Clinical Department for Farm Animals and Food System Science

University of Veterinary Medicine Vienna

Nutritional Physiology, Centre for Veterinary Systems Transformation and Sustainability

(Head: Univ.-Prof. Dr. sc. agr. Barbara Metzler-Zebeli)

Development and modulation of gut microbe-host interactions

in chickens and piglets

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Dedication

To my wife Dwi Supriyanti and my son Ferozka A. Yozadi

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Contribution of authors

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Yosi F	Experiment performance, interpretation of the data, writing the manuscript
Sharma S	Experiment performance, writing the manuscript
Sener-Aydemir A	Experiment performance, writing the manuscript
Koger S	Experiment performance, writing the manuscript
Baskara AP	Experiment performance, writing the manuscript
Metzler-Zebeli BU	Study design, experiment performance, data analysis, interpretation of the data, writing the manuscript

Dietary probiotics modulate gut barrier and immune-related gene expression and histomorphology in broiler chickens under non- and pathogen-challenged conditions: A meta-analysis. 2023. Animals, 13(12), 1970, doi: 10.3390/ani13121970

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Metzler-Zebeli BU	Study design, experiment performance, data analysis, interpretation of the data, writing the manuscript

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Koger S	Experiment performance, writing the manuscript
Verhovsek D	Experiment performance, writing the manuscript
Metzler-Zebeli BU	Study design, experiment performance, data analysis, interpretation of the data, writing the manuscript

Declaration

I confirm that I have followed the rules of good scientific practice in all respects.

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Abbreviations

AGPs	antimicrobial growth promoters
AMPK	AMP-activated protein kinase
BCFA	branched-chain fatty acids
cAMP	cyclic adenosine monophosphate
CLDN	claudins
CP	crude protein
CSF	competence and sporulation factors
DoL	day of life
DPI	days post-infection
DPP	day postpartum
FAs	fatty acids
FFARs	free fatty acid receptors
FXR	farnesoid X receptor
GLP1	glucagon-like peptide
GPRs	G protein-coupled receptors
HCARs	hydroxy-carboxylic acid receptors
HDACs	histone deacetylases
IFNs	interferons
ILs	interleukins
LA	lactic acid
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCFA	medium-chain fatty acids
MCTs	monocarboxylate transporters
ME	metabolizable energy
MUC	mucin
NF-κB	nuclear factor kappa-B
NODs	nucleotide-binding oligomerization domain receptors
OCLN	occludins
PK	protein kinase
PRRs	pattern recognition receptors
PUFA	polyunsaturated fatty acids

PYY	peptide YY
SCFA	short-chain fatty acids
SMCTs	sodium-coupled monocarboxylate transporters
TJP	tight junction protein
TLRs	toll-like receptors
TNFs	tumor necrosis factors
ZO	zonula occludens

1. SUMMARY

1.1. English

Increasing evidence suggests that gut microbes and their metabolites, i.e., short-chain fatty acids (SCFA), have beneficial effects on the gut function and health of monogastric farm animals, particularly chickens and pigs. Nevertheless, more research is still needed to fully understand the complex relationship between gut microbes and their hosts, especially in the following three aspects. First, investigations into the modulating effects of mixed SCFA on intestinal barrier function and contractibility in chickens are scarce. Second, the role of direct-fed microbes (probiotics) in improving gut health and growth in chickens is still controversial, as evidenced by the different results in various studies. Third, research on the mother-offspring axis and its role in the establishment of gut colonization in neonatal piglets is still limited.

We applied three complementary study models, including *ex vivo* and *in vivo* experiments, and meta-analysis, to investigate these topics: The *ex vivo* study aimed to investigate the local effect of mixed SCFA, with different acetate:butyrate ratios and SCFA concentrations, on the jejunal and cecal contractibility and jejunal barrier function in laying hens as an animal model. By implementing Ussing chamber and organ bath techniques, we discovered that increasing the butyrate proportions and SCFA concentrations improved jejunal ion uptake and barrier function, as well as stimulated cecal muscle contraction in laying hens.

The aim of the *in vivo* study was to investigate the changes in bacterial and fungal communities in sow feces during the lactation period as well as in the gastric and cecal digesta of piglets from suckling to one week after weaning. Additionally, the correlation between fecal consistency scores and cecal microbial taxa in the weaned piglets was evaluated. It was shown that the progressing lactation influenced the bacterial and fungal communities in sow feces. Moreover, sow feces and piglet gut digesta were found to share bacterial and fungal taxa, providing evidence that maternal microbes contribute to the gut colonization of neonatal piglets. In addition, potential bacterial and fungal markers for softer and firm feces postweaning have been identified that may serve as indicators of gut homeostatic conditions.

The meta-analysis study aimed to assess the modulatory effects of dietary probiotics on gut barrier and immune-related gene expression, histomorphology, and growth performance in broiler chickens, both with and without pathogen challenge. Our meta-analysis revealed that dietary probiotics with various genera/species improved gut integrity and structure in broiler chickens, without affecting growth performance. Furthermore, the effectiveness of probiotics was found to be modified by dietary metabolizable energy, crude protein, and days post-infection.

Overall, these studies provide new insights into the early gut microbial colonization in piglets as well as into roles of SCFA, and dietary probiotics for gut barrier function and immune responses of the host in chickens. The results obtained may help to develop dietary strategies to improve gut health in these monogastric farm animals.

1.2. German

Es gibt vermehrt Hinweise, dass Darmmikroben und ihre Stoffwechselprodukte, z.B. kurzkettige Fettsäuren (SCFA), positive Auswirkungen auf die Darmfunktion und die Gesundheit von monogastrischen Nutztieren, wie Hühnern und Schweinen, haben. Dennoch bedarf es noch weiterer Forschung, um die komplexe Beziehung zwischen Darmmikroben und dem Wirtstier vollständig zu verstehen, insbesondere in den folgenden drei Aspekten. Erstens gibt es kaum Untersuchungen zu den Darm-modulierenden Wirkungen, wenn die SCFA in einer Mischung vorliegen, auf die Barrierefunktion und Kontraktibilität bei Hühnern. Zweitens gibt es in der Literatur widersprüchliche Angaben zur Rolle von direkt gefütterten Mikroben (Probiotika) für die Verbesserung der Darmgesundheit und des Darmwachstums bei Hühnern. Drittens ist die Forschung zur Mutter-Nachkommen-Achse und ihre Rolle bei der Etablierung der mikrobiellen Gemeinschaft im Darm bei neugeborenen Ferkeln noch begrenzt.

Um die drei Aspekte zu untersuchen, wurden drei Studienmodelle angewendet: ex-vivo- und in-vivo-Experimente sowie Metaanalysen. Das Ziel der ex-vivo-Studie war es, die lokale Wirkung von SCFA-Mischungen mit unterschiedlichen Azetat:Butyrat-Verhältnissen und Konzentrationen auf die Kontraktibilität von Jejunum und Zäkum sowie die Barrierefunktion im Jejunum bei Legehennen als Tiermodell zu untersuchen. Mittels Anwendung von Ussing-Kammern und dem Organbad zeigte sich, dass ein höherer Butyratanteil und SCFA-Konzentrationen die Aufnahme von Ionen im Jejunum sowie die Barrierefunktion verbesserte sowie die Kontraktionen der Zäkummuskulatur bei Legehennen stimulierte.

Das Ziel der in-vivo-Studie war es, die Veränderungen in Bakteriom und Mycobiom im Sauenkot im Verlauf der Laktation sowie im Magen- und Zäkumchymus bei Ferkeln von der Geburt bis eine Woche nach dem Absetzen zu untersuchen. Des Weiteren wurden eine Korrelationsanalyse zwischen der Kotkonsistenz und den Abundanzen der mikrobiellen Taxa im Zäkum durchgeführt. Die Ergebnisse zeigten, dass sich mit fortschreitender Laktation die Zusammensetzung der Bakterien- und Pilzgemeinschaften im Sauenkot veränderte. Darüber hinaus zeigte das gemeinsame Vorkommen von Bakterien- und Pilz-Taxa im Sauenkot und Chymus im Magen und Zäkum der Ferkel, dass das Mikrobiom im Sauenkot zur Darmbesiedlung der neugeborenen Ferkel beigetragen hat. Darüber hinaus wurden Bakterien

und Pilze identifiziert, die mit weicherem oder festen Kot nach dem Absetzen einhergingen und als Marker-Taxa für den Zustand der Darmhomöostase dienen könnten.

Das Ziel der Metaanalyse war es, die modulierenden Wirkungen von Probiotika auf die Darmbarriere und Genexpression zu Immunparametern, der Histomorphologie und der Wachstumsleistung bei Masthühnern zu evaluieren. Es wurde jeweils der Einfluss auf die genannten Parameter mit und ohne Pathogen-Exposition untersucht. Die Metaanalyse zeigte, dass Probiotika die Darmintegrität und -struktur bei Masthühnern verbesserten, jedoch ohne Effekt auf die Wachstumsleistung. Des Weiteren ließen die Daten abschätzen, dass die Wirksamkeit von Probiotika durch die Nährstoffdichte der Nahrung (umsetzbare Energie und Rohproteingehalt) sowie die Anzahl der Tage nach der Infektion beeinflusst wird.

Zusammengefasst liefern die durchgeführten Versuche neue Erkenntnisse über die frühe mikrobielle Besiedlung des Darms bei Ferkeln. Sie liefern ebenfalls neue Einsichten über die Rolle von SCFA und die Wirksamkeit von Probiotika für die Darmbarrierefunktion und die -immunrespons bei Hühnern. Die vorliegenden Ergebnisse können dazu beitragen, Ernährungsstrategien zur Verbesserung der Darmgesundheit von monogastrischen Nutztieren zu optimieren.

2. GENERAL INTRODUCTION

2.1. Gut health in monogastric farm animals

The term "healthy gut" is often used in animal nutrition, referring to the combination of physiological, microbiological, and physical functions of the gastrointestinal tract that work collectively to maintain homeostasis (Diaz Carrasco et al., 2019; Kogut, 2019; Wickramasuriya et al., 2022). The gastrointestinal tract plays an important role in supporting livestock productivity and health, as it not only serves as a site for digestion and absorption of nutrients, but also functions as a metabolic and immunological organ (Scanes and Pierzchala-Koziec, 2014). Gut health disorders caused by both nutritional and non-nutritional factors are a major cause of lost productivity in the monogastric industry, including poultry and swine farms (Jayaraman and Nyachoti, 2017; Kogut, 2019). In pigs, piglets are highly susceptible to intestinal health disorders, especially during the weaning transition, resulting in high rates of diarrhea (Upadhaya and Kim, 2021; Huting et al., 2021). To improve the gut health in these two monogastric farm animal species, especially through improved nutritional factors, it is crucial to understand the constitution of a healthy gastrointestinal tract and its role in nutrient metabolism and immune function.

2.2. Gastrointestinal tract and its function in chickens and piglets

Both in chickens and piglets, feed enters the esophagus after being digested in the mouth. Especially in chickens, the digested feed from the mouth passes through the esophagus into the crop, which serves as a temporary storage site for feed materials (Scanes and Pierzchala-Koziec, 2014). The digested feed is then transferred to the stomach, which is further digested by the action of hydrochloric acid and digestive enzymes to form a digested feed mixture called chyme. In chickens, they have two types of stomachs, i.e., the proventriculus (glandular stomach) and the gizzard (muscular stomach). Here, the proventriculus is responsible for enzymatic digestion, whereas the gizzard, which is located posterior to the proventriculus, is the main site of mechanical digestion of feed materials (Scanes and Pierzchala-Koziec, 2014). In addition, the development of the gizzard helps to increase the secretion of pancreatic enzymes in the small intestine, improve gastrointestinal motility, and increase nutrient digestibility, thereby improving intestinal function. After chyme is formed, it then flows into the small intestine which is divided into three segments in order from proximal to distal parts: duodenum, jejunum, and ileum. The small intestine is the primary site of nutrient breakdown with the help of digestive enzymes secreted by the pancreas and bile released by the liver (Scanes and Pierzchala-Koziec, 2014; Pluske, 2016). Once digested, nutrients are

absorbed through the wall of the small intestine with the assistance of finger-like epithelial villi. After the small intestine, feed digestion continues in the cecum, of which chickens have a pair. Here, microbial-assisted fermentation of fiber or undigested nutrients occurs to produce volatile fatty acids, such as lactic acid and short-chain fatty acids. In addition to their function in digestion and absorption of nutrients, the small intestine, and ceca also play an important role in supporting the immune function (Scanes and Pierzchala-Koziec, 2014; Pluske et al., 2018). The next part of the digestive tract is the large intestine or colon, which is anatomically different and shorter in chickens than in pigs.

2.3. Gut microbiota and factors affecting their colonization in chickens and piglets

As widely recognized, the gastrointestinal tract is home to a complex and dynamic microbial community that includes bacteria, fungi, archaea, protozoa, and viruses (Shang et al., 2018; Yadav and Jha, 2019). Among these microbial communities, bacteria are the most dominant and their interaction with the host is necessary to support gut health and development (Wei et al., 2013; Shang et al., 2018). It is important to note that each part of the gastrointestinal tract has different metabolic, physiological, and immune functions that shape the microbial community (Yeoman and White, 2014; Chen et al., 2021). More specifically, differences in available substrates for growth, redox potential, pH, mucus, and host antimicrobial secretions, as well as the transit time of substrates from the upper to the lower intestine, are considered to be the main factors leading to gut region-specific differences in microbial distribution and abundance (Lan et al., 2005; Guevarra et al., 2019; Metzler-Zebeli, 2022). In addition, other factors, such as genetics, age, diet, rearing system, and health status, are also presumed to influence the colonization of the gut commensal microbiota (Rehman et al., 2007; Yadav and Jha, 2019; Chen et al., 2019; Rychlik, 2020), thus it is necessary to consider these factors when assessing parameters related to the gut microbiota.

2.3.1. Gut microbial composition in chickens

In chickens, many studies have been performed to investigate the composition of the gut microbiota in each gut segment. The chicken crop is predominantly colonized by Firmicutes, such as *Lactobacillus*, followed by Actinobacteria (e.g., *Bifidobacterium*) and Proteobacteria (e.g., *Enterobacter*) (Yeoman et al., 2012; Shang et al., 2018). Similarly, *Lactobacillus* is also the dominant genus identified in the proventriculus and gizzard, followed by lactose-negative *Enterobacter, Enterococcus*, and coliforms (Shang et al., 2018; Rychlik, 2020). Although the concentration of bacteria is similar to the crop (Shang et al., 2018), the bacterial fermentation

activity in the stomach is lower, mainly due to the lower pH, making it less ideal for bacterial development (Rehman et al., 2007). In the small intestine, the concentration of bacteria varies from segment to segment and increases distally (Fathima et al., 2022). The different phyla of bacteria that colonize the small intestine include Firmicutes (e.g., *Bacillus, Enterococcus, Clostridium, Lactobacillus, Candidatus, Ruminococcus, Eubacterium, Staphylococcus, Streptococcus, Turicibacter,* and *Methylobacterium*), Proteobacteria (e.g., *Ochrobaterium, Alcaligenes, Escherichia, Campylobacter, Hafnia,* and *Shigella*), Cytophaga/Flexibacter/Bacteroides (e.g., *Bacteroidetes, Flavibacterium, Fusobacterium,* and *Bifidobacterium*), as well as Actinobacteria/Cyanobacteria (e.g., *Corynebacterium*) (Shang et al., 2018; Rychlik, 2020).

Figure 1. The composition of major microbial phyla in each gut segment in chickens. The information included in the figure are extracted from Yeoman et al. (2012), Shang et al. (2018) and Rychlik (2020). The figure was created with biorender.com.

The duodenum has a lower pH than the jejunum and ileum, which limits the growth of bacteria and most pathogens (Xiao et al., 2021). In addition, shorter transit times, higher activity of digestive enzymes, and/or dilution of the digesta by bicarbonate and bile secretions result in lower microbiota density in the duodenum (Lan et al., 2005; Shang et al., 2018; Yadav and Jha, 2019). Compared to the duodenum and jejunum, the ileal microbiota is more abundant

and predominantly composed of *Lactobacillus* (Shang et al., 2018). The abundance and diversity of the gut microbiota is considerably increased in the cecum. The cecal microbiota is mainly composed of the phyla Firmicutes (e.g., *Anaerotruncus, Ruminoccoccus, Faecalibacterium, Lachnospirceae, Bacillus, Streptococcus, Clostridium, Megamonas, Lactobacillus, Enterococcus, Weisella, Eubacterium, Staphylococcus, Streptococcus*) and Bacteroides/Cytophaga/Flexibacter (e.g., *Bacteroidetes, Fusobacterium, Bifidobacterium, Flavibacterium, Alistipes, and Odoribacter*), followed by the minor phyla Actinobacteria (e.g., *Corynebacterium, Olsenella*, and *Collinsella*), Proteobacteria (*Ochrobaterium, Alcaligenes, Escherichia,* and *Campylobacter*), and methanogenic archaea (e.g., *Methanobacterium, Methanobrevibacter, Methanococcos, Methanosphaera, Methanothermobacter, Methanopyrus,* and *Methanothermus*) (Shang et al., 2018; Rychlik, 2020). The chicken colon is quite short and does not retain much digesta. The types of microbes that colonize the colon are influenced by the digesta from the preceding intestinal segments, such as the ceca and ileum. From several studies, Firmicutes (e.g., *Lactobacillus*) and Proteobacteria (e.g., *Escherichia coli*) are the bacterial phyla found in the colon (Shang et al., 2018; Rychlik, 2020).

2.3.2. Gut microbial composition in piglets

While the fecal microbiota composition of suckling and weaned piglets has been extensively studied (Bian et al., 2016; Chen et al., 2017), there is information on the microbiota composition of different gastrointestinal segments in piglets throughout the suckling and early postweaning periods. Similar to chickens, microbial abundance in the small intestine of piglets is lower than in the large intestine, due to the shorter transit time in this compartment. Nevertheless, their presence is still important for microbiome-gut-host interactions (Metzler-Zebeli, 2022). Luminal pH is another factor that also determines the microbiota community in gut segments. Furthermore, there are major changes in the abundance and diversity of gut microbial species in piglets from birth to weaning, which are closely related to the sow's milk intake and environmental factors (Metzler-Zebeli, 2022). This clearly indicates that the sow-piglet relationship is critical for establishing the microbiota in the piglet gut. During the suckling period, milk-oriented microbial species are more predominant in the gut, which are strongly influenced by milk glycans, i.e., lactose and oligosaccharides. In contrast, after weaning, the number of milk glycan-oriented bacteria is reduced and more microbes utilizing plant carbohydrates and proteins are detected (Holman et al., 2021; Metzler-Zebeli, 2022). Overall, more is known about on the evolution of the bacterial microbiota, whereas less has been described for other microbial groups, such as fungi, protozoa, and viruses.
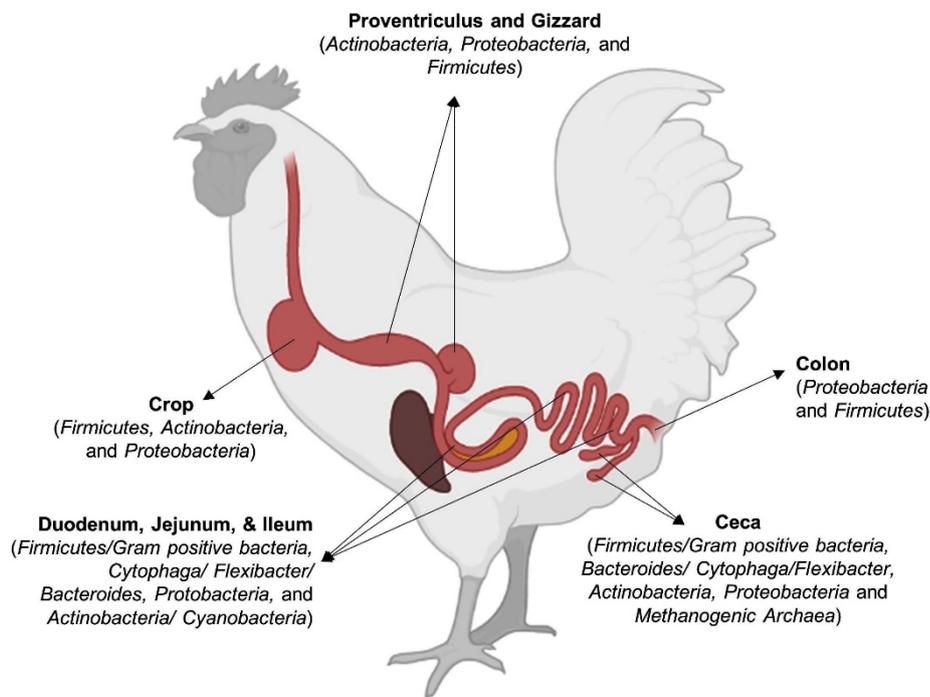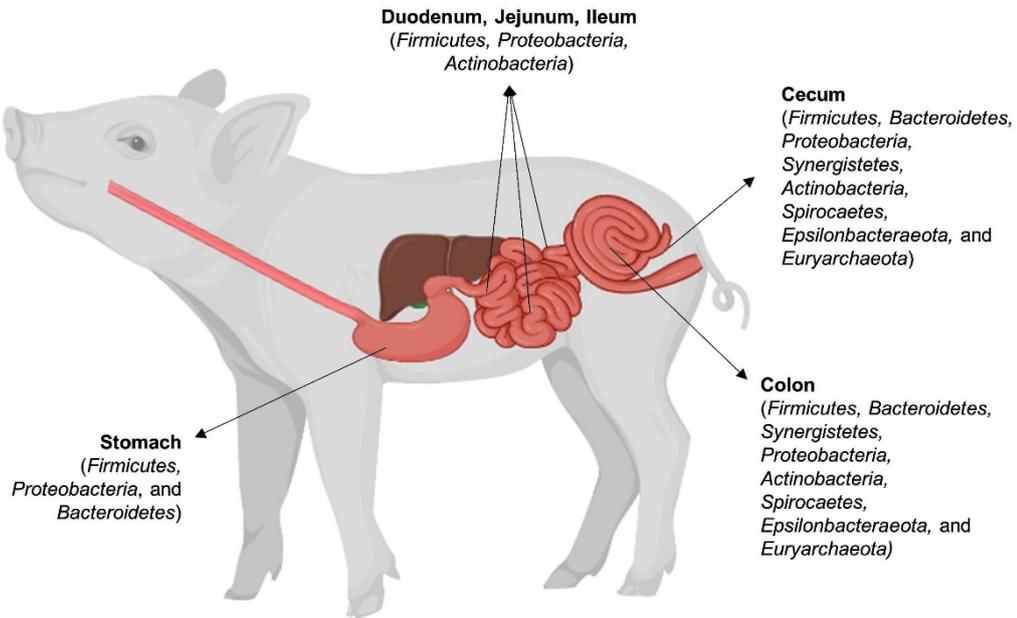
Figure 2. The composition of major microbial phyla in each gut segment in neonatal piglets. The information included the figure are extracted from Li et al. (2018), Gresse et al. (2019), Gryaznova et al. (2022), and Lerch et al. (2023), The figure was created with biorender.com.

