CORE GUT MICROBIOTA IN RABBIT: OPPORTUNITIES TO STRENGTHEN THE INTESTINAL BARRIER

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ABSTRACT

The symbiotic relationship between the intestinal microbiota and its host is crucial to the development and functioning of both partners. The microbiota plays a key role in the development and physiology of its host (nutrition, growth, health, and cognition). In turn, the host shapes the microbiota, according to factors that are intrinsic or dependent on its environment. However, the definition of an optimal microbiota that maximises ecosystem services (host benefits) has yet not be established. The symbiotic relationship between the microbiota and its host is based on a complex molecular dialogue at the level of the intestinal epithelium and the underlying mucosal immune system. These interactions condition the establishment of an intestinal barrier, limiting colonisation by microbial pathogens and thereby guaranteeing health. In this review, we propose a 'core' rabbit microbiota definition through a re-analysis of available open-source data. Based on the association between the abundance of bacterial taxa and host traits, we attempt to identify microbiota key species that would likely be involved in growth performance and health. Then, we describe the components of the intestinal barrier and the host-microbiota interaction mechanisms. Finally, we propose early in life nutritional levers to strengthen this intestinal barrier and thereby enhance the health of young rabbits before weaning.

Key words: intestinal epithelium, immune system, feed transition, meta-analysis.

INTRODUCTION

The microbiota is composed of commensal and symbiotic microorganisms that occupy all the different accessible niches in the host's body (Kundu et al., 2017). In rabbits, as in other mammals, next-generation sequencing has facilitated our knowledge of the microbiota taxonomic composition and diversity. According to the results obtained with the latest technologies, the rabbit gastrointestinal microbiota represents the richest and most diverse microbial community inhabiting the rabbit's body (Kundu et al., 2017; Hu et al., 2021). The microbial community varies widely from the stomach to the colon (Cotozzolo et al., 2020; Hu et al., 2021), with the cecum and large intestine showing the highest richness and diversity in bacterial species, while the highest interindividual variability is found in the upper digestive tract. High similarity in alpha and beta diversity is observed between the bacterial communities inhabiting adult rabbit cecum, colon, appendix and hard feces. These results suggest that the lower digestive tract microbiota structures are close (Velasco-Galilea et al., 2018; Hu et al., 2021; Curone et al., 2022). With regard to the taxonomic composition, the most notable differences between feces and cecal content are observed at the genus level, suggesting that hard feces may serve as a relevant proxy for studying the bacterial community composition of the main fermenter, the cecum (Velasco-Galilea et al., 2018).

At the interface between feed and the intestinal epithelium, this microbial community plays a role in the development and health of its host. The intestinal microbiota is involved in the metabolization of dietary nutrients that escape host digestion and substrates derived from host secretions. The gut microbiota produces essential compounds for rabbit nutrition, such

as vitamins (Carabaño et al., 2010), volatile fatty acids (García et al., 2002) and secondary bile acids (Kasbo et al., 2002). In addition, the microbiota plays a role in direct and host-mediated defence against pathogens (Sonnenburg & Bäckhed, 2016). The action of intestinal bacteria on the maturation of the mucosal and systemic immune system has been extensively documented (Pott & Hornef, 2012). All of the microbiota ecosystem services related to host development and health are based on the establishment of a specific host-microbiota dialogue and allows the intestinal barrier functions to be maintained.

By providing favorable life conditions and food, the host in turn shapes its gut microbiota. Ontogenic factors (Beaumont et al., 2022) and the physiological state (Abecia et al., 2007; Savietto et al., 2020) drive the community establishment and its further evolution during adulthood. In addition, host-specific genetic characteristics filter out microbial metacommunities that are able to live in the hindgut (Velasco-Galilea et al., 2022). With regards to extrinsic factors, diet is one of the major levers in the structuring of the gut microbiota (Beaumont et al., 2022). In rabbits, numerous studies have shown that fibre content (Zhu et al., 2015; Li et al., 2023b), and the nature of the fibre (Gómez-Conde et al., 2009; Yang et al., 2020; Liu et al., 2022; Ma et al., 2022; Paës et al., 2022) have an impact on the composition and function of the gut microbiota. Finally, host environment (Velasco-Galilea et al., 2020; Ye et al., 2023) and the presence of littermates (Abecia et al., 2007; Fang et al., 2019) and cage comates (Velasco-Galilea et al., 2022) contribute to further shaping the gut microbiota.

Altogether, the extrinsic and intrinsic capabilities of the host to shape its own intestinal microbiota suggest a high degree of hosting plasticity. This plasticity has thus been identified by research groups as an opportunity to drive the establishment of a particular microbiota that maximizes the ecosystem services rendered to its host mainly in terms of digestive efficiency and health, that in turn impact on growth performances. For instance, Fang et al. (2019) demonstrated that 16% of the variation in weaning weight was attributable to the gut microbiota. Furthermore, moderate correlations have been observed between microbiota composition and growth traits (Velasco-Galilea et al., 2022). The optimal microbial community would be capable of optimising these ecosystem services. However, the specific characteristics of an optimal gut microbiota (diversity, core taxonomic composition and/or function) for an individual or group of individuals remain to be defined.

This review firstly aims to provide insight into the definition of the core cecal microbiota diversity and composition in post weaning rabbits and to identify key taxa that may be associated with improved rabbit health or growth. Given that the symbiotic relationship between the microbiota and its host is based on a fine molecular dialogue at the level of the intestinal epithelium and the underlying mucosal immune system, we secondly review the current understanding of host-microbiota cross-talk in order to promote health in rabbits, with a focus on the intestinal barrier function. Finally, we assess the nutritional strategies aimed at enhancing the host intestinal barrier-microbiota interactions, thereby promoting health.

DEFINITION OF THE CORE RABBIT GUT MICROBIOTA

The first studies investigating the composition and function of the rabbit gut microbiota were based on culture techniques, which were therefore limited to describing the cultivable fraction of the microbiota. This was constrained by the culture medium and conditions available at the time (for a review, see Combes et al., 2013). The use of molecular microbiology techniques has allowed considerable progress in our knowledge of the species inhabiting the rabbit digestive tract, in particular by highlighting taxonomic specificities not observed in other species (undescribed species, dominance of the Firmicutes phylum, Combes et al., 2013). Results presented in the present review focus on the numerous results obtained in the last decade, using sequencing technologies widespread among research groups interested in rabbits. The prevalent techniques combine amplification of a portion of the hypervariable

region of the 16S small subunit ribosomal RNA gene with short-read sequencing. These approaches have the advantage of being relatively simple, cheap and fast. They are based on a number of databases that attempt to be as rich and diverse as possible, and bioinformatics and statistical analysis pipelines are well established. However, these technologies are subject to biases and limitations. These biases include extraction, PCR amplification, sequencing error, bioinformatics analysis, and database for taxonomic affiliation biases. Moreover, 16S RNA gene amplicon short-read sequencing technologies produce compositional data that do not take into account the variable number of copies in the bacterial genome (from 1 to 21 copies, https://rrndb.umms.med.umich.edu/) and exhibit low taxonomic resolution (genus level), which is limited by the short-read lengths (Poretsky et al., 2014). Finally, the last limitation relates to their inability to distinguish between the active and the dead or dormant microbial fractions. Recently, shotgun metagenomics technics (i.e. whole metagenome sequencing) were used in two studies to explore rabbit microbiota (Casto-Rebollo et al., 2023; Zhao et al., 2024). Compared to 16S sequencing, these techniques provide access to bacterial genes, enabling inference of the functional characteristics of the microbiota. However, they only overcome the biases associated with PCR amplification and 16S copy number.

To date, the production of 16S sequence data, combined with its release as part of open science, allows data from different research teams to be re-analysed together to provide a comprehensive description of the rabbit gut microbiota. A literature search over the last 10 years identified 93 studies dealing with the variations in composition of the rabbit gut microbiota in relation to changes in rearing conditions (housing and diet), genetics, growth performance or health status. Of these studies, 70 used 16S RNA gene amplicon sequencing technologies and two used a shotgun metagenomic technology to characterize the rabbit gut microbiota. From these articles, we collected publicly available raw sequencing data (either from NCBI and CNBG repositories or from supplementary data in papers). Overall, concatenation with the available metadata enabled us to collect data from 15 studies, 11 of which included cecal microbiota analyses (Table 1, see GitHub link for detailed procedure https://lcauquil.pages.mia.inra.fr/review_wrc2024/).

To define a core microbiota based on data from the literature, we limited our analysis to the 11 studies where cecal microbiota data were available (Table 1). To avoid the variability associated with the establishment of the microbiota and onset of solid feed intake, we only included rabbits older than 49 days (Combes et al., 2011). According to our pipeline using FROGS tools (Escudié et al., 2018) and the Silva 138 taxonomic database (Quast et al., 2013), the average value of the cecal microbiota Shannon index of a grown rabbit (> 48 days of age) was of 5 (Figure 1). Shannon index median values ranged from 4.2 to 5.5. A change in such an index indicates the establishment of a dominance or an increase/decrease of the number of species. These changes may be related to microbiota establishment in the young or unbalanced microbiota in post-weaning rabbit. Indeed, using the same analysis pipeline, the Shannon values were around 3 in 18-day old pups (Paës et al., 2020b, 2022; Beaumont et al., 2022). With the exception of outlier samples in some studies, the Shannon index showed a good overlap between studies suggesting that the diversity was evaluated similarly between studies despite differences in experimental conditions or sample process preparation.

