# Increased intake of fermentable carbohydrates induces IBS-like symptoms; a complementary understanding of mechanisms involved

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# THÈSE

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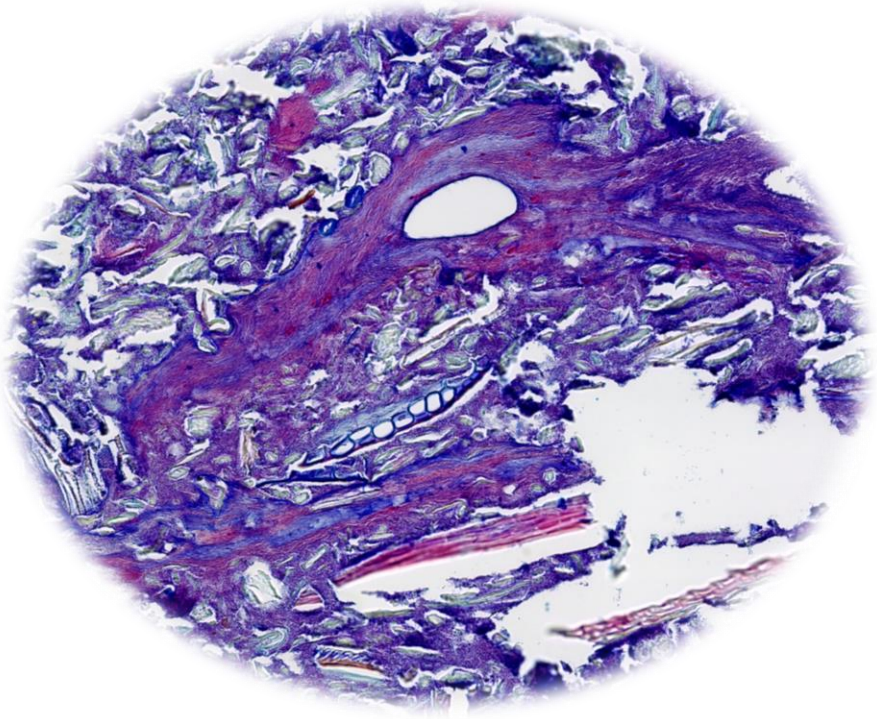
# Increased intake of fermentable carbohydrates induces IBS-like symptoms; a complementary understanding of mechanisms involved

Visceral sensitivity, intestinal barrier function,
bacterial metabolites, and organisation of mucus secretions

Jasper Kamphuis, 2019



"....By this the Earth it self, which lyes so near us, under our feet, shews quite a new thing to us, and in every little particle of its matter, we now behold almost as great a variety of Creatures, as we were able before to reckon up in the Whole Universe it self...."

~Robert Hooke in Micrographia, 1665

*"A'j't oaweral met eens bint, bi'j rap oetkek'n" **

To my parents, who have supported me throughout 30 years of learning

To my friends, who are my friends

* "Als je het overal met eens bent, ben je snel uitgekeken"
* "If you'd agree with everything, you would soon cease to wonder"
* " Si vous étiez d'accord avec tout, vous ne vous poseriez plus de questions "

Peering into a microscope in the dark invites the mind to make new friends,
some of which are displayed on the Chapter pages of this dissertation; the *"Faces in Faeces"*.

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## Summary

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder (FGID) characterized by abdominal pain, bloating, and erratic bowel habits. It is an affliction with a high prevalence of around 11% worldwide. It carries a significant economic cost in lost productivity and work absence, and more importantly, it has a strong negative impact on quality of life. Because it is a functional disorder of which the causes are not well understood, treatment is difficult. In recent years, a low-FODMAP diet (low in Fermentable Oligo-, Di-, Mono-saccharides And Polyols) has been successfully used to reduce symptoms of IBS. The efficacity of this approach is not completely understood, but a reduction in enteric distension by reduced gas production and small intestinal water bulk by osmotic effects are most often cited. The bacterial metabolic toxin hypothesis, proposed by Campbell *et al*. poses that anaerobic fermentation of unabsorbed carbohydrates by the colonic gut microbiota, producing such metabolites as alcohols, ketones, and aldehydes, are responsible for food intolerances such as lactose intolerance. We hypothesized that this same mechanism could be extended to FODMAPs to explain the efficacity of the low-FODMAP diet.

In this thesis, we looked for complementary mechanisms on how FODMAPs could influence IBS symptoms, besides distension related complaints. Our studies in a healthy mouse model show a complex role for FODMAPs in IBS physiopathology; FODMAP treatments cause a visceral and abdominal hypersensitivity, and a mucus barrier dysregulation, characterized using an innovative approach.

We hypothesized that this is due to generation of glycating agents by the intestinal microbiota, and the prevention of these effects by co-treatment with pyridoxamine indicates that this hypothesis is correct. Mucosal mast cell counts were increased in FODMAP treated animals, but not in animals co-treated with pyridoxamine. Mast cells are implicated in visceral hypersensitivity, as well as in mucus barrier dysregulation, and increased mucosal mast cell numbers or activity are often linked to IBS.

This work thus offers a link between the efficacity of the low-FODMAP diet and the involvement of intestinal mast cells in IBS.

## Résumé

Le syndrome de l'intestin irritable (SII) est un trouble gastro-intestinal fonctionnel caractérisé par des douleurs abdominales, des ballonnements et des troubles du transit intestinal. Cette pathologie digestive a une prévalence mondiale importante d'environ 11%. Elle entraîne un coût économique important : perte de productivité et absentéisme au travail. De plus, elle entraine une forte dégradation de la qualité de la vie des patients. Les causes de ce trouble fonctionnel ne sont pas bien comprises rendant le traitement thérapeutique difficile. Au cours des dernières années, un régime alimentaire à faible teneur en FODMAPs (Fermentable Oligo-, Di-, Mono-saccharides And Polyols) s'est révélé efficace dans la réduction des symptômes du SII. Sur le plan mécanistique ces effets positifs restent à élucider. Toutefois, on cite le plus souvent une réduction de la distension entérique due à une réduction de la production de gaz et du volume d'eau intestinale par des effets osmotiques. Campbell *et al.* pose l'hypothèse qu'une fermentation anaérobie de carbohydrates non-absorbés par le microbiote intestinal provoque la formation endoluminale de métabolites tels que les alcools, les cétones et les aldéhydes, responsables d'intolérances alimentaires comme l'intolérance au lactose. Nous avons émis l'hypothèse que ce mécanisme pourrait être étendu aux FODMAPs pour expliquer l'efficacité du régime alimentaire pauvre en FODMAPs chez les patients SII.

Nos études montrent un rôle complexe des FODMAPs sur des modèles murins qui reflètent la physiopathologie du SII. Les traitements par FODMAPs (lactose et fructo-oligosaccharides) provoquent une hypersensibilité viscérale et abdominale et une dysfonction de la barrière de mucus au niveau de la muqueuse intestinale.

Nous avons démontré que ces effets étaient dûs à la production d'agents de glycation par le microbiote intestinal. En effet, ces effets étaient prévenus par un co-traitement à la pyridoxamine. Le nombre de mastocytes muqueux était également augmenté chez les animaux traités par FODMAPs et significativement réduit par un co-traitement à la pyridoxamine. Les mastocytes sont connus pour être impliqués dans l'hypersensibilité viscérale et dans la dysrégulation de la barrière de mucus de l'intestin. Par ailleurs, une augmentation du nombre et/ou de l'activité des mastocytes est retrouvée sur des biopsie de patients SII.

Ce travail de thèse original permet donc de faire un lien entre l'efficacité du régime alimentaire à faible teneur en FODMAPs, la symptomatologie et l'implication des mastocytes intestinaux chez le patient SII.

## Abbreviations

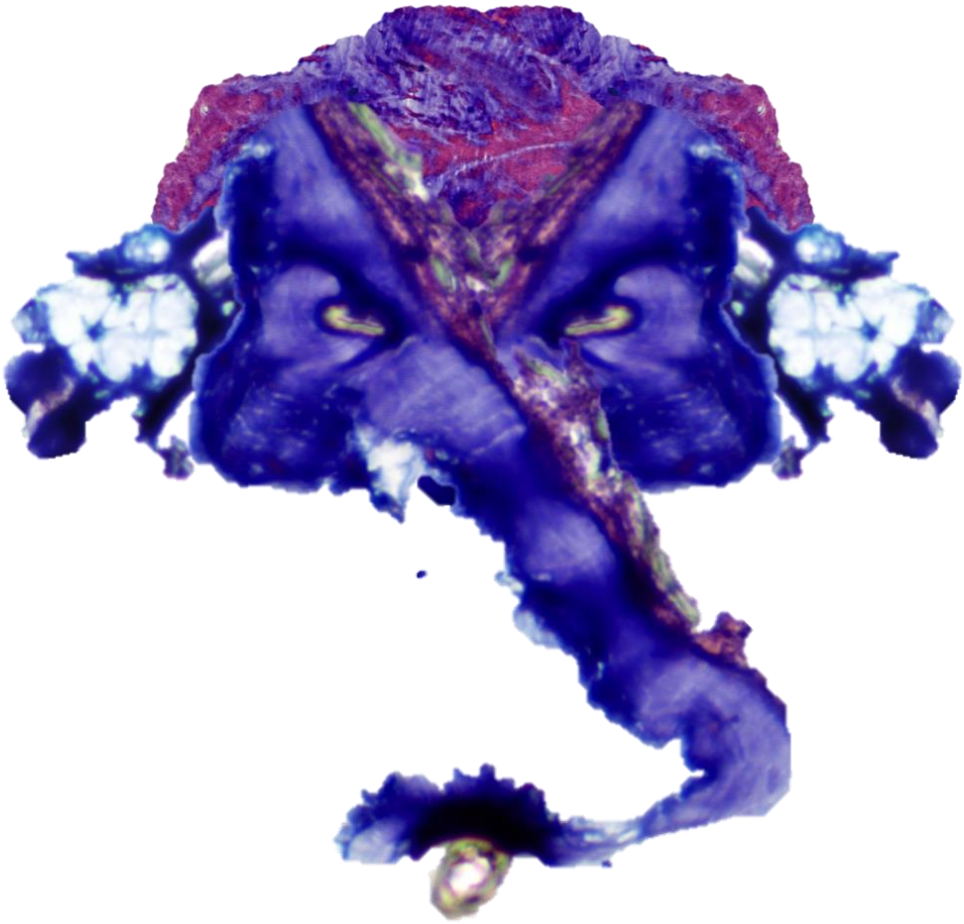
| | |
|---|---|
| 4-HNE | 4-hydroxynonenal |
| ACTH | Adrenocorticotropic hormone |
| AGEs | Advanced glycation end products |
| ANS | Autonomous nervous system |
| APC | Antigen-presenting cell |
| CFAP | Chronic Functional Abdominal Pain |
| cfu | colony forming units |
| cGMP | Cyclic guanosine monophosphate |
| CGRP receptor | Calcitonin gene-related peptide |
| CNS | Central nervous system |
| CRF | Corticotropin releasing factor |
| DAMP | Damage-associated molecular pattern |
| DC | Dendritic cell |
| DSCG | Disodium cromoglycate |
| DSS | Dextran sodium sulphate |
| EMG | Electromyographic |
| ENS | Enteric nervous system |
| FAE | Follicle-associated epithelium |
| FGIDs | Functional gastrointestinal disorders |
| FISH | Fluorescent in situ hybridisation |
| FITC | Fluorescein isothiocyanate |
| FODMAP | Fermentable Oligo-, Di-, Mono-saccharides and Polyols |
| FOS | Fructo-oligosaccharides |
| GABA | γ-aminobutyric acid |
| GALT | Gut-associated lymphoid tissue |
| GAP | Goblet cell-associated antigen passages |
| GC | Goblet cell |
| GI | Gastrointestinal |
| Glo | Glyoxalase |
| GOS | Galacto-oligosaccharides |
| GR | Glucocorticoid receptors |
| GSH | Glutathione |

| | |
|---|---|
| HPA-axis | Hypothalamic-pituitary-adrenal axis |
| IBD | Inflammatory Bowel Disease |
| IBS | Irritable Bowel Syndrome |
| IEC | Intestinal epithelial cells |
| Ig | Immunoglobulin |
| IL | Interleukin |
| LAB | Lactic acid bacteria |
| LFD | Low-FODMAP diet |
| LPS | Lipopolysaccharide |
| M cell | Microfold cell |
| MAMP | Microbe-associated molecular pattern |
| MAPK | Mitogen-activated protein kinase |
| MDA | Malonaldehyde |
| MG | Methylglyoxal |
| MGS | Methylglyoxal synthase |
| MLCK | Myosin Light Chain Kinase |
| MR | Mineralocorticoid receptors |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NGF | Nerve growth factor |
| NLRP6 | Nod-like receptor family pyrin domain containing 6 |
| OR | Odds ratio |
| PAR-2 | Protease-activated receptor 2 |
| PI-IBS | Post-infectious Irritable Bowel Syndrome |
| PM | Pyridoxamine |
| PRR | Pattern recognition receptor |
| PRS | Partial restraint stress |
| QOL | Quality of Life |
| RAGE | Receptor for advanced glycation end products |
| RCT | Randomized controlled trial |
| ROS | Reactive oxygen species |
| SAM | Sympathetic adrenomedullary system |
| SCFA | Short-chain fatty acid |
| senGC | Sentinel goblet cell |

| | |
|---|---|
| SIBO | Small intestinal bacterial overgrowth |
| SP | Substance P |
| TGFβ | Transforming growth factor beta |
| TJ | Tight junction |
| TLR | Toll-like receptor |
| TNBS | 2,4,6-trinitrobenzene sulfonic acid |
| TNF-α | Tumour Necrosis Factor α |
| Tregs | Regulatory T-cells |
| UC | Ulcerative Colitis |
| UWL | Unstirred water layer |
| WAS | Water avoidance stress |
| WHO | World Health Organization |
| ZO1 | Zona occludens-1 / tight junction protein 1 |

# 1 General Introduction

## Background Information, Thesis Context



# 1 General Introduction

This thesis project was part of the Initial Training Network (ITN) NeuroGUT, financed by the People Programme of the European Union's Seventh Framework Programme. This training network aimed to offer training in neurogastroenterology and complementary skills to young researchers, to help form the next generation of scientists dedicated to the rapidly developing field of neurogastroenterology. The projects financed in this way all pertain to functional gastrointestinal disorders (FGIDs) and investigate such topics as the involvement of gut-brain interaction, nutritional challenges of the immune system, low-grade inflammation, and post-infectious changes to the enteric nervous system as possible pathophysiological mechanisms. The network contains both clinical and basic science partners, and fellows with both medical and research science backgrounds were recruited. During the project, fellows met several times per year for training activities and annual meetings, as well as for scientific conferences. One such training activities was a Summer School organised around the Bologna IBS Days 2016, and the results of training activities during this summer school were published as a Position Paper in the Neurogastroenterology and Motility journal (Albusoda, Barki *et al.*, 2017), which is included as the first section after this general introduction.

The goal of this thesis project was to investigate and describe mechanisms by which FODMAPs (Fermentable Oligo-, Di-, Mono-saccharides and Polyols) can induce symptoms of Irritable Bowel Syndrome (IBS), based on the facts that a low-FODMAP diet can successfully reduce symptoms in IBS patients (Gibson and Shepherd, 2005; Halmos, Power *et al.*, 2014; Böhn, Störsrud *et al.*, 2015; Marum, Moreira *et al.*, 2016a; Prince, Myers *et al.*, 2016; McIntosh, Reed *et al.*, 2017), and that fermentation of certain non-absorbable carbohydrates can lead to the formation of methylglyoxal, with negative local and systemic effects (Campbell, Waud *et al.*, 2005; Campbell, Matthews *et al.*, 2010). We hypothesized that FODMAP fermentation could have sensitizing and immuno-modulatory effects in the colon by production of toxic microbial metabolites, which supplements the prevailing idea that the efficacity of the low-FODMAP diet is related to the increased intestinal distension via osmotic effects and gas production (Barrett, Gearry *et al.*, 2010; Ong, Mitchell *et al.*, 2010) induced by FODMAP intake, in an already hypersensitive system. To get more insight into this matter and test our hypothesis, we used an animal model to measure relevant markers in colonic tissues and digesta, such as mastocyte counts, mucus barrier function, and aldehyde contents of faeces, as well as more systemic parameters, such as visceral and abdominal sensitivity.

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POSITION PAPER

WILEY Neurogastroenterology & Motility NGM

# A fresh look at IBS—opportunities for systems medicine approaches

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**Funding information**
NeuroGUT

## Abstract

NeuroGUT is a EU-funded initial training network (ITN) of 14 research projects in neurogastroenterology that have employed an equal number of early-stage researchers. Neurogut trainees have—among other activities—attended an international conference on irritable bowel syndrome (IBS) in Bologna in 2016 and were asked to critically review and evaluate the current knowledge on IBS for their respective research activities, and to state what they were missing. Most appreciated were the topics brain imaging of gut activity, the role of the gut microbiota, the pharmacology of gut functions, the IBS-IBD interrelation, the new Rome IV criteria, the role of gas, and the placebo response in functional disorders. Missed were more detailed coverage of high-resolution manometry, functional brain imaging, advanced "systems medicine" approaches and bioinformatics technology, better sub-classification of IBS patients, and the development of disease biomarkers, extended at the molecular (genetic/epigenetic, proteonomic) level. They summarize that despite excellent specialized research, there is a gap open that should be filled with systems medicine. For this, it would be necessary that medical research learns even more from the data sciences and other basic disciplines, for example, information technology and system biology, and also welcomes a change in paradigm that enhances open sharing of data, information, and resources.

**KEYWORDS**
functional bowel disorders, irritable bowel syndrome

## 1 | THE NEUROGUT TRAINING NETWORK

NeuroGUT is an initial training network (ITN) of the European Union's 7th Framework Programme (7FP) (2007-2013), according to the rules released by the EU.[1] Its proposal was submitted for review in the years 2010, 2011, 2012, and 2013, before it was approved in May 2013, and received funding by the end of 2013. NeuroGUT started February 1st, 2014 and will last for 4 years, with a total budgetary EU contribution of 3.687 Mio €.

In agreement with the rules for ITNs, NeuroGUT proposed a training network of 14 projects in the area of neurogastroenterology and motility, stating that "neurogastroenterology is a new and emerging medical/scientific subspecialty that currently has no formal training opportunities in medicine and related disciplines despite its existence as scientific community for more than 20 years. It includes basic science aspects (neurophysiology, neurobiology, neuropsychology, psychophysiology, pharmacology, biotechnology) as well as clinical

All authors contributed equally to this paper.

  

aspects (gastroenterology, neurology, internal medicine, surgery, psychology, psychosomatic medicine) of the neural control of intestinal functions (motility, secretion, absorption, immunity, sensitivity, food intake) in health and disease."[2]

Initial training networks are thought as training networks, not as much as research projects: this implies that the focus is—beside scientific excellence—on training of a future generation of scientists and clinicians, to qualify them for academic, clinical, or industrial work in the related areas. In consequence, the funding received is to allow completion of 3-year PhD training for early stage researchers (ESRs) at each participating partner laboratory, with supplementary money for the participating centres. Among others, the requirement to work as ESR in one of the projects is mobility of the researcher: he/she cannot have resided in the country of his/her host institution for more than 1 year in the 3 years prior to his/her recruitment; in most cases he/she has to change the country of residency. ITNs within FP7 also included experienced (post-doctoral) researchers (ERs) especially to work with industrial partners (small and medium-size enterprises, SME) for 2 years. NeuroGUT involves 11 ESRs and 3 ERs (see Table 1).

All ESRs had to enroll in local PhD programs in addition to their NeuroGUT training activities; these included seminars in paper and grant writing, didactic courses in oral presentation skills, a mini-MBA course to foster business activities, summer, and winter schools on specific topics of general interest, self-organized researcher camps, and secondments (short-term stays in other laboratories, etc.). One such activity was the participation in the international IBS Bologna Days 2016.[3]

The principle investigators and supervisors of the ESRs/ERs of the NeuroGUT network consist of internationally leading experts in the respective research fields, coming from academia and from the private sector, that have designed 14 state-of the-art research subprojects to explore the neuronal and immunological control of gut functions in health and in major functional gastrointestinal disorders such as the irritable bowel syndrome (IBS).

These 14 projects were distributed to three work packages (basic science, translational science, clinical science), each supplemented with one industry-based project. All projects were pre-arranged in a kind of cycle (called the "digestive cycle") starting and ending with the process of food ingestion, as organizing principle (see Figure 1).

The research project for each ESR covered a period of 3 years, while projects for ERs were planned for 2 years. For details of the individual projects, we refer to the NeuroGUT website.[2]

### KEY POINTS

- NeuroGUT trainees attended an international conference on irritable bowel syndrome (IBS) in Bologna in 2016.
- They critically evaluated the current knowledge on IBS presented for their respective research activities.
- They summarize that there is a gap open that should be filled with systems medicine.

## 2 | THE TASK

Both ESRs and ERs of NeuroGUT are exposed to a number of learning events beyond the individual PhD programs they are involved in

**TABLE 1** Short titles of the 14 subprojects (SP) of the ITN NeuroGUT with principle investigator (PI) and ESR/ER (initials); see www.neurogut.eu for further details

| Project title | PI | ESR/ER |
|---|---|---|
| Projects for early state researchers (ESR), 36 months | | |
| SP1: Central representation of food intake in health & disease | Enck | IL |
| SP2: Epithelial barrier function and micro-inflammation | Theodorou | JBJK |
| SP3: Mucosa-ENS-signaling in chronic bowel diseases | Schemann | ML |
| SP4: Dysfunction of esophagus & esophagogastric junction | Smout | TH |
| SP5: Luminal bacteria, immune system and enteric nerves | Stanghellini | TBK |
| SP6: Neuro-immune mechanisms in visceral pain perception | Boeckxstaens | EP |
| SP7: Inflammation and Pain | Grundy | FU |
| SP8: Pathophysiological alterations/symptoms in FBD | Simren | AP |
| SP9: Gastrointestinal motility/sensitivity as a key to FBD | Azpiroz | TP |
| SP10: Autonomic nervous system in visceral hypersensitivity | Aziz | AA |
| SP11: Processing of visceral sensation & the gut microbiome | Enck | HW |
| Projects for experienced researcher (ER), 24 months | | |
| SP12: Intestinal motility by endoluminal image analysis | Rabinovitz/Horn (Given) | NN* |
| SP13: 5HT and other receptors in visceral hypersensitivity | Schemann/Grundy | NB |
| SP14: Bacterial flora in health and functional bowel disorder | Zimmermann (Symbio) | NM |

*Position empty after 11 months.

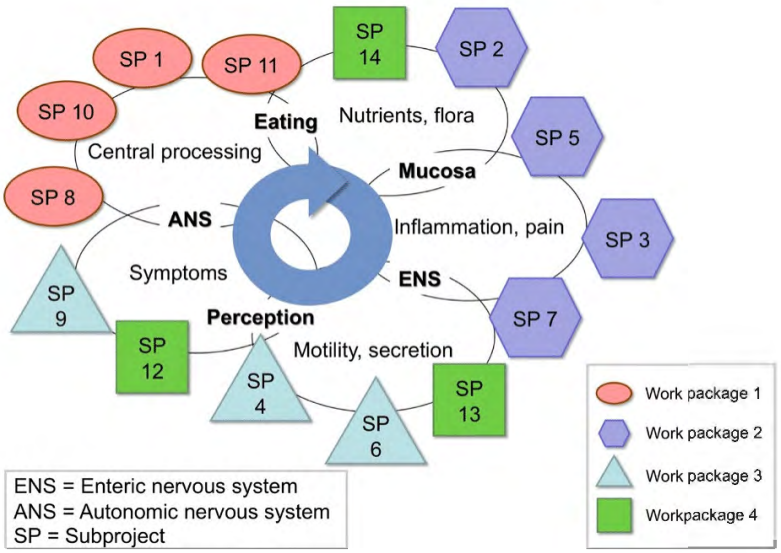


**FIGURE 1** Arrangement of the subprojects (SPs) of the NeuroGUT network. Colors and shapes represent work packages (see text below) and are related to the collaborative aspects of the NeuroGUT networking

at their home institutions, and among them are training seminars and summer schools. With respect to the latter, rather than inviting them to special events (usually 3-day courses with a specific topic and faculty), NeuroGUT attempted to test new formats of learning by integrating them into scientific events of a larger scale, for example, international conferences such as the European Neurogastroenterology and Motility conference in Istanbul 2015 and the international IBS Bologna Days in 2016. The respective tasks assigned to them during the conferences were adjusted to their respective level of training achieved so far.

During the Bologna meeting, their task was twofold: to report back (i) which of the presentation during the conference was the most valuable for their own and specific PhD project, and (ii) which topic or aspect they missed most for their work, and for functional gastrointestinal disorders in general and IBS specifically. Therefore, this report allows a fresh-men's and fresh-women's view on the current research on IBS.

## 3 | THE TOPICS MOST APPRECIATED

As can be predicted, the appreciation of individual talks at the IBS meeting was co-determined by the research focus of the single ESR/ER researcher, but not surprisingly as well, the ranking also reflects the areas less well covered by NeuroGUT: the talks by Lucas Oudenhoven (on brain imaging of gut activity) and Steven Collins (on the role of the gut microbiota) found high appreciation, as did the pharmacology of gut functions (by Jan Tack), the IBS-IBD interrelation (by Thierry Piché), the new Rome IV criteria (by Douglas Drossman), the role of gas (by Fernando Azpiroz) and the placebo response (by Paul Enck) for functional disorders.

## 4 | THE TOPICS MISSED

More important than the topics covered during the conference may be the ones that the NeuroGUT trainees missed in the presentations of the conference; they can be subdivided into 3 categories: Research Techniques, Symptom Assessments, and Mechanisms of Action:

### 4.1 | Research techniques

It was felt that high-resolution manometry deserved a wider application than is currently implemented for esophageal and anorectal diagnostics, for example, for recording activity of the colon, a widely ignored area for many years. It was also stated that functional brain imaging is grossly underestimated due to the complexity of the technology on the one hand and a lack of understanding of its options on the other; many more laboratories should cooperate with imaging facilities to enhance this applications. With the number of putative biomarkers of IBS constantly increasing, and with inclusion of "big data" from "omics"-sciences (genomics, microbiomics, metabolomics, proteomics) the need for advanced "systems medicine" approaches and bioinformation technology appears evident, while current methodology still looks "old fashion" and lacks the options these approaches allow nowadays. The same applies to further integration of other disciplines of biomedical research (systems biology, microbiology, genetics, psychology, neuroscience) into neurogastroenterology.

### 4.2 | Symptom assessment

Among the symptoms not receiving enough attention in the IBS debate was heartburn, and among other deficits mentioned was the insufficient sub-classification of IBS patients, beyond the ongoing Rome (IV) differentiation. Furthermore, it was felt that symptom differentiation needs supplementation by the development of disease biomarkers.

### 4.3 | Methodological deficits

The biomarker discussion needs to be extended at the molecular (genetic/epigenetic, proteonomic) level and linked to visceral

hypersensitivity, which still represents a major clinical characteristic of IBS. A further exploration of the gut-to-brain pathway (and the inclusion of the role of the microbiota) is needed, as the current discussion is focusing too much on brain-to-gut mechanisms, and is in part ignoring the reciprocal role. It was also proposed to consider the link of IBS to IBD (inflammatory bowel disease) as a kind of "inflammatory IBS" in the future.

## 5 | SUMMARY

As one participant stated, "the magnitude of pathomechanisms presented … shows the importance of viewing IBS as a multifactorial, complex and systemic condition. Such a condition cannot be fully understood by viewing only its single parts. What could be seen on the conference was that despite excellent specialized research there is a gap open that should be filled with systems medicine approaches. For this it is necessary that medical research learns even more from the data sciences and other basic disciplines, and also welcomes a change in paradigm that enhances open sharing of data, information, and resources … medicine and biomedical research has now reached a level where these strategies can be of tremendous help to better understanding of these complex disorders …"

## ACKNOWLEDGMENTS

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## DISCLOSURE

No competing interest declared.

## REFERENCES

1. see: http://ec.europa.eu/research/mariecurieactions/index_en.htm
2. see: http://www.neurogut.eu
3. see: http://www.ibsbolognadays2016.it

## 1.2 IBS, FODMAPs

The main subject of this thesis concerns the mechanism of effect by which a low-FODMAP diet reduces symptoms in irritable bowel syndrome (IBS). In this chapter, the current state of knowledge concerning IBS and some other functional gastrointestinal disorders (FGIDs) will be discussed.

### 1.2.1 Functional gastrointestinal disorders (FGIDs)

Functional gastrointestinal disorders (FGIDs) are a group of distinct disorders of different sections of the gastrointestinal tract with the commonality that they have no clear pathophysiological cause (hence 'functional'). FGIDs are common among the population, with Irritable Bowel Syndrome (IBS) and Functional Dyspepsia the most frequent. On a whole, FGIDs are the most commonly diagnosed disorders by gastroenterologists. Because they are difficult to define and, for the moment, have no clear pathological cause, their treatment is complicated, consisting of a variety of pharmacological, psychological, dietary, and complementary medical treatments (Whitfield and Shulman, 2009). In an ongoing effort to classify and define the FGIDs for diagnosis, the international "Rome process" has yielded the 4$^{th}$ edition of the Rome criteria for FGIDs in 2016 (Drossman, 2016).

#### 1.2.1.1 Rome Process and criteria

The Rome Foundation first issued criteria for the diagnosis of Irritable Bowel Syndrome in 1989 (Thompson et al. 1989), following with the Rome Classification System for FGIDs in 1990 (Drossman, 2007). Since then, 4 classifications have been published; Rome I (1994), Rome II (1999-2000), Rome III (2006), and Rome IV (2016) (Drossman, 2016). It has grown from being mostly a tool for researchers of FGIDs to better classify experimental subjects, to a robust diagnostic tool for clinical practitioners. The current Rome IV criteria have been updated to specifically take 'pain' into account, changing from 'discomfort' which was often difficult for patients to respond to, and doesn't translate well between cultures (Drossman, 2016). A full overview of Functional Gastrointestinal Disorders specified by Rome IV can be found in Table 1.

There are some limitations to using the Rome criteria in clinical practice. For example, they may exclude some treatable patients who do not strictly fall within the definitions set for each disorder, and patients with multiple FGIDs might not be recognized as such (Oświęcimska, Szymlak *et al.*, 2017). Additionally, changing diagnosis criteria from one iteration to the next might cause some patients to be 'cured' of their disease on rediagnosis, without any change in their health situation.

Although the Rome criteria are increasingly useful and accepted for clinical practice, its background as a tool for patient selection for trial purposes means its first goal was mostly to select for those patients who were the most clearly includable and thus likely to respond to treatment, preventing false positives.

*Table 1 – Overview of FGIDs specified by Rome IV (Drossman, 2016)*

| Functional Gastrointestinal Disorders: Disorders of Gut–Brain Interaction | |
|---|---|
| **A. Esophageal Disorders** | |
| A1. Functional chest pain | A4. Globus |
| A2. Functional heartburn | A5. Functional dysphagia |
| A3. Reflux hypersensitivity | |
| **B. Gastroduodenal Disorders** | |
| B1. Functional dyspepsia | B3. Nausea and vomiting disorders |
| B1a. Postprandial distress syndrome (PDS) | B3a. Chronic nausea vomiting syndrome (CNVS) |
| B1b. Epigastric pain syndrome (EPS) | B3b. Cyclic vomiting syndrome (CVS) |
| B2. Belching disorders | B3c. Cannabinoid hyperemesis syndrome (CHS) |
| B2a. Excessive supragastric belching | B4. Rumination syndrome |
| B2b. Excessive gastric belching | |
| **C. Bowel Disorders** | |
| C1. Irritable bowel syndrome (IBS) | C2. Functional constipation |
| IBS with predominant constipation (IBS-C) | C3. Functional diarrhea |
| IBS with predominant diarrhea (IBS-D) | C4. Functional abdominal bloating/distension |
| IBS with mixed bowel habits (IBS-M) | C5. Unspecified functional bowel disorder |
| IBS unclassified (IBS-U) | C6. Opioid-induced constipation |
| **D. Centrally Mediated Disorders of Gastrointestinal Pain** | |
| D1. Centrally mediated abdominal pain syndrome (CAPS) | |
| D2. Narcotic bowel syndrome (NBS)/ | |
| Opioid-induced GI hyperalgesia | |
| **E. Gallbladder and Sphincter of Oddi (SO) Disorders** | |
| E1. Biliary pain | |
| E1a. Functional gallbladder disorder | |
| E1b. Functional biliary SO disorder | |
| E2. Functional pancreatic SO disorder | |
| **F. Anorectal Disorders** | |
| F1. Fecal incontinence | F2c. Proctalgia fugax |
| F2. Functional anorectal pain | F3. Functional defecation disorders |
| F2a. Levator ani syndrome | F3a. Inadequate defecatory propulsion |
| F2b. Unspecified functional anorectal pain | F3b. Dyssynergic defecation |
| **G. Childhood Functional GI Disorders: Neonate/Toddler** | |
| G1. Infant regurgitation | G5. Functional diarrhea |
| G2. Rumination syndrome | G6. Infant dyschezia |
| G3. Cyclic vomiting syndrome (CVS) | G7. Functional constipation |
| G4. Infant colic | |
| **H. Childhood Functional GI Disorders: Child/Adolescent** | |
| H1. Functional nausea and vomiting disorders | H2a1. Postprandial distress syndrome |
| H1a. Cyclic vomiting syndrome (CVS) | H2a2. Epigastric pain syndrome |
| H1b. Functional nausea and functional vomiting | H2b. Irritable bowel syndrome (IBS) |
| H1b1. Functional nausea | H2c. Abdominal migraine |
| H1b2. Functional vomiting | H2d. Functional abdominal pain – NOS |
| H1c. Rumination syndrome | H3. Functional defecation disorders |
| H1d. Aerophagia | H3a. Functional constipation |
| H2. Functional abdominal pain disorders | H3b. Nonretentive fecal incontinence |
| H2a. Functional dyspepsia | |

#### 1.2.1.2 Irritable Bowel Syndrome

Irritable Bowel Syndrome (IBS) is one of the FGIDs defined by Rome IV; it is a mild to serious disease with a high prevalence in many societies, ranging from 7% (South-East Asia) to 21% (South America), with a pooled prevalence of 11% (Lovell and Ford, 2012b), see Table 2.

*Table 2 – Pooled prevalence of IBS according to geographic location (Lovell and Ford, 2012b)*

| | No. of studies | No. of subjects | Pooled prevalence (%) | 95% CI | $I^2$ (%) | *P* value for $I^2$ |
|---|---|---|---|---|---|---|
| All studies | 80 | 260,960 | 11.2 | 9.8–12.8 | 99.3 | <.001 |
| North European studies | 21 | 72,031 | 12.0 | 9.0–15.0 | 99.4 | <.001 |
| Southeast Asian studies | 19 | 55,545 | 7.0 | 5.0–9.0 | 98.5 | <.001 |
| North American studies | 10 | 52,790 | 11.8 | 7.4–17.2 | 99.6 | <.001 |
| South European studies | 9 | 36,577 | 15.0 | 11.0–20.0 | 98.5 | <.001 |
| Middle Eastern studies | 8 | 32,374 | 7.5 | 3.5–12.8 | 99.5 | <.001 |
| South Asian studies | 4 | 5857 | 17.0 | 5.0–33.0 | 99.5 | <.001 |
| South American studies | 4 | 1272 | 21.0 | 18.0–25.0 | 54.7 | .004 |
| Australasian studies | 3 | 3739 | 14.0 | 13.0–15.0 | N/A[a] | N/A[a] |
| African studies | 2 | 775 | 19.0 | 2.0–46.0 | N/A[a] | N/A[a] |

N/A, not applicable.
[a]Too few studies to assess heterogeneity.

IBS is characterized by abdominal pain, bloating, erratic bowel habits, and variable changes in the consistency of stools (Enck, Aziz *et al.*, 2016; Spiller, 2016). It is a heterogeneous disorder, with 4 defined sub-types; Diarrhoea predominant (IBS-D), Constipation Predominant IBS (IBS-C), Mixed bowel habits (IBS-M), or Unclassified (IBS-U) (Drossman, 2016). Even though it is a functional disorder, IBS has a high impact on the quality of life (QOL), akin to that suffered by patients of (organic) inflammatory bowel disorders such as Crohn's disease or ulcerative colitis (UC) (Pace, Molteni *et al.*, 2003). An important risk factor to IBS is female sex (Lovell and Ford, 2012a) as well as occurrence in relatives (Saito, Petersen *et al.*, 2010), which can be linked to genetics, shared environment, or social learning behaviour (Levy, Jones *et al.*, 2001). Differences in reported prevalence among the sexes is likely due to differences in hormonal regulation, though a difference could also be linked to differential usage of healthcare services (Enck, Aziz *et al.*, 2016). Alternatively, a subset of IBS patients develops their symptoms acutely, following an infectious gastroenteritis. In this case, the syndrome is termed post-infectious IBS (PI-IBS) (Ghoshal, Park *et al.*, 2010). 84% of IBS patients self-report that symptoms are elicited by certain foods (Bohn, Storsrud *et al.*, 2013), which influences their eating behaviour, with potentially negative social and nutritional effects. Additionally, there are some psychiatric comorbidities noted in IBS patients, such as depression or anxiety, though the majority of IBS patients do not warrant a diagnosis of a mood or anxiety disorder (Janssens, Zijlema *et al.*, 2015).

#### 1.2.1.3 Types of IBS Classification and Diagnostics

The Rome IV criteria are used to diagnose IBS and distinguish IBS from transient gut symptoms, and gut disorders with an organic origin. To qualify as IBS, symptoms must have occurred for the first time ≥6 months before presentation for diagnosis (Longstreth, Thompson *et al.*, 2006), and have occurred ≥1 days per week during the last 3 months. Additionally, symptoms get better or worse after defecation, and stools show changes in frequency and form (Lacy, Mearin *et al.*, 2016). The subtype is characterised as IBS-C if ≥25% of stools is hard or lumpy and <25% loose or watery; IBS-D if ≥25% of stools is loose or watery, and <25% hard or lumpy; IBS-M ≥25% of stools is hard or lumpy or ≥25% of stools is loose or watery; and IBS-U if there is insufficient abnormality to classify one of the other types (see Figure 1).

A tool often used to characterise stool form is the Bristol Stool Form Scale (Lewis and Heaton, 1997a), displayed in Table 3. According to the authors, the shape and form of stools is directly related to the transit time of stools through the gastro-intestinal tract, and is a better representation than frequency of defecation (Lewis and Heaton, 1997a). However, a later study using more modern methods to measure transit time found no correlation between whole gut transit and stool form in healthy adults, though moderate correlations with stool form were observed for constipated patients (Saad, Rao *et al.*, 2009).

*Table 3 - Bristol Stool Form Scale, adapted from (Lewis and Heaton, 1997a)*

| | |
|---|---|
| **Type 1** | Separate hard lumps, like nuts |
| **Type 2** | Sausage-shaped but lumpy |
| **Type 3** | Like a sausage or snake but with cracks on surface |
| **Type 4** | Like a sausage or snake, smooth and soft |
| **Type 5** | Soft blobs with clear-cut edges |
| **Type 6** | Fluffy pieces with ragged edges, a mushy stool |
| **Type 7** | Watery, no solid pieces |

To measure the severity of IBS, the IBS-Severity Scoring System (IBS-SSS) questionnaire is used. By asking about the incidence, severity of pain, severity of bloating, satisfaction with bowel function, and interference in personal life related to the IBS symptoms, a score for the patient's IBS severity is calculated. On a scale from 0-500, mild (75-175), moderate (175-300) and severe (≥300) categories are defined. Scores below 75 can be considered as non-IBS, or in remission (Francis, Morris *et al.*, 1997).

A type of IBS characterized not by its symptoms, but by its onset is post-infectious IBS (PI-IBS). PI-IBS has an incidence of approximately 10% within a year after acute infectious gastroenteritis (Klem, Wadhwa *et al.*, 2017), but is less likely after 'traveller's diarrhoea' at 5,4% (Schwille-Kiuntke, Mazurak *et al.*, 2015). PI-IBS is often diarrhoea predominant (Ghoshal and Ranjan, 2011). It is defined as acute onset IBS after a gastroenteritis in patients not previously defined as having IBS according to the Rome Criteria. PI-IBS, because of its clear organic origin, is renewing interest in the role of impaired intestinal permeability function in IBS (Matricon, Meleine *et al.*, 2012; Piche, 2014).

Apart from the different symptoms, there is a differential impact on disease specific Quality of Life (QOL) scores between the sub-groups of IBS. IBS-M and IBS-D score lower on IBS-QOL questionnaire assessment than IBS-C, indicating a greater subjective impact on their life (Singh, Staller *et al.*, 2015), which is consistent with the expected urgency of the main symptoms.

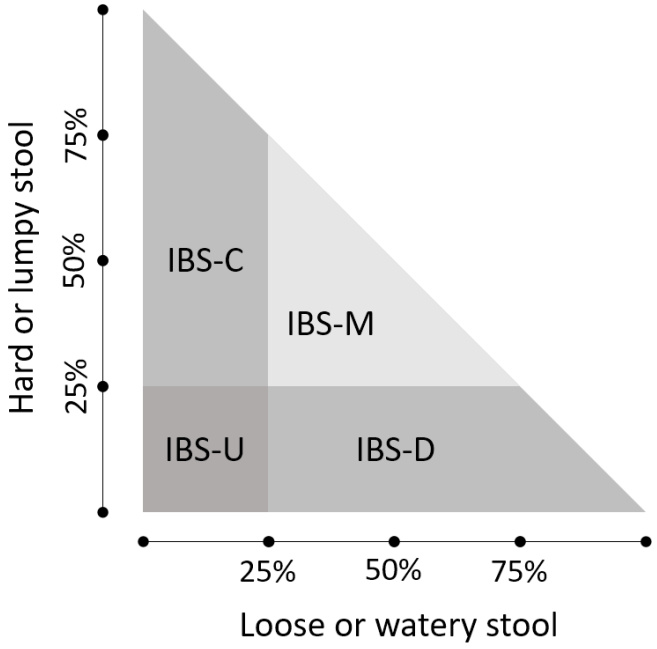



*Figure 1 - Subtypes of IBS*
*Adapted from (Longstreth, Thompson et al., 2006)*

#### 1.2.1.4 Chronic Functional Abdominal Pain

Chronic Functional Abdominal Pain (CFAP) is another FGID characterized by abdominal pain. The difference with IBS is that CFAP is not related to changes in bowel habit or stool form (Drossman, 2016). The absence of changes in bowel habit in CFAP can be taken as indication that the pain component in CFAP, but likely also in subgroups of IBS patients, is not related to motility disorders or other defects likely to influence stool transit. An altered visceral sensitivity and changes to the function of the brain-gut axis are therefore more likely causes.

### 1.2.2 Aetiology IBS and physiopathology

As mentioned before, IBS has an unclear aetiology and pathophysiology. However, a growing number of publications point to several factors implied in causing and/or perpetuating it. A combination of visceral hypersensitivity, genetics, psychological stress, dysfunctional intestinal permeability, and involvement of the microbiota are all possibly implicated in IBS.

#### 1.2.2.1 Visceral sensitivity

A key component of IBS is abdominal pain. A logical and probable cause for this abdominal pain is an increased visceral sensitivity to different stimuli of the gastrointestinal tract. The first to describe the increased sensitivity to distension of IBS patients was James Ritchie, in 1973 (Ritchie, 1973), and he considered this to be a likely factor in the aetiology of IBS. Bouin *et al.* (Bouin, Plourde *et al.*, 2002) again showed that IBS patients, but not controls or patients with functional constipation (without pain) show a lower pain threshold, using a barostat (Table 4), indicating a hypersensitivity of the rectum.

*Table 4 – Rectal balloon distension by barostat*
*Adapted from Bouin et al. (Bouin, Plourde et al., 2002)*

| | Controls | IBS | Functional constipation |
|---|---|---|---|
| Pain threshold (mmHg) | 44.5 ± 5 | 30.4 ± 6.7 | 45.4 ± 5.3 |

Azpiroz, Bouin *et al.* (Azpiroz, Bouin *et al.*, 2007) report three criteria of altered rectal perception in IBS patients; (1) a decreased pain threshold (33 instead of 40 mmHg), (2) a sensitisation to repeated distensions, where a second set of distensions shows a threshold of 28, vs 41 mmHg for controls, and (3) referral of pain to aberrant sites instead of the sacral area in 83% of IBS patients, versus 10% in controls. The authors point to the concept of dysregulation of the neural sensory system to explain these observations. Furthermore, it is highly likely that this dysregulation is peripheral in nature (Azpiroz, Bouin *et al.*, 2007), indicated, among other reasons, by the infiltration of inflammatory cells into the gut mucosa or enteric plexuses often observed in IBS patients (Barbara, Stanghellini *et al.*, 2004). It is likely that there are different mechanisms of visceral hypersensitivity at play between regular IBS patients, and PI-IBS patients which developed symptoms after irritation of the gut by infectious agents. Visceral hypersensitivity seems to be correlated with the severity of other gastrointestinal (GI) symptoms (Simrén, Törnblom *et al.*, 2017), implying that visceral hypersensitivity contributes to GI symptom generation, or that third contributors are responsible for both GI symptoms and

visceral hypersensitivity. Illustrating the possible common cause of visceral sensitivity and GI symptoms, a reduction of visceral sensitivity by blocking the histamine receptor HRH1 is accompanied by a reduction in GI symptoms (Wouters, Balemans *et al.*, 2016).

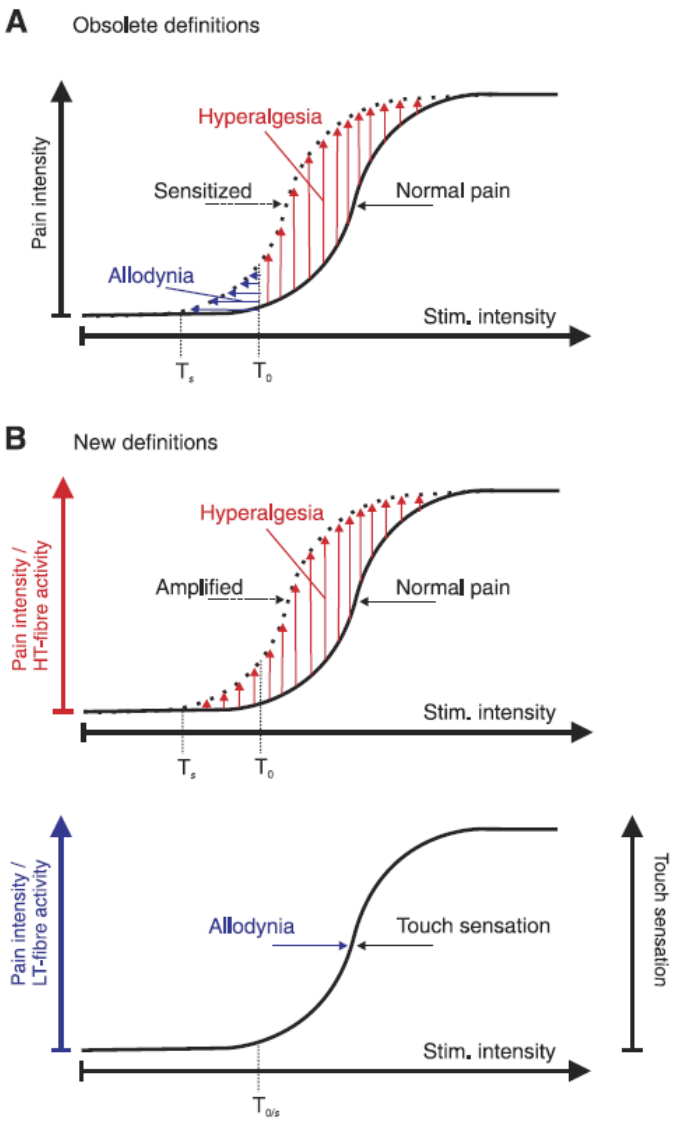



*Figure 2 – Hyperalgesia and allodynia (Sandkühler, 2009)*

Hypersensitivity issues can be seen to occur as either allodynia, in case there is pain in response to a non-painful stimulus, or hyperalgesia, an increased sensitivity to pain. In 2008, the International Association for the Study of Pain (IASP) redefined the terms allodynia and hyperalgesia. Since then, the terms are differentiated based on the possible involvement of nociceptors. In this new definition, allodynia refers to pain in response to interaction with low-threshold sensory nerve fibres, whereas hyperalgesia involves high-threshold fibres; nociceptors (Figure 2) (Sandkühler, 2009).

There is a clear link between mental stress and an increase of visceral sensitivity, and this effect is mainly mediated by the release of corticotropin-releasing factor (CRF) by the hypothalamus. This activates release of adrenocorticotropic hormone (ACTH) from the pituitary gland into the bloodstream, which then leads to cortisol release from the adrenal glands. Cortisol has many effects in the body, for example, it is involved in increasing blood-glucose concentration, and suppression of the immune system. We will however focus on the effects of Hypothalamic-Pituitary-Adrenal (HPA)-axis activation on visceral sensitivity.

In rats, visceral hyperalgesia can be induced by repeated water-avoidance stress treatments, a model of psychological stress. This effect can be prevented by injection of CRF antagonists, showing that CRF-receptors play a main role in the generation of hyperalgesia by psychological stress (Larauche, Bradesi *et al.*, 2008). Similarly, it has been shown that mast cells play a role in the generation of visceral hypersensitivity due to stressful conditions, through central pathways involving CRF (Gue, Del Rio-Lacheze *et al.*, 1997). The nervous system can directly convey signals resulting from psychological stress to mast cells by CRF and/or Substance P (SP) release, which can trigger them to release mediators via piecemeal degranulation, in response to crowding stress (Vicario, Guilarte *et al.*, 2010).

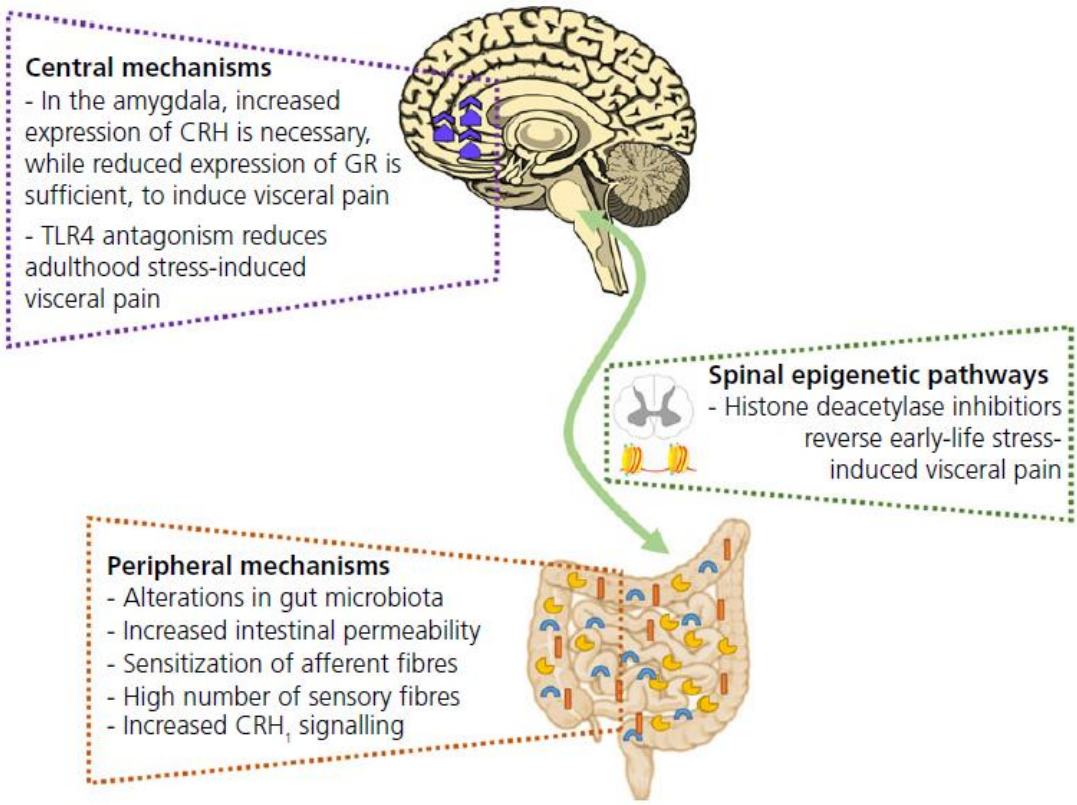



*Figure 3 – Pathophysiology of visceral pain. GR – Glucocorticoid receptor; CRH – Corticotropin releasing hormone (CRF) (Greenwood-Van Meerveld, Moloney et al., 2016)*

#### 1.2.2.2 Genetics/ epidemiology

Regardless of the largely unclear aetiology and pathophysiology of IBS, epidemiology can shed light on correlated and possibly causative factors. Research into this topic has focused on relation to family connections, comorbidity with psychological afflictions, and the local occurrence of infectious diseases.