Recently, Lerch et al. (2023) demonstrated that the number of bacteria, fungi, yeasts, protozoa, and archaea in the gastric digesta of piglets increased from days 7 to 35 of age. Likewise, bacterial species richness and diversity in the stomach increased during the suckling period but decreased after weaning. This study also showed that *Lactobacillaceae* bacteria dominated the stomach during the suckling period followed by *Streptococcaceae* and *Pasteurellaceae*. Shortly after weaning at 4 weeks of age, the abundance of *Lactobacillaceae* and *Streptococcaceae* in the stomach decreased, providing a niche for the unclassified families *Rikettsiales* and *Pasteurellaceae*. About 1 week after weaning, this microbial composition changed again, with *Lactobacillaceae* increasing in abundance and the unclassified families *Rikettsiales* and *Pasteurellaceae* decreasing in abundance. The cecal community is generally more diverse than the gastric microbiota. In the study by Lerch et al. (2023), the abundance of bacteria reached its highest on day 7 of age, whereas the number of protozoa increased on days 21 and 31 of age. However, the abundance of archaea, fungi, and yeast did not change during the pre- and postweaning periods. Similar to the stomach, the bacterial species richness and diversity in the cecum also increased during suckling but decreased after weaning, wheras the fungal community did not change. It was also reported that *Prevotellaceae* predominated in the cecum during the suckling and postweaning periods,

whereas *Bacteroidaceae* and *Pasteurellacae* were replaced by *Lachnospiraceae* and *Acidaminococcaceae* after weaning. This study investigated the fungal composition of cecal digesta, which was dominated by *Dipodascaceae* at 2 weeks of age. Their numbers declined with age and were present only in low numbers after weaning. The abundance of *Erysiphaceae* in the cecum was almost absent in the first 2 weeks of age but increased at week 4 of age, just before weaning, and became dominant after weaning. *Saccharomycetaceae* in the cecum started to appear at week 4 of age but declined after weaning. In general, this study concluded that the effect of creep feeding was small, indicating the importance of sow milk components for the microbial succession in the gut.

Another study by Gryaznova et al. (2022) showed that the number of *Lactobacillaceae* was significantly higher in the ileum than in the cecum and rectum within 1 week of age. In addition, the abundance of *Fusobacteriaceae* seemed to increase in the direction from the small to the large intestine. The abundance of *Bacteroidaceae* was also higher in the cecum and colon than in the ileum. Likewise, Li et al. (2018) found that *Prevotellaceae* were the dominant bacteria identified in the colonic digesta of piglets fed a corn/soybean-based diet at around 3 weeks of age, followed by *Ruminococcaceae, Lachnospiraceae, Porphyromonadaceae,* and *Bacteroidaceae.* Moreover, *Lachnospiraceae, Negativicutes, Selenomonadales, Campylobacterales*, and some other species increased in weaned piglets, whereas *Porphyromonadaceae, Alloprevotella, Barnesiella*, and *Oscillibacter* decreased in their study. Furthermore, a study by Gresse et al. (2019) on the composition of the gastrointestinal microbiota in newly weaned piglets at week 4 of age showed that both the stomach and small intestine were predominantly colonized by *Lactobacillaceae* and *Pasteurellaceae*, whereas higher numbers of *Peptostreptococcaceae* and *Streptococcaceae* were found in the small intestine. In the cecum and colon, the *Ruminococcaceae, Prevotellaceae*, and *Lachnospiraceae* dominated, followed by *Lactobacilaceae, Bacteroidaceae, Rikenellaceae, Tannerellaceae, Muribaculaceae, Christensenellaceae, Acidaminococcaceae, Clostridiaceae, Enterobacteriaceae*, and *Spirochaetaceae.* For the archaeal community, *Methanobacteriaceae* dominated in the cecum and colon, followed by a small number of *Methanomethylophilaceae.*

2.4. Understanding the mother-offspring axis in establishing microbial colonization

Ensuring optimal microbial establishment early in life is important to improve gut immunity and growth performance in chickens and pigs. According to a previous study in chickens, a diverse microbiota colonization was detected in the cecal contents of 1-day-old chicks using 16S rRNA

(Jiang et al., 2015). This study showed that Proteobacteria was the most dominant phylum, followed by Firmicutes and Actinobacteria, whereas Tenericutes, Bacteroidetes, Acidobacteria, Verrucomicrobia, Nitrospirae and Cyanobacteria were identified at very low levels. These results indicate that beneficial or commensal bacteria may be transferred from mother to offspring. Subsequently, *in ovo* techniques were developed to further investigate this potential pathway, allowing the transfer of various biological materials and supplements to chick embryos. For instance, early inoculation of young chicks with native microbiota from healthy adult birds could promote the development of an early gut microbiome (Roto et al., 2016). Other studies had also revealed that air sac injections with supplements such as prebiotics modulated the growth of indigenous microflora in the egg along with embryo development, such as *Bifidobacterium* spp and *Lactobacillus* spp. (Slawinska et al., 2019; Das et al., 2021).

While *in ovo* techniques have been extensively studied in chickens, the investigation of the mother-offspring axis in shaping the microbial colonization of newborn piglets during the suckling period has not been fully elucidated. Microbial colonization of the piglet gut is thought to begin at birth during the vaginal passage. In addition, sow feces may also serve as a source of other microbes that could potentially influence gut microbial colonization. A study by Berry et al. (2021) showed that maternal parity was able to alter the numbers of *Akkermansia muciniphila, Prevotella stercorea,* and *Campylobacter coli* in the piglet intestine 10 days after birth. Accordingly, modification of the sow's microbiome may affect the bacterial colonization of the piglets. In order to understand the mechanism of direct differentiation in the piglet gut microbiota, it would be beneficial to verify whether these microbial shifts and susceptibilities are related to the microbiota of sows postpartum, as well as pre- and post-weaned piglets.

2.5. The importance of balancing the host gut microbial community for gut health

As mentioned earlier, the gastrointestinal tract harbors a diverse commensal microbiota that has been shown to have a mutually beneficial relationship with the host (Broom and Kogut, 2018). To establish a mutually beneficial coexistence between the gut microbiota and the host, balanced gut microbiota and homeostatic conditions are required as the main prerequisites (Perry and Arsenault, 2022). In this reciprocal relationship, the gut microbiota plays beneficial roles related to aspects of metabolism and physiology, such as facilitating digestion and nutrient absorption, regulating intestinal epithelial proliferation, and modulating the intestinal immune response and barrier function (Tomkovich and Jobin, 2016; Rowland et al., 2018). In return, the host intestinal tract provides a comfortable environment and sufficient nutrients for

the gut microbiota to grow and proliferate (Rinttilä and Apajalahti, 2013; Perry and Arsenault, 2022). In addition, under homeostatic and balanced conditions, beneficial gut microbes also act synergistically to prevent the colonization and proliferation of pathogenic microbes through competitive exclusion, in part by synthesizing antimicrobial compounds (Shang et al., 2018; Kogut, 2019; Perry and Arsenault, 2022). However, when the composition and function of the gut commensal microbiota is disrupted, referred to as dysbiosis, it leads to an imbalance between beneficial and harmful microbes (Shang et al., 2018). Such conditions increase the host's susceptibility to infection and inflammation, leading to decreased metabolic activity, impaired gut structure, and reduced intestinal epithelial barrier function, which is commonly known as "leaky gut" (Teirlynck et al., 2011; Forgie et al., 2019; Shehata et al., 2022). As a result, the performance and productivity of chickens and pigs are drastically reduced, ultimately leading to economic losses.
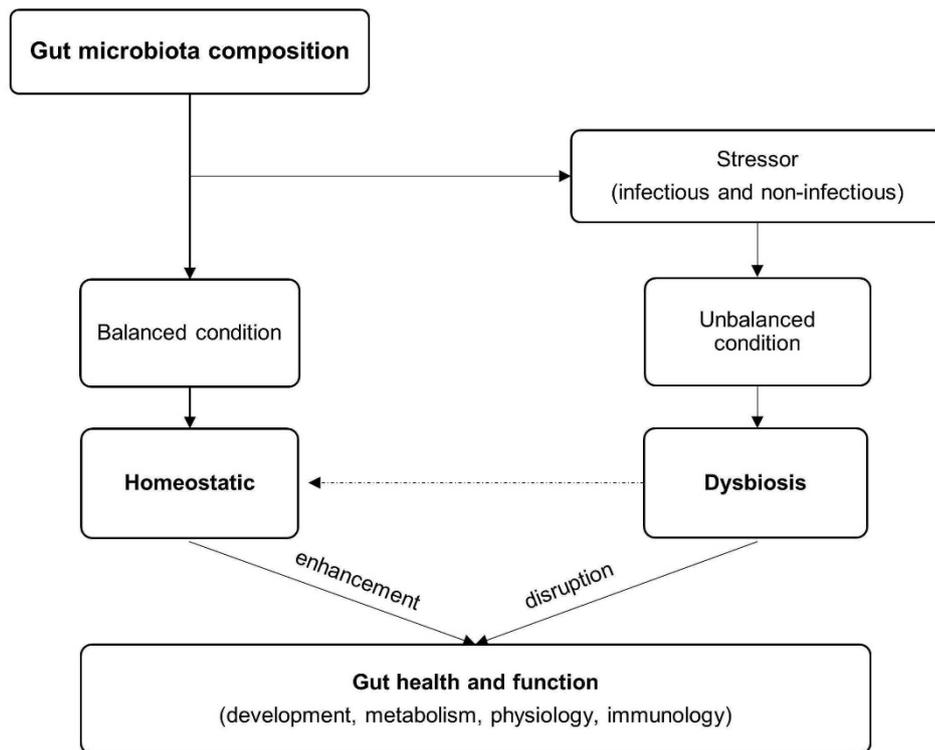
Figure 3. A flow chart describing the relationship between microbiota balance and gut health. This chart is constructed based on information from Shang et al. (2018), Forgie et al. (2019), and Kogut (2019).

In general, dysbiosis can be caused by two main factors, namely non-infectious and infectious factors. One of the main causes of non-infectious dysbiosis comes from dietary aspects, such

as changes in feed type and composition, nutrient content, or feeding pattern (Teirlynck et al., 2009; Stanley et al., 2013). For example, changes in the amino acid composition of dietary protein may alter the composition of the gut microbiota. Or high dietary protein can promote the overgrowth of protein-fermenting bacteria which can increase susceptibility to disease (Ma et al., 2017; Forgie et al., 2019; Fathima et al., 2022). Likewise, diets high in simple and refined carbohydrates or high in saturated fat have also been shown to affect gut microbial homeostasis, leading to gut barrier dysfunction and increased risk of infection (Thaiss et al., 2018; Forgie et al., 2019). Infection-induced dysbiosis results from the infiltration of toxic agents and/or pathogenic microbes such as bacteria, viruses, protozoa, and fungi. For example, *Eimeria* spp. and *Clostridium perfringens*, have been shown to disrupt the balance and homeostasis of the gut microbiota, with *Eimeria* replicating in gastrointestinal epithelial cells and *C. perfringens* producing toxins that damage epithelial cells in poultry (Broom and Kogut, 2018). In piglets, postweaning diarrhea is the most common disease in weaned piglets, primarily caused by pathogens or enterotoxins (Pluske et al., 2018). A previous study showed a disruption in the composition of the gut microbiota after weaning, which may lead to intestinal inflammation and ultimately slower growth rate (Dou et al., 2017).

2.6. The gut microbe-host crosstalk in chickens and piglets

Maintaining the balance of gut commensal microbes is essential to preserve the normal metabolic and physiological functions in farm animals. One mechanism by which the host recognizes the presence of commensal bacteria is through the regulation of pattern recognition receptors (PRRs), including: 1) toll-like receptors (TLRs), 2) nucleotide-binding oligomerization domain receptors (NODs), 3) C-type lectin receptors, and 4) retinoic acid-inducible gene 1-like receptors (Amarante-Mendes et al., 2018; Metzler-Zebeli et al., 2022a). This recognition mechanism subsequently provides appropriate responses according to specific microbe-derived ligands, such as peptidoglycan, lipoprotein, lipopolysaccharide and flagellin (Amarante-Mendes et al., 2018; Forgie et al., 2019). Likewise, commensal bacteria produce microbial fatty acids (FAs), i.e., short-chain fatty acids (SCFA), lactic acid (LA), and polyunsaturated fatty acids (PUFA), to interact with the intestinal epithelium to support specific intestinal metabolic and physiological functions (Silva et al., 2020; Gomez-Osorio et al., 2021; Metzler-Zebeli, 2021; Metzler-Zebeli et al., 2021a).

Short-chain fatty acids are the most important microbial fatty acids which are produced from the fermentation of polysaccharides, such as dietary fiber and (resistant) starch, and protein (Dai et al., 2011; Makki et al., 2018; Silva et al., 2020; He et al., 2020). Acetate, butyrate, and

propionate are the major SCFA that contribute significantly as energy substrates for intestinal epithelial cells, improve intestinal structure, and enhance defense mechanisms (De Vadder et al., 2014; Forgie et al., 2019; Vasquez et al., 2022). Promoting butyrate fermentation in the gut is attractive to researchers because it is the preferred energy source for enterocytes and stimulates intestinal epithelial cell differentiation and proliferation (Kien et al., 2007; Rinttilä and Apajalahti, 2013; Zhong et al., 2019). Some examples of butyrate-producing bacteria in pigs include *Ruminococcaceae, Faecalibacterium, Lachnospiraceae, Blautia, Roseburia, Clostridium* and *Eubacterium* (Gardiner et al., 2020; Vasquez et al., 2022).

Gut microbes also produce branched-chain fatty acids (BCFA), which are derived from the fermentation of branched-chain amino acids, such as valine, leucine, and isoleucine (Rist et al., 2013; Pieper et al., 2016). As a marker of protein fermentation, BCFA synthesis is has been negatively correlated with dietary fiber levels (He et al., 2017; Zhang et al., 2020a; Vasquez et al., 2022). Isobutyrate and isovalerate are considered as the major BCFA in the gastrointestinal tract (Rios-Covian et al., 2020; Vasquez et al., 2022). Bacteria such as *Clostridium, Propionibacterium, Streptococcus*, and *Bacteroides* are BCFA producers in pigs (Rist et al., 2013; Gardiner et al., 2020; Vasquez et al., 2022). In addition, isobutyric acid-producing taxa have also been positively correlated with feed efficiency, with higher abundances of *Christensenellaceae, Oscillibacter, Cellulosilyticum, Rothia, Subdoligranulum,* and *Leeia* observed in pigs with high feed efficiency (McCormack et al., 2017; Reyer et al., 2020).

Another important microbial metabolite is lactic acid (LA), which is mainly produced from carbohydrate fermentation (Gardiner et al., 2020; Vasquez et al., 2022). Lactic acid helps lower the pH of the stomach, thereby preventing the growth of pathogenic bacteria, such as *Escherichia coli* (Vasquez et al., 2022). In addition, lactic acid can be utilized by SCFA-producing microbiota to produce SCFA, particularly propionate and butyrate, via a metabolite cross-feeding mechanism (Brestenský et al., 2017; Vasquez et al., 2022).

The interaction between microbial FAs and the host is facilitated by the activation of host receptors, which are G protein-coupled receptors/free fatty acid receptors (GPRs/FFARs) and hydroxy-carboxylic acid receptors (HCARs) (Offermanns, 2017; Priyadarshini et al., 2018; Kimura et al., 2020; Metzler-Zebeli, 2021). Among the GPRs/FFARs family, GPR43/FFAR2 and GPR41/FFAR3 have been identified as the major receptors of SCFA which are expressed on white adipocytes, immune cells, and enterocytes (Brown et al., 2003; Mielenz, 2017; He et al., 2020). These two receptors also have different affinities for SCFA types in the following order: acetate=propionate>butyrate for FFAR2 and propionate=butyrate>acetate for FFAR3

(He et al., 2020). Because of the tremendous benefits of microbial FAs, it is important to further understand their signaling pathways in promoting gut homeostasis, especially in relation to transport systems, nutrient metabolism, gut motility and development, immune responses, and barrier functions.

2.6.1. SCFA-host signaling associated with transport system

Once microbial SCFA are produced by the commensal gut microbiota, they are then transferred to the intestinal cells by a specific transport system. Two main mechanisms of SCFA absorption that have been proposed: passive and active absorption. Passive transport is the transport of undissociated SCFA by diffusion across the cell membrane. Active transport of the dissociated SCFA anions occurs via several transporters, namely: 1) SCFA-HCO_3^- exchange, 2) SCFA-monocarboxylate transporters (MCTs), and 3) SCFA-sodium-coupled monocarboxylate transporters (SMCTs) (Vidyasagar et al., 2005; Sivaprakasam et al., 2017; Ali et al., 2022; Jadhav et al., 2022).

In the SCFA-HCO_3^- exchange transport mechanism, SCFA anions bind to HCO_3^- to form SCFA-HCO_3^-, followed by transporter-mediated exchange (Harig et al., 1991; 1996; Vidyasagar et al., 2005). The second SCFA transport mechanism involves monocarboxylate transporters (MCTs), which function as H^+-coupled electroneutral transporters (Ali et al., 2022; Jadhav et al., 2022). For instance, *MCT1* plays an important role in butyrate transport and showed high expression in lymphocyte cells (Hadjiagapiou et al., 2000; Ali et al., 2022). The third transport mechanism of SCFA anions across intestinal epithelial cells is carried out by sodium-coupled monocarboxylate transporters (SMCTs) (Takebe et al., 2005; Ali et al., 2022). Two types of SMCTs have been identified, namely *SMCT1* (electrogenic transport) and *SMCT2* (electroneutral transport). In pigs, *SMCT1* and *SMCT2* are expressed on the apical membrane of the small intestine (Metzler-Zebeli, 2021; Metzler-Zebeli et al., 2022a). Both *SMCT1* and *SMCT2* have different affinities for SCFA, with the corresponding Na^+ stoichiometry: SCFA = 2:1 and 1:1, thus *SMCT2* contributes more to lactate transport (Sivaprakasam et al., 2017). Furthermore, the affinity of *SMCT1* is highest for butyrate, followed by propionate and then acetate (Miyauchi et al., 2004; Iwanaga and Kishimoto, 2015).