As previously widely described in the literature, the most abundant phyla in the rabbit cecal microbiota are Firmicutes (76.4%), Bacteroidota (16.5%), Verrucomicrobiota (3%), Proteobacteria (1%) and Actinobacteriota (0.9%) (Figure 2).The overwhelming dominance of the Firmicutes phylum is a notable feature of rabbit gut microbiota compared to other farm mammals (cattle & sheep: Szeligowska et al., 2021; pigs: Mach et al., 2015 ; horses: Grimm et al., 2019) or human gut microbiota (Arumugam et al., 2011), but is also strikingly observed in chickens (Allaoua et al., 2022). The mechanisms behind the preferential selection of microbes from the Firmicutes phylum in the rabbit cecum remain to be elucidated, although it

is likely to be related to the specificity of its digestive strategy (hindgut fermentor, herbivorous and caecotrophic species) (Ley et al., 2008). In rabbits, the relative abundance of Firmicutes has been shown to increase with age at the expense of Bacteroidota. Read et al. (2019) have suggested that the ratio of Firmicutes to Bacteroidota would be a suitable index of microbiota maturity, while Chen et al. (2019) evidenced that this ratio steadily decreased in severe intestinal disorder compared to a healthy rabbit. In the re-analysis of data from healthy rabbits, this ratio shows a high variability between studies (from 1.7 to 17.9), according to the metadata. Although there is a confounding effect between age and study, Firmicutes to Bacteroidota ratio may reflect animal age, but this high variability between studies may indicate a low robustness of the absolute value (Figure 3).

Table 1: Studies included in our re-analysis for cecal microbiota composition evaluation

Study	Data accession	16S RNA gene hypervariable regions	Age (days)	Experimental conditions	Number of samples
Read et al. 2019	PRJNA315608	V3-V4	49	Preweaning diet modulation	30
Wang et al. 2019	PRJNA512067	V3-V4	58, 70, 82	Water drinking temperature	82
Paës et al. 2020	PRJNA589727	V3-V4	57	Early access to diet in the nest	40
Cotozolo et al. 2021	PRJNA1069001	V3-V4	110	Digestive segment	14
Dabou et al. 2021	PRJNA645756	V3-V4	77	Insect fats dietary supplementation	24
Feng et al. 2022	PEERJ_13068	V3-V4	76	Environmental enrichment	12
Liu et al. 2022	PRJNA781070	V3-V4	87	Dietary fibre modulation	12
Mora et al. 2022	PRJNA524130	V4-V5	66	Microbiota and growth causal relationship modelling	407
Paës et al. 2022	PRJNA615661	V3-V4	58	Early access to diet in the nest	29
Li et al. 2023	CPN0003860	V3-V4	73	Dietary non-fibrous carbohydrate to neutral detergent fibre ratio	30

Lachnospiraceae (19.6%), Oscillospiraceae (17.9%), Ruminococcaceae (13.3%), Muribaculaceae (7.2%), and Christensenellaceae (6.7 %) were the 5 dominant families found in almost all the studies (Figure 2). Interestingly, among the 288 genera observed, only 36 genera were found in all 11 studies. Oscillospiraceae NK4A214 (13.8%), Ruminococcus (10.4%), Christensenellaceae R-7 (9.2%), Lachnospiraceae NK4A136 (7.6%), and Ruminococcaceae V9D2013 (5%) groups were the 5 most abundant genera (Figure 4). Using whole metagenomic sequencing based techniques, microbiota exploration at the species level has recently been reported (Casto-Rebollo et al., 2023; Zhao et al., 2024). Several species have been reported as the characteristic species of 60 and 90 days old rabbit (Zhao et al., 2024) or associated with selection for resilience (Casto-Rebollo et al., 2023).

In terms of beta diversity evaluated at the genus level on the 771 samples across the 11 studies, cecal community structure seems to cluster according to study and even to research group, but interestingly without strong opposition or outlier samples (Figure 5). Most of the variability of the bacterial community at the genus level could be attributed to the relative

abundance of *Akkermensia*, Christensenellaceae R-7, and Ruminococcaceae NK4A214 groups. With the exception of the study conducted by Mora et al. (2022), all studies employed the 16SrRNA hypervariable V3V4 region for sequencing (Table 1). The observed variability, including the absence of the *Akkermensia* genus in our own studies (Read et al., 2019; Paës et al., 2020b, 2022), can be explained by the differences in the primer V3-V4 sequence with different degenerated nucleotides at different positions (see GitHub link for primer list https://lcauquil.pages.mia.inra.fr/review_wrc2024/).

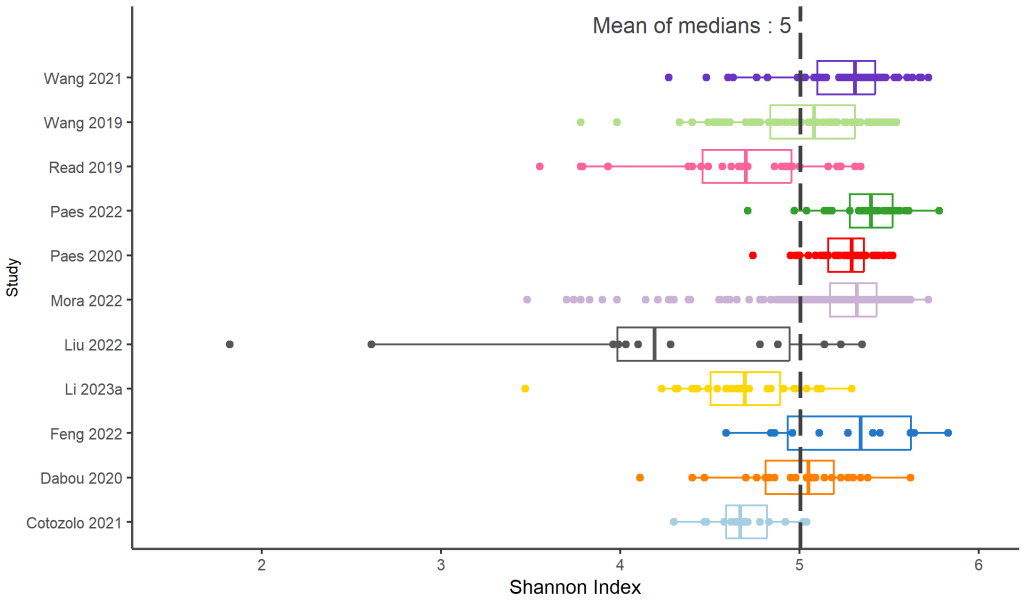

Figure 1: Shannon index calculated in rabbit cecal microbiota from 11 publicly available studies providing 16S RNA gene amplicon data (see github link for detailed procedure https://lcauquil.pages.mia.inra.fr/review_wrc2024/)

A. B.

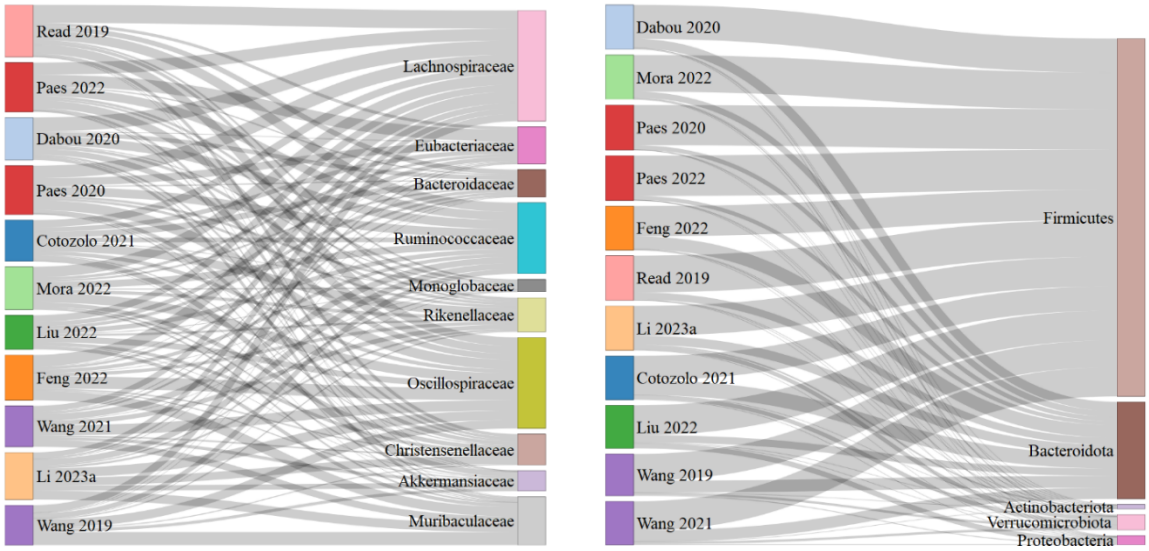

Figure 2: Relative abundances of (A) phyla and (B) top 10 families in rabbit cecal content using 11 freely available studies providing 16S RNA gene amplicon data (see github link for detailed calculation procedure https://lcauquil.pages.mia.inra.fr/review_wrc2024/).