Firstly, looking at social connections, IBS aggregates heavily to families. It has a high odds ratio (OR) between siblings (3.12), children (2.12), and parents (1.91). Interestingly, there was no correlation between spouses (OR 0.89), indicating that genetics and not shared environment might be responsible for the observed effect (Saito, Petersen *et al.*, 2010). This effect could then be explained by genes directly responsible for IBS, or genes increasing the likelihood of a related trait, such as lactose intolerance, depression or anxiety, somatization, or an immune system that increases risk of infection (Saito, Petersen *et al.*, 2010). In a recent study done on twins, familial and intra-uterine factors were shown to affect the co-occurrence of IBS and psychological factors such as depression and anxiety. They were able to show that the comorbidity of IBS with mental disorders (depression and anxiety) were only present for those twins in a lower weight group of smaller than 2,5kg (Bengtson, Aamodt *et al.*, 2015). This has led to the hypothesis that the HPA-axis links these two afflictions in intrauterine growth restriction and might explain the often-observed comorbidity, because an intrauterine physical stress could pre-program the HPA-axis to be more responsive (Bengtson, Aamodt *et al.*, 2015).

Looking further into the genetic aspects of this family aggregation it is important to realize that the susceptibility of the majority of IBS patients is caused by a complex interaction of many genes and the environment of the patient (Henström and D'Amato, 2016). However, in some cases, single genes may be responsible for symptom generation. A loss-of-function mutation of the SCN5A gene, encoding for the $Na_V1.5$ ion channel present on interstitial cells of Cajal, seems to be heavily related to IBS-C (Beyder, Mazzone *et al.*, 2014). Because of the more well-known function of the $Na_V1.5$ ion channel, these patients were initially identified because of cardiac arrhythmia. Another gene of interest is TNFSF15, which codes for TNF-like ligand 1A, which is a clear susceptibility related gene for IBS (Zucchelli, Camilleri *et al.*, 2011). This gene is also involved in the development of colitis in mice by DSS treatment, by supporting TH1 and TH17 effector functions (Takedatsu, Michelsen *et al.*, 2008), furthermore, it is an important susceptibility locus in for example Crohn's Disease (CD) (Barrett, Hansoul *et al.*, 2008) and arthritis of the spine (Zinovieva, Bourgain *et al.*, 2009). That this gene is related to IBS can be

seen as an indication that IBS is related to an altered immune response in patients with positively correlated alleles.

#### 1.2.2.3 Stress and anxiety

Because IBS and certain psychological profiles seem to be related, it is interesting to investigate the effects of mental stress and anxiety on IBS symptom generation and perception.

Early life trauma, such as childhood abuse, has a significantly higher prevalence in IBS patients than in healthy controls, particularly in women. A history of emotional abuse specifically has a high predicting power; feeling ignored, and sexual abuse increase the odds of developing IBS with 2.08 and 3.05 times, respectively (Bradford, Shih *et al.*, 2012). IBS patients meeting criteria for any psychiatric disorder range from 40% to 94% in some studies, though this is most likely an overestimation due to tertiary care patient selection. It is clear however that IBS patients have a higher frequency of depressive, panic, or generalized anxiety disorders than other patients in the same clinics (Surdea-Blaga, Băban *et al.*, 2012).

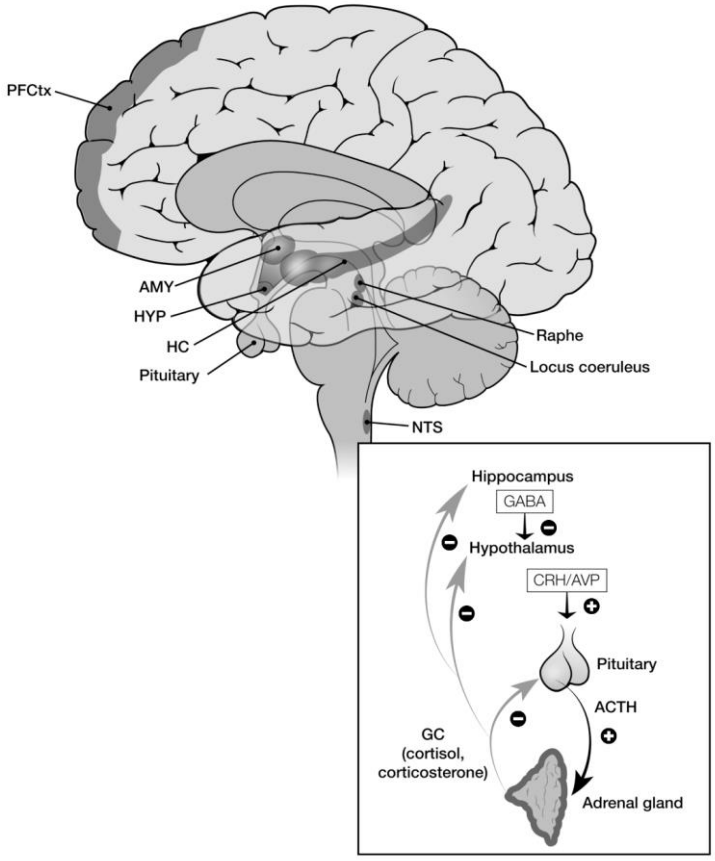



*Figure 4 - HPA axis*
AMY (amygdala), HC (hippocampus, HYP (hypothalamus), NTS (nucleus of the tractus solitarius, PFDtc (prefrontal cortex). ACTH (adrenocorticotropic hormone), AVP (arginine vasopressin) CRH (corticotropin releasing hormone) GABA (gamma aminobutyric acid)
*(Gunnar and Vazquez, 2006)*

The mechanisms for this observed association are likely to involve both true psychological factors, as well as the gut-brain and HPA axes (see Figure 4). In rats, psychological stress can increase visceral hypersensitivity by corticotropin releasing factor (CRF) release, which involves mast cell activation (Gue, Del Rio-Lacheze *et al.*, 1997). Similarly, stress can increase the paracellular permeability through corticotropin releasing factor (CRF), stimulating the release of nerve growth factor (NGF) by mast cells (Barreau, Cartier *et al.*, 2007) and the increase of mucus

production in response to stress is mast cell dependent as well (Castagliuolo, Lamont *et al.*, 1996). Alternatively, somatization is considered an important factor in IBS, correlating with visceral hypersensitivity (Grinsvall, Törnblom *et al.*, 2017). Because of this, cognitive behavioural therapy can improve GI symptoms through pathways improving anxiety levels (Jones, Koloski *et al.*, 2011). Logically, improving stress levels in this way would improve both HPA-axis and psychological mediators.

While an understanding of the proximate causes of the link between stress and visceral sensitivity is crucial in research, appreciating a more ultimate cause of this link is important too. Stress, either physical or psychological, induces adaptive responses by activation of both the HPA axis, as already discussed, and the sympathetic adrenomedullary system (SAM). Activation of SAM is very rapid, directly responsible for adaptations related to the flight-or-fight response and characterized by the release of adrenaline and noradrenaline by chromaffin cells innervated by the sympathetic preganglionic neurons, leading to acute adaptations such as increased cardiac output and blood glucose levels. Interestingly the noradrenaline:adrenaline release ratio seems to be dependent on neural integration in response to the nature and magnitude of stimulus (Vollmer, 1996). In contrast, activation of the HPA axis and subsequent increased cortisol levels have more chronic effects, and work mainly through regulating gene transcription (Sapolsky, Romero *et al.*, 2000). Proper HPA axis function permits effective flight-or-flight by optimising the response to adrenaline (the SAM response). Glucocorticoids released in HPA axis activation interact with 2 types of receptors in the brain, glucocorticoid receptors (GR) and mineralocorticoid receptors (MR), activation of which often have opposite effects. The balance of activation is determined by the concentration of glucocorticoids; at basal concentrations, only the MRs are activated, supporting maintenance processes such as ensuring responsiveness of neurons to neurotransmitters and regulating the circadian rhythm (Sapolsky, Romero *et al.*, 2000), these processes permit effective responses to stress by optimising responses to adrenaline/noradrenaline (Gunnar and Vazquez, 2006). At peak concentrations GRs are engaged as well, which leads to stress responses, but with deleterious effects on brain function, such as inhibiting glucose utilisation by neurons, and impairing neural plasticity, learning and memory (Gunnar and Quevedo, 2007). These negative effects are likely meant to reverse the acute response to stressors, and support a return to homeostasis (Sapolsky, Romero *et al.*, 2000). The balancing act between the activity of the HPA axis and SAM function to maintain homeostasis is called 'allostasis' (McEwen and Seeman, 1999), where the intended equilibrium is not at a fixed level, but dependent on context.

In case of prolonged or repeated stress, this allostatic system begins to become deleterious, due to negative health effects of otherwise adaptive responses (Gunnar and Quevedo, 2007; Greenwood-Van Meerveld, Moloney *et al.*, 2016), with the gastrointestinal tract particularly vulnerable to negative effects (Mayer, 2000).

#### 1.2.2.4 Gut permeability in IBS

An increase in gut permeability has been seen in IBS patients, particularly in PI-IBS, and can be associated to inflammatory processes and increased visceral sensitivity. In this section, we will investigate the role of increased permeability in IBS. For more information on intestinal permeability in general, please refer to section 1.5.2.

It has been shown that the increased visceral sensitivity in response to acute (restraint) stress in rats is mediated by an increase in intestinal permeability, and prevention of this increase in permeability prevents the effect on sensitivity (Ait-Belgnaoui, Bradesi *et al.*, 2005). In short, activation of stress pathways leads to mast cell activation, which produces mediators that lead to an increase in paracellular permeability, by opening tight junctions. The resulting increase in mucosal uptake of bacterial fragments and toxins activate the mucosal immune system, and together with the mast cell mediators, this causes visceral hyperalgesia (see Figure 5) (Ait-Belgnaoui, Bradesi *et al.*, 2005). In the same vein, it has been shown that public thesis defence stress increases small bowel permeability, an effect which can be blocked by mast-cell stabilizer cromoglicate (Vanuytsel, van Wanrooy *et al.*, 2014), again showing that corticotropin releasing factor (CRF) release acts on intestinal permeability via mast cell activation. Volunteers were injected with CRF, which increased small intestinal permeability, but treatment with both CRF and cromoglicate did not do so.

In IBS patients, paracellular permeability of colonic biopsies has been found to be increased in all subtypes of IBS, and this increase can be transferred *in vitro* to Caco-2 cell monolayers via soluble mediators derived from patient biopsies (Piche, Barbara *et al.*, 2009). This shows that, like stressed animals, IBS patients have an increased paracellular permeability, and this increase is due to soluble mediators in the mucosa. The involvement of mast cells is implied both by their described capability of increasing intestinal permeability (Santos, Yang *et al.*, 2001), as well as their role in increasing sensitivity (Barbara, Stanghellini *et al.*, 2004; Barbara, Wang *et al.*, 2007),

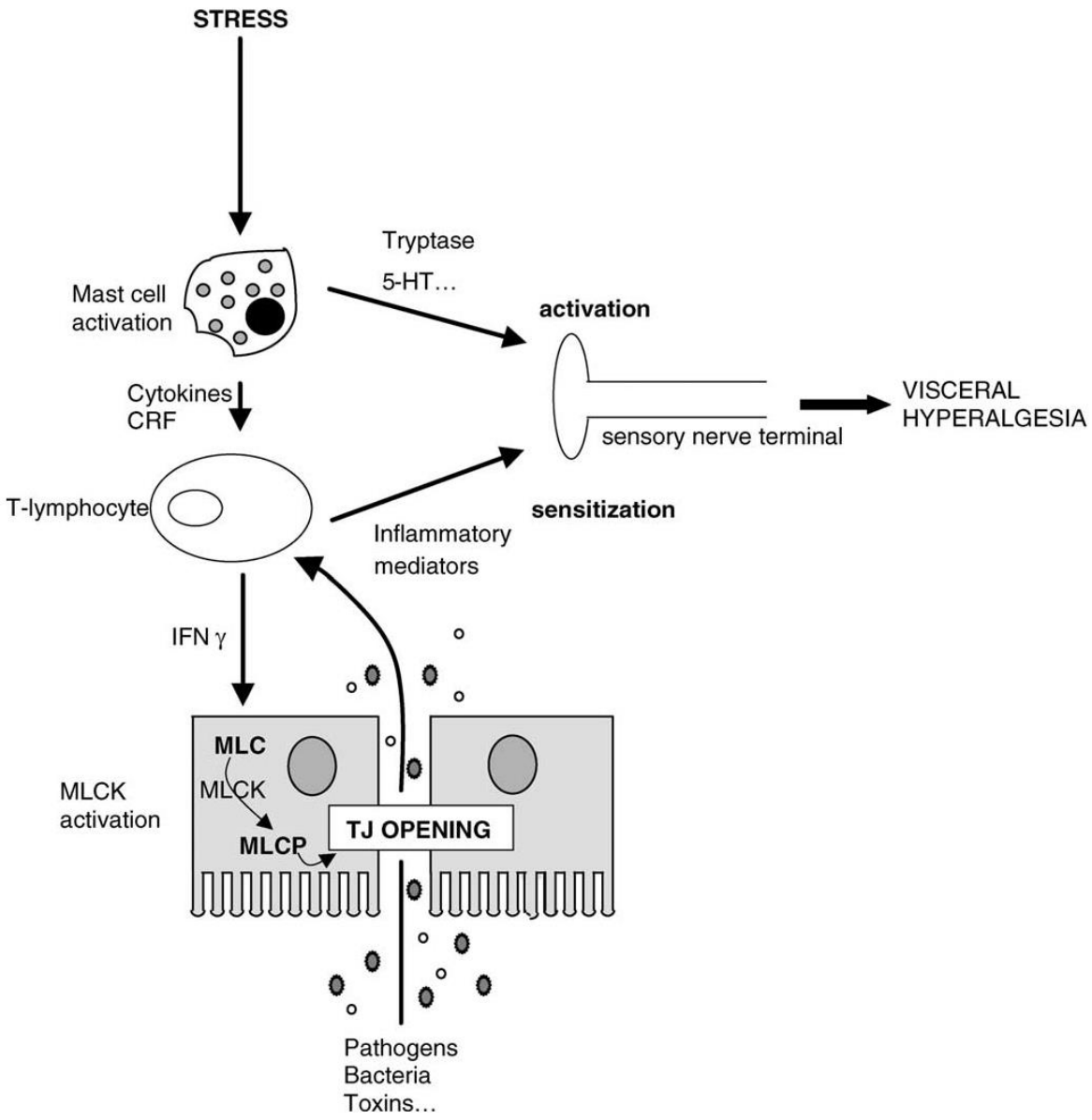



*Figure 5 – Possible mechanism by which stress can lead to increased visceral sensitivity through increased intestinal permeability. (Ait-Belgnaoui, Bradesi et al., 2005)*

and the observation that IBS patients have increased mucosal mast cell counts (Matricon, Meleine *et al.*, 2012), which holds up in a recent meta-analysis (Bashashati, Moossavi *et al.*, 2017). Another recent study claims that the colonic barrier function is uncompromised in female IBS-C patients (Peters, Edogawa *et al.*, 2017), in contradiction to earlier findings claiming the opposite (Piche, Barbara *et al.*, 2009). Additionally, they found no differences in expression of the important tight junction proteins Occludin, ZO-1, ZO-2, or ZO-3 (Peters, Edogawa *et al.*, 2017).

A possible explanation for these conflicting reports might be the use of different fluorescent markers in the *ex vivo* biopsy study; fluorescein–5.6 sulfonic acid (478 Da) for Piche, versus 4kDa FITC–dextran for Peters, and that the earlier study conducted by Piche, Barbara *et al.* used a cohort of IBS patients of all subtypes, diagnosed using Rome 2 criteria including slightly less IBS-C patients, whereas the study by Peters, Edogawa *et al.* only looked at IBS-C patients diagnosed using Rome 3 criteria. This underlines the importance of distinguishing subgroups of IBS not only on their symptoms, but possibly also on the underlying causes for their affliction, although the study by Piche, Barbara *et al.* found no differences between subgroups.

There are other possible mechanisms that could be responsible for the observed increase in permeability in IBS; a variety of proteases, whether derived from the host or the microbiota, have been described as involved in increasing the paracellular permeability of the host (Van Spaendonk, Ceuleers *et al.*, 2017). Especially mast-cell derived trypsin and tryptase can be responsible for an increase in permeability due to PAR-2 activation, at the same time causing an increase in visceral sensitivity (Annahazi, Ferrier *et al.*, 2013). Likewise, in the context of PI-IBS, an increased intestinal permeability plays an important role. Following *Campylobacter* infection, approximately 25% of patients develop 'post-dysenteric irritable bowel syndrome' (later called post-infectious IBS, or PI-IBS), characterized by increased intestinal permeability (Spiller, Jenkins *et al.*, 2000). Furthermore, a study investigating possible genetic risk factors in the development of PI-IBS shows that certain alleles of IL-6, TLR9, and CDH1 rendered the patient more susceptible (Villani, Lemire *et al.*, 2010). CDH1 (cadherin-1/E-cadherin) is a trans-membrane glycoprotein involved in cell-cell adhesion, and tight-junction formation by facilitating Adherens junctions (Hartsock and Nelson, 2008). Disruption of epithelial tight-junctions by enteric pathogens such as *Campylobacter* or *E.coli* causes an increase in intestinal permeability during infection (Berkes, Viswanathan *et al.*, 2003; Wu, Rhee *et al.*, 2007); so it is interesting that certain variants of the E-cadherin gene could be linked to development of PI-IBS. Either an impaired ability to adequately restore tight-junctions after infection, or a pre-existing higher permeability caused by this genetic variation could increase the likelihood of PI-IBS.

#### 1.2.2.5 Immune System

As mentioned before, the immune system is often seen as a key player in the physiopathology of IBS, and not only in the context of post-infectious IBS. Here, some possible mechanisms of involvement of the immune system in IBS will be discussed.

Firstly, the clearest link between IBS and the immune system is represented by post-infectious IBS. After an infectious gastroenteritis, the risk of developing IBS is increased six times, which lasts for up to three years after the infection is resolved (Thabane, Kottachchi *et al.*, 2007). Apparently, the activation of the immune system during the infection has long lasting effects on gut function, even after elimination of the infectious agent. In a similar fashion, IBD patients in remission often have IBS-like complaints (Barbara, Cremon *et al.*, 2014), which indicates that even after the inflammation is abated, the recent immune activation has leftover effects resembling IBS. The reason for this link between inflammation and IBS symptoms might be related to the increased permeability of the tissue induced by it, as discussed in the previous section.

A marker of the innate immune system, β-defensin 2, produced by neutrophils, monocytes and lymphocytes is increased in IBS patients, like in Ulcerative Colitis patients (Langhorst, Junge *et al.*, 2009). Anti-microbial β-defensin 2 production is induced by pro-inflammatory cytokines in response to microbial invasion, after activation of toll-like receptors (TLRs) (Selsted and Ouellette, 2005), possibly indicating a partially shared origin of these maladies, or merely indicating an increase in permeability permitting more bacterial components to trigger TLRs in both cases. IBS symptoms in quiescent IBD seem to be related to undetectable inflammation involving increased numbers of intraepithelial lymphocytes and TNF-α, which were absent in IBS patients and quiescent IBD patients without IBS symptoms (Vivinus-Nebot, Frin-Mathy *et al.*, 2014).

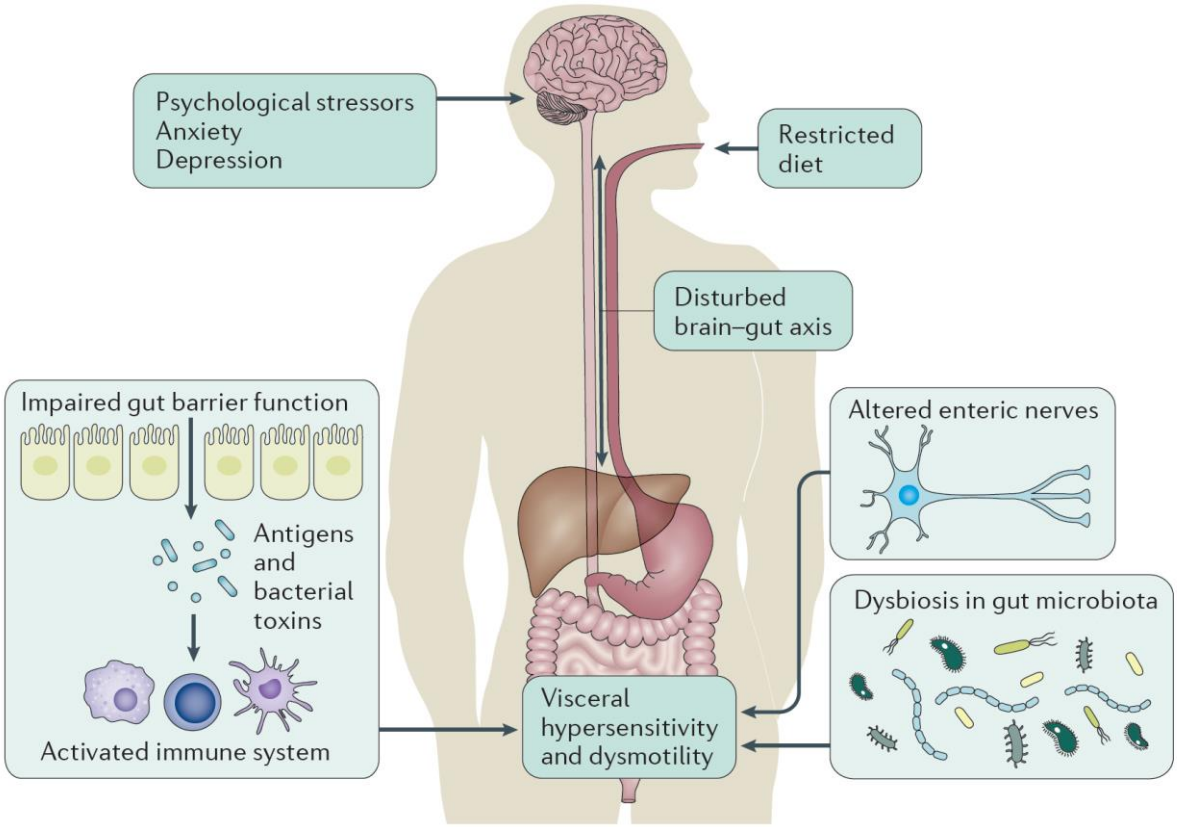



*Figure 6 - Possible shared mechanisms generating IBS symptoms in IBS and IBD in remission (Spiller and Major, 2016)*

Similarly, IBD patients with IBS symptoms show increased faecal calprotectin levels compared to IBD patients without IBS symptoms, again indicating occult inflammation (Keohane, O'Mahony *et al.*, 2010). Increased calprotectin levels are indicative of neutrophil activation and thus inflammation and are generally not significantly increased in IBS. This would indicate that the IBS symptoms in IBD patients have a different origin than those of IBS patients (neutrophil mediated inflammation *v* mast cell mediated inflammation), though mechanisms such as subsequent increased permeability are similar in both cases. Alternatively, IBS has been described as a syndrome characterized by a low-grade inflammation, with a number of immune markers modestly but significantly increased compared to healthy controls, though still with a significant overlap with controls (Barbara, Cremon *et al.*, 2011). In this view, IBS and IBD are positioned on different parts of a single spectrum of inflammation.

IBD patients in remission that do not show occult inflammation, i.e., without increased faecal calprotectin levels but showing IBS symptoms, make up 31% of total IBD patients in remission (Berrill, Green *et al.*, 2013), which is a higher proportion than observed in the general population. Figure 6 shows the possible mechanisms that could explain these patients, with mechanisms that largely correspond to PI-IBS, which is not surprising, considering the shared history of inflammation in PI-IBS and IBD patients. A subtle but interesting difference exists between the sanguine cytokine levels of healthy subjects and IBS patients. While no distinguishing profiles could be characterized, subgroups of IBS patients showed an increased immune activity, and cytokine profiles were more variable in the IBS group (Bennet, Polster *et al.*, 2016). This further indicates the involvement of the immune system, at least in subsets of IBS patients.

Inflammatory processes can lead to increased sensitivity by disconnecting nerves with their targets, which then causes reconnection and remodelling of these nerves (Byers, Suzuki *et al.*, 2003; Spiller and Major, 2016). For example, following recovery from TNBS induced colitis, substance P levels remained significantly increased in mucosal nerves in the medium term, and mucosal nerve galanin and muscular nerve substance P remained elevated on the long term together with a generally increased nerve innervation throughout the affected tissues, indicative of an increased potential for nociception (Simpson, Sundler *et al.*, 2008). Relatedly, visceral sensitivity in IBS-D patients has been linked to mast-cell derived nerve growth factor (NGF) (Xu, Zhang *et al.*, 2017), which could play a role in similar ways; promoting nerve growth and differentiation. However, the increased mucosal mast cell counts in IBS-D patients found in the same work might also be related to the increased visceral sensitivity independent of concurring increased levels of NGF. A recent meta-analysis indicates that mucosal mast cell numbers in the descending and rectosigmoid colon are indeed significantly increased in IBS patients, but no significant increase is observed in the ascending colon, additionally, $CD3^+$ T cells show a significant increase in the rectosigmoid region, in IBS patients (Bashashati, Moossavi *et al.*, 2017).

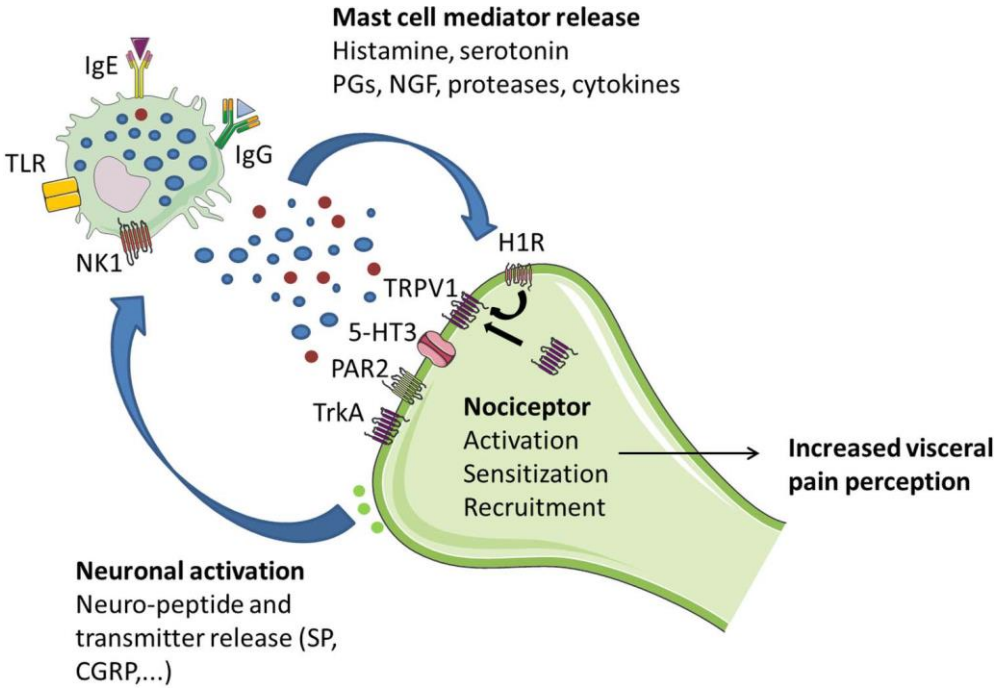



*Figure 7 - Bidirectional interaction between mast cells and enteric nociceptors (Wouters, Vicario et al., 2016)*

Mast cell mediators such as histamine and proteases are known to activate enteric nerves (Schemann, Michel *et al.*, 2005), and the close relation between mast cells and nerve endings has been linked to visceral pain (Barbara, Stanghellini *et al.*, 2004; Barbara, Wang *et al.*, 2007). The interaction between mast cells and nerves in the intestinal mucosa is a two-way process, with mast cells activating and being activated by nerves (Van Nassauw, Adriaensen *et al.*, 2007; Wouters, Vicario *et al.*, 2016), which is illustrated in Figure 7. This bi-directionality is key to understanding the link between the nervous system and gastrointestinal function. Not only chemical signalling, but also psychological stress can activate mast cells (Gue, Del Rio-Lacheze *et al.*, 1997; Overman, Rivier *et al.*, 2012). Recent unpublished yet interesting work indicates that mast cell activation in the gastrointestinal tract might also induce anxiety-like behaviour (Cordner, Liu *et al.*, 2017; Liu, Cordner *et al.*, 2017) in rats, and such psychological factors are a common occurrence in IBS, as seen in previous chapters. Since other mast cell products besides histamine, such as NGF and tryptase, can also be responsible for IBS symptoms, it makes sense that pure histamine receptor antagonists might be less useful in IBS than compounds with a general mast cell stabilizing effect. Such compounds, such as ketotifen (Klooker, Braak *et al.*, 2010) and cromolyn sodium (Stefanini, Prati *et al.*, 1992), do indeed show promise in the treatment of IBS symptoms, and can also prevent the increase in intestinal permeability following psychological stress (Vanuytsel, van Wanrooy *et al.*, 2014).

#### 1.2.2.6 Microbiota particularities/ dysbiosis

Starting with the recognition of the association of gastroenteritis, as discussed before, and the use of antibiotics as a risk factor for developing IBS (Villarreal, Aberger *et al.*, 2012), the microbiota is increasingly recognized as a possible factor in IBS. The microbiota profiles of IBS patients tend to differ from those of healthy patients (Rajilic-Stojanovic, Biagi *et al.*, 2011), and they can be used to predict the responsiveness of IBS patients to dietary intervention with a low-FODMAP diet (Bennet, Böhn *et al.*, 2017; Valeur, Smastuen *et al.*, 2018). However, because IBS is characterized by changes in bowel habits, which are very likely to influence the microbiota composition, specific differences are difficult to interpret. Additionally, it is not yet clear whether a specific microbiota pattern exists for IBS, but in general, a reduction of bacterial diversity and an increased instability have been consistently found (Collins, 2014).

It is very likely that the two-way signalling between microbiota and epithelium which can regulate secretion of mucus and other molecules involved in host-microbe interactions is involved in IBS, because IBS patients show a dysregulation of the mucus layer and β-defensin-2 peptides (Swidsinski, Loening-Baucke *et al.*, 2008; Langhorst, Junge *et al.*, 2009; Simren, Barbara *et al.*, 2013). Illustrating a role for microbiota in IBS, patients show a modulated expression of certain Toll-like receptors (TLRs), which perceive pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) for the innate immune system, indicating involvement of microbiota-immune interactions in IBS. Both IBD and IBS patients show alterations in the composition of the gut microbiota (Spiller and Lam, 2011; Casen, Vebo *et al.*, 2015), also known as 'dysbiosis', though it is not immediately clear whether this dysbiosis is cause, effect, or both.

Transit time and stool consistency directly influence microbiota composition (Vandeputte, Falony *et al.*, 2015), and both these factors are altered in IBS and IBD, making it difficult to relate microbiota differences as a cause. Additionally, microbiota composition is affected by dietary patterns (Rajilic-Stojanovic, Jonkers *et al.*, 2015), and patients often change their dietary habits in an effort to mitigate symptoms. Perhaps unsurprisingly, the measure of dysbiosis found in IBS patients is correlated to the gravity of their symptoms (Tap, Derrien *et al.*, 2017), and interestingly, these differences in microbiota composition were not correlated to diet.

It is not straightforward to clearly link certain phyla or species of bacteria to IBS, but certain trends have been consistently observed; such as a depletion of Bifidobacteria in both faecal and mucosal microbiota (Malinen, Rinttila *et al.*, 2005; Kerckhoffs, Samsom *et al.*, 2009). Duplicate HITChip microarray analyses of faecal samples of IBS patients and healthy controls in Finland showed that IBS patients had 1.5-fold decreased Bifidobacteria counts, and a 2-fold increased ratio of Firmicutes/Bacteroidetes (Rajilic-Stojanovic, Biagi *et al.*, 2011). Additionally, a Japanese study showed that a pooled group of IBS-subtypes had significantly higher counts of *Veillonella* and *Lactobacillus* than healthy controls (Tana, Umesaki *et al.*, 2010).

That it is possible for the microbiota to have a causative effect in generating IBS symptoms has been shown in animal experiments. Inoculating germ-free rats with a microbiota from IBS patients renders them hypersensitive to colorectal distension, compared to conventional rats, but being inoculated with healthy human microbiota does not have this effect (Crouzet, Gaultier *et al.*, 2013), and similarly, a decreased transit time, impaired intestinal permeability, and anxiety-like behaviour can be transferred to germ-free mice by inoculating them with IBS-D patient microbiota (De Palma, Lynch *et al.*, 2017), results which indicate a possible causative effect of a disorder-associated microbiota profile.

#### 1.2.2.7 Small intestinal bacterial overgrowth

Small intestinal bacterial overgrowth (SIBO) is a syndrome often linked to IBS. It consists of an increased number of bacteria in the small bowel, defined as numbers over $1*10^3$cfu/ml proximal jejunal contents (Khoshini, Dai *et al.*, 2008), while the small bowel should normally contain small bacterial populations (Bouhnik, Alain *et al.*, 1999; Bures, Cyrany *et al.*, 2010). The expansion of bacteria from the large into the small intestine is held responsible for such symptoms as bloating, abdominal discomfort, and changes in stool form (Pimentel and Lezcano, 2008), which is reminiscent of IBS, but with an organic cause. However, a higher proportion of diagnosed IBS patients than healthy controls are reported to present with SIBO (Lupascu, Gabrielli *et al.*, 2005), which makes it questionable whether they actually have true IBS, or it might indeed indicate a causative role for SIBO in IBS, depending on interpretation.

This possible causal relationship between SIBO and (all cases of) IBS has been hotly contested, among other reasons, because there are issues with the methods used to characterize SIBO; the lactulose hydrogen breath test (LHBT) to determine abnormally early carbohydrate fermentation is flawed, and it has recently been shown that in most (88%) of the IBS patients for who the test is positive, the bolus was already in the caecum, as measured by oro-caecal scintigraphy (Yu, Cheeseman *et al.*, 2010). Additionally, bacterial numbers in the jejunum used to determine severity of SIBO do not seem to correlate to IBS symptom severity (Grover, Kanazawa *et al.*, 2008). In short, it is unlikely that SIBO has a clear causal role to play in IBS (Spiegel, 2011).

It is often beneficial for patients to exclude lactose from the diet and to reduce other simple sugars (Bures, Cyrany *et al.*, 2010), which resembles the low-FODMAP diet in form and function, i.e., lower the amount of available fermentable carbohydrates to the gut microbiota. SIBO is often mis- or underdiagnosed due to the wide range of clinical manifestations, and understanding its mechanisms better will hopefully lead to future improvements to patient care (Sachdev and Pimentel, 2013).

We will talk more about the functions of the microbiota in a later chapter (1.3) devoted to the matter.

#### 1.2.2.8 Biomarkers IBS

To better characterize patients, and to facilitate diagnosis, a lot of effort has been targeted to identify biochemical markers (biomarkers) for IBS and its subgroups, with varying success. Though IBS is increasingly seen as having organic characteristics, its history as a functional disorder has not yielded much in the way of biomarkers. However, there are some promising developments to help determine appropriate treatment of patients; microbiota profiles can apparently serve to predict responsiveness to dietary intervention (Bennet, Böhn *et al.*, 2017), and genetic markers can be used to predict responsiveness to certain drugs (Enck, Aziz *et al.*, 2016). Additionally, technical advancements such as the pressure-, temperature-, and pH-sensitive SmartPill (Given Imaging Ltd, Yoqneam, Israel) can be used to get detailed insights into gastrointestinal motility of patients (Rao, Kuo *et al.*, 2009), which can be used for patient characterisation and help to identify targets for antispasmodics (Enck, Aziz *et al.*, 2016). Another possible approach is the use of cytokine profiles (Bennet, Polster *et al.*, 2016), though as of yet, there are no biomarkers that can be used to reliably diagnose IBS.

### 1.2.3 Clinical practice

Treatment of IBS is complicated, with many treatments not sufficiently effective. Because the underlying causes for IBS are not well understood, it has proven difficult to design evidence-based therapies with a clear mechanism of effect. Additionally, scientific underpinning for treatment of functional disorders in general suffers from a high placebo effect to treatment.

Diagnosis of IBS depends mostly on excluding any other cause for the symptoms. Diagnostic algorithms for diagnosis of IBS must therefore make sure that there are no more serious origins of complaints (Enck, Aziz *et al.*, 2016).

What follows now is an oversight of current clinical practice in IBS patients.

#### 1.2.3.1 IBS – Medication

Several pharmaceutical treatments of IBS have been developed over the years, mostly aimed at tackling one or the other symptom, in accordance with the functional nature of the syndrome. To treat the constipation in a subset of IBS patients, laxatives can be effective, and the laxative and analgesic linaclotide has proven useful in treating constipation in IBS (Chey, Lembo *et al.*, 2012), while at the same time reducing pain by activating epithelial guanylate cyclase-C receptor (GC-C), which increases extracellular cGMP, thereby reducing nociceptor sensitivity (Castro, Harrington *et al.*, 2013). To reduce painful smooth muscle spasms, common in IBS, antispasmodic drugs are used. However, these drugs can cause constipation, and should not be tried in already constipated patients (Enck, Aziz *et al.*, 2016). One such antispasmodics is peppermint oil, which blocks calcium channels, thereby inhibiting contractions (Hawthorn, Ferrante *et al.*, 1988). It is specifically effective when administered in capsules which release throughout the small intestine (Cash, Epstein *et al.*, 2016). It follows from what we know about the involvement of mast cells in IBS, that mastocyte stabilizers can be expected to have beneficial effects in IBS. An animal IBS model inducing visceral sensitivity in rats by partial restraint stress (PRS), shows that mast cell stabilizer doxantrazole suppresses stress-induced visceral hypersensitivity (Gue, Del Rio-Lacheze *et al.*, 1997). Similarly, an animal IBS model using chemical induction of visceral hypersensitivity by acetic acid-induced colitis shows that the same mast cell stabilizer can significantly attenuate the hypersensitivity (La, Kim *et al.*, 2004). In humans, trials with anti-inflammatory drugs have proven more ambiguous, with mast-cell stabiliser ketotifen showing promise in reducing visceral hypersensitivity and improving QOL (Klooker, Braak *et al.*, 2010), but mesalazine, another mast cell stabilizer, no better than

placebo, after a pilot study initially yielded hopeful results (Corinaldesi, Stanghellini *et al.*, 2009; Barbara, Cremon *et al.*, 2016).

#### 1.2.3.2 IBS – Probiotics

Because of the often-cited link to the intestinal microbiota, there is a significant interest in treating IBS with probiotics. Oral probiotics are defined as 'live micro-organisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition' (Guarner and Schaafsma, 1998), and later by the WHO, very similarly, as 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host' (Joint FAO/WHO Working Group, 2002) although it has to be considered that non-live microbial preparations and subcellular components are often sufficient to induce beneficial effects (Collins, 2014).

In a maternal deprivation early life stress rat model of IBS, and similarly in the more acute partial restraint stress (PRS) model, it has been clearly shown that certain probiotics are superior to others in preventing the increase in gut permeability and sensitivity induced by these stressors. *Lactobacillus paracasei* NCC2461, but not *Bifidobacterium lactis* NCC362 or *Lactobacillus johnsonii* NCC533, was effective in ameliorating gut health impacted by stress, showing both that probiotics can be effective in these cases, and that this efficacity is strain-specific (Eutamene, Lamine *et al.*, 2007). Similarly, a recent *in vitro* study has shown that potential probiotic strains of lactic acid bacteria (LAB) can modulate goblet cell gene expression in a treatment time- and strain-specific manner (Ren, Dokter-Fokkens *et al.*, 2018). Extrapolation of these results to *in vivo* situations would indicate that these LAB can exert an effect on the gut barrier function by modulating mucus-related genes. Interpretation is complicated due to the strong fluctuations, both positive and negative, of gene expression profiles on short-scale treatment time differences reported in this paper. This makes it entirely unclear how to translate such findings to an *in vivo* setting, in which exact exposure time cannot be controlled, but progresses naturally.

A meta-analysis of randomised controlled trials (RCTs) of probiotic treatment of IBS patients showed a general positive effect, in contrast to prebiotics and synbiotics, which were not shown to be effective (Ford, Quigley *et al.*, 2014). However, these kinds of meta-analyses often cite a lack of high-quality data, whilst claiming a positive effect for probiotics, according to a meta-analysis from a year later, identifying reasons for difficulties to reach a strong overall conclusion on the efficacity of probiotics in treating IBS (Mazurak, Broelz *et al.*, 2015). They found that the grouping of different probiotic species and strains as 'probiotics' is a major hurdle to a strong

conclusion (Mazurak, Broelz *et al.*, 2015), which supports the idea of strong variability between strains and species.

Additionally, to better treat IBS patients with probiotics, it would be helpful to first analyse their microbiota and thereby characterise their possible dysbiosis. In a patient for which the bacterial function to be supplemented is not lacking, doing so should not be expected to bring about significant improvement, and no normalisation of microbiota function should be expected by this approach.

It is not clear that the efficacy of probiotics indicates an important role for the microbiota in IBS, because oral administration of probiotics can be viewed as simply delivering substances of therapeutic value (of microbial origin), with little effect on the host microbiota. Upon reaching the colon, where the majority of the intestinal microbiota resides, the concentration of probiotics alive after passing through the preceding gastrointestinal tract is orders of magnitude less abundant than the endogenous bacteria, and after stopping treatment with probiotics, the comparatively small effect on microbiota composition is soon lost (Collins, 2014).

#### 1.2.3.3 IBS – Diet

Around 60% (Brandt, Chey *et al.*, 2009) to 82% (Bohn, Storsrud *et al.*, 2013) of IBS patients say that their symptoms worsen with the intake of certain foods, which, together with the inadequacy of pharmaceutical treatments, has led to the development of dietary interventions. A common dietary intervention aims to increase the dietary fibre intake, often through supplementation, to improve bowel movements (Enck, Aziz *et al.*, 2016). However, these interventions have often not shown a convincing increased effect over placebo diet treatments, and a meta-analysis of 14 randomised controlled trials (RCTs) yielded a weak recommendation of these kinds of treatments in IBS patients (Ford, Moayyedi *et al.*, 2014). Additionally, it is noted that fibres can lead to increased fermentation in the intestines, leading to production of gases, a variety of other metabolites, as well as increasing the microbial population (Flamm, Glinsmann *et al.*, 2001; Eswaran, Muir *et al.*, 2013). These effects of increased dietary fibre intake can be problematic in IBS patients, the corresponding increase in distension can be painful in patients with increased visceral sensitivity. One systematic review found that they can be beneficial in some patients, but ineffective or even worsen symptoms in other patients, with especially insoluble fibres proven problematic (Bijkerk, Muris *et al.*, 2004) shown also by a randomised placebo controlled trial, where (poorly fermentable) soluble psyllium proved useful, while insoluble bran proved problematic enough for patients to drop-out of the trial at an increased rate (Bijkerk, De Wit *et al.*, 2009). On the other hand, fermentable soluble fibres have also been linked to gastrointestinal troubles, such as bloating, flatulence, and painful cramps. Fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) for example, are rapidly fermented in the terminal ileum and early colon under production of a lot of gas which can induce gastrointestinal symptoms in IBS patients (Eswaran, Muir *et al.*, 2013). It is too broad to state the efficacy of fibres in general, because their properties are not uniform; fermentable or non-fermentable, soluble or insoluble.

Currently, dietary advice most often dispensed are the 'traditional IBS diet', and the 'low-FODMAP diet'. A traditional IBS dietary approach generally calls for increased attention to when and how to eat rather than focusing on specific dietary choices (Böhn, Störsrud *et al.*, 2015). Nicely summarised in this paper: "the participants were instructed to regularly eat 3 meals and 3 snacks a day, never too much or too little each time, never to be hungry or too full; to eat in peace and quiet and to chew thoroughly; reduce intake of fatty or spicy foods, coffee, alcohol, onions, cabbage, and beans; avoid soft drinks and carbonated beverages, chewing gums, and sweeteners that end with -ol, and to eat fibres but distribute the intake evenly during the

day." (Böhn, Störsrud *et al.*, 2015). The low-FODMAP diet calls for avoidance of 'fermentable oligo-, di-, mono-saccharides and polyols', which are represented by dietary carbohydrates such as fructose, galactans, and mannitol; for an extensive list of prevalent foodstuffs containing sizeable amounts of FODMAPs, see Table 5.

It is noteworthy that both diets call for avoidance of soft drinks, which are generally rich in fructose, vegetables rich in fermentable fructans and galactans, and polyol-type sweeteners. The low-FODMAP diet is sometimes claimed to work better than traditional IBS dietary advice (Staudacher, Whelan *et al.*, 2011), while a more recent study claims they are equally effective (Böhn, Störsrud *et al.*, 2015). Of course, it is difficult to control for pre-existing dietary differences between trials performed in different cultures, or the differences in advice provided by different dieticians, to make a real assessment on the efficacity of a low-FODMAP diet versus a traditional IBS diet.

*Table 5 - Examples of foods rich in specific FODMAPs (adapted from (Gibson and Shepherd, 2010))*

| FODMAP | Excess fructose | Lactose | Oligosaccharides (fructans and/or galactans) | Polyols |
|---|---|---|---|---|
| Problem high FODMAP food source | *Fruits:* apples, pears, nashi pears, clingstone peaches, mango, sugar snap peas, watermelon, tinned fruit in natural juice<br><br>*Honey*<br><br>*Sweeteners:* fructose, high fructose corn syrup<br><br>*Large total fructose dose:* concentrated fruit sources; large serves of fruit, dried fruit, fruit juice | *Milk:* cow, goat and sheep (regular & low-fat), Ice cream<br><br>*Yoghurt* (regular & low-fat)<br><br>*Cheeses:* soft & fresh (e.g. ricotta, cottage) | *Vegetables:* artichokes, asparagus, beetroot, Brussels sprout, broccoli, cabbage, fennel, garlic, leeks, okra, onions, peas, shallots.<br><br>*Cereals:* wheat & rye when eaten in large amounts (e.g. bread, pasta, couscous, crackers, biscuits)<br>*Legumes*: chickpeas, lentils, red kidney beans, baked beans<br>*Fruits:* watermelon, custard apple, white peaches, rambutan, persimmon | *Fruits:* apples, apricots, cherries, longon, lychee, nashi pears, nectarine, pears, peaches, plums, prunes, watermelon<br>*Vegetables:* avocado, cauliflower, mushrooms, snow peas<br><br>*Sweeteners:* sorbitol(420), mannitol(421), xylitol(967), maltitol (965), isomalt (953) & others ending in '-ol' |

### 1.2.4 FODMAP diet

As already introduced before, in recent years a low-FODMAP diet has been successfully used to reduce symptoms of IBS, with a proposed mechanistic basis of effect (Gibson and Shepherd, 2010; Halmos, Power *et al.*, 2014). Because it is the main target of investigation in this thesis, we will take a more in-depth look into this diet and the expected effects, positive and/or negative.

#### 1.2.4.1 Theoretical background; list of FODMAPS/ diet specifics

To begin with, many symptoms of IBS, principally abdominal pain, but also bloating, are noted in response to luminal distension. Gibson and Shepherd proposed that minimizing the consumption of those dietary compounds that lead to distension of the intestine would lead to improvement of symptoms of FGIDs. A group of dietary components the same authors had previously coined 'FODMAPs', for Fermentable Oligo-, Di-, Mono-saccharides And Polyols (Gibson and Shepherd, 2005) have those properties that can lead to distension; they are poorly absorbed in the small intestine, osmotically active, and are rapidly fermented by the gut microbiota upon reaching the colon (Gibson and Shepherd, 2010), see Figure 8. It is generally claimed that FODMAPs themselves are not responsible for symptoms in IBS patients, but induce adverse effects due to inherent responses in these patients (Molina-Infante, Serra *et al.*, 2016).

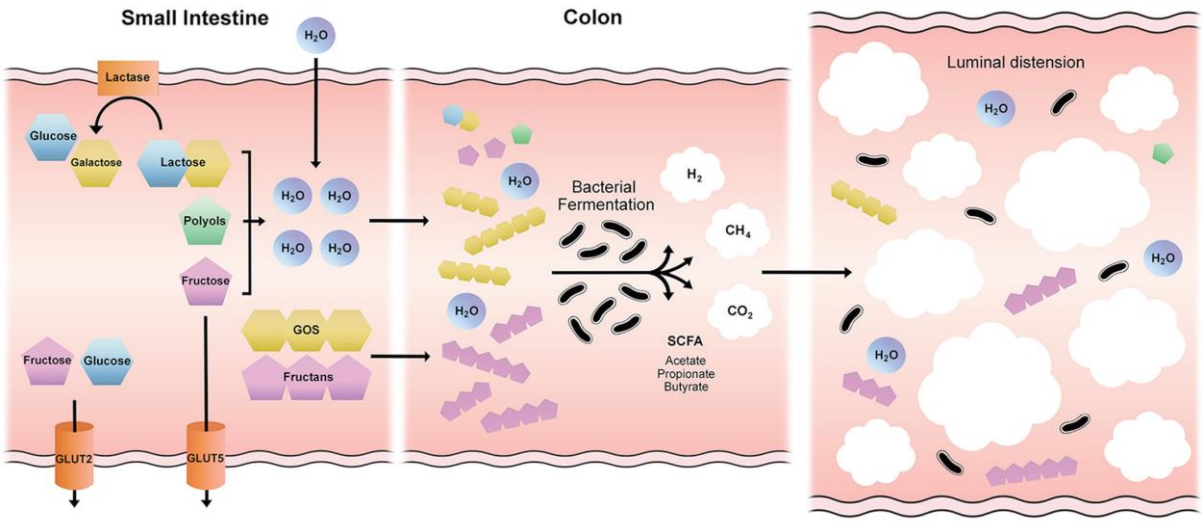



*Figure 8 - Mechanisms by which FODMAPs can induce gastrointestinal symptoms. In the small intestine, unabsorbed FODMAPs can increase small intestinal water, thereby causing distension. In the large intestine, fermentation of FODMAPs leads to production of gases and ultimately luminal distension, and SCFAs. (Staudacher and Whelan, 2017)*

*Table 6 - Trials investigating the efficacy of a low-FODMAP diet*

| *Ref* | Study design | Participants | N | Duration | Symptom scoring | Findings |
|---|---|---|---|---|---|---|
| | ***Controlled trials*** | | | | | |
| *(Staudacher, Lomer et al., 2016)* | Placebo-controlled dietary advice RCT (single blind) | Rome III IBS-D, IBS-M, IBS-U | LFD n=51<br>Sham diet n=53 | 4 weeks | AR, IBS-SSS, IBS-QOL | Primary outcome: No difference in AR (LFD 57% vs control 38%; p=0.051)<br>Secondary outcomes: Lower IBS-SSS score (LFD 173 vs control 224; p=0.001) and greater numbers achieving MCID for IBS-QOL (LFD 51% vs control 26%; p<0.023) |
| *(Staudacher, Lomer et al., 2012)* | Dietary advice RCT (unblind) | Rome III IBS with bloating or diarrhoea | LFD n=19<br>Habitual diet n=22 | 4 weeks | AR, GSRS, Bristol Stool Form | Primary outcome: Lower luminal abundance of Bifidobacteria<br>Secondary outcomes: Greater numbers reporting AR (LFD 68% vs control 23%; p=0.005) Lower bloating, borborygmi, overall symptoms LFD versus control (p<0.05) Greater number of normal stools (LFD 24% vs control 7%; p=0.02) |
| *(Schultz, Harvie et al., 2013)* | Dietary advice RCT (unblind) | Rome III IBS | LFD n=23<br>Waiting list n=27 | 3 months | IBS-SSS, IBS-QOL | Outcomes: Greater reduction in IBS-SSS (LFD 276 to 129 pt. vs control 247 to 204 pt.; p<0.01), frequency of pain episodes (p<0.01) Greater increase in IBS-QOL score for LFD versus control (p<0.0001) |
| *(Pedersen, Andersen et al., 2014)* | Dietary advice RCT (unblind) | Rome III IBS | LFD n=42<br>Probiotic n=41<br>Habitual diet n=40 | 6 weeks | IBS-SSS, IBS-QOL | Primary outcome: Greater reduction in IBS-SSS (LFD −75 pt. vs control −32 pt.; p<0.01)<br>Secondary outcome: No change in IBS-QOL for all groups |
| *(Halmos, Power et al., 2014)* | Placebo-controlled feeding RCT, crossover (single blind) | Rome III IBS | LFD n=27,<br>Typical diet n=27 | 21 days | 100 mm symptom VAS, Stool frequency, Stool water content | Primary outcome: Lower overall GI symptoms (LFD 23 mm vs control 45 mm; p<0.001). Secondary outcome: Lower stool frequency in IBS-Din LFD versus control |
| | ***Comparative trials*** | | | | | |
| *(McIntosh, Reed et al., 2017)* | Dietary advice RCT (single blind) | Rome III IBS | LFD n=20, HFD n=20 | 3 weeks | Responder: ≥50 pt. reduction IBS-SSS | Primary outcome: area under the curve for lactulose breath test<br>Secondary outcomes: Greater number of responders (LFD 72% vs HFD 21%; p<0.009) Lower IBS-SSS in LFD versus HFD (p=0.01) |
| *(Hustoft, Hausken et al., 2017)* | Dietary advice RCT, crossover (double blind) | Rome III, IBS-D, IBS-M | LFD+placebo n=20<br>LFD+fructans n=20 | 6 weeks | IBS-SSS, 100 mm symptom VAS | Outcomes: Lower IBS-SSS (LFD 80% vs control 30%; p=0.014) and severity of nausea/vomiting, belching, flatulence in LFD versus control (p<0.05) |
| *(Eswaran, Chey et al., 2016)* | Dietary advice RCT (unblind) | Rome III IBS-D | LFD=45<br>Modified NICE guideline n=39 | 4 weeks | Responder: AR ≥50% of weeks 3,4; Composite pain and stool score | Primary outcome: No difference in numbers of responders (LFD 52% vs control 41%; p=0.31) Secondary outcomes: No difference in those achieving composite score endpoint (LFD 27% vs control 13%; p=0.13) Greater reduction in pain (LFD 51% vs control 23%; p=0.008) |
| *(Peters, Yao et al., 2016)* | Dietary advice RCT (unblind) | Rome III IBS | LFD n=24<br>Hypnotherapy n=25<br>LFD + hypnotherapy n=25 | 6 weeks | Responder: ≥20 mm VAS improvement in symptoms, IBS-QOL | Primary outcome: No difference in numbers of responders (LFD 71% vs hypnotherapy 72% vs combination 72%; p=0.67).<br>Secondary outcomes: Lower symptom severity in LFD and hypnotherapy versus baseline (p<0.05) and higher IBS-QOL scores in all groups compared with baseline (p<0.001) but no differences between groups for symptoms or IBS-QOL |
| *(Böhn, Störsrud et al., 2015)* | Dietary advice RCT (single blind) | Rome III IBS | LFD n=38<br>NICE guideline n=37 | 4 weeks | Responder: ≥50 pt. reduction IBS-SSS, Stool frequency and consistency | Primary outcome: No difference in number of responders (LFD 50% vs control 46%; p=0.72) |

AR, adequate relief; FFQ, Food Frequency Questionnaire; GSRS, Gastrointestinal Symptom Rating Scale; HFD, high FODMAP diet; IBS-QOL, IBS Quality of Life Questionnaire; IBS-SSS, IBS Severity Scoring System; LFD, low FODMAP diet; MCID, minimal clinically important difference; NICE, National institute for Health and Care Excellence; RCT, randomised controlled trial; VAS, visual analogue scale.
*Adapted from Staudacher and Whelan, 2017 (Staudacher and Whelan, 2017)*

However, these dietary components can induce gastrointestinal problems in healthy persons too. A monosaccharide FODMAP, fructose, can lead to a variety of gastrointestinal symptoms,

such as diarrhoea, cramps and flatulence (Ravich, Bayless *et al.*, 1983; Major, Pritchard *et al.*, 2017), but fructose malabsorption is ameliorated when fructose is taken together with glucose, due to the facilitating effect to small intestinal absorption of these two compounds together which prevents symptom generation (Truswell, Seach *et al.*, 1988). These days, fructose is consumed at increasing doses as a result of the growing usage of high-fructose corn syrup in many products, likely increasing the concentration reaching the colon for microbial processing. In lactase non-persistent persons, the disaccharide FODMAP lactose reduces the oro-caecal transit time, independently from the increased luminal distension of the small intestine induced by the osmotically active lactose (He, Venema *et al.*, 2008). Instead, it is claimed that colonic fermentation of lactose in maldigesters could be partly responsible for symptoms of lactose intolerance (He, Priebe *et al.*, 2006). In relatively high doses, inulin, a fructo-oligosaccharide, can lead to gastro-intestinal symptoms such as flatulence (Pedersen, Sandström *et al.*, 1997; Major, Pritchard *et al.*, 2017). Sorbitol, a polyol FODMAP, too is known to induce gastrointestinal symptoms at higher doses (>10 g) (Hyams, 1983). However, simultaneous malabsorption of modest doses of multiple of these compounds is relatively rare (Ladas, Grammenos *et al.*, 2000), as measured by the occurrence of gastrointestinal symptoms, showing that generally, reasonable doses are tolerated in healthy subjects. As mentioned before, the low-FODMAP diet intends to reduce the intake of these compounds (see Table 5), to lessen intestinal distension, and thus, lessen the provocation of symptoms in IBS patients. The diet has shown promising results since it has been introduced, see Table 6.

