarrheal episodes per child during their first 3 years of life (Bourgeois, Wierzba and Walker 2016). The attack rate then declines thereafter suggesting that protective immunity develop following infection (Qadri *et al.* 2005). Interestingly, studies have shown that ST-containing ETEC isolate would be more associated with infant diarrhea than non-ST containing strains (Kotloff 2017).

Finally, we cannot exclude that in low-income and remote areas, the inventory of ETEC diseases and / or deaths might be difficult due to data gaps or absence of health-care facilities.

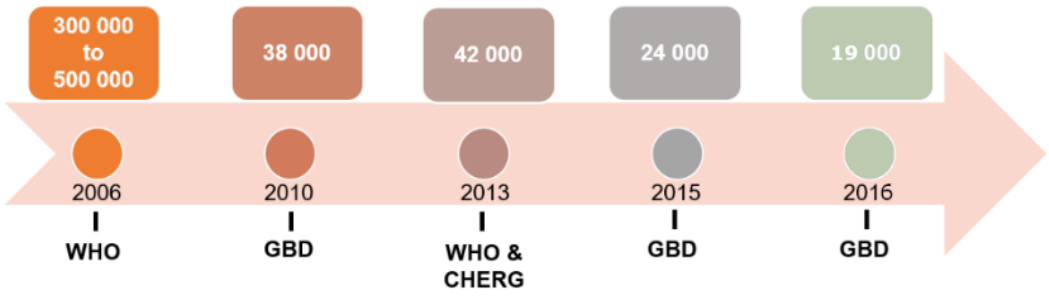


Figure 4.4. Timeline of infants (< 5 years old) deaths due to ETEC around the world.
Each year, the corresponding number of infant's deaths and the entity who collected the data are indicated. Different methodologies were used to assess the number of ETEC related deaths according to the entity in charge. Thus, biases are not excluded and can create a limitation in the interpretation.
CHERG: Child Health Epidemiology Reference Group, GBD: Global Burden of Diseases, WHO: World Health Organization. Compiled from Kotloff *et al.*, 2013, Troeger *et al.*, 2017 and Khalil *et al.*, 2018.
Modified from Charlene's Roussel PhD manuscript, 2017.

4.2.2.2. Traveler's diarrhea

4.2.2.2.1. Epidemiology

Beyond living populations in developing countries, travelers constitute as well a significant at-risk population in less developed destinations, especially those visiting Africa, Asia and Latin America (**Fig 4.5**). Diarrheal illnesses in travelers are termed traveler's diarrhea (TD) or other non-exhaustive fanciful synonyms (e.g. Turista, Montezuma's revenge or Delhi Belly) (Leung *et al.* 2019).

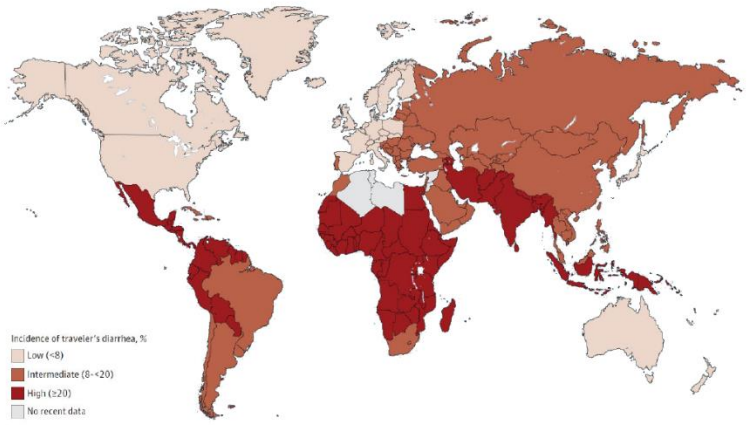


Figure 4.5. Incidence rate of traveler's diarrhea in the world.
Incidence rates (percentages) of travelers' diarrhea in the initial 2 weeks period in various regions of the world from 1996–2008.
Modified from Fedor *et al.* 2019.

Travelers' diarrhea is the most common disorder in travelers (Greenwood *et al.* 2008). Among 64 million people traveling to endemic countries each year, 22 million people contract a diarrheal episode. In some areas, up to 60% travelling persons contract the disease (Greenwood *et al.* 2008) (**Fig. 4.6**). It is noteworthy that in the last two decades, the number of cases during a 2-week trip has decreased substantially (Steffen, Hill and DuPont 2015), most probably because of the increasing hygiene standards in developing countries and the raising of traveler's awareness.

The pathogen causing traveler's diarrhea is identified in only 40–60% of travelers with symptoms, as the symptoms often disappear spontaneously (Al-Abri, Beeching and Nye 2005). Total Bacteria account for up to 90% of identified pathogens for travelers' diarrhea, viral pathogens up to 10% and protozoal parasites are even very rare (Leung *et al.* 2019). ETEC is the most common pathogen identified in traveler's diarrhea accounting for 30-40% of cases in travelers to Latin America and Asia, respectively (Jiang and DuPont 2017; Boxall *et al.* 2020) (**Fig. 4.6**). In average, nearly one out of every six travelers to endemic regions is infected by

ETEC (**Fig. 4.6**). After ETEC, EAEC, *Campylobacter jejuni*, *Shigella* and *Salmonella* are the most common bacterial pathogens involved (Giddings, Stevens and Leung 2016).

4.2.2.2.2. Clinical manifestations

In term of symptoms, travelers' diarrhea is defined as the passage of more than 3 unformed stools per 24 hours plus at least one additional symptom (such as nausea, vomiting, abdominal cramps, fever, blood/mucus in the stools, or fecal urgency) that develop while abroad or within 10 days of returning from any resource-limited destinations. The onset of diarrhea is usually quick, with an incubation period between 6-50 hours after ingestion if the pathogen is a bacteria or a virus (Leung, Robson and Davies 2006).

The inconvenient traveling situations due to diarrhea required to alter planned activities in 40% of the total cases, to stay in bed for at least one day in 20%, to seek for medical care in 10%, and to require hospitalization in 3% (**Fig. 4.6**) (Steffen *et al.* 2006; Giddings, Stevens and Leung 2016).

In 1% of cases, traveler's diarrhea can evolve from an acute distress to a chronic disease and sometimes it may have long-term consequences on the overall health of the patient (Steffen, Hill and DuPont 2015; Steffen 2017; Fedor, Bojanowski and Korzeniewski 2019). The consequences results in post-infectious (PI) sequelae ranging from functional gastrointestinal disorder to irritable bowel syndrome (IBS) (Halvorson, Schlett and Riddle 2006) or reactive musculoskeletal symptoms like reactive arthritis (Tuompo *et al.* 2020).

ETEC associated traveler's diarrhea specifically present mainly with watery diarrhea without bloody stools or fever (Leung, Robson and Davies 2006). Still, the clinical manifestations can range from this mild-watery diarrhea manifestation, without important dehydration, to a profuse watery diarrhea similar to Cholera syndrome (*Vibrio cholerae*) (Qadri *et al.* 2005). Symptomatic subjects infected with ETEC experienced the worst symptoms between day 2 and 4 post-infection with an average incubation time that varied between 10 and 60 hours depending on the strains and dose tested (McKenzie *et al.* 2008; Harro *et al.* 2011; Yang *et al.* 2016).

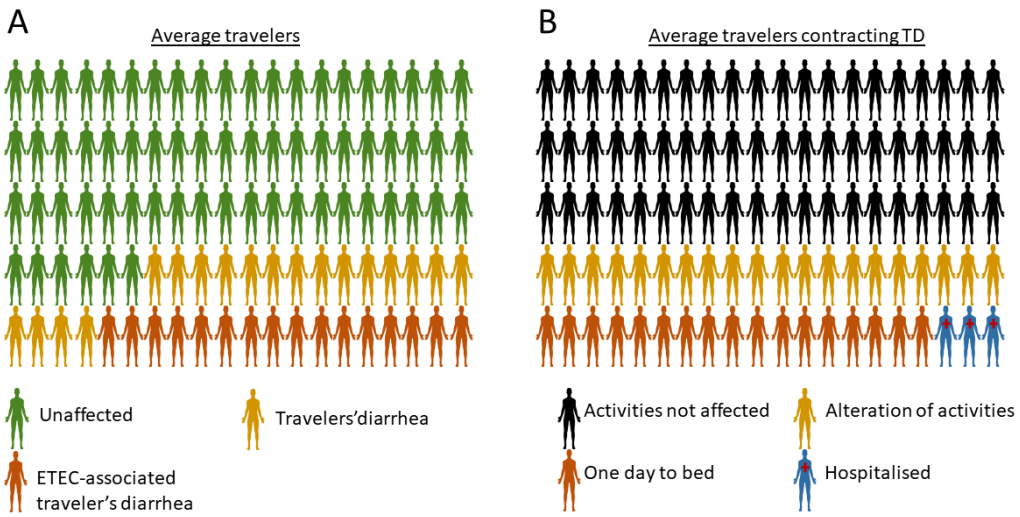


Figure 4.6. Schematic representations of travelers' diarrhea impact on travelers.
100 average travelers (A) or travelers impacted (B) by travelers' diarrhea are represented.
Built from personal source.

Bullet points, ETEC epidemiology

- Human ETECs are responsible for more than 2 hundred million cases and 51,000 deaths annually
- Children under 5 years of age in endemic countries and travelers are considered are one of the two main groups affected by human ETEC isolates, with a huge proportion (up to 60%) of visitors affected in some areas.
- ETEC is often considered as the most common agent of traveler's diarrhea. In average, nearly 1 out of 6 travelers to endemic regions would be infected by ETEC.

4.3. Exploring the virulence function of ETEC

In order to avoid removal from the organism and initiate an enteric infection, ETEC pursue a sophisticated strategy, supported by several virulence factors and deeply affecting the host intestinal physiology. This section will review the structure, function and genetics of the predominant and valuable ETEC virulence factors (e.g. colonisation factors, enterotoxins, mucinases), related to human infection. Due to the lack of structural and mechanistic information, putative factors (e.g. EAST1, ClyA a pore forming cytotoxin) related to few ETEC strains will be not presented (Yamamoto and Echeverria 1996; Ludwig *et al.* 2004).

4.3.1 ETEC survival in the gastrointestinal tract

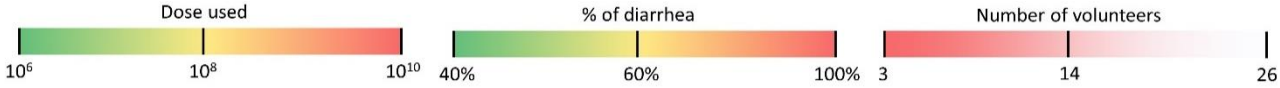
4.3.1.1. Infectious doses

In order to realize its infectious cycle and triggers symptoms, ETEC have to be ingested in relative high numbers. Volunteer's studies are maybe the best way to investigate these numbers. Studies conducted with strains other than the H10407 prototypical one are generally conducted with a low number of subjects, impeding interpretation (**Table 4.2**). Still, we can observe that the attack rates largely depend of the strain considered (**Table 4.2**).

Table 4.2. Different ETEC percentages of induced diarrhea in volunteers depending on the dose and strain used.
To illustrate the disparate nature of the presented ETEC strain, the toxins they produce, their known colonisation factors and their serogroups are represented (when known).
ST: heat stable toxin, LT: heat labile toxin, CS: Coli Surface antigen, CFA: colonisation factor antigen.
Built from personal source.

Strain	Toxin	Colonisation Factor(s)	Serogroup	Dose used (ingested CFU)	% of diarrhea in volunteers	Type of symptoms considered	Total number of volunteers	Reference.
B7A	STh and LT	CS6	O148:H28	1.97×10^{10}	71%	Moderate-to-severe	7	Talaat *et al.* 2020b
B7A	STh and LT	CS6	O148:H28	1.4×10^{10}	75%	All diarrhea cases	8	Coster *et al.* 2007
B7A	STh and LT	CS6	O148:H28	10^{10}	80%	All diarrhea cases	5	DuPont *et al.* 1971
B7A	STh and LT	CS6	O148:H28	10^{10}	63%	All diarrhea cases	8	Clements *et al.* 1981
TD225-C	LT	Unknown	O75:H9	10^{10}	40%	All diarrhea cases	5	Clements *et al.* 1981
TW10722	STh	CS5, CS6	O115:H5	10^{10}	78%	All diarrhea cases	21	Sakkestad *et al.* 2019
B2C	ST and LT	CFA/II, CS2, CS3	O6:H16	10^{10}	60%	All diarrhea cases	5	DuPont *et al.* 1971
WS0115A	STp and LT	CS19	O114:H-	9×10^{9}	44%	All diarrhea cases	9	McKenzie *et al.* 2011
LSN03-016011/A	LT	CS17	O8:H-	6×10^{9}	88%	All diarrhea cases	8	McKenzie *et al.* 2011
WS0115A	STp and LT	CS19	O114:H-	3×10^{9}	33%	All diarrhea cases	6	McKenzie *et al.* 2011
E24377A	STh and LT	CS1, CS3	O139:H28	4×10^{9}	80%	All diarrhea cases	10	McKenzie *et al.* 2008
B7A	STh and LT	CS6	O148:H28	1.9×10^{9}	62%	Moderate-to-severe	26	Talaat *et al.* 2020b
B7A	STh and LT	CS6	O148:H28	1.9×10^{9}	43%	Moderate-to-severe	7	Talaat *et al.* 2020b
B7A	STh and LT	CS6	O148:H28	1.5×10^{9}	63%	All diarrhea cases	8	Coster *et al.* 200)
LSN03-016011/A	LT	CS17	O8:H-	7×10^{8}	60%	All diarrhea cases	5	McKenzie *et al.* 2011
WS0115A	STp and LT	CS19	O114:H-	4×10^{8}	20%	All diarrhea cases	5	McKenzie *et al.* 2011
B7A	STh and LT	CS6	O148:H28	1.66×10^{8}	43%	Moderate-to-severe	7	Talaat *et al.* 2020b
B7A	STh and LT	CS6	O148:H28	10^{8}	20%	All diarrhea cases	5	DuPont *et al.* 1971
B7A	STh and LT	CS6	O148:H28	10^{8}	100%	All diarrhea cases	3	Clements *et al.* 1981
214-4	STp	CS6	O167:H5	10^{8}	80%	All diarrhea cases	5	Clements *et al.* 1981
B7A	STh and LT	CS6	O148:H28	10^{8}	64%	All diarrhea cases	11	Levine *et al.* 1979
TD225-C4	LT	unknown	O75:H9	10^{8}	63%	All diarrhea cases	11	Levine *et al.* 1980
E2528-C1	LT	CS8, CS14	O25:NM	10^{8}	40%	All diarrhea cases	6	Levine *et al.* 1979
B2C	ST and LT	CFA/II, CS2, CS3	O6:H16	10^{8}	40%	All diarrhea cases	5	DuPont *et al.* 1971

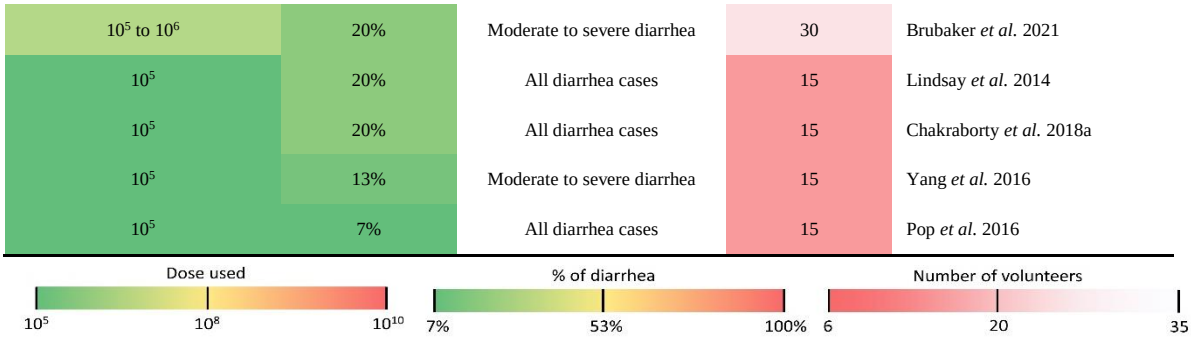
DS26-1	LT	CS19	O8:H9	10^8	0%	All diarrhea cases	5	McKenzie *et al.* 2011
TW11681	STh	CFA/I, CS21*	O19:H45	10^6 to 10^8	22%	All diarrhea cases	9	Sakkestad *et al.* 2019
B7A	STh and LT	CS6	O148:H28	10^6	50%	All diarrhea cases	6	Levine *et al.* 1979



Studies conducted with the ETEC strain H10407 are usually conducted with a higher number of subjects and trends are more obvious. We can observe that the ETEC strain H10407 triggers symptoms depending on the ingested doses (Table X). Thus, between 10^6 and 2.10^7 CFU of the reference strain, are necessary to trigger diarrhea in 50% of the volunteers. The dose of 2.10^7 CFU of ETEC strain H10407 has been considered by some authors as the optimal dose to induce diarrhea in around 70% of the volunteers using the lowest possible inoculum to maximize safety and sensitivity (Chakraborty *et al.* 2018a). In comparison to other ETEC strains, the strain H10407 is considered to induce a relatively high proportion of severe symptoms (Harro *et al.* 2011). Studies conducted with other ETEC strains rarely used a dose inferior to 2.10^7 CFU (only 2 studies in Table X) and even with such dose, the attack rates do not necessarily reach 70% (**Table 4.3**).

Table 4.3. Percentage of diarrhea induced by ETEC strain H10407 in volunteers depending on the dose used.
The ETEC strain H10407 (serotype O78:H11) is STh, STp, STh and CFA/I positive.
CFU: Colony forming unit.
Built from personal source.

Dose of ETEC strain H10407 ingested (CFU)	% of diarrhea in volunteers	Type of symptoms considered	Total number of volunteers	Reference.
5 x 10^9	89%	All diarrhea cases	9	Evans *et al.* 1988
1.4 x 10^9	75%	All diarrhea cases	8	Coster *et al.* 2007
10^9	70%	All diarrhea cases	10	Freedman *et al.* 1998
6 x 10^8	100% - (75%)	All diarrhea cases - (moderate to severe diarrhea)	20	McKenzie *et al.* 2007
1.2 x 10^8	75%	All diarrhea cases	7	Coster *et al.* 2007
10^8	100%	Moderate to severe diarrhea	9	Brubaker *et al.* 2021
10^8	83%	All diarrhea cases	6	McArthur *et al.* 2017
2 x 10^7	74%	All diarrhea cases		Harro *et al.* 2019
2 x 10^7	80%	All diarrhea cases	35	Harro *et al.* 2011
10^7	75%	moderate to severe diarrhea	24	Brubaker *et al.* 2021
10^6	40%	All diarrhea cases	15	Lindsay *et al.* 2014
10^6	33%	All diarrhea cases	15	Chakraborty *et al.* 2018a
10^6	27%	All diarrhea cases	15	Pop *et al.* 2016
10^6	27%	Moderate to severe diarrhea	15	Yang *et al.* 2016



Globally, whatever the strain, ETEC ingested dose does not only correlate with diarrhea severity, but also with incidence of others symptoms as nausea and vomiting (McKenzie *et al.* 2008; Porter *et al.* 2016). It is noteworthy that vulnerable populations such as infants are considered susceptible at lower dose of ingested ETEC such as 10^6 CFU (Levine *et al.* 1979; Gupta *et al.* 2008).

4.4.1.2. Survival through the gastro-intestinal tract

ETEC action site is considered by most studies to be the distal part of the small intestine, from the jejunum to the ileum (Al-Majali *et al.* 2000, 2007; Allen, Randolph and Fleckenstein 2006; Al-Majali and Khalifeh 2010; Gonzales *et al.* 2013; Rodea *et al.* 2017). Of note, all of these data have been gathered in very diverse animal models (e.g. camels, calves, mice), but not directly in humans.

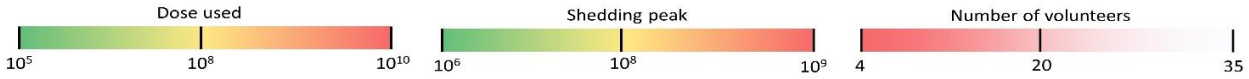
The first stress that food and water-borne pathogens find upon ingestion is the very acidic pH of the stomach, which decreases during digestion from around 5 to 2. Its final endpoint is to reach the small intestinal niche with pH close to the neutrality. Several batch studies have investigated the effects of pH on ETEC survival. Masters et al. (1994) have shown that after static exposure to pH 2, ETEC became undetectable by plate counting after 2 hours (Masters, Shallcross and Mackey 1994). Another study using flow cytometry indicated that there was no significant difference in the percentage of live bacteria when ETEC were subjected either to pH 5 or pH 7 (Gonzales *et al.* 2013). Supporting this negative effect of acidic pH in the gastric compartment, it has been acknowledged that giving acid-neutralizing sodium bicarbonate to volunteers before challenge increases ETEC attack rates (Levine *et al.* 1980; Chakraborty *et al.* 2018a). However, static batches are far from human physiology in which gastric emptying is continuous and consequently, only part of the ingested bacteria is exposed to acidic pH at the end of digestion (Roussel *et al.* 2020a). The most complete work about ETEC survival has been conducted in the multi-compartmental TIM-1 (TNO gastrointestinal model 1) model (described in part 5.2.2) (Roussel *et al.* 2020a), which accurately captures the spatio-

temporal events occurring during gastric and small intestinal digestion. In this model, ETEC did suffer from this exposure to acidic pH. After 45 min of gastric digestion, when pH dropped below 2, the number of viable ETEC in the gastric compartment was 3 log lower than expected with the theorical transit marker representing bacterial survival without multiplication or destruction. However, the ETEC population did not die entirely in the stomach compartment. Part of it reached the duodenum, where the bacteria is exposed to high levels of bile salts, resulting in a progressive decrease of ETEC survival from 10^7 to 10^5 CFU.mL^{-1} at the end of the digestion (Roussel *et al.* 2020a). Only one study has specifically investigated the impact of a bile mixture (30 g.L^{-1}) containing sodium choleate, taurocholic, glycocholic, deoxycholic, and cholic acids on the survival of ETEC *in vitro*. Despite the known bactericidal effect of bile in the intestine, no difference in term of growth were reported in Luria Bertani (LB) media supplemented or not with bile salts (Sahl and Rasko 2012). In the TIM-1 jejunal and ileal compartments, reabsorption of bile salts and further pH increase contributed to ETEC's increased survival, which reaches 5.2 % and 26 % of the initial inoculum at the end of the jejunal and ileal digestion (300 min), respectively (Roussel *et al.* 2020a).

Concerning human ETEC survival in the lower intestinal compartment where the microbiota prevails in high numbers (10^{10}–10^{11} CFU.mL^{-1} in the colon), Moens and colleagues have shown that ETEC was able to grow from 10^8 to 1.5 10^9 CFU.mL^{-1} in fecal batch experiments. However, these batchs were set in "dysbiotic conditions" with poor fecal donor inoculation (0.02% v/v) (Moens *et al.* 2019). In the M-SHIME (Mucosal-SHIME) model (described in part 5.3), ETEC strain H10407 attachment to the mucin beads helped to maintain luminal concentrations above 10^6 copies.mL^{-1} in both ileum and ascending colon up to 5 days post-infection. At day 4 post infection, the number of ETEC gene copies were even significantly 3-fold higher in the mucosal phase compared to the luminal phase of the in vitro model (Roussel *et al.* 2020a). These data showing ETEC gaining an edge in colonic prevalence would be in accordance with the follow-up of the fecal microbiota composition of soldiers affected by DEC (EPEC, EAEC, EPEC), in which a bloom of *Enterobacteriaceae* is concomitant to the infection (Walters *et al.* 2020). The bloom in excreted ETEC after infection is also reported in studies in volunteer challenges, which are listed in **Table 4.4**. ETEC shedding in feces could be seen as the ultimate goal of the infection, allowing the pathogen to contaminate the environement as much as possible.

Table 4.4. Differents ETEC shedding depending on the dose used in volunteers' studies. The table indicates the dose used, the shedding peak and the duration of shedding when provided.
LT: heat labile toxin, ST: heat stable toxin, CS: Coli Surface antigen, CFA: colonisation factor antigen, CFU: colony forming unit.
Built from personal source.

Strain	Toxin	Colonisation Factor(s)	Serogroup	Dose used (ingested CFU)	Shedding peak ($CFU.g^{-1}$)	Time of shedding peak (days post-inoculation)	total number of volunteers considered	Reference.
B7A	STh and LT	CS6	O148:H28	1.97×10^{10}	7.00×10^{8}	2	7	Talaat *et al.* 2020b
B7A	STh and LT	CS6	O148:H28	1.00×10^{10}	7.50×10^{7}	2	8	Talaat *et al.* 2020a
E24377A	STh and LT	CS1, CS3	O139:H28	3.00×10^{9}	1.00×10^{7}	2	16	McKenzie *et al.* 2008
E24377A	STh and LT	CS1, CS3	O139:H28	3.00×10^{9}	4.00×10^{8}		10	McKenzie *et al.* 2008
H10407	STh, STp and LT	CFA/I	O78:H11	1.00×10^{9}	4.50×10^{8}		10	Freedman *et al.* 1998
H10407	STh, STp and LT	CFA/I	O78:H11	6.00×10^{8}	2.00×10^{8}	3	20	McKenzie *et al.* 2007
H10407	STh, STp and LT	CFA/I	O78:H11	10^{8}	3.00×10^{8}	2-4	7	Levine *et al.* 1980
H10407	STh, STp and LT	CFA/I	O78:H11	10^{8}	2.00×10^{8}		9	Harro *et al.* 2011
214-4	STp	CS6	O167:H5	10^{8}	1.05×10^{8}	2-4	4	Levine *et al.* 1980
H10407	STh, STp and LT	CFA/I	O78:H11	2.00×10^{7}	5.00×10^{7}	2	34	Darsley *et al.* 2012
TW11681	STh	CFA/I, CS21	O19:H45	10^{6}-10^{8}		3.5	9	Vedøy *et al.* 2018
H10407	STh, STp and LT	CFA/I	O78:H11	10^{7}	8.00×10^{7}		35	Harro *et al.* 2011
H10407	STh, STp and LT	CFA/I	O78:H11	10^{6}	1.80×10^{6}		11	Chakraborty *et al.* 2018a
H10407	STh, STp and LT	CFA/I	O78:H11	10^{5}	10^{8}		13	Chakraborty *et al.* 2018a



Usually, the prototypical strain shedding peak is reported to be from Day 2 and 4 as for the other ETEC strains (**Table 4.4**). Whatever the strains, it is noteworthy that the concentration of ETEC shed in the feces is positively associated with the severity of the diarrheal symptoms (Pop *et al.* 2016; Talaat *et al.* 2020a, 2020b). In conclusion, whatever the ETEC inoculated dose, development of symptoms and shedding are all correlated reflecting probably the pathogen strategy to multiple in its diverse ecological niches.

Bullet points, ETEC epidemiology

- Different studies indicate that ETEC is well adapted to the gastrointestinal tract conditions and in particular to the mucus compartment. Despite some challenges (e.g. physico-chemical conditions of digestion, microbiota) the bacteria are able to prevail in high numbers in the lower part of the intestine.
- ETEC have to be ingested in high number to reach significant attack rates. Dose, symptoms severity and bacterial fecal shedding are all correlated.
- Among ETEC strains, the strain H10407 is considered to be particularly virulent to humans.

4.3.2. ETEC Intestinal adhesion

Upon ingestion and after reaching the gastrointestinal tract, ETEC colonise the small intestine through an interaction of fimbrial and non-fimbrial adhesins with specific receptors present in the apical membrane of the small intestinal epithelium (Vipin Madhavan and Sakellaris 2015). Most of the adhesins are pili (fimbriae) or pilus-related molecules with polymeric structures. However, few adhesins are not related to pili and are simple outer membrane proteins that do not form macromolecular structures. Some other adhesins are also associated to ETEC such as flagella. These adhesins seem to work in a concerted manner for optimal adhesion (Sheikh *et al.* 2017).

4.3.2.1. Colonization factors and pili

Colonisation factors (CF) are necessary for the bacteria to colonise and stabilize in the gut (Satterwhite *et al.* 1978). CF differ in morphology, serotype, amino acid sequence, and receptor binding specificity. To date, at least 25 distinct CF have been identified in human ETEC strains (Kharat *et al.* 2017; von Mentzer *et al.* 2017). The genes encoding ETEC CF are organized in operons, and all the genes needed for the assembly of functional CF are carried by plasmids, suggesting that ETEC acquired the whole operons by horizontal gene transfer (Vipin Madhavan and Sakellaris 2015). Based on the morphology, four main types of ETEC CF have been described: (i) fimbrial (typical rod-like morphology): CFA/I, CS1, CS2, CS4, CS8, CS12, CS14, CS17-21 and CS26; (ii) fibrillary (CS3, CS11, CS13 and CS22); (iii) helical (CS5 and CS7); and (iv) afimbrial (CS6, CS10, CS15 and CS23). The CF CS6 and CFA/I are usually found to be the most prevalent among ETEC strains (Mondal *et al.* 2021). However, the distribution of certain CF among ETEC strains can vary geographically and over time. As well, an estimated number of 30-50% of ETEC strains might be still CF uncharacterized, thus questioning the real number of CF that the pathogen can produce (Begum *et al.* 2014). Most CF are pili-related structures. Pili are hair-like proteinaceous appendages that protrude from the bacterial cell surface and, in general, mediate the attachment of bacteria to surfaces. CF pili structures show either homopolymeric or heteropolymeric conformations.

Among the well-known ETEC CF, CFA/I was the first identified and is the most predominant one. Mature CFA/I consists of two pili subunits: a major pilin subunit named CfaB, and one or a few copies of the tip-residing adhesive minor subunit CfaE (Li *et al.* 2009). The minor pillin CfaE has also been identified as one of the main protein mediating the ETEC strain H10407 colonisation of mouse intestine (Abd El Ghany *et al.* 2021). Encoded by the

highly conserved *fim* chromosomal operon, the type 1 pili is also involved in ETEC pathogenesis by promoting ETEC-host interaction (Sheikh *et al.* 2017). This highly conserved and widely spread pilus is also found in commensal *E. coli*, highlighting their shared need for mucosal adherence to maintain in the gut. Type 1 pili usually play a critical role in virulence, especially the key adhesin FimH tipped at the apex of the pili structure. In ETEC, FimH plays a role in delivery of both enterotoxins in a rabbit ileal loop assay (Sheikh *et al.* 2017) (**Fig. 4.7**).

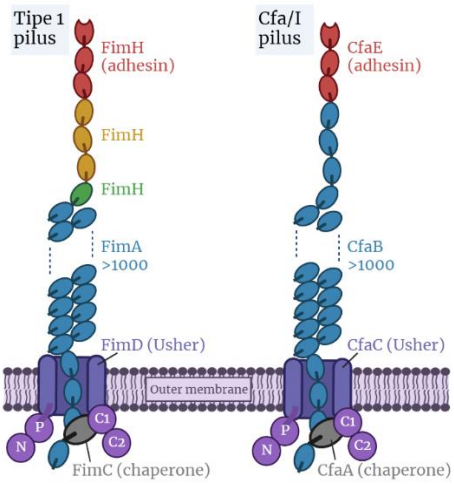


Figure 4.7. Schematic structure of the assembly of Type I and CFA/I pili in *E. coli*.
Each pilus is built up by and with a different combination of subunits (Fim, Cfa), represented by different colors. These pili expose their binding sites at the cell surface and are anchored at the bacterial outer membrane by the usher platform. The usher platform is an outer membrane complex composed of a transmembrane pore domain and four soluble domains: the N-terminal domain (N), the plug domain (P), and the C-terminal domains (C1 and C2). The usher platform is required for secretion of pilins and assembly of the pilus on the bacterial cell surface.
Modified from Busch *et al*., 2015.

4.3.2.2. Non-pili adhesins

Uncommon non-fimbrial and pathogenicity islands encoded adhesins have also been found in some ETEC strains but remain poorly elucidated in terms of molecular structure and function.

The enterotoxigenic invasion locus B (TibA) is an autotransporter glycoprotein (104-kDa) mediating bacterial attachment to intestinal epithelial cells, autoaggregation and biofilm formation, encoded within the *tibDBCA* gene cluster (Elsinghorst and Weitz 1994; Lindenthal and Elsinghorst 1999). TibA mediates bacterial autoaggregation and biofilm formation in a glycosylation-independent manner (Sherlock, Vejborg and Klemm 2005). The enterotoxigenic locus invasion A (Tia) is a 25-kDa outer membrane protein (Fleckenstein *et al.* 1996).

Interaction of Tia with the intestinal epithelium would be at least partially mediated *via* binding to heparin sulfate proteoglycans (Fleckenstein, Holland and Hasty 2002).

The two adhesins TibA and Tia were first identified in the prototypical ETEC strain H10407 for promoting invasion of HCT-8 cells *in vitro*. However, the bacteria is unable to replicate inside the cell (Elsinghorst and Kopecko 1992; Fleckenstein *et al.* 1996; Lindenthal and Elsinghorst 1999). As well, expression of Tia and TibA in non-pathogenic *E. coli* strains HB101 and DH5α confers the invasive phenotype, suggesting that this protein would not require additional factors to exert its function. So far, the invasion mechanism has been described in the 90s and no extended investigation has been recently performed, highlighting the incertitude of this discovery. In fact, the invasion rates of ETEC strain H10407 in cultured intestinal epithelial cells are far lower than those reported for intracellular pathogens such as *Salmonella enterica* (Torres 2016). Furthermore, epidemiological studies performed in Latin America revealed that *tia* and *tib* genes were found in only 17% of the total isolates (Guerra *et al.* 2014).

4.3.2.3. EtpA and the ETEC flagellum involvement in adherence

Some clues indicate that ETEC flagellum participate directly in ETEC adherence to IECs. (Roy *et al.* 2009; Kansal *et al.* 2013). ETEC strain H10407 adhesion to intestinal epithelial cells *in vitro* is associated to bacteria morphological changes. At early time point (15 min), long peritrichous flagella seek contact with the cell, for, at later time point (60 min), engulf themself in it (Kansal *et al.* 2013). In support of the role of the flagellum in ETEC adherence, deletion of the FliC (flagellin subunit) results in decreased adherence to HCT-8 and Caco-2 cells (Roy *et al.* 2009). Furthermore, the EtpA adhesin has been highlighted to mediate flagellum binding to epithelial surface (Roy *et al.* 2009) (**Fig 4.8**). EtpA is thus also necessary for both adhesion and colonisation of the epithelial cell lines and murine intestine, respectively (Roy *et al.* 2008, 2009).

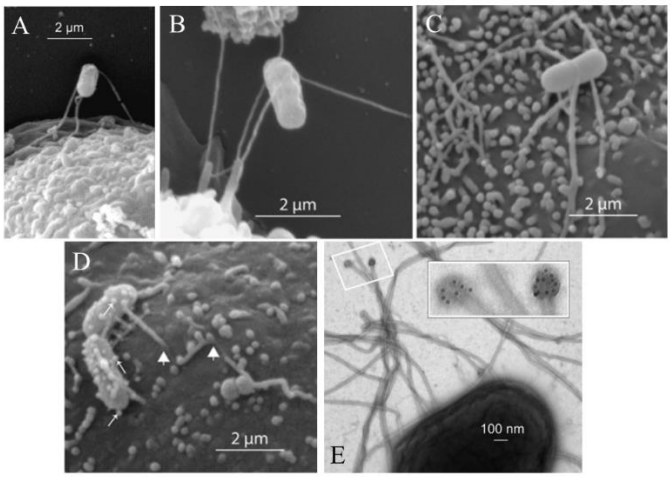


Figure 4.8. Scanning electron microscopy of ETEC strain H10407 flagella and its interactions with intestinal epithelial cells *in vitro*.
A and **B.** Long distance engagement of ETEC flagella with epithelial cells. **C** and **D.** Progressive engulfment of ETEC flagella within the cells. **D**. Large arrows indicate the course of flagellum that has been partially engulfed by the cell surface, small arrows indicate the appearance of blebs on the surface of ETEC strain H10407. **E.** Immunogold labelling of EtpA *in situ* showing the localization of EtpA adhesin at the tip of the ETEC strain H10407 flagella. All micrographs have been obtained with HCT-8 or Caco-2 cells.
Micrographs A and E originate from Roy *et al*., 2009, and micrographs B, c and D from Kansal *et al*., 2013. They are printed with author's permissions.

To summarize, ETEC adhesins have been shown to possess at least three locations on the bacterial cell as presented below (**Fig. 4.9**). Adhesins can be associated to pili, to the bacterial surface, or to the flagella.

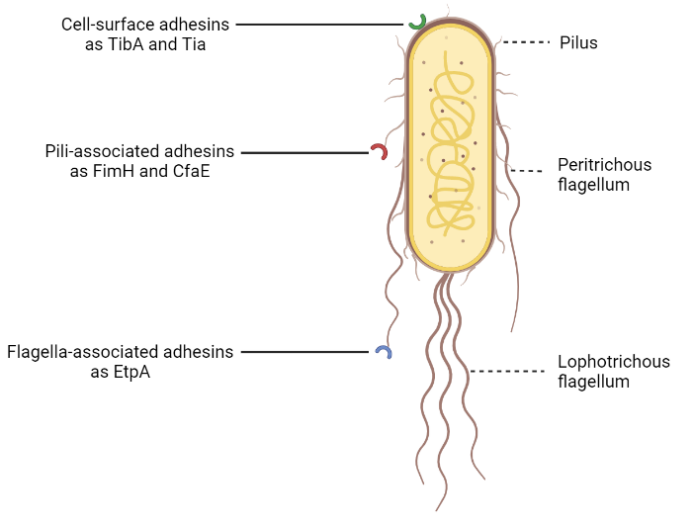


Figure 4.9. Schematic repartition of known ETEC adhesins.
The three-known repartition of ETEC adhesins have been represented. Pili- and flagella-associated adhesins have been found to localize at the tip of the appendixes.
CFA: colonisation factor antigen, CS: Coli Surface antigen, LT: heat labile toxin, ST: heat stable toxin.
Built from personal source.

4.3.2.3 Host cell receptors to ETEC adhesins

Recognition of cellular receptors by ETEC pili and non-pili adhesins is part of the infectious mechanism to facilitate resilient host-pathogen interaction. Nonetheless, most of the molecular receptors for ETEC CF remain scarcely known, especially in human (Kumar *et al.* 2014). Additional aspects to consider, such as receptor-binding specificity, distribution and stability of the receptors along the gut make the understanding even more complex. In general, these receptors are composed by sugars found in glycoproteins or glycolipids in the villous epithelium of the small intestine and are able to hemagglutinate (Morabito 2016).

The major subunit of CFA/I, CfaB, has been found to bind asialo-glycosphingolipids from Caco-2 cells (Madhavan *et al.* 2016). In relation with the FimH stereochemical specificity for mannose residues (Li *et al.* 2009; Sheikh *et al.* 2017), a study using enteroids derived from healthy human intestinal stem cells has shown that the adhesion of FimH, tipped on type I pili, enhanced production of highly mannosylated proteins on intestinal epithelial cells (Sheikh *et al.* 2017). Further, binding of CS6 to fibronectin has been reported, indicating that extracellular matrix proteins could also serve as a focal contact point prior to reach rabbit epithelial cells (Chatterjee *et al.* 2011). CS21, which is expressed on E9034A (a wild-type ETEC strain originally isolated from an outbreak of diarrhea in the Caribbean) mediates the bacteria adherence to IPEC-1 and IPEC-J2 (porcine) cell lines and would recognise specifically neuraminic acid residues (Guevara *et al.* 2013).

4.3.2.4. ETEC adhesion to mucus

Under healthy conditions, mucosal surfaces composed of gel-forming mucins lining the gastrointestinal tract prevent penetration by pathogens as ETEC (see section 2.1.1). Still, data about ETEC adhesion to mucus and mucus receptor to adhesins are scarce. Kerneis and colleagues, in 1994, showed that the ETEC strain H10407 adhesion to HT29-MTX and HT29-FU, two mucus secreting cell lines, co-localize more with the brush border of the cells than with their mucus patches (Kerneis *et al.* 1994), which is in contradiction with cellular studies conducted with other intestinal pathogens (Gagnon *et al.* 2013; Hews *et al.* 2017).

Few studies have investigated ETEC adhesins specificity for mucus polysaccharides. The CF CS2, CS5 and CS6 bind to components of rabbit intestinal mucus and this interaction is prevented by treatment with sodium metaperiodate salt, suggesting recognition of specific carbohydrates (Helander, Hansson and Svennerholm 1997). Besides this observation, as already quoted in section 2.1.2, human blood group antigens (Group-A and/or -B) can be found in

mucosal secretions. It has been shown that CFA/I adhesion can adhere to blood group A-terminated glycosphingolipids but not to blood group B-terminated glycosphingolipids expressed on host cells (Jansson *et al.* 2006; Ahmed *et al.* 2009). Supporting this finding, ETEC strains expressing CFA/I group of CF infect more frequently blood group A children compared to blood group B (Ahmed *et al.* 2009). In the same way, EtpA binds preferentially to N-acetylgalactosamine expressed in the context of A blood group glycans on the intestinal mucosa (Qadri *et al.* 2007; Kumar *et al.* 2018; Kuhlmann *et al.* 2019). Thus, ETEC possess two blood group-A specific adhesins. It was shown that EtpA locates preferably in close proximity to mucin-producing cells in frozen section of mouse ileum (Roy *et al.* 2009).

Bullet points, ETEC intestinal adhesion

- Human ETEC strains possess numerous adhesins localized on pili appendixes, the flagella or the cell surface.
- Epithelial and especially mucus receptors to human ETEC adhesins are scarcely known and need further investigations, *in vitro* and *in vivo*.

4.3.3. Mucin-degrading proteins

In the case of ETEC infection, two mucin-degrading enzymes or mucinases have been identified over the last five years. These mucinases allow temporary access to cell membrane, then promoting close contact of the pathogen to the intestinal epithelial cells.

4.3.3.1. EatA

EatA is a member of serine protease autotransporters of the *Enterobacteriaceae* (SPATE). According to two different studies, the level of detection of this mucinase gene in ETEC isolates would be between 55-70% (Kuhlmann *et al.* 2019; Mondal *et al.* 2021). *EatA* gene is not specific of the ETEC pathotype, as a study also detected this gene in 8.8% of EPEC strains (Abreu *et al.* 2013). EatA is able to degrade MUC2, a major protein present in the human mucus layer of the small intestine (Sheikh *et al.* 2021). In a model using LS174T colonic cell lines producing abundant MUC2, EatA accelerates the removal of MUC2, thereby facilitating the access of ETEC enterotoxins to the enterocyte surface (Kumar *et al.* 2014). In human enteroids also, EatA engages and degrades MUC2, promoting ETEC access to the epithelium and toxin action (Sheikh *et al.* 2021). EatA has also another contradictory function. By degrading the EtpA adhesin, it would decrease the pathogen mucosal colonization in a murine

model, in favor of fecal shedding (Roy *et al.* 2011). Still, in ETEC strains, EatA accelerates the LT enterotoxin effect on Caco-2 cells (Roy *et al.* 2011). In general, adhesion by bacteria is thought to be an important prerequisite for delivery of bacterial effectors such as enterotoxins. However, the ability to negatively modify adhesion events also appears to be an important virulence trait, not yet clearly understood. In anyway, EatA seems to be a particularly important virulence factor for the development of symptoms since *eatA* seems to be correlated with the presence of *stx* gene, at least in children below 2 years old (Kuhlmann *et al.* 2021). Lastly, a recent study using transposon mutagenesis and transcriptomic analysis identifies EatA mucinases as one of the important genes for the capability of ETEC strain H10407 to survive in the gut of mice (Abd El Ghany *et al.* 2021).

4.3.3.2. YghJ

YghJ (also known as SsLE for secreted and surface associated lipoprotein) is a cell surface associated and secreted lipoprotein harboring M60 metalloprotease domain (Tapader, Bose and Pal 2017). This lipoprotein is able to forms amyloid-like fibrils *in vitro* (Belousov *et al.* 2018). The gene is conserved among both pathogenic and commensal *E. coli* isolates, but the expression and secretion of YghJ is higher among diverse *E. coli* pathotype including ETEC (Nakjang *et al.* 2012; Luo *et al.* 2014; Tapader *et al.* 2016). YghJ was found to be secreted by 89% of ETEC isolates (Luo *et al.* 2014). Furthermore, according to a recent study, YghJ from ETEC origin would be more glycosylated and more immunogenic (Thorsing *et al.* 2021). YghJ is able to degrade MUC2 and MUC3 mucins (Luo et al 2014). YghJ is co-transcripted with the type 2 secretion system by which the mucinases are also secreted (Yang *et al.* 2007; Luo *et al.* 2014). Interestingly, YghJ promotes access to LS174T cells and optimal delivery of LT enterotoxin (Luo *et al.* 2014). More strikingly, purified YghJ alone causes extensive tissue damage and is also able to induce significant fluid accumulation in a mouse ileal loop assay (Tapader, Bose and Pal 2017), indicating an intrinsic enterotoxic effect not yet elucidated.

Bullet points, mucin degrading proteins

- Two mucin-degrading enzymes, or mucinases, have been identified in human ETEC strain recently, EatA and YghJ.
- These two enzymes are widely spread in ETEC strains but not restricted to them.
- Even if mechanistic data about these enzymes are scarce, their role in ETEC adhesion and toxin delivery has been demonstrated.

4.3.4. Outer membrane vesicles

Outer membrane vesicles (OMVs) are nanoscale proteoliposomes (20–250 nm) secreted from the cell envelope of all Gram-negative bacteria (**Fig. 4.10**). They are produced by a controlled budding of the bacterial outer membrane *via* different mechanisms (**Fig 4.11**). As a result, OMVs are surrounded by a single membrane bilayer and contain mostly components of the bacterial outer membrane and the periplasm (Kulp and Kuehn 2010). Concerning pathogens, OMVs can carry both bacterial toxins (Horstman and Kuehn 2000; Kesty *et al.* 2004) and other virulence factors such as adhesins, invasins, outer membrane proteins, LPS, flagellin, and proteases (Ellis and Kuehn 2010; Rueter and Bielaszewska 2020). In general, OMVs are released in increased amounts from pathogenic bacteria, suggesting that OMV secretion is an additional virulence mechanism of pathogens (Horstman and Kuehn 2000; Ellis and Kuehn 2010). Pathogenic *E. coli* such as ETEC and EHEC produce OMVs under laboratory conditions as well as during infection (Rueter and Bielaszewska 2020), as they may serve as vehicles for toxin delivery into host cells and inducers of inflammatory responce (Chutkan and Kuehn 2011). Among the virulence factor associated to ETEC, OMVs are reported to sustain the transport of the LT toxin, EtpA, CeXE and tibA (Roy *et al.* 2010). In particular, the OMV-LT toxin association has been demonstrated by several groups (Wai, Takade and Amako 1995; Horstman and Kuehn 2000).

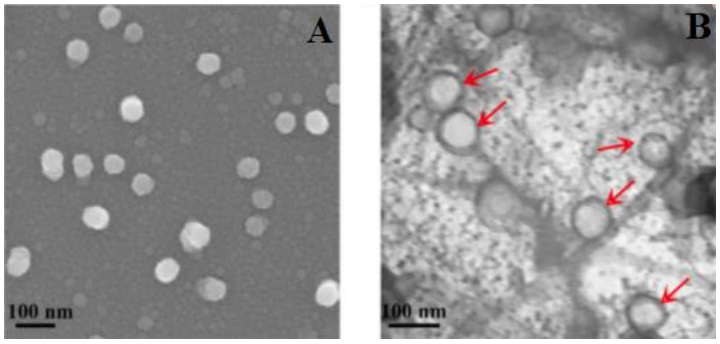


Figure 4.10. Scanning electron (A) and transmission electron (B) micrographs characterizing OMVs secreted by an *E. coli* strain (Avian pathogenic *E. coli* O2).
On the transmission electron microscopy panel (B), the red arrows indicate the OMV.
The figure has been reprinted with permission from Hu *et al.* 2020a.

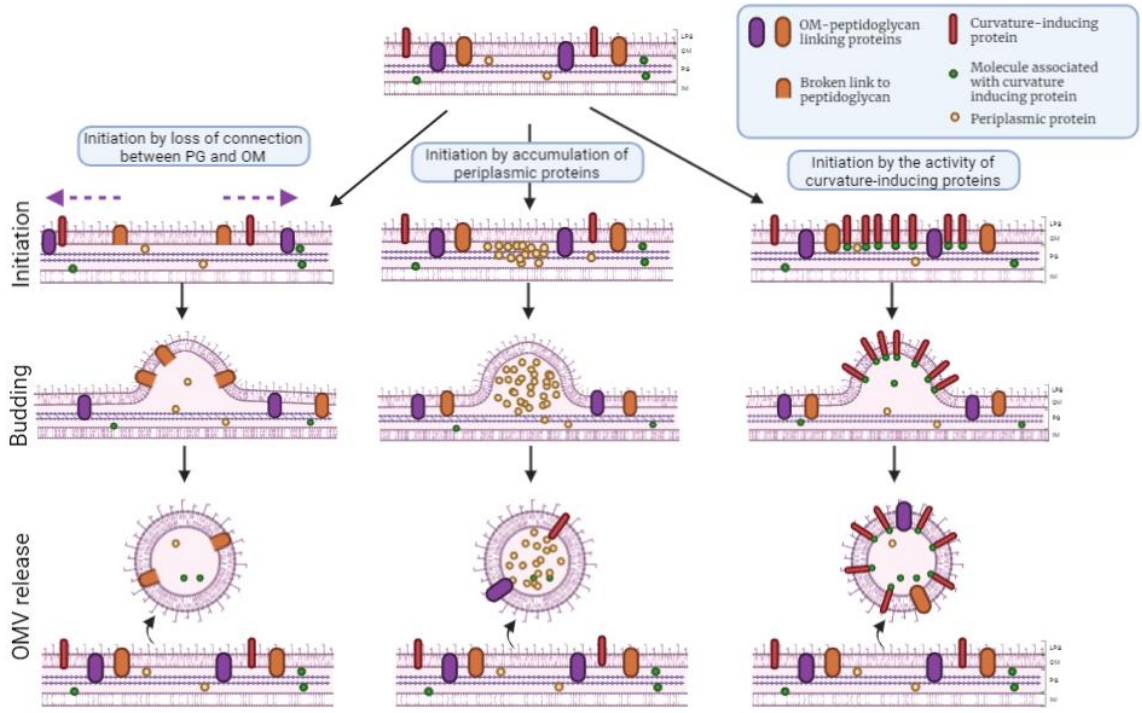


Figure 4.11. Different mechanisms of OMV formation.
Different mechanisms can initiate the budding of Gram-negative bacteria outer membrane. In left column, links between the OM and the peptidoglycan are lost, either by movement of the linking protein (purple ovals) or by breaking the connections directly (orange half-ovals). In the central column, gathering of periplasmic proteins (yellow dots) are responsible for the initial curvature. This ultimately results in OMV enriched in periplasmic proteins. In the right column, the initiative come from curvature-inducing OM proteins (red stick). These proteins and the one associated to them (green dots) are enriched in the released OMV. The OMV formation can also be triggered by LPS remodeling not represented here. These multiple budding mechanisms are not exclusive.
LPS: lipopolysaccharides, OM: Outer membrane, PG: peptidoglycan, IM: inner membrane.
Built according to Kulp and Kuehn 2010 and Schwechheimer and Kuehn 2015.

4.3.5. Enterotoxins production

4.3.5.1 Heat-labile enterotoxin (LT)

As its name suggests, LT is sensitive to heat treatment and easily breaks down at 70°C for 10 minutes (Gill *et al.* 1981). This large enterotoxin (84 kDa) encoded by *eltAB* gene shares 80% homology of structure and function with Cholera toxin from *Vibrio cholerae*. LT is a multimeric AB5 toxin, composed of a single catalytic A subunit (LTA), associated with a five B subunits (LTB) necessary for binding and internalization (Dubreuil 2012). The LTA subunit consists of a large A1 domain and a short A2 domain (Sánchez and Holmgren 2005).

The genes encoding LT are located on a large plasmid called pEnt (Ochi *et al.* 2009). A study revealed that approximately 60% of ETEC isolates associated with human diarrhea expressed either LT alone (27%) or LT with ST (33%) (Isidean *et al.* 2011). LT is further

categorized as either LT-I type or LT-II type (LT-IIa, b, c) depending on its antigenic capacity and associated genetic sequence (Hajishengallis and Connell 2013). The two toxin LT-I and LT-II share less than 14% amino-acid sequence identity in the amino-acid sequence of their B subunits, resulting in differential binding to ganglioside receptors (Liang and Hajishengallis 2010). LT-I can be further divided in LTIh and LTIp, produced respectively by human and porcine and human ETEC strains (Dubreuil, Isaacson and Schifferli 2016). LT-II toxins have mainly been observed causing disease in humans and calves (Connell and Holmes 1992; Nagy and Fekete 2005).

ETEC contact with host cell is required for efficient LT toxin delivery (Dorsey, Fischer and Fleckenstein 2006). Both LTA and LTB subunits feature signal sequences directing them to the periplasm of *E. coli* through the Sec translocation machinery (Spicer and Noble 1982; Yamamoto *et al.* 1982). In the periplasm, monomers assemble spontaneously or by disulfide bond protein A (DsbA) disulfide oxidoreductase activity, for the AB5 toxin to accumulate. Then, part of the LT toxin is secreted through a type II secretion system (T2SS) to the external environment (Hirst *et al.* 1984; Ellis and Kuehn 2010). ATPase activity is required for this secretion (Mudrak and Kuehn 2010). Once in the external environment, LT binds to the bacterial outer membrane via interaction of LT-B subunit with lipopolysaccharide S (Ellis and Kuehn 2010). Thus, LT accumulates both in the bacterial periplasm and on the bacterial surface. Subsequently, the toxin is released from the bacterial cells by budding of OMVs (Dubreuil, Isaacson and Schifferli 2016). In the H10407 strain, the LeoA protein has been shown to favor LT secretion. Actually, this bacterial GTPase is a dynamin-like protein (DLP) which uses energy to remodel membranes and would help the formation of OMVs (Michie *et al.* 2014). However, only around 3% of ETEC strains carry the *leoA* gene (Turner *et al.* 2006a), suggesting that the role of LeoA in LT secretion is certainly not a universal one. *Via* interaction of another site of its B subunit, the LT associated to the external side of vesicle membrane recognises the GM1 receptor, which is a ganglioside (composed of a glycosphingolipid with one or more sialic acids) expressed on the host cells surface (Chatterjee and Chaudhuri 2011). OMV endocytosis will be dependent of cholesterol-rich lipid rafts found on the surface of intestinal epithelial cells (Kesty *et al.* 2004). Therefore, the endocytosed vesicle associated LT will traffic through the Golgi apparatus and endoplasmic reticulum. Following internalization into the endoplasmic reticulum, the LTA peptide is cleaved into A1 and A2 fragments (Gill and Richardson 1980; Epstein *et al.* 1989). The A1 domain harbors its catalytic function via ADP-ribosylation of G proteins, resulting in activation of adenylate cyclase and elevated intracellular cyclic AMP (cAMP) levels. This is followed by the PKA phosphorylation of cystic fibrosis transmembrane

regulator (CFTR), a chloride channel present at the apical membrane of intestinal epithelial cells brush-border. Activation of CFTR provokes the opening of this anion channel and results in the secretion of chloride (Cl^-) and bicarbonate ($HCO3^-$) ions from the cells into the intestinal lumen (Hug, Tamada and Bridges 2003), eliciting massive watery diarrhea (Ellis and Kuehn 2010). CTFR activation is considered as the major player in LT-induced secretions. The canonical action mode of the LT toxin in the H10407 strain is summarized in **Figure 4.12**. Of note, the action of LT toxin on cells can also occurs directly, without involvement of ETEC OMVs.

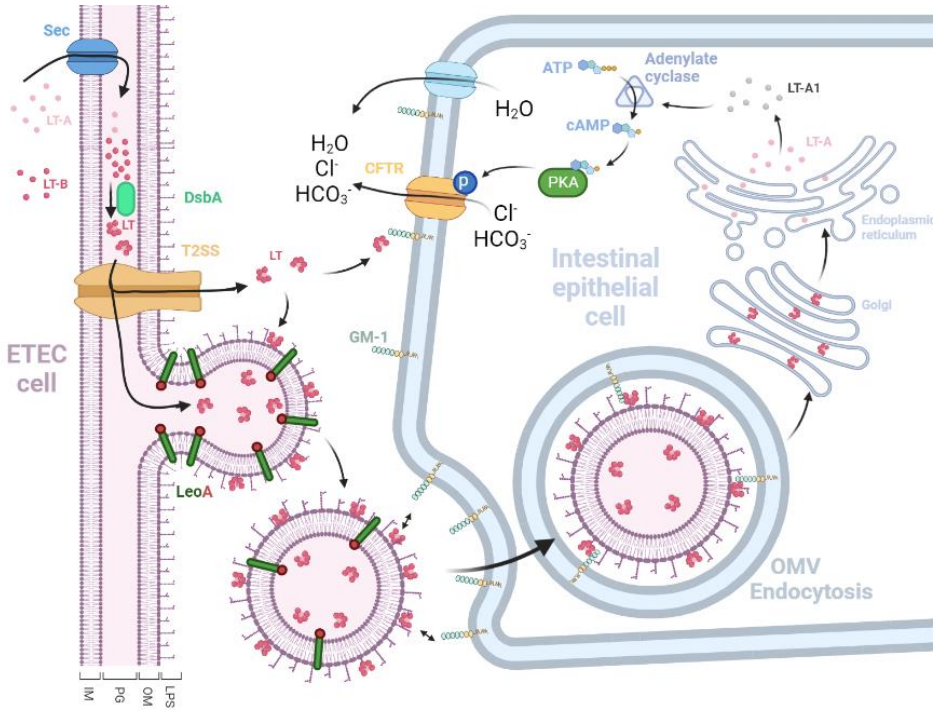


Figure 4.12. Model of heat-labile enterotoxin (LT) secretion, internalization and mode of action in intestinal epithelial cells.
The figure presents the model for LT mode of action from ETEC secretion to minerals release, as presented in the main text.
CFTR: Cystic Fibrosis Transmembrane Regulator, DsbA: disulfide bond protein A, LeoA: labile enterotoxin output A, GM1: monosialotetrahexosylganglioside, IM: inner membrane, LT: Heat labile toxine, LPS: lipopolysaccharide, OM: outer membrane, PG: peptidoglycan, PKA: Phosphokinase A, Sec: sec machinery, OMV: outer membrane vesicle, T2SS: type 2 secretion system, Sec: Sec machinery.
Built from personal source.

Concerning LT effect on ETEC pathogenesis, it has been very well described in piglets how functional LT toxin from animal ETEC strains favors the bacteria adherence and subsequent intestinal colonisation (Santiago-Mateo *et al.* 2012; Fekete *et al.* 2013). Concerning ETEC strains from human origin, the data are scarce. LT can enhance adhesion to Caco-2 cells (Johnson *et al.* 2009) and to HCT-8 cells *via* MAPK signaling pathway (Wang, Gao and

Hardwidge 2012). Interestingly, pure cAMP (the main product of LT toxin catabolism) also stimulates the LT-dependent adherence of ETEC to enterocytes *in vitro* (Johnson *et al.* 2009). The LT toxin also alters the structure and composition of the intestinal epithelial mucin layer by reducing MUC4 expression (Verbrugghe *et al.* 2015) and enhancing MUC2 expression (Duan *et al.* 2019), both effect resulting in increased adhesion of the pathogen. Recently, the induction of MUC2 secretion by LT toxin was confirmed at the protein level in human enteroids (Sheikh *et al.* 2021). Among other LT toxin effect, one study reported that the toxin decreases intestinal epithelial cells viability, inducing apoptosis in a dose and time dependent manner in HCT-8 cells, Caco-2 cells and mouse model (Lu *et al.* 2017).

4.3.5.2. Heat-stable enterotoxin (ST)

E. coli heat-stable enterotoxins came to attention in the 1970s after it was observed that heat-inactivation of bacterial cultures from patients and animals suffering from diarrhea failed to eliminate enterotoxigenic activity (Smith and Gyles 1970; Burgess *et al.* 1978). Indeed, these toxins remain active after 60 min of heating at 95°C. Their small size and 3D-structure are responsible for resistance to boiling.

ETEC isolates can express two distinct ST families, differing in structure and function: the methanol soluble protease resistant STa (synonyms ST1), and the methanol insoluble and protease sensitive STb (synonyms ST2) (Chapman *et al.* 2006; Weiglmeier, Rösch and Berkner 2010; Loos *et al.* 2012). The STa and STb peptides are encoded by two distinct genes, *estA* and *estB* (Harnett and Gyles 1985). The STb variant is almost always associated to animal infected with ETEC and its role in human infection is very debated (Weiglmeier, Rösch and Berkner 2010; Loos *et al.* 2012). The STb variant will no longer be mentioned in this manuscript.

Within STa, two variants associated with human disease have been described, STh and STp, originally found in human and pig, respectively (Weiglmeier, Rösch and Berkner 2010). One ETEC strain can possess several variants of the ST toxin, as the ETEC strain H10407 strain possess both STh and STp toxin variants (Haycocks *et al.* 2015).

The STa polypeptide is a non-antigenic, low molecular weight protein (2 kDa). STa is synthesized as 72 amino acid proteins consisting of a 19 amino acids signal peptide signal peptide, a 34 amino acids pro peptide and a 18-19 amino acids carboxy terminal region, forming the mature and active enterotoxin (Wang *et al.* 2019a). STa polypeptide is translocated across the inner membrane to the periplasm *via* the signal peptide, and cleaved into the mature STa peptide by Sec machinery-dependent export pathway (Weiglmeier, Rösch and Berkner 2010). Then, the toxin is folded to its mature tertiary structure *via* the action of DsbA. Then the toxin

is efficiently secreted into the lumen through the TolC channel, a multidrug pump efflux system (Yamanaka *et al.* 2008). After its secretion, STa will bind the guanylate cyclase C (GC-C) receptor on intestinal epithelial cells. GC-C is a glycoprotein that is expressed on the brush border of villous and crypt intestinal cells (Steinbrecher 2014). It is well demonstrated, at least in animal study, that the GC-C receptor concentration is maximum in the jejunum and ileum (Al-Majali *et al.* 2007; Al-Majali and Khalifeh 2010). STa binding to GC-C leads to increase intracellular formation of cyclic GMP (cGMP) (Weiglmeier, Rösch and Berkner 2010; Sunuwar *et al.* 2020). Increased levels of cGMP activate the cGMP-dependent protein kinase II (PKGII), which co-localizes with the CFTR transporter and phosphorylates it (Vaandrager *et al.* 1997, 1998), thus promoting the release of Cl^- into the lumen. In addition, cGMP is able to inhibit phosphodiesterase 3 (PDE3) that hydrolyzes cAMP, resulting in cAMP accumulation. In turn, cAMP activates protein kinase A, which will also phosphorylate CFTR (Chao *et al.* 1994). The Na^+ /H^+-exchanger (NHE) is a second target of cGMP action in intestinal epithelium. The protein kinase A inhibits the re-absorption of sodium by NHE (He and Yun 2010). The canonical action mode of the ST toxin is detailed in **Figure 4.13**.

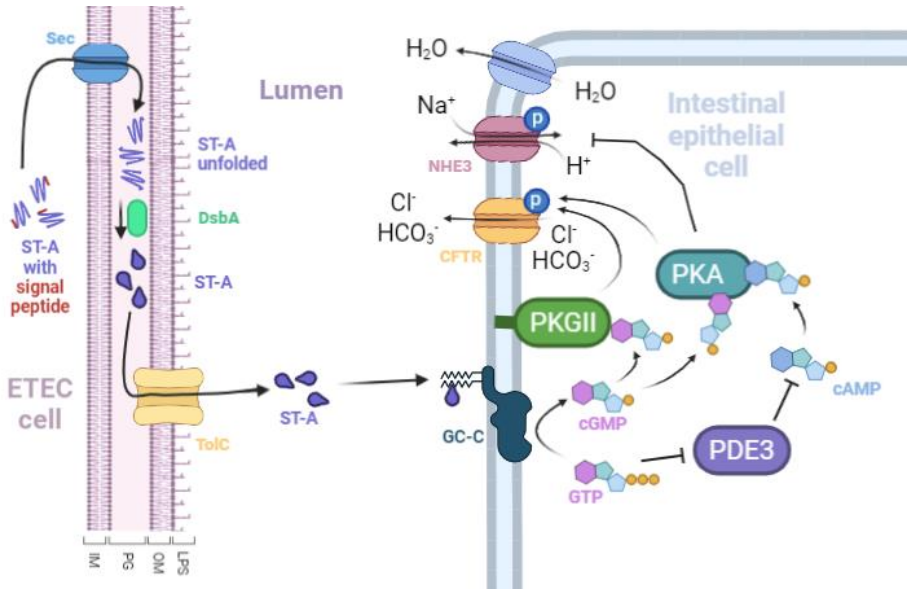


Figure 4.13. Model of heat-stable enterotoxin variant A (ST-A) secretion, internalisation and mode of action in intestinal epithelial cells.
The figure presents the model for ST-A action mode from ETEC secretion to minerals release, as presented in the main text.
CFTR: Cystic Fibrosis Transmembrane Regulator, DsbA: disulfide bond protein A, GC-C: guanylate cyclase C, IM: inner membrane, PDE3: phosphodiesterase 3, PKA: protein kinase A, PKGII: cGMP-dependent protein kinase II, LPS: lipopolysaccharide, NHE3: Na+ /H+-exchanger 3, OM: outer membrane, PG: peptidoglycan, ST: Heat stable toxine, Sec: sec machinery, TolC: TolC efflux protein.
Built from personal source.

Concerning ST effect on host physiology, it has been observed that treating T-84 polarized cell monolayers with STa elicited a reduction in TEER, indicating an increased permeability (Nakashima, Kamata and Nishikawa 2013). In this sense, ETEC challenge in volunteer's increases fecal intestinal fatty acid-binding protein (I-FABP) concentration, an indicator of compromised intestinal epithelial integrity (Brubaker *et al.* 2021).

Bullet points, ETEC enterotoxins

- Human ETEC possess at least one of the two heat-labile (LT) and heat-stable (ST) toxins.
- Each toxin possesses variants that are more or less associated with human infection, even if this classification is actually debated. The LTIh, LTII and STa variants could be considered as the classical ones associated to human infection.
- All these variants elicit a profuse watery diarrhea notably by activation of the CFTR canal and secretion of chloride (Cl^-) and bicarbonate ($HCO3^-$) ions from the cells into the intestinal lumen.
- Among the other toxins well- characterized effects, the LT toxin would favor ETEC adhesion, while the ST toxin increases cellular permeability.

4.3.6. ETEC virulence: regulatory networks

4.3.6.1. Genetic features of the reference strain ETEC H10407

ETEC strain H10407 serotype O78:H11:K80, originally isolated in 1973 from an adult case of severe cholera-like diarrheal illness in Dacca (Bangladesh), is to date the most extensively characterized strain of the pathotype. This isolate is LT+, ST+, CFA/I+, EtpA+, tia +, YghJ+, eatA+. Although ETEC strain H10407 contains all of these classical virulence factors, there are not all necessary to cause symptomatic disease (Levine *et al.* 1977).

The complete genomic and plasmidic sequences of ETEC strain H10407 are available and represented in **Figure 4.14** (Evans *et al.* 1977; Crossman *et al.* 2010; Haycocks *et al.* 2015) (EMBL database accession number FN649414). The ETEC H10407 genome consists of a circular chromosome of 5,153,435 bp and four plasmids designated pETEC948, pETEC666, pETEC58, and pETEC52. The two larger plasmids (pETEC948 and pETEC666) are reminiscent of conjugative plasmids that are often associated with the carriage of virulence factors, whereas the two smaller plasmids (pETEC58 and pETEC52) are homologous to mobile plasmids frequently encountered in a variety of bacterial species (Crossman *et al.* 2010; Haycocks *et al.* 2015; Hazen *et al.* 2017).

This reference strain has been used in the framework of this PhD work.

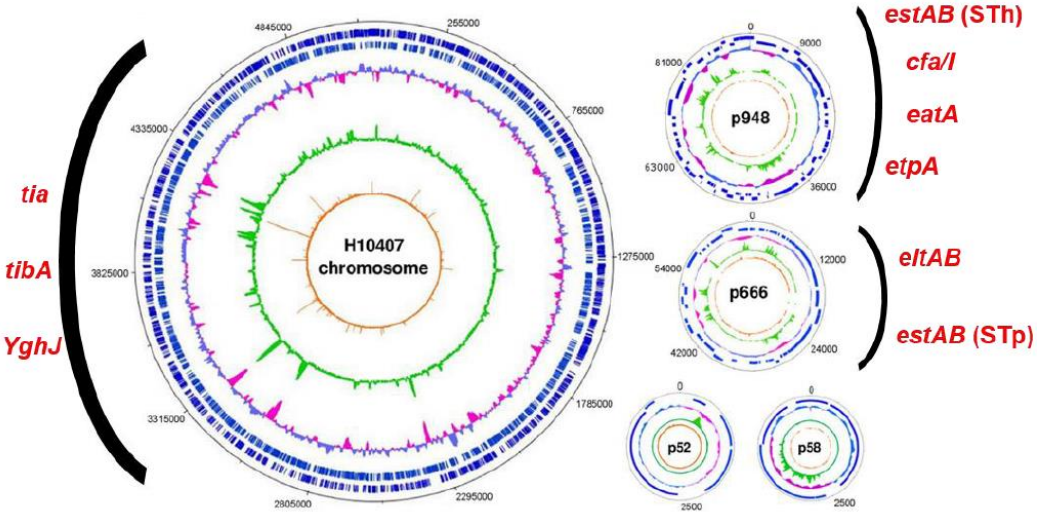


Figure 4.14. Genome and plasmids mapping of ETEC H10407.
The main virulence genes of ETEC H10407 are represented in red.
CFA/I: colonisation factor antigen I, eatA: ETEC autotransporter A, tia: enterotoxigenic locus invasion A, tibA: Enterotoxigenic invasion locus B, ST: Heat-stable toxin.
Modified from Haycocks et al., 2015.

4.3.6.2. Intrinsic transcriptional modulations

Pathogens in general have deployed mechanisms to sense the environment in which they evolve. In response to the signals received, they will act accordingly by turning off or on the expression of their virulence genes. However, prior to describing ETEC genes regulation according to the gastrointestinal cues (developed in section 4.3.6.3), intrinsic network governing ETEC genes modulations will be discussed.

To date, the ETEC virulence networks are largely unknown and need to be unraveled. The majority of transcriptional studies have focused on ETEC *rns* regulon (related to ToxT, found in *V. cholera*) (Midgett *et al.* 2021). RNS can regulate the expression of almost half of the known pili in ETEC and also non pili adhesins, such as EtpA (Basturea *et al.* 2008; Bodero, Harden and Munson 2008)

The heat-stable nucleoid-structural (H-NS) protein for its part negatively controls the transcription of *eltAB* gene, encoding for LT toxin. Interestingly, H-NS also regulates the *gspCDEFGHIJKLM* gene cluster and *Yghj* (mucinase gene) (Yang *et al.* 2005, 2007). As GspD is a pore-forming protein helping the LT translocation through the T2SS (Ellis and Kuehn, 2010), it appears that the transcriptional machinery responsible for the production and secretion of LT is governed by H-NS.

The cAMP receptor protein (CRP) senses cAMP, and regulates the toxins genes accordingly. When cAMP is high, CRP represses transcription of *eltAB* gene while it positively

regulates *estAB* gene (Bodero and Munson 2009; Haycocks *et al.* 2015). This illustrates that the two toxins genes can have differential regulation patterns as already reported (Roussel *et al.* 2020b). The FNR regulon is another transcriptional factor that would inhibit virulence genes as *fimH* and CFA/I adhesin genes, and the toxin genes *eltAB*, *sta1* and *sta2* (Crofts *et al.* 2018), in anaerobic condition (Crofts *et al.* 2018).

Finally, *leoABC* genes and *tia* locus are tightly regulated together. *LeoA* is encoded within the *tia* locus, itself within a pathogenicity island (Fleckenstein *et al.* 2010).

4.3.6.3. Virulence modulation in the gastrointestinal tract

To be fully pathogenic, bacteria must not only survive in the human GIT but also coordinate expression of virulence determinants in response to localized gut microenvironments. An increased number of *in vitro* or animal studies have shown that ETEC is able not only to resist the stressful conditions encountered in the gut (see section 4.3.1), but rather respond or utilize various GI cues to modulate the expression of its virulence factors (Gonzales-Siles and Sjöling 2016; Sistrunk *et al.* 2016; Roussel *et al.* 2020b).

4.3.6.3.1. Modulation by physicochemical parameters of the human gut

In the TIM-1 model, upon human gastric simulated digestion, the gene *eltB* from ETEC strain H10407 encoding LT toxin production was repressed at a pH below 3.6 and no production of LT toxin was observed (Roussel *et al.* 2020b). This observation is in accordance with studies conducted in much more simpler *in vitro* models, which have shown that extracellular pH has an influence on the release of LT toxin, increasing with alkalinity (Kunkel and Robertson 1979; Hegde, Bhat and Mallya 2009). Apart from the *eltB* gene, other virulence genes as *leoA*, *fimH* and *tia* tended to be induced in the gastric compartment. These gastric observations are in opposition with virulence gene expression patterns in the ileal compartment. There, the *eltB* toxin tended to be expressed at the beginning of the digestion and repressed at the end, and all the other virulence genes assayed (*estP, leoA, tolC, fimH, tia and CfaIb*) were repressed. This global anti-virulence profile induced in the ileum of the TIM-1 (while it supposed to be the colonisation site of ETEC) could be related to some limitations of the model such as high oxygen concentrations and lack of gut microbiota.

Concerning oxygen, LT would be secreted efficiently under anaerobic or microaerobic conditions only in presence of terminal electron acceptors (e.g. trimethylamine N-oxide dihydrate or nitrate). Precisely, GspD protein, a secretin subunit of the T2SS required for LT secretion is assembled under anaerobic conditions in presence of terminal electron acceptors

only (Lu *et al.* 2016). Another *in vitro* study reported that in anaerobic condition, the FNR regulator inhibits *eltAB* genes (encoding for LT toxin) and *cfa* (encoding for CFA/I adhesin). This inhibition could be lost near the epithelium, where microaerobic conditions prevail (Crofts *et al.* 2018). In accordance, a study in the M-SHIME model, which reproduces anaerobic conditions of the gut, showed that most of ETEC strain H10407 virulence genes were inhibited in ileum and colonic compartments. Of note, despite the absence of oxygen, it was also reported that *eltB* was punctually promoted (Roussel *et al.* 2020b).

Digestive enzymes and bile salts are other component that could act as chemosensors and allow ETEC to locate itself in the gut. In *in vitro* studies, trypsin has been shown to increase LT release (Kunkel and Robertson 1979) and its secretory activity (Rappaport *et al.* 1976). Numerous studies have investigated the impact of bile salts on ETEC virulence but leading to contradictory results. Bile salts have been found to prevent the binding of LT toxin to the GM1 at 2 $g.L^{-1}$ (Chatterjee and Chaudhuri 2011) but to up-regulate *estA*, *eltA* or *etpA* genes at 30 $g.L^{-1}$ (encoding STa, LTa and EtpA, respectively) (Sahl and Rasko 2012). Sodium deoxycholate and sodium glycocholate at 1.5 $g.L^{-1}$ have been shown to induce *in vitro* the expression of CS5 encoding gene (Nicklasson *et al.* 2012) but to downregulate the CS1 and CS3 encoding genes at 30 $g.L^{-1}$ (Sahl and Rasko 2012). Finally, it appears that the modulation of ETEC virulence genes expression by bile salts may be strain dependent (Sahl and Rasko 2012). The different concentrations and products used to reproduce the bile secretion in these numerous studies also impede common interpretation.

4.3.6.3.2. Modulation by interactions with intestinal epithelial cells

Pathogen-host cell interactions are finely orchestrated by ETEC. When cAMP is recognised by the cAMP receptor protein, the transcription of *eltAB* is suppressed. As cAMP is one of the main products of the LT toxin activity on epithelial cells, this would constitute a feedback loop by which the production of LT can be downregulated once a certain amount of cAMP has been released by intestinal epithelial cells (Bodero and Munson 2009; Haycocks *et al.* 2015).

To date, only one study has investigated transcriptional modifications of ETEC when interacting with intestinal epithelial cells (Kansal *et al.* 2013). This study was first conducted with the ETEC strain E24377A and the Caco-2 cells model. Among the genes regulated during ETEC-cell contact, *crp* gene, the repressor of *eltAB,* was down-regulated in adhered ETEC compared to ETEC grown in cell culture media alone. Surprisingly, genes encoding for LT, ST-1b toxin and CF were found to be down-regulated in adhered ETEC cells compared to ETEC

in cell culture media. Then, still on Caco-2 cells, the authors showed with another ETEC strain (H10407) that although *crp* expression was down-regulated in attached bacteria, *eltA* was increased when compared to planktonic organisms (average, 7,2 ± 3,8-fold; P = 0.047). The authors also demonstrated that the modulation of ETEC adhesion-related genes on cell contact was time-dependent, with a clear adhesion promoting profile reported at 15 min and a more mixed expression profile at 60 min. In support of this sequential genes activation, at early time points, ETEC strain H10407 appears to engage host cells at a distance *via* their flagella (15 min) while at later time points (30, 60 min), flagella appear shortened and/or engulfed by the host cell. Interestingly, at 60 min, vesicle-like structures that look like OMVs are visible on the bacterial surface. These results suggest that ETEC strains may react to cells adhesion proximity in a strain specific manner (Kansal *et al.* 2013).

4.3.6.3.3. Modulation by microbiota and metabolic activities

Very few data on how human gut microbiota may influence ETEC virulence are available to date. Roussel and colleagues demonstrated using the M-SHIME model that virulence genes, among which *eltB*, are switched off in the ileum and the ascending colon, where microbiota populations and associated activities are important. A study has shown that addition of SCFA (e.g. acetate, propionate and butyrate) at of 2 $mg.mL^{-1}$, a concentration relevant of the colonic levels (Cummings 1981), the culture medium significantly reduced or even abolished LT production (Takashi, Fluita and Kobari 1989). Moens and colleagues found that inoculation of ETEC strain H10407 in human colonic batch system does not result in a clear increase of LT toxin concentration (Moens *et al.* 2019). Thus, microbial activity cues could be inhibitor of ETEC virulence, which is coherent with an ETEC site of action in the distal part of the small intestine. At the opposite, free glucose (that is absorbed in the upper GIT and thus is not supposed to be present in high quantity in the colonic compartment) has been found to stimulate LT toxin production (Mudrak and Kuehn 2010).

To our knowledge, only one study addresses how specific members of the human gut microbiota (apart from probiotic, addressed in section 4.4.3.) could impact ETEC virulence. In volunteers challenged with 10^5 or 10^6 CFU of ETEC strain H10407, some phylogroups of the gut microbiota have been associated with disease severity. The most robust predictors of symptoms development included *Bacteroides dorei*, *Prevotella* sp., *Alistipes onderdonkii*, *Bacteroides* sp. (ovatus), and *Blautia* sp., while the predictors of resistance included *Sutterella* sp., *Prevotella copri*, and *Bacteroides vulgatus* (Pop *et al.* 2016).

Bullet points, ETEC virulence modulation in the gastro-intestinal tract

- Few studies have focused on ETEC virulence modulation by specific physicochemical parameters found in the gut, making difficult any conclusion.
- Surprisingly, Roussel's work reported that ETEC virulence genes encoding for the toxins machinery, CFA/I, tia and fimH are switched- on and switched-off by physicochemical parameters of the stomach and ileum, respectively.
- First clues indicate that ETEC virulence gene expression seems to be down regulated by microbial activity occurring in the colon.

4.3.7. ETEC and inflammation

4.3.7.1. General ETEC effect on host inflammation

In cellular model, ETEC infection leads to a global inflammation pattern. In T-84 cells, infection with ETEC strain H10407 at a multiplicity of infection (MOI) 20 triggers LPS-dependent pro-inflammatory IL-8 secretion (He *et al.* 2016), while in HCT-8 cells, infection at MOI 50 activates both NF-κB and MAPK signaling pathways. In Caco-2 cells, it has been shown that ETEC strain H10407 at MOI 100 triggers an IL-8 secretion (Roussel *et al.* 2018b). In the C57BL/6 mouse model, single oral challenge with ETEC strain H10407 (10^9 CFU) leads to an increased fecal excretion of lipocalin-2 (Lcn-2) and myeloperoxidase (MPO) (Bolick *et al.* 2018). In humans, travelers with ETEC diarrhea have been reported to present higher markers of enteric inflammation, such as fecal blood, leukocytes, lactoferrin, fatty acid-binding protein and increased levels of fecal cytokines as IL-8, IL-1β, and IL-1ra (Rodrigues *et al.* 2000; Greenberg *et al.* 2002). Serum inflammatory markers like the cytokines IL-17A and IFN-γ are also increased (Brubaker *et al.* 2021). Children in endemic countries are also concerned and exhibit an increased inflammation as reported by fecal leukocytes and lactoferrin and serum Reg1b, which can be induced after epithelial barrier injuries (Mercado *et al.* 2011; Iqbal *et al.* 2019). A study conducted on Mexican children reported that increased of both pro- or anti-inflammatory cytokines loads in feces are associated with increased ETEC infection duration (Long *et al.* 2010). In adults volunteers challenged with ETEC strain H10407 (doses ranging from 10^6 to 10^8 CFU), increase fecal MPO and serum IL-17A were reported (Brubaker *et al.* 2021). Surprisingly, it has also been reported that challenge with ETEC H10407 in volunteers induces an expression profile in blood similar to the one of anti-inflammatory drugs. This could represent an attempt by the host to limit the inflammatory intestinal response (Yang *et al.* 2016). It is noteworthy to indicate that even asymptomatic infections with the ETEC strain H10407 cause significant inflammation in humans as reported by increased levels of MPO in stool and

intestinal fatty acid-binding protein (an indicator of compromised intestinal epithelial integrity) in serum (Brubaker *et al.* 2021). All the observed effect of ETEC on induced-inflammation are summarized in **Figure 4.15**.

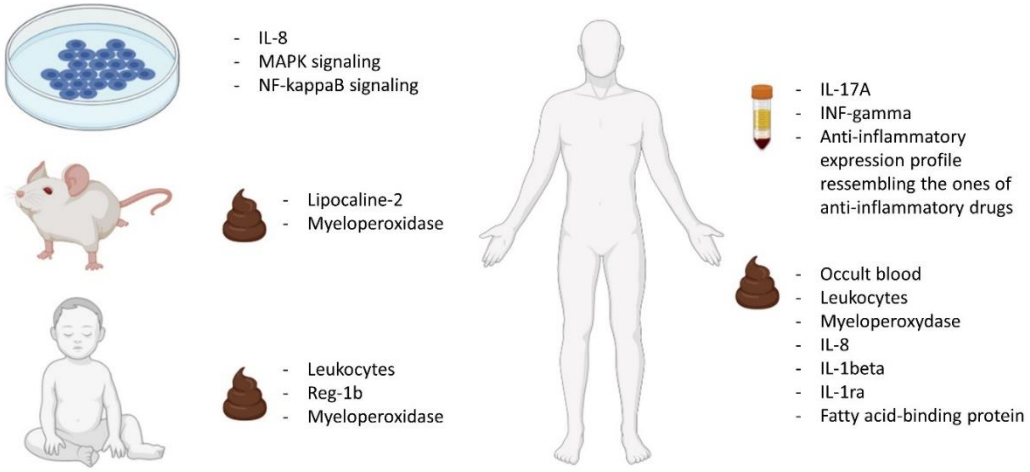


Figure 4.15. Summary of observed inflammatory effects associated with ETEC infection. All inflammatory-related markers reported to be induced by ETEC infection in cellular models, in mice, in children below 5 years old and in adults.
Il: Interleukin, IFN: interferon, MAPK: Mitogen-activated protein kinases, Reg-1b: Lithostathine-1-beta
Built from personal source.

4.3.7.2. Virulence factors associated to ETEC-induced inflammation

Different virulence factors have been specifically associated with ETEC-induced inflammation in *in vitro* studies. First, the LT-I toxin and more particularly its B subunit have been extensively studied as potent vaccine adjuvants to strengthen immune responses. Among the possible mechanism, it seems that the LT toxin interacts with macrophages and other innate immune cells to induce expression of IL-1 (Bromander, Holmgren and Lycke 1991; Foss and Murtaugh 1999; Williams, Hirst and Nashar 1999), a cytokine which displays potent mucosal adjuvant activity (Staats and Ennis 1999). Supporting the role of LT in inflammation, in the HCT-8 cellular model, ETEC H10407 activates both NF-κB and MAPK signaling pathways through mechanisms that are primarily dependent upon LT presence (Kosek *et al.* 2017). Depending on the LT toxin variants, various cytokines could be modulated through TLR2 signaling (Hajishengallis *et al.* 2005) but associated mechanisms are poorly described to date.

Compared, to the LT toxin, the ST toxin is non-immunogenic in its natural form (Taxt *et al.* 2010). Still, some early clues indicate that both STa and STb could induce inflammation at least in piglets (Loos *et al.* 2012; Loos, Hellemans and Cox 2013). The STa peptide in particular has been reported to induce a secretion of pro-inflammatory cytokines and chemokines, as IL-6 and IL-8 (Loos, Hellemans and Cox 2013).

The mucinase YghJ has also been highlighted as an inflammation inducer. In infected humans, challenged volunteers and mice exposed to ETEC strain H10407, YghJ was identified as an immunogen (Roy *et al.* 2010; Chakraborty *et al.* 2018c). In consequence, YghJ has been proposed as one of the key proteins triggering immune response during ETEC infection (Luo *et al.* 2015; Tapader, Basu and Pal 2019). YghJ induces production of pro-inflammatory cytokines such as IL-8, IL-1α, IL-1β and TNF-α and down-regulates anti-inflammatory cytokine production in HT-29 cells and mouse macrophages (Tapader *et al.* 2016, 2018). It is noteworthy that the other ETEC mucinase, EatA, is also immunogenic, according to a recent study in human volunteers (Chakraborty *et al.* 2018c).

Finally, OMVs from the H10407 strain are able to induce an inflammatory response, in particular a secretion of IL-6 and IL-8 in T-84 cells (Chutkan and Kuehn 2011). Interestingly, even OMVs from the ETEC strain H10407, in which LT toxin encoding gene has been knocked out, are able to trigger a modest interleukin-6 production in human T-84 cells (Chutkan and Kuehn 2011), probably by wearing other inflammation inducing cell-surface component as LPS.

Bullet points, ETEC induced inflammation

- ETEC initiates a mid-inflammatory response in travelers (e.g. IL-8, IL-1β, IL-1ra)
- This inflammation pattern, notably characterized by an increased IL-8 production, has been also described in cellular models (T-84, Caco-2, HCT-8).
- Different virulence factors as the LT toxin, the ST toxin or the mucinase YghJ are recognised to be involved in the onset of ETEC-induced inflammation.
- By carrying the LT toxin, outer membrane vesicles are able to induce cellular inflammation, but also on their own.