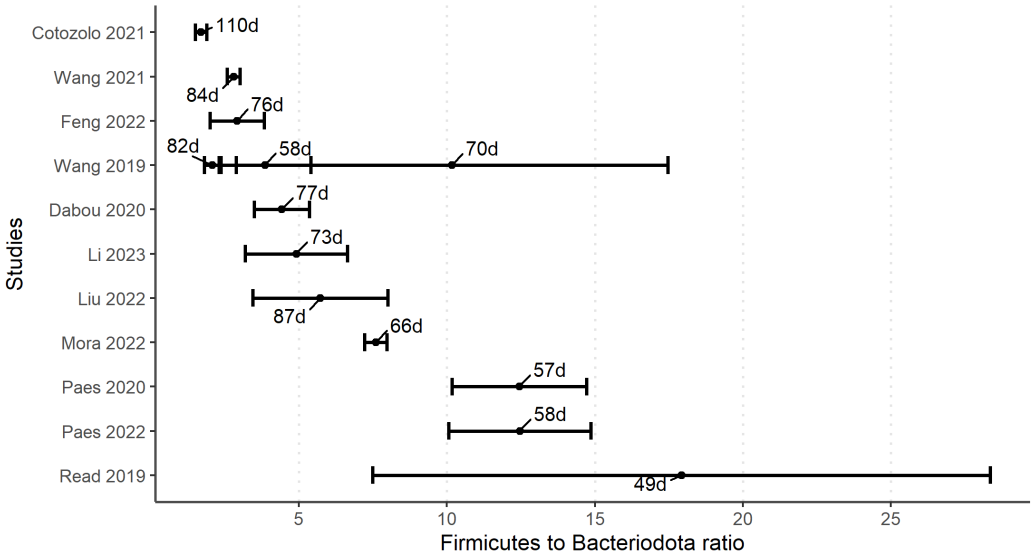

Figure 3: Firmicutes to Bacteroidota ratio calculated in rabbit cecal microbiota from 11 publicly available studies providing 16S RNA gene amplicon data (see GitHub link for detailed calculation procedure https://lcauquil.pages.mia.inra.fr/review_wrc2024/)

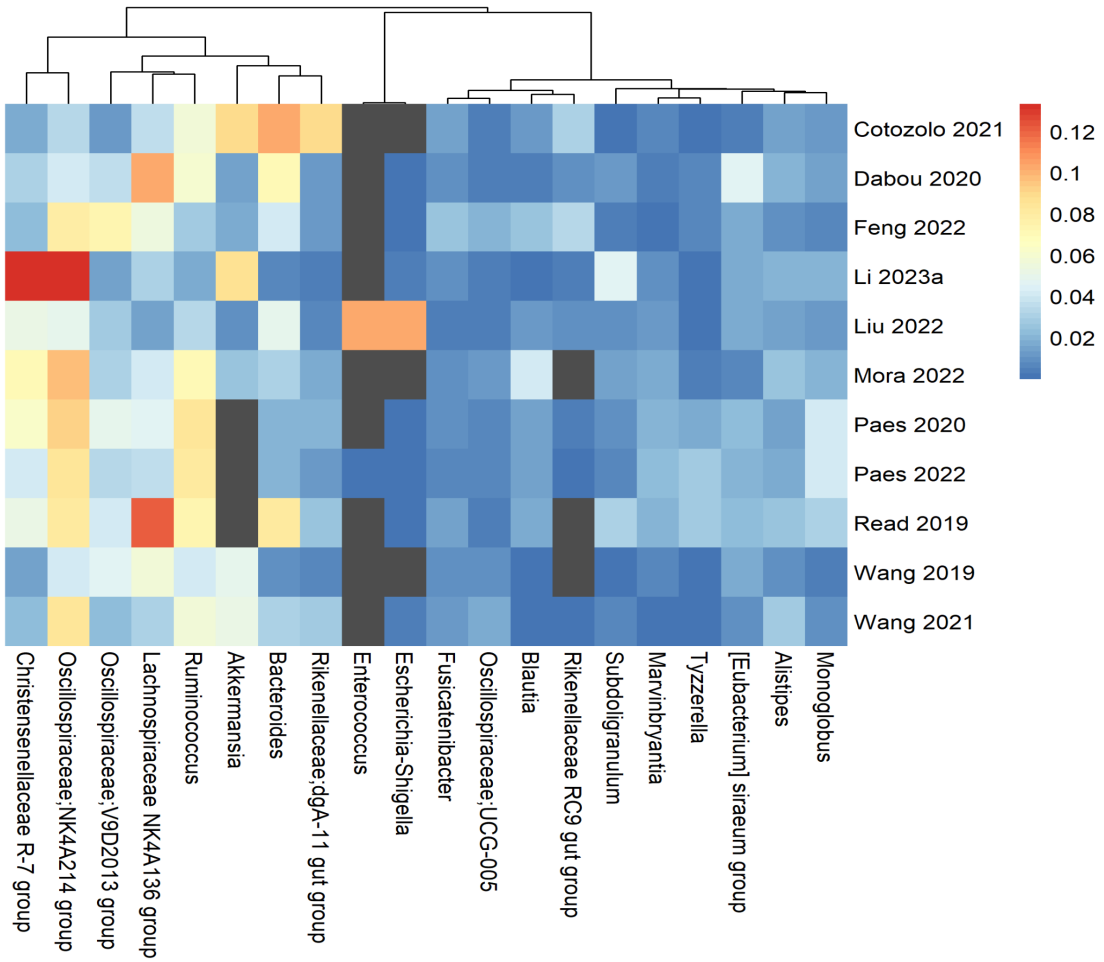

Figure 4: Relative abundances of top twenty genera in rabbit cecal content using 16S RNA gene amplicon data from 11 publicly available studies (see GitHub link for detailed calculation procedure https://lcauquil.pages.mia.inra.fr/review_wrc2024/). The colors represent the relative abundance from low (blue) to high values (red). Grey color indicates the absence of the genus.

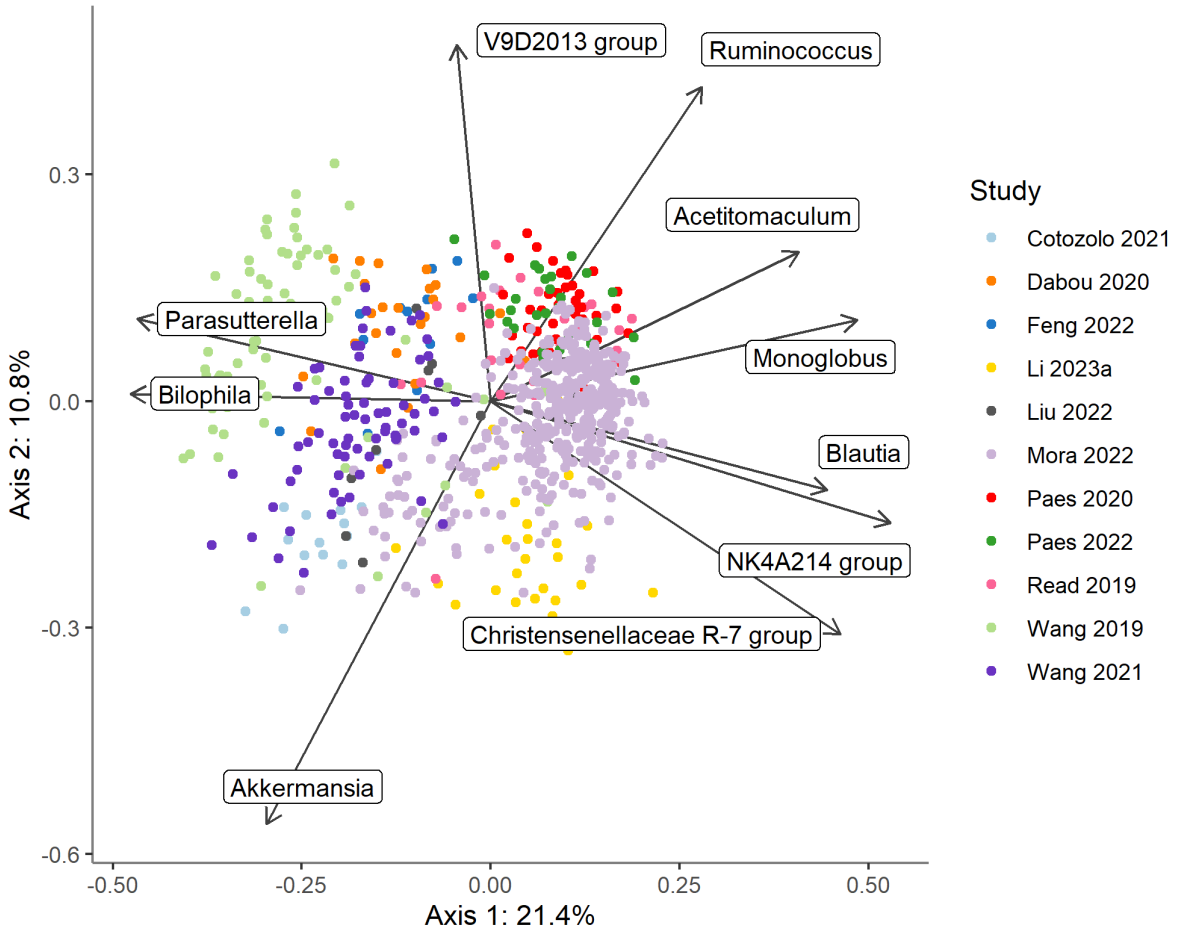

Figure 5: PCoA analysis of cecal bacterial communities at the genus taxonomic level. Dots are colored according to the the study from which 16S RNA gene amplicon data were extracted. (see GitHub link for detailed procedure, https://lcauquil.pages.mia.inra.fr/review_wrc2024/)