Generally, these studies show that the low-FODMAP is effective in reducing IBS symptoms, though it is not superior to NICE guidelines (traditional IBS diet) (Böhn, Störsrud *et al.*, 2015), or hypnotherapy (Peters, Yao *et al.*, 2016). Although another study shows an increased effect on abdominal pain, bloating, stool consistency, stool frequency, and urgency in IBS-D patients, compared to modified NICE guidelines, but not on general adequate relief (AR) (Eswaran, Chey *et al.*, 2016). A meta-analysis from 2015 covering 6 RCT and 16 non-randomized interventions supports the efficacy of low-FODMAP diet compared to non-treatment, with pooled odds ratios for significant improvement of IBS-SSS questionnaire scores of 0.44 for RCTs and 0.03 for the non-randomized trials (Marsh, Eslick *et al.*, 2016). Generally, we can conclude that the low-FODMAP diet delivers a significant improvement to the situation of IBS patients.

#### 1.2.4.2 Possible adverse effects low-FODMAP diet

In most cases, the low-FODMAP approach calls for drastic changes to the day-to-day diet. Here, we will look at the possible adverse effects of following a low-FODMAP diet.

First, since FODMAPs are fermentable, they have a prebiotic function, meaning that they provide the intestinal microbiota with nutrients, and therefore support the bacterial populations in the gut. Drastically reducing these compounds logically influences the composition and density of the intestinal microbiota. Because of this, the main concern with the low-FODMAP approach are unintended long-term consequences to the intestinal microbiota (Halmos, Christophersen *et al.*, 2015), as well as possible nutritional deficiencies due to the restrictive diet (Staudacher and Whelan, 2017), although in a comparative study, only calcium intake was slightly impacted, due to the reduced intake of lactose-containing dairy products (Staudacher, Lomer *et al.*, 2012). However, since a low-FODMAP diet is supported by a professional dietician, we can expect nutritional sufficiency to be better safeguarded than in patients who try to adapt their diet by themselves to prevent adverse events; which is common among IBS patients (Monsbakken, Vandvik *et al.*, 2006).

Concerning microbiota composition, several studies have been undertaken, showing a low-FODMAP diet can be responsible for a reduction in Bifidobacteria (Staudacher, Lomer *et al.*, 2012; Halmos, Christophersen *et al.*, 2015; McIntosh, Reed *et al.*, 2017; Staudacher, Lomer *et al.*, 2017), *F.prausnitzii, Clostridium* cluster IV, and total bacterial numbers (Halmos, Christophersen *et al.*, 2015). However, it is still unclear whether there are health effects to these changes and what these would be, though Bifidobacteria are generally considered to be beneficial, and have been shown to already be reduced in untreated IBS patients (Malinen, Rinttila *et al.*, 2005; Kerckhoffs, Samsom *et al.*, 2009; Rajilic-Stojanovic, Biagi *et al.*, 2011). The patients in these studies were not on specified diets, so it is possible that they have lower Bifidobacteria counts due to evasion of prebiotic FODMAPs on their own initiative.

Apart from effects on the composition of the microbiota, a reduction in supposedly beneficial bacterial metabolites, such as short-chain fatty acids (SCFA) is an additional possible concern.

Technical difficulties and conflicting results have left this consideration to be clarified for the time being. Faecal SCFA concentrations have been shown to be actually higher in IBS patients than in control patients, with total SCFA, propionic acid, and acetic acid significantly increased, which was correlated with IBS symptoms (Tana, Umesaki *et al.*, 2010), although the direction of the possible causation is not clear. In animal experiments however, butyrate enemas can induce a visceral hypersensitivity in rats mediated by CGRP receptor, offering a non-inflammatory hypersensitivity model of IBS, according to the authors (Bourdu, Dapoigny *et al.*, 2005). Conversely, SCFA produced by bacteria play an important role in host energy provision (Bergman, 1990), enterocyte energy provision in particular (Basson, Turowski *et al.*, 1996), and colonic $T_{reg}$ cell homeostasis (Smith, Howitt *et al.*, 2013) among other important functions. Consistent with the decreased intake of prebiotic material, faecal SCFA concentrations are reduced in IBS-M and IBS-D patients on a low-FODMAP diet (Hustoft, Hausken *et al.*, 2017).

However, it has to be noted that faecal SCFA analysis is not a reliable method, because it reflects colonic absorption better than production (Cook and Sellin, 1998; Sakata, 2018), and is heavily influenced by transit time (Ringel-Kulka, Choi *et al.*, 2015), which we know can be highly variable, in particular between IBS patients with either diarrhoea or constipation, and healthy patients. In short, without measuring SCFA concentrations where they are produced, mostly in the ascending colon, it will not be possible to clearly identify the link between SCFA concentrations, IBS symptoms, and possible effects of the low-FODMAP diet (Staudacher and Whelan, 2017), so most of these results can be interpreted as a mix of artefacts of sampling method and less than useful correlations.

## 1.3 Intestinal microbiota

The intestinal microbiota plays a vital role in vertebrate health. In this chapter we will look into the many functions the gut microbiota has, but also how it can be co-responsible for some health issues.

### 1.3.1 Function

Our gastrointestinal tract is populated by microbes whose numbers, complex population composition, and ecology boggle the mind. They do not limit themselves to being impressive though; they are important in digestion, immunity, and resistance to pathogens as well.

The gut microbiota is needed for the correct development of innate and adaptive immunity, the integrity of the intestinal epithelium, provision of the host with both energy sources and vitamins, prevention of colonization of our gastrointestinal tract by pathogenic microbes as well (Dave, Higgins *et al.*, 2012). Research into the microbiome has become more and more translational in recent years, now driving investments coming from *big pharma* and other private institutions to benefit from the many expected applications of knowledge to treat health conditions with probiotics, as well as of identification of useful microbiota diagnostic biomarkers (Gilbert, Blaser *et al.*, 2018).

#### 1.3.1.1 Symbiosis; never alone

The human gut microbiota has evolved together with us, and due to this long-shared history, there is a high level of symbiosis. Vertebrates benefit greatly from their microbiota, although, as shown in the possibility of rearing germ-free animals, it is not essential in the strict sense. Normally, we are inoculated by gut microbes during natural birth (Mandar and Mikelsaar, 1996), a process which does not take place during Caesarean section, with possible ramifications to later health (Di Mauro, Neu *et al.*, 2013; Jakobsson, Abrahamsson *et al.*, 2014). A healthy microbiota contains many different phylogenetic types; molecular analysis of stool samples of 124 subjects shows that there are over 1000 bacterial species associated to the human microbiota, with at least 160 species per individual (Qin, Li *et al.*, 2010). This large microbial diversity contains many unique genes, which, by modest estimation, contains at least 9 million unique genes (Yang, Xie *et al.*, 2009) though this relates to the totality of known gut microbiota species, which are not all present in every individual. Still, seeing as the human genome contains a maximum of approximately 39 thousand genes (including hypothetical and unannotated genes) (Venter, Adams *et al.*, 2001), the bacterial genes associated to our gut microbiota are at least 200 times more abundant than eukaryotic host genes. We have to keep in mind that most of these genes regulate normal functioning of bacterial lifecycles and metabolism and that many are homologous and/or analogous between different species and strains, indicating a substantial redundancy. It is often claimed that there are 10 times more bacterial cells in the intestines than there are animal cells in our bodies (Savage, 1977), a claim that is eagerly repeated in many article introductions. However, this number is based on two very rough estimates for host and microbial cell numbers. In 2016, using more informed calculations, it has been estimated that the ratio is closer to 1:1,3, wherein bacteria still outnumber host cells (Sender, Fuchs *et al.*, 2016). They have not recalculated the number of host cells, which might still change this ratio.

What drives the relation between host and microbe is a common interest in metabolism as a requisite for life. The host provides the microbiota with nutrients by its animal mobility and motivations, as well as a highly beneficial anaerobic, warm, and humid environment represented by the gastrointestinal tract. Our gut and its microbial ecology are so suitable for the guest organisms that we can cultivate the majority of gut microbiota exclusively inside of ourselves. However, considerable advancements have recently been made on the cultivability of gut bacteria, based on the extensive sporulation taking place. This sporulation and the subsequent

reactivation under suitable conditions is key to the colonisation of and transit through inhospitable environments (Browne, Forster *et al.*, 2016).

A good host always makes sure that their guests are well-fed, even at a possible cost to themselves. A very nice example of this is reported in a study from 2014, where it is shown that animals suffering from an infection and going through a temporary anorexia to deal with this infection will increase fucosylation of the intestinal epithelium to support the commensal bacteria, which would otherwise have no energy provision (Pickard, Maurice *et al.*, 2014). This underlines that the host is a pro-active actor in their relationship with the gut microbiota.

Genetics and co-evolution play a role in host-microbe interactions, already visible in the exclusivity of bacterial capabilities to colonize mammals, indicating the necessity of a certain suitable set of traits. Within our species, genetical differences between people can influence which microbes feel at home, though this effect is limited; there is a small but significant increase in similarity in microbiota composition between homozygotic twins compared to dizygotic twins (Goodrich, Waters *et al.*, 2014).

#### 1.3.1.2 Metabolism; collecting the rent

Of course, a host does not always accept guests without expecting something in return. The microbiota pays us back by indispensable metabolic processes, digesting components which are indigestible to us, producing simpler molecules that we can use. Additionally, the gut microbiota is an important source of vitamins, such as riboflavin (vitamin $B_2$), biotin (vitamin $B_7$), folic acid (vitamin $B_9$), cobalamin (vitamin $B_{12}$), and vitamin K (Hill, 1997). In herbivore animals, cellulose and other cell wall components are processed by microbes to provide otherwise unavailable energy, particularly important in ruminants (Flint, 1997), but also in omnivores such as humans, the microbiota plays an important role in breaking down dietary carbohydrates (Cockburn and Koropatkin, 2016). Interestingly, the mucus produced by the host supports a metabolic network based on mucolytic activities of bacteria such as *Akkermansia muciniphila* that produces butyrate and vitamin $B_{12}$ as well (Belzer, Chia *et al.*, 2017). Butyrate produced by the microbiota is an important source of energy especially for colonocytes, who forego glucose from the blood in favour of butyrate produced by the microbiota (Roediger, 1980), other short-chain fatty acids produced are also absorbed and transported to be metabolised by rest of the body (den Besten, van Eunen *et al.*, 2013). It is estimated that in humans, 6 to 10 per cent of energy requirements are provided by microbiota-derived SCFAs, based on the intake of a standard British diet (McNeil, 1984; Bergman, 1990), the authors point out however, that fermentable carbohydrate consumption differs greatly around the world, and therefore these energy contributions must vary substantially as well (Bergman, 1990).

The metabolites produced during microbial processing of intestinal contents are not only utilised by the host, but a complex ecology exists between different microbes as well. Many species are dependent on other species with complementary activities, and in this way, a complex metabolic network forms with mutualistic, amensalistic, or parasitic relationships between different species, where mutualism in the gut is particularly increased under anoxic conditions (Heinken and Thiele, 2015). Depending on the variety of resources they can utilize, species can be classified as either generalists or specialists. While we would normally expect generalists to be widely distributed and specialists to occur mostly in smaller niches (Cockburn and Koropatkin, 2016), in the gut, this does not necessarily hold true (Carbonero, Oakley *et al.*, 2014), with specialists often being more widely distributed than generalist species.

#### 1.3.1.3 House rules; reciprocal control

In the relation between the host and the intestinal microbiome, both actors influence each other and exert a modicum of control. Under healthy conditions, the host enforces sterility where it is necessary, and supports the microbiota where desired. The microbiota in its turn, communicates with the host to support immune tolerance and optimal conditions to its prosperity.

The microbiota, through their gene expression and products, but also metabolites such as SCFAs, influences the host immune cells and cytokine production (Geuking, Koller *et al.*, 2014), while it itself is subject to change by host-produced antimicrobial peptides and IgA secretions, which have regulatory functions, which can be, but are not always, microbicidal (Neish, 2009). The innate and adaptive immunity both play a significant role in monitoring and regulating the microbiota. For the innate immune system, some of these are produced independently from circumstances, such as α- and β-defensins (Pütsep, Axelsson *et al.*, 2000), and Crohn's disease patients exhibit a defect in α-defensin production by Paneth cells, underlining its important function (Wehkamp, Salzman *et al.*, 2005),the abundance of other secretions, such as C-type-lectin Reg3γ, is regulated by the activity of pattern recognition receptors (PRRs) (Gong, Xu *et al.*, 2010), important regulators of innate immunity. Reg3γ is poorly expressed in germ-free mice, and introduction of a mixed mouse microbiota increases this expression 20-fold, related to the ability of the bacteria to reach the epithelium (Cash, Whitham *et al.*, 2006), indicating the involvement of epithelial sensing, most likely through PRRs. Many of these PRRs belong to the toll-like receptor family (TLRs), but Nod1 and Nod2, as well as several other types of receptors are important PRRs too. Many PRRs are activated by microbe-associated molecular patterns (MAMPs), which are represented among others by lipopeptides, lipoproteins, flagellins, and lipopolysaccharides; i.e., molecules that indicate the presence of microbes (Medzhitov and Janeway, 2002). Upon recognition of such a molecule, the PRR is activated and, depending on which PRR it is, activates regulatory pathways such as the mitogen-activated protein kinase (MAPK), nuclear factor κB (NF-κB)/Rel pathways, or the inflammasome (Neish, 2009). PRRs can be present on the cell membrane, on endosomes, or in the cytoplasm (Neish, 2009). In most tissues, PRRs activate inflammatory pathways, but because of the high microbial presence in the gut, PRRs there are also used to retain tolerance. For example, while activation of basolaterally positioned TLR9 leads to NF-κB activation by degradation of IκBα, apical activation leads to stabilisation of IκBα and p105, preventing activation of the NF-κB pathway (Lee, Mo *et al.*, 2006). $TLR9^{-/-}$ mice have a lower NF-κB activation threshold, and consequently are more susceptible to experimental colitis induced by DSS (Lee, Mo *et al.*, 2006), further indicating the

vital role of apical PRRs in maintaining immune tolerance in the gut. Similarly, flagellin-sensitive TLR5 is located only basolaterally to be activated when bacteria are present on the 'wrong' side of the epithelium, inducing inflammatory processes upon activation by bacteria penetrating the epithelium (Gewirtz, Navas *et al.*, 2001).

On the side of the adaptive immune system, there is an impressive production of IgA-type antibodies, which are secreted into the lumen. Production of bacterial antigen specific IgAs is induced upon colonisation of the gut, and the variety of specificities of these antibodies is related to the composition of the microbiota (Hapfelmeier, Lawson *et al.*, 2010). In mice, a deleterious mutation in AID, leading to defective somatic hypermutation and thus loss of affinity-maturated antibodies, the composition of the microbiota is altered, indicating the capacity of specific antibody production to shape microbiota composition (Wei, Shinkura *et al.*, 2011). In animals lacking both immunoglobulin isotype switching and somatic hypermutation, leading to the lack of specific antibody production and the absence of IgA isotypes, overgrowth of anaerobes in the small intestine takes place (Fagarasan, Muramatsu *et al.*, 2002), indicating a role for this IgA production in maintaining a favourable symbiotic relation with the gut microbiota. To regulate the response to the intestinal microbiota, the immune system uses a variety of cell types, informed by the intestinal epithelial cells, such as dendritic cells (DCs), intra-epithelial lymphocytes (IELs), and macrophages. Based on epithelial cell signals in the form of retinoic acid, thymic stromal lymphopoietin (TLSP), and TGFβ, tolerogenic dendritic cells (DCs) and macrophages are generated. Migrating $CD103^{+}$ DCs present microbial antigens to the adaptive immune system in a context of tolerance, while intestine-resident macrophages stay in close contact with epithelial cells to rapidly clear any microbes that pass the intestinal epithelial barrier (Peterson and Artis, 2014) (see Figure 9).

Microbial metabolites, for their part, regulate host immune responses as well. For example, microbiota-produced SCFAs play a crucial role in regulation and proper resolution of the inflammatory response through their interaction with G-protein-coupled receptor 43 (GPR43), evidenced by an exacerbated DSS-induced colitis in GPR43-deficient mice, and in germ-free mice (Maslowski, Vieira *et al.*, 2009). Similarly, the increase in SCFAs (e.g., butyrate) connected to increased dietary fibre intake can help reduce inflammation in ulcerative colitis (UC) (Hallert, Bjorck *et al.*, 2003), based on the notion that application of butyrate can reduce inflammation in UC patients (Scheppach, Sommer *et al.*, 1992).

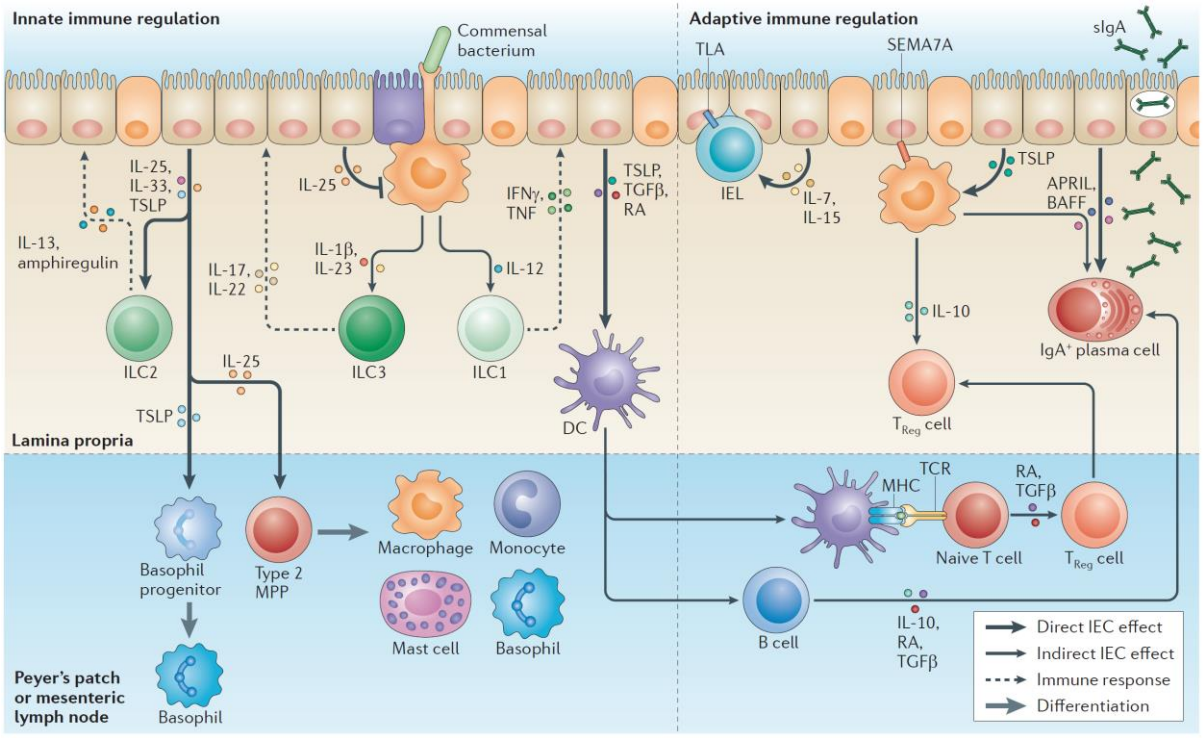



*Figure 9 - Immune regulation in relation to intestinal microbes; (Peterson and Artis, 2014)*

Supporting this anti-inflammatory effect of SCFAs, microbiota-derived butyrate induces the peripheral generation of regulatory T cells (Tregs) (Arpaia, Campbell *et al.*, 2013), pointing to a credible mechanism for the observed beneficial effects. However, the microbiota has a strong capacity to be pro-inflammatory as well, and, due to the passage of endotoxins such as LPS into the general circulation, this is not exclusive to the gut. Colonization of germ-free mice with the common gut bacterium *E.coli* increases macrophage infiltration and inflammatory cytokine production (Caesar, Reigstad *et al.*, 2012), and oral treatment of mice with LPS increases adipose tissue inflammation and induces insulin resistance (Cani, Amar *et al.*, 2007), mirroring the increased systemic LPS levels and adipose tissue inflammation observed in type 2 diabetes patients (Creely, McTernan *et al.*, 2007). A high-fat diet causes metabolic disease among others by increasing circulating LPS due to its absorption in chylomicrons (Cani, Amar *et al.*, 2007), the formation of which is increased in the presence of more fatty acids (Ghoshal, Witta *et al.*, 2009).

### 1.3.2 Toxic metabolite hypothesis

As written in previous chapters, the gut microbiota produces many useful metabolites. However, it also has the capacity to make products with negative effects on gut function and general health. For example, bacteria-produced proteases can induce inflammation and break down the mucus barrier (Steck, Mueller *et al.*, 2012), and production of methane by bacteria such as *Methanobrevibacter smithii* increases gut transit time, which can lead to constipation (Bennet, Öhman *et al.*, 2015).

Some particularly toxic products can be generated during anaerobic fermentation of unabsorbed carbohydrates; methylglyoxal and other reactive aldehydes and ketones (Campbell, Matthews *et al.*, 2010). Campbell *et al.* hold these metabolites responsible for a wide range of local and systemic effects observed in lactose malabsorption, such as headache, fatigue, heart palpitations, and depression. The mechanisms by which methylglyoxal can induce health complications are elucidated more deeply, which will be further discussed in the coming section 1.4. Methylglyoxal, or pyruvaldehyde, affects both prokaryote cells, e.g., impacting growth rates and thus changing the microbiota, and eukaryotic cells, e.g., changing cell proliferation, gene expression patterns and cell signalling processes (Campbell, Matthews *et al.*, 2010). Fermentation products of non-absorbed carbohydrates such as lactose can induce symptoms reminiscent of IBS, both in terms of gut symptoms, and more systemic symptoms, but other metabolites, such as those produced by putrefaction of proteins and amino acids are often toxic as well (Campbell, Waud *et al.*, 2005). Another way by which bacterial fermentation can impact gut health is by changes in pH. In IBS patients, colonic intraluminal pH is decreased compared to healthy controls, as measured using a pH-measuring ingested SmartPill system, which indicates an increased fermentation (Ringel-Kulka, Choi *et al.*, 2015).

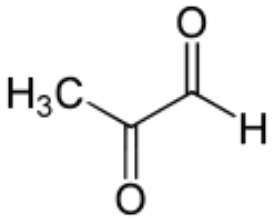
## 1.4 Bacterial Metabolites; aldehydes/ methylglyoxal

We have already seen the many functions of the microbiota, and its possible association to IBS. In this chapter we will take a more in-depth look into the role bacterial fermentation of carbohydrates and the resulting reactive metabolites can play in gastrointestinal health, particularly in the context of reactive glycating metabolites.

As we have seen before (in 1.3.2), the production of methylglyoxal by the intestinal microbiota is hypothesised to play a role in a wide variety of health issues. Here we will look at the properties of methylglyoxal and other reactive dicarbonyl metabolites.

### 1.4.1 Chemical properties of methylglyoxal

Methylglyoxal (Figure 10), also known as pyruvaldehyde, chemical formula $CH_3C$ (O)CHO, is a derivative of pyruvic acid, and a possible side-product of glycolysis. Its molecular mass is 72,06266 Da. Methylglyoxal is the most reactive dicarbonyl compound produced during glycolysis, it glycates lysine and cysteine residues (Price, Hassi *et al.*, 2010) but prefers arginine and thus causes the formation of hydroimidazolone residues in proteins (Rabbani and Thornalley, 2012). Methylglyoxal can be formed by the spontaneous degradation of triosephosphates, glyceraldehyde-3-phosphate (GA3P), and dihydroxyacetone phosphate (DHAP) (Phillips and Thornalley, 1993), as well as by oxidation of acetone during ketone body catabolism (Reichard, Skutches *et al.*, 1986).




Methylglyoxal

*Figure 10 - Formula of methylglyoxal*

### 1.4.2 Biological properties of methylglyoxal

During bacterial anaerobic fermentation of carbohydrates, methylglyoxal is produced by the activity of methylglyoxal synthase (MGS), which is practically universally present among bacterial species (Figure 11). In the expansive but incomplete NCBI database of characterized proteins, the search key "Methylglyoxal synthase" returns >83000 results from a wide variety of prokaryote species and phyla, not necessarily directly reflective of particularly active species, but perhaps more of particularly active researchers interested in those species. It had already been shown that different culturable species isolated from human faeces express MGS and are able to produce methylglyoxal in culture (Baskaran, Rajan *et al.*, 1989). During bacterial glycolysis, dihydroxyacetone phosphate can be transformed to methylglyoxal, when it actives MGS. On the other hand, one of the products of this transformation, phosphate, inhibits MGS (Hopper and Cooper, 1971).


*Figure 11 - Results of NCBI Protein search for "Methylglyoxal synthase"*

Like the production of ethanol is a limiting factor to the vitality of yeast in the production of wine and beer, the production of methylglyoxal poses problems to its producers, and higher concentrations (starting from 1mM for *E.coli*) will negatively impact bacterial growth rates (Campbell, Matthews *et al.*, 2010). Because not all species are equally sensitive, this can change the composition of the microbiota. For example, *Prevotella ruminicola* will produce high

concentrations (3-4 mM) of methylglyoxal when offered an excess of glucose, which will kill the culture, but different strains of this species were not equal producers (Russell, 1993). Additionally, the activity of the glyoxalase detoxification system in bacteria is predictive of their ability to cope with methylglyoxal, and glyoxalase knockout mutants become very sensitive to even modest concentrations (Ferguson, Totemeyer *et al.*, 1998). It seems that for bacteria, the production of methylglyoxal serves to balance phosphate metabolism, and a MGS knockout mutant *E.coli* reaches growth inhibition more rapidly due to accumulation of toxic phosphorylated metabolites (Totemeyer, Booth *et al.*, 1998).

Similarly, growing *E.coli* in phosphate-limited medium elevates methylglyoxal levels, which can be increased by overexpressing MGS (Booth, Ferguson *et al.*, 2003), again indicating an intended increase in phosphate turnover. An important observation is that abundant methylglyoxal production is expected in case of a sudden influx of carbon sources, causing a loss of control over carbon entry in the bacterial cells (Totemeyer, Booth *et al.*, 1998) which is indeed the situation caused by the ingestion of significant amounts of unabsorbable carbohydrates, prevented by a low-FODMAP diet.

In diabetic humans, the methylglyoxal levels in the blood are increased, which is unlikely to be directly related to activity of the microbiota. The generally accepted hypothesis explaining the increased MG levels in diabetic patients points to hyperglycaemia, leading to an increased production of methylglyoxal in blood cells (Kalapos, 2013). Even brief periods of hyperglycaemia can significantly increase MG levels (Beisswenger, Howell *et al.*, 2001), leading to accumulation of dicarbonyl metabolites and the modification of DNA and proteins (Rabbani, Xue *et al.*, 2016a), which will be elaborated upon in the next section.

Apart from methylglyoxal, glyoxal and 3-deoxyglucosone (3-DG) are important reactive dicarbonyl compounds as well, with similar effects and detoxification processes (Hellwig, Gensberger-Reigl *et al.*, 2018).

### 1.4.3 Deleterious effects of dicarbonyl stress

Methylglyoxal is one of several highly reactive dicarbonyl metabolites naturally present in physiological systems. Chemical reactions of these reactive carbonyls with proteins, DNA, and phospholipids lead to the formation of Advanced Glycation End products (AGEs) (Thornalley, Yurek-George *et al.*, 2000), which are stable, and can accumulate in tissues in certain health conditions, such as diabetes, obesity, cardiovascular disease, and renal failure, or during the natural process of ageing (Rabbani, Xue *et al.*, 2016a). In diabetes, insulin resistance is worsened by hyperglycaemia, because the resulting increase in MG and AGEs dampen the response of insulin receptors (Riboulet-Chavey, Pierron *et al.*, 2006), leading to a vicious circle of worsening hyperglycaemia and less responsive insulin signalling. *In vitro*, methylglyoxal at non-cytotoxic levels inhibited beta-cell insulin response and secretion, indicating the capacity of methylglyoxal to induce beta cell failure (Fiory, Lombardi *et al.*, 2011). Typically, MG concentrations in human plasma are 50-150 nM, the concentration in cells is higher, at 1-4 µM. Concentrations above this level risk impairing health, causing protein and cell dysfunction (Rabbani, Xue *et al.*, 2016a).

The generation of Advanced Glycation End Products (AGEs) (see Figure 12), which lead to Receptor for Advanced Glycation End products (RAGE) activation (Thornalley, Langborg *et al.*, 1999), cause ROS generation and a pro-inflammatory state through NF-κB pathway signalling (Wautier, Guillausseau *et al.*, 2016). Advanced Glycation End products thus produced in conditions of heightened dicarbonyl stress can activate NF-κB in RAGE expressing cells, leading to prolonged, instead of transient, activation by increased NF-κBp65 production, which

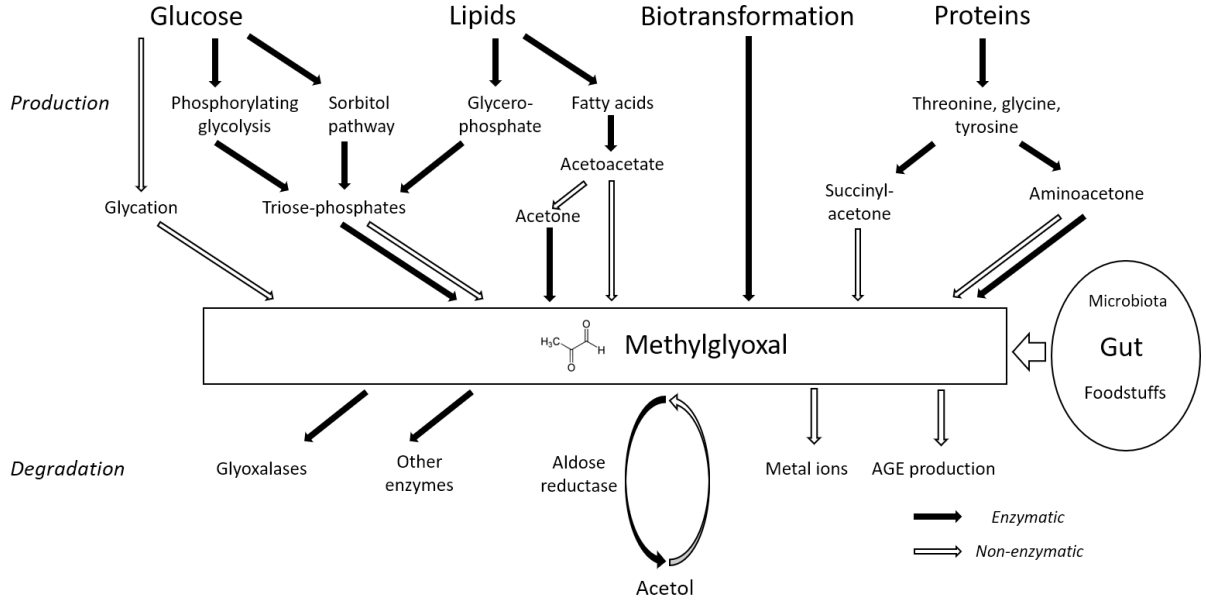



*Figure 12 – Methylglyoxal metabolism (adapted from (Kalapos, 2013))*

could be a cause of long-term low-grade inflammation in diabetes (Bierhaus, Schiekofer *et al.*, 2001). Furthermore, RAGE functions as a counterreceptor for leukocyte integrins, meaning it supports leukocyte recruitment in inflammatory processes. This mechanism can be relevant in disorders with increased RAGE expression (Chavakis, Bierhaus *et al.*, 2003).

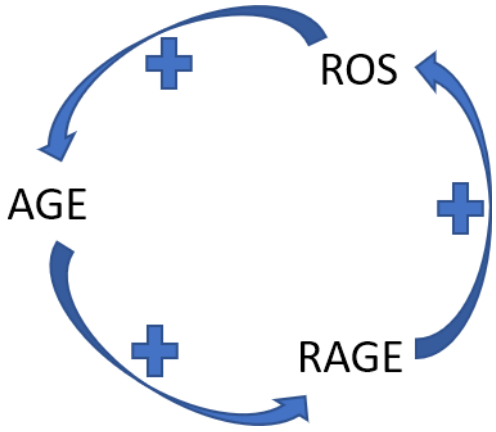



*Figure 13 - Positive feedback loop between increased AGE, RAGE activation, and ROS. AGEs activate RAGE, which leads to inflammatory processes and the production of ROS, which creates a favourable environment for the generation of more AGEs.*

Since an increase in AGE increases the expression of RAGE (He, Zheng *et al.*, 2000; Simm, Casselmann *et al.*, 2004; Bierhaus and Nawroth, 2009), and the activation of RAGE leads to ROS production, which favours generation of AGE (Baynes, 1991; Ott, Jacobs *et al.*, 2014), a positive feedback loop can occur (Figure 13), which can be interrupted by detoxification processes, which will be discussed in the next section, 1.4.4.

### 1.4.4 Detoxification – glyoxalase system

Reflective of its basic nature, methylglyoxal as a metabolite is common to prokaryotes and eukaryotes. For the same reason, mechanisms to cope with this toxic molecule have emerged very early in the history of life, and mechanisms are conserved across Kingdoms. Such a mechanism is the glyoxalase system, which converts methylglyoxal to D-lactate, and consists of glyoxalase I (Glo1), (lactoylglutathione lyase) and glyoxalase II (Glo2) (hydroxyacylglutathione hydrolase). The conversion is dependent on glutathione (GSH) as a catalyser (Figure 14). This same reaction takes place in Animals, Plants, Fungi, and even Prokaryotes (Thornalley, 1993). The enzymes are active in the cytosol, with Glo2 also active in organelles such as the mitochondria, in plants and animals (Thornalley, 1993), and additionally in chloroplasts (Schmitz, Dittmar *et al.*, 2017). Glo1 is in the top 13% in the human proteasome, reflective of its key role in life (Rabbani, Xue *et al.*, 2016b).

In some bacteria, particularly in gram-negative ones, Glyoxalase 1 enzymes are more effectively activated by $Ni^{2+}$ and $Co^{2+}$ ions, but inhibited by $Zn^{2+}$, while in animals, this metalloenzyme is maximally active with $Zn^{2+}$ (Suttisansanee and Honek, 2011). This varying metal-dependency ($Zn^{2+}$-activated v non-$Zn^{2+}$-activated) could open up possibilities to selectively inhibit microbial species by limiting the availability of certain metal ions in conditions of dicarbonyl stress.

A third glyoxalase (Glyoxalase 3; Glo3) can be present in plants (Ghosh, Kushwaha *et al.*, 2016), fungi (Zhao, Su *et al.*, 2014), and bacteria (Misra, Banerjee *et al.*, 1995). Glyoxalase 3 is glutathione-independent and thought to catalyse the conversion from methylglyoxal directly to D-lactate (Misra, Banerjee *et al.*, 1995). However, more recent work indicates that what was known as glyoxalase 3 in *E.coli* actually deglycates proteins by attacking the early glycation intermediates hemithioacetals and aminocarbinols, generating repaired proteins, and lactate and glycolate (Mihoub, Abdallah *et al.*, 2015). In that way, glyoxalase 3 uses early glycation intermediates as reaction intermediates and can help to prevent the formation of AGEs.

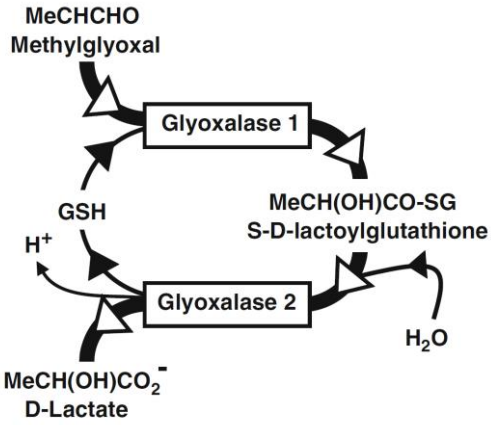



*Figure 14 - Glyoxalase system (Rabbani and Thornalley, 2012)*

It has been suggested in literature that the high expression of glyoxalases in the epithelium of the intestine is related to the production of methylglyoxal by enteric bacteria (Ferguson, Totemeyer *et al.*, 1998), for which they cite

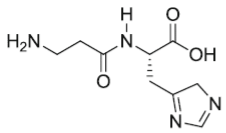
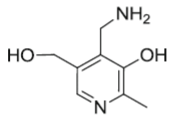
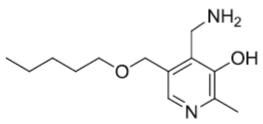
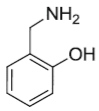
Baskaran *et al.* (Baskaran, Rajan *et al.*, 1989) who demonstrated the presence of Glyoxalase 1 in simian intestine (Baskaran and Balasubramanian, 1987). Their claim that this expression may protect against methylglyoxal produced by enterobacteria is based on an article that shows glyoxalase expression in erythrocytes and liver cells (Aronsson and Mannervik, 1977).

While the hypothesis has merit, this train of inferences skips several logical steps, as none of the sources show a particularly high glyoxalase expression in the intestine which would support it, and findings have been mischaracterised from one publication to the next. Comparative expression levels of glyoxalase throughout animal tissues are hard to come by, but one study on bovines show the highest level of glyoxalase 1 expressed in the liver, though regrettably they did not analyse intestinal tissues (Hayes, Milner *et al.*, 1989). However, based on the general understanding that glyoxalase is ubiquitously expressed, and the microbiota is capable of producing methylglyoxal, intestinal detoxification of this chemical would indeed be necessary to prevent health complications.

In the brain, where glucose is the major energy substrate, and detoxification of by-products of glycolysis is important, it can equally be expected that the glyoxalases are highly expressed. Indeed, the glyoxalase system seems to be important in protecting the brain, with expression in the brain increasing until 55 years of age as levels of dicarbonyl stress rise with age, after which expression levels drop and neurodegenerative processes associated with ageing show a significant increase (Kuhla, Boeck *et al.*, 2006), and AGEs accumulate (Luth, Ogunlade *et al.*, 2005). There is a significantly higher expression of Glo1 in astrocytes compared to neurons (9.8 times higher) (Belanger, Yang *et al.*, 2011), which corresponds to their higher rate of glycolysis, after which they supply derived energy substrates to the neurons, which have a higher energy expenditure (Allaman, Belanger *et al.*, 2015). This mechanism might be effective in shielding neurons, which are less replaceable, from glycation damage.

To help protect against the negative effects of dicarbonyl stress in a clinical or medical setting, some interesting strategies have been envisioned. For example, quite a lot of attention has been spent on dicarbonyl scavengers, drugs that are meant to chemically capture and neutralise reactive dicarbonyls, such as aminoguanidine and phenacylthiazolium bromide. While showing promise, issues with toxicity and molecular stability prevented further employment of these compounds (Rabbani, Xue *et al.*, 2016a). Pyridoxamine can be used to prevent the formation of AGEs *in vitro* in lens proteins and *in vivo* in diabetic rat plasma proteins (Nagaraj, Sarkar *et al.*, 2002). Other MG scavengers are the imidazole based carnosine, and the 2-aminomethylphenols pyridoxamine (PM), 5'-O-pentyl-pyridoxamine (PPM), and 2-methoxy-benzylamine (2-HOBA) (Davies and Zhang, 2017) (see Table 7).

*Table 7 - Different methylglyoxal scavengers with pre-clinical or clinical application (adapted from (Davies and Zhang, 2017))*

| Scavenger by class | Reaction rate | *In vivo* applications or studies |
|---|---|---|
| **Imidazole-based** | | |
| Carnosine  | Acrolein = ONE > HNE > MGO > others | • Pilot studies show improved glucose tolerance and insulin sensitivity in overweight and obese subjects (de Courten, Jakubova *et al.*, 2016)<br>• Improved symptoms of neurodegeneration; Parkinson's (Boldyrev, Fedorova *et al.*, 2008), and Alzheimer's (Cornelli, 2010) |
| **2-aminomethylphenols** | | |
| PM  | IsoLG > ONE > MGO > Acrolein = MDA | • Mildly improves diabetic nephropathy in humans (Williams, Bolton *et al.*, 2007), marked improvements in renal function (Tanimoto, Gohda *et al.*, 2007) and plasma glucose handling (Maessen, Brouwers *et al.*, 2016) in animal models<br>• Efficient in other diabetes models like atherosclerosis (Watson, Soro-Paavonen *et al.*, 2011) and cardiac function (Cao, Chen *et al.*, 2015) |
| PPM  | IsoLG > ONE > MGO > Acrolein = MDA | • Efficient in mouse model of hypertension (Kirabo, Fontana *et al.*, 2014) |
| 2-HOBA  | IsoLG > ONE > MGO > Acrolein = MDA | • Efficient in animal models of Alzheimer's (Davies, Bodine *et al.*, 2011), epilepsy (Pearson, Warren *et al.*, 2017), aging (Nguyen, Caito *et al.*, 2016), and hypertension (Kirabo, Fontana *et al.*, 2014) |

Another suitable strategy to alleviate dicarbonyl stress in patients is by increasing Glo1 expression, and a synergetic effect of 2 clinically applicable substances (trans-resveratrol (tRES) and hesperetin (HESP)) have been shown to induce this effect. A treatment with these 2 compounds could increase expression of Glo1 by 27%, thus reducing total body methylglyoxal-protein glycation by 14%, in highly overweight subjects (Xue, Weickert *et al.*, 2016). In metabolic syndrome and diabetes, such a treatment could help prevent complications, but it is possibly less optimal for situations which present more topical dicarbonyl stress.

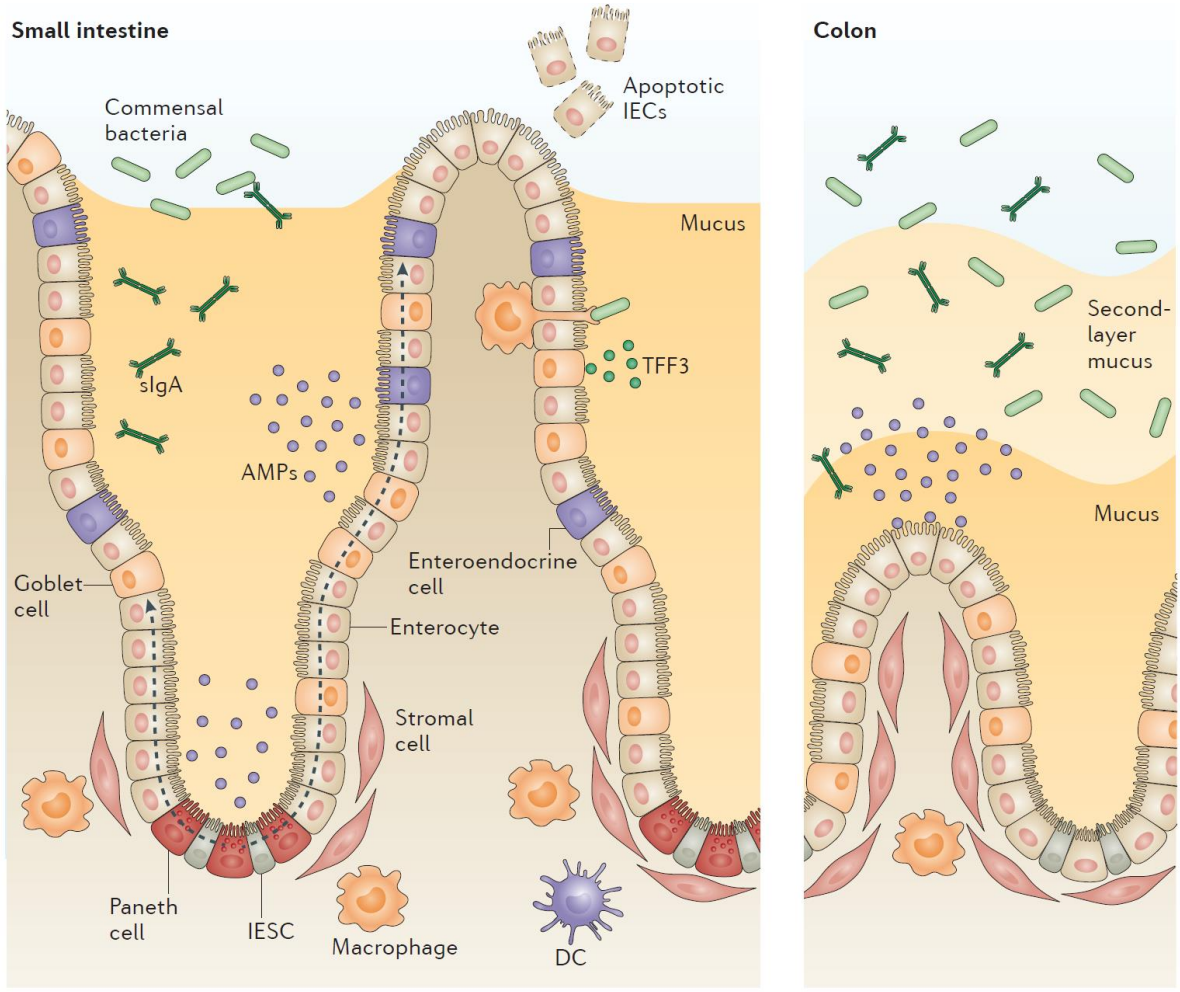



*Figure 15 - Intestinal Epithelial Cells (IECs) of the small and large intestine.*
*IESC, Intestinal Epithelial Stem Cell; AMP, antimicrobial proteins; sIgA, secretory immunoglobulin A; TFF3, trefoil factor 3; DC, dendritic cell*

*Epithelial cells are generated from the IESC at the bottom of the crypts and differentiated cells then migrate upwards through the crypt, except for Paneth cells, which stay at the bottom. The release of AMPs, sIgA, TFF3, as well as mucins, which form a mucus layer, all contribute to the separation of the luminal milieu from the mucosal immune system.*

*Adapted from (Peterson and Artis, 2014)*

## 1.5 Intestinal barrier function

We have seen that diet together with the intestinal microbiota can generate problems, which can impact the crucial barrier that separates the self from the environment. In this chapter, we will look at the functioning of the intestinal barrier, and some pathophysiologically relevant complications of its disturbance.

### 1.5.1 Epithelial barrier

When we think of our intestines, we usually picture it as part of our inside. However, the gastrointestinal lumen is part of the external environment, with our bodies forming a continuous tube. To keep external and internal separated, the mucosal lining of our gastrointestinal tract has an important barrier function. The first cellular components in contact with the exterior are the epithelial cells, which form the luminal part of the mucosal lining of the gastrointestinal tract. Underneath the epithelium are located the lamina propria, and the muscularis mucosa. The epithelial cells are tightly connected by so-called tight and adherens junctions which regulate passage in between cells. The cells themselves are impermeable to most hydrophilic components by virtue of their cell plasma membranes, unless there is a specific transporter protein (-complex) present. Epithelial cells in the small intestine consist of absorptive enterocytes, goblet cells, enteroendocrine cells, and Paneth cells. In the large intestine, there are enterocytes, enteroendocrine cells, and goblet cells (see Figure 15). These components together form a dynamic barrier that protects the organism from harmful inflammatory stimuli, while at the same time allowing digestion and absorption of nutrients.

#### 1.5.1.1 Small intestine barrier function and morphology

The small intestine's tasks are digestion of chyme and absorption of nutrients, and the barrier function in this part of the intestinal tract mirrors these functions. The microbiota is kept in check by the production of antibodies and antimicrobial peptides such as α-defensins, RegIIIγ (Cash, Whitham *et al.*, 2006), and lysozyme (Dommett, Zilbauer *et al.*, 2005).

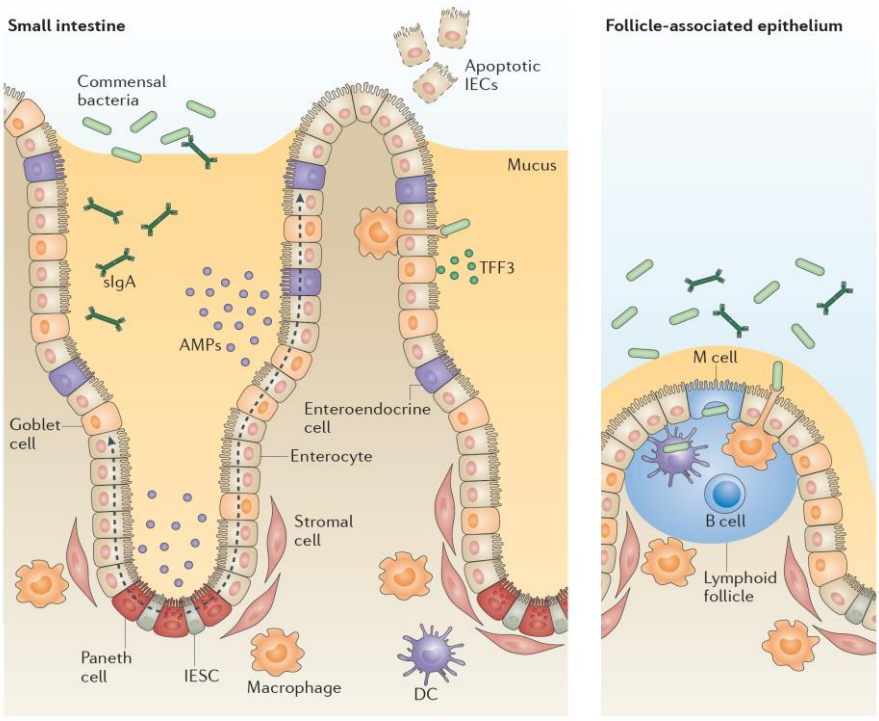



*Figure 16 - Epithelial barrier of the small intestine*
*Adapted from (Peterson and Artis, 2014)*

The epithelium in the small intestine covers so-called *villi*, projections into the lumen that greatly increase the surface area of the small intestinal lining, to help with digestion, through increasing contact area of digestive enzymes on cellular surfaces, and absorption, through increased contact area of nutrients with absorptive enterocytes. The enterocytes themselves are covered apically by *microvilli*, projections on the cell surface that, again, increase the effective surface area of the small intestine. Capillary blood vessels and lymph channels called *lacteals* project through the villi and export absorbed nutrients. Covering nodes of lymphatic tissue in the small intestine called Peyer's patches, follicle-associated epithelium is a layer of epithelial cells that include microfold cells (M cells) that facilitate antigen uptake from the lumen, inducing an immune response or tolerance in the immune cells in the underlying Peyer's patch (Gebert, Rothkötter *et al.*, 1996). The mucus produced here is loose and patchy and easily removed (Atuma, Strugala *et al.*, 2001; Ermund, Schutte *et al.*, 2013), it thus mostly serves as a

matrix for antimicrobial peptides and antibodies. Structural integrity and barrier functions demand that the epithelial cells are tightly connected, which is the responsibility of tight junctions, adherens junctions, and desmosomes, protein complexes which extrude from the cell membrane and interconnect and pull together cells, which will be discussed more in the paragraph dedicated to paracellular permeability.