2.6.2. SCFA-host signaling associated with nutrient metabolism

Microbial SCFA are also involved in nutrient signaling and metabolism, such as glucose, proteins, lipids, and electrolytes, via multiple signaling pathways, such as: 1) FFARs/FXR-glucagon-like peptide 1 (GLP1)/ peptide YY (PYY) pathway, 2) FFAR-AMP-activated protein

kinase (AMPK) pathway, 3) FFARs-cyclic adenosine monophosphate (cAMP)/ protein kinase A (PKA) pathway, and 4) FFARs-mitogen-activated protein kinase (MAPK)/ protein kinase C (PKC) pathway activation (Cantó and Auwerx, 2010; Flint et al., 2012; Rinttilä and Apajalahti, 2013; den Besten et al., 2013; He et al., 2020).

Accumulating evidence suggests that SCFA may modulate glucose and lipid metabolism by activating FFARs. Based on *in vitro* and *in vivo* experiments, SCFA-induced FFAR2 effectively regulates glucose and protein levels by producing several regulatory hormones, such as GLP1, insulin, and/or leptin, and by secreting peptide YY from SCFA-induced FFAR3 (Pingitore et al., 2019; Gardiner et al., 2020; He et al., 2020). Likewise, activation of SCFA-induced FFAR2 can also improve the uptake of electrolytes such as calcium (Ca^{2+}) (Kimura et al., 2014; He et al., 2020). In contrast, SCFA-induced GLP1 secretion can be inhibited by other factors, such as bile acid secretion (Ducastel et al., 2020). A recent study showed that activation of the farnesoid X receptor (*FXR*), a bile acid receptor, modulated SCFA-induced GLP1 secretion in human and mouse cell lines, as indicated by reduced FFAR2 gene expression (Ducastel et al., 2020).

Bile acids play an important role in lipid emulsification and digestion as well as in the absorption of fat-soluble vitamins (Hofmann, 2009; Lin et al., 2019; Lin et al., 2020; Vasquez et al., 2022). In general, there are different types of bile acids produced in the digestive tract for lipid metabolism, namely: primary, secondary, and tertiary bile acids (Bai et al., 2022; Shi et al., 2023). Primary bile acids are conjugated bile acids produced in the liver with taurine or glycine and are mostly reabsorbed in the distal ileum (Grüner and Mattner, 2021; Vasquez et al., 2022). A small fraction of primary bile acids is then converted to secondary and tertiary bile acids, such as deoxycholic acid and lithoxycholic acid, which are produced for instance via 7α-hydroxylation by *Clostridium* and *Eubacterium* (Zhan et al., 2020; Guo et al., 2020; Grüner and Mattner, 2021; Shi et al., 2023) or via 7β-dehydroxylation by specific gut bacteria (Ridlon et al., 2014; 2016). Primary and secondary bile acids can activate *FXR,* which is highly expressed in the small intestine and liver, and subsequently modulate lipid metabolism (Fiorucci et al., 2009; Ding et al., 2015; Grüner and Mattner, 2021). In addition, some bacterial genera such as *Lactobacillus*, *Clostridium*, *Bacteroides*, *Bifidobacterium*, and *Enterococcus* are involved in the production of free primary bile acids via bile salt hydrolases by deconjugating taurine or glycine from conjugated bile acids (Wahlström et al., 2016; Lin et al., 2019; Lin et al., 2020; Vasquez et al., 2022). Several studies had also shown that microbial hydrolysis of bile acids indirectly regulated the lipid homeostasis by lowering serum cholesterol and triglyceride levels (Begley et al., 2006; Shokryazdan et al., 2017; Vasquez et al., 2022).

This clearly indicates that changes in the gut microbiota can lead to altered bile acid composition in the gut and consequently affect bile acid signaling and digestion.

The second SCFA-induced metabolic signaling pathway is through regulation of the AMP/ATP ratio via activation of AMP-activated protein kinase (AMPK) (He et al., 2020; Tang and Li, 2021). This pathway affects lipolysis by regulating the expression of key lipolytic enzymes, such as hormone-sensitive lipase and adipose triglyceride lipase (Cantó and Auwerx, 2010; Tang et al., 2020). In addition, AMPK activation also modulates glycogen and protein synthesis and glucose transport, thereby affecting protein and glucose levels (Hardie, 2004; He et al., 2020). According to other previous studies, SCFA-induced AMPK activation may contribute to lipid metabolism by increasing the expression of peroxisome proliferator-activated receptor-gamma coactivator -1alpha in adipose tissue (Mollica et al., 2017; Tang and Li, 2021). In this context, this receptor regulates the transcriptional activity of peroxisome proliferator-activated receptors (Jäger et al., 2007; He et al., 2020). Decreased plasma levels of cholesterol, triglycerides, or free fatty acids resulting from inhibited intracellular lipolysis suggest a regulatory effect of SCFA on lipid metabolism via GPRs signaling (He et al., 2020).

The next signaling mechanism that also contributes to the regulation of lipid metabolism is the FFAR-cAMP/PKA pathway (He et al., 2020). Here, once stimulated by SCFA, FFARs then couple with beta receptors, such as the beta-adrenergic receptor, to generate cAMP through activation of adenylyl cyclase. This is then followed by the activation of cAMP/PKA, which leads to the phosphorylation and activation of hormone-sensitive lipase. Finally, this enzyme catalyzes the breakdown of triglycerides and diglycerides, resulting in the release of free fatty acids and glycerol (Carmen and Víctor, 2006). The last potential signaling pathway associated with SCFA-induced lipid metabolism is the activation of protein kinase C (PKC), which is stimulated by phorbol ester PMA both independently and dependently via mitogen-activated protein kinase (MAPK). It is suggested that activation of the MAPK/PKC pathway may stimulate the hormone-sensitive lipase activity to metabolize lipids (Langfort et al., 2003).

2.6.3. SCFA-host signaling associated with gut histomorphology and motility

SCFA contribute significantly to the development of intestinal structure and motility. Basically, the development of intestinal structure is closely related to epithelial cell proliferation and highly influenced by the activity of intestinal microbiota (e.g., *Lactobacillaceae* and *Ruminococcaceae*) through the production of SCFA (Zhong et al., 2019; Martin-Gallausiaux et al., 2021; van der Hee and Wells, 2021; Vasquez et al., 2022). Several studies had shown that SCFA, especially butyrate, effectively improved intestinal histomorphological measures, such

as increased villus height and decreased crypt depth, thereby improving nutrient absorption (Liu, 2015; Park et al., 2016; Shang et al., 2018). Likewise, butyrate also has been shown to have another beneficial effect in the form of reduced apoptosis (Liu, 2015). In addition, MCFA and PUFA are also known to be involved in improving intestinal histomorphology. For example, Dierick et al. (2004) reported that administration of MCFA to weaned pigs significantly increased villus length in the small intestine, combined with decreased crypt depth and intraepithelial lymphocyte count. Similarly, Liu et al. (2012) demonstrated that weaned piglets supplemented with long-chain (n-3) PUFA had greater villus height and villus height: crypt depth ratio.

In addition to influencing gut histomorphology, SCFA play a role in the regulation of gut motility, which aids in the transfer of feed material along the gastrointestinal tract. Growing evidence suggests that gut motility is influenced in part by SCFA through multiple signaling pathways, such as 1) the FFARs-cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway, and 2) the FFARs-peptide YY (PYY) pathway (Forbes et al., 2015; Blakeney et al., 2019; Gardiner et al., 2020). A study investigating gut motility showed that branched-chain fatty acids, i.e., isovaleric acid, can modulate the contractibility of lower intestinal smooth muscle cells via the GPRs-activated cAMP/PKA pathway (Blakeney et al., 2019). SCFA affect gut motility by releasing intestinal peptides, especially PYY (Cherbut et al., 1997; Yuan et al., 2020). Studies have shown that SCFA could stimulate the secretion of PYY by activating FFAR, resulting in the inhibition of motility in the lower part of the intestinal tract (Cherbut et al., 1997; 1998; Psichas et al., 2015).

2.6.4. SCFA-host signaling associated with gut immune response

Gut commensal microbiota play an important role in modulating host innate and adaptive immune responses (Read and Holmes, 2017). This modulatory effect can be achieved through several potential SCFA-activated signaling pathways, including: 1) FFARs-PRRs-NF-kB pathway, 2) FFARs-MAPK pathway, 3) FFARs-histone deacetylases (HDACs) pathway, and 4) SMCTs-HDACs pathway (Wu et al., 2012; Sun et al., 2017).

The host recognizes harmful and non-harmful agents at the intestinal mucosa via the activation of PRRs, including Toll-like receptors (TLRs) and nucleotide binding oligomerization receptors (NODs) (Perry and Arsenault, 2022). Upon activation by their respective ligands, these receptors initiate inflammatory responses, for example, via NF-κB and MAPK pathways (He et al., 2020). Among various ligands, lipopolysaccharide (LPS) on the cell wall of Gram-negative bacteria is an inflammatory stimulator. After LPS induces TLRs (e.g., *TLR4*/*TLR2*) in epithelial

and immune cells, the NF-κB or MAPK pathway is initiated, leading to the release of various gut inflammatory chemokines and cytokines, such as tumor necrosis factors (*TNFs*), interleukins (*ILs*), and interferons (*IFNs*) (Liu and Malik, 2006; Park and Lee, 2013; He et al., 2020). While LPS induces inflammation through the TLR signaling, SCFA blocks LPS-induced inflammation through different pathways, i.e., GPRs/FFARs and HDACs pathways. Several studies also demonstrate that activation of SCFA-induced FFARs, especially acetate, propionate and butyrate, can inhibit NF-kB and MAPK signaling pathways and subsequently suppress the intestinal expression of pro-inflammatory of *TNFα, IL1β, IL4, IL5*, and *IL6* (Nakajima et al., 2017; Pirozzi et al., 2018; Xu et al., 2019; Metzler-Zebeli et al., 2022a), and upregulates anti-inflammatory *IL10* expression from Treg cells (Zeng and Chi, 2015; Sakaguchi et al., 2008; Vasquez et al., 2022). Likewise, BCFA also show ameliorative effects on the pro-inflammatory cytokines *TNFα* and *IFNγ* in pigs (Boudry et al., 2013; Vasquez et al., 2022). Moreover, SCFA, especially butyrate, can stimulate B cells to produce secretory immunoglobulin A to counteract harmful microbial infections (Liu et al., 2021). Apart from inhibiting NF-κB and MAPK activity through the LPS-induced TLRs pathway, SCFA are also considered to reduce inflammation by inhibiting HDACs activity, either via stimulation of FFARs or transported SMCTs (Chang et al., 2014; Gurav et al., 2015; Sun et al., 2017). Among the SCFA, butyrate has been extensively studied, and its ability to inhibit HDACs is greater than that of propionate and acetate (Davie, 2003; Thangaraju et al., 2009). The host immune response is also modulated by the activation of lactic acid-induced HCARs. A study by Shin et al. (2017) revealed that activation of HCARs effectively suppressed macrophage migration to reduce inflammation.

2.6.5. SCFA-host signaling associated with gut barrier function

SCFA are also considered to have an essential role in improving intestinal barrier function. Mechanistically, microbiota colonizing the gastrointestinal tract, either gram-positive or gram-negative, are recognized by host receptors, i.e., TLRs. After activating the TLRs-NF-kB signaling pathway, microbes stimulate mucus and antimicrobial secretion, mucin (MUC) transcription, and regulate tight junction protein (TJP) expression in epithelial cells (Hedemann et al., 2009; Groschwitz and Hogan, 2009; Liu et al., 2021). Concurrently, SCFA control these signaling pathways after initially stimulating fatty acid-specific receptors, i.e., FFARs or HCARs (Priyadarshini et al., 2018; Metzler-Zebeli et al., 2022a).

As the first line of defense, the epithelial mucus layer provides a lubricant that helps trap invasive bacteria or toxins and facilitates their removal through the luminal flow (Zhang et al.,

2015; Broom and Kogut, 2018). The intestinal mucus layer is largely composed of mucins, which are secreted by mature goblet cells (Broom and Kogut, 2018). Several types of mucins have been identified in the gastrointestinal tract of chickens and pigs, but the most studied are mucin-2 (*MUC2*) and mucin-4 (*MUC4*) (Deplancke and Gaskins, 2001; Chen et al., 2015). Mucin secretion along the intestinal epithelium has been found to increase distally, which appears to reflect an increase in the microbial population (Broom and Kogut, 2018). Several studies showed that SCFA, especially butyrate, strongly influenced mucus secretion and mucin transcription in the intestinal epithelial cells (Shimotoyodome et al., 2000; Willemsen et al., 2003; Hedemann et al., 2009).

Another potential mechanism by which SCFA improve gut integrity is by regulating the expression of tight junction proteins, mainly claudins (*CLDN*), occludins (*OCLN*), and zonula occludens (*ZO*) (Turner, 2009; Groschwitz and Hogan, 2009). *CLDN* and *OCLN* are major transmembrane proteins that form a selective paracellular barrier, whereas *ZO* is a major cytoplasmic protein located at the peripheral membrane (Robinson et al., 2015). Studies have shown that SCFA such as butyrate and propionate, could upregulate intestinal TJPs, such as *CLDN1, CLDN3, CLDN4, OCLN,* and *ZO1* in pigs (Grilli et al., 2016; Diao et al., 2019). Likewise, a recent study by Metzler-Zebeli et al. (2022a) revealed that SCFA modulated the expression of *OCLN*, *CLDN4,* E-cadherin and junction adhesion molecule-like protein in the jejunum of porcine fetuses. Futhermore, the increased SCFA-activated barrier function may also reduce the risk of cell death, known as apoptosis. It is important to note that LPS-induced dysbiosis is one of the causes of apoptosis (Negroni et al., 2015). The reducing effect of SCFA on intestinal apoptosis can be identified from the expression of apoptosis inhibitory proteins (Ruemmele et al., 2003). Several studies have also suggested that SCFA, especially butyrate, play an important role in modulating the apoptosis inhibitory protein expression in epithelial cells (Meng et al., 1999; Ruemmele et al., 2003).

2.6.6. SCFA-host signaling associated with oxidative status

It is widely assumed that oxidative stress is one of the major causes of the adverse consequences of stress in poultry and pigs. In response to this oxidative stress, the antioxidant defense system uses several mechanisms to restore a homeostatic state, one of which is to increase the production of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase. In addition to the diet, LPS-induced dysbiosis has also been identified as another factor affecting oxidative stress in poultry, characterized by decreased expression of antioxidant enzymes (Surai et al., 2019). To mitigate these negative effects, microbially

derived SCFAs play an important role in regulating oxidative status by modifying the production and activity of antioxidant enzymes. This is supported by several studies in mice showing that SCFA effectively suppressed oxidative stress induced by either LPS or other stressors by modulating the activity of the antioxidant enzymes (Ranganna et al., 2014; Liu et al., 2017; Huang et al., 2017).

2.7. Application of Ussing chamber and organ bath to investigate SCFA-host signaling

The Ussing chamber and organ bath are two *ex vivo* techniques that can be implemented in animal studies and provide valuable insights into intestinal permeability and muscle contractibility in many animal species (Blakeney et al., 2019; Baskara et al., 2021). Technically, the Ussing chamber technique allows the measurement of electrophysiological parameters, including short-circuit current and tissue conductance as main indicators for net ion flux and tissue permeability (Metzler-Zebeli et al., 2017a; Baskara et al., 2021). In the measurement process, the intestinal mucosal layer is commonly used, whereas the outer serous layers (*Tunica serosa* and *Tunica muscularis*) are often stripped away, depending on the thickness of the serosa itself (Baskara et al., 2021). For calculation, the short-circuit current is determined as the net amount of electrogenic charge transfer across the epithelium, whereas the tissue conductance is calculated as the inverse of the transepithelial resistance (Baskara et al., 2021). To date, this technique has been increasingly used to investigate the microbial transport of fatty acids, such as short-, medium-, and long-chain fatty acids, by various monocarboxylate transporters, such as the sodium-dependent monocarboxylate transporters (SMCTs) or the monocarboxylate transporters (MCTs) in porcine intestine (Metzler-Zebeli et al., 2021b; 2022a; Lerch et al., 2022). It was also found that SCFA treatment can modify the short-circuit current and tissue conductance in the jejunum of fetal pigs (Metzler-Zebeli et al., 2022a).

The organ bath is an *ex vivo* technique that helps to study gut contractibility by measuring changes in intestinal muscle tension (Cai, 2015; Baskara et al., 2021; Metzler-Zebeli et al., 2021b). Briefly, the organ bath is equipped with force transducers, which are connected to a four-channel bridge amplifier, and data are collected using a kymograph data acquisition software (Baskara et al., 2021; Metzler-Zebeli et al., 2021b; Lerch et al., 2022). It is important to note that, the viability of intestinal cut samples must be ensured prior to measurement, one of the ways being the addition of acetylcholine (Baskara et al., 2021; Metzler-Zebeli et al., 2021b). The contraction or relaxation of the intestinal muscles is recorded as a positive or negative change in tension (Metzler-Zebeli et al., 2021b). According to a recent study using this technique, the addition of SCFA reduced muscle contraction in fetal porcine jejunal tissue

(Metzler-Zebeli et al., 2022a). Likewise, a study by Blakeney et al. (2019) showed that the branched-chain fatty acid, i.e., isovaleric acid, caused muscle relaxation in the rat colon. To date, information on the modulatory effects of microbial fatty acids (e.g., SCFA/BCFA and lactic acid) on host mucosal signaling using these two *ex vivo* techniques is still limited, both in pigs and chickens, hence more experiments are required to gain a broader understanding.

2.8. Probiotics as an alternative to antibiotics and their role in modulating gut microbes

For decades, antimicrobial growth promoters (AGPs) in feed have been used intensively in chicken and pig farms due to their benefits in improving the growth and performance of these monogastric animals. However, since the emergence of antibiotic-resistant gut microbes and residues in animal products, the use of antibiotics in feed has been restricted in many countries (Gadde et al., 2017). Under these conditions, probiotics have been recommended as alternative feed additives to replace antibiotics (Fathima et al., 2022). Indeed, some evidence suggests that AGPs effectively inhibit the growth and proliferation of pathogenic microbes and prevent toxic interference (Gaskins et al., 2002; Gadde et al., 2017). However, AGPs also have some drawbacks, such as reducing the total number and diversity of gut microbes (Alagawany et al., 2018; Forgie et al., 2019), which indirectly affects the disruption of the gut microbial balance. Furthermore, there is some evidence that AGPs have the potential to disrupt the production of antimicrobial peptides, tight junction proteins, and immune factors due to gut microbial imbalance (Gadde et al., 2017; Forgie et al., 2019; Raheem et al., 2021). Probiotics are considered to address these drawbacks, where they can eliminate pathogenic bacteria without disrupting the balance of the gut microbial community (Jha et al., 2020).

In recent years, the use of probiotics as feed additives has increased rapidly in both poultry (Raheem et al., 2021; Shini and Bryden, 2021) and swine (Barba-Vidal et al., 2018; Zhang et al., 2023) farms. Probiotics are defined as live microorganisms that provide health benefits to the host when administered in adequate cell counts (Yang et al., 2018; Chen et al., 2022a). Probiotics can be administered as a single or multiple strains/species with a combination of different microorganisms such as bacteria and yeast (Neveling and Dicks, 2021). Some genera of bacteria commonly used as probiotics in studies include *Lactobacillus* spp., *Bacillus* spp., *Bifidobacterium* spp., *Pediococcus* spp., *Streptococcus* spp., *Paenibacillus* spp., and *Enterococcus* spp., whereas the genus for yeast is *Saccharomyces* spp. (Barba-Vidal et al., 2018; Markowiak and Śliżewska, 2018; Neveling et al., 2020). Studies in chickens and piglets have shown that probiotics can restore gut microbiota homeostasis by inhibiting pathogen

invasion and promoting the growth of beneficial bacteria. For instance, administration of *Bacillus*-based probiotics increased the numbers of *Lactobacillus, Bifidobacterium* and *Bacillus* and reduced the colonization of *Escherichia coli* and *Salmonella* spp. in the small intestine and ceca of broiler chickens at either the starter or finisher phase (Zhen et al., 2018; Arif et al., 2021; Wang et al., 2021c). Similarly, supplementation of lactic acid-based probiotics, such as *Lactobacillus* spp*., Bifidobacterium* spp. and *Enterococcus* spp., has been shown to suppress the development of the pathogens *Campylobacter* spp. and *Salmonella* spp. in the ceca of broiler chickens (Ghareeb et al., 2012; Prado-Rebolledo et al., 2017; El-Sharkawy et al., 2020). In piglets, the provision of lactic acid-based probiotics or *Bacillus*-based probiotics in the diet can modify the abundance of certain bacteria, such as *Lactobacillus*, *Clostridium, Peptococcus, Streptococcus, Erysipelotrichaceae, Coprococcus,* and *Oscillibacter* either in the intestinal digesta or feces of weaned piglets (Upadhaya et al., 2017; Shin et al., 2019; Xin et al., 2020; Moturi et al., 2021; Lee et al., 2022; Pang et al., 2022) and inhibit the development of pathogenic bacteria such as *Escherichia coli* and *Salmonella* spp. (Trevisi et al., 2011; Hu et al., 2014; Guerra-Ordaz et al., 2014; Naqid et al., 2015). Consequently, probiotics can reduce the incidence of diarrhea in weaned piglets (Pupa et al., 2022).