Given the average composition of the cecal microbiota, we wanted to identify the taxa that were associated with improved health or growth in rabbits. Out of the 93 recorded studies, our analysis identifies 29 articles, describing positive or negative associations with microbiota composition. These 29 studies represent a wide range of experimental conditions (age, dietary challenge, drinking water study, use of prebiotics or antibiotics, modification of environmental conditions, healthy *vs.* epizootic rabbit enteropathy (ERE) rabbits). Interestingly, some taxa have a more consistent link to a negative or positive effect in relation to the phenotype measured (Table 2). For example, increased relative abundance of *Bacteroides* in rabbits appears to be detrimental to growth and health (Baüerl et al., 2014; Chen et al., 2019; Velasco-Galilea et al., 2021; Liu et al., 2022; Puón-Peláez et al., 2022). *Bacteroides* is a dominant genus in suckling rabbits, which abundance sharply decreases with the introduction of solid feed and may therefore not be adapted to plant carbohydrate substrates. Health and growth performance are also likely to be positively associated with *Ruminococcus* relative abundance (Baüerl et al., 2014; Ye et al., 2022; Li et al., 2023a; Wu et al., 2023). Conversely, for some taxa the phenotype association is not consistent across studies (Table 3). For instance, *Rikenella* relative abundance is associated with positive effects in three studies (Luo et al., 2020; Wang et al., 2021; Du et al., 2023), but is also associated with ERE compared to healthy rabbits (Baüerl et al., 2014). Regarding cecal

microbiota taxonomic composition, it seems difficult to draw a conclusion on possible key taxa improving health or growth in rabbits. Several explanations can be found: **(i)** First, rabbit bacterial strains inhabiting the cecum are far from being well defined. Indeed, re-analysis of our 11 studies revealed that, at the genus level, 20 to 49 % of sequences were affiliated to "unknown genus". Furthermore, some members (OTU, operational taxonomic unit; or ASV, amplicon sequence variant) of a same genus could show either positive or negative associations with a given phenotype. For example, Velasco-Galilea et al., (2021) reported that *Ruminococcus*, *Butyricicoccus*, and *Bacteroides* genera displayed this inconsistency, suggesting functional and/or physiological taxonomic heterogeneity. Altogether, improving our knowledge of the microbiota at the strain level is essential to discover key gut bacterial species involved in rabbit health and/or growth. **(ii)** Secondly, in many studies the sample size may be too small to detect a significant difference between treatments regarding the size effect and taxa abundance variability (Table 2). Furthermore, the sensitivity of the sequencing technique must also be considered. Paës et al. (2020) demonstrated that the relative quantification of OTUs with low abundance (under 0.5 %) is poorly reproducible. **(iii)** Finally, the beneficial or detrimental role of bacteria may depend on life experience of host and environmental factors. Symbiosis between the microbiota and its host encompasses different forms of relationships (Belkaid & Harrison, 2017). Indeed, the same microorganism can evolve as a mutualist, commensal or parasite, depending on the genetic background, nutritional status or co-infection of its host.

While it appears difficult to identify key species or microbial communities that are beneficial to health or growth phenotype, perturbations in microbiota composition or diversity are often described in association with disease. These changes are commonly referred to as dysbiosis (Sekirov & Finlay, 2009). In rabbits, several studies have shown an association between digestive diseases of unknown aetiology and microbiota dysbiosis (Baüerl et al., 2014; Jin et al., 2018; Chen et al., 2019). In the case of ERE, the microbial diversity is significantly reduced, resulting in a decrease in the relative abundance of *Alistipes* and *Ruminococcus* genera, while that of *Bacteroides*, *Akkermansia*, *Rikenella* and *Clostridium* increased (Baüerl et al., 2014; Jin et al., 2018), as well as that of *Escherichia* and *Lysinibacillus* (Jin et al., 2018). Overall, disruption of gut microbiota leads to the proliferation of opportunistic or pathogenic bacteria occupying commensal bacterial niches with access to previously unavailable nutrients and creating an environment that maximizes their pathogenicity. How specific microbes or microbial communities help to prevent the onset of dysbiosis is far from being fully understood. However, it is clear that the microbiota plays a key role in gut barrier function through two main mechanisms: limiting colonization by opportunistic bacteria and strengthening the epithelial barrier and the gut immune system. The following section ("Components of the gut barrier") defines the gut barrier and then describes what is known about the three components of rabbit gut barrier: microbiota role, intestinal epithelium and mucosal immunity. Then, we review the current understanding of host-microbiota cross-talk in order to promote gut barrier function and thus digestive health in rabbits (chapter "Microbial influence on host gut barrier").

Table 2: Genus level taxa of the gut microbiota associated with growth or health in rabbits.

Taxa[1]	**Associated effects (+: positive in red; - negative in blue), Author, Aim of the study; (segment if not cecum) (number of rabbits involved)**
Akkermansia	(+) Huang 2021, Clostridium butyricum supplementation & health (n=9) (-) Li et al 2023 Ammonia exposure (n=6) (-) Li et al 2023, NFC/NDF growth & health (n=5) (-) Puón-Peláez et al 2022, ERE vs healthy rabbit (n = 12) (-) Chen et al 2019, Deficient fiber diet & health (n = 43) (-) Baüerl et al 2014, ERE (n=30)
Alistipes	(+) Chen et al 2021, Bacitracin supplementation & health (n=8) (-) Li et al 2023, Ammonia exposure (n=6) (-) Wang et al 2021, feeding rhythm & health (n=72); (-) Drouilhet et al 2016, Genetic selection & growth (n=18); (-) Baüerl et al 2014, ERE (n=30)
Bacteroides	(-) Liu 2022, Dietary Fiber & growth (n=6); (-) Velasco-Galilea et al 2021, growth and feeding regime (n=201) (-) Puón-Peláez et al 2022, ERE vs healthy rabbit (n = 12); (-) Chen et al 2019, deficient fiber diet & health (n = 43) (-) Baüerl et al 2014, ERE (n=30)
Blautia	(+) Li et al 2023, Temperature and humidity index & growth (n=6) (-) Fang et al 2019, Weaning weight (feces) (n= 135)
Butyricicoccus	(-) Velasco-Galilea et al 2021, Growth and feeding regime (n=201) (-) Fang et al 2019, Weaning weight (feces) (n= 135)
Christensenellaceae_R-7	(-) Li et al 2023, Ammonia exposure (n=6) (-) Fang et al 2020, ADG (feces), (n=180)
Clostridium	(+) North et al 2019, Quercetin supplementation and sex & growth (n = 12) (-) Puón-Peláez et al 2022, ERE vs healthy rabbit (n = 12)
Clostridium XlVb	(-) Baüerl et al 2014, ERE (n=30) (-) Chen et al 2019, deficient fiber diet & health (n = 43)
Desulfovibrio	(-) Mora et al 2022, Growth in restricted or ad libitum feeding (n=206); (-) Wang et al 2021, feeding rhythm & health (n=72)
Dorea	(+) Ma et al 2022 Oat β-glucan & growth (n=6); (+) Chen et al 2021, Bacitracin supplementation & health (n=8)
Erysipelatoclostridium	(+) Feng et al 2022, Environmental enrichment & behaviour (n=6) (-) Puón-Peláez et al 2022 ERE vs healthy rabbit (n = 12)
Escherichia-Shigella	(+) Ma et al 2022, Oat β-glucan & growth (n=6);

Taxa[1]	**Associated effects (+: positive in red; - negative in blue), Author, Aim of the study; (segment if not cecum) (number of rabbits involved)**
	(+) Ye et al 2022 Clostridium butyricum supplementation & growth (n=6)
Lachnospiraceae_NK4A136	(+) Du et al 2023 Copper supplementation & health (n=4) (+) Li et al 2023 Ammonia exposure (n=6); (+) Li et al 2023 NFC/NDF growth Health (n=5) (-) Li et al 2023 Temperature and humidity index & growth (n=6)
Papillibacter	(+) Chen et al 2022, Chlorogenic acid & growth & health (n= 4) (+) Yasob et al 2021, Heat stress and Moringa oleifera leaf supplementation & health (n=21)
Rikenella	(+) Du et al 2023, Copper supplementation & health (n=4) (+) Wang et al 2021, Feeding rhythm & health (n=72) (+) Luo et al 2021, Bacillus subtilis HH2 supplementation induced colitis model & health (colon) (n=16) (-) Baüerl et al 2014 ERE (n=30)
Ruminococcaceae_NK4A214	(+) Li et al 2023, NFC/NDF growth Health (n=5); (+) Liu et al 2022, Dietary Fiber & growth (n=6) (-) Ye et al 2022, Clostridium butyricum supplementation & growth, (n=6)
Ruminococcaceae_UCG-005	(+) Chen et al 2022, Chlorogenic acid & growth & health (n= 4); (+) Ye et al 2022, Clostridium butyricum supplementation & growth (n=6);
Ruminococcaceae_UCG-013	(+) Li et al 2023, NFC/NDF growth Health (n=5); (+) Kong et al 2022, Enzymolytic soybean meal inclusion & health (n=4); (-) Ye et al 2022, Clostridium butyricum supplementation & growth (n=6)
Ruminococcaceae_V9D2013	(+) Liu et al 2022, Dietary Fiber & growth (n=6) (+) Wang et al 2021, feeding rhythm & health (n=72)
Ruminococcus	(+) Wu et al 2023, Carvacrol supplementation & health (n=4); (+) Li et al 2023 Ammonia exposure (n=6) (+) Ye et al 2022, Clostridium butyricum supplementatin & growth (n=6) (+) Baüerl et al 2014, ERE (n=30)
Subdoligranulum	(+) Li et al 2023 Ammonia exposure (n=6) (+) Li et al 2023 NFC/NDF growth Health (n=5)
Synergistes	(-) Wang et al 2021, feeding rhythm & health (n=72) (-) Puón-Peláez et al 2022 ERE vs healthy rabbit (n = 12)

[1]Only genus level taxa observed to be differentially abundant according to treatment in at least two studies are listed.