#### 1.5.1.2 Large intestine barrier function and morphology

Compared to the small intestine, the large intestine has a larger volume and a smaller surface area, related to its functions, which are fermentation of chyme by the microbiota, resorption of water and condensation of chyme into faeces, absorption of useful fermentation products, and storage of faeces before defecation. Likewise, the barrier function in this part of the intestine mirrors these function in several distinct ways. In the colon, Paneth cells are absent, and to retain separation of luminal microbes and the host tissues, mucus plays a more important role than in the small intestine, at the same time lubricating the passage of the increasingly dry and abrasive stool. Colonocytes, enterocytes of the colon, have a greater capacity to actively absorb water against the ion gradient compared to enterocytes of the small intestine, and have a lower expression of digestive brush border enzymes (Real, Xu *et al.*, 1992). The colon has a large capacity to absorb water of around 5.7 litre/ day, tested by direct infusion of water into the cecum of healthy volunteers (Debongnie and Phillips, 1978). Of course, since most water is already absorbed in the small intestine (see Figure 18), the colon normally absorbs just over one litre/day (Thiagarajah and Verkman, 2018), but against a much stronger osmotic gradient due to the lower water content of colonic contents compared to ileal contents. Fermentation products of the microbiota, which are a lot more abundant in this part of the intestine, are absorbed here, together with water. In the colon, mucus is a more important component of the barrier compared to the small intestine, goblet cell secretions in this part of the intestine form a dense mucus layer devoid of bacteria, separating the epithelium from the microbes present in the stool (Johansson, Phillipson *et al.*, 2008).

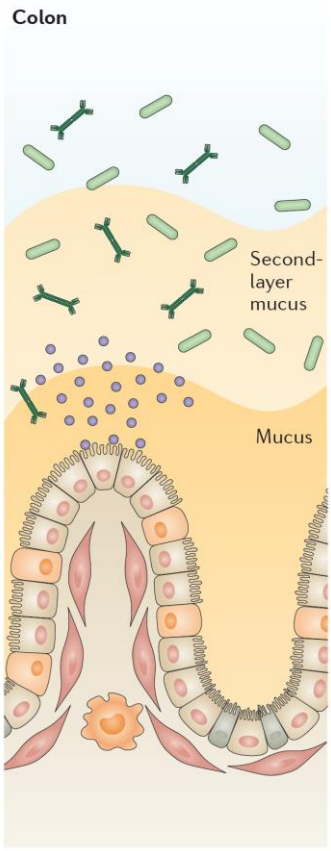



*Figure 17 - Epithelial barrier of the large intestine*
*Adapted from (Peterson and Artis, 2014)*

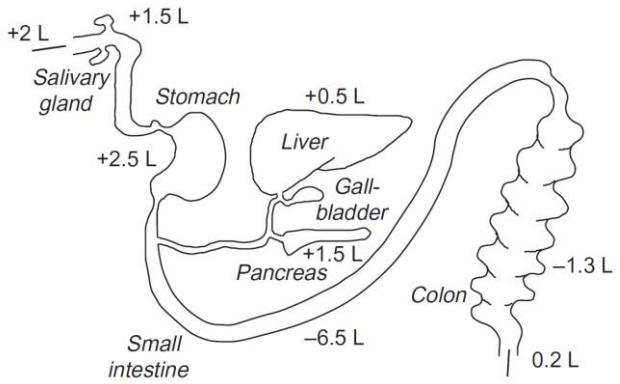



*Figure 18 - Water excretion/absorption throughout the GI-tract (Thiagarajah and Verkman, 2018)*

This mucus is generally reported to be around 95% water (Strugala, Allen *et al.*, 2003), though the original source for this number is hard to pin down. Stools are around 70% water (Aichbichler, Wenzl *et al.*, 1998; Bliss, 1999), with up to 55% bacterial mass (Stephen and Cummings, 1980) from which water is not freely available to be extracted, so if colonic mucus is also 95% water, the question arises how it is possible to extract water from the drier stools through this mucus layer. On first glance, we would expect the mucus layer to lose humidity together with the contents, changing its rheological properties.

The large intestine is characterized by the most concentrated microbial presence of any part of the human body, and the barrier function in this part of the gastrointestinal tract is adapted to cope with this particular challenge. In the absence of a mucus barrier, such as caused by a knockout mutation for muc2 ($Muc2^{-/-}$ mice), a spontaneous and lethal colitis occurs, but fewer problems occur in the earlier compartments of the intestines (Van der Sluis, De Koning *et al.*, 2006), although muc2 is the main component of excreted mucus in these parts too. In the colon, antimicrobial peptides specific for Paneth cells in the small intestine, are much more rare, due to the absence of Paneth cells. However, in chronic inflammation, the colon can generate Paneth cells (Paterson and Watson, 1961), and these metaplastic Paneth cells produce human α-defensin 5 (HD-5), lysozyme, and secretory phospholipase 2 (sPLA2) (Cunliffe and Mahida, 2004). Apart from the excreted mucus barrier, another barrier awaits for any elements passing through; the glycocalyx. The glycocalyx is a carbohydrate-rich network of glycolipids and glycoproteins, anchored to the cell membrane (Okumura and Takeda, 2017). Transmembrane mucins such as MUC1, MUC4, and MUC13 together form the cell surface mucin layer (Linden, Sutton *et al.*, 2008). Transmembrane mucins can shed in reaction to pathogens, acting as decoys, binding bacteria, to prevent adhesion to the cell surface (Ashida, Ogawa *et al.*, 2011). For this reason, $Muc1^{-/-}$ mice are more susceptible to *H.pylori* (Linden, Sheng *et al.*, 2009).

In the next sections (1.5.2 and 1.5.3) we will look further into the regulation of cellular permeability and physical mucus barrier function.

### 1.5.2 Permeability

The intestinal barrier is meant to be a selective filter for nutritional and immunological purposes. Simultaneously, it must facilitate passage of useful resources; nutrients, and information; e.g., antigen sampling, while impeding free passage of bacteria, toxins, and antigens, over a considerable surface. The surface area of human intestine is about $32m^2$, according to the latest calculations (Helander and Fandriks, 2014), which is approximately 10 times smaller than the number often cited in literature (Macdonald and Monteleone, 2005; Niess and Reinecker, 2006).

#### 1.5.2.1 Paracellular

The paracellular pathway of passage through the epithelium involves elements moving through the space in between cells. As mentioned before, IECs form a tight network through the adhesive function of the tight and adherens junction complexes as well as desmosomes (see Figure 19). Claudin, Occludin, and ZO1 proteins form an adhesive network that limits the passage of solutes through the space in between cells, regulated by actin and myosin, which can tighten or loosen the bonds (Turner, 2009). Additionally, tight junctions safeguard cell polarity, by preventing diffusion of apically located receptors in the basolateral direction (König, Wells *et al.*, 2016). Adherens junctions, formed between cadherins, serve to initiate the construction of a fully functional paracellular barrier, and are necessary for the formation of tight junctions (Hartsock and Nelson, 2008). Desmosomes, lastly, are responsible for the strong resistance to mechanical stress that most tissues have. They are specialised in keeping cell-cell bonds intact by anchoring to intracellular filamentous cytoskeleton elements (Garrod and Chidgey, 2008). The paracellular route of passage then is mostly available to small hydrophilic solutes (He, Murby *et al.*, 1998).

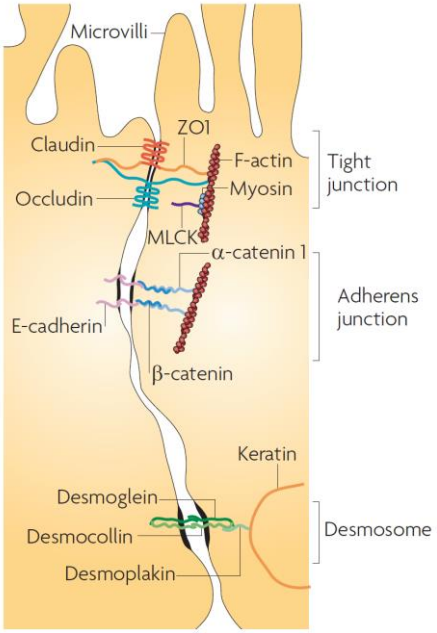



*Figure 19 - Cell-cell junctions between IECs Adapted from (Turner, 2009)*

In short, the paracellular pathway functions as a semi-selective sieve, allowing for passive diffusion of small hydrophilic molecules across the epithelial lining, following concentration gradients.

#### 1.5.2.2 Transcellular

"Transcellular pathway" refers to the passage of molecules through cells. It involves passive transport by hydrophobic molecules partitioning in and out of lipid bilayers, active transport by specific transporter proteins and channels, or endocytosis by the cell membrane (Keita and Söderholm, 2010). The transcellular pathway is mostly inaccessible to hydrophilic elements, due to the lipid bilayer of the cell plasma membranes. Instead, membrane-bound receptors and pores on the one hand, and endocytosis on the other hand can provide access to components, to take this route through the epithelium. Transcellular transport is partially dependent on the integrity of the paracellular barrier, because the established concentration gradient would cause passive diffusion to re-establish equilibrium otherwise (Turner, 2009).

Transcellular transport is performed by a set of highly regulated and complex processes, and apart from the uptake of nutrients, serves to transport antigens to be processed by the immune system.

### 1.5.3 Mucus barrier

An often-misunderstood component of the intestinal barrier is the mucus barrier, which consists of excreted and membrane-bound mucins which form a semi-permeable chemical, physical, and biological barrier, in interplay with other barrier components. Mucins, glycoproteins containing galactose, fucose, N-acetylglucosamine, N-acetylgalactosamine, and sialic acids, are the essential component of the mucus barrier. The property essential to their character is the structure of the sugars attached to a central protein core; hundreds of sugars branching out, resembling a bottle brush (Allen, 1983). The mucus barrier consists of the glycocalyx, apically located transmembrane mucins and secreted elements covering the epithelial cells, and excreted mucins which form a net-like gel of disulphide-linked macromolecules (Johansson, Sjövall *et al.*, 2013).

This mucus lubricates the passage of stools, supports the intestinal microbiota by providing a matrix as well as energy/carbon source, and it serves as a barrier protecting against luminal threats, such as pathogens and potentially harmful particles. In the gastrointestinal tract, it has different properties based on the region of the intestine, as depicted in Figure 20.

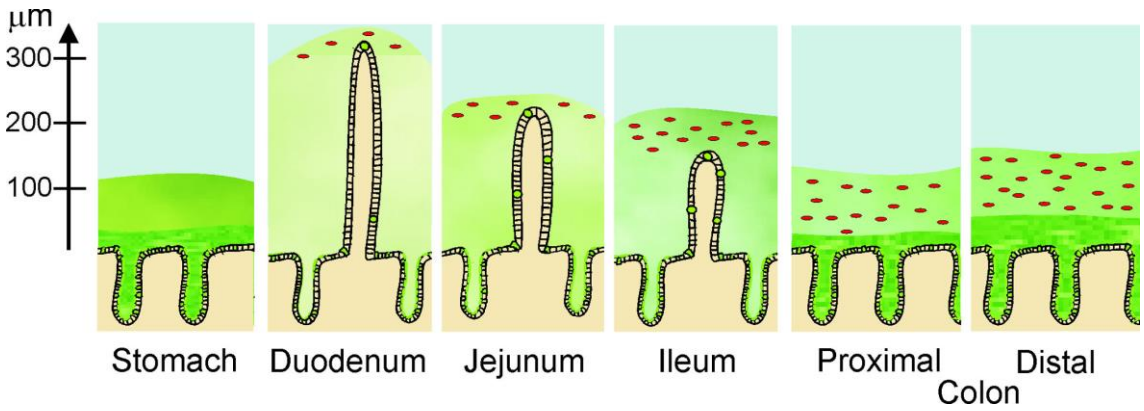



*Figure 20 - Mucus layer thickness in different regions of the gastrointestinal tract (Pelaseyed, Bergstrom et al., 2014)*

#### 1.5.3.1 Mucus types; functions

There are many different types of mucins expressed in the GI-tract, with their own functions and expression patterns (Table 8). As mentioned before, there are membrane-bound and secreted mucins, which already strongly determines their function. What follows is a short description of mucins expressed in the intestinal tract;

*Table 8 - Mucin gene expression in the GI-tract (adapted from (McGuckin, Lindén et al., 2011))*

| TISSUE | EXCRETED | MEMBRANE BOUND |
|---|---|---|
| ***ORAL CAVITY*** | MUC5B, MUC7, MUC19 | MUC1, MUC4, MUC16 |
| ***STOMACH*** | MUC5AC, MUC6 | MUC1, MUC16 |
| ***SMALL INTESTINE*** | MUC2 | MUC1, MUC3A, MUC3B, MUC4, MUC12, |
| ***LARGE INTESTINE*** | MUC2, MUC5AC, MUC6 | MUC13, MUC15, MUC16, MUC17 |

In the oral cavity, the excreted mucins mostly serve to retain antimicrobial peptides, and interact directly with microbes (Frenkel and Ribbeck, 2015), while the membrane bound mucins have regular glycocalyx functionality, covering and protecting the mucous tissues, providing scaffolding for excreted mucins and signal transduction (Liu, Lague *et al.*, 2002). Additionally, saliva acts as a lubricant for food to pass the oesophagus (Wickstrom, Davies *et al.*, 1998). The stomach has a dual mucus layer protecting the epithelium from being damaged by the stomach acid, while simultaneously allowing for renewal of this stomach acid, but also buffering the pH and retaining $HCO_3^-$ (Taherali, Varum *et al.*, 2017).

In the small intestine, the excreted mucin (muc2) forms a gel, which manifests as loose and patchy, serving mostly to prevent bacterial adherence to the epithelium, retain the plethora of antimicrobial products, and prevent microbes from entering crypt (Schneider, Pelaseyed *et al.*, 2018). In the large intestine, the excreted mucins, the most abundant by far being muc2, form a more impenetrable barrier, separating luminal contents from intestinal epithelium (Johansson, Larsson *et al.*, 2011). The membrane bound mucins in the small and large intestine are numerous, reflecting the increasingly complex interactions with an expanded microbial presence. Transmembrane mucins can act as decoys for bacterial adherence (Ashida, Ogawa *et al.*, 2011), they can provide steric hindrance to any element set to penetrate (Cone, 2009; Boegh and Nielsen, 2015), and they can serve receptor functions (van Putten and Strijbis, 2017).

Secreted mucins may also function to cover, sequester, and transport particles out of the body (Florey, 1962), and as decoys for pathogens (Linden, Mahdavi *et al.*, 2004; Linden, Bierne

*et al.*, 2008), in a way that reminds somewhat of airway mucins, which capture and remove environmental pollutants and pathogens (Knowles and Boucher, 2002).

Excreted mucus plays an important role in modulating infectious disease severity. For example, Muc2, the main mucin of intestinal excreted mucus, is key in controlling *Salmonella* infection, as evidenced by use of a Muc2-deficient mouse model (Zarepour, Bhullar *et al.*, 2013). The researchers showed that O-glycosylation properties of muc2 is essential for this functionality, as mutants with aberrant glycosylation show increased susceptibility (Zarepour, Bhullar *et al.*, 2013). The mechanism behind protection by mucus is more complex than just physical obstruction; secreted antibodies like IgM are slightly mucophilic, but diffuse through the mucus, accumulating on antigen-covered particles, such as pathogens. Due to this accumulation, even normally motile mucus-penetrating bacteria become immobile in the mucus, with antigens forming polyvalent low affinity bonds to the gel (Cone, 2009).

It is often said that contact between bacteria and the epithelium leads to inflammation, and that the excreted mucus barrier prevents this contact (Johansson, Gustafsson *et al.*, 2014). While it is clear that muc2$^{-/-}$ mice without an excreted mucus barrier develop severe colitis (Van der Sluis, De Koning *et al.*, 2006), it has not been clearly shown that this is solely due to epithelium-bacterial contact. Since it is known that in the small intestine the mucus layer is patchy and easily removed (Atuma, Strugala *et al.*, 2001; Ermund, Schutte *et al.*, 2013), the microbial presence there should lead to inflammation too if contact alone would be enough, even when taking into account that the small intestine has its own array of anti-microbial defences, as discussed in 1.5.1.1. Still, small intestinal bacterial overgrowth (SIBO), should cause extensive contact between epithelium and bacteria, it is however not specifically characterized by ileitis.

Apart from protecting against microbial threats, the excreted mucus barrier is also involved in protecting against chemical agents, such as food-borne environmental pollutants and possibly harmful food additives, although this has not been sufficiently studied and documented until today (Gillois, Leveque *et al.*, 2018).

Besides protective functions, in the intestine, mucus also serves a supportive purpose for the resident microbes; mucus produced by the host supports a microbial network of mucolytic bacteria, such as *Akkermansia muciniphila* and associates (Derrien, Vaughan *et al.*, 2004; Belzer, Chia *et al.*, 2017). Intestinal mucus thus provides a suitable niche to the commensal microbiota, supported by beneficial fucosylation of mucins to support the microbiota (Pickard, Maurice *et al.*, 2014), as well as differing glycosylation patterns throughout the intestine, which seem to have role in selecting microbial population composition, while at the same time being influenced by the microbiota (Sekirov, Russell *et al.*, 2010; Johansson, Jakobsson *et al.*, 2015). For example, fucosylation of the glycocalyx is increased by signalling from *B. thetaiotamicron*, and this fucose is then used by this bacterium (Hooper and Gordon, 2001). This example nicely demonstrates that host and microbe communicate to shape a suitable niche for the microbiota through mucin modifications.

The goblet cell to total cell ratio in mice progresses upwards throughout the intestine, approximately 2:50 in duodenum, 3:50 in jejunum, 6:50 in ileum (Cheng, 1974), and 8:50 in the distal colon (Chang and Leblond, 1971), which correlates to the density of microbes in these intestinal compartments, which increases from proximal to distal regions. This holds well with the general idea that mucus is a main mediator in host-microbe interactions, as part of the variety of adaptations of the metaorganism to exist in eubiosis, which are studied in the rapidly upcoming field of metaorganism or holobiont research (Esser, Lange *et al.*, 2018).

#### 1.5.3.2 A sticky history of organisation

Much has been written about the organisation, and even the existence, of the gastrointestinal mucus barrier. The transparent mucus of the internal, difficult to access, mucosae is prone to dry out and notoriously hard to study. It has been debated extensively whether a permanent barrier even covers the internal mucosae or not.

In 1955, in the Croonian Lecture, Sir Howard Florey laid out the current understanding of various mucus secretions of mammals at that time. For intestinal mucus, he presented mainly a lubricative and protective function. Mucus was thought able to entrap food particles and bacteria touching the epithelium "…wrapping them up as it were in an envelope and expediting their forward movement…", but without impeding diffusion of smaller molecules and not impeding absorption. Rationale behind this understanding is the higher concentration of goblet cells in the large intestine compared to the small intestine, together with the liquid chyme in the small intestine and the solid stools in the large intestine, where lubrication is thus of higher importance (Florey, 1955). Additionally, questions were raised about whether mucus release in response to irritants such as mustard oil is mediated by the central nervous system or autonomously by the tissue. Some years later, starting from 1965, a 'surface coat' in the intestine covering epithelial cells and the microvilli was described, separately from the mucus produced by goblet cells, observed using electron microscopy (Ito, 1965; Rifaat, Iseri *et al.*, 1965; Mukherjee and Williams, 1967; Monis, Candiotti *et al.*, 1969). This surface coat is produced by each cell individually and can vary quite abruptly from cell to the next (see Figure 21). This layer is known as the glycocalyx, a term proposed by Stanley Bennett in 1962 (published in 1963) meaning 'sweet husk', from Greek, in reference to the polysaccharides present in this layer (Bennett, 1963) in many species across the biological Kingdoms.

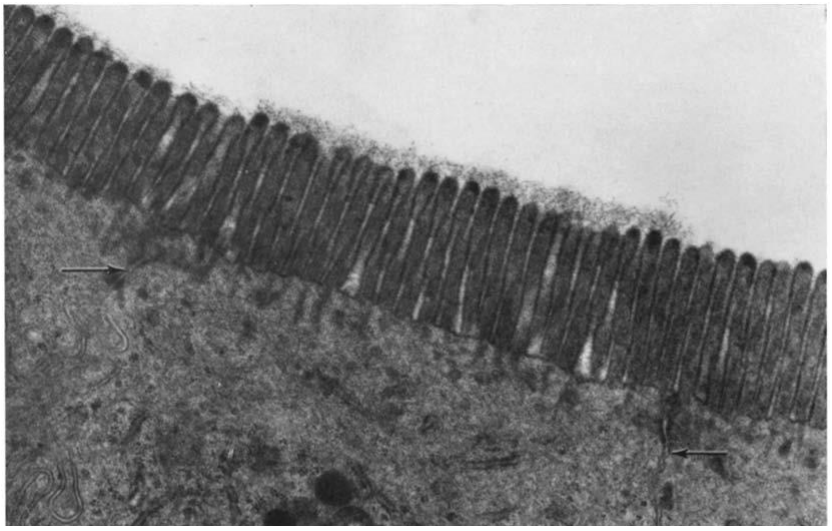


*Figure 21 - Surface coat of microvilli can vary strongly from one cell to the next. Adapted from (Mukherjee and Williams, 1967)*

Up until the eighties, the diffusion barrier of the intestinal barrier was thought to be an 'unstirred water layer' (UWL), water that is not stirred due to proximity to cells, and therefore forces absorption to occur only through diffusion, specifically negating the role of the glycocalyx and the mucus secretions in absorption (Thomson and Dietschy, 1984). This role was asserted earlier, in part because the available space in rat intestine is not sufficient for an UWL of adequate thickness to explain the slow diffusion rate observed (Smithson, Millar *et al.*, 1981). During this debate, people started to further conceptualize the nature of the mucus gel covering gastrointestinal epithelia, and it was proposed that the already familiar UWL covered by a lipid membrane is actually hydrated goblet-cell mucin (Nimmerfall and Rosenthaler, 1980). It was proposed that the mucus is secreted in a condensed form that subsequently expands in the lumen (Allen, 1983). It seems logical that a possible unstirred water layer would be incorporated in the mucus layer, based on the strong gel-forming properties and interactions with water of this condensed mucin secretion. At the same time, literature started to report on the properties of a 'luminal mucin layer' (LML), covering epithelia throughout the colon of different species. Notably, it was reported that the proximal colon has a thicker but highly variable LML, not continuous and mixed with food particles and bacteria, while the distal colon has a thinner layer of very compact non-penetrated LML covering the epithelium and separating it from the contents, observed in cryo-sections (Sakata and Engelhardt, 1981).

Later again, in the nineties, the adherent mucus layer of human colon biopsies was investigated by sectioning fresh material after removing the mucosa from the *muscularis mucosa*, and observed on filter paper, while keeping the tissue moist with saline. In this way, a mucus layer of around 150µm is observed, see Figure 22 (Pullan, Thomas *et al.*, 1994).

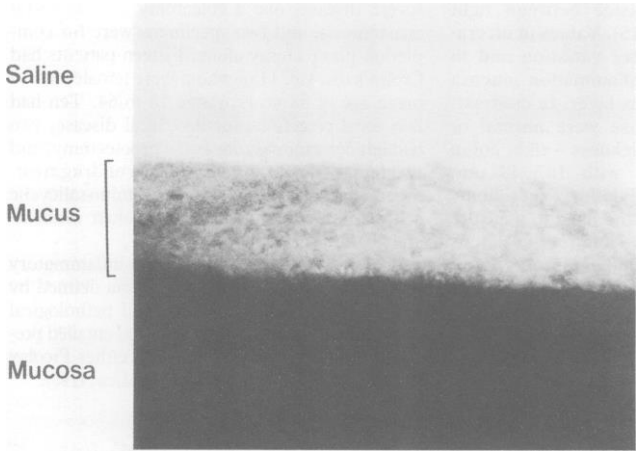



*Figure 22 - Mucus covering the mucosa of a prepared fresh human colonic biopsy. (Pullan, Thomas et al., 1994)*

Similarly, Matsuo *et al.* showed an adherent mucus layer on human colonic biopsies, by rinsing, pinning on board, and coloration after fixation with Carnoy's solution. They reported a 2-layer organisation, with an inner layer ranging from approx. 4,5 µm to 12,7µm, and an outer layer ranging from approx. 31 to 89µm (Matsuo, Ota *et al.*, 1997). Studies using paraffin-embedded

rodent intestine fixed in Carnoy's solution in combination with *ex vivo* measurements using explants mounted in specially designed mucus chambers would then clarify more about the function of this mucus covering the epithelial lining on the intestines. In 2008, it was reported that *ex vivo*, the outer layer covering mouse colon is about 100µm, and is easily removed, and the inner around 50µm, which is firmly attached to the epithelium. In the same study, in histological material, it is shown that the outer layer contains bacteria, while the inner layer does not (Johansson, Phillipson *et al.*, 2008). This notion, together with the severe and often lethal colitis observed in muc2$^{-/-}$ mice (Van der Sluis, De Koning *et al.*, 2006), which lack a mucus barrier, led to the understanding that the mucus barrier is necessary to keep bacteria away from the epithelium, and thus essential in preventing inflammation.

A proper understanding of the nature and functions of the intestinal mucus barrier is important, because it impacts the design and veracity of theoretical, practical and mathematical models of interactions in the gut. For example, results obtained using promising systems such as gut-on-a-chip (Kim, Huh *et al.*, 2012) have to be compared to a precise understanding of physiological conditions *in vivo*, to allow realistic interpretation. Additionally, pharmacological approaches take interactions of medicinal compounds with the mucosal barrier into account (Netsomboon and Bernkop-Schnurch, 2016), and to effectively do this, correct estimations of mucus barrier organisation and regulation are essential.

One thing is sure, mucosae produce mucus…

#### 1.5.3.3 Regulation

Even though the general description of the mucus layer is fairly static, there are several known important mechanisms that regulate mucus secretion, and finetune the production in relation to different situations and challenges. Since presence of a pathogen is one of the most acute naturally occurring challenges to the intestinal epithelium, and protection against pathogens of the main functions of the mucus barrier, it stands to reason that it influences mucus barrier regulation. NLRP6 inflammasome activity is strongly implied in proper barrier function, as NLRP6-deficient mice have a defective mucus barrier, which renders them more susceptible to *Citrobacter rodentium* (Wlodarska, Thaiss *et al.*, 2014). Inflammasomes are multiprotein complexes that regulate innate immune responses of cells, integrating information from cytokine signalling, microbial products, and damage-associated molecular patterns (DAMPs) (Rathinam, Vanaja *et al.*, 2012). Signalling from TLR4 in response to bacterial lipopolysaccharides (LPS) causes mucin granule exocytosis of goblet cells, thereby causing an increase in its thickness, and flushing away the bacteria from the epithelial surface (Dharmani, Srivastava *et al.*, 2009). An insufficient mucus barrier, such as observed in germ-free animals, due to lack of signals from microbial products, renders mice exceptionally sensitive to chemically induced colitis by treatment with dextran sodium sulphate (DSS) (Kitajima, Morimoto *et al.*, 2001), indicating both that the microbiota and microbial signalling is necessary for optimal mucus barrier regulation, and that the mucus barrier protects against harmful toxic chemicals.

Colonic crypts are 'guarded' by sentinel goblet cells (senGC), which reacts strongly to the presence of bacteria by NLRP6 activation by PRR signalling, in response to LPS and flagellin. This causes compound exocytosis of muc2 mucus, and triggers mucus release in neighbouring goblet cells as well, through an intercellular gap-junction signal (Birchenough, Nyström *et al.*, 2016). In the ileum, other PRRs are expressed, and crypts there are not responsive to LPS or flagellin (Birchenough, Nyström *et al.*, 2016). This could reflect the different situations in these compartments; whereas bacteria reaching the epithelium in the colon is a danger that warrants a reaction, in the ileum, where no true separating mucus barrier exists, this is a normal situation.

Apart from signalling from microbes or luminal chemicals, mucus release is also promoted by cholinergic signalling (Specian and Neutra, 1980) and histamine (Castagliuolo, Lamont *et al.*, 1996). Though both small and large intestine mucus production is activated by acetylcholine, the small intestine is by far more sensitive to this (Birchenough, Johansson *et al.*, 2015).

Together with the observation that the large intestine is more responsive to TLR activation than the small intestine, this reinforces the idea that the mucus release is regulated differently in these two different organs; the small intestine mucus barrier more responsive to parasympathetic activity, the large intestine more to luminal signalling.

#### 1.5.3.4 Dietary effects

The mucus barrier can be impacted by the diet in several ways. What we eat can have effects on the expression of mucins and the regulation of mucus release, but the physical properties of the bolus and faeces also have an impact on the properties of the mucus layer, and the rheology of mucus excretions. Here, we will look at some examples of known effects of the diet on the mucus barrier.

A major influence on the mucus barrier is dietary fibre; an *in vivo* rat model testing different dietary fibre contents showed that mucus turnover as well as production was reduced by fibre deficiency, and different fibres have different effects on these factors (Brownlee, Havler *et al.*, 2003). In the same vein, fibre deficiency in a mouse model causes the expansion and increased activity of mucus-degrading bacterial species, leading to a reduced mucus barrier, and a reduced resistance to *C. rodentium* (Desai, Seekatz *et al.*, 2016). In a way, it speaks for itself that nutrients that lead to the production of SCFAs by the microbiota, such as these dietary fibres, have an effect on the mucus barrier, because SCFAs have effects on mucus release and production (Sakata and von Engelhardt, 1981; Shimotoyodome, Meguro *et al.*, 2000; Hedemann, Theil *et al.*, 2009), as well as possible modulation of mucin gene expression (Paturi, Butts *et al.*, 2012).

Dietary lipid intake has an effect on the mucus barrier as well, changing the permeability of this barrier, decreasing the mobility of particles (Yildiz, Speciner *et al.*, 2015). Contrary to the effects of SCFAs, these effects of lipids play a direct role in interaction with the mucus itself.

Apart from these effects, because we have already described microbiota interactions with the host and its effects on mucus barrier regulation, diets with specific impacts on microbiota composition or activity, logically also have an effect on the mucus barrier.

## 1.6 Aims and Outline of Thesis

### 1.6.1 Hypotheses

Based on the available literature and the theoretical framework, we propose that FODMAP fermentation by the intestinal microbiota can have harmful effects which increase the tendency to display gastrointestinal symptoms. This is complementary to the known mechanisms by which FODMAP ingestion provokes these symptoms; an increased distension of the intestine by an increase of the osmotic load and water volume in the small intestine, and of gas volume in the large intestine, generated during fermentation. As functional parameters to these harmful effects, we selected to investigate the visceral sensitivity, intestinal barrier function, and microbial metabolite profiles of the colonic contents in relation to treatment with selected representatives of FODMAPs. We formulate the following **hypotheses**:

1. **FODMAP ingestion can induce gastrointestinal symptoms of IBS by itself, in an otherwise healthy system**
2. **This effect relies on the production of harmful bacterial fermentation metabolites, among which methylglyoxal**
3. **Methylglyoxal and other strong glycation agents lead to an increase in AGEs and a pro-inflammatory state through activation of RAGE**

A recurring theme and major field of interest in research of gastrointestinal disease and function is the relation of the host with their microbiota. In this context, the contact between intestinal microbes and host surfaces are considered important, and an understanding of the properties of the excreted mucus barrier, which separates microbes from the epithelium in the colon where the concentration of microbes is highest, is key to understanding host-microbe relations in the intestine. For this reason, to better understand the mucus barrier function, we have identified some situations in which information regarding this interesting topic was missing and designed experimental approaches to fill in these gaps of knowledge.

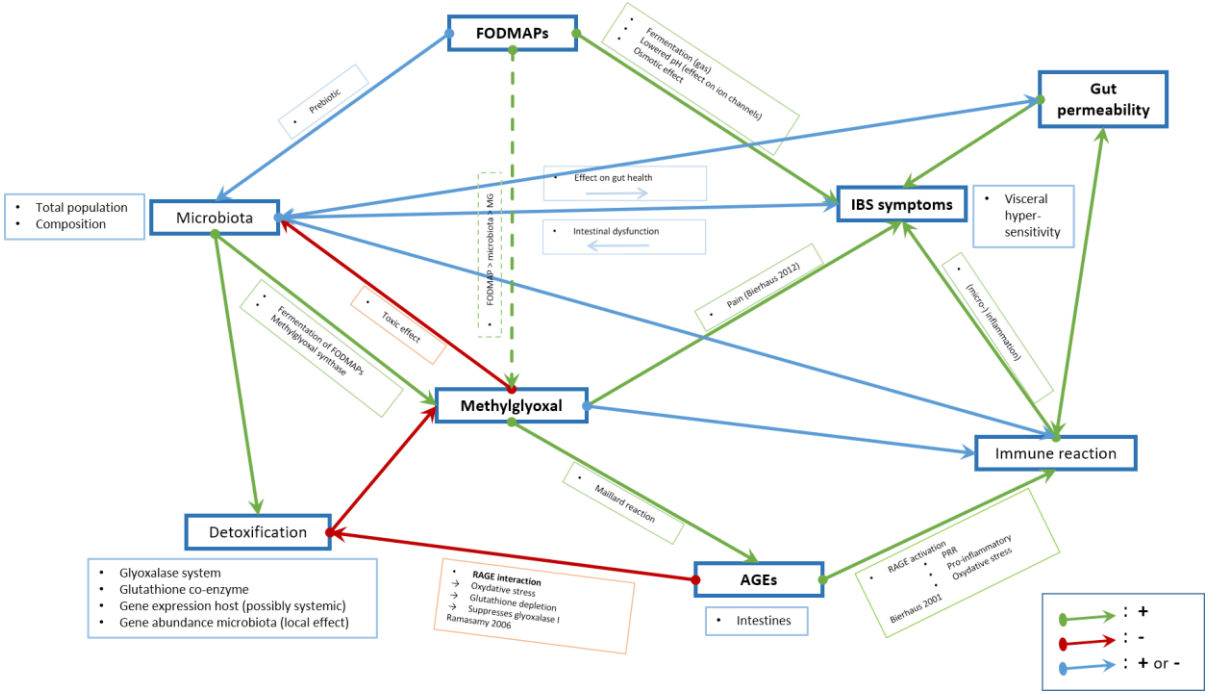



*Figure 23 - Web of possible causal relations involved in the effects of FODMAPs on IBS symptoms*

**+** *: positive relation, i.e., increases*

**-** *: negative relation, i.e., decreases*

**+ or -** *: either positive or negative relation*

To identify approaches of interest to better understand the subject, a mind map of putative causal relationships was designed based on literature and logical connections. The fermentation of FODMAPs by the gut microbiota leads to the production of methylglyoxal, which in return can impact the composition and functionality of the gut microbiota (Russell, 1993; Totemeyer, Booth *et al.*, 1998; Tuohy, Hinton *et al.*, 2006; Campbell, Matthews *et al.*, 2010). Because methylglyoxal has been named as an important toxic bacterial metabolite (Campbell, Naseem *et al.*, 2007; Campbell, Matthews *et al.*, 2010), it has a central place. Its activity as a dicarbonyl leads to the production of Advanced Glycation End products (AGEs) which themselves lead to immune activation, because they're recognized as DAMPs by the innate immune system. This in turn can lead to symptom generation, and can increase gut permeability, which is also implicated in the development of IBS symptoms. In general, it can be inferred that either by direct impact or effect on an intermediate actor, most of these factors influence each other in various feedback loops.

### 1.6.2 Approach

To investigate the relations between FODMAP intake, IBS symptoms, immune activation, and bacterial fermentation products, we have designed and performed animal experiments related to each hypothesis; male C57Bl/6 mice have been given FODMAP representatives (lactose and fructo-oligosaccharides) for 3 weeks, to induce FODMAP related changes to their physiology. Additionally, an anti-glycation agent (pyridoxamine) was administered intended to counteract the effects of glycating elements produced during bacterial fermentation of said FODMAPs. Next, analyses were performed to answer our questions;

**Hypothesis 1**: To test whether FODMAPs can induce IBS symptoms we have measured the visceral sensitivity, gut permeability, mast cell counts in the colon, and colonic mucus barrier dysregulation in the treated animals.

**Hypothesis 2**: To test whether these effects were caused by glycation processes, we have analysed whether administration of anti-glycation agent pyridoxamine protects against these consequences, additionally, generation of reactive aldehydes and ketones in the colonic contents was analysed by HPLC/MS after derivatisation with BBHA (1-((aminooxy)ethyl)-2-bromobenzene hydrochloride).

**Hypothesis 3**: To identify the effect of these glycation processes, we have measured the occurrence of advanced glycation end-products (AGEs), and the expression of Receptor for AGEs (RAGE) in the colon of treated animals.

### 1.6.3 Experimental procedures

#### 1.6.3.1 Treatment setup

C57Bl/6 mice are housed in groups and treated with daily gavages of lactose and/or pyridoxamine, or put on a custom FOS diet (AIN-93M +/-10% FOS, starch partly replaced). Animals are weighed 2 times per week. Animals for permeability assays and mechanical behavioural testing are also used for histological purposes, animals for visceral sensitivity assays are not.

#### 1.6.3.2 Visceral sensitivity: EMG, von Frey

Since a central IBS symptom relieved with the low-FODMAP diet is abdominal pain/discomfort, we set out to measure the possible induction of increased sensitivity by FODMAP ingestion. The reference method of recording of electromyographic response to colorectal balloon distension, as well as an alternative method of mechanical behavioural testing using von Frey filaments were performed. This alternative was necessary as FOS-treatment lead to increased amounts of intestinal content that caused the colorectal cavity to remain full even after fasting and habituation periods, which would impede reliable data gathering.

##### Visceral sensitivity (Electromyography (EMG))

Electrodes are implanted into the abdominal muscles and colorectal distensions are used as noxious stimuli to evaluate visceral hyperalgesia by electromyographic (EMG) recording. Striated muscle's EMG activity was recorded and analysed according to (Larsson, Arvidsson *et al.*, 2003). Basal EMG activity was subtracted from the EMG activity registered during the periods of distension.

This procedure is considered the gold standard in visceral sensitivity analysis, measuring the abdominal contractions in response to a painful stimulus in a quantifiable way. Visceral hypersensitivity is observable through an increased response to the colorectal distension stimulus, both by a response to stimuli that do not provoke a reaction in normosensitive subjects (allodynia), as well as an increased response to painful stimuli (hyperalgesia).

Mechanical behavioural testing (von Frey)

Three von Frey filaments with bending force 0.16, 0.6 and 1.4 g are applied to the mid-plantar area of each hind paw and nociceptive specific behaviours noted.

This procedure allows us to compare the central sensitivity of animals, by valuing the responsiveness to mechanical stimuli on the hind paws, as well as the abdominal region. Animals that have an increased sensitivity exhibit a higher responsiveness. Animals with an increased visceral sensitivity will have a higher central sensitivity through sensitization processes mediated through the spinal cord (Lamb, Zhong *et al.*, 2006), probably through convergence of primary afferents on the same neurons in the spinal cord.

#### 1.6.3.3 Intestinal permeability

An important parameter of intestinal health is its permeability, which is known to play a role in IBS as well (Dunlop, Hebden *et al.*, 2006; Bischoff, Barbara *et al.*, 2014; Enck, Aziz *et al.*, 2016; König, Wells *et al.*, 2016). To investigate the effects of our FODMAP interventions on this parameter, we have performed *in vivo* measurements of intestinal permeability.

In vivo measurement of permeability using FITC

Fasted mice are gavaged with 250µl of fluorescein isothiocyanate (FITC) solution (1mg/ml). After 4 hours, when the entirety of the gastrointestinal has been passed by the solution, 100µl of blood is taken and kept in the dark on ice. Serum is recovered and analysed in a spectrophotometer. FITC concentration of blood samples is calculated using the standard curve.

This *in vivo* procedure is designed to measure the passage of a fluorescent marker to the blood, reflecting the paracellular or transcellular permeability of the gastrointestinal tract, depending on the size and nature of the marker used. We used fluorescein isothiocyanate (FITC), a small (~390Da) molecule, meant to measure predominantly the paracellular permeability.

#### 1.6.3.4 Immunofluorescence/ histology

##### Histological sample preparation

Collected tissues were fixated for 1 day in Carnoy's fixative and included in paraffin, and subsequently microsectioned and attached to glass microscopical slides.

##### Immunofluorescence

To visualize marker proteins and measure their expression, microscopic immunofluorescence assays were used.

AGE: Slides were deparaffinated and incubated overnight at 4°C with primary antibodies (Polyclonal Rabbit-anti-AGE (ab23722), followed by incubation with secondary antibody (Alexa Fluor 488 Donkey-anti-Rabbit (A21206).

Following our hypothesis that FODMAP fermentation leads to the production of reactive carbonyl species, we sought to quantify the occurrence of the retained, and cumulative, marker of glycation processes, advanced glycation end products (AGEs).

RAGE: Slides were deparaffinated and incubated overnight at 4°C primary antibodies (Polyclonal Goat-anti-RAGE (ab7764), followed by incubation with secondary antibody (Alexa Fluor 488 Donkey-anti-Goat (A-11055).

An increased occurrence of AGEs in the tissue can be expected to activate Receptor for AGEs (RAGE), which consecutively leads to the increased expression of this receptor (Bierhaus and Nawroth, 2009).

MMCP: Slides were deparaffinated and incubated overnight at 4°C with primary antibodies (Sheep anti-mMCP1 (MS-RM8), followed by incubation with secondary antibody (Alexa Fluor 594 Donkey-anti-Sheep (A-11016).

Mouse Mast Cell Protease-1 was chosen as a selective marker of (mucosal) mast cells (Dai and Korthuis, 2011), to visualize and quantify the mucosal mast cells in histological slides. Because it is easy to trigger mast cell degranulation and exocytosis of this marker, particular care was taken not to disturb the tissue before fixation, i.e., no touching of the tissue, no flushing of contents, no cutting to size, until after fixation.

Muc2: Slides were deparaffinated and incubated overnight at 4°C with primary antibodies (Rabbit anti-mucin 2 (sc-15334), followed by incubation with secondary antibody (Alexa Fluor 488 Donkey-anti-Rabbit (A21206).

Muc2 is the predominant mucin in small intestinal and colonic mucus, and this antibody staining is used to very clearly visualize mucus in immunofluorescent slides. This can be combined with FISH.

AB/H&E: Slides were deparaffinated and stained by 5 min in Hematoxylin, 10 min in running water, 30 min in Alcian Blue solution (pH 3.0) followed by 5 min in running water, 3 min in Eosin.

The haematoxylin and eosin stain (H&E) is a standard staining to visualize general morphology and recognize immune cell infiltrates in tissues, while also staining bacterial masses, the combination with Alcian blue (AB) staining visualizes goblet cells and mucus. The combination AB/H&E thus allows to analyse a diverse set of parameters.

Fluorescent *in situ* Hybridization (FisH): FISH staining for all bacteria was performed using probes EUB338I (5' CGTGCCTCCCGTAGGAGT 3'), EUB338II (5' GCAGCCACCCGTAGGTGT 3'), and EUB338III (5' GCTGCCACCCGTAGGTGT 3') conjugated to DY-590. Slides were deparaffinated and incubated with the FISH probes at 48°C overnight.

These 3 probes (EUB338I, EUB338II, EUB338III) together target virtually all true bacteria (Daims, Brühl *et al.*, 1999), so this mix can be used to reliably stain all bacteria found in the intestine, to clearly visualize the presence and location of bacteria.

#### 1.6.3.5 Markers of immune activation

##### Lipocalin-2

Faecal supernatants were analysed for lipocalin-2 content by ELISA.

Lipocalin-2 (LCN-2) is a very sensitive marker for intestinal inflammation, even subclinical low-grade inflammation, where histopathology is not clear (Chassaing, Srinivasan *et al.*, 2012). LCN2 is produced by neutrophils and macrophages.

##### Microscopic score

Composite micrographs were scored according to protocol on a scale from 0-4 (Neurath, Fuss *et al.*, 1995); 0: no signs of inflammation, 1: very low level of leukocytic infiltration, 2: low level of leukocytic infiltration, 3: high level of leukocytic infiltration, high vascular density, thickening of the colon wall, 4: transmural infiltrations, loss of goblet cells, high vascular density, thickening of the colon wall.

This scoring system for the mouse colon evaluates signs of overt inflammation, and thus grades severity of colitis.

#### 1.6.3.6 Physiological markers of transit

##### Transit time

Mice were temporarily housed in individual cages, carmine red solution was administered by intragastric gavage, and starting from 2 hours after administration, every 15 minutes or when the researcher noted defecation, the cages were checked for red-coloured droppings, the time of first appearance of a red dropping was used to determine the transit time.
This test tells us how long it takes for material to pass through the gastrointestinal tract, starting at the stomach.

##### Faecal water content

Faecal droppings were weighed, dried for 2 days at 80°C, and weighed again. Faecal humidity is the percentage of weight lost between wet and dry weights.

Faecal water content is a reasonable marker of colonic transit time, because a faster transit leads to higher percentages of water in faeces (Degen and Phillips, 1996; Lewis and Heaton, 1997b). We were interested in transit time and faecal water content because FODMAPs can be expected to influence these markers, which is important to consider in relation to further effects of treatment.

#### 1.6.3.7 Analysis of microbiota composition

##### Faecal DNA extraction, 16S rRNA gene sequencing, and bacterial community analysis

Genomic DNA was obtained from frozen faeces, 16S rRNA was amplified by PCR. Pooled amplicon libraries were sequenced employing an Illumina MiSeq. For further data on analysis of microbiota, please refer to section 2.2.

We analysed microbiota profiles to see the effect of lactose treatment on gut microbiota composition, as well as the effect of the anti-glycation agent pyridoxamine, because FODMAPs have a known prebiotic function, and could therefore be expected to impact microbiota profile composition, even at 5 mg daily.

#### 1.6.3.8 Analysis of carbonyl compounds in colonic content

Colonic content extracts were incubated with 1-((ammoniooxy)methyl)-2-bromobenzene chloride (BBHA) to generate brominated aldehyde derivates which were analysed by high-performance liquid chromatography coupled to high-resolution mass spectrometry (HPLC-HRMS). Resulting semi-quantitative mass spectra were analysed statistically to characterize differential ions and construct heat-map profiles for the different treatment groups. For a complete description, please refer to section 2.2.

This analysis allows us to follow the effects of our dietary interventions on carbonyl metabolite profiles.

#### 1.6.3.9 Mucus barrier function analysis

##### Imaging; Manual-ultra high-resolution Composite Image Overview (MUCIO)

Manual Ultra-high-resolution Composite Image Overview (MUCIO) approach: datasets of overlapping microscope images covering entire slides were generated by manual microscope photography and stitched together using dedicated software. The generated composite images contain all the information of the data set in a single ultra-high-resolution file. No selection for 'representative regions' is necessary, only obvious artefacts (e.g., possibly folded double/ heavily damaged sections) are evaded. Subsequent downscaling is necessary to facilitate interaction with images for further applications, but close-up views in full quality can be extracted from the original composite.

### Mucus layer thickness measurements

The faecal mucus layer thickness was measured every 100 micrometres along the entire imaged faecal surface generated with the MUCIO approach. Data interpretation: The hundreds of measurements generated were interpreted by calculating several statistics. First of all, of course, the average mucus layer thickness is calculated, but this does not tell us anything about the variation, and the standard deviation or confidence interval is calculated between individuals, not on multiple measurements per individual. To mitigate this, the Coefficient of Variation (CoV) is calculated for the dataset of each individual, and these values are compared for treatment groups.

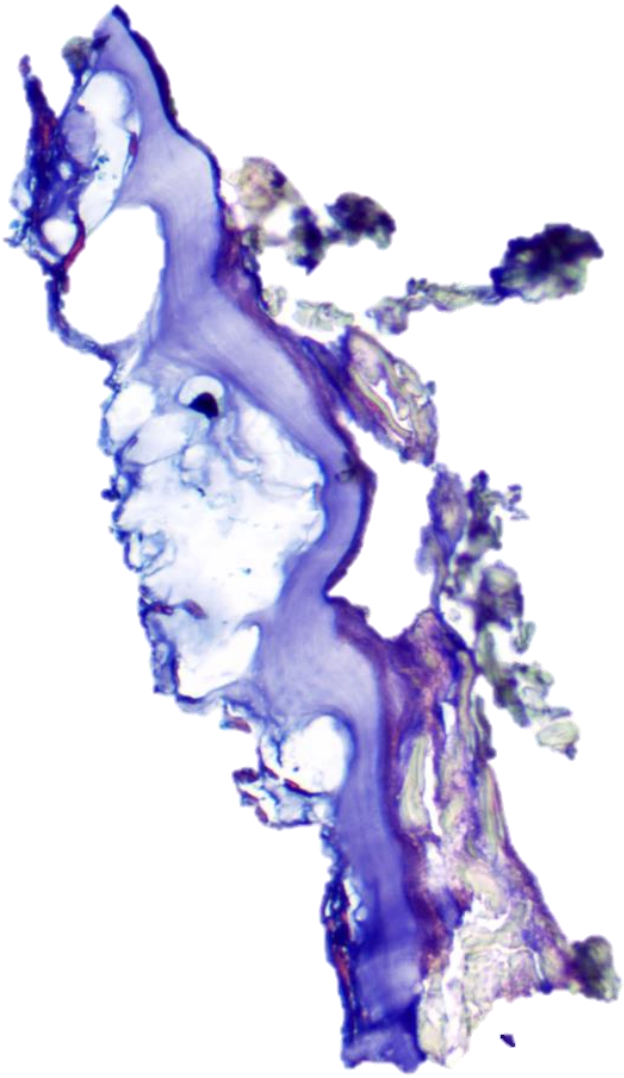
### Active goblet cell numbers

One ratio discharging goblet cell:crypt per mouse based on analysis of 25-90 crypts in transversal sections of empty colon, dependent on availability of suitable visual material

By calculating the average mucus layer, the CoV for this mucus layer, as well as the active goblet cells in empty colon, we can characterize the mucus barrier function, considering the variable situations that naturally occur in the distal colon.

# 2 Project Results

## Published Work, under Review, under Preparation



*"To see the world in a grain of sand, and to see heaven in a wild flower, hold infinity in the palm of your hands, and eternity in an hour."*

~William Blake, 1757-1827

# 2 Project Results

## 2.1 Mucus organisation is shaped by colonic content; a new view

While analysing the possible effects of FODMAPs on mucus barrier function, analysing histological sections in both regions with and without faeces, some observations were made that warranted further investigation. Notably, in sections lacking a faecal pellet, there is a marked absence of the classical inner-outer mucus layer organisation. This absence has been described before, and explained by the supposed protection from washing off of the mucus layer by the faecal pellet (Johansson, Larsson *et al.*, 2011). However, since we routinely acquire and fix several centimetres of colon, containing regions with and without faeces, and there is no flushing of this intact intestinal tube either before or after fixation, we wanted to test whether this absence is due to washing off of mucus, as postulated in literature.

Therefore, we decided to make longitudinal sections, and image the entire section, to see what is going on in the transition from empty to full sections of the colon, and *vice versa*. Additionally, we reasoned that if a possible collapse of the mucus was responsible for the absence of the described two-layer system, the bacteria should still be present, but now probably in closer contact to the epithelium. To visualize these bacteria, we performed fluorescence *in situ* hybridisation (FisH) of longitudinal and transversal sections of empty and full sections of proximal and distal colon.

The results of this investigation were published in Scientific Reports (Springer Nature).