2.9.   Potential action mode of probiotics in promoting gut health in chickens and piglets

As previously mentioned, probiotics exert health-promoting effects on the host by balancing the gut microbial composition and inhibiting pathogens. This positive effect is inseparable from the favorable effects of probiotics in supporting the growth of beneficial microbes and their ability to produce antimicrobial substances and fermentable metabolites, such as SCFA/BCFA and lactic acid (LeBlanc et al., 2017; Oh et al., 2021; Ding et al., 2021; Vasquez et al., 2022). In addition, there is growing evidence that probiotics can improve intestinal barrier function and immune function in both chickens and piglets, whether challenged with pathogens or not, and can effectively alleviate postweaning diarrhea in piglets (Su et al., 2022). Although there have been many studies investigating the effects of probiotics on gut microbial colonization and intestinal development have been intensively conducted. However, these observations have focused on the postweaning period, whereas their effects during the suckling period have not been elucidated. Therefore, opportunities for further research are wide open.

To date, much research is being conducted to discover the possible mechanisms by which probiotics may improve gut function and prevent diarrhea. According to the results of previous studies, improved immunity and gut integrity after probiotic supplementation may be correlated with increased concentrations of gut microbial fatty acids, especially SCFA (Sakata et al., 2003;

Wang et al., 2019; Peng et al., 2022). Such microbial fatty acids can trigger ligand-sensing host receptors and initiate various intracellular signaling pathways to overcome dysbiosis and restore intestinal homeostasis (Raheem et al., 2021).

Therefore, to understand how probiotics act in maintaining gut homeostasis and overcoming LPS-induced dysbiosis, it is necessary to further undestand the possible signaling pathways from different types of probiotics to the host. In the following, probiotics were categorized into 4 groups, namely: 1) lactic acid-based probiotics, 2) *Bacillus*-based probiotics, 3) *Clostridium*-based probiotics, and 4) yeast-based probiotics.
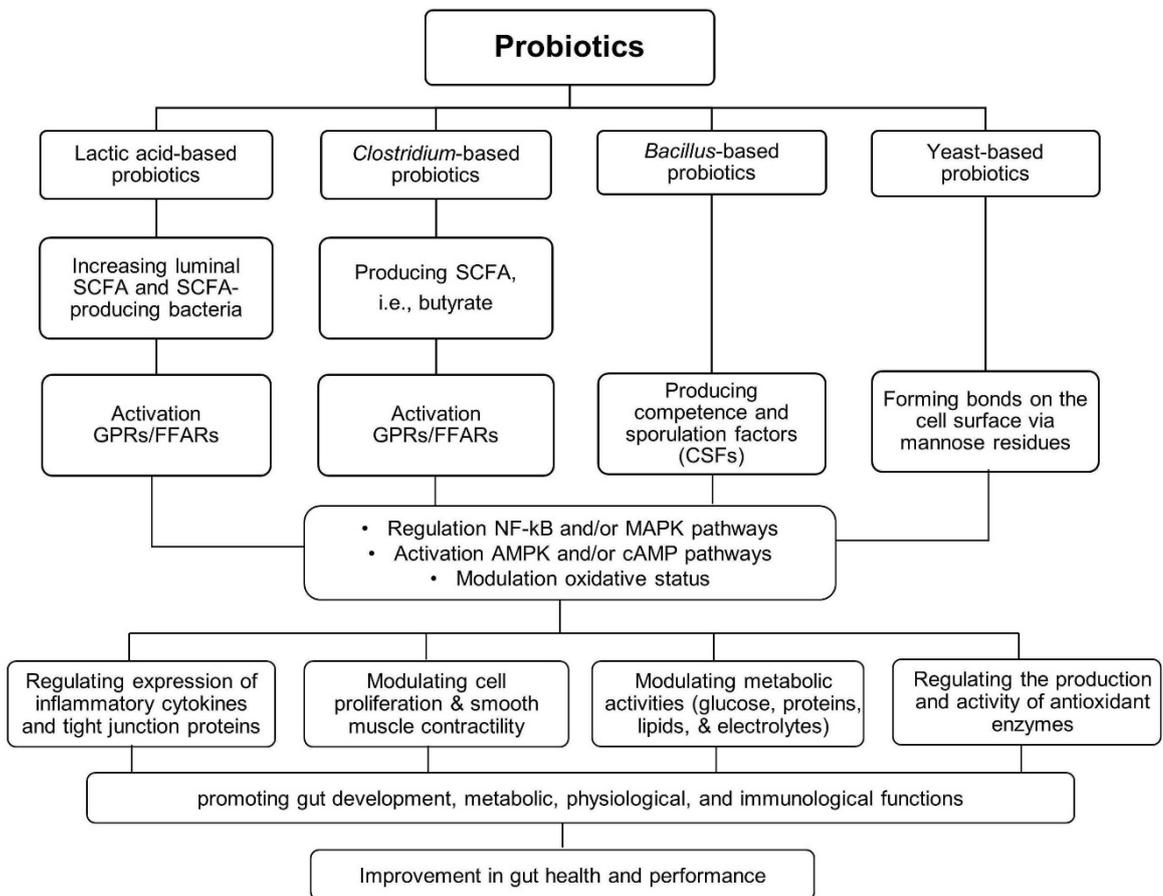
Figure 4. A flow chart showing the potential action mode of probiotics in improving gut health and performance in chickens and piglets.

2.9.1. Lactic acid-based probiotics

Lactic acid-based probiotics are a type of probiotics that are widely used in research. Some of the bacterial genera included in this type of probiotics are *Lactobacillus*, *Enterococcus,*

Bifidobacterium, Pediococcus, and *Streptococcus.* These probiotics can produce lactic acid as their main fermentation product, which functions to lower the lumen pH, thus potentially inhibiting pathogens (Wu et al., 2018). In addition to lactic acid production, this type of probiotic can also increase luminal SCFA concentrations. For example, several studies have shown that administration of *Lactobacillus* spp. can increase the concentrations of lactic acid and SCFA, but not BCFA, including total SCFA, acetate, propionate and/or butyrate in the digesta and feces of grower-finisher broilers, either challenged or not with pathogens and mycotoxins (Wu et al., 2018; Wang et al., 2021a; Chen et al., 2022c). Likewise, a study by Barba-Vidal et al. (2017a) also showed that weaned piglets receiving *Bifidobacterium* spp. had higher levels of total SCFA in the colon when challenged with pathogens. These results clearly indicate that the mechanism of lactic acid-based probiotics in overcoming pathogen-induced dysbiosis is by producing lactic acid and increasing SCFA levels. In this regard, the increase in SCFA concentrations is likely due to the enrichment of SCFA-producing microbes in the gut, in which these microbes utilize lactic acid to convert to SCFA through a cross-feeding mechanism (Vasquez et al., 2022). A study by Wang et al. (2020) showing that broilers supplemented with *Enterococcus faecium* had a higher abundance of butyrate-producing bacteria, such as *Ruminococcaceae* and *Eubacterium*.

Once produced, SCFA are thought to activate GPRs/FFARs and subsequently inhibit the LPS-induced NF-κB and MAPK pathways (He et al., 2020; Vasquez et al., 2022). This, in turn, regulates pro- and anti-inflammatory cytokines, modulates the expression of TJPs, and increases antioxidant enzyme levels to counteract gut inflammation (Liu et al., 2021; Ali et al., 2022). As confirmed by previous studies, dietary administration of lactic acid-based probiotics, e.g., *Enterococcus* spp. or *Pediococcus* spp., significantly increased the expression of first line of defense genes, e.g., *MUC2* and *CLDN1*, as well as upregulated *IL10* and downregulated pro-inflammatory cytokines, such as *NF-κB, TNFα, TLR4, IL1β, IL4*, and *IL8* in broiler chickens challenged with pathogens, such as *E. coli*, *Salmonella* spp. and *Clostridium perfringens* (Huang et al., 2019; Wu et al., 2019; Ateya et al., 2019; Lan et al., 2020). Similar findings were also found in piglet studies, showing that *Lactobacillus spp.* improved intestinal morphology, antioxidant enzymes, and TJPs (e.g., *OCLN, ZO1,* and *CLDN1*) and increased the expression of anti-inflammatory *IL10*, but decreased pro-inflammatory cytokines (e.g., *TNFα* and *IL1β*) in weaned piglets challenged with or without LPS (Li et al., 2019; Sun et al., 2020; Chen et al., 2020). It has also been reported that *Lactobacillus* spp. supplementation effectively reduced diarrhea scores in weaned piglets (Dowarah et al., 2017).

2.9.2. *Clostridium*-based probiotics

The following groups of probiotics are *Clostridium*-based probiotics, of which *Clostridium butyricum* is the most used species in studies (Guo et al., 2020). *C. butyricum* produces butyrate and acetate, thereby contributing to the intestinally produced SCFA concentrations in the gut. In terms of mode of action, these probiotics mainly affect the host by activating the butyrate/SCFA-stimulated FFARs/GPRs in the epithelial cells and then blocking LPS-induced TLRs-NF-κB signaling pathway to improve gut immunity and integrity (Guo et al., 2020). Apart from the NF-κB pathway, it has also been suggested that *C. butyricum*-derived butyrate/SCFA may affect gut development by modulating the AMP-activated protein kinase (AMPK) or Akt signaling pathway (Yan and Ajuwon, 2017; Mishra et al., 2020). According to previous studies, *C. butyricum* supplementation markedly increased cecal SCFA content in broiler chickens and subsequently improved innate immune response, barrier function, and oxidative status, as indicated by downregulation of pro-inflammatory cytokines (e.g., *IL1β, IL6,* and *TNFα*) as well as increasing TJPs (e.g., *OCLN* and *ZO1*) and antioxidant enzymes (e.g., *SOD* and *GPx*) in the small intestine (Liao et al., 2015; Li et al., 2021; Liu et al., 2022). Similarly, *C. butyricum* has been shown to suppress the growth of pathogens, such as *E. coli*, *Salmonella* and *Clostridium perfringens* in the grower-finisher broilers (Yang et al., 2012). In addition, dietary administration of *C. butyricum* increased the levels of butyric acid, propionic acid, acetic acid, and total acid in the cecum of weaned piglets. At the same time, *C. butyricum* also improved intestinal morphology, increased first-line defense genes (e.g., of *MUC1, MUC4, OCLN, ZO1, ZO2,* and *CLDN1*), modulated genes related to the immune system (e.g., *IL1β, TNFα, IL6, IL8*, and *1L10*), and decreased the rate of diarrhea in weaned piglets (Zong et al., 2019; Fu et al., 2021).

2.9.3. *Bacillus*-based probiotics

Another type of probiotic that has been extensively studied is *Bacillus*-based probiotics, including *B. coagulans, B. subtilis, B. pumilus, B. amyloliquefaciens,* and *B. licheniformis*. Unlike lactic acid- or *Clostridium*-based probiotics, *Bacillus* spp. probiotics are considered to exert immunoregulatory effects involving not only through fermentation acids, but also via the production of quorum-sensing peptides, such as competence and sporulation factors (CSF) (Solomon et al., 1996; Lazazzera et al., 1997). CSF is a key molecule that is crucial for interbacterial communication and for bacterial proliferation and sporulation (Tam et al., 2006; Okamoto et al., 2012). To improve host immune function, CSF is proposed to activate the MAPK pathways, i.e., Akt and p38 MAPK, and subsequently increase the expression of anti-

inflammatory of *IL10*, and decrease some pro-inflammatory mediators, such as *IL4, IL6,* and *TNFα* in the intestinal cells (Okamoto et al., 2012). In addition, CSF has been shown to protect epithelial cells from oxidative stress (Okamoto et al., 2012; Suva et al., 2016). According to studies in chickens, dietary supplementation of *Bacillus* spp., such as *B. coagulans*, *B. pumilus* and *B. subtilis*, could enhance the immune response as indicated by an increase in *IL10* and a decrease in *IFN-γ* expression (Zhen et al., 2018; Bilal et al., 2021). These probiotics also increased the abundance of SCFA-producing bacteria in the ceca, such as *Lactobacillus* and *Clostridium*, and suppressed the growth of pathogenic *E. coli* and *Salmonella*. The similar response was also observed in piglet studies where supplementation of probiotic *Bacillus* spp., i.e., *B. licheniformis* and *B. subtilis*, could modify SCFA/BCFA levels and regulate the expression of pro-inflammatory cytokines, such as *TLR4, IL2, IL6, IL1β,* and *TNFα* in both the small and large intestine of weaned piglets (Wang et al., 2021b). *Bacillus* spp. were also able to increase the number of *Lactobacillus*, suppress pathogens, i.e., *Salmonella* spp. and *E. coli*, and reduce the incidence of diarrhea in piglets after weaning (Hu et al., 2014; Barba-Vidal et al., 2017b; Sun et al., 2022).

2.9.4. Yeast-based probiotics

The last group of probiotics are yeast-based probiotics, including *Saccharomyces cerevisiae* and *Saccharomyces boulardii. Saccharomyces* spp. probiotics have been shown to effectively suppress intestinal inflammation by binding to specific pathogens or toxins via mannose residues on their cell surface. This formed binding has the potential to inhibit MAPK and/or NF-kB signaling pathways which are induced by LPS-producing pathogens such as *E. coli* and *Salmonella* spp. or by *Fusarium*-derived mycotoxins (Zanello et al., 2011; Martins et al., 2011; Chang et al., 2017). In addition, supplementation with *Saccharomyces*-based probiotics can increase the abundance of short-chain fatty acid-producing bacteria (Kiros et al., 2019). It has been reported that inclusion of *Saccharomyces* spp. in the diet of chickens could improve histomorphological measures, increase the abundance of beneficial bacteria, i.e., *Enterococcus* and *Brevibacillus*, and inhibit *E. coli*-induced upregulation of proinflammatory *TLR4, NF-κB,* and *IL1β* in the small intestine (Wang et al., 2016). A similar response was also observed in weaned piglets, where the administration of dietary *Saccharomyces* spp. increased the concentrations of SCFA, i.e., isobutyrate and valerate, decreased the pro-inflammatory of *IL6* and *TNFα*, and improved the antioxidant capacity, in both the small and large intestine (Zanello et al., 2011; Zhang et al., 2020a). In addition, the incidence of diarrhea

in weaned piglets was significantly reduced after receiving *Saccharomyces* spp. in their diet (Zhang et al., 2020a).

2.10. Factors influencing the efficacy of probiotics

The use of probiotics as an alternative to antibiotics in chicken and pig production has become increasingly popular in recent decades (Jha et al., 2020; Abd El-Hack et al., 2022). However, results obtained from several studies show that the effects of probiotics on gut health and performance parameters are still inconsistent. The inconsistency of the results observed in different studies may be due to several factors, including: 1) the type and dose of probiotics used (e.g. strain/species, single/multi-species), 2) the age of the animals (e.g. suckling or weaning phase for piglets; starter, grower, or finisher phase for chickens), 3) the duration of probiotic supplementation, 4) the diet provided (e.g., feed type and composition, nutrient content, and feeding pattern), and 5) the health status of the animals (e.g. healthy or pathogen-infected state) (Lambo et al., 2021; Vasquez et al., 2022).

The type and dosage of probiotics is considered as a crucial factor in influencing the efficacy of probiotics (Lambo et al., 2021). Several studies have shown that different probiotic species or strains are revealed to have different capacities to promote gut health and livestock production (Wang and Gu, 2010; Al-Shawi et al., 2020). In addition, multi-species or strain probiotics exert greater effects and benefits compared to mono-species or strain probiotics (Timmerman et al., 2004; Lambo et al., 2021). Different combinations in multi-species or strain probiotics have also been shown to have different modulatory effects on growth performance, intestinal histomorphology, immune response, and the ability to inhibit pathogen colonization of the gastrointestinal tract of chickens and piglets (Lu et al., 2018; Neveling and Dicks, 2021). The use of low and high doses of probiotics also showed differential expression of TJPs and microbial abundance in the small and large intestine (Li et al., 2012; Bilal et al., 2021). The efficacy of probiotics is also strongly influenced by age, which may be related to differences in gut microbial abundance and diversity between young and old animals (Ocejo et al., 2019). The duration of probiotic administration is also another factor to consider as it determines the efficacy of the probiotics (Vasquez et al., 2022). Different results have been observed in several variables between short-term and long-term administration, such as the expression of genes related to the gut barrier and immune response, histomorphology, and growth performance (Musa et al., 2019; Kan et al., 2021).

2.11. Meta-analytical approach to address inconsistency in probiotic studies

There are many qualitative reviews on the effect of probiotics in chickens and pigs (Shini and Bryden, 2021; Barba-Vidal et al., 2018). However, the different results between the original studies due to changes in direct and indirect factors cannot be considered in this way (Sales, 2014). In this regard, conducting a meta-analysis is considered the most appropriate method to address this complexity by generalizing the overall treatment effect (Metzler-Zebeli et al., 2019a). This method allows data from existing randomized controlled animal trials to be statistically combined, and the results can then be summarized under different conditions. In recent years, the trend towards meta-analysis studies has grown rapidly in various fields of study. In animal science, meta-analysis is mainly concerned with the relationship between quantitative variables to predict the average response of a dependent variable in an experiment, to one, or more, independent variables or covariables (Sauvant et al., 2020).

In general, there are several main steps in conducting a meta-analysis, namely, 1) literature search, 2) database construction, and 3) data analysis (Metzler-Zebeli et al., 2017c; 2019a). Literature search is the first step, in which all relevant articles published in scientific journals are searched and collected using search engines. Once collected, studies are selected according to strict criteria, which is then followed by database construction. In this step, a minimum requirement of 3 studies and 10 single observations (treatment means) along with the standard error for each dependent variable is determined as a prerequisite for estimating the combined effect size (Lipsey and Wilson, 2001; Metzler-Zebeli et al., 2017c; 2019a).

The next step is data analysis, where descriptive statistics are first performed on the predictor and dependent variables. The data on the dependent variables are then subjected to mixed modeling analysis. To avoid positive correlation between intercepts and slopes, an unstructured variance-covariance matrix is used (St-Pierre, 2001). To account for unequal variance across studies, the dependent variable is weighted by the inverse of the square of the standard error (Metzler-Zebeli et al., 2017c; 2019a). The estimates, root mean square error and coefficient of determination are also calculated and used to assess the fit. To obtain a more precise prediction of the factors affecting the dependent variable that are influenced by the independent variable, backward elimination analysis is used (Metzler-Zebeli et al., 2017c; 2019a). In addition, overparameterization of the model is considered with a variance inflation factor of less than 10 for each independent variable tested (Metzler-Zebeli et al., 2019a).

3. AIMS AND HYPHOTHESIS OF THE STUDY

According to the thoughts and considerations presented in the introduction part, some experiments were conducted in this PhD thesis, which were divided into three parts. The first part included a short-chain fatty acids (SCFA) experiment to clarify SCFA-host signaling in the jejunum and ceca of laying hens using *ex vivo* models. The second part was to assess the modulating effects of probiotics on gut mucosal gene expression, histomorphology, and growth performance in broiler chickens using a meta-analytical approach. The third part was to investigate the mother-offspring axis in establishing gut microbial colonization in piglets throughout the suckling and early postweaning periods using an *in vivo* model. The detailed objectives and hypotheses of the three experiments are as follows:

Experiment 1	:	Investigating the local effect of specific microbial metabolites (i.e., short-chain fatty acids) on the gut barrier function and contractibility in laying hens as an animal model using *ex-vivo* models ((Yosi et al. 2022), further on referred to as manuscript 1)
Objective 1	:	To determine the effect of different molar acetate: butyrate ratios, and SCFA concentrations on the jejunal and cecal contractibility and jejunal barrier function in laying hens *ex vivo*.
Hypothesis 1	:	Greater contractibility of the jejunal and cecal muscle and mucosal barrier function will be noted with higher molar butyrate proportions and total SCFA concentrations.

Key questions that will be addressed in Experiment 1:

1. To what extent do different acetate: butyrate molar ratios and SCFA concentrations affect the contraction of mid-jejunal and cecal muscles in laying hens using the organ bath system?