COMPONENTS OF THE GUT BARRIER

According to Viggiano et al (2015), the gut barrier is a functional unit, organized as a multi-layer system, made up of two main components: a physical barrier that prevents bacterial translocation, and a functional immune barrier that is able to discriminate between pathogens and commensal microorganisms. From the outer layer to the inner layer, the intestinal barrier is composed of the gut microbiota, the mucus layer, the epithelial cells and the innate and adaptive immune cells forming the gut-associated lymphoid tissue.

Gut microbiota barrier

The commensal gut microbiota acts as a barrier to colonization by pathogenic and opportunistic microbes through several mechanisms (McKenney & Kendall, 2016). **(i)** As a result of the host selection pressure, commensal microbes adapted to the intestinal environment conditions, are ecological gatekeepers in healthy guts. Indeed, commensal microbes compete with pathogens for the nutrients and adhesion sites. For instance, members of the Muribaculaceae, Lachnospiraceae, Rikenellaceae, and Bacteroidaceae families, dominant in the rabbits gut (Figure 2), are able to reduce *Clostridioides difficile* colonization in mice based on competition for mucosal sugars (Pereira et al., 2020). **(ii)** A second mechanism relies on the antimicrobial properties of metabolites produced by commensal bacteria, such as short-chain fatty acids (SCFA) or secondary bile acids, which contribute to colonization resistance. For instance, in rabbits, SCFA limit the effects of *Shigella* infection by reducing its abundance (Rabbani et al., 1999). **(iii)** Commensal bacteria also produce bacteriocins, which are peptides that inhibit the growth or kill other bacteria. In rabbits, fecal *Enterococcus* isolates have been shown to produce enterocins (entP, entB and entA), which inhibit the growth of Gram-positive strains (Simonová & Lauková, 2007; Lengliz et al., 2021). **(iv)** Cell-to-cell signalling, such as quorum sensing systems, can have a profound impact on the microbial community structure. It has been shown that molecules produced by the gut microbiota both stabilize the microbiota and suppress bacterial virulence (McKenney & Kendall, 2016). In rabbits, the impact of quorum sensing systems has received little attention, despite the fact that their gut microbiota produce metabolites such as indole, which play a key role in this chemical communication between bacteria (Kim & Park, 2015). However, there is some evidence to suggest that quorum sensing molecules are involved in bacterial pathogenesis in rabbits (*Clostridium perfringens* type C strain (Vidal et al., 2012); rabbit enteropathogenic *Escherichia coli* (Zhu et al., 2007)).

Gut epithelial barrier

The intestinal epithelium is the first layer of cells exposed to the gut microbiota and its products (Figure 6). Indeed, this monolayer of cells is located at the surface of the mucosa and forms a physical and immunological barrier between the intestinal lumen and the organism (Peterson et Artis, 2014). In rabbits, stem cells located at the bottom of crypts renew epithelial cells within a few days (Grant et Specian, 2001), which is a critical mechanism for maintaining the integrity of the epithelial barrier that is exposed to harmful microbial products. The formation of tight junctions at the apical side of epithelial cells is another key mechanism preventing the entry of microbial compounds into the organism (Figure 6). During their migration to the top of the crypt, epithelial precursors differentiate into the absorptive or the secretory lineage.

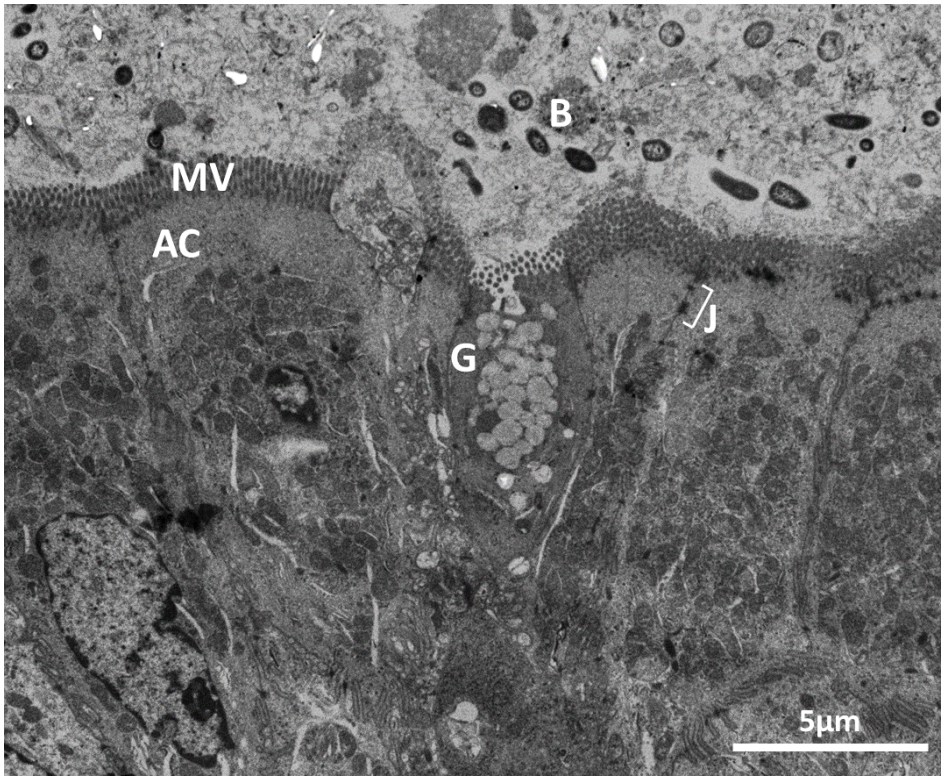

Figure 6: Transmission electron microscopy observation of the rabbit cecal epithelium. AC: absorptive epithelial cell, B: bacteria in the luminal content, G: goblet cell filled with mucin granules, J: cell junctions, MV: microvilli.

The majority of epithelial cells belong to the absorptive lineage and are characterized by the presence of densely packed microvilli decorated with transmembrane mucins (e.g. MUC1, MUC13) that form the glycocalyx, which is both a protective layer for the organism and an attachment site for bacteria (Figure 6) (Pelaseyed & Hansson, 2020). A rare subset of absorptive cells called microfold (M) cells is also present in the rabbit intestinal epithelium associated with lymphoid follicles, as described below (Lelouard et al., 2001). Our recent single-cell RNA-sequencing analysis of the rabbit cecal epithelium also revealed the presence of a rare subset of mature absorptive cells characterized by the expression of the ion channel bestrophin 4 (BEST4) (Malonga et al., 2024). The putative functions of BEST4+ cells (e.g. sensing and responding to changes in luminal pH, hydration of mucus and secretion of antimicrobial peptides) suggest their implication in the cross-talk with the microbiota but this hypothesis remains to be experimentally validated. The most abundant secretory cells are goblet cells, which secrete mucus and play an important role in immune regulation, conferring them the role of gatekeepers of the microbiota (Figure 6) (Birchenough et al., 2015). Paneth cells are a subset of secretory epithelial cells located at the base of crypts and have been identified in the rabbit small intestine (Satoh et al., 1990; Cui et al., 2023). Paneth cells are specialized for the secretion of antimicrobial peptides (e.g. LYZ, REG3G, DEFB1), but other types of epithelial cells may play this role in the large intestine. Finally, tuft cells are a rare subset of epithelial secretory cells that play an important role in mouse and human intestinal immune response triggered by the microbiota (Silverman et al., 2024), but to our knowledge their presence has not been demonstrated in rabbits.

A reliable model to study the rabbit intestinal epithelium has long been lacking due to the absence of epithelial cell lines in this species. Recently, organoid models have been developed to culture the rabbit intestinal epithelium *in vitro* (Mussard et al., 2020; Kardia et al., 2021). In this culture system, stem cells isolated from the rabbit intestine produce 3D-structures formed by a single layer of polarized epithelial cells composed of the major lineages found *in vivo*, such as absorptive and goblet cells. Moreover, rabbit organoid cells

can be dissociated and seeded on cell culture inserts (2D monolayers) in order to facilitate the access to the apical side, where interactions between the epithelium and the microbiota occur.

Gut immune barrier

Besides the intestinal epithelium, the immune system plays a key defence role in the gut. Indeed, the intestinal mucosa is associated with a dense lymphoid tissue known as the gut-associated lymphoid tissue (GALT). All immune cells are derived from pluripotent cells of the bone marrow, which derive myeloid lineage cells (granulocytes, macrophages, mastocytes and dendritic cells) and lymphoid lineage cells (B and T lymphocytes) that migrate towards primary lymphoid organs to mature before homing to secondary lymphoid organs such as the GALT. In the GALT, the immune cells are gathered in inductive and effector immune sites and act in cooperation to recognize, capture, neutralize and destroy pathogens.

The inductive sites are organized lymphoid tissue, ranging from simple follicles mainly composed mainly of B lymphocytes to specialized aggregates of lymphoid follicles such as Peyer's patches (PP). In rabbits, two to ten PP can be found in the small intestine, increasing in size and number from the duodenum to the ileum (Fortun-Lamothe & Boullier, 2007). PP are covered by the follicle-associated epithelium (FAE), containing M cells. M cells take up antigens from the gut lumen and present them to underlying cells, mainly dendritic cells and macrophages, which are able to recognize pathogen-associated molecular patterns (PAMP) on the surface of pathogens through pattern recognition receptors (PRR). In rabbits, a very large PP called the *sacculus rotundus* is located in the terminal ileum. Another unique feature of the rabbit immune system is the *vermiform appendix*, located at the caudal end of the cecum (Tizard, 2023) (Figure 7). Usually considered as a secondary lymphoid organ in other species, the appendix is a primary lymphoid organ in rabbits. *Sacculus rotundus* and *vermiform appendix* represent more than 50 % of the lymphoid tissue in rabbit gut (Arrazuria et al., 2018). These structures are thought to play a major role in inducing immune response to the gut microbiota in rabbits.