4.3.8. ETEC and intestinal epithelial permeability

Surprisingly, apart from the studies isolating the ST toxin as a permeability promoting factor in the 70s (Evans, Evans and Gorbach 1973b, 1973a, 1974), to our knowledge, very few work focusing on intestinal permeability induced by ETEC strains from human origin have been conducted to date. One *in vitro* study showed that the treatment of T-84 monolayers with the STa variant (at a concentration of 4 µM) did not increase paracellular permeability to fluorescein isothiocyanate (FITC)-dextran, but reduced TEER within 2 hours (Nakashima, Kamata and Nishikawa 2013). The huge majority of studies investigating intestinal permeability have been conducted with the porcine F4+ ETEC strain K88 (serotype O149:K91,

K88ac, LT1+, ST1+, ST2+), by Chinese research groups (Wang *et al.* 2018; Xiong *et al.* 2020; Xu *et al.* 2020). All results provided by cell models, mice and pig *in vivo* studies, support that this ETEC strain promotes permeability, as measured by different outputs as TEER, molecular permeability (mostly to FITC-dextran), serum markers of permeability (e.g. D-lactate and diamine oxidase), expression and production of tight junctions' proteins (Claudin-1, Occludin and Zonula-occuldens-1, ZO-1) and epithelial histological scores. However, one study conducted with the same strain in Ussing chambers set-up with intestinal samples from piglet's jejunum reported an increased TEER and decreased permeability to fluorescein (Lodemann *et al.* 2017). Two other studies conducted in mice and piglets with other ETEC strains from animal origin reported conflicting results (Wu and Su 2018; Choi *et al.* 2020). The first study showed an increase of tight junction proteins (increase in Claudin-1, Occludin and ZO-1 expression) by multiple ETEC strains (Wu and Su 2018). The second study demonstrated an increase of occludin expression by the ETEC strain P4 (Choi *et al.* 2020). These conflicting findings question the relevance of the generally accepted ETEC- induced permeability, which is mostly supported by work conducted with the ETEC K88, i.e. from animal and not from human origin.

4.3.9. ETEC modulation of human gut microbiota

During passage through the human gut, enteric pathogenic bacteria such as ETEC also have to face a high number of commensal bacteria that compete with them for nutrients and space, as previously mentioned in the section 3.2.3.

Only three studies have directly evaluated gut microbiota composition modulation upon ETEC infection in humans (David *et al.* 2015; Youmans *et al.* 2015; Pop *et al.* 2016). Overall, in these studies, ETEC infections were associated with a rapid and reversible change in gut microbial community structure as well as a significant decrease in overall bacteria diversity, as measured by Shannon and Simpson indexes (Youmans *et al.* 2015; Pop *et al.* 2016). ETEC-induced microbiota changes varied greatly from individual to individual, whether or not diarrhea occurred (David *et al.* 2015; Youmans *et al.* 2015; Pop *et al.* 2016). However, the original structure of gut microbiota is largely restored at 1 and 3 months post-challenge (depending on the studies follow-up program), showing the resilience of gut microbiota following perturbation by a bacterial pathogen (David *et al.* 2015; Pop *et al.* 2016).

By dissociating the host part, *in vitro* studies enable a description of ETEC direct effect on the gut microbiota. Moens and colleagues conducted a 48-h batch experiment in healthy and dysbiotic conditions, the dysbiotic conditions differing from the healthy ones by a lower microbiota inoculation (500-fold decreased in volume) and addition of ETEC strain (LMG2092

at 3.10^7 CFU.mL^{-1}). These ETEC dysbiotic conditions result in overall decrease in fermentation metabolites, such as major SCFA (acetate, propionate and butyrate) and ammonium(Moens *et al.* 2019). However, one cannot dissociate between the effect due to lesser microbiota inoculation and the ones due to ETEC presence. Importantly, one of the other limitations of the above studies is the fact that fecal samples are not representative of the niche encountered in the ileum, suggested as the site of action for ETEC (see section 4.3.1). By using environmental conditions mimicking the human ileal and colonic compartments, the M-SHIME *in vitro* system alleviates this limit. Surprisingly, inoculating ETEC strain H10407 (10^{10} CFU) in the M-SHIME did not result in profound shifts in the microbiota composition (Roussel *et al.* 2020a). Yet, correlations between ETEC administration and specific microbial genera were observed. Key changes in the luminal phases were found in the Firmicutes phylum with a decrease in *Clostridium butyricum* and an increase in *Clostridium scindens* (only the ascending colon). The mucosal ileum showed blooms of taxa recognised as opportunistic pathogens as *Klebsiella variicola* and non-tuberculous *Mycobacterium* (Stecher, Berry and Loy 2013; Martin and Bachman 2018). Roussel and colleagues also documented changes in SFCAs concentration following ETEC infection, mainly through the increase of propionate concentration, reflecting changes in microbial metabolism/activity (Roussel *et al.* 2020a). All the reported effects of ETEC from human origin on human gut microbiota are resumed bellow in **Table 4.5**.

Table 4.5. Summary of studies reporting ETEC impact on human gut microbiota composition/activity.
Built from personal source.

Experimental design	Number of subjects/donors	Observations	Sample origin	Reference
Travelers to Central America or India affected by ETEC	35	α-diversity not impacted (Simpson index). β-diversity decreased (Yue and Clayton distance metric). ↗in Firmicutes and Proteobacteria. ↘ in Bacteroidetes.	Fecal	Youmans *et al*. 2015
Infected patients after confirmation of ETEC infection by PCR	18	At day 0 and 1, ↗ in *Streptococcus* and *E. coli*. At day 7, ↗ in *Bacteroides* in some patients.	Fecal	David *et al*. 2015
Volunteers challenged with ETEC strain H10407 (10^5 or 10^6)	12	Decrease in α-diversity (Shannon index). From 1 to 3 days post- challenge: ↗ in *Escherichia Bacteroides dorei*, *Bacteroides* ovatus, *Barnesiella intestinihominis*; ↘ in *Bacteroides vulgatus*, *Bacteroides xylanisolvens*, *Parabacteroides distasonis.*	Fecal	Pop *et al.*2016
Fecal batch with ETEC strain H10407 ($10^{7.5}$ CFU.mL^{-1}) and with 50-fold lower fecal inoculum	1	↗ in ethanol. ↘ in acetate, propionate, butyrate, lactate and ammonium.	48 hours batch inoculated with human feces	Moens *et al.*2019

M-SHIME experiment challenged with ETEC strain H10407 (10^{10})	6	↘ of α-diversity (Simpson index) in ileum lumen and mucus. ↘ *Clostridium butyricum, Proteobacteria OTU*s (lumen colon and ileum) ↗ *Bacillus Xiaoxiensis, Clostridium scindens, Bacteroides* OTUs (lumen colon) ↗ opportunistic pathogens as *Citrobacter, Klebsiella variicola* and *Mycobacterium (ileum mucus).* ↗ in propionate production (lumen colon and ileum).	Luminal and mucosal phases of ileal and ascending colon of M-SHIME model inoculated with human feces	Roussel *et al.* 2020

4.4. ETEC anti-infectious strategies

I-4

A better understanding of ETEC pathogenesis in the human gastro-intestinal tract will help to develop novel therapeutic approaches. Ideally, such therapeutics, either prophylactic or curative must be safe, well tolerated and respond greatly in patients. This section will draw up a state of the art of both the current and under development strategies to remediate ETEC infections in humans. So far, treatments to cure ETEC infections are not specific to the pathogen, but rather follow the general recommendations given for diarrheal diseases in both children and adults. Unanimously, oral rehydration solution is the key treatment, often prescribed to prevent dehydration and loss of electrolytes. In the case of mild to moderate diarrhea, anti-secretory agents such as Bismuth subsalicylate or pepto-bismol© may decrease the frequency of bowel movements and the posology varies according to the age-range. While for acute diarrhea, it is recommended to use antimotility drugs such as Loperamide within 48 hours, but only in adults.

4.4.1. Antibiotics

Although traveler's diarrhea is a self-limited illness commonly resolved within 5 days when untreated, antibiotic therapy has been proved to be effective in treating patients by significantly reducing associated symptoms and shortening the illness duration (de Bruyn, Hahn and Borwick 2000; Diemert 2006). As antibiotic therapy remains the most effective treatment for bacterial diseases, it is common for clinicians to prescribe antibiotics to international travelers for self-treatment if they experience diarrheal symptoms while abroad. However, due to this selective pressure of antibiotics for treatment, recent studies have expressed concerns about the potential for acquisition and subsequent carriage of multidrug-resistant pathogens (Kennedy and Collignon 2010; Ruppé *et al.* 2015). Antibiotic resistance is widely regarded as one of the major public health concerns of the 21st century, leading to longer hospital stays, higher medical costs and increased mortality. ETEC and other pathogens associated with traveler's diarrhea have progressively gain resistance to antibiotics (Riddle *et al.* 2017; Guiral

et al. 2019; Boxall *et al.* 2020). A recent study on 61 ETEC strains isolated in UK travelers reported that 65.6% of the strains were antibiotic resistant to one antibiotic and 32.8% were multi-resistant (Boxall *et al.* 2020). Nowadays, ampicillin, trimethoprim-sulfamethoxazole, and doxycycline, which are the antibiotic generally used to treat DEC infections, meet significant antibiotic resistance by ETEC strains. Fluoroquinolones are still an effective therapy according to the Center for Disease Control and Prevention (CDC, https://www.cdc.gov/ecoli/etec.html, consulted on 03/2022). However, some studies recent studies have shown that fluoroquinolones and azithromycin resistance level have climbed during the last decades (Tribble 2017).

4.4.2. Vaccines

Among other prophylaxis strategies investigated against ETEC infection, vaccines are good candidates. In contrast to piglets, which can be protected by a live oral vaccine (comprising a mixture of F4+ and F18+ *E. coli*), currently no vaccine is licensed to protect against human ETEC infections (Nadeau *et al.* 2017). Nevertheless, Dukoral®, an oral whole-cell/recombinant B-subunit vaccine, originally directed against *Vibrio cholerae*, has been found to provide short-term efficacy (67% of protection) in some serotypes of ETEC diarrhea, involving the virulotype LT (Jelinek and Kollaritsch 2008). The prescription of this vaccine is however limited to Europe, Canada and Australia (CDC, https://www.cdc.gov/ecoli/etec.html, consulted on 03/2022).

Vaccines designed to prevent ETEC infection have employed different strategies. Majority of the investigations focused on ETEC CF and the enterotoxin LT as immunogenic agents. The most recent Etvax oral vaccine is employing four inactivated *E. coli* strains over-expressing some major CF and the LT-B subunit. In 2020, this vaccine has passed the phase 2B of clinical trials in adult volunteers from Finland travelling to endemic areas (Bourgeois, Wierzba and Walker 2016; Lundgren, Jertborn and Svennerholm 2016). The study showed a significant protective efficacy of 56% against all severe diarrhea, independently of the pathogen (most likely because ETEC was preponderant as found in 75% of all severe diarrhea cases) (https://cordis.europa.eu/project/id/778253/fr, consulted on 03/2022).

Facing the recognised increased pathogenicity of ST+ ETEC strain, some authors also bet on the induction of ST neutralizing antibodies (Taxt *et al.* 2010; Fleckenstein, Sheikh and Qadri 2014). To palliate the ETEC strain diversity, some studies investigate other conserved ETEC proteins as vaccine antigens (Fleckenstein and Rasko 2016). For instance, YghJ has been identified as a novel glycosylated vaccine candidate and a novel antigen, the Skp protein, has been reported to be immunongenic in mice. Some authors are also currently investigating *in*

silico approaches to find new potential immunogenic epitopes among 4915 proteins of the ETEC strain E24377 (Barry *et al.* 2019). Some investigations are carried on to use ETEC OMV (genetically detoxified or not) to induce adaptive immunity in mice (Leitner *et al.* 2015; Beikzadeh and Nikbakht Brujeni 2018; Matías *et al.* 2020). The use of OMV have been proven a valuable tool as they carry both adjuvant factors (LT toxin, LPS, lipid-A) and virulence factors (LT toxin, CF).

4.4.3. Bacteriophages

Up to now, around 10 studies have reported isolation of phages targeting (specifically or not) ETEC strains (Bourdin *et al.* 2014; Nobrega *et al.* 2015; Zhou *et al.* 2015; Chakraborty *et al.* 2018b; Manohar *et al.* 2018; Sváb *et al.* 2018; Piya *et al.* 2019a; Kaczorowska *et al.* 2021). Interestingly, an enterobacteria-targeting phage T7, designated as IMM-002, showed a significant specificity towards CF CS3-expressing ETEC isolates (Chakraborty *et al.* 2018b). The lytic phage JS09, isolated from a swine farm in China, could infect antibiotic-resistant APEC and ETEC (Zhou *et al.* 2015). In another study, some cocktails of T4-like phages achieved 30% to 53% coverage efficiency against ETEC isolates from Bangladesh (Bourdin *et al.* 2014). Importantly, a bacteriophage effective against ETEC (among other *E. coli*) is already commercialized. The phage LL12 is included in a mix of bacteriophages, commercialized as a prebiotic named PreforPro, which is supposed to support general intestinal health, and does not specifically target ETEC (Piya *et al.* 2019b) (Deerland Enzymes®, https://deerland.com/preforpro/the-difference/, consulted the 03/2022).

4.4.4. Probiotics

Probiotic are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Syngai *et al.* 2016; Riddle *et al.* 2017). In the past 10 years, efforts have been undertaken to develop anti-ETEC probiotics as an alternative to antibiotics (Roussel *et al.* 2017). Although the underlying mechanisms associated with probiotic prevention or alleviation of enteric pathogens are still largely unclear, the modes of action thought to contribute to human and/or animal health fall into three general classes of anti-pathogenic mechanisms: direct antagonism, immunomodulation and competitive exclusion (Preidis *et al.* 2011). The huge majority of studies about probiotic and ETEC have been conducted with ETEC strains from animal origin. Concerning human ETEC strains, the probiotic investigations are scarcer. One study reported the inhibition of the growth of different ETEC strains using various *in vitro* tests (inhibition zone on agar plates, co-culture) following co-incubation of the pathogen

with culture supernatant of *Lactobacillus* species. The inhibition of ETEC growth was due to the antimicrobial activity of the probiotic *via* production of lactic acid (Tsai, Lin and Hsieh 2008). It has also been shown that *Lactobacillus rhamnosus* inhibits LT toxin production in co-culture with the ETEC strain MTCC723 (Anand, Mandal and Tomar 2019). *Pediococcus pentosaceus* OZF I is able to reduce the adhesion of the ETEC strain LMG 3083 to Caco-2 cells from about 99% when incubated with the cells prior to the pathogen infection (Osmanagaoglu, Kiran and Ataoglu 2010). *Bifidobacterium longum* SBT2928 impedes ETEC strain Pb 176 adhesion on HCT-8 cells (Fujiwara *et al.* 2001). *Saccharomyces cerevisiae* CNCM I-3856 presets many anti-infectious properties against the ETEC strain H10407 *in vitro*, among which reduction in ETEC growth, toxin production and cellular adhesion to mucin-agar and Caco-2 cells and inflammation induction as reported by IL-8 secretion. Interestingly, the yeast anti-adhesive effect could be due to mannose residue on its surface, potentially involving mannan polysaccharides (Roussel *et al.* 2018b). The yeast anti-infectious properties have also been tested in the TIM-1 and M-SHIME models. In the TIM-1 model, toxin encoding genes were repressed and a lower LT toxin production was noted (around 2-fold decrease in the ileal effluent along the experiment) when the yeast was co-administered with the ETEC strain H10407. In the M-SHIME model, the probiotic pre-treated microbiota displayed a higher robustness in composition following ETEC challenge compared to the non-treated condition (Roussel *et al.* 2021). This could be the first anti-infectious property of probiotic against human-infecting ETEC passing through microbiota modulation ever evidenced. The yeast also decreases ETEC colonisation of streptomycin-treated mice in the ileum and colon (Roussel *et al.* 2018b).

4.4.5. Dietary fibers

As demonstrated in section 2.4.2, dietary fibers present numerous potential anti-infectious properties. Actually, most of the studies investigating their anti-infectious properties on ETEC have been conducted on strains from animal origin. Firstly, chitosan nanoparticles at 0.0125 $mg.L^{-1}$ and raw chitosan at 64 $mg.L^{-1}$ were shown to be lethal to ETEC strain K88. Then, numerous adhesion assays have demonstrated adhesion inhibition by dietary fibers. The tested fibers were milk oligosaccharides (Martín, Martín-Sosa and Hueso 2002; Cilieborg *et al.* 2017), microbial-derived polysaccharides (Wang, Gänzle and Schwab 2010; Badia *et al.* 2012; González-Ortiz *et al.* 2013; Chen *et al.* 2014; Zhu *et al.* 2018), plant based dietary fibers (Roubos-van den Hil *et al.* 2009, 2010; González-Ortiz *et al.* 2014) and human synthetized neoglycans (Sarabia-Sainz *et al.* 2013). The tested product concentrations range from 0.3 $g.L^{-1}$

to 10 $g.L^{-1}$ for adhesion inhibition up to 95% (González-Ortiz *et al.* 2014). The adhesion surface used for tests are mucin extracted from pig stomach (Sarabia-Sainz *et al.* 2013; González-Ortiz *et al.* 2014), erythrocytes (Roubos-van den Hil *et al.* 2009; Wang, Gänzle and Schwab 2010; Chen *et al.* 2014), or cell models, such as (surprisingly) human Caco-2 cells (Roubos-van den Hil *et al.* 2009, 2010; Badia *et al.* 2012), but also pig PSIc1 (Cilieborg *et al.* 2017) or IPEC-J2 cells (González-Ortiz *et al.* 2013; Zhu *et al.* 2018). *In vivo* assays in pigs have also been conducted. The tested fibers included chitosan, alginate, psyllium, inulin, cellulose, dextran, microorganisms derived beta-glucans and lactulose, with doses ranging from 0.2 $g.kg^{-1}$ to 80 $g.kg^{-1}$ (Hayden 1998; Wellock *et al.* 2008; Halas *et al.* 2009; Stuyven *et al.* 2009; Chen *et al.* 2014; Guerra-Ordaz *et al.* 2014; Xiao *et al.* 2014; Wan *et al.* 2018). The dietary fiber beneficial outputs were various as improvement of diarrhea, decreased ETEC shedding, decreased immune response (IL-1β and IL-6 expression and calprotectin production). On study conducted in mice showed that chitosan at 0.3 $g.kg^{-1}$ was able to reduce of nearly 1 log ETEC SEC470 fecal shedding and jejunum colonisation at day 7 post-infection. Intestinal inflammation markers also decreased (expression of IL-1β, IL-6, IL-17, IL-18, TNF-α and TLR4 abundance) (Liu *et al.* 2016a).

Studies specifically targeting dietary fiber effects upon ETEC from human origin are scarce and have investigated very few dietary fibers. Milk oligosaccharides and plantain soluble fibers at concentrations of 1$g.L^{-1}$ and 5 $g.L^{-1}$ respectively were proven to reduce ETEC adhesion to Caco-2 cells up to 80% (Idota and Kawakami 1995; Roberts *et al.* 2013; Salcedo *et al.* 2013). Lastly, diverse studies have investigated the ability of dietary fibers to decoy ETEC toxins from its receptor (Otnaess, Laegreid and Ertresvåg 1983; Newburg *et al.* 1990; Idota *et al.* 1995; Paton *et al.* 2005). These studies have all been conducted with HMO, due to HMO structure similarities with toxin receptors. Indeed, the LT GM1 receptor consists in an oligosaccharide molecular pattern also found in milk (Otnaess, Laegreid and Ertresvåg 1983). Hence, the GM1 oligosaccharide but also siallylactose have been shown to inhibit LT binding to its receptor in ELISA (Enzyme-Linked Immunoabsorbent Assay) assay and fluid secretions in rabbits intestinal loops (Otnaess, Laegreid and Ertresvåg 1983; Idota *et al.* 1995). Another human milk component, certainly a fucosylated oligosaccharide, is able to inhibit ST binding to the extracellular domain of its GC-C receptor and to reduce diarrhea in mice (Newburg *et al.* 1990; Crane *et al.* 1994). In line with these observations, mother's milk oligosaccharide richness is associated with infant resistance to many pathogens, notably ETEC (Newburg, Ruiz-Palacios and Morrow 2005). Lastly, facing these results, Paton and colleagues developed some genetically modified versions of *E. coli* bearing on their surface GM2 (monosialicganglioside

2) and other oligosaccharides. These HMO-presenting probiotics also inhibit toxin binding and reduce fluid secretion in rabbits (Paton *et al.* 2005).

As recurrently demonstrated along the section 3.2.3, ETEC infectious cycle passes through multiple interactions with the mucus layer (e.g. adhesion, colonisation, expression of mucinases) and access to epithelium is a preponderant step in the infectious cycle (modulation of virulence genes expression, secretion and binding of toxins, bacteria engulfment…). Furthermore, it has been demonstrated that dietary fibers can decoy pathogen from mucus binding, preserve the mucus layer from microbiota degradation and the host from subsequent intestinal infection (section 2.4.2.3). However, to date, no study has addressed the potential of dietary fibers to interfere with ETEC interactions with the mucus layer specifically.

Figure 4.16 summarizes the alternative anti-infectious therapies against ETEC presented in the last section.

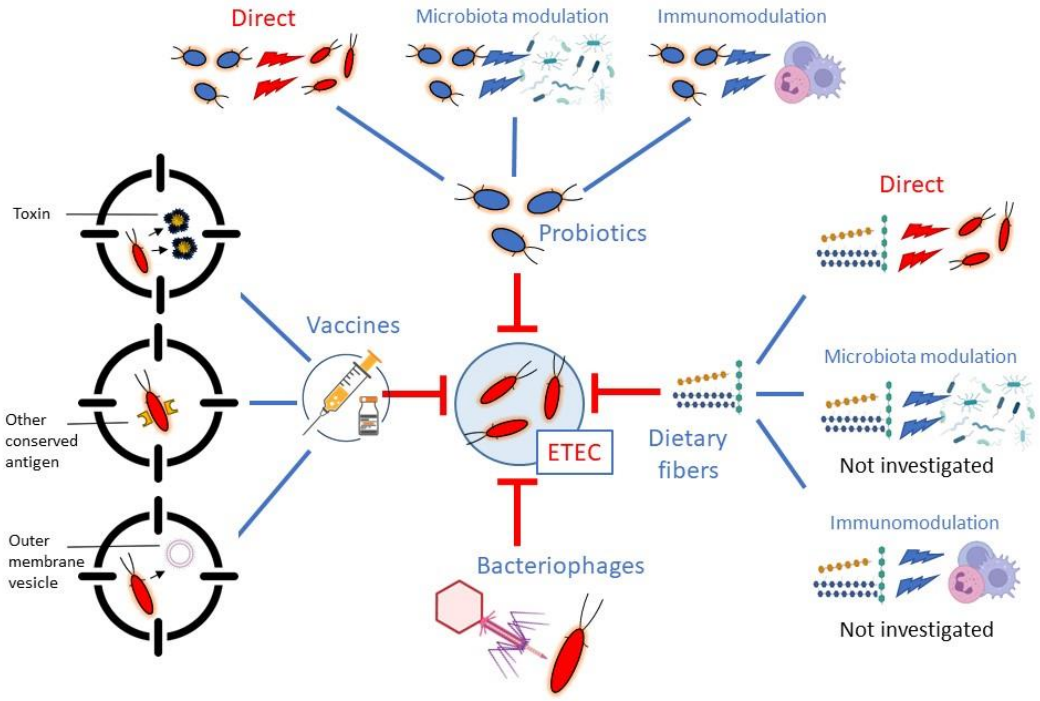


Figure 4.16. Alternative anti-infectious approaches against human ETEC infections.
Facing the raise of antibiotic resistance and the necessity to develop alternative way to fight ETEC infection in humans, different approaches were investigated. Both probiotics and dietary fibers potentially present anti-infectious properties though direct antagonism (e.g. growth or adhesion inhibition), microbiota modulation or immunomodulation. Vaccines can target different antigens as toxins, other conserved antigens or OMV. Bacteriophages targeting human infecting ETEC are also regularly isolated.
Not investigated = not investigated according to the available literature on human ETEC strains.
Built from personal source.

Bullet points, ETEC therapeutic strategies

- Traveler's diarrhea is a self-limited illness commonly resolving within 5 days when untreated. However, Physicians routinely prescribed antibiotic therapy for the most serious cases and for patients' comfort. As antibiotic resistance raises, other prophylactic strategies have to be considered.
- Despite numerous studies investigating the use of vaccines, bacteriophages, or probiotic strategies, to date no specific treatment is currently available to treat ETEC infection in humans.
- Concerning the use of dietary fiber, up to now very few studies are available concerning ETEC strains from human origin impeding relevant conclusions on their potential beneficial effects.

5. Available *in vitro* gut and cellular models to investigate the interactions between dietary fiber, mucus and enteric pathogens

As described in Section 3, dietary fiber intakes could be an interesting way to fight against enteric infections by different means, notably, by preventing pathogen and mucus interactions. Nevertheless, as aforementioned in Section 4.4.4, very few data have been gathered concerning dietary fiber anti-infectious properties against ETEC strains from human origin. The incoming section will review the available *in vitro* gut and cellular models that could be used to study ETEC survival and virulence function and its modulation by dietary fibers in the human simulated gut. As the study of pathogen interactions with the mucus layer is particularly relevant when assaying dietary fiber impact on pathogens, a particular emphasis will be given on integration of a mucus compartment in these models.

5.1. *In vitro* human gut models as a relevant alternative to *in vivo* studies

First, question could be asked on why choosing *in vitro* gut models upon other *in vivo* approaches. *In vivo* approaches in humans obviously represent the gold standard to investigate the interactions between dietary fiber, gut microbiota and enteric pathogens. However, several limits could hamper their use. First, the biological interpretation is complicated due to a myriad of factors among which inter-individual variability is one of the main challenges. In human clinical trials, there is a huge discrepancy between the studies due to dietary habits, genetic background, lifestyle and geographical origin of participants. Strict compliance of participants to the tested diet in interventional studies is also a factor difficult to monitor. Thus, any specific effect related to dietary fiber interventions is difficult to measure in healthy people. Secondly,

for evident ethical reasons, access to the different segments of the GIT (from the stomach to the distal colon) is very limited and collection of mucus layer from human biopsies remains difficult (Hansson 2012). To minimize invasive procedures, human gut microbiota studies are usually performed using fecal samples and measured as endpoints, thus making it is difficult to decipher where in the GIT the effects of a specific treatment occur (Riva *et al.* 2019). Lastly, human interventional studies are limited in scope or are even impossible depending on the pathogenic microorganisms involved. In the case of the study of ETEC pathogenesis, studies involving non-attenuated pathogens or physiological dose in human are obviously not recommended. Still, in 2019, 14 different ETEC strains have been tested in at least one human trial and some authors argue for more methodological development to control human infection models (Hanevik *et al.* 2019). In addition, when considering that ETEC and DEC infections are associated with increased risks of long-term diseases like IBS (Bourgeois, Wierzba and Walker 2016) or musculoskeletal symptoms as reactive arthritis (Tuompo *et al.* 2020), these human trials raise ethical concerns.

A widespread alternative to clinical studies is the use of *in vivo* models. Animal models are undoubtedly very useful to study physiological or pathological conditions at the level of the entire organism. For decades, their use has been essential for a better understanding of various infectious diseases. To investigate the involvement of gut microbiota on host functions, the use of gnotobiotic animals is particularly relevant, even if these experiments remain *expensive* and time-consuming (Kirk 2012). Nevertheless, more and more attention should be paid to reduce dependence on animal studies considering the societal demand to limit experiments on animals and the increasing ethical constraints (European parlmeent procedure file 2021/2784). Also, important caution should be applied when translating data obtained in animal models to humans. Importantly, *in vivo* approaches involving laboratory animals can be hampered by differences between animal and human digestive physiology including resident microbiota and susceptibility to infection by pathogens (Hugenholtz and de Vos 2018). For illustration, rats have a lower capacity to digest polysaccharides from fibers than h uman (Knudsen *et al.* 1994). There is also a lack of relevant animal models for reproducing human ETEC infection. As previously underlines, the animal associated toxin variants bind to different receptors compared to the human ones (Dreyfus *et al.* 1993; Dubreuil 2012; Dubreuil, Isaacson and Schifferli 2016; Wang *et al.* 2019a). Adhesins from human-infecting ETEC strains also differ from the animal's ones (Dubreuil, Isaacson and Schifferli 2016; Kharat *et al.* 2017; von Mentzer *et al.* 2017). This proves that ETEC strain virulence factors are adapted to mucosal receptors at an inter-species level.

Another alternative is the use of *in vitro* models simulating the human digestive environment. These in *vitro* human gut models can be divided in 3 main types, digestion simulators, fermentation systems and mucosal simulators. *In vitro* digestion simulators reproduce with more or less accuracy the different *in vivo* digestive parameters found in the luminal side of the gut. Fermentation systems integrate the microbial activity of the gut microbiota. Finally, mucosal simulators give emphasis on the gut mucosal surfaces. All these types of models have their advantages depending on the parameters studied and the boundaries between them are less and less clear, as increasingly efforts are made to combine them.

5.2. *In vitro* models for human digestion simulation

As the human digestive tract is not directly accessible and human studies are not recommended with pathogens, reproducing the physicochemical parameters of the human gut is a first key to unravel ETEC spatio-temporal behavior under digestive conditions and the potential impact of dietary fiber on the pathogen behavior. Diverse *in vitro* digestive models are described in the literature (**Table 5.1**).

5.2.1. Gastric monocompartmental simulators

Static mono-compartmental models (also termed batchs) are the simplest option (**Table 5.1**). In such model, only one or two digestive conditions are fixed at start in a unique vessel, underestimating thereby the complexity of the GI physiology. Due to their simplicity, these models have been extensively used to simulate gastric digestion and do not necessary own a name (Bengoa *et al.* 2018). We can still quote the Simulated Gastric Fluid (SGF), which simulates peptide hydrolysis by stirring the food sample with pepsin and pH maintenance between 2 and 3 for 30 to 120 min (Egger *et al.* 2016). Nonetheless, in this case the complexity of the gastric physiology is undervalued since the gastric pH is not progressively dropped, and the enzymatic cocktail, classically found in the stomach is not included. These simple-to-use and cheap models offer the flexibility to test unlimited conditions and are often used for screening assays.

To cleverly appreciate the physical and/or chemical changes affecting microbiological agent in the stomach, dynamic models have also been developed (**Table 5.1**). Depending on the *in vitro* systems considered, they offer a largr spectrum of parameters to follow such as the continuous changes in pH (pH drop) and secretion flow rates (e.g. pepsin, lipase), the peristalsis, and physical breakdown or the gastric emptying as well. Different models are available that

differs especially by their mixing and mechanical breakdown patterns. We can quote the human gastric simulator (Kong and Singh 2010), the dynamic gastric model (Mercuri *et al.* 2011), the TIM- advanced gastric compartment (TIM-agc) (Minekus 2015) and the Gastric Digestion Simulator (Kobayashi *et al.* 2017).

5.2.2. Multi-compartmental simulators

To study with precision the fate of an enteric pathogen, it is required to simulate further steps of the human digestion, the intestinal digestive secretion deliveries and the associated transit time. In response, bi-regionalized or multi-compartmental simulators have been developed (**Table 5.1**). Bi-compartmental models mimic only the stomach and duodenum by controlling the pH, temperature, transit time and bile concentration (Mainville, Arcand and Farnworth 2005; Tompkins, Mainville and Arcand 2011; Levi and Lesmes 2014; Ménard *et al.* 2014). Then, to increase the complexity and relevance of those digesters, only few systems became innovative by combining both bi-regionalization and dynamism of the GI digestion. The well-known TIM-1 faithfully reproduces the physicochemical parameters of the human upper GIT (e.g. stomach, duodenum, jejunum and ileum) and integrates the passive absorption of small molecules thanks to a dialysis system in the jejunal and ileal compartments (Blanquet-Diot *et al.* 2012). This system allows the mimicking of the body temperature, the temporal and longitudinal changes in gastric and intestinal pH kinetics, the dynamism of the chime transit and mixing, and the sequential delivery of intestinal, pancreatic and biliary secretions (**Fig. 5.1**). Nonetheless, to date this model does not integrate anaerobiosis nor intestinal microbiota. However, the TIM-1 model has already shown its relevance to study the behavior of *E. coli* pathotypes, such as EHEC or ETEC, in the stomach and the three compartments of the small intestine, and the impact of both serotypes and food components (Etienne-Mesmin *et al.* 2011; Miszczycha *et al.* 2014; Roussel *et al.* 2018a). Despite the absence of metabolisation of dietary fiber in the stomach and small intestine, their viscosity and water holding capacity have to be considered (Taghipoor *et al.* 2014). Therefore, evaluating the detrimental or beneficial influence of dietary fiber on pathogen survival and/or virulence in the stomach and small intestine would be a future challenge. In the same way, meeting the challenge to add a mucus compartment in these upper gut models, could allow one to decipher the direct effect of mucus on pathogen survival and virulence.

Apart from the TIM-1 model, other models have been developed around the world. To overcome some limitations encountered in the TIM-1, the Engineered Stomach and Small Intestine (ESIN) currently developed by MEDIS lab would allow an accurate reproduction of

real food size particles entering the stomach and reproduces a differential gastric emptying between liquid and solid particles (Guerra *et al.* 2016). Current development will integrate the gut microbiota of jejunal and ileal compartments. We can also mention the SHIME model developed by Ghent University, which enable to include all the compartments from the stomach to the colon (Molly *et al.* 1994; Van de Wiele *et al.* 2015).

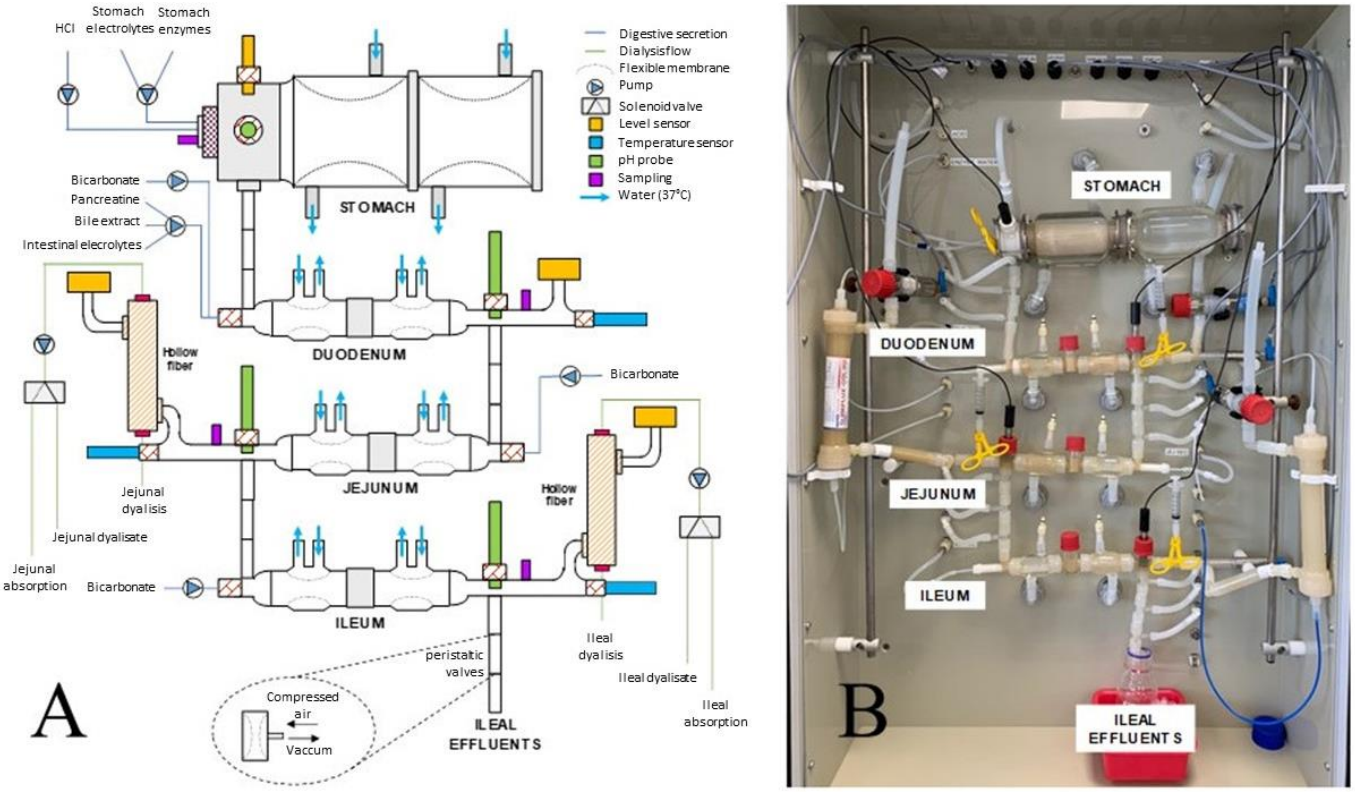


Figure 5.1 Schematic representation (A) and picture (B) of the TIM-1 system.
Built from personal source.

5.2.3. Limits of digestive simulators

As seen with these previous examples, the *in vitro* simulators of human digestion are more and more accurate to simulate human digestion. However, they still present some limits. The chemical products used (e.g. enzymes, bile salts) are never from human origin and thus, potentially far from human physiology. More importantly, the host part is missing. In consequence, the absorption phenomenon, when included, is limited to passive absorption. The cross talk between the human microbiota and the immunity cannot obviously be reproduced in those *in vitro* systems. Actually, only few models respect the anaerobic status of the GIT compartments and include the human gut microbiota. The SHIME system is one of these models, combining digestive simulators and fermentation systems. It will be further developed in the next section alongside with other fermentation systems.

5.3. *In vitro* human fermentation models

5.3.1. Description of the main models

As the preponderant role of the gut microbiota in human digestion has raised over the years, *in vitro* models including this key player have been developed in the last decades. The inclusion of the human microbial fermentation is particularly relevant to study of dietary fiber interactions in the gut. The developed models range from simple (single stage batch systems) to more complex and representative three-stage semi-continuous and continuous reactor models, maintained for several weeks (Miller and Wolin 1981; Gibson, Cummings and Macfarlane 1988; Blanquet-Diot *et al.* 2012; McDonald *et al.* 2013; Van den Abbeele *et al.* 2013). Again, static systems or batchs represent the simplest option (**Table 5.1**). In these models, the fecal microbiota is inoculated in a close anaerobic compartment containing an anaerobic medium recapitulating the colonic condition and allowing the microbiota follow-up for up to 48h. The rapidity and high throughput of batch models render them very useful for large screening studies of dietary fiber (Pham and Mohajeri 2018; Pérez-Burillo *et al.* 2021), but this approach is limited by short-time fermentation but also accumulation of metabolites and pH decrease that could impede microbial activities (Payne *et al.* 2012). To palliate to these limits, single-stage fermentors have been developed (**Table 5.1**). These semi-continuous or continuous fermentation models recapitulate the main biotic and abiotic parameters of the human colon, such as temperature, pH, residence time, supply of nutritive medium reproducing the composition of ileal effluents, therefore enabling the study of a stable, complex and metabolically active gut microbiota under anaerobiosis conditions. Among the available models, we can cite the ARtificial COLon (ARCOL), a device integrating anaerobiosis guaranteed by the sole activity of the gut microbes whereas most fermentor systems are maintained anaerobically by regular flushing with CO_2 and/or N_2 gases (Thévenot *et al.* 2013). These mono-compartmental colonic models are interesting, but do not consider the spatial regionalization of the colon observed *in vivo*. Different multi-stage models have been developed for this purpose (**Table 5.1**), among which the continuous three-stage colon system developed by Gibson, Cummings and MacFarlane (Gibson, Cummings and Macfarlane 1988) or the PolyFermS which can include up to 5 compartments depending on the level of regionalization needed (Cinquin *et al.* 2004; Fehlbaum *et al.* 2015). Finally, multi-stage continuous fermentor systems have been also extended to include the anterior segments of the GIT. They allow to perform microbiota studies not only in the colonic compartment but also in the upstream's ones, notably the ileum. SHIME and SIMGI (SIMulator Gastro-Intestinal) are the most complete of

these multi-stage bioreactors including the microbiota. They simulate nearly entirely the GI transit from the stomach to the descending colon(Van de Wiele *et al.* 2015; Verhoeckx *et al.* 2015). Importantly, by integrating upper compartiments of the gut and their physiological impact on the whole GIT, these models gathered digestive simulators and fermentation models. Among these two models, the SHIME remains the originally and internationally known system, with special features that are constantly developed in this system. For example, the SHIME system has been adapted to facilitate a rapid, anaerobic, frequent sampling of insoluble dietary substrate. This system therefore opens the possibility to investigate microbial interactions with this additional niches (De Paepe *et al.* 2018).

5.3.2. Limits of fermentation models for the human gut

Despite their diversity and flexibility, fermentation models undoubtedly present some limits. Fermentation models exhbitit lower gut microbiota diversity compared to human fecal samples, suggesting that they are not yet capable of supporting the full range of species that are living in the human gut (Van de Wiele *et al.* 2015; Pham and Mohajeri 2018). Similarly, SCFA that play a major role in gut homeostasis are not absorbed by most of these models, or only by passive mechanisms, which may have an impact on gut microbiota or tested pathogenic microorganisms (Pham and Mohajeri 2018). Finally, and very importantly in this PhD context, the host part is generally absent, or reduced to the possible presence of mucin matrices (mucin beads or microcosms), which remain far from human physiology, even if some attempts are undertaken to alleviate this limit (detailed in the section below).

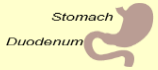
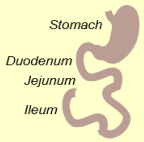
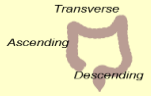
The main non-static digestive simulators and fermentation models are presented alongside in **Table 5.1**.

Bullet points, *in vitro* digestive simulators and fermentation systems

- Numerous *in vitro* digestive simulators and fermentation systems with different complexity levels simulate the human GIT and associated physicochemical parameters and gut microbiota activity, respectively.
- Some models, as the SHIME, combine these two types of models (namely digestive and fermentation models).
- Low complexity models enable high throughput studies, while the most complete systems enable simultaneous inclusion of multiple parameters of the human gut physiology (e.g. physicochemical parameters, microbiota, absorption, anaerobiosis).

Table 5.1. Main *in vitro* systems of the human digestion and/or fermentation. Batch system are included, but they do not necessarily have a name due to their wide availability.

Modified from Charlene Roussel's PhD thesis manuscript.

Main features	Digestive simulators of the Upper GI tract(No integration of fermentation)			Digestive similators/Fermentation systems	Fermentation systems	
	Mono-compartmental gastric simulators 	Bi-compartmented 	Multi-compartmented 	Upper + lower GI tract 	Lower GI tract (No simulation of the digestion)	
					Single-stage 	Multi-stage 
In vitro models	**Static models or batch** Simulated Gastric Fluid (SGF) Simple stirring of the food with pepsin *Egger et al., 2016*	In Vitro Digestive System (IViDiS) Mimic the stomach and duodenum (pH, temperature, transit time and bile concentration) *Thompkins et al., 2011* *Mainville et al., 2005*	TNO gastrointestinal Model 1 (TIM-1) Mimic from Stomach to the ileum (pH, temperature, enzymes, peristaltic movements, enzymes secretions, passive absorption) *Blanquet-Diot et al., 2012* *Minekus et al., 1995*	Mouth, stomach, duodenum, jejunum and three stages of the large intestine M-SHIME includes a mucosal phase (mucin-agar microcosms) *Van de Wiele et al., 2015* *Molly et al., 1994*	**Static models or batch** Simple culture of fecal inccolum in anaeroby medium up to 48h *Pham and Mohajeri 2018, Moens et al. 2019*	PolyFermS 1-5 bioreactors *Fehlbaum et al., 2015*
				Multicompartmental Dynamic Model of the Gastrointestinal System (SIMGI) Stomach, small intestine and three stages of the large intestine *Barroso et al., 2015*		EnteroMix 4 low volume-bioreactors (from 6 to 12 mL) *Makivuokko et al., 2005*
	Dynamic models Human Gastric Simulator (HGS) and Gastric digesiton Simulator (GDS) Mixing by peristaltic motion and mechinal breakdown by a roller rotation system *Kong and Singh, 2010* *Kobayashi et al., 2017*		Tiny-TIM Simplified and downscaled TIM *Havenaard et al., 2013*		**Dynamic models** ARtificial COLon (ARCOL) Anaerobiosis guaranteed by the sole activity of the gut microbes (no flushing) M-ARCOL includes a mucosal phase (mucin-alginate beads) *Thévenot et al., 2013*	Copenhagen MiniGut (CoMiniGut) Five low volume-bioreactors (5mL) for high throughput *Wiese et al., 2018*
	Dynamic Gastric Model (DGM) Mixing by water presser piston *Mercuri et al., 2011*		Engineered Stomach and small Intestine (ESIN) Mimic from mouth to the ileum Progressive meal intake with conservation of particles and differential stomach emptying between liquid and solid particles *Guerra et al., 2016*			TNO gastrointestinal Model 2 (TIM-2) 1-3 compartments Integratation of peristalsis and passive absorption *Minekus et al., 1999*
	TIM-advanced gastric compartment (TIM-agc) Mixing by peristaltic motion between proximal and distal antrum and no mechanical breakdown *Minekus, 2015*					The smallest Intestine (TSI) Reproduce the small intestine and incorporates the ileal microbiota *Cielpak et al., 2018*
						The human colonic model (HCM) New chinese colonic model, including pH, temperature and anaeroby control within the 3 colonic compartments *Zhang et al. 2022*

5.4. Models to specifically study mucosal interactions with emphasis on the mucus compartment

To specifically address host-microbiota interactions, different models integrating a mucus compartment have been developed. These models include the use of purified mucins, mucin-secreting cells or tissues, as described below.

It is worth to note that this state of the art has been published in a review article in the FEMS Microbiol. Rev. journal (Impact Factor: 16.408) and redrafted / updated for the present section.

Review: ETIENNE-MESMIN L, CHASSAING B, DESVAUX M, DE PAEPE K, GRESSE R, SAUVAITRE T, FORANO E, VAN DE WIELE T, SCHUELLER S, JUGE N, BLANQUET-DIOT S. Experimental models to study intestinal microbes-mucus interactions in health and disease. FEMS Microbiol Rev. 2019 Sep 1;43(5):457-489. doi: 10.1093/femsre/fuz013.

5.4.1. *In vitro* mucus/mucin binding assays

Mucus/mucin binding assays are considered as the simplest models to study microbial interactions with mucus and/or mucin. Even if the mucin used is often far from human physiology and no real host part is present, these models are still widely used nowadays for their high screening abilities and cheap costs. Purified mucins or mucus are immobilized on a support. Often, nons-pecific interactions are inhibited by the use of a blocking such as bovine serum albumin (BSA). Bacteria are allowed to interact during a precise time with the support, after which binding can be determined by different means. The revelation methods include crystal violet staining, use of specific antibodies, measurement of viable counts after plating of the cells or by quantitative PCR, radioactive probe, a fluorescent dye, or fluorescent tag in combination with flow cytometry (Etienne-Mesmin *et al.* 2019).

5.4.2. *In vitro* cell models

5.4.2.1. Mono-culture models

Compared to simpler binding assay described above, the use of cells allows to study bacteria interactions with the mucosal barrier in a more physiological manner, integrating host-cell reaction to the bacteria.

5.4.2.1.1. Non-mucus secreting cell lines

Before introducing the different mucus-secreting cell lines and their relevance for the study of pathogen interactions with the mucus compartment, we must highlight that non-mucus secreting cell lines are also relevant for such studies. Firstly, mucus non-secreting cells represent a useful model to study pathogen adhesion/invasion of epithelial cells. Human ETEC adhesins notably recognise molecular patterns on these epithelial cells, indicating that close epithelial interactions are relevant in bacterial pathogenesis. FimH, TibA, Tia, EtpA and CFA/I are all ETEC adhesins that favor adhesion/invasion of Caco-2 or HCT-8 cells by recognizing specific patterns on cell surfaces (Elsinghorst and Kopecko 1992; Madhavan *et al.* 2016; Sheikh *et al.* 2017). Secondly, mucus non-secreting cells allow the study of ETEC enterotoxins effect. T84 cells have been extensively used in this regard. As they are known to react properly to the toxins by an increase of the intracellular cGMP and cAMP levels, they establish themselves as the referent cell line in this domain (Zhang *et al.* 2010; Beltrán *et al.* 2015). Thirdly, mucus non-secreting cells allow the integration of signals from the innate immune response (Kern *et al.* 2017), as their cytokines production adapts upon contact with pathogen as ETEC cells or their virulence factors (Wang and Hardwidge 2012; Klingspor *et al.* 2015; He *et al.* 2016). For all these reasons, non-mucus secreting cell lines can be used to investigate the effect of dietary fibers on pathogen adhesion, toxin production and induction of immune response.

5.4.2.1.2. Mucus-secreting cell lines

The use of mucus-secreting cell lines integrates the mucus compartment in the study of pathogen interactions with the intestinal epithelium. Many colon carcinoma cell lines (e.g. Caco-2) express mRNAs encoding surface-associated and/or secreted intestinal mucins (Deplancke and Gaskins 2001). However, few of them actually secrete MUC2 or form a mucus layer (Lindén, Driessen and McGuckin 2007; Navabi, McGuckin and Lindén 2013; Hews *et al.* 2017). Most mucus-secreting cell lines are derived from the heterogeneous adenocarcinoma cell line HT-29 which can be differentiated into a mucus-secreting phenotype by growth under metabolic stress conditions. HT29–18N2 cells are often used as a model system for goblet cell differentiation and mucin secretion. These cells have been established by growth under glucose deprivation in galactose-containing culture medium (Phillips *et al.* 1988). In contrast, HT29-MTX cells and their clonal derivatives have been obtained by sequential adaptation to increasing concentrations of methotrexate (Lesuffleur *et al.* 1990) When grown on Transwell filter supports, some HT-29 MTX clones (e.g. MTX-D1 and MTX-E12) form polarized monolayers mostly constituted of mature goblet cells secreting an adherent mucus layer of 50–

150 µm thickness as revealed by Alcian Blue staining (Behrens *et al.* 2001). In addition, the mucin-secreting clonal cell line HT-29.cl16E emerged from parental HT-29 cells after subculture in sodium butyrate whilst HT29-FU cells were established by treatment with 5-fluorouracil (Lesuffleur *et al.* 1991). These mucus-producing HT-29 derivatives have been widely used to investigate the adherence of commensal and pathogenic bacteria to host cells (Gagnon *et al.* 2013; Naughton *et al.* 2013; Martins *et al.* 2015) and/or evaluate the effect of commensal bacteria on infection with enteropathogens (Zivkovic *et al.* 2015; Vazquez-Gutierrez *et al.* 2016). Some studies investigated the direct effect of commensal or pathogenic bacteria on host cell mucin synthesis and/or composition of the mucus layer (Vieira *et al.* 2010; Graziani *et al.* 2016). Among others, atypical EPEC increased expression of secreted MUC2, MUC3, MUC4 and MUC5AC as well as membrane-bound MUC3 and MUC4 in HT29-MTX cells, thereby enhancing bacterial growth by providing nutrients for adherent bacteria (Vieira *et al.* 2010). Another study showed that apical infection with *Listeria monocytogenes* stimulated mucus secretion by polarised HT29-MTX cells (Coconnier *et al.* 1998) which reduced bacterial invasion and colonisation of the host epithelium (Liévin-Le Moal, Servin and Coconnier-Polter 2005). Apart from all these HT-29 cell derivatives, the LS174T colon carcinoma cell line also secretes mucus,notably secrete mature MUC2 and MUC5AC (van Klinken *et al.* 1996) but do not produce an adherent mucus layer (Navabi, McGuckin and Lindén 2013). Again with this cell line, studies have been conducted to investigate the effect of commensal/pathogen bacteria activity on mucus structure, composition and barrier function (Wang *et al.* 2014; Leon-Coria *et al.* 2018).

5.4.2.2. Co-culture models

To model human intestinal epithelia, mixed cultures of enterocyte-like Caco-2 cells and mucus-producing HT29-MTX cells have been widely used in drug absorption and permeability studies (Hilgendorf *et al.* 2000; Lozoya-Agullo *et al.* 2017). This model has the benefit to combine the Caco-2 cells, with characteristic more relevant of the small intestine (Devriese *et al.* 2017), with mucus secreting cells. Some studies reported that this co-culture model secretes mucus patches upon itself rather than a proper continuous mucus layer (Dorier *et al.* 2017; García-Rodríguez *et al.* 2018; Gillois *et al.* 2021) (**Fig. 5.2**), while some others state that co-cultures seeded out on transwell inserts form a continuous mucus layer similar to cultures of HT29-MTX cells grown alone (Poquet, Clifford and Williamson 2008; Béduneau *et al.* 2014). This co-culture model has also been used to study the specificity of pathogen/commensals adhesion for mucus secreting cells (Laparra and Sanz 2009) and the effect of pathogen toxins

on cell physiology (Wang *et al.* 2014; Hoffmann *et al.* 2021). For instance, the LT toxin at a concentration of 7.5 $\mu g.ml^{-1}$ do not impact the permeability to Lucifer yellow of this co-culture models after 45 min exposure (Hoffmann *et al.* 2021). Interestingly, some authors have recently started to integrate innate immune cells in their co-culture models to study host-pathogen interaction. Vernay and colleagues showed that the supernatant of *B. fragilis* inhibited *Salmonella* translocation across a co-culture model composed of Caco-2 cells, HT29-MTX and differentiated M cells (Caco-2 are able to differentiate into M-cells following addition of Raji-B lymphocytes) (Vernay *et al.* 2020). *Vibrio parahaemolyticus* has the ability to translocate more easily across Caco-2/Raji B co-culture compared to Caco-2 monoculture (Finn *et al.* 2014).

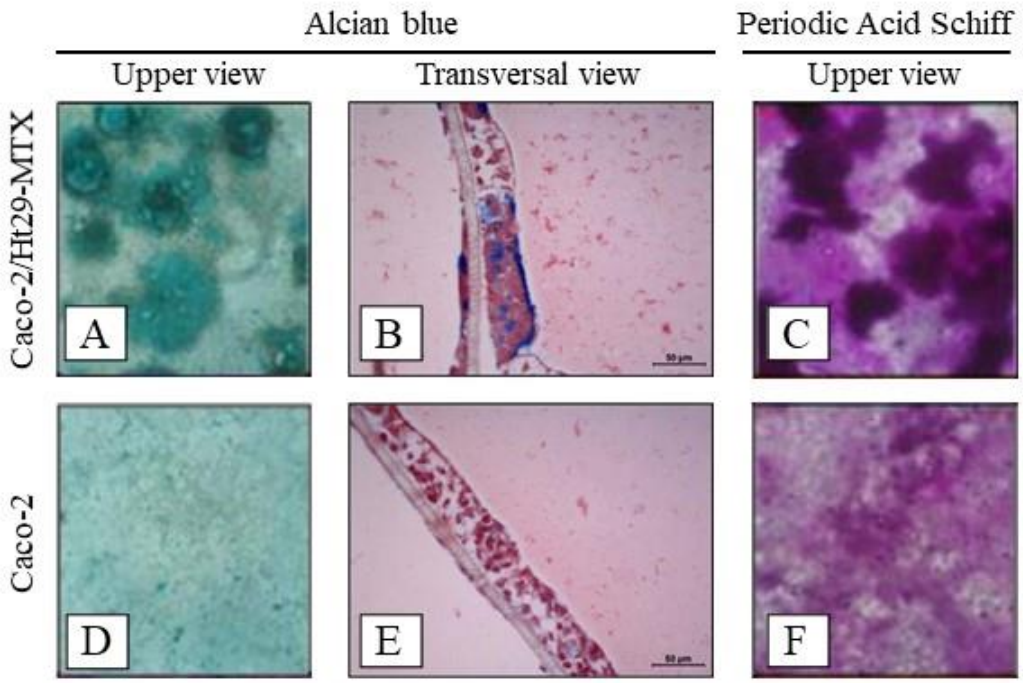


Figure 5.2. Microscopic caracterisation of the mucus secretion by Caco-2/HT29-MTX compared to Caco-2 models by two staining technics.
Mucus secretion is compared between the Caco-2/HT29-MTX (A, B, C) and the Caco-2 (D, E, F) cell models. Mucus secretion is revealed by Alcian Blue (A, B, D, E) and Periodic Acid Schiff (C, F) stainings on upper (A, C, D, F) and transversal view (B, E). Transversal views have been obtained on cells grown on transwell.
Figures A, C, D and F come from Dorier *et al*. 2017 and figures B and E from García-Rodríguez *et al*. 2018. They are printed with authors permission.

5.4.2.3. Gut-on-a-chip

Another cellular approach to simulate a mucin-producing human intestinal epithelium is the 'Gut-on-a-Chip' system, where Caco-2 cells are grown on a porous membrane support in a microfluidic device. While the cell membrane support is maintained under cyclic strain mimicking peristaltic motion, the chambers above and below the cell membrane are constantly perfused with medium, thereby generating low shear stress. These stream disturbances upon the cells are particularly relevant of the *in vivo* conditions, where the mucosal surface is under turbulent flow (De Weirdt and Van de Wiele 2015). Furthermore, this turbulent environment stimulates the formation of 3D intestinal villi similar to those found in the small intestine (Kim *et al.* 2012a), and the differentiation of Caco-2 cells into four types of differentiated epithelial cells including enterocytes, mucus-secreting, enteroendocrine and Paneth cells (Kim and Ingber 2013). In addition, Caco-2 epithelium grown in the Gut-on-a-Chip model displays enhanced barrier function and mucus production as compared to static Caco-2 cell cultures (Kim and Ingber 2013). Mimicking shear forces conditions is extremely important for opportunistic pathogen colonisation and virulence as demonstrated for *Salmonella*, *Shigella* and *E. coli* (De Weirdt and Van de Wiele 2015). The gut-on-a-chip devices have also been specifically developed to follow intestinal inflammation (Shin and Kim 2018). The HuMix (Human Microbial Cross-talk) model is another microfluidic device enabling the culture of human cell lines like Caco-2 cell with commensal bacteria under anaerobic conditions. In contrast to the Gut-on-a-Chip system, the epithelial cells, which do not produce mucus, are separated from the bacteria by a membrane coated with porcine gastric mucin (Shah *et al.* 2016). EIEC have been added to these gut-on-a-chip devices to study the host response to pathogens. Compared to non-pathogenic *E. coli*, EIEC cells exhibited an excessive growth at the apical surface of human cells which resulted in rapid injury of the epithelium, loss of normal villus morphology, and disruption of cell–cell junctions, which compromises the use of this model for the pathogen study (Kim *et al.* 2016).

5.4.3. Human enteroids/colonoids and intestinal organoids

New technologies have been developed which enable the generation of self-propagating spheres of primary intestinal epithelial cells ("mini-guts"). Enteroids or colonoids are derived from adult stem cells isolated from the crypts of human small intestinal or colonic tissues, respectively (Jung *et al.* 2011; Sato *et al.* 2011). As these enteroids are not derived from cancer cells, their differentiated cells are supposedly closer to human physiology. The apical side of

the epithelium can be on the inside or on the outside side of the enteroids (Co *et al.* 2019). If the apical side of the intestinal epithelial cells is orientated towards the inside of the sphere, then infection with bacteria requires microinjection. Enteroids have been used to study pathogens / commensals interactions with the epithelial barrier and its innate immune system (Engevik *et al.* 2015; Hill *et al.* 2017; Karve *et al.* 2017). Their relevance has been validated by different sets of observations. For instance, in enteroids, *Salmonella enterica* serovar Typhimurium invades the enteroid cells and induces actin ruffles as already observed in murine model but not in other models including intestinal epithelial cells (Co *et al.* 2019). Futhermore, in micro-injected facing-in enteroids, *Salmonella* Typhimurium intraepithelial replication has been shown to promote luminal colonisation by pathogen reemergence through infected intestinal epithelial cells expulsion, explaining how bacterial colonisation of the epithelium potently fuels expansion also into the luminal compartment (Geiser *et al.* 2021). Enteroids can also be used to study commensals/pathogens interactions with mucus. *E. coli* pathotypes as EAEC and EHEC have been highlighted to better adhere to mucus droplet on human enteroids than to the brush border. In particular, EAEC seem to adhere to specific mucins at the epithelial surface (Rajan *et al.* 2020). The ETEC mucinase EatA degrades the MUC2 mucin barrier formed by enteroids and promotes bacterial access to target enterocytes, as observed in other classic cell models (Kumar *et al.* 2014; Sheikh *et al.* 2021). To facilitate incubations with bacteria, 2D enteroid systems have been developed, where primary intestinal cells are grown as monolayers on permeable membrane supports (Nickerson *et al.* 2021). They contain MUC2-producing goblet cells and a mucus layer of more than 25 µm thickness (VanDussen *et al.* 2015; In *et al.* 2016). As the 3D enteroids, 2D enteroid systems support EAEC, EHEC and EPEC binding, and can be used to study pathogen interactions with the intestinal barrier notably (VanDussen *et al.* 2015; In *et al.* 2016; Zachos *et al.* 2016; Noel *et al.* 2017). In the support of their relevance, it has been shown that EHEC infection resulted in the formation of characteristic attaching and effacing lesions and mucus degradation, EPEC infection in piedestrals formation and EAEC infection in aggregative adhesion patterns (In *et al.* 2016; Nickerson *et al.* 2021). Futhermore, on these 2D-models also, the *E. coli* pathotypes seem to adhere specifically to mucus patches (Nickerson *et al.* 2021).

5.4.4. *Ex vivo* organ cultures

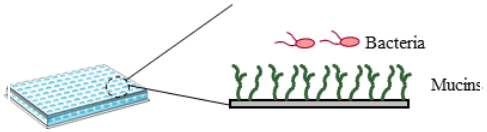
As illustrated above, cell lines models and their diverse complexity levels provide valuable tools for prediction of human physiology, pathology, and therapeutic responses. However, all these models are still limited by the absence of the tissue microenvironment.

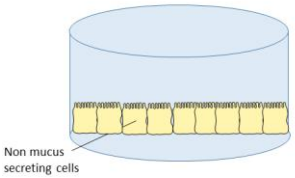
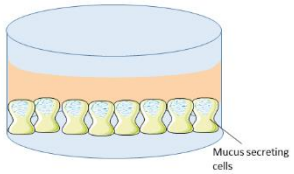
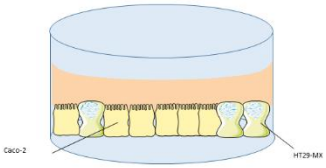
Culture approaches using human intestinal biopsy samples therefore represent an upscale platform to investigate the involvement of the mucus layer in pathogens and commensals interactions with their host. The advantages of *in vitro* organ culture (IVOC) of intestinal biopsies versus cell line culture models include the presence of healthy non-transformed cells including all major intestinal epithelial cell types (enterocytes, goblet cells, Paneth cells and neuroendocrine cells), underlying basement membrane and mucosal tissue, and the production of mucus. IVOC of human biopsy samples has been used to investigate adherence of pathogenic bacteria as EPEC, EHEC, ETEC and *C. jejuni* (Knutton *et al.* 1989; Grant, Woodward and Maskell 2006; Schüller *et al.* 2007; Lewis *et al.* 2015), cytotoxic effects of bacterial toxins (Schüller, Frankel and Phillips 2004), impact of enteropathogenic bacteria and their mucinase on mucus production (Andrade, Freymüller and Fagundes-Neto 2011; Hews *et al.* 2017).

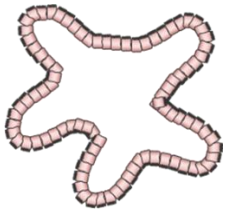
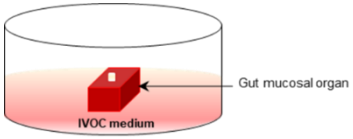
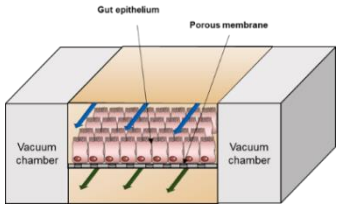
5.4.5. Limits of *in vitro* cell assays including a mucosal phase

Intestinal mucosal models provide valuable tools to study the interaction of microbes (and more particularly pathogens) with the host. However, despite their diversity, as very model, they present some limitations (Etienne-Mesmin *et al.* 2019) (**Table 5.2**). Intestinal mucosal models do not integrate a nervous or endocrinal system and a complete host immune response, showing the inability to precisely monitor host-microbe interactions and colonisation resistance determinants which are particularly important (Payne *et al.* 2012; Etienne-Mesmin *et al.* 2019). Furthermore, all the models aforementionned (except the simple mucus/mucin binding assays) require high levels of oxygen and some of them suffer from the toxicity induced by close contact with high load of microbes, limiting the possibility to study interactions with complex intestinal anaerobic communities. As an answer, attempts have been made to combine fermentation models with mucosal simulators, as presented below.

Table 5.2 Summary of the different models used to simulate a mucosal phase and potentially used in the study of pathogens interactions with the epithelium.
Modified from Etienne-Mesmin *et al.* 2019.

Types of models	Description	Applications	Advantages	Limitations	References
***In vitro* mucus/mucin binding assays**					
Microplates - Flow chambers					
	* Immobilization of mucus/mucin on the microtiter plate	* Evaluation of bacterial adhesion (commensals and pathogens) to mucins and molecular mechanisms associated	* Fast, quantitative and high throughput method to study mucus-microbe interactions independently from other *in vivo* conditions * Identification of molecular determinants involved in adhesion of microbes * Coupling with biophysical techniques (Surface Plasmon Resonance, Atomic Force Microscopy)	* Influence of experimental conditions (antibiotics, mechanical treatments, growth conditions, hydrophobic interactions) * Limited availability of purified mucins (mainly use of pig gastric mucin) * Absence of gut microbiota	McNamara *et al.*, 2000, Gusils *et al.* 2004, Ringot-Clyne *et al.* 2017, Dunne *et al.* 2018

Types of models	Description	Applications	Advantages	Limitations	References
***In vitro* cell models**					
Monoculture models					
Non-mucus secreting cell lines 	* Gut–derived epithelial cells resembling intestinal tissue consisting of enterocytes	* Adherence of commensal and pathogenic bacteria to host cells * Effect of bacteria on cell immunity * Assessment of bacteria toxin effect	* Reproducible and easily handled in laboratories * Identification of molecular determinants involved in adhesion of microbes and host cell mucin synthesis * Good platform for screening and characterizing probiotic activity	* Derived from cancer cells, different from healthy tissue * Not representative of various cell types recovered in mucosal epithelial tissues * Absence of gut microbiota * Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes * Difficulty to maintain for long-term experiments (>1 month) * No mucus compartment	Beltrán *et al.*2015, Di *et al.* 2017, Roussel *et al.* 2017, Leong *et al.* 2019
Mucus secreting cell lines 	* Gut–derived epithelial cells resembling intestinal tissue consisting mainly of mature goblet cells that secrete an adherent mucus layer	* Same as above but with integration of a mucus compartment * Effect of commensals/pathogens on host cell mucin synthesis and/or composition of the mucus layer * Impact of the mucus layer on bacterial physiology	* Same as above * Mucus composition closer to human physiology than model using commercially available mucin	* Same as above with mucus compartment * Not representative of appropriate *MUC* gene expression	Linden *et al.* 2007, Gagnon *et al*. 2013, Navabi *et al.* 2013, Hews *et al.* 2017
Co-culture models					
	* Mixed culture of enterocytes and mucin secreting cells	* Adherence of commensal and pathogenic bacteria to host cells * Effect of commensals/pathogens on host cell mucin synthesis and / or composition of the mucus layer	* Better representation of cell-type ratio recovered in mucosal epithelial tissues * Simple model, well described in literature	* Absence of M-cells (development of triple co-culture Caco-2/HT29-MTX/Raji B) * Variations in seeding ratios of HT29 MTX/Caco-2 can impede results interpretation * Modulation of mucus production by culture conditions * Absence of gut microbiota * Difficulty to maintain for long-term experiments (>1 month) * Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes)	Hilgendorf *et al.*, 2000, Lozoya-Agullo *et al.* 2017

Types of models	Description	Applications	Advantages	Limitations	References
***Ex-vivo* organ cultures**					
Intestinal organoids					
	* Generation of self-propagating spheres of primary intestinal epithelial cells * Enteroids = derived from adult stem cells isolated from the crypts of human small intestinal * Colonoids = derived from adult stem cells isolated from the crypts of human colonic tissue	* Study of advanced aspects of mucus development in a more complex scenario * Study of host–commensals and pathogens interactions	* Often collected from mice tissues, possible use of patient-derived tissues * Assay that more accurately mimics *in vivo* conditions * Amenable to long-term culture	* Highly expensive and requires specialized expertise * Requires access to biopsies/tissues * Donor-to-donor variability * Requirement of injection to infect organoids with bacteria * Absence of gut microbiota * No reproduction of peristalsis motions and GI stressful events * Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes)	Jung *et al.* 2011, Sato *et al.* 2011
In vitro organ culture *(IVOC)*					
	* Whole organs maintained *in vitro*	* Study of host–commensals and pathogens interactions	* Better maintenance of tissue architecture * Presence of non-transformed cells including all major cell types (enterocytes, goblet cells, Paneth cells and endocrine cells) * Often collected from animal tissues, possible use of patient-derived tissues	* Requires access to biopsies/tissues * Expensive and requires expertise * Donor-to-donor variability * Difficulty to maintain for long-term experiments * No reproduction of peristalsis motions and GI stressful events * Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes)	Browning & Trier 1969, Schüller *et al.* 2007
Gut-on-a chip					
	* Reproduction of the multicellular structures, cell–cell and tissue–tissue interactions, and the native microenvironment * Closely reproduction of the *in vivo* situation	* Study of the complex physiological and pathophysiological responses of tissues at an organ level * Study of host–commensals and pathogens interactions	* Presence of non-transformed cells including all major cell types (enterocytes, goblet cells, Paneth cells and endocrine cells) * Reproduction of peristalsis like motions * Possible use of biopsies from disease patients (e.g. IBD)	* Expensive and requires dedicated expertise and instrumentation * Stem cell differentiation is difficult to achieve * Flow rate of the medium can influence cell metabolism * Absence of gut microbiota * No input from immune and nervous system * Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes) * No reproduction of the full complexity of the human gut microbiota	Kasendra *et al.* 2018

5.5. Inclusion of mucosal phase in digestive/fermentation systems

Coupling digestive/fermentation systems and mucosal simulators would be the ultimate goal to simulate the complete GIT physiology, allowing a model to integrate the human digestive physicochemical parameters of the human gut, its microbiota, and the host. First attempts in this direction have already been made, as described below.

5.5.1. The added value of the mucus compartment in fermentation systems

Historically, addition of mucin in solution has been frequently included in the nutritive medium (mimicking the composition of ileal effluents) of fermentation models to provide a nutrient source to the gut bacteria. However, the study of mucus colonisation by gut bacteria in these systems has been revolutionized by the ability to simulate the viscoelastic gel-like nature of the mucus layer through the incorporation of mucus carriers (Gibson, Cummings and Macfarlane 1988; Macfarlane, Hay and Gibson 1989; Macfarlane, Woodmansey and Macfarlane 2005; Van Herreweghen *et al.* 2017). These mucus carriers simulating the mucosal phase are similar to the ones used in mucus/mucin binding assays. As these are inert surfaces without any human cells, they can stand direct contact with the microbiota. MacFarlane was the first to demonstrate a rapid colonisation of an agar mucus layer during a 48-hour incubation in a two-stage continuous fermentor system by a mixture of *Bacteroides*, *Enterobacteria* and facultative anaerobes (Macfarlane, Woodmansey and Macfarlane 2005) (**Fig. 5.3**). However, the use of glass tubes in this set-up, containing this agar-mucus layer, did not permit a practical long-term implementation (Van den Abbeele *et al.* 2009). Since then, mucus coated beads (mixture of 5% porcine mucin type II and 1% agar or 2% alginate) have been identified as crucial platform in sustaining microbial diversity by selectively enriching species, which are not thriving in the luminal environment. As already mentioned in Section 4.3.1, this mucus interphase has already been introduced in batch models, in the form of mucin-agar microcosms in a polyethylene netting, to study pathogen interactions with the mucus layer (Moens *et al.* 2019), but more complex models are also concerned. Mucin-agar microcosms were introduced in the colonic compartments of the SHIME resulting in the M-SHIME (for Mucosal-SHIME) configuration (Van den Abbeele *et al.* 2012, 2013) (**Fig. 5.3**). The M-SHIME model has already been used to detect mucus-associated species (De Paepe *et al.* 2018), follow dietary fiber

metabolism (De Paepe *et al.* 2019, 2020) and study ETEC survival/virulence (see sections 4.3.1. and 4.3.6.4.3.) (Roussel *et al.* 2018a, 2020a). Based on the same technology, a supplementary module containing mucin-alginate beads has been added to the ARCOL mono-compartmental system. This external module is maintained under a continuous flow of fermentation medium coming from the main bioreactor and can be renewed every two days without any flushing with CO_2 or N_2, allowing study of mucus-orientated microbiota under more physiological anaerobic conditions (Deschamps *et al.* 2020; Gresse *et al.* 2021b, 2021a) (**Fig. 5.3**).

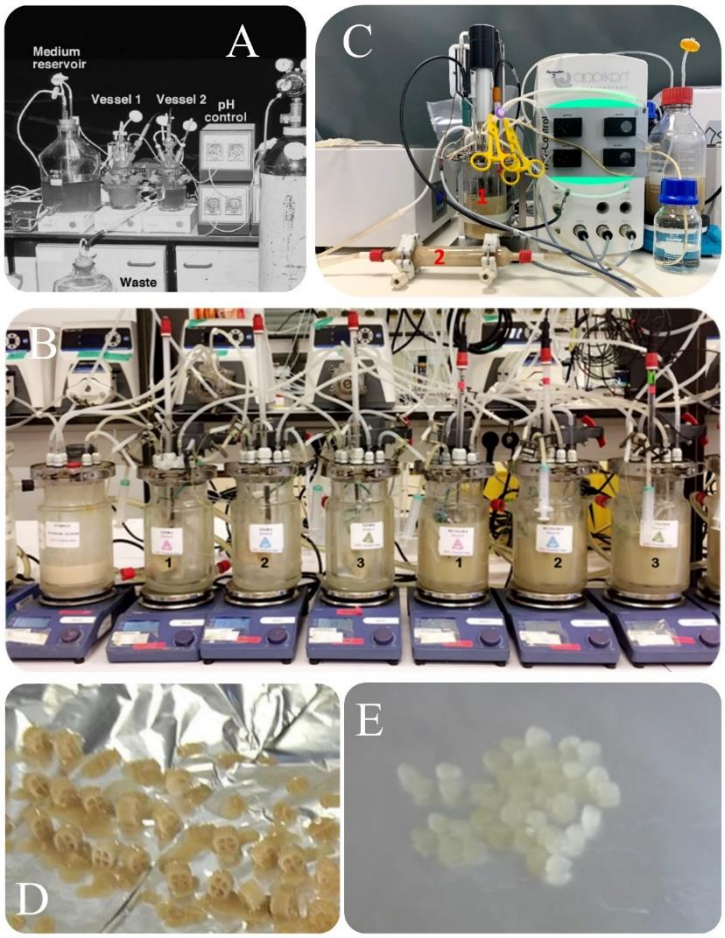


Figure 5.3. Mucus matrix inclusion in fermentation models.
Pictures of the three continuous models of fermentation in which a mucus matrix has been included and their mucin carriers. **(A)** The two-stage continuous culture system of Mac Farlane in which vessel 1 and 2 simulate the proximal and distal colon, respectively. Glass tubes containing mucin-agar gelose were added directly into the vessels. **(B)** The M-SHIME model containing mucin-agar microcosms placed inside the ileal or colonic compartments (#1-3). (**C**) The M-ARCOL model is a one-stage fermentation system composed of a single glass vessel (1) and an external module with mucin-alginate beads (2), which can be temporary disconnected from the main fermentor container to allow sampling and beads renewal without flushing of the system. (**D**) Mucin-agar microcosms (9 mm diameter) used in the M-SHIME model models and (**E**) mucin-alginate beads (around 4 mm diameter) are used in the M-ARCOL model.
The photographs A is issued from Mac Farlane *et al.* 2005 and printed with permission. The others come from personal source.

5.5.2. Coupling fermentation models with cell models

In vitro fermentation models with a mucosal phase are a first step in deciphering microbiota interactions with the intestinal mucus. However, as shown in the previous section, the use of human cells/tissues allows to study bacteria interactions with the mucosal barrier in a more physiological close manner, integrating host reaction to the bacteria. Despite the fact that human cells need high oxygen levels, recent advances have been undertaken to combine *in vitro* fermentation models with cellular systems (Bahrami *et al.* 2011; Marzorati *et al.* 2014; Defois *et al.* 2018). The easiest way is to directly apply filtered or diluted samples from the fermentation system onto mucosal simulators, such as co-cultures of enterocytes and immune cells in transwell systems (Defois *et al.* 2018; Calatayud *et al.* 2021). *In vitro* gut models can also be coupled with complex cell-containing units, such as the Host Microbiota Interaction (HMI) module (Marzorati *et al.* 2014). The HMI module has been specifically designed to be connected to continuous fermentation models such as the SHIME model. It consists of two separated compartments, one containing mixed microbiota and the other Caco-2 cells. It incorporates (micro)-environmental parameters from the mucosa such as microaerophilic conditions and shear forces (Marzorati *et al.* 2014). Regarding their particular relevance in the follow-up of intestinal inflammation, gut-on-a-chip devices could also be coupled to fermentation models to further unravel enteric infections interactions with the innate immune responses (Shin and Kim 2018). Recent upgrades in those chips have integrated the oxygen gradient microenvironment allowing robust and long-term co-culture of obligate anaerobic gut microbiota with the cells (Jalili-Firoozinezhad *et al.* 2019; Shin *et al.* 2019).

Bullet points, *in vitro* models to study pathogen interactions with mucosa and their coupling with digestive/fermentation systems

- Numerous models with different levels of complexity could allow to specifically addressing pathogen interactions with the mucosal phase.
- Among them, the most common methods rely on the use of mucus/mucin binding assays and mucus-secreting intestinal cellular models.
- Efforts have been made to add a mucosal compartment to complex fermentation models, in order to study the microbial communities of the human gut mucosa and their interactions with the host.

6. Context, research questions and outline of the PhD

This PhD work was performed, in the continuity of that of Charlène Roussel, in the frame of a partnership between the UMR MEDIS (Université Clermont Auvergne, France) and the CMET (Ghent University, Belgium). These two laboratories benefit from more than 20 years of internationally recognized expertise and knowledge in the fields of *in vitro* human gut simulation. They have both developed a technological platform around the digestive environment associating *in vitro* digestion and fermentation tools, cellular models of the human intestinal epithelium, and molecular tools of genomics and post-genomics. The major interest of this collaboration was to associate the in-depth expertise of UMR MEDIS in the upper gut simulation and the one from CMET in lower gut models and host-microbe interactions.

This PhD initially planned a two-year period in UMR 0454-MEDIS (Université Clermont Auvergne, France), followed by one year at CMET (Ghent University, Belgium). Due to the pandemic situation in 2019, the departure to Belgium was delayed, resulting in only 9 months in CMET at the end of the PhD framework. Fiber products screening program, cellular experiments and TIM-1 experiments were realized in France, while fecal batch experiments were performed in CMET (see details below).

Beyond this academic partnership, this PhD work was part of a collaborative project with 4 industrial companies from the Auvergne-Rhône-Alpes region, which financially supported the project through FEDER funding. Lallemand SAS is a world leader company in the development, production and marketing of yeasts, bacteria and derived ingredients for animal and human purposes. Limagrain is a French agricultural cooperative group specialized in seed selection, vegetable seeds and cereal products. PiLeJe manufactures and distributes micronutrition, phytonutrition and microbiotic-based solutions. HARi&CO is a start-up commercializing various vegetables and fiber-based foods. These companies have provided most of the fiber-containing products tested in this PhD project.

The literature review hereinabove has redrawn the current knowledge on the three-way relationship between gut microbiota, dietary fibers and mucus layer and how they could unravel the capacity of enteric pathogens to colonize the human digestive tract and ultimately lead to infection. However, scarce studies have already investigated the role or mucus or even its associated microbiota in the pathophysiology of ETEC infections, as well as the anti-infectious activity of dietary fibers against the pathogen, considering its interactions with the mucus compartment.

Therefore, using the prototypical ETEC strain H10407, the ultimate goal of the present joint PhD was to:

(i) Better decipher the interactions of ETEC pathogen with the mucus compartment in the human digestive environment (axis I).

(ii) Investigate the potential of dietary fiber-containing products as innovative anti-infectious strategies against ETEC (axis II)

In the two axes, different aspects of ETEC physiopathology have been addressed using various complementary *in vitro* approaches.

- Colonisation: Do mucus and dietary fiber-containing products impact ETEC growth/survival in the presence or not of the human microbiota?
- Adhesion: Does ETEC present a propensity to adhere to the mucins or mucus-secreting cells and can dietary fiber-containing products inhibit such adhesion?
- Virulence gene expression: Does the mucus compartment shape ETEC virulence genes in the human simulated digestive environment and can dietary fiber-containing products products module it?
- Host-pathogen interactions: Are the mucus compartment and dietary fiber-containing products able to modulate innate immunity, mucus secretion and intestinal permeability related-genes?
- Microbiota-pathogen interactions: Do the presence of a mucus compartment and dietary fiber-containing products impact ETEC-induced modulation of gut microbiota composition/activity?

The following chapters in section II will present the results of each stage of the experimental work and are structured around three main chapters (**Fig. 6.1**). Chapter 1 reports the work investigating the role of mucus in the modulation of ETEC virulence and survival. Chapter 2 focuses on the screening program conducted to select two fiber-containing products among eight tested for their anti-infectious properties against ETEC. Chapter 3 focuses on further studies investigating the anti-infectious properties of the two selected fiber products, namely the lentil extract and specific yeast cell walls.

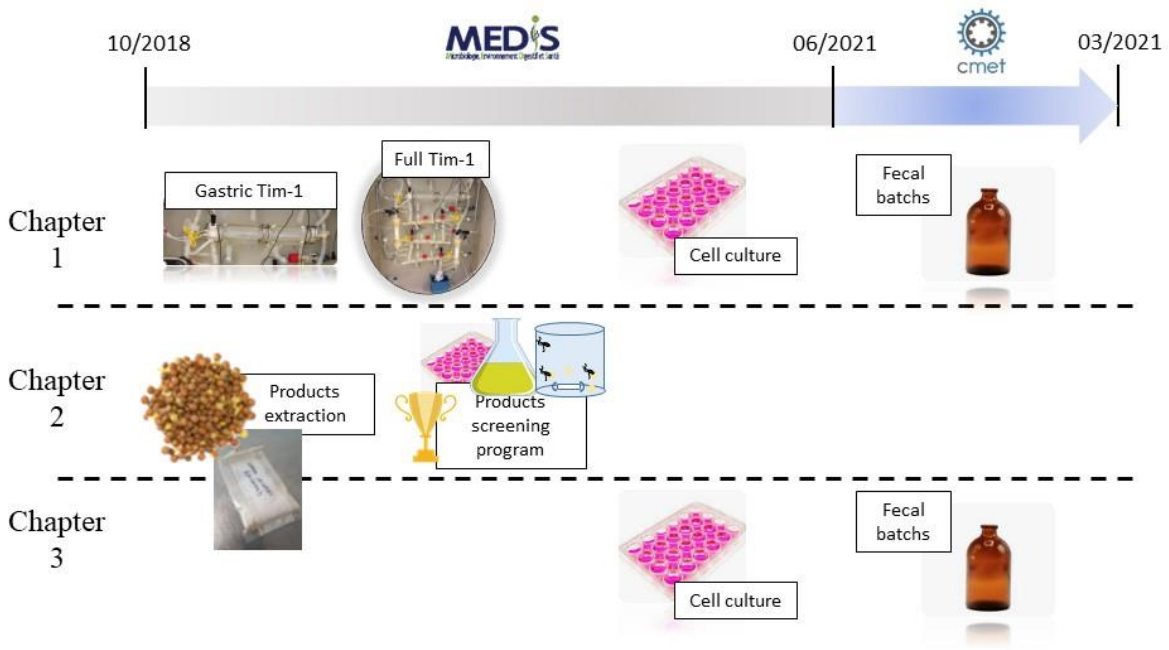


Figure 6.1. Outline of the experimental work during the joint PhD.
The main stages of the experimental work are summarized in this figure.
From October 2018 to June 2021, experiments were performed at MEDIS laboratory (Université Clermont Auvergne, Clermont-Ferrand, France), while from June 2021 to March 2022, experiments were conducted at CMET laboratory (Ghent University, Gent, Belgium).

Section II – Experimental work

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Chapter I - Role of mucus-bacteria interactions in Enterotoxigenic *Escherichia coli* (ETEC) H10407 virulence

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Deciphering the role of mucus-bacteria interactions in Enterotoxigenic *Escherichia coli* physiopathology in the human digestive environment was the aim of the first axis of this PhD project. Using various complementary *in vitro* approaches, the interactions of the ETEC strain H10407 with intestinal mucus was investigated. Among the parameters tested: (i) the survival and/or virulence of ETEC in the complex physicochemical and microbial background of the human gut, using the TIM-1 model and fecal batch experiments simulating the upper and lower GI tract, respectively; (ii) the adhesion to mucosal surface using mucin beads and cell-culture assays. All those experiments were completed with a large set of techniques including plate counts, molecular-based methods to assay ETEC survival and virulence gene expression (qPCR, qRT-PCR) and gut microbiota composition (16S rRNA gene amplicon sequencing, flow FISH), then subsequent toxin production and induction of innate immune responses were evaluated by ELISA assays and PCR-based methods.

The results have been subjected to the writing of an original research article, to be submitted in npj Biofilms and Microbiomes (Impact Factor: 6.769) and redrafted for the present chapter.

Role of mucus-bacteria interactions in Enterotoxigenic *Escherichia coli* (ETEC) H10407 virulence and interplay with gut microbiome.
Thomas Sauvaitre, Josefien Van Landuyt, Claude Durif, Charlène Roussel, Adeline Sivignon, Sandrine Chalancon, Ophélie Uriot, Florence Van Herreweghen, Tom Van de Wiele, Lucie Etienne-Mesmin[#], Stéphanie Blanquet-Diot[#]

Submitted in npj Biofilms and Microbiomes
 # co-last authors

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1. Abstract

The intestinal mucus layer has a dual role in human health constituting a well-known microbial niche that supports gut microbiota maintenance but acting as a physical barrier against enteric pathogens. Enterotoxigenic *Escherichia coli* (ETEC), the major agent responsible for traveler's diarrhea, is able to bind and degrade intestinal mucins, representing an important but understudied virulent trait of the pathogen. Using complementary in vitro approaches simulating the human digestive environment, this study aimed to describe how the mucus compartment could shape different aspects of the human ETEC strain H10407 pathophysiology, namely its survival, adhesion, virulence gene expression, innate immunity induction and interactions with human gut microbiota. Using the TIM-1 model, simulating the physicochemical conditions of the human upper gastro-intestinal (GI) tract, we reported that mucus secretion and physical surface sustained ETEC survival, probably by helping it to face GI stresses. When integrating the host part in Caco2/HT29-MTX co-culture model, we demonstrated an adhesion propensity of ETEC to mucus, an up-regulation of virulence genes at cell vicinity, but no significant effect on ETEC-induced inflammation. Furthermore, we demonstrated that mucosal surface did not favor ETEC colonisation in a complex gut microbial background simulated by batch fecal experiments. However, mucus-specific microbiota was widely modified upon ETEC challenge suggesting its role in pathogen infectious cycle. Using multi-targeted in vitro approaches, this study supports the major role played by mucus in ETEC pathophysiology, opening avenues in the design of new treatment strategies.