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# Mucus organisation is shaped by colonic content; a new view

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**The colonic mucus barrier is commonly described as a continuous double layer covering the epithelium, separating the microbiota from the intestinal tissue. This model is currently considered valid throughout the colon. The colon is characterised by regional anatomo-functional specificities such as presence and consistency of contents and location. In this study, we characterised the organisation of the colonic mucus barrier in proximal and distal colon of rodents by histological and FISH staining, taking into account aforementioned specificities. By using longitudinal sections and imaging extensive areas of tissue with and without colonic contents, we have obtained a spatiotemporal overview of mucus organisation in the colon. We describe for the first time that the colonic mucus layer covers the faeces instead of the epithelium in the distal colon. This faecal mucus layer confines the microbiota to the faeces and prevents it from remaining in empty distal colon. In the proximal colon, the mucus did not form a separating layer between bacteria and epithelium. We conclude that the organisation of colonic mucus is reliant on the presence of the colonic content, and the location within the colon. Our findings reopen the discussion on the nature of the colonic mucus barrier.**

The intestinal mucus layer has a critical role in gut health. It facilitates the passage of faeces through the intestine, reducing the risk of damage to the gut epithelium[1]. The critical importance of intestinal mucus to gut health is underlined by the spontaneous development of colitis in Muc2 knockout ($Muc2^{-/-}$) mice[2], and their increased susceptibility to pathogens[3]. Bacteria of the intestinal microbiota profit the host with their metabolic activities[4], and modulation of the immune system[5]. Limiting the contact between intestinal microbes and the colonic epithelium, a thick mucus layer is described that separates the two[6]. Johansson *et al.*[7] reported that the distal colonic mucus layer is organised in 2 parts, a firm component and a loose one, both built around Muc2 mucin protein. This layer has been described to be organised as a loose layer inhabited by bacteria, and a layer firmly attached to the epithelium, devoid of bacteria[7]. Primarily, this understanding is based on histological observations of transversal sections of colon, imaging the mucus layers separating microbes from the epithelium. It became evident that it was impossible to image the mucus barrier in histological sections lacking a faecal pellet, so the method of reference became to section samples containing a faecal pellet to investigate the properties of the mucus layer. A given explanation for this necessity was that the faecal pellet protects and conserves the mucus barrier during histological processing[7]. Additionally, to further investigate mucus barrier properties, *ex vivo* experiments involving explant tissue[8], and *in vivo* experiments have been designed and performed[6,9]. The general organisation is described to be constantly sustained[7], though specifics are variable over time, and influenced by harmful factors such as inflammation[10] or even the time frame of microbiota colonisation[11], emphasising the intimate relationship between intestinal mucus and gut microbiota. Furthermore, microscopic analysis of the biostructure of human faeces shows mucus irregularly intersecting into the faecal mass[12], and a mucus layer is observed on expelled faeces from rodents as well as humans[12,13]. These results obtained from faecal material illustrate the organisation of the contents of distal colon, which does not necessarily reflect the situation in the proximal colon. Apart from the morphological differences between the proximal and distal colon, the contents too are dissimilar in their consistency and humidity; the proximal colon content is liquid whereas the contents of distal colon normally form pellets with a firmer consistency. Changes in the physical and biochemical properties of colonic mucus have been documented in pathophysiological conditions. For instance, colonic mucus from ulcerative colitis (UC) patients in the active phase of the disease is thinner and more penetrable to fluorescent beads compared to healthy subjects[14] and glycosylation of colonic mucins is found to be correlated with the severity of inflammation in UC patients[15]. Likewise, O-glycosylation of mucins is strongly affected by chronic psychological stress in rats, associated with a flattening and a loss of cohesive properties of the mucus layer[16]. Despite data indicating that the

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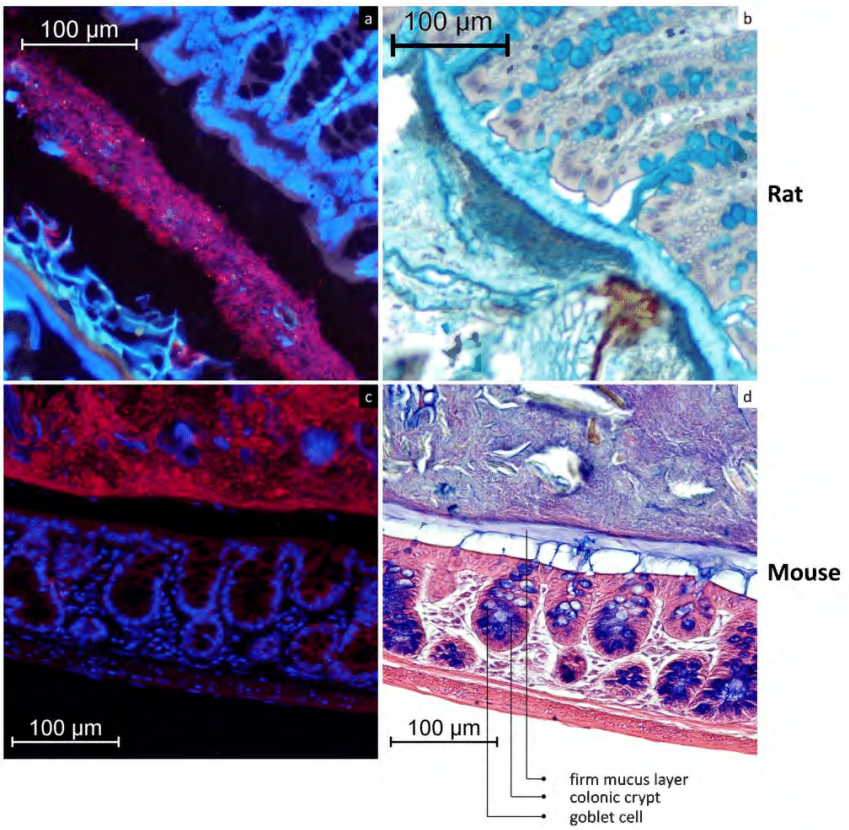


**Figure 1.** A firm mucus layer separates the bacteria from the epithelium. (**a**) FISH stained transversal section (Bacteria: red; nuclear staining DAPI: blue) of rat distal colon. (**b**) AB/H/E stained transversal section of rat distal colon. (**c**) FISH stained transversal section (Bacteria: red; nuclear staining DAPI: blue) of mouse distal colon. (**d**) AB/H/E stained longitudinal section of mouse distal colon. The FISH staining indicates where the bacteria are located, and that this corresponds to the dark blue/purple staining in AB/H/E stained samples.

properties of intestinal mucus are dynamic and influenced by a disruption of intestinal homeostasis, modification of the microbiota, altered food intake, and disturbances in gut motility, little is known about the dynamics of the mucus barrier during normal gastrointestinal transit, reflected by the periodic presence and absence of faeces. This study is meant to clarify the influence of the colonic load on mucus layer structure and organisation in physiological conditions in the colon. We performed longitudinal and transversal sections of distal and proximal colon of rat and mouse in samples covering regions with and without luminal content. We used classical histology to characterise tissue and mucus morphology, combined with 'Fluorescent *in situ* Hybridisation' (FISH) staining to localise the gut microbiota. Instead of depicting select microscopic fields, we generated images covering the entirety of both transversal and longitudinal sections using a Manual Ultra-high resolution Composite Image Overview (MUCIO) approach, creating comprehensive overviews of tissue sections, rendering them more understandable as well as preventing an image selection bias in our results.

## Results

**Organisation of mucus in distal colon is influenced by the presence of colonic content.** In transversal sections of full distal colon, a sterile mucus barrier separated the epithelium from the faeces and the microbiota (Fig. 1). Bacteria were almost absent from this layer of about 35 µm (Fig. 1a,b) in rat, and 20 µm (Fig. 1c,d) in mouse. Changing the direction of sectioning, in longitudinal sections covering both empty and full sections of rat distal colon, faecal pellets were completely covered by a sterile mucus layer of variable thickness (36 µm ± 9 µm), with a mix of bacteria and mucus present underneath (Fig. 2a–d). The mucus layer only covers the faecal pellet, and is not attached to the epithelium (Fig. 2a,c). The dark blue layer (Fig. 2c,d) is rich in bacteria, as corroborated by Fig. 1. In transversal sections of empty distal colon mucus was not organised in a similar fashion; instead, 'luminal' mucus without an apparent layer structure was present (Fig. 3). In these empty sections the tissue was folded up neatly, with opposing epithelia very close to each other, sometimes in contact. In rat tissue, the distance between opposite epithelia in empty distal colon averages 17 ± 9 µm (Fig. 3a), in mice it averages 16 ± 7 µm (Fig. 3c). In contrast to the situation in colon containing faeces, we have not observed bacteria-colonised mucus

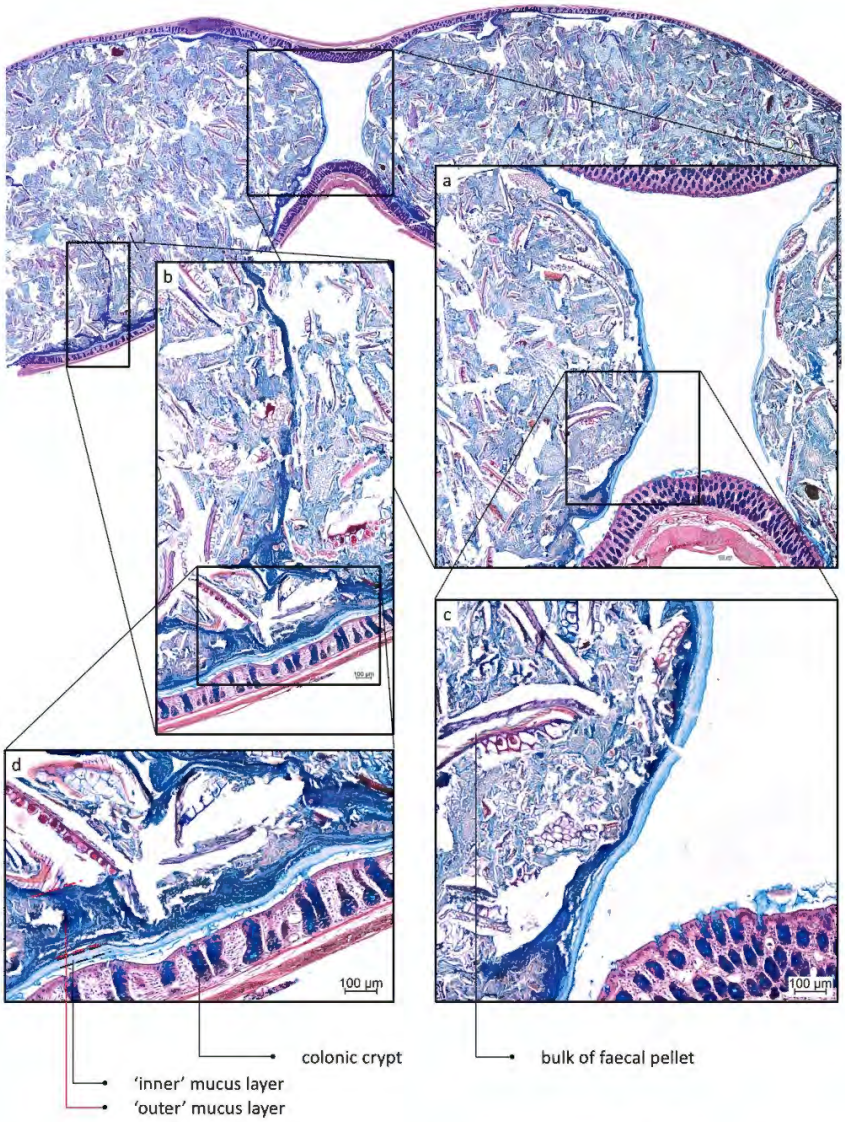


**Figure 2.** AB/H/E stained longitudinal section of distal rat colon containing faecal pellets. A sterile mucus layer covers the pellet completely. (**a**) The mucus layers attached to the faeces cover the entire pellet. (**b**) A line of bacteria-overgrown mucus penetrates the faecal pellet. (**c**) Sterile (light blue) and hacteria-overgrown mucus layers (dark blue) can be recognised, mucus production by goblet cells in the lumen is visible. (**d**) Sterile ('inner') and bacteria-overgrown ('outer') mucus layers, are recognised in a manner commonly observed in transversal sections.

in empty distal colon (Fig. 3b,d). A longitudinal section of mouse distal colon containing faeces shows that bacteria are confined to the pellet, which consists mostly of alimentary residues embedded in microbiota-colonised material (Fig. 4). The sterile mucus layer can be internalised by the colonic contents in mouse distal colon (Fig. 5). In this situation, the internalised firm mucus layer remains sterile, seen from the lack of signal in a FISH staining (Fig. 5c,d).

**Excreted faeces are covered by a faecal mucus layer.** On expelled faecal pellets of mice, a mucus layer ($24 \pm 4.5\,\mu m$) devoid of bacteria covers a mix of bacteria and mucus directly (Fig. 6), as well as the rest of the faecal material. This faecal mucus layer has the same appearance and general thickness as the one observed previously (Figs 1 and 2) in distal colon, which covers faecal pellets and separates contents from the epithelium.

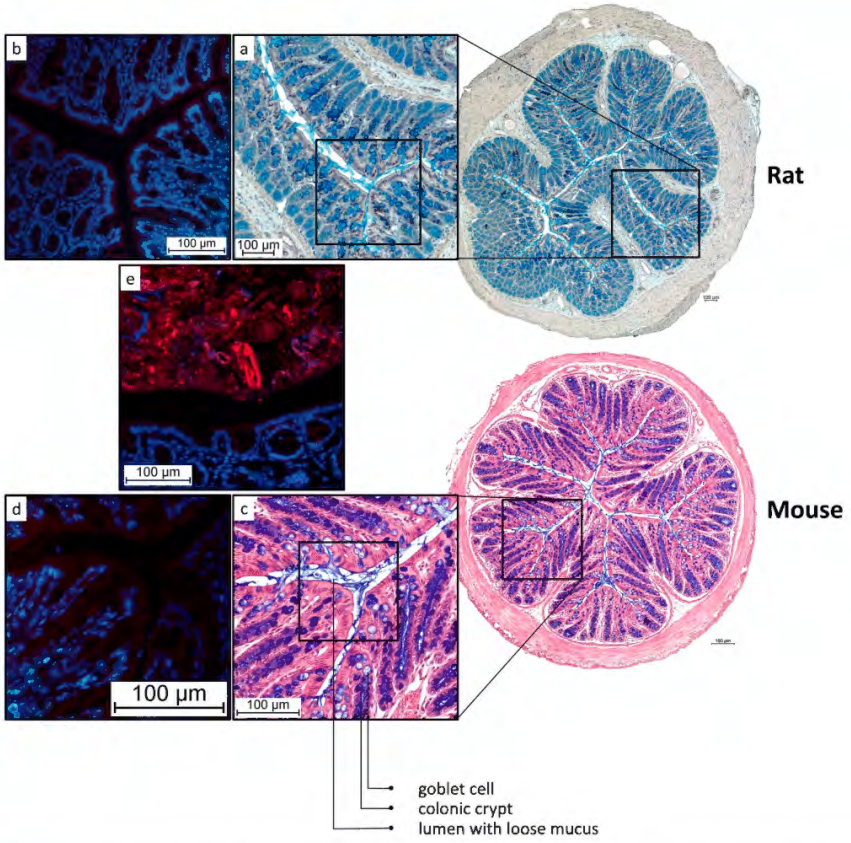


**Figure 3.** AB/H/E stained transversal sections of distal rat and mouse colon without faeces. The lumen contains loose mucus, but no bacteria are detected. (**a**) Close-up of rat empty distal colon (**b**) FISH staining indicating the absence of bacteria in the lumen of collapsed rat colon. (**c**) Close-up of mouse empty distal colon. (**d**) FISH staining indicating the absence of bacteria in the lumen of collapsed mouse colon. (**e**) FISH staining of rat colon containing faeces (Bacteria: red; nuclear staining DAPI: blue) serving as a positive control.

**Mucus barrier in proximal colon is affected by consistency of contents.** In the proximal colon, the microbiota was in contact with the epithelium with a noticeable absence of a significant mucus barrier (Fig. 7). The colonic contents were seen to form the first pellet in the distal end of the proximal colon, on the transition towards the distal colon. Here, the first establishment of a mucus barrier can be observed, in multiple streaks on the most distal part of the newly formed pellet (Fig. 7b). In proximal colon, mucus containing bacteria was found in direct contact with the colonic tissue, both in more folded collapsed tissue (Fig. 7c) and in tissues where the lumen is distended by colonic contents (Fig. 7d). In mostly or completely collapsed proximal colon, bacteria-colonised mucus was found in contact with the intestinal tissue, unseparated by a sterile mucus barrier (Figs 7c and 8c,d), in contrast to the situation described earlier for distal colon. In full proximal colon, we observed bacteria in contact with the epithelium too, with the microbiota present in an increasing gradient towards the tissue (Figs 7d and 8a,b); the highest concentration of bacteria was present around and especially below the tissue folds specific to the proximal colon.

## Discussion

Longitudinal sections of distal colon reveal that the colonic load is a determining factor in the organisation of the colonic mucus. The model of dual mucus layers covering the colonic epithelium is based on observations in transversal sections of colon containing a faecal pellet[6]. We obtained similar observations in transversal sections (Fig. 1). To our knowledge, this organisation has never been observed in bistological sections of untouched tissue lacking faecal pellets, reportedly because the faecal pellet is a necessary factor for mucus conservation during the histological procedures[7]. Our results point out that the faecal pellet is indeed prerequisite for observation of the firm mucus layer, but for different reasons. In longitudinal sections, it is noticed that the observed mucus layers cover the faecal surface instead of the epithelium, and so are not attached to the epithelial surface (Fig. 2). Further supporting this implication, the sterile mucus layer produced by the intestinal mucosa is sometimes found inside a faecal pellet, where it can only have ended up after a collision between two faecal pellets (Fig. 5). Taken together, these data suggest that the use of transversal sections to observe mucus layer organisation has led

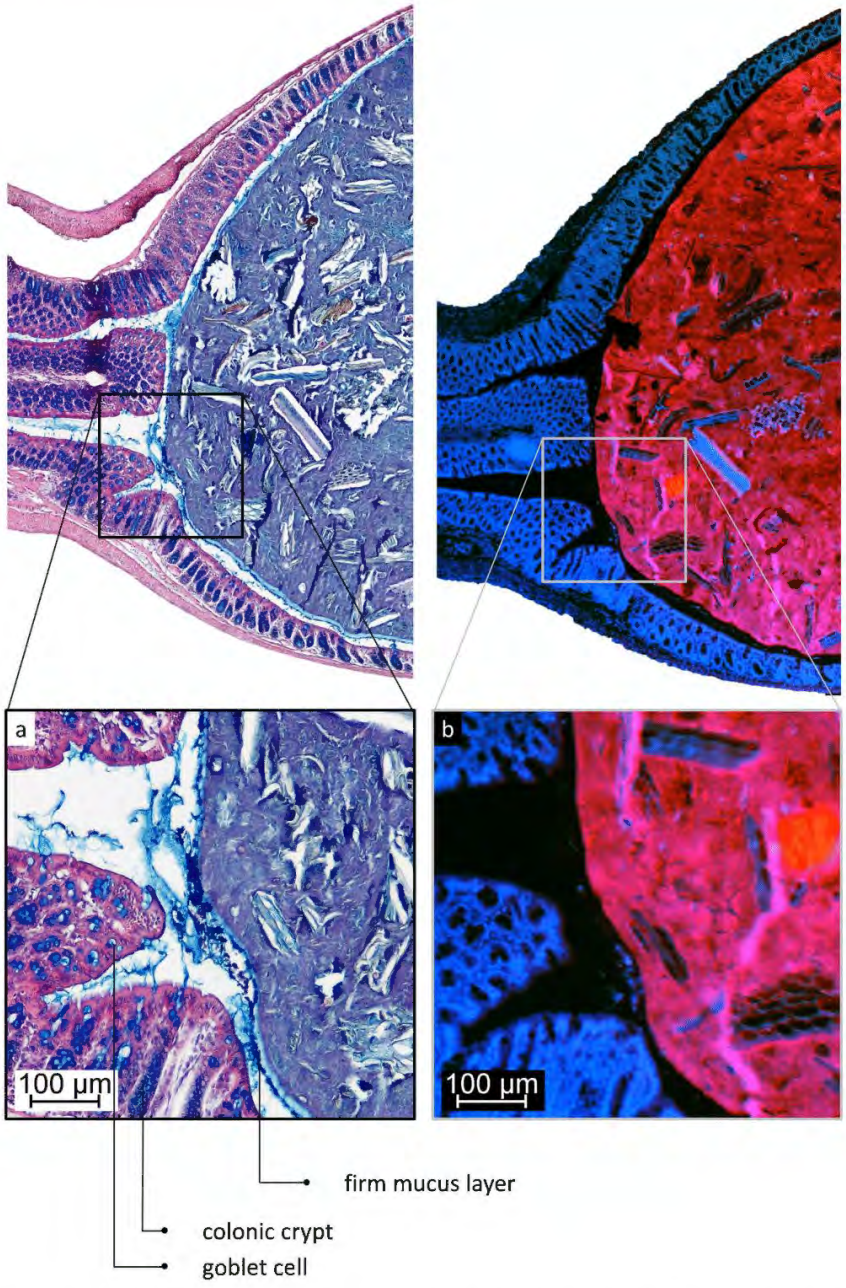


**Figure 4.** In distal colon, the microbiota is confined to the faecal pellet. (**a**) AB/H/E stained longitudinal section of mouse distal colon. (**b**) FISH stained (Bacteria in red; nuclei in blue) longitudinal section of mouse distal colon. The bacteria are located in the faecal pellet, the lumen of the empty colonic part is almost completely devoid of bacteria, but does contain some loose mucus. A mucus layer devoid of bacteria, confining the bacteria to the faeces, covers the pellet.

to the misinterpretation that the faecal mucus layer is attached to the epithelium. Instead, the organisation of the mucus layers seems to be determined by faecal pellet transit within the gut. In our experiments, we found that the sterile mucus barrier of the distal colon is not continuous, but depends on the presence of a faecal pellet. Still, the conditions in empty distal colon are strongly devoid of bacteria, because a clear majority of microbes are removed together with the faecal pellet and its mucus. A schematic overview of our proposed mucus layer organisation in distal colon can be found in Fig. 9.

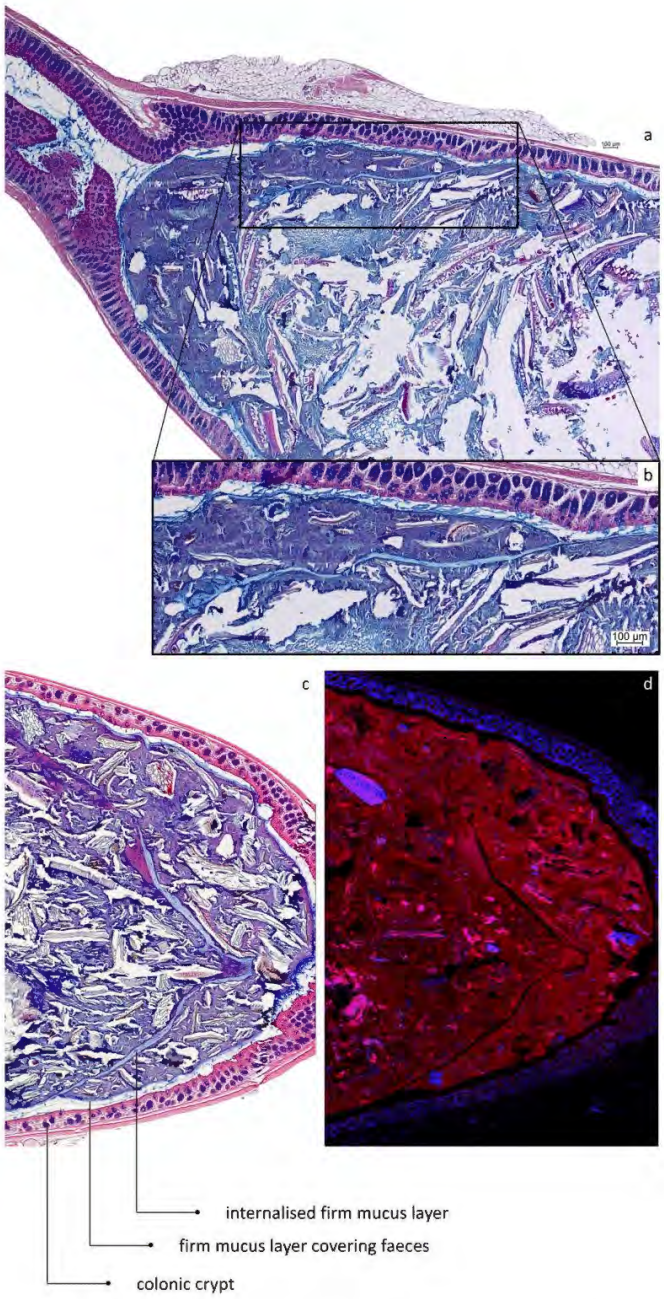


**Figure 5.** After collisions between faecal pellets in the distal colon, the mucus layers covering their surface can be internalised into the resulting composite pellet. (**a**) AB/H/E stained longitudinal section of distal mouse colon. (**b**) Close-up showing the internalised firm mucus layer. (**c**) AB/H/E stained longitudinal section of distal mouse colon. A firm mucus layer devoid of bacteria is found inside the pellet. (**d**) FISH stained (Bacteria in red; nuclei in blue) longitudinal section of mouse distal colon, the internalised firm mucus layer is recognised by the absence of bacteria.

As supported by literature[8], mucus is excreted from colonic crypts both in the presence and absence of luminal content, indicating a continuous baseline excretion. In *ex vivo* experiments, mucus produced by the tissue forms a firm inner layer of fresh mucus attached to the epithelium and a loose outer layer of older, more disintegrated

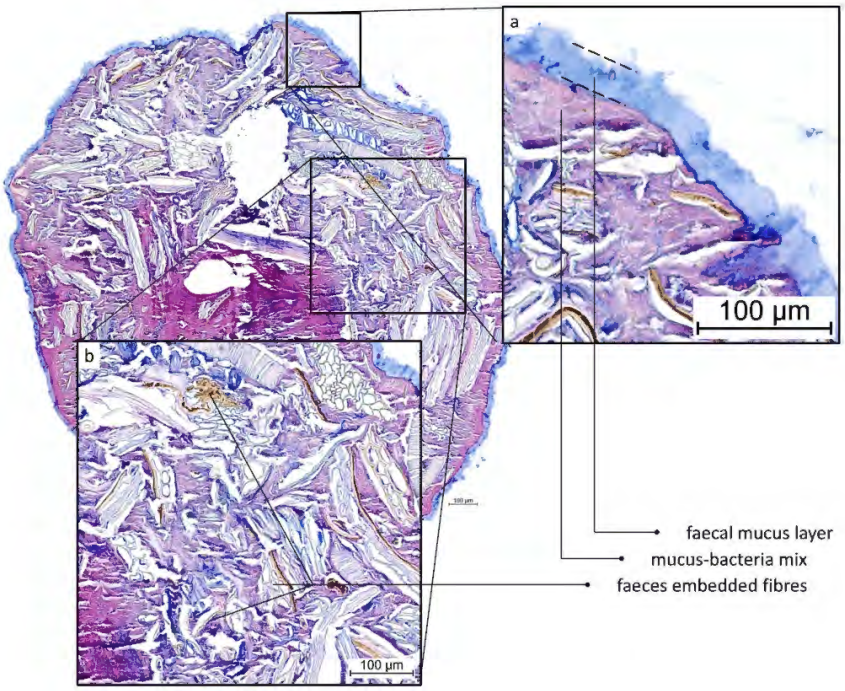


**Figure 6.** AB/H/E stained transversal cross-section of an expelled mouse faecal pellet. (**a**) A mucus layer devoid of bacteria covers the entire pellet. (**b**) Apart from alimentary residues and microbiota mixed with mucus, there are undigested plant fibres embedded in the faeces, as commonly observed in faecal matter in the gut as well.

mucus[6,9]. In these conditions, the firm adherent mucus layer of rat colon is reported to be 116 µm[9], or 101 µm in *in vivo* experiments[17]. In *in vivo* measurements in mice, the firm mucus layer thickness is stated to be 49 µm[6]. In histological sections of empty distal colon, these loose and firm layers are not observed. In these conditions, the lumen is collapsed and the tissue neatly folded, leaving insufficient space to harbour an equally thick mucus layer. Indeed, collapsed distal colon of both rat and mouse has epithelia at around 16 µm from each other, leaving just 8 µm on average per epithelial surface for this supposed mucus layer (Fig. 3). The disparity of these data seems linked to the experimental approach; opening the colon and submerging the epithelial surface in buffer solution, such as usually practiced in *ex vivo* and *in vivo* experiments, might increase the thickness of the observed mucus layer. Mucus swells in a wet environment, based on its properties[18,19], so it is important to notice that the humidity levels of the colonic contents in distal colon are normally around 55%, a strong deviation from these experimental conditions. Additionally, it is likely that mucus production and release are increased in response to mechanical and chemical perturbations of the tissue induced by the experiments. Unfolding of the colonic tissue, which is a condition shared between these *in vivo* and *ex vivo* experiments and physiological pellet transit, might influence mucus secretion. An increased mucus secretion can also be induced by the high concentration of microbial products in the faeces, known stimuli of mucus excretion such as short chain fatty acids (SCFAs)[20], lipopolysaccharides (LPS) and peptidoglycans (PGN)[21]. Specifically LPS was recently shown to trigger crypts to release mucus by activating Nlrp6[22]. These last points are particularly interesting because, as we have shown here, the arrival of the faecal pellet to empty distal colon reintroduces a large microbial presence, and thus increases the presence of LPS and other microbial factors; this could represent an important regulatory mechanism by which the mucus barrier is formed around the faecal pellet. We posit that colonic crypts release additional mucus onto the faecal pellet during its passage, covering it in a mucus layer. Mucus is notoriously difficult to fix for paraffin inclusion. Standard formalin fixation causes the mucus to be completely lost[9]. However, fixation with Carnoy's solution is currently the most suitable option for conservation of the mucus layers for histological purposes[23], and although shrinkage does still occur[24], mucus layers are observed sufficiently well. As discussed earlier, the faecal mucus layer is less hydrated than mucus in an aqueous environment; the resulting increase in density could increase the efficiency of fixation and subsequent staining, which means that a possible highly hydrated surface mucus layer could remain undetected due to restraints inherent in histological techniques. However, we still see that the folding of empty distal colon does not leave enough space for double mucus layers of the thickness classically reported. In the proximal colon, in a similar fashion, the complete absence of a separating mucus layer can not be explained by this type of artefact, instead of empty space left behind after shrinkage, which is normally reported[24], we see close contact between bacteria and epithelia. Matsuo *et al.*[23] have shown that it is possible to fix mucus layers covering the intestinal epithelium if they are present, even in the absence of intestinal contents, in apparent contradiction to our findings. In their experiments, surgically removed human intestinal

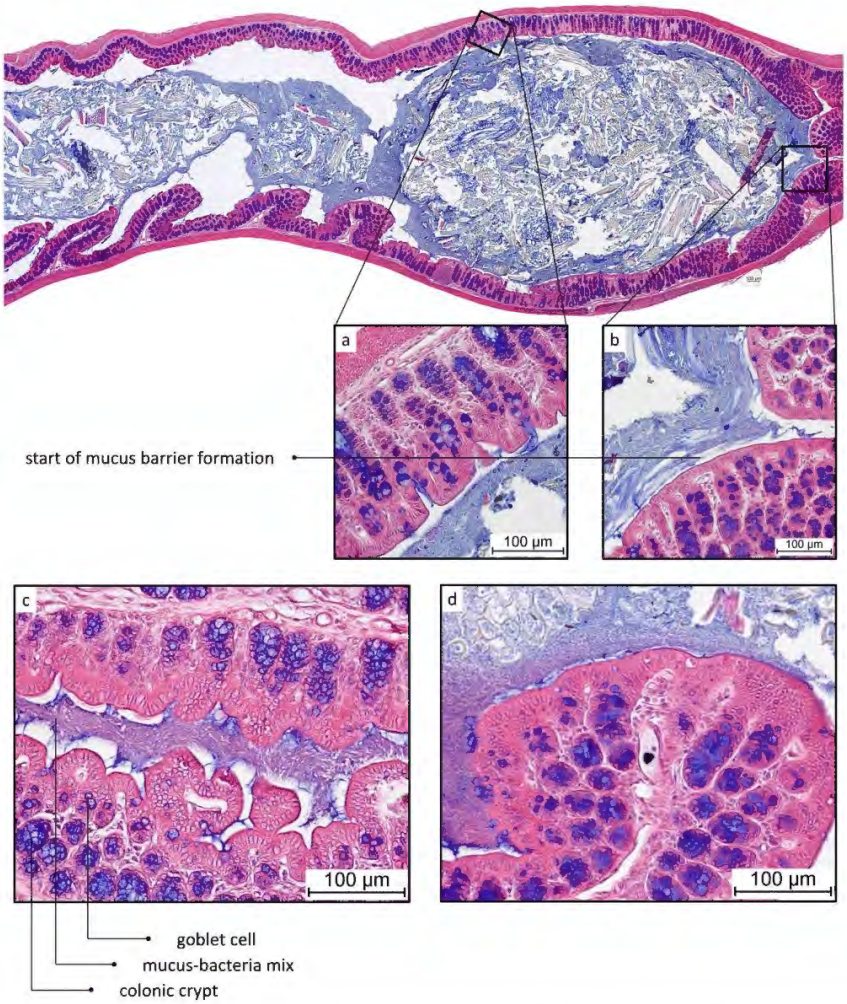


**Figure 7.** AB/H/E stained longitudinal section of mouse proximal colon. Proximal side of tissue on the left, distal on the right. (**a**) Close-up of proximal part of newformed pellet. (**b**) Close-up of distal part of newformed pellet. (**c**) AB/H/E stained longitudinal section of mouse proximal colon. No mucus layer separates bacteria from epithelium. (**d**) AB/H/E stained longitudinal section of mouse proximal colon. No mucus layer separates bacteria from epithelium.

tissue is opened longitudinally and the tissue with mucus layers is fixed using Carnoy's solution and subsequently histologically visualised. According to our understanding, the patients' preparation for surgery with laxatives removing the intestinal contents, the opening of the intestinal tube, and the inevitable passage of time between resection and fixation might have led to the formation of attached mucus layers. However, their ability to visualise the mucus layers in these conditions using Carnoy's solution seems to indicate that the lack of these attached layers in our results is due to actual absence rather than technical artefact. Apparently, surface mucus layers can be absent or present, ohservable or undetectable, depending on the experimental approach and physiological circumstances. These data show the necessity to further characterise both the regulation and the presence of these secretions in variable conditions, to make sure that a presence is not due to induced formation, and that an ahsence is not due to technical artefact.

We have no reason to doubt the general functions of the formation of a mucus layer in the distal colon in our adjusted model, which are lubrication[1] and physical separation of bacteria and epithelium[6]. Additionally, this layer, which covers the entire faecal pellet (see also Fig. 6), in effect isolates the bacteria from the intestinal milieu and confines them to the pellet, as we know from previous research that the mucus layer is mostly impenetrahle to bacteria[6]. This explains why no typical bacteria-colonised mucus is observed in empty distal colon (Figs 3b,d and 4). This heavily diminished presence of microbes in empty distal colon is of relevance to the host, hecause, in the absence of digesta, a large microbial population could be deleterious in the distal colon. Our data suggest

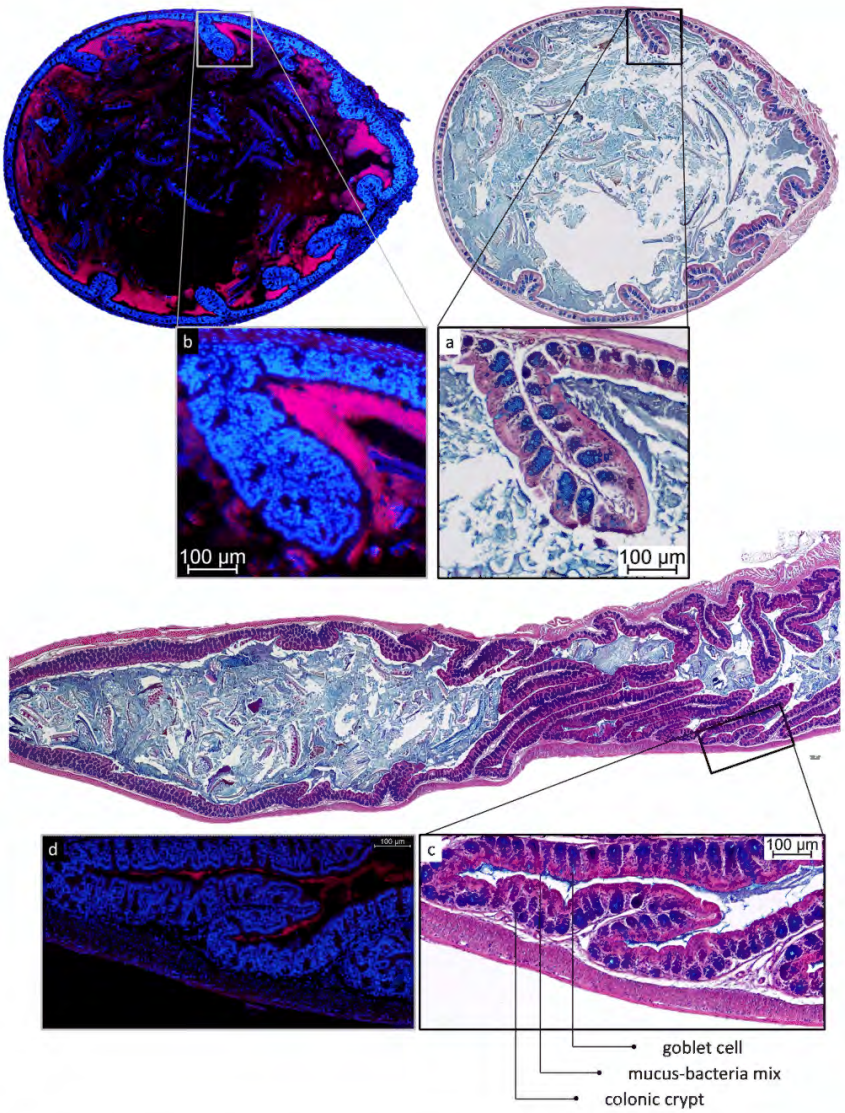


**Figure 8.** Localisation of microbiota in mouse proximal colon. (**a**) AB/H/E staining of transversal section of mouse proximal colon. (**b**) FISH staining of consecutive transversal section of mouse proximal colon. Bacteria are observed in direct contact with the epithelium (**a**), without a mucus layer to separate them from the tissue (**a**,**b**). Significantly less bacteria are observed in the middle of the contents than near to the mucosa. A high concentration of bacteria is detected particularly under the tissue folds specific to the proximal colon. (**c**) AB/H/E staining of longitudinal section of mouse proximal colon. (**d**) FISH staining of consecutive longitudinal section of mouse proximal colon. In collapsed proximal colon, bacteria mixed with mucus remain present in the lumen of folded tissue.

that the distal colon contains a mostly transient microbiota, confined to the faeces, which means that most of the resident gut microbiota is hosted higher in the gut, in the proximal colon and cecum, as we have indeed observed. This study, like many other studies on mucus organisation, is performed on rodents, which have markedly drier faeces than humans do. However, the faecal mucus layer is also observed on human stool[12,13], so it is likely that this organisation of mucus is shared by rodents and humans. The current widely accepted model has been based on largely the same techniques and experimental practices that we have used in our study, which supports the reliability and relevance of these results.

In proximal colon, released mucus is directly mixed into the chyme, and virtually no mucus is found without bacteria, except at the moment of secretion. This is indicated by the abundance of producing goblet cells in the proximal colon, combined with the lack of a significant excreted mucus barrier. Layer formation in the proximal

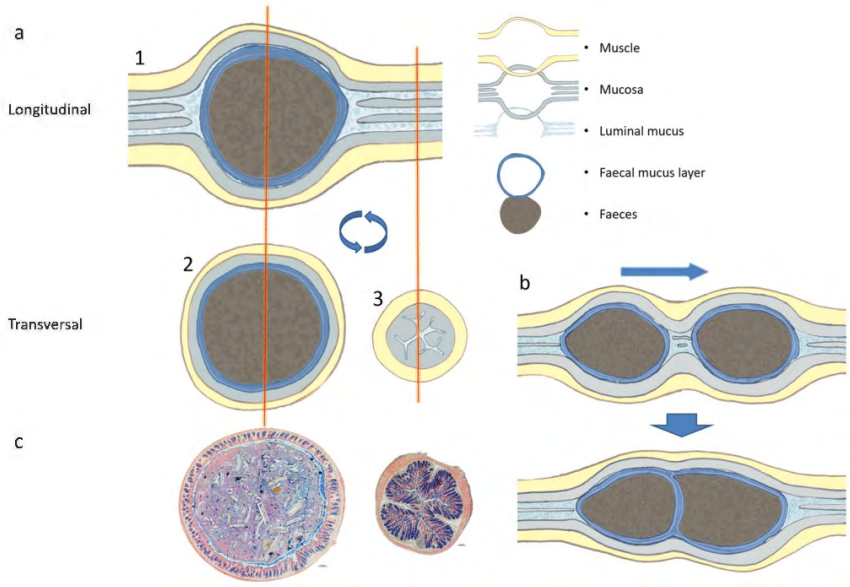


**Figure 9.** Schematic overview of mucus organisation situations as observed in different sections. (**a1**) In longitudinal sections the mucus layer is found attached to the faecal pellet. (**a2**) In transversal sections, we see the firm mucus layer around the faeces in sections containing faeces. (**a3**) This cannot be observed in transversally sectioned empty colon. (**b**) Further evidence of adherence of the mucus layer to the faecal pellet is provided by the internalisation of the sterile mucus layer inside faecal pellets by collisions between pellets; an observation which can not be explained in the classical model of mucus organisation in which the observed mucus layer is attached to the epithelium. Additionally, the observation of the same mucus layer covering expelled faecal pellets (Fig. 6) indicates clearly that this mucus layer covers the faeces. (**c**) AB/H/E stained sections of full and empty mouse distal colon corresponding to the schematic overview of (**a**).

colon is only observed further towards the transition to distal colon, after the chyme has been shaped into a rudimentary pellet under influence of water extraction by normal functioning of the colon (Fig. 7b). It is likely that the sterile mucus barrier needs a firmer pellet to form, in conditions that prevent it from directly mixing into the chyme. It seems that the classical mucus layer organisation depends on the presence of a firm faecal pellet. This indicates that the faecal mucus layer as reported by Shimotoyodome *et al.*[13], and again shown by us (Fig. 6), is already, and continuously, deposited on the faeces relatively soon after it passes the proximal colon, and not, as might be expected, starting from the rectum. Simultaneously, we see that mucus mixed with bacteria remains in contact with the epithelium in the collapsed lumen of proximal colon (Fig. 8c,d). In contrast, in collapsed distal colon, we find mucus devoid of bacteria instead (Fig. 3). That the proximal and distal colon do not have an equally thick mucus barrier has also been noted by Ermund *et al.*[24]. The use of transversal sections through a formed pellet has likely prevented them from observing the lack of a mucus barrier in the proximal colon further towards the cecum. To sum up, in the early proximal colon, production of mucus does not lead to a sterile layer, instead mixing into the liquid contents, while in the distal colon, mucus excretion leads to a layer covering the drier faecal pellet.

Because there is a difference in organisation of colonic mucus depending on the location and local stage of transit, the functions we can attribute to colonic mucus secretions depend on these same factors. The intestinal mucus has a modulating effect on the microbiota[25], together with specific compounds excreted into it, such as defensins, c-type lectins, cathelicidins, and IgA; for reviews on this subject, see for example Duerkop *et al.*[5], and Liévin-Le Moal and Servin[26]. In proximal colon, the bacteria are mostly growing near to the mucus-producing mucosa, whereas further inwards the luminal contents are made up of coarser materials with less bacteria (Fig. 8). The high density of microbes close to the epithelium indicates that these secretions might support the growth of the microbial population. This could increase the efficiency of the microbial digestive functions; if the population can quickly propagate on easily available substrate, the resulting larger population will have a stronger capacity to utilise less available compounds in the faeces. Additionally, the excretion of mucus in proximal colon, combined with the absorption of water by the mucosa, seems to lead to a change in consistency and shape of the colonic contents (Fig. 7). Mucus being released from goblet cells will sequester water from the environment[18,19,27] while forming a gel, reducing the amount of free water. Mucus released into the chyme can be expected to have a shaping role in the consistency of the colonic contents, where it would serve as a matrix for the microbial community. We hypothesize that mucus released into the chyme in the proximal colon serves to support the intestinal microbiota, and through the gel-like properties of the mucus helps in giving a firmer consistency to the contents. We do indeed see that the bulk of many mouse faecal pellets is made up of microbe-colonised mucus (Figs 1c,d, 4 and 5),

indistinguishable from the described[6] loose mucus layer containing bacteria. Regarding resource and energy efficiency, it seems indeed more expedient to cover the faecal pellet in a thick mucus layer than to cover the entire colonic epithelium, to separate these two components. Clearly, a lubricating mucus layer becomes more crucial as the pellet gets drier and more compact as it progresses through the colon, to prevent damaging abrasion of the tissue. In the proximal colon, when the contents are still liquid, a mucus barrier does not form, even though there is significant production. Direct exposure of the colonic epithelium to the microbiota and digesta is generally expected to cause a strong immune reaction and tissue damage, the prevention of which is often cited as an important function of the mucus barrier. The apparent lack of these effects in these physiological conditions in the proximal colon warrants further investigation.

Our MUCIO approach to histology, in combination with multiple orientation sectioning, has allowed for a comprehensive overview of the evolution of the mucus barrier throughout the colon. Until now, histological results are commonly depicted in single-image microscopic views, which can complicate a thorough understanding of the full results, might underrepresent possible variations throughout the section, and can give rise to a selection bias for 'suitable regions' on a slide.

In conclusion, we have shown that a mucus layer is attached to and covers faecal pellets in the distal colon, isolating the faecal bacteria from the intestinal lumen. Mucus is mixed into the chyme and faeces in the proximal colon and in collision events between faeces segments in the distal colon. In the proximal colon, in contrast to the distal colon, no firm mucus barrier is formed until the chyme starts to gain a pellet structure. Before the mucus layer is established, bacteria are in contact with the epithelial surface, and in these conditions, we should reconsider the nature of the barrier function in this region of the colon.

## Materials and Methods

**Animals and sample collection.** 8 adult male Wistar rats (Janvier, Le Genest St Isle, France) were individually housed in polypropylene cages and offered unlimited access to standard rodent food (Mucedola Global Diet 2018, Harlan, Italy) and water. 8 adult male C57BL/6 mice (Janvier, Le Genest St Isle, France) were housed in polypropylene cages in groups of 4 and offered unlimited access to standard rodent food (Mucedola Global Diet 2018, Harlan, Italy) and water. Rats were euthanized by decapitation, after which 3 cm of distal colon containing faeces was removed and stored in Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid) overnight. Mice were euthanized by cervical dislocation, after which both 1.5 to 2 cm of distal colon and of proximal colon covering regions with and without contents were removed and stored in Carnoy's fixative overnight. Mouse faecal pellets were collected directly from the anus, and fixated immediately in Carnoy's fixative overnight. All animal experiments were performed in accordance with EU directive 2010/63/EU and approved by the local Animal Care and Use Committee of Toulouse Midi-Pyrénées (agreement CEEA-86).

**Tissue processing.** The mice and rat tissues were automatically processed by a Shandon™ Excelsior™ ES Tissue Processor using the following program: 2 × 60 min anhydrous ethanol, 2 × 60 min butanol, 480 min butanol, 3 × 80 min paraffin at 60 °C. Tissue samples were included in paraffin blocks using a Thermo Scientific™ HistoStar™ Embedding Workstation. Tissues were oriented for longitudinal or transversal sectioning. 6 µm tissue sections, and 4 µm faecal sections were made using a Microm™ HM 340 E microtome and attached to Thermo Scientific Menzel-Gläser Superfrost® Plus slides.

**Histological staining (AB/H/E staining).** Paraffin embedded sections were deparaffinised using American Mastertech Clearify™ and subsequent passage through an increasingly diluted ethanol-water series, starting with anhydrous ethanol. Staining was performed by 5 min in Hematoxylin, 10 min in running water, 30 min in Alcian Blue solution (pH 3.0) followed by 5 min in running water, 3 min in Eosin, 10 min in 95% ethanol, followed by dehydration in an ethanol series of increasing purity, finishing with dry ethanol, ending with 3 baths of American Mastertech Clearify™, followed by mounting with Diamount mountant.

**Fluorescent *in situ* Hybridisation.** FISH staining for all bacteria was performed using probes EUB338I (5′ CGTGCCTCCCGTAGGAGT 3′), EUB338II (5′ GCAGCCACCCGTAGGTGT 3′), and EUB338III (5′ GCTGCCACCCGTAGGTGT 3′). After deparaffinising as described for '*Histological staining*' (see above), the slides were incubated with FISH hybridisation solution (0.9 M NaCl, 20 mM Tris/HCl, 0.01% (v/v) SDS, with 5 ng/µL of EUB338(I/II/III) probes at 48 °C overnight, followed by a washing step with the hybridisation solution without probe at 49 °C for 25 min. Slides were rinsed with demineralised water and briefly air-dried, followed by mounting using ProLong Gold® antifade reagent with DAPI (Thermo Fisher Scientific, USA).

**Imaging; Manual Ultra-high resolution Composite Image Overview (MUCIO).** Manual Ultra-high resolution Composite Image Overview (MUCIO) approach: datasets of 50 to 500 overlapping microscope views covering entire slides were generated by manual microscope photography (single photo resolution: 1280 × 1024 pixels) and stitched together using Microsoft Image Composite Editor (MICE), specifying 'planar motion' in the program interface, to best fit the movement of the microscope camera relative to the microscopic slide. The generated composite images contain all the information of the data set in a single ultra-high resolution file. No selection for 'representative regions' is necessary, only obvious artefacts (e.g. possibly folded double/heavily damaged sections) are evaded. Samples were imaged using a Nikon Eclipse 90i microscope fitted with a DXM 1200 F Digital Camera. Image sets were taken with 100x or 200x magnification, overlapping and covering complete sections. Resulting images were saved uncompressed in TIFF format. To prevent scaling issues, image resolutions were only downscaled after the appropriate scale bar was integrated. Subsequent downscaling is necessary to

facilitate interaction with images for further applications, but close-up views in full quality can be extracted from the original composite.

**Data availability.** The data generated and analysed during the current study are available from the corresponding author upon reasonable request.

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## Author Contributions

J.B.J.K., H.E., and V.T. designed the study; J.B.J.K. developed the methodology and performed the research; J.B.J.K., M.M.B., H.E., and V.T. analysed and interpreted the data, and wrote the paper.

## Additional Information

**Competing Interests:** The authors declare that they have no competing interests.

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## 2.2 FODMAPs increase visceral sensitivity in mice through glycation processes, increasing mast cell counts in colonic mucosae

This work regroups the main findings of the thesis work, i.e., the effects of FODMAP administration on visceral sensitivity, the mucosal mast cell population, the expression of Receptor for AGEs (RAGE), and the prevention of these effects by anti-glycation agent pyridoxamine, indicating the involvement of glycating processes. Analysis of the gut microbiota composition showed no significant effects of our lactose treatment on population composition, and no effect for pyridoxamine either. Carbonyl metabolite analysis showed differences between control and FODMAP treated groups, but no additional effect by co-treatment with pyridoxamine.

These results and this manuscript are currently under a second round of reviewing in the Gastroenterology journal (Elsevier) as an original article.

# FODMAPs increase visceral sensitivity in mice through glycation processes, increasing mast cell counts in colonic mucosae

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## Conflict of interest statement

The authors do not have any conflicts of interest to declare.

## Author Contributions

JBJK, FG, LD, MO, HE, and VT designed the experiments; JBJK, BG, ML, IJ, SY, VTo, PR, SC, M-HN-M performed the experiments and analysed data; MO, J-FM analysed data; JBJK, HE, and VT analysed data and wrote the manuscript; HE and VT supervised the project.