2. To what extent do different acetate: butyrate molar ratios and SCFA concentrations affect short-circuit current and transepithelial tissue conductivity in the mid-jejunum of laying hens as indicators for net ion flux and barrier function by using the Ussing chamber?

Experiment 2 : Assessing the modulatory effect of dietary probiotics on gut barrier and immune response, histomorphology, and growth performance in broiler chickens using a meta-analytical approach ((Yosi et al. 2023), further on referred to as manuscript 2)

Objective 2 : To evaluate the effects of probiotics on the gut barrier and immune-related gene expression, histomorphology, and growth in broiler chickens either challenged with pathogens or not.

Hypothesis 2 : It was hypothesized that the meta-analysis will confirm the results from individual studies both at the structural and gene expression levels that probiotics can support gut histomorphology, barrier function, and immune response in broilers under non- or pathogen-challenged conditions.

Key questions that will be addressed in Experiment 2:

1. Does the meta-analysis confirm the findings from the individual studies that probiotics can modulate the expression of genes related to gut barrier function and immune response in broiler chickens either with or without pathogen challenge?

2. Does the meta-analysis confirm the findings from the individual studies that probiotics can improve the histomorphological measures in broiler chickens either with or without pathogen challenge?

3. Does the meta-analysis confirm the findings from the individual studies that probiotics can improve growth performance in broiler chickens either with or without pathogen challenge?

4. Does the meta-analysis confirm the findings from the individual studies that probiotics exert different responses in broiler chickens under non-challenged and challenged conditions, both at the level of gut structure and gene expression?

5. Does the meta-analysis confirm the findings from the individual studies that probiotics exert different responses in broiler chickens between different ages and gut segments, both at the level of gut structure and gene expression?

6. Does the meta-analysis confirm the findings from the individual studies that dietary metabolizable energy, crude protein, and days post-infection modify the modulating effects of probiotics on the gut barrier and immune-related gene expression, histomorphology, and growth performance in broiler chickens?

Experiment 3 : Investigating the mother-offspring axis in establishing gut microbial colonization in suckling and newly weaned piglets using *in vivo* model (further on referred to as manuscript 3)

Objective 3 : To assess: 1) the changes that occur in the bacterial and fungal communities in sow feces during the lactation period as well as in gastric and cecal digesta of piglets from day 3 of life until one week after weaning; and 2) bacterial and fungal taxa in cecal digesta of the piglets postweaning that associate with the fecal consistency

Hypothesis 3 : 1) microbial communities in piglet's gut would reflect the microbial composition in sow feces during the suckling phase; and 2) the association of higher fecal scores with microbial taxa in cecal digesta will let identify taxa that are linked to a lower gut homeostatic state postweaning.

Key questions that will be addressed in Experiment 3:

1. To what extent does progressing lactation influence the composition and abundance of bacterial and fungal communities in sow feces?

2. To what extent do microbes from sow feces contribute to the composition and abundance of bacterial and fungal communities in the gastric and cecal digesta of piglets during the suckling and early postweaning periods?

3. How do the bacterial and fungal communities of the gastric and cecal digesta of piglets develop from suckling to early postweaning?

4. What is the fecal consistency score of piglets within one week after weaning?

5. To what extent do bacterial and fungal taxa in the cecal digesta correlate with fecal consistency score in newly weaned piglets?

4. MANUSCRIPTS

4.1. Manuscript 1

The citation of the published paper is: Yosi, F., Sharma, S., Sener-Aydemir, A., Koger, S., Baskara, A.P., Metzler-Zebeli, B.U. Short-chain fatty acids promote jejunal barrier function and caecal muscle contractibility in laying hens *ex vivo*. Br Poult Sci. 2022; 63(3): 406–413. https://doi.org/10.1080/00071668.2021.2008312
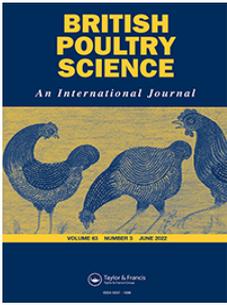
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Short-chain fatty acids promote jejunal barrier function and caecal muscle contractibility in laying hens *ex vivo*

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ABSTRACT

1. Short-chain fatty acids (SCFA) exert beneficial actions in the gut; nevertheless, information about the effect of SCFA on physiological responses in the small intestine of chickens is rare.

2. The aim of this study was to assess the effect of 1) different molar acetate:butyrate ratios (Ac:But; Experiment 1; 78.5% acetate and 7.3% butyrate versus 71.4% acetate and 14.0% butyrate) and 2) SCFA concentrations (Experiment 2; final concentration in chambers: 70.5 versus 141 µmol SCFA/ml buffer) on the jejunal and caecal contractibility and jejunal barrier function in laying hens. The change in muscle contractibility due to the SCFA was measured in mid-jejunal and caecal segments ($n = 4$ each per hen) from four laying hens using the organ bath system after precontraction with acetylcholine for 15 min. Changes in short-circuit current (I_{SC}) and transepithelial tissue conductivity (G_T) as indicators for net ion flux and barrier function, respectively, were measured in mid-jejunal tissue ($n = 3$/hen and treatment), mounted into Ussing chambers.

3. In Experiment 1, the addition of SCFA, irrespective of the Ac:But ratio, decreased jejunal muscle tension ($P < 0.05$), jejunal G_T as well as caused a less negative I_{SC} ($P < 0.05$). In Experiment 2, the increasing SCFA concentrations increased the caecal muscle contraction and jejunal I_{SC} by 75.6% while decreasing the G_T by up to 19.6% ($P < 0.05$).

4. In conclusion, results demonstrate that increasing butyrate proportions and SCFA concentrations stimulate caecal muscle contraction, thereby increasing caecal mixing and emptying *in vivo*. Jejunal I_{SC} and G_T support a strong SCFA sensing capacity in the jejunum, as both, more butyrate and higher SCFA, increased mucosal ion uptake and barrier function.

Introduction

Short-chain fatty acids (SCFA) are the major primary products from fermentation, and contribute to the animal's energy supply when absorbed (Byrne et al. 2015; Stumpff 2018). The main SCFA produced in the gastrointestinal tract are straight-chain SCFA, namely acetate, butyrate, and propionate, originating from the microbial degradation of carbohydrates (Macfarlane and Macfarlane 2012). These have beneficial effects in relation to gut function, inflammatory signalling, regulation of glucose homoeostasis and metabolism, appetite regulation and inhibition of colon cancers (Corrêa-Oliveira et al. 2016; Larraufie et al. 2017). Simultaneously, branched-chain fatty acids are produced by microbial activity on dietary and host-derived proteins; specifically, branched amino acids valine, isoleucine, and leucine are fermented to isobutyric acid, 2-methylbutyric acid, and isovaleric acid, respectively (Macfarlane and Macfarlane 2012). Branched-chain fatty acids are less well characterised than SCFA and are commonly considered to be damaging to the gut (Blakeney et al. 2018). Nevertheless, during microbial fermentation in the intestines both types of SCFA are produced. Therefore, intestinal digesta comprises a mix of straight- and branched-chained fatty acids and their actual molar proportions depend on the respective microbial substrate, which should be considered when investigating effects of single SCFA.

Acetate and propionate show potential to promote small intestinal barrier function, as demonstrated in the duodenum of mice (Wan Saudi and Sjöblom 2017). Moreover, in mice, butyrate accelerated gastrointestinal transit (Touw et al. 2017), whereas branched-chain fatty acids caused relaxation of colonic smooth muscles (Blakeney et al. 2018). Evidence exists that butyrate strengthens the epithelial barrier, reduces inflammation and increases the production of mucins and antimicrobial peptides in the distal chicken gut (Onrust et al. 2015), whereas it is unclear whether butyrate has similar actions in the small intestine of chickens. As both single and mixed SCFA can be used as dietary supplements, it should be clarified whether SCFA alters gut contractibility, especially in the small intestines. Changes in gut motility are associated with alterations in nutrient digestion and passage rate; the latter generally being faster in the small rather than in the large intestine (Kolakshyapati et al. 2019). The extent of digestion and retention time in the respective gut segments, in turn, influences the intestinal substrate availability for microbial activity and time for fermentation, thereby modulating the production and profile of SCFA.

Overall, more evidence exists for straight- and branched-chained SCFA effects in the colon, whereas similar roles in the jejunum and caecum have been less well investigated. These two intestinal segments, however, play crucial roles in birds, because the jejunum is the longest part of the intestine and is important for nutrient assimilation, whereas the caeca are the gut section in which the highest microbial activity can be found (Oakley et al. 2014; Metzler-Zebeli et al. 2019). The objective of the following trial was to investigate the effects of different molar ratios of SCFA (i.e. acetate:butyrate ratio) and luminal SCFA concentrations on the jejunal and caecal motility and jejunal barrier function in chickens *ex vivo*. It was hypothesised that greater contractibility of the jejunal and caecal muscles and mucosal barrier function would be seen with higher molar butyrate proportions and total SCFA concentrations.

Material and methods

Animals and experimental procedures

The protocol relating to the handling of experimental animals was approved under the Good Scientific Practice Guidelines of the Institutional Ethics Committee of the University of Veterinary Medicine Vienna (ETK-54/03/2019). Two separate *ex vivo* trials were performed to investigate the effect of butyrate concentration (Experiment 1) and the effect of the total SCFA concentration (Experiment 2). Each of the two experiments comprised an electrophysiological element performed in the Ussing chamber and gut motility was measured in tissues in an organ bath. In each of the two experiments, the jejunum and caeca from four hens from a commercial strain (Lohmann Luna) at the beginning of their laying period from a local organic layer farm were used. The hens arrived two days before the experiments began and were housed in pens together. Until the morning of the organ bath and Ussing chamber experiments, hens had free access to a commercial, corn-soybean meal-based layer diet (Königshofer, Ebergassing, Austria; 17.8% crude protein, 12.0% ash, 5.8% ether extracts, 4.2% crude fibre, 3.7% calcium, 0.8% lysine, 0.6% phosphorus, 0.4% methionine and 0.17% sodium on an as-fed basis) and fresh water.

Gut tissue collection was similar to that described in Baskara et al. (2021). Hens were euthanised with an overdose of sodium pentobarbital (20 mg/kg; Thiopental, Sandoz GmbH, Vienna, Austria) by i.v. injection into the caudal tibial vein followed by exsanguination. After opening the abdomen, the whole gastrointestinal tract was removed. In order to distinguish between distal jejunum and ileum, the distribution of the mesenteric arteries (McLelland 1975) was followed, which categorises the gut section distal to the Meckel's diverticulum as distal jejunum. Two 25-cm gut sections were taken from the jejunum of each hen, one proximal and one distal to Meckel's diverticulum towards the ileum, and both caeca were collected. The gut samples were immediately placed into ice-cold modified Krebs-Henseleit (KH) buffer for the organ bath and Ussing chamber experiments. Buffers were gassed with carbogen gas (95% O_2 and 5% CO_2) to allow respiration of the tissues. The time elapsing from euthanasia of the hen until the start of the equilibration phase in Ussing chambers and tissue bath was not longer than 20 min.

Short-chain fatty acid solutions

To assess molar proportions and concentration-dependent effects, two SCFA solutions were prepared, containing physiological concentrations of the predominant SCFA acetate, propionate, butyrate, valerate and caproate, as well as the branched-chain SCFA iso-butyrate and iso-valerate (Sigma-Aldrich, Vienna, Austria; Table 1). The stock solution contained 1.96 mmol SCFA/ml and was added to the organ bath and Ussing chambers. In Experiment 1, two different molar ratios of acetate:butyrate were tested, which were 78.5% acetate and 7.3% butyrate (SCFA solution 1; low butyrate) versus 71.4% acetate and 14.0% butyrate (SCFA solution 2; high butyrate) in the analysed SCFA solutions. Table 1 illustrates the analysed concentrations of the single SCFA in the organ bath and Ussing chambers. Irrespective of the acetate: butyrate ratio, the SCFA solutions added to the Ussing and organ bath chambers reached a final concentration of 140 µmol/ml buffer in each chamber. Similar concentrations can be found in the caeca of laying hens, whereas concentrations in the jejunum are normally below 100 µmol/ml. To provoke a physiological response in the jejunal tissue and to have a direct comparison to the caecal tissue in the present study, the same SCFA concentration as for the caeca for the jejunal tissue was used in Experiment 1 and 2. In Experiment 2, two different concentrations of SCFA were tested, which were created by adding different amounts of the SCFA solution 2 from Experiment 1 to the chambers. The low SCFA concentration resembled that found in the distal parts of the small intestine in chickens.

Measurement of gut motility

The experimental procedure to monitor gut motility was performed according to previously published studies (Cai et al. 2015; Blakeney et al. 2018; Baskara et al. 2021). The jejunal sample proximal to the Meckel's diverticulum and one caeca were used. Contraction measurements were performed as described in Baskara et al. (2021). Four samples per gut segment from each hen were excised (1 cm in length) and their lumen flushed with KH buffer (Table 2), warmed to 37°C and gassed with carbogen to remove digesta residues. Both ends of the gut samples were tied with silk suture without occluding the lumen, and placed in a 20 ml water-jacketed organ bath chamber maintained at 37°C (ISO-02, MDE Heidelberg, Germany) and continuously gassed with carbogen (95% O_2–5% CO_2). The lower silk suture was fixed at the bottom of the chamber, whereas the upper silk suture was attached to a force transducer (SEN-03-34, MDE Heidelberg, Germany) which was connected to a four-channel bridge amplifier (EXP-SG-4, MDE Heidelberg,

Table 1. Composition of short-chain fatty acids (SCFA) in the organ bath and Ussing chamber in Experiments 1 and 2.

Item	Experiment 1		Experiment 2	
	Low butyrate	High butyrate	Low SCFA	High SCFA
Concentration (µmol/mL)				
Acetate	110	100	50.4	100.7
Propionate	15	15	7.7	15.4
Butyrate	10	20	10.2	20.4
Isobutyrate	1	1	0.5	0.9
Valerate	2	2	0.9	1.9
Isovalerate	1	1	0.5	1.0
Caproate	0.5	0.5	0.3	0.5

Table 2. Composition of Krebs-Henseleit (KH) buffer and modified KH buffer used in the organ bath and Ussing chamber.

	Concentration	
Item	g/L	mmol/L
KH buffer used in organ bath[a]		
NaCl	6.90	118.1
NaHCO$_3$	2.10	25.0
KCl	0.35	4.7
MgSO$_4$	0.30	1.2
CaCl$_2$	0.17	1.2
KH$_2$PO$_4$	0.16	1.2
D-Glucose	1.50	8.3
Modified KH buffer used in Ussing chamber[a]		
NaCl	6.72	115.0
NaHCO$_3$	2.10	25.0
Na$_2$HPO$_4$.2H$_2$O	0.42	2.4
KCl	0.37	5.0
CaCl$_2$.2H$_2$O	0.17	1.2
MgCl$_2$	0.11	1.2
NaH$_2$PO$_4$.H$_2$O	0.05	0.4
Mannitol	0.36	2.0
D-Glucose	1.80	10.0
HEPES	1.19	5.0
Kanamycin sulphate	0.10	0.2

[a]Buffer pH 7.4.

Germany). Continuous data were collected using advanced kymograph data acquisition software S.P.E.L. (Advanced ISOSYS, MDE Heidelberg, Germany). The tension of each tube piece was adjusted to an initial tension of 1.0 g or 10 mN, and the segment was equilibrated for 20 min. After this, the KH buffer was replaced three times in 5 min intervals. The viability of the jejunal and caecal samples was tested by contraction stimulated by the addition of acetylcholine (ACh) to reach a final concentration of 10 µM in the chamber buffer before testing the effect of the SCFA solutions. When the contractions became stable in all chambers, which took about 10 min, the SCFA solution was added and the response was recorded as positive or negative change in tension. The response was measured for 15 min. Following this, the chambers were cleaned three times using a brush and rinsed in at least three-bath volumes of KH buffer at 5 min intervals to remove the previous solution before the addition of the next SCFA solution. Responses to the SCFA were calculated as the decrease or increase in tension compared to the mean muscle tension prior to addition. The mean tension, measured at peak increase or decrease in tone after the addition of the respective test SCFA solution, was compared to the mean tension 1 min before addition. The increase or decrease in muscle tone was measured, and the contraction induced by the respective SCFA was calculated as the percentage decrease or increase from the level of tone prior to the addition of the respective SCFA solution. Positive changes in muscle tension indicated increased contraction of the muscles, whereas a negative value indicate muscle relaxation.

Measurement of gut electrophysiological parameters

Effects of SCFA on jejunal electrophysiology were tested from four gut sample replicates per hen, using a similar procedure as described in Baskara et al. (2021). In omitting the first centimetre, 12 consecutive jejunal gut samples, distal to Meckel's diverticulum, were prepared, which were opened at the mesentery, rinsed with modified KH buffer (Table 2) to remove digesta particles and stripped of the outer serosal

layers (Metzler-Zebeli et al. 2017; Baskara et al. 2021). After mounting in the chambers, the jejunal tissue was allowed to rest to allow equilibration for 10 min under open-circuit conditions. In both experiments, either the respective SCFA solution or concentration or modified KH buffer as control were added to the mucosal side. Modified KH buffer was added to allow volume adjustment on the serosal side. Electrophysiological measurements, including short-circuit current (I_{SC}) and transepithelial tissue conductance (G_T), which are indicators for net ion flux and tissue permeability, respectively, were recorded for 20 min after the addition of the respective solutions. As the major response occurred in the first minutes after the addition, the chemical effect on mucosal nutrient flux and permeability was assessed by comparing the I_{SC} and G_T for 1 min before the addition of the test SCFA. The peak current and resistance response of the exposed tissue (ΔI_{SC} and ΔG_T) obtained within 5 min after the addition of the SCFA or buffer was used as the control treatment. At the beginning and end of the experiment buffer samples from the mucosal and serosal sites of the Ussing chamber were collected to determine the SCFA concentration.

Analysis of SCFA

Total SCFAs in the stock solutions, as well as in the mucosal and serosal buffers from the Ussing chambers, were determined using gas chromatography (GC). For this, 200 µl of 1.8 mol hydrochloric acid and the internal standard 4-methylvaleric acid (Sigma-Aldrich) were added to 600 µl of the test sample. Samples were vortexed and centrifuged at 20,000 × g for 20 min at 4°C and the clear supernatant was transferred into glass vials for GC. The measurement of SCFA was conducted according to the method of Qumar et al. (2016) using GC apparatus (Shimadzu GC Plus with FID detector, Shimadzu, Kyoto, Japan) that was equipped with a 30 m × 0.53 mm i.d. × 0.53 µm capillary column (Trace TR Wax, Thermo Fisher Scientific, Waltham, MA). The injector and detector had temperatures of 170°C and 220°C, respectively. Helium was used as the carrier gas.

Statistical analysis

Data were tested for normal distribution and outliers using the Shapiro–Wilk test and UNIVARIATE procedure in SAS (Version 9.4; SAS Stat Inc.). To compare treatment effects on intestinal muscle contraction and permeability, the respective data were subjected to ANOVA using the PROC MIXED in SAS. The data of each of the two experiments were analysed separately. For gut motility parameters, the data were analysed as repeated measures over time to determine whether SCFA modified the basal tension of the intestinal tissue pieces after addition of the test solution. Fixed effects included gut segment, SCFA treatment, basal versus post-addition of SCFA, and the respective two-and three-way-interactions. In the second model, gut motility data were analysed per gut segment to account for the differing treatment effects across gut segments. In this model, the fixed effect of SCFA treatment and the random effect of hen and chamber was included. The second model was used to analyse the data from intestinal electrophysiology, i.e. I_{SC} and G_T. A third model was used to analyse the mucosal and serosal SCFA concentrations from Experiment 2, using the

MIXED procedure in SAS. This model included the fixed effects of SCFA treatment and gut section and their two-way-interaction. In all models, the experimental unit was the chamber replicate within each hen sample. Degrees of freedom were approximated using the method of Kenward-Roger (ddfm = kr). Least square means were computed and significance declared at P ≤ 0.05 and trends at 0.05 < P ≤ 0.10. Pairwise comparisons among least square means were performed using the probability of difference option in SAS. The orthogonal polynomial contrast statement was used to establish linear (Experiment 1 and 2) and quadratic (only Experiment 2) relationships for ac:but-ratios (Experiment 1) and SCFA concentrations (Experiment 2) for gut electrophysiological data.

Results

Experiment 1

In the organ bath, the average stable muscle tension after stimulation with ACh differed between gut segments and amounted to 1.36 and 1.58 g for the jejunal and caecal segments, respectively, across acetate:butyrate ratios (P < 0.001; Table 3). The addition of the SCFA solutions, irrespective of the acetate:butyrate ratio, caused a decrease in muscle tension which corresponded to an average decrease in muscle contractibility of 12.8% in the jejunum compared to before the addition of the SCFA solutions (P < 0.05; Table 4). The SCFA addition to the caecal gut samples, in turn, tended (P < 0.10) to increase the muscle contractibility by up to 59.3% for the high butyrate solution after addition.