2. Introduction

Continuously produced and secreted by goblet cells, the intestinal mucus is a complex viscoelastic adherent secretion composed of water, electrolytes, lipids and proteins (Etienne-Mesmin *et al.* 2019). Its main structural components (around 5 %) are large glycoproteins called mucins (Etienne-Mesmin *et al.* 2019; Sauvaitre *et al.* 2021b). Due to its location at the interface between the digestive lumen and the host compartment, accumulating evidence has shown the mucus layer to be a key feature in the modulation of gut health, including the role it plays for several members of the gut microbiome (Martens, Neumann and Desai 2018; Daniel, Lécuyer and Chassaing 2021). From one side, the mucus layer is a well-known microbial niche, colonized by a specific associated microbiota enriched in Firmicutes, Proteobacteria and Actinobacteria, enabling the establishment of particular environmental conditions and nutrient sources (Chassaing and Gewirtz 2019). Especially, mucosal communities are highly enriched in *Bacteroides acidifaciens*, *Bacteroides fragilis*, the mucin-degrader *Akkermansia muciniphila* and in species belonging to the *Lachnospiraceae* taxa (Donaldson, Lee and Mazmanian 2016; Pereira and Berry 2017). On the other side, mucus acts as a barrier against physical, chemical and biological stressors (Sicard *et al.* 2017; Sauvaitre *et al.* 2021b). Notably, enteric pathogens have to interact with and penetrate this line of defense in order to colonize the intestinal epithelium (Turner 2009; Peterson and Artis 2014; Desai *et al.* 2016). In accordance, several studies showed that genetic or environmental defects in mucus integrity increase pathogen susceptibility (Bergstrom *et al.* 2010; Desai *et al.* 2016). Among enteric pathogens, the food and water-borne enterotoxigenic *Escherichia coli* (ETEC) is the most important cause of travelers' diarrhea, with hundreds of millions of diarrheal episodes worldwide (Khalil *et al.* 2018). Infecting at numerous concentration, this bacterium has to penetrate the mucus layer to fulfil its infection cycle(Kumar *et al.* 2014; Tapader, Bose and Pal 2017). However, the conditions in the gastro-intestinal tract modulating the virulence and pathology are poorly understood. Once ingested, ETEC first has to withstand the stringent conditions (e.g. acidic pH, bile acids and competition with gut microbes) encountered in the human digestive environment (Roussel *et al.* 2017, 2020a), to reach its action site, most probably the distal parts of the small intestine (Stintzing and Möllby 1982; Allen, Randolph and Fleckenstein 2006; Rodea *et al.* 2017). Then, two ETEC mucin-degrading proteins (namely mucinases), EatA and YghJ promote pathogen access to the underlying epithelium (Kumar *et al.* 2014; Tapader, Bose and Pal 2017) and a panel of fimbrial (e.g. FimH) and non-fimbrial (e.g. Tia) adhesins supports bacterial attachment to mucosal surface through the recognition of specific surface receptors

(Lindenthal and Elsinghorst 1999; Vipin Madhavan and Sakellaris 2015; Sheikh *et al.* 2017). Some of these surface receptors are patterns specific to mucus. As an example, the EtpA adhesin preferentially binds to N-acetylgalactosamine motifs, which are expressed in blood group A antigens (Qadri *et al.* 2007; Kumar *et al.* 2018; Kuhlmann *et al.* 2019). ETEC degradation of the mucus layer and adhesion to the mucosal surface facilitate the production and delivery of LT and/or ST enterotoxins, that play a major role in ETEC pathogenesis and leading to profuse watery diarrhea (Qadri *et al.* 2005). In turn, the LT toxin also alters the structure and composition of the intestinal epithelial mucin layer by decreasing MUC4 expression (Verbrugghe *et al.* 2015) and increasing MUC2 expression and secretion, that results in an increased pathogen adhesion (Duan *et al.* 2019; Sheikh *et al.* 2021). Besides, ETEC also induces an intestinal inflammatory response (notably an Interleukin-8 (IL-8) secretion) that correlates with disease severity (Long *et al.* 2010; Mercado *et al.* 2011; He *et al.* 2016). Such phenomenon can be in turn modulated by ETEC virulence factors, as LT/ST toxins and the YghJ mucinase (Huang *et al.* 2004; Ma 2016; Tapader *et al.* 2016; Motyka *et al.* 2021). Lastly, several human clinical trials and *in vitro* reports have also shown that ETEC infection is associated with alterations of gut microbiota in terms of structure and activity (David *et al.* 2015; Youmans *et al.* 2015; Pop *et al.* 2016; Moens *et al.* 2019; Roussel *et al.* 2020a), suggesting its possible involvement in host susceptibility to the pathogen (Stevens, Bates and King 2021).

Given the modulatory role of mucus in enteric pathogen virulence, but also the scarcity of data regarding ETEC pathotype, the present study aims to decipher more closely the role of bacteria-mucus interactions in ETEC infection. Using complementary *in vitro* approaches simulating the human gastro-intestinal tract, we investigated the role of the mucus compartment on various facets of ETEC reference strain H10407 physiopathology, namely survival, adhesion, inflammation, virulence and interactions with gut microbiota.

3. Materials and methods

3.1. ETEC strain and growth conditions

The prototypical ETEC strain H10407 serotype O78:H11:K80 (ATCC® 35401, LT^+, ST^+, CFA/I^+), isolated in Bangladesh from a patient with a cholera-like syndrome (Evans *et al.* 1977) was used in this study. Bacteria were routinely grown under agitation (37°C, 120 rpm, overnight) in LB broth.

3.2. Growth kinetics in M9 culture medium

ETEC strain H10407 (initial concentration of 10^7 CFU.mL^{-1}) was allowed to grow under aerobic conditions (37°C, 5 hours, 120 rpm), in M9 medium (minimum medium for *E. coli* pH 6.8) (Elbing and Brent 2002), with or without 3 g.L^{-1} mucin from porcine stomach type II or III (Sigma-Aldrich, St. Louis, MO, USA). Medium was regularly sampled and plated onto LB agar for ETEC numeration (n=3).

3.3. Mucin-agar adhesion plate assays

Adhesion experiments were adapted from Tsilia et al. (2016) as previously described (Tsilia *et al.* 2016; Roussel *et al.* 2018b). Briefly, mucin-agar consisted of 5% porcine stomach mucin type II (Sigma-Aldrich, St. Louis, MO, USA) and 1% bacteriological agar (Sigma-Aldrich, St. Louis, MO, USA), with a pH adjusted to 6.8 to mimic human small intestinal pH. Six-well plates containing mucin-agar were inoculated with ETEC strain H10407 (initial concentration of 10^7 CFU.ml^{-1}). After 1-hour incubation (37°C, 120 rpm), each well was rinsed twice with phosphate buffer saline (PBS) to remove non-adherent bacteria. Separation of adhered bacteria was mechanically performed by transferring aseptically the whole mucin layer into a sterile bag containing PBS and further homogenization in a 400P BagMixer® for 10 min (Interscience, Bread, Netherlands). Adhered ETEC bacteria were quantified by plating on LB agar. Experiments were performed in triplicate and agar without mucin was used as a negative control.

3.4. Mucin beads preparation

Mucin-alginate beads were obtained as already described (Deschamps *et al.* 2020). The mixture containing 5% (w/v) porcine gastric mucin type III (Sigma-Aldrich, Saint-Louis, MO, USA) and 2% (w/v) sodium alginate (Sigma-Aldrich, Saint-Louis, MO, USA) was dropped using a peristaltic pump into a sterile solution of 0.2 M $CaCl_2$ under agitation (100 rpm). Control beads with the same density but without mucin were produced using alginate at a final concentration of 4.5%. Beads (diameter: 4.5 mm in average, data not shown) were then stored at 4°C (no more than 24 hours prior experiments).

3.5. *In vitro* static and dynamic digestion procedures

Static *in vitro* gastro-jejunal digestions were performed before mucin-bead adhesion assay (50 beads were added in the duodenum-ileum vessel) or to simulate upper gastrointestinal

stresses experienced by ETEC before colonic batch fermentation experiments (without mucin beads), as previously described (Roussel *et al.* 2020a) (**Table 1.1**). For adhesion assays, ETEC strain H10407 was inoculated at 10^7 CFU.mL^{-1} and experiments were performed in triplicate. Inoculation rates were calculated to ensure a 10^8 CFU.mL^{-1} initial concentration in batch experiments (n=6).

Table 1.1. Static *in vitro* gastro-ileal digestion procedure.
A static batch incubation (Erlenmeyer) was used to reproduce the physicochemical parameters of a gastro-ileal digestion according to parameters set-up in the TIM-1 system. Digestive secretions and solutions for pH adjustment were manually added during the 90 min digestion. Adapted from TIM-1 parameters presented bellow.

Parameters of static *in vitro* digestion	**Gastric vessel**	**Duodenum-Ileal vessels**
pH	From 6 (T0) to 2.1	Maintained at 6.8
Volume (mL)	50	90
Secretions	(**i**) 5.36 mg pepsin (727 U.mg^{-1}) (**ii**) 4.28 mg lipase (32 U.mg^{-1}) (**iii**) HCl 0.3 M (**iv**) $NaHCO_3$ 0.5 M if necessary	(**i**) 0.9 g bile salts (27.9 mM in solution) (**ii**) 1.8 g of pancreatin 4 USP (**iii**) Trypsin 2 mg.mL^{-1} (**iv**) $NaHCO_3$ 0.5 M if necessary
Time period in batch (min)	30	60
Chyme mixing	100 rpm (magnetic stirrer)	100 rpm (magnetic stirrer)
[Total microbes]	Sterile	Sterile
Oxygen level (%)	20	20
Temperature (°C)	37	37

Dynamic digestions were also performed using the TIM-1 system, which consists in four successive compartments simulating the human stomach and the three parts of the small intestine (duodenum, jejunum, and ileum). This *in vitro* system integrates the main physicochemical parameters of human digestion, such as body temperature, temporal and longitudinal changes in gastric and intestinal pH levels, peristaltic mixing and transport, gastric, biliary, and pancreatic secretions, and passive absorption of small molecules and water (Cordonnier *et al.* 2015; Roussel *et al.* 2016). For the first time, to simulate the mucus compartment, porcine stomach mucin type III secretion (Sigma-Aldrich, St. Louis, MO, USA) was added in the initial meal and delivered into the duodenum (final concentration of 3 g.L^{-1} throughout the digestive tract). In addition, two polyester pockets containing 40 beads of mucin-alginate beads were placed in each of the four compartments of TIM-1 to provide physical surface for bacterial adhesion. In the present study, the TIM-1 was set-up to reproduce, based on in *vivo* data, the digestive conditions observed in a healthy adult after ingestion of a glass of

200 mL mineral water (**Table 1.2**), contaminated with ETEC strain H10407 at a final level of 10^{10} CFU. Two types of *in vitro* digestions were performed: (i) gastric digestions where only the gastric compartment was set-up (total duration of 60 min) and (ii) gastro-intestinal digestions using the complete TIM-1 model (total duration of 300 min). During digestion, samples were regularly collected from each compartment (digestive lumen and mucin-alginate beads) to determine ETEC survival and adhesion. Gastric and ileal effluents were kept on ice and pooled on 0–10, 10–20, 20–40 and 40–60 min for gastric digestion and hour-by-hour for whole digestion (0–60, 60-120, 120-180, 180-240 and 240-300 min). Gastric and ileal effluents, as well as mucin beads, were kept at -20°C in RNAlater™ Stabilization Solution (Invitrogen, Waltham, MA, USA) for further RNA extraction. All digestions were run in quadruplicate and control digestions were performed without any mucin secretion nor mucin beads.

Table 1.2. Parameters of the TIM-1 system when simulating digestive conditions of a healthy adult after intake of a glass of water.
TIM-1 model was set-up to simulate the digestive conditions of a healthy adult after ingestion of a glass of water. $T_{1/2}$ represents the half time of gastric or ileal deliveries.
Defined by Cordonnier *et al.* 2015.

	TIM-1			
Parameters of *in vitro* digestion of a glass of water	Gastric compartment	Duodenal compartment	Jejunal compartment	Ileal compartment
pH	From 6 (T0) to 1.5 (90 to 300 min)	Maintained at 6.4	Maintained at 6.9	Maintained at 7.2
Volume (mL)	200 (initial)	30	115	115
Secretions	(**i**) 130 U.min^{-1} of pepsin (**ii**) 5 U.min^{-1} of lipase (**iii**) HCl 0.3M	(**i**) 20 mg.min^{-1} of bile salts 27.9 mM (first 30 min of digestion) then 10 mg.min^{-1} of bile salts 9.3 mM (**ii**) 20 mg.min^{-1} of pancreatic juice 4 USP (**iii**) Trypsin 2 mg.min^{-1} (15156 units/mg protein) (**iv**) $NaHCO_3$ 0.5M if necessary (**v**) Mucin Type III (final concentration 3 g.L^{-1})	(**i**) $NaHCO_3$ 0.5M if necessary	(**i**) $NaHCO_3$ 0.5M if necessary
Half-emptying time (min) / Residence time (h)	$T_{1/2}$ = 15 min	-	-	$T_{1/2}$ = 150 min
Chyme mixing	Water pressure	Water pressure	Water pressure	Water pressure
Passive absorption	-	-	Yes	Yes
[Total microbes]	Sterile	Sterile	Sterile	Sterile
Oxygen level (%)	20	20	20	20
Temperature (°C)	37	37	37	37

3.6. ETEC survival during gastrointestinal passage

During TIM-1 experiments, samples were taken in the initial bacterial suspension (T0 used for inoculation) and regularly collected in each compartment during *in vitro* digestions to determine ETEC survival by plating on LB agar ("planktonic" bacteria). Results were expressed as percentages of initial intake and cross-compared to those obtained with an inert and non-absorbable transit marker indicating 100% survival rate for ETEC bacteria.

3.7. Mucin-alginate beads adhesion assays during *in vitro* digestion

During static *in vitro* digestions, ETEC bacteria were allowed to adhere for 1 hour while mucin-alginate beads were collected from the TIM-1 system at 20 and 60 min in the stomach, 120 and 240 min in the duodenum, and 180 and 300 min in both jejunum and ileum. At the end of experiments, beads were washed three times with ice-cold sterile physiological water and crushed with an ultra-turrax apparatus (IKA, Staufen, Germany). The resulting suspensions were then serially diluted and plated onto LB agar for ETEC numeration ("adhered" cells).

3.8. Caco-2 and HT29-MTX cell culture assays

Caco-2 and HT29-MTX cells were cultivated as already reported (Sauvaitre *et al.* 2021a). Both Caco-2 mono culture and Caco-2/HT29-MTX co-culture (ratio 70-30) were maintained for 18 days to reach the full differentiation stage. Cells were then infected with ETEC strain H10407 at MOI 100 for 3 hours (37°C, 5% CO_2). At the end of experiment, cell supernatants were collected for monitoring ETEC virulence gene expression ("planktonic" bacteria). After three washes with ice-cold PBS pH 7.2 (Thermo Fisher Scientific, Waltham, MA, USA), intestinal cells were lysed with 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). Serial dilutions of the lysed cells were plated onto LB agar plates to determine the number of adherent ETEC bacteria ("adhered" bacteria). Cell supernatants and lysates were also centrifuged (3000 g, 5 min, 4°C) to discard remaining bacterial cells. Resulting supernatants were used to measure IL-8 cytokine extra- and intracellular levels, respectively. Bacterial pellets were stored in RNA later at -20°C for downstream analysis. All experiments were performed at least in triplicate.

3.9. Interleukin-8 measurement by ELISA

Pro-inflammatory IL-8 cytokine concentrations were determined in the supernatants and cell lysates from the monoculture and co-culture models according to the manufacturer's

instructions (DuoSet ELISA, human CXCL8/IL-8 ref DY208, RnD Systems, Minneapolis, MI, USA). Results were expressed as fold changes compared to control experiments without bacteria.

3.10. RNA extractions and quality controls

Total eubacteria RNA from TIM-1 samples (from digestive lumen and mucin beads) and cell culture experiments (planktonic and adhered bacteria) were extracted using the TRIzol® method (Invitrogen, Waltham, MA, USA) as described (Roussel *et al.* 2020a), with an additional purification step with MinElute Cleanup Kit (Qiagen, Hilden, Deutschland). Nucleic acid purity was checked and RNA was quantified using the NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). To remove any contaminating genomic DNA, DNAse treatment was performed as described (Roussel *et al.* 2020a).

3.11. Quantitative reverse transcription (RT-qPCR) analysis of ETEC virulence genes

RT-qPCR was performed as previously described (Roussel *et al.* 2020a). cDNA amplification was achieved using a CFX96 apparatus (Bio-Rad, Hercules, CA, USA), using primers and conditions (40 cycles) listed in **Table 1.3**. qPCR data were analyzed using the comparative $E^{-\Delta\Delta Ct}$ method and normalized with the reference genes *tufA* and *ihfB*. The amplification efficiency of each primer pair was determined by the generation of a standard curve based on a serial dilution of an ETEC cDNA sample. Differences in the relative expression levels of each virulence gene were calculated as follows: $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{reference gene}})_{\text{tested condition}} - (Ct_{\text{target gene}} - Ct_{\text{reference gene}})_{\text{reference condition}}$ and data were derived from $E^{-\Delta\Delta Ct}$.

3.12. Colonic batch experiments

Batch experiments were carried out for 24 hours in 60 mL penicillin bottles containing 20 mL nutritive medium and 60 mucin-alginate beads or 60 alginate beads as a control. The nutritive medium was composed per L of: 0.5 g guar gum, 1 g pectin, 0.5 g xylan, 1 g potato starch, 1 g yeast extract, 1 g proteose peptone, and 1g of pig gastric mucin type III (all from Sigma Aldrich, St. Louis, MO, USA), suspended into 0.1 M phosphate buffer (pH 6.8) and autoclaved before use. To examine the inter-individual variability of ETEC interactions with mucin and gut microbiota, penicillin bottles were inoculated with fecal samples collected from six healthy individuals. These donors were three males (donors 1, 2, 3) and three females

(donors 4, 5, 6), ranging in age from 20 to 30 years, without any history of antibiotic use six months prior to the study. The Research incubation work with fecal microbiota from human origin was approved by the ethical committee of the Ghent University hospital under registration number BE670201836318. Fecal collection and fecal slurry preparation were already described (De Paepe *et al.* 2017). Inoculation at a 1:5 dilution ratio of the 20% (w/v) fecal slurry resulted in a final concentration of 4% (w/v) fecal inoculum in the penicillin bottles. ETEC was pre-digested (as described above) and introduced at the final concentration of 10^8 CFU.mL^{-1}. The penicillin bottles were flushed with a mixture of N_2/CO_2 (80%/20%) during 20 cycles to obtain anaerobic conditions. The cycle was stopped at overpressure; and before starting experiments, the bottles were set at atmospheric pressure. Penicillin bottles were incubated at 37°C and 120 rpm on an orbital shaker KS 4000 i (IKA, Staufen, Germany) and aliquots were taken immediately after the start of the incubation (T0) and at 24 hours of fermentation from the liquid and atmospheric phases. Mucin-beads were collected 24h post-inoculation and washed twice in ice-cold physiological water before storage. All samples were immediately stored at -20°C, except samples for flow cytometry that were fixed before storage.

3.13. Gut microbiota metabolite analysis

SCFA production was measured using capillary gas chromatography coupled to a flame ionization detector after diethyl ether extraction as already described (Anderson, Ellingsen and McArdle 2006: 200; De Paepe *et al.* 2017). The gas phase composition was analyzed with a Compact gas chromatograph (Global Analyser Solutions, Breda, The Netherlands), equipped with a Molsieve 5A pre-column and Porabond column (CH_4, O_2, H_2 and N_2) or a Rt-Q-bond pre-column and column (CO_2). Concentrations of gases were determined with a thermal conductivity detector. Total pressure in the penicillin bottles was analyzed using a tensiometer (Greisinger, Regenstauf, Germany).

3.14. DNA extraction

DNA extraction was performed from samples collected at T0 and T24 during batch experiments as already reported (De Paepe *et al.* 2017; Miclotte *et al.* 2020). DNA quality and quantity were verified by electrophoresis on a 1.5% (w/v) agarose gel (Life technologies, Madrid, Spain) and spectrophotometer DENOVIX ds-11 (Denovix, Delaware, Wilmington).

3.15. ETEC quantification by qPCR and RNA fluorescent *in situ* hybridization

qPCR was performed using StepOnePlus real-time PCR system (Applied Biosystems, Waltham, MA, USA). Reactions were conducted in a total volume of 20 µL consisting of 10 µL of 2x iTaq universal SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA, USA), 2 µL of DNA template, 0.8 µL (10 µM) of each primer, and 6.4 µL nuclease-free water. Primers used to amplify cDNA are listed in **Table 1.3**. Data were analyzed using the comparative $E^{-\Delta\Delta Ct}$ method. The amplification efficiency of the primers pair was determined by the generation of a standard curve based on serial dilution of five ETEC-infected samples. Differences in number of copies of the *eltB* gene was calculated as follows: $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{reference gene}})_{\text{sample of interest}} - (Ct_{\text{target gene}} - Ct_{\text{reference gene}})_{\text{reference sample}}$ and data were derived from $E^{-\Delta\Delta Ct}$. All qPCR analyses were conducted in triplicate.

Flow cytometry samples were fixed and prepared for RNA fluorescent *in situ* hybridization, as already described (Huang *et al.* 2007). Briefly, cells were fixed by addition of three volumes of 4% paraformaldehyde in PBS and incubated at 4°C for 3 h. Subsequently, cells were washed in PBS prior to resuspension in a 1:1 (vol: vol) mix of PBS and 96% (vol: vol) ethanol. Cells were hybridized in 100 µL hybridization buffer for 3 h at 46°C. The hybridization buffer consisted of 900 mmol.L^{-1} NaCl, 20 mmol.L^{-1} Tris–HCl (pH 7.2), 0.01% sodium dodecyl sulfate, 20% deionized formamide, 5mM EDTA. The buffer also contained the two *E. coli* targeting probes at the final concentration of 2 ng.μl^{-1} and a combination of probes targeting eubacteria at the final concentration of 1 ng.µl-1 each (**Table 1.3**) (Baudart and Lebaron 2010). After hybridization, samples were washed with wash buffer (900 mmol.L^{-1} NaCl, 20 mmol.L^{-1} Tris–HCl pH 7.2, 0.01% sodium dodecyl sulfate) for 15 min at 48°C. After washing, cells were resuspended in 50 µL of PBS. Samples were diluted and stained with SYBR® Green I (SG, 100x concentrate in 0.22 µm-filtered dimethyl sulfoxide, Invitrogen) and incubated for 20 min at 37°C (Props *et al.* 2016). Samples were analyzed immediately after incubation with a Attune NxT BRXX flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA). The flow cytometer was operated with Attune™ FocusingFluid, as sheath fluid. Threshold was set on the primary emission channel of blue lasers (488 nm). The Attune Cytometric Software was used to draw the gates, but also the percentage of active *E. coli* in the total bacteria population was expressed as the number of cells showing the *E. coli* probe fluorescence out of the number of cells fluorescently labelled with the Eubacteria probes and SYBR green fluorescence.

3.16. 16S Metabarcoding analysis of gut microbial communities

Next-generation 16S rRNA gene amplicon sequencing of the V3-V4 region was performed by LGC Genomics (Berlin, Germany) on an Illumina MiSeq platform (Illumina), as previously described (De Paepe *et al.* 2017), excepted that luminal and mucosal samples had undergone respectively 30 and 33 amplification cycles.

All data analysis was performed in R (4.1.2). The DADA2 R package was used to process the amplicon sequence data according to the pipeline tutorial (Callahan *et al.* 2016). In a first quality control step, the primer sequences were removed and reads were truncated at a quality score cut-off (truncQ=2). Besides trimming, additional filtering was performed to eliminate reads containing any ambiguous base calls or reads with high expected errors (maxEE=2.2). After dereplication, unique reads were further denoised using the DADA error estimation algorithm and the selfConsist sample inference algorithm (with option pooling = TRUE). The obtained error rates were further inspected and after approval, the denoised reads were merged. Subsequently, the ASV (Amplicon Sequence Variant) table obtained after chimera removal was used for taxonomy assignment using the Naive Bayesian Classifier and the DADA2 formatted Silva v138 ASV's mapping back to anything other than 'Bacteria' as well as singletons were excluded and considered as technical noise(McMurdie and Holmes 2014).

3.17. Data availability

The sequence data have been deposited at NCBI Sequence Read Archive database with accession number PRJNA802327.

3.18. Statistical analysis

All statistical analysis, except the one conducted on the microbiota diversity composition results were performed using GraphPad Prism v8.0.1. Statistical data analysis on microbiota diversity was performed using in R, version 4.1.2 (R Core Team, 2016), using statistical packages as Phyloseq (v1.38)(McMurdie and Holmes 2013) for ASV's data handling, vegan v2.5.7 (Dixon 2003), betapart v 1.5.4 for diversity analysis of ASV's (Baselga and Orme 2012), deseq2 v1.34 (Love, Huber and Anders 2014: 2) for significant higher/lower abundance of ASV. The evolution of the microbial community α-diversity between conditions was followed by computing the richness (Observed ASV) and evenness indexes (Shannon, Simpson, Inverse Simpson, Fisher) using vegan. To highlight differences in microbial community composition between conditions, ordination and clustering techniques were applied

and visualized with ggplot2 (v3.3.5)(Ramette 2007). Non-metric multidimensional scaling (NMDS) was based on the relative abundance-based Bray-Curtis dissimilarity matrix (Legendre, Borcard and Peres-Neto 2005). The influence of ETEC infection and the type of beads used was determined by applying a distance-based redundancy analysis (db-RDA) using the abundance-based Bray-Curtis distance as a response variable (Legendre and Anderson 1999; Ramette 2007). db-RDA was performed both including and excluding ASV1 (attributed to *Escherichi*/*Shigella*) from the ASV table. The significance of group separation between conditions was also assessed with a Permutational Multivariate Analysis of Variance (permANOVA) using distance matrixes (Ramette 2007). Prior to this formal hypothesis testing, the assumption of similar multivariate dispersions was evaluated. In order to find statistically significant differences in ASV abundance between infected and non-infected conditions, a Wald test (corrected for multiple testing using the Benjamini and Hochberg method) was applied using the DESeq2 package. The metabolic response (measured SCFA and pH) was modelled in function of the beads and infection conditions in a db-RDA analysis.

Table 1.3. ETEC primers used in the study.

Gene	Target	Primer sequence 5'-3'	Amplicon length (pb)	References
Genes to monitor ETEC survival ETEC by qPCR (in fecal batches)				
eltB	LT toxin	F-GGCAGGCAAAAGAGAAATGG R-TCCTTCATCCTTTCAATGGCT	117	Lothigius *et al.* 2008
16S	Reference gene	F- NNNNNNNNNTCCTACGGGNGGCW GCAG R- NNNNNNNNNNTGACTACHVGGGTA TCTAAKCC	464	Klindworth *et al.* 2013
Genes for RT-qPCR analysis of ETEC virulence genes				
tufA	Reference gene	F-GACATGGTTGATGACGAAGA R-GCTCTGGTTCCGGAATGTA	199	Delmas *et al.* 2019
ihfB	Reference gene	F-CTGCGAGGCAGCTTCCAGTT R-GCAGCAACAGCAGCCGCTTA	419	Zhou *et al.* 2011
eltB	LT toxin	F-GGCAGGCAAAAGAGAAATGG R-TCCTTCATCCTTTCAATGGCT	117	Lothigius *et al.* 2008
leoA	Labile enterotoxin output	F-AAACGGTGCATATCCTCGTC R-AAATGCTGCCACCGAAATAC	168	Roussel *et al.* 2020
estP	ST toxin	F-TCTTTCCCCTCTTTTAGTCAG R- ACAGGCAGGATTACAACAAAG	165	Rodas *et al.* 2009
tolC	TolC outer membrane protein (ST toxin secretion)	F-AAGCCGAAAAACGCAACCT R-CAGAGTCGGTAAGTGACCATC	101	Swick *et al.* 2011
tia	Adhesin	F-ACAGGCTTTTATGTGACCGGTAA R-GACGGAAGCGCTGGTCAGT	67	Nicklasson *et al.* 2012
fimH	Minor component of Type I pili	F-GTGCCAATTCCTCTTACCGTT R-TGGAATAATCGTACCGTTGCG	164	Hojati, Molaie and Gholipour 2015
yghJ	Mucinase	F-CCCTGTTAGCCGGTTGTGAT R-GGTATCGGTTCTGGCGTAGG	166	This study
eatA	Mucinase	F-AACGGAAGCACCGTCATTCT R-CAGAGTCAGGGAGGCGTTTT	363	This study
rpoS	Environmental stresses response	F-GCGCGGTAGAGAAGTTTGAC R-GGCTTATCCAGTTGCTCTGC	229	Rahman *et al.* 2006
ETEC gene quantification by RNA fluorescent *in situ* hybridization in batch fermentation				
16S	Eubacteria 16S rRNA	1- GCTGCCTCCCGTAGGAGT 2- CGGCGTCGCTGCGTCAGG 3- MCGCARACTCATCCCCAAA	N/A	Amann *et al.* 1990
16S	*E. coli* 16S rRNA	1- GCAAAGGTATTAACTTTACTCCC *(Cy5 in 5')* 2-GCAGCAACAGCAGCCGCTTA *(Helper probe)*	N/A	Baudart and Lebaron 2010

F: Forward, LT: Heat-labile enterotoxin, R: Reverse, ST: Heat-stable enterotoxin

4. Results

4.1. ETEC is able to grow on mucin as sole substrates

ETEC strain H10407 ability to use purified mucin as substrate was first assayed following pathogen growth kinetics in M9 minimum culture medium supplemented with or without commercially available mucins (Type II and III) (**Suppl. Fig. 1.1**). After a 5-hour incubation period, compared to the control condition, the number of cultivable ETEC cells was multiplied by 56 and 32 with commercial mucins type II and type III, respectively ($p < 0.05$). The capacity of ETEC to grow on mucin type II was significantly higher compared to type III, with $6.6x10^8$ *versus* $3.9x10^8$ CFU.mL^{-1} at the end of the experiment ($p < 0.05$, n=3, Tukey's multiple comparison tests).

4.2. ETEC shows a tropism for mucin and mucus-secreting intestinal cells

Specificity of ETEC adhesion to mucus was evaluated using different *in vitro* models (**Fig 1.1**). First, in a simple plate assay, ETEC strain H10407 showed an enhanced adhesion for mucin-agar layer (with mucin type II) compared to agar alone (**Fig. 1.1.A**), with an average of $1.8x10^8$ *versus* $2.4x10^7$ CFU.mL^{-1} adhered bacteria ($p < 0.05$). Then, in order to integrate the host component, we performed cell adhesion experiments using monoculture Caco-2 (enterocytes) or Caco-2/HT29-MTX (enterocyte and mucus-secreting goblet cells) co-culture models (**Fig. 1.1.B**). After 3-hour exposure period, the number of adherent bacteria was one log higher in the co-culture of Caco-2/HT29-MTX cells compared Caco-2 alone (5.47 *versus* 4.60), suggesting tissue tropism of ETEC towards these mucus-producing cells ($p < 0.001$).

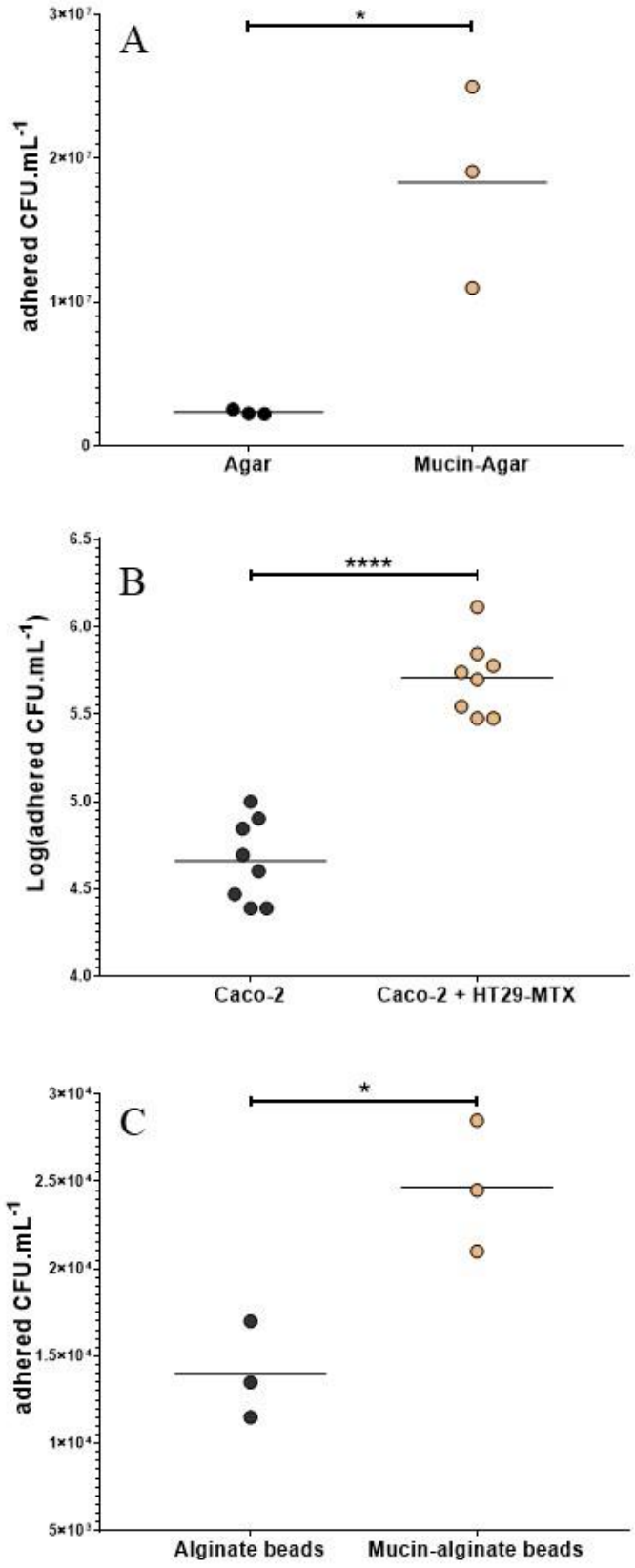
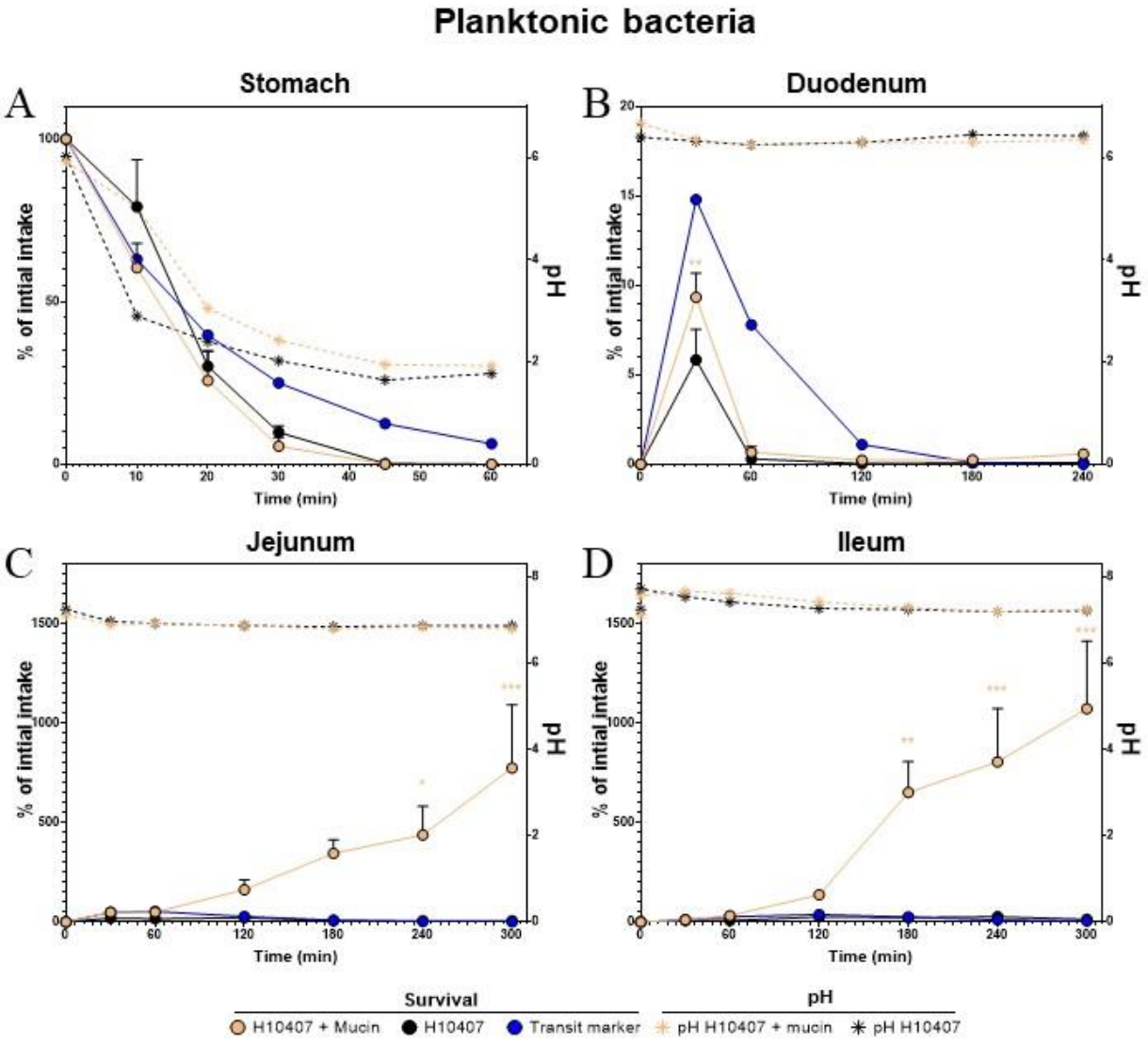


Figure 1.1. ETEC adhesion to the mucus compartment.
Adhesion of the ETEC strain H10407 to the mucus compartment was analyzed by three different *in vitro* assays.
A. ETEC bacteria (initial concentration: 10^7 CFU.ml^{-1}) adhered in plate assays to type II mucin-agar layer (orange dots) or agar without mucin used as a negative control (black dots).
B. ETEC adhesion to Caco-2/HT29-MTX co-culture model (orange dots) or Caco-2 cells only (black dots) after infection at MOI 100 for 3 h.
C. ETEC bacteria (initial concentration: 10^7 CFU.ml^{-1}) adhered to type III mucin-alginate beads (orange dots) or alginate without mucin used as a negative control (black dots), during static gastro-jejunal digestion procedure.
Figures represent the results of three independent experiments (in **B**, all technical replicates are represented). Means are indicated by black bars. *p*-values are provided by unpaired *t* test with Welch's correction (* $p<0.05$; **** $p<0.0001$).

4.3. Mucin allows ETEC to better cope with upper gastrointestinal stresses

To evaluate the impact of physicochemical parameters (pH, digestive secretions) of the upper human gastrointestinal tract on ETEC adhesion specificity to mucin, we first performed bead adhesion assays using a simple static *in vitro* digestion process (**Fig. 1.1.C**). After 180 min of digestion, ETEC strain H10407 showed a 1.8-fold higher adhesion on mucin type III containing alginate beads compared to control condition with alginate beads ($p < 0.05$). Then, the dynamic and multi-compartmental TIM-1 model was used to simulate more closely human physiological digestive conditions. In this model, we assessed the effect of mucin secretion on ETEC survival in the digestive lumen but also pathogen ability to adhere to mucin-alginate beads as a physical surface during transit. In the TIM-1 gastric compartment (**Fig. 1.2.A**), despite a delay in pH adjustment (certainly due to buffer effect), mucin addition did not significantly modify planktonic bacteria survival resulting in significant mortality independent of the tested condition. In the duodenum of TIM-1 (**Fig. 1.2.B**), mucin addition attenuated ETEC-associated death to stringent conditions of this compartment (in particular high bile salts concentration). Such phenomenon was significant at T30 min with survival percentage of 9.3% of the initial intake with mucin compared to 5.8% in the control condition ($p < 0.01$). At the end of digestion (240 min), ETEC even began to multiply, especially when mucin was present where 0.58% of bacterial intake is viable compared to 0.10% in the non-mucin condition and 0.01 % for the transit marker. In the jejunal and ileal compartments (**Fig. 1.2.C, 1.2.D**), mucin addition allowed a sharp increase in ETEC survival, especially from 120 min of digestion. In these two compartments, the areas under the curve in the mucin condition is significantly different at the 95% confidence interval from both non-mucin control and the theoretical marker conditions. The ultimate time points (240 and 300 min) are also significantly different between the mucin and non-mucin conditions according to Sidak multiple comparison test ($p < 0.05$). ETEC global survival in the ileal effluents reached 28.9- and 0.6-fold of the initial intake under the mucin and control conditions, respectively (data not shown). The number of adherent bacteria on mucin-alginate beads was also determined throughout *in vitro* digestions. The highest adhesion levels were found in the jejunal and ileal compartments, reaching nearly 2% of the initial bacterial intake at 300 min in the jejunum (**Fig. 1.2.E**). When compared to the total amount of bacteria in each digestive compartment (planktonic and adherent bacteria), the highest percentages of adhesion were observed in the stomach at 60 min (when pH reached 1.9) and the duodenum at 240 min, with 90% and 60% adhered bacteria, respectively (**Fig. 1.2.F**).


Planktonic bacteria
A
Stomach
B
Duodenum
C
Jejunum
D
Ileum
% of intial intake
pH
Time (min)
Survival
pH
H10407 + Mucin
H10407
Transit marker
pH H10407 + mucin
pH H10407

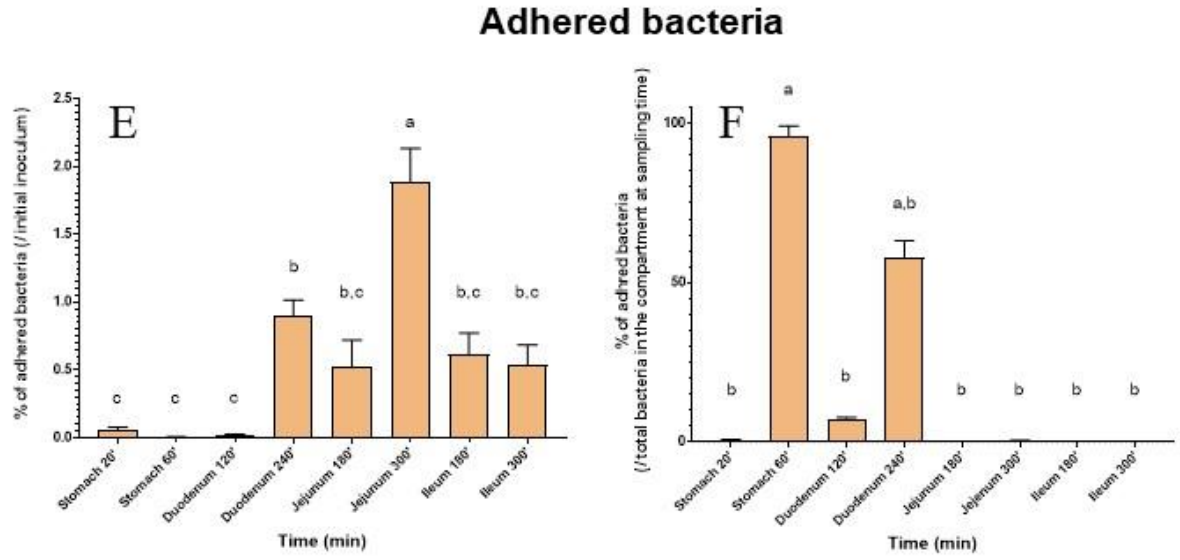

Adhered bacteria
E
% of adhered bacteria (/ initial inoculum)
F
% of adhered bacteria
(/ total bacteria in the compartment at sampling time)
Stomach 20'
Stomach 60'
Duodenum 120'
Duodenum 240'
Jejunum 180'
Jejunum 300'
Ileum 180'
Ileum 300'
Time (min)

Figure 1.2. Dynamics of ETEC survival and adhesion to mucin in the successive TIM-1 compartments.
(A-D) After introduction of a glass of ETEC-contaminated water (10^{10} CFU) in the TIM-1 model, the number of cells in the lumen ("planktonic" bacteria) of stomach (**A**), duodenum (**B**), jejunum (**C**) and ileum (**D**) compartments was determined by plate counting. Results are expressed as mean percentages ± SEM (n = 4) of initial intake. Bacterial survival kinetics with (orange dots) and without (black dots) mucin are compared with an inert and non-absorbable transit marker indicating 100% survival (blue dots). The evolution of pH in each compartment is also indicated with (orange star) or without mucin (black star). Indicated *p*-value correspond to times at which the survival in the mucin condition was found to be statistically different from the non-mucin condition according to Sidak multiple comparison tests (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).
(E, F) ETEC adhesion to mucin beads was also analyzed by sampling and plating at different time points in each compartment of TIM-1. Results are expressed as mean percentages ± SEM (n = 4) of initial intake (**E**) or of total bacteria (planktonic + adhered) in the compartment (**F**). Results that are no significantly different from each other according to Tukey's multi-comparison test are grouped under the same letter ($p < 0.05$)

4.4. Adhesion to mucin surface has a poor impact on ETEC virulence during gastrointestinal passage

To assess how ETEC adhesion to a mucus surface could affect bacterial virulence during gastrointestinal passage, we investigated the expression of 9 genes from planktonic (bacteria found in the digestive lumen) and adhered bacteria (on the mucin beads) in the TIM-1 model. In the gastric compartment, ETEC strain H10407 virulence gene expression splits in two profiles, with a global increase until 40 min into the digestion (up to 2.9- fold) and a return to baseline afterwards, except for *fimH* and *yghJ* (**Fig. 1.3.A**). The overexpression of *eltB* in beads-adhered bacteria at 20 min and *eatA* in planktonic bacteria between 10 and 20 min reached significance ($p < 0.05$). Globally, adhesion to mucin-beads had a minor impact on ETEC-associated virulence in the stomach (**Fig. 1.3.A**). No statistical difference had been observed except for *eatA*, since its expression is decreased at 20 min on mucin beads compared to luminal bacteria between 10 and 20 min ($p < 0.05$). In the ileal effluents, virulence gene expression was globally repressed all along the course of digestion, except for *eatA* and *yghJ*, the two ETEC mucinase genes, with around a 2-fold increase in expression (**Fig 1.3.B**). In particular, the up-regulation of *eatA* reached significance on mucin beads at both 180 and 300 min, with respectively 2.7- and 4.8-fold increases ($p < 0.05$). However, again, the effect of adhesion on mucin beads is subtle with only a significant 2.6-fold increase for *eatA* in adhered bacteria at 300 min when compared to the luminal ones ($p < 0.05$).

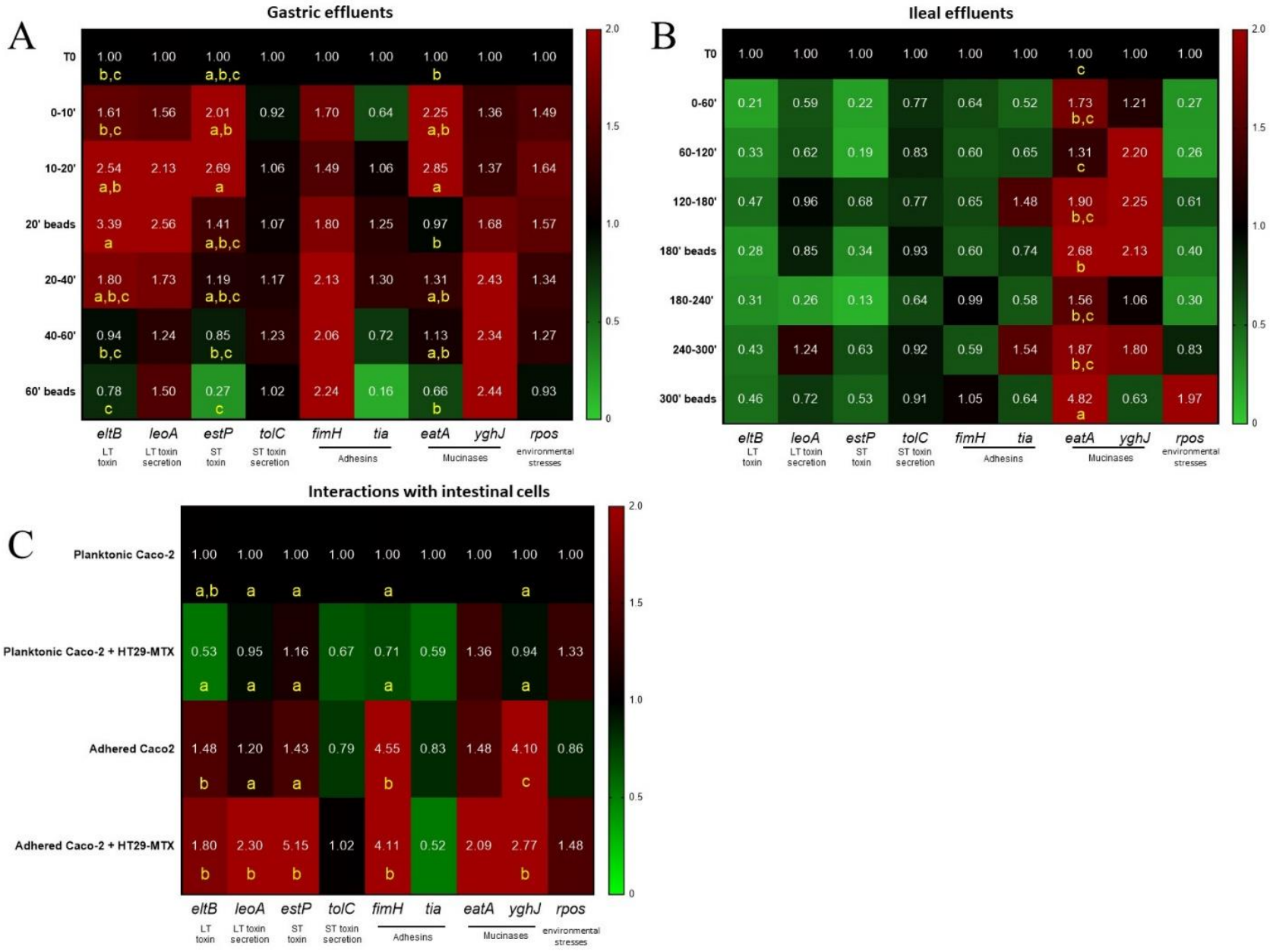

A
Gastric effluents
T0 1.00 b,c 1.00 1.00 a,b,c 1.00 1.00 1.00 b 1.00 1.00
0-10' 1.61 b,c 1.56 2.01 a,b 0.92 1.70 0.64 2.25 a,b 1.36 1.49
10-20' 2.54 a,b 2.13 2.69 a 1.06 1.49 1.06 2.85 a 1.37 1.64
20' beads 3.39 a 2.56 1.41 a,b,c 1.07 1.80 1.25 0.97 b 1.68 1.57
20-40' 1.80 a,b,c 1.73 1.19 a,b,c 1.17 2.13 1.30 1.31 a,b 2.43 1.34
40-60' 0.94 b,c 1.24 0.85 b,c 1.23 2.06 0.72 1.13 a,b 2.34 1.27
60' beads 0.78 c 1.50 0.27 c 1.02 2.24 0.16 0.66 b 2.44 0.93
eltB LT toxin
leoA LT toxin secretion
estP ST toxin
tolC ST toxin secretion
fimH tia Adhesins
eatA yghJ Mucinases
rpos environmental stresses
2.0 1.5 1.0 0.5 0
B
Ileal effluents
T0 1.00 1.00 1.00 1.00 1.00 1.00 1.00 c 1.00 1.00
0-60' 0.21 0.59 0.22 0.77 0.64 0.52 1.73 b,c 1.21 0.27
60-120' 0.33 0.62 0.19 0.83 0.60 0.65 1.31 c 2.20 0.26
120-180' 0.47 0.96 0.68 0.77 0.65 1.48 1.90 b,c 2.25 0.61
180' beads 0.28 0.85 0.34 0.93 0.60 0.74 2.68 b 2.13 0.40
180-240' 0.31 0.26 0.13 0.64 0.99 0.58 1.56 b,c 1.06 0.30
240-300' 0.43 1.24 0.63 0.92 0.59 1.54 1.87 b,c 1.80 0.83
300' beads 0.46 0.72 0.53 0.91 1.05 0.64 4.82 a 0.63 1.97
eltB LT toxin
leoA LT toxin secretion
estP ST toxin
tolC ST toxin secretion
fimH tia Adhesins
eatA yghJ Mucinases
rpos environmental stresses
2.0 1.5 1.0 0.5 0
C
Interactions with intestinal cells
Planktonic Caco-2 1.00 a,b 1.00 a 1.00 a 1.00 1.00 a 1.00 1.00 1.00 a 1.00
Planktonic Caco-2 + HT29-MTX 0.53 a 0.95 a 1.16 a 0.67 0.71 a 0.59 1.36 0.94 a 1.33
Adhered Caco2 1.48 b 1.20 a 1.43 a 0.79 4.55 b 0.83 1.48 4.10 c 0.86
Adhered Caco-2 + HT29-MTX 1.80 b 2.30 b 5.15 b 1.02 4.11 b 0.52 2.09 2.77 b 1.48
eltB LT toxin
leoA LT toxin secretion
estP ST toxin
tolC ST toxin secretion
fimH tia Adhesins
eatA yghJ Mucinases
rpos environmental stresses
2.0 1.5 1.0 0.5 0

Figure 1.3. Dynamics of virulence gene expression in planktonic and adhered ETEC bacteria during gastrointestinal transit and interactions with intestinal cell
ETEC virulence gene expression was analyzed by RT-qPCR in the gastric (**A**) and ileal (**B**) effluents of the TIM-1 model inoculated with 10^{10} CFU and in cellular experiments (MOI 100) involving Caco-2 cells cultivated with or without HT29-MTX mucus-secreting cells (**C**). Gene expression was analyzed over time in the TIM-1 on planktonic bacteria or bacteria adhered to mucin beads (**A-B**) or intestinal cells (**C**). Results were expressed and colored according to fold-change expression compared to ETEC gene expression in the glass of water used to inoculate the TIM-1 model (T0) (**A**, **B**) or planktonic bacteria upon Caco-2 cells (**C**). Assayed genes were *estP* (ST toxin), *eltB* (LT toxin), *leoA* (LT toxin output), *tolC* (ST toxin outpout), *tia* (adhesin), *fimH* (minor component of type I pilus), *yghJ* (mucinase), *eatA* (mucinase) and *rpos* (environmental stress response). Results that are significantly different from each other according to Tukey's multi-comparison are grouped under different yellow letters ($p < 0.05$).

4.5. Adhesion to mucus-secreting cells favors ETEC virulence gene expression

To decipher the role of the mucus compartment in ETEC virulence during host interactions, virulence gene expression was further measured on both planktonic and adhered bacteria during infection of Caco-2 or Caco-2/HT29-MTX cells. Whatever the cell model (mono- or co-culture), adhesion of ETEC strain H10407 was associated with a global increase in virulence gene expression (**Fig 1.3.C)**. When considering the monoculture model only, the expression of *fimH* (4.6 fold) and *YghJ* (4.1 fold) increased significantly with adhesion ($p < 0.05$). In the co-culture model, significance was reached with upregulation of *eltB*, *leoA*, *estP*, *fimH* and *YghJ* genes in adhered cells (3.7-, 2.4-, 4.4-, 5.8- and 3.0- fold, respectively, $p < 0.05$). When focusing on the adhered bacteria populations, with exception of *YghJ*, the expression of all virulence genes assayed was higher in the Caco-2/HT29-MTX model compared to the Caco-2 model alone. These increases reach significance for *leoA* and *estP* ($p < 0.05$) (**Fig. 1.3.C**). Inversely, when focusing on planktonic bacteria only, the expression for most virulence genes, except *estP* and *eatA*, tended to decrease with the co-culture when compared to the monoculture, yet without reaching significant differences (**Fig 1.3.C)**.

4.6. Mucus-secreting cells contribute to ETEC-induced inflammation

To assess if differences in adhesion and virulence gene expression associated with the mucus compartment would affect ETEC induced-inflammation, pro-inflammatory interleukin-8 (IL-8) was measured prior and following infection in cell assays (**Fig 1.4**). Bacterial infection was associated with a significant rise in IL-8 levels, but only for intracellular production ($p < 0.001$). Whatever the infection status (infected or non-infected cells), co-culture with mucin-

secreting cells condition led to a significant increase in IL-8 intracellular production (**Fig 1.4.B**) and secretion (**Fig 1.4.A**) compared to Caco-2 alone ($p < 0.001$). After a 3-hour infection period with ETEC strain H10407, intracellular and extracellular IL-8 levels significantly dropped by 1.8- and 3.7-fold respectively from monoculture to co-culture conditions ($p < 0.001$).

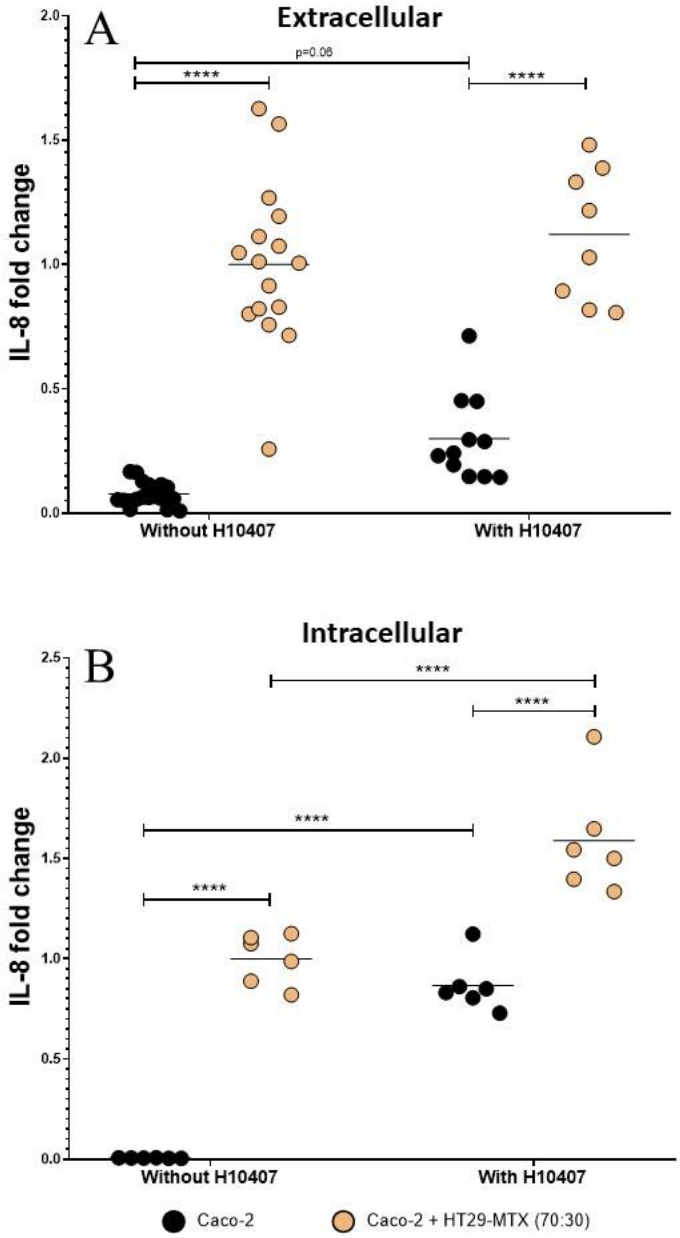


Figure 1.4. ETEC induction of Interleukin-8 production by mucin secreting or non-secreting intestinal cells.
Interleukin-8 (IL-8) extracellular secretion (**A**) and intracellular production (**B**) by ETEC-infected Caco-2 (black dots) or Caco-2/HT29-MTX (orange dots) cells were measured by an ELISA assay. Intestinal cells were infected for a 3 h period with 10^7 CFU.mL^{-1} (MOI 100) with ETEC strain H10407. Control experiments were performed without the bacteria. Results are expressed as fold changes compared to non-infected Caco-2/HT29-MTX cells. The data represents the replicates of at least 3 independent experiments with their means (black line). Statistical differences provided by Tukey's multiple comparisons test are indicated (**** $p<0.0001$).

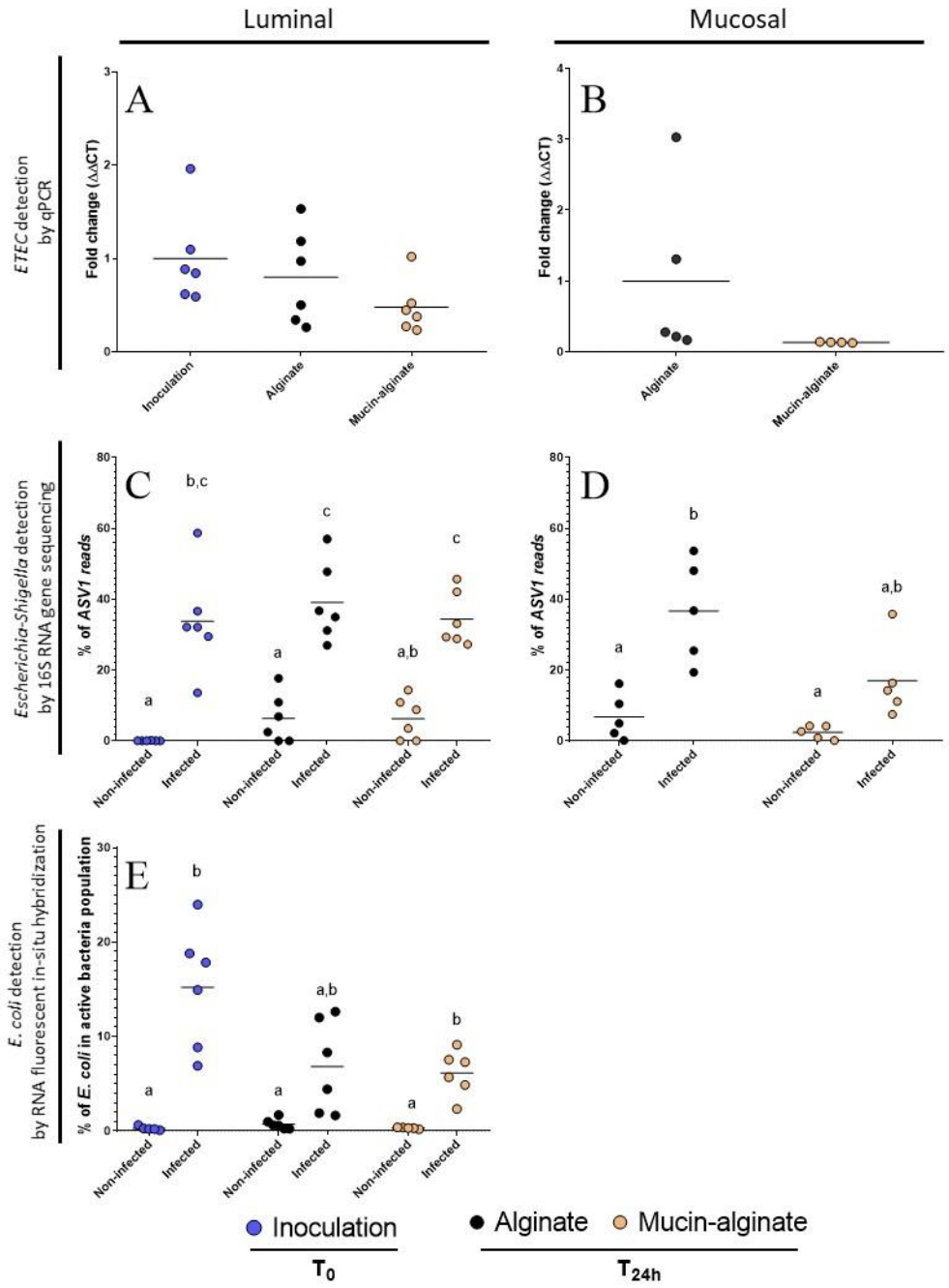


Figure 1.5. Impact of mucin on ETEC survival in *in vitro* fecal fermentation batches.
Penicillin bottles with mucin-alginate beads or alginate beads (as a control) were inoculated with feces from 6 healthy donors and challenged or not with pre-digested ETEC strain H10407 at 10^8 $CFUmL^{-1}$. Blue, black and orange dots represent individual biological replicates at the beginning of the experiment after ETEC inoculation (inoculation T0) or after 24 h fermentation with alginate (alginate T24) and mucin-alginate (mucin-alginate T24) beads, respectively. (**A, B**) qPCR detection of ETEC strain H10407 among total bacterial populations expressed as fold changes compared to inoculation T0 (luminal phase) or alginate condition (mucosal phase). (**C, D**) Percentages of ASV1 reads detected by 16S RNA gene amplicon sequencing in planktonic and adhered ETEC bacteria. ASV1 is the ASV with the highest reads abundance in all samples and its reads have been assigned to the *Escherichia*/*Shigella* genus and to *Escherichia albertii/boydii/coli/dysenteriae/fergusonii/flexneri/marmotae/sonnei* species. (**E**) Proportion of active *E. coli* in the total bacterial populations as detected by RNA fluorescent *in situ* hybridization. Black bars represent the mean of data (n=6). Results that are no significantly different from each other according to Tukey's multi-comparison are grouped under the same letter ($p < 0.05$).
ASV: amplicon sequence variant

4.7. Mucin surface does not favor ETEC colonisation in a complex microbial background

To investigate how mucus physical surface may impact ETEC interactions with human gut microbiota, batch experiments inoculated with human fecal microbiota were conducted by addition of mucin-alginate beads or alginate beads as a control. ETEC strain H10407 as well as *E. coli* populations were investigated using high-throughput analyses. As expected, after inoculation, the *E. coli* population became predominant in the luminal phase of infected bottles and represented on average 34% of the detected bacterial ASV reads by *16S rRNA* gene sequencing (**Fig. 1.5.C**) and 15% of the active bacteria assayed by RNA fluorescent *in situ* hybridization (**Fig. 1.5.E**). According to quantitative PCR and *16S rRNA* gene sequencing data, under both conditions (alginate and mucin-alginate beads), ETEC strain H10407 and *Escherichia-Shigella* population did not significantly decrease in the luminal phase of ETEC-infected bottles during the timecourse of the experiment (**Fig. 1.5.A** and **1.5.C**), while RNA flow-FISH showed a non-significant 2-fold decrease of the *E. coli* active population (**Fig. 1.5.E**). Whatever the molecular analytical technique used, mucin did not impact ETEC survival nor *Escherichia* abundance after a 24-hour fermentation period (**Fig. 1.5.A, 1.5.C** and **1.5.E**). Even if no significance was reached due to important donor variation, results are more striking in the mucosal compartment. According to qPCR results at T24h, ETEC level tended to be on average 7.4-fold lower in the mucin-alginate beads compared to alginate conditions (**Fig. 1.5.B**). Accordingly, the proportion of *Escherichia-Shigella* population tended to be 2.2-fold lower with mucin according to *16S rRNA* gene sequencing analyses (**Fig. 1.5.D**).

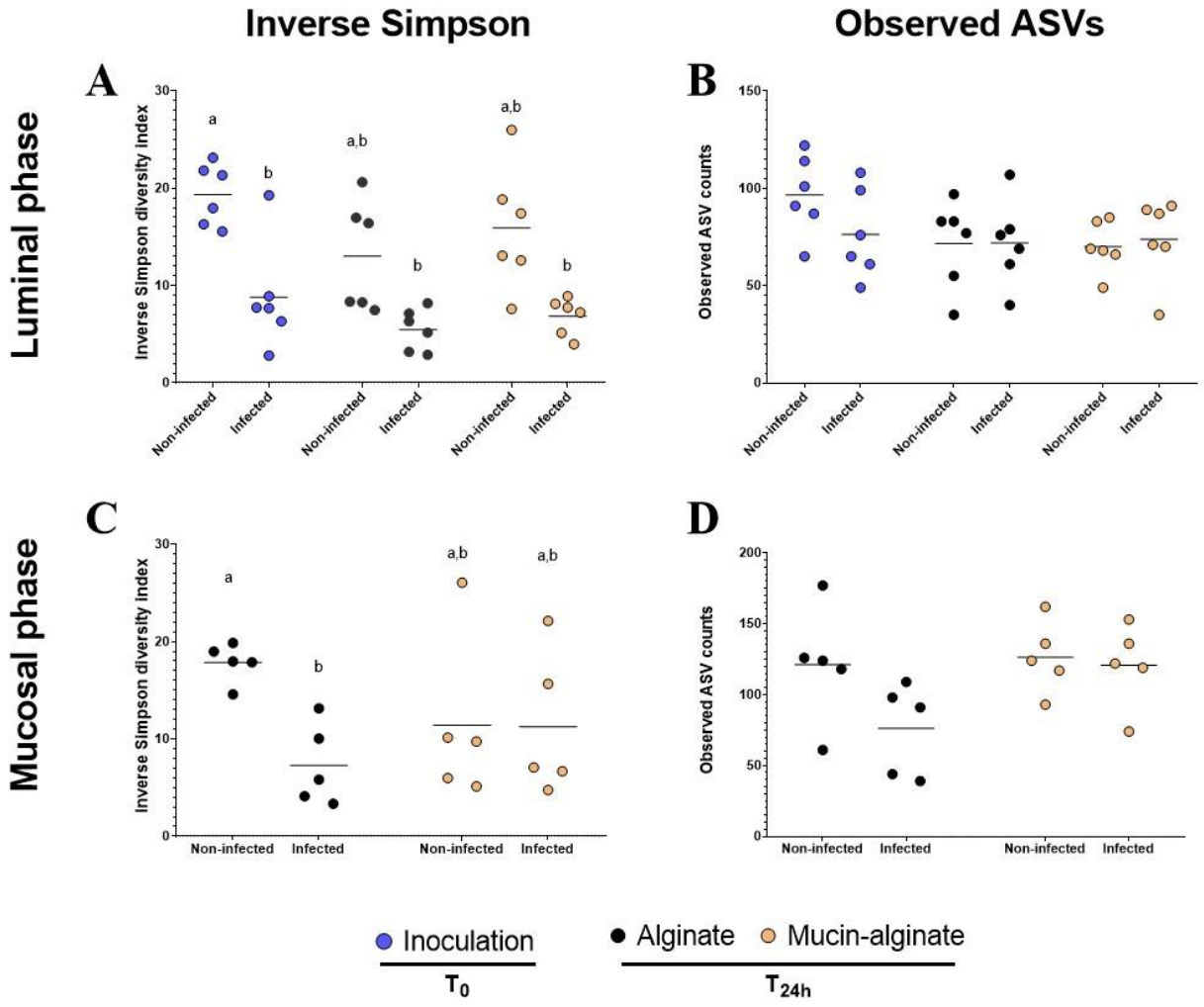


Figure 1.6. Impact of mucin on ETEC modulation of microbial communities as determined by α-diversity.

Batch experiments were performed using feces from 6 healthy donors, challenged or not with ETEC strain H10407, with mucin-alginate beads or alginate beads (as a control). The graphs represent the variation of the microbiota α-diversity at the ASV level at inoculation (T0) and after 24h (T24h) between bottles including mucin-alginate beads and alginate beads in luminal (**A**-**B**) and mucosal compartments (**C**-**D**). The parameters analyzed included species richness represented by Observed ASV (**B, D**) and evenness represented by Inverse Simpson index (**A, C**). Blue, black and orange dots represent individual biological replicates at the beginning of the experiment (T0) or after 24 hours (T24h) in the alginate and mucin-alginate beads conditions, respectively, while black bars represent the mean. Results that are no significantly different from each other according to Tukey's multi-comparison are grouped under the same letter ($p < 0.05$).

ASV: amplicon sequence variant

4.8. Mucin-associated microbiota is particularly affected by ETEC colonisation

To further explore how mucus surface would modulate ETEC impact on gut microbiota composition, we performed Illumina *16S rRNA* gene amplicon sequencing and bacterial community analysis. Concerning α-diversity, ETEC inoculation tended to reduce bacterial species evenness in the luminal compartment whatever the tested conditions (**Fig. 1.6**). Notably, the Inverse Simpson index tends to be reduced following ETEC challenge by 2.2-, 2.4- and 2.3-fold at inoculation (T0) and 24 hours post-infection with alginate and mucin-alginate beads, respectively ($p < 0.05$) (**Fig. 1.6.A**). Whatever the considered index (Shannon, Simpson, Inverse Simpson or observed ASV counts), addition of mucin did not impact the α-diversity (**Fig. 1.6.A** and **1.6.B**, **Suppl. Fig. 1.2**). Results are more striking in the mucosal phase. In the control condition (alginate beads), ETEC inoculation reduced species evenness (2.4-fold decrease for the Inverse Simpson index, $p < 0.05$, **Fig. 1.6.C**) and tended to reduce richness (1.6-fold decrease of observed ASV, **Fig. 1.6.D**). Interestingly, these decreases were not observed with mucin-alginate beads (**Fig. 1.6.C** and **1.6.D**). Regarding β-diversity, NMDS analysis showed that stool donor is the predominant explanatory variable for dissimilarities in gut microbiota composition, both in the luminal and mucosal compartments (**Fig. 1.7.A**). Still, permANOVA analysis conducted on the samples at T24h and excluding ASV1 (attributed to *Escherichia/Shigella*), reported that 'infection' and 'mucin' significantly accounted for 10.4% ($p < 0.001$) and 3.8% ($p < 0.05$, 999 permutations) of the dissimilarities, respectively (**Suppl. Fig. 1.3**). To go further, db-RDA was performed using mucin condition and ETEC challenge ('infection') as explanatory variables. ASV1 (attributed to the *Escherichia*/*Shigella* genus) was also excluded from the analysis to efficiently capture the impact of different conditions towards the microbiota community (**Fig. 1.7.B**). The db-RDA was able to cluster more efficiently samples from mucin condition *versus* alginate condition in the mucosal compartment (**Fig. 1.7.B, Suppl. Fig 1.3**), indicating that the effect of mucin on gut microbiota composition is greater on the mucus-associated microbiota than the luminal one. Non-infected mucin beads display a specific microbiota that was particularly enriched in *Clostridium*, *Roseburia* and *Lactobacillus* ASV (**Fig .1.7.C** and **1.7.D, Suppl. Fig. 1.4**), even if *Lactobacillus* colonisation appear to be donor-dependent (**Suppl. Fig. 1.5 and 1.6**). ETEC infection tended to specifically influence this mucin-associated microbiota, with decreases in *Clostridium*, *Lactobacillus* and *Bifidobacterium* and increase in *Roseburia* ASV (**Fig. 1.7.C, 1.7.D** and **Suppl. Fig 1.5**). In this

sense, the mucosal phase of mucin-alginate beads was the only condition for which ASV were found to be significantly modulated by ETEC infection (**Suppl. Fig 1.7**).

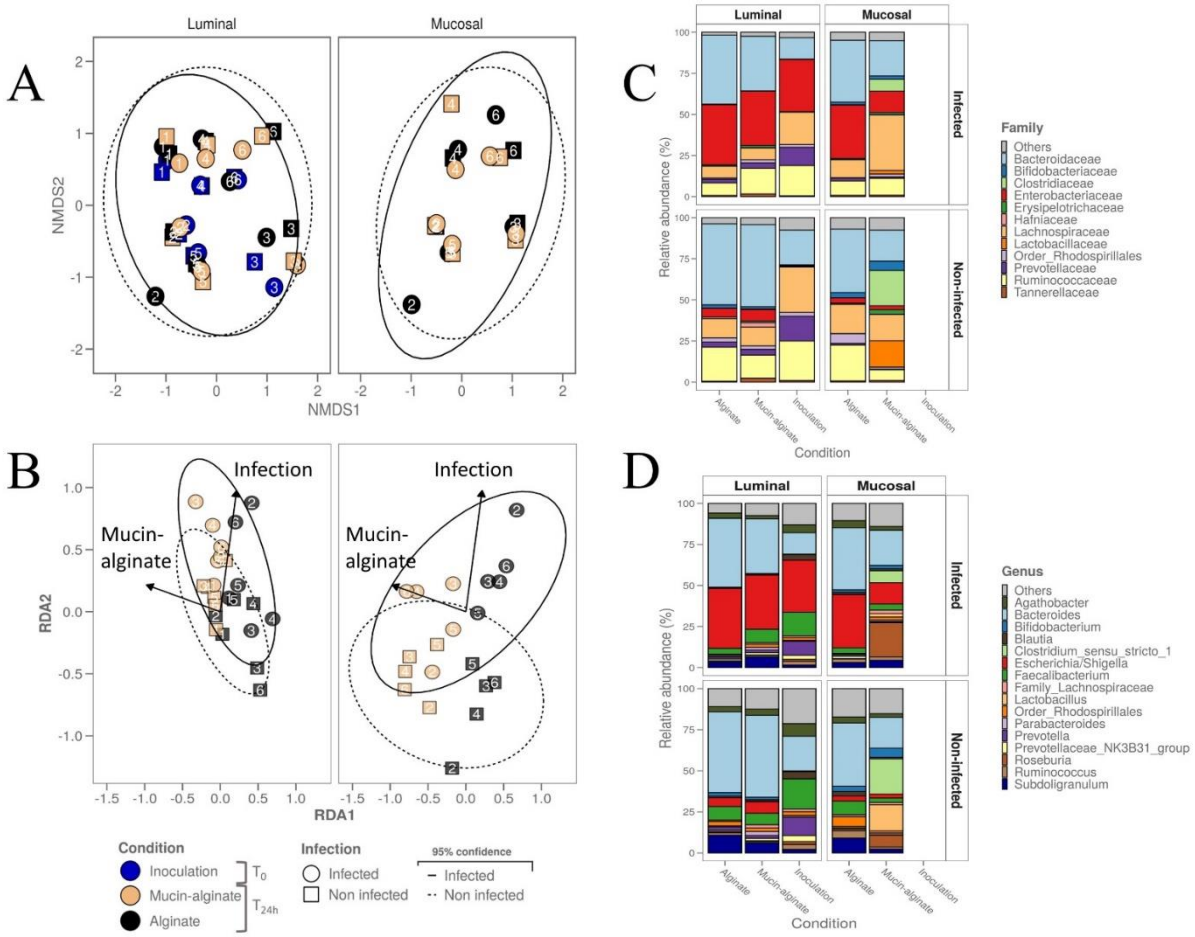


Figure 1.7. Impact of mucin on ETEC modulation of microbial communities as determined by β-diversity.
Batch experiments were performed using feces from 6 healthy donors, challenged or not with ETEC strain H10407, with mucin-alginate beads or alginate beads (as a control). (**A**) Nonparametric multidimensional scaling (NMDS) and (**B**) Distance-based redundancy analysis (db-RDA) two-dimension plot visualization report the microbial community β-diversity at the ASV level, as determined by 16S rRNA gene amplicon sequencing. For db-RDA, ASV1 (attributed to the *Escherichia*/*Shigella* genus) was excluded from the relative ASV table and "infection" and "mucin" were provided as sole environmental variables (binary) and plotted as vectors (arrows). Blue, black and orange dots represent individual biological replicates (donor numbers are indicated) at the beginning of the experiment (inoculation, T0) or after 24 hours of fermentation (T24h) with alginate and mucin-alginate beads, respectively. Samples are represented by dot shape and square shape for the infected and non-infected conditions, respectively. The 95% confidence ellipse area is also indicated in continuous line for the infected condition and in dotted line for the non-infected condition. Cumulative bar plots of the relative microbial community composition at the family (**C**) and genus (**D**) levels. The area graphs show the relative abundance of the 12 most abundant families and 16 most abundant genera with all six different donors confounded.

4.9. Mucin has a moderated impact on gut microbial activities

In a last step, the effect of mucin on gut microbial activity during ETEC infection was assessed by following various indicators such as SCFA and gas production, pH acidification and gas pressure. Compared to control alginate beads, mucin-alginate led in non-infected condition to a significant increase in the amount of SCFA, as reported by the levels of acetic (1.4-fold increase) and propionic (1.6-fold increase) acids ($p < 0.05$). Under ETEC-infected conditions, mucin addition led also to a significant increase ($p < 0.05$) in acetic, propionic, and butyric acids by 1.5, 1.8, and 2.4-fold, respectively (**Fig 1.8.A**). Regarding pH acidification, addition of mucin-alginate beads resulted in a lower pH at T24h of fermentation compared to the control beads, in both non-infected and ETEC-infected bottles ($p < 0.05$). Surprisingly, the infection by ETEC tended ($p=0.08$) to limit the pH decrease at T24h independent of mucin presence (**Fig 1.8.B**). Interestingly, ETEC infection induced a significant increase in gas pressure with control alginate beads ($p < 0.05$). Gas pressure was also influenced by mucin addition, with a non-significant 10% increase in both infected and non-infected conditions (**Fig 1.8.C**). Regarding gas production (**Fig 1.8.D**), ETEC infection induced significant changes when mucin was added, as shown with increasing CO_2 percentage by 2.1% while decreasing N_2 level by 3.4% ($p < 0.05$). Mucin presence also influenced headspace gas profiles, by increasing both CO_2 and H_2 and decreasing N_2. Yet, a mucin-dependent significant effect was not reached due to high donor variabilities. To further investigate how microbial metabolite production could be associated to changes in microbial community structure, 'SCFA' and 'pH' were included as explanatory variables in a db-RDA analysis performed on the whole ASV table of the luminal samples. Samples were then clustered according to 'infection' and 'mucin', proving that these variables accounted for some of the differences in β-diversity taxonomy structure between tested conditions (**Fig 1.8.E**). Supporting the data presented in this section, pH increase correlated with the taxonomy structure of the alginate beads and infected samples, while SCFA production only correlated with mucin bead taxonomy structure.

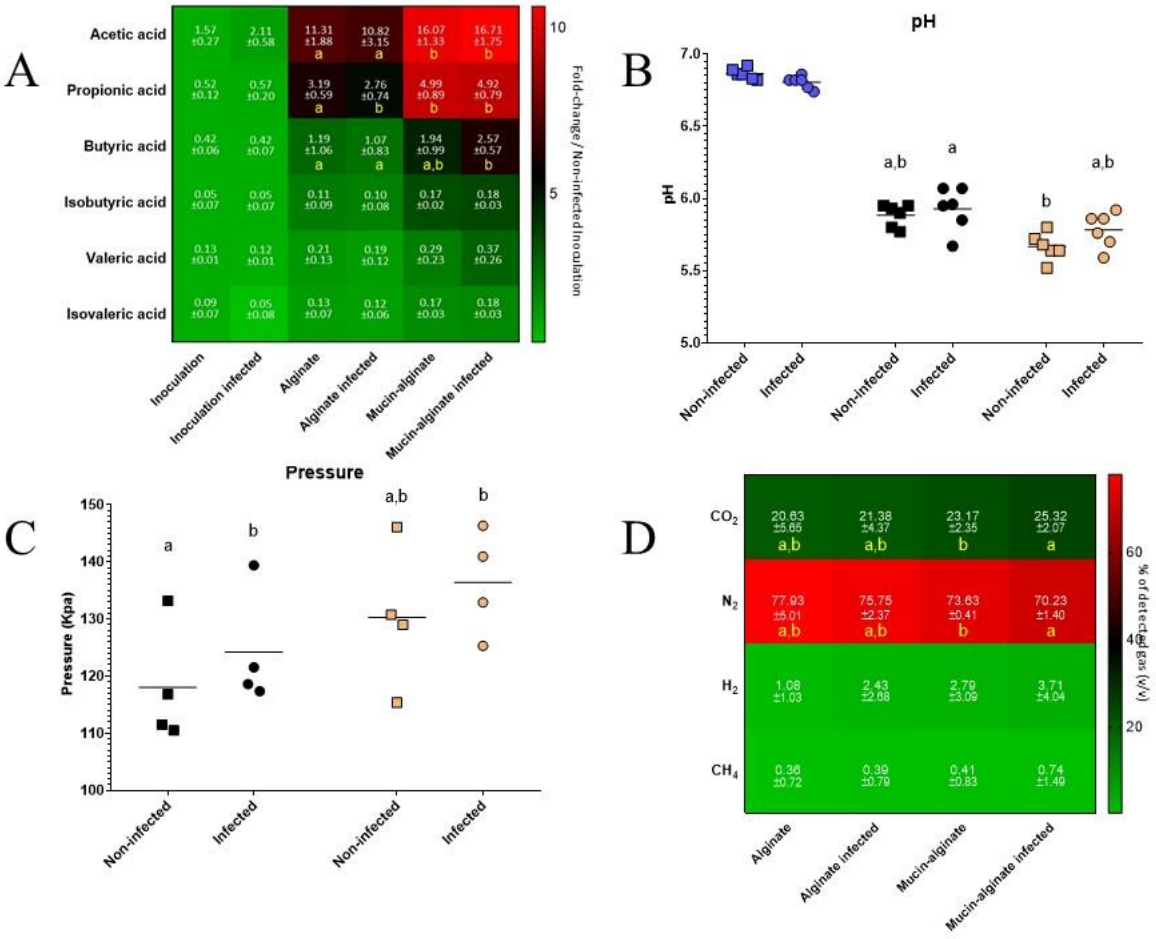

A
Acetic acid
Propionic acid
Butyric acid
Isobutyric acid
Valeric acid
Isovaleric acid
Inoculation
Inoculation infected
Alginate
Alginate infected
Mucin-alginate
Mucin-alginate infected
Fold-change / Non-infected Inoculation
B
pH
Non-infected
Infected
C
Pressure
Pressure (Kpa)
D
CO2
N2
H2
CH4
% of detected gas (V/V)
E
RDA1
RDA2
pH
valeric.acid
acetic.acid
isobutyric.acid
propionic.acid
butyric.acid
isovaleric.acid
Condition
Inoculation
Mucin-alginate
Alginate
T0
T24h
Infection
Infected
Non infected
95% confidence

Figure 1.8. Impact of mucin on ETEC modulation of gut microbial activity.
The impact of ETEC inoculation (infected *versus* non-infected) and mucin (mucin-alginate versus alginate beads) on gut microbiota activity in batch fermentations were assayed by the measurement of SCFA production (**A**), pH acidification (**B**), gas pressure (**C**) and gas composition (**D**). Experiments were performed using fecal samples from 6 healthy donors. Blue, black and orange dots represent samples collected at the beginning of the experiment (inoculation, T0) or after 24 h fermentation (T24h) with alginate and mucin-alginate beads, respectively. (**A**) SCFA production in the luminal phase was analyzed by liquid chromatography. Results were expressed in mmol (n=6) and colored according to fold change compared to the control condition (non-infected, T0). (**B**) pH of the fermentation medium was recorded over-time at T0 and T24h and biological replicates are represented as dots with their means (black line). (**C**) Gas pressure was measured at T24h and biological replicates are represented as dots with their means (black line). **(D)** Gas composition was determined by gas chromatography at T24h. Results were expressed as mean percentages ± standard deviation (n=6) and accordingly colored. (**E**) The 2 dimensions-plot reports the β-diversity structure of the whole microbial community taxonomy at the ASV level in the luminal phase according to db-RDA according to metabolites variables (namely SCFA and pH). Individual samples are represented by dot and square shapes for the infected and non-infected conditions, respectively. The 95% confidence ellipse zone is also indicated in continuous line for the infected condition and in dotted line for the non-infected condition. The donor number is indicated for each sample.
Results that are no significantly different from each other according to Tukey's multi-comparison are grouped under the same letter ($p < 0.05$).

5. Discussion

The particular relationship between enteric pathogens and the mucus layer, which represents both a physical barrier and an anchorage surface to adhere to, is currently underexplored. Using complementary *in vitro* models of the human lumen and cellular digestive environment, we showed for the first time the key role of the mucus compartment in ETEC H10407 survival, adhesion, virulence and interactions with gut microbiome.