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## What you need to know

### Background and context

- A low-FODMAP diet is effective in reducing IBS symptoms
- FODMAPs increase intestinal bloating and increase small intestinal water
- Recent research indicates additional mechanisms of action

### New findings

- This study indicates a role for glycation agents produced during FODMAP fermentation by the intestinal microbiota as an additional mechanism by which FODMAPs can worsen IBS symptoms

### Limitations

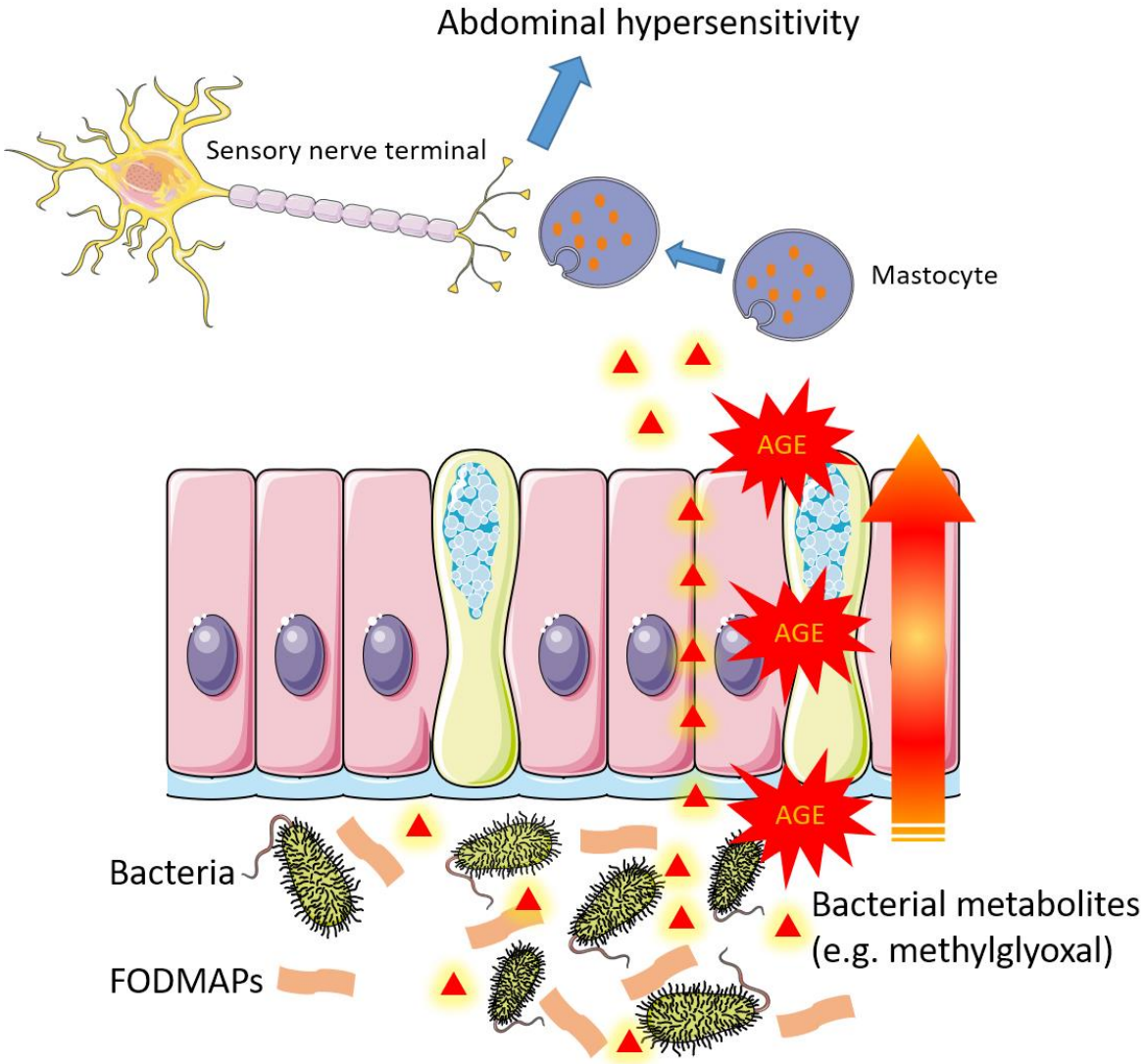
- This is a study using an animal model, and it does not contain information on human IBS patients

### Impact

- The findings of this study contribute to a deeper understanding of the efficacy of the low-FODMAP diet, and can help to develop new functional nutritional strategies focused on the prevention of glycation reactions caused by microbial toxic metabolites

## Short Summary

Dietary FODMAPs can induce an increase in abdominal sensitivity in healthy mice through previously unknown processes, indicating a complementary and original explanation for the efficacy of the low-FODMAP diet.




*Graphical Abstract*

## Abstract

Background and Aims: Irritable Bowel Syndrome (IBS) is characterized by abdominal pain, bloating, and erratic bowel habits. In recent years, a low-FODMAP (Fermentable Oligo-, Di-, Mono-saccharides And Polyols) diet has been successfully used to reduce IBS symptoms. Microbial fermentation products have been implicated in this symptom generation. We hypothesized that FODMAP ingestion can induce IBS-like visceral hypersensitivity, mediated by fermentation products of the intestinal microbiota.

Methods: We have used dietary interventions with lactose and fructo-oligosaccharides (FOS) in a mouse model to investigate the role of reactive bacterial metabolites produced in FODMAP fermentation in symptom generation, by characterizing abdominal sensitivity, colonic mucosal mast cell counts, and expression of receptor for advanced glycation end products (RAGE). We have also analysed colonic microbiota and content carbonyl profiles.

Results: Chronic oral administration of lactose and FOS increases abdominal sensitivity in mice, associated with an increase in colonic mucosal mastocyte counts, and an increased RAGE expression in proximal colon epithelium. Pyridoxamine as an anti-glycation agent prevented all these effects, without affecting phylogenetic composition of the microbiota. Mass spectrometric analysis of carbonyl compounds of colonic content extracts was able to distinguish signatures of control and FODMAP-treated groups.

Conclusion: Our study indicates a possible mechanism by which FODMAPs can increase abdominal sensitivity, pointing to a role for glycating agents produced by the intestinal microbiota, a complementary explanation for low-FODMAP diet efficacity in functional gastrointestinal disorders (FGIDs). These findings could contribute to the development of new functional nutritional therapeutic treatments focused on the prevention of glycation reactions in FGIDs.



Keywords: IBS, mastocytes, advanced glycation end products

## Introduction

Irritable Bowel Syndrome (IBS) is a functional gastrointestinal disorder (FGID) characterized by abdominal pain, bloating, erratic bowel habits, and variable changes in the consistency of stools [1, 2]. It is a heterogeneous disorder, with 4 defined sub-types; Diarrhoea (IBS-D) or Constipation Predominant IBS (IBS-C), Mixed bowel habits (IBS-M), or Unclassified (IBS-U) [3]. Because the underlying causes for IBS are not well understood, it has proven difficult to design evidence-based therapies with a clear mechanism of effect. However, in recent years a low-FODMAP (Fermentable Oligo-, Di-, Mono-saccharides And Polyols [4]) diet has been successfully used to reduce symptoms of IBS [5-8]. Dietary FODMAPs have properties that can lead to distension; they are poorly absorbed in the small intestine, osmotically active, and are fermented by the gut microbiota upon reaching the colon [9]. These dietary components, characterized for IBS patients, can also induce gastrointestinal symptoms in healthy subjects. For example, inulin, a fructo-oligosaccharide, can lead to gastro-intestinal symptoms such as flatulence and gut cramps[10].

At first sight, evasion of dietary FODMAPs helps to prevent problematic gut distension, thus alleviating symptom generation in IBS patients[11], but it is not necessarily expected to ameliorate underlying reasons for the increased sensitivity to distension. Dietary carbohydrate fermentation has been shown to influence seemingly unrelated symptoms as well. A link between perceived food intolerances (mostly to fermentable carbohydrates, or gluten) and IBS symptoms, but also musculoskeletal pain, and fatigue has been described [12]. Lactose and fructose intolerant FGID patients report headaches and tiredness in response to a challenge with these carbohydrates at an even higher rate than the more classical symptoms, such as diarrhoea or gastrointestinal cramps [13]. This indicates that dietary carbohydrate fermentation might promote symptoms through other pathways than intestinal distension alone. In addition, to our knowledge, it has not been shown that the low-FODMAP diet is only or more effective in IBS-patients with visceral hypersensitivity, as it has been shown that visceral sensitivity is not omnipresent in IBS patients[14, 15]. Apart from gas production and osmotic distension due to FODMAP ingestion, microbial fermentation products have been raised as factors involved in symptom generation.

The bacterial metabolic toxin hypothesis, proposed by Campbell et al.[16] poses that harmful bacterial fermentation products, particularly those produced in anaerobic fermentation of unabsorbed carbohydrates by the gut microbiota, are responsible for effects observed from food intolerances such as lactose intolerance. Particularly such metabolites as alcohols, ketones, and aldehydes are held responsible. Indeed, methylglyoxal, a highly reactive dicarbonyl compound, increases visceral sensitivity when administered by enema to female Wistar rats, as well as inducing behaviour indicative of headache [17]. The idea that increased methylglyoxal concentrations can lead to increased sensitivity

is supported by the finding that methylglyoxal drives neuropathic pain in diabetic patients, in part through the activation of transient receptor potential ankyrin 1 (TRPA1)[18, 19].

Moreover, reactive carbonyl compounds like methylglyoxal or glyoxal are major precursors to Advanced Glycation End Products (AGEs) [20]. Protein conformation modifications by formation of dicarbonyl adducts could interfere with the function of host proteins, and AGEs are recognized by the innate immune system as damage-associated molecular patterns (DAMPs), which activates a pro-inflammatory signalling pathway [21]. An increase in such glycating agents during microbial processing of FODMAPs in the gut could be expected to enhance the formation of AGEs, and in that way, support a pro-inflammatory state. Interestingly, mast cells can be activated by AGEs through RAGE activation[22], or even by aldehydes (acetaldehyde) directly [23]. These processes should be universal, so to explain why FODMAPs can cause problems in FGID patients, but not everyone, we propose that increased susceptibility due to genetic or environmental factors, variations in intestinal permeability and microbiota, and differences in the handling of carbohydrates are likely responsible.

In this study, we have tested the hypothesis that production of carbonyl compounds responsible for increased non-enzymatic glycation reactions produced during fermentation of certain FODMAPs can directly or indirectly induce symptoms of IBS. We used lactose and fructo-oligosaccharides (FOS) as representatives of FODMAPs in an animal model, to evaluate the effects of chronic increased FODMAP intake on visceral sensitivity and low-grade inflammation through activation of Receptor of AGEs (RAGE). Finally, we investigated whether mast cells, known to be key players in IBS symptoms and susceptible to RAGE activation, were implicated in the effects of this chronic intervention.

# Materials and Methods

## Animals and sample collection

Lactose experiments: 32 adult male C57Bl/6 mice (Janvier, Le Genest St Isle, France) of 6 weeks old were housed in polypropylene cages in groups of 8 without mixing treatment groups, mice were distributed randomly to groups upon arrival, and offered unlimited access to standard rodent food (Mucedola Global Diet 2018, Harlan, Italy) and water. After a 4 days adjustment period, the lactose-treated group received a daily oral gavage, every morning, of 3mg, 5mg, or 15mg lactose (β-lactose, Sigma Aldrich, France) in 200 µl saline solution, the control group received only saline, for 3 weeks.

Lactose-pyridoxamine experiment: 80 adult male C57Bl/6 mice (Janvier, Le Genest St Isle, France) of 6 weeks old were housed in polypropylene cages in groups of 8 without mixing treatment groups, mice were distributed randomly to groups upon arrival, and offered unlimited access to standard rodent food (Mucedola Global Diet 2018, Harlan, Italy) and water. After a 4 days adjustment period, the lactose-pyridoxamine treated group received a daily oral gavage, every morning, of 5mg lactose (β-lactose, Sigma Aldrich, France) and/or 5mg pyridoxamine (pyridoxamine dihydrochlorate, Sigma Aldrich, France) in 200 µl saline solution, the control group received only saline, for 3 weeks.

FOS experiments: 50 adult male C57Bl/6 mice (Janvier, Le Genest St Isle, France) of 6 weeks old were housed in polypropylene cages in groups of 10 without mixing treatment groups, mice were distributed randomly to groups upon arrival, Animals received a custom modified AIN-93M diet ad libitum, containing 0% or 10% fructo-oligosaccharides (corn-starch partly substituted for FOS) (supplementary data table S1), complemented with or without 1mg/mL pyridoxamine in drinking water.

Mice scheduled for immunofluorescence assays were euthanized by cervical dislocation, after which both 1.5 to 2 cm of distal colon and of proximal colon covering regions with and without contents were removed and stored in Carnoy's fixative overnight. Caeca were resected and weighed. Mouse fecal pellets were collected directly from the anus on the last day of treatment, and collected in 0,5 mL Eppendorf tubes, frozen in liquid nitrogen, and stored at -80°C for downstream analyses.

All animal experiments were performed in accordance with EU directive 2010/63/EU and approved by the local Animal Care and Use Committee of Toulouse Midi-Pyrénées (agreement CEEA-86).

## Abdominal sensitivity

*Visceral sensitivity (Electromyography (EMG))*

Lactose/ Lactose-pyridoxamine experiments: Under xylazine/ketamine anaesthesia (both 1.2 mg, subcutaneously), two nickel–chrome electrodes were implanted into the abdominal external oblique

muscle and a third into the abdominal skin and exteriorised on the back of the neck. On the fifth to seventh postoperative day, colorectal distensions were used as noxious stimuli to evaluate visceral hyperalgesia by electromyographic (EMG) recording. Polyethylene perfusion and distension catheters (Fogarty catheter for arterial embolectomy, 4F, Edwards Lifesciences, Nijmegen, The Netherlands) were inserted into the colon. The colorectal distension procedure started 60 min after habituation to the tunnel, progressively increasing in 0.02 mL steps, from 0 to 0.08 mL, each step lasting 10 s with 5 min non-distension periods in between. During the distension periods, the striated muscle's EMG activity was recorded and analysed according to Larsson, Arvidsson, Ekman, *et al.* [24]. Basal EMG activity was subtracted from the EMG activity registered during the periods of distension. Method adapted from Gecse, Roka, Ferrier, *et al.*[25]. Statistical analysis: Two-way ANOVA, multiple comparisons between all groups of the same distension, Tukey's correction for multiple comparisons.

*Mechanical behavioural testing (von Frey)*

FOS experiment: Animals were placed upon an elevated mesh floor surrounded by a clear plastic enclosure (10 × 10 × 10 cm). Mechanical sensitivity was assessed using three von Frey filaments with bending force 0.16, 0.6 and 1.4 g (Bioseb Inc., France). In ascending order of force, each filament was applied for a duration of 2 seconds to the mid-plantar area of each hind paw five times, with 3 seconds between each application. Rapid retraction, shaking and/or licking of the hind paw were considered to represent nociceptive specific behaviours and only one of these responses needed to be displayed to be considered as a positive withdrawal response. Applications were applied to both hind paws, counted and then expressed as an overall percentage response. The performing researcher was kept unaware to which treatment groups mice belonged. Statistical analysis: Two-way ANOVA, multiple comparisons between all groups for the same filament, Tukey's correction for multiple comparisons.

## Microbiota analysis

*Fecal DNA extraction, 16S rRNA gene sequencing, and bacterial community analysis*

For materials and methods, please refer to the supplementary data.

## Immunofluorescence

*Histological sample preparation*

Collected tissues from animals that did not undergo the visceral sensitivity protocol were rinsed in 100% ethanol after 1 day in Carnoy's fixative and automatically processed using a Shandon Excelsior ES Tissue Processor by the following program: 2x 60min 100% ethanol, 2x 60min butanol, 480min butanol, 3x 80min paraffin at 60 °C. Tissue samples were included in paraffin blocks using a Thermo Scientific HistoStar Embedding Workstation. 5µm tissue sections were made using a Microm HM 340 E microtome and attached to Superfrost Plus microscope slides (Thermo Scientific, USA).

MMCP: 5µm paraffin embedded sections were deparaffinated by using 3x 5min baths of American Mastertech Clearify followed by 3x 5min 100% ethanol, 3x 5min 95% ethanol, 2x 5min 70% ethanol, 5min demineralised water. Slides were washed 2x in PBS for 5min, followed by a 2-hour blocking step with 10% donkey serum in PBS, and washed 3x 5min under light agitation in PBS. Slides were incubated overnight at 4°C with primary antibodies (Sheep anti-mMCP1 (MS-RM8 (Moredun Group, UK)) diluted 1:400), followed by 2x 5min rinsing steps in PBS. Secondary antibody (Alexa Fluor 594 Donkey-anti-Sheep (A-11016 (Molecular Probes, USA)) diluted 1:400 in 1% donkey serum PBS) incubation was performed for 2 hours, followed by 3x 5min washing steps in PBS, a quick rinse with tap water, followed by mounting using ProLong Gold® antifade reagent with DAPI (Thermo Fisher Scientific, USA).

RAGE: 5µm paraffin embedded sections were deparaffinated by using 3x 5min baths of American Mastertech Clearify followed by 3x 5min 100% ethanol, 3x 5min 95% ethanol, 2x 5min 70% ethanol, 5min demineralised water. Slides were washed 2x in PBS for 5min, followed by a 2-hour blocking step with 10% donkey serum in PBS, and washed 3x 5min under light agitation in PBS. Slides were incubated overnight at 4°C with primary antibodies (Polyclonal Goat-anti-RAGE (ab7764 (Abcam, UK)) diluted 1:400), followed by 2x 5min rinsing steps in PBS. Secondary antibody (Alexa Fluor 488 Donkey-anti-Goat (A-11055 (Molecular Probes, USA)) diluted 1:300 in 1% donkey serum PBS) incubation was performed for 2 hours, followed by 3x 5min washing steps in PBS, a quick rinse with tap water, followed by mounting using ProLong Gold® antifade reagent with DAPI (Thermo Fisher Scientific, USA).

*Microscopy*

Samples were imaged using a Nikon Eclipse 90i microscope fitted with a DXM 1200 F Digital Camera. Image sets were taken at 200x magnification.

*Immunofluorescence analyses and statistics*

Mast cell count analyses: One ratio mastocyte:crypt per mouse based on analysis of 50-250 crypts, dependent on availability of suitable visual material. Image sets were coded, randomised and analysed blindly. Statistical analysis: One-way ANOVA, multiple comparisons with Tukey's correction for multiple comparisons.

RAGE expression analyses: Fluorescence intensity of 3 suitable regions of epithelium was analysed per microscope field, 4 microscope fields per mouse were analysed. Only epithelial cells were selected, without goblet cells, because these presented high unspecific staining throughout. Image sets were coded, randomised and analysed blindly. Statistical analysis: One-way ANOVA, multiple comparisons with Tukey's correction for multiple comparisons.

*Physiological markers of colonic content transit*

Transit time: For this experiment, after 2.5 weeks of treatment, 5 mice per group were temporarily housed in individual cages, 0.15 mL paper-filtered saturated carmine red in physiological salt solution was administered by intragastric gavage, and starting from 2 hours after administration, every 15 minutes or when the researcher noted defecation, the cages were checked for red-coloured droppings, the time of first appearance of a red dropping was used to determine the transit time. Statistical analysis: One-way ANOVA, multiple comparisons with Tukey's correction for multiple comparisons.

Faecal water content: Droppings were collected in weighted tubes, weighed to determine wet weight, and dried in an oven at 80°C for 48 hours. Tubes were weighed again and used to determine the dry weight. Faecal humidity is the percentage of weight lost between wet and dry weights. Statistical analysis: One-way ANOVA, multiple comparisons with Tukey's correction for multiple comparisons. To compare Ctrl v FOS: Student's t-test.

*Verification of intestinal inflammation*

Lipocalin-2 ELISA: Faecal supernatants were prepared by grinding 0.2g faeces in 1 mL demineralised water with 5 ceramic beads using a Fast-Prep (MP Biomedicals, Illkirch, France) (3x 15sec 6m/s with 1 min breaks on ice) followed by 20min centrifugation at 8000x, supernatants were collected in 1.5 mL Eppendorf tubes and stored at -20°C until use. ELISAs were performed according to instructions provided by the manufacturer (DuoSet ELISA, Mouse Lipocalin-2/NGAL (DY1857)) (R&D Systems, MN, USA). One-way ANOVA, multiple comparisons with Tukey's correction for multiple comparisons.

Microscopic score: For materials and methods, please refer to the supplementary data.

## Results

### Increase in sensitivity after FODMAP treatment

Oral treatment with lactose for 3 weeks increased visceral sensitivity compared to control. These increases were statistically significant for the two highest lactose concentrations for both 0.06 mL and 0.08 mL volume of distension ($P \leq 0.05$) (Supplemental Figure 1). Based on these results and to reflect a modest lactose consumption, subsequent experiments with lactose were performed using 5mg lactose daily. In animals treated with 5mg lactose and/or 5mg pyridoxamine, visceral sensitivity was also increased in the lactose-treated group by ±70 % ($P<0.01$), ±55% ($P<0.0001$) versus control, at a distension volume of 0.06 mL and 0.08 mL respectively (Figure 1). Co-treatment with pyridoxamine effectively reversed the effect of lactose to basal visceral sensitivity response, such as observed in the control group (no significant differences between Lact+Pyr, Pyr and control), Lact+Pyr decreased ±38% ($P<0.01$), ±36% ($P<0.0001$) v Lact at a distension volume of 0.06 mL and 0.08 mL respectively, and Pyr

was reduced ±30% (P<0.05), ±31% (P<0.0001) v Lact at distension volumes of 0.06 mL and 0.08 mL respectively.

Fructo-oligosaccharide (FOS) significantly increased the abdominal sensitivity in response to mechanical stimulation of the hind paw (29% FOS v 14% control response rate for 0.6g filament (P<0.05), and 47% v 30% response rate for 1.4g filament (P<0.05)). These increased abdominal sensitivities were reduced by pyridoxamine (21% FOS-Pyr v 29% FOS for 0.6g filament, and 39% FOS-Pyr v 47% control response rate for 1.4g filament)(Figure 2), but not significantly so.

### Mast cell analysis of colonic mucosae

Both lactose and FOS treatments significantly (P<0.01 v Ctrl and Lact+Pyr, P<0.001 v Pyr, P<0.0001 v Ctrl and FOS+Pyr) increased the number of mucosal mast cells in the proximal colon(Figure 3) versus their respective other groups. Lactose treatment did not significantly increase mast cell counts in the distal colon (Supplemental Figure 4), while the FOS diet increased mast cells in both proximal and distal colon (Supplemental Figure 5).

### Expression of RAGE in epithelial cells of the proximal colon

RAGE immunofluorescence intensities in proximal colon epithelial cells were higher for lactose and FOS groups (P<0.0001), compared to all other groups (Figure 4). The FOS+pyridoxamine group showed significantly lower RAGE expression than the respective control group (P<0.01).

### Transit time, fecal water content, and cecal weight

The FOS-enriched diet increased the fecal water content and cecal weight and reduced the transit time compared to the control diet. 5mg/d lactose treatment had no significant impact on these parameters. Additive pyridoxamine treatment did not impact these characteristics in control or FODMAP-treated animals (Figure 5).

### Microbiota

Lactose and/or pyridoxamine did not induce significant alterations in the composition of the fecal microbiota, as demonstrated quantitatively at the OTU level by the prevalence and abundance of the detected OTUs (Figure 6A) and phyla (Figure 6B) respectively. Whichever index of alpha-diversity tested, neither lactose nor pyridoxamine nor a combination of the two significantly altered the number of OTUs, as indicated by the index of richness (chao-1) and evenness (Shannon) (Figure 6C). Multidimensional scaling analysis of unweighted or weighted UniFrac distances revealed no inter-sample difference linked to treatments (Figure 6D). None of the OTUs agglomerated at the genus rank were significantly affected by treatment by using DESeq2.

## Intestinal inflammation

Levels of the sensitive inflammation biomarker Lipocalin-2 were at low, non-inflamed, levels in all animals (Figure 7). Similarly, microscopic scoring revealed no inflammation in any of the treatment groups (Supplementary Figures 2 & 3).

## Discussion

We have shown that both lactose treatment and a high-FOS diet can lead to pro-nociceptive effects, as well as an increase in colonic mucosal mast cell counts. Interestingly, these effects were common to treatment with two different FODMAPs at different doses. This indicates that the intake of FODMAPs alone can increase these sensitivities. The dose of lactose (5mg per day) used was chosen to correspond to a modest intake of milk (relative amount of 1 glass of milk for a human of average size), where it has to be noted that mice do not retain lactase activity after weaning and are therefore lactose malabsorbers[26]. For FOS, we chose to administer FOS as 10% of the total diet based on a previously reported pertinent dose in mice [27]. Another recent study comparing the effects of different fibres on short-chain fatty acids (SCFA), using doses of 10%, showed that FOS had different effects on SCFA production than lignin or resistant starch[28]. Because rodent diets naturally contain a great deal more fructans and glycans than a conventional human diet, to be able to see the effects of an oligosaccharide in mice, we chose this higher dose than would be used in humans.

The observed effects of lactose and the FOS diet were prevented by co-treatment with pyridoxamine, a recognized anti-glycation agent [29-34], indicating the involvement of glycation processes in the generation of the effects observed for both FODMAPs. Accordingly, the expression of RAGE on colonic epithelial cells increased in response to the FODMAP treatments, and was, likewise, prevented by pyridoxamine co-administration.

The generation of glycating agents in the colonic lumen was evaluated by LC-MS analysis of carbonyl compounds in colonic contents (Supplementary Figure 4). Indeed, a subset of these compounds in colonic contents was significantly increased for FODMAP treated animals versus their respective controls. Taking into account the high chemical reactivity of certain carbonyl compounds with proteins, we can speculate that the amounts of such compounds produced in the colonic lumen of the FODMAP treated animals are sufficient to generate glycation end products, contributing to the FODMAP-induced effects. Interestingly, despite the prevention of the FODMAP-induced effects by pyridoxamine, no specific differences were detected in the carbonyl compound profiles of the FODMAP versus the FODMAP+pyridoxamine groups, in the colonic content supernatants (Supplemental Figure 3). This can be explained by the strong capacity of the small intestine to absorb pyridoxamine[35], leading to systemic, rather than local protection of the gut tissues against glycating

reactions, in a similar way as observed in work studying the prevention of glycation reactions by pyridoxamine in animal models of diabetes[32, 36, 37]. This also implies that the generation of AGEs *in vivo* by interaction with absorbed carbonyl compounds generated during FODMAP fermentation is at the basis of the observed effects, rather than the AGE load of the luminal contents.

We observed an increase in RAGE expression in the mucosa of animals treated with lactose or FOS, which was, again, reversible by co-treatment with pyridoxamine, indicating that glycation processes and activation of the receptor of AGEs (RAGE) are involved in the induction of visceral and abdominal sensitivity in our animal model. *In vitro*, AGEs have been shown to interact directly with mast cells, rapidly triggering mast cell exocytosis dose-dependently. This interaction could be prevented by blocking access to RAGE by using an antibody, showing it depends on the interaction between AGEs and RAGE [22]. The observed hypermastocytosis in our animals was prevented by co-treatment with pyridoxamine too, indicating it is part of the same pathway that is responsible for the increased sensitivity, and indeed an increased mast cell count in tissues is reported as a possible factor in increased visceral sensitivity of IBS patients [38, 39]. In IBS patients on a low FODMAP diet, histamine levels in the urine dropped eight-fold compared to a high-FODMAP intervention[40], supporting the idea that dietary FODMAPs can be responsible for an increase in mastocyte proliferation. Mast cell counts and mast cell mediator production are associated [41], and mast cell mediator release in turn can lead to mast cell hyperplasia [42]. It has been reported that histamine levels are increased in the mucosa of IBS patients [39, 43] and it can be directly involved in the sensitization of TRPV1 by its action on HRH1, which can cause symptom generation in IBS patients [44]. In short, our findings support the idea that anaerobic microbial fermentation of FODMAPs can lead to production of glycating agents, which increase the AGE-load locally in the colon, inducing expansion of the mucosal mastocyte population, and by mastocyte-nerve cell interactions, increasing visceral sensitivity.

Analysis of intestinal microbiota profiles of lactose experiment groups did not uncover significant differences between the profiles of the 4 groups; control, lactose, lactose-pyridoxamine, and pyridoxamine. It is well-known from literature that FOS changes the microbiota composition [45, 46], but as indicated by the reversibility of the effects by pyridoxamine in both treatment groups, and the lack of effect of pyridoxamine on microbiota composition, we assess that the effects of the FODMAP treatments were mostly due to microbial metabolic changes, rather than a possible dysbiosis. This could alternatively indicate that no significant malabsorption occurs at this dose of lactose, but it's unlikely that we would have observed effects on visceral sensitivity and mast cell numbers if lactose were completely hydrolysed. Additionally, as mentioned before, we did find differences in carbonyl compound profiles between control and FODMAP-treated animals, which would be surprising if lactose were hydrolysed and absorbed completely in the small intestine. Conversely, it has recently

been reported that a diet high in FODMAPs can induce intestinal inflammation represented by increased mucosal expression of IL-1β, IL-6, IL-17, TNF-α, and IFN-γ, a visceral hypersensitivity, and an increased intestinal permeability in rats, by changing the gut microbiota composition and increasing levels of lipopolysaccharides[47]. We have not observed such changes in the microbiota in our work, nor have we observed intestinal inflammation in either lactose or FOS-treated groups, according to fecal lipocalin-2 levels (Figure 7) and general histology (Supplemental Figure 2), and while IBS does not normally present with these kind of inflammatory markers [48], this interesting work of Zhou *et al.* indicates that FODMAPs can induce symptoms in even more ways than previously expected.

We have not observed changes in bowel movement characteristics (fecal water content, output, transit time) in our mice treated with lactose, in contrast to the mice treated with FOS, which showed both a higher water content and decreased transit time compared to control, though the basic diet between these 2 groups was not the same, and basal characteristics between the 2 control groups were not identical. In our experiments, the application of the FODMAP representatives is dissimilar between lactose and FOS. Lactose was administered once daily, diluted in saline, whereas FOS was present in the animal feed, as a percentage of the regular diet. This means that lactose represented chronic acute challenges over 3 weeks, while FOS had a permanent and bulkier presence. It is not surprising then, that FOS had significantly more effects on transit time and fecal output than lactose. It is also for this reason that we used the alternative testing of sensitivity in the FOS-experiment, as FOS-treatment lead to increased amounts of intestinal content that prevented emptying of the colorectal cavity even after fasting and habituation periods, impeding a reliable distension procedure. In contrast to effects related to aldehyde generation, pyridoxamine did not reverse fecal water content differences between control and FOS diets (data not shown), indicating that, like for the lactose-treated groups, differences in transit time were not responsible for the observed symptom generation for this group either, although modification of transit time itself can be seen as a symptom too.

It has been clearly demonstrated that FODMAP ingestion increases water and fermentable material to the proximal colon [49], and can lead to distension through increased chyme volume and production of gas [50]. However, it has been found that increased sensitivity rather than increased distension is the cause for complaints in IBS patients related to FODMAP consumption [11] not taking away the fact that decreasing the FODMAP intake should reduce this distension. The short-term effects observed upon reducing FODMAP intake in patients are probably thanks to a reduction in distension obtained, whereas the effects obtained in our animal model are on a longer time-scale, in which FODMAPs are involved in modulating sensitivity itself. If FODMAP fermentation products increase mast cell numbers, factors that activate mast cell product release such as psychological stress[51, 52] will have a

greater effect because there is a larger population to receive these activating signals. It is unlikely that FODMAPs can cause IBS by themselves, but by the effects that we describe, we can conclude that they can cause physiological changes possibly responsible for symptoms of IBS.

Our study shows that the role of FODMAPs in IBS is multifactorial; apart from the previously reported osmolarity and distension related symptom-generation caused by dietary FODMAPs, reactive carbonyl fermentation products of their microbial processing can cause physiological changes in the colon, specifically reminiscent of IBS. These insights may contribute to the development of functional nutritional strategies focused on the prevention of glycation reactions caused by microbial toxic metabolites.

## Acknowledgments

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## Figure legends

Figure 1: Increase in visceral sensitivity after daily treatment with lactose and/or pyridoxamine for 3 weeks, in response to increasing volumes of distension. Sensitivity is expressed as a percentage of the maximum average control response. N=12

Figure 2: Increase in sensitivity to mechanical stimulation of the hind paw, after following a diet high in FOS, with or without pyridoxamine (1mg/mL) in drinking water. N=10

Figure 3: (A) Representative images of mucosal mast cell immunofluorescent staining (mast cells: red; DAPI nuclear counterstain: blue) of proximal colon of animals treated with 5mg lactose and/or pyridoxamine for 3 weeks. (B) Mucosal mast cell (MC) counts of proximal colon in animals treated daily with 5mg lactose and/or pyridoxamine for 3 weeks, expressed as MC/crypt. (C) Representative images of mucosal mast cell immunofluorescent staining (mast cells: red; DAPI nuclear counterstain: blue) of proximal colon of animals following a diet high in FOS, with or without pyridoxamine (1mg/mL) in drinking water. (D) Mucosal mast cell (MC) counts of proximal colon of animals following a diet high in FOS, with or without pyridoxamine (1mg/mL) in drinking water for 3 weeks, expressed as MC/crypt.

Figure 4: (A) Representative images of epithelial RAGE immunofluorescent staining (RAGE: green; DAPI nuclear counterstain: blue) of proximal colon of animals treated with 5mg lactose and/or pyridoxamine for 3 weeks. (B) Intensity of RAGE staining in mucosal epithelial cells of proximal colon of lactose experiment. (C) Representative images of epithelial RAGE immunofluorescent staining (RAGE: green; DAPI nuclear counterstain: blue) of proximal colon of animals following a diet high in FOS, with or without pyridoxamine (1mg/mL) in drinking water. (D) Intensity of RAGE staining in mucosal epithelial cells of proximal colon of FOS experiment.

Figure 5: Fecal water contents of animals on FODMAP treatments. (A) Fecal water content (B) cecal weight and (C) transit time of lactose groups. (D) Fecal water content (E) cecal weight and (F) transit time of animals following a control, or a FOS-enriched diet.

Figure 6: Effect of lactose and/or pyridoxamine on the community distribution and diversity of the fecal microbiota as determined by 16S rRNA gene Illumina Miseq sequencing of animals treated with 5mg lactose and/or pyridoxamine daily. (A) Prevalence per OTU in samples according to groups (B) Relative abundance (%) per phylum according to groups (C) Richness (α-diversity) measured by Chao1 and Simpson Indexes (D) Unweighted (left) and Weighted (right) Unifrac Multidimensional Scaling (MDS) plots representing structural changes between groups (β-diversity). The fraction of diversity captured by the coordinate is given as percentage.

Figure 7: Lipocalin-2 concentrations of faeces, expressed as pg/mg faeces, of animals treated with lactose (A), or FOS (B) and/or pyridoxamine. None of the animals showed increased lipocalin levels indicative of inflammation.

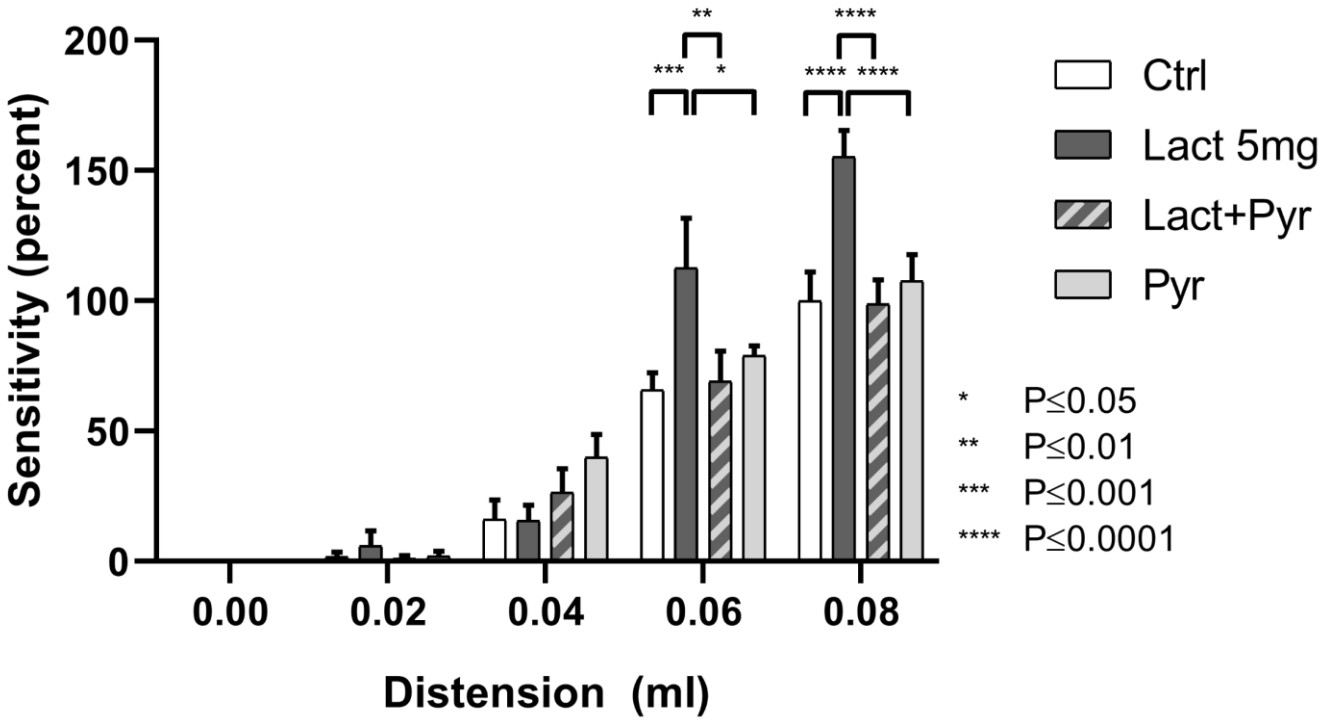

Sensitivity (percent)
200
150
100
50
0
0.00
0.02
0.04
0.06
0.08
Distension (ml)
**
****
***
*
****
****
Ctrl
Lact 5mg
Lact+Pyr
Pyr
* P≤0.05
** P≤0.01
*** P≤0.001
**** P≤0.0001

Figure 1

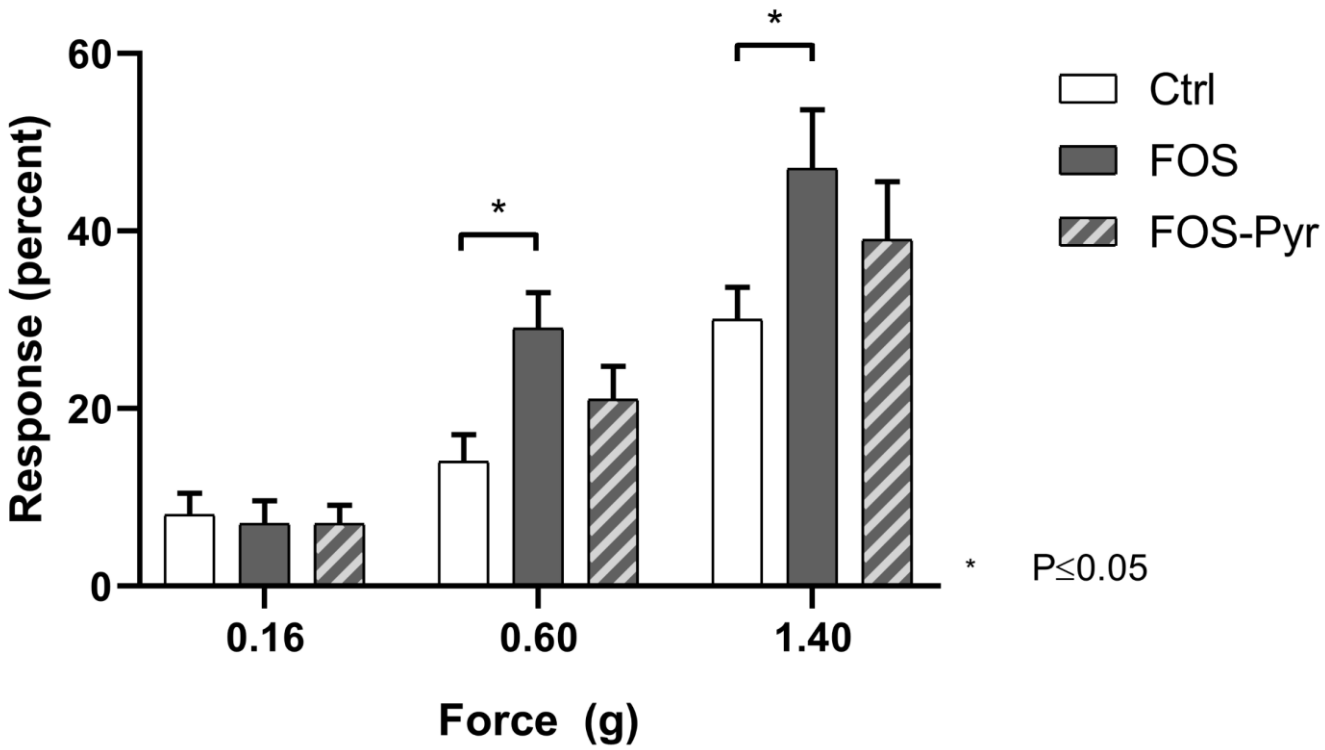

60
40
20
0
Response (percent)
*
*
0.16
0.60
1.40
Force (g)
Ctrl
FOS
FOS-Pyr
* P≤0.05

Figure 2

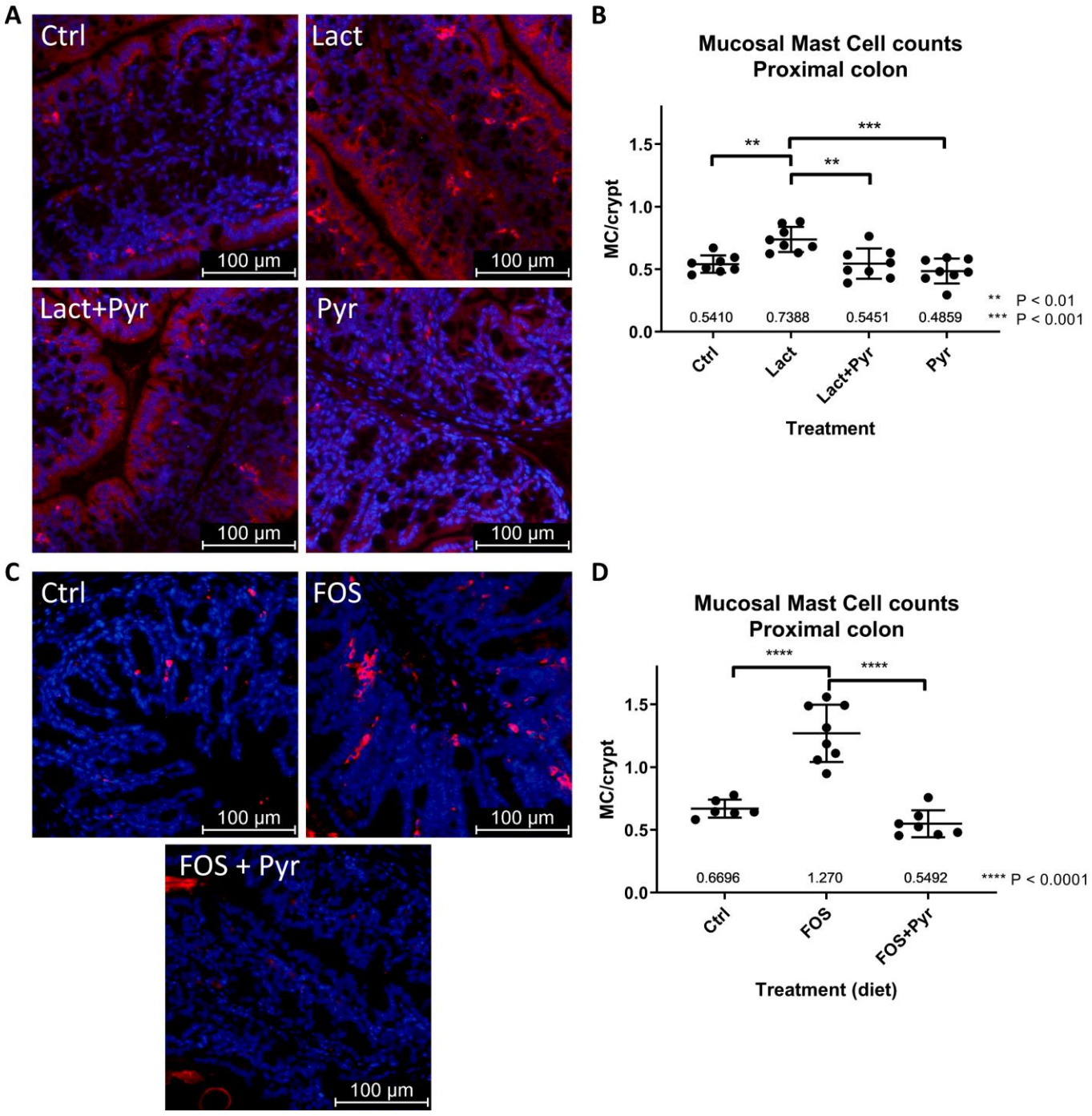
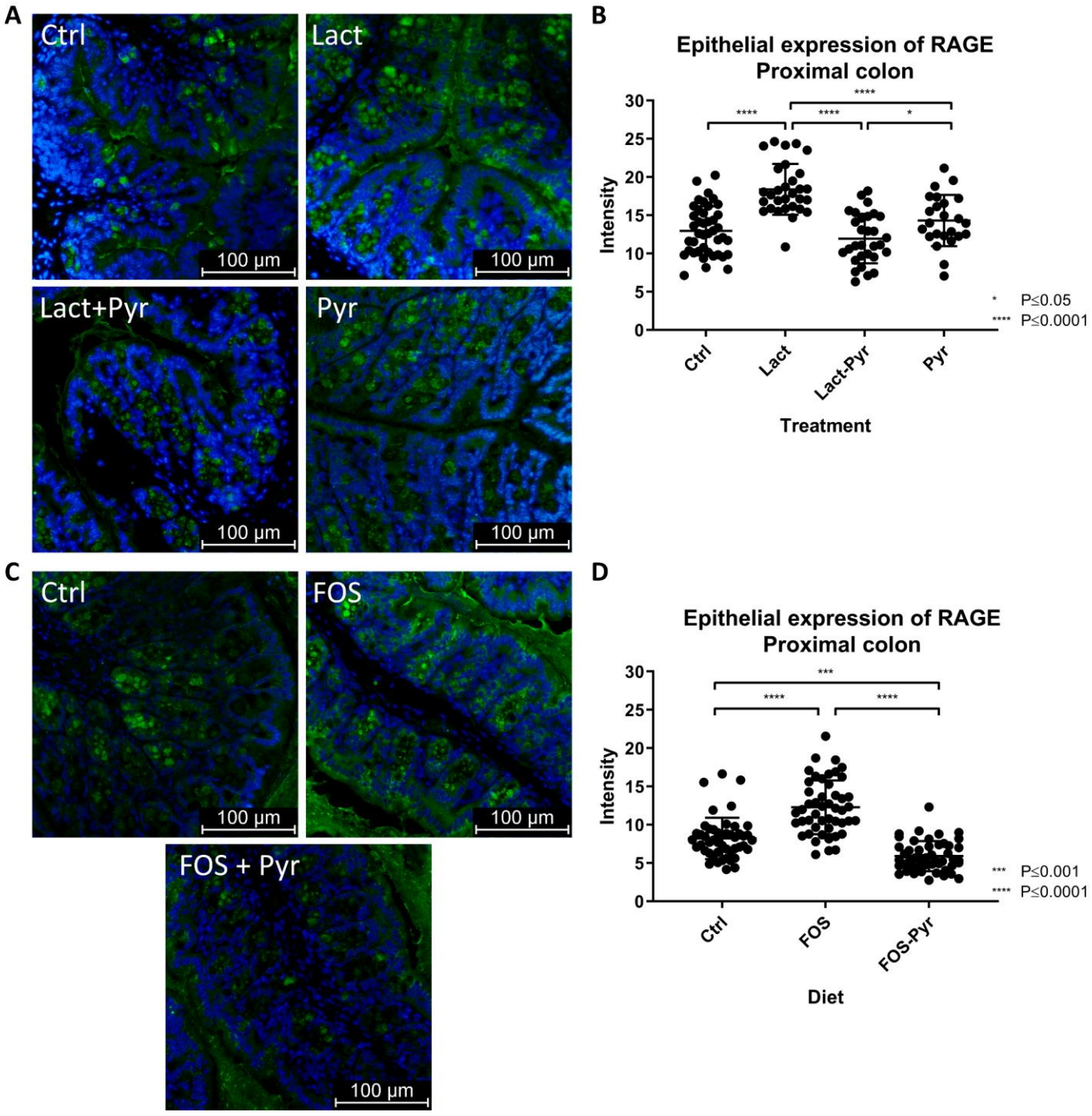

A
Ctrl
Lact
Lact+Pyr
Pyr
100 µm
B
Mucosal Mast Cell counts
Proximal colon
MC/crypt
0.0
0.5
1.0
1.5
**
**
***
0.5410
0.7388
0.5451
0.4859
** P < 0.01
*** P < 0.001
Ctrl
Lact
Lact+Pyr
Pyr
Treatment
C
Ctrl
FOS
FOS + Pyr
100 µm
D
Mucosal Mast Cell counts
Proximal colon
MC/crypt
0.0
0.5
1.0
1.5
****
****
0.6696
1.270
0.5492
**** P < 0.0001
Ctrl
FOS
FOS+Pyr
Treatment (diet)

Figure 3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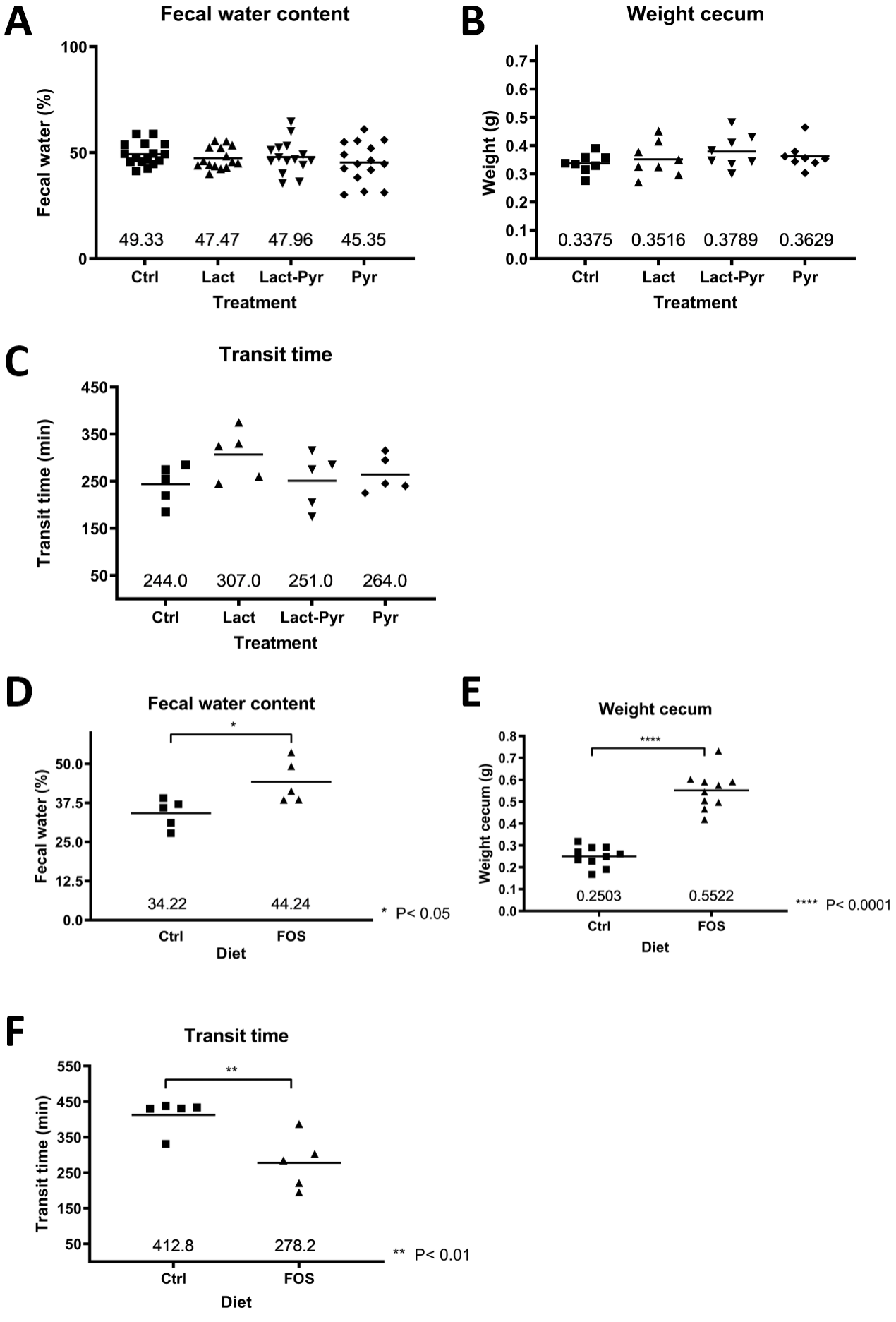
Ctrl
Lact
Lact+Pyr
Pyr
100 µm
B
Epithelial expression of RAGE
Proximal colon
Intensity
Ctrl
Lact
Lact-Pyr
Pyr
Treatment
* P≤0.05
**** P≤0.0001
C
Ctrl
FOS
FOS + Pyr
D
Epithelial expression of RAGE
Proximal colon
Intensity
Ctrl
FOS
FOS-Pyr
Diet
*** P≤0.001
**** P≤0.0001

Figure 4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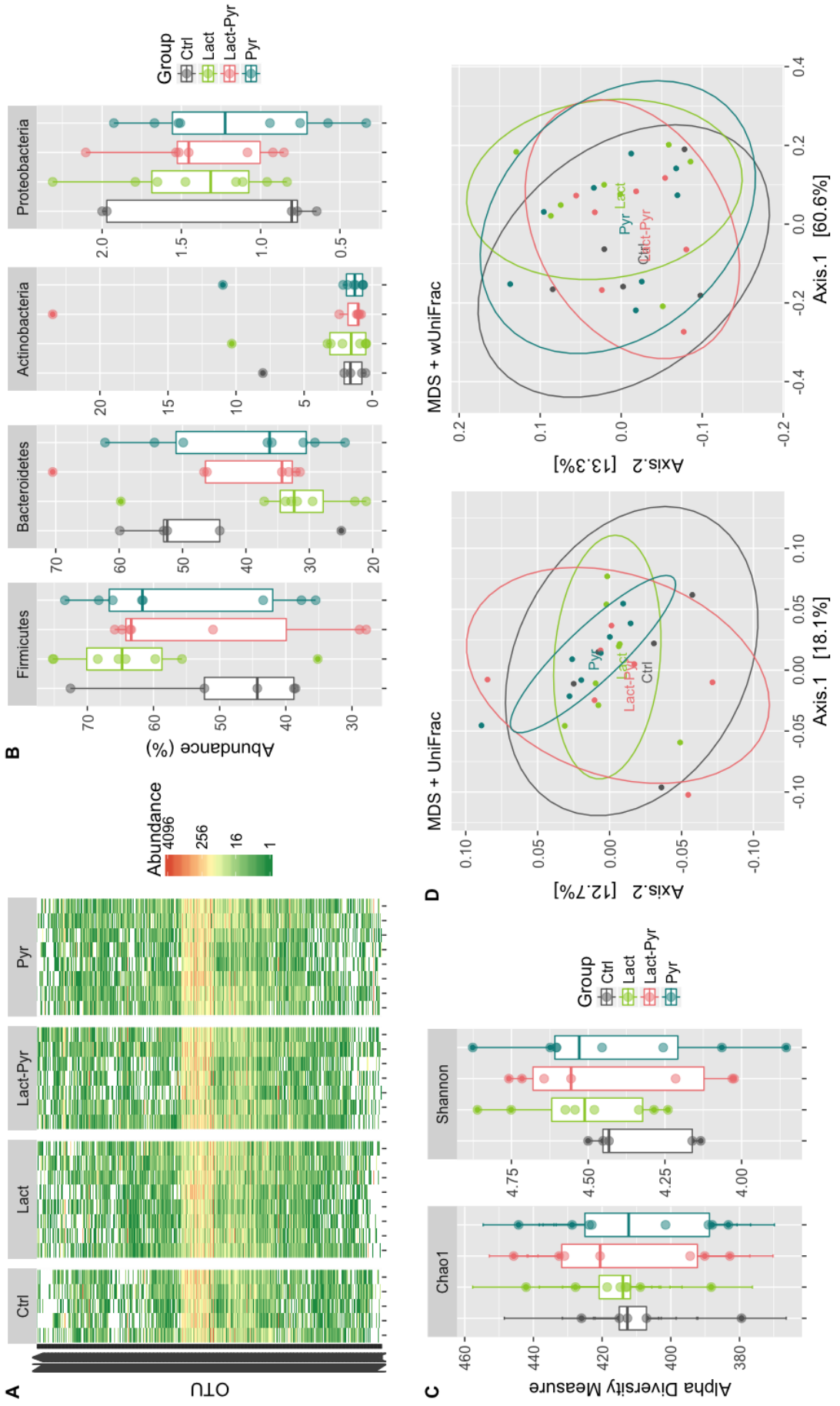
Fecal water content
Fecal water (%)
100
50
0
49.33
47.47
47.96
45.35
Ctrl
Lact
Lact-Pyr
Pyr
Treatment
B
Weight cecum
Weight (g)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.3375
0.3516
0.3789
0.3629
Ctrl
Lact
Lact-Pyr
Pyr
Treatment
C
Transit time
Transit time (min)
450
350
250
150
50
244.0
307.0
251.0
264.0
Ctrl
Lact
Lact-Pyr
Pyr
Treatment
D
Fecal water content
*
Fecal water (%)
50.0
37.5
25.0
12.5
0.0
34.22
44.24
* P< 0.05
Ctrl
FOS
Diet
E
Weight cecum
****
Weight cecum (g)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.2503
0.5522
**** P< 0.0001
Ctrl
FOS
Diet
F
Transit time
**
Transit time (min)
550
450
350
250
150
50
412.8
278.2
** P< 0.01
Ctrl
FOS
Diet

Figure 5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OTU
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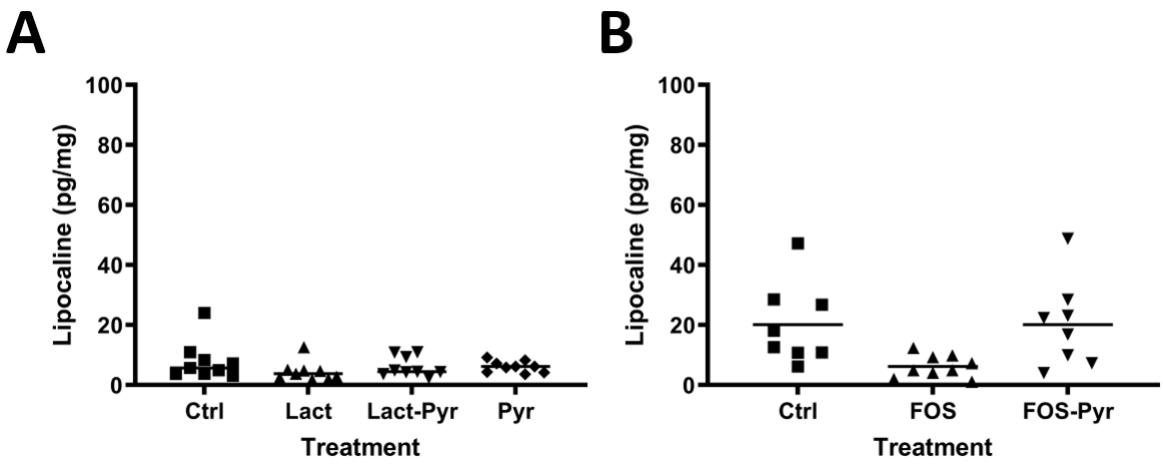
Lact
Lact-Pyr
Pyr
Abundance
4096
256
16
1
B
Abundance (%)
Firmicutes
Bacteroidetes
Actinobacteria
Proteobacteria
Group
Ctrl
Lact
Lact-Pyr
Pyr
C
Alpha Diversity Measure
Chao1
Shannon
Group
Ctrl
Lact
Lact-Pyr
Pyr
D
MDS + UniFrac
Axis.2 [12.7%]
Axis.1 [18.1%]
MDS + wUniFrac
Axis.2 [13.3%]
Axis.1 [60.6%]

Figure 6


A
100
80
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Lipocaline (pg/mg)
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B
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40
20
0
Lipocaline (pg/mg)
Ctrl
FOS
FOS-Pyr
Treatment

Figure 7

## Supplementary data

### Materials and Methods - Microbiota analysis

Faecal DNA extraction, 16S rRNA gene sequencing, and bacterial community analysis; Genomic DNA was obtained from frozen faeces using the ZR Faecal DNA Miniprep™ kit (Zymo Research). The microbial 16S rRNA gene was amplified during the first PCR step with adapter fusion primers targeting the V3 to V4 regions (corresponding to a 460-bp region of *Escherichia coli* 16S rRNA gene, GenBank number J01695 with bacterial forward 343F (TACGGRAGGCAGCAG[1]) and reverse 784R (TACCAGGGTATCTAATCCT[2]) primers. Pooled amplicon libraries were sequenced employing an Illumina MiSeq (2 x 250 bp) at the GeT-PlaGe platform in Toulouse (France).