With regards to the gut electrophysiological measures, the addition of SCFA resulted in a less negative I_{SC} as indicated by the greater ΔI_{SC} compared to the control. This effect was irrespective of the acetate:butyrate ratio (P < 0.05; Table 5). Expressing this difference as the percentage change to the basal measurement showed a linear decrease (P < 0.05) in ΔI_{SC}, amounting to 165% for the higher butyrate ratio compared to the control. In contrast, the G_T only showed an effect after the SCFA addition when expressed as a proportional change, as indicated by the more negative ΔG_T (P < 0.001).

Experiment 2

The SCFA analysis confirmed that the buffer in each organ bath and Ussing chamber contained either 70.5 (low SCFA) or 141 μmol SCFA/ml buffer (high SCFA; Table 1). With respect to gut motility, the average muscle tension after the pre-contraction with ACh was similar among gut segments and amounted to 1.65 and 1.44 g for jejunal and caecal tissues, respectively, for the two SCFA concentrations (Table 6).

Muscle tension linearly increased (P < 0.05) with higher SCFA concentrations in the caecum (Table 6). When expressing the data as the percentage change in contractibility in the caeca, there was a trend for a linear increase with the increasing SCFA concentrations (P < 0.10; Table 7).

The response of jejunal tissue in the Ussing chambers showed a less negative I_{SC} after the addition of the increasing SCFA concentrations (P < 0.001; Table 8). Orthogonal poly-nominal contrasts showed a quadratic effect for the change in I_{SC} (P < 0.001), which indicated that the difference in jejunal I_{SC} mainly existed between the control and SCFA solutions, irrespective of the SCFA concentration.

In contrast, the ΔG_T linearly decreased (P < 0.001) after the addition of the increasing SCFA concentrations. In order to characterise changes in the SCFA absorption, SCFA concentrations in mucosal (Table 9) and serosal buffers (Table 10) of the Ussing chambers were measured. As the modified KH buffer was used as the control and SCFA solutions were added to the mucosal side, results for the serosal side are mainly presented.

Serosal propionate was lowest for the low SCFA concentration compared to the other two treatments, as indicated by the quadratic relationship (P = 0.040). Serosal buffer contained similar concentrations of isobutyrate, isovalerate, valerate and caproate between added SCFA concentrations, but less of the respective SCFA compared to the control (P < 0.05). Changes in the molar SCFA profiles on the serosal side among the three treatments indicated a higher proportion of acetate and butyrate in the high concentration, but less propionate, isobutyrate and isovalerate, valerate and caproate in the buffer, compared to the other two treatments (P < 0.05). For the low SCFA concentration, the serosal buffer contained proportionally the most isobutyrate, isovalerate, valerate and caproate compared to the control and high SCFA concentration (P < 0.05).

Discussion

There is much research on the importance of the microbiota in the upper digestive tract of chickens. Nevertheless, a literature search revealed a lack of knowledge regarding whether microbial metabolites, such as SCFA, could modulate gut motility and barrier function in the small intestines of chickens. Due to the beneficial actions of butyrate (Liao et al. 2020), various strategies have been used in poultry nutrition to stimulate intestinal function, but focus has been on the hindgut to date (Ravn et al. 2018; Ząbek et al. 2020). Fermentation in the gut produces a mixture of SCFA, whereas, in *in vitro* studies, typically single SCFA were investigated (Larraufie et al. 2017; Blakeney et al. 2018). Therefore, this trial investigated the effects of SCFA mixtures

Table 3. Effect of SCFA solutions with different acetate:butyrate ratios on muscle tension in jejunum and caeca of laying hens before (basal) and after the addition of the SCFA (Experiment 1).

Muscle tension (g)	Basal contraction		SCFA addition[a,b]			P-value		
	Low butyrate	High butyrate	Low butyrate	High butyrate	SEM	Basal vs. SCFA addition	Ac:But-ratio	Basal vs. SCFA ×Ac:But ratio
Jejunum	1.44	1.29	1.13	1.11	0.154	0.037	0.450	0.567
Caeca	1.74	1.42	1.97	2.29	0.290	0.032	0.989	0.202

Values are least squares means and standard error of means (SEM), n = 16 per gut site and treatment. Ac, acetate; But; butyrate; SCFA, short-chain fatty acids; vs., versus.
[a]Low butyrate = acetate:butyrate-ratio of 78.5% acetate and 7.3% butyrate in the analysed SCFA solution; high butyrate = acetate:butyrate-ratio of 71.4% acetate and 14.0% butyrate in the analysed SCFA solution.
[b]SCFA concentration in the organ bath chamber was 140 μmol SCFA/mL buffer.

Table 4. Effect of SCFA solutions with different acetate:butyrate ratios on muscle contractibility in the jejunum and caeca of laying hens (Experiment 1).

Contractibility (%)[a-c]	Low butyrate	High butyrate	SEM	P-value
Jejunum	−16.4	−9.2	3.66	0.125
Caeca	27.0	59.3	17.48	0.060

Values are least squares means and standard error of means (SEM), n = 16 per gut site and treatment. SCFA, short-chain fatty acids.
[a]Low butyrate = acetate:butyrate-ratio of 78.5% acetate and 7.3% butyrate in the analysed SCFA solution; high butyrate = acetate:butyrate-ratio of 71.4% acetate and 14.0% butyrate in the analysed SCFA solution.
[b]SCFA concentration in the organ bath chamber was 140 μmol SCFA/mL buffer.
[c]The response to the SCFA solutions was calculated as the proportional decrease or increase in tension within 15 min after the addition of the SCFA solution. The tension measured at peak decrease or increase in tone after the addition of the SCFA solution was compared to the mean tension 1 min prior to the addition.

Table 5. Basal electrophysiological measurements of the jejunum and changes in the tissue response to the addition of SCFA solutions with different acetate:butyrate ratios in laying hens (Experiment 1).

Item[a,b]	Control	Low butyrate	High butyrate	SEM	P-value
Basal measurements					
I_{SC} (μA/cm^2)	−18.9	−22.0	−17.3	4.76	0.593
G_T (mS/cm^2)	10.7	8.8	10.6	3.25	0.746
Response to SCFA addition					
ΔI_{SC} (μA/cm^2)	0.17^e	32.2^d	30.2^d	8.71	0.006
ΔI_{SC} (% of basal value)^c	−1.3^d	−110.4^e	−165.2^f	44.38	0.015
ΔG_T (mS/cm$^{2)}$	−0.85	−2.2	−3.3	1.23	0.142
ΔG_T (% of basal value)	−3.8^d	−22.7^e	−20.0^e	3.63	<0.001

Values are least squares means and standard error of means (SEM), n = 16 per treatment. SCFA, short-chain fatty acids.
[a]Low butyrate = acetate:butyrate-ratio of 78.5% acetate and 7.3% butyrate in the analysed SCFA solution; high butyrate = acetate:butyrate-ratio of 71.4% acetate and 14.0% butyrate in the analysed SCFA solution.
[b]ΔI_{SC} is the difference between the maximal I_{SC} value obtained from 5 min after addition of the SCFA solution and the basal value determined 1 min before the addition; ΔG_T is the difference between the G_T value obtained from 5 min after the addition of the SCFA solution and the basal G_T 1 min before the addition.
[c]Orthogonal polynominal contrast: linear effect, P < 0.001.
[d,e,f]Least squares means within a row with different lowercase superscripts differ (P < 0.05).

with different acetate:butyrate ratios (Experiment 1) and concentrations (Experiment 2) on gut physiological features using jejunal and caecal samples from laying hens. The present results demonstrated a strong effect of SCFA on caecal muscle contraction, as well as jejunal ion flux (I_{SC}) and G_T, whereas the effect of SCFA on jejunal contractibility was equivocal. Dose-response relationships indicated that higher luminal butyrate and total SCFA concentrations can promote jejunal barrier function. When interpreting the results for gut contractibility, only 1 cm segments were used, which possibly interrupted the action of the migrating motor complexes, segmentation and propulsion movements, which may have been especially the case for the jejunal segmental pieces. This may have contributed to the ambiguous results for

jejunal motility seen in Experiment 1 and 2. Since concentrations of SCFA that corresponded to caecal concentrations were used, but were higher than typically seen in the jejunum, the protocol ensured that the local concentration would not be too small to cause any effect. Despite such differences, results from both experiments confirmed that the jejunum in chickens reacts to microbial metabolites, modifying its physiological response.

In Experiment 1, the results for jejunal motility showed immediate muscle relaxation as result of the SCFA addition, which, if the same for the intact mid-jejunum, may modify digestive and absorptive processes in this part of the gut. However, slower contractions may have reduced segmentation and peristalsis, although slower digesta movement would have allowed more time for enzymes to act on feed particles and may have promoted increased nutrient availability for absorption. The SCFA solution with the higher butyrate proportion was used to test its effects in Experiment 2, whereby the high SCFA concentration corresponded to the amount added in Experiment 1. The low SCFA concentration was only half the high concentration, and was closer to the concentration expected in the distal small intestine of chickens (Rehman et al. 2007). Therefore, it may be that the lower SCFA concentration did not reach the threshold concentration to trigger a response in the jejunal tissue, but the higher concentration should have generated a response similar to Experiment 1. When comparing basal muscle tension from both experiments, the tension was approximately 0.3 g higher in Experiment 2 after pre-contraction with ACh than in Experiment 1. This may have interfered with any potential SCFA effect in addition to an interruption in nerve conduction. Although animals were kept and treated similarly and originated from the same farm, the hens used in Experiment 1 and 2 showed a different stress response. The ACh addition in Experiment 2, although being at the same concentration as in Experiment 1 and in previous recent experiments (Baskara et al. 2021; Metzler-Zebeli et al. 2021) greatly excited the muscles, thereby depleting intracellular Ca and preventing further contractions. However, the replenishment of Ca in the muscles occurs directly after the addition of ACh. The ACh concentration used in the present study was calculated to balance the muscle tonus and has been used previously, thereby not inhibiting potential treatment effects (Baskara et al. 2021; Metzler-Zebeli et al. 2021).

Increased caecal contractions after the addition of SCFA suggest that this promoted caecal digesta mixing and emptying, and may have progressed fermentation. Of note, caecal contractibility tended to be enhanced in a dose-dependent fashion in both experiments. Hence, higher concentrations of SCFA and increased butyrate fermentation stimulated caecal functionality. At this point, it is only possible to speculate

Table 6. Effect of increasing SCFA concentrations on muscle tension in jejunum and caeca of laying hens before (basal) and after the addition of the SCFA (Experiment 2).

Muscle tension (g)	Basal contraction		SCFA addition[a-c]		SEM	P-value		
	Low SCFA	High SCFA	Low SCFA	High SCFA		Basal vs. SCFA addition	SCFA amount	Basal vs. SCFA addition × amount
Jejunum	1.67	1.64	1.75	1.82	0.189	0.249	0.836	0.635
Caeca	1.44	1.44	1.80	1.91	0.290	0.012	0.723	0.737

Values are least squares means and standard error of means (SEM), n = 16 per gut site and treatment. SCFA, short-chain fatty acids; vs., versus.
[a]Low SCFA, SCFA concentration in the organ bath chamber equalled 70.5 μmol SCFA/mL buffer.
[b]High SCFA, SCFA concentration in the organ bath chamber equalled 141 μmol SCFA/mL buffer.
[c]The response to the SCFA solutions was calculated as the proportional decrease or increase in tension within 15 min after the addition of the SCFA solution. The tension measured at peak decrease or increase in tone after the addition of the SCFA solution was compared to the mean tension 1 min prior to the addition.

Table 7. Effect of increasing SCFA concentrations on muscle contractibility in jejunum and caeca of laying hens before (basal) and after the addition of the SCFA (Experiment 2).

Contractibility (%)[a-c]	Low SCFA	High SCFA	SEM	P-value
Jejunum	4.6	9.7	8.08	0.335
Caeca	12.7	34.8	12.17	0.089

Values are least squares means and standard error of means (SEM), n = 16 per gut site and treatment. SCFA, short-chain fatty acids.
[a]Low SCFA, SCFA concentration in the organ bath chamber equalled 70.5 μmol SCFA/mL buffer.
[b]High SCFA, SCFA concentration in the organ bath chamber equalled 141 μmol SCFA/mL buffer.
[c]The response to the SCFA solutions was calculated as the proportional decrease or increase in tension within 15 min after the addition of the SCFA solution. The tension measured at peak decrease or increase in tone after the addition of the SCFA solution was compared to the mean tension 1 min prior to the addition.

Table 8. Basal electrophysiological measurements of the jejunum and changes in the tissue response to the addition of increasing SCFA concentrations in laying hens (Experiment 2).

Item[a-c]	Control	Low SCFA	High SCFA	SEM	P-value
Basal measurements					
I_{SC} (μA/cm^2)	−54.1	−43.8	−35.2	10.97	0.259
G_T (mS/cm^2)	13.5	9.7	11.1	1.91	0.214
Response to SCFA addition					
ΔI_{SC} (μA/cm^2)	0.54[g]	29.7[f]	29.6[f]	5.79	<0.001
ΔI_{SC} (% of basal value)[d,e]	−2.5[f]	−77.2[g]	−74.0[g]	4.76	<0.001
ΔG_T (mS/cm^2)[d]	−0.82[f]	−1.7[g]	−3.3[g]	0.77	0.046
ΔG_T (% of basal value)[d]	−4.6[f]	−15.9[g]	−19.6[g]	2.56	<0.001

Values are least squares means and standard error of means (SEM), n = 16 per treatment. SCFA, short-chain fatty acids.
[a]Low SCFA, SCFA concentration in the organ bath chamber equalled 70.5 μmol SCFA/mL buffer.
[b]High SCFA, SCFA concentration in the organ bath chamber equalled 141 μmol SCFA/mL buffer.
[c]ΔI_{SC} is the difference between the maximal I_{SC} value obtained from 5 min after addition of the SCFA solution and the basal value determined 1 min before the addition; ΔG_T is the difference between the G_T value obtained from 5 min after the addition of the SCFA solution and the basal G_T 1 min before the addition.
[d]Orthogonal polynominal contrast: linear effect, P < 0.05.
[e]Orthogonal polynominal contrast: quadratic effect, P < 0.05.
[f,g]Least squares means within a row with different lowercase superscripts differ (P < 0.05).

Table 9. Concentrations and molar proportions of SCFA in the buffer at the mucosal side after the addition of increasing concentrations of SCFA to the mucosal side of the jejunal tissue in the Ussing chamber (Experiment 2).

Item	Control	Low SCFA	High SCFA	SEM	Fixed effect, P-value SCFA conc.	Contrasts[d], P-value Linear	Quadratic
Concentration (μmol/mL)							
Total SCFA	4.6[c]	91.5[b]	172.0[a]	7.58	<0.001	<0.001	0.730
Acetate	1.9[c]	67.2[b]	126.1[a]	5.90	<0.001	<0.001	0.665
Proprionate	0.74[c]	9.9[b]	18.6[a]	0.82	<0.001	<0.001	0.807
Isobutyrate	0.37[c]	0.68[b]	1.2[a]	0.04	<0.001	<0.001	0.123
Butyrate	0.61[c]	11.6[b]	22.5[a]	0.85	<0.001	<0.001	0.954
Isovalerate	0.33[c]	0.67[b]	1.1[a]	0.05	<0.001	<0.001	0.338
Valerate	0.30[c]	1.0[b]	1.9[a]	0.07	<0.001	<0.001	0.533
Caproate	0.40[c]	0.48[bc]	0.64[a]	0.04	0.003	<0.001	0.344
Molar proportions (% of total SCFA)							
Acetate	40.6[b]	73.0[a]	73.1[a]	0.77	<0.001	<0.001	<0.001
Proprionate	16.1[a]	10.9[b]	10.8[b]	0.13	<0.001	<0.001	<0.001
Isobutyrate	8.1[a]	0.87[b]	0.70[b]	0.16	<0.001	<0.001	<0.001
Butyrate	13.2	12.9	13.2	0.26	0.606	0.960	0.320
Isovalerate	7.2[a]	0.77[b]	0.67[b]	0.28	<0.001	<0.001	<0.001
Valerate	6.3[a]	1.2[b]	1.1[b]	0.20	<0.001	<0.001	<0.001
Caproate	8.5[a]	0.55[b]	0.39[b]	0.20	<0.001	<0.001	<0.001

Values are least squares means and standard error of means (SEM), n = 16 per treatment. SCFA, short-chain fatty acids; conc., concentration.
[a,b,c]Least squares means within a row with different lowercase superscripts differ (P < 0.05).
[d]Orthogonal polynominal contrasts.

about the signalling pathways triggered by SCFA, including various types of receptors such as muscarinic acetylcholine receptors, L-type calcium channels, histamine, nicotine or serotonin receptors (Rehman et al. 2012; Cai et al. 2015). Moreover, different activation routes are feasible that may have mediated the SCFA effects, such as activation of transcription factors (e.g. peroxisome proliferator-activated receptors) or G-protein receptors, which have different affinities for various SCFA (Kumar et al. 2020). Consequently, these receptors may have responded according to various acetate:butyrate ratios and SCFA concentrations in the present study.

Results for the less negative I_{SC} of the jejunal tissue after addition of SCFA (irrespective of the acetate:butyrate ratio) in Experiment 1 suggested an increased mucosal-to-serosal cation flux or reduced anion flux. As SCFA were added on the mucosal side, increased co-transport of sodium (Na$^+$) ions due to the activation of the sodium monocarboxylate transporters (SMCT)-1 and −2 may have been feasible, being expressed at the apical membrane of the small intestine (Sivaprakasam et al. 2018; Metzler-Zebeli et al. 2019). In considering the different stoichiometry that SMCT-1-mediated transport is electrogenic (Na$^+$:SCFA stoichiometry = 2:1), whereas SMCT-2-mediated transport is electroneutral (Na$^+$:SCFA

stoichiometry = 1:1; Den Besten et al. 2013; Sivaprakasam et al. 2018), this may explain the ΔI_{SC} of 31 μA/cm^2 measured across acetate:butyrate ratios in Experiment 1. This trial showed a similar increase in I_{SC} in Experiment 2 (ΔI_{SC} = 29 μA/cm^2) irrespective of the SCFA concentration, which indicated that a certain saturation of the mucosal-to-serosal Na$^+$ symport may have occurred, not allowing any further increase in SCFA uptake. Accordingly, the low SCFA concentration appeared to be sufficient to saturate mucosal transport capacity. As the focus of the present study was on actual changes in jejunal functioning, the contribution of the paracellular to the transcellular transport still needs to be verified in future research. The buffer concentrations on the serosal side in Experiment 2 may have supported a certain saturation of the SCFA transport mechanisms when comparing the low and high concentrations. However, the buffer reservoirs in the Ussing chambers were covered, but not hermetically sealed, allowing some evaporation leading to concentration of the buffers. Therefore, results for the SCFA concentrations in the buffers should be interpreted with care. This, for instance, may explain the variation observed for the low and high SCFA concentrations compared to the stock solutions and calculated amounts in each chamber. Moreover, in order not to destroy the mucosal layer, each sample was carefully cleaned but not stripped, allowing microbes to be present and active during the incubation. Consequently, the results indicated that some microbial activity (especially in the serosal buffers from the control treatment) was present in the mucosa, along with epithelial metabolism of SCFA (Schroeder 2019).

The dose-response relationship towards the lower acetate and higher butyrate proportion in Experiment 1 was noted, when results for the ΔI_{SC} were expressed as percentage change, which indicated increased transport of butyrate and higher affinity for SCFA transporters for butyrate. By contrast, the

Table 10. Concentrations and molar proportions of SCFA in the buffer at the serosal side after the addition of increasing concentrations of SCFA to the mucosal side of the jejunal tissue in the Ussing chamber (Experiment 2).