5.1. ETEC survival throughout the gastrointestinal tract

Low gastric pH is the first challenge ETEC faces upon ingestion (Smith 2003). In this environment, mucin addition did not seem to reduce pathogen mortality overtime in the luminal comparment, despite its obvious buffering effect on gastric pH. However, such phenomenon is associated with a lower fraction of ETEC exposed to lethal pH values in the stomach, resulting in more viable bacteria subsequently reaching subsequently the duodenum (significant at T60 min). The next hurdle during gastrointestinal passage is the released of bile salts in the duodenum, with a well-known deleterious effect on enteric pathogen survival by disrupting bacterial membranes(Thanassi, Cheng and Nikaido 1997; Begley, Gahan and Hill 2005). In

accordance with previous studies performed in the TIM-1 on various *E. coli* pathotypes (Etienne-Mesmin *et al.* 2011; Miszczycha *et al.* 2014; Roussel *et al.* 2020a), ETEC H10407 viability dropped in the duodenal compartment. Nevertheless, mucin addition led to a slight increase in viable bacteria from 120 min to the end of the duodenal digestion. Then in the distal part small intestine, less stringent conditions (i.e. pH close to neutrality, lower bile salts concentrations due to re-absorption and/or longer residence times) allowed ETEC to grow, as previously shown for other *E. coli* strains (Gänzle *et al.* 1999; Miszczycha *et al.* 2014; Roussel *et al.* 2020a). This was particularly striking with mucin, with an exponential increase in cell concentration in both the jejunal and ileal compartments. Such growth can certainly be explained by the presence of nutrients brought by mucin and supported by the capacity of ETEC strain H10407 to grow on minimal culture medium supplemented with type III mucin. It is likely that release of mucin-derived sugars could represent an important reservoir of nutrients that promotes the growth of ETEC as previously shown *in vitro* for other pathotypes (Conway and Cohen 2015; Le Bihan *et al.* 2017). Lastly, ETEC survival was evaluated during short-term *in vitro* batch incubations as a simple model of human colonic conditions. Even if the pathogen action site is generally considered to be the distal part of the small intestine (Al-Majali *et al.* 2000, 2007; Allen, Randolph and Fleckenstein 2006; Al-Majali and Khalifeh 2010; Gonzales *et al.* 2013; Rodea *et al.* 2017), shedding after infection remains particularly important (Chakraborty *et al.* 2018a; Talaat *et al.* 2020b) and studying survival in an environment where the gut microbiota prevails in high number is undoubtedly relevant. Some authors already performed fermentation experiments with human ETEC strains in *in vitro* models including a mucus compartment (mucin-agar microcosms), both in batch experiments, yet with a poor microbial background simulating “dysbiotic” condition (Moens *et al.* 2019), and in the SHIME model (Roussel *et al.* 2020a). Nevertheless, these authors did not specifically address the impact of the mucosal compartment on ETEC survival by comparison to a proper control condition. Here, we report that ETEC and/or *E. coli* are able to maintain their relative presence during 24h in normally-inoculated batch experiments (i.e normal microbial background) and that the replacement of control condition by mucin-beads did not significantly impact ETEC survival in the luminal compartment. We argue that mucin-derived substrates released in the luminal phase are negligible compared to the nutrients supplied by the colonic medium and therefore have less impact on these parameters, as supported by microbiota composition results. It could be hypothesized that mucin has a minor impact on competition between endogenous microbiota and enteric pathogen (Pacheco and Sperandio 2015; Berkhout, Plugge and Belzer 2021; Huus *et al.* 2021) .

5.2. ETEC adhesion on the mucus compartment

Besides survival in the luminal environment, adhesion to mucus as a physical surface is a next challenge for pathogens to colonize the gastrointestinal tract. The high percentages of ETEC-associated bacteria to mucin-alginate beads in the TIM-1 stomach and duodenum at the end of digestion indicated that the presence of a mucus surface constitutes a significant protective micro-niche enabling longer survival under stringent conditions (e.g. pH, bile acids), with specific physicochemical-parameters different from the lumen (Daniel, Lécuyer and Chassaing 2021)**.** *In vivo*, gastric mucus is already well-known to harbor a pH gradient protecting the epithelium from the acidic pH (Lewis, Keener and Fogelson 2017). It could be envisioned that gastric mucin polymers have the ability to sequestrate proton (Schreiber and Scheid 1997). *Helicobacter pylori* has previously been described to benefit from this mucus layer shelter to maintain its gastric presence (Schreiber *et al.* 2004; Ansari and Yamaoka 2017). The present study suggests for the first time that this concept might be extended to the survival of pathogens that display a more distal tissue tropism in the upper gut. Lastly, in colonic batch experiments, we reported that ETEC gene copy numbers tended to be lower on mucin-alginate beads compared to control beads, particularly in the presence of certain individual donor microbiota. Thus, in a complex microbial background, specific colonisation of mucin beads by resident microbiota could certainly protect from ETEC engraftment.

As access to the mucin surface was shown to be meaningful for the pathogen survival during gastrointestinal transit in the TIM-1 system, specificity of ETEC adhesion to mucus compartment was therefore investigated by using different *in vitro* assays including control conditions without mucin. A significantly higher adhesion of ETEC H10407 was shown on mucin-alginate compared to alginate beads after a simple gastro-intestinal digestion procedure, as well as an increased adhesion to mucus-secreting cells compared to non-secreting ones. Our results are in accordance with previous studies showing that i*n vitro* adherence of *Salmonella enterica* serovar Typhimurium and EHEC was higher on high-mucus producing cells (e.g. HT29-MTX or LS174T) than in non- or low-mucus producing cells (e.g. Caco-2 or HT29) (Gagnon *et al.* 2013; Hews *et al.* 2017). All together these data suggest that *Enterobacteriaceae* pathogens are well adapted to the intestinal mucus barrier. Such idea was strengthened by a recent study showing that Enteroaggregative *E. coli* (EAEC) and EHEC specifically adhere to mucus droplets in human enteroids (Rajan *et al.* 2020). Of note, Kerneis and colleagues showed that ETEC H10407 binding on HT29-MTX cells did not necessarily co-localize with mucus,

suggesting that strain affinity could also be due to recognition of HT29-MTX surface receptors (Kerneis *et al.* 1994).

5.3. ETEC virulence gene expression

To achieve its infectious cycle, ETEC has to efficiently express a large panel of virulence genes, especially those related to toxin secretion. In simple broth media, mucin has already shown to influence virulence and motility of pathogens such as *Campylobacter jejuni* and EHEC (Tu, McGuckin and Mendz 2008; Kim *et al.* 2012b) and concerning ETEC, to support CFA/I and CS1/CS3 CF expression but to decrease LT toxin secretion (Haines *et al.* 2015). By incorporating mucin beads in the TIM-1 model, we were able to investigate the impact of adhesion to a physical mucosal surface on ETEC virulence expression in a relevant model of the human upper gut. Our results showed that the impact of mucin-beads adhesion on ETEC virulence was quite subtle, indicating that adherence to a mucin surface has a minor influence on ETEC H10407 virulence regulation than changes in digestive physicochemical parameters during gastrointestinal passage, as previously shown by Roussel et al (Roussel *et al.* 2020a). This modest effect of adhesion can be due to the presence of mucin in the luminal phase of the TIM-1, added to accurately simulate mucus constant shedding. Using cellular culture approaches, we further investigated how host-bacteria interactions modulate ETEC virulence. Cell adhesion was strongly associated to virulence gene expression, even more with mucus-secreting cells. Our results are in accordance with a study from Kansal and colleagues showing that cell contact enhanced transcription of LT and Type 1 pilus encoding genes in ETEC H10407 (Kansal *et al.* 2013). It is noteworthy that the same authors found that another ETEC strain decreased its virulence with cellular proximity, indicating such data are strain-specific (Kansal *et al.* 2013). The greater impact of mucus-secreting cells on ETEC virulence could be attributed to specific glycoproteins secreted by HT29-MTX. Indeed, porcine gastric mucin, mainly composed of MUC5AC and MUC5B mucin (Haines *et al.* 2015; Padra *et al.* 2018), which are also among the main mucins secreted by HT29-MTX cells (Wikman-Larhed and Artursson 1995; Smirnova *et al.* 2001; Huang *et al.* 2019), positively influences CF expression in various ETEC strains including H10407 (Haines *et al.* 2015). We can also envision that the co-culture model could induce more stressful conditions for the bacteria as evidenced by the highest induction of *rpos* gene expression, an environmental stress regulator.

5.4. ETEC-induced inflammation

We next considered if addition of mucus-secreting cells, for which ETEC H10407 has an obvious adhesion affinity, could result in changes in pro-inflammatory IL-8 cytokine production. Despite an increased basal production in the co-culture model, we were able to measure significant IL-8 induction by ETEC infection. This is in line with previous studies showing that ETEC toxins and YghJ mucinase induce cellular inflammation (Ma 2016; Wang *et al.* 2019a; Motyka *et al.* 2021), as depicted in our study with all the associated genes being overexpressed by adherent bacteria in the co-culture model. These results also suggest that the mucus layer (or rather mucus patches phenotype) (Dorier *et al.* 2017; García-Rodríguez *et al.* 2018; Gillois *et al.* 2021) in HT29-MTX cells does not sufficiently decoy bacteria from epithelial close contact to inhibit IL-8 induction.

5.5. ETEC impact on microbiota composition

Several studies have already demonstrated the impact of human ETEC strains on fecal microbiota composition *in vivo* (David *et al.* 2015; Youmans *et al.* 2015; Pop *et al.* 2016: 2016). As gut microbiota alterations could modulate infections outcomes (Ghosh *et al.* 2011; Hopkins and Frankel 2021), it is crucial to better decipher the impact of such changes on the infection process. To our knowledge, this work is the first one investigating the combined modulatory effects from the presence of a mucus compartment and ETEC presence towards simulated human gut microbiota. Our finding of ETEC infection to be associated with a bloom of *Escherichia*/*Shigella* (most probably ETEC), a decrease in microbiota evenness, and a modest impact on β-diversity is a confirmation of previous observations in humans (Youmans *et al.* 2015; Pop *et al.* 2016: 2016; Walters *et al.* 2020). Second, regarding the effect of mucin, addition of mucin-alginate beads had a minor impact on α and β-diversities indices in batch colonic incubation in the luminal compartment. However, we report the colonisation of mucin beads by a specific microbiota characterized by increase in *Clostridium* and *Bacillus* species, as previously shown in the mucosal compartment of the SHIME model (Van den Abbeele *et al.* 2012, 2013). This specific microbiota might be responsible for the inhibition of the observed ETEC colonisation and the maintenance of α-diversity on mucin beads. We also evidenced that specific mucus-associated microbiota is particularly impacted by ETEC inoculation. Given the known health-related properties of some impacted phylogroups (e.g. *Clostridium, Lactobacillus* and *Bifidobacterium*) (Arboleya *et al.* 2016; Heeney, Gareau and Marco 2018; Stoeva *et al.*

2021) and the clear association between mucosal microbiota in health and diseases (Daniel, Lécuyer and Chassaing 2021), the impact of ETEC challenge on the mucosal microbiota would deserve further investigations. It remains to be investigated whether the colonic mucus layer together with a complex microbiota would contribute to enhance pathogen susceptibility or how this effect might vary between individual microbial communities in large cohort.

5.6. ETEC impact on microbiota activity

To date, few studies have focused on ETEC impact on gut microbiota-derived metabolites. In colonic batch experiments, Moens and colleagues reported a decrease in SCFA with ETEC infection (Moens *et al.* 2019), while Roussel et al. showed an increase in propionic acid production in the M-SHIME model (Roussel *et al.* 2020a). Here, we reported that ETEC infection increases gas production (increased pressure and CO_2 level) but also limits the pH drop associated with fermentation activity. We argue that this feature could be due to *E. coli* acid resistance systems which notably consume H^+ to produce H_2O, H_2 and CO_2 (Kanjee and Houry 2013). Unsurprisingly, we reported that the use of mucin beads, rich in nutritive substrates, resulted in increased production of fermentation end products. More interestingly, mucin beads seemed to boost ETEC impact on microbiota activity (higher level of CO_2). This could be due either from higher requirement for acid resistance to counterbalance fermentation acidification or from ETEC mucinases activity leading to higher availability of substrates for commensal bacteria.

6. Conclusion

Using complementary *in vitro* models of the digestive lumen and host intestinal cells, our integrated approach covering the upper and lower gastrointestinal tract sheds more light on the dynamic interactions between ETEC H10407 reference strain and the mucus compartment in a human-related context. The mucus niche is usually and accurately seen as an efficient barrier against pathogenic invaders (Bergstrom *et al.* 2010; Zhang *et al.* 2021). In that sense, we showed that presence of a mucus-specific microbiota might be an effective mean against ETEC mucosal colonisation in human simulated colonic conditions. In this work, we also reported some ETEC pathophysiological features where mucus presence does not necessarily represent an advantage for the host (**Fig 1.9**). Taken together, our findings propose that the presence of a mucus niche in the simulated upper gastrointestinal conditions favors ETEC survival in the digestive lumen and its adhesion to physical surface thereby increasing the

pathogen's resilience against the harsh conditions of gastrointestinal passage. Adhesion to mucus-secreting intestinal cells also led to a sharp increase in virulence gene expression. Thus, we can argue that ETEC strains may have adapted to this mucus barrier and to some extent benefit from it. Further complementary *in vivo* studies in animal models are needed to confirm these promising *in vitro* results, opening avenues to better understand the role of mucus in ETEC physiopathology and should be paramount to develop new strategies to fight against these infections in humans.

Author Contributions

TS designed the experimental work, performed the experiments and drafted the manuscript. CD, SC, OU and LEM performed the experiments. AS, OU, FVH, CR and LEM supervised the experimental work. TS and JVL performed the analysis and interpretation of the Illumina data. TVW, LEM and SBD conceived the study and supervised the project. TVW, LEM and SBD revised the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest

T.V.d.W. is advising ProDigest as a science officer and is a member of the scientific advisory board of MRM Health. S.B.-D. is advising NexBiome as a science officer. The other authors declare no conflict of interest.

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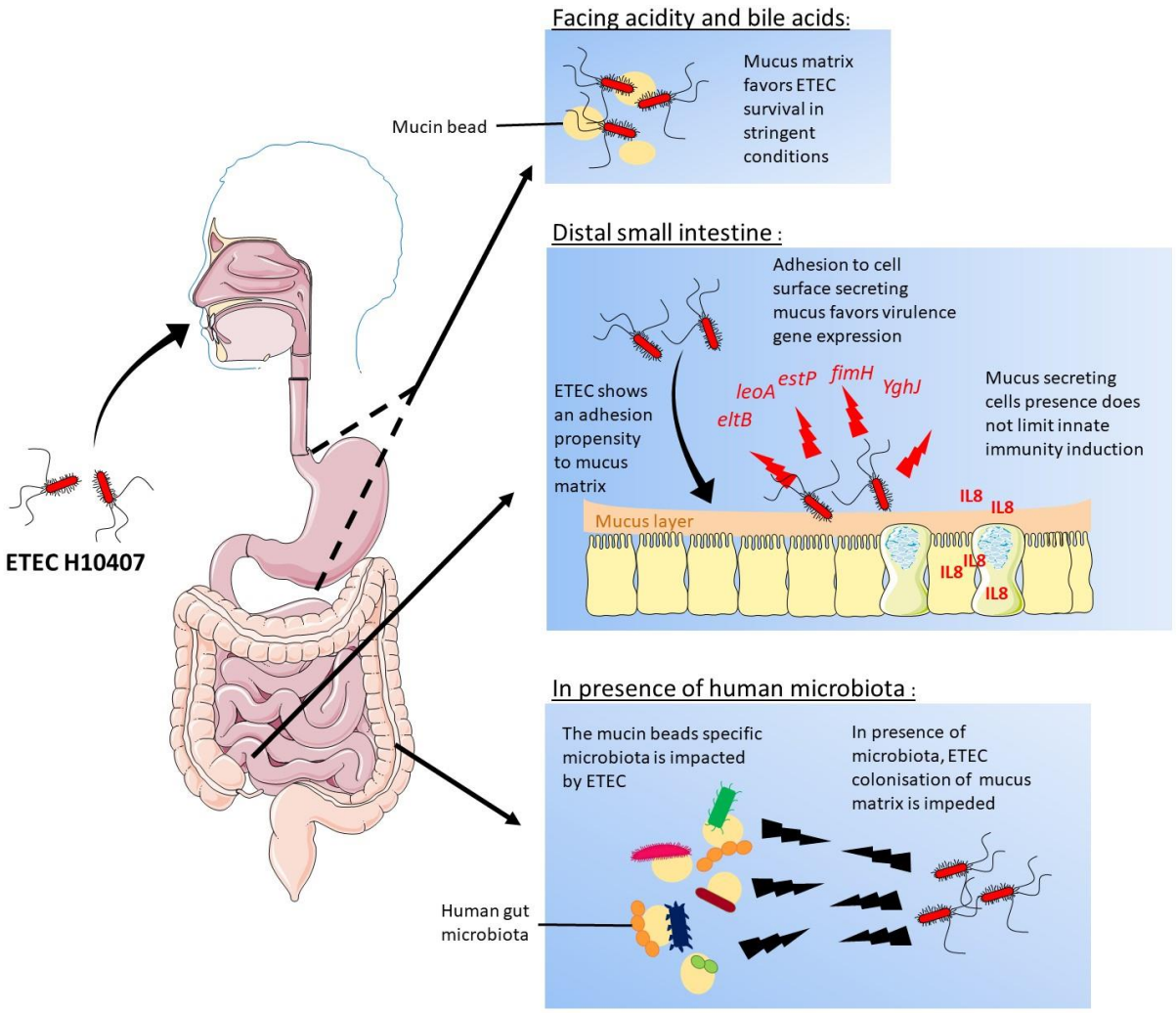
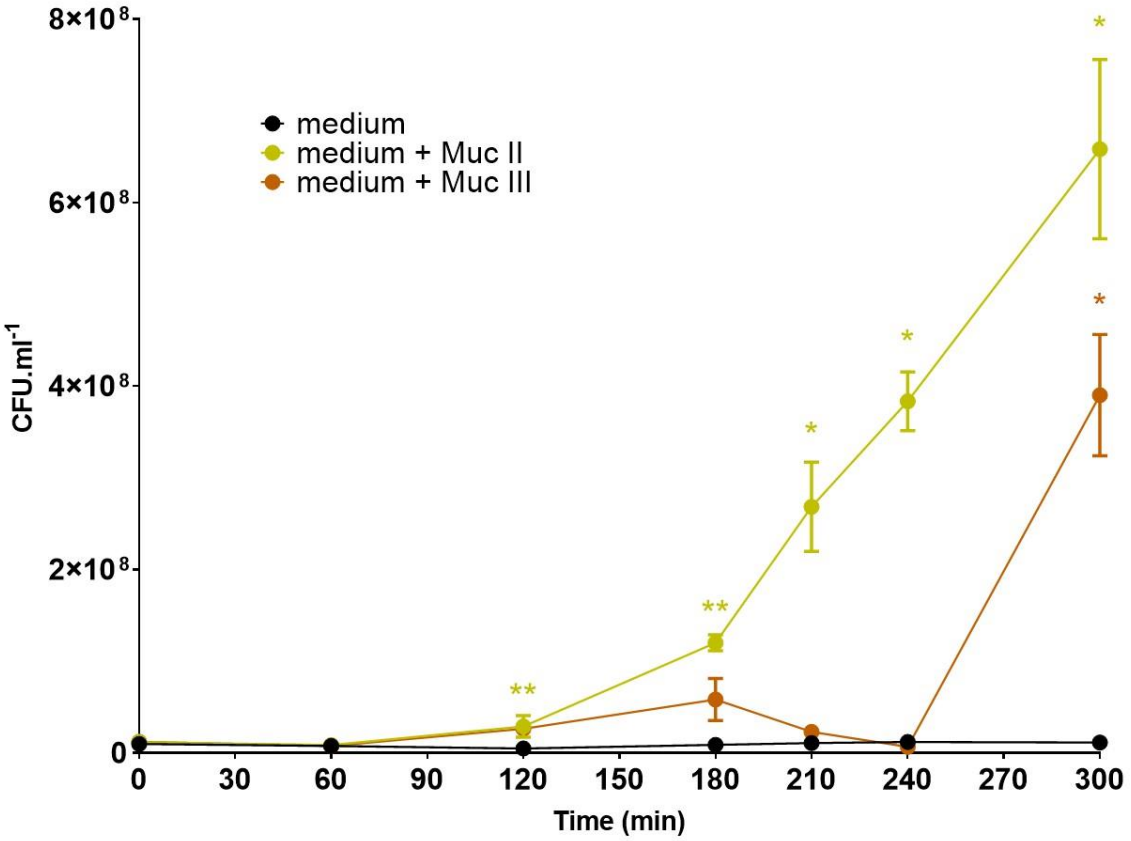


Figure 1.9. General view of mucus impact on ETEC strain H10407 physiopathology according to main results obtained in this study.

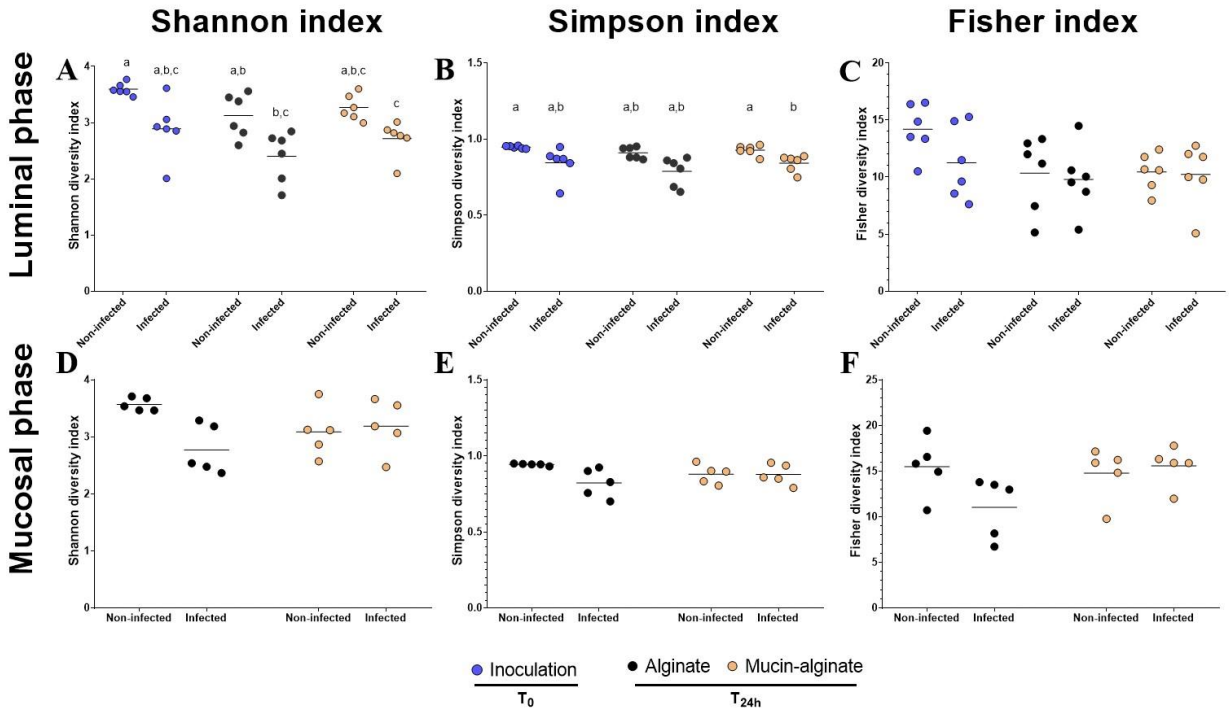
The main results of the study from Chapter I are summarized and organized according to the location in the human gastro-intestinal tract.

IL8: Interleukin-8.

7. Supplementary Figures

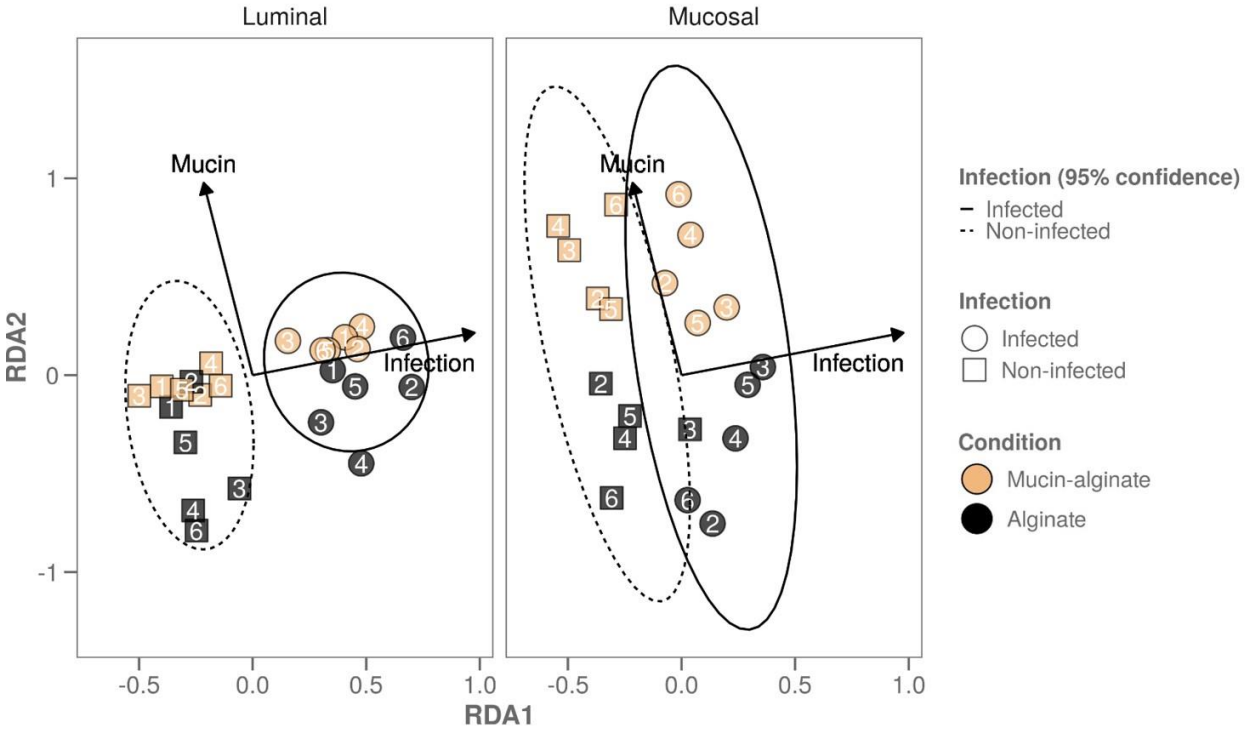


Suppl. Figure 1.1. Impact of mucin on ETEC growth in minimal culture medium. Growth kinetics of ETEC strain H10407 (inoculation at 10^7 CFU.ml^{-1}) in M9 minimal medium supplemented with type II mucin (yellow line), type III mucin (brown line) at 3g.L^{-1} or not supplemented (black line). Samples were regularly collected and plated on LB agar. Results are expressed as CFU.mL^{-1} (mean ± SD, n=3). Statistical differences with the control condition are indicated and provided by Tukey's multiple comparisons test (*: p<0.05; **: p<0.01).

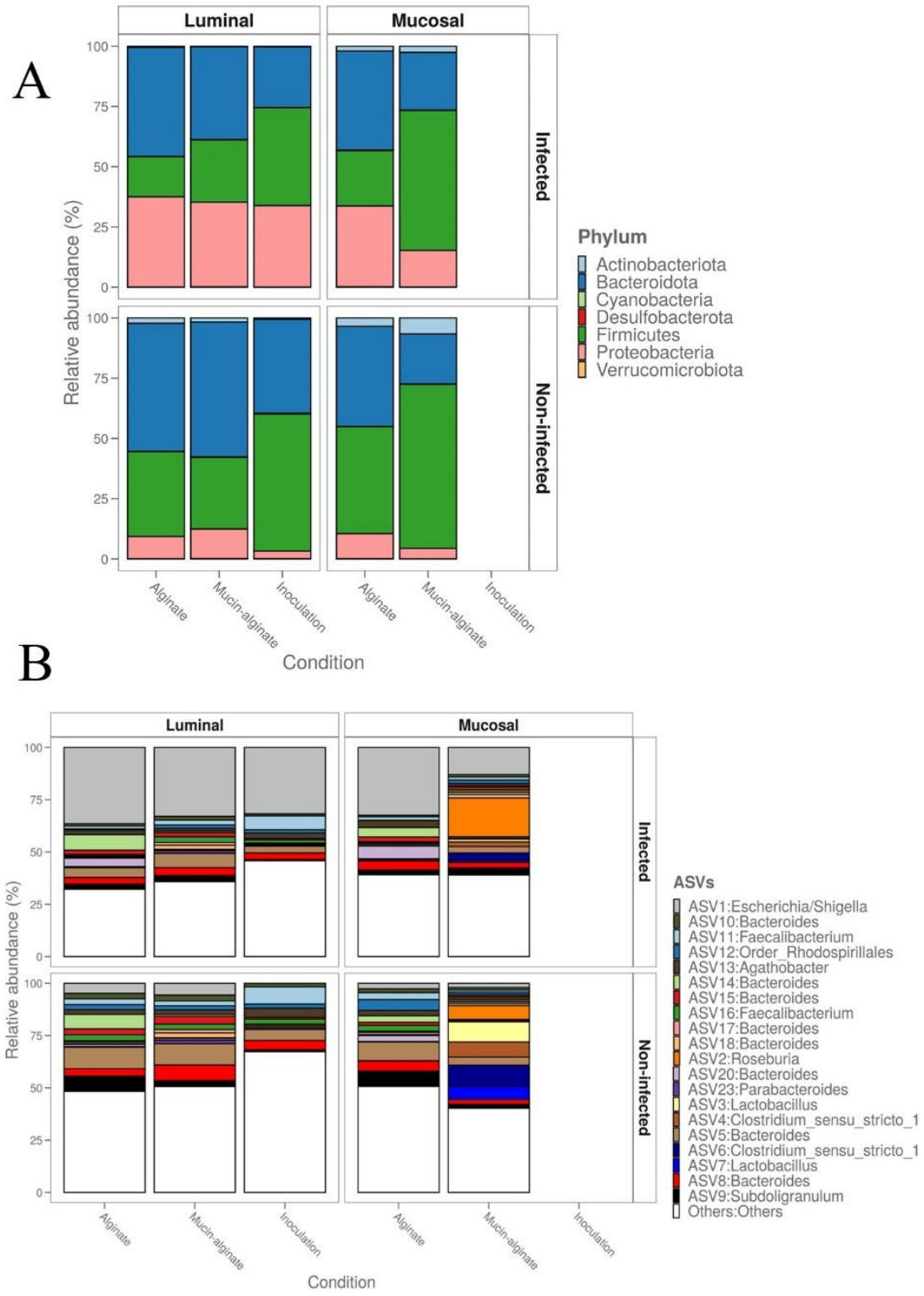


Suppl. Figure 1.2. Impact of mucin on ETEC modulation of microbial communities evenness.

Batch experiments were performed using feces from 6 healthy donors, challenged or not with ETEC strain H10407, with mucin-alginate beads and alginate beads (as a control). The graphs represent the variation of the microbiota α-diversity at the ASV level in the luminal (**A-C**) and mucosal (**D-F**) compartments. The species evenness is represented by Shannon (**A, D**), Simpson (**B, E**) and Fisher (**C, F**) indexes. Blue, black and orange dots represent individual biological replicates at the beginning of the experiment (T0) or after 24 hours (T24h) in the alginate and mucin-alginate beads conditions, respectively, while black bars represent the mean. Results that are no significantly different from each other according to Tukey's multi-comparison are grouped under the same letter ($p < 0.05$).

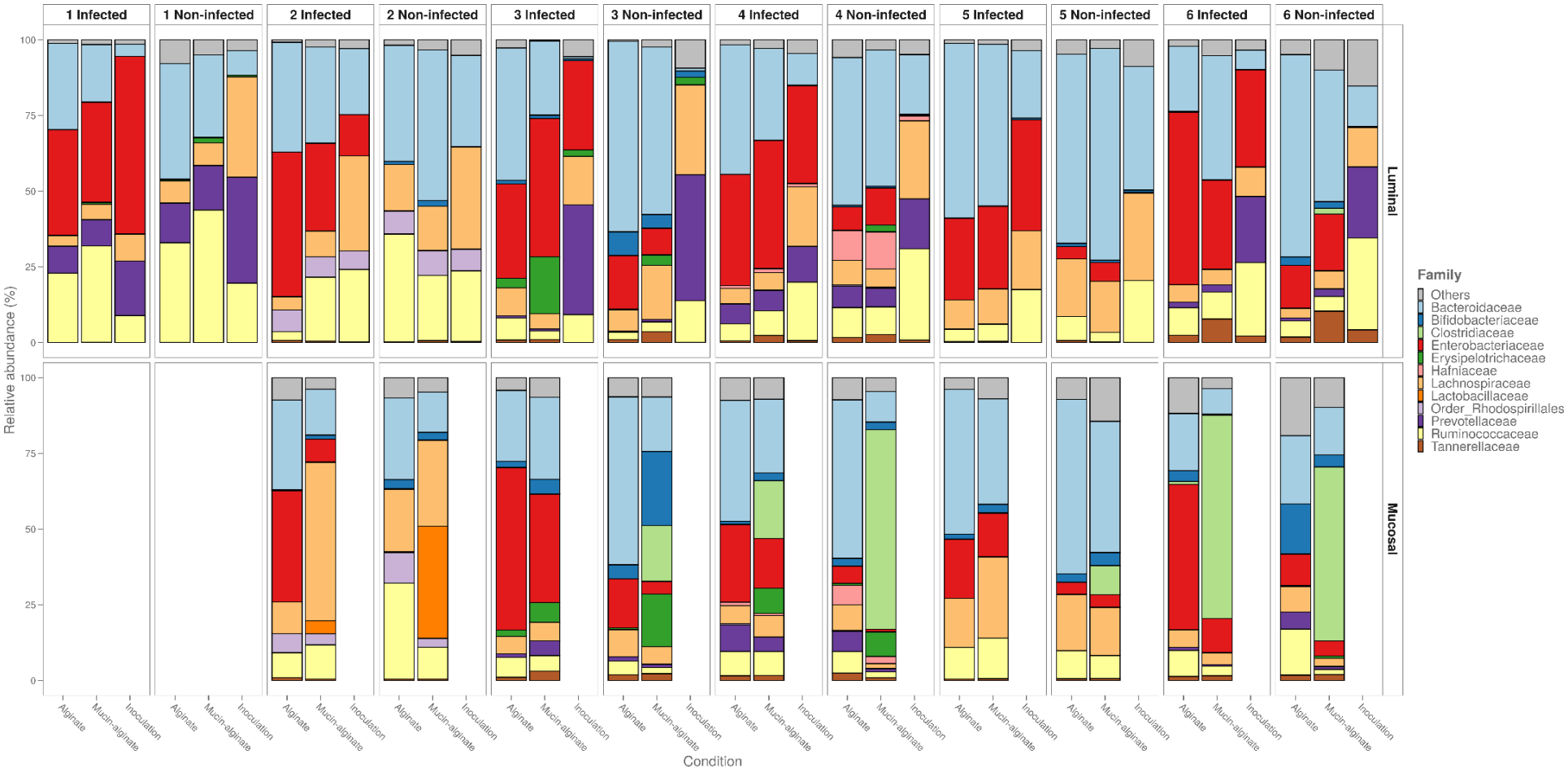


Suppl. Figure 1.3. Distance-based redundancy analysis of the modulation of microbiota β-diversity structure with the whole ASV table.
Batch experiments were performed using feces from 6 healthy donors, challenged or not with ETEC strain H10407, with mucin-alginate beads or alginate beads (as a control). db-RDA allows the two-dimension plot visualization of the microbial community β-diversity at the ASV level, as determined by 16S rRNA gene amplicon sequencing. Compared to the db-RDA presented in Figure 7, this one includes ASV1 (attributed to the *Escherichia*/*Shigella* genus) in the relative ASV table. 'Infection' and 'mucin' were provided as sole environmental variables (binary) and plotted as vectors (arrows). Blue, black and orange dots represent individual biological replicates (donor numbers are indicated) at the beginning of the experiment (inoculation, T0) or after 24 h of fermentation (T24h) with alginate and mucin-alginate beads, respectively. Samples are represented by dot shape and square shape for the infected and non-infected conditions, respectively. The 95% confidence ellipse area is also indicated in continuous line for the infected condition and in dotted line for the non-infected condition.

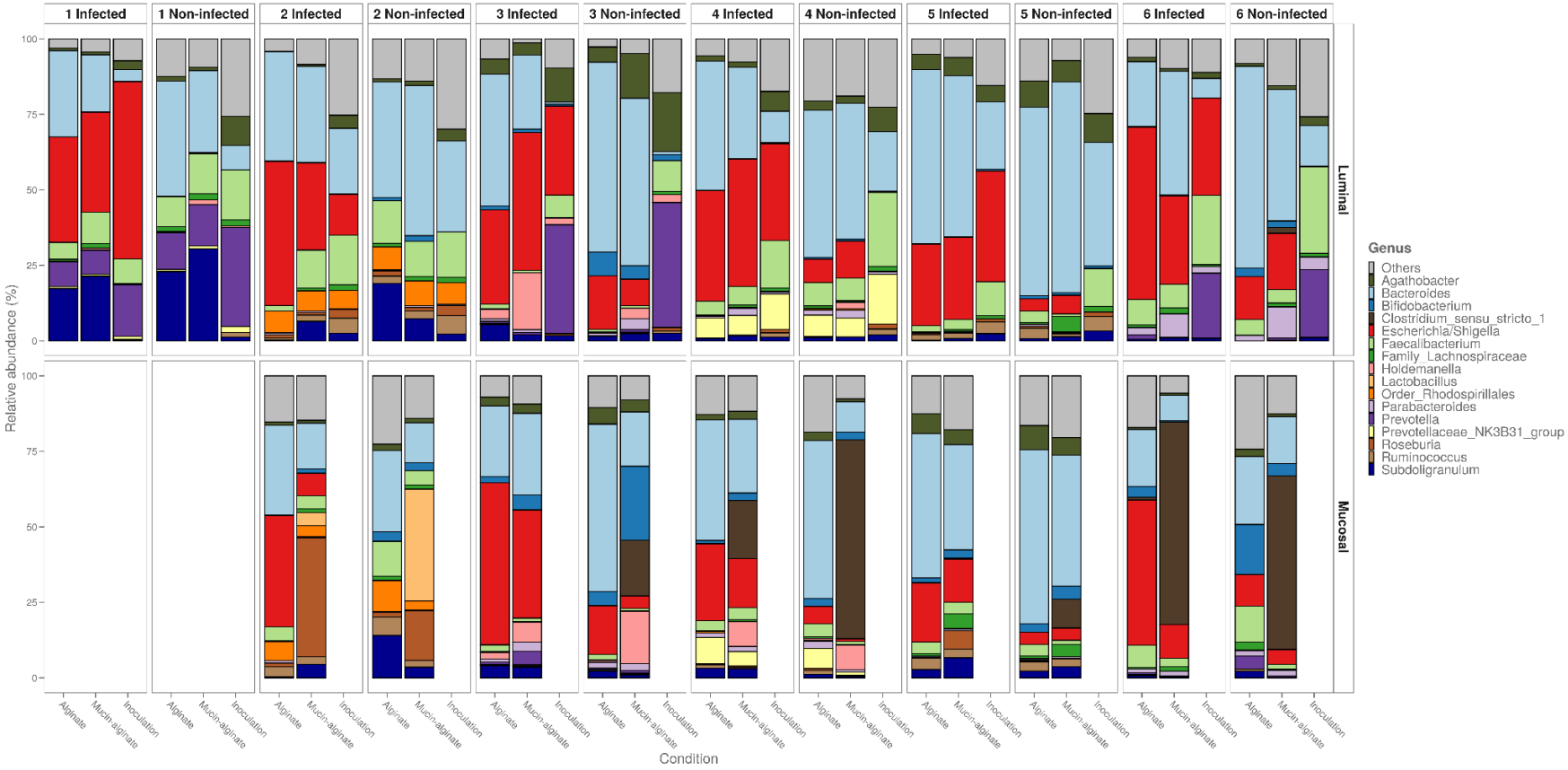


Suppl. Figure 1.4. Impact of mucin on ETEC modulation of microbial communities at the phylum and ASV levels.

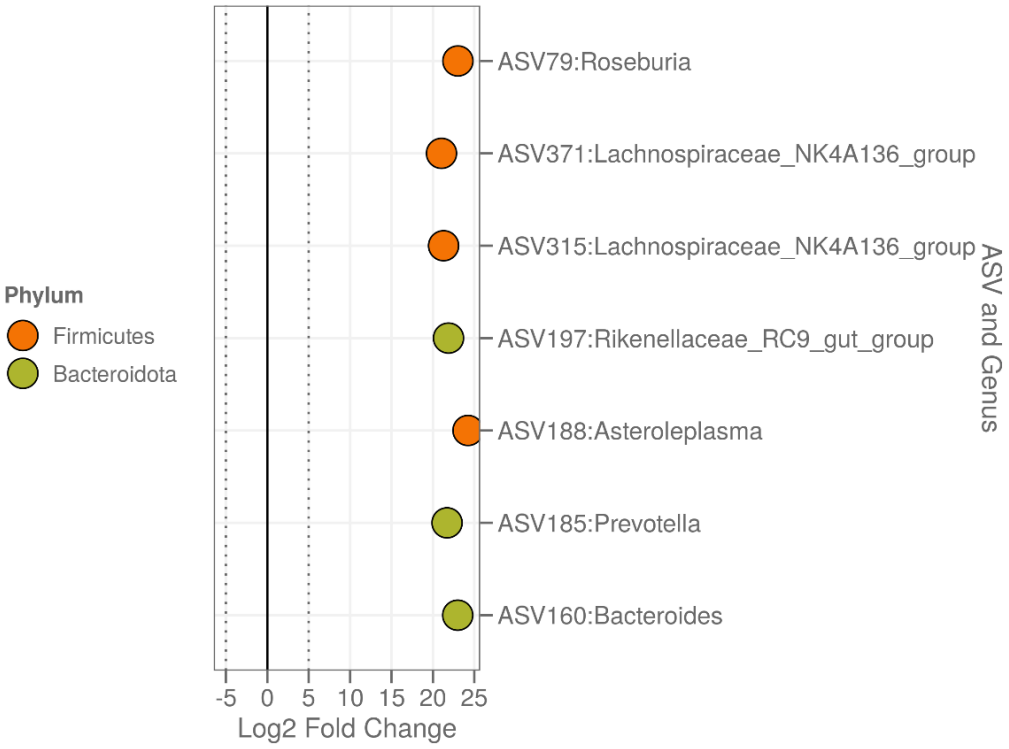
Batch experiments were performed using feces from 6 healthy donors, challenged or not with ETEC strain H10407, with mucin-alginate beads or alginate beads (as a control). The graph shows cumulative bar plots of the relative microbial community composition at the phylum (**A**) and ASV (**B**) levels. The area graphs show the relative abundance of the 5 most abundant phyla and 20 most abundant ASV with all six different donors confounded.



Suppl. Figure 1.5. Donor-specific impact of mucin on ETEC modulation of microbiota β-diversity at the family level.
Cumulative bar plots of the relative microbial community composition in fecal batch experiments at the family level. The graphs show the relative abundance of the 12 most abundant families in the luminal and mucosal phases for the six different donors, as determined by amplicon sequencing. Mucosal phase data are missing for donor 1 due to technical problem during sampling.



Suppl. Figure 1.6. Donor-specific impact of mucin on ETEC modulation of microbiota β-diversity at the genus level.
Cumulative bar plots of the relative microbial community composition in fecal batch experiments at the family level. The graphs show the relative abundance of the 16 most abundant genera in the luminal and mucosal phases for the six different donors, as determined by amplicon sequencing. Mucosal phase data are missing for donor 1 due to technical problem during sampling.



Suppl. Figure 1.7. Log2 fold change of relative ASV abundances significantly impacted by ETEC infection on mucin-beads in batch experiments.
Multivariate analysis was performed to detect which ASV were significantly impacted by ETEC infection in the different conditions (log-transformed adjusted *p* value = 0.05). The conditions tested were the luminal phase or mucosal phase of alginate or mucin-alginate beads conditions at T24h. Only the mucosal phase of mucin beads reported some ASV with significantly affected prevalence. A positive log2 fold change indicates ASV positively correlated with ETEC infection.

Chapter II - *In Vitro* Evaluation of Dietary Fiber Anti-Infectious Properties against Food-Borne Enterotoxigenic *Escherichia coli*

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Investigate in depth the potential antagonistic properties of dietary fiber-containing products as relevant anti-infectious strategies against ETEC infection was the aim of the second axis of this PhD project. This chapter presents the screening of eight dietary fiber-containing products (from cereals, legumes or microbes) for their antagonistic properties against the prototypical human ETEC strain H10407. Inhibitory effects of these products on the pathogen were tested through bacterial growth, toxin production and mucus/cell adhesion inhibition assays.

This work has been published in Nutrients (Impact Factor: 5.429) in 2021 and redrafted for the present chapter II.

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Sauvaitre T, Durif C, Sivignon A, Chalancon S, Van de Wiele T, Etienne-Mesmin L[#], Blanquet-Diot S[#]. Nutrients. 2021 Sep 14;13(9):3188. doi: 10.3390/nu13093188.

co-last authors

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1. Abstract

Dietary fibers have well-known beneficial effects on human health, but their anti-infectious properties against human enteric pathogens have been poorly investigated. Enterotoxigenic *Escherichia coli* (ETEC) is the main agent of travelers' diarrhea, against which targeted preventive strategies are currently lacking. ETEC pathogenesis relies on multiple virulence factors allowing interactions with the intestinal mucosal layer and toxins triggering the onset of diarrheal symptoms. Here, we used complementary *in vitro* assays to study the antagonistic properties of eight fiber-containing products from cereals, legumes or microbes against the prototypical human ETEC strain H10407. Inhibitory effects of these products on the pathogen were tested through growth, toxin production and mucus/cell adhesion inhibition assays. None of the tested compounds inhibited ETEC strain H10407 growth, while lentil extract was able to decrease heat-labile toxin (LT) concentration in culture media. Lentil extract and specific yeast cell walls also interfered with ETEC strain H10407 adhesion to mucin beads and human intestinal cells. These results constitute a first step in the use of dietary fibers as a nutritional strategy to prevent ETEC infection. Further work will be dedicated to the study of fiber/ETEC interactions within a complex gut microbial background.

2. Introduction

Dietary fibers are carbohydrate polymers with 10 or more monomeric units, which are not hydrolyzed by endogenous enzymes in the human small intestine, thus providing preferential substrates for gut microbes (Gill *et al.* 2021). Most of dietary fibers consumed by humans are of plant origin but some of them are also derived from animals, fungi or bacteria (Porter and Martens 2017). They have well-known beneficial health effect in humans, such as transit regulation, slowing down of glucose absorption, immune system modulation and support of gut microbiota diversity (Hooper, Littman and Macpherson 2012; Makki *et al.* 2018). Insoluble dietary fiber particles have even been recently shown to constitute a microbiota niche on their own (De Paepe *et al.* 2019, 2020). Another understudied effect of dietary fibers is their ability to prevent enteric infections (Sauvaitre *et al.* 2021b). Scarce *in vitro* studies have already shown the antagonistic properties of fibers against various enteric bacterial pathogens, mostly through a direct bacteriostatic effect, anti-adhesion properties on intestinal cells (Chantarasataporn *et al.* 2014; Ma *et al.* 2016; Vardaka, Yehia and Savvaidis 2016; Garrido-Maestu *et al.* 2018) or a decoy for pathogen/toxin binding to mucosal polysaccharides (Idota *et al.* 1995; Di *et al.* 2017; Liu *et al.* 2017; Leong *et al.* 2019). Interestingly, dietary fibers could also lure the resident gut microbiota from mucus consumption, thereby impeding access to the underlying epithelium to pathogen like *Citrobacter rodentium* (Desai *et al.* 2016). Therefore, dietary fibers may be considered as a promising alternative strategy when available therapy for the management of enteric infection is limited, as met with Enterotoxigenic *Escherichia coli* (ETEC) (Taylor, Hamer and Shlim 2017). ETEC is the main agent of traveler's diarrhea, responsible for hundreds of millions of diarrheal episodes worldwide (Fedor, Bojanowski and Korzeniewski 2019; Kotloff *et al.* 2019). ETEC has a preferential tropism for the distal part of the small intestine (Stintzing and Möllby 1982; Allen, Randolph and Fleckenstein 2006), where bacteria have to degrade mucus and adhere to the intestinal epithelium using mucinases and a myriad of adhesins (Kumar *et al.* 2014; Vipin Madhavan and Sakellaris 2015; Tapader, Bose and Pal 2017),. Then, the hallmark of ETEC infection is the production of two toxins, the LT and ST enterotoxins, which through binding to their respective receptors, are both leading to hypersecretion of H_2O and Cl^- at the root of watery cholera-like diarrhea (Qadri *et al.* 2005; Turner *et al.* 2006b). To date, very few studies have addressed dietary fiber effects upon ETEC strains from human origin. Only milk oligosaccharides and plantain soluble fibers were proven to reduce ETEC adhesion to Caco-2 intestinal epithelial cells (Idota and Kawakami 1995; Roberts *et al.* 2013; Salcedo *et al.* 2013). Here, we investigated using complementary *in vitro*

approaches the potential antagonistic properties of eight fiber-containing products against the prototypical human ETEC strain H10407. We assessed the effect of dietary fiber-containing products on bacterial growth and LT toxin production in broth media, as well as their anti-adhesive properties on mucins and human intestinal epithelial cells.

3. Materials and methods

3.1. Dietary fiber-containing products

The main characteristics of the eight-dietary fiber-containing products tested in this study are summarized in **Table 2.1**. Among them, some were kindly provided by local companies, while others were purchased from supplier (Merck, Darmstadt, Deutschland) or extracted in the laboratory from raw products. Before this extraction, lentils, red beans and oat were prepared according to their usual households of consumption. Briefly, red beans and oat flakes were soaked in water overnight and 10 minutes, respectively. Red beans and lentils were separately boiled (30 min). Then, all products were washed in sterile distilled water, grinded at maximum speed in a blender (8010S, Waring, Torrington, Connecticut, USA) until homogeneity, and filtered through a 0.9 mm diameter pore filter. Per 200 g of raw products, 10 g of pancreatin (P1750, Merck, Darmstadt, Deutschland) was added to 200 mL of sterile distilled water and centrifuged (8000 g, 30 min, 4°C). The supernatant was collected and added to the grinded material with 3.2 mg trypsin (T0303, Merck, Darmstadt, Deutschland). Digestion was performed for a total duration of 6 hours (100 rpm, 37°C). To precipitate soluble fibers, 3 volumes of 96% ethanol were then added to the mixture under agitation (4°C, 100 rpm, 1 hour). The solution was centrifuged (2500 g, 15 min, 4°C) and the pellet was washed 3 times in 75% ethanol. Finally, the pellet was dried in an incubator (overnight, 42°C) and then finely grounded at full speed under sterile conditions in a blender (8010S, Waring, Torrington, Connecticut, USA). Fiber content of the eight products was analyzed by an external company (CAPINOV, Landerneau, France) according to the AOAC 985.29 method (Stephen *et al.* 2017), except for wheat starch (resistant starch content was directly indicated by the provider). If the products were not sterile as determined by plating on plate counting agar, they were autoclaved (121°C, 15 min). In all *in vitro* experiments, products were used at final fiber concentration of 2 $g.L^{-1}$.

Table 2.1. Characteristics of dietary fiber-containing products.
The product origin and source, their solubility at 2 $g.L^{-1}$ in water, their fiber content, as well the analysis method used for determining fiber content are indicated in the table.

Extract/product	Origin	Product source	Solubility at 2g.L^{-1} in water	Fiber content (g.100g^{-1})	Analysis method
Green lentils	Plants	Home made	Insoluble	41.4	AOAC 985.29
Guar gum	Plants	Commercially available	Soluble	84.3	AOAC 985.29
Locus bean gum	Plants	Commercially available	Soluble	83.3	AOAC 985.29
Oat	Plants	Home made	Insoluble	19.8	AOAC 985.29
Oat bran	Plants	Provided by local companies	Insoluble	44.4	AOAC 985.29
Red beans	Plants	Home made	Insoluble	53	AOAC 985.29
Wheat starch	Plants	Provided by local companies	Soluble	17	Resistant starch content communicated by provider
Specific yeast cell walls (from *Saccharomyces cerevisiae*)	Microorganisms	Provided by local companies	Insoluble	62.6	AOAC 985.29

3.2. ETEC strain and growth conditions

The prototypical ETEC strain H10407 serotype O78:H11:K80 (ATCC® 35401, LT+, ST+, CFA/I+) isolated in Bangladesh from a patient with a cholera-like syndrome (Evans *et al.* 1977) was used in this study. Bacteria were routinely grown under agitation (125 rpm, overnight, 37°C) in LB broth until OD 600nm = 0.6 (stationary phase).

3.3. Growth assay

ETEC strain H10407 (10^6 $CFU.mL^{-1}$) was allowed to grow aerobically for 6 hours at 37°C under 100 rpm agitation, in complete LB (Sigma, St. Louis, USA) or minimal M9 medium, with or without each fiber-containing product. Medium was regularly sampled and plated onto LB agar for ETEC numeration. Three independent biological replicates were performed.

3.4. LT toxin overnight production

LT production was assayed by cultivating ETEC strain H10407 with or without fiber-containing products in overnight Casamino Acids-Yeast Extract (CAYE) medium at 37°C under agitation (100 rpm) (Fontes *et al.* 1982). After overnight culture, medium was centrifuged

(3000 g, 5 min, 4°C,) and toxin concentrations were measured in the supernatant by ELISA assay as previously described (Salimian *et al.* 2010; Roussel *et al.* 2020b). Optical density was read at 450 nm using an EPOCH multiplate spectrophotometer (BIOTEK, Winooski, Vermont, USA). Three independent biological replicates were performed.

3.5. Mucin beads

Mucin beads were obtained as already described (Deschamps *et al.* 2020). Mucin from porcine stomach type III (Sigma-Aldrich, Saint-Louis, USA) was diluted in sterile distilled water, at a concentration of 5 % (w/v). Sodium alginate (Sigma-Aldrich, Saint-Louis, USA) was added at a concentration of 2 % (w/v). To produce mucin alginate beads, the mixture was dropped using a peristaltic pump into a 0.2 M solution of sterile $CaCl_2$ under agitation (100 rpm). Beads (diameter: 4.5 mm in average) were then kept at 4°C (no more than 24 hours prior use).

3.6. Mucin bead adhesion assay

Adhesion assays were carried out with mucin beads resuspended in 50 mL PBS pH 6.8 with or without fiber-containing products. ETEC strain H10407 was added at 10^7 or 10^8 $CFU.ml^{-1}$ for a 30 minutes or 1-hour contact period, respectively. Mucin-beads were then washed three times with 40 mL sterile physiological water and crushed in 19.8 mL physiological water with an ultra turrax apparatus until homogeneity (IKA, Staufen, Germany). Adhered bacteria were numerated by plating onto LB agar. Each experiment was repeated at least four times.

3.7. Caco-2/HT29-MTX cell cultures

Caco-2 and HT29-MTX cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Life Technologies Ltd, Paisley, UK) containing glucose and glutamine, supplemented with non-essential amino acid (Gibco, Life Technologies Ltd, Paisley, UK) and antibiotic-antimycotic solution (Gibco, Life Technologies Ltd, Paisley, UK). Media were also supplemented with 20 and 10 % Foetal Bovine Serum (FBS, Thermo Fisher Scientific, Waltham, Massachusetts, USA) for Caco-2 and HT29-MTX cells, respectively. For experimental studies, Caco-2 and HT29-MTX cells were seeded at a density of 10^5 cells/well on 12 wells plates (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at a ratio 70:30. The coculture was allowed to differentiate for 18 days in medium with 20% heat-inactivated FBS in an atmosphere of 5% CO_2 at 37°C. The growth medium was replaced every 2 days.

3.8. Adhesion tests on Caco-2/HT29-MTX co-culture model

Cells were pre-treated or not with fiber-containing products for a 3-hour period. Cells were then infected with ETEC strain H10407 at MOI 100 for 3 hours in antibiotic-antimycotic free medium. After three washes with PBS pH 7.2 at 4°C (Thermo Fisher Scientific, Waltham, Massachusetts, USA), cells were lysed with 1 mL of 1% Triton X-100 (Sigma, St. Louis, USA). Serial dilutions of lysed cells were plated onto LB agar to determine the number of adhered bacteria. Each experiment was repeated at least six times.

4. Results

4.1. All Fiber-Containing Products Have No Effect on ETEC Growth in Complete Nutritive Medium

When ETEC bacteria were grown in LB-rich medium (**Fig. 2.1.A**), no statistical difference was observed between each fiber-supplemented condition and the negative control (no fiber) according to Dunnett's multiple comparisons test. The growth curves were similar whatever the conditions tested, with all culture reaching between 6.10^8 and 8.10^8 $CFU.mL^{-1}$ at the end of the experiment. Therefore, none of the eight screened products was able to reduce ETEC growth in complete culture medium. In M9 minimal medium, all of the fiber-containing products showed a tendency to sustain ETEC growth compared to the non-treated condition, with a clear product effect (**Fig. 2.1.B**). In particular, lentils and the specific yeast cell walls from *Saccharomyces cerevisiae* AQP 12,260 led to more than 1-log difference with the control condition after 5 h. These differences became statistically different at 240 and 300 min according to Dunnett's multiple comparisons test ($p < 0.05$).

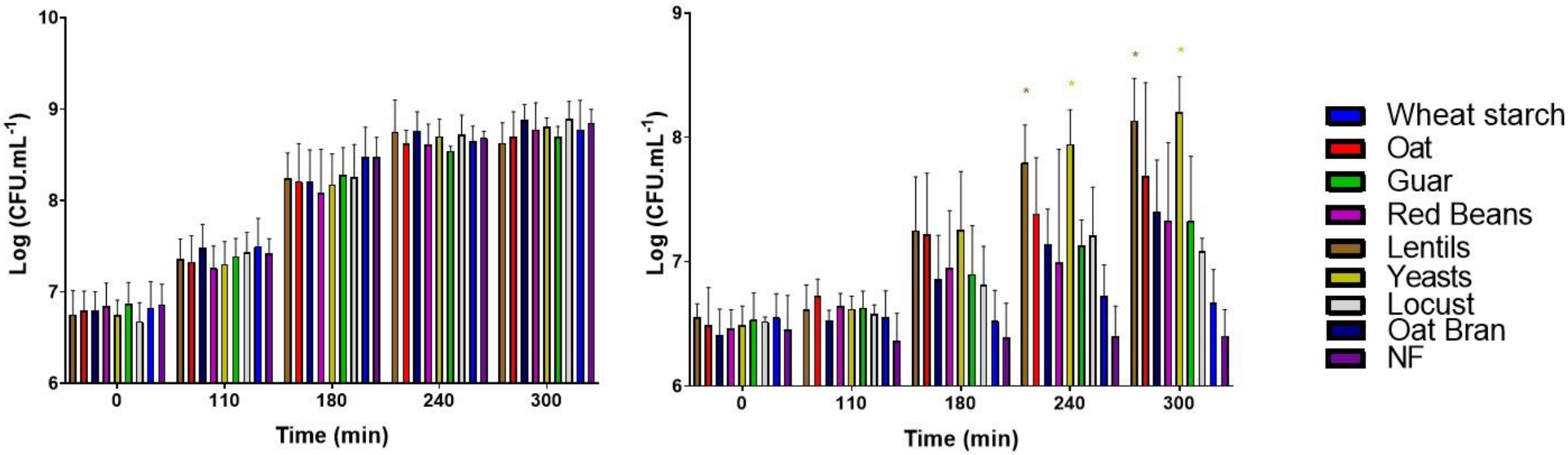

Figure 2.1. Effect of fiber-containing products on the time course of ETEC strain H10407 bacterial growth in complete LB medium (**A**) or in M9 minimal medium (**B**). Fiber-containing products were tested at 2 $g.L^{-1}$ of final fiber content. Each bar represents the mean of three biological independent replicates (±SD). Results are expressed as mean log10 $CFU.mL^{-1}$. Significance with the control condition was determined by Dunnett's multiple comparisons test (*: $p < 0.05$). NF=control condition with no product added, SD=standard deviation.

4.2. Lentil Fibers Decreases LT Toxin Production

We also evaluated the direct effect of the eight fiber-containing products on LT toxin concentration in CAYE medium, known to support toxin production (Fontes *et al.* 1982). In the absence of fiber-containing product (control condition), ETEC strain H10407 produced on average 69.7 ± 7.4 ng.mL^{-1} of LT toxin (**Fig. 2.2**). With most of the tested products, a higher LT toxin concentration was measured (from 90.0 ± 2.9 ng.mL^{-1} for red beans to 174.5 ± 15.7 ng.mL^{-1} for specific yeast cell walls). In sharp contrast, the lentil-derived fibers widely reduced LT toxin concentrations. One biological replicate showed a concentration of 16 ng.mL^{-1} of toxin, much lower than the non-treated condition (NF) and the toxin was not detected in the two other replicates (levels below the detection threshold). When incubated with pure LT toxin (500 ng.L^{-1}), lentil extracts (from 0 to 8 g.L^{-1} of fiber content) significantly decreased toxin amount at the highest dose ($p < 0.05$) but had no effect at the 2 g.L^{-1} concentration used throughout this study (data detailled in Chapter 3 of the experimental section, **Fig. 3.2**)

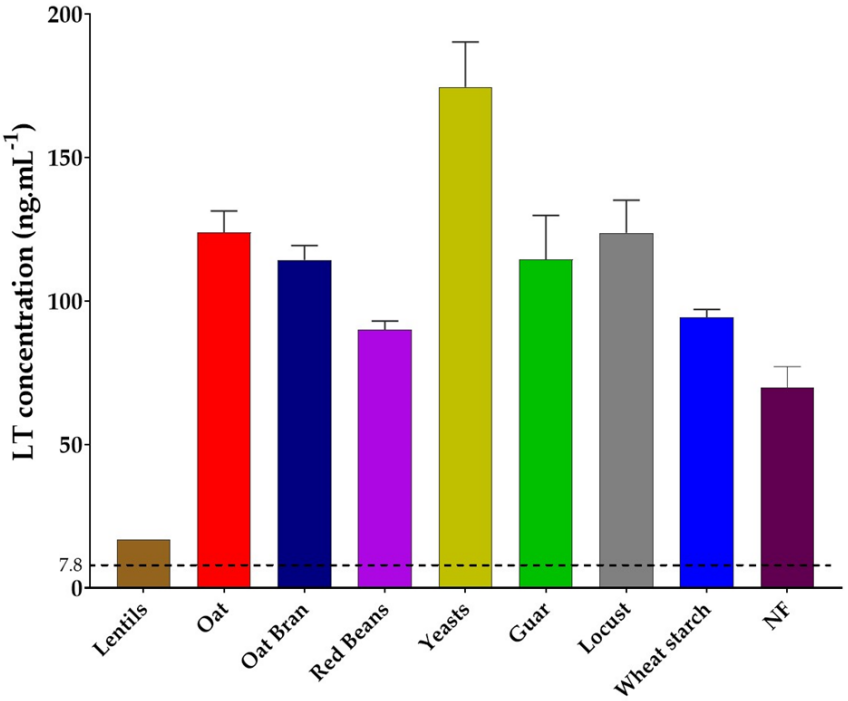

Figure 2.2. Effect of fiber-containing products on LT toxin concentration after an overnight culture of ETEC strain H10407 in CAYE medium. Fiber-containing products were tested at 2 g.L-1 of final fiber content. Results are expressed as mean ng·mL^{-1} of toxin (±SD) of three independent biological replicates. For lentil extracts, only one biological replicate is represented as toxin was not detected in the two other replicates. No statistical test was performed because of these two under-threshold results. The detection threshold (7.8 ng.mL^{-1}) is indicated by a dotted black line. NF=control condition with no product added, SD=standard deviation.

4.3. Specific Yeast Cell Walls and Lentil Fibers Inhibit ETEC Adhesion to Mucin Beads

Next, a mucin-bead adhesion assay aimed at investigating whether some fiber-containing products were able to reduce pathogen adhesion to intestinal mucus. Different experimental conditions were tested: initial bacterial concentration of 10^7 CFU.mL^{-1} and a 30 min contact time in the first experiment (**Fig. 2.3.A**), parameters that were both increased in the second assay with 10^8 CFU.mL^{-1} and a 60 min contact time (**Fig. 2.3.B**). Both experiments showed a clear tendency of lentils, oat, oat bran, red beans and specific yeast cell walls containing fiber products to reduce ETEC adhesion to mucin beads (**Fig. 2.3.B**). In particular in the first assay (**Fig. 2.3.A**), specific yeast cell walls reduced ETEC adhesion more than 6-fold. In the second assay (**Fig. 2.3.B**), lentils reduced ETEC adhesion of more than 4-fold.

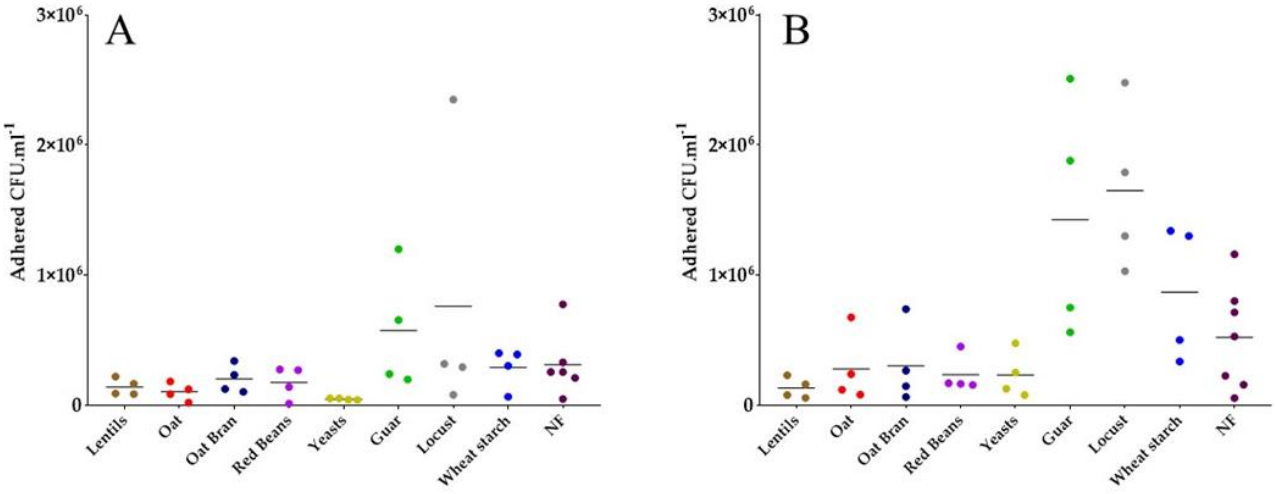

Figure 2.3. Effect of fiber-containing products on ETEC adhesion on mucin beads, when inoculating with 10^7 (A) or 10^8 (B) CFU.mL^{-1}, during 30 minutes (A) or 1 h (B) contact period. Fiber-containing products have been tested at 2 g.L^{-1} of final fiber content. Each biological replicate and their mean are represented (n= 4-7). Results are expressed as adhered CFU.mL^{-1} of crushed bead solution. No significance with the control condition was found according to Tukey's multiple comparison tests. NF = control condition with no product added.

4.4. Most Fiber-Containing Products Inhibit ETEC Adhesion to Caco-2/HT29-MTX Co-Culture

To further address the potential of fiber-containing products to modulate ETEC adhesion to the human intestinal epithelium, we performed adhesion experiments on a co-culture model of Caco-2/HT29-MTX cells, respectively differentiating in enterocytes and mucus-secreting goblet cells. These experiments were performed with a lower number of products, selected from the results of previous *in vitro* experimentations. All tested products

except oat bran showed a trend in reducing ETEC adhesion levels (**Fig. 2.4**). Average adhesion levels were 50-, 30- and 15-fold lower compared to the control condition (no fiber-containing product added), for wheat starch, guar and specific yeast cell walls, respectively.

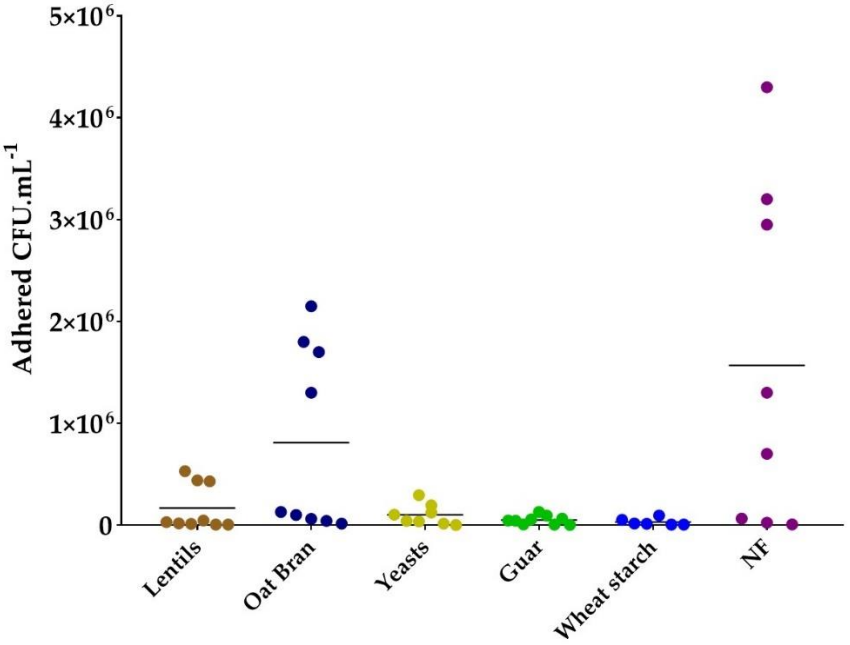

Figure 2.4. Modulation of ETEC adhesion to co-culture of Caco-2/HT29-MTX cells by fiber-containing products. Fiber-containing products were tested at 2 $g.L^{-1}$ of final fiber content. Each biological replicate and their mean are represented (*n*=6-9). Results are expressed as adhered $CFU.mL^{-1}$ of cellular lysate. No significance with the control condition was found according to Tukey's multiple comparison tests. NF=control condition with no product added.

5. Discussion

The current study aimed at evaluating the antagonistic properties of a broad range of dietary fiber-containing products against the prototypical human ETEC strain H10407 using various complementary *in vitro* assays. Fibers from lentils and specific yeast cell walls showed the most interesting inhibitory properties, because of their LT toxin lowering and mucosal adhesion inhibiting properties, respectively. The products tested herein have different origin (vegetal or microbes) and thus contain different types of soluble and/or insoluble fibers, among which resistant starch for wheat starch, beta-glucans for oats and specific yeast cell walls, mannans for specific yeast cell walls, galactomannan for guar and locust bean gums, celluloses and hemicelluloses for lentils (Deehan *et al.* 2018). All the products were tested at the physiological dose of 2g of fibers per liter, considering both the ingested amount in Westernized diet that ranges from 10 to 30 grams per day (King, Mainous and Lambourne 2012; Holscher 2017; Scientific Opinion on the substantiation of health claims related to dietary fibre (ID 744,

745, 746, 748, 749, 753, 803, 810, 855, 1415, 1416, 4308, 4330) pursuant to Article 13(1) of Regulation (EC) No 1924/2006) and the dilution by digestive fluids of nearly 10 liters per day (Kiela and Ghishan 2016). This amount of $2g.L^{-1}$ is in the range of tested fiber concentrations against intestinal pathogens in vitro (up to $10g.L^{-1}$ for complex polysaccharides in cellular assays), as recently reviewed (Makki *et al.* 2018). ETEC strain was also used in the in vitro assays at a physiological concentration as infectious dose in humans varies from 10^5 to 10^{10} ingested bacteria (Levine *et al.* 1979; Yang *et al.* 2016: 201; Mirhoseini, Amani and Nazarian 2018; Brubaker *et al.* 2021).

The beneficial effects of dietary fibers on human health is now well acknowledged, but their ability to exert antagonistic effects against enteric pathogens remains poorly studied (Deehan *et al.* 2018; Asadpoor *et al.* 2020; Forgie, Fouhse and Willing 2019; Davis *et al.* 2020; Sauvaitre *et al.* 2021b). To date, the vast majority of studies investigating the potential of fibers in the fight against ETEC-associated infections has been performed on porcine ETEC strains (Roubos-van den Hil *et al.* 2010; Wang, Gänzle and Schwab 2010; González-Ortiz *et al.* 2013, 2014; Zhu *et al.* 2018) while studies involving ETEC strains from human origin are scarce (Roberts *et al.* 2013; Salcedo *et al.* 2013; Liu *et al.* 2016a).

Fibers can act at different levels of ETEC pathological process. A first target in our study was the observed reduction in the number of bacteria able to reach the pathogen's site of action in the distal part of the human small intestine (Allen, Randolph and Fleckenstein 2006). Then, we first investigated the direct antagonist effect of fiber-containing products on ETEC strain H10407 growth in classical culture media. None of the tested products was able to reduce pathogen growth in LB complete medium. This is not surprising since to our knowledge only chitosan, a human engineered fiber was previously shown to inhibit pathogen growth among which EHEC (Chantarasataporn *et al.* 2014). Besides, tested products were all sustaining ETEC growth when using M9 minimal medium, most probably due to the presence of non-fiber components in the fiber-containing products, as *E. coli* strains are not known to be able to degrade complex polysaccharides (Muñoz-Gutiérrez and Martinez 2013). When translated to the complex nutritional background of the human gut, this is certainly not an issue since ETEC will encounter many other nutrient sources than the ones provided by our fiber-containing products.

In a second step, since toxin production is a key feature in ETEC physiopathology, we assessed the effect of fiber-containing products on LT toxin production. To our knowledge, only one study has previously reported an indirect effect of dietary fibers on ETEC toxin. Indeed, SCFA, that are major end products of dietary fiber metabolism by gut microbiota,

significantly reduced or even abolished LT toxin production at a concentration of 2 $g.L^{-1}$ in CAYE culture medium (Takashi, Fluita and Kobari 1989). In our study, lentils extract induced a decrease in LT enterotoxin concentration in the supernatant of overnight ETEC strain H10407 culture in CAYE medium. Several hypotheses have been raised related to the mechanisms of action. First, lentils extract may repress ETEC LT toxin production at the transcriptional or translational levels, but there is no data in the literature to support such hypothesis. Then, if the LT toxin production is not impacted by lentils extract, we can imagine that the inhibition can occur at the detection step. To challenge this hypothesis, we evaluated the effect of lentil extracts on pure LT toxin solutions and showed that the inhibitory effect was partially conserved but not significant at the dose of 2 $g.L^{-1}$ of fiber used in this study. Such inhibition can occur at different steps of the ELISA assay, but we can speculate that the toxin binds to some lentils components that act as decoy, preventing interactions with antibodies. In a next step, we could investigate the inhibitory effect of fiber-containing products on ETEC toxin production *via* gut microbiota modulation.

Lastly, fibers can also favor the exclusion of pathogens from mucosal surface by presenting potential binding site and thus acting as a decoy. Many dietary fibers originating from milk, plant and microorganisms, have already proven efficiency in reducing adhesion to mucus (Sarabia-Sainz *et al.* 2013; González-Ortiz *et al.* 2014), erythrocytes (Wang, Gänzle and Schwab 2010: 201; Chen *et al.* 2014), Caco-2 cell line (Roubos-van den Hil *et al.* 2009, 2010) or cells from porcine jejunal epithelium (González-Ortiz *et al.* 2013; Cilieborg *et al.* 2017; Zhu *et al.* 2018: 201) for ETEC strains from animal origin. Here, we investigated the ability of some of our fiber-containing products to distract the human ETEC strain H10407 from mucosal-like surfaces. First, by using a mucin bead adhesion assay we demonstrated that lentil extracts and yeast cell walls could decoy ETEC strain H10407 from mucus polysaccharides adhesion. Compared to other mucus integrating models used to test inhibitory properties of fibers (Sarabia-Sainz *et al.* 2013; González-Ortiz *et al.* 2014), the use of mucin beads under constant agitation eliminates the possibility of non specific bacterial exclusion by fiber sedimentation on the mucus compartment. Second, to integrate the host part, we used cellular adhesion experiments with Caco-2/HT29-MTX cellular co-culture model. This model includes Caco-2 enterocytes-like cells and HT29-MTX cells secreting mucus polysaccharides (Dorier *et al.* 2017; García-Rodríguez *et al.* 2018; Gillois *et al.* 2021). To date, only milk oligosaccharides (HMOs) (Idota and Kawakami 1995; Salcedo *et al.* 2013) and soluble plantain fibers at a dose of 5 $g.L^{-1}$ (Roberts *et al.* 2013) have already shown efficiency to reduce adhesion of human ETEC strains other than H10407 to Caco-2 cell line. Our experiments showed a global trend

towards an inhibitory effect of yeast specific cell walls and lentils, but also of other fiber-containing products such as guar gum and wheat starch. As previously observed, such effects can be due to a decoy effect from mucus adhesion. Besides, it is well known that ETEC adhesins are able to recognize specific receptors on cell surface, such as glycosphingolipids (Jansson *et al.* 2006; Ahmed *et al.* 2009; Madhavan *et al.* 2016), mannosylated proteins (Sheikh *et al.* 2017) and fibronectin (Chatterjee *et al.* 2011). This implies that some components found in all tested compounds, probably fibers, may be able to bind to such cellular receptors, thereby blocking bacterial attachment. Lastly, we checked using Trypan blue exclusion assay that specific yeast walls and lentils extract had no effect on ETEC-induced cytotoxicity in intestinal Caco-2 and HT29-MTX cells (data not shown).

6. Conclusion

Taken together, our results suggest that among the tested fiber-containing products, lentils and yeast specific cell walls could present promising anti-infectious activities against the human reference ETEC strain H10407. These effects seem to be mediated through a multi-targeted pathway, namely inhibition of toxin production and reduction of adhesion to mucins and intestinal epithelial cells. The associated mechanism of action obviously needs to be further investigated. Next steps will be dedicated to the testing of selected fiber-containing products in more complex experimental set-ups reproducing the physiological conditions of the human digestive environment in a more representative manner and including gut microbiota, which is a key player in gut homeostasis and fiber degradation. This study is the first step in the use of dietary fibers as a new nutritional strategy to prevent ETEC-induced traveler's diarrhea.

Author Contributions

Conceptualization, S.B.-D, L.E.-M. and T.V.d.W.; methodology, T.S., S.B.-D., L.E.-M. and T.V.d.W.; formal analysis, T.S.; investigation, T.S., C.D., A.S. and S.C.; writing—original draft preparation, T.S.; writing—review and editing, T.S., S.B.-D., L.E.-M. and T.V.d.W.; supervision, S.B.-D., L.E.-M. and T.V.d.W.; project administration, S.B.-D.; funding acquisition, S.B.-D. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

T.V.d.W. is advising ProDigest as a science officer and is a member of the scientific advisory board of MRM Health. S.B.-D. is advising NexBiome as a science officer. The other authors declare no conflict of interest.

Chapter III - Lentils and yeast fiber-containing products: a new strategy to mitigate Enterotoxigenic *Escherichia coli (ETEC)* strain H10407 virulence?

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As a follow-up of the results from chapter II highlighting that the lentil extracts and the specific yeast cell wall extracts appear as the most relevant candidates for their anti-infectious properties against ETEC, this chapter III focuses on digging more deeply the antagonistic properties of the two selected products. Especially, we investigated how lentils and yeasts can play in the human digestive environment (i) a direct anti-infectious effect such as inhibition of growth, adhesion or toxin production or (ii) an indirect antagonistic role through the modulation of luminal and mucosal microbiota or host responses.

The results have been subjected to the writing of an original research article, to be submitted in Nutrients (Impact Factor: 5.429) in the special issue “Prebiotics and Prebiotics in Immune health” under invitation and redrafted for the present chapter.

Lentils and yeast fibers: a new strategy to mitigate Enterotoxigenic Escherichia coli (ETEC) strain H10407 virulence?
Sauvaitre T, Van Herreweghen F, Delbaere K, Durif C, Van Landuyt J, Fadhlaoui K, Huile S, Chaucheyras-Durand F, Etienne-Mesmin L, Blanquet-Diot S[#], Van de Wiele T[#]
Nutrients. 2022 May 21;14(10):2146. doi: 10.3390/nu14102146.

last co-authors

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1. Abstract

Dietary fibers exhibit well-known beneficial effects on human health, but their anti-infectious properties against enteric pathogens have been poorly investigated. Enterotoxigenic *Escherichia coli* (ETEC) is a major food-borne pathogen that causes acute traveler's diarrhea. Its virulence traits mainly rely on adhesion to an epithelial surface, mucus degradation, and the secretion of two enterotoxins associated with intestinal inflammation. With the increasing burden of antibiotic resistance worldwide, there is an imperious need to develop novel alternative strategies to control ETEC infections. This study aimed to investigate, using complementary in vitro approaches, the inhibitory potential of two dietary-fiber-containing products (a lentil extract and yeast cell walls) against the human ETEC reference strain H10407. We showed that the lentil extract decreased toxin production in a dose-dependent manner, reduced pro-inflammatory interleukin-8 production, and modulated mucus-related gene induction in ETEC-infected mucus-secreting intestinal cells. We also report that the yeast product reduced ETEC adhesion to mucin and Caco-2/HT29-MTX cells. Both fiber-containing products strengthened intestinal barrier function and modulated toxin-related gene expression. In a complex human gut microbial background, both products did not elicit a significant effect on ETEC colonization. These pioneering data demonstrate the promising role of dietary fibers in controlling different stages of the ETEC infection process.

2. Introduction

The food and water-borne enterotoxigenic *Escherichia coli* (ETEC) is the first agent responsible for travelers' diarrhea, with hundreds of millions of diarrheal episodes worldwide (Khalil *et al.* 2018). The site of action for ETEC is mostly localized in the distal part of the human small intestine (Stintzing and Möllby 1982; Allen, Randolph and Fleckenstein 2006; Rodea *et al.* 2017). There, a myriad of virulence factors supports its infectious cycle (Vipin Madhavan and Sakellaris 2015; Mirhoseini, Amani and Nazarian 2018). The mucus-degrading proteins (YghJ and EatA) and adhesins (like FimH and Tia) facilitate ETEC access to the epithelial brush border and promote ETEC attachment, respectively (Kumar *et al.* 2014; Tapader, Bose and Pal 2017). Then, ETEC's close proximity with the intestinal epithelium favors the action of the LT and/or ST toxins. These enterotoxins trigger water and ions secretion in the intestinal lumen leading to cholera diarrhea-like symptoms (Turner *et al.* 2006; Qadri *et al.* 2007). In parallel, an inflammatory response elicited by different pro-inflammatory serologic and fecal markers (Greenberg *et al.* 2002; Brubaker *et al.* 2021) and microbiota composition changes (David *et al.* 2015; Pop *et al.* 2016) are also reported in infected patients, suggesting their role in ETEC physiopathology.

To date, the treatment for ETEC-associated diarrhea is the same as for any acute secretory diarrheal disease. Antibiotic therapy is often prescribed to patients, albeit not routinely recommended due to the rise of antimicrobial resistance and potential side effects on human health (Taylor, Hamer and Shlim 2017). Potential alternative strategies are therefore being investigated including bacteriophages (Piya *et al.* 2019b), probiotics (Roussel *et al.* 2018b), or in a less well-known way with dietary fibers (Roberts *et al.* 2013; Sauvaitre *et al.* 2021a).

Dietary fibers are generally defined as carbohydrate polymers with 10 or more monomeric units, which are not hydrolyzed by the endogenous enzymes in the small intestine of humans, thus providing preferential substrates for gut microbes (Jones 2014). They can be divided into subgroups according to their origin, structure and physicochemical properties (Porter and Martens 2017; Deehan *et al.* 2018). Most of dietary fibers consumed by humans are of plant origin (e.g. from vegetables, legumes or cereals), but some of them are also derived from animal products (e.g. milk), fungi or bacteria (Porter and Martens 2017). They are subdivided into soluble and insoluble fibers: the soluble fraction is degraded faster by microbes in the gastrointestinal tract, while the insoluble one constitutes a physical microbial attachment surface on its own during intestinal transit (Deehan *et al.* 2018; De Paepe *et al.* 2019, 2020). Dietary fibers have well-known beneficial health effects in humans, such as transit regulation,

slowing down of glucose absorption, immune system modulation and support of gut microbiota diversity (Hooper, Littman and Macpherson 2012; Makki *et al.* 2018).

More recently, *in vitro* studies have also shown the antagonistic properties of fibers against various enteric bacterial pathogens, mostly through a direct bacteriostatic effect (Chantarasataporn *et al.* 2014; Ma *et al.* 2016; Vardaka, Yehia and Savvaidis 2016; Garrido-Maestu *et al.* 2018), anti-adhesion properties on intestinal cells or a decoy for pathogen/toxin binding to mucosal polysaccharides (Idota *et al.* 1995; Di *et al.* 2017; Liu *et al.* 2017; Leong *et al.* 2019; Sauvaitre *et al.* 2021a). The effects from dietary fiber can also be indirectly mediated by gut microbiota modulation, e.g. by supporting probiotic species showing anti-infectious properties (Fooks and Gibson 2003). Recently, a novel potential mechanism of action was also suggested: by presenting another nutrient source to the resident microbiota, fibers could also lure it from mucus consumption, thereby impeding pathogen access to the underlying epithelium (Desai *et al.* 2016; Martens, Neumann and Desai 2018). Since ETEC needs to interact with the mucus layer to fulfil its infection cycle, this strategy could be particularly relevant to investigate. However, up to now, studies specifically addressing dietary fiber affects ETEC strains from human origin are really scarce. Only milk oligosaccharides and plantain soluble fibers were proven to reduce adhesion of ETEC strains Pb-176, CECT 685 and C410 to human Caco-2 intestinal epithelial cells (Idota and Kawakami 1995; Roberts *et al.* 2013; Salcedo *et al.* 2013). Faced with this lack of data, we previously conducted a short screening program to select the most promising fiber-containing products among eight candidates, i.e. an homemade lentil extract and specific yeast cell walls from *Saccharomyces cerevisiae* AQP 12260 (Sauvaitre *et al.* 2021a).

The aim of the present study is to investigate more deeply the anti-infectious potential of these fiber structures against the human ETEC H10407 reference strain. By using complementary *in vitro* approaches simulating the human gastrointestinal tract, we investigated the direct and indirect effect of lentil- and yeast wall fiber-containing products on various stages of ETEC physiopathology, namely bacterial growth, adhesion to mucus and intestinal epithelial cells, toxin production and regulation of main virulence genes, impact on intestinal barrier integrity, induction of innate immunity and human gut microbiota modulation.

3. Materials and methods

3.1. Preparation and characterization of dietary fiber-containing products

The specific yeast cell wall from *Saccharomyces cerevisiae* AQP 12260 and the raw green lentils were provided by Lallemand Inc. (Blagnac, France) and HARi&CO (Lyon, France), respectively. Lentils were prepared according to their usual household of consumption and extracted by a digestion step followed by ethanol precipitation, as previously described (Sauvaitre *et al.* 2021a). Briefly lentils were boiled (30 min), washed in sterile distilled water, ground at maximum speed in a blender 8010S (Waring, Stamford, USA) until homogeneity, and filtered through a 0.9 mm diameter pore filter. For each 200 g of raw products, 10 g of pancreatin (P1750, Merck, Darmstadt, Deutschland) was added to 200 mL of sterile distilled water and centrifuged (8000× *g*, 30 min, 4°C). The supernatant was collected and added to the ground material with 3.2 mg of trypsin (T0303, Merck, Darmstadt, Deutschland). Digestion was performed for a total duration of 6 h (37°C, 100 rpm). To precipitate soluble fibers, three volumes of 96% ethanol were then added to the mixture under agitation (4°C, 100 rpm, 1 h). The solution was centrifuged (2500× *g*, 15 min, 4 °C), and the pellet was washed three times in 75% ethanol. Finally, the pellet was dried in an incubator (42°C, overnight) and then finely ground at full speed under sterile conditions in a blender 8010S (Waring, Stamford, USA). The lentil extract was found to be sterile by plating on plate counting agar. The specific yeast cell walls were autoclaved (121°C, 15 min) prior to use in *in vitro* experiments.

The detailed nutritional analysis of the two fiber-containing products used in this study was performed by an external company (CAPINOV, Landerneau, France) and the results are provided in **Table 3.1**. In all experiments, the products were used at the final fiber concentration of 2 $g.L^{-1}$ otherwise stated.