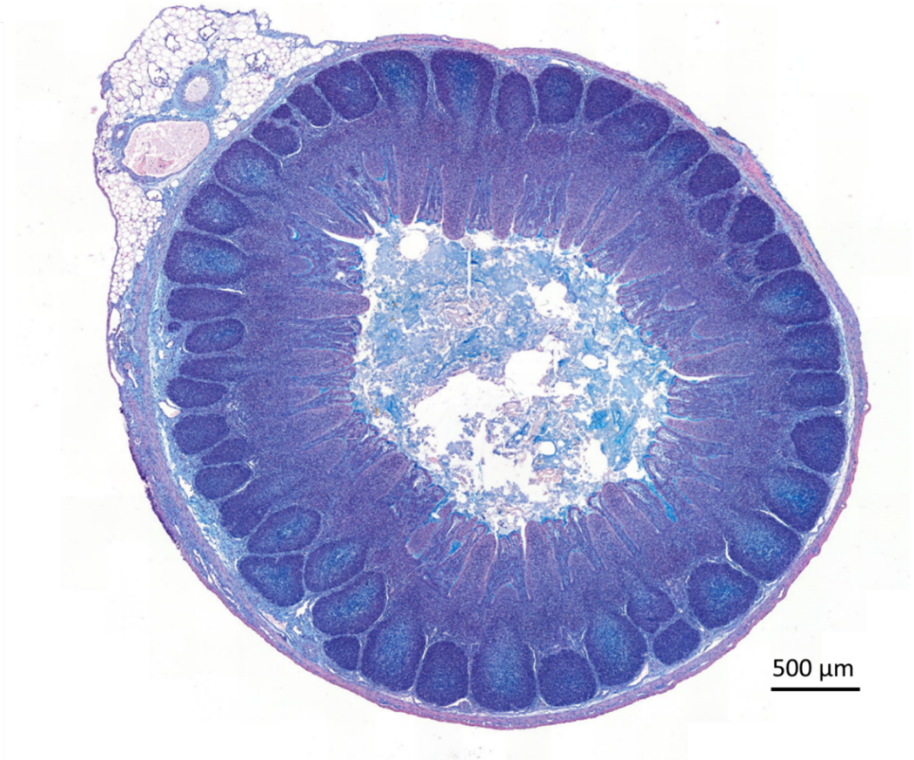

Figure 7: Histological observation of the rabbit vermiform appendix. The tissue section was stained with Alcian blue and periodic acid-Shiff. Large lymphoid follicles are seen around the appendix circumference.

The detection of external aggressions by innate immune cells at inductive sites leads to the phagocytosis of pathogens and the production of mediator molecules, cytokines and chemokines, which relay inflammatory messages and engage immune responses at the effector sites. The effector immune sites are diffusely located in the epithelium (intra-epithelial lymphocytes) and in the underlying *lamina propria* (Wagner et al., 2022). A recent single cell analysis of the rabbit cecum revealed the diversity of cells of haematopoietic origin (CD45+) in the *lamina propria,* including dendritic cells (DC), macrophages, B and T lymphocytes, basophils and natural killer cells (Knudsen et al., 2022). Macrophages and DC are also referred to as antigen presenting cells (APC) because they will present microbial products to B and T lymphocytes (Drouet-Viard et Fortun-Lamothe, 2010). Lymphocytes exert a specialized response targeted towards specific antigens recognized thanks to their cell receptor (B or T cell receptor) acquired during their maturation in primary lymphoid organs. This recognition activates two types of response. The humoral mediated response, characterized by the release of antibodies by B lymphocytes, also known as immunoglobulins (Ig). The predominant immunoglobulin produced in the gut is secretory IgA (70 to 90%), which are transported through epithelial transcytosis to the gut lumen, where they cross-link microorganisms to keep them away from of the epithelium. The cellular mediated response is mediated by CD8+ cytotoxic and CD4+ regulatory T lymphocytes. The coordinated action of these cells is critical for the balance between regulatory and inflammatory responses in order to tolerate commensal microorganisms while defending the host against pathogens.

MICROBIAL INFLUENCE ON HOST GUT BARRIER

The gut microbiota plays a crucial role in regulating the host intestinal barrier mainly through the release of metabolites and the recognition of microbial molecular patterns (Hertli & Zimmermann, 2022).

Microbiota-derived metabolites tune the gut barrier function

The release of metabolites by gut bacteria is a central mechanism mediating the action of the gut microbiota on host cells. These metabolites can be produced from substrates available in the gut lumen (i.e. derived from the diet, from host secretions or from microbial products). In rabbits, as in other species, the main metabolites produced by gut bacteria are the SCFA acetate, butyrate and propionate which are mainly produced by fibre fermentation. These SCFA are generally considered to be beneficial for health as they can i) inhibit the growth of pathogens, ii) serve as energy substrates in host cells, iii) enhance the intestinal barrier function (Ghosh et al., 2021). Other minor SCFA derived from amino acid fermentation can also be detected in the rabbit gut contents (e.g. valerate, isobutyrate, isovalerate and isocaproate) (Li et al., 2022), but their effects on host cells remain poorly characterized. Bacterial deamination of amino acids also releases ammonia, which is present in high concentrations (mM) in the rabbit gut (Beaumont et al., 2022).

A recent untargeted shotgun metagenomic study, predicting the microbial origin of metabolites revealed that the rabbit cecal microbiota is capable of producing a large diversity of metabolites (Casto-Rebollo et al., 2023). Numerous rabbit cecal metabolites were derived from amino acids such as tyrosine (e.g. 3-(4-hydroxyphenyl)lactate), tryptophan (e.g. indole, indole-lactate, indole-propionate), phenylalanine (e.g. phenyl-lactate, 3-hydroxyphenylacetate), lysine (e.g. 5-aminovalerate) and histidine (e.g. imidazole propionate). Among these, indole derivatives are well characterized for their ability to enhance gut barrier function (Ghosh et al., 2021). Rabbit microbiota also produce metabolites derived from plant components (e.g. equol) or from benzoate metabolism (e.g. 3-phenylpropionate, 3-(4-hydroxyphenyl)propionate, 3-(3-hydroxyphenyl)propionate). These compounds are considered to have anti-inflammatory effects and protect against oxidative stress (Ghosh et al., 2021). The rabbit gut microbiota is also capable of metabolizing host-secreted bile acids into secondary bile acids (e.g. glycodeoxycholate, chenodeoxycholate,

deoxycholate, ursodeoxycholate) (Kasbo et al., 2002; Casto-Rebollo et al., 2023; Zhao et al., 2024). Secondary bile acids have antimicrobial properties and regulate the barrier function (Ghosh et al., 2021). In suckling rabbits, the gut microbiota are also known to produce metabolites derived from choline (e.g. trimethylamine) (Beaumont et al., 2020, 2022), but the consequences for the gut barrier function are not well defined. Other metabolic intermediates of microbial fermentations can be detected in rabbits cecal contents such as ethanol, succinate and formate (Beaumont et al., 2020, 2022). It is important to note that most of these metabolites found in the rabbit gut lack absolute quantification, which would be required to test their effects on rabbit intestinal cells at physiological concentrations.

The effects of microbiota-derived metabolites on the gut barrier have mostly been characterized in human, rodent and porcine cells, while only a few studies have evaluated their effects in rabbit intestinal cells. The global effect of metabolites produced by the gut microbiota before or after the introduction of solid food was evaluated by treating rabbit cecal organoids with sterile supernatants of cecal content from 18- or 25-day old rabbits (Beaumont et al., 2020). The results showed that compounds present in the rabbit cecum after the onset of solid feeding were able to modulate the expression of components of epithelial defence (antimicrobial peptides, toll-like-receptors), which reflected the maturation of the gut barrier observed *in vivo* at the transition from suckling to weaning. Another study showed that serotonin, which can be produced by both the host and the microbiota, increased the expression of the tight junction protein claudin-1 (CLDN1) in rabbit intestinal epithelial cells cultured in vitro (Wang et al., 2021). Despite these findings, additional studies are required to further investigate the effects of microbiota-derived metabolites on rabbit intestinal cells.

Microbiota-derived metabolites also act on intestinal immune cells, either directly or through epithelial-mediated signalling. Although data are lacking in the rabbit species, these aspects have been characterized in humans, rodents and, to a lesser extent, in pigs. SCFA have been thoroughly investigated and butyrate has been shown to be the most potent SCFA to regulate intestinal immune responses. Butyrate enhances the tolerogenic response of macrophages and dendritic cells. For instance, in macrophages, butyrate induces antimicrobial peptide production and reduces the inflammatory response. By stimulating DCs, butyrate subsequently induces the differentiation of naive T cells. Butyrate and propionate also act directly on naive T cells, controlling their differentiation into regulatory T cells (Martin-Gallausiaux et al., 2021, for review). In pigs, *in vitro* studies showed that in peripheral blood mononuclear cells (PBMC) co-cultured with gut epithelial cells and stimulated with lipopolysaccharides (component of Gram-negative bacteria cell wall), NF-κB expression was strongly downregulated by acetate and propionate supplementation, demonstrating a protective effect of these SCFA on acute inflammation (Andrani et al., 2023). Although less well documented, SCFA such as acetate also appear to influence B cell maturation (Martin-Gallausiaux et al., 2021; for review) and support the expansion of innate lymphoid cells (Sepahi et al., 2021). Tryptophan-derived metabolites and secondary bile acids have also been shown to affect DCs, macrophages and regulatory T lymphocytes, in particular by inhibiting NF-κB pathway and downstream inflammatory response (Hosseinkhani et al., 2021). However, the specific effects of microbial metabolites on rabbit immune cells remain to be explored, notably by using primary culture of immune cells isolated from the rabbit gut.