					Fixed effect, *P*-value	Contrasts[d], *P*-value	
Item	Control	Low SCFA	High SCFA	SEM	SCFA conc.	Linear	Quadratic
Concentration (µmol/mL)							
Total SCFA	9.6	3.3	11.3	3.82	0.319	0.753	0.144
Acetate	4.5	1.3	7.5	2.84	0.327	0.464	0.194
Proprionate	1.9[a]	0.49[b]	1.3[ab]	0.40	0.073	0.292	0.040
Isobutyrate	0.55[a]	0.31[b]	0.27[b]	0.02	<0.001	<0.001	0.001
Butyrate	1.1	0.44	1.5	0.50	0.308	0.566	0.158
Isovalerate	0.52[a]	0.24[b]	0.22[b]	0.03	<0.001	<0.001	0.002
Valerate	0.46[a]	0.21[b]	0.26[b]	0.04	0.001	0.002	0.009
Caproate	0.54[a]	0.31[b]	0.25[b]	0.02	<0.001	<0.001	0.011
Molar proportions (% of total SCFA)							
Acetate	46.9[b]	39.8[c]	57.2[a]	1.78	<0.001	0.001	<0.001
Proprionate	19.6[a]	14.6[b]	12.0[c]	0.24	<0.001	<0.001	<0.001
Isobutyrate	5.8[b]	9.4[a]	4.6[c]	0.36	<0.001	0.031	<0.001
Butyrate	11.8[c]	13.4[b]	14.4[a]	0.32	<0.001	<0.001	0.472
Isovalerate	5.4[b]	7.2[a]	3.7[c]	0.44	<0.001	0.010	0.000
Valerate	4.8[b]	6.3[a]	3.7[c]	0.32	<0.001	0.029	<0.001
Caproate	5.7[b]	9.4[a]	4.4[c]	0.41	<0.001	0.040	<0.001

Values are least squares means and standard error of means (SEM), *n* = 16 per treatment. SCFA, short-chain fatty acids; conc., concentration.
[a,b,c]Least squares means within a row with different lowercase superscripts differ (P < 0.05).
[d]Orthogonal polynominal contrasts.

jejunal G_T did not respond in a dose-dependent fashion in Experiment 1, which suggested similar signalling for acetate and butyrate in relation to mucosal barrier function, as both SCFA solutions equally reduced the paracellular permeability. However, the dose-response dependency for ΔG_T in Experiment 2 due to actual SCFA concentration added at the mucosal side seemed to be more important for the paracellular permeability than the acetate:butyrate ratio. The existence of this dose-response may indicate that signalling pathways were independent from the epithelial SCFA uptake capacity in the jejunum.

In conclusion, the results demonstrated dose-response relationships for the stimulation of caecal muscle contraction by increasing butyrate proportions and SCFA concentrations, which, in the *in vivo* condition, may increase caecal digesta mixing and emptying. In contrast, results for any SCFA effect on jejunal contractibility were equivocal, but they may point towards a certain relaxation effect on the jejunal muscles. Meanwhile, gut electrophysiological parameters, including ΔI_{SC} and ΔG_T, supported a strong capacity in the jejunal epithelium for SCFA. The data suggested saturation of the SCFA transport processes and improved barrier function in the jejunal epithelium with more butyrate and higher luminal SCFA concentrations. This should be followed up at molecular level in future studies.

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4.2. Manuscript 2

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Article

Dietary Probiotics Modulate Gut Barrier and Immune-Related Gene Expression and Histomorphology in Broiler Chickens under Non- and Pathogen-Challenged Conditions: A Meta-Analysis

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Simple Summary: The supplementation of diets for broiler chickens has increased due to the increasing demand of consumers for antibiotic-free broiler products. Nevertheless, the benefits of probiotics for intestinal barrier and immune functions, as well as on growth performance in chickens, are still controversially discussed. In performing a meta-analysis, we found that dietary supplementation with probiotics of various genera/species can enforce intestinal barrier function. Moreover, our meta-regressions indicated that in pathogen-challenged birds, probiotics might effectively help reduce gut inflammation by suppressing the expression of pro-inflammatory cytokines. Probiotics further sustained the intestinal histomorphology and hence digestive and absorptive processes in challenged and non-challenged chickens.

Abstract: Data published in the literature about the favorable effects of dietary probiotics on gut health in broiler chickens are inconsistent. To obtain a more comprehensive understanding, we conducted a meta-analysis to assess the effects of probiotics on the gut barrier and immune-related gene expression, histomorphology, and growth in chickens that were either challenged or non-challenged with pathogens. From the 54 articles published between 2012 and 2022, subsets of data, separately for non-challenged and challenged conditions, for response variables were created. The mean dietary probiotic concentrations ranged from 4.7 to 6.2 and 4.7 to 7.2 $\log_{10}$ colony-forming unit/kg under non-challenged and challenged conditions, respectively. Probiotics increased the expression of genes for mucins and tight junction proteins in the jejunum and ileum at weeks 3 and 6. The stimulatory effect of probiotics on tight junction protein expression was partly stronger in challenged than in non-challenged birds. Meta-regressions also showed an anti-inflammatory effect of probiotics under challenged conditions by modulating the expression of cytokines. Probiotics improved villus height at certain ages in the small intestine while not influencing growth performance. Dietary metabolizable energy, crude protein, and days post-infection modified the effects of probiotics on the observed variables. Overall, meta-regressions support the beneficial effects of probiotics on gut integrity and structure in chickens.

Keywords: barrier function; broilers; gastrointestinal tract; growth; histomorphology; immune response; meta-analysis; pathogen; probiotics

1. Introduction

The use of antibiotic growth promoters in chicken farms has been banned in many countries worldwide. Probiotics are considered a promising alternative for livestock animals, including poultry, as they seem to exert a favorable effect on gut health [1]. To

date, various microbial genera have been investigated for use as probiotics in poultry diets, including *Lactobacillus*, *Bacillus*, *Enterococcus*, *Bifidobacterium*, and *Saccharomyces* [1]. Several studies show potential beneficial effects of probiotics on growth performance, absorptive and secretory processes, as well as expression of genes related to host defense mechanisms, barrier function, and inflammation in broiler chickens [2–4]. For instance, dietary probiotics, such as *B. subtilis* and *B. pumilus*, have been shown to stimulate host defense mechanisms at the ileal epithelium by modulating tight junction protein expression in the grower and finisher phases [2]. Other probiotics, such as *L. acidophilus* and *L. plantarum*, have been reported to exert anti-inflammatory effects in the small intestine by moderating pro-inflammatory nuclear factor-kappa B (NF-kB) signaling, which, in turn, leads to lower transcript levels pro-inflammatory cytokines in the jejunum and ileum [3,4]. However, the reported effects of probiotics on the gut epithelial response in chickens are inconsistent [5,6]. Multiple factors may be behind the controversial findings, including direct (e.g., strain and level of probiotics) and indirect factors (e.g., age of birds, intestinal sampling spot, and health status). Although the relationship between dietary probiotics and gene expression levels related to intestinal integrity and immunity in chickens has been described in recent qualitative reviews [7,8], the variation in results of the dependent variable due to influencing factors cannot be assessed in this manner [9]. The conductance of a meta-analysis is considered the most suitable method to address this complexity by generalizing the overall treatment effect, in our case, the effect of probiotics, presented in published studies [10,11]. To obtain a more comprehensive understanding of the efficacy of probiotics, investigations on each response variable were performed separately between original studies with or without pathogen challenge. Therefore, the present meta-analysis aimed to investigate the effects of dietary supplementation of probiotics on the expression of genes associated with intestinal barrier function and immune response, histomorphology, and growth performance in broiler chickens under non-challenged or pathogen-challenged conditions. Furthermore, we assessed the effects of dietary metabolizable energy, crude protein, and days post-infection as additional predictors to obtain a more accurate prediction on the observed variables.

2. Materials and Methods

2.1. Literature Search

For the identification of original articles, a literature search was conducted using 5 public search generators, including Scopus, PubMed, Web of Science, Science Direct, and Google Scholar (Figure 1). Research articles investigating the effects of dietary probiotics on the expression levels of genes related to intestinal barrier function and immune response in broiler chickens that were published in scientific journals between January 2012 and July 2022 were considered for data extraction. To identify adequate articles, the following search terms were used in different combinations: probiotic, direct-fed microbes, gut, intestine, barrier function, gut permeability, gut integrity, tight junction proteins, immune response, inflammatory cytokines, gut inflammation, intestinal immunity, chicken, and broiler.

2.2. Selection of Studies

Stringent criteria were applied in the decision to exclude or include the research articles in the present meta-analysis (Figure 1). The quality assessment criteria used in this study included detailed information on probiotics (type and level of dietary probiotics), chicken strain, body weight and age of chickens, rearing period, and number of chickens per treatment, diet composition, experimental design, including randomization of treatments, description of statistical analysis, and intra-study error (if standard deviation was provided, then it was converted to standard error). Only probiotics that were administered via the diet were considered in this study. In addition, studies investigating the combined effects of dietary probiotics with other treatments on target parameters were also included. From these studies, only data for control and probiotic treatments were considered. Regarding gene expression measurements, only studies that applied quantitative real-time PCR

analysis and in which the relative gene expression was calculated using the 2^{-ddCt} method were included. Moreover, only literature data from in vivo experiments was considered.
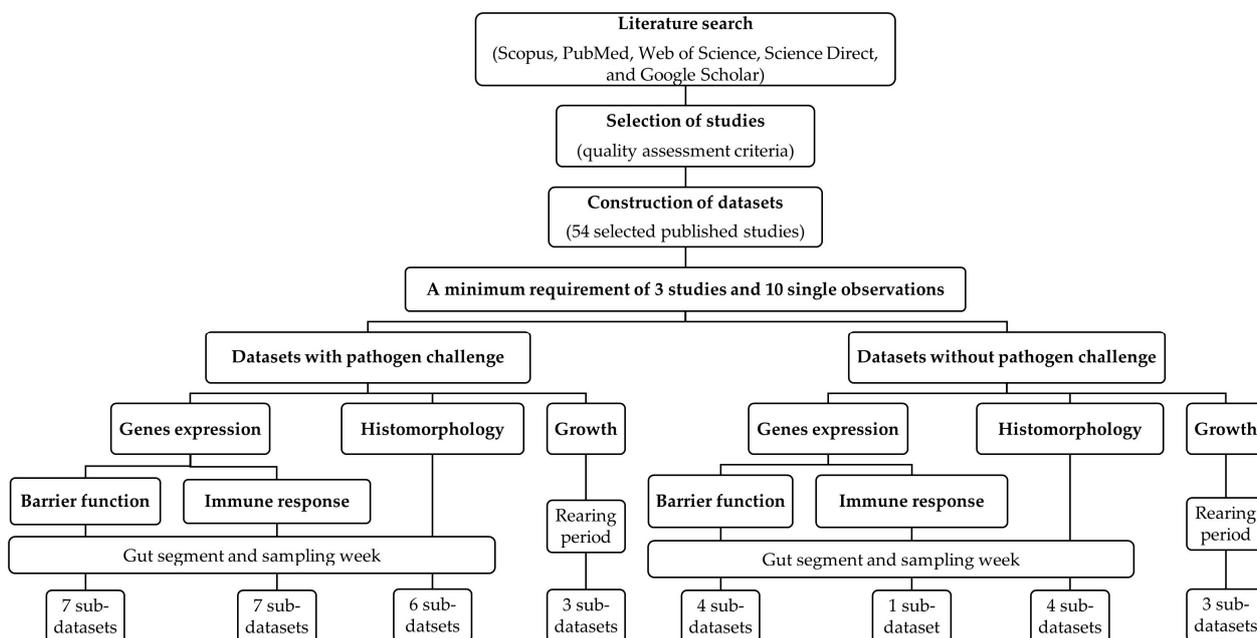
Figure 1. Flowchart showing the process of the collection and selection of original studies as well as the construction of databases in the current meta-analysis.

2.3. Construction of Database

After screening the literature, we identified 54 eligible research articles that met the quality criteria (Figure 1). A minimum requirement of 3 studies and 10 single observations (treatment means) along with the standard error (SE) for each dependent variable was set as requirement for calculating the combined effect size [10,11]. The main predictive variable was the dietary probiotic concentration. Information about the probiotic species used was mandatory. Reported expression levels of genes related to intestinal barrier function and immune response in various intestinal segments (e.g., duodenum, jejunum, ileum, and ceca) were extracted as dependent variables. Moreover, details provided on the chickens (strain, sex, age, and start body weight), experimental setup (experimental design, number of treatments, rearing period, number of chickens per group, and sampling days), pathogen challenge (species or strain of pathogen, administration route, and days post-infection), ingredients and nutritional composition of the diet, and gene expression analysis (e.g., reference genes) were extracted as probable additional prediction variables in the regression analysis. When available, histomorphology data such as villus height, crypt depth, and villus height/crypt depth ratio, as well as growth performance, including average daily feed intake (ADFI), average daily body weight gain (ADG), and feed conversion ratio (FCR) were also extracted. If data from the articles were presented in graphical form, they were extracted using Web Plot Digitizer software (Version 4.5; Ankit Rohatgi, Pacifica, CA, USA).

Two databases were constructed: one for data from research with pathogen challenge and the other for data from studies without pathogen challenge (Figure 1). The next step was to construct datasets for individual dependent variable categories separately for data with or without pathogen challenge, i.e., one dataset each for gut barrier and immune response-related gene expression, histomorphology measures (i.e., villus height, crypt depth, and villus height/crypt depth ratio), and growth performance (i.e., ADG, ADFI, and FCR). Datasets for gut barrier and immune-related gene expression and histomorphology were further subdivided; one sub-dataset was created for each gut segment. For each gut segment, the sub-sets were then grouped by age of the chicken. The dataset for growth performance was divided into sub-datasets based on the stage of the rearing period: starter

(1–3 weeks), finisher (4–6 weeks), and overall (1–6 weeks) periods. As there were not enough studies available to investigate each probiotic strain or species separately, results for the various species/strains of probiotics were analyzed together in this meta-analysis. A reference list of the sub-datasets of broiler studies is presented in Table S1.

The screening for the non-challenge studies showed that the minimum number of studies and observations for gene expression variables related to intestinal barrier function and immune response were fulfilled for the jejunum and ileum at weeks 3 and 6 of life. Adequate numbers of studies and observations for histomorphology variables were available for the jejunum and ileum at weeks 3 and 6 of life. For growth performance variables, the extracted data for the starter, finisher, and entire rearing period also met the requirement. For the studies with pathogen challenge, with respect to the expression of genes related to the intestinal barrier, the variables for the jejunum at weeks 2 to 5 of life, ileum at weeks 3 and 4 of life, and ceca at week 4 of life provided the required number of studies and observations. The variables related to the immune response met the requirement for the jejunum and ileum at weeks 2 to 4 and ceca at week 2 of life. For histomorphology variables, the minimum requirement of studies and observations existed for the data with pathogen challenge for the duodenum at week 5 of life, jejunum at weeks 2, 3, and 5 of life, and ileum at weeks 3 and 5 of life. Adequate numbers of studies and observations were also available for growth variables of starter, finisher, and overall periods. Only dependent variables that met the minimum requirements will be presented.

To create comparability among response variables across studies, the $\log_2$fold values for the gene expression data were calculated in each sub-dataset between control and probiotic treatment for un-challenged and pathogen-challenged data. Positive and negative $\log_2$fold values indicate increased and decreased expression, respectively. Data were processed and displayed as fold change, which was calculated using logarithmic scale to base 2. As dietary metabolizable energy (ME) and crude protein (CP) can affect nutritional metabolism and growth performance, these variables were included as additional predictor variables for both databases. Specifically for the data from pathogen challenge studies, days post-infection (DPI), defined as the interval from the first day of pathogen administration to sample collection, was also incorporated as an additional predictor.

2.4. Data Analysis

Descriptive statistics on the predictive variable (dietary probiotic concentration) and dependent variables (expression of gut barrier and immune-related genes in the jejunum, ileum, and ceca; histomorphology measures in the duodenum, jejunum, and ileum; and growth parameters) were performed separately for the dataset with or without pathogen challenge using the SAS MEANS procedure (version 9.4; SAS Inst. Inc., Cary, NC, USA), as previously described [10,11]. Mixed modeling of each dependent variable was established using the MIXED procedure similar to Metzler-Zebeli et al. [10,11].

$$Y_{ij} = \alpha_0 + \beta_1 X_{ij} + s_i + b_i X_{ij} + e_{ij}$$

where Y_{ij} = expected outcome for the dependent variable Y observed at level j ($j = 2, \ldots, n$) of the predictor variable X in the study i, whereas n is the number of treatment means in study i, α_0 = overall intercept across all studies (fixed effect), β_1 = overall regression coefficient of Y on X across all studies (fixed effect), X_{ij} = the value j of continuous variable X in study i, s_i = random effect of the study i ($i = 1, \ldots$), b_i = the random effect of study i on the regression coefficient of Y on X in study i, and e_{ij} = the unexplained error. Thus, the model's random effect components consist of $s_i + b_i X_{ij} + e_{ij}$, and the distributions are displayed below as follows:

$$e_{ij} \sim iid\ N\left(0, \sigma_e^2\right) \text{ and } \begin{bmatrix} S_i \\ b_i \end{bmatrix} \sim iid\ N\left[\begin{pmatrix} 0 \\ 0 \end{pmatrix}, \Sigma\right],$$

which assumes that e_{ij} is normally distributed with a mean of 0 and constant variance and that s_i and b_i are normally distributed, have means of 0, and Σ is their variance–covariance matrix:

$$\Sigma = \begin{bmatrix} \sigma_s{}^2 & \sigma_{sb} \\ \sigma_{sb} & \sigma_b{}^2 \end{bmatrix}$$

As predictor variables for both study and dietary probiotic concentration were examined. The initial random effects included the slope and intercept based on the study and concentration of dietary probiotics. To prevent positive correlation between intercept and slope, an unstructured variance–covariance matrix (type = UN) was used [12]. The dependent variable was weighted by the inverse of its squared SE (SE of the treatment mean taken directly from the studies) to consider unequal variance between studies. The squared terms of the predictor variables were entered into the model to check for a quadratic relationship if significant ($p < 0.05$). The variance–covariance matrix, in this case, was modeled as variance components (TYPE = VC). For the current data set, there was no significant quadratic correlation; instead, the predictor and response variables showed only linear relationships. The GPLOT technique was used to display the data. To assess the quality of fit, estimates, root mean square error (RMSE), and R^2 were calculated. For established relationships, alteration in the quantity of the dependent variables as affected by dietary probiotic concentration was shown for an assumed probiotic concentration in the diet of $4 \log_{10}$ colony forming units (CFU)/kg.

We performed backward elimination analyses for the datasets with and without pathogen challenge to obtain more accurate predictions of the factors influencing the dependent variables that were affected by the dietary probiotic concentration [10,11]. This enabled us to simultaneously assess how the response variable was affected by the predictors of dietary probiotic concentration, dietary probiotic concentration squared, and dietary ME and CP level, as well as DPI specifically for pathogen challenge datasets. Consideration of variance inflation factors smaller than 10 (which presupposes no substantial multicollinearity among the tested predictor variables) for each continuous independent variable served to limit model over-parameterization [10,11].

3. Results

3.1. Database Description

The main characteristics of the 54 studies that met the selection criteria are presented in Table S1. Of the 54 studies, 14 and 28 studies were without and with pathogen challenge, respectively, whereas 12 studies provided data for challenged and un-challenged conditions. Overall, nine different genera and various species within these genera were administrated as probiotics in the included studies (Figure 2): *Bacillus* (29 studies) and *Lactobacillus* (19 studies) were predominantly used, followed by *Enterococcus* (6 studies), *Saccharomyces* (4 studies), *Pediococcus* (4 studies), *Clostridium* (3 studies), *Bifidobacterium* (3 studies), *Paenibacillus* (2 studies), and *Streptococcus* (1 study). Eight different *Bacillus* species (*B. subtilis*, *B. licheniformis*, *B. coagulans*, *B. amyloliquefaciens*, *B. mesentericus*, *B. methylotrophicus*, *B. tequilensis*, and *B. pumilus*), nine species for *Lactobacillus* (*L. acidophilus*, *L. plantarum*, *L. fermentum*, *L. reuteri*, *L. casei*, *L. animalis*, *L. gallinarum*, *L. johnsonii*, and *L. salivarius*), three species for *Bifidobacterium* (*B. animalis*, *B. bifidum*, and *B. thermophilum*), two species each for *Enterococcus* and *Pediococcus* (*E. faecium*, *E. fecalis*, *P. acidilactici*, and *P. pentosaceus*), one species each for *Clostridium*, *Streptococcus*, *Paenibacillus*, and *Saccharomyces* (*C. butyricum*, *S. faecalis*, *P. polymyxa*, and *S. cerevisiae*) were administered. In addition, 36 studies used only one mono-species probiotic, 7 studies used more than one mono-species probiotic, 8 studies used multi-species probiotics, and 3 studies used both mono- and multi-species probiotics. The experimental diets were mainly composed of corn, wheat, barley, bran, rice, distiller grain, and sorghum, with soybean meal, fish meal, corn gluten meal, corn protein powder, rapeseed meal, peanut meal, and cottonseed meal as protein feedstuffs (Table S1). The experimental diets did not contain other bioactive compounds.