Table 3.1. Detailed composition of the two fiber-containing products tested.

Parameters tested	Results (+/- incertitude) ($g.100g^{-1}$)		Analytical methods
	Lentils	Specific yeast cell wall	
Moisture at 70°C and low pressure	6.1 (+/- 0.5)	7.7 (+/- 0.5)	Steaming
Ashes	4.1 (+/- 0.2)	3.0 (+/- 0.2)	Incineration
Protein "N*6.25"	36.1 (+/- 1.0)	13.3 (+/- 0.6)	Kjeldahl
Fat "B"	0.90 (+/- 0.50)	15.9 (+/- 1.3)	Soxhlet

Total carbohydrates	52.7	60.2	Calculation
Total dietary fibers	34.8 (+/- 2.0)	57.7 (+/- 2.0)	Enzymatic method
Carbohydrates	17.9	2.5	Calculation

3.2. ETEC strain and growth conditions

The prototypical ETEC strain H10407 serotype O78:H11:K80 (ATCC® 35401, LT^+, ST^+, CFA/I^+), isolated in Bangladesh from a patient with a cholera-like syndrome (Evans *et al.* 1977) was used in this study. Bacteria were routinely grown under agitation (37°C, 120 rpm, overnight) in LB broth.

3.3. Growth kinetics assays in broth media

ETEC strain H10407 (initial concentration of 10^7 $CFU.mL^{-1}$) was allowed to grow aerobically (37°C, 5 hours, 100 rpm) in complete LB or M9 minimal media (Sigma, St. Louis, MO, USA), with or without each fiber-containing products (2 $g.L^{-1}$). Medium was regularly sampled and plated onto LB agar for ETEC numeration (n=3).

3.4. Toxin measurement in broth media

LT concentration was assayed by cultivating ETEC strain H10407 in CAYE medium (37°C, 100 rpm) with or without fiber-containing products at various concentrations (ranging from 0.0625 to 8 $g.L^{-1}$). After overnight culture, medium was centrifuged (3000 g, 5 min, 4 °C) and toxin concentrations were measured in the supernatant by GM1-ELISA assay as previously described (Sauvaitre *et al.* 2021a). Pure LT toxin detection inhibition assays were also carried out as aforementioned with pure LT-Cholera B toxin sub-unit (Sigma-Aldrich, Saint-Louis, MO, USA) added in CAYE medium at a concentration of 500 $ng.mL^{-1}$ without ETEC bacteria. The absence of negative effect of various doses of fiber-containing products on ETEC growth was verified by plating on LB agar plates at the end of LT experiments. Three independent biological replicates were performed for each assay.

3.5. Mucin bead adhesion assays

Mucin-alginate beads were obtained as already described (Deschamps *et al.* 2020). Briefly, the mixture containing 5% (w/v) porcine gastric mucin type III and 2% (w/v) sodium alginate (Sigma-Aldrich, Saint-Louis, MO, USA) was dropped using a peristaltic pump into a sterile solution of 0.2 M $CaCl_2$ under agitation (100 rpm). Beads were stored at 4°C no more

than 24 hours prior experiments. For yeast-alginate beads, mucin was replaced by the specific yeast cell wall product at the same concentration (5% w/v). Adhesion assays on beads were carried out as followed: ETEC was inoculated at a dose of 10^7 CFU.mL^{-1} and allowed to adhere for a 1-hour contact period. At the end of experiment, beads were washed three times with ice-cold sterile physiological water and crushed with an ultra-turrax apparatus (IKA, Staufen, Germany). The resulting suspensions were then serially diluted and plated onto LB agar plates for ETEC numeration ("adhered" cells). On order to test adhesion inhibition by mannose residues, D-mannose (Sigma-Aldrich, Saint-Louis, MO, USA) was added at a final concentration of 10 g.L^{-1} in the medium prior to ETEC inoculation. Three independent biological replicates were performed.

3.6. Caco-2 and HT29-MTX cell culture assays

Caco-2 and HT29-MTX cells were cultivated as already reported (Sauvaitre *et al.* 2021a). Caco-2/HT29-MTX co-culture (ratio 70:30) was maintained for 18 days to reach the full differentiation stage. Cells were pre-treated or not with fiber-containing products (2 g.L^{-1}) for a 3-hour period. Cells were then infected with ETEC strain H10407 at MOI 100 for 3 additional hours (37°C, 5% CO_2) in antibiotic-antimycotic free medium. At the end of experiment, to monitor ETEC "planktonic" bacteria cells, culture medium was collected and centrifuged (3000 g, 5 min, 4°C). The resulting pellet was kept in RNA later (Invitrogen, Waltham, MA, USA) at -20°C for downstream RNA extraction and RT-qPCR analysis of ETEC virulence genes. To monitor ETEC "adhered" bacteria, cell layers were washed three times with ice-cold PBS pH 7.2 (ThermoFisher, Waltham, MA, USA). In a first set of experiments, Caco-2/HT29-MTX cells were lysed with 1% Triton X-100 (Sigma-Aldrich, Saint-Louis, MO, USA). Cell lysates were plated onto LB agar to determine the number of ETEC bacteria adhered to the cells or further centrifuged (3000 g, 5 min, 4°C). The resulting supernatant was used to measure intracellular pro-inflammatory IL-8 levels, while pellet cells were stored in RNA later (Invitrogen, Waltham, MA, USA) at -20°C for further prokaryote RNA extraction. In a second set of experiments, RNAs from adhered bacteria were extracted for eukaryotic gene expression analysis (ETEC virulence genes). Control experiments were also performed with non-infected Caco-2/HT29-MTX cells and in the DMEM medium devoid of intestinal cells for virulence gene expression analysis. The impact of both ETEC strain H10407 and fiber-containing products on intestinal cell viability was controlled during a 3 h-time course using Trypan blue exclusion assay. For each set of experiments, at least three independent biological replicates were performed.

3.7. Measurement of Caco-2/HT29-MTX permeability on transwells

For permeability experiments, Caco-2/HT29-MTX cells were rinsed with PBS pH 6.8 and incubated with an apical concentration of caffeine (1 $g.L^{-1}$) or atenolol (50 $mg.L^{-1}$) in fresh DMEM medium containing or not dietary fiber-containing products (2 $g.L^{-1}$). Medium was collected after 2h- incubation at both apical and basolateral sides of the transwells. Caffeine and atenolol concentrations were determined by HPLC (Elite LaChrom, Merck HITACHI, USA) using a Onyx™ Monolithic C18 LC column 100 x 4.6 mm at 20°C (Phenomenex, Torrance, CA, USA) and a Interchimm C18 column of 250 x 4.6 mm at 40°C (Interchim, Montluçon, France), respectively. Mobile phase was composed of acetonitrile/PBS pH 6.5 (10:90, v/v) and acetonitrile/water (20:80, v/v) with 10mM ammonium acetate for caffeine and atenolol, respectively. Data were obtained and analyzed by the EZChrom Elite software at 235 and 275 nm for caffeine and atenolol, respectively. Caffeine and atenolol concentrations were calculated from standard curves established from known serial dilutions of each compound. The molecular absorption was defined as the percentage of basal molecules/total molecules introduced. TEER was measured regularly during the time course of experiment (total duration = 3 hours) with a volt/ohmmeter (World Precision Instruments, Hessen, Germany). Three independent biological replicates were performed.

3.8. RNA extractions

Eukaryotic RNAs were extracted with the RNeasy Plus Mini Kit (Qiagen, Germany). Total bacterial RNAs were extracted using the TRIzol® method (Invitrogen, Waltham, MA, USA) as already described (Roussel *et al.* 2020a), with an additional purification step with MinElute Cleanup Kit (Qiagen, Hilden, Deutschland). Nucleic acid purity was checked and RNA was quantified using the NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). To remove any contaminating by genomic DNA, DNAse treatment was performed (Roussel *et al.* 2020a).

3.9. Quantitative reverse transcription (RT-qPCR) analysis of ETEC virulence genes

cDNA amplification was achieved using a CFX96 apparatus (Bio-Rad, Hercules, CA, USA) and q-PCR was performed using primers listed in **Table 3.2**. qPCR data were analyzed using the comparative $E^{-\Delta\Delta Ct}$ method and normalized with the reference genes *TufA* and *ihfB*. The amplification efficiency of each primer pair was controlled from the slope of the standard

curves $E = 10^{(-1/slope)}-1$ based on a serial dilution of a pool of 3 ETEC cDNA sample. Differences in the relative expression levels of each virulence gene were calculated as follows: $\Delta\Delta Ct = (Ct_{target\ gene} - Ct_{reference\ gene})_{\text{in the tested condition}} - (Ct_{target\ gene} - Ct_{reference\ gene})_{\text{in the reference condition}}$ and data were derived from $E^{-\Delta\Delta Ct}$.

3.10. Quantitative reverse transcription (RT-qPCR) analysis of selected intestinal cell genes

Reverse transcriptions were first performed with the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). Then, quantitative PCR was carried out using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and a TaqMan 7900 Fast instrument (Thermo Fisher Scientific, Waltham, MA, USA), with primers listed in **Table 3.2**. Expression of host genes related to mucin synthesis, tight junction's proteins and inflammation were investigated. Data were analyzed with SDS 2.3 software (Thermo Fisher Scientific, Waltham, MA, USA) using the comparative $2^{-\Delta\Delta Ct}$ method and normalized with the reference genes *GAPDH*, *HPRT* and *PPIA*. The amplification efficiency of each primer pair was controlled from the slope of the standard curves $E = 10^{(-1/slope)}-1$ based on a serial dilution of a pool of six RNA samples from the experiments.

Table 3.2. Primers used in this study.

Gene	Target	Primer sequence 5'-3'	Amplicon length (pb)	References
Genes to monitor ETEC survival by qPCR (in fecal batches)				
eltB	LT toxin	F-GGCAGGCAAAAGAGAAATGG R-TCCTTCATCCTTTCAATGGCT	117	Lothigius *et al.* 2008
16S	Reference gene	F- NNNNNNNNNTCCTACGGGNGGCWGCAG R- NNNNNNNNNNTGACTACHVGGGTATCTAAKCC	464	Klindworth *et al.* 2013
Genes for RT-qPCR analysis of ETEC virulence genes				
tufA	Reference gene	F-GACATGGTTGATGACGAAGA R-GCTCTGGTTCCGGAATGTA	199	Delmas *et al.* 2019
ihfB	Reference gene	F-CTGCGAGGCAGCTTCCAGTT R-GCAGCAACAGCAGCCGCTTA	419	Zhou *et al.* 2011
eltB	LT toxin	F-GGCAGGCAAAAGAGAAATGG R-TCCTTCATCCTTTCAATGGCT	117	Lothigius *et al.* 2008
leoA	LT enterotoxin output	F-AAACGGTGCATATCCTCGTC R-AAATGCTGCCACCGAAATAC	168	Roussel *et al.* 2020
estP	ST toxin	F-TCTTTCCCCTCTTTTAGTCAG R-ACAGGCAGGATTACAACAAAG	165	Rodas *et al.* 2009
tolC	TolC outer membrane protein (ST toxin secretion)	F-AAGCCGAAAAACGCAACCT R-CAGAGTCGGTAAGTGACCATC	101	Swick *et al.* 2011
tia	Tia Adhesin	F-ACAGGCTTTTATGTGACCGGTAA R-GACGGAAGCGCTGGTCAGT	67	Nicklasson *et al.* 2012
fimH	Minor component of Type I pili	F-GTGCCAATTCCTCTTACCGTT R-TGGAATAATCGTACCGTTGCG	164	Hojati, Molaie and Gholipour 2015

yghJ	Mucinase	F-CCCTGTTAGCCGGTTGTGAT R-GGTATCGGTTCTGGCGTAGG	166	This study
eatA	Mucinase	F-AACGGAAGCACCGTCATTCT R-CAGAGTCAGGGAGGCGTTTT	363	This study
rpoS	Environmental stresses response	F-GCGCGGTAGAGAAGTTTGAC R-GGCTTATCCAGTTGCTCTGC	229	Rahman *et al.* 2006
Genes for bacterial quantification by RNA fluorescent *in situ* hybridization in batch fermentation				
16S	Eubacteria 16S rRNA	1-GCTGCCTCCCGTAGGAGT 2-CGGCGTCGCTGCGTCAGG 3-MCGCARACTCATCCCCAAA	N/A	Amann *et al.* 1990
16S	*E. coli* 16S rRNA	1-GCAAAGGTATTAACTTTACTCCC *(Cy5 in 5')* 2-GCAGCAACAGCAGCCGCTTA *(Helper probe)*	N/A	Baudart and Lebaron 2010
Genes for RT-qPCR analysis of host response				
Mucin related genes				
MUC1	Mucin 1	F-AGACGTCAGCGTGAGTGATG R-CAGCTGCCCGTAGTTCTTTC	172	Dorier *et al.* 2019
MUC2	Mucin 2	F-CAGTGTGTCTGTAACGCTGG R-AATCGTTGTGGTCACCCTTG	160	This study
MUC5AC	Mucin 5AC	F-GTTTGACGGGAAGCAATACA R-CGATGATGAAGAAGGTTGAGG	278	Huang *et al.* 2019
MUC5B	Mucin 5B	F-GTGACAACCGTGTCGTCCTG R-TGCCGTCAAAGGTGGAATAG	171	Huang *et al.* 2019
MUC12	Mucin 12	F-ACCTTAGCACCAGGGTTGTG R-GGAGGATGCGTCATTCATCT	204	Dorier *et al.* 2019
MUC17	Mucin 17	F-TGCAGAACAGGACCTCAGTG R- AGGTCATCTCAGGGTTGGTG	206	Dorier *et al.* 2019
TFF3	Trefoil factor 3	F-AGGAGTACGTGGGCCTGTCT R-AAGGTGCATTCTGCTTCCTG	175	Dorier *et al.* 2019
KLF4	Kruppel-like-factor 4	F-CTCACCCCACCTTCTTCACC R-AAGGTTTCTCACCTGTGTGG	202	This study
Tight junctions related genes				
CLDN1	Claudin 1	F-TGGAAGACGATGAGGTGCA R-AAGGCAGAGAGAAGCAGCA	206	Dorier *et al.* 2019
CLDN2	Claudin 2	F-CATTTGTACCTGCAAGGTCTTCT R-GCCTAGGATGTAGCCCACAA	236	This study
OCLN	Occludin	F-ACTTCAGGCAGCCTCGTTAC R-CCTGATCCAGTCCTCCTCCA	170	Dorier *et al.* 2019
TJP1	Zonula occludens 1	F-GTGCTGGCTTGGTCTGTTTG R-TCTGTACATGCTGGCCAAGG	159	Dorier *et al.* 2019
Inflammation related genes				
TNF	Tumor necrosis factor α	F-GCCCATGTTGTAGCAAACCC R-AGGAGGTTGACCTTGGTCTG	242	This study
IL6	Interleukin 6	F-CCAGAGCTGTGCAGATGAGTACA R-GGCATTTGTGGTTGGGTCAGG	101	Ponce de León-Rodríguez, 2019
IL8	Interleukin 8	F-TCTGCAGCTCTGTGTGAAGG R-TGAATTCTCAGCCCTCTTCAA	252	This study
IL10	Interleukin 10	F-GGCGCTGTCATCGATTTCTTC R-CACTCATGGCTTTGTAGATGCC	108	Ponce de León-Rodríguez, 2019
IL1β	Interleukin 1*β*	F-AGCCATGGCAGAAGTACCTG R-TGGTGGTCGGAGATTCGTAG	171	Netsch *et al.* 2006
Housekeeping genes				
GADPH	Housekeeping gene	F-GGAGTCCACTGGCGTCTT R-GAGTCCTTCCACGATACCAAA	235	Huang *et al.* 2019
HPRT	Housekeeping gene	F-TTGCTGACCTGCTGGATTAC R-AGTTGAGAGATCATCTCCAC	149	Berger *et al.* 2017
PPIA	Housekeeping gene	F-TGCTGACTGTGGACAACTCG F-TGCAGCGAGAGCACAAAGAT	136	Raveschot *et al.* 2020

F: Forward, LT: Heat-labile enterotoxin, R: Reverse, ST: Heat-Stable enterotoxin.

3.11. Measurement of interleukin-8 by ELISA

Pro-inflammatory IL-8 cytokine concentrations were determined in cell lysates from Caco-2/HT29-MTX co-culture experiments according to the manufacturer's instructions (DuoSet ELISA, human CXCL8/IL-8, RnD Systems, Minneapolis, MN, USA). Results were expressed as fold changes compared to control experiments performed without ETEC (non-infected) nor fiber-containing product (non-treated).

3.12. Batch experiments

Batch experiments were carried out for 24 hours in 60 mL penicillin bottles containing 20 mL of nutritive medium containing 60 mucin-alginate beads. The medium was composed of per L: 1 g potato starch, 1 g yeast extract, 1 g proteose peptone, and 1g type III pig gastric mucin (all from Sigma Aldrich, St. Louis, MO, US) suspended into 0.1 M phosphate buffer (pH 6.8) and autoclaved before use. The lentil extracts and yeast cell walls products were added at the final fiber concentration of 2 $g.L^{-1}$. In the control condition with no dietary fiber-containing product (non-treated), the composition of the nutritive medium was compensated with addition of 0.5 g guar gum, 1 g pectin and 0.5 g xylan (same final total fiber concentration).

To examine the inter-individual variability of ETEC interactions with dietary fiber-containing products and human gut microbiota, experiments were replicated with fecal samples from six healthy individuals. These donors were three males (donors 1, 2, 3) and three females (donors 4, 5, 6), ranging in age from 20 to 30 years, without any history of antibiotic use six months prior to the study. Consent for fecal collection was obtained under registration number BE670201836318 (Gent University). Fecal collection and fecal slurry preparation were performed as already described (De Paepe *et al.* 2017). Inoculation at a 1:5 dilution of the 20% (w/v) fecal slurry resulted in a final concentration of 4% (w/v) fecal inoculum in the penicillin bottles. To reproduce stresses that pathogens have to face during transit in the stomach and small intestine in humans, ETEC strain H10407 was pre-digested using a simple static gastro-intestinal procedure as described in **Table 3.3**. ETEC was inoculated at the final concentration of 10^8 $CFU.mL^{-1}$. The penicillin bottles were flushed with N_2/CO_2 (80%/20%) during 20 cycles to obtain anaerobic conditions. The cycle was stopped at overpressure and before the start of the experiment, the bottles were set at atmospheric pressure. Penicillin bottles were incubated (37°C, 120 rpm) on an orbital shaker KS 4000 i (IKA, Staufen, Germany) and aliquots were taken immediately after the start of the incubation (T0) and at 24h- fermentation (T24h) from the liquid and atmospheric phases. Mucin-alginate beads were collected 24h post-inoculation

and washed twice in ice-cold physiological buffer before storage. All aliquots were immediately stored at -20°C, except samples for flow cytometry that were fixed before storage.

Table 3.3. Static *in vitro* gastro-ileal digestion procedure.
A static batch incubation (Erlenmeyer) was used to reproduce the physicochemical parameters of a gastro-ileal digestion. Digestive secretions and solutions for pH adjustment were manually added during the 90 min digestion.

Parameters of static *in vitro* digestion	Gastric vessel	Duodenum-Ileum vessels
pH	From 6 (T0) to 2.1	Maintained at 6.8
Volume (mL)	50	90
Secretions	**(i)** 5.36 mg pepsin (727 $U.mg^{-1}$) **(ii)** 4.28 mg lipase (32 $U.mg^{-1}$) **(iii)** HCl 0.3 M **(iv)** $NaHCO_3$ 0.5 M if necessary	**(i)** 0.9 g bile salts (27.9 mM in solution) **(ii)** 1.8 g of pancreatin 4 USP **(iii)** Trypsin 2 $mg.mL^{-1}$ (15156 U/mg protein) **(iv)** $NaHCO_3$ 0.5 M if necessary
Time period in batch (min)	30	60
Chyme mixing	100 rpm (magnetic stirrer)	100 rpm (magnetic stirrer)
[Total microbes]	Sterile	Sterile
Oxygen level (%)	20	20
Temperature (°C)	37	37

3.13. Gut microbiota metabolite analysis

SCFA production was measured using capillary gas chromatography coupled to a flame ionization detector after diethyl ether extraction as previously described (Anderson, Ellingsen and McArdle 2006; De Paepe *et al.* 2017). The gas phase composition was analyzed with a Compact gas chromatograph (Global Analyser Solutions, Breda, The Netherlands), equipped with a Molsieve 5A pre-column and Porabond column (CH_4, O_2, H_2 and N_2) or a Rt-Q-bond pre-column and column (CO_2). Concentrations of gases were determined with a thermal conductivity detector. Total pressure in the penicillin bottles was analyzed using a tensiometer (Greisinger, Regenstauf, Germany).

3.3.14. DNA extraction

DNA extraction and quality controls were performed from samples collected at T0 and T24 during batch experiments as previously described (De Paepe *et al.* 2017; Miclotte *et al.* 2020). DNA quality and quantity were verified by electrophoresis on a 1.5% (w/v) agarose gel and analysis on spectrophotometer DENOVIX ds-11 (Denovix, Wilmington, DE, USA).

15. ETEC quantification by qPCR

qPCR was performed using StepOnePlus real-time PCR system (Applied Biosystems, Waltham, MA, USA). Reactions were conducted in a total volume of 20 µL consisting of 10 µL of 2x iTaq universal SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA, USA), 2 µL of DNA template, 0.8 µL (10 µM stock) of each primer, and 6.4 µL nuclease-free water. Primers used for ETEC quantification are listed in **Table 3.2**. Data were analyzed using the comparative $E^{-\Delta\Delta Ct}$ method. The amplification efficiency of the primer pairs was determined by the generation of a standard curve based on serial dilution of five ETEC-infected samples. Differences in number of copies of the *eltB* gene was calculated as follows: $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{reference gene}})_{\text{sample of interest}} - (Ct_{\text{target gene}} - Ct_{\text{reference gene}})_{\text{reference sample}}$ and data were derived from $E^{-\Delta\Delta Ct}$. All qPCR analyses were conducted in triplicate.

3.16. ETEC quantification by RNA fluorescent *in situ* hybridization

Flow cytometry samples were fixed and prepared for RNA fluorescent *in situ* hybridization, as already described (Huang *et al.* 2007). Briefly, cells were fixed by addition of three volumes of 4% paraformaldehyde in PBS and incubated at 4°C for 3 h. Subsequently, cells were washed in PBS prior to resuspension in a 1:1 (vol: vol) mix of PBS and 96% (vol: vol) ethanol. Cells were hybridized in 100 µL hybridization buffer for 3 h at 46°C. The hybridization buffer consisted of 900 $mmol.L^{-1}$ NaCl, 20 $mmol.L^{-1}$ Tris–HCl (pH 7.2), 0.01% sodium dodecyl sulfate, 20% deionized formamide, 5mM EDTA. The buffer also contained the two *E. coli* targeting probes at the final concentration of 2 $ng.µl^{-1}$ and a combination of probes targeting eubacteria at the final concentration of 1 $ng.µl^{-1}$ each (**Table 3.2**). After hybridization, samples were washed with wash buffer (900 $mmol.L^{-1}$ NaCl, 20 $mmol.L^{-1}$ Tris–HCl pH 7.2, 0.01% sodium dodecyl sulfate) for 15 min at 48°C. After washing, cells were resuspended in 50 µL of PBS. Samples were diluted and stained with SYBR® Green I (100x concentrate in 0.22 µm-filtered dimethyl sulfoxide, Invitrogen) and incubated for 20 min at 37°C (10.1111/2041-210X.12607). Samples were analyzed immediately after incubation with a Attune NxT BRXX flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA). The flow cytometer was operated with Attune™ FocusingFluid as sheath fluid. Threshold was set on the primary emission channel of blue lasers (488 nm). The Attune Cytometric Software was used to draw the gates, but also the percentage of active *E. coli* in the total bacteria population was expressed as the number of cells showing the *E. coli* probe fluorescence out of the number of cells fluorescently labelled with the Eubacteria probes and SYBR green fluorescence.

3.17. 16S Metabarcoding analysis of gut microbial communities

Next-generation 16S rRNA gene amplicon sequencing of the V3-V4 region was performed by LGC Genomics (Berlin, Germany) on an Illumina MiSeq platform (Illumina, San Diego, California), as previously described (De Paepe *et al.* 2017), excepted that luminal and mucosal samples had undergone respectively 30 and 33 amplification cycles.

All data analysis was performed in R (4.1.2). The DADA2 R package was used to process the amplicon sequence data according to the pipeline tutorial (Callahan *et al.* 2016). In a first quality control step, the primer sequences were removed and reads were truncated at a quality score cut-off (truncQ=2). Besides trimming, additional filtering was performed to eliminate reads containing any ambiguous base calls or reads with high expected errors (maxEE=2.2). After dereplication, unique reads were further denoised using the DADA error estimation algorithm and the selfConsist sample inference algorithm (with option pooling = TRUE). The obtained error rates were further inspected and after approval, the denoised reads were merged. Subsequently, the ASV table.obtained after chimera removal was used for taxonomy assignment using the Naive Bayesian Classifier and the DADA2 formatted Silva v138 ASV's mapping back to anything other than 'Bacteria' as well as singletons were excluded and considered as technical noise (McMurdie and Holmes 2014).

3.18. Data availability

The sequence data have been deposited at NCBI Sequence Read Archive database with accession number PRJNA802368.

3.19. Statistical analysis

All statistical analysis, except the one conducted on the microbiota diversity composition results were performed using GraphPad Prism v8.0.1. Statistical data analysis on microbiota diversity was performed using in R, version 4.1.2 (R Core Team, 2016), using statistical packages as Phyloseq (v1.38)(McMurdie and Holmes 2013) for ASV's data handling, vegan v2.5.7(Dixon 2003), betapart v 1.5.4 for diversity analysis of ASV's (Baselga and Orme 2012), deseq2 v1.34 (Love, Huber and Anders 2014: 2) for significant higher/lower abundance of ASV. The evolution of the microbial community α-diversity between conditions was followed by computing the richness (Observed ASV) and evenness indexes (Shannon, Simpson, Inverse Simpson, Fisher) using vegan. To highlight differences in microbial community composition between conditions, ordination and clustering techniques were applied

and visualized with ggplot2 (v3.3.5) (Ramette 2007). NMDS was based on the relative abundance-based Bray-Curtis dissimilarity matrix (Legendre, Borcard and Peres-Neto 2005). The influence of ETEC infection and the type of beads used was determined by applying a db-RDA using the abundance-based Bray-Curtis distance as a response variable(Legendre and Anderson 1999; Ramette 2007). db-RDA was performed both including and excluding ASV1 (attributed to *Escherichi*/*Shigella*) from the ASV table. The significance of group separation between conditions was also assessed with a permANOVA analysis using distance matrixes (Ramette 2007). Prior to this formal hypothesis testing, the assumption of similar multivariate dispersions was evaluated. In order to find statistically significant differences in ASV abundance between infected and non-infected conditions, a Wald test (corrected for multiple testing using the Benjamini and Hochberg method) was applied using the DESeq2 package. The metabolic response (measured SCFA and pH) was modelled in function of the beads and infection conditions in a db-RDA analysis.

4. Results

4.1. Fiber-containing products do not impede ETEC growth in complete culture medium

When ETEC strain H10407 was grown in LB rich medium (**Fig. 3.1.A**), no statistical difference was observed between the conditions supplemented with the lentil extract (lentils) and the specific yeast cell walls (‘yeast’) compared to the negative control (‘non-treated’). Therefore, none of the two fiber-containing products was able to impede ETEC growth in complete culture medium. In M9 minimal medium (**Fig. 3.1.B**), both products were able to sustain ETEC growth compared to the non-treated condition, leading to almost 2-log difference with the control condition after 5 h incubation. This overgrowth became statistically different at 240 and 300 min according to Dunnett’s multiple comparisons test ($p < 0.05$).

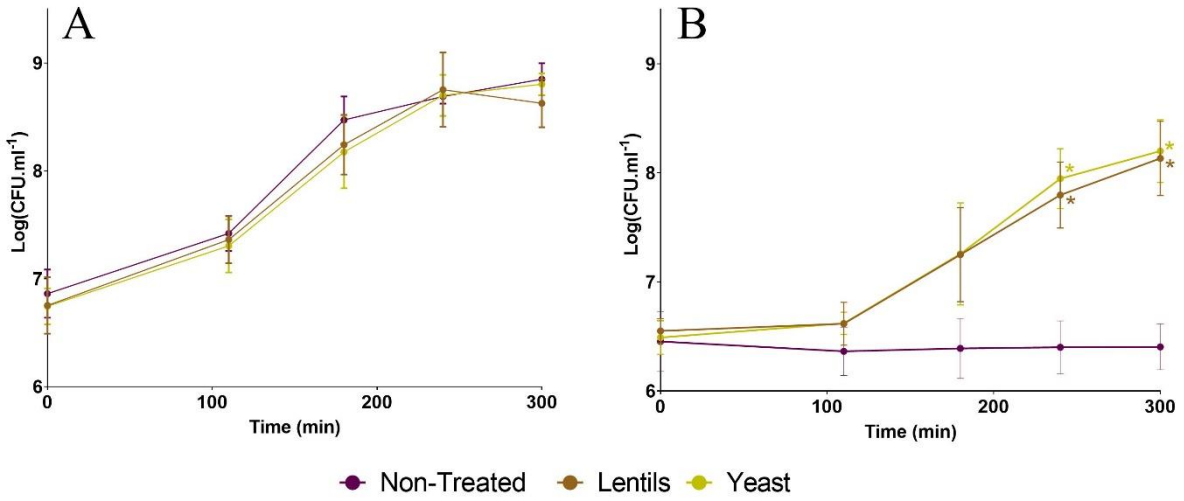

Figure 3.1. Impact of fiber-containing products on ETEC growth in broth media. Growth kinetics of ETEC strain H10407 (inoculation at 10^7 CFU.ml^{-1}) in LB medium **(A)** or in M9 minimal medium **(B)** supplemented with specific yeast cell walls (yellow line, 'yeast'), lentils (brown line, 'lentils') at 2 g.L^{-1} or not supplemented (purple line, 'non-treated'). Samples were regularly collected and plated on LB agar. Results are expressed as Log_{10} CFU.ml^{-1} (mean ± SD, n=3). Statistical differences with the control condition are indicated and provided by Dunnett's multiple comparisons test (*: p<0.05).

4.2. Lentil extracts decrease LT toxin concentrations in a dose-dependent manner

Irrespective of the dose tested, specific yeast walls had no effect on LT toxin concentrations (**Fig. 3.2.A**). In contrast, lentil extract significantly decreased LT toxin concentrations in a clear dose-dependent manner (**Fig. 3.2.B**). This inhibitory effect was significant from the low fiber dose tested of 0.065 g.L^{-1} (1.64 fold decrease, p < 0.05). LT toxin was even no longer detected when lentil concentration exceeded 1 g.L^{-1}. To dig into the possible mechanism of inhibition, we incubated the pure B sub-unit of the LT toxin at 500 ng.mL^{-1} with various doses of lentil extracts in the absence of ETEC (**Fig. 3.2.C**). Lentil extracts tended to inhibit LT toxin detection by the GM1-ELISA assay in a dose-dependent manner. At the highest fiber dose tested (8.0 g.L^{-1}), LT concentrations were 36-fold lower (6.0 ± 9.1 ng.mL^{-1}) compared to the lowest dose (0.0625 g.L^{-1}, 214.8 ± 158.9 ng.mL^{-1}, p = 0.08). Finally, we verified that the lentil extracts had no effect on ETEC growth in the CAYE medium during LT assays (**Fig. 3.2.D**).

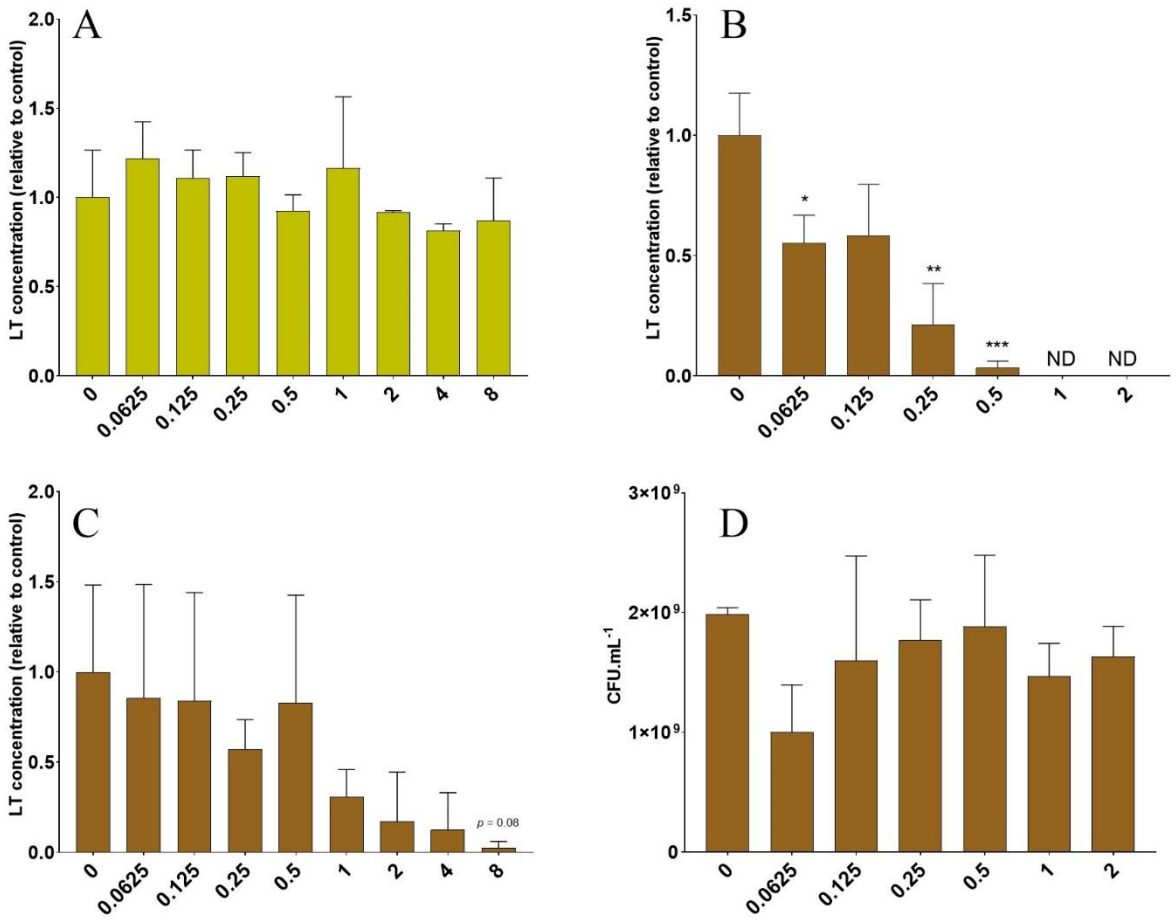

Figure 3.2. Dose effect of fiber-containing products on LT toxin concentrations in broth media.
LT concentrations were measured in CAYE medium after overnight incubation with ETEC strain H10407 (**A, B**) or pure LT-Cholera B toxin sub-unit (**C**) and increasing doses of specific yeast cell walls **(A)** or lentil extracts **(B, C)**. Fiber-containing product concentrations are expressed in $g.L^{-1}$ of final fiber content. Results are expressed as mean variations (± SD) compared to the control condition (no product added). The data represent the replicates of three independent experiments. **(D)** The dose effect of the lentil extracts on ETEC growth in CAYE medium after overnight incubation. Results are expressed as mean $CFU.mL^{-1}$ (± SD) of three independent replicates. Statistical difference with the non-treated control condition were provided by Tukey's multiple comparisons tests (*: $p < 0.05$; **: $p < 0.01$; $p < 0.001$ ***). *ND = non-detected*

4.3. Yeast cell walls inhibit ETEC adhesion to mucin and mucus-secreting intestinal cells

First, the absence of deleterious effect of both ETEC strain H10407 and fiber-containing products on intestinal cell viability was confirmed (**Suppl. Fig. 3.1**). The lentil extract and yeast cell walls were able to significantly reduce ETEC adhesion to mucin-alginate beads by about 6- and 3-fold, respectively (**Fig. 3.3.A**, $p < 0.05$). Compared to the control condition, the yeast

cell walls reduced the number of adhered ETEC bacteria to Caco-2/HT29-MTX cells from nearly one log compared to the non-treated condition ($p < 0.001$, **Fig. 3.3.B**). Additional experiments were performed with yeast-alginate beads to challenge ETEC affinity for yeast cell walls (**Fig. 3.3.C**). ETEC adhesion on yeast-alginate beads was significantly increased compared to mucin-alginate beads (nearly one-log increase, $p < 0.01$). The addition of mannose at 10 $g.L^{-1}$ in the medium did not affect ETEC adhesion on yeast-alginate beads (non-significant 33% inhibition, $p > 0.05$), while it had a significant impact on the number of adherent bacteria on mucin-alginate beads (64% inhibition, $p < 0.01$).

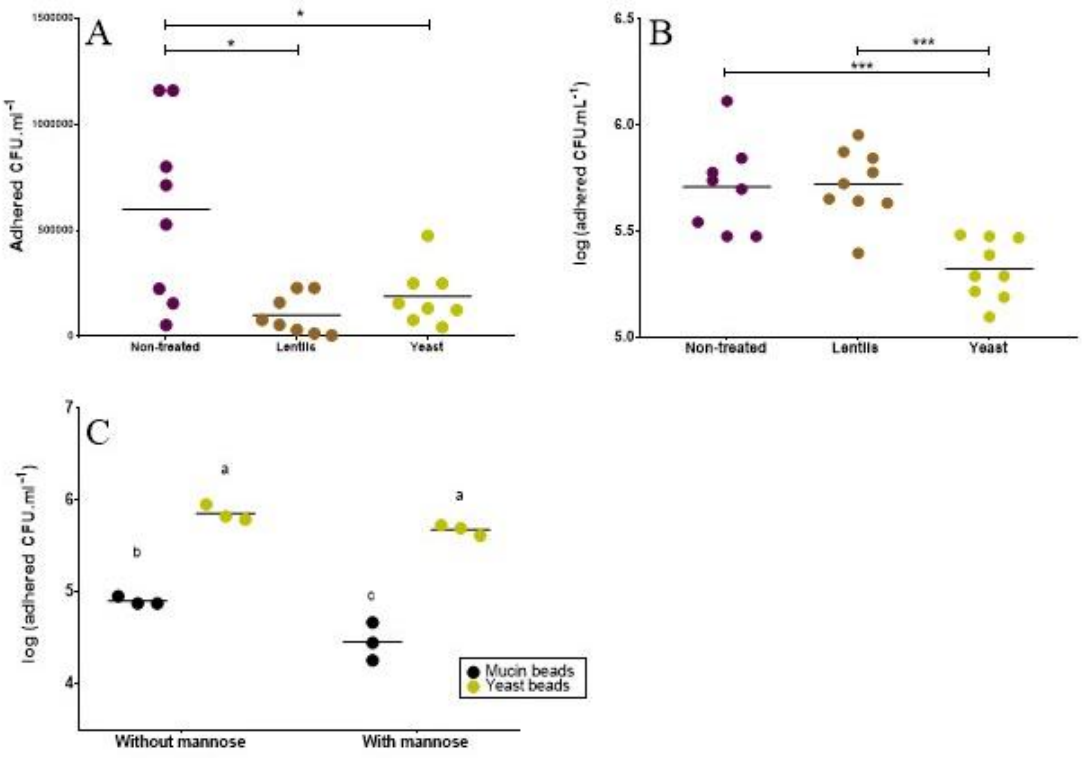

Figure 3.3. Effects of fiber-containing products on ETEC adhesion on mucin and mucus-secreting intestinal cells.

(A, B) The impact of lentil extract (brown, 'lentils') and specific yeast cell walls (yellow, 'yeast') on ETEC adhesion (initial concentration: 10^7 $CFU.mL^{-1}$) was investigated using two different *in vitro* assays, and compared to the non-treated control condition (purple). **(A)** ETEC adhesion to mucin beads after a 1h infection period. Results are expressed as adhered cells ($CFU.mL^{-1}$). **(B)** ETEC adhesion to Caco-2/HT29-MTX co-culture model after a 3 h pre-treatment with fiber-containing products followed by an additional 3-h infection period. Results are expressed as adhered cells (Log_{10} $CFU.mL^{-1}$). Figure represents all technical replicates from three independent experiments and means are indicated by black bars. Indicated *p*-values are provided by Tukey's multiple comparisons tests (*: p <0.05, ***: p <0.001). **(C)** Adhesion of ETEC strain H10407 (initial concentration: 10^7 $CFU.mL^{-1}$) on specific yeast-alginate beads (yellow dot) or mucin-alginate beads (black dot), with or without mannose (10 $g.L^{-1}$). Each point represents one of three independent biological replicates and means are indicated by black

bars. Results that are not different from each other according to Tukey's multiple comparisons tests are grouped under a same letter ($p < 0.05$).

4.4. Both fiber-containing products modulate ETEC toxin-related virulence gene expression

The impact of the fiber-containing products on ETEC strain H10407 virulence genes was analyzed using two different experimental set-ups: with Caco-2/HT29-MTX cells (**Fig. 3.4.A**) or in the DMEM medium devoid of intestinal cells (**Fig. 3.4.B**). Overall, lentil extract and specific yeast cell walls had a strong effect on virulence gene expression of planktonic ETEC bacteria (i.e non-adhered), whether in presence or absence of intestinal cells. Interestingly, lentil extract upregulated the expression of *fimH* adhesin (5.3- to 8.6-fold) and *YghJ* mucinase (2.3- to 10.3-fold) genes (**Fig. 3.4.A** and **4.B**) while also downregulating the expression of the two toxin genes *eltB* and *estp*, as well as *tolC* which participates in ST toxin secretion and *rpoS* gene involved in environmental stress responses. The presence of intestinal cells did not impact the modulatory effect from lentils towards ETEC gene expression. The yeast cell walls increased the expression of the two adhesins *fimH* and *tia*, as well as the genes involved in LT toxin production and secretion, *eltB* and *leoA*, from 1.32- to 4.47-fold, depending on the genes (**Fig. 3.4.A** and **4.B**). In the non-treated conditions, cell adhesion increased virulence gene expression as reported by *fimH, eltB* and *estP* respective 5.5-, 2.3- and 3.0-fold increases ($p < 0.05$, **Fig. 3.4.A**). Compared to planktonic bacteria, the modulation of adhered bacteria virulence by dietary fiber-containing products was more subtle (**Fig. 3.4.A**). The two compounds reduced *eltB* and *estP* toxin genes induction to a maximum of 1.7-fold compared to the non-treated control (**Fig. 3.4.A**). In particular, yeast walls significantly reduced *estP* gene induction in adhered bacteria by 90% ($p < 0.05$). In contrast, none of the fiber products succeeded in reducing the 5-fold *fimH* induction by cell adhesion (**Fig. 3.4.A**), with a slight promoting effect for yeast cell walls (1.28-fold increase, $p < 0.05$). Lastly, both lentil extract and yeast walls tended to reduce the environmental stresses encountered by adhered ETEC, as reported by the respective 60% and 70% decreases in *rpos* expression (**Fig. 3.4.A**).

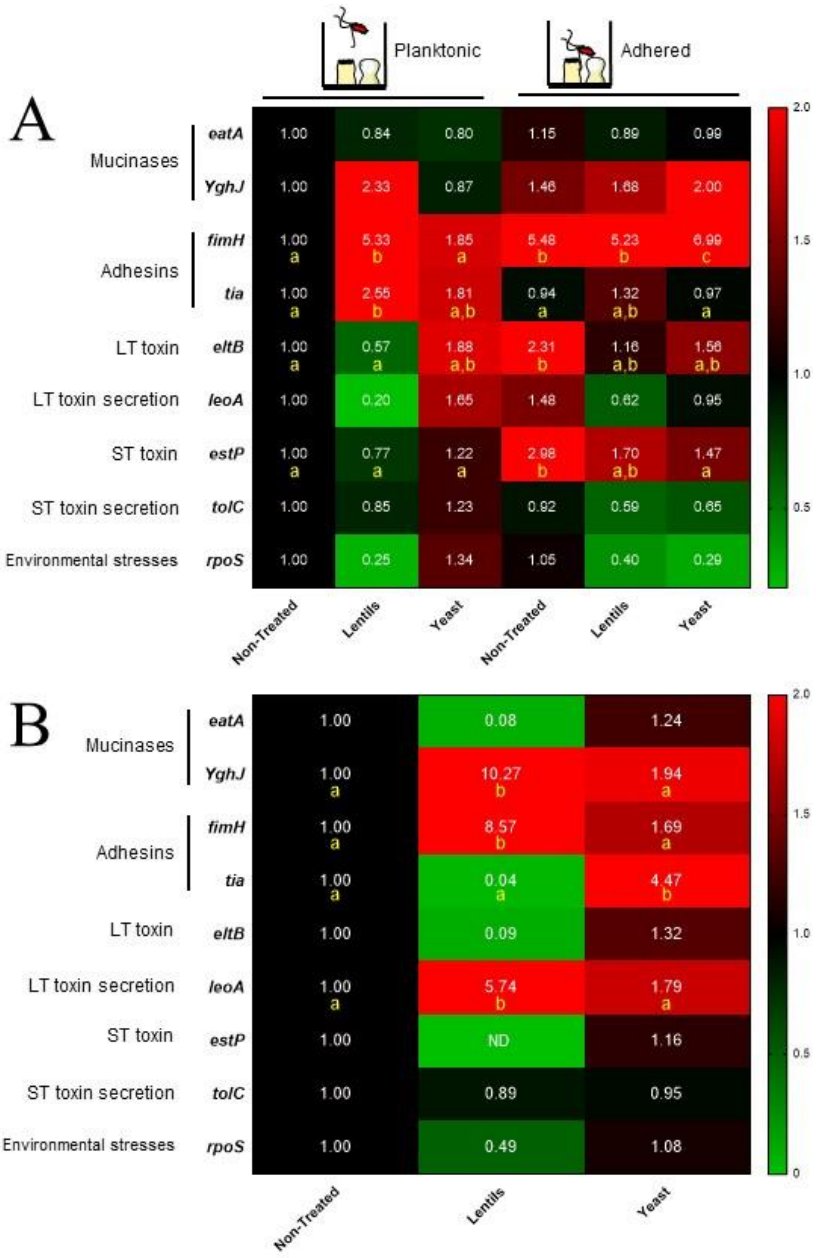

Figure 3.4. Impact of fiber-containing products on virulence gene expression of planktonic and adherent ETEC cells. ETEC virulence gene expression was analyzed by RT-qPCR in Caco-2/HT29-MTX cells infected at MOI 100 (**A**) or in DMEM medium as a control condition devoid of intestinal cells (**B**), with or without lentil extract ('lentils') and specific yeast cell walls ('yeast') at a final concentration of 2 $g.L^{-1}$. Results are expressed and colored according to fold-change expression compared to the control condition (planktonic bacteria non-treated in (**A**), non-treated medium in (**B**)). Figure represents at least three independent experiments. If a statistical difference was reached, results that are significantly different from each other according to Tukey's multiple comparisons tests are grouped under different yellow letters ($p < 0.05$). NT = non-treated, ND = non-detected.

4.5. The lentil extract limits ETEC-induced inflammation

Host innate immune response related genes (cytokines) were selected and analyzed during Caco-2/HT29-MTX experiments. ETEC infection of intestinal cells triggered the expression of all cytokine genes, as reported by the respective 65-, 5-, 63-, 244- and 2-fold increases in *TNF-α*, *IL-1β*, *IL-6*, *IL-8* and *IL-10* expressions ($p < 0.05$, **Fig. 3.5**). The lentil extract tended to reduce induction of all of these genes, with significance reached for *IL-1β*, *IL-6* and *IL-10* ($p < 0.05$), with decreases of 52, 52 and 41%, respectively (**Fig. 3.5.A, 3.5.C and 3.5.D**). The results were more mitigated with the specific yeast cell walls which only reduced

IL-10 expression ($p < 0.01$) (**Fig. 3.5A**). We further analyzed IL-8 concentration to assess the impact of fiber-containing product on cytokine induction at the protein level. As expected, ETEC inoculation induced a significant ($p < 0.001$) 1.6-fold increase in intracellular IL-8 production (**Fig. 3.5.F**). Both the yeast walls and lentil extract were able to significantly decrease IL-8 intracellular production under non-infected conditions ($p < 0.05$). In the infected condition, the protective effect was mostly preserved for lentils ($p < 0.001$) , with relative IL-8 levels comparable to the control condition without any fiber nor bacteria (0.85 ± 0.07 *versus* 1.00 ± 0.12), while results obtained with yeast walls almost reached significance ($p = 0.06$).

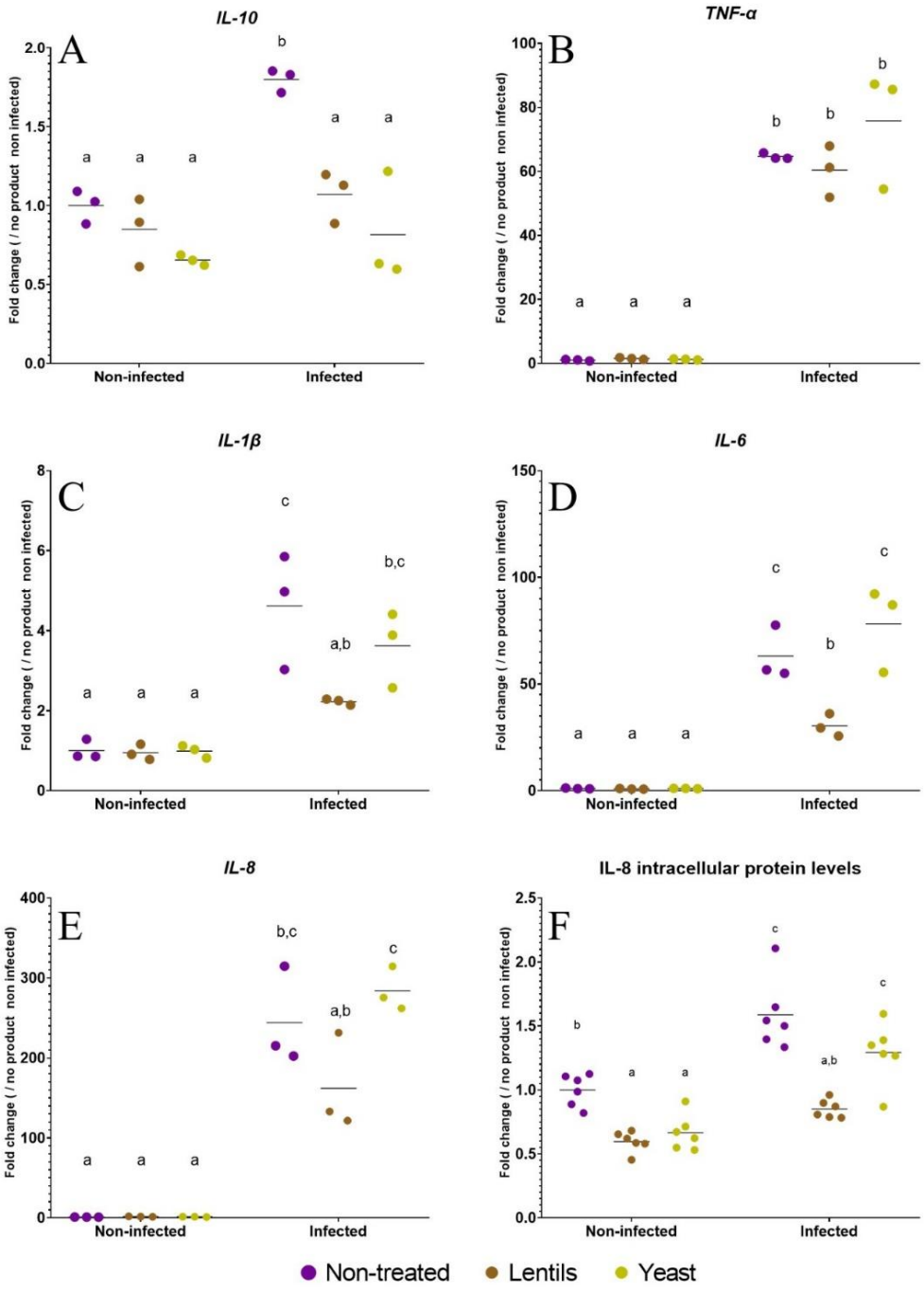

Figure 3.5. Modulation of host innate immune related genes by fiber-containing products. Caco-2/HT29-MTX were infected with ETEC strain H10407 (10^7 CFU.mL^{-1}, MOI 100) after a 3-hour pre-treatment with the lentil extract (brown dots, 'lentils') or specific yeast cell walls (yellow dots, 'yeast'). Non-infected and non-treated conditions (purple dots, 'non-treated') were used as control experiments. Cytokines (*IL-10*, *TNF-α*, *IL-β*, *IL-6* and *IL-8*)-related gene expressions were analyzed by RT-qPCR **(A-E)** and the interleukin-8 (IL-8) intracellular protein level was measured by an ELISA assay **(F)**. Results are expressed as fold changes compared to the non-infected and non-treated control condition. The data represent the replicates of at least three independent experiments with their means. Results that are not different from each other according to Tukey's multiple comparisons tests are grouped under a same letter ($p < 0.05$).

4.6. The lentil extract modulates ETEC induction of mucus-related genes expression

Furthermore, mucus-related gene expression was assayed as a witness of the innate effector response. Inoculation with ETEC strain H10407 tended to induce all selected genes except *TTF3* (**Fig. 3.6**). This induction was significant ($p < 0.05$) for *MUC17* (3-fold) and *KLF4* (2-fold) only. The lentil extract tended to mitigate ETEC induction of *MUC1*, *MUC2*, *MUC5AC*, *MUC5B* and *KLF4* with significance reached for *MUC1* and *KLF4* ($p < 0.05$, **Fig. 3.6**). *MUC1* and *KLF4* expression was induced by 1.5- and 2.3-fold under infected condition, and returned at 0.9- and 1.2-fold of their basal expression levels with the lentil extract, respectively (**Fig. 3.6.A** and **6.H**). Contrarily, the lentil extract favored the basal expression of *MUC17* (2.4-fold induction, $p < 0.05$), and this effect was conserved after ETEC inoculation (1.3-fold compared to non-treated control, $p < 0.05$, **Fig. 3.6.F**).

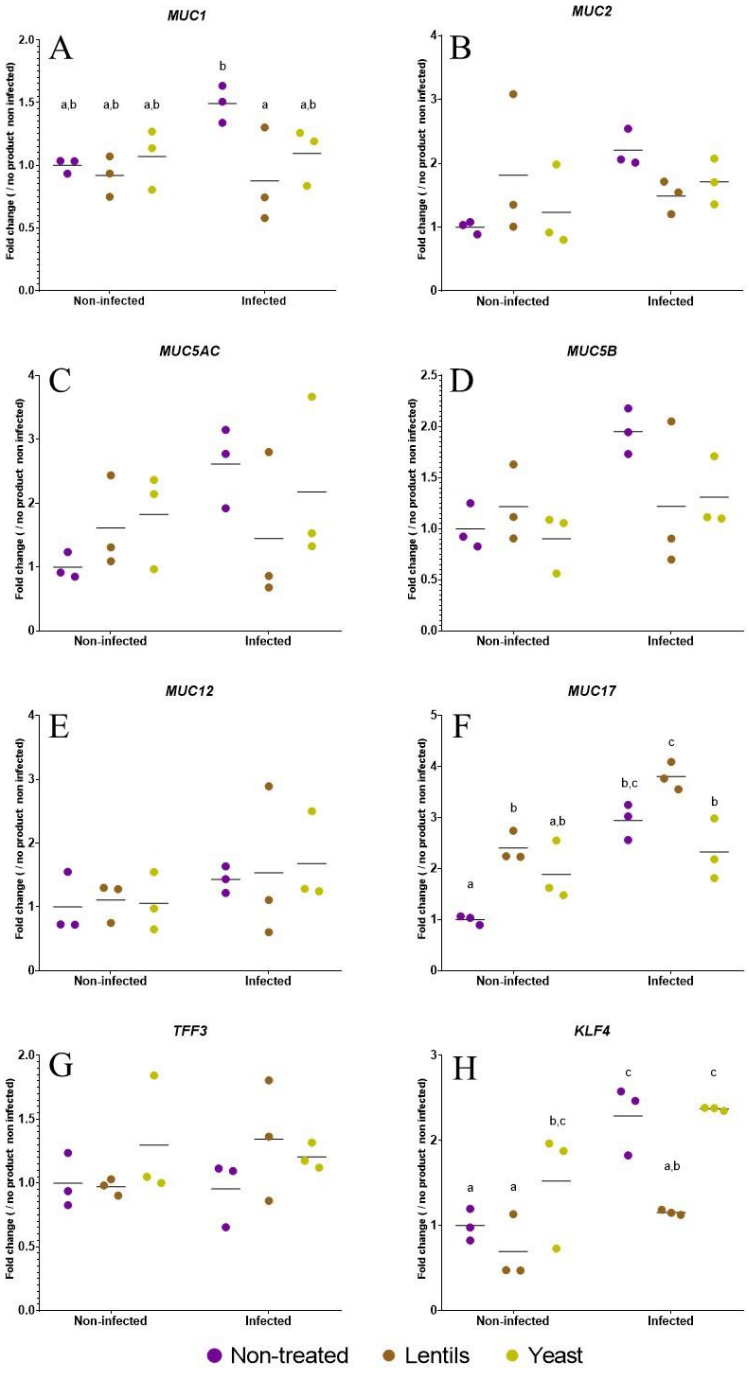

Figure 3.6. Modulation of mucus-related gene expression by fiber-containing products. Caco-2/HT29-MTX were infected with the ETEC strain H10407 (10^7 CFU.mL^{-1}, MOI 100) after a 3-hour pre-treatment with the lentil extract (brown dots, 'lentils'), specific yeast cell walls (yellow dots, 'yeast'). Non-infected and non-treated conditions (purple dots, 'non-treated') were used as control experiments. Expression of mucus-related genes (*MUC1*, *MUC2*, *MUC5AC*, *MUC5B*, *MUC12*, *MUC17* and *TFF3*) **(A-G)** and *KLF4* (Kruppel-like factor 4) involved in goblet cell differentiation (**H**), were analyzed by RT-qPCR. The results are expressed as fold changes compared to non-infected and non-treated control condition. The data represent the replicates of three independent experiments with their means. Results that are not different from each other according to Tukey's multiple comparisons tests are grouped under a same letter ($p < 0.05$).

4.7. Yeast cell walls strengthen intestinal barrier function

As human ETEC strains and their virulence factors can potentially impact the epithelial barrier, the expression of tight junction related-genes were also followed during cellular experiments. Among the four genes that were studied (**Fig. 3.7**), only *CLDN1* was significantly induced by ETEC infection (1.6-fold induction, $p < 0.05$). Interestingly, this induction was reduced by the yeast cell walls to almost return to the basal level ($p < 0.05$, **Fig. 3.7.A**). *TJP1* expression was also triggered by lentil extract, but only when ETEC strain H10407 was inoculated (3.4-fold induction, $p < 0.05$, **Fig. 3.7.C**). Considering these mitigated results, we decided to assess the effect of fiber-containing products on epithelial barrier permeability. When applied to the apical side of Caco-2/HT29-MTX transwells, after 2h- contact, none of the tested products increased the absorption of caffeine (**Fig. 3.7.E**) or atenolol (**Fig. 3.7.F**), which were used as markers for transcellular and paracellular permeability {Citation}(Libuse Smetanova, Xiaomei Chen 2017), respectively. Yeast cell walls even significantly decreased caffeine absorption from 21.2 to 17.0% ($p < 0.05$, **Fig. 3.7.E**) and both products strongly reduced ($p < 0.05$) atenolol absorption, with 3.0- and 5.8- fold reductions for lentil extract and yeast cell walls, respectively (**Fig. 3.7.F**). Accordingly, fiber-containing products led to a rise in TEER over time, with significant 1.3- and 1.4-fold increases for lentil extract compared to the non-treated condition at 120 and 180 min, respectively ($p < 0.05$, **Fig. 3.7.G**).

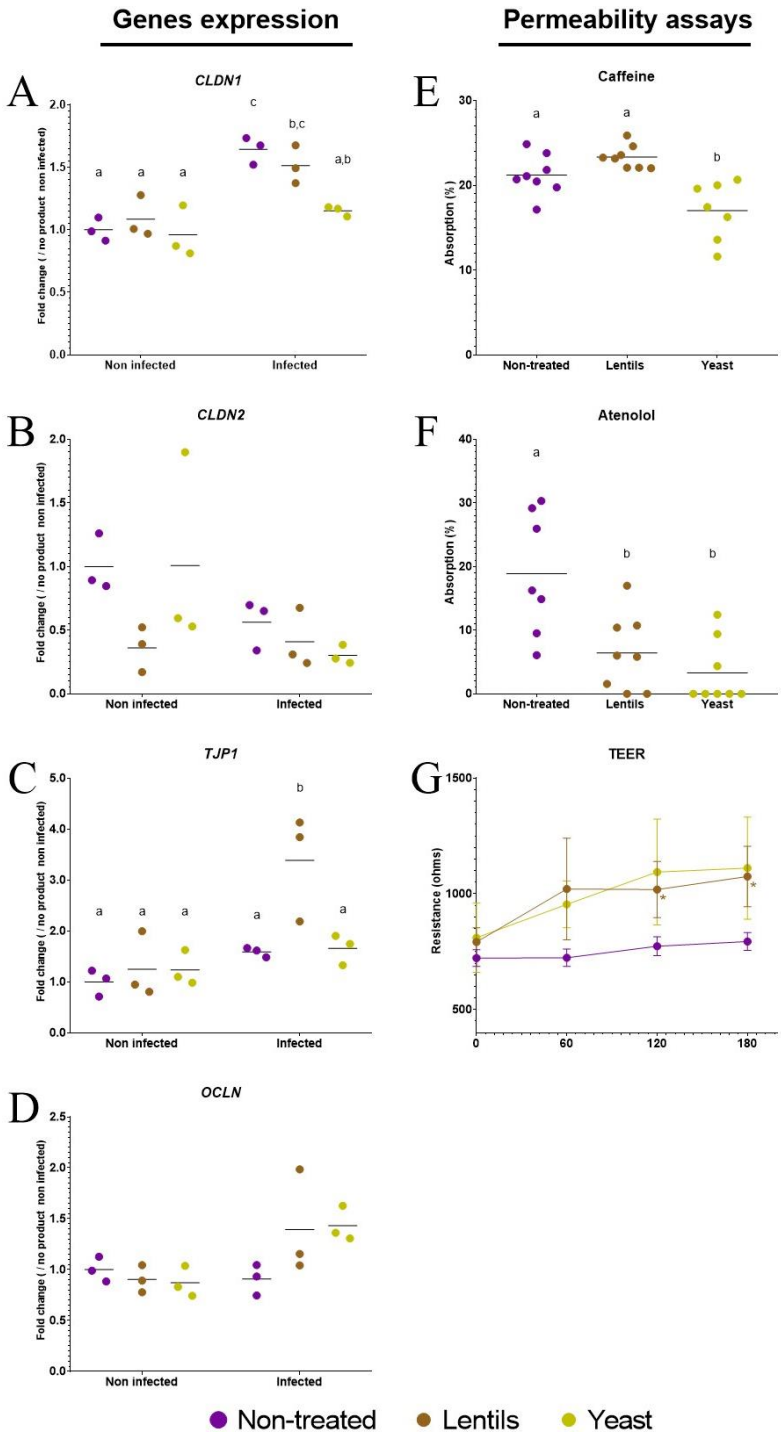

Figure 3.7. Modulation of intestinal epithelial permeability by fiber-containing products. Caco-2/HT29-MTX were infected with the ETEC strain H10407 (10^7 CFU.mL^{-1}, MOI 100) after a 3-h pre-treatment with the lentil extract (brown dots, 'lentils'), specific yeast cell walls (yellow dots, 'yeast'). Non-infected and non-treated conditions (purple dots, 'non-treated') were used as control experiments.

(**A-D**) The expression of tight junction-related genes (*CLDN1*, *CLDN2*, *TJP1* and *OCLN*) was analyzed by RT-qPCR. The results are expressed as fold changes compared to non-infected and non-treated control condition.

(**E-F**) The absorption of caffeine (1g.L^{-1}) (**E**) and atenolol (50 mg.L^{-1}) (**F**) after a 2-hour co-incubation of Caco-2/HT29-MTX cultured on transwells with or without fiber-containing products. Permeability is given as a percentage of the initial apical concentration.

(**G**) Transepithelial resistance (TEER) measured during a 3-hour incubation period of Caco-2/HT29-MTX cultured on transwells with or without fiber-containing products. (**A-F**) represent individual replicates of 3 independent experiments with their means, while (**G**) represents the mean resistance (± SD) of three independent experiments. Conditions that are not different from each other according to Tukey's multiple comparisons tests are grouped under a same letter ($p < 0.05$). In panel (**G**), statistical differences with the non-treated control condition provided by Tukey's multiple comparisons tests are indicated on graph (*: $p < 0.05$).

4.8. Yeast cell walls mostly impact mucus-associated microbiota during ETEC infection

To investigate the impact of dietary fiber-containing products on ETEC interactions with human luminal and mucosal gut microbiota, batch experiments inoculated with human feces were performed in flasks containing mucin-alginate beads. As expected, at the start of the experiment, the *Escherichia/Shigella* population became predominant in the luminal phase of infected bottles and represented 34% of the detected bacterial ASV reads by *16S rRNA* gene sequencing (**Fig. 3.8.C**) and 15% of active bacteria by RNA fluorescent *in situ* hybridization (**Fig. 3.8.E**). The proportion of ETEC or *Escherichia/Shigella* in the luminal phase remained stable. during the experimental time course, regardless of the detection technic used (**Fig. 3.8A, 8.C** and **8.E**). Dietary fiber-containing products had no significant effect on *Escherichia/Shigella* or ETEC proportions in the luminal phase (**Fig. 3.8.A, 8.C** and **8.E**), yet a decreasing trend in ETEC levels (1.7-fold lower) with yeast cell walls was observed (**Fig. 3.8.A**). Concerning the mucosal compartment, in infected conditions, the number of adherent ETEC as reported by qPCR, tended to be respectively 1.2- and 1.7-fold lower with the lentil extract and yeast cell walls compared to the non-treated control, but again, no significance was reached (**Fig. 3.8.B**). *16S rRNA* gene sequencing showed a non-significant 33% decrease in adhered *Escherichia/Shigella* ASV under yeast cell walls condition compared to non-treated one (**Fig. 3.8.D**).

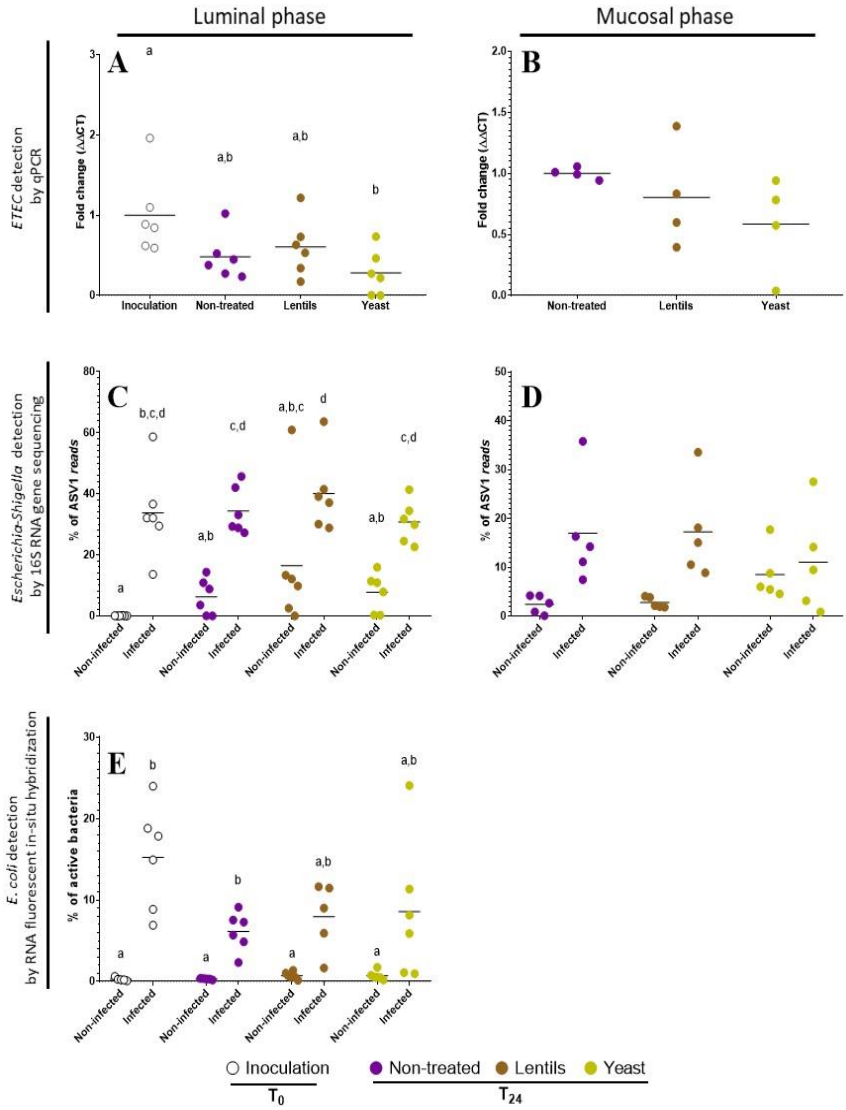

Figure 3.8. Impact of dietary fiber-containing products on ETEC survival in *in vitro* batch colonic conditions.
Penicillin bottles containing nutritive medium enriched in dietary fiber-containing products were inoculated with feces from six healthy donors and then challenged with pre-digested ETEC strain H10407 at 10^8 CFU.mL^{-1}. Control experiments were performed under non-treated and non-infected conditions. White, purple, brown and yellow dots represent individual biological replicates at the beginning of the experiment after ETEC inoculation (Inoculation, T_0) or after 24h-fermentation in the non-treated (Non-treated, T_{24}), lentil extract (Lentils, T_{24}) or specific yeast cell walls (Yeast, T_{24}) conditions, respectively. (**A, B**) qPCR detection of H10407 ETEC strain among total bacterial populations expressed as fold changes compared to inoculation T_0 (luminal phase) or non-treated T_{24} (mucosal phase) conditions. (**C, D**) Percentages of ASV1 reads detected by 16S RNA gene amplicon sequencing in luminal and mucosal bacteria. ASV1 is the ASV with the highest reads' abundance in all samples and its reads have been assigned to the *Escherichia*/*Shigella* genus and to *Escherichia albertii/boydii/coli/dysenteriae/fergusonii/flexneri/marmotae/sonnei* species. (**E**) Proportion of active *E. coli* in the total bacterial populations as detected by RNA fluorescent *in situ* hybridization. Bars represent the mean of data (n = 6). Results that are not significantly different from each other according to Tukey's multi-comparison are grouped under the same letter ($p < 0.05$).
ASV: amplicon sequence variant

4.9. Fiber products have no significant effect on ETEC colonization in a complex microbial background

To further explore the effects of dietary fiber-containing products on gut microbiota composition, we performed Illumina *16S rRNA* gene amplicon sequencing and bacterial community analysis. Regarding α-diversity, ETEC infection was associated to a significant decrease in α-diversity evenness in the luminal phase but supplementation with fiber-containing products had no effect (**Fig. 3.9.B** and **9.C**). Both infection by ETEC and supplementation with fiber products had no influence on species richness in the luminal phase (**Fig. 3.9A**) and on both species' richness and evenness in the mucosal phase (**Fig. 3.9.D, 9.E** and **9.F**).

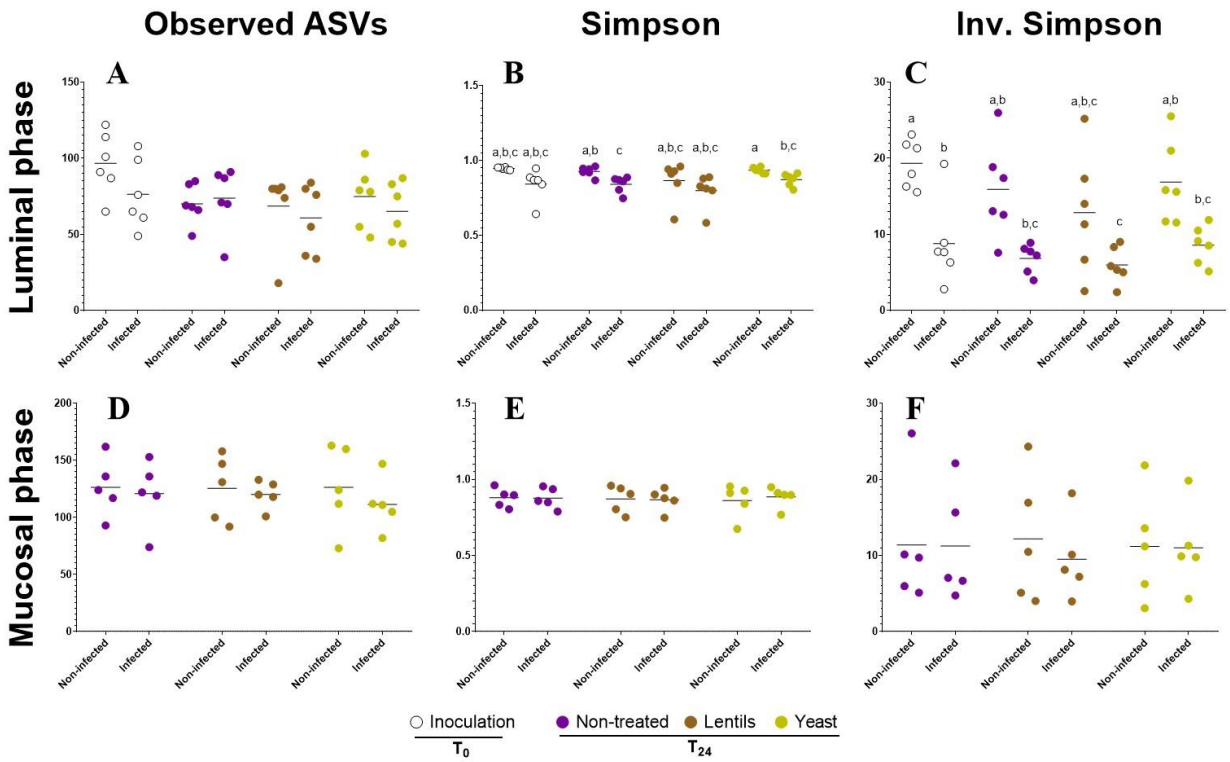

Figure 3.9. Impact of the dietary fiber-containing products on ETEC modulation of microbial community α-diversity.
Batch experiments were performed using feces from six healthy donors, challenged or not with ETEC strain H10407, and treated or not with fiber-containing products. The graphs represent the variation of the microbiota species richness (**A, D**) and species evenness represented by Simpson (**B, E**) and Inverse Simpson indexes (**C, F**) at the ASV level. Samples were collected both in the luminal (**A-C**) and mucosal compartments (**D-F**). White, purple, brown and yellow dots represent individual biological replicates at the beginning of the experiment after ETEC inoculation (Inoculation T_0) or after 24 h-fermentation in the non-treated (Non-treated, T_{24}), lentil extract (Lentils, T_{24}) or specific yeast cell walls (Yeast, T_{24}) conditions, respectively. Black bars represent the mean (n=6). Results that are not significantly different from each other according to Tukey's multi-comparison are grouped under the same letter ($p < 0.05$).

Concerning β-diversity, an NMDS analysis showed that the stool donor was the predominant explanatory variable for dissimilarities in gut microbiota compositionin both in luminal and mucosal compartments (**Fig. 3.10.A**). A PermANOVA analysis performed on the samples at T24h and excluding ASV1 (attributed to *Escherichia/Shigella*) confirmed that donor origin accounted for 10.0% of the dissimilarities ($p < 0.001$, 999 permutations). ETEC infection was also a significant source of variations and accounted for 6.0% of the dissimilarities ($p < 0.001$, 999 permutations), but dietary fiber-containing products was not (p=0.51). To go further, a db-RDA analysis was performed on samples at 24h using "yeast", "lentil" and "infection" as explanatory variables. The db-RDA was able to cluster more efficiently infected sample from non-infected ones more efficiently in the mucosal phase (**Fig. 3.10.B**). If none of the tested products was able to modify the impact of infection on the gut microbiota structure, yeast samples clustered away from the rest in both luminal and mucosal compartments, suggesting that the yeast cell walls product was responsible for some variations in the microbiota community structure, although only modest (**Fig. 3.10.B**). In the luminal phase, ETEC infection induced a global increase in *Escherichia/Shigella* (**Fig. 3.8A**) to the detriment of other groups such as *Bacteroides* (**Fig. 3.10.C** and **10.D, Suppl. Fig. 3.2**). At the genus and family levels, no clear difference in phylogenetic groups' relative abundances was observed between the control and treated conditions at 24 hours in the luminal phase, apart from a light but consistent increase in *Tannerellaceae/Parabacteroides* by yeast cell walls, whatever the infection status (**Fig. 3.10.C** and **10.D, Suppl. Fig. 3.3** and **4**). Compared to the luminal microbiota, the mucosal non-infected microbiota was depleted of *Faecalibacterium* and enriched in *Clostridium*, *Roseburia*, *Bifidobacterium* and *Lactobacillus*, even if *Lactobacillus* colonization appeared to be donor-dependent (**Fig. 3.10.C** and **10.D, Suppl. Fig. 3.4**). In the non-treated condition, ETEC infection tended to be constantly detrimental to the *Clostridium* and *Bifidobacterium* species representation on mucin-beads and the dietary fiber-containing products tended to limit the *Clostridium* disappearance (**Fig. 3.10.C** and **10.D, Suppl. Fig. 3.4)**. In the luminal compartment, yeast cell walls seemed to reduce *Faecalibacterium* and *Ruminococcaceae* prevalence and to favor *Tannerellaceae/Parabacteroides*, while in the mucosal compartment, they appeared to favor *Tannerellaceae/Parabacteroides* and commensal *Escherichia/Shigella* colonization. No clear trend was identified for the lentil extract (**Fig. 3.10.C** and **10.D, Suppl. Fig. 3.3** and **4**).

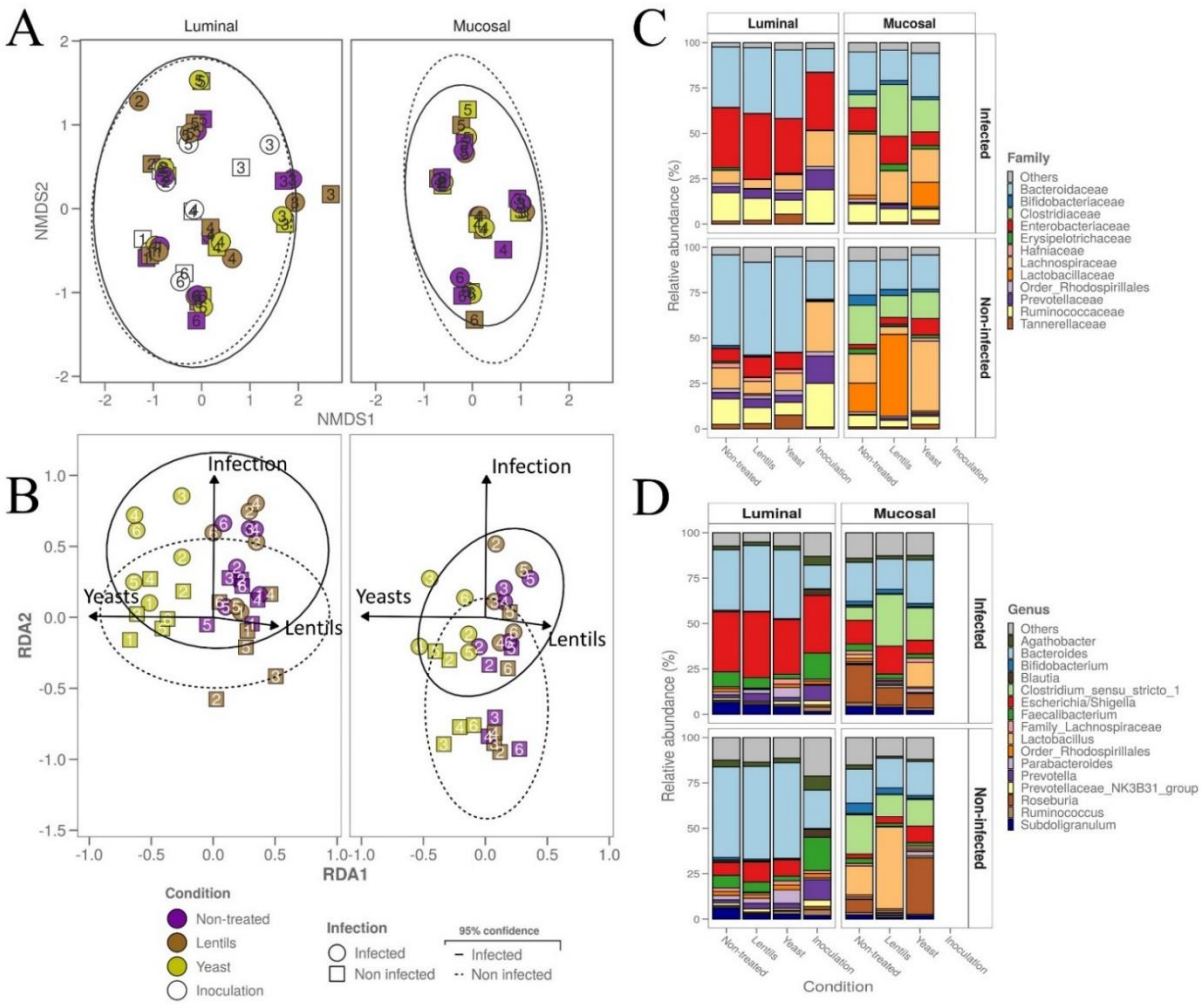

Figure 3.10. Impact of dietary fiber-containing products on ETEC modulation of microbial communities β-diversity.
Batch experiments were performed using feces from six healthy donors, challenged or not with ETEC strain H10407, and treated or not with fiber-containing products.
(A-B) Non-parametric multidimensional scaling (NMDS) (**A**) and Distance-based redundancy analysis (db-RDA) (**B**).
Two-dimensional plots visualizations report the microbial community β-diversity at the ASV level, as determined by *16S rRNA* gene amplicon sequencing. The db-RDA was performed on the ASV table excluding the inoculation samples (T_0) and ASV1 (attributed to the *Escherichia*/*Shigella* genus). Infection and fiber products have been provided as sole environmental variables (binary), and are plotted as vectors (arrows). White, purple, brown and yellow dots represent individual biological replicates at the beginning of the experiment after ETEC inoculation (Inoculation, T_0) or after 24h-fermentation in the non-treated (Non-treated, T_{24}), lentil extract (Lentils, T_{24}) or specific yeast cell walls (Yeast, T_{24}) conditions, respectively. The samples are represented by dot shapes and square shapes for the infected and non-infected conditions, respectively. The 95% confidence ellipse area is also indicated in a continuous line for the infected condition and in dotted line for the non-infected conditions. The donor number is indicated for each sample. **(C-D)** Cumulative bar plots of the relative microbial community composition at the family (**C**) and genus (**D**) levels. The area graphs show the relative abundance of the 12 most abundant families and 16 most abundant genera in all six different donors confounded.

4.10. Fiber-containing products slightly affect gut microbial activities during ETEC infection

In a last step, the effect of dietary fiber-containing products on gut microbial activity during ETEC infection was assessed by following various indicators such as SCFA, gas production, pH acidification, and gas pressure. We also investigated mucin-alginate beads degradation as a measure of the mucosal microbiota degrading capability. ETEC inoculation significantly impacted butyric acid production (p <0.05, two-way ANOVA), with 1.3-, 1.4- and 1.2- fold increases in non-treated, lentils and yeast conditions, even if no individual significances were reached (**Fig. 3.11.A**). When added, lentil extract and yeast cell walls increased propionic acid production by 10-20% and 30-40%, respectively, with only yeast condition reaching significance (p < 0.05, **Fig. 3.11.A**). Regarding pH acidification, at 24h-fermentation, the pH tended to be increased with around 0.1 when ETEC was inoculated (p = 0.07, two-way ANOVA), with no significant effect from fibers (**Fig. 3.11.B**). ETEC inoculation also tended to be associated with an increased pressure in the bottles at the end of the experiment (p = 0.08, two-way ANOVA, **Fig. 3.11.C**), with again no significant impact of fibers. Gas analysis showed that CO_2 levels were significantly impacted by both ETEC and fiber-containing products compared to the non-treated and non-infected control conditions (p <0.05, **Fig. 3.11.D**). However, the addition of fiber products exhibited no significant impact on gas composition under the infected condition. Lastly, dietary fiber-containing products led to a decrease in mucin bead weight at 24h, with reached significance for the yeast cell walls in the infected condition (p <0.01, **Fig. 3.11.E**). Yeast supplementation was indeed associated with an increase in bead degradation by 22 and 23% in the non-infected and infected conditions, respectively. In accordance with our observations, the microbial community structure of the infected samples correlated with pH and butyric acid production and dietary fiber-containing products had no effect (**Fig. 3.12**).

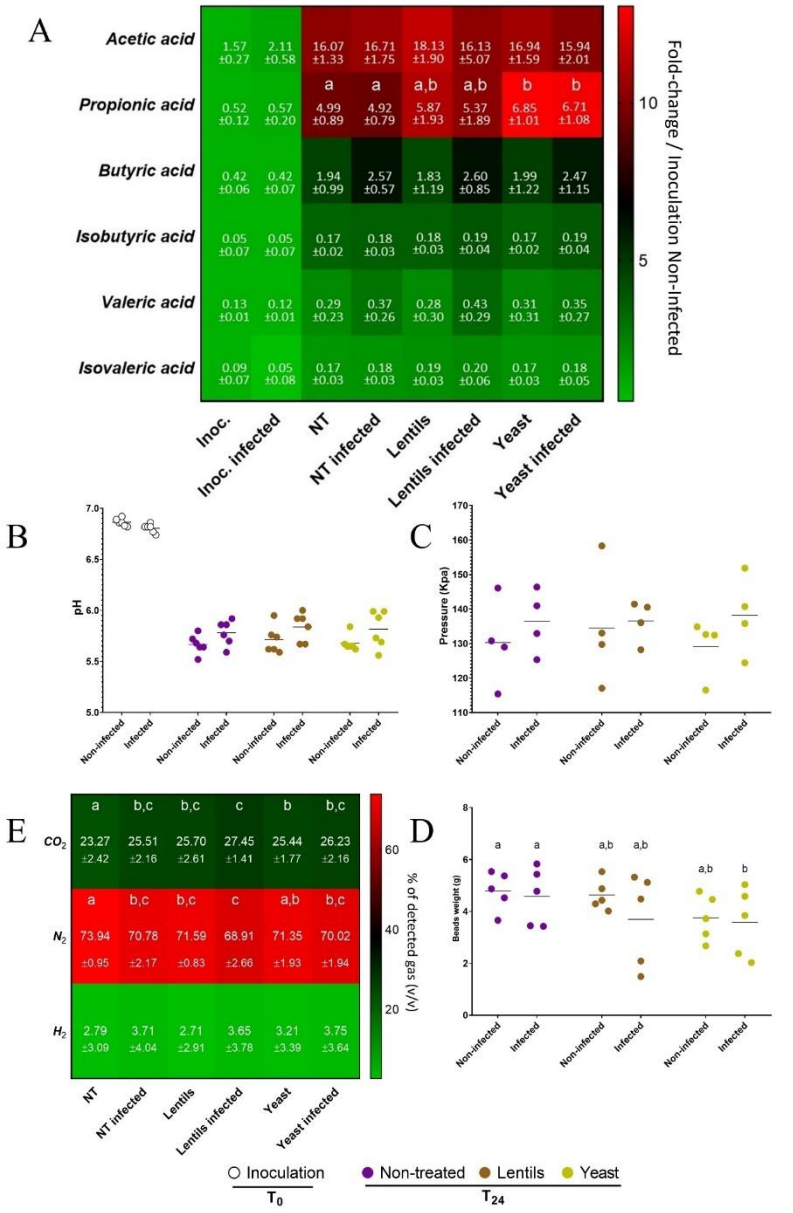

Figure 3.11. Modulation of gut microbial activity by ETEC infection and dietary fiber-containing products.