Modulation of the gut barrier function via recognition of microbial molecular patterns and direct cell contact

Besides metabolites, the gut microbiota is able to modulate the host gut barrier function through direct contact, or through the recognition of microbial/ PAMP by PRR, including toll-like receptors (TLR) (in rabbits: Chen et al., 2014; De Vos et al., 2022). The TLRs expressed on the epithelial cell surface (TLR 1, 2, 4, 5 and 6) mainly recognize components of the bacteria cell wall, such as peptidoglycan, lipoprotein, and lipopolysaccharide in the case of

Gram-negative bacteria. TLR recognition of bacterial polysaccharides usually elicits strong antibody responses. However, this bacterial-host recognition in commensal interactions is not well defined. The large structural diversity of bacterial polysaccharides, due to the variety of monosaccharide composition, glycosidic linkages, and conformation of the polymers might likely contribute to induce, suppress or modulate the immune response (Comstock & Kasper, 2006).

In addition to recognising bacterial surface polysaccharides, the protein Amuc_1100 expressed on the outer membrane of *Akkermansia muciniphila* has been shown to signal via TLR2 and reduce colitis (Wang et al., 2020). *Akkermensia* genus has been observed in the rabbit gut in several studies (Figure 4), but its abundance has been alternatively associated with positive or negative effects (Table 2). These discrepancies among studies underlines that the same microbe may act as a mutualist, commensal or pathobiont depending on its environment.

Bacteroides upregulates fucosylation in the host epithelium, thereby modulating the barrier function. *Bacteroides* cleaves host fucose residues, which can then be internalized and catabolized by the bacteria for energy or for incorporation into capsular polysaccharides or bacterial glycoproteins. This host-bacteria interaction probably has significant consequences for the success of *Bacteroides* in the gut (Comstock & Kasper, 2006). In rabbits, the abundance of *Bacteroides* is particularly high in young animals before weaning (Combes et al., 2014; Read et al., 2019; Paës et al., 2020b, 2022). The rabbit would therefore be an interesting model for studying this positive host-microbiota interaction related to the maturation of the gut barrier function.

Segmented filamentous bacteria (SFB), belonging to the Clostridiaceae family, have been shown to attach directly to intestinal epithelial cells in several species, including rabbits (Heczko et al., 2000). SFB harbor a nipple-like appendage that is inserted into the epithelium, particularly in the follicle-associated epithelium of Peyer's patches (Caselli et al., 2010). This attachment does not damage the host cell and plays a crucial role in the postnatal maturation of the mucosal immune system. In rabbits, the presence of SFB has been observed by light and electron microscopy and its presence has been correlated with the absence of rabbit enteropathogenic *Escherichia coli* (EPEC) 0103 ileal colonization (Heczko et al., 2000). Age-related patterns of SFB in rabbits have never been reported. In rodents and humans, colonization occurs at the onset of the weaning process and decreases thereafter (Grant & Specian, 2001). Characterizing the prevalence of SFB in the rabbit gut microbiota could provide insight into their potential role in facilitating the maturation of the gut barrier function and easing the weaning transition.

NUTRITIONAL OPPORTUNITIES TO STRENGTHEN POSITIVE INTESTINAL HOST-MICROBIOTA INTERACTIONS IN EARLY LIFE TO PROMOTE INTESTINAL BARRIER AND HEALTH

Under breeding conditions, rabbits are particularly susceptible to digestive disorders, especially around weaning. "Positive" host-microbiota interactions are essential for the co-maturation of the two symbiotic partners to ensure the development of the barrier function and thus preserve digestive health. Strengthening the gut barrier by stimulating host-microbiota interactions prior to weaning could be an effective lever to maintain digestive health in young rabbits.

Early in life, the microbiota in rabbits, as in other mammals, is characterized by a low microbial diversity at the individual level but greater phylogenetic diversity between individuals (human: Yatsunenko et al., 2012; pig: Mach et al., 2015; calf: Rey et al., 2014; and rabbit: Read et al., 2019). In contrast, after weaning, when solid feed intake is well established, the composition of the microbiota is more homogeneous between individuals,

with less phylogenetic diversity. The postnatal period therefore represents a window of permissiveness for engineering the microbiota architecture, when the richness and diversity of the microbiota are low and the selective pressure exerted by the host on its microbiota is reduced due to an immature mucosal immunity. As it has been widely demonstrated, diet is one of the most effective ways to shape microbiota composition and function (Gómez-Conde et al., 2009; Zhu et al., 2015; Yang et al., 2020; Liu et al., 2022; Ma et al., 2022; Paës et al., 2022; Li et al., 2023b). Several studies have been conducted on innovative preweaning nutritional strategies to modulate microbiota and promote host-microbiota interactions. The following section focuses on results on effects of milk with a focus on milk oligosaccharide components, solid feed intake and coprophagic behavior in the nest, and cessation of milk intake (i.e. weaning), on microbiota and host gut barrier functions.

Milk intake and its prebiotic action through milk oligosaccharides

Milk provides the newborn with essential nutritional components (protein and fat rich milk) and non-nutritional bioactive components. Among the latter, rabbit milk is particularly rich in short-chain fatty acids with antibacterial properties. Several proteins also exert antimicrobial activities such as transferrins, α and β-caseins and immunoglobulins, which are also involved in the passive immunity (Maertens et al., 2006). Recently, other bioactive components have been studied for their immunomodulatory functions and effects on the gut microbiota, namely the milk oligosaccharides (MO). They consist of three to ten monosaccharide units and are composed of five monosaccharides: glucose, galactose, N-acetyl-glucosamine, fucose and sialic acid residues. The combinations of monosaccharides and their linkages result in the formation of linear or branched structures of great diversity. Host enzymes do not have the capacity to digest these molecules, so they turn to gut bacteria, being the first prebiotics available to newborns. *Bacteroides* has been shown to metabolize MO (Marcobal et al., 2011). Bacterial MO consumption results in metabolite production, such as butyrate, an essential energy source for colonic epithelial cells (Walsh et al., 2020). Other studies have shown the potential of neutral fucosylated and non-fucosylated MO to directly enhance intestinal epithelial barrier integrity in a Caco-2-cell line (Boll et al., 2024). Kong et al. (2019) also used a Caco-2-cell line to show the stimulatory effect of fucosylated milk oligosaccharides on glycocalyx development of intestinal epithelial cells. MO structure in itself plays antimicrobial and anti-adhesive roles. They act as decoys for infectious microorganisms by mimicking gut epithelial binding receptors used by pathogens to invade the host gut (Walsh et al., 2020). In particular, the 3'-sialyllactose has been shown to bind the pathogen *Helicobacter pylori* in an intestinal epithelial cell line model (Simon et al., 1997) while 2'-fucosyllactose inhibited adhesion of *Campylobacter jejuni* in a cell line model (Yu et al., 2016). Finally, MO can educate and modulate the host immune system, via direct linkage or indirectly through bacterial metabolites (Ayechu-Muruzabal et al., 2018). It is believed that MO, especially the sialylated types are able to bind to specific lectin receptors present on the surface of numerous immune cells such as dendritic cells and macrophages (Rousseaux et al., 2021).

The MO composition, has been reported in human milk and for a wide range of non-human mammals, highlighting significant variations in concentration and composition between species and lactation stage. However, MO has not been characterized in rabbits. Our recent analysis of MO from 67 female rabbits has shown that sialylated forms predominate (83 %) (Combes et al., 2023). However, the diversity of MO in rabbit milk is much lower than in human milk. A further characterization of MO in rabbits is necessary to elucidate their potential direct and microbiota-mediated role in strengthening the intestinal barrier. This research could lead to the development of health-promoting strategies in rabbits, including i) the use of dietary supplementation with MO as a novel type of prebiotic and as a tool to enhance the barrier function, ii) the optimization of MO composition in milk by genetic selection or dietary modulation, iii) the provision of bacteria used as probiotics (e.g. *Bacteroides* strains) capable of degrading MO.

Introduction of solid food

Under rearing conditions, suckling rabbits have access to solid food from 12-15 days of age (Scapinello et al., 1999) as soon as they are able to leave the nest and reach their mother's feeder. Rabbit pups gradually move from milk, which is highly digestible by the hydrolytic action of the host's enzymes, to a more complex and less digestible solid food, containing in particular indigestible plant polysaccharides. The arrival of these new substrates leads to the creation of new ecological conditions in the gut, allowing the establishment and dominance of new microbial species better adapted to their degradation. The introduction of solid feed thus contributes to a drastic change in the composition and metabolic activity of the gut microbiota (Padilha et al., 1999; Read et al., 2019; Beaumont et al., 2020; Zhao et al., 2024). This maturation in the microbiota composition and metabolic activity are closely linked to the development of the epithelial barrier function, as observed with the altered expression of genes involved in tight junctions, mucin production and in innate immunity (production of cytokines and antimicrobial peptides) (Beaumont et al., 2020). In particular, it has been shown that the butyrate produced by the bacteria after the introduction of solid food plays a key role in the development of the epithelial barrier *in vitro* (Beaumont et al., 2020). Optimizing the nutritional composition of the feed available before weaning is undoubtedly a relevant way to strengthen the gut barrier function. At the onset of solid feed intake, dietary modulation has been shown to alter the dynamics of microbiota establishment (dietary modulation of energy and crude protein content; Read et al. 2019), favoring the Lachnospiraceae family over the Ruminococcaceae family (dehydrated alfalfa: Mattioli et al., 2019).