Sequence reads were quality controlled and high quality filtered reads were further processed using FROGS pipeline (Find Rapidly OUT with Galaxy Solution) to obtain OTUs and their respective taxonomic assignment thanks to Galaxy instance (http://sigenae-worbench.toulouse.inra.fr)[3]: an initial FROGS pre-processing step which allows to select overlapping reads with expected length without N. Swarm clustering method was applied by using a first run for denoising with a distance of 1 and then a second run for clustering with a maximal aggregation distance of 3 on the seeds of the first Swarm. Putative chimerae were removed using Vsearch combined with cross-validation (GitHub repository. DOI:10.5281/zenedo.15524). Cluster abundances were filtered at 0,005%[4] and/or had to be present at least in 3 samples. 100% of clusters were affiliated to OTU by using the silva132 16S reference database and a taxonomic multi-affiliation procedure (Blast+ with equal multi-hits[5]). Taxonomic assignment at the lowest phylogenetic level and prevalence-based filtering step allowed to obtain of 468 OTUs (after correcting multi-affiliations and some misleading affiliations). Between 15 000 and 22 518 valid sequences per sample were counted.

Richness and diversity indexes of bacterial community, as well as clustering and ordinations, were computed using the Phyloseq package (v 1.19.1) in RStudio software[6, 7]. Within sample community alpha diversity was assessed using Chao-1 and Shannon indexes. Divergence in community composition between samples was quantitatively assessed by calculating both weighted and unweighted UniFrac distance matrices. Unconstrained ordination was visualized using multidimensional scaling (MDS) and compared using Adonis test (9999 permutations).

In order to evaluate differential abundance in response to treatment and identify important taxa modulated by lactose and prevented by pyridoxamine, OTUs were agglomerated at the genus rank. Univariate differential abundance of taxa was tested using a negative binomial noise model for overdispersion as implemented in the R package DESeq2 (v1.14.1[8, 9]). A 2x2 factor design combined with a Wald test was applied. Taxa were considered significantly differentially abundant between

groups if their adjusted P-value was below 0.05 and if estimated change was log2FC>|1.5|. Tests were corrected for multiple inferences using the Benjamini-Hochberg method to control the false discovery rate.

Sequences are available on MG-RAST [10], Project name JK_Lactose, temporary project ID: mgp 90034.

## Dietary information

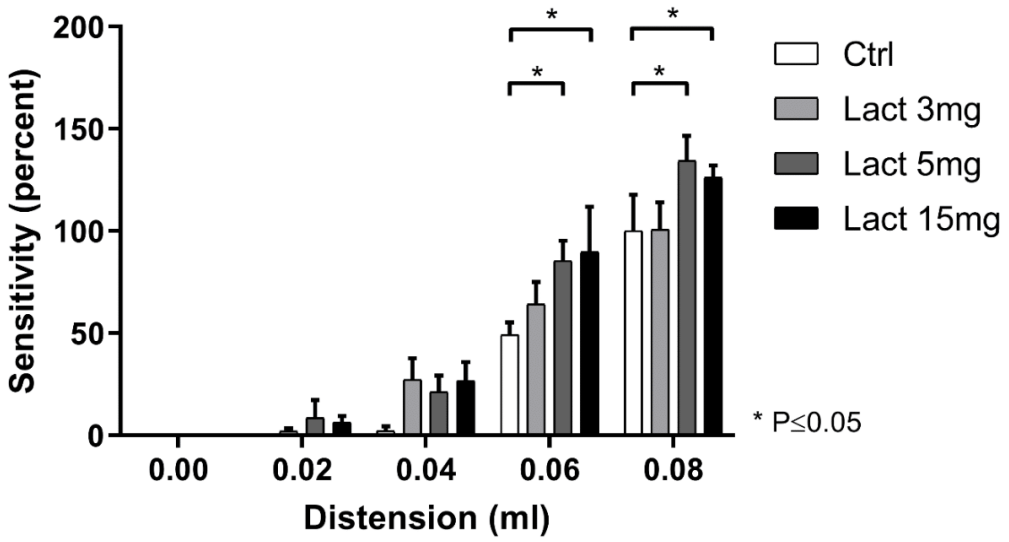
### Composition custom AIN93-M +/- FOS diets

*Table S1 - Diet composition AIN93M +/- FOS (g/kg)*

| | **AIN-93M** | **AIN93M-FOS** |
|---|---|---|
| **Corn-starch** | 465.692 | 365.692 |
| **Fructo-oligosaccharides** | 0 | 100 |
| **Casein** | 140 | 140 |
| **Dextrinized corn-starch** | 155 | 155 |
| **Sucrose** | 100 | 100 |
| **Soybean oil** | 40 | 40 |
| **Powdered cellulose** | 50 | 50 |
| **Mineral mix (AIN-93M-MX)** | 35 | 35 |
| **Vitamin mix (AIN-93-VX)** | 10 | 10 |
| **l-Cystein** | 1.8 | 1.8 |
| **Choline bitartrate** | 2.5 | 2.5 |
| **Tert-butylhydroquinone** | 0.008 | 0.008 |

*Reference:* Reeves, P. G., F. H. Nielsen and G. C. Fahey, Jr. (1993). "AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet." J Nutr **123**(11): 1939-1951.[11]

Diets were prepared and mixed at the UE300 'Unité de Préparation des Aliments Expérimentaux' (UPAE) INRA Jouy-en-Josas




*Supplemental figure 1 - Increase in visceral sensitivity after daily treatment with different concentrations of lactose for 3 weeks, in response to increasing volumes of distension. Sensitivity is expressed as a percentage of the maximum average control response. N=8*

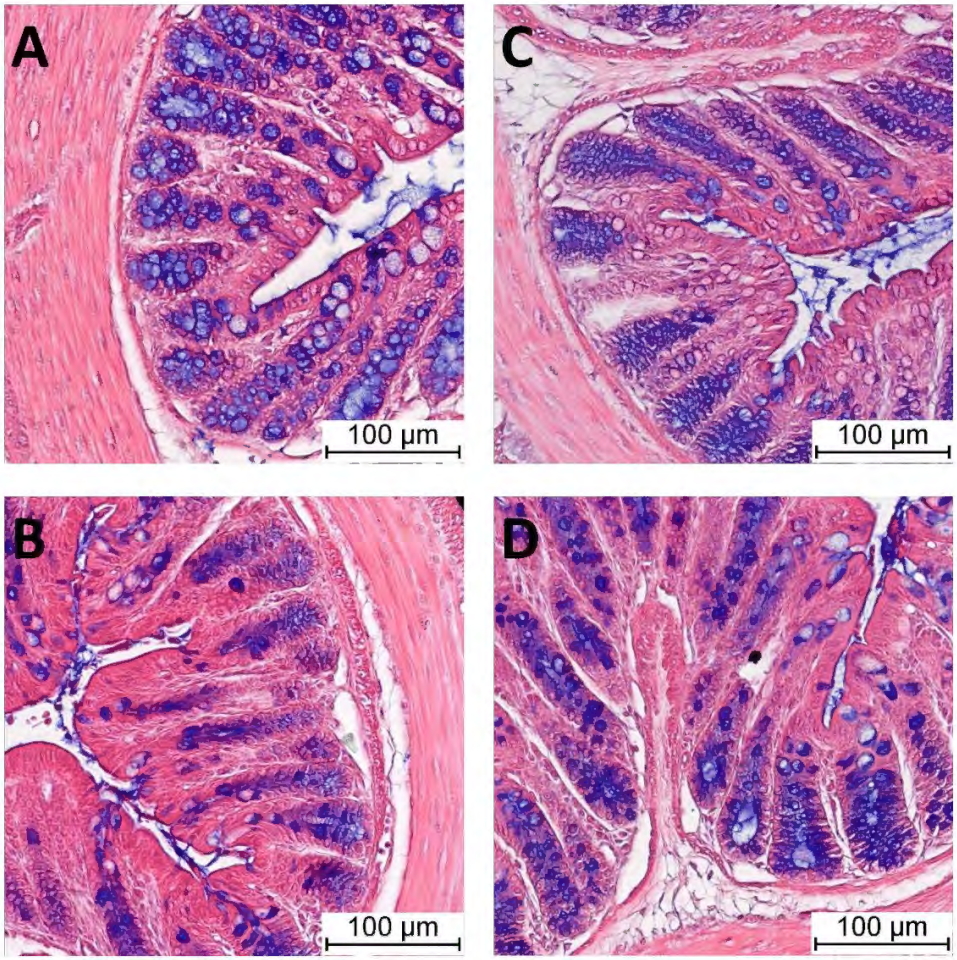
## Histological scoring

### M&M

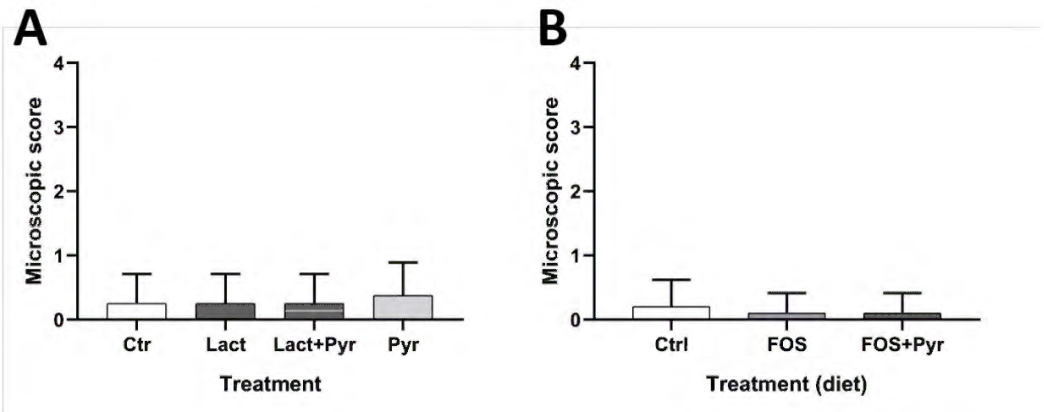
Histological processing, ABHE staining; 5µm paraffin embedded sections were deparaffinated by using 3x 5min baths of American Mastertech Clearify followed by 3x 5min 100% ethanol, 3x 5min 95% ethanol, 2x 5min 70% ethanol, 5min demineralised water. Staining was performed by 5 min in Hematoxylin, 10 min in running tap water, 30 min in Alcian Blue solution (pH 3.0) followed by 5 min in running water, 3 min in Eosin, 10 min in 95% ethanol, followed by dehydration (2x 4min 70%ethanol, 2x 5min 95% ethanol, 2x 5min 100% ethanol, 3x 5min American Mastertech Clearify), and finally mounted using Diamount mountant.

Manual Ultra-high resolution Composite Image Overview (MUCIO); datasets of overlapping microscope views covering entire slides were generated by manual microscope photography (single photo resolution: 1280 × 1024pixels) and stitched together using Microsoft Image Composite Editor (MICE), as originally described in Kamphuis, Mercier-Bonin, Eutamène, Theodorou (2017)[12]. Samples were imaged using a Nikon Eclipse 90i microscope fitted with a DXM 1200 F Digital Camera.

Microscopic scoring; composite micrographs were scored according to protocol on a scale from 0-4[13]; 0: no signs of inflammation, 1: very low level of leukocytic infiltration, 2: low level of leukocytic infiltration, 3: high level of leukocytic infiltration, high vascular density, thickening of the colon wall, 4: transmural infiltrations, loss of goblet cells, high vascular density, thickening of the colon wall. Statistical analysis: Scores were averaged per treatment group; One-way ANOVA, multiple comparisons with Tukey's correction for multiple comparisons.




*Supplemental figure 2 - Representative images of sections of colon showing no signs of inflammation. (A,B): Controls of lactose, FOS respectively. (C,D) Lactose-, and FOS-treated, respectively.*




*Supplemental figure 3 – Microscopic scores of Lactose-pyridoxamine experiments (A) (N=8), and FOS-pyridoxamine experiments (B) (N=10). Apart from a very mild presence of leukocytes in 1 or 2 individuals in each group, no signs of inflammation were observed in any group.*

No macroscopic (at moment of tissue collection) or microscopic signs of inflammation have been observed in response to FODMAP treatments, further excluding the presence of overt active inflammation in these experiments.

## LC/ MS analysis of aldehydes

### M&M

#### Chemicals

Methanol (HPLC grade) and acetonitrile (Optima LC/MS grade) were purchased from Fisher (Illkirch, France), formic acid from Sigma Aldrich (St Quentin Fallavier, France). Ultra-pure water was obtained using a Milli-Q system (Millipore, St Quentin en Yvelines, France).

1-((ammoniooxy)methyl)-2-bromobenzene chloride (BBHA) was purchased from Interchim (Montluçon, France). Piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) and trifluoroacetic acid (TFA) were obtained from Acros organics (Geel, Belgium). Glyoxal, Methyglyoxal and 3-Deoxyglucosone were purchased from Sigma Aldrich (St Quentin Fallavier, France) and benzaldehyde-d5 (Internal standard) from CDN isotopes (Quebec, Canada). BBHA derivatives were synthesized in house according to previously published methods[14]. Briefly, BBHA (50-100 µmol, 1-2 eq) was added to standard solutions of aldehydes (50 µmol) in PIPES buffer (0.1 M, pH 6.5, 1 mL), and the mixture was stirred at 6-8°C for one hour. Each BBHA derivative was then purified by SPE.

#### Sample treatment

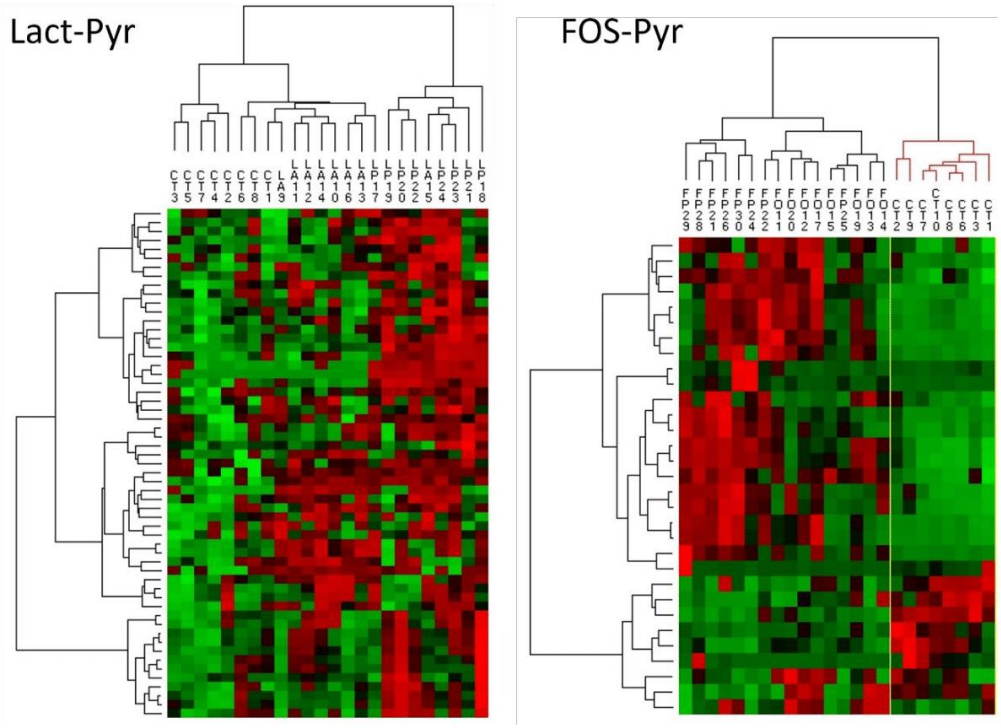
Intestinal content samples were prepared by homogenising 0.2g intestinal content in 1 mL demineralised water with 5 ceramic beads using a Fast-Prep (MP Biomedicals, Illkirch, France) (3x 15sec 6m/s with 1 min breaks) followed by 20min centrifugation at 8000x, supernatants were collected in 1.5 mL Eppendorf tubes and stored at -20°C until use.

Intestinal content samples (100 µl) were added to 60 µL of PIPES buffer (0.1 M, pH 6.5) and derivatised with 120 µL BBHA (50 nmol / µL) in presence of 20 µL of internal standard (Benzaldehyde-d5, 1 ng/µL). The samples were stirred at 6-8°C for one hour. SPE was conducted on a Visiprep SPE Vacuum manifold (Supelco, St Quentin Fallavier, France), using Agilent C18 Bond Elut (100mg, 1 mL) cartridges. The sorbent was conditioned with 1 mL of $CH_3OH$, then 1 mL water. The derivatized samples were vortexed and then deposited on the SPE cartridge. Washing was performed with first 1 mL PIPES (rinse the container and deposit on cartridge) and then 0.05% TFA/$CH_3OH$ (3/2). The cartridge was then dried under vacuum (1min), eluted with 400 µL $CH_3OH$ and collected in glass tubes. Finally, the volume was adjusted to exactly 400 µL with $CH_3OH$, and the extracts were stored at -20°C until analysis.

#### Liquid chromatography – mass spectrometry

Sample extracts were analyzed by high-performance liquid chromatography coupled to high-resolution mass spectrometry (HPLC-HRMS). The HPLC system consisted in an Ultimate 3000 RS pump fitted with the Ultimate 3000 autosampler (Dionex-Thermo Scientific, Les Ulis, France). The flow rate was 0.2 mL/min with the following elution gradient program: 0min 0% of B, from 3min to 15min 100%

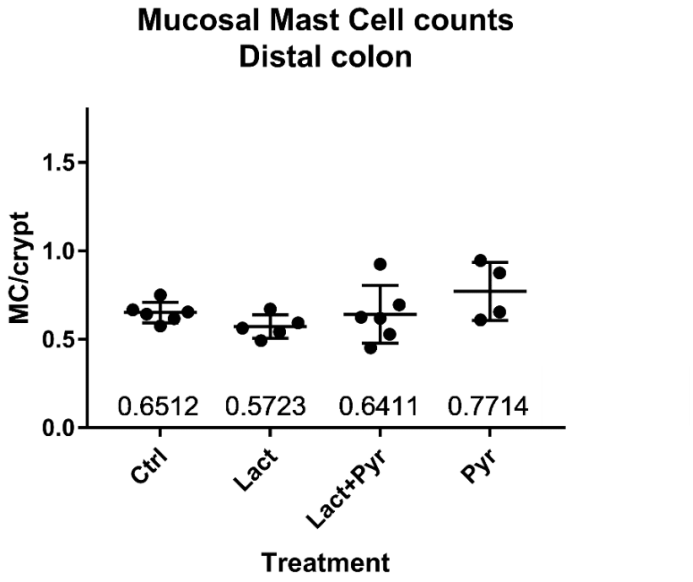
of B, and from 15 to 25min 100% of B. Mobile phases were composed of (A) $H_2O/CH_3CN/HCOOH$ 95/5/0.1 (v:v:v) and (B) $CH_3CN/H_2O/HCOOH$ 95/5/0.1 (v:v:v). 5µL of sample were injected on a Kinetex Core-Shell C18 (150 x 2.1mm, 2.6µm) column (Phenomenex, Le Pecq, France) maintained at 40°C. Detection was achieved on an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific) equipped with an electrospray ionization source used in the positive mode. Ionization parameters were set at +4.5kV for the spray voltage, 35 arbitrary units (au) for the sheath gas flow rate ($N_2$), 5 au for the auxiliary gas flow rate ($N_2$) and 300°C for the capillary temperature. High-resolution mass spectra were acquired at a resolution power of 30,000 from m/z 80 to 800 in centroid mode. Identifications were performed by tandem mass spectrometry experiments ($MS^n$) using the ion trap mass analyzer of the LTQ-Orbitrap mass spectrometer. Solutions of synthesized standard glyoxal-BBHA, methyglyoxal-BBHA and 3-deoxyglucosone-BBHA at different concentration levels were used to characterize the method in terms of linearity of response, repeatability and sensitivity. Statistical analysis: From the lactose-pyridoxamine experiment raw data files, ions were extracted using xcms software[15]. Signals corresponding to brominated compounds were filtered based on the HRMS signal of the exact mass of each $[M+H]^+$ ion, according to a mass measurement error of ± 5 ppm, and to the occurrence of two signals of equivalent intensities with ΔM = 1.998 corresponding to the mass difference between the two bromine isotopes. Then isotopic ratio between isotopes was checked. For the FOS-pyridoxamine experiment, the same extraction process was carried out. Supervised multivariate partial least squared discriminant analysis with orthogonal signal filtration (OSC-PLS-DA) and univariate non-parametric Kruskal Wallis tests were carried out on these ions. Discriminant models were validated if PLS Q2 criterion was greater than 0.4 and a permutation test was validated. Significant potential aldehyde ions were selected if PLS variables importance on projection (VIP) was greater than 1 and univariate Kruskal Wallis p-values with false discovery rate correction was lower than 0.05. Heatmap with hierarchical clustering analysis (HCA) using Euclidian distance and Ward method as aggregation criterion was used to present the results of both experiments.



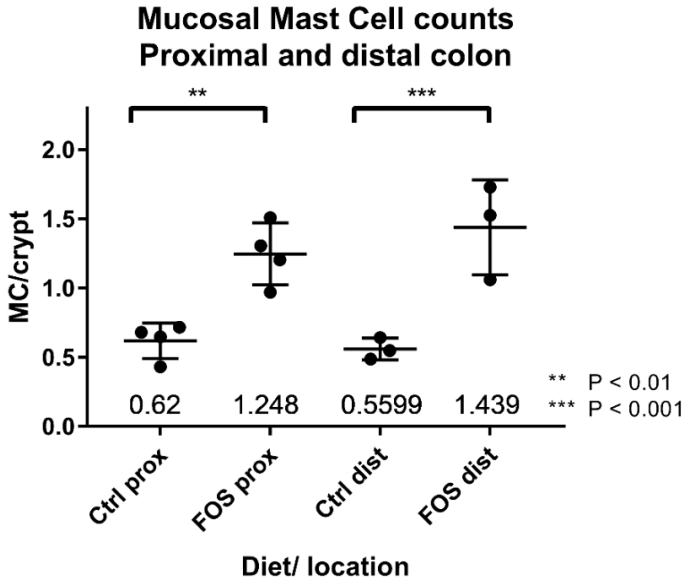

*Supplemental figure 3 - Derivatised ions (carbonyl compounds) for which the control group had lower values than the FODMAP treated groups*

## HPLC/MS analysis of aldehydes in colonic contents

In lactose- and FOS-treated animals, a metabolomic analysis by HPLC-MS of colonic contents after BBHA derivatization identified a distinct clustering of global reactive carbonyl compound profiles versus control (Figure S3). Additive treatment with pyridoxamine of lactose- and FOS-treated animals did not modify internal clustering between these groups.




*Supplemental figure 4 - Mucosal mast cell (MC) counts of distal colon in animals treated daily with 5mg lactose and/or pyridoxamine for 3 weeks, expressed as MC/crypt.*




*Supplemental figure 5 - Mucosal mast cell (MC) counts of distal colon in animals treated daily with 5mg lactose and/or pyridoxamine for 3 weeks, expressed as MC/crypt.*

## References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## 2.3 Increased FODMAP intake alters colonic mucus barrier function through glycation processes and increased mastocyte counts

This work reports the results of our investigation of the effects on the colonic mucus barrier of FODMAP treatment, as a reflection on intestinal barrier function. We used our MUCIO approach and considered our new model of mucus organisation developed in the earlier work presented in section 2.1. Apart from finding that FODMAPs can dysregulate the colonic mucus barrier function through effects on mucosal mast cells and production of glycating agents described in section 2.2 we show that the increased production of mucus in the absence of faecal matter goes together with an increased variability and decreased coverage of the faecal mucus layer, indicative of a less functional mucus barrier.

These results and this manuscript are ready to be submitted to the American Journal of Physiology – Gastrointestinal and Liver Physiology, as a Rapid Report.

# Increased FODMAP intake alters colonic mucus barrier function through glycation processes and increased mastocyte counts

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## Abstract:

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder for which dietary interventions can be a useful treatment. In recent years, the low-FODMAP approach is gaining traction in this regard. The fermentation of FODMAPs by the gut microbiota can generate toxic glycating metabolites, such as methylglyoxal. These metabolites can have harmful effects by their role in the generation of Advanced Glycation End Products (AGEs), which activates Receptor for AGEs (RAGE). Mast cells can be stimulated by AGEs, play a role in IBS, and their main mediator histamine is reduced in patients on a low-FODMAP diet. Additionally, it is known that dietary components can influence intestinal mucus barrier function, and mast cells likewise can influence mucus barrier function. We hypothesized that an increased intake of dietary FODMAPs is implicated in mucus barrier particularities. We have found that an increased intake of FODMAPs induces a dysregulation of the colonic mucus barrier, increasing mucus discharge in empty colon, while increasing variability and decreasing average thickness of the fecal mucus layer. Changes were correlated with increased mast cell counts, pointing to a role for the cross-talk between these and goblet cells. Observed effects were prevented by co-treatment with anti-glycation agent pyridoxamine, indicating a role for glycation processes in the negative impact of FODMAP ingestion. This study shows that FODMAP ingestion can cause colonic mucus barrier dysregulation in mice, by a process that involves glycating agents and increased mastocyte counts.

## Introduction

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder characterized by abdominal pain, erratic bowel habits, and variable changes in stool consistency(11). It is a common affliction, with a global prevalence of 11,2% according to a 2012 meta-analysis(20). Because IBS is a functional disorder, and causes are incompletely understood, treatment has proven difficult. Dietary interventions prove useful, with particularly the low-FODMAP approach(14) gaining traction in recent years. Apart from gas production and osmotic distention due to FODMAP ingestion, microbial fermentation products have been raised as factors possibly involved in symptom generation. The bacterial metabolic toxin hypothesis, proposed by Campbell *et al*.(6) poses that harmful bacterial fermentation products are responsible for the wide range of systemic effects observed from food intolerances such as lactose intolerance. Particularly anaerobic fermentation of unabsorbed carbohydrates by the colonic gut microbiota, producing such metabolites as alcohols, ketones, and aldehydes, is held responsible. A particularly toxic metabolite is methylglyoxal, which affects both prokaryote cells and eukaryotic cells, e.g. changing cell proliferation, gene expression patterns and cell signaling processes(6). Relatedly, methylglyoxal can have harmful effects by its role in the generation of Advanced Glycation End Products (AGEs), because of its properties as a strong glycating agent(28). Increasing AGE concentrations lead to activation of the Receptor for Advanced Glycation End Products (RAGE), which in turn leads to ROS generation and a pro-inflammatory state through NF-κB pathway signalling(31). A potential increase in aldehydes and other glycating agents during microbial processing of FODMAPs in the gut can be expected to enhance the formation of AGEs, and in that way, support a pro-inflammatory state. Mast cells too can be activated by AGEs(25) through RAGE activation, or by (acetaldehyde) aldehydes directly(12). We have worked to integrate the bacterial metabolic toxins hypothesis by Campbell *et al.*(6) as a complementary mechanism of action to explain the efficacity of the low-FODMAP diet; our work has indicated that glycating agents produced in the microbial processing of FODMAPs can be responsible for the emergence of IBS-like traits in a mouse model, by increasing mucosal mast cell counts in the colon(18). Mast cells can also impact mucus

barrier function, for example by inducing mucus release from goblet cells during immobilization stress in mice(8). Jalanka-Tuovinen *et al.*(17) have found that PI-IBS and IBS-D patients report passage of mucus *per rectum*, and the IBS-type microbiota profile that they describe was positively correlated with both mast cell and goblet cell counts in rectal biopsy samples. It has also been established that diet by itself can modulate intestinal mucus barrier function, in the absence of pathophysiological processes. Male rats on 6% FOS supplemented fiber-free semi purified (FFP) diets showed heavily impaired cecal epithelial mucus barrier function(13). On the other hand, dietary fiber deficient diets decrease the colonic mucus barrier(5, 10), an effect which is mediated by the intestinal microbiota(10). Inulin supplementation given to rats immediately after weaning modifies mucin gene expression, colonic crypt depth and goblet cell numbers(22).

Thus, it has been established that the diet can influence intestinal mucus barrier function. We used an animal mouse model to investigate whether an increased intake of dietary FODMAPs is implicated in mucus barrier particularities, which could be involved in further epithelial barrier dysfunction, to study complementary mechanisms of effect of a low-FODMAP diet.

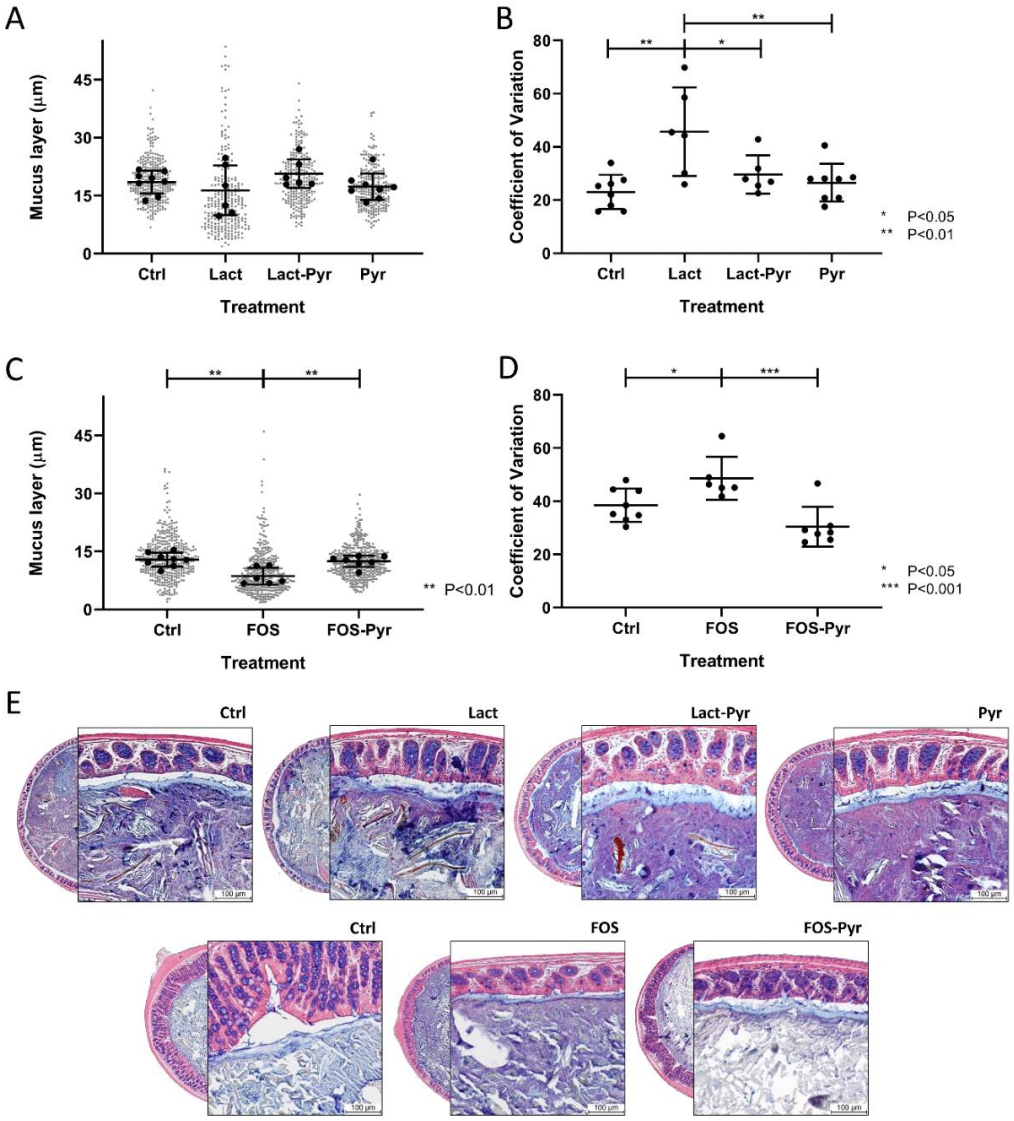


*Figure 1* - Fecal mucus layer thickness in distal colon of mice treated with FODMAPs +/- pyridoxamine. (A) The average colonic fecal mucus layer thickness (black), and the pooled complete data-set that generated these averages (grey) of mice treated daily with 5mg lactose +/- 5mg pyridoxamine. (B) The Coefficients of Variation (CoVs) of the mucus layer thickness averages for mice treated daily with 5mg lactose +/- 5mg pyridoxamine. (C) The average colonic fecal mucus layer thickness (black), and the pooled complete data-set that generated these averages (grey) of mice fed with a FOS-augmented diet +/- pyridoxamine-enriched drinking water (1mg/ml). (D) The CoVs of the mucus layer thickness averages for mice fed with a FOS-augmented diet +/- pyridoxamine-enriched drinking water (1mg/ml). (E) Representative images showing the fecal mucus layers of all treatment groups.

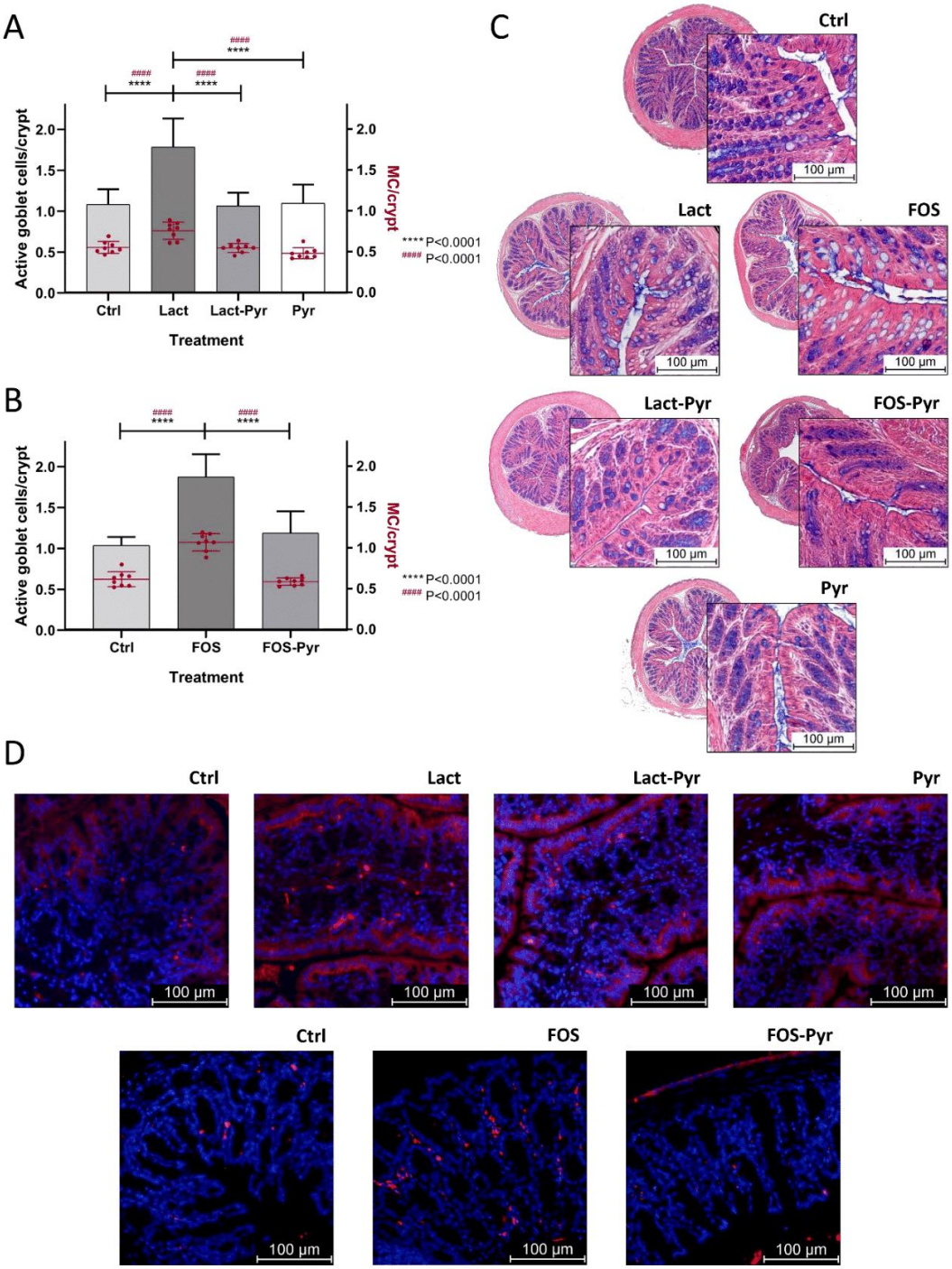


*Figure 2* - Active goblet cells per crypt in the distal colon of mice treated with FODMAPs +/- pyridoxamine. (A) Ratio active goblet cells/crypt for distal colon of animals treated with lactose and/or pyridoxamine. In red, the ratio mastocyte/crypt is represented. (B) Ratio active goblet cells/crypt for distal colon of animals treated with FOS and/or pyridoxamine. In red, the ratio mastocyte/crypt is represented. (C) Representative images showing the increase in active goblet cells in the colon of FODMAP-treated animals, and its reversal by pyridoxamine. (D) Representative images showing the increased colonic mucosal mastocytes in FODMAP-treated animals, and the normalization obtained by pyridoxamine co-treatment.

Treatment with 5 mg/day lactose caused a slight diminution of the average fecal mucus layer thickness (-11.9% *v* control, P<0.68), but with a markedly altered dispersion of measured values (grey point-cloud, Figure 1A), which shows in the significantly increased Coefficient of Variation (CoV) for the lactose-treated group (+98% *v* control, P<0.01), which was normalized by co-treatment with pyridoxamine (Figure 1A,B). Similarly, treatment with a FOS-enriched diet caused a diminution of the average fecal mucus layer thickness (-33% *v* control, P<0.01), with a significantly increased CoV (+26% *v* control, P<0.05%), which, again, was lowered by co-treatment with pyridoxamine (Figure 1C,D). Measurements were performed using the MUCIO approach(19) on AB/HE stained distal colon sections, thus obtaining complete overviews, and numerous measurement values per sample and individual (Figure 1E).

To further investigate the cause of these changes in fecal mucus layer thickness, we next analyzed the activity of goblet cells in empty colon, without the presence of feces, as we previously demonstrated that the colonic mucus layer depends on the presence of luminal contents(19), and we questioned how the production of mucus in the absence of contents might differ between groups. FODMAP-treatment with either lactose or FOS caused an increase in the number of discharging goblet cells in the empty distal colon (lactose: +70% *v* control, P<0.0001; FOS: +81% *v* control, P<0.0001) and this effect was lost or moderated when cotreated with anti-glycation agent pyridoxamine (lactose-pyridoxamine: -1.7% *v* control, P=0.9979; FOS-pyridoxamine: +14.5% *v* control, P=0.4622) (Figure 2A,B,C). At the same time, FODMAP-treatment caused an increase in colonic mucosal mastocyte counts in proximal colon (lactose: +45% *v* control, P<0.0001; FOS: +73% *v* control, P<0.0001), which was similarly lost through co-treatment with pyridoxamine (lactose-pyridoxamine: -1.2% *v* control, P=0.9978; FOS-pyridoxamine: -4.8% *v* control, P=0.7663) (Figure 2A,B,D).

## Discussion:

We observed an increase in mucus production in both lactose- and FOS-treated animals, as demonstrated by the increased goblet cell discharge in empty distal colon (Figure 2), which has been described before in response to the intake of FOS in rat(27) as well as in man(26). Ten Bruggencate *et al*. take this increased mucus production to be an indicator of mucosal irritation in response to an increased luminal concentration of irritants produced by the microbiota(26, 27), a claim that has not been readily accepted by everyone(15). A mucosal insult by fermentation of FODMAPs is however supported by our results, and the increased mucus production is somewhat paradoxically accompanied by a reduced fecal mucus barrier formation, and a higher variability in this mucus layer (Figure 1), something more readily understood in the model of colonic mucus organization we previously described(19). In short, the separating mucus barrier is formed in the presence of feces and covers the fecal pellet. In case mucus is liberated at an increased rate due to mucosal irritation, even in the absence of feces, there seems to be a hypervariable and insufficient coverage of the feces, and the barrier separating the fecal material from the epithelium of distal colon is compromised. We saw a prevention of these negative effects by co-treatment with pyridoxamine, which is a known anti-glycation agent(7, 9, 16, 30), which points to the involvement of glycation processes in the generation of these effects, most likely stemming from microbial fermentation products, as proposed in the toxic bacterial metabolite hypothesis by Campbell *et al.*(6). Reactive glycation agents interact with protein to produce glycated residues, leading to the formation of Advanced Glycation End Products (AGEs)(28), which can directly stimulate mast cells(25), and mast cells in turn are capable of causing mucus discharge by goblet cells(8). The aberrant mucus release and passage *per rectum* of mucus in IBS patients(17) might be explained in this way too, since IBS patients show increased mast cell numbers(2, 3, 33), and mast cells can induce mucus release from goblet cells(8). Histamine levels are increased in the mucosa of IBS patients(1, 2), while evasion of dietary FODMAPs lowers urinary histamine levels(21). We therefore hypothesize the following cascade explaining the effects observed in this work: FODMAP fermentation causes production of reactive glycation agents by the intestinal

microbiota, which leads to generation of AGEs locally in tissues exposed to these toxic metabolites. This in turn leads to RAGE activation, activating mast cells and increasing their population locally. At the same time, increased mast cell numbers and mediator production causes goblet cells to discharge more, even in the absence of contents, causing the observed dysregulation (Figure 3). Previously, it has been demonstrated that a 10% FOS diet decreases the cecal mucus barrier in rats(29) and similarly, a 6% FOS-diet induces an increased cecal permeability in rats on an otherwise fiber-free diet, probably due to a disturbed mucus layer, which caused a transient inflammatory state, which abated in a timely fashion, and was interpreted by the authors as an adaptative response to develop the immune system(13). We observed no overt inflammation in our mice after 3 weeks of treatment with either 5mg daily of lactose or diet containing 10% FOS, by fecal lipocalin-2 quantification, macroscopic or microscopic observation (data not shown). It is likely that an increase in short-chain fatty acids (SCFAs) caused by our FODMAP interventions has a significant effect on mucus barrier function as well, as previously reported(32, 34) for SCFAs. However, in contrast to our study, these effects are generally described to be positive for mucus function, and if similar processes were responsible in our experiments, the observed reversibility by anti-glycation agent pyridoxamine is not easily explained. The SCFAs acetate, butyrate, and propionate can also directly stimulate mucus release(24); the periodic arrival of the colonic contents in the distal colon would bring increased levels of SCFAs with it, stimulating release of mucus in the presence of feces, while we have observed an increased discharge in the absence of contents, thus likely during moments where the SCFAs concentrations were not particularly increased.

The specific predisposition of the immune system to react or to tolerate gut microbes becomes more important in case of a dysfunctional mucus barrier and increased intestinal permeability, and it is indeed known that serum cytokine profiles of IBS patients have a tendency to show more IL-6, IL-8, and less IFN-γ, while mucosal mRNA expression of IL-10 and FOXP3 tend to be decreased(4). These tendencies could indicate the relatively higher predisposition of the IBS patient intestine to retain low-grade inflammation, in particular in the context of a dysfunctional mucus barrier, which we showed

can be caused by a badly tolerated FODMAP intake. In our view, the dysregulation of the intestinal mucus barrier observed herein is unlikely to cause systemic or even local symptoms unless other factors are present that exacerbate or exploit this dysregulation.

We have shown that FODMAP ingestion can cause mucosal irritation, and dysregulation of the intestinal mucus barrier in mice, by a process that involves glycating agents, and a subsequent increase in mastocyte counts.

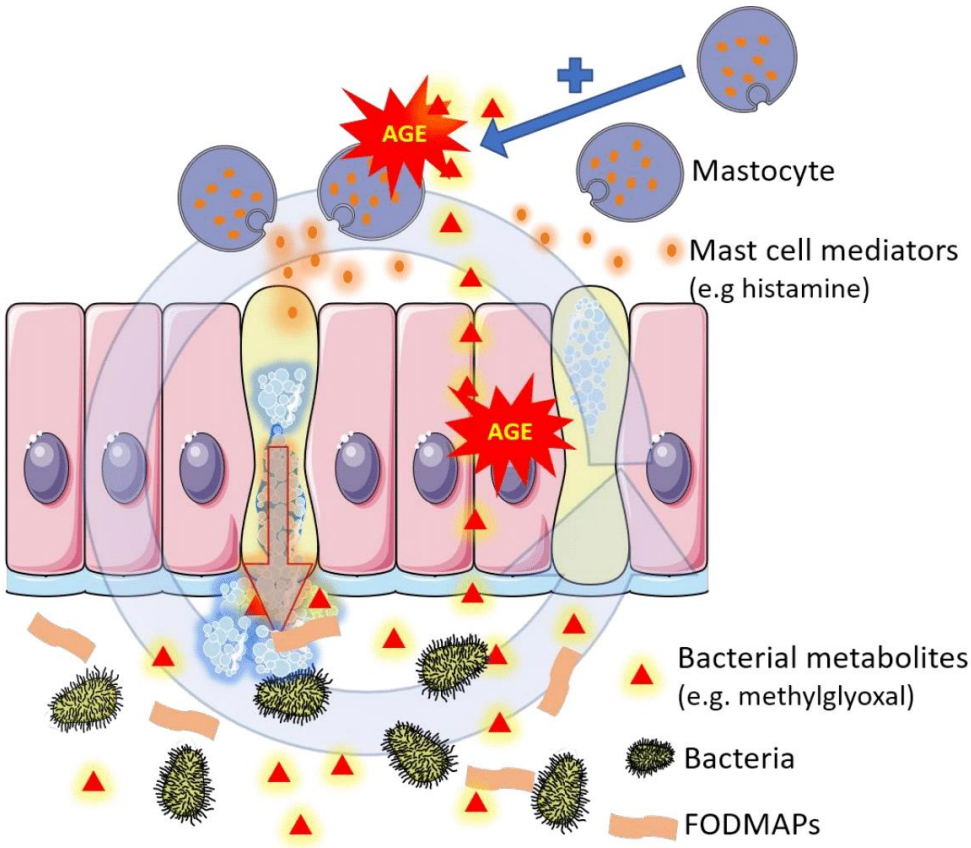


*Figure 3* - Hypothesized cascade by which FODMAP ingestion could dysregulate the colonic mucus barrier. Toxic bacterial metabolites produced during FODMAP fermentation lead to AGE production which cause mastocyte number increases and activation, interaction between mastocytes and goblet cells lead to mucus discharge dysregulation.