The impact of ETEC strain H10407 inoculation (infected *versus* non-infected) and fiber-containing product (non-treated *versus* lentils or yeast) on microbiota activity in batch experiments were assayed by the measurement of SCFA production (**A**), pH acidification (**B**), gas pressure (**C**) and gas composition (**D**). Mucin-beads weight was also measured at the end of the experiment (**E**). Batch experiments were performed using feces from six healthy donors. White, purple, brown and yellow dots represent individual biological replicates at the beginning of the experiment after ETEC inoculation (Inoculation, T_0) or after 24 hours of fermentation in the non-treated (Non-treated, T_{24}), lentil extract (Lentils, T_{24}) or specific yeast cell walls (Yeast, T_{24}) conditions, respectively. (**A**) SCFA production in the luminal phase was analyzed by liquid chromatography. Results are expressed in mM ± SD (*n=6*) and colored according to fold changes compared to the control condition (non-infected, non-treated, T0). (**B**) pH of the fermentation medium was followed-up over-time and biological replicates are represented as dots with their means (black line). (**C**) Gas pressure was measured at T24h and biological replicates are represented as dots with their means (black line). **(D)** Gas composition was determined by gas chromatography at T24h. Results are expressed as mean percentages ± SD (*n=6*) and accordingly colored (**E**) Mucin-beads collected at T24h were weighted and biological replicates are represented as dot plots with their means (black line). Results that are not significantly different from each other according to Tukey's multi-comparison are grouped under the same letter ($p < 0.05$).
NT: Non-treated.

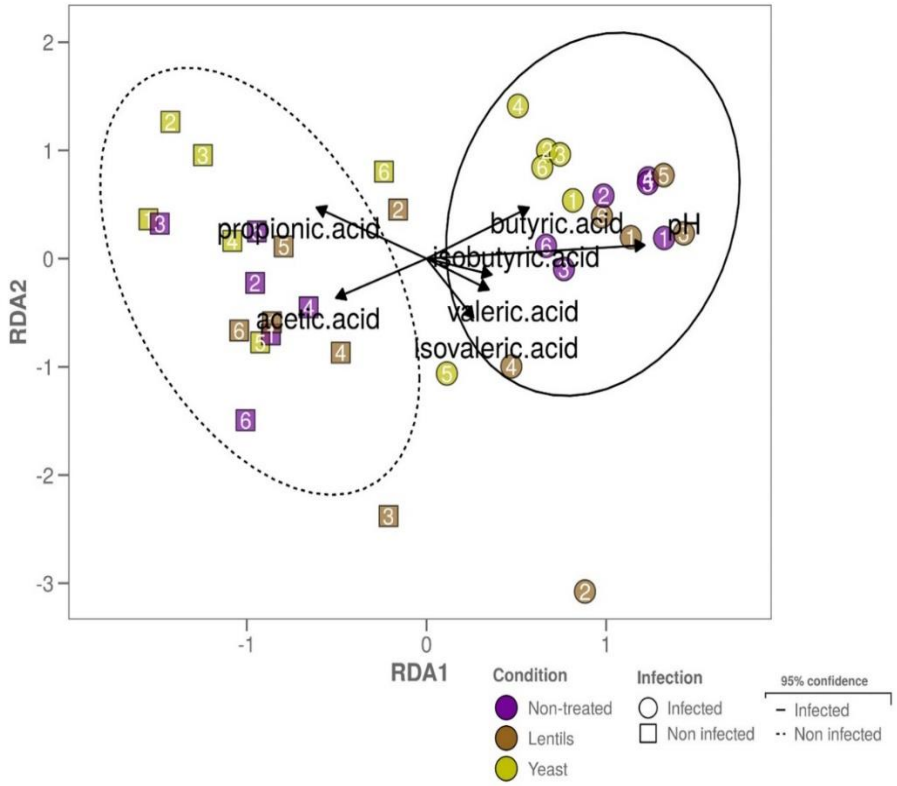

Figure 3.12. **Distance-based redundancy analysis modeling of the microbiota community structure according to pH and SCFA production**.
The two-dimensional plot reports the β-diversity structure of the whole microbial community taxonomy at the end of the experiment (T24) at the ASV level in the luminal phase according to db-RDA and explained by metabolite variables (SCFAs and pH). Individual samples are represented by dot shapes and square shapes for the infected and non-infected conditions, respectively. The 95% confidence ellipse zone is also indicated in a continuous line for the infected condition and in a dotted line for the non-infected conditions. The donor number is indicated for each sample.

5. Discussion

To date, only few studies have investigated the potential anti-infectious properties of dietary fibers against ETEC strains responsible for traveler's diarrhea in humans (Idota and Kawakami 1995; Drakoularakou *et al.* 2010; Roberts *et al.* 2013; Salcedo *et al.* 2013; He *et al.* 2016). Using a large panel of complementary *in vitro* models, we showed that two fiber-containing products from legumes and microbes, namely, a lentil extract and a specific yeast cell wall from *Saccharomyces cerevisiae*, selected in our previous study (Sauvaitre *et al.* 2021a), were able to exert antagonistic effects towards the ETEC reference strain H10407 at various stages of the pathological process. These products from different origins contain various types of soluble and

insoluble fibers, mainly resistant starch, cellulose, and hemi-cellulose for lentils (Dodevska *et al.* 2013) and mannans and β-glucans for yeast cell walls (Liu *et al.* 2021). This variation could explain their differences in terms of the anti-infectious properties found in the present study. The two fiber products were tested at the *in vivo* relevant concentration of 2 grams per liter of final fiber content. This value was calculated based on the 10 to 30 grams of fibers consumed per day in industrialized countries (King, Mainous and Lambourne 2012; Holscher 2017) and the approximately 10 liters of fluid passing through the GI tract daily (Kiela and Ghishan 2016). Of note, as the tested products were not pure, we cannot exclude that components other than fibers could exert anti-infectious properties against ETEC (Wijemanne and Moxley 2014).

5.1. Bacterial colonization

A first target in our study was to investigate if fiber products could affect pathogen growth in classical broth media. None of the tested compounds was able to impact the growth of ETEC strain H10407. This is not unexpected since, to our knowledge, only the human-engineered fiber chitosan has been reported to exert a bacteriostatic effect *in vitro* on diverse bacterial pathogens such as enterohemorrhagic *Escherichia coli* (EHEC) (Chantarasataporn *et al.* 2014). We also showed that the lentil extract and yeast cell walls were able to sustain ETEC growth in M9 minimal medium, most likely due to the presence of non-fiber components, as *E. coli* strains are not able on their own to degrade complex polysaccharides (Muñoz-Gutiérrez and Martinez 2013; Patnode *et al.* 2019, Onyango *et al*., 2021). We argue that this positive effect on pathogen growth may not be an issue in the context of the complex nutritional and microbial background of the distal small intestine, the main site of ETEC colonisation (Al-Majali *et al.* 2000, 2007; Allen, Randolph and Fleckenstein 2006; Al-Majali and Khalifeh 2010; Gonzales *et al.* 2013; Rodea *et al.* 2017). In the human gut, fibers are degraded in smaller carbohydrates by the endogenous microbiota, providing substrates for pathogens as ETEC which generally behave as secondary degraders (Sauvaitre *et al.* 2021b). By performing fecal batch experiments including microbiota from human origin, we confirmed that dietary fiber-containing products had no significant effect on ETEC colonisation in a complex milieu, with only a slight tendency of yeast cell walls to reduce pathogen levels in both the luminal and mucosal compartments.

5.2. LT toxin production and virulence gene expression

As toxin production is a key feature in ETEC physiopathology, our next step was to study the impact of fiber products on LT toxin. To our knowledge, only one study has previously reported an indirect effect of dietary fibers on ETEC toxins. SCFA, major end-products of dietary fiber metabolism by gut microbiota, have been shown to significantly reduce or even abolish LT toxin production at a concentration of 2 $g.L^{-1}$ in CAYE culture medium (Takashi, Fluita and Kobari 1989). Here, we showed that LT toxin concentration was significantly reduced in culture medium by the lentil extract in a dose-dependent manner. This effect seems to be partly due to toxin binding to some lentil components acting as decoy, as previously reported by other groups with GM1 ELISA assays used with other carbohydrates (Verhelst *et al.* 2013). Despite the involvement of several virulence genes in the ETEC infectious process (including those encoding for toxin production), data investigating the direct impact of dietary fibers on ETEC virulence gene expression are clearly missing in the literature. In this study, we investigated a panel of ETEC virulence genes in cellular assays. We demonstrated that such compounds could be used to modulate the induction of ETEC virulence gene expression by cellular proximity. Such induction was already reported by previous study for ETEC strain H10407, but on non-mucus secreting Caco-2 cells (Kansal *et al.* 2013). Here, we showed that, at the transcriptional level, the *eltB* gene was consistently inhibited by the lentil extract. Dietary fiber supplementation is known to modulate the expression of genes involved in fiber degradation (Scott *et al.* 2011; Patnode *et al.* 2019). Only a few studies investigated the modulation of virulence genes. As an example, chitosan significantly modified *Campylobacter jejuni* genes involved in motility, quorum sensing, stress response and adhesion (Wagle *et al.* 2019). Here our study indicates that toxin concentration decrease could be mediated by a direct inhibitory effect of lentil extract on LT toxin encoding gene expression.

5.3. Adhesion on mucus and epithelial cells

Getting access to the epithelium is a crucial step for most intestinal pathogens to fulfill their infection cycle (Ribet and Cossart 2015). To this sole purpose, ETEC strain H10407 possesses two mucus-degrading enzymes (Kumar *et al.* 2014; Luo *et al.* 2014) and numerous adhesins allowing mucosal adhesion (Vipin Madhavan and Sakellaris 2015; Mirhoseini, Amani and Nazarian 2018). To date, only milk oligosaccharides (Idota and Kawakami 1995; Salcedo *et al.* 2013) and soluble plantain fibers at a dose of 5 $g.L^{-1}$ (Roberts *et al.* 2013) have shown the ability to reduce adhesion

of human ETEC strains (others than H10407) to Caco-2 cell line. Here, we rather used a co-culture of enterocytes and mucus-secreting cells to more accurately mimic the physiological situation in the human intestine (Dorier *et al.* 2017; García-Rodríguez *et al.* 2018; Gillois *et al.* 2021). We first observed the inhibition of ETEC adhesion by both fiber-containing products on mucin beads. This anti-adhesive property cannot be explained by the sedimentation effect observed with insoluble fiber particles, as beads were always maintained under agitation. Only the yeast cell walls were able to reduce ETEC adherence using the more complex Caco-2/HT29-MTX model. Microorganism-derived polysaccharides have already shown adhesion inhibition properties against enteric pathogens (Kim, oh and Kim 2009; Wang, Gänzle and Schwab 2010; Chen *et al.* 2014; Liu *et al.* 2017), but this is the first time that yeast cell walls reduced mucosal adhesion of an ETEC strain from human origin. By using yeast-alginate beads, we showed that ETEC strain H10407 presented a greater adhesion specificity for the yeast cell walls than for mucin, supporting a potential decoying effect of the product during pathogen adhesion. However, this observed decoying effect did not seem to involve mannose residues, as previously shown when the whole living probiotic yeasts are used (Roussel *et al.* 2018b).

5.4. Host innate immunity and mucus-related genes

ETEC, as well as its virulence factors, are well known to be linked to innate immunity activation and induction of inflammation in epithelial cell lines, animals and humans (Rodrigues *et al.* 2000; Greenberg *et al.* 2002; Park *et al.* 2010; Chutkan and Kuehn 2011; Loos *et al.* 2012; Wang, Gao and Hardwidge 2012; Tapader *et al.* 2016: 201), which could be positively associated to infection severity (Long *et al.* 2010; Brubaker *et al.* 2021). Here, as expected, we observed a general induction of cytokines related genes upon ETEC H10407 exposure in cellular experiments (He *et al.* 2016). Interestingly, the lentil extract showed a significant inhibitory effect on those genes, while the influence of yeast cell walls was more subtle. The most striking effect was observed on the pro-inflammatory *IL-8* for which inhibition by fiber products was observed not only at the gene but also at the protein level. The underlying mechanisms of dietary fibers on their ability to modulate innate immune response are not clear. A study from He and colleagues, performed on a human ETEC strain, performed on human ETEC strain, showed that the HMO 2'-fucosyllactose could modulate CD14 expression in infected enterocytes, thus attenuating LPS-induced inflammation (He *et al.* 2016). Here, our results showed that the products exerted a basal

anti-inflammatory effect (as shown with IL-8 production) but also led to an inhibition of the innate immune response activation, regardless of the inflammatory status (as shown with IL-10 expression), which could be the result of decreased interactions with innate immune receptors. The activation of innate immune receptors is known to ultimately stimulate mucus secretion (McNamara and Basbaum 2001; Birchenough *et al.* 2016). Accordingly, we found in this study that mucus-related genes tended to be activated following ETEC infection and that this activation was limited by both fiber products, with a more significant effect of the lentil extract. Of note, as mucus secretion is involved in pathogen clearance from the mucosal epithelium (Birchenough *et al.* 2016), an inhibition of mucus-related genes by the lentil extract may be considered to be unfavorable in the fight against the ETEC pathogen.

5.5. Host tight junction-related genes and cellular permeability

The regulation of tight junctions in intestinal epithelial cells is one of the main means for the host to control epithelial permeability (Farré *et al.* 2020). ETEC ST toxin variants have been largely described as modulators of paracellular permeability and more specifically of tight junctions (Nakashima, Kamata and Nishikawa 2013; Ngendahayo Mukiza and Dubreuil 2013; Nassour and Dubreuil 2014). In contrast, few studies have investigated the effect of whole ETEC bacteria on cell permeability. Kreisberg and colleagues reported that some human ETEC strains including H10407 elicited a reduction in TEER in T84 epithelial cell monolayers, mediated by the LT toxin which induced paracellular permeability (Harper *et al.* 2011).

In the present study, we showed that only claudin-1 encoding gene was up-regulated following ETEC challenge. Generally, upregulation of tight junction related genes is regarded as beneficial for the host (Che *et al.* 2017; Kim *et al.* 2022). Meanwhile, we could presume that our observation may result from an activation of innate immunity interacting especially with tight junctions following ETEC infection (Schwarz *et al.* 2007; Weber *et al.* 2010; Shen *et al.* 2011; Han *et al.* 2016). When fiber-containing products were added, the most remarkable effects were observed with yeast cell walls, which abolished ETEC-induction of *CLDN1*, but also significantly decreased transcellular and paracellular permeability and increased TEER values. Up to now, no study has ever reported an attempt to modulate human targeting-ETEC induced changes in epithelial integrity with dietary fiber-containing products. Contrarily, *in vivo* studies in pigs have already shown a beneficial effect on intestinal barrier disruption of dietary fibers such as chitosan

or fructooligosaccharides (Li *et al.* 2019a; Wan *et al.* 2019; Liu *et al.* 2020). This positive effect may result from a lower innate immunity activation, as reported by decreases in TLR4 and CD14 expression (Li *et al.* 2019a; Wan *et al.* 2019) and serological cytokines (Liu *et al.* 2020). However, we cannot also exclude a sedimentation effect of the fiber products upon the intestinal cells or a binding with the molecules used as permeability markers. We argue that, at least, the products are unlikely to be detrimental to cellular integrity. Of note, some authors also reported detrimental effects of other fibers like cellulose and arabinoxylans (van Hees *et al.* 2021), indicating that the outcomes are most probably fiber-specific.

5.6. Gut microbiota composition and activity

Evidences from previous *in vitro* and *in vivo* studies support an influence of ETEC strains on human gut microbiota (David *et al.* 2015; Youmans *et al.* 2015; Pop *et al.* 2016; Moens *et al.* 2019; Roussel *et al.* 2020). As microbiota alterations can even more favor enteric infections (Ghosh *et al.* 2011; Hopkins and Frankel 2021), we investigated the impact of ETEC strain H10407 on gut microbiota structure and activity and how it can be further modulated by supplementation with fiber-containing products. None of the tested products was able to restore microbiota evenness that was, according to human *in vivo* data, decreased with ETEC infection (Pop *et al.* 2016). We showed that ETEC inoculation was particularly detrimental to mucosal-associated *Clostridium* species, as already reported by Roussel et al. (Roussel *et al.* 2020a). Supplementation with dietary fiber-containing products enable a slight but consistent (in most of individuals) maintenance of *Clostridium*. Yeast cell walls also induced ETEC-unrelated changes in microbiota composition, with increases in *Parabacteroides* in both the luminal and mucosal compartments. This result would deserve more attention since *Parabacteroides* species have already been highlighted as potential new generation probiotic species in intestinal inflammation-related diseases like metabolic syndrome (Wang *et al.* 2019b; Wu *et al.* 2019) and colorectal cancer (Koh *et al.* 2020). Up to now, only *Lactobacillaceae* have been regularly highlighted as probiotic species with anti-infectious properties against human ETEC strains (Tsai, Lin and Hsieh 2008; Osmanagaoglu, Kiran and Ataoglu 2010; Anand, Mandal and Tomar 2019). Here, one donor was particularly colonized by *Lactobacillaceae* and this bacterial population was found to be enriched on mucin beads by yeast cell walls under infected condition. Interestingly, this donor was also the one having the lowest proportion of *Escherichia*/*Shigella* on mucin beads. Regarding gut microbial

activity, we showed that ETEC inoculation had contradictory effects on fermentation activities, increasing butyric acid production, gas pressure and CO_2 level, but limiting pH acidification. This may result from ETEC mucinase activities leading to higher substrate availability for fermentation, combined with *E. coli* acid resistance systems which notably consume H^+ to produce H_2O, H_2 and CO_2 (Kanjee and Houry 2013). Up to now only two *in vitro* studies had evaluated the effect of ETEC on human gut microbial activity (Moens *et al.* 2019; Roussel *et al.* 2020). However, major differences in experimental conditions hampered any comparison. When added, fiber-containing products had low impact on ETEC-induced changes in microbiota activity. Unsurprisingly, they only seem to favor even more fermentation activities (e.g. fermentation gases). Lastly, since previous studies have elegantly shown in mice that dietary fiber intakes limited pathogen infection by protecting the mucus layer from degradation (Desai *et al.* 2016; Schroeder *et al.* 2018; Neumann *et al.* 2021), we measured the total weight of mucin beads at the end of batch experiments. However, this previous hypothesis was not confirmed here, certainly because of the use of simple batch experiments, which do not include goblet cells nor allow the continuous supply of fiber sources and renewal of luminal content.

6. Conclusion

Using a large panel of in vitro models, this study demonstrated that fiber-containing products, namely, a lentil extract and yeast cell walls, can exert anti-infectious activities against the human reference strain ETEC H10407. Tested products were found to interfere with the ETEC infection process during virulence gene expression, cell adhesion, cross talk with intestinal host cells, and interactions with gut microbiota. Even if the products were not pure fibers, these results are encouraging for further mechanistic investigations. Next steps should be dedicated to the study of dietary fibers/ETEC interactions in more complex and dynamic multi-compartmental models of the human GI tract, such as the TNO intestinal model (TIM) or the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) before going further in animal models, where we can evaluate their effect on the whole organism (e.g., prevention of diarrhea). These findings reveal important implications regarding how our immediate diet history may modify susceptibility to some enteric diseases but also provide meaningful insights in the use of low-cost dietary-fiber-containing products as a relevant prophylactic strategy in the fight against ETEC infections and traveler's diarrhea (**Figure 3.13**).

Author Contributions

Conceptualization, T.S., F.V.H., L.E.-M., S.B.-D., and T.V.d.W.; methodology, T.S., F.V.H., K.F., L.E.-M., S.B-D., and T.V.d.W.; software, T.S. and J.V.L.; formal analysis, T.S. and J.V.L.; investigation, T.S., K.D., C.D., and L.E.-M.; resources, F.C.-D., S.H., L.E.-M., S.B-D., and T.V.d.W.; writing—original draft preparation, T.S.; writing—review and editing, T.S., L.E.-M., S.B.-D., and T.V.d.W.; visualization, T.S. and J.V.L.; supervision, F.V.H., L.E.-M., S.B.-D., and T.V.d.W.; project administration, S.B.-D.; funding acquisition, S.B.-D. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

Consent for fecal collection was obtained under registration number BE670201836318 (Ghent University).

Informed Consent Statement

Written informed consent was obtained from all donors prior to fecal collection.

Data Availability Statement

The 16S RNA gene amplicon sequencing data were deposited and are publicly available in the NCBI Sequence Read Archive database with accession number PRJNA802368.

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Conflicts of Interest

T.V.d.W. is advising ProDigest as a science officer and is a member of the scientific advisory board of MRM Health. S.B.-D. is advising NexBiome as a science officer. S.H. is an employee of HARi&CO. F.C.D. is an employee of Lallemand SAS. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as potential conflicts of interest.

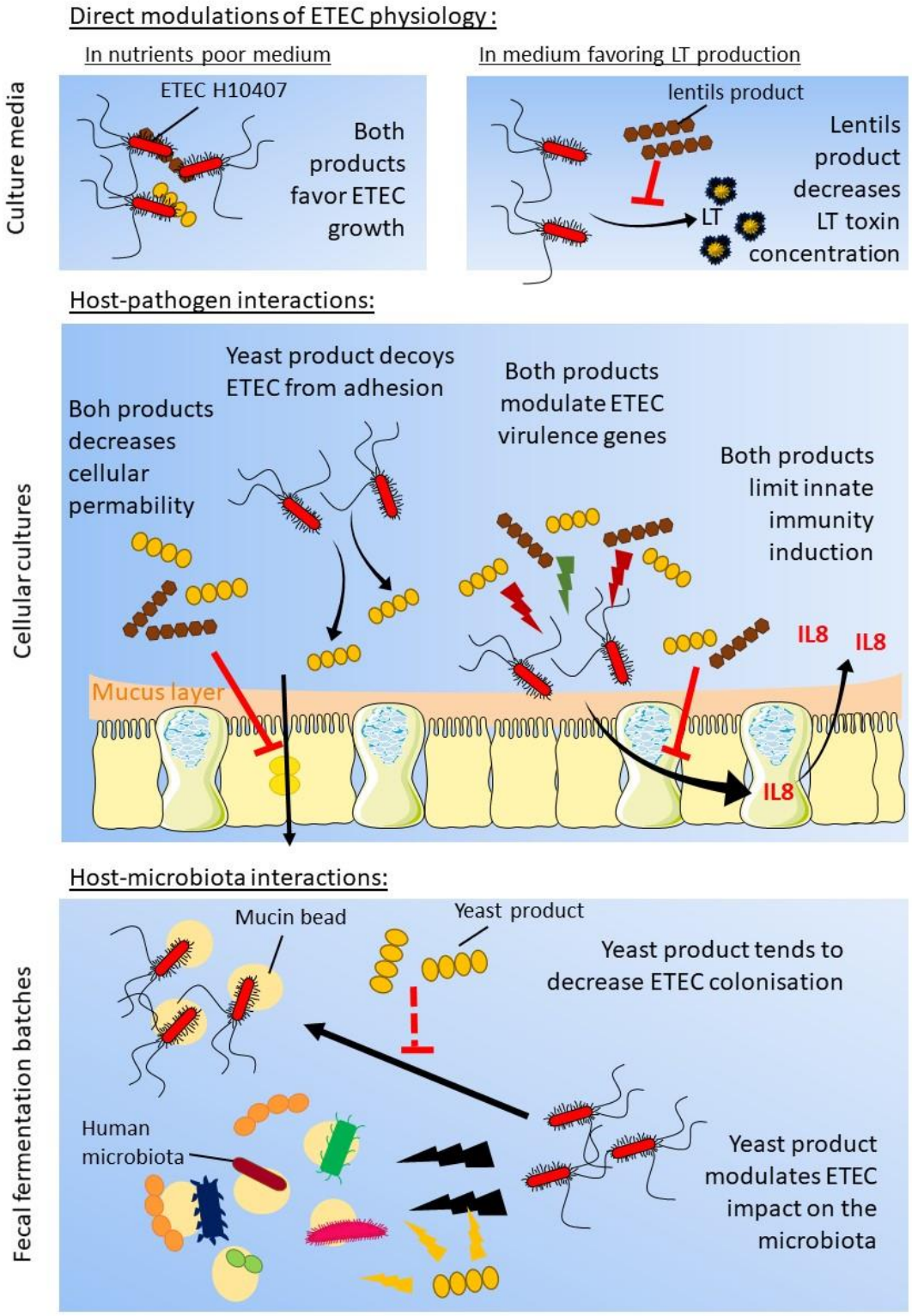
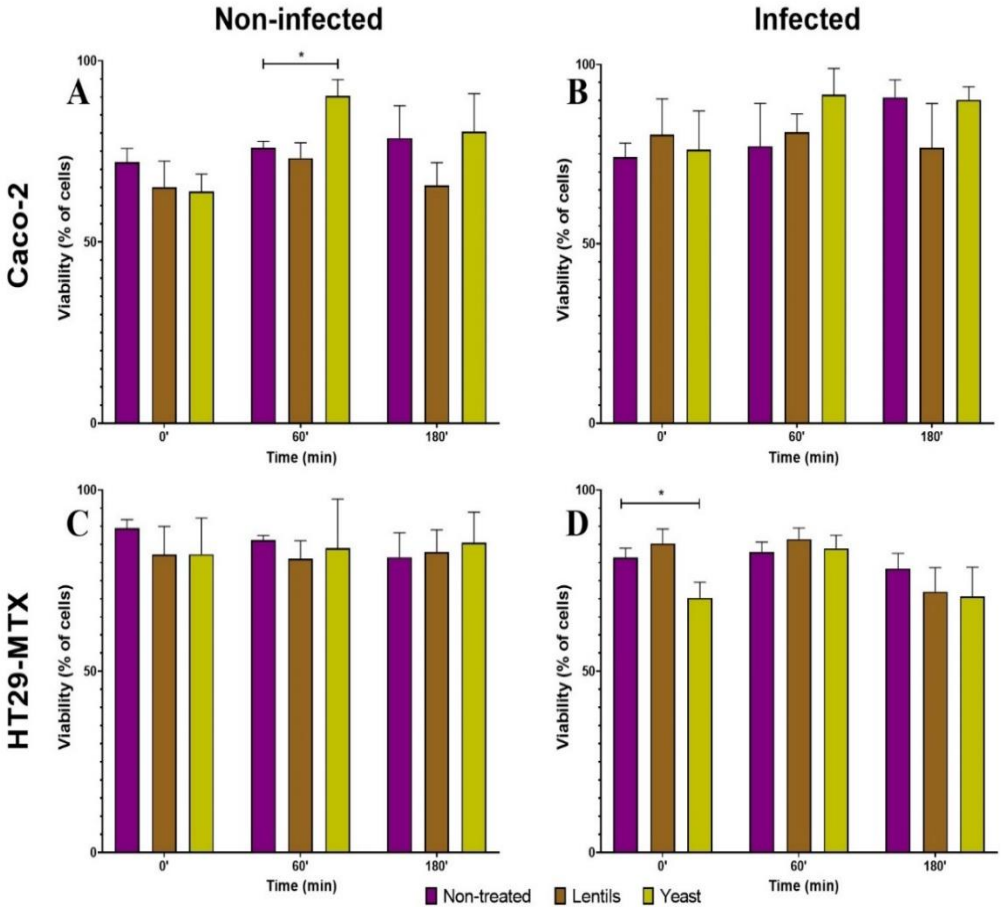


Figure 3.13. Overview of dietary-fiber containing products impact on ETEC strain H10407 virulence.

The main results obtained regarding dietary fiber containing product modulation of ETEC virulence are recapitulated according to the methods used.

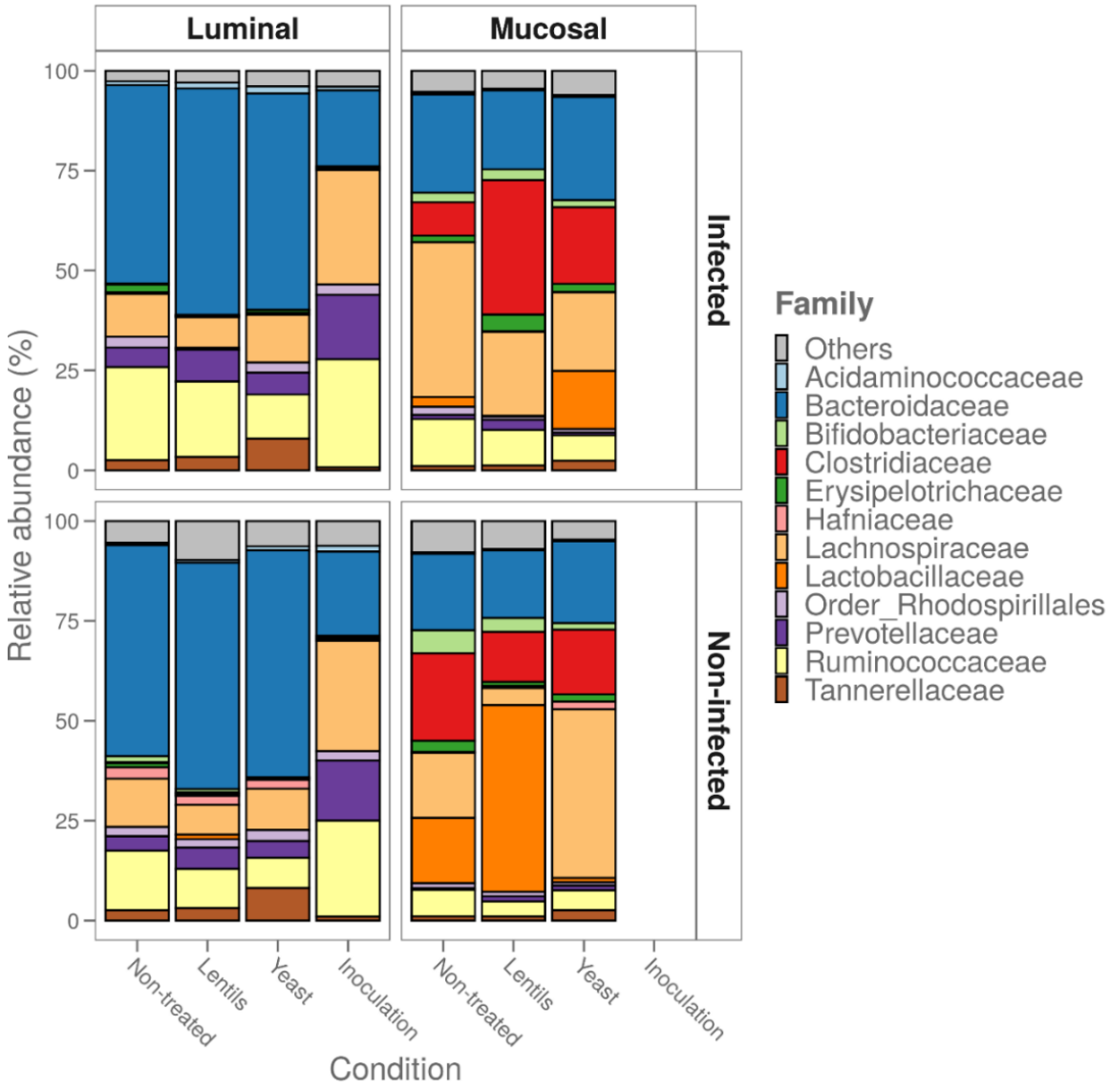
IL-8: interleukin 8 ; LT: LT toxin.

7. Supplementary Figures

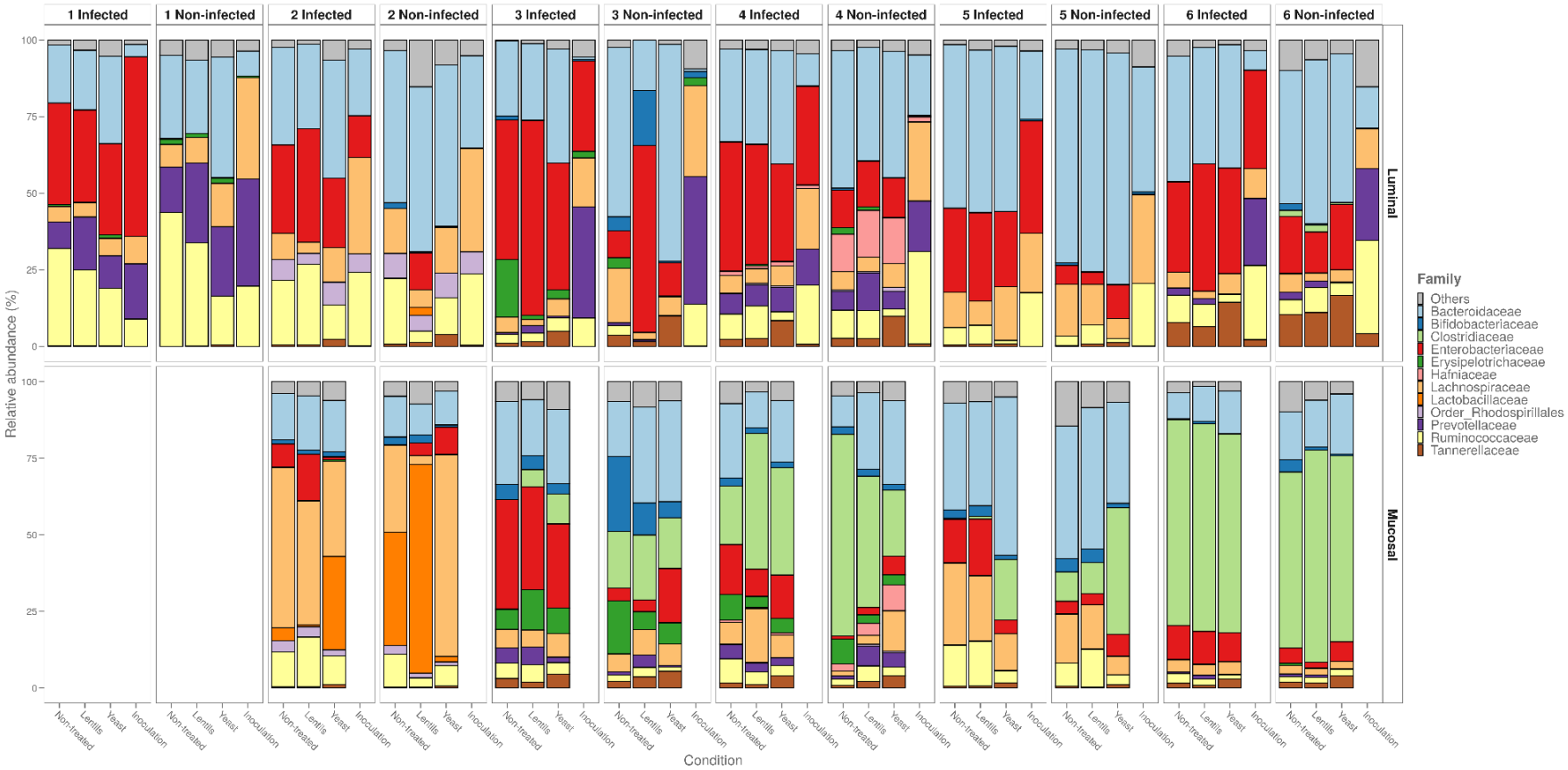


Suppl. Figure 3.1. Effect of dietary-fiber products and ETEC H10407 infection on intestinal cell viability.

Cell medium containing dietary fiber containing-products (lentils or yeast, 2 g. L^{-1}) or not was added on the apical side of the transwell of Caco-2 (**A, B**) and HT29-MTX (**C, D**) cells and infected (**B, D**) or not with ETEC strain H10407 (10^7 CFU.mL^{-1}) (**A, C**). Cell viability was then analyzed for 3 hours using Trypan blue exclusion assay. Graphs present the mean ± SD of three independent experiments. Statistical differences with the control condition (not infected, non-treated) were found by Dunnett's multiple comparisons (*: $p < 0.05$).

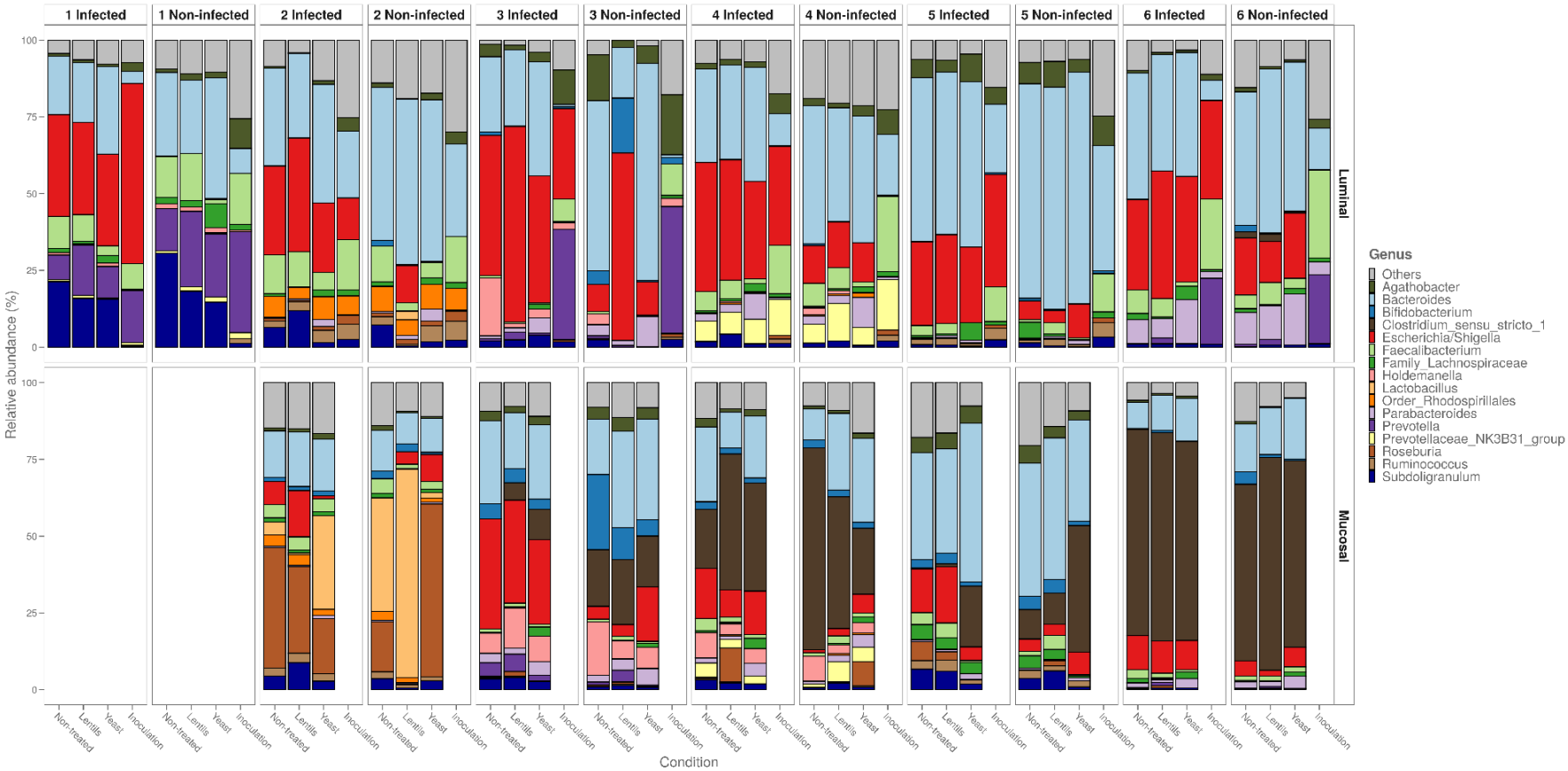


Suppl. Figure 3.2. Cumulative bar plots of fiber-containing products and ETEC modulation of microbiota composition at family level, excluding ASV1.
Batch experiments were performed using feces from six healthy donors, challenged or not with ETEC strain H10407, and treated or not with the dietary fiber-containing products. The graphs show cumulative bar plots of the relative microbial community composition at the family level excluding ASV1 belonging to *Escherichia*/*Shigella*, which has the highest number of reads. The area graphs show the relative abundance of the 12 most abundant families in all six different donors confounded.



Suppl. Figure 3.3. Donor specific impact of dietary-fiber containing products on ETEC modulation of microbiota β-diversity at the family level.

Cumulative bar plots of the relative microbial community composition in fecal batch experiments at the family level. The graphs show the relative abundances of the 12 most abundant families in the luminal and mucosal phases for the six different donors, as determined by amplicon sequencing. Mucosal phase data are missing for donor 1 due to a technical problem.



Suppl. Figure 3.4. Donor specific impact of dietary-fiber containing products on ETEC modulation of microbiota β-diversity at the genus level.

Cumulative bar plots of the relative microbial community composition in fecal batch experiments at the genus level. The graphs show the relative abundance of the 16 most abundant genera in the luminal and mucosal phases for the six different donors, as determined by amplicon sequencing. Mucosal phase data are missing for donor 1 due to a technical problem issue.

8. Additional Results

Effect of ETEC and toxins on cAMP induction

1. Material and methods

1.1. Maintenance of T-84 cells

T-84 cells were maintained in DMEM/F12 medium, supplemented with non-essential amino acid, antibiotic-antimycotic solution and 10% FBS. For experimental studies, T-84 cells were seeded at a density of 5.10^5 cells/well on 12 well-plates and allowed to differentiate for 2 days. All supplies were purchased from Gibco (Life Technologies, Paisley, UK) except for FBS and wells plates purchased from Thermo Fisher Scientific (Waltham, MA, USA).

1.2. Measurement of intracellular cAMP induction

To assay dietary fiber-containing products modulation of toxins/bacteria effect on cells, supernatant of an overnight culture of ETEC strain H0407 in CAYE medium was collected (3000g, 4°C, 5min), filtered (0.22µm) and submitted to ultracentrifugation (100 000g, 4°C, 1 hour). After addition of dietary fiber-containing products (lentils and yeasts, 2 $g.L^{-1}$), 1 mL of Cholera toxin of *Vibrio cholerae* (10 $ng.mL^{-1}$) or 1 mL of ETEC supernatant or ETEC (10^7 $CFU.mL^{-1}$) was deposited on T-84 cells. After 2 hours incubation (37°C, 5% CO_2), cells were washed twice with ice-cold PBS and intracellular cAMP levels were measured with cAMP Direct Biotrak ELISA kit (Cytiva, Marlborough, MA, USA) according to manufacturer's instructions.

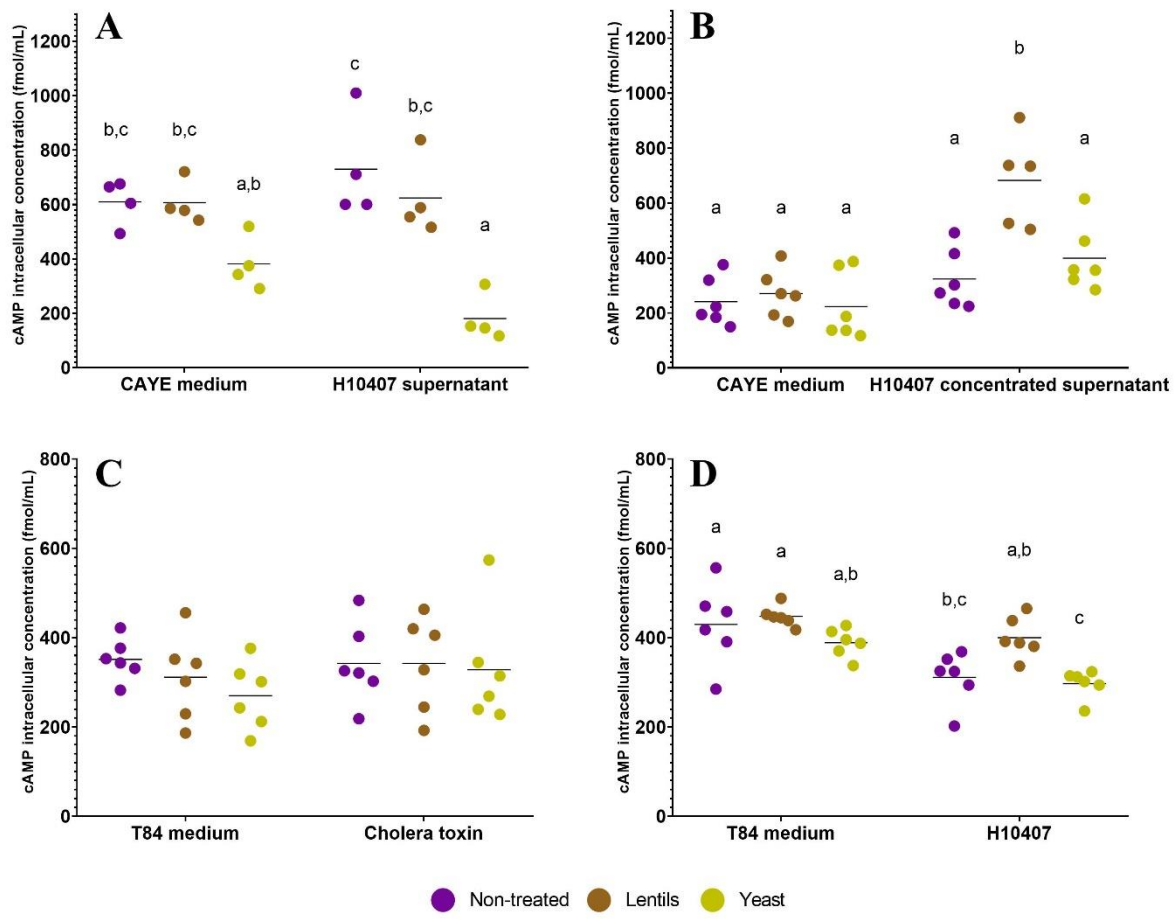


Figure 3.14 Impact of dietary fiber-containing products on the modulation of cAMP induction by toxins and ETEC whole cells.
Following overnight culture of ETEC strain H10407 in CAYE medium, T-84 cells were treated with filtered supernatant (**A**), ultra-centrifuged and filtered supernatant (**B**), pure cholera toxin at 10 ng.mL^{-1}(**C**) or with the ETEC strain at 10^7 CFU.mL^{-1} (**D**). Concomitantly, dietary fiber-containing products were added at 2 g.L^{-1} of final fiber content. Control experiments were performed without fibers ('non-treated'). After two hours, cAMP intracellular concentrations were measured by ELISA assay. The graphs represent the results of one (**A**) or three (**B-C**) independent experiments. Results that are not different from each other according to Tukey's multiple comparisons tests are grouped under a same letter ($p < 0.05$).

2.1. Toxins and ETEC fail to induce cAMP induction in T-84 cells

As lentil extract seemed to decrease LT toxin concentrations in the ELISA assay, we decided to investigate if this product could modulate toxin effects on human cells. Both LT and ST effects on intestinal cells are mediated through intracellular cAMP accumulation (Chao *et al.* 1994; Ellis and Kuehn 2010; Verhelst *et al.* 2013; Beltrán *et al.* 2015). In a first experiment, we detected a slight 1.2-fold non-significant induction of cAMP levels by an overnight culture in

CAYE medium of ETEC filtered supernatant (**Fig. 3.14.A**). As the LT toxin is known to be secreted mostly by ETEC OMVs (Ellis and Kuehn 2010), we decided to concentrate ETEC supernatant by ultracentrifugation (Horstman and Kuehn 2000). Despite a 2-fold increase in LT toxin concentration following ultracentrifugation (data not shown), concentrated ETEC supernatant only induced a non-significant 1.3-fold induction of intracellular cAMP levels (**Fig. 3.14.B**). Surprisingly, the Cholera toxin (80% amino acids sequence homology with ETEC LT toxin and a similar mode of action (Dubreuil 2012) and ETEC bacteria itself also failed to increase intracellular cAMP levels (**Fig. 3.14.C and 3.14.D)**. The ETEC bacteria appeared to even lower cAMP intracellular levels (70% decrease when compared to T-84 non-infected cells (**Fig. 3.14.D**). However, despite the inability of LT toxin from ETEC or the bacteria itself to induce significant increase of cAMP levels, it is worth noticing that the yeast cell walls constantly tended to lower cAMP intracellular levels (decrease varying between 10 to 40%), while the lentils did not.

3. Discussion

Such data contrary to expectation impede any conclusion on the dietary fiber-containing products effects and particularly on the lentil extract capacity to hinder LT toxin effects. This result is surprising, as both LT and ST toxins have been regularly found to trigger cAMP production in different cell lines and especially T-84 cells (Zhang *et al.* 2010; Verhelst *et al.* 2013; Beltrán *et al.* 2015). As highlighted with the absence of induction of cAMP levels by pure Cholera toxin (10 $ng.mL^{-1}$) for 2 hours, we could argue that this probably comes from issues with the cells or the commercial kit used to measure cAMP levels.

Section III – General discussion

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1. Positioning the research

Diarrheal diseases strike populations of all ages, across all geographies and was responsible in 2016 diarrhea for more than 1.6 million human deaths worldwide (Khalil *et al.* 2018). Among the 13 recognized etiological agents of diarrheal diseases (e.g. bacteria, parasites, viruses), ETEC alone annually accounts for hundreds of millions of diarrheal episodes over the world (Khalil *et al.* 2018). In 2016, ETEC was the eighth leading cause of diarrhea mortality, accounting for an estimated 51,000 deaths (Khalil *et al.* 2018). Noteworthy, ETEC burden attests considerable disparities according to age range as well as socioeconomic status and living conditions of the population. Two at-risk groups for ETEC infections are distinguished with (i) infants living in resource-limited countries, and (ii) adults traveling in endemic zones such as Asia, Africa and Latin America. Among the millions of people traveling to endemic countries each year, nearly one third contracts traveler's diarrhea. In some areas, this number goes up to 60% (Greenwood *et al.* 2008). The inconvenient traveling situations due to diarrhea require to alter planned activities in 40% of the total cases, to stay in bed for at least one day in 20%, to seek for medical care in 10%, and to require hospitalization in 3% (Steffen *et al.* 2006; Giddings, Stevens and Leung 2016). Furthermore, in 1% of cases, traveler's diarrhea can evolve from an acute distress to a chronic disease and sometimes it may have long-term consequences on the patient overall health (Steffen, Hill and DuPont 2015; Steffen 2017; Fedor, Bojanowski and Korzeniewski 2019). The consequences results in post-infectious (PI) sequelae ranging from functional gastrointestinal disorder to IBS (Halvorson, Schlett and Riddle 2006). ETEC is the most common pathogen identified in traveler's diarrhea accounting for 30-40% of travelers cases to Latin America and Asia, respectively (Jiang and DuPont 2017; Boxall *et al.* 2020). In average, nearly one out of six travelers to endemic regions is infected by ETEC (Giddings, Stevens and Leung 2016).

However, no specific treatment targeting ETEC is currently available. Alongside with symptoms management, antibiotic therapy remains the most effective treatment for severe cases of traveler's diarrhea and clinicians commonly prescribe antibiotics to international travelers for self-treatment in case of diarrheal symptoms while abroad. However, due to this selective pressure of antibiotics for treatment, recent studies have expressed concerns about the potential acquisition and subsequent carriage of multidrug-resistant pathogens (Kennedy and Collignon 2010; Ruppé *et al.* 2015). Antibiotic resistance is widely regarded as one of the major public health concerns of the 21st century, leading to longer hospitalization, increased medical costs and mortality. Thus, it primordial to develop new therapeutic strategies. Different solutions are

proposed and tested in the literature such as vaccines (Khalil *et al.* 2021), probiotics (Roussel *et al.* 2021) and bacteriophages (Piya *et al.* 2019b), but are still under development. The use of dietary fibers could be another alternative strategy since they have already shown antagonistic effects against enteric pathogens by different means. Some of these mechanisms are direct like bacteriostatic effect or anti-adhesion properties (Chantarasataporn *et al.* 2014; Ma *et al.* 2016; Vardaka, Yehia and Savvaidis 2016; Garrido-Maestu *et al.* 2018). Some dietary fibers can act as a decoy for pathogen/toxin binding to mucosal polysaccharides (Idota *et al.* 1995; Di *et al.* 2017; Liu *et al.* 2017; Leong *et al.* 2019). The dietary fiber effect can also pass through microbiota modulation, for instance by supporting probiotic species with anti-infectious properties (Fooks and Gibson 2003). Interestingly, dietary fibers could also lure the resident gut microbiota from mucus consumption, thereby impeding access to the underlying epithelium to pathogen such as *Citrobacter rodentium* (Desai *et al.* 2016; Neumann *et al.* 2021).

The protection of the mucus compartment from interactions with ETEC could be particularly relevant, sine this pathogen seems to be adapted to this host barrier. Some clues indicate that ETEC possess adhesins specifically targeting mucus patterns (Qadri *et al.* 2007; Ahmed *et al.* 2009; Kumar *et al.* 2018; Kuhlmann *et al.* 2019). ETEC possess also at least two mucinases able to promote access to the epithelium by degrading the core of mucin glycoproteins (Kumar *et al.* 2014; Luo *et al.* 2014: 20). The LT toxin also stimulates mucin production in the human small intestine (Sheikh *et al.* 2021). Furthermore, the mucus compartment has already been suggested to help ETEC maintenance in the lower intestinal tract conditions *in vitro* where the microbiota prevails in high numbers (Roussel *et al.* 2020a).

Despite this mucus dependency, studies specifically targeting anti-infectious effects of dietary fibers upon ETEC from human origin are scarce and have investigated very few different fibers, while carbohydrates are considered as the most diverse group of biological molecules Milk oligosaccharides and plantain soluble fibers were proven to reduce human ETEC strain adhesion to Caco-2 intestinal epithelial cells (Idota and Kawakami 1995; Roberts *et al.* 2013; Salcedo *et al.* 2013).

In this context, this joint doctoral research aimed to **(i) better decipher the interactions of the prototypical ETEC strain H10407 with the mucus compartment in the simulated human gastrointestinal tract (axis I)** and **(ii) investigate more widely the potential of dietary fiber-containing products as a relevant anti-infectious strategy (axis II).**

To answer these research questions, the PhD work was designed to rely on various complementary *in vitro* models, which shows numbers of advantages to study pathogenic agent

such as flexibility, safety, absence of ethic issue, low cost and high screening capacities. For the second time, a joined PhD on the ETEC pathogen was initiated between UMR MEDIS, Université Clermont Auvergne, France (33 months) and the CMET laboratory, Ghent University, Belgium (9 months), both with a leadership in *in vitro* human gut simulation from more than 20 years. In addition, a partnership with industrial companies (Lallemand Animal Nutrition, Limagrain, PiLeJe and HARi&CO) was achieved and they provided the fiber-containing products tested in the second and third chapter.

Prior to initiate the experimental work, an exhaustive review of the literature was made on how mucus, dietary fiber, gut microbiota and their interactions shape pathogenic infection and the methods used to investigate these interactions. This literature review led to the publications of two review papers remodeled in the bibliographic section of this manuscript. This state-of-the art confirmed the necessity to further assess ETEC adhesion affinity for the mucus. The publications about mucus modulation of ETEC virulence gene expression were very limited, with only LT and a few CF encoding genes investigated in simple batch experiments (Haines *et al.* 2015). In her PhD, Charlène Roussel integrated a mucus compartment in fermentation models to study ETEC pathophysiology in colonic human conditions (Roussel *et al.* 2020a), but no one has never specifically addressed the impact of this compartment on ETEC survival in the human gastrointestinal tract or on the modulation of microbiota composition/activity. Lastly, no report was found on how mucus could specifically impact innate immunity induction or intestinal barrier function in an ETEC context. Our literature review also shed light on the multiple mechanisms by which dietary fibers could limit enteric infections, but also that research papers usually do not investigate more than one mechanism at the same time. In particular, a limited number of studies were found on how dietary fibers could directly shape virulence gene expression in human enteric pathogens. Only chitosan was reported to modulate *Campylobacter jejuni* genes involved in motility, quorum sensing, stress response and adhesion (Wagle *et al.* 2019). Regarding ETEC strain from human origin, the studies only investigate the effects of fibers on toxin or pathogen binding. The use of dietary-fiber containing products as anti-infectious strategy against ETEC seemed particularly relevant as ETEC action is considered to be the distal part of the small intestine, where dietary fiber degradation is not completed. **Table 1** summarizes the studies investigating the effect of mucus or fibers on ETEC human strains.

Table. 1 Summary of the main literature findings about mucus and dietary fiber modulations of ETEC strain virulence and survival from human origin.
Built from personal sources.

	Mucus compartment effect	Dietary fiber effect
Survival	Despite no comparison to a control matrix, inclusion of mucin-beads in the M-SHIME model participates to ETEC H10407 maintenance in ileal and colonic compartments (Roussel *et al.* 2020).	If chitosan, a human engineered fiber, has been regularly reported for its wide spectrum of bactericidal activity, its effect has never been assayed on human-targeting ETEC survival (Liu *et al*. 2000, Qi *et al*. 2004, Chantarasataporn *et al*. 2014, Jeon *et al*. 2014, Ma *et al*. 2016, Garrido-Maestu *et al*. 2018).
Adhesion	• ETEC strain H10407 adhesion to HT29-MTX and HT29-FU, two mucus secreting cell lines, shows a better co-localization pattern for the brush border than for mucus patches (Kerneis *et al*. 1994). • Coli surface antigens (CS2, CS5 and CS6) bind to components of rabbit intestinal mucus (Helander, Hansson and Svennerholm 1997). • CFA/I adheres to blood group A-terminated glycosphingolipids, which can be expressed in human mucus (Jansson *et al*. 2006, Ahmed *et al*. 2009).	Milk oligosaccharides and plantain soluble fibers at concentrations of $1 g.L^{-1}$ and $5 g.L^{-1}$ respectively were proven to reduce ETEC adhesion to Caco-2 cells up to 80% (Idota and Kawakami 1995, Roberts *et al*. 2013, Salcedo *et al*. 2013).
Toxin effect	Pig gastric mucin inhibits LT toxin secretion of ETEC strain 258909–3 in simple batch culture (Haines *et al*. 2015).	• GM1 oligosaccharide and siallylactose inhibit LT binding and dowstream fluid secretions in rabbit intestinal loops (Otnaess, Laegreid and Ertresvåg 1983, Idota *et al*. 1995). • A human milk fucosylated oligosaccharide inhibits ST binding and subsequently reduces diarrhea in mice (Newburg *et al*. 1990, Crane *et al*. 1994).
Virulence gene expression	Pig gastric mucin favors colonisation factors (CFA/I and CS1/CS3) expression from ETEC strains H10407 and 258909–3 in simple batch culture (Haines *et al*. 2015).	None
Innate immune response	If human targeting ETEC infections are well known to be associated with intestinal inflammation and disease severity (Mercado *et al*. 2011, Iqbal *et al.* 2019, Brubaker *et al*. 2021), no data have been found about mucus and dietary fibers potential implications.	
Mucus related genes modulations	Non-relevant	None
Changes in epithelial barrier permeability	Compared to the summary of report in pig models, studies reported human targeting-ETEC induced changes in epithelial barrier permeability are scarce. The ETEC strain H10407 elicited a reduction in trans-epithelial electrical resistance in T84 epithelial cell monolayers, mediated by the LT toxin (a know inducer of paracellular permeability) (Harper *et al*. 2011). No data have been found regarding mucus or dietary fiber potential implications.	
Gut microbiota modulations	Despite no comparison to a control matrix in the M-SHIME model, mucin-beads associated human microbiota is significantly impacted by ETEC strain H10407 (Roussel *et al*. 2020).	None

Thus, in the first experimental chapter, we investigated more deeply how mucus shapes ETEC virulence and survival in the human gastrointestinal tract, notably questioning the missing pieces of the puzzle: adhesion propensity, survival, virulence gene expression, innate immunity, intestinal barrier function and microbiota modulation. The knowledges acquired in the first chapter would allow us to better adapt the models used to investigate the fiber anti-infectious properties. The second chapter was then designed to screen out of the eight fiber-containing products the two ones with the most promising properties. The third chapter was the

one really settled to decipher if the fiber selected products could present a wide range of anti-infectious properties, considering ETEC growth/survival, adhesion, virulence gene expression, induction of innate immunity, effect of intestinal barrier function and gut microbiota modulations. The next section of this discussion will then go through all the parameters of the ETEC pathophysiology studied in the two PhD axes, discussing for each of them together the research outcomes and the potential perspectives

2. The research outcomes discussion and associated perspectives

2.1. Dietary fiber-containing product selection and extraction

In the frame of the DYSFIBRE project, industrial partners provided 6 products from diverse origins (vegetal or microbes), namely green lentils, red beans, oat, an oat bran extract, wheat starch and specific yeast cell walls from *Saccharomyces cerevisiae*. These products contain different types of soluble and/or insoluble fibers, among which are resistant starch for wheat starch, beta-glucans for oats and specific yeast cell walls, mannans for specific yeast cell walls, celluloses and hemicelluloses for lentils and read beans (Deehan *et al.* 2018). Two additional commercial products were added in the screening program, i.e. locust bean gum and guar gum containing galactomannans. Indeed, galactose containing dietary fibers, and in particular galactomannans have already shown to reduce Enterobactericeae pathogen mucosal colonisation in different *in vitro* models such as epithelial cells line or the M-SHIME (Shoaf *et al.* 2006; Badia *et al.* 2012; Sarabia-Sainz *et al.* 2013; Van den Abbeele *et al.* 2016; Leong *et al.* 2019). Concerning galactomannans, at doses from 0.5 to 20 $g.L^{-1}$, they reduce up to 70% *Salmonella enterica* serovar Typhimurium and ETEC K88 adhesion to IPI-2I cells and Caco-2 cells, respectively, decrease IL6 and CXCL8 inflammation markers (Badia *et al.* 2012).

Some of the products provided were raw material and therefore need further extraction. As dietary fibers are polysaccharides (or oligosaccharides) not digested by the human gut (Jones 2014; Porter and Martens 2017), we chose first to hydrolyze proteins and simple sugars, then precipitate the soluble dietary fibers by ethanol addition, and finally centrifuge the suspension to pellet both soluble and insoluble dietary fiber fractions. This technic was inspired from the AOAC official methods of dietary fiber measurement in food (McCleary *et al.* 2013) and digestion performed in the TIM-1 model (Cordonnier *et al.* 2015). Contrarily to AOAC method that usually requires filtration, we choose centrifugation in order to keep high amount of material (**Fig. 2**). After extraction of raw materials, all the products were tested for sterility.

Homemade extracts and wheat starch were sterile, while others were contaminated. To avoid any bias due to contamination in further experiments, all contaminated products were autoclaved before any testing, despite the know effect of heating on dietary fibers, notably by changing starch polymerization (Kapusniak *et al.* 2021).

Dietary fiber content of the eight products was then analyzed by CAPINOV, according to the AOAC 985.29 method (Stephen *et al.* 2017), except for wheat starch for which the resistant starch content was directly provided by the industrial partner. None of the products were pure, ranging from 17% (w/w) of resistant starch content for wheat starch, to 84% (w/w) of dietary fiber content for guar gum. To consider these differences, we decided to normalize our conditions depending on the fiber content. In consequence, different amounts (weight) of products were applied depending on the product considered. According to different sources, dietary fiber intakes in industrialized countries would be around 10 to 30 grams of fibers per day (King, Mainous and Lambourne 2012; Holscher 2017). The gut lumen does not represent a continuous watery compartment (Schiller *et al.* 2005), but it is generally acknowledged that nearly 10 L of fluid pass through the GI tract daily (Kiela and Ghishan 2016). The bulk transport of these fluids and electrolytes occurs along the small intestine, decreasing the amount of fluid in the distal parts (Kiela and Ghishan 2016). We decided to test the anti-infectious properties of fiber-containing products at the *in vivo* relevant concentration of 2g.L^{-1} of final fiber content.

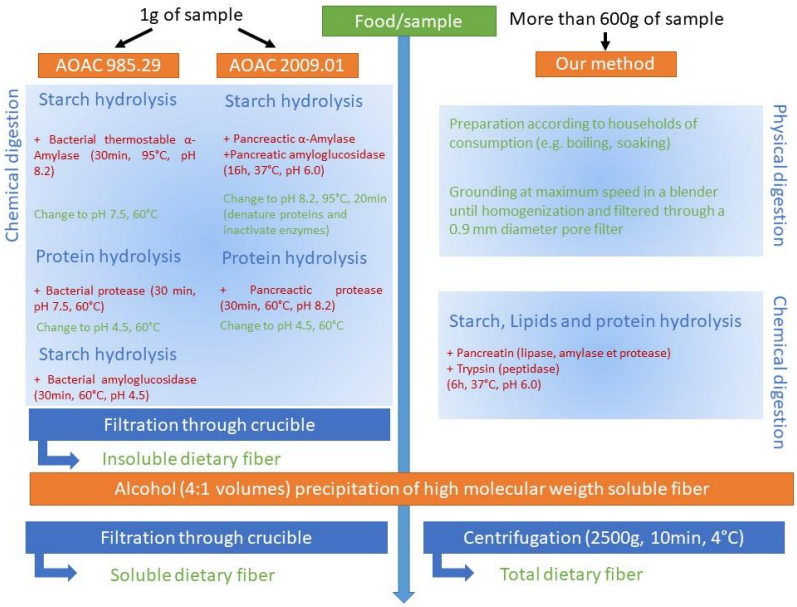

Figure 2. Comparison of the dietary fiber extraction method used in this study to the official method (AOAC) for dietary fiber measurement.
Built according to McCleary *et al.* 2013

2.2. Investigation of ETEC physiopathology parameters

2.2.1. Growth/survival in the upper gastrointestinal tract

In the temporality of enteric infection, the capacity of the pathogen to reach its action is the first parameter to consider. ETEC action site is assumed to be the distal part of the human small intestine, as evidenced from animal studies (Al-Majali and Khalifeh 2010; Gonzales *et al.* 2013; Rodea *et al.* 2017). It would be interesting to address this question in human patients, for instance by visualizing ETEC-induced inflammation by sensitive imagery technic as computed tomography, magnetic resonance imaging or PET imaging (Brewer *et al.* 2008; Le Fur *et al.* 2020).

The integration of mucin secretion and physical surface in the TIM-1 model indicated that the mucus compartment could help ETEC to maintain when facing harsh upper GI tract conditions. Indeed, mucin beads constitute a dense physical niche in which type III mucin is concentrated at 50 $g.L^{-1}$, which prevent ETEC from disappearance in TIM-1 stomach and duodenum. This is probably due to the high buffering capacity of mucin glycoproteins (Lewis, Keener and Fogelson 2017), partly due to their ability to sequestrate hydrogen (Schreiber and Scheid 1997). We thus postulate that the presence of a mucus niche in the upper part of the GI tract is not necessarily an advantage against pathogen with more distant action site. The mucus could allow bacteria to hold better conditions where they can proliferate (Vesper *et al.* 2009; Rahman *et al.* 2020). In the lower intestinal compartment, we evidenced an exponential ETEC growth, most probably due to its ability to use mucin as nutritive substrate. Such growth evidenced in the TIM-1 is not directly transposable to the *in vivo* situation, where mucin availability is limited due to mucus layer structural properties and competition with resident microbiota (**Fig. 3**). Still, it confirms that ETEC seems to be well adapted to maintain in the distal small intestine, as already demonstrated in the TIM-1 model (Roussel *et al.* 2020a). To go further inside the mucus impact in the lower intestinal compartments, we can imagine to compare ETEC survival in the M-SHIME and SHIME configuration, in which microbiota competition would maybe inhibit ETEC exponential growth observed in the TIM-1. Concerning *in vivo* animal studies, such investigations are not recommended since impacting the mucus layer by gene knock out (for example) would induce a bias due to over-innate immunity activation (Morampudi *et al.* 2016). One can also argue that we could knock out ETEC mucus-adhesin genes to evidence their involvement in ETEC animal colonisation, but keeping in mind that adhesin receptors to ETEC differ between humans and animals.

Concerning the second axis, we reported that none of our dietary fiber-containing product could impede ETEC viability counts in simple culture media, as a fiber-like chitosan does (Chantarasataporn *et al.* 2014; Ma *et al.* 2016). This is not surprising as chitosan is a human engineered fiber, which possesses very specific anti-bacterial properties (Jeon *et al.* 2014). We also reported that fiber products sustain ETEC growth in minimal medium, which is most probably due to the non-fiber fraction of the products, since pathogens as ETEC are not known to degrade complex polysaccharides and generally behave a cross-feeders (Pacheco *et al.* 2012; Ng *et al.* 2013; Conway and Cohen 2015, Onyango *et al*. 2021). This non-fiber fraction may be degraded and absorbed in the human upper intestinal tract, limiting ETEC ability to feed on in the distal parts of the intestine. Controlling the effect of fiber-containing products on ETEC growth in more *in vitro* (TIM-1 or M-SHIME models) or *in vivo* (e.g. piglets) relevant conditions integrating digestion, absorption and endogenous microbiota should be necessary in a next future.

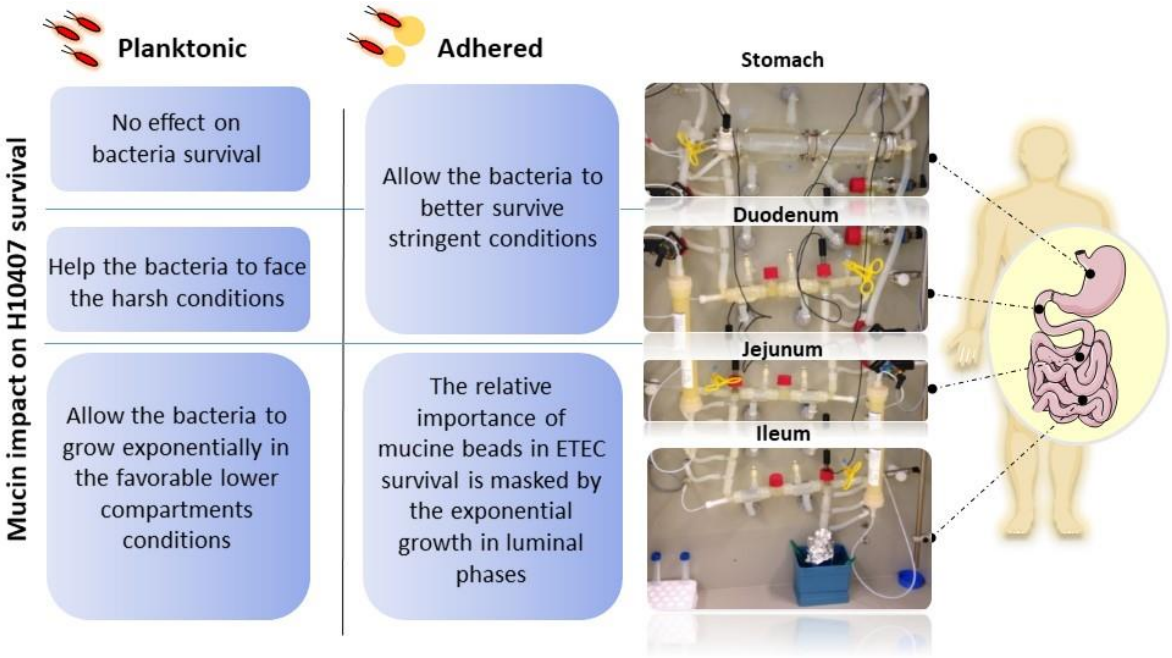

Figure 3. Summary of mucin impact on ETEC H10407 survival in the TIM-1 model.
The impact of mucin secretion and mucin-beads on the ETEC H10407 strain survival are discussed according to phase (planktonic *versus* adhered) and compartment considered.
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2.2.2. Virulence gene expression

Enteric pathogens like ETEC have deployed mechanisms to sense their evolving environment, notably the digestive environment (Roussel *et al.* 2020a). In response to signals received, they will act accordingly by turning “on” or “off” their virulence genes expression. Roussel and

colleagues provided the most complete survey of ETEC virulence genes regulation facing the human GI tract conditions, investigating ETEC behavior in both the TIM-1 and M-SHIME model (Roussel *et al.* 2020a). Surprisingly, most of ETEC virulence genes were not induced at the presumed ETEC action site (distal small intestine). More studies are therefore needed to decipher the requirements for ETEC to turn on its evil machinery. Our contribution to the field rely on investigating mucus-compartment and dietary-fiber products impact on ETEC virulence.

Regarding the impact of mucus, the sole work available in the literature reported that pig gastric mucin favored CFA/I and CS1/CS3 expression in the human ETEC strains H10407 and E24377A respectively, but decreased LT toxin secretion in the E24377A strain (Haines *et al.* 2015). This work was performed in simple culture media, far from human gut physiology. Using the complex TIM-1 model, we showed that ETEC virulence genes are not activated in the ileum in presence of mucin. The two only genes found to be activated were mucinase-encoding genes, not described in Roussel's study (Roussel *et al.* 2020a: 20). We also reported that ETEC adhesion to mucin-beads in the TIM-1 model does not have a significant impact on virulence genes activation. This could be due to the presence of mucin in both mucin beads and mucin secretion, added to simulate mucus shedding similarly to *in vivo* situation. Divergence between *in vitro* results and the potential site of ETEC colonisation potentially points out a sequential activation of virulence genes expression occurring *in vivo* but not observed in the TIM-1 model, or the absence of virulence gene activation could result from absence of of host cells. Kansal and colleagues showed that, with the H10407 strain specifically, adhesion to non-mucus secreting Caco-2 cells promoted LT encoding gene expression while the expression of CF was time-dependent (Kansal *et al.* 2013). This strengthens the hypothesis that a real host part is required for virulence promotion. Our cell experiments confirmed that cellular adhesion promoted global virulence genes expression of ETEC strain H10407, especially when mucus-secreting cells were present. This observation is the first one showing the importance of mucus-secreting cells in virulence gene induction of ETEC. Still, it would be interesting to assay the specific involvement of the mucus layer, by comparing cell lines as the HT29-MTX and HT29 (Gagnon *et al.* 2013). Coupling digestive simulators like the TIM-1 or SHIME and cellular models (Marzorati *et al.* 2014) could also bring further insights on the relative importance of host part relative in the triggering of ETEC virulence.

Considering these results, it was chosen to test the impact of dietary fiber-containing products on ETEC virulence gene expression using mucus secreting-cells. The literature concerning fiber modulation of pathogen virulence genes is really scarce, only one found about

a human intestinal pathogen (Wagle *et al.* 2019). Despite low variations in gene expression, we showed that dietary fiber-containing products tended to increase ETEC colonisation-related and decreased toxin-related gene expression (**Fig. 4**). Considering that we did not observed an increase in the adhered ETEC population, these modulations are encouraging for the development of a fiber-based strategy. Further mechanistic studies would be welcomed to highlight the significance of these observations in ETEC pathophysiology. First, the observed modulations could be investigated at the protein level, for instance, by ELISA or western-blot technics (Rocha *et al.* 2013). Second, methods as gene knock-out could emphasize the different virulence factors involvement in ETEC epithelial colonisation (Johnson *et al.* 2009; Wang, Gao and Hardwidge 2012). Of note, we also showed that, in the non-adhered ETEC populations, toxin-related genes were increased and decreased by the yeast cell walls and lentil extracts, respectively. This is consistent with the effect of fibers on toxin production measured in simple culture media (see section bellow), suggesting it could pass (at least partially) by virulence gene modulation. The analysis by RT-qPCR of ETEC genes expression involved in LT production in CAYE medium would allow to confirm this hypothesis. Finally, all the modulations we highlighted would deservedto be investigated in more *in vitro* or *in vivo* relevant contexts including the product digestion.

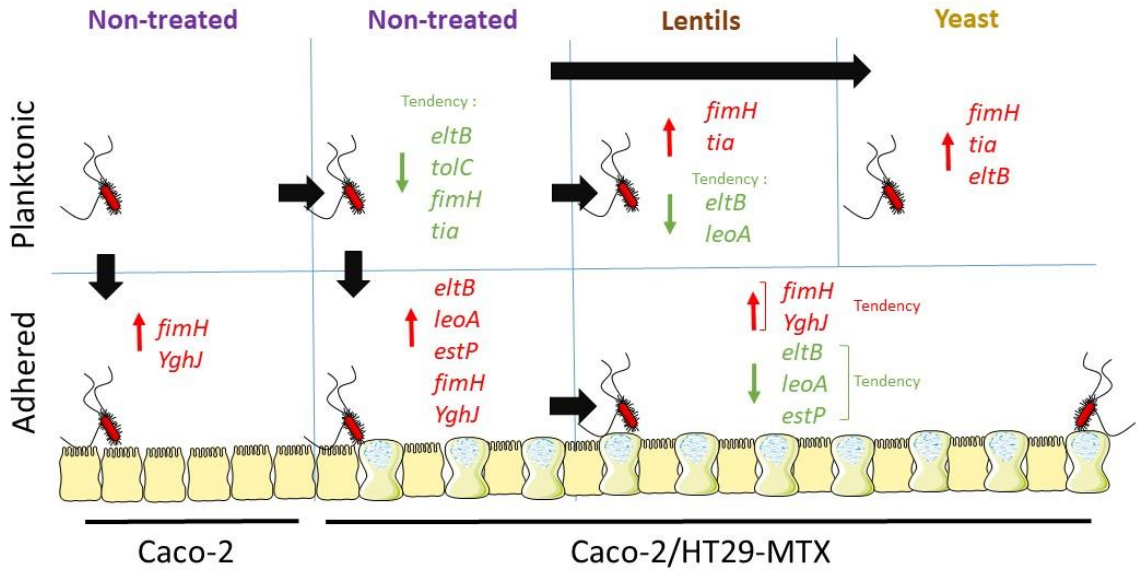

Figure. 4 Summary of mucus-secreting cells and dietary fiber-containing products impact on ETEC strain H10407 virulence gene expression.
The effect of mucus-secreting cells and fiber products on ETEC strain H10407 virulence is summarized. Type of comparison is indicated by a black arrow and promoted virulence genes are indicated in red while inhibited are mentioned in green. When the depicted changes were no significant; "tendency" is indicated.
Built from personal source.

2.2.3. Toxin production and their effect on intestinal cells

Since ST and LT toxins are the main agents responsible for ETEC-induced diarrhea (Ruan *et al.* 2012), they are certainly the important output to monitor when studying ETEC pathogenicity. Whatever the axes considered, only LT toxin was studied in this PhD work by GM1-ELISA assay (Verhelst *et al.* 2013), Unfortunately, this ELISA assay was not very sensitive, impeding investigations in media other than overnight culture supernatant. In the future, this assay should be improved to increase sensibility and enable investigations in more complex samples as in *in vitro* digestas. As ST toxin was regularly considered more virulent than LT (Troeger et al. 2017), a collaboration was initiated with the Bacteriology Laboratory of Butantan Institute (São Paulo, Brasil), which provided us anti-ST antibodies (Rocha *et al.* 2013). Unfortunately, the time was running out to fully to optimize the ST ELISA assay. Since ST dosage is uncommon in the literature, developing a reliable method allowing ST quantification would also be a real advance to study of ETEC physiopathology.

In the frame of the first axis, we did not investigated modulation of LT toxin production by mucus at the protein level and this could constitute a nice perspective of our work, since to our knowledge, no one has ever performed such experiments.

Regarding the second axis, this work is the first one reporting that a fiber-containing product could reduce LT toxin concentration. We also reported that this antagonistic effect of lentil extracts could be partially mediated by a direct inhibition mechanism, due to toxin binding by some lentils components that act as a decoy (Otnaess, Laegreid and Ertresvåg 1983; Newburg *et al.* 1990; Idota *et al.* 1995; Verhelst *et al.* 2013). This toxin binding by fibers could inhibit their adhesion to mucosal receptors and thus their downstream effect. Intracellular levels of cAMP and cGMP are usually considered as good indicators of toxin effects on human intestinal cells (Zhang *et al.* 2010; Beltrán *et al.* 2015). For an unknown reason, we failed to detect cAMP or cGMP induction in T-84 cells whatever the tested conditions (i.e. ETEC strain H10407, concentrated over-night culture supernatant, or pure Cholera toxin). We could imagine these results from technical issues either with the cells or the kits used for detection. To solve this problem, different intestinal cells (Caco-2, IPEC-J2, Vero cells), different ways to detect cAMP/cGMP intracellular concentration, or both (Zhang *et al.* 2010; Kern *et al.* 2017) could be tested. If the use of other human cell lines fails to induce intracellular cAMP/cGMP, other approaches should be envisionned, such as *ex vivo* organ culture. In particular, the rabbit ileal loop technic had been regularly used to monitor luminal liquid secretions following ETEC/*Vibrio cholerae* or their toxins administration (Bailey and Sangwan 1986). As already

mentioned, animal models could also be considered to follow-up at the whole organism level the effect of toxins and/or ETEC administration, on different outputs such as diarrhea, ETEC colonisation or microbiota modulation (Zhang and Francis 2010).

2.2.4. Mucus and cellular adhesion

Once ETEC has reached its action site, adhesion to the intestinal mucosa and mucus layer is primordial to fulfil its infection cycle. Even if ETEC exhibits mucus-recognizing adhesins (Jansson *et al.* 2006; Qadri *et al.* 2007; Ahmed *et al.* 2009; Kumar *et al.* 2018) and mucinases (Kumar *et al.* 2014; Luo *et al.* 2014), only one previous work had focused on the propensity of human ETEC strains to adhere to the mucus layer. Kerneis and colleagues showed that adhered ETEC strain H10407 co-localize more with the brush border than mucus patches of HT29-MTX and HT29-FU cell lines (Kerneis *et al.* 1994). This is in opposition with all the works performed on other *Enterobactericeae* pathogens (Gagnon *et al.* 2013; Hews *et al.* 2017; Rajan *et al.* 2020; Nickerson *et al.* 2021). Here, we showed that ETEC presents an adhesion affinity towards porcine mucin and mucus-secreting cell lines. This adhesion affinity was conserved after simulated human digestion, which is of great importance as cellular adhesion of pathogens is known to be modified by digestion (Bengoa *et al.* 2018) and previous studies on ETEC did not integrate this parameter (Gagnon *et al.* 2013; Hews *et al.* 2017; Rajan *et al.* 2020; Nickerson *et al.* 2021). Increased adhesion to mucus-secreting cells may be linked to specific adhesion to the secreted mucus (Nickerson *et al.* 2021) or goblet cell receptors or modulation of ETEC virulence gene associated to colonisation, as described above. The use of microscopic technics, such as scanning electron microscopy (SEM), transmission electron microscopy (TEM) or periodic Acid Schiff/ Alcian Blue staining (Rajan *et al.* 2020), would allow to decipher if ETEC increased adhesion to mucus-secreting cell line is due to specific adhesion to mucus patches. Another option would be to compare ETEC adhesion to HT29 *versus* HT29-MTX cell lines (Gagnon *et al.* 2013; Naughton *et al.* 2013; Martins *et al.* 2015). Our findings confirmed the relevance of mucus-secreting models to assay the impact of fiber products on ETEC adhesion.

To our knowledge, the screening program presented in chapter II is the first work assessing the effect of a wide panel of fiber-containing products on ETEC human strain mucosal adhesion. To avoid any possible sedimentation effect of insoluble particles, we used mucin beads maintained under constant agitation. This precaution was not applied to cellular experiments, which would gain in the future by being performed upside down, in order to avoid the fiber particles sedimentation effect. In the chapter II, yeast cell walls product was evidenced

as the first fiber product from non-plant origin to present an anti-adhesion effect against human-infecting ETEC, after HMO and plantain soluble fibers (Idota and Kawakami 1995; Roberts *et al.* 2013; Salcedo *et al.* 2013). The down-regulation of bacterial adhesins is probably not involved as their expression is promoted by the product. In contrast, the high adhesion propensity of ETEC for the yeast product indicates most probably a decoy mechanism, as already reported with whole living yeast probiotic cells (Roussel *et al.* 2018b).

We showed ETEC adhesion affinity to the mucus compartment and the antagonistic effect of fiber products (**Fig. 5**), but we did not investigate the specific involvement of adhesins and mucosal receptors in these phenomena. In the future, this could be achieved by using mucosal polysaccharides degrading enzymes, blocking antibodies, addition of decoying saccharides motives or combination of them (Sheikh *et al.* 2017). Bacterial or eukaryotic genes knockouts would also be helpful for further mechanistic insights (Sheikh *et al.* 2017).

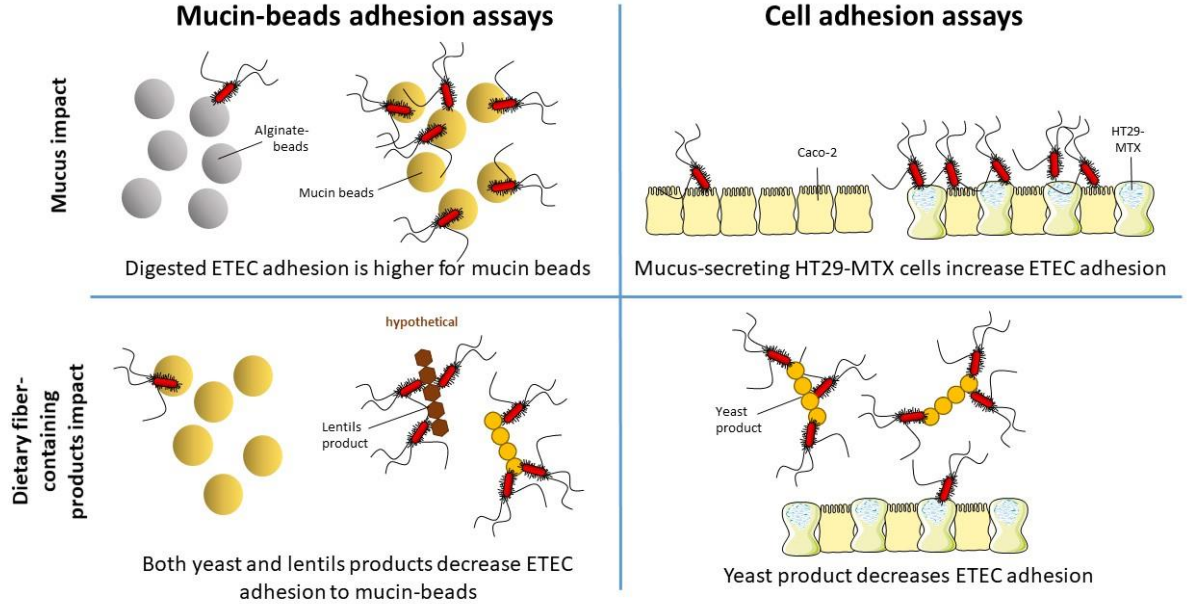

Figure 5. Summary of the main findings about mucin and dietary fiber-containing products impact on ETEC adhesion.
The figure summarizes the findings about the modulation of ETEC adhesion by mucus and fibers in mucin beads and cell adhesion assays. If ETEC adhesion affinity for yeast products has been proven, lentil extracts attractiveness is still hypothetical and has to be further investigated.
Built from personal source.