Early introduction of solid food (before 12-15 days of age in the nest), while maintaining milk consumption, has been evaluated as a strategy to optimize host-microbiota interactions. In rabbits, the possibility of stimulating early feed intake in the nest was first demonstrated by Kacsala et al. (2018). To optimize this early solid feed intake, we have developed a nutritional substrate in the form of a gel, taking into account the food preferences of suckling rabbits and without altering milk intake (Paës et al., 2020a). This early stimulation of solid food ingestion, significant from 7 days of age, leads to an increased bacterial diversity before weaning and an increased abundance of Lachnospiraceae and Ruminococcaceae at the expense of Bacteroidaceae, the latter being characteristic of the microbiota of exclusively suckling rabbits. These changes in the microbiota were amplified with higher levels of feed intake and were also related to the litter weight (Paës et al., 2020b). These observations reflect an acceleration in the maturation of the microbiota and can be explained by a targeted seeding of the digestive microbiota at the beginning of life, which favours the development of bacteria specialized in the degradation of complex parietal carbohydrates. Early feed intake was also associated with an increase in the cecal levels of 7 amino acids, as well as acetate and butyrate in the intestinal lumen of rabbits, which indicated a change of bacterial metabolism (Paës et al., 2022). This maturation of the gut microbiota induced by early solid food ingestion could therefore improve rabbit health. Indeed, in piglets, changes in the microbiota associated with early feeding have been shown to reduce diarrhoea and improve growth performance (Choudhury et al., 2021). In rabbits, it remains to be determined whether changes in microbiota composition and in the luminal concentrations of metabolites, molecular intermediates in the microbiota-host crosstalk, are sufficient to preserve the health of the young rabbits and help them to cope with weaning.

Coprophagia behavior in the nest

The ingestion of solid substrate early in life also includes coprophagia behavior in the nest, i.e. the ingestion of feces deposited by the mother during nursing (Hudson & Distel, 1982; Kovács et al., 2006; Combes et al., 2014). Coprophagia early in life has a protective effect on rabbit health by increasing survival rate (Combes et al., 2014). This beneficial effect is associated with an acceleration of the implantation dynamics of the microbiota in the cecum, allowing bacteria belonging to the Ruminoccocaceae family to become predominant at the expense of those belonging to the Bacteroidaceae family. Furthermore, coprophagia is also

associated with transcriptomic changes in the gut that reflect immune development in the ileum during the first two weeks after weaning. These changes specifically involve the type I interferon signalling, but also include innate immune responses (antimicrobial peptides, mucin and cytokine secretions) and adaptive immune responses (transcriptional regulation of IgA secretion) (Cauquil et al., 2024). Altogether, these results suggest that coprophagia in the nest may favor early host-microbiota interactions with long-lasting effects after weaning. Further studies should be conducted to better understand how coprophagia drives its effect on the mucosal immune system, thereby strengthening the host gut barrier. For instance, it would be interesting to determine whether specific bacterial strains or metabolites present in maternal feces drive these protective effects of early life coprophagia in rabbits.

Cessation of milk intake

Weaning is commonly defined as the transition from exclusive milk intake to exclusive solid feed intake. In rabbit rearing conditions, the term “weaning” is associated with suckling cessation, and the separation of the pups from the doe, and usually occurs between 28 and 35 days of age. Several studies evidence a lower growth when animals are weaned early, between 21 and 25 days of age, compared to 34-35 days of age (Gallois et al., 2008; Cesari et al., 2009; Kovács et al., 2012), which could be associated with a higher susceptibility to digestive disorders, evidenced by an increased mortality (Cesari et al., 2009). Interestingly, early weaning (i.e. suckling cessation) is not associated with changes in microbiota composition or metabolic activity (Kovács et al., 2012; Beaumont et al., 2022). These observations indicate that solid food ingestion, rather than milk intake cessation, shapes the rabbit gut microbiota. Regarding microbial activity, increased levels of SCFA, namely butyrate (Kovács et al., 2012) and acetate (Gallois et al., 2008), are evidenced in rabbits weaned at 21 days of age compared to rabbits weaned at 35 days of age. Beaumont et al. (2022) found no changes in SCFA but higher levels of the polyphenol-derived metabolite 3-phenypropionate at 25 days of age in rabbits weaned at 18 days of age compared to their suckling counterparts. Altogether, early weaning does not induce considerable changes in the digestive microbial compartment. Conversely, early weaning appears to have a strong effect on host physiology and mucosal development. Gutiérrez et al., (2002) showed that pups that were weaned at 25 days of age had shorter villi and deeper crypts in the jejunum at 35 days of age than their suckling counterparts, which was associated with decreased brush border enzyme activity (lactase, maltase and sucrase) and increased alpha-amylase activity. Beaumont et al., (2022) also evidenced strong gene expression modulations in the cecal epithelium of 25 days old rabbits weaned at 18 days of age compared to their suckling counterparts. Namely, stem cell and proliferation markers were down-regulated in early weaned rabbits, whereas the expression of PIGR, which is involved in immunoglobulin transport, was strongly up-regulated, possibly due to the loss of maternal passive immunity. In both aforementioned studies, most of the changes induced by early weaning were consistent with an enhancement of spontaneous development with age, suggesting that early weaning could accelerate gut mucosal development. However, it is important to consider that disrupting the kinetics of microbiota and mucosal maturation can enhance the susceptibility to diseases later in life (Al Nabhani et al., 2019), as evidenced by the increased mortality associated with early weaning in rabbits (Cesari et al., 2009).

CONCLUSIONS

The reanalysis of 16S amplicon sequencing data from different research groups has allowed us to establish the core microbiota of the post-weaning rabbit cecum in a robust manner, including technical and experimental variability. Nevertheless, this analysis highlights that there is still a considerable lack of knowledge regarding the microbes that inhabit the cecal ecosystem, their functions and taxonomy at the species and strain level. Studies based on the sequencing of all bacterial genes (shotgun metagenomics) are already available. These technologies allow the integration of taxonomic knowledge with the functions present in the ecosystem. The advent of long-read sequencing, applied to both 16S amplicon sequencing

and shotgun metagenomics, will increase taxonomic resolution while reducing taxonomic errors and ambiguity in assigning sequences to specific taxa. It will also allow a better detection of rare species due to increased sensitivity and coverage of sequenced microbial genomes (Eisenhofer et al., 2024). The review of the literature over the past decade has enabled the identification of some key species in the rabbit gut ecosystem that are associated with beneficial or detrimental traits related to health and growth. However, we were unable to reach a consensus between the different studies, which precluded us from defining the optimal rabbit microbiota. The large variability in experimental conditions between studies explains much of this difficulty. Another reason may be that, beyond knowing the species, the activity of the microbiota needs to be further studied, either by inference through metagenomic studies or by characterizing the metabolome. Culture of bacterial strains isolated from the rabbit microbiota will also be instrumental in identifying their role in symbiosis.

The microbiota plays a pivotal role in gut health, and more specifically as a central component of the intestinal barrier. The underlying mechanisms of its role as a barrier itself still remain to be investigated in rabbits. Given the taxonomic specificity of the rabbit microbiota, it would be valuable to validate the generic bacterial gatekeeper mechanisms already demonstrated in other mammalian models. The epithelial component of the intestinal barrier also exhibits remarkable features in rabbits, such as the presence of $BEST4^+$ absorptive epithelial cells, which role remains to be elucidated. The development of *in vitro* intestinal organoid models in rabbits offers promising avenues for elucidating the functional interactions between the epithelium and gut bacteria and their products. Additionally, the immune component of the intestinal barrier remains relatively understudied, due to the lack of suitable tools in rabbits, despite the presence of unique lymphoid tissues in this species. Further investigations are therefore warranted.

A deeper understanding of the symbiotic relationship between the microbiota and its host is essential to strengthen the intestinal barrier and consequently digestive health. Top-down approaches such as metagenomics, metabolomics, transcriptomics and proteomics, which do not require cultures of individual species, have provided genomic and metabolic signatures within a complex community, and together generate hypotheses about existing microbe-microbe and microbe-host interactions. Testing hypotheses, and going from these association studies to proven causality, will require performing genetic engineering studies and applying bottom-up synthetic biology approaches combined with functional assays using models of gut barrier host cells. Genetic engineering, i.e. loss, gain and modulation of functions, provides opportunities to probe the intrinsic molecular host-microbiota interactions in rabbits. It will provide a new source of fundamental knowledge and strategic guidance for understanding the role of the gut microbiota and its metabolites on the intestinal barrier.

We have shown that prior to weaning, modulation of dietary intake during the transition from milk to solid food has a strong influence on the microbiota and, concurrently, the host's health. By understanding the causality in microbe-host interactions, it will thus be possible to engineer the establishment of the microbiota in the young rabbit and strengthen the intestinal barrier. For instance, innovative nutritional strategies before weaning might be proposed, such as (i) dietary prebiotic supplementation that focus on targeted health-promoting bacteria, (ii) probiotic supplementation derived from, and thus specific to, the rabbit gut, which is known to strengthen host intestinal barrier function, (iii) symbiotic supplementation (supply of live bacteria with their substrate) or postbiotics, i.e. beneficial metabolites for the host's intestinal barrier produced by cultivated and lysed bacteria. Altogether, it is evident that the microbiota plays a pivotal role in the digestive health of rabbits. Consequently, to optimize its composition and functional capabilities and to enhance the intestinal barrier function, it is necessary to adopt multidisciplinary approaches that integrate diverse methodologies at various scales. These include *in vitro* cellular culture models, notably cell models that combine epithelial and immune cells and bacteria, and *in situ* validation models.

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