## Materials and Methods:

### Animals and sample collection

Adult male C57/Bl6 mice (Janvier, Le Genest St Isle, France) were housed in polypropylene cages in groups of 8 and offered unlimited access to standard rodent food (Mucedola Global Diet 2018, Harlan, Italy) and water. Lactose-pyridoxamine treated group received a daily oral gavage of 5mg lactose and/or 5mg pyridoxamine in 200 µl saline solution, the control group received only saline, for 3 weeks. The FOS-pyridoxamine treated group received a custom modified AIN-93 diet, with or without 10% fructo-oligosaccharides (composition in Table 1), complemented with or without 1mg/ml pyridoxamine in drinking water. Mice were euthanized by cervical dislocation, after which both 1.5 to 2 cm of distal colon and of proximal colon covering regions with and without contents were removed and stored in Carnoy's fixative overnight. All animal experiments were performed in accordance with EU directive 2010/63/EU and approved by the local Animal Care and Use Committee of Toulouse Midi-Pyrénées (agreement CEEA-86).

*Table 1 - Composition custom AIN93-M +/- FOS diets*

| | **AIN-93M** | **AIN93M-FOS** |
|---|---|---|
| **Corn-starch** | 465.692 | 365.692 |
| **Fructo-oligosaccharides** | 0 | 100 |
| **Casein** | 140 | 140 |
| **Dextrinized corn-starch** | 155 | 155 |
| **Sucrose** | 100 | 100 |
| **Soybean oil** | 40 | 40 |
| **Powdered cellulose** | 50 | 50 |
| **Mineral mix (AIN-93M-MX)** | 35 | 35 |
| **Vitamin mix (AIN-93-VX)** | 10 | 10 |
| **l-Cystein** | 1.8 | 1.8 |
| **Choline bitartrate** | 2.5 | 2.5 |
| **Tert-butylhydroquinone** | 0.008 | 0.008 |

*Reference:* Reeves, P. G., F. H. Nielsen and G. C. Fahey, Jr. (1993) J Nutr **123**(11): 1939-1951.(23)

Diets were prepared and mixed at the UE300 'Unité de Préparation des Aliments Expérimentaux' (UPAE) INRA Jouy-en-Josas

### Histological sample preparation

Collected tissues were rinsed in 100% ethanol after 1 day in Carnoy's fixative and automatically processed using a Shandon Excelsior ES Tissue Processor by the following program: 2x 60min 100% ethanol, 2x 60min butanol, 480min butanol, 3x 80min paraffin at 60 °C. Tissue samples were included in paraffin blocks using a Thermo Scientific HistoStar Embedding Workstation. 5µm tissue sections were made using a Microm HM 340 E microtome and attached to Superfrost Plus microscope slides (Thermo Scientific, USA).

### Visualization

Mouse Mucosal Mast Cell Protease (MMCP) staining: 5µm sections were deparaffinated by using 3x 5min baths of American Mastertech Clearify followed by 3x 5min 100% ethanol, 3x 5min 95% ethanol, 2x 5min 70% ethanol, 5min demineralized water. Slides were washed 2x in PBS for 5min, followed by a 2-hour blocking step with 10% donkey serum in PBS, and washed 3x 5min under light agitation in PBS. Slides were incubated overnight at 4°C with primary antibodies (Sheep anti-mMCP1 (MS-RM8 (Moredun Group, UK)) diluted 1:400), followed by 2x 5min rinsing steps in PBS. Secondary antibody (Alexa Fluor 594 Donkey-anti-Sheep (A-11016 (Molecular Probes, USA)) diluted 1:400 in 1% donkey serum PBS) incubation was performed for 2 hours, followed by 3x 5min washing steps in PBS, a quick rinse with tap water, followed by mounting using ProLong Gold® antifade reagent with DAPI (Thermo Fisher Scientific, USA).

Alcian Blue/Hematoxylin/Eosin staining (AB/HE): 5µm sections were deparaffinated by using 3x 5min baths of American Mastertech Clearify followed by 3x 5min 100% ethanol, 3x 5min 95% ethanol, 2x 5min 70% ethanol, 5min demineralized water. Staining was performed by 5 min in Hematoxylin, 10 min in running tap water, 30 min in Alcian Blue solution (pH 3.0) followed by 5 min in running water, 3 min in Eosin, 10 min in 95% ethanol, followed by dehydration (2x 4min 70%ethanol, 2x 5min 95%

ethanol, 2x 5min 100% ethanol, 3x 5min American Mastertech Clearify), and finally mounted using Diamount mountant.

## Microscopy and image analysis

Manual Ultra-high resolution Composite Image Overview (MUCIO): datasets of overlapping microscope views covering entire slides were generated by manual microscope photography (single photo resolution: 1280 × 1024pixels) and stitched together using Microsoft Image Composite Editor (MICE), as originally described in Kamphuis, Mercier-Bonin, Eutamène, Theodorou (2017)(19). Samples were imaged using a Nikon Eclipse 90i microscope fitted with a DXM 1200 F Digital Camera.

Mucus layer thickness measurements: The fecal mucus layer thickness was measured using ImageJ software; a measurement perpendicular to the surface of the colonic contents was taken every 100 micrometers along the entire imaged surface.

Active goblet cell count analyses: One ratio discharging goblet cell:crypt per mouse based on analysis of 25-90 crypts in transversal sections of empty colon, depending on availability of suitable visual material. Image sets were coded, randomized and analyzed blindly. Statistical analysis: One-way ANOVA, multiple comparisons with Tukey's correction for multiple comparisons.

Fluorescence microscopy: Samples were imaged using a Nikon Eclipse 90i microscope fitted with a DXM 1200 F Digital Camera. Image sets were taken at 200x magnification.

Mast cell count analyses: One ratio mastocyte:crypt per mouse based on analysis of 50-250 crypts, dependent on availability of suitable visual material. Image sets were coded, randomized and analyzed blindly. Statistical analysis: One-way ANOVA, multiple comparisons with Tukey's correction for multiple comparisons.

## Author Contributions

JBJK, HE, and VT designed the experiments; JBJK performed the experiments, analyzed data, and wrote the manuscript; HE and VT supervised the project.

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# 3 General Discussion

## Discussion, Perspectives, and Conclusion

# 3 General Discussion

As mentioned at the start in the introduction, this thesis is part of the NeuroGut project, which ran from 2014 to 2018. This Initial Training Network, financed by the European Commission, has led to interesting findings, many of which have been published in renowned international scientific journals, such as Neurogastroenterology and Motility, Scientific Reports, Alimentary Pharmacology and Therapeutics, Neurotherapeutics, and The American Journal of Clinical Nutrition.

More importantly, it has allowed a selection of young researchers to develop their professional and personal skills, and international network. For our part in this project, we investigated the effects of certain alimentary compounds on intestinal health, in relation to functional gastrointestinal disorders, particularly irritable bowel syndrome (IBS), which represents an important negative factor on quality of life (QOL) of patients, as well as costing society billions of euros in lost work-hours and productivity. IBS patients are increasingly following a low-FODMAP diet, which is quite effective in improving QOL and appearance of symptoms, but it remains somewhat difficult to explain the reason for this efficacity.

In this thesis dissertation, complementary mechanisms of effect behind the efficacity of the low-FODMAP diet are explored, by using a mouse model exposed to an increased FODMAP intake. We have shown that FODMAP ingestion by itself can induce certain physiological effects reminiscent of IBS, such as an increased visceral and central sensitivity (section 2.2), and intestinal barrier function dysregulation (section 2.3), and these effects seem related to an increase in glycating agents caused by microbial fermentation of these FODMAPs by the gut microbiota, indicated by the increased expression of Receptor for AGEs (RAGE), and the prevention of observed effects by administration of anti-glycation agent pyridoxamine, a form of vitamin B6. The negative effects on visceral sensitivity and barrier function seem to be mediated by an increased mucosal mast cell population (sections 2.2, 2.3).

## 3.1 Fermentable carbohydrates and IBS symptoms

Since FODMAPs are defined by their 'unabsorbed' status, individual characteristics, such as lactase deficiency or fructose malabsorption, can influence which carbohydrates should be considered FODMAPs in different individuals. Some FODMAPs, such as many polyols, fructo-oligosaccharide (FOS) or fructans, and galacto-oligosaccharide (GOS) are undigestible/unabsorbable for all humans, while for others this depends on factors such as dose, intestinal health status, transporter complexes, and (brush border) enzyme expression, such as for lactose and fructose (Gerbault, Liebert *et al.*, 2011; Brown-Esters, Mc Namara *et al.*, 2012). We have used lactose as a representative of FODMAPs, because mice, like all mammals except a sub-population of humans, lose lactase activity after weaning, and should be considered lactose-malabsorbers.

The insight that FODMAPs can induce an increased abdominal sensitivity without further challenge is interesting, because it indicates that it might render patients even more sensitive to the distension that it causes. Because stress is an important factor in IBS symptom generation (Qin, 2014; Greenwood-Van Meerveld, Moloney *et al.*, 2016), and mast cells are an important mediator in stress-induced visceral hypersensitivity (Gue, Del Rio-Lacheze *et al.*, 1997) and permeability (Santos, Yang *et al.*, 2001; Barreau, Cartier *et al.*, 2007; Vanuytsel, van Wanrooy *et al.*, 2014), it can be expected that the increased mast cell population observed in FODMAP-treated animals renders them more susceptible to mast cell mediated effects of psychological stress.

Our finding that the performed FODMAP treatments can increase visceral but also central sensitivity fits with the frequent association of FGIDs with non-GI symptoms, such as for fibromyalgia and chronic fatigue syndrome (Mathieu, 2009; Wilder-Smith, Olesen *et al.*, 2018), all of which are often exacerbated by stress (Mathieu, 2009). The increase in mastocyte counts could provide a link between these GI and non-GI symptoms in combination with an increased vulnerability to psychological stress, because mast cells can be activated by mental stress through nerve system signalling (Castagliuolo, Lamont *et al.*, 1996; Barbara, Stanghellini *et al.*, 2004; Barbara, Wang *et al.*, 2007; Moon, Befus *et al.*, 2014; Carabotti, Scirocco *et al.*, 2015; Zhang, Song *et al.*, 2016).

Related to the psychological aspects of IBS and fermentable carbohydrates, lactose and fructose malabsorption has been linked to a decreased L-tryptophan metabolism, precursor to serotonin (Ledochowski, Sperner-Unterweger *et al.*, 1998; Ledochowski, Widner *et al.*, 2000),

suggesting a reduced serotonin producing capacity. Indeed, the tryptophan plasma level of fructose malabsorbers is reduced (Ledochowski, Widner *et al.*, 2001). Moreover, in depressed patients, reduced levels of plasma tryptophan are found (Ogawa, Fujii *et al.*, 2014). In contrast, a recent study has found that fructose malabsorption is associated to depression, without a significant relation between circulating tryptophan levels and depression (Enko, Wagner *et al.*, 2018). Conversely, Burokas *et al.* report that prebiotic unabsorbed carbohydrates FOS and GOS exert anti-depressant and anxiolytic effects in mice (Burokas, Arboleya *et al.*, 2017). However, in this study plasma tryptophan levels were reduced for the prebiotic groups GOS and FOS+GOS. These data indicate a link between disturbed tryptophan-metabolism and non-absorbed carbohydrates. While none of these studies deeply explore the mechanism behind these effects, Ledochowski *et al.* propose that non-absorbed carbohydrates react with peptides and amino-acids, forming complexes that interfere with normal metabolism (Ledochowski, Widner *et al.*, 2001).

It is not yet clear whether the effects of FODMAPs on sensitivity and the mucus barrier function reported in sections 2.2 and 2.3 are linked, i.e. the mucus barrier dysfunction aggravates the increase in sensitivity, or whether both are downstream effects of the increase in mast cells, without significantly influencing each other. It is known that barrier defects are correlated to an increased sensitivity (Annahazi, Ferrier *et al.*, 2013), in this case increased paracellular permeability. A mucus barrier dysregulation could thus also have similar effects on gut sensitivity, but the effects observed on this barrier in our model were relatively mild and are not likely to have a significant impact on visceral sensitivity without further challenge.

Reactive microbial fermentation products of unabsorbed carbohydrates could have the same effects on tryptophan levels as FOS/GOS, but are generally more prone to strong and fast glycation reactions (Campbell, Matthews *et al.*, 2010), and it would be interesting to characterize tryptophan levels in FODMAP-treated animals, as well as ethology related to depressive and anxious behaviour. We have not observed any obvious differences in mouse behaviour between treatment groups, but a dedicated study might be interesting. It would therefore also be interesting to see how increased FODMAP intake impacts the chronic stress response of mice, for example by using a water avoidance stress (WAS), or maternal separation model. It has been shown that the increase in visceral sensitivity and induction of intestinal inflammation by WAS is aggravated by FOS in mice (Chen, Du *et al.*, 2017), though in this work,

contrary to ours, FOS alone did not have effects, and was administered at lower doses (8g/kg b.w., i.e. +/- 0.2g per day, versus our 10% of food intake, i.e. +/- 0.4g per day).

In IBS patients too, it would be interesting to investigate tryptophan levels on normal and low-FODMAP diets and relate this to the (improvement in) psychological well-being.

To definitely prove that glycating carbonyls that cause the physiological effects in our experiments are produced in fermentation and not in spontaneous breakdown of carbohydrates, a germ-free or microbiota depleted animal model should be used to compare metabolite profiles and effects of the treatments *an sich*. However, the physiology of germ-free animals is significantly different from conventional mice. For example, the transit time of germ free Swiss-Webster mice is about 60% longer than for conventional Swiss-Webster mice (Kashyap, Marcobal *et al.*, 2013). At the same time no beneficial metabolites of microbial processing will be produced, which causes an underdeveloped immune system (Parker, Lawson *et al.*, 2017).

An experiment using non-absorbed antibiotics to deplete the microbiota would alter aspects of normal digestion and furthermore induce a dysbiosis, which of course has its own effects on gut health which could confound the findings. It is important to realize that we have not found microbiota profile changes in our experiments involving lactose, and that a reduced or absent microbiota by itself could induce visceral hypersensitivity (Verdu, 2006; Luczynski, Tramullas *et al.*, 2017), which complicates the reliability of an experiment into these features.

Confusingly, contrary to what is observed in entirely germ-free animals, some review literature report a decrease in intestinal transit time after antibiotic use (Gaskins, Collier *et al.*, 2002). In fact, the source reference states "… and the subsequent changes in the intestine, including a slower intestinal passage rate,…" (emphasis mine), while the table in the same report does indeed imply a reduced transit time (Sweden Ministry of Agriculture, 1997). Other primary research do however report an increased whole gut transit (Anitha, Vijay-Kumar *et al.*, 2012; Ge, Ding *et al.*, 2017), caused by activation of TLR4 receptors by microbial products (Anitha, Vijay-Kumar *et al.*, 2012). Apart from supporting the general idea that microbial depletion does indeed bring the physiology closer to that of germ-free animals, this illustrates that it can be important to check the sources of claims presented in review articles.

Concerning visceral sensitivity and the importance of the microbiota, it has proven impossible to find information showing the basal sensitivity of germ-free animals, while antibiotic treatment (not necessarily microbial depletion) induces an increased visceral sensitivity that can be prevented by co-treatment with the probiotic *L. paracasei*, or its spent medium without live bacteria (Verdu, 2006). On the other hand, using intracellular recordings of myenteric plexus nerve cells showed that the absence of the microbiome in germ-free mice makes these nerves hypo-excitable, and the microbiome is thus necessary for normal excitability (McVey Neufeld, Mao *et al.*, 2013), pointing to a less sensitive system in these animals. Until comparable techniques are used to compare the visceral sensitivity of germ-free and conventional animals, it is not entirely clear whether the microbiome generally increases or decreases this sensitivity.

In relation to the increased visceral and central sensitivity observed in IBS patients, it would be interesting to see if these factors ameliorate after following a low-FODMAP diet for a longer period, for example by barostat testing such as used to show visceral hypersensitivity (Bouin, Plourde *et al.*, 2002), and a Cold Water Tolerance Test, such as performed in FGID patients (Bouin, Meunier *et al.*, 2001) and in fibromyalgia patients (Reyes del Paso, Garrido *et al.*, 2011). Interestingly, there is a comorbidity between IBS and fibromyalgia (FM) (Martinez-Martinez, Mora *et al.*, 2014), where some authors even propose it's the same disease but through the lens of different medical fields (Kindler, Bennett *et al.*, 2011; Clauw, 2015), FM patients seem to benefit from a low-FODMAP diet as IBS patients (Marum, Moreira *et al.*, 2016b), and this affliction too has been linked to mast cell activation (Tsilioni, Russell *et al.*, 2016).

## 3.2 Immune activation

We have not observed clear immune activation by our FODMAP treatments, apart from mastocyte population increases; faecal lipocalin-2 levels were not significantly different between any groups but instead tended to be slightly decreased through FOS-treatment. In IBS patients, 3 weeks of low-FODMAP treatment however, lowered pro-inflammatory cytokines IL-6 and IL-8, but not TNF-α, from levels that never were significantly different from baseline for the healthy population, and 10-day FOS supplementation following this low-FODMAP intervention did not impact these cytokines in these patients (Hustoft, Hausken *et al.*, 2017). This indicates that FODMAPs, but not FOS in particular, can influence cytokine profiles and inflammatory processes. Indeed, it is known that FOS as a prebiotic can improve rather than worsen inflammation in the intestine (Capitan-Canadas, Ocon *et al.*, 2016), even though it has not proven to be effective in improving true inflammatory disorders such as Crohn's disease (Marteau, 2011). Additionally, the low-FODMAP diet has effects on gut microbiota profile composition (Staudacher, Lomer *et al.*, 2012; Halmos, Christophersen *et al.*, 2015; Hustoft, Hausken *et al.*, 2017; McIntosh, Reed *et al.*, 2017; Staudacher, Lomer *et al.*, 2017), and FOS supplementation can help to normalise the microbiota after an increased dysbiosis index induced by a low FODMAP intake, although this did worsen the IBS symptom severity (Hustoft, Hausken *et al.*, 2017). This indicates that normalising the microbiota profile in this case should not be of primary concern, and a 'dysbiosis' mainly indicates a change from baseline, without necessarily representing a dysfunction.

To see if the improved inflammatory profile of IBS-patients relies on the same mechanisms as described in this thesis, it would be interesting to test whether anti-glycation agents such as described in section 1.4.4 can mimic the positive effects observed for the low-FODMAP diet.

## 3.3 Permeability

We have performed *in vivo* permeability tests, using oral gavage of the fluorescent marker fluorescein thiocyanate (FITC), with non-conclusive results. Differences were striking from time to time, but treated groups showed sometimes the highest, and sometimes the lowest permeability, and this was not reproducible. This is probably in part due to a mismatch with our question; FITC is absorbed throughout the gastrointestinal tract, in which the small intestine definitely represents the greatest exchange surface by a large margin, with the colon representing 300 $cm^2$, and the small intestine 13400 $cm^2$ of the total of 14100 $cm^2$ surface of mouse intestine effective surface (Casteleyn, Rekecki *et al.*, 2010). Of course, we are not sure our treatments induced an alteration of permeability, but for previously mentioned reasons, our negative results do not necessarily indicate that there is not.

Increased mast cell counts do imply an increased permeability in basal conditions, as previously described (Lee, Park et al., 2013). Further work to test colonic permeability in this model could be done using Ussing chambers. However, the observed differences in mast cell populations between the tissues will likely produce results that could be considered artefacts. Effectively, tissue preparation for mounting in Ussing chambers will degranulate mast cells, increasing gut permeability (Moriez, Leveque et al., 2007). Therefore, *ex vivo* permeability procedures are likely to be sub-optimal for our model.

## 3.4 Role of glycation in efficacy of low-FODMAP diet

To measure the production of reactive dicarbonyls that lead to AGE production, we set out to measure AGE content in intestinal tissues. It turned out that the antibodies against AGE also have high cross-reactivity with glycosylated proteins, such as those found in mucus (see Figure 24). Apart from complicating immunofluorescent analysis, this makes it impossible to use commercial "anti-methylglyoxal" ELISA kits to analyse intestinal contents. These kits use anti-AGE antibodies and a standard curve based on BSA-AGE, and because we have shown that FODMAP treatments influence the production of mucus, observed differences between groups will not necessarily be reflective of different AGE contents.

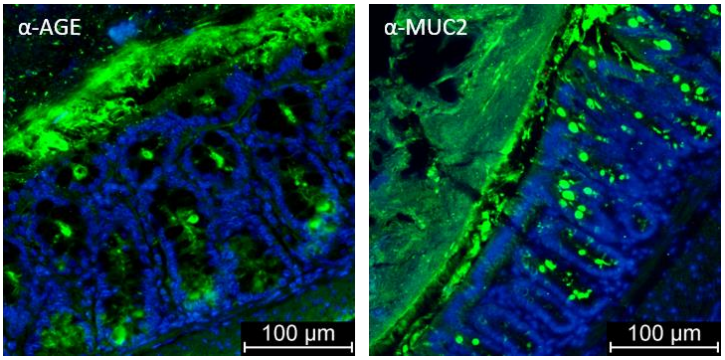


*Figure 24 - anti-AGE staining (left), anti-MUC2 staining (right). Both strongly stain mucus.*

In addition, we are not sure which other antigens might be recognized by these antibodies. It makes sense that no antibodies against methylglyoxal itself exist, because it is a small non-antigenic ketone. Because of these complications, we went one step further and quantified epithelial RAGE expression, which is upregulated by activation with AGEs (Bierhaus and Nawroth, 2009), as presented in section 1.4.3. Even though this is quite an indirect way of inferring AGE production, it is of considerable interest because it is the main mediator of its effects, and it helps to explain the observed increase in mast cells and mucus production.

An increase in mastocytes in adipose tissue is seen in a disease heavily linked to AGE production, type-2 diabetes (Divoux, Moutel *et al.*, 2012). RAGE activation does indeed lead to mastocyte activation (Yang, Yan *et al.*, 2007), and preventing mastocyte activation pharmacologically by disodium cromoglycate (DSCG) or by using a mast cell deficient knockout model $Kit^{W\text{-}sh/W\text{-}sh}$ attenuates diabetes, improving glucose tolerance (Liu, Divoux *et al.*, 2009). This might indicate that disorders involving generation of AGEs can be mediated by mast cell activation, caused by increased RAGE activation and expression. In our model, methylglyoxal is not increasingly produced systemically, but locally during fermentation in the gut, and its likely for this reason that obvious effects were limited to physiological effects in the intestinal tract.

It would be interesting to use a RAGE antagonist to directly see specific involvement of RAGE activation in the cascade, and to verify that the increase in mastocytes is mediated by RAGE activation in our model, like it is in the case of allergic airway sensitization (Ullah, Loh et al., 2014). Another way to test our proposed model of the mechanistic cascade would be to repeat the FODMAP challenges in a mast cell deficient mouse model, these animals should not show an increase in visceral sensitivity, although the complete lack of mast cells likely has an effect on other components of health, or basal sensitivity, as it is known that mast cells are involved in regulation of visceral sensitivity (van Diest, Stanisor *et al.*, 2012; Wouters, Balemans *et al.*, 2016; Wouters, Vicario *et al.*, 2016), although in rats, mast cell deficiency apparently does not influence the response to anorectal distension (Ohashi, Sato *et al.*, 2008).

Additionally, it would be of great interest to measure the RAGE expression in intestinal biopsies from healthy controls, and IBS patients on normal and low-FODMAP diets, to see whether similar mechanisms are at play in humans as we have observed in our work.

If these mechanisms are shown to play a role in IBS patients, it will be relevant to evaluate the efficacy of agents like aminoguanidine to counteract the effects of luminal production of glycating agents. Additionally, to support the glyoxalase system, a treatment with tRES and HESP such as described in section 1.4.4 would be possibly effective and highly interesting, since this ameliorates the health status of obese and diabetic patients on similar principles.

Explorative results observed in AGE staining of caecal sections show a starkly delineated region of high-intensity signal next to the tissue (see Figure 25) in control conditions. This delineated line is disturbed in lactose-treated (5mg/day) animals. Besides, in an ABHE staining performed on the same region no discernible difference can be observed. Moreover, a FISH staining does not show differences in bacterial population density, and neither does a muc2 staining indicate a high mucin concentration in this region (data not shown). Even though the staining for AGEs is not specific, this

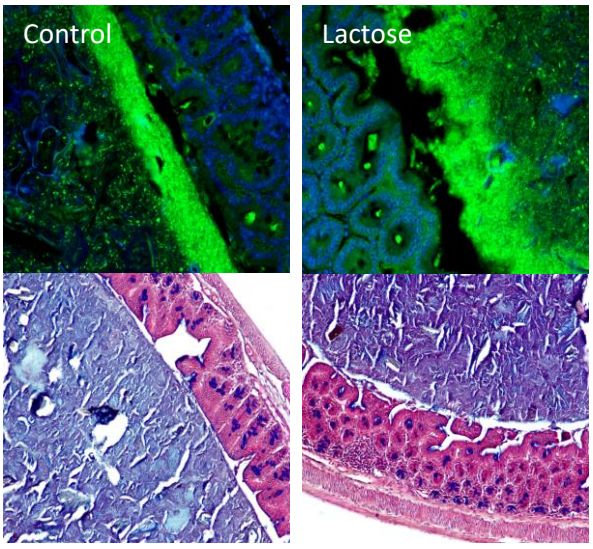



*Figure 25 - anti-AGE staining in cecum, in control (left), or lactose-treated (right) animals. ABHE staining (below) non-conclusive, but AGE staining shows clear line of highly positive material.*

interesting region highly suggests a bacterial organisation, resembling a biofilm.

Currently, we propose 3 hypotheses, based on these results;

1) Effectively, the AGE staining could have stained a glycosylated extracellular protein belonging to the biofilm matrix.

2) Otherwise, maybe the host tissue produces chemicals that support AGE formation, such as ROS.

3) It is also possible that (partial) bacterial breakdown of mucus makes the muc2 staining lose specificity, while the less specific AGE staining still stains these glycosylated protein(s) (fragments).

It would be immensely interesting to find/develop a way to determine whether this is a biofilm or not, to get more insights in host-microbe interactions in the caecum. Additionally, this might help to understand the implications of its possible disruption in response to FODMAP treatments.

## 3.5 Mucus barrier

Concerning the colonic mucus barrier, we have shown that the double layer itself is not a continuous barrier covering the entire colonic epithelium (see chapter 2.1). First of all, at least in rodents, which are a widely used model for studies on the intestinal mucus barrier (Johansson, Phillipson *et al.*, 2008; Johansson, Gustafsson *et al.*, 2010; Johansson, Larsson *et al.*, 2011; Petersson, Schreiber *et al.*, 2011; Ermund, Schutte *et al.*, 2013; Holmen Larsson, Thomsson *et al.*, 2013; Rodriguez-Pineiro, Bergstrom *et al.*, 2013; Birchenough, Nyström *et al.*, 2016; Schneider, Pelaseyed *et al.*, 2018), it is highly variable between the different compartments of the colon, with extensive contact between the microbiota and the epithelium in the proximal colon, and an effective separation starting from the particular moment a pellet with a higher consistency is formed. Secondly, the mucus barrier is dependent on the presence of a faecal pellet, and in the absence of contents, no double mucus layer is observed. We can conclude that this organisation depends on both space (location), and time (periodic presence of contents), and should not be considered stable and uniform.

These findings have several interesting and possibly important implications. For example, we should realize that the microbiota of contents in the distal colon is mostly transient and isolated from the host by a covering of sterile mucus that is constantly being deposited on the outside of the faecal pellet until defecation, at least in case the stools are solid.

This can change the comprehension of how bacteria approach the epithelium, instead of penetrating the 'inner' mucus layer covering the mucosae, they would have to escape the faecal pellet through the faecal mucus layer (see Figure 26).

Our analyses of FODMAP impact on mucus barrier function are based on this novel model of colonic mucus organisation, differentiating the situation between empty and faeces-containing distal colon, which is variable. This means that colon epithelium, and the state of its mucus barrier, is constantly alternating between situations where a barrier is separating host and microbes, and a situation where both barrier and microbiota are apparently mostly absent.

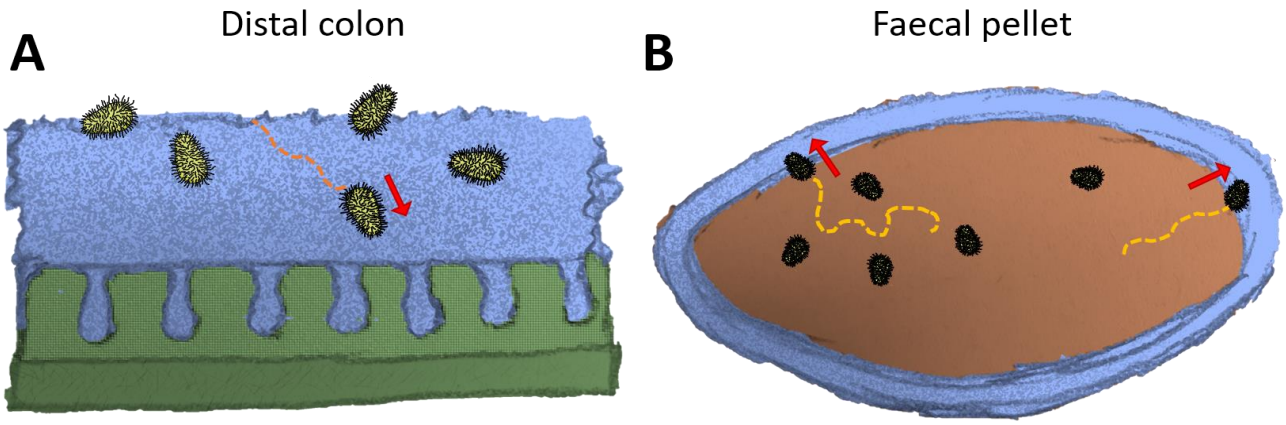



*Figure 26 - Two models of microbiota penetrating the mucus barrier.*
*A: Classical model of bacteria approaching the epithelium through a mucus barrier.*
*B: Bacteria escaping the faeces through the faecal mucus barrier to gain access to the epithelium.*

We have found that our FODMAP treatments induce a dysregulation in this balance, causing excessive production of mucus in empty colon, probably due to epithelial irritation as described before (Ten Bruggencate, Bovee-Oudenhoven *et al.*, 2006), which then causes an increased variability in the faecal mucus barrier, deposited during the passage of faecal pellets. This could be interpreted as resembling a system functioning (too) close to capacity. Our approach to include the individual CoV calculated from a large dataset of measurements of the mucus layer has allowed us to characterize a new factor that can play a role in mucus barrier function characterisations. An animal that has a higher CoV but a similar average mucus layer thickness, logically has more fragile regions in this barrier, which means it is less well protected. This factor (CoV) also seems more sensitive in situations where effects are mild or subtle, as our FODMAP treatment has clear effects on the CoV of our treated groups, normalized with pyridoxamine co-treatment, while average mucus layer thickness was not strongly modified.

It is unlikely that these effects on the intestinal mucus barrier have serious consequences in otherwise healthy systems, but it could imply an increased vulnerability to challenges.

For the proximal colon, where the chyme does not form high-consistency pellets, future research to investigate the barrier function and immune tolerance evident from the lack of inflammation despite extensive contact between bacteria and host in this compartment of the colon will be of great interest.

In light of a possible increased vulnerability, it would be interesting to challenge FODMAP-treated mice with pathogens, such as *Citrobacter rodentium*, a model that shares pathogenic mechanisms with putative human pathogens such as *E. coli* (Collins, Keeney *et al.*, 2014).

Considering that in the absence of a faecal pellet, the microbiota is strongly diminished, the periodic presence of contents and microbial signalling could cause the activity of immune and goblet cells, and possibly tissue regeneration, to display waveform functions instead of a continuous or stochastic expression, which in itself is an interesting topic for future research.

We have shown that the mucus layer classically measured in histology is attached to the faecal pellets, which means that it should be feasible to measure the mucus layer on Carnoy-fixated paraffin-embedded droppings, instead of intestinal sections. The faecal mucus layer on droppings is well-conserved and has the same general characteristics as when measured in colonic sections (see figure 6 of section 2.1, page 104). This has several important benefits; it is non-invasive, non-terminal, and technically easier to perform. Additionally, the same individual's droppings can be analysed throughout an experiment, collected at different timepoints, allowing for characterisation of the progressive effects of treatment. In the context of the 3 Rs (Replacement, Reduction, Refinement) of animal research, this approach would allow us to both Reduce and Refine mucus barrier measurements, obtaining more information from each individual, while causing them less harm.

In patients too, it represents an important improvement, because it can be performed on stools, and does not necessitate invasive procedures. It should be verified however that the mucus barrier in humans is organised similarly to that of rodents. In any case, a mucus layer covers human stools, and it can be analysed, as shown in literature (Shimotoyodome, Meguro *et al.*, 2005; Swidsinski, Loening-Baucke *et al.*, 2008; Swidsinski, Loening-Baucke *et al.*, 2008). As it becomes more widely accepted that the distal colon mucus layer observed in histology is identical to the faecal mucus layer, it will become more attractive to use this approach.

## 3.6 Concluding remarks

Finally, we come back to our hypotheses:

**1. FODMAP ingestion can induce gastrointestinal symptoms of IBS by itself, in an otherwise healthy system**

**2. This effect relies on the production of harmful bacterial fermentation metabolites, among which methylglyoxal**

**3. Methylglyoxal and other strong glycation agents lead to an increase in AGEs and a pro-inflammatory state through activation of RAGE**

Our current understanding of the FODMAP-induced effects is summarised in Figure 27.

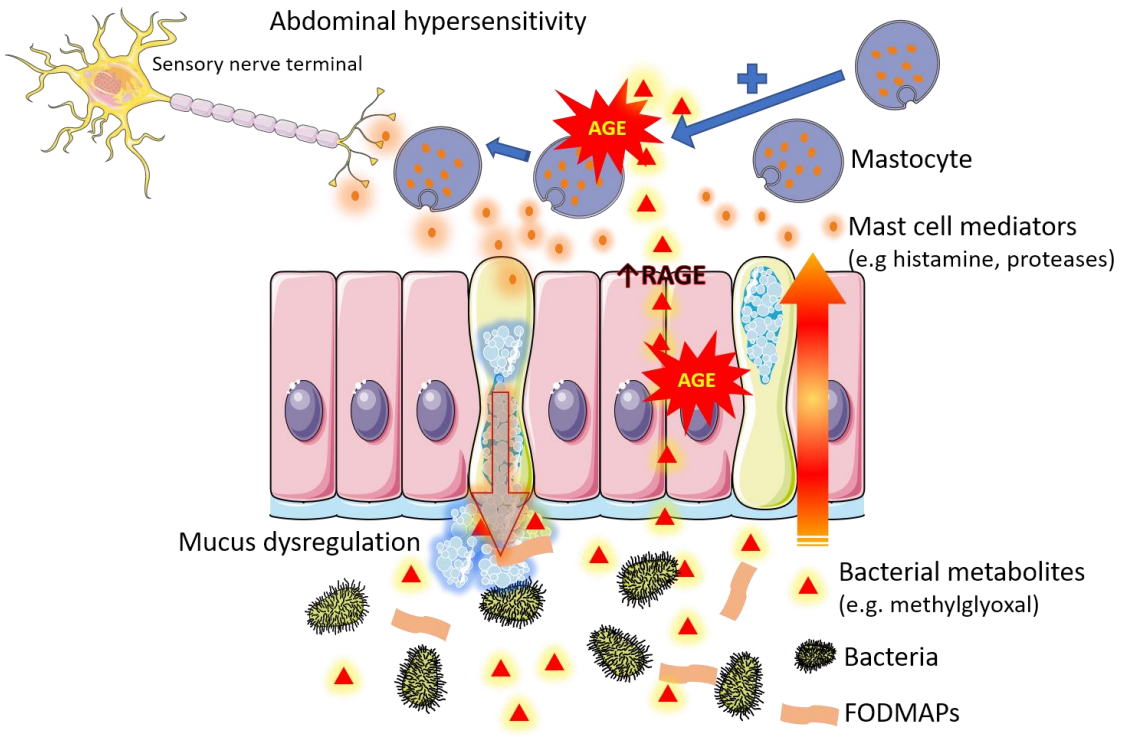



*Figure 27 - Graphical abstract of hypothetical mechanisms responsible for FODMAP-induced symptom generation presented in this thesis.*

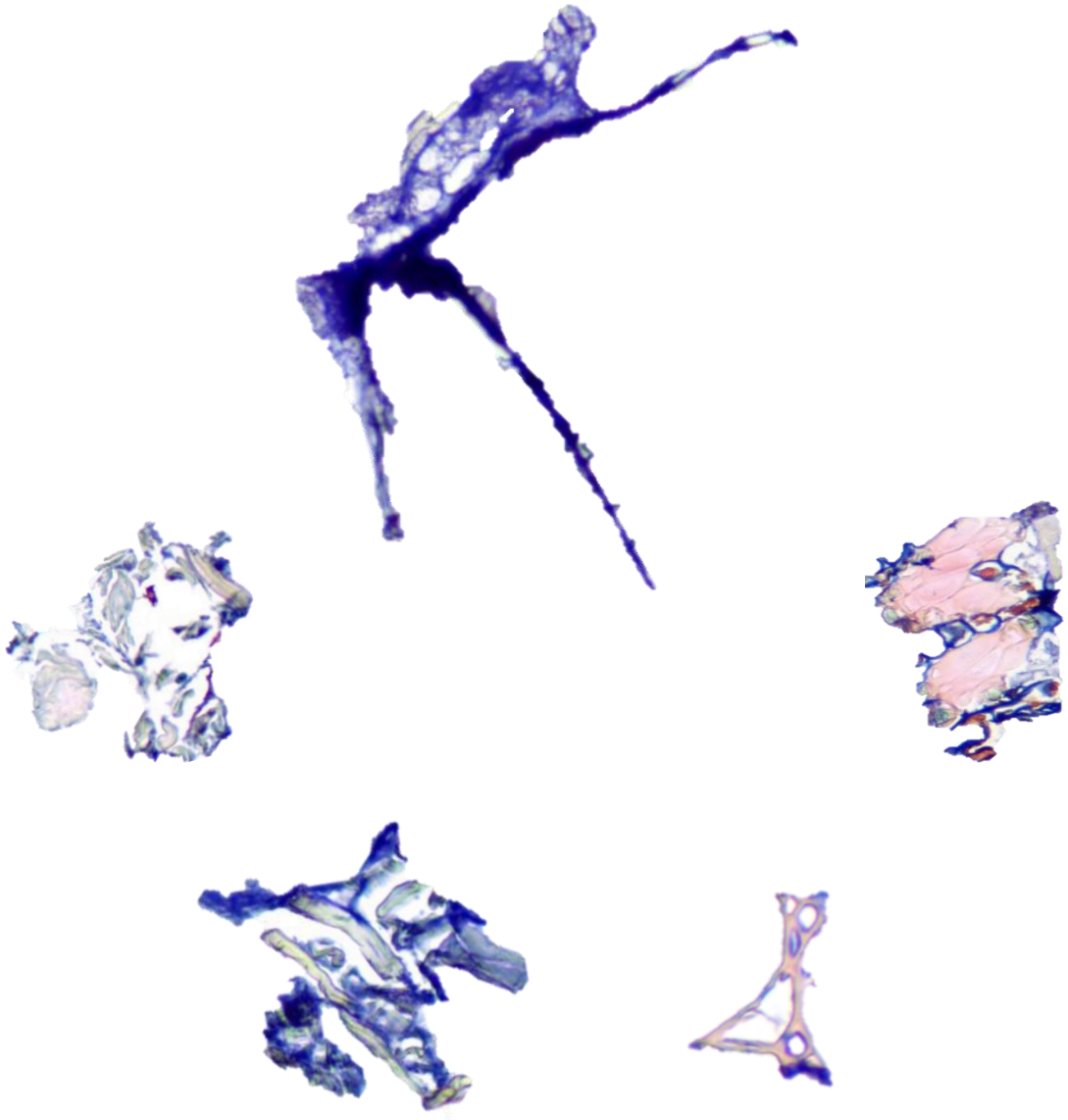
*FODMAP fermentation in the intestinal lumen leads to the production of harmful bacterial metabolites (i.e., reactive carbonyls such as methylglyoxal). These metabolites cause carbonyl stress and the formation of AGEs in the tissue, which activates and upregulates RAGE. RAGE activity induces NF-κβ pathways and increases mast cell activity and population size, which leads to increased excitability of nerves, and an increased discharge of mucus by goblet cells, which dysregulates the intestinal mucus barrier.*

We have shown that FODMAPs can indeed induce GI symptoms of IBS by itself, in a healthy mouse model. Visceral and central sensitivity were increased, and the colonic mucus barrier disrupted. That this effect relies on the generation of reactive dicarbonyls was shown indirectly by a preventive effect obtained by co-treatment with pyridoxamine. Due to technical constraints, we have not shown directly that AGEs were increased, but the expression of their receptor (RAGE) increased in response to treatment and normalized with pyridoxamine co-treatment. A plausible explanation for both the increased sensitivity and mucus barrier dysregulation is the observed increase in mastocyte counts, probably mediated by RAGE, interactions that have been reported in literature for other situations.

Knowing that FODMAPs can influence these factors and a better understanding of the mechanisms responsible for the efficacity of the low-FODMAP diet will hopefully lead to development of novel approaches to benefit FGID patients, for example by improving dicarbonyl stress status in the intestine using pharmacologicals, or by using probiotic bacteria that have an increased capacity to detoxify methylglyoxal *in situ*.

The work presented in this thesis describes a novel supplementary mechanism by which FODMAPs are involved in GI-symptom generation and opens up new approaches to research and possibly treat IBS and other FGIDs.

# 4 Acknowledgments



# 4 Acknowledgments

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*SSS; snakeskin stress

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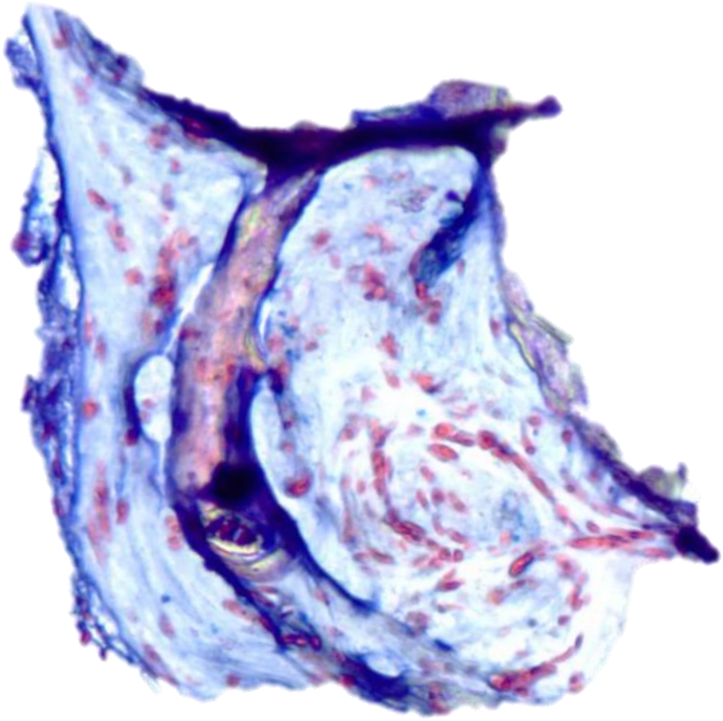
**Everyone**, named and unnamed, thanks for the great experience and all the memories gained during my years at Toxalim, merci beaucoup!

# 5 Publications, dissemination, and training



# 5 Publications, Dissemination and Training Activities

## 5.1 Publications part of this thesis

- Kamphuis, J.B.J., M. Mercier-Bonin, H. Eutamene and V. Theodorou (2017). **"Mucus organisation is shaped by colonic content; a new view."** Scientific Reports **7** (1): 8527.

- Albusoda, A., N. Barki, T. Herregods, J.B.J. Kamphuis, T. B. Karunaratne, M. Lazarou, I. Lee, N. Mazurak, E. Perna, A. Polster, T. Pribic, F. Uhlig, H. Wang and P. Enck (2017). **"A fresh look at IBS-opportunities for systems medicine approaches."** Neurogastroenterology and Motility **29** (3).

- Kamphuis, J.B.J., B. Guiard, M. Leveque, M. Olier, I. Jouanin, S. Yvon, V. Tondereau, P. Rivière, F. Guéraud, S. Chevolleau, M.-H. Noguer-Meireles, J.-F. Martin, L. Debrauwer, H. Eutamène, V. Theodorou (2019). **"FODMAPs increase visceral sensitivity in mice through glycation processes, increasing mast cell counts in colonic mucosae."** *Under review in Gastroenterology*

- Kamphuis, J.B.J., H. Eutamène, V. Theodorou (2019) **"Increased FODMAP intake alters colonic mucus barrier function through glycation processes and increased mastocyte counts"**
  *Manuscript under preparation*

## 5.2 Other Publications

- Radziwill-Bienkowska, J.M., P. Talbot, J.B.J. Kamphuis, V. Robert, C. Cartier, I. Fourquaux, E. Lentzen, J. N. Audinot, F. Jamme, M. Refregiers, J. K. Bardowski, P. Langella, M. Kowalczyk, E. Houdeau, M. Thomas and M. Mercier-Bonin (2018). **"Toxicity of Food-Grade TiO2 to Commensal Intestinal and Transient Food-Borne Bacteria: New Insights Using Nano-SIMS and Synchrotron UV Fluorescence Imaging."**
  Frontiers in Microbiology **9**: 794.

- Talbot, P., J.M. Radziwill-Bienkowska, J.B.J. Kamphuis, K. Steenkeste, S. Bettini, V. Robert, M. L. Noordine, C. Mayeur, E. Gaultier, P. Langella, C. Robbe-Masselot, E. Houdeau, M. Thomas and M. Mercier-Bonin (2018). **"Food-grade TiO2 is trapped by intestinal mucus in vitro but does not impair mucin O-glycosylation and short-chain fatty acid synthesis in vivo: implications for gut barrier protection."**
  Journal of Nanobiotechnology **16**(1): 53.

## 5.3 Oral presentations

- ***JFHOD 2018, Paris, France***

  *22 March 2018*

  "L'administration de FODMAPS de type Fructo-oligosaccharides (FOS) augmente la sensibilité abdominale et le nombre de mastocytes muqueux via la production d'agents de glycation par le microbiote intestinal."

- ***Mucins in Health and Disease (14$^{th}$ International Workshop), Cambridge, UK,***

  *24-28 July 2017*

  "Mucus organisation is shaped by colonic content; a new view"

- ***CECED, JFHOD Joint Conferences 2017, Paris, France***

  *22-26 March 2017*

  "Colonic load differentiates organization of mucus in colon of mouse and rat; a remodelling"

- ***PA3S conference, Toulouse, France***

  *20 January 2017*

  "Un régime enrichi en FODMAPs induit la production de métabolites toxiques par le microbiote intestinal responsables d'une hypersensibilité viscérale."

- ***GFNG 2016 conference, Rouen, France***

  *23 June 2016*

  "Effects of a high FODMAPs diet on visceral sensitivity: involvement of advanced glycation end products and colonic mast cells"

## 5.4 Poster presentations

- ***UEGW 2018, Vienna, Austria,***

  *20-24 October 2018*

  1) "Dietary FODMAPs such as fructo-oligosaccharides and lactose can increase abdominal sensitivity and the number of mucosal mastocytes in mice; a study on underlying mechanisms." *J.B.J. Kamphuis, B. Guiard, P. Rivière, S. Yvon, V. Tondereau, H. Eutamène, V. Theodorou*

  2) *"*Dietary FODMAPs can lead to microbial production of glycation agents, increasing mucosal mastocytes and impacting colonic mucus barrier function."
  *J.B.J. Kamphuis, S. Yvon, V. Tondereau, H. Eutamène, V. Theodorou*
  *Abstract was awarded €750 Travel Grant*

- ***FNM 2018, Amsterdam, the Netherlands,***

  *29 August – 1 September 2018*
  "The effects of a diet rich in fructo-oligosaccharides on abdominal sensitivity; a study on underlying mechanisms of action." *J.B.J. Kamphuis, B. Guiard, P. Rivière, S. Yvon, V. Tondereau, H. Eutamène, V. Theodorou*

- ***DDW 2018, Washington, USA,***

  *2-5 June 2018*
  *in absentia*
  "Dietary FODMAPs such as fructo-oligosaccharides can increase abdominal sensitivity and the number of mucosal mastocytes in mice through production of glycating agents by the intestinal microbiota." *J.B.J. Kamphuis, B. Guiard, P. Rivière, S. Yvon, V. Tondereau, H. Eutamène, V. Theodorou*
  *Abstract selected for Oral ePoster presentation*

- ***DDW 2017, Chicago, USA***

  *6-9 May*
  *in absentia*
  "Colonic load differentiates organization of mucus in colon of mouse and rat; a remodelling"
  *J.B.J. Kamphuis, H. Eutamène, V. Theodorou*

- ***FNM 2016, San Francisco, USA***

  *24-28 August 2016*

  "Effects of a high FODMAPs diet on visceral sensitivity: involvement of advanced glycation end products and colonic mast cells"

  *J.B.J. Kamphuis, P. Rivière, S. Yvon, V. Tondereau, H. Eutamène, V. Theodorou*

- ***JFHOD 2016, Paris, France,***

  *17-20 March 2016*

  *in absentia*

  "Effets et mécanismes des régimes contenant des 'oligosaccharides, disaccharides, monosaccharides fermentescibles et polyols' (FODMAPs) sur la sensibilité viscérale"

  *J.B.J. Kamphuis, P. Rivière, S. Yvon, V. Tondereau, H. Eutamène, V. Theodorou*

## 5.5 Other training and dissemination activities

**2014**

***Utrecht, the Netherlands,***

***8-19 December***

*Cursus Proefdierkunde, Universiteit Utrecht*

*(Animal Experimentation Course)*

**2015**

***London, UK,***

***4-6 March***

*First Annual NeuroGut Meeting and*

*Progress Report*

*Project update presentation and discussion with network partners*

***Tübingen, Germany,***

***13-17 April***

*Entrepreneurship Academy NeuroGut*

*Module 1*

***Istanbul, Turkey,***

***4-6 June***

*NeuroGASTRO 2015*

*Summer School NeuroGut*

***Toulouse, France,***

***17 September***

*Journée des Doctorants INP- EI-Purpan*

*Oral + poster presentation*

***Tübingen, Germany,***

***21-25 September***

*1st NeuroGut Complementary Training Course, University of Tübingen*

*Intellectual Property, Scientific Writing Skills, Scientific Presentation Skill*

***Tübingen, Germany,***

***22-26 November***

*Entrepreneurship Academy NeuroGut*

*Module 2*

**2016**

***Brussels, Belgium,***

***27-28 February***

*Mid-term Review Meeting*

*Progress Report with Officer of European Commission*

*Project update oral + poster presentation; Discussion with network partners and European Commission Officer*

***Heidelberg, Germany,***

***11-13 March***

*COST Genieur Training School,*

*+ NeuroGut Spring School*

"Molecular Methods in IBS Research: genetics, epigenetics and microbiota research"

***Tübingen, Germany,***

***17-21 April***

*Entrepreneurship Academy NeuroGut Module 3*

***Toulouse, France,***

***2 May***

*CODIR EI-Purpan*

*Project update, oral presentation and discussion*

***Bologna, Italy,***

***8-10 June***

*Bologna IBS Days*

*NeuroGut Summer School*

***Toulouse, France,***

***30 September***

*La Nuit Européenne des Chercheurs*

Prepared microscopy and educational material & assisted at the Toxalim educational stand

**2017**

***Toulouse, France,***

***5 January***

*Demi-journée de la Recherche EI-Purpan*

*Oral presentation*

"PhD student experience, my thesis "

***Lille, France,***

***12-18 February***

*Lab Exchange CR1 INSERM – Lille*

Study to clarify mucus barrier function using a $Muc2^{-/-}$ mouse model.

***Toulouse, France,***

***20-24 February***

*Chirurgie Générale de l'Animal de Laboratoire, École Nationale Vétérinaire Toulouse*

***Gothenburg, Sweden,***

***8-11 March***

*NeuroGut Young Researcher Camp*

'Functional Gastrointestinal Disorders and Networking Opportunities'

***Toulouse, France,***

***27 March***

*Journée des doctorants SEVAB*

*Oral presentation*

"Colonic content shapes mucus organization; a dynamic view"

***Toulouse, France,***

***17 November***

*Invited to discuss recent publication at seminar of Institut de Recherche en Santé Digestive (IRSD) – INSERM UMR 1220, INRA UMR 1416, ENVT, UPS*

*Oral presentation*

"Mucus organisation is shaped by colonic content; a new view."

**2018**

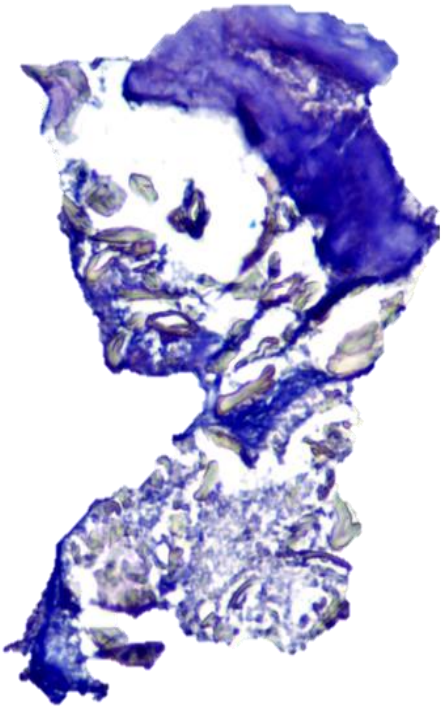
***Toulouse, France,***

***28 September***

*La Nuit Européenne des Chercheurs*

Assisted the Toxalim stand "Le fabuleux voyage dans l'intestin"

# 6 References



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