2.2.5. Impact on host innate immunity

Even if some authors have considered ETEC induced inflammation to be “mild”, more and more publications show that this feature should not be neglected and can have a huge impact on patients, especially in children (Brubaker *et al.* 2021). In this PhD work, no less than 17 genes related to innate immune response have been investigated in the infected Caco-2/HT29-MTX cells. ETEC seems to generally activate those genes regardless of their pro-inflammatory or anti-inflammatory (IL-10) status, supporting a classic activation of the innate immune response to bacteria proximity (Couper, Blount and Riley 2008). Interestingly, this concomitant pro- and anti-inflammatory response has already been documented in human patients, supporting the relevance of the Caco-2/HT29-MTX model (Long *et al.* 2010; Yang *et al.* 2016). The induction of IL-10 could be due to ETEC trying to limit host reaction and its clearance, as already shown with other enteric pathogens (Redpath, Ghazal and Gascoigne 2001). Of note, some of the ETEC-induced genes were encoding mucus proteins. This was not surprising as the activation of innate immune receptors is known to elicit cascades simulating mucus secretion (McNamara and Basbaum 2001; Birchenough *et al.* 2016; Wang *et al.* 2016). LT toxin was also previously shown to stimulate MUC2 production (Sheikh *et al.* 2021). To complete our work, the induction of mucus production by ETEC could be investigated at the protein levels by Western blot assays (Damiano *et al.* 2018). Microscopy technics could also help to evidence increase in mucus or mucin glycoproteins density. As mucinases will counterbalance ETEC-induced mucus production, mucinases mutants could be considered for further mechanistic insights (Sheikh *et al.* 2021). Lastly, we reported a minor impact of ETEC strain H10407 on tight junction protein encoding genes. This observation questions the limited number of studies reporting modulation of intestinal permeability by human ETEC strains, essentially associated to ST toxin effect (Evans, Evans and Gorbach 1973a, 1973b, 1974; Nakashima, Kamata and Nishikawa 2013; Zhou *et al.* 2021). Most of the work performed on ETEC-induced intestinal epithelial permeability has been conducted in pigs (Dubreuil 2017) and more human specific reports are needed. Based on the only other study reporting a human-targeting ETEC whole cell effect on permeability (Nakashima, Kamata and Nishikawa 2013), TEER measurement should be the main out-put to monitor. More human clinical studies investigating serological markers of epithelial permeability would be also welcomed (Dubreuil 2017).

In the first axis, we postulated that whether ETEC increased adhesion was restricted on mucus patches of mucus-secreting cell lines, the induction of the innate immune response could be reduced due to distance with the epithelium. Nevertheless, the analysis of intracellular IL-8

did not show any inhibition in the mucus-secreting model. This may consolidate the hypothesis raised by Kerneis and colleagues suggesting ETEC adhesion to the brush border (Kerneis *et al.* 1994). Other explanations have also to be considered such as the involvement of induced virulence genes (toxins, mucinases) in the mucus-secreting model. Comparison between a monoculture model secreting a continuous mucus layer (e.g. HT29-MTX) and a non-mucus secreting one (e.g. HT29) will help to definitely decipher if the sole presence of a mucus layer can modulate ETEC-induced inflammation. Mucinase mutants would also allow to decipher their involvement in inflammatory process.

Previous works have already investigated the effect of probiotics on ETEC-induced inflammation (Roussel *et al.* 2018), but none has explored dietary fiber impact. In this PhD work, dietary fiber-containing products globally reduced ETEC induced-innate immune response. As yeast cell walls reduced ETEC adhesion and both products modulated virulence gene expression, it is tempting to link those effects to the observed reduction in innate immunity action. However, a direct immunomodulation effect of fiber products cannot be excluded (Van den Ende, Peshev and De Gara 2011; Noll *et al.* 2016). Further analysis of the cellular redox status and innate immune response pathways could be envisaged (Wang, Gao and Hardwidge 2012; He *et al.* 2016; Hu *et al.* 2020b). To confirm these results in more relevant conditions, we can imagine the use of digestive simulator combined to cellular models, or animal studies with non-invasive methods based on the dosage of fecal Lcn-2 and MPO (Bolick *et al.* 2018) or serological markers (cytokines). We also showed that fiber products were able to reduce ETEC induction of mucus-related genes, which is not surprising as mucus production is an effector response of the innate immune response (McNamara and Basbaum 2001; Birchenough *et al.* 2016; Wang *et al.* 2016). Lastly, we also demonstrated fiber products capability to reduce epithelial barrier permeability. This could be due to the modulation of the innate immunity, as already shown (Li *et al.* 2019a; Wan *et al.* 2019; Liu *et al.* 2020) but a sedimentation effect cannot be excluded. Then, other models should be envisaged, as reversed epithelial cell lines (Calatayud *et al.* 2019). To summarize, this work proved that human ETEC strain H10407 can trigger a strong innate-immune response and that fiber products and especially lentil extracts could interfere with this activation (**Fig. 6**).

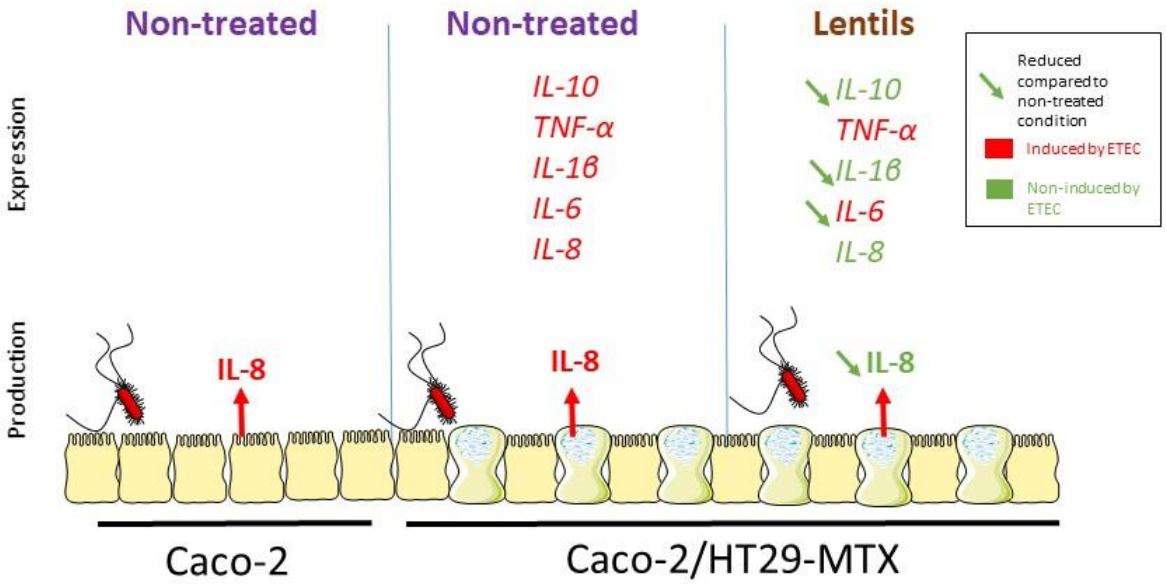

Figure 6. Main findings regarding ETEC-induced cytokine expression/production. The main findings concerning the effect of fiber products and mucus on ETEC-induced changes in cytokine expression/production are summarized. Only the effects of lentil products are represented, as they were more significant than those of yeast cell walls.
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2.2.6. Survival within a complex microbiota background

ETEC shedding at the end of the human GI tract may be the ultimate goal of the infectious cycle as it increases environmental contamination and the possibility to re-infect new hosts. In human volunteers, ETEC shedding peak is observed between 2 and 4 days after ingestion, with levels around 10^8 CFU.g^{-1} of feces. Furthermore, the analysis of the fecal microbiota composition of soldiers affected by diarrheagenic *E. coli* (EPEC, EAEC, ETEC) have shown that a relative bloom of Enterobacteriaceae is concomitant to the infection symptoms (Walters *et al.* 2020). Altogether these observations tend to support not only a maintenance, but also a possible multiplication of ETEC in the human GI tract. Thus, even if ETEC action site was suggested to be the distal part of the small intestine, it appeared meaningful to investigate the pathogen behavior in *in vitro* models where gut microbiota prevails in high numbers. In line with this idea, Moens and colleagues have shown using short time batch experiments (48 hours) inoculated with human fecal samples that the ETEC strain H10407 was able to overgrow. However, their batch incubations were conducted under a very low fecal inoculation (Moens *et al.* 2019). When the same strain was introduced into the more complex M-SHIME model, the pathogen level decreased over-time (Roussel *et al.* 2020a). In accordance with these results, we did not report an ETEC growth in our batch assays (performed

using normal inoculation rate of fecal samples and pre-digestion of ETEC strain to mimic upper GI transit). We thus argue that the observations reported by Moens and colleague was due to the poor inoculation levels, most probably because of the loss of gut microbiota barrier effect. Such simple batch experiments are particularly relevant to perform screening assays but are limited to a short period, as pH is not controlled and metabolite products accumulate. More studies are clearly needed to confirm the spatio-temporal dynamic of ETEC survival throughout the human GI tract in the presence of a complex microbial background (not found in the TIM-1). The ESIN model currently under development at MEDIS lab (Guerra *et al.* 2016) could be a useful *in vitro* tool as it will combine for the first time the complexity of the TIM-1 with a small intestinal microbiota from a human origin.

Concerning the axis I objectives, we reported that mucin beads were not able to modulate ETEC survival in the luminal compartment of batch flasks. However, due to the simplicity of our *in vitro* approach (no medium renewal), we cannot really appreciate if those beads are acting as a colonisation reservoir for the lumen as mucus does *in vivo*. Contrarily, this can be studied in continuous fermentation systems (as M-SHIME or M-ARCOL). In the previous study in M-SHIME by Roussel and colleagues, the authors showed that ETEC colonisation was higher on ileum beads than in the luminal content at day 4 post infection, suggesting this reservoir role (Roussel *et al.* 2020a). Of note, these authors did not perform any experiments with control alginate beads (as done in this PhD work), hampering a real conclusion on the role of mucus. Regarding the adhered population, we reported a tendency of ETEC to decrease on mucin-alginate beads compared to control alginate ones. This can be linked to a colonisation by a specific mucus-associated microbiota (notably *Clostridium* and *Lactobacilli* species), as already described in the M-SHIME (Van den Abbeele *et al.* 2012, 2013) and discussed in the next section. As *Lactobacilli* species have been regularly highlighted as probiotic species with anti-infectious properties against human ETEC strains, it suggests a probable protective effect from that mucus-specific microbiota. We also have to acknowledge that our study would be strengthened by a raw number quantification of ETEC (and not a relative one), as the microbiota colonisation of alginate beads is probably inferior to mucin ones. Such raw quantification could be achieved using flow-cytometry combined either with RNA fluorescent *in-situ* hybridization or with quantitative PCR relative measurement (Roussel *et al.* 2020a; Minnebo *et al.* 2021).

By being degraded into smaller carbohydrate fragments by the gut microbiota, dietary fiber can provide a substrate for pathogens like ETEC, which behave as secondary degraders (Onyango *et al*. 2021, Sauvaitre *et al.* 2021b). We reported the ability of ETEC strain H10407

to growth on dietary fiber-containing products in simple culture media, however we did not observe any outgrowth in batch fermentation assays. This probably indicates that ETEC cannot outcompete the human fecal microbiota for growth on fiber products, which is reassuring for product safety. Unfortunately, we did not report any significant beneficial effect of fiber products, which may be due to the compensation of fiber content in the control conditions. We only observed a tendency of yeast cell walls to decrease ETEC mucosal colonisation. Dietary fibers can limit pathogen colonisation through microbiota modulations, including prebiotic support of probiotic species or modulation of microbiota activity/composition (Sauvaitre *et al.* 2021b). In line with these mechanisms, we showed that the yeast products induced some interesting changes in terms of microbiota composition and an increase of microbial activity (as detailed below). We thus propose that the yeast products may increase the pool of microorganisms ready to switch to mucin consumption when the fiber pool run-out. This increased pool of microorganisms on the mucin beads could also explain why ETEC colonisation is less important with the yeast cell walls. Lastly, it is worth noticing that the beneficial effects of fibers we previously highlighted (e.g. inhibition of toxin production, mucosal adhesion and innate immune response) are all dependent of the host. As shown with other member of *Enterobacteriaceae* such as *Salmonella*, enteric pathogens can take advantage of inflammation to colonize their host (Liu *et al.* 2012; Behnsen *et al.* 2014, 2015). In this regard, integrating the host part in *in vitro* or *in vivo* assays may be necessary to evidence a protective effect of the dietary fiber-containing products mediated by the gut microbiota. Interestingly, some authors already created genetically modified bioluminescent ETEC from human origin (strain FMU073332), which colonisation can be tracked in space and time within a complex microbial background (Rodea *et al.* 2017).

2.2.7. Modulation of gut microbiota composition

In humans, ETEC infection is associated with a rapid and reversible change in gut microbial community structure with significant decrease in overall bacteria diversity, as measured by Shannon and Simpson indexes (community evenness). ETEC-induced microbiota changes varied greatly from individual to individual, whether or not diarrhea occurred and studies have reported that microbiota changes are not dissociable from the diarrhea effect (David *et al.* 2015; Youmans *et al.* 2015; Pop *et al.* 2016). As microbiota alterations can favor infection (Ghosh *et al.* 2011; Hopkins and Frankel 2021), it is crucial to better study the impact of these changes on the infection process.

In this PhD work, we investigated the direct (i.e. not mediated by the host) modulation of the human microbiota by ETEC using batch experiments. As observed in humans, we reported a decrease in α-diversity and more especially in community evenness. We argue that this observation is not surprising as ETEC was inoculated in high numbers (10^8 CFU.mL^{-1}), but relevant compared to the *in vivo* infectious dose, and represented thereafter a substantial part of the microbiota. Supporting that the impact on community evenness would be due to the sole over-representation of ETEC, we did not report any change in microbiota community richness. Accordingly, Youmans and colleagues reported a non-significant decrease in observed OTU in ETEC-infected travelers compared to healthy ones (Youmans *et al.* 2015). Concerning β-diversity, we did not observe any profound change in the community structure following ETEC infection in the luminal compartment. This is in accordance with studies conducted on human feces, where the shift in microbiota β-diversity structure induced by ETEC is not always easy to evidence due to high inter-individual variabilities (Pop *et al.* 2016) or not distinguishable from the diarrhea effect (David *et al.* 2015; Youmans *et al.* 2015). Besides, our results highlighted more profound shifts in the mucosal phase than in the luminal ones following ETEC inoculation. Despite the lower representation of *Escherichia coli* in the mucosal compartment, some mucosal-specific phylogroups tended to be impacted by ETEC infection as *Clostridrium*, *Lactobacillaceae* and *Bifidobacterium*. These *in vitro* data cannot be compared to *in vivo* ones, since no human study has ever investigated ETEC modulation of mucus-associated microbiota.

This work is the first one specifically addressing how mucus affect ETEC microbiota modulations. Our results highlighted the colonisation of mucin beads by a specific microbiota, enriched in Firmicutes, and notably in *Lactobacillaceae* and *Clostridium* species, as previously shown in mucin-agar microcosms from the M-SHIME (Van den Abbeele *et al.* 2012, 2013). Interestingly, these groups, and especially *Clostridium*, were negatively impacted by ETEC inoculation. These results are in line with those previously observed by Roussel and colleagues following ETEC infection in the M-SHIME, both the ileal and colonic compartments of the system (Roussel *et al.* 2020a). As the mucosal phylogroups modulated by ETEC (Arboleya *et al.* 2016; Heeney, Gareau and Marco 2018; Stoeva *et al.* 2021), and more generally the whole mucosal microbiota, are known to exert some effects on human health (Daniel, Lécuyer and Chassaing 2021), this study raises awareness on the potential deleterious effect of ETEC on human mucosal microbiota. Deciphering if the observed ETEC induced changes in mucosal microbiota is involved in disease etiology is a real challenge. To try to answer this question, an option would be to inoculate species from the mucosal phylogroups conjointly with ETEC to see if they impede or not pathogen colonisation in *in vitro* models or in animals. On a technical

point of view, it appears important to incorporate the mucus compartment when investigating an enteric pathogen, as this micro-environment seems to play a major modulator of gut microbiota composition.

Concerning the second axis, we highlighted that dietary fiber-containing products could limit the decrease in mucosal *Clostridium* species. Furthermore, fiber products were able to support other mucosal phylogroups not necessarily impacted by ETEC, as *Escherichia*/*Shigella* and *Parabacteroides*. Those two groups could play a role in the control of the infection, as commensal *Escherichia*/*Shigella* may occupied the same niches as ETEC as already seen with other pathogens (Rendón *et al.* 2007; Maltby *et al.* 2013), and *Parabacteroides* have been highlighted for their anti-inflammatory properties in a panel of non-infectious diseases (Wang *et al.* 2019b; Wu *et al.* 2019; Koh *et al.* 2020). To decipher if the observed microbiota changes induced by fiber products are benefic or not, non-filtered fermentation samples could be applied on gut-on-a-chip modules and the inflammatory status of the cells assayed (Jalili-Firoozinezhad *et al.* 2019; Shin *et al.* 2019).

2.2.8. Modulation of gut microbiota activity

Only two previous *in vitro* studies are available on the modulation of gut microbiota activity by human ETEC strains, including one with non-physiological condition (Moens *et al.* 2019; Roussel *et al.* 2020). Here, we reported a surprising impact of ETEC strain H10407 on gut microbiota activity. Whatever the conditions studied (with or without mucin beads and dietary fibers), ETEC promoted some parameters associated with fermentation activity (like butyric acid production, gas pressure and increase in CO_2/H_2 levels) while limiting pH drop. This pH increase could be due to the activity of *E. coli* acid resistance systems, which under anaerobic conditions, consume H^+ to produce dihydrogen (H_2), carbon dioxide (CO_2) and H_2O (Kanjee and Houry 2013). In particular, the glutamic acid dependent acid resistance (GDAR) system, considered as one of the most efficient in *E. coli*, consumes one molecule of H^+ through decarboxylation of glutamate to γ-aminobutyric acid (GABA) (Capitani 2003; Foster 2004). GABA can then be metabolized by some gut bacteria resulting in the production of diverse metabolites as butyrate and/or acetate (Strandwitz *et al.* 2019). Interestingly, when using NCBI BLAST (Basic Local Alignment Search Tool) with amino acids sequence of the enzymes necessary for GABA metabolism (Strandwitz *et al.* 2019), we found that the ETEC strain H10407 genome encodes for similar enzymes, and especially the first one of the chain responsible for the conversion of GABA in succinate semi-aldehyde, suggesting that the ETEC strain itself can metabolize GABA. Further investigations are needed to identify acid resistance

systems expressed by ETEC strain H10407 and their potential involvement in the modulation of gut microbiota activity. The most relevant solution would be to perform the same batch experiments with a mutant strain where acid resistance system would be knocked-down (Damiano *et al.* 2018). If ETEC acid resistance system importance is confirmed, these observations will need to be confirmed in *in vivo* colonic conditions, where bicarbonate secretion (Boland 2016) and other microbial acid resistance system counterbalance pH decrease (Feehily and Karatzas 2013).

In the first axis, the integration of mucin beads allowed even more a discrimination of ETEC impact on microbial activity, with namely higher pH increase, and increased butyric acid and gas production. This could be due to the higher amount of carbohydrates provided by mucin-alginate beads (compared to alginate beads) available for gut microbiota fermentation, leading to higher acidity to be countered balance by ETEC. This assumption could be verified by transcriptomic analysis of ETEC genes focusing on acid-resistance systems (Hirakawa *et al.* 2010).

The addition of fiber products did not mitigate ETEC impact on gut microbial activity. Addition of fiber containing-products even tended to increase some parameters linked to fermentation activity (propionate production, CO_2 levels, pressure), suggesting that the amount of fermentable components was increased with the treatment (maybe brought by the non-fiber fraction of the products). As dietary fibers seem to be able to preserve mucus from gut microbiota degradation in animals (Desai *et al.* 2016; Schroeder *et al.* 2018; Neumann *et al.* 2021), we measured mucin bead degradation in batch flasks. Counterintuitively, we showed that our fiber products tended to increase their degradation. The most probable explanation is that the preservation of the mucus layer is not visible in our experiment due to compensation of fiber content in the control condition. This would imply that the fibers contained in our products are not more efficient than that from the batch nutritive medium to preserve the mucus from consumption. A control condition without fiber compensation would have helped to better highlight the general effect of our products on bead degradation. We can also hypothesize that addition of our fiber products (and their non-fiber components) increased the pool of microorganisms ready to switch to mucin consumption when fibers are consumed. This could be confirmed by microbiota raw quantification on mucin beads by combination of flow cytometry and quantitative PCR. It is also worth mentioning that, unlike continuous *in vitro* models, batch fermentation models are limited by the non-continuous supply of nutritive medium, which thus cannot continuously decoy the microbiota from mucus consumption (Pham and Mohajeri 2018; Pérez-Burillo *et al.* 2021). Finally, to unravel if the modulations of

microbiota activity observed in batch experiments are benefic or not, filtrated samples from the assays could be directly applied to cellular cultures to measure immune response modulations (Defois *et al.* 2018; Calatayud *et al.* 2021). The **Figure 7** summarizes our findings relating to the effect of mucus and dietary fiber-containing products on ETEC survival within a complex microbial background.

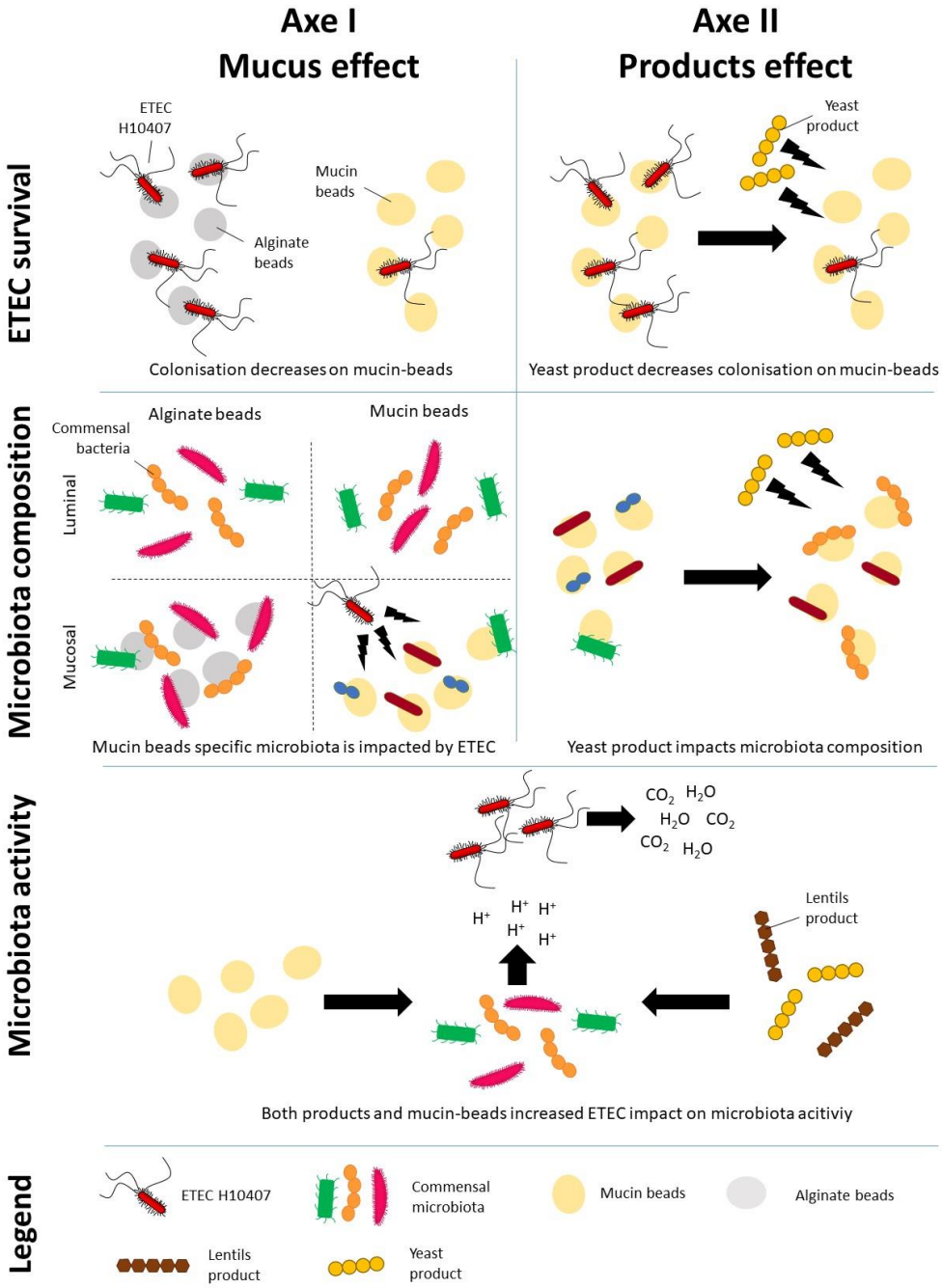

Figure 7. Summary of the main findings concerning the modulation of ETEC interaction in presence of the human gut microbiota.
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Figure 8 summarizes the main findings regarding mucus and dietary-fiber containing products modulations of ETEC interactions with human gut microbiota. Regarding the microbiota activity, the increased activity of ETEC acid resistance systems in response to substrate fermentation remains hypothetical.

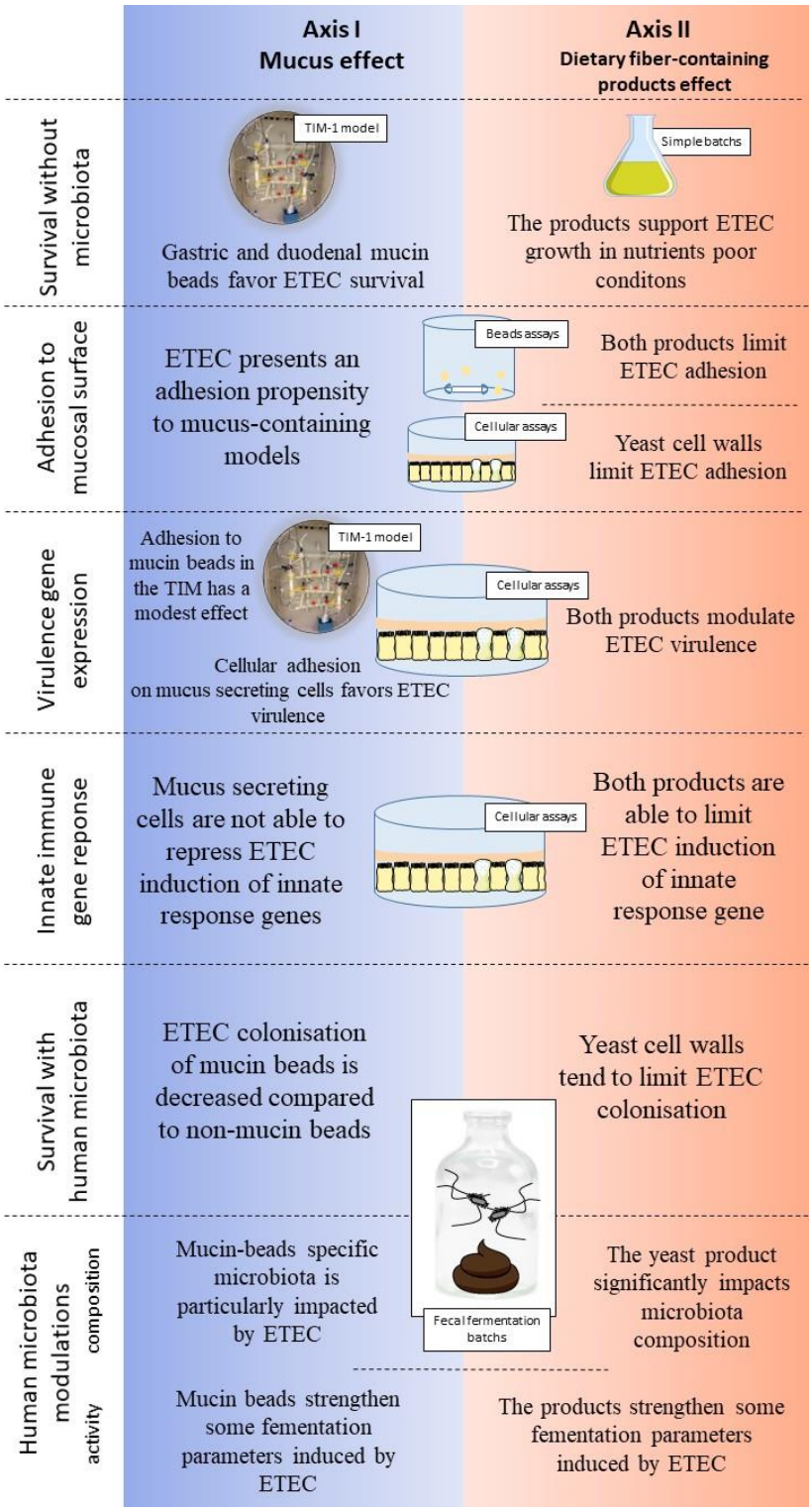

Figure 8. Main findings of the PhD two axis.
This figure illustrates the main findings from the two axes of the PhD work.
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Table 2. Limits, technical issues, interrogations concerning ETEC pathophysiology and associated perspectives.

For each aspect of the ETEC pathophysiology investigated, the table represents the main limits, technical issues or interrogations encountered and the proposed perspectives to circumvent.

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		ASPECT of ETEC PATHOPHYSIOLOGY INVESTIGATED								
		Growth/survival in the upper GI tract	**Adhesion**	**Virulence gene expession**	**Toxin production and effect on cells**	**Host response**	**Survival in presence of gut microbiota**		**Modulation of gut microbiota diversity**	**Modulation of gut microbiota activity**
GENERAL - both axes	Main limits, technical problems or interrogations encountered	No precise human data about ETEC action site impeding conclusions	No mechanistic investigations of adhesins and receptors involved in	Our results support a sequential activation of ETEC virulence gene expression	ST production has not been assayed	No assessment of bacteria effect on mucus secretion and cellular permeability	No data about the requirements for ETEC to thrive in intestinal conditions	Only relative bacterial quantification on mucin-alginate beads	No consensus about ETEC (and its toxins) effect on luminal microbiota. No data about ETEC effect on mucosal microbiota *in vivo*	Potential involvement of ETEC acid resistance systems
	Envisaged perspectives	Human clinical studies allowing to visualize ETEC-induced inflammation (computed tomography, Magnetic Resonance Imaging, PET imaging)	Mucosal polysaccharides degrading enzymes, blocking antibodies, addition of decoying saccharides motives or combination of them	Time- and spatial-scaled resolution of ETEC virulence gene expression in animal models	Development and optimisation of a reliable ST toxin ELISA assay	Mucin specific -ELISA or -Western blot assays, microscopy technics, TEER, absorption of molecules (atenolol, caffeine)	Coupling between cellular models and fermentation models Animal models inoculated with ETEC strains producing toxin(s), or toxin(s) alone or mutant ETEC strains	Flow-cytometry coupling with RNA fluorescent *in-situ* hybridization or with qPCR relative	Multiply imicrobiota vomposition studies with increased number of subjects Animal models inoculated with ETEC toxin(s) or toxin(s) knock out ETEC	Transcriptomic analysis Experiments with mutant ETEC strains (acid resistance systems knockout)
	References	Brewer *et al.* 2008; Le fur *et al.* 2021	Sheikh *et al.* 2017	Yang *et al.* 2010	Rocha *et al.* 2013	Rajan *et al.* 2020	Erume *et al.* 2008; Bolick *et al.* 2018; Wang *et al.* 2019	Minnebo *et al.* 2021	David *et al.* 2015; Youmans *et al.* 2015; Pop *et al.* 2016, Roussel *et al.* 2020	Jin *et al.* 2009; Zhao *et al.* 2018;
	Methodology used	TIM-1 (Upper GI model)	Beads and cellular adhesion assays	TIM-1 and cellular experiments	None	Cellular experiments (IL-8 ELISA)	Fecal fermentation batches (Lower GI model)			
AXIS 1	Main limits, technical problems or interrogations encountered	Exponential growth on luminal mucin has hampered conclusions on the relative importance of mucin beads in lower compartments of the TIM-1	No proof of mucus patches involvement in ETEC adhesion propensity for the Caco-2/HT29-MTX coculture	No definitive conclusions on the role played by the mucus compartment compared to physicochecimal conditions	No clear assessment of mucus impact on toxins production (LT and ST)	No clear assessment of mucus patches impact in the modulation of innate immunity	No renewal of the nutritive medium in the luminal phase impeding conclusions about the mucosal reservoir		Batchs are simplified *in vitro* models far from human GI physiology	Non-specific effect of mucus addition by increasing the amount of fementable substrates
	Envisaged perspectives	TIM-1 experiments without mucin secretion	Microscopy technics (SEM, TEM, PAS/AB stainings)	TIM-1 experiments with and without mucin-alginate beads	GM1-ELISA assays	Comparison of HT29-MTX *vs* HT29 culture in combination or not with mucosal polysaccharides degrading enzymes	Continous fementations models as M-ARCOL or M-SHIME		Confirmation of the results in more *in vitro* relevant systems (M-SHIME, M-ARCOL)	Performing the experiments in nutrients rich medium
		M-SHIME *versus* SHIME experiments	Comparison of HT29-MTX *vs* HT29 culture	TIM-1 model coupled with mucus secreting cellular models	Intracellular cAMP/cGMP ELISA assays on T-84 cells	Mutants ETEC strains (mucinase genes knock-out)	Animal models (mice or pig)			
	References	None	Gagnon *et al* . 2013; Rajan *et al* . 2020	None	None	Gagnon *et al.* 2013	Roussel *et al.* 2020; Gresse *et al.* 2021		Roussel *et al.* 2020; Gresse *et al.* 2021	None

		ASPECT of ETEC PATHOPHYSIOLOGY INVESTIGATED							
		Growth/survival in the upper GI tract	**Adhesion**	**Virulence gene expession**	**Toxin production and effect on cells**	**Host response**	**Survival in presence of gut microbiota**	**Modulation of gut microbiota diversity**	**Modulation of gut microbiota activity**
AXIS 2	Methodology used	Simple culture media assays	Cellular adhesion assays	Cellular experiments	GM1-ELISA assays, intracellular cAMP/cGMP ELISA assays on T-84 cells	Cellular experiments (ELISA, transcriptomic analysis, permeability assays)	Fecal fermentation batches (Lower GI model)		
	Main limits, technical problems or interrogations encountered	Human and bacterial digestion of containing products have not been taken into consideration	Sedimentation effect has not been considered in cell experiments	More mechanistic insights are needed to support the impact of the products on ETEC physiology	No induction of intracellular cAMP (T-84 cells) by toxins or bacteria	No mechanistic investigations of the anti-inflammatory effect of the dietary-fiber containing products	Absence of statistically significant effect of the dietary-fiber containing products	No data about potential beneficial effect (on ETEC colonisation or host physiology)	Absence of statistically significant effect of the dietary-fiber containing products (particularly on mucin-alginate beads degradation)
	Envisaged perspectives	TIM-1 experiments	Invert cellular experiments (performed upside down)	Investigation at the proteins level (ELISA, Western blots)	Performing the experiments with new cells, or new detecting kits for toxins	Oxydation assays (e.g. ELISA assays)	New biological replicates	Coupling of fermentor model with cell line or gut-on-a-chip module	Control conditions without dietary-fiber containing products to illustrate potential unspecific effects
		M-SHIME *vs* SHIME experiments		Mutants ETEC strains (knock-out of virulence genes) or co-administration of virulence factors (mucinases, toxins) in cellular experiments	Performing the experiments with new cells, or new detecting kits for toxins	Innate immune pathways analysis by proteomic or transcriptmic analysis	Including the host part : coupling fermentation and cellular cultures models, animal experiments	Targetting the highlighted phylogroups to assess their involvement in the resistance to ETEC colonisation (phage therapy)	Beads measurement at earlier time points
		Animal experiments		Including the products digestion to decipher if the products effects are conserved (coupling between *in vitro* models, animal experiments)	Rabbit ileal loop assyas	Innate immune pathways analysis by proteomic or transcriptmic analysis	Control conditions without dietary-fiber containing products to illustrate potential unspecific effects	Symbiotic combinations of dietary-fiber containing products plus probiotics in fermentation experiments	Continous fementations models as M-ARCOL or M-SHIME
	References	None	None	Johnson *et al.* 2009; Wang *et al.* 2012; Rocha *et al.* 2013	Johnsson *et al.* 2009; Zhang *et al.* 2010; Svennerholm *et al.* 2011; Kern *et al.* 2018	He *et al.* 2016; Hu *et al.* 2020	Roussel *et al.* 2018; Calatayud *et al.* 2021	Fooks and Gibson 2003; Shin *et al.* 2019; Jalili-Firoozinezhad *et al.* 2019	None

3. Towards further developments of anti-infectious strategies against ETEC

This PhD project has been built on two main axis, the first one aiming to have a glimpse on the extent to which the mucus compartment impact ETEC physiopathology, and the second focusing on the anti-infectious properties of dietary-fiber containing products. Still, this PhD project opens new avenues in the development of therapeutic strategies, even if several steps are obviously required before any product development.

3.1. Questioning the relevance of studying a single ETEC strain

Both axes are concerned by a main limit, namely the testing of a single human-targeting ETEC strain, the H10407 strain considered as the reference strain for the modelling of adult ETEC infection (Evans 1977). The results obtained on this strain cannot be extrapolated to other strains isolated from adults as the panel of virulence factors of ETEC strains is very large and varied (different adhesins or toxins) (Isidean *et al.* 2011; Vipin Madhavan and Sakellaris 2015)) and pathophysiology modulations have been reported to be strain-dependent (Kansal *et al.* 2013). It is also impossible to transpose our results to infant targeting ETEC, as ETEC strains seem to be specialized between infant and adult (Gyles and Fairbrother, 2008).

3.2. A better description of the mucus role can help in the development of alternative anti-infectious strategies

3.2.1. Towards better inclusion of mucus in ETEC infection models

Even if ETEC pathogen seems adapted to the mucus layer, to date *in vitro* studies investigating ETEC behavior in the human GI tract rarely integrate this component (Roussel *et al.* 2018b, 2020a, 2021; Moens *et al.* 2019). In the same way, anti-adhesion strategies against human-infecting ETEC are typically investigated with the Caco-2 model (Bernet *et al.* 1993, 1994; Coconnier *et al.* 1993), and not with mucus secreting-HT29 derivatives or the LS174T cell lines (van Klinken *et al.* 1996; Dorier *et al.* 2017; Gillois *et al.* 2021). With our *in vitro* exploration of mucus impact on ETEC virulence and survival, we aim to reinforce the need to integrate the mucus component in ETEC related experiments aiming to test or develop anti-infectious strategies and pay more attention to mucus-ETEC interactions in animal models and ultimately in human trials.

3.2.2. Towards new anti-infectious strategies exploiting mucus environment

This work could lead to the development of new anti-infectious strategies specifically targeting ETEC-mucus interactions. First, it could be envisaged to inhibit ETEC-mucus interactions. One possibility would be to target ETEC adhesin, such as type I pilus FimH which is overexpressed on adhered bacteria. Mannose residues, that are interestingly well known to be presented by yeast cell walls (Sivignon *et al.* 2017; Roussel *et al.* 2018b), have already been proposed as a therapy target against other *E. coli* pathotypes, such as AIEC and (uropathogenic *E. coli*) UPEC (Mydock-McGrane, Hannan and Janetka 2017; Sivignon *et al.* 2017; Chevalier *et al.* 2021). Another possible strategy is the reinforcement of the mucus barrier prior to ETEC infection. For instance, some medical drugs as Rebamipide have already been reported to increase gastric mucus secretion in human (Iijima *et al.* 2009), while some early clues supported butyrate stimulation of mucin gene expression in the human colon, (Blaak *et al.* 2020) and dopamine increase of mucus secretion in the colon of rats (Li *et al.* 2019b). Of course, more efforts are needed to develop compounds allowing the reinforcement of small intestinal mucus secretion. As explained extensively in the literature review, dietary fiber intakes decoy the microbiota from mucus consumption and Desai and colleagues were pioneers in extending this notion to pathogens (Desai *et al.* 2016; Neumann *et al.* 2021).

3.3. Dietary fiber-containing product as a new anti-infectious strategy

Up to now, dietary fibers potential against human-targeting ETEC has been poorly investigated and the only mechanisms studied were anti-adhesion properties against both the pathogen and its toxins (Otnaess, Laegreid and Ertresvåg 1983; Newburg *et al.* 1990; Idota and Kawakami 1995; Roberts *et al.* 2013; Salcedo *et al.* 2013). This PhD highlights the potential use of dietary fibers as a multi-targeted alternative strategy.

3.3.1. Better characterization of fiber products and their active components

First, the major limit of our study resides in the dietary fiber-containing products impurities. Consequently, we cannot be sure that the fiber fraction is only responsible of the anti-infectious effects that we observed. For instance, the two extracts contain around 3-4% of minerals, which may contain for example zinc, already known to increase the ileal burden and shedding of ETEC in mice (Bolick *et al.* 2018). Dietary fibers are also known to form complex with other innate immunity modulating molecules as polyphenols which could be responsible

for some of the observed effect (Li *et al.* 2020). Thus, it appears meaningful to confirm if dietary fibers are the sole active components in our products. One solution reside in a new extraction protocol for a better isolation of fibers before further testing. This could be achieved by a more rigorous adaptation of the official methods for dietary fiber quantifications, like the original AOAC method 985.29 or the more advanced method 2009.01 which better takes into account resistant starch and low-molecular weight soluble fibers (McCleary *et al.* 2013; Stephen *et al.* 2017). Notably, the filtration step in these methods should not be skipped for a centrifugation, even if the quantity obtained will be lower. Another possibility resides in the better characterization of the fiber fraction contained in the products, which can be achieved by different manners, as chromatography techniques (Anudeep *et al.* 2016; Xue *et al.* 2020), fiber-specific pre-design kits (McCleary *et al.* 2013) and AOACs specific methods. When the main fibers contained in the products would be identified, one option (if possible) is to purchase them from commercial suppliers and test them separately at relevant concentrations. This option present two limits. First, the potential differences in fiber structures between plants, as already shown with pectin (Dranca and Oroian 2018) that could impact the results. Second, using purified fibers could result in the loss of the product natural complexity and potential synergic effects (Yamada 2017).

3.3.2 Necessary scale-up to more *in vivo* relevant models

Most of the results obtained during this PhD work have been obtained in simple *in vitro* models. Next steps would be to perform new experiments in more complex systems as M-SHIME or ESIN. Depending on the objectives, these simulators could be coupled to cellular models to integrate the host components (Marzorati *et al.* 2014; Shin and Kim 2018). Animal models could be used in the next pre-clinical steps to integrate the whole organism and contribute to product safety assessment, before moving to clinical trials.

3.3.3. Fiber product development strategies

In this PhD work, we brought some preliminary proofs that fiber-containing products could present anti-infectious properties against ETEC. This section discusses the product development strategy that could be adopted in a next future if these beneficial effects are confirmed. The available possibilities are compared in **Table 3** and **Figure 9** presents a possible decision-make tree.

Among them, medical devices, dietetic food and medical drugs appeared as the less suitable and/or are based on data that have been not explored in this PhD work. Indeed, a

medical device can be used for "diagnosis, prevention, monitoring, prediction, prognosis, treatment or alleviation of disease" but it "does not achieve its principal intended action by pharmacological, immunological or metabolic means, in or on the human body, but which may be assisted in its function by such means" (Van Norman 2016). This definition means that our product could be commercialized uniquely under a mechanical heath claim, like a feces bulking capacity, which has not been investigated in the present study. **Dietetic food** are intended to correct nutritional deficiencies due for example to impairment in the patient feeding capacities, but they cannot claim or prevent any disease (Coppens and Pettman 2014). Again, the potential nutritional benefice of our products for travelers' diarrhea patients has not been studied. Lastly, even if **medical drug** are not limited concerning health claims and new drug developers are automatically granted at 10 years monopoly after authorization, their development according to European Union rules is constraining without common measure. Among others, the active pharmaceutical ingredient must be fully qualitatively and quantitatively characterized, the quality of the product must be assured at all steps of the product development, which of course will be difficult to achieve, especially with our lentil extract (Reis *et al.* 2015).

Next possibility would be to consider our product as **prebiotics**, which are defined as "substrates that are selectively utilized by host microorganisms conferring a health benefit" (Slavin 2013). Following the introduction of the Nutrition and Health Claims Regulation (NHCR) in 2006, the European Commission has classified the term prebiotic as health claims. This implies that prebiotics require authorization in order to be commercialized that could theoretically be granted by providing strong scientific evidences (Laser Reuterswärd 2007). Probiotics are submitted to the same regulation. Among the 400 health claim applications submitted for authorization since 2006, only one was authorized. Considering both the uncompromising EU evaluations and the modest effect of our products on microbiota, developing our products as prebiotics for traveler's diarrhea prevention is certainly not the best option.

Last possibility to study is **food supplements**. According to EFSA, food supplements can contain a wide range of ingredients, including vitamins, minerals, amino acids, essential fatty acids, fiber and various plants and herbal extracts (https://www.efsa.europa.eu/en/topics/topic/food-supplements consulted in January 2022). However, to ensure consumer safety, EFSA edited a list of substances authorized as food supplements. This list contains very few fiber-containing products (inulin, chitosan, wheat bran, guar gum) and yeast cell walls and lentil extracts are not part of them. Thus, our products would have to undergo authorization procedure to be approved

(https://www.efsa.europa.eu/en/topics/topic/food-supplements consulted in January 2022). Furthermore, food supplements are not intended “to treat or prevent diseases in humans or to modify physiological functions” (https://www.efsa.europa.eu/en/topics/topic/food-supplements consulted in January 2022). Thus, it is impossible to distribute a food supplement with a health claim for traveler’s diarrhea prevention. The only clinically demonstrated health benefits associated with fiber supplements are cholesterol lowering, improved glycemic control, satiety, weight loss, constipation/stool softener, diarrhea/stool normalizer, and IBS, any another claim will have to be supported by solid scientific evidences. Therefore, one alternative solution could be to pick the “diarrhea/stool normalizer” claim, and communicate around the products to refer to our studies and traveler’s diarrhea.

To conclude, none of the strategies mentioned above are ideal and all have pros and cons, even if at the current stage, food supplement would appear as the most feasible one. We argue that most of strategies would benefit from identification of the active ingredients, but this would be mandatory for medical drugs. In any case, identification of active compounds would allow to test the most physiological dose and move toward more specific anti-infectious effect. Next point would be to confirm if large scale purification of the active products is achievable at a reasonable cost.

Table 3. Example of legislative requirements for the different product development strategies

Built according to Laser Reuterswärd 2007, Coppens and Pettman 2014, Reis *et al.* 2015, Van Norman *et al*. 2016 and EFSA website (consulted on March 2022)

	Complete characterization of the product necessary	Post-market survaillance	Clinical phase	Safety has to be assayed	Require a dose/daily intake to be provided	Claims	
						Limitations	Demonstration requirements prior to distribution
Medical drugs	X	X	X	X	X	Not limited	Strong scientifical evidences
Medical device		X	X	X	X	Limited to mechanical effects	Strong scientifical evidences
Prebiotic				X	X	Limited to a few allegations (passing by the microbiota)	Strong scientifical evidences
Food supplement				X	X	Limited to a few allegations (do not prevent diseases)	Strong scientifical evidences
Dietetic foods					X	limited to nutritional deficiencies corrections	None

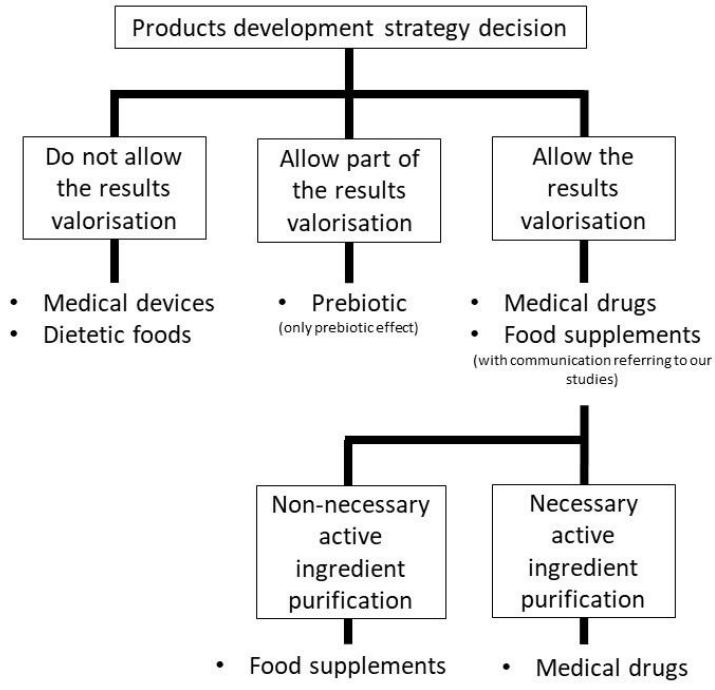

Figure 8. Decision-making tree about the possible product development strategies

Built according to personal sources.

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Appendix

Appendix 1 (Publication). Etienne-Mesmin, L., B. Chassaing, M. Desvaux, K. De Paepe, R. Gresse, T. Sauvaitre, E. Forano, T. V. de Wiele, S. Schuller, N. Juge and S. Blanquet-Diot (**2019**). Experimental models to study intestinal microbes-mucus interactions in health and disease. *FEMS Microbiol Rev* **43**(5): 457-489.

Appendix 2 (Poster). Sauvaitre, T., Roussel, C., Sivignon, A., Chalancon, S., Durif, C., Huille, S., Chaucheyras-Durand, F., Van de Wiele, T., Etienne-Mesmin, L., Blanquet-Diot, S. (**2021**). Use of a fiber-based strategy to prevent enterotoxigenic *Escherichia coli* infections: *in vitro* investigation on their antagonistic effects. 12th international symposium on Gut Microbiology, October 2021, Clermont-Ferrand, France

Appendix 3 (Poster). Sauvaitre, T., Etienne-Mesmin, L., Roussel, C., Chalancon, S., Durif, C., Uriot, O., Van de Wiele, T., Blanquet-Diot, S. (**2021**). Use of a fiber-based strategy to prevent bacteria-mucus interactions in enterotoxigenic *Escherichia coli* infections? Beneficial Microbes meeting, March 2021, Amsterdam, The Netherlands

Appendix 1

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REVIEW ARTICLE

Experimental models to study intestinal microbes–mucus interactions in health and disease

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One sentence summary: The review summarises the state of the art for studying gut microbes-mucus interactions using *in vitro*, *ex vivo* and *in vivo* experimental models.

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ABSTRACT

A close symbiotic relationship exists between the intestinal microbiota and its host. A critical component of gut homeostasis is the presence of a mucus layer covering the gastrointestinal tract. Mucus is a viscoelastic gel at the interface between the luminal content and the host tissue that provides a habitat to the gut microbiota and protects the intestinal epithelium. The review starts by setting up the biological context underpinning the need for experimental models to study gut bacteria-mucus interactions in the digestive environment. We provide an overview of the structure and function of intestinal mucus and mucins, their interactions with intestinal bacteria (including commensal, probiotics and pathogenic microorganisms) and their role in modulating health and disease states. We then describe the characteristics and potentials of experimental models currently available to study the mechanisms underpinning the interaction of mucus with gut microbes, including *in vitro*, *ex vivo* and *in vivo* models. We then discuss the limitations and challenges facing this field of research.

Keywords: intestinal mucus; gut microbiota; experimental models; mucin O-glycosylation

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INTRODUCTION

The human gastrointestinal (GI) tract harbours a complex and diverse community of microbes, including 10 trillion of microorganisms, collectively referred to as the gut microbiota (Sender, Fuchs and Milo 2016). Several regulatory mechanisms cooperate to maintain intestinal homeostasis and a disturbance of the relationship between the gut microbiota and the host can result in several disorders including chronic inflammatory diseases and metabolic syndromes (Rooks and Garrett 2016). While the intestinal microbiota provides important benefits to the host, such as calorie extraction and immune system maturation, it also holds the power to activate various innate and adaptive immune signalling which can lead to uncontrolled and deleterious intestinal inflammation (Pickard *et al.* 2017). A key component in maintaining a beneficial relationship between the commensal microbes inhabiting the intestine and the host is the presence of an appropriate barrier that prevents bacteria to reach and persist on the epithelial surface (Johansson and Hansson 2016; Sicard *et al.* 2017; Bretin, Gewirtz and Chassaing 2018). It is well acknowledged that intestinal epithelial cells (IECs) provide a physical and biochemical barrier that prevents the translocation of commensal bacteria to the underlying host tissue. In addition, there is an emerging paradigm that the mucus layer is an important modulator of human health in mediating the homeostatic relationship between the gut microbiota and the host. On the luminal side, the mucus layer provides the first physical, chemical and biological line of defence against large particles, including commensal bacteria and invading pathogens, segregating them from IECs (Turner 2009; Peterson and Artis 2014). Furthermore, mucus provides a biological niche for a microbial community, referred to as mucus-associated microbiota, which is likely to have a major influence on human health (Martens, Neumann and Desai 2018). However, advances in this field of research have been hampered by the lack of suitable model systems recapitulating all the interactions occurring at the mucosal interface. This review provides an overview of currently available experimental models to study the interplay between gut bacteria and intestinal mucus at a mechanistic level, and summarizes their main applications and the challenges remaining in this field of research.

OVERVIEW OF MUCUS STRUCTURE AND FUNCTION IN THE GASTROINTESTINAL (GI) TRACT

Mucus structural organisation

Mucus structure

Mucus is a highly hydrated gel made up of more than 98% water that makes it totally transparent, microscopically invisible and difficult to study. This aqueous viscoelastic secretion also contains electrolytes, lipids and various proteins (Bansil and Turner 2018). Mucus is found throughout the entire GI tract from the stomach to the large intestine, with its thickness and structure varying depending on the location, reflecting its various protective functions.

The mucus in the small intestine consists of one layer, while the stomach and colon have a bi-layered mucus. In human stomach, the mucus is about 200–400 μm in thickness and consists of an inner layer loosely attached to the epithelial surface, keeping the surface neutral (pH 7) while the gastric lumen pH is acidic (pH 2), and an outer layer which is mobile on the luminal side. Only few bacteria have evolved strategies to colonise the stomach, among which *Helicobacter pylori* is a specialist (Atuma *et al.* 2001; Juge 2012). In the small intestine, mucus fills up the space between the villi but is not attached to the epithelium and is somewhat permeable to bacteria (Atuma *et al.* 2001). In the colon, the two layers mediate opposite interactions with the microbiota; whereas the outer layer (up to 800 μm) is densely colonised by an important microbial biomass, the inner layer (> 200 μm in humans) is virtually devoid of bacteria leaving a space virtually free of microbes (commensals and or pathogens) leaving a space virtually free of microbes above the epithelium (Johansson, Sjovall and Hansson 2013). However, single-cell imaging at tissue scale in mice revealed the presence of bacteria in close proximity of the epithelium (Earle *et al.* 2015). Among commensal microorganisms, Segmented Filamentous Bacteria (SFB) are immunomodulatory commensals with the ability to adhere to IECs and to invade this mucus layer without invading the host (Hedblom *et al.* 2018; Ladinsky *et al.* 2019). Of note, a recent study revealed differences in mucus organization between the proximal and distal colon of rodents (Kamphuis *et al.* 2017): in the later, the mucus layer is attached to the faecal pellet and absent from the surface of the epithelium (Kamphuis *et al.* 2017).

Other studies demonstrated that the mucus thickens as the microbiota become more diverse, as particularly evident in the colon (Jakobsson *et al.* 2015). This is also supported by studies using germ free mice showing an impairment in mucus structure (Johansson *et al.* 2008; Johansson, Sjovall and Hansson 2013; Jakobsson *et al.* 2015). Gnotobiotic mice colonized with human faecal microbiota present a mucus layer structure resembling that of conventional mice by day 7 post-colonization (Hayes *et al.* 2018). Animals housed in distinct rooms of the same animal facility exhibit distinct microbiota profiles that are associated with large differences in the inner colon mucus layer, thereby affecting mucus barrier properties (Jakobsson *et al.* 2015). Also, it has been demonstrated in mice that mucus becomes thinner with age (Elderman *et al.* 2017). Variations in the mucus thickness and spatial organisation of the gut microbiota in mice were also found to be dependent of the diet (Earle *et al.* 2015). Interestingly, the thickness of the mucus layer has been shown to undergo circadian fluctuations, with highest microbial proximity to the mucosal surface during the dark phase (Thaiss *et al.* 2016).

Mucus secretion

The mucus is produced and secreted by specialized cells namely goblet cells located in the crypt in the small intestine and in higher numbers in the upper crypt in the colon (Johansson and Hansson 2013; Johansson and Hansson 2016; Sicard *et al.* 2017). Before secretion in the gut lumen, mucin polymers are stored in mucin granulae within the goblet cells (Johansson, Larsson and Hansson 2011; Johansson, Sjovall and Hansson 2013). The function of goblet cells varies depending on their localisation in the small intestinal or colonic crypts (Pelaseyed *et al.* 2014). Apart from their role in secreting mucus, small intestinal goblet cells can play a role in delivering luminal material to the immune system (Pelaseyed *et al.* 2014). Interestingly, a study from Gunnar Hanson's laboratory identified a subpopulation of goblet cells called 'sentinels' goblet cells (Birchenough *et al.* 2016). These cells are able to sense Toll-like receptor (TLR) microbial ligands at the entrance of colonic crypts and trigger the activation of

NLRP6 inflammasome, leading to mucus secretion from neighbouring goblet cells to defend the colon against bacterial invasion (Birchenough et al. 2016).

Renewal of the mucus is an important factor to preserve epithelial damage and bacterial exposure. The colonic mucus has a rapid turnover, since the inner mucus layer is renewed within 1 hour (Johansson 2012), while the gut epithelium renewal takes around 4–5 days (De Weirdt and Van de Wiele 2015).

Gastro-intestinal mucins

The main structural components of mucus are large glycoproteins called mucins. The protein sequences of mucin domains share a common core structure rich in the amino acids proline (P), threonine (T) and serine (S) called the PTS domain. These domains are then decorated by O-linked glycans made up of *N*- acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), galactose (Gal) and usually terminated by sialic acid and fucose (Juge 2012; Johansson and Hansson 2016; Sicard et al. 2017). These O-glycans render the mucin domains highly resistant to protease degradation and confer mucins their high-water binding capacity.

Mucins are produced as transmembrane mucins or secreted gel-forming mucins (Juge 2012; Johansson, Sjovall and Hansson 2013). In the stomach, MUC1 and MUC5AC are produced by the superficial epithelium, while MUC6 are secreted by the stomach glands (Johansson, Sjovall and Hansson 2013; Johansson and Hansson 2016). In the small intestine and colon, mucus is structurally built around the mucin-2 glycoprotein (MUC2). The folding and dimerization of MUC2 is a demanding process owing to the large number of disulfide bonds, and a defect during this process may affect the structure and function of intestinal mucus (Johansson, Larsson and Hansson 2011). Proteolytic cleavages of MUC2 catalysed by the host as well as bacteria enzymes favour the transition from firm to loose layer form and allow bacteria to penetrate into the mucin net-like structure of the outer mucus layer (Johansson et al. 2008). In addition to this proteolytic activity, the degradation of mucin glycan chains by bacterial glycosidases contribute to the establishment of a microbial community in the outer mucus layer (Johansson et al. 2008; Pelaseyed et al. 2014).

Mucin glycosylation

Glycosylation is the most frequent post-translational modification of proteins and can occur in *N*-linked and O-linked form, and O-glycosylation is the main modification of mucins (Arike and Hansson 2016). Mucin-type O-glycans are built from eight core structures, with core 1, core 2, core 3 and core 4 glycans most commonly found in intestinal mucins (Brockhausen, Schachter and Stanley 2009). O-glycosylation is initiated in the Golgi apparatus by the addition of a GalNAc residue to the hydroxyl group of serine and threonine of the mucin backbone. Further elongation and branching of the O-glycan chains is governed by a large family of glycosyltransferase enzymes (Bennett et al. 2012). The oligosaccharides can be further modified by addition of histo-blood group antigens (ABO, Lewis), secretor (H) epitopes and sialic acids and sulfate (Rossez et al. 2012; Bansil and Turner 2018). Mucin glycosylation varies along the GI tract (Robbe et al. 2003; Robbe et al. 2004; Holmen Larsson et al. 2013) and is linked to microbial colonization (Juge 2012; Bergstrom and Xia 2013; Tailford et al. 2015; Arike, Holmen-Larsson and Hansson 2017). Mounting evidence suggests that mucin glycosylation is critical to the biological and physical role played by mucus in the gut by influencing the physico-chemical properties and penetrability of mucus and by modulating the composition of the associated mucus-associated microbiota (see section 1.3). Not surprisingly, an alteration of mucin O-glycosylation profile has been reported in intestinal diseases associated with an impaired gut barrier function such as inflammatory bowel disease (IBD) and colorectal cancer (Larsson et al. 2011; Theodoratou et al. 2014) as also supported by work in animal models (Bergstrom et al. 2017) (see also section 4.5).

Mucus function in the gut

For decades, mucus has been considered to act as a simple physical barrier protecting the host, but mounting evidence suggests that mucus plays additional biological and immunological roles in maintaining gut homeostasis. The coating gel of mucus is acting, in concert with the immune system, the intestinal epithelium and the gut microbiota, to provide a physical, biological and chemical line of defence against potentially harmful invaders while harbouring a distinct microbial community having a major influence on host health.

Throughout the gut, the viscous mucus secretion acts as a lubricant that helps the progress of digestive matter along the GI tract and protects the underlying epithelium from excessive mechanical or chemical stresses. In the stomach, the mucus coating creates a pH gradient that protects the epithelium against the crude acidic gastric environment. Mucus acts as a size exclusion filter for larger compounds while selectively allowing transport of small molecules such as gases, ions, nutrients and many proteins to reach the enterocytes (De Weirdt and Van de Wiele 2015), but the mucus lining would prevent digestive enzymes from attacking these cells.

In the colon, the outer mucus layer serves as a biological habitat for various microorganisms. Indeed, the glycan structures in the mucus provide potential binding sites and constitute a carbon and energy source to support the growth of commensal but also pathogenic bacteria (Tailford et al. 2015) (see sections 2.1 and 2.2). It is believed that the mucin glycosylation patterns along the GI tract contribute to the microbial tropism of certain taxa in the mucus (Tailford et al. 2015).

The mucus layer also helps in the protection of the epithelium and, in association with the immune system, plays a crucial role in intestinal homeostasis. This gel is an important retention matrix for non-mucin proteins with immune regulatory molecules such as antimicrobial molecules (e.g. bactericidal RegIII$_\gamma$, α-defensins, secretory immunoglobulins IgAs, etc), therefore limiting the number of bacteria that can reach the epithelium and the underlying immune system (Peterson and Artis 2014; Johansson and Hansson 2016). This physical and biological barrier helps to keep the tremendous amount of bacteria that reside in the lumen as well as enteric pathogens at a safe distance from the epithelium (Chassaing, Ley and Gewirtz 2014; Johansson et al. 2014; Chassaing et al. 2015a). However, this system can be subverted and invading pathogens or pathobionts have evolved strategies to circumvent this barrier by e.g. degrading mucins and/or influencing mucin secretion (Rolhion and Chassaing 2016). In summary, mucus has a dual role in relation to the gut microbiota, it is an ecological niche for bacteria by providing adhesion sites and nutrients, while protecting the underlying epithelium from microbial aggressors that can breach this barrier.

The mucus-associated microbiota

The gut microbiota composition is known to differ along the longitudinal axis of the GI tract but it also varies transversally from the lumen to the mucosa due differences in key physiological parameters such as nutrient availability or oxygen gradient. The colonic epithelium is made of crypts with specific oxygen conditions and various concentrations of glycans that is a niche for mucin-degrading bacteria such as *Bacteroides fragilis* (Pereira and Berry 2017). The use of Carnoy fixative to preserve the mucus layer has been a crucial step for the detection of bacteria in the mucosal environment (Johansson *et al.* 2008). It is now well appreciated that the faecal microbiota community differs from the luminal, mucosa- or mucus-associated bacterial communities (Swidsinski *et al.* 2005; Li *et al.* 2015).

Studies in humans demonstrated that the abundance of Bacteroidetes appears to be higher in faecal/luminal samples than in the mucosa (Eckburg *et al.* 2005). Members of Firmicutes phylum and in particular *Clostridium* cluster XIVa are significantly enriched in the mucus layer compared to the lumen (Van den Abbeele *et al.* 2013). Analysis of human colonic biopsies have also shown a distinct mucosal community enriched in Actinobacteria and Proteobacteria compared to the luminal community (Albenberg *et al.* 2014). Certain species such as *Bacteroides acidifaciens*, *B. fragilis* and *Akkermansia muciniphila* are enriched in the outer layer of colon mucus (Derrien *et al.* 2004; Donaldson, Lee and Mazmanian 2016).

Similar findings have been observed in animals. Indeed, mice studies have shown that Firmicutes were enriched in the mucosa-associated microbiota, especially members of the Lachnospiraceae and Ruminococcaceae families (Tailford *et al.* 2015). Bacterial species such as *Bacteroides thetaiotaomicron* or *Escherichia coli* display specific genomic repertoires to persist in the outer mucus layer compared with the same species in the intestinal lumen (Li *et al.* 2015). This spatial localisation may be reflective of the radial oxygen gradient that shapes the mucus-associated and faecal microbiota, since oxygen can favour or impede certain microorganisms (Albenberg *et al.* 2014). Moreover, laser capture microdissection (LCM) in combination with metagenomics studies provided new insights into the composition of the mucus-associated microbiota (Wang *et al.* 2010). The use of LCM in mouse models revealed that this microbial community is especially dominated by Acinetobacter in the colonic crypts (Pedron *et al.* 2012). Using LCM coupled to DNA sequencing based analysis, Chassaing and Gewirtz recently reported profound differences at the phyla level between the inner mucus communities comprising 20% – 60% Proteobacteria and a concomitantly marked reduction in Bacteroidetes as compared to faecal microbiota (Chassaing and Gewirtz 2019).

Due to a high polysaccharide content (up to 80% of the mucin biomass), mucus provides an ecological niche for the intestinal microbiota. Mucus-associated bacteria are able to use oligosaccharides from mucins as binding sites through specific bacterial adhesins that promote their colonisation (Section 2.1) or as an energy source to support their growth (Section 2.2). Robbe and colleagues first suggested that the important repertoire of potential ligands and/or carbon sources in mucins could explain the pattern of bacterial colonisation in the different gut regions (Robbe *et al.* 2004). Mucin degradation has been extensively studied in pathogenic bacteria and more recently investigated in commensal bacteria including *A. muciniphila*, *Bacteroides* spp., Bifidobacteria and *Ruminococcus* spp. (Derrien *et al.* 2004; De Weirdt and Van de Wiele 2015). A disproportion of bacterial taxa able to invade mucus could further play a role in the development of the dysbiotic microbiota associated with the onset of various intestinal diseases (see section 3).

MUCIN-BACTERIA INTERACTIONS

Mechanisms of mucin binding by commensal and pathogenic microorganisms in the gut

Cell-surface proteins of pathogens and probiotics/commensal strains have been implicated in mediating the binding of microbes to intestinal mucus (Fig. 1). These include (i) specialized cell-surface adhesins or lectins, (ii) appendages such as pili and flagella or (iii) moonlighting proteins (see (Juge 2012) for a review). In particular, a considerable amount of research has been devoted to the characterization of these adhesins in *Lactobacillus* species (as extensively reviewed in (Van Tassell and Miller 2011; Nishiyama, Sugiyama and Mukai 2016)).

Mucus binding proteins

Mucus-binding proteins (MUBs) containing a variable number of Mub repeats are unique to gut inhabiting Lactobacilli and these proteins have been thoroughly characterised in *Lactobacillus reuteri*, a gram-positive bacterial species inhabiting the GI tract widely used as a probiotic (Frese *et al.* 2011). MUB from *L. reuteri* ATCC 53608 is one of the best-studied examples of mucus adhesins in commensal bacteria. It is a large protein consisting of six type 1 repeats (Mub1) and eight type 2 repeats (Mub2) with each repeat divided into a mucin binding (MucBP) domain and an immunoglobulin binding protein domain (Kuznetsova ; MacKenzie *et al.* 2009; Etzold *et al.* 2014b). The Mub repeats mediate binding to mucin glycans, through interactions with terminal sialic acid (Etzold *et al.* 2014a; Gunning *et al.* 2016), and Igs (MacKenzie *et al.* 2009). MUB has the shape of a long, fibre-like structure, of around 180 nm in length (Etzold *et al.* 2014b), and forms appendices reminiscent to pili found in pathogenic and, more rarely, other commensal bacterial species. However, in contrast to pathogenic pili which adhesin is restricted to the N-terminal tip, MUB interactions with mucin glycans occur through its long and linear multi-repeat structure, as shown by atomic force spectroscopy (Gunning *et al.* 2016). This multivalent binding would restrict penetration through mucus and limit access of the bacteria to the epithelium surface. In addition, MUB from *L. reuteri* ATCC 53608 was recently shown to modulate inflammatory responses in human monocyte-derived dendritic cells *via* interaction with DC-SIGN (Bene *et al.* 2017). The presence of mucus adhesins was also shown to mediate the binding of *L. reuteri* strains to both HT-29 and mucus-producing LS174T cells. The binding of *L. reuteri* to mucus led to a decreased enteropathogenic *E. coli* (EPEC) adherence to small intestinal biopsy epithelium (Walsham *et al.* 2016). Recombinant Mub proteins containing Mubs5s6 domains from Lp-1643 protein of *L. plantarum* Lp9 have been shown to adhere to human intestinal tissue sections (Singh *et al.* 2017) and inhibited the adhesion of enterotoxigenic *E. coli* (ETEC) to cultured intestinal HT-29 and Caco-2 cell lines, probably through the recognition of cell-surface mucins (Singh *et al.* 2018). Together, these findings show that the nature and function of these adhesins are strain-specific with the potential to target either the epithelium or the mucus layer and compete with pathogens.

Flagella

Several microorganisms have evolved strategies, in particular extracellular appendages such as flagella, pili and fimbriae, to

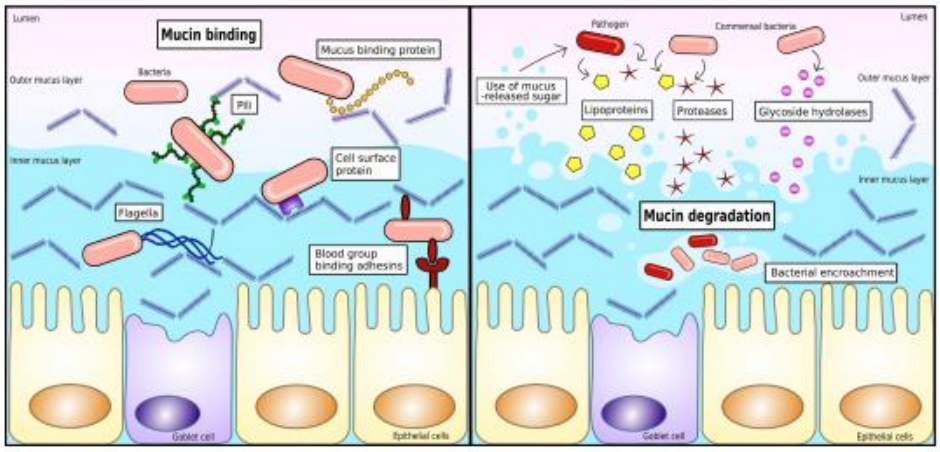


Figure 1. Mucin-bacterial interactions in the digestive tract.

Left panel: Mucins display various and diverse oligosaccharide structures representing potential binding sites for microbial adhesion. Commensal and pathogenic microbes can use cell-surface appendages, such as pili, flagella or fimbriae or adhesins to bind to mucus.

Right panel: Mucin glycans are an important energy source for microbes inhabiting the mucus niche that further confer them with an ecological advantage over other members of the gut microbiota. Commensal and pathogenic microorganisms can degrade mucin glycan chains leading to the release of mono- or oligosaccharides from that can be subsequently metabolized by other gut microbes in the mucosal environment.

attach to and to penetrate the mucus layer (Juge 2012). Pili and flagella are large polymeric proteins that form long surface structures involved in bacterial adhesion. Flagella are composed of several thousand copies of flagellin subunits and have been extensively studied in EPEC and enterohemorrhagic *E. coli* (EHEC) for their role in virulence and motility, but their role in mucus binding remains unclear. The adhesive properties of bacterial flagella to mucus were previously reported for *Clostridium difficile* where crude flagella, recombinant flagellar FliC and FliD proteins were shown to bind to murine mucus (Tasteyre *et al.* 2001). In pathogenic *E. coli* strains, the H6 and H7 flagella EPEC E2348/69 and EHEC EDL933 and their flagellin monomers were shown to bind to mucins and to bovine mucus (Erdem *et al.* 2007). Further studies then showed that EPEC and EHEC O157:H7 adherence to HT-29 cells is related to mucin-type core 2 *O*-glycan, facilitating invasion into host cells (Ye *et al.* 2015; Ye *et al.* 2015). However, flagella are involved in the ability of these pathogenic strains to cross the mucus layer, conferring a selective advantage in penetrating the mucus layers and reaching the epithelial surface, as demonstrated with Adherent-Invasive *E. coli* (AIEC) LF82 (G368). It is therefore tempting to speculate that in EPEC and EHEC, the flagella have a preference for cell-surface mucins rather than secreted mucus, in line with their ability to penetrate the mucus layer and attach onto the cell surface before invasion. In the probiotic *E. coli* strain Nissle 1917, a direct interaction was observed between isolated flagella from EcN and porcine MUC2 and human mucus but not murine mucus. The mucus component gluconate was identified as one receptor for the binding of EcN flagella (Troge *et al.* 2012). EcN was therefore proposed to confer the probiotic strain the ability to compete for binding sites on host tissue with bacterial pathogens.

Pili

Pili have been identified in *Lactobacillus rhamnosus* GG where they confer binding to mucus (Kankainen *et al.* 2009; von Ossowski *et al.* 2011) and are predicted to exist in other *Lactobacillus* species including *L. casei* and *L. paracasei*, based on genomics analyses (Douillard *et al.* 2013; Aleksandrzak-Piekarczyk *et al.* 2015; Nissila *et al.* 2017). In *L. rhamnosus* GG, these are composed of a three-protein complex SpaCBA, which has been involved in adhesion to mucus, IECs, and immunomodulatory interactions with IEC (Lebeer *et al.* 2012; von Ossowski *et al.* 2013; Ganguli *et al.* 2015; Vargas Garcia *et al.* 2015; Bene *et al.* 2017). The mucus-binding pili of *L. rhamnosus* GG shares immunological and functional similarities with those of the clinical *Enterococcus faecium* strain E1165. The binding of *E. faecium* E1165 to mucus could be prevented by the addition of the mucus-binding SpaC protein or antibodies against *L. rhamnosus* GG (Tytgat *et al.* 2016). Collectively, these studies show the potential of using mucus adhesins from probiotic strains to prevent the binding of enteric pathogens to the host.

Although not a resident member of the gut microbiota, several *Lactococcus lactis* strains have also been shown to exhibit mucus-binding properties through bacterial surface proteins such as mucin-binding proteins and pili (as recently reviewed in (Mercier-Bonin and Chapot-Chartier 2017)). The mechanisms of adhesion have been extensively studied by atomic force spectroscopy demonstrating a comparable role played by these two surface proteinaceous components in adhesion of *L. lactis* TIL448 to pig gastric mucin (PGM) neutral oligosaccharides under static conditions, whereas a more important contribution of the MUBs than the pili one was observed under shear flow (Le *et al.* 2013).

Other cell surface proteins

Other cell surface proteins implicated in the binding of commensal bacteria to mucin include aggregation-promoting factors (APFs) from *L. plantarum* NCIMB 8826 (Bolonkin 1990) or *L. lactis* (Lukic *et al.* 2012; Lukic *et al.* 2014), mucus-binding protein A (CmbA) from *L. reuteri* ATCC PTA 6475 (Etzold *et al.*

2014a; Jensen *et al.* 2014), Lam29 from *L. mucosae* ME-340 (Watanabe *et al.* 2010), mucus adhesion-promoting protein (MapA) from *L. fermentum/reuteri* 104R (Rojas, Ascencio and Conway 2002), a mucus-binding factor (MBF) from *L. rhamnosus* GG (von Ossowski *et al.* 2011; Nishiyama *et al.* 2015), a MucBP-containing mannose-specific adhesin protein (Msa) from *L. plantarum* WCFS-1 (Pretzer *et al.* 2005), a 32-Mmubp from *L. fermentum* BCS87 (Macias-Rodriguez *et al.* 2009), an extracellular transaldolase (Tal) from *Bifidobacterium bifidum* DSM20456 (Gonzalez-Rodriguez *et al.* 2012) and a recently-characterised serine rich repeat protein (SRRP) from *L. reuteri* ATCC 53 608 (Sequeira *et al.* 2018). It is expected that adhesion of these commensal or probiotic bacteria to mucus may favour their persistence within the gut in order to exert their beneficial effects to the host. Furthermore, it was recently suggested that carbohydrate binding modules (CBMs) appended to glycoside hydrolases could contribute to the tropism of gut bacteria to glycan-rich area of mucins in the colon, as shown for *Ruminococcus gnavus* sialic-acid-specific CBM40 (Owen *et al.* 2017).

Blood group binding adhesins

In addition, several human enteric pathogens bind to human histo-blood group antigens (HBGAs) expressed on the gut mucosa, including *Campylobacter jejuni*, *Norwalk virus* and *H. pylori*. The role of HBGA recognition to mucin binding has been extensively studied in the gastric pathogen *H. pylori* where *Helicobacter* adhesins have been reported to play a critical role in the attachment of the pathogen to both the glycosylated gastric epithelial cell surface and to glycosylated mucins. The binding of *H. pylori* to gastric mucins through blood group binding adhesin (BabA) and sialic acid-binding adhesin (SabA) revealed a complex charge/low pH-dependent mechanism involving four modes of *H. pylori* adhesion to MUC5B, MUC7 and MUC5AC mucins (Linden *et al.* 2008; Skoog *et al.* 2017). More recently, a novel outer membrane protein adhesin named LabA has been identified in *H. pylori* and shown to bind to LacdiNAc, a structure, which is also expressed on MUC5AC (Rossez *et al.* 2014). Binding of *H. pylori* to gastric mucins therefore is determined both by the mucin glycosylation and also by the adhesins expressed by individual strains. A chitin-binding protein GbpA from *Vibrio cholerae* shown to bind to N-acetyl-D-glucosamine residues of intestinal mucin has been proposed as an important factor mediating intestinal colonisation and pathogenesis by *V. cholerae* (Bhowmick *et al.* 2008; Wong *et al.* 2012).

Moonlighting proteins

Unexpectedly, several primarily cytoplasmic proteins have been reported to play a role in mucin binding. Due to their dual function, these proteins are referred to as moonlighting proteins (Henderson and Martin 2011; Henderson and Martin 2013; Henderson 2014). In *L. acidophilus*, *L. plantarum* and *Mycoplasma genitalium* for instance, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was clearly demonstrated to play a role in bacterial adhesion and bind mucins (Alvarez, Blaylock and Baseman 2003; Kinoshita *et al.* 2008; Patel *et al.* 2016). While the exact domain responsible for mucin binding remains to be elucidated, GAPDH is suggested to play a similar role in other commensal or pathogenic microorganisms (Kinoshita *et al.* 2013). In *L. reuteri*, elongation factor-Tu (EF-Tu) was found to bind the PGM when exposed at the bacterial cell surface (Nishiyama *et al.* 2013). Here, the sulfated carbohydrate moieties of mucins were demonstrated to play a significant role in EF-Tu-mediated bacterial adhesion to PGM and mucosal surfaces (Nishiyama *et al.* 2013). Proteosurfaceome analyses in a range of microorganisms have revealed a large repertoire of cytoplasmic proteins present at the bacterial cell surface but their implications in binding to various extracellular matrix (ECM) proteins, including mucins, remain to be more systematically investigated (Chagnot *et al.* 2012; Desvaux, Candela and Serror 2018).

Mechanisms of mucin degradation by commensal and pathogenic microorganisms in the gut

Several enzymatic activities are required for the degradation of mucins by pathogens or commensal bacteria including glycoside hydrolases (GHs), sulfatases, or proteases (Fig. 1) as described below.

Glycoside hydrolases

Mucin glycan degradation in bacteria relies on the expression of GHs such as sialidases (GH33), α-fucosidases (GH29, GH95), exo- and endo-β-N-acetylglucosaminidases (GH84 and GH85), β-galactosidases (GH2, GH20, GH42), α-N-acetylglucosaminidases (GH89), endo-β1,4-galactosidases (GH98) and α-N-acetylgalactosaminidases (GH101, GH129) (www.cazy.org). These enzymes have been functionally characterised in resident members of the gut microbiota able to forage on mucins, including *A. muciniphila*, *B. thetaiotaomicron*, *B. bifidum*, *B. fragilis* and *R. gnavus*, as recently reviewed (Tailford *et al.* 2015; Ndeh and Gilbert 2018). The released mono- or oligosaccharides derived from mucus degradation by these commensal bacteria can be utilised by the bacteria itself or scavenged by other bacteria inhabiting the mucus niche including pathogenic species such as *Salmonella* species, *C. difficile*, diarrhoeagenic *E. coli* or *Vibrio cholerae* through cross-feeding interactions (Fabich *et al.* 2008; Abyzov *et al.* 2012; Ng *et al.* 2013). In addition, some of these pathogens have the glycolytic potential to release mucus-derived sugars for their own consumption (Mondal *et al.* 2014; Arabyan *et al.* 2016).

Sulfatases

Sulfatases are being increasingly investigated for their role in modulating the gut microbial ecosystem in health and disease. Some members of the gut microbiota such as *B. thetaiotaomicron*, *Bacteroides ovatus* and Prevotella sp. strain RS2 *Bifidobacterium breve* UCC2003, or *B. fragilis* possess mucin-desulfating sulfatases or glycosulfatases (Salyers *et al.* 1977; Berteau *et al.* 2006; Benjdia *et al.* 2011; Egan *et al.* 2016; Praharaj *et al.* 2018). Mucin sulfatase activity of these species may provide them a competitive advantage in the infant gut and/or the adult gut. The mucin-desulfating sulfatases that have been characterised so far include sulfatases specific for the -D-galactopyranosyl 3-sulfate, -Dgalactopyranosyl6-sulfate and 2-acetamido-2-deoxy-D-glucopyranosyl6-sulfate (6-SO3-GlcNAc) building blocks of the oligosaccharide chains. GlcNAc-6-S can be found in terminal or branched positions of mucin oligosaccharide. The desulfation of mucin by bacterial sulfatases may be a rate-limiting step in mucin-degradation mechanism, allowing glycosidases to access and act on the mucins by other members of the gut microbiota. The release of sulfate from mucins may also contribute to the expansion of Sulfate-reducing bacteria (SRB) in the gut (Rey *et al.* 2013). SRB are able to produce hydrogen sulfide (H_2S) which can reduce disulfide bonds present in the mucus network, leading to mucus erosion and access of bacteria to the epithelium, therefore contributing to epithelial damage and inflammation This mechanism has been proposed to be involved in the aetiology and/or severity of IBD (Ijssennagger, van der Meer and van Mil 2016). In addition, Hickey and colleagues showed that sulfatases

of B. thetaiotaomicron are required for its outer membrane vesicles to transit to underlying host immune cells and cause colitis (Chatzidaki-Livanis and Comstock 2015). Together these data highlight the complex role of bacterial sulfatases in the gut.

Proteases

Bacterial proteases from commensal or pathogenic E. coli have also been implicated in the recognition and degradation of mucins. In EHEC, StcE (secreted protease of C1 esterase inhibitor from EHEC) was originally described as specifically cleaving C1 esterase inhibitor (C1-INH) (Lathem et al. 2002; Grys, Walters and Welch 2006) but later showed to be even more active against MUC7 (Lathem et al. 2002). This soluble enzyme is important in reducing mucin levels. StcE has been suggested to have a dual role during human infection, (i) by promoting the penetration of bacterial cells through the mucus barrier lining the GI tract and thus facilitating the intimate EHEC adherence to IECs, which is an essential step in colonisation (Hews et al. 2017), and (ii) by acting as an anti-inflammatory agent protecting bacterial and host cell surfaces from complement-mediated lysis (Grys et al. 2005; Abreu and Barbosa 2017). StcE is secreted by a Type II, subtype a, secretion system (T2aSS) (Monteiro et al. 2016; Hay et al. 2018). This mucinase is a metalloprotease belonging to the peptidase M66 family (IPR019503) carrying one zinc atom per protein but no structural calcium, which is a reported feature of metalloproteases (Yu, Worrall and Strynadka 2012). Recently, EHEC StcE metalloprotease was shown to reduce the inner mucus layer in human colonic mucosal biopsies and the MUC2 glycoprotein levels in mucin-producing LS174T colon carcinoma cells (Hews et al. 2017).

Pic (protein involved in intestinal colonisation), also previously known as Shmu (Shigella mucinase), is a secreted protease identified in Shigella flexneri and enteroaggregative E. coli (EAEC) (Henderson et al. 1999a). Pic is secreted by a Type V, subtype a, secretion system (T5aSS) and belongs to the subfamily of serine protease autotransporters (SPATEs), with a catalytic domain corresponding to the peptidase S6 family (IPR030396). This enzyme was reported to display proteolytic activity against gelatin as well as bovine and murine mucin but not hog gastric mucin (Henderson et al. 1999a). PicU was also shown to exhibit mucinolytic activity in uropathogenic E. coli (Parham et al. 2004).

Hbp (hemoglobin-binding protease), also previously known as Tsh (temperature-sensitive haemagglutinin), is capable of cleaving bovine submaxillary mucin but not hog gastric mucin, which so far would appear as a feature of mucinolytic serine protease autotransporter of Enterobacteriaceae (SPATE) of the peptidase S6 family (Dutta et al. 2002). In some EHEC strains, a SPATE of the peptidase S6 family exhibiting mucinolytic activity was identified on plasmid pO113, namely EpeA (EHEC plasmid-encoded autotransporter) (Leyton et al. 2003). In AIEC, a Vat (vacuolating autotransporter) homologue belonging to the SPATE of the peptidase S6 family was demonstrated to exhibit a mucinolytic activity (Gibold et al. 2016). Vat-AIEC appears to significantly contribute to the colonisation ability of AIEC by decreasing mucus viscosity as well as enhancing bacterial penetration in mucus and access to IECs (Gibold et al. 2016).

In some non-O157 EHEC strains, a subtilase cytotoxin (SubAB) was identified (Paton et al. 2006; Wang, Paton and Paton 2007) and appeared to contribute to mucin depletion as shown with a Shiga-toxin encoding E. coli (STEC) O113:H21 strain (Gerhardt et al. 2013). While the A subunit harbours the enzymatic activity with a subtilase-like serine protease domain belonging to the peptidase S8/S53 family (IPR000209), the mucinolytic activity of SubAB remains to be clearly established.

Other proteins have been described in V. cholerae. Among them, TagA is a secreted protease of V. cholerae that specifically cleaves mucin glycoproteins (Szabady et al. 2011). The V. cholerae extracellular chitinase ChiA2 secreted in the intestine hydrolyzes intestinal mucin to release GlcNAc, and the released sugar is successfully utilized by V. cholerae for growth and survival in the host intestine (Mondal et al. 2014).

SslE (secreted and surface associated lipoprotein), previously known as YghJ, is a secreted and cell-surface lipoprotein degrading the major mucins in the small intestine, namely MUC2 and MUC3, thus facilitating bacterial penetration of the mucus layer and ultimately adhesion to host cells (Luo et al. 2014; Valeri et al. 2015; Tapader, Bose and Pal 2017). SslE is secreted via a T2aSS and appears inactive against the mucin-like CD43, bovine submaxillary mucin, gelatin, or IgG (Luo et al. 2014). This Zn-metalloprotease, belonging to the peptidase M60 family (IPR031161), is found in pathogenic and commensal E. coli, including ETEC, EHEC O104:H4, E. coli SE-11 or Nissle 1917 strains. AcfD (accessory colonisation factor D) from V. cholerae is homologous to SslE but its putative mucinolytic activity remains to be investigated (Peterson and Mekalanos 1988). Of note, SslE is also considered as a relevant target for the development of vaccines against intestinal pathogenic E. coli (Nesta et al. 2014; Naili et al. 2016; Naili et al. 2017).

Importance of mucus-bacteria interactions in health and disease

In the colon, the outer mucus layer offers a niche to commensal bacteria by providing preferential binding sites (Section 2.1) and nutrients (Section 2.2). Due to its proximity to host cells and the immune system, the mucus-associated microbiota, sometimes also referred to as the mucobiome (Belzer et al. 2017), has been proposed as an important modulator of health. The integrity of the mucosa relies on a combination of factors including the gut microbiota composition, the diet and host genetic factors (Fig. 2) (Martens, Neumann and Desai 2018). The mucus and mucus-associated bacterial community play a key role in limiting access of invading pathogens to the underlying epithelial cells and in limiting the progression of intestinal and extra-intestinal diseases (Donaldson, Lee and Mazmanian 2016).

Effect of bacteria and bacterial products on mucus production

A number of animal studies (using antibiotic-treated, germ-free or gnotobiotic mice) suggest that the presence of bacteria triggers the development of the protective mucus layer. Mice treated with the antibiotic metronidazole, but not streptomycin, display an altered goblet cell function and thinning of the inner mucus layer (Wlodarska et al. 2011). However, another study reported that depletion of the intestinal microbiota following a 3 week-antibiotic period (cocktail of four antibiotics) did not modify mucus penetrability (Johansson et al. 2015).

Compared to conventionally housed animals, germ-free mice have fewer goblet cells, which are smaller in size (Kandori et al. 1996) and harbour an impaired mucus layer, indicating that the formation of the protective mucus layer depends upon the presence of bacteria (Rodriguez-Pineiro and Johansson 2015). Johansson and colleagues demonstrated that the mucus of germ-free mice displayed a significant decrease in Muc2 level and was more penetrable to bacterium-size fluorescent beads as compared to conventionally raised mice (Johansson et al. 2015). The gut microbiota composition of germ free animals is normalized two weeks after colonisation in terms of microbiota composition, but up to 8 weeks are needed to reach

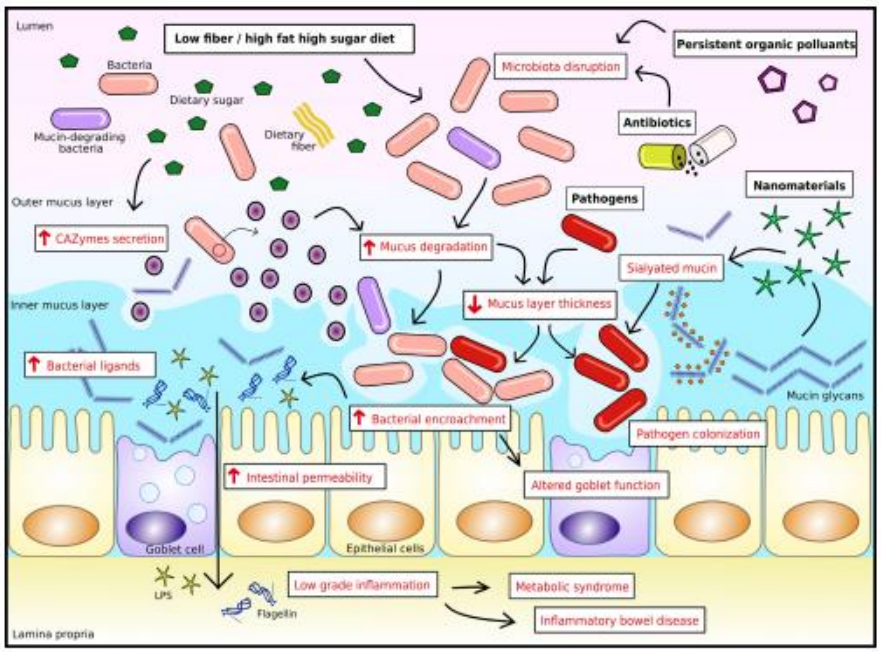


Figure 2. Perturbations of the mucus barrier in response to environmental and microbial stimuli.

This figure represents an overview of the various factors (diets, nanomaterials, pollutants, antibiotics or invading pathogens) affecting the gut microbiota composition and/or the thickness, structure and composition of the mucus barrier. Disruption of the mucus layer promotes bacterial encroachment leading to the subsequent development of low-grade inflammation, associated with inflammatory bowel diseases and metabolic disorders.

a normalized mucus phenotype (Johansson *et al.* 2015; Hayes *et al.* 2018). In support of this, fortification of the mucus layer and increased diversity of mucin glycosylation was observed within 48 hours of human intestinal organoid colonization with human-derived, non-pathogenic *E. coli* (Hill *et al.* 2017). Some bacteria, in particular Anaerostipes, have been shown to display mucus-stimulating properties (Jakobsson *et al.* 2015). *Lactobacillus* species can also stimulate MUC2 production and secretion by the goblet cells in the human gut (Sicard *et al.* 2017). Representative members of the two main phyla of the gut microbiota, *B. thetaiotaomicron* and *Faecalibacterium prauznitzii* can modulate goblet cell differentiation and thus mucus production (Wrzosek *et al.* 2013). A recent study showed that *Streptococcus thermophilus*, a transient food-borne bacterium, was able to induce mucus pathway in gnotobiotic rodents despite its poor capacity for mucus adhesion and mucin glycan degradation *in vitro* (Fernandez *et al.* 2018). Some of the mechanisms mediating mucin production and secretion by gut bacteria have been elucidated as described below.

Pathogen associated molecular patterns such as lipopolysaccharide (LPS) or peptidoglycan are known to induce mucus production (Petersson *et al.* 2011). LPS and flagellin purified from Gram-negative bacteria as well as lipoteichoic acid from Gram-positive bacteria have been shown to induce mucin upregulation via the Ras-signalling pathway (McNamara and Basbaum 2001). LPS also increases the production of interleukin (IL)-8 by goblet cells, which further promotes mucin secretion (Smirnova *et al.* 2003). TLR family members play an important role in mucus formation. Mice lacking the TLR adaptor protein MyD88 show a decreased production of mucus (Bhinder *et al.* 2014). Mice engineered to lack the flagellin receptor, TLR5 deficient mice, have a disorganised mucus layer and lack a well-defined inner layer when compared to wild type animals with an increase abundance of Proteobacteria in close contact with the epithelial surfaces (Carvalho *et al.* 2012; Chassaing, Ley and Gewirtz 2014; Chassaing *et al.* 2015a). Lastly, it has been shown *in vitro* using various human-derived cell lines that bacterial metabolites such as short-chain fatty acids (SCFA) and especially butyrate can stimulate MUC2 production in the absence of other energy sources (Willemsen *et al.* 2003; Gaudier *et al.* 2004). The effect of butyrate on MUC2 gene expression is mediated by epigenetic modifications (acetylation/methylation of histones) on the MUC2 promoter as demonstrated *in vitro* using human goblet cell-like LS174T cells (Burger-van Paassen *et al.* 2009). Fernandez and colleagues suggested that lactate produced by *S. thermophilus* in the GI tract could stimulate mucus production via a signalling pathway dependent of KLF4, a transcription factor involved in the differentiation of goblet cells (Fernandez *et al.* 2018). Some other bacterial effectors have been identified to mediate mucin expression and glycosylation such as small peptides from *R. gnavus* and *B. thetaiotaomicron* (see section 2).

Interactions of pathogens with mucus

The mucus barrier provides a bulwark against intestinal pathogens (Johansson, Sjovall and Hansson 2013; Sicard *et al.* 2017; Martens, Neumann and Desai 2018). The importance of

intestinal mucus in controlling enteric infection has been widely documented in *Muc2* knockout mice (*Muc2*$^{-/-}$ mice) (see section 4.5), which do not produce mucus in the small and large intestine, thus leading to a close contact between bacteria and the epithelium. Bergstrom and colleagues reported that *Muc2*$^{-/-}$ mice exhibit an increase susceptibility to murine bacterial pathogen *Citrobacter rodentium* (Bergstrom *et al.* 2010). Likewise, Muc2 plays a crucial role in controlling *Salmonella* infection (Zarepour *et al.* 2013). In a similar way, *H. pylori* has evolved mechanisms allowing its residence in the gastric mucus layer (Moore, Boren and Solnick 2011). As previously described for bacteria, *Muc2*$^{-/-}$ mice are also more susceptible to enteric parasitic infection with *Trichuris muris* since they exhibit a delayed expulsion of the parasite compared to wild type animals (Hasnain *et al.* 2010). Clearance of parasitic infection is associated with exclusion of helminths via a TH2 cell-mediated goblet cell increase and mucus release (Artis and Grencis 2008). *Entamoeaba histolytica* also possesses lectins binding to mucins and secretes proteases responsible for the cleavage of Muc2, allowing the protozoan to invade the underlying epithelium (Lidell *et al.* 2006). Recently, a detailed investigation of the cooperative roles for colonic microbiota and Muc2 in mediating innate host defence against *E. histolytica* was carried out using *Muc2*$^{-/-}$ mice, germ free mice and mucus-secreting LS174T cells, demonstrating that mucus secretion and pro-inflammatory responses were microbiota-specific (Leon-Coria *et al.* 2018). Lastly, as shown with *S. flexneri* and *H. pylori*, some pathogenic bacteria are able to reshape mucin structures by remodelling their glycosylation pattern in a type III secretion system-dependent manner (Sperandio *et al.* 2013; Magalhaes *et al.* 2015).

Emerging data suggest that pathogenic bacteria can benefit from the capacity of commensal microorganisms to release mucin degradation products that can be used to support their proliferation within the mucus niche. For example, *B. thetaiotaomicron* can release free sialic acid from colonic mucus glycans that can be utilized by *C. difficile* and *Salmonella* Typhimurium to promote their own colonisation and persistence in the gut (Ng *et al.* 2013). Another study indicates that EHEC bacteria colonise the mucus layer within the cooperation of local bacterial communities including *B. thetaiotaomicron* and other anaerobes which are able to cleave host glycan-derived sugar and produce fucose (Pacheco *et al.* 2012). EHEC then senses fucose produced by *B. thetaiotaomicron* to control expression of its type III secretion system (Pacheco *et al.* 2012; Cameron *et al.* 2018).

Mucus-pathogen interactions have also been evidenced in the extra-digestive area. *Pseudomonas aeruginosa*, a Gram-negative-flagellated pathogen, is the main causal agent for the development of pneumonia in immunocompromised patients and patients with cystic fibrosis (CF). This infection is associated with a genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride and bicarbonate ion channel protein with a key role in protecting the small intestine from bacterial invasion. CF conducts to blockage airway, mucus hypersecretion leading to chronic bacterial lung infections and inflammation. It has been shown that *P. aeruginosa*, via LPS, upregulate MUC2 and MUC5AC gene expression contributing to the excessive mucus production and airway blockage seen in CF (Bellu *et al.* 2013).

Effect of diet on mucus

Recent evidences have demonstrated that the diet can influence the properties of colonic mucus and thereafter interfere with the gut microbiota.

Fibres

Living in symbiosis with the host, the gut microbiota depends mostly on non-digestible fibres and polysaccharides as energy source. In the absence of fibres in the diet, the gut microbiota shifts towards the utilisation of host glycans such as those provided by mucins, resulting in a thinner protective colonic mucus (Sonnenburg and Sonnenburg 2014; Earle *et al.* 2015). Accordingly, Desai and colleagues demonstrated that a low-fibre diet promotes the enrichment of mucin-degrading bacteria and the overexpression of carbohydrate-active enzymes (CAZymes) that degrade the colonic mucus barrier (Desai *et al.* 2016; Martens, Neumann and Desai 2018). In these mice fed with a deprived-fibre diet, infection with *C. rodentium* promotes greater epithelial access and lethal colitis (Desai *et al.* 2016).

A lack of fermentable fibres in the diet also leads to a reduction in epithelial cell proliferation resulting in a thin mucosa with encroached bacteria (Chassaing *et al.* 2015b). Further studies in mice reported that inulin supplementation increases the number of colonic goblet cells, which correlates with a thicker mucus layer and an increase proportion of the *Akkermansia* genus (Kleessen, Hartmann and Blaut 2003; Everard *et al.* 2013). Similarly, studies in mice showed that a diet enriched in inulin fibre prevents mucus deterioration (Schroeder *et al.* 2018). It was recently showed that inulin but not cellulose protects against diet-induced obesity by reducing microbiota encroachment in a cytokine IL-22-dependent manner (Zou *et al.* 2018), demonstrating the importance of dietary factors, especially soluble fibre, in the homeostasis of host–microbiota relationship. Considering the increased mucus foraging activity occurring when mice are fed with a low-fibre diet, a recent study showed that supplementation with probiotic bifidobacteria (*B. longum*) or prebiotic fibre (inulin) could reduce such mucus defect. Notably, administration with *B. longum* was sufficient to restore mucus growth, while administration with inulin could prevent the increase of mucus penetrability in mice fed a western style diet (WSD) (Schroeder *et al.* 2018).

Western diet and food additives

Besides fibres, other nutrients within a WSD can modulate intestinal barrier function. A WSD is a rich in saturated fats and simple carbohydrates but depleted in dietary fibres. As a result, a diet-induced obesity in mice leads to colon mucosal barrier dysfunction with a thinner mucus layer (as described above) and treatment with A. muciniphila appears to counteract this effect by improving mucus thickness (Everard *et al.* 2013). Similarly, mice fed a high-fat and high-sugar diet exhibit an increased abundance of mucin-degrading species leading to a decrease in mucus thickness (Martinez-Medina *et al.* 2014). The diet of modern societies has dramatically changed as evidenced by a steady increase in the consumption of processed foods concomitantly with an increase in the use of food additives (Chassaing *et al.* 2015a). Mice treated with dietary emulsifiers (polysorbate 80 or carboxymethylcellulose) show a reduced mucus thickness and increased gut permeability. In these animals, some bacteria appear in close contact with the epithelium. Emulsifier-treated mice have an altered microbial composition associated with increased levels of mucolytic bacteria including *R. gnavus* and a marked reduction in microbial diversity, with a bloom in *Verrucomicrobia* phyla, especially *A. muciniphila* (Chassaing *et al.* 2015a). This may further contribute to the intestinal passage of bacterial constituents such as LPS and flagellin, which participates in the development of low-grade inflammation and metabolic disorders in wild type mice and of colitis in susceptible host animals (Chassaing, Ley and Gewirtz 2014; Chassaing *et al.* 2015a;

Chassaing et al. 2017b). Mice fed with diets enriched in maltodextrin, a filler and thickener used in food processing, show a reduction of Muc2 expression, making the host more sensitive to low-grade inflammation but with no significant change in mucosa-associated microbiota (Laudisi et al. 2018).

Effect of food contaminants on mucus

The intestinal mucosa is increasingly appreciated as a key player in the emerging field of gut toxicology of environmental pollutants, as recently reviewed (Gillois et al. 2018). Human contamination mainly occurs via the oral route through consumption of food but also through polluted water and soil exposure.

Nanomaterials

The use of nanotechnology in many common consumer products, especially in food products, is growing. Scarce studies have evaluated the interactions of food nanoparticles with the microbiota and mucus (Mercier-Bonin et al. 2018). It was shown *in vitro* that common nanoparticles of Titanium dioxide (TiO_2) are trapped into mucus, leading to areas with a high local concentration (Talbot et al. 2018). Silver nanoparticles are widely used in food industry to colour the surface of confectionary and pastries. Rats fed with these particles exhibit higher numbers of goblet cells and a modification of the glycosylation pattern of mucins with a decreased proportion of sulfated mucins and an increased proportion of sialyated mucins (Jeong et al. 2010). Repeated silver nanoparticle-exposure may therefore produce pathological regions in the lamina propria (Jeong et al. 2010).

Persistent Organic Pollutants

A recent study showed that mice chronically exposed to benzo[a]pyrene (BaP) which is the most toxic member of the polycyclic aromatic hydrocarbons family display significant shifts in the composition and relative abundance of stool and mucosa-associated bacterial communities (decrease of *Verrucomicrobiaceae*, represented by *A. muciniphila*) (Ribiere et al. 2016). Furthermore, exposure to perfluorooctane sulfonate (PFOS, environmental contaminant used as a surfactant and repellent) in a mouse model of *C. rodentium* infection led to a significant reduction in mucin gene expression and a failure to clear the bacterial infection (Suo et al. 2017). Smoke exposure also significantly affects the mucosa-associated bacterial community and alters the expression of mucins in the murine gut (Allais et al. 2016).

Mucus and inflammatory-related diseases

Inflammatory bowel diseases (IBD)

Barrier disturbances including alterations in the thickness or composition of the intestinal mucus layer are recognized to play a crucial role in the onset of GI disorders such as Crohn disease (CD) or ulcerative colitis (UC). The mucus layer in UC patients is thinner and has an altered glycosylation profile making it more penetrable to bacteria (Johansson et al. 2014). To better understand the onset of IBD, several murine models of intestinal inflammation (genetically or chemically induced) have been established. The most common experimental model of colitis relies on the administration of Dextran Sodium Sulfate (DSS) in the drinking water. Mice orally administered with DSS display an inner mucus layer which is more penetrable by bacteria within 12 hours (Johansson et al. 2010). Similarly, $IL\text{-}10^{-/-}$ and $TLR5^{-/-}$ mice that develop spontaneous colitis have a thicker mucus layer and more penetrable inner mucus layer when compared to wild type animals (Johansson et al. 2014). $Muc2^{-/-}$ mice develop intestinal inflammation with diarrhoea, rectal bleeding and prolapse (Johansson et al. 2008) and are more susceptible to DSS-induced colitis; these animals exhibit a massive number of bacteria in close contact with host tissues, further promoting inflammation (Van der Sluis et al. 2006). Moreover, abnormal mucin *O*-glycosylation has been associated with an increased inflammation, highlighting the importance of mucin glycans in the maintenance of gut homeostasis (Johansson et al. 2014) (Bergstrom and Xia 2013; Bergstrom et al. 2016).

These changes in mucus composition were also mirrored by changes in the gut microbiota composition at the mucosal surface. IBD patients exhibit a disproportion of mucin-degrading (or mucinolytic) bacteria with an increased abundance of *Ruminococcus torques* and *R. gnavus*, but a decreased abundance of *A. muciniphila*. In addition, the expansion of certain pathobionts and in particular AIEC exhibiting mucinolytic activity has been reported to favour gut colonisation and further induce inflammation in CD (Palmela et al. 2018). Taken together, these data suggest that mucus-bacteria interactions contribute to the intestinal barrier dysfunction in IBD patients and future work is needed to better understand the influence or consequence of these interactions on the disease.

Obesity and metabolic-related disorders

A correlation between adiposity, dysglycemia and microbiota encroachment has been reported in a number of animal studies. $Muc2^{-/-}$ mice fed a High Fat Diet (HFD) are protected from diet-induced weight gain, fatty liver, and insulin resistance as they displayed less inflammation and increased systemic levels of IL-22 (Hartmann et al. 2016). This study supports a role of Muc2 during obesity and highlights the importance of the crosstalk between microbiota, mucus and immune mediators. In mice fed a HFD, mucus secretion is altered in the ileum but not in the duodenum and jejunum, largely in response to an alteration of PPAR-γ signalling. In these mice, Muc2 accumulates at the apical side of goblet cells, leading to a reduction in the expansion capacity of the mucins, thus strongly altering the phenotype of the mucus layer (Tomas et al. 2016). Studies by Chassaing and colleagues in different mouse models of metabolic syndrome and in humans demonstrated that bacteria have the ability to infiltrate the mucus layer and reach the epithelium (Chassaing, Ley and Gewirtz 2014; Chassaing et al. 2015a; Chassaing et al. 2017a). Further, measurement of bacterial-epithelial distance reveals that microbiota encroachment is a feature of insulin resistance-associated dysglycemia in humans that may promote inflammation (Chassaing et al. 2017a). Several studies demonstrated that *A. muciniphila* is less abundant in the intestinal microbiota of both genetic and diet-induced obese and diabetic mice, as well as in individuals with obesity, when compared to the faecal microbial population of healthy individuals (Everard et al. 2013; Shin et al. 2014). *A. muciniphila* treatment has been shown to reverse fat gain, serum LPS levels, gut barrier function and insulin resistance. In addition, oral administration of an outer-membrane protein from *A. muciniphila* led to reduced fat mass and metabolic syndrome in mice fed an obesity-induced diet (Plovier et al. 2017). Conversely, anti-diabetic treatments such as metformin administration led to an increase in the *Akkermansia* spp. population (Shin et al. 2014).

Human studies have shown that alcohol abuse induced alcoholic liver diseases (ALD) are associated with an increase in intestinal mucus thickness in patients, using wheat germ agglutinin staining on duodenal biopsies (Hartmann et al. 2013). Animal studies demonstrated that when compared to wild type

animals, *Muc-2*$^{-/-}$ mice are protected from alcoholic steatohepatitis in an experimental alcohol-induced liver disease model (Hartmann et al. 2013). In addition, *Muc-2*$^{-/-}$ mice are protected from Non Alcoholic Fatty Liver Disease (NAFLD) when fed a high-fat diet inducing liver steatosis (Hartmann et al. 2016). Altogether, these data highlight the role of mucus and mucins in the gut-liver axis.

Cancer

The role of mucins in cancer progression has been extensively reviewed (Hollingsworth and Swanson 2004; Kufe 2009). *Muc2*$^{-/-}$ mice displayed spontaneous development of adenomas in the small intestine that progressed to invasive adenocarcinoma, as well as rectal tumours (Velcich et al. 2002). In humans, high levels of expression of MUC2 by pancreatic and biliary tumours has been associated with a low degree of invasiveness, malignancy and a better prognosis as compared to tumours not expressing MUC2 (Hollingsworth and Swanson 2004). An abnormal mucin *O*-glycosylation has been associated with an increased inflammation that could contribute to the development of colitis-associated colon cancer in mice (Bergstrom and Xia 2013; Bergstrom et al. 2016). Together these studies support the role of MUC2 as a tumour suppressor.

POTENTIAL OF EXPERIMENTAL MODELS TO STUDY MUCUS/MUCIN INTERACTIONS WITH GUT MICROBES

As mounting evidences highlight the importance of mucus in the cross-talk between the gut microbiota and the host, a wide range of experimental models has been developed to study mucus-bacteria interactions (Table 1). These include the use of purified mucins, mucin-secreting cells or tissues, or mucin-containing fermentation models, as described below.

In vitro mucus/mucin binding assays

Microplate assays

Several microtiter plate assays have been developed for testing bacterial adhesion to mucus and/or mucin (McNamara, Sack and Fleiszig 2000; Gusils, Morata and Gonzalez 2004). These generally rely on the immobilisation of mucins or mucus to the wells of microtiter plates following incubation overnight at 4°C or at room temperature in buffers such as such as PBS (pH 7–7.5), HBSS (pH 7–7.5) or carbonate buffer (pH 9.6) (Gusils, Morata and Gonzalez 2004; Dague et al. 2010; Mackenzie et al. 2010; Chagnot et al. 2013). BSA (bovine serum albumin) is generally used as a negative control for assessing the specificity of the binding to mucus and/or mucin. Binding assays are usually performed at 37°C and the contact time with bacterial cells generally ranges between 30 min to 3 hours before washing to remove non-adhered bacteria. Antibiotic at growth inhibiting concentration, such as chloramphenicol, or sometimes thermic treatment can be applied to prevent the growth of microorganisms in the course of the adhesion assay. Binding can be determined using crystal violet staining of the adhered microbial biomass (Azeredo et al. 2017) or by Enzyme-linked Immunosorbent Assay (ELISA) when specific antibodies against bacteria are available (Skoog et al. 2012), by measurement of viable counts after plating of the cells (McNamara, Sack and Fleiszig 2000) or by quantitative PCR (Skoog et al. 2012). Alternatively, bacteria can be labelled with a radioactive probe or a fluorescent dye before inoculation and the binding quantified using a scintillation counter or a fluorometer, respectively (Gusils, Morata and Gonzalez 2004; Mackenzie et al. 2010). Microbial cells can also be labelled by biotinylation and further assayed using streptavidin-HRP by ELISA (Sheng et al. 2012). Quantification of microbial binding to mucin can also be performed by flow cytometry, where microbial cells are put in contact with mucin labelled with a fluorescent tag (de Repentigny et al. 2000).

Dot blot assay

More recently, a dot-blot method was developed for the sensitive and rapid detection of microorganisms able to bind to mucins (Ringot-Destrez et al. 2018). In brief, purified mucins were spotted on a nitrocellulose membrane, whereas the bacterial cells were labelled using a fluorescent dye, such as 4',6-diamidino-2-phenylindole (DAPI), Syto9 or Fluorescein isothiocyanate (FITC), before being overlaid (Ringot-Destrez et al. 2018). The adhesion capacities of the microorganisms tested differed depending on the nature of the mucins including purified GI tract mucins, PGM and mucins from the mucus-secreting cell line such as HT29-MTX (see detailed description of this cell line in Section 4.2) (Ringot-Destrez et al. 2018).

Mucin microarrays

The carbohydrate microarray technology offers a powerful platform where natural or synthetic glycans are immobilized onto a solid support. (Poole et al. 2018). Microarrays incorporating mucins from various sources onto different chips surfaces provide a high-throughput approach to screen bacteria-mucin interactions as well as identify glycan-binding proteins and glycan epitopes involved in this interaction (Clyne et al. 2017). For example, the use of mucin microarrays revealed that *C. jejuni* and *H. pylori* recognised distinct mucin receptors despite being closely related phylogenetically (Naughton et al. 2013). Recently, *H. pylori* was shown to interact with trefoil factor family (TFF) protein TFF1 (Reeves et al. 2008), and that TFF1 specifically interacts with human gastric mucin but not with human colonic mucins nor mucins from other animal sources as shown using mucin microarrays (Dunne et al. 2018). This indicates that TFF1 may play an important role in the development of gastric cancer in *H. pylori* infections (Reeves et al. 2008; Dunne et al. 2018). Mucin microarrays were also used to identify the interactions of commensal strains with mucus (Lactobacillus salivarius AH102 and Bifidobacteria longum AH1205), highlighting the importance of mucin glycans in the preference of the two bacteria to mucins (Naughton et al. 2013; Flannery et al. 2015).

Binding assays in flow chamber

As a consequence of fluid shear gradient in the gut, the bacteria located in the outer mucus layer are exposed to a more turbulent flow compared to those that reside between the microvilli of the epithelial cells and therefore less exposed to physical perturbation (De Weirdt and Van de Wiele 2015). While the assays described above correspond to adhesion under static conditions, experiments can also be performed in dynamic conditions using flow chamber, where the shear force can be controlled (Le et al. 2013). Low-fluid shear environments and high shear rates are known to provide laminar pattern. Over time and with different laminar flow rates, the surface coverage of microbial cells to coupons coated with mucin provides an estimate detachment profile as a function of the shear stress.

Table 1. Experimental models available to study mucus-bacteria interactions.

GI : Gastro-Intestinal, HMI : Host–Microbe Interactions, IBD : Inflammatory Bowel Ddiseases, IVOC : *In vitro* organ culture, M-SHIME : Mucus Simulator of the Human Intestinal Microbial Ecosystem.

Types of models	Description	Applications	Advantages	Limitations	References
In vitro mucus/mucin binding assays Microplates—Flow chambers	∗ Immobilization of mucus/mucin on the microtiter plate ∗ Microtiter plate: adhesion in static conditions ∗ Flow chambers: adhesion under dynamic conditions (fluid shear)	∗ Evaluation of bacterial adhesion (commensals and pathogens) to mucins and molecular mechanisms associated	∗ Fast, quantitative and high throughput method to study mucus-microbe interactions independently from other *in vivo* conditions ∗ Identification of molecular determinants involved in adhesion of microbes ∗ Coupling with biophysical techniques (Surface Plasmon Resonance, Atomic Force Microscopy)	∗ Influence of experimental conditions (antibiotics, mechanical treatments, growth conditions, hydrophobic interactions) ∗ Limited availability of purified mucins (mainly use of pig gastric mucin) ∗ Absence of gut microbiota	McNamara *et al.* 2000; Gusils *et al.* 2004; Ringot-Destrez *et al.* 2018; Clyne *et al.* 2017; Dunne *et al.* 2018
In vitro cell models Monoculture models	∗Gut–derived epithelial cells resembling intestinal tissue consisting mainly of mature goblet cells that secrete an adherent	∗Adherence of commensal and pathogenic bacteria to host cells ∗ Effect of commensals/pathogens on host cell mucin synthesis and/or composition of the mucus layer	∗Reproducible and easily handled in laboratories ∗ Identification of molecular determinants involved in adhesion of microbes and host cell mucin synthesis ∗ Good platform for screening and characterizing probiotic activity	∗Derived from cancer cells, different from healthy tissue ∗ Not representative of various cell types recovered in mucosal epithelial tissues ∗ Not representative of appropriate *MUC* gene expression ∗ Modulation of mucus production by culture conditions ∗ Absence of gut microbiota ∗ Difficulty to maintain for long-term experiments (> 1 month) ∗ Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes)	Linden *et al.* 2007; Navabi *et al.* 2013; Hews *et al.* 2017

Table 1. Continued

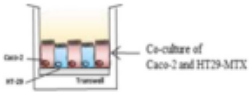
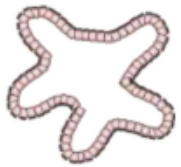
Types of models	Description	Applications	Advantages	Limitations	References
Co-culture models 	•Mixed culture of enterocytes and mucin secreting cells	•Adherence of commensal and pathogenic bacteria to host cells • Effect of commensals/pathogens on host cell mucin synthesis and/or composition of the mucus layer	•Better representation of cell-type ratio recovered in mucosal epithelial tissues • Simple model, well described in literature	•Absence of M-cells (development of triple co-culture Caco-2/HT29-MTX/Raji B) • Variations in seeding ratios of HT29 MTX/Caco-2 can impede results interpretation • Modulation of mucus production by culture conditions • Absence of gut microbiota • Difficulty to maintain for long-term experiments (> 1 month) • Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes)	Hilgendorf *et al.* 2000; Lozoya-Agullo *et al.* 2017
Ex-vivo organ cultures *Intestinal organoids* 	•Generation of self-propagating spheres of primary intestinal epithelial cells • Enteroids = derived from adult stem cells isolated from the crypts of human small intestinal • Colonoids = derived from adult stem cells isolated from the crypts of human colonic tissue	•Study of advanced aspects of mucus development in a more complex scenario • Study of host–commensals and pathogens interactions	•Often collected from mice tissues, possible use of patient-derived tissues • Assay that more accurately mimics *in vivo* conditions • Amenable to long-term culture	•Highly expensive and requires specialized expertise • Requires access to biopsies/tissues • Donor-to-donor variability • Requirement of injection to infect organoids with bacteria • Absence of gut microbiota • No reproduction of peristalsis motions and GI stressful events • Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes)	Jung *et al.* 2011; Sato *et al.* 2011

Table 1. Continued

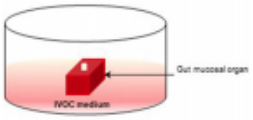
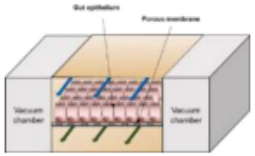
Types of models	Description	Applications	Advantages	Limitations	References
In vitro organ culture (IVOC) 	∗Whole organs maintained *in vitro*	∗Study of host–commensals and pathogens interactions	∗Better maintenance of tissue architecture ∗Presence of non-transformed cells including all major cell types (enterocytes, goblet cells, Paneth cells and endocrine cells) ∗ Often collected from animal tissues, possible use of patient-derived tissues ∗ Possible use of biopsies from disease patients (e.g. IBD)	∗Requires access to biopsies/tissues ∗ Expensive and requires expertise ∗Donor-to-donor variability ∗ Difficulty to maintain for long-term experiments ∗ No reproduction of peristalsis motions and GI stressful events ∗ Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes)	Browning and Trier 1969; Schüller *et al.* 2007
Gut-on-a chip 	∗Reproduction of the multicellular structures, cell–cell and tissue–tissue interactions, and the native microenvironment ∗ Closely reproduction of the *in vivo* situation	∗Study of the complex physiological and pathophysiological responses of tissues at an organ level ∗ Study of host–commensals and pathogens interactions	∗Presence of non-transformed cells including all major cell types (enterocytes, goblet cells, Paneth cells and endocrine cells) ∗ Reproduction of peristalsis like motions ∗ Possible use of biopsies from disease patients (e.g. IBD)	∗Expensive and requires dedicated expertise and instrumentation ∗ Stem cell differentiation is difficult to achieve ∗ Flow rate of the medium can influence cell metabolism ∗ Absence of gut microbiota ∗ No input from immune and nervous system ∗ Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes) ∗ No reproduction of the full complexity of the human gut microbiota	Kasendra *et al.* 2018

Table 1. Continued

Types of models	Description	Applications	Advantages	Limitations	References
In vitro human colonic models involving a mucosal phase					
M-SHIME 	∗Series of bioreactor modeling the different parts of the human gut ∗ Introduction of mucus-coated carriers (Mucus SHIME)	∗Study the fine-scale spatial organization of the gut microbial ecosystem ∗ Investigation of the interactions between commensals, pathogens, probiotics and luminal/mucosal gut microbiota	∗Integration of human GI -related parameters and possibility to modulate them depending on diet, age and diseases (e.g. ulcerative colitis) ∗ Capture dynamics by time-resolved analyses ∗ Capture inter-individual variability of human gut microbiota ∗ Possible long term experiments ∗ Possible coupling with cell culture models and Host-Microbe Interactions (HMI) module	∗Expensive and requires expertise and specialized instrumentation ∗ Use of pig gastric mucin ∗ No reproduction of immune and nervous system ∗ No reproduction of the full complexity of the human gut microbiota ∗ Donor-to-donor variability	Van den Abbeele *et al.* 2012; Van den Abbeele *et al.* 2013; De Paepe *et al.* 2018
In vivo animal models 	∗Whole organism models ∗ Development of genetically modified mice with impaired mucin production (comparison with wild type animals)	∗Study of the functional roles of mucin and mucus under physiological or pathological conditions at the level of entire organism ∗ Investigation of downstream consequences of mucin modulation in mucosal barrier defense ∗ Investigation of the interactions between commensals, pathogens, probiotics and luminal/mucosal gut microbiota	∗Physiological model ∗ Allow targeting of a specific gene/pathway in the complex gut-microbiota—host interactions ∗ Amenable to diet or microbiome-based interventions ∗ Possible long-term experiments	∗Requires housing facility and adequate agreements ∗ Expensive to maintain colonies ∗Housing husbandries and diets can modulate mouse microbiota ∗ Murine gut microbiota different from the human gut microbiota ∗ Mucin glycosylation profile of mice different from human intestinal mucins ∗ No reproduction of the full complexity of the human gut microbiota ∗ Limited translational capacity to human situation ∗ Mice generally inbred so no reproduction of the genetic variations found in the human population	Velcich *et al.* 2002; Van der Sluis *et al.* 2006

Biophysical assays

In order to gain further molecular insights into the interactions of microbial cells with mucin, various biophysical techniques have been developed and applied over the years. Optical biosensors based on resonant mirrors have been used to determine the binding kinetics of *H. pylori* cells to mucin (Hirmo *et al.* 1999). Following competition binding assays, the recognition of sialylated and sulphated moieties of mucin by *H. pylori* was demonstrated. Surface plasmon resonance (SPR) has been used to evaluate the adhesion abilities of a range of *Lactobacillus* species (Uchida *et al.* 2004; Kinoshita *et al.* 2007). In these studies, human colonic mucin (HCM) was immobilised on the sensor chip whereas bacterial cells were eluted as analytes. Using sialidase or sulfatase, it was further possible to discriminate some strains of lactobacilli and bifidobacteria that could specifically bind to the sialic acid or sulphate residues of HCM respectively (Huang *et al.* 2013). Single-cell force spectroscopy (SFCS) has been used to quantify the adhesion forces of *L. rhamnosus* with mucin at a single-cell level, pinpointing heterogeneities in the bacterial population (Sullan *et al.* 2014). More recently, further molecular details of mucin-bacteria interactions were investigated using atomic force microscopy (AFM). Such an approach was used for the first time to accurately quantify the force of adhesion of *L. lactis* cells immobilised on the AFM tip to PGM at nanoscale level (Dague *et al.* 2010). Surprisingly, it was found that PGM coating strongly reduced the bacterial adhesion force compared to bare polystyrene, highlighting the interplay between electrostatic, hydrophilic and steric repulsions, and that both specific and non-specific interactions need to be considered (Dague *et al.* 2010). These results were consistent with a previous investigation of the muco-adhesive properties of *L. lactis* using quartz crystal microbalance with dissipation monitoring (Le *et al.* 2012). Using bacteria mutant strains, AFM was also used to provide molecular insights into the respective role and contribution of mucus-binding proteins and surface organelles (pili or flagella) in muco-adhesion (Le *et al.* 2013). Interactions at the protein-protein level were further investigated by AFM to study the adhesive properties of *L. reuteri* Mub with mucins (Gunning *et al.* 2016).

In vitro mucin-secreting cell models

Monoculture models

While many colon carcinoma cell lines express mRNAs encoding surface-associated and/or secreted intestinal mucins (Deplancke and Gaskins 2001), few of them secrete MUC2 or form a mucus layer (Linden, Driessen and McGuckin 2007; Navabi, McGuckin and Lindén 2013; Hews *et al.* 2017). Most mucus-secreting cell lines are derived from the heterogeneous adenocarcinoma cell line HT-29 which can be differentiated into a mucus-secreting phenotype by growth under metabolic stress conditions. After an initial phase of cell mortality, adapted subpopulations of highly differentiated cells emerge (Lievin-Le Moal and Servin 2013). HT29–18N2 cells are often used as a model system for goblet cell differentiation and mucin secretion; these cells have been established by growth under glucose deprivation in galactose-containing culture medium (Phillips *et al.* 1988). In contrast, HT29-MTX cells and their clonal derivatives have been obtained by sequential adaptation to increasing concentrations of methotrexate (Lesuffleur *et al.* 1990). When grown on Transwell filter supports, some HT-29 MTX clones (e.g. MTX-D1 and MTX-E12) form polarised monolayers mostly constituted of mature goblet cells secreting an adherent mucus layer of 50–150 μm thickness as revealed by Alcian Blue staining (Behrens *et al.* 2001). In addition, the mucin-secreting clonal cell line HT-29.cl16E emerged from parental HT-29 cells after subculture in sodium butyrate whilst HT29-FU cells were established by treatment with 5-fluorouracil (Lesuffleur *et al.* 1991).

These mucus-producing HT-29 derivatives have been widely used to investigate the adherence of commensal and pathogenic bacteria to host cells (Coconnier *et al.* 1992; Bernet *et al.* 1993; Eveillard *et al.* 1993; Bernet *et al.* 1994; Kerneis *et al.* 1994; Favre-Bonte, Joly and Forestier 1999; Gopal *et al.* 2001; Schild *et al.* 2005; Barketi-Klai *et al.* 2011; Dolan *et al.* 2012; Gagnon *et al.* 2013; Naughton *et al.* 2013; Martins *et al.* 2015; Martins *et al.* 2016) and/or evaluate the effect of commensal bacteria on infection with enteropathogens (Bernet *et al.* 1993; Bernet *et al.* 1994; Coconnier *et al.* 1998; Gopal *et al.* 2001; Alemka *et al.* 2010; Zihler *et al.* 2011; Zivkovic *et al.* 2015; Vazquez-Gutierrez *et al.* 2016). Some studies investigated the direct effect of commensal or pathogenic bacteria on host cell mucin synthesis and/or composition of the mucus layer. Infection with atypical EPEC increased expression of secreted MUC2 and MUC5AC as well as membrane-bound MUC3 and MUC4 in HT29-MTX cells, thereby enhancing bacterial growth by providing nutrients for adherent bacteria (Vieira *et al.* 2010). Another study showed that apical infection with *Listeria monocytogenes* stimulated mucus secretion by polarised HT29-MTX cells. This effect was mediated by binding of the toxin listeriolysin O to a receptor on the epithelial brush border (Coconnier *et al.* 1998) and reduced bacterial invasion and colonisation of the host epithelium (Lievin-Le Moal, Servin and Coconnier-Polter 2005). Interestingly, probiotic *Lactobacillus* strains which adhering to mucus-producing HT-29 cells upregulated the transcription and secretion of MUC3 which reduced adherence of EPEC in co-incubation experiments (Mack *et al.* 2003). Modulation of mucus production and mucin glycosylation by commensal bacteria can also occur independently of adhesion. For example, a small soluble peptide of the gut commensal *R. gnavus* E1 strain has been shown to increase HT-29 MTX cell glycosylation via enhanced transcription of glycosyltransferases and MUC2-encoding genes (Graziani *et al.* 2016). Similarly, a soluble low molecular weight compound from *B. thetaiotaomicron* has been reported to enhance galactosylation in HT29-MTX cells. While no change in transcription was detected, galactosyltransferase activity was increased in HT29-MTX cells treated with soluble bacterial extract suggesting post-translational mechanisms of regulation (Miguel *et al.* 2001).

In addition to HT-29 cell derivatives, mucus-producing LS174T colon carcinoma cells have been used to study host-bacteria interactions. LS174T cells secrete mature MUC2, MUC5AC and human gallbladder mucin (van Klinken *et al.* 1996) but do not produce an organised adherent mucus layer (Navabi, McGuckin and Lindén 2013). Recent studies using this cell line showed that the secreted metalloprotease StcE reduced MUC2 levels during infection with EHEC and thereby facilitated bacterial adherence to the intestinal epithelium (Hews *et al.* 2017). In addition, the soluble protein p40 from *L. rhamnosus* GG stimulated MUC2 mRNA and protein expression in LS174T cells, and this effect was dependent on the epidermal growth factor receptor (Wang *et al.* 2014). Furthermore, treatment with butyrate, a product of bacteria fermentation, increased mucin production in LS174T cells (Burger-van Paassen *et al.* 2009; Jung *et al.* 2015). Recently, LS174T cells were used to decipher *E. histolytica*-elicited suppressed goblet cell transcription (Leon-Coria *et al.* 2018).

Co-culture models

To model human intestinal epithelia, mixed cultures of enterocyte-like Caco-2 cells and mucus-producing HT29-MTX

cells have been widely used in drug absorption and permeability studies (Hilgendorf *et al.* 2000; Lozoya-Agullo *et al.* 2017). Co-cultures prepared with different ratios of Caco-2 and HT29-MTX cells seeded out on Transwell inserts formed a continuous mucus layer similar to cultures of HT-29-MTX cells grown alone (Poquet, Clifford and Williamson 2008; Beduneau *et al.* 2014). Notably, the probiotic strains *L. rhamnosus* GG or bifidobacteria as well as pathogenic strains of *E. coli* or *L. monocytogenes* adhered better to mucus-deficient Caco-2 cells than to mucus-producing HT-29MTX cells or Caco-2/HT-29 co-cultures (Laparra and Sanz 2009). Considering the 'closed' oxygen-restricted environment in the human gut, Chen and colleagues developed a 3D porous silk scaffolding in the shape of a hollow tube. While the inner tube wall was coated with Caco-2/HT29-MTX epithelia, primary human intestinal myofibroblasts were grown in the tube scaffold space underneath to support epithelial growth and differentiation. Notably, epithelia grown on 3D scaffolds demonstrated increased MUC2 production compared to Transwell cultures resulting in the formation of a mucus layer of 11–17 μm thickness (Chen *et al.* 2015).

Organ-on-a-chip

Another approach to simulate a mucin-producing human intestinal epithelium is the 'Gut-on-a-Chip' system, where Caco-2 cells are grown on a porous membrane support in a microfluidic device. While the cell membrane support is maintained under cyclic strain mimicking peristaltic motion, the chambers above and below the cell membrane are constantly perfused with medium, thereby generating low shear stress. This environment stimulates the formation of 3D intestinal villi similar to those found in the small intestine (Kim *et al.* 2012), and the differentiation of Caco-2 cell into absorptive enterocytes, and also includes enteroendocrine cells, Paneth cells and mucus-producing goblet cells (Kim and Ingber 2013). In addition, Caco-2 epithelium grown in the Gut-on-a-Chip model display enhanced barrier function and mucus production as compared to static Caco-2 cell cultures (Kim and Ingber 2013). This system has recently been developed further to allow co-culture with strict anaerobes (Shin *et al.* 2019). Although the Gut-on-a-Chip devices have been mostly used for long-term co-culture of IECs with commensal microbes under healthy conditions, they are now being employed to model intestinal inflammation (Kim *et al.* 2016). Using a Gut-on-a-Chip model, the pathophysiological manifestation and dysregulated barrier function observed during inflammation could be recapitulated which may help to gain insights into disease mechanisms and assess potential therapeutic strategies (Shin and Kim 2018). Notably, probiotic VSL#3 targeted restoration of the mucosal barrier did not effectively control the local inflammation nor improve mucus production (Shin and Kim 2018). The HuMix (Human Microbial Cross-talk) model is another microfluidic device enabling the co-culture of Caco-2 cell monolayers with commensal bacteria under anaerobic conditions. In contrast to the Gut-on-a-Chip system, the epithelial cells which do not produce mucus are separated from the bacteria by a membrane coated with porcine gastric mucin (Shah *et al.* 2016).

Ex vivo organ cultures

As described above, traditional culture of human cells represents a valuable predictor of human physiology, pathology, and therapeutic responses but is limited by the absence of the tissue microenvironment. Culture approaches using human intestinal biopsy samples therefore represent an upscale platform to investigate the involvement of the mucus layer in healthy conditions or in the onset of various diseases.

In vitro organ culture (IVOC)

In 1969, Browning and Trier were the first to establish a technique to culture human mucosal biopsies *ex vivo*. By using a specific culture medium and incubation of the samples in 95% O_2, 5% CO_2 at 37°C, mucosal biopsies from the duodenojejunal junction were kept alive for up to 24 hours demonstrating epithelial cell proliferation, fat absorption and active mucus secretion by goblet cells (Browning and Trier 1969). The advantages of IVOC of intestinal biopsies versus cell line culture models include the presence of healthy non-transformed cells including all major IEC types (enterocytes, goblet cells, Paneth cells and neuroendocrine cells), underlying basement membrane and mucosal tissue, and the production of mucus. While it is problematic to maintain the loose outer mucus layer of colonic biopsies during sampling, the inner colonic and small intestinal mucus layers are generally well preserved as evidenced by microscopy (Haque *et al.* 2004; Walsham *et al.* 2016; Hews *et al.* 2017). IVOC of biopsy samples has been used to investigate adherence of pathogenic bacteria such as EPEC (Knutton, Lloyd and McNeish 1987; Schüller *et al.* 2007), EHEC (Phillips *et al.* 2000; Fitzhenry *et al.* 2002; Lewis *et al.* 2015), ETEC (Knutton *et al.* 1989; Baker, Moxley and Francis 1997) and *C. jejuni* (Grant, Woodward and Maskell 2006) to human intestinal mucosa. In addition, IVOC demonstrated cytotoxic effects of bacterial toxins, such as Pet toxin from EAEC (Henderson *et al.* 1999b), Shiga toxin from EHEC (Schüller, Frankel and Phillips 2004) and *C. difficile* toxin A (Mahida *et al.* 1996) on intestinal epithelium or mucosa. Interactions of enteropathogenic bacteria with mucus production were observed in small intestinal and colonic biopsy tissue infected with EAEC where bacteria were predominantly associated with a thick mucus layer above the epithelium, which was not present in non-infected control samples (Hicks, Candy and Phillips 1996; Andrade, Freymuller and Fagundes-Neto 2011). This suggests that EAEC stimulates mucus secretion which agrees with the production of mucoid stools during EAEC diarrhoea (Croxen *et al.* 2013). Similarly, stimulation of mucus secretion and bacterial binding to the mucus layer were observed in biopsy samples from the terminal ileum infected with S. Typhimurium. This was followed by *Salmonella* adherence and invasion of the epithelium accompanied by ruffling of the host cell membrane (Haque *et al.* 2004). Recently, the IVOC system was used to show that the metalloprotease StcE diminishes the inner mucus layer and enhances EHEC adherence to human colonic biopsy epithelium (Hews *et al.* 2017).

Polarised IVOC (pIVOC)

While the traditional IVOC system allows bacterial access to the mucosal and submucosal side of the biopsy, polarised organ culture models have been developed which limit bacterial contact to the mucosal side of the tissue. This is particularly relevant when studying host responses to bacterial infections where artificial interactions with immune cells in the lamina propria might confound experimental readouts. Using a pIVOC approach by mounting colonic tissue explants between two Perspex disks in a Snapwell plate, Raffatellu and colleagues demonstrated that *Salmonella* Typhi reduced mucosal expression of the pro-inflammatory cytokine interleukin (IL)-8 by production of a capsule which masked pathogen-associated molecular patterns such as LPS and flagellin (Raffatellu *et al.* 2005). In addition, pIVOC showed that apical exposure to EPEC or purified H6 flagellin induced IL-8 expression in duodenal biopsies (Schüller *et al.*

2009). Furthermore, infection with *C. jejuni* stimulated the production of reactive oxygen species (ROS) in duodenal and colonic mucosa (Corcionivoschi *et al.* 2012). The pIVOC system has also been used to study the interaction of probiotic bacteria with mucosal tissue, and incubation of duodenal explants with *L. reuteri* demonstrated localisation of bacteria in the mucus layer but not in the epithelium. Nevertheless, pre-incubation with *L. reuteri* reduced EPEC adherence to the epithelium (Walsham *et al.* 2016). A different approach to restrict bacterial access to the epithelial surface was developed by Tsilingiri and colleagues by gluing a perspex cylinder to the mucosal side of colonic resection tissue (Tsilingiri *et al.* 2012). Surprisingly, apical incubation with probiotic *L. plantarum* resulted in degeneration of mucosal tissue from healthy donors, whilst all three strains studied (*L. paracasei*, *L. rhamnosus*, *L. plantarum*) caused tissue damage in resections from patients with IBD. In contrast, supernatants from *L. paracasei* reduced inflammation in *Salmonella*-infected and IBD tissue. As the maintenance of larger tissue samples requires incubation in high levels of oxygen (95–99%), the use of IVOC to study interactions of oxygen-sensitive bacteria with human intestinal mucosa remains problematic. However, a novel murine 3D-intestinal organ culture system was recently developed whereby an intact intestinal fragment was luminally perfused with degassed medium containing anaerobic bacteria while the serosal side of the tissue was maintained under humidified oxygenated conditions. Whilst preserving gut tissue architecture, the system also supported the growth of commensal microbes (*Clostridium ramosum* and SFB) and allowed assessment of their impact on the immune and nervous system (Yissachar *et al.* 2017).

Human enteroids/colonoids and intestinal organoids

New technologies have been developed which enable the generation of self-propagating spheres of primary intestinal epithelial cells ('mini-guts'). Enteroids or colonoids are derived from adult stem cells isolated from the crypts of human small intestinal or colonic tissue, respectively (Jung *et al.* 2011; Sato *et al.* 2011). In contrast, human intestinal organoids (HIOs) are established by differentiation of embryonic or, more often, induced pluripotent stem cells (genetically reprogrammed adult stem cells) (Spence *et al.* 2011). In comparison to enteroids, HIOs lack maturation and more closely resemble foetal than adult intestine. In addition, they are devoid of functional intestinal stem cells and surrounded by a mesenchyme which is absent in enteroids (Sinagoga and Wells 2015; Leslie and Young 2016). As the apical side of the epithelium is facing inwards, infection of spheroid enteroids/HIOs with bacteria requires microinjection. Studies on the anaerobic pathogen *C. difficile* showed that injected bacteria remained alive in HIOs for up to 12 hours and caused disruption of epithelial barrier function *via* secretion of the toxin TcdA. Interestingly, oxygen measurements indicated reduced oxygen levels in the lumen of HIOs (5 to 15%). Furthermore, infection with *C. difficile* resulted in reduced MUC2 and mucus production in HIOs (Engevik *et al.* 2015). HIOs also supported growth of EHEC and commensal *E. coli*. Infection with EHEC induced ROS production and an inflammatory response associated with recruitment of external neutrophils into HIO spheres (Karve *et al.* 2017). Interestingly, colonisation of HIOs with commensal *E. coli* (ECOR2) stimulated enterocyte maturation, antimicrobial peptide secretion, production of a MUC2-containing mucus layer and increased epithelial barrier function, thereby indicating the establishment of stable host–microbe symbiosis (Hill *et al.* 2017).

To facilitate incubations with bacteria, 2D enteroid systems have now been successfully developed where primary intestinal cells are grown as monolayers on permeable membrane supports. Previous studies showed that differentiated human enteroid and colonoid monolayers contained MUC2-producing goblet cells and formed a mucus layer of more than 25 μm thickness (VanDussen *et al.* 2015; In *et al.* 2016). Two-dimensional enteroids and colonoids supported binding of EAEC, EHEC and EPEC (VanDussen *et al.* 2015). More specifically, apical EHEC infection of colonoids resulted in the formation of characteristic attaching and effacing lesions, mucus degradation and reduced expression of the microvillar protein protocadherin 24, which was mediated by the secreted serine protease EspP (In *et al.* 2016). The 2D enteroid model was further refined by adding primary human macrophages to the basolateral side of the membrane support. Intriguingly, enteroid monolayers grown in the presence of macrophages exhibited increased cell height and barrier function. In addition, underlying macrophages were able to capture and kill EPEC and ETEC by extending projections across the epithelial monolayer (Noel *et al.* 2017).

In another approach to mimic the gut environment more closely, cells from human small intestinal enteroids were seeded on tubular silk sponge scaffolds and supported by primary human intestinal myofibroblasts as described for Caco-2/HT29-MTX (Section 4.2). The resulting intestinal model epithelium contained all four major epithelial cell types and exhibited tight junction formation, microvillus polarisation, digestive enzyme secretion and low oxygen tension in the lumen. Moreover, infection with a laboratory strain of *E. coli* resulted in a significant innate immune response (Chen *et al.* 2017). Recently, a Gut-on-a-Chip model based on primary intestinal epithelial cells has been developed which also includes co-culture of an underlying endothelium. Human enteroids are cultured on a side of a porous membrane within a microfluidic device whereas the intestinal microvascular endothelium is established on the other side of the filter. This device reproduces the epithelial cells proliferation and host defenses more accurately (Kasendra *et al.* 2018). Kim and colleagues showed that a human Gut-on-a-Chip micro device colonized by non-pathogenic bacteria (commensal and probiotic bacteria) was able to induce production of a key set of pro-inflammatory cytokines. This device enabled high level of mucus production on micro engineered intestinal villi, therefore providing a protective barrier to maintain long-term stable host–microbe coexistence (Kim *et al.* 2016).

In vitro human fermentation models involving a mucosal phase

As aforementioned, the spatial positioning of gut microorganisms in the mucus layer is important with respect to their functional role in the human gut ecosystem. The microbial community residing in the mucus layer across the length of the GI tract is, however, hard to study given the difficulty to sample this region *in vivo*, especially in human (Macfarlane, McBain and Macfarlane 1997; Flint *et al.* 2012; Donaldson, Lee and Mazmanian 2016). *In vitro* colonic models involving a mucosal phase are a valuable alternative to study the fine-scale spatial organisation of the gut microbial ecosystem.

Multiple colon *in vitro* models have been developed over the years, ranging from simple, single stage batch incubations to more complex and representative three stage continuous and semi-continuous reactor models (Miller and Wolin 1981; Gibson, Cummings and Macfarlane 1988; Allison, McFarlan and MacFarlane 1989; Blanquet-Diot *et al.* 2012; McDonald *et al.* 2013; Van den Abbeele *et al.* 2013). These continuous fermentation models, inoculated with faecal samples of donors, recapitulate the main

biotic and abiotic parameters of the human colon, such as temperature, pH, residence time, supply of nutritive medium reproducing the composition of ileal effluents, therefore enabling the study of a complex and metabolically active gut microbiota under anaerobiosis conditions. In these fermentation models, the bioreactors can be inoculated with fresh or frozen stools provided by individual or pool of different donors being healthy human volunteers with no history of antibiotic treatment 2 to 6 months before the beginning of the study.

Introduction of mucus carriers in human in vitro colonic models

Mucins in solution have been frequently included in these colonic models to provide a nutrient source to the gut bacteria, but the study of mucus colonisation by gut bacteria in these systems has been revolutionized by the ability to simulate the viscoelastic gel-like nature of the mucus layer through the incorporation of mucus carriers (Gibson, Cummings and Macfarlane 1988; Macfarlane, Hay and Gibson 1989; Macfarlane, Woodmansey and Macfarlane 2005; Van Herreweghen *et al.* 2017). MacFarlane first demonstrated a rapid colonisation of an agar-mucus layer during a 48-hour incubation in a two-stage continuous fermentor system by a mixture of *Bacteroides*, enterobacteria and facultative anaerobes (Macfarlane, Woodmansey and Macfarlane 2005). However, the use of glass tubes, containing this agar-mucus layer, in this set-up did not permit a practical long-term implementation (Van den Abbeele *et al.* 2009). Mucus-coated beads (mixture of 5% porcine mucin type II and 1% agar) have since been identified as crucial platforms in sustaining microbial diversity by selectively enriching species, which are not thriving in the luminal environment. This mucus interphase was introduced in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) resulting in the M-SHIME (Mucus SHIME) configuration (Van den Abbeele *et al.* 2012). This system was first used to assess the fitness of potential probiotic lactobacilli, revealing a pronounced enrichment of *Lactobacillus mucosae* and *L. rhamnosus* GG in the mucus beads compared to the luminal environment, contributing to their long-term persistence in M-SHIME (Van den Abbeele *et al.* 2012). The use of next-generation 16S rRNA gene amplicon sequencing methods and the incorporation of mucus beads in both proximal and distal colon conditions in the M-SHIME, further resulted in the detection of several additional mucus-associated species (De Paepe *et al.* 2018). Besides *Roseburia faecis/Enterococcus rectale*, a strong enrichment of *Ruminococcus inulinivorans*, *Clostridium*, *Bilophila*, *Anaeroglobus* and *Veillonellaceae* species was observed in the proximal mucus layer compared to the lumen (De Paepe *et al.* 2018). *A. muciniphila*, *Cloacibacillus evryensis*, *Pyramidobacter piscolens*, *Eubacterium contortum* and species belonging to *Odoribacter*, *Enterobacteriaceae* and *Desulfovibrio* were predominantly residing in the distal mucus layer (De Paepe *et al.* 2018). It can be expected that the list of species inhabiting the mucus layer will continue to expand in the future M-SHIME experiments, by continuing to explore the inter-individual variability of faecal samples.

Another major advantage of *in vitro* fermentation models is the possibility to capture dynamics by time-resolved analyses. To minimize disturbance of the system during such analyses, the M-SHIME system was adapted to facilitate a rapid, anaerobic, frequent sampling by mounting sampling ports with an airlock system on top of the SHIME lids. These sampling ports moreover enable the anaerobic addition, transfer and sampling of any insoluble dietary substrate. The adapted model was correspondingly termed Dietary Particle-Mucosal-Simulator of the Human Intestinal Microbial Ecosystem (DP-M-SHIME). The DP-M-SHIME offers a closer proxy for the diversity of *in vivo* GI microenvironments, as aside from the mucus layer, insoluble dietary particles present an interesting additional niche for microorganisms to physically interact with and colonise (De Paepe *et al.* 2018).

In addition, these colonic *in vitro*-fermentation models enable the study of severe gut microbiome perturbations such as antibiotic therapy or pathogen invasion, which cannot be performed in humans for obvious ethical reasons. Using M-SHIME, the mucus bead carriers were shown to confer resilience to lactobacilli species against a combined treatment of antibiotics including tetracycline, amoxicillin and ciprofloxacin (Van den Abbeele *et al.* 2012). In addition, the M-SHIME model has been used to demonstrate the antagonistic effects of probiotics and prebiotics such as *L. reuteri*, long-chain arabinoxylans or inulin towards AIEC colonisation in the mucosal environment (Van den Abbeele *et al.* 2016). Another study reported the importance of mucus for providing a protective environment to beneficial gut microbes, such as *L. reuteri*, to help them escape from stress induced by high loads of linoleic acid, the most common polyunsaturated fatty acid in a WSD (De Weirdt *et al.* 2013). The M-SHIME model has also been shown to preserve disease signatures, as illustrated by a reduced luminal and mucosal *Clostridium* cluster XIVa colonisation in colon vessels inoculated with the faecal material of ulcerative colitis patients compared to healthy donors (Vermeiren *et al.* 2012). The mucin-beads technology has recently been transferred into the Artificial Colon (ARCOL), with adaptations to avoid flushing the bioreactor with nitrogen when mucin-coated beads are being renewed (Cordonnier *et al.* 2015; Thevenot *et al.* 2015).

Combining colon fermentation models with in vitro cell culture

Recent advancements in the field of *in vitro* fermentation involve the combination of gut microbiota models with a host compartment to assess host–microbiota interactions (Bahrami *et al.* 2011; Marzorati *et al.* 2014; Defois *et al.* 2018). The three-way interactions between host, microbiome and dietary interventions can be examined by applying the supernatants of colonic samples onto human cell lines or combinations thereof, such as co-cultures of enterocytes and immune cells, in Transwell systems (Marzorati *et al.* 2014). Cytokine and TNF-α production are followed up as markers of intestinal inflammation, whereas the trans-epithelial electrical resistance (TEER) and Lucifer yellow translocation give an indication of epithelial barrier function (Geirnaert *et al.* 2017). While typically, in human cell line experiments, the short-term effects of a single treatment application onto differentiated cells with a disrupted barrier are being evaluated, recently, a method was developed to test the effects of a probiotic treatment on the development of epithelial barrier integrity during cell differentiation, which is more representative of the *in vivo* situation (Geirnaert *et al.* 2017). In order to further improve the *in vivo* relevance, a host module, such as the Host Microbiota Interaction (HMI) Module (Marzorati *et al.* 2014), can be coupled to the colonic *in vitro* fermentation systems described above to directly and continuously recirculate the colonic microbial suspension over a mucus layer that is in indirect contact with enterocytes and/or immune cells.

In vivo animal models

As described above, *in vitro* mucin-secreting cell cultures, *ex-vivo* organ cultures as well as *in vitro* fermentation models have yielded fundamental insights into the role of mucins and mucus in bacterial interactions with the host. However, the use of *in vivo* models is necessary to study the biological roles of mucins

under physiological or pathological conditions at the level of entire organism. Genetically modified mouse models with an impaired mucin production or glycosylation have been developed to assess the role of mucus in the interaction between gut bacteria and the host *in vivo*.

Muc2$^{-/-}$ *mouse model*

Many *in vivo* animal studies investigating the role of mucus in gut homeostasis have relied on the use of *Muc2*$^{-/-}$ mice, lacking the major intestinal mucin Muc2.

The first studies based on *Muc2*$^{-/-}$ mice showed that these animals displayed an impaired epithelial barrier function characterised by aberrant intestinal crypt morphology and altered cell maturation and migration, and that the mice frequently developed adenomas in the small intestine, as well as rectal tumours (Velcich *et al.* 2002). The microscopic analysis of the colon indicated mucosal thickening, increased proliferation and superficial erosions (Van der Sluis *et al.* 2006). The development of spontaneous colitis in Muc2 deficient mice indicated that Muc2 is critical for colonic protection (Van der Sluis *et al.* 2006). A gut microbiota dysbiosis was also observed in the *Muc2*$^{-/-}$ mice which harboured a pro-inflammatory-like microbiota profile, characterized by an increase in *Clostridiales* and a decrease in *Lactobacillaceae* (Huang *et al.* 2015). Furthermore, it was shown that the spatial compartmentalization of bacteria in the intestine of *Muc2*$^{-/-}$ mice was compromised and transcriptomic analysis revealed a downregulation of TLR, immune and chemokine signaling pathways compared to wild type mice (Sovran *et al.* 2015) Also, the expression of the network of IL-22-regulated defense genes was increased in *Muc2*$^{-/-}$ mice (Sovran *et al.* 2015). Recent work also confirmed a clear shift in the microbiota composition of *Muc2*$^{-/-}$ mice, with the Firmicutes phylum enriched and the Bacteroidetes phylum decreased, as well as an increase in genera considered as potential pathogens also (Wu *et al.* 2018).

Muc2$^{-/-}$ mice have been used to test the effect of the probiotic mixture VSL#3 on colonic inflammation and intestinal barrier function (Kumar *et al.* 2017). This probiotic mixture contains eight strains belonging to *Lactobacillus*, *Bifidobacterium* and *Streptococcus* genera which are usually found in the human intestinal microbiota. In *Muc2*$^{-/-}$ mice, VSL#3 reduced basal colonic proinflammatory cytokine levels and improved epithelial barrier function. In addition, VSL#3 reduced the level of proinflammatory chemokines and upregulated tissue regeneration growth factors leading to a faster resolution of colitis symptoms in *Muc2*$^{-/-}$ mice with DSS-induced colitis. This was associated with the restoration of antimicrobial peptide gene expression in the small intestine, and an increased abundance of commensal bacteria in the gut. The authors proposed that these beneficial effects were mediated by acetate, produced by the gut bacteria (Kumar *et al.* 2017). Treatment of *Muc2*$^{-/-}$ mice with *Lactobacillus* spp. could ameliorate spontaneous colitis and led to an increased production of SCFA (Morampudi *et al.* 2016).

Muc2$^{-/-}$ mice have also been used to investigate the role of this mucin to prevent bacterial and parasite infection. Upon infection with *C. rodentium*, a murine pathogen related to diarrhoeagenic attaching-effacing *E. coli*, *Muc2*$^{-/-}$ mice exhibited a rapid weight loss and up to 90% mortality (Bergstrom *et al.* 2010). Mucin secretion was increased in wild type mice during infection as compared to the uninfected controls, suggesting that mucin production is critical to clear the mucosal surface from pathogenic bacteria. In *Muc2*$^{-/-}$ mice, commensal bacteria were also found to interact with *C. rodentium* and host tissues, indicating that Muc2 regulates all forms of intestinal microbiota at the gut surface (Bergstrom *et al.* 2010). When *Muc2*$^{-/-}$ mice were infected with *Salmonella*, they showed a dramatic susceptibility to infection, carrying significantly higher caecal and liver pathogen burdens, and developing significantly higher barrier disruption and higher mortality rates than wild type mice (Zarepour *et al.* 2013). Colonisation of *Muc2*$^{-/-}$ mice by enterotoxigenic *B. fragilis*, a causative agent of acute diarrhoea in humans, led to lethal disease (Hecht *et al.* 2017). The protective function of Muc2 was also demonstrated in models of *T. muris* parasitic infection (Hasnain *et al.* 2010). *T. muris* is a murine infecting nematode which is used as model of T. trichiura infection in humans, a threat in developing countries. After infection, *Muc2*$^{-/-}$ mice showed a delayed expulsion of the worms from the intestine compared to wild type mice. In addition, an increase in Muc2 production, observed exclusively in resistant mice, correlated with worm expulsion. The nematodes demonstrated a decrease in their energy status in wild type mice compared to the susceptible *Muc2*$^{-/-}$ mice (Hasnain *et al.* 2010). *E. histolytica* is a human parasite infecting the colon and responsible of amoebic dysentery and/or liver abscesses. *E. histolytica* specifically colonises the mucus layer by adhering to galactose and GalNAc residues present in Muc2 (Kissoon-Singh *et al.* 2013). The parasite also induces potent hypersecretion from goblet cells. Kissoon-Singh and colleagues showed that *E. histolytica* induced a pronounced time-dependent secretory exudate with increased gross pathology scores and serum albumin leakage in *Muc2*$^{-/-}$ mice. Colonic pathology, secretory responses and increased pro-inflammatory cytokine secretions were also correlated with altered expression of tight junction proteins (Kissoon-Singh *et al.* 2013). These results demonstrate that colonic mucins confer both luminal and epithelial barrier functions and that, in the absence of Muc2, mice are more susceptible to *E. histolytica*-induced secretory and pro-inflammatory responses. A recent study using antibiotic treated *Muc2*$^{-/-}$ and *Muc2*$^{+/+}$ littermates showed that *E. histolytica* elicited robust mucus and water secretions, enhanced pro-inflammatory cytokines and chemokine expression and higher pathology scores as compared to the modest response observed in non-antibiotic treated littermates. Host responses were microbiota specific as mucus secretion and pro-inflammatory responses were attenuated following homologous faecal microbial transplants in antibiotic-treated *Muc2*$^{+/+}$ quantified by secretion of ^{3}H-glucosamine newly synthesized mucin, Muc2 mucin immunostaining and immunohistochemistry (Leon-Coria *et al.* 2018). The mechanism controlling mucus release in the presence of *E. histolytica* was further studied by Cornick and colleagues who identified vesicle-associated membrane protein 8 (VAMP8) present on mucin granules as orchestrating regulated exocytosis in human goblet cells in response to the presence of *E. histolytica* (Cornick *et al.* 2017). In *Vamp8*$^{-/-}$ mice, *E. histolytica* induced enhanced killing of epithelial cells and aggressive proinflammatory response with elevated levels of IL-1α, IL-1β and TNF-α secretion, highlighting the downstream consequences of improper mucin secretion in mucosal barrier defence. Taken together, these results demonstrate the critical involvement of Muc2 in host protection from nematode infection, by constituting an effective physical and biological barrier against pathogenic infection.

Muc1$^{-/-}$ *mouse model*

Mice impaired in the production of cell surface mucins have also been engineered. The *Muc1*$^{-/-}$ mouse model revealed the role played by Muc1 in *H. pylori* infection, a pathogen involved in gastric ulcers and adenocarcinoma (McGuckin *et al.* 2007; Linden *et al.* 2009). *Muc1*$^{-/-}$ mice displayed a 5-fold increase in *H. pylori* colonisation as compared to wild type mice (McGuckin

et al. 2007). This study further demonstrated the ability of *H. pylori* to bind to purified Muc1 *in vitro*, suggesting that Muc1 limits the access of *H. pylori* to the epithelial surface thereby providing protection from infection and proinflammatory bacterial products. Muc1 deficiency also resulted in increased epithelial cell apoptosis in *H. pylori* infected mice (Linden et al. 2009). More recently, the long-term consequence of Muc1 deficiency on *H. pylori* pathogenesis was investigated in *Muc1*$^{-/-}$ mice (Ng et al. 2016). *Muc1*$^{-/-}$ mice began to die 6 months after *H. pylori* challenge, indicating that a deficiency in Muc1 leads to lethal infection. This study also revealed that *Muc1* was an important, previously unidentified negative regulator of the NLRP3 inflammasome, and loss of this regulation resulted in the development of severe pathology (Ng et al. 2016). Consistent with these studies, *Muc1*$^{-/-}$ mice have a higher rate of systemic infection in a murine *C. jejuni* model of gastroenteritis (McAuley et al. 2007).

Muc13$^{-/-}$ *mouse model*

The MUC13 transmembrane mucin is highly and constitutively expressed in the small and large intestines and MUC13 polymorphisms have been associated with human IBD and susceptibility to *E. coli* infection in pigs. While Muc13-deficient mice did not show intestinal pathology, they developed more severe acute colitis than wild type mice after DSS challenge, as reflected by increased weight loss, rectal bleeding, diarrhoea and histological colitis scores (Sheng et al. 2011).

Mouse models with an altered mucin glycosylation

Loss of O-glycans impairs the expression and function of several intestinal mucins, thereafter causing more profound defects in the function of the intestinal barrier than a flaw caused by the deficiency of an individual mucin. A number of transgenic mouse models have been developed to decipher the mechanisms underpinning the role of mucin glycosylation in gut homeostasis. Mice lacking core 3–derived O-glycans (also known as *C3GnT*$^{-/-}$ mice) display a substantial reduction of Muc2 protein and an increased susceptibility to DSS-induced colitis and accelerated colorectal tumorigenesis (An et al. 2007). In addition, core 3 O-glycosylation was shown to play a major role in controlling Salmonella intestinal burdens in *C3GnT*$^{-/-}$ mice (Zarepour et al. 2013). Similarly, *C2GnT2*$^{-/-}$ mice (mice lacking core 2-derived O-glycans) displayed an increased susceptibility to DSS-induced colitis but with no change in Muc2 expression (Stone et al. 2009). Mice with intestinal epithelial cell–specific deficiency of core 1–derived O-glycans (IEC *C1galt1*$^{-/-}$) develop spontaneous colitis (Fu et al. 2011). Mice lacking both core 1- and core 3-derived O-glycans (DKO mice) have an impaired mucus barrier function and develop colitis-associated colon cancer in which the dysbiotic microbiota promote inflammation and cancer (Bergstrom et al. 2016). In a water avoidance model in rats, psychological stress lead to less-protective mucus layer. In particular, O-glycosylation of mucins was strongly affected and these changes were associated with flattening and loss of the mucus layer cohesive properties (Da Silva et al. 2014). Altogether, these data suggest that the lack of a proper O-glycosylation impairs Muc2 expression or secretion and alters gut barrier function of the mucus layer.

In addition to modifications of mucin core glycans, mouse models have been developed targeting epitope modifications of the mucin glycans chains. Dawson and colleagues reported that deletion of the sulfate transporter NaS1 in mice (*Nas1*$^{-/-}$ mice) resulting in a decrease in mucin sulfation, enhanced susceptibility to experimental DSS colitis and systemic infection by *C. jejuni* (Dawson et al. 2009). In addition, mice with a deletion of the sulfo-transferase GlcNAc6ST2 enzyme adding sulfate to GlcNAC residues on O-mucin glycan chains exhibited an increased susceptibility to DSS-induced colitis (Tobisawa et al. 2010). Mice deleted for Sat-1 (sulphate anion transporter-1) were more susceptible to chronic infection by parasite *T. muris* (Hasnain et al. 2017). Collectively, these findings indicate that mucin abnormalities can initiate the onset of inflammatory related diseases in the gut.

In addition to mice harbouring a deletion in genes encoding proteins directly involved in mucin expression or glycosylation, several transgenic mouse models have been shown to display alterations in mucus properties. These include the Winnie and Eeyore mice which carry single missense mutations in two different D-domains of Muc2 (Heazlewood et al. 2008). These mice display fewer goblet cells and a reduction in secreted mucus with O-glycosylated and non-O-glycosylated Muc2. The misfolding results in endoplasmic reticulum stress, goblet cell apoptosis, depletion of the secreted mucus layer and development of chronic intestinal inflammation (Heazlewood et al. 2008). In combination with mucus, a large population of intraepithelial lymphocytes (IELs) bearing the $\gamma\delta$ T cell receptor is mediating immune protection against invading bacteria. In $\gamma\delta$ T-cell-deficient (*TCR*$\delta^{-/-}$) mice, mucin expression and glycosylation is altered, mucus-secreting goblet cells are significantly reduced in number and those animals are more prone to DSS-induced colitis (Kober et al. 2014). Mouse models deficient in TLR5, IL-10 and Sodium hydrogen antiporter 3 (Slc9a3 or Nhe3) revealed bacteria in contact with the epithelium. Additional analysis of the less inflamed *IL-10*$^{-/-}$ mice revealed a thicker mucus layer but a more penetrable inner mucus allowing bacteria to penetrate and reach the epithelium (Johansson et al. 2014).

Non-rodent models

Differences in mucus thickness and composition have been observed between rats, pigs and rabbits, and suggests that the pig mucus pattern resembles more closely that of humans (Varum et al. 2012). The zebrafish larva is an emerging model system for investigating components of the innate immune system, including mucus physiology. It has been shown that five gel-forming secreted mucin genes are found in zebrafish with a high degree of homology to other vertebrate mucins regarding their genomic and protein domain organisation, as well as their tissue specific expression (Jevtov et al. 2014).

Limitations of current experimental models involving mucus and future challenges

Given the importance of the gut microbiota as a modulator of health and disease, increasing attention has been devoted to the role played by mucus in the interaction with gut bacteria (Juge 2019). As described above (section 4), various experimental models increasing in complexity from simple *in vitro* assays to cell lines, organ-on-chips, *in vitro* colon fermentation systems or animal models have been developed and successfully applied to the study of gut microbe-mucus interactions. However, one of the limitations common to most *in vitro* models is the origin of mucins used to assess the interactions with bacteria doubled by the inability to reproduce a colonic mucus gel recapitulating the *in vivo* situation. This is important as the nature and origin of purified mucins used in these assays greatly influence the outcome of binding as demonstrated using microtitre plate (Owen et al. 2017), dot-blot (Ringot-Destrez et al. 2018) or mucin microarrays (Clyne et al. 2017). Mucin glycosylation plays a critical role in the interaction between gut bacteria and mucus and significant

glycosylation differences occur between purified mucins used in *in vitro* assays from different sites of the murine GI tract or from goblet cells (e.g. LS174T), as analysed by mass spectrometry (Leclaire *et al.* 2018; Ringot-Destrez *et al.* 2018). In addition, the purification steps alter the properties of native glycoproteins and purified mucins used in these assays lack the ability to form viscoelastic hydrogels (Kocevar-Nared, Kristl and Smid-Korbar 1997). A similar situation occurs with mucus secreting cell lines where the type of mucins and structure of mucus differ from the colonic environment. For example, the HT29 cell line secretes mostly MUC5AC whereas MUC2 is the main mucin secreted in the small and large intestines. These differences are due to the use of cancer cells which show an alteration in the expression and glycosylation of mucins. In addition, the production of mucus by epithelial cell lines can be influenced by culture conditions. For example, growing cells on Transwell filters with a small amount of apical medium (semi-wet interface culture) in combination with mechanical stimulation (on a rocking platform) and addition of the Notch γ-secretase inhibitor DAPT resulted in polarisation and secretion of MUC2 and MUC5AC by HT29 MTX-P8, HT29 MTX-E12 and LS513 cells (Navabi, McGuckin and Lindén 2013). Additionally, the mucus produced by goblet cells in *in vitro* co-culture cell models is not continuous nor homogenate which is not fully representative of the *in vivo* situation. Lastly, the formation of the bi-layered mucus found in the colon remains a challenge in these models. Novel strategies such as multiple cell layers, 2D-organoid techniques or Organ-on-a-Chip devices are currently being developed to better mimic the human intestinal epithelial microenvironment. Such multiple cell models exhibit intestinal villus morphogenesis associated with mucus production. These models are also needed to recapitulate antimicrobial defense and inflammatory reactions normally occurring in mucosal tissues. Another advantage of these systems is that, unlike cell lines, organoids can be used to evaluate long-term interactions between mucus and gut microbes. However, these more advanced biopsy- based models remain low throughput and expensive as compared to *in vitro* assays and are limited by the availability and variability of clinical specimens.

In addition, to the host side, several microbial factors must be taken into consideration when assaying the interactions between the gut bacteria and mucus. These include the handling and labelling microbial cells which may affect the surface molecular determinants potentially involved in mucus/mucin interactions (e.g. cell-surface adhesins, pili or flagella) (Chagnot *et al.* 2014). The growth conditions (e.g. growth media with different nutrient compositions; temperature, pH, osmolarity or redox potential) can also influence the expression of the bacterial receptors mediating the interactions with mucins. To date, most studies have focused on the interactions between mucus and probiotic or pathogenic strains and assessing strictly anaerobe gut symbionts or complex microbial communities remain a challenge in this field of research.

An alternative to the systems described above is the use of dynamic *in vitro* fermentation models of the human gut, such as the M-SHIME (Marzorati *et al.* 2014) or DP-M-SHIME (De Paepe *et al.* 2018) models. In these *in vitro* colonic models, the introduction of mucin-covered beads allows to study the long-term *in vitro* microbial colonisation of mucin, in the presence of a complex anaerobic intestinal microbiota (Marzorati *et al.* 2014; Shah *et al.* 2016). These models provide a mean to study gut microbiota functionality and niche differentiation, during treatments with xenobiotics (for example antibiotics, synthetic chemicals such as food additives, environmental pollutants like persistent organic pollutants (POPs)), pathogens or functional foods. Future developments in this field will be the introduction of mucus secretion and/or a mucus surface layer in dynamic *in vitro* models of the upper GI tract, such as in the gastric and small intestinal TNO Gastro-Intestinal model (TIM) (Guerra *et al.* 2012). This is important so to take into account the successive stressful events (e.g. acidic gastric pH, bile salts) that commensal or pathogenic microbes undergo in the human GI tract before reaching the intestinal epithelium and that may greatly influence their physiological stage, virulence and/or activity.

However, as mentioned above, a limitation of these *in vitro* GI models is that they rely on commercially available mucins used for the mucin bead technology. These secretory mucins, usually MUC5AC and MUC6 porcine gut gastric mucin, differ in terms of structure and glycosylation from intestinal MUC2 and cannot form a bi-layered mucus gel. It has been proposed that in the future, *in vitro* engineered mucus may be used to mimic human-derived mucus in a more reproducible manner. The colonic *in vitro* models could also be improved by including immunoglobulins, specific antimicrobial peptides, or secreted phosphatidylcholine, which have been shown to modulate mucus surface properties, thereby influencing bacterial adhesion (Martens, Neumann and Desai 2018). Future *in vitro* colon models should also better mimic the *in vivo* transit, and particularly retrograde movements (Hiroz *et al.* 2009), as back-flow was recently suggested to be crucial for the persistence of gut microbes in the GI tract (Cremer *et al.* 2016). Current technological advances include the coupling of these fermentation models to intestinal epithelial cells or more complex units such as the HMI module. A next step will be to couple the digestive/fermentation models with enteroids/colonoids or HIO. However, despite their increased complexity, most of these approaches remain limited by the absence of important host functions, such as variable peristalsis-like motions. This is a critical limitation because mechanical deformations resulting from peristalsis both influence normal epithelial cell differentiation and control microbial overgrowth in the living intestine (Gayer and Basson 2009; Benam *et al.* 2015). The development of microfluidic systems and organ-on-chips is currently addressing this important technological gap (Kim and Ingber 2013; Kim *et al.* 2016).

The development of these advanced *in vitro* systems is essential to help reduce dependence on animal studies. Due to the invasive nature of the experiments, the mechanisms underpinning microbe-mucus interactions *in vivo* have mainly been investigated in animal models, mostly rodents. Genetically engineered mice impaired in mucin secretion or glycosylation have been instrumental to decipher the role of mucins and mucus in the protection of the intestinal epithelium and the interactions between pathogenic bacteria, commensal microbiota and the mucus barrier. However, although the domain organisation and expression pattern of mucins appear largely conserved between human and mouse (Joshi *et al.* 2015), mucin glycosylation and gut microbiota (Nguyen *et al.* 2015) differ between these two species. It has been speculated that differences in mucin glycosylation between mammalian species may underlie some of the differences in infectivity and/or pathogenicity for individual microbial pathogens (Linden *et al.* 2008) or the different commensal microbiota (Thomsson *et al.* 2012). Therefore, caution should be applied when translating data obtained in mouse models to humans. Lastly, unlike *in vitro* assays, *in vivo* studies are restricted to end-point measurements.

Recent years have witnessed unprecedented technological advances in the development of *in vitro* GI models that more

closely resemble the gut mucosal interface. Our next challenge will be to simulate these models at different stages of development or disease conditions (e.g. IBD, obesity or CF). Special attention should be paid to inter-individual differences and intra-individual variability in gut microbiota composition and intestinal biopsies from different donors or patients. This is important to better understand the role of gut microbe–mucus interactions in the aetiology of a particular disease or condition and determine the microbial and biochemical signature that could differentiate between diseased and healthy status. In particular, more research is warranted to determine how the physicochemical properties and/or thickness of the mucus layer and mucin glycosylation are altered during a specific disease. In the future, these pre-clinical models will help screen novel therapeutic strategies aimed at restoring gut barrier function and tailored to the individual patient as a step towards personalised medicine.

Conflict of Interest. None declared.

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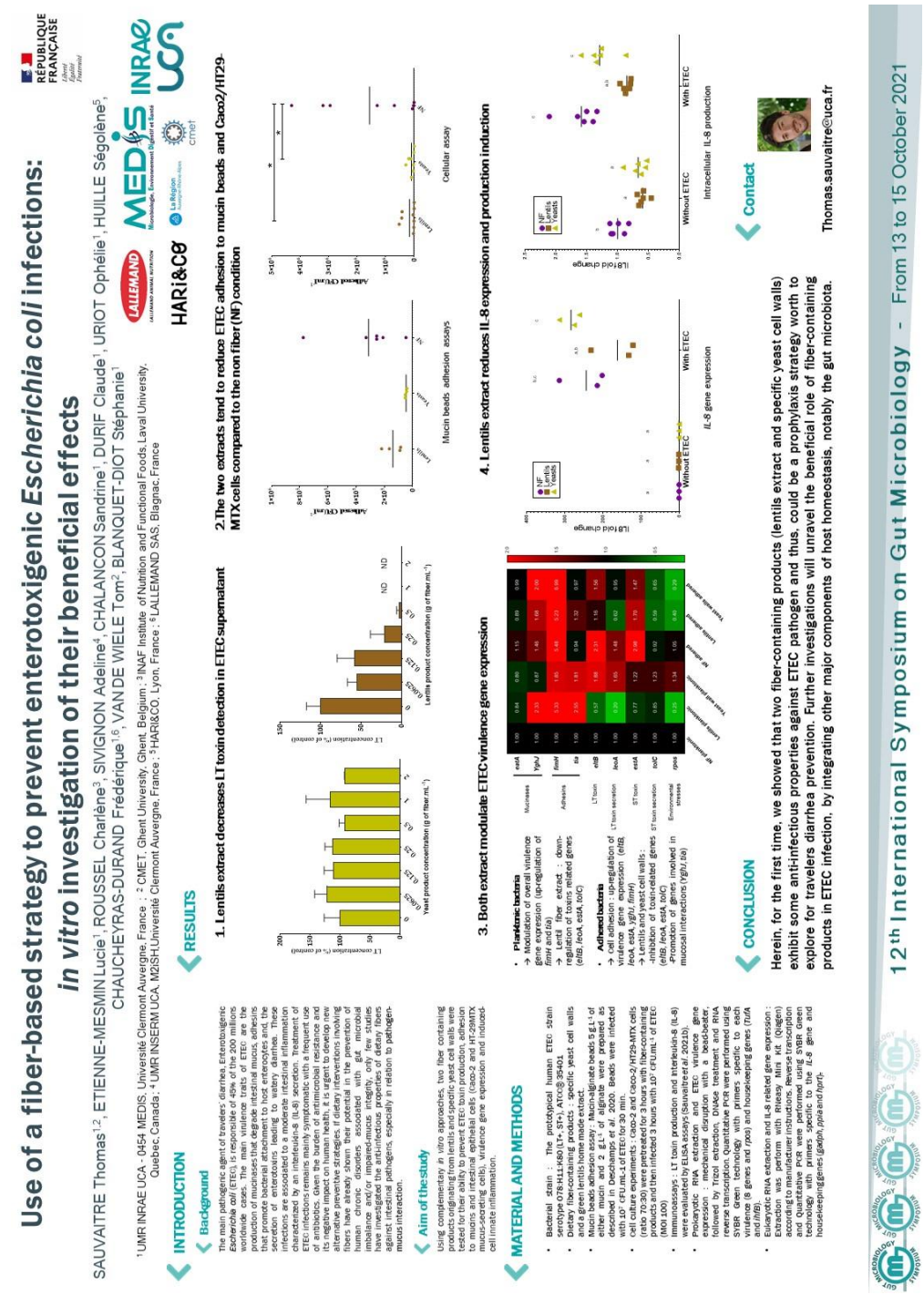
Appendix 2

Sauvaitre, T., Roussel, C., Sivignon, A., Chalancon, S., Durif, C., Huille, S., Chaucheyras-Durand, F., Van de Wiele, T., Etienne-Mesmin, L., Blanquet-Diot, S. (**2021**). Use of a fiber-based strategy to prevent enterotoxigenic *Escherichia coli* infections: *in vitro* investigation on their antagonistic effects. 12th international symposium on Gut Microbiology, October 2021, Clermont-Ferrand, France



Appendix 3

Sauvaitre, T., Etienne-Mesmin, L., Roussel, C., Chalancon, S., Durif, C., Uriot, O., Van de Wiele, T., Blanquet-Diot, S. (**2021**). Use of a fiber-based strategy to prevent bacteria-mucus interactions in enterotoxigenic *Escherichia coli* infections? Beneficial Microbes meeting, March 2021, Amsterdam, The Netherlands