Dietary ME/CP ratios were constant, with a mean of 0.6 and 0.7 for the starter and finisher diets, respectively, for the various response variables.
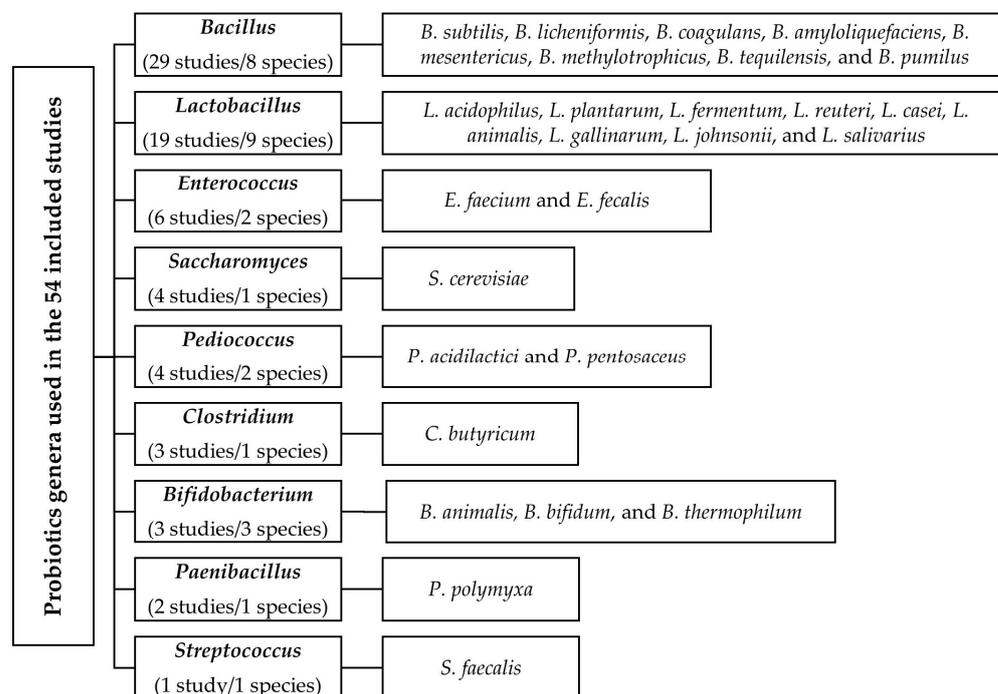
Figure 2. Overview of genera and species within genera administered as probiotics in the included studies.

Descriptive statistical results of the predictor and dependent variables for the database without pathogen challenge are presented in Tables S2–S4. For these data, means of dietary probiotic concentrations across genera/species for the starter phase (1–3 weeks of age) ranged from 4.7 to 5.7 $\log_{10}$ CFU/kg, and those for the finisher phase (4–6 weeks of age) ranged from 5.7 to 6.2 $\log_{10}$ CFU/kg for the various categories of response variables. The means of dietary ME levels for the starter period were 12.3–12.5 MJ/kg, whereas those for the finisher period were 12.8–13.0 MJ/kg. Dietary CP levels for the starter and finisher phases showed means of 21.3–21.6% and 19.4–19.6%, respectively, for the various response variables.

The results of descriptive statistics for predictor variables and dependent variables of the database with pathogen challenge are presented in Tables S5–S8. Several pathogens were included in the original studies, such as *Escherichia coli, Clostridium perfringens, Eimeria (E. tenella, E. maxima, E. acervulina, E. mivati, E. brunetti, E. mitis,* and *E. praecox), Salmonella (S. enteritidis, S. pullorum,* and *S. minnesota), Listeria monocytogenes,* as well as the fungi *Fusarium graminearum* and aflatoxins. For these data, the means of dietary probiotic concentrations across genera/species for the starter and finisher phases ranged from 4.6 to 5.6 $\log_{10}$ CFU/kg and 4.6 to 7.2 $\log_{10}$ CFU/kg, respectively, for the various categories of response variables. The respective means for the dietary ME level for starter and finisher periods were 12.0–12.6 MJ/kg and 12.4–12.8 MJ/kg for various dependent variables. The dietary CP levels in the starter phase showed a mean of 20.8–21.9%, whereas those in the finisher phase were 19.0–20.5% for a different category of response variables. In addition, the mean DPI for measuring gut barrier and immune gene expression for the starter and finisher ages ranged from 3.4 to 14.3 days and 7.6 to 28.7 days, respectively, for various intestinal segments. The mean DPI for the histomorphology variables were 5.2–10.3 days for the starter phase and 30.6–32.0 days for the finisher phase. For growth variables, the mean DPI for the starter and finisher ages were 10.8 and 33.2 days, respectively.

3.2. Probiotic Effects on Gut Barrier and Immune-Related Gene Expression

The results for the meta-regressions between probiotics and gut barrier and immune-related gene expression without pathogen challenge are presented in Table 1, whereas those with pathogen challenge can be found in Table 2. Irrespective of the pathogen challenge, most relationships between probiotics and gene expression levels were established for the jejunum and ileum.

Table 1. Prediction of relative expression (fold change) of jejunal and ileal expression of genes related to gut barrier function and immune response in broiler chickens at weeks 3 to 6 of life without pathogen challenge.

Response Variable (Y) [1,2]	n_Treat	Parameter Estimates				Model Statistics		
		Intercept	SE_Intercept	Slope	SE_Slope	RMSE	R^2	*p*-Value
Jejunum, Week 3								
MUC2	10	0.95	0.149	0.053	0.023	0.336	0.40	0.049
ZO1	11	0.99	0.046	0.019	0.007	0.104	0.46	0.023
OCLN	13	0.96	0.247	0.084	0.037	0.612	0.32	0.044
CLDN1	11	0.99	0.111	0.041	0.017	0.251	0.39	0.041
IL1B	11	1.01	0.060	−0.012	0.009	0.135	0.15	0.241
IFNG	14	1.02	0.058	−0.003	0.009	0.154	0.01	0.773
TLR2	11	1.07	0.181	0.014	0.028	0.407	0.03	0.621
Jejunum, Week 6								
MUC2	10	0.99	0.449	0.156	0.065	0.902	0.42	0.044
ZO1	14	1.00	0.170	0.069	0.022	0.382	0.45	0.009
OCLN	16	0.94	0.245	0.067	0.032	0.604	0.24	0.056
CLDN1	12	1.00	0.266	0.060	0.034	0.533	0.23	0.112
Ileum, Week 3								
MUC2	10	0.89	0.217	0.095	0.030	0.439	0.57	0.012
ZO1	11	0.97	0.171	−0.016	0.023	0.347	0.05	0.510
OCLN	13	0.85	0.150	0.064	0.020	0.338	0.47	0.009
CLDN1	10	0.96	0.115	0.036	0.016	0.233	0.39	0.054
Ileum, Week 6								
MUC2	15	0.98	0.538	0.130	0.074	1.211	0.19	0.103
ZO1	14	0.86	0.463	0.127	0.062	0.930	0.26	0.061
OCLN	16	0.97	0.115	0.034	0.015	0.259	0.26	0.043
CLDN1	11	0.94	0.362	0.087	0.049	0.728	0.26	0.112

n_Treat, number of treatment means; SE, standard error; RMSE, root mean square error; *MUC2*, mucin−2; *ZO1*, zonula occludens-1; *OCLN*, occludin; *CLDN1*, claudin-1; *IL1B*, interleukin-1beta; *TLR2*, Toll-like receptor-2; *IFNG*, interferon-gamma. [1] Probiotic genera included for these response variables were *Bacillus*, *Bifidobacterium*, *Lactobacillus*, *Clostridium*, *Enterococcus*, *Pediococcus*, *Paenibacillus*, and *Saccharomyces*. [2] Data were calculated as $\log_2$fold change between probiotic and control treatments and expressed in fold change using a logarithmic scale to base 2.

Without the pathogen challenge (Table 1), increasing probiotic concentrations linearly increased the jejunal expression of *MUC2*, *ZO1*, *OCLN*, and *CLDN1* at week 3 of life ($R^2 = 0.32$–0.46; $p < 0.05$). For a probiotic concentration of 4 $\log_{10}$ CFU/kg, this would correspond to an increase in expression levels of these genes by 0.21-, 0.08-, 0.34-, and 0.16-fold, respectively. Likewise, at 6 weeks of life, increasing probiotic concentrations linearly increased the jejunal expression of *MUC2* and *ZO1* ($R^2 = 0.42$–0.45; $p < 0.05$). Accordingly, the administration of a probiotic concentration of 4 $\log_{10}$ CFU/kg in the diet would increase the jejunal *MUC2* and *ZO1* expression levels by 0.62- and 0.28-fold, respectively. In the ileum, increasing probiotic concentrations linearly increased the expression of *MUC2* and *OCLN* of life at week 3 of life and of *OCLN* at week 6 of life ($R^2 = 0.26$–0.57; $p < 0.05$), which corresponds to an upregulation of the *MUC2* and *OCLN* expressions by 0.38-, 0.26- and 0.14-fold, respectively, for an assumed dietary probiotic concentration of 4 $\log_{10}$ CFU/kg.

Table 2. Prediction of relative expression (fold change) of jejunal, ileal, and cecal expression of genes related to barrier function and immune response in broiler chickens from weeks 2 to 5 of life with pathogen challenge.

Response Variable (Y) [1,2,3,4]	n_Treat	Parameter Estimates				Model Statistics		
		Intercept	SE_Intercept	Slope	SE_Slope	RMSE	R^2	*p*-Value
Jejunum, Week 2								
ZO1	14	0.99	0.026	0.015	0.004	0.070	0.51	0.004
OCLN	16	0.71	0.909	0.170	0.137	2.420	0.10	0.234
CLDN1	14	0.94	0.226	0.041	0.035	0.601	0.10	0.264
CLDN3	10	1.00	0.040	0.103	0.007	0.089	0.97	<0.001
IL1B	10	1.00	0.015	−0.009	0.003	0.035	0.63	0.006
IL10	14	1.04	0.247	0.015	0.039	0.606	0.01	0.707
IFNG	10	1.00	0.037	−0.037	0.006	0.083	0.82	<0.001
Jejunum, Week 3								
MUC2	10	0.93	0.238	0.051	0.037	0.538	0.19	0.205
ZO1	17	0.97	0.098	0.036	0.014	0.260	0.31	0.021
OCLN	17	0.94	0.177	0.062	0.025	0.473	0.28	0.028
CLDN1	14	0.80	0.617	0.138	0.091	1.524	0.16	0.155
IL1B	17	1.00	0.074	−0.042	0.010	0.198	0.53	0.001
IL6	12	1.01	0.096	−0.030	0.013	0.216	0.35	0.044
IL10	13	0.91	0.290	0.152	0.042	0.719	0.54	0.004
IFNG	18	1.00	0.106	−0.022	0.016	0.303	0.11	0.190
TNFA	10	1.01	0.066	−0.026	0.010	0.150	0.45	0.033
Jejunum, Week 4								
ZO1	12	1.06	0.085	0.034	0.013	0.192	0.40	0.026
OCLN	12	0.99	0.283	0.056	0.043	0.634	0.15	0.220
IL1B	10	0.98	0.113	0.076	0.017	0.227	0.72	0.002
IFNG	14	0.99	0.186	0.038	0.028	0.458	0.13	0.198
Jejunum, Week 5								
MUC2	13	0.97	0.206	−0.004	0.026	0.358	0	0.890
Ileum, Week 2								
IFNG	10	1.04	0.231	−0.015	0.036	0.517	0.02	0.677
TLR4	10	1.03	0.048	−0.035	0.007	0.107	0.75	0.001
Ileum, Week 3								
ZO1	16	0.96	0.196	0.050	0.028	0.483	0.19	0.096
OCLN	16	0.98	0.207	0.056	0.029	0.509	0.21	0.077
CLDN1	11	0.98	0.239	0.010	0.035	0.479	0.01	0.785
IL10	10	0.97	0.178	0.013	0.026	0.358	0.03	0.626
IFNG	12	1.01	0.043	−0.032	0.006	0.097	0.71	0.001
Ileum, Week 4								
ZO1	11	0.98	0.062	0.042	0.009	0.141	0.71	0.001
OCLN	11	0.93	0.143	0.070	0.021	0.325	0.56	0.008
CLDN1	11	0.89	0.263	0.082	0.038	0.597	0.34	0.059
TNFA	10	0.95	0.121	−0.023	0.016	0.244	0.21	0.185
Ceca, Week 2								
IL6	18	0.96	0.093	−0.034	0.013	0.270	0.31	0.017
IL8	10	0.83	0.251	−0.001	0.031	0.518	0	0.967
IL10	10	1.12	0.571	0.213	0.081	1.014	0.47	0.030
Ceca, Week 4								
ZO1	10	1.21	0.198	0.119	0.033	0.401	0.62	0.007

n_Treat, mean number of treatments; SE, standard error; RMSE, root mean square error; *MUC2*, mucin-2; *ZO1*, zonula occludens-1; *OCLN*, occludin; *CLDN1,-3*, claudin-1,-3; *IL6,-8,-10,-1B*, interleukin-6,-8,-10,-1beta; *TLR4*, Toll-like receptor-4; *IFNG*, interferon-gamma; *TNFA*, tumor necrosis factor-alpha. [1.] Probiotic genera included for these response variables were *Bacillus*, *Bifidobacterium*, *Lactobacillus*, *Paenibacillus*, *Clostridium*, *Enterococcus*, *Pediococcus*, *Streptococcus*, and *Saccharomyces*. [2.] Pathogens included for these response variables were *E. coli*, *C. perfringens*, *S. enteritidis*, *E. maxima*, *E. tenella*, *E. acervulina*, *E. mivati*, *E. brunetti*, *E. mitis*, *E. praecox*, *F. graminearum*, *S. pullorum*, *S. minnesota*, *L. monocytogenes*, and Aflatoxin B1. [3.] Means of days post-infection ranged from 3.4 to 28.7 days for various ages and gut segments. [4.] Data were calculated as $\log_2$fold change between probiotic and control treatments and expressed in fold change using a logarithmic scale to base 2.

Regarding the meta-regressions with data from the pathogen challenge (Table 2), a positive linear relationship could be established between jejunal *CLDN3* expression and probiotic concentration at week 2 of life ($R^2 = 0.97$; $p < 0.001$). Here, an assumed dietary probiotic concentration of 4 $\log_{10}$ CFU/kg would increase the jejunal *CLDN3* expression by 0.41-fold. Meta-regressions showed that increasing probiotic concentrations linearly increased the jejunal *ZO1* expression from weeks 2 to 4 of life ($R^2 = 0.31$–0.51; $p < 0.05$) and that of *OCLN* from week 3 of life ($R^2 = 0.28$; $p = 0.028$). Likewise, dietary probiotics positively influenced the expression of *ZO1* and *OCLN* in the ileum at week 4 of life ($R^2 = 0.56$–0.71; $p < 0.05$), amounting to an increase of 0.17- and 0.28-fold with an assumed probiotic concentration of 4 $\log_{10}$ CFU/kg, respectively. In the ceca, expression of *ZO1* linearly increased with increasing dietary probiotic concentrations at week 4 of life ($R^2 = 0.62$; $p = 0.007$), which corresponded to a 0.48-fold increase with a probiotic concentration of 4 $\log_{10}$ CFU/kg.

Under pathogen-challenged conditions, increasing dietary probiotic concentrations linearly decreased jejunal *IFNG* expression at week 2 of life ($R^2 = 0.82$; $p < 0.001$; Table 2), which would correspond to a 0.15-fold decrease with a probiotic concentration of 4 $\log_{10}$ CFU/kg in the diet. Similarly, a negative linear relationship existed between the jejunal *IL1B* expression with increasing probiotic concentrations at weeks 2 and 3 of life ($R^2 = 0.53$–0.63; $p < 0.05$). In contrast, a dietary probiotic concentration of 4 $\log_{10}$ CFU/kg would increase the jejunal *IL10* expression by 0.61-fold at week 3 of life ($R^2 = 0.54$; $p = 0.004$). Moreover, expression of jejunal *IL6* and *TNFA* linearly decreased at week 3 of life ($R^2 = 0.35$–0.45; $p < 0.05$), amounting to 0.12- and 0.10-fold, respectively, with a probiotic concentration of 4 $\log_{10}$ CFU/kg. Like in the jejunum, increasing concentrations of dietary probiotics linearly downregulated the expression of *TLR4* and *IFNG* in the ileum at weeks 2 and 3 of life, respectively ($R^2 = 0.71$–0.75; $p = 0.001$; Table 2). At the cecal mucosa, higher probiotic concentrations decreased *IL6* expression ($R^2 = 0.31$; $p = 0.017$; Table 2) but increased the expression of *IL10* ($R^2 = 0.47$; $p = 0.030$) by 0.14- and 0.85-fold, respectively, at week 2 of life, with an assumed dietary probiotic concentration of 4 $\log_{10}$ CFU/kg.

3.3. Probiotic Effects on Gut Histomorphology

For the data without pathogen challenge (Table 3), increasing probiotic concentrations linearly increased jejunal villus height at weeks 3 and 6 of life ($R^2 = 0.28$–0.66, $p < 0.05$), and the jejunal villus height/crypt depth ratio at week 3 of life ($R^2 = 0.42$; $p = 0.009$). In the ileum, a similar positive linear relationship between the probiotic concentration and villus height was observed at week 6 of life ($R^2 = 0.58$; $p < 0.001$) and ileal villus height/crypt depth ratio at week 3 and 6 of life ($R^2 = 0.41$–0.65; $p < 0.05$). For the results with the pathogen challenge (Table 4), increasing probiotic concentrations linearly increased the villus height in the duodenum at week 5 of life ($R^2 = 0.53$; $p = 0.002$). A similar relationship was found for the jejunal villus height at week 3 of life ($R^2 = 0.42$; $p = 0.005$). Moreover, dietary probiotic concentrations showed a negative relationship with crypt depth ($R^2 = 0.28$–0.71; $p < 0.05$) but a positive linear relationship with jejunal villus height/crypt depth ratio ($R^2 = 0.29$–0.40; $p < 0.05$) at weeks 2, 3, and 5 of life. In the ileum, increasing probiotic concentrations linearly increased the crypt depth and decreased the villus height/crypt depth ratio at week 5 of life ($R^2 = 0.37$–0.41; $p < 0.05$).

Table 3. Prediction of jejunal and ileal histomorphology (fold change) in broiler chickens at weeks 3 and 6 of life without pathogen challenge.

Response Variable (Y) [1,2]	n_{Treat}	Parameter Estimates				Model Statistics		
		Intercept	$SE_{Intercept}$	Slope	SE_{Slope}	RMSE	R^2	*p*-Value
Jejunum, Week 3								
Villus Height	15	1.00	0.030	0.022	0.004	0.080	0.66	<0.001
Crypt Depth	15	0.99	0.041	−0.005	0.006	0.110	0.05	0.411
Villus Height/Crypt Depth	15	1.00	0.064	0.029	0.009	0.171	0.42	0.009

Table 3. *Cont.*

Response Variable (Y) [1,2]	n_Treat	Parameter Estimates				Model Statistics		
		Intercept	SE_Intercept	Slope	SE_Slope	RMSE	R^2	*p*-Value
Jejunum, Week 6								
Villus Height	19	1.00	0.032	0.011	0.004	0.084	0.28	0.020
Crypt Depth	19	1.00	0.061	0.008	0.008	0.163	0.05	0.348
Villus Height/Crypt Depth	19	1.01	0.050	0.004	0.007	0.133	0.02	0.529
Ileum, Week 3								
Villus Height	11	0.98	0.037	0.003	0.005	0.083	0.03	0.585
Crypt Depth	11	1.00	0.058	−0.016	0.008	0.130	0.29	0.088
Villus Height/Crypt Depth	11	0.99	0.065	0.023	0.009	0.147	0.41	0.034
Ileum, Week 6								
Villus Height	17	0.99	0.023	0.014	0.003	0.058	0.58	0.000
Crypt Depth	17	1.00	0.048	−0.004	0.006	0.119	0.02	0.570
Villus Height/Crypt Depth	17	1.01	0.022	0.015	0.003	0.055	0.65	<0.001

n_Treat, number of treatment means; SE, standard error; RMSE, root mean square error. [1] Probiotic genera included for these response variables were *Bacillus*, *Bifidobacterium*, *Lactobacillus*, *Clostridium*, *Enterococcus*, and *Saccharomyces*. [2] Data were calculated as log$_2$ fold change between probiotic and control treatments and expressed in fold-change using a logarithmic scale to base 2.

Table 4. Prediction of duodenal, jejunal, and ileal histomorphology (fold change) in broiler chickens from weeks 2 to 5 of life with pathogen challenge.

Response Variable (Y) [1,2,3,4]	n_Treat	Parameter Estimates				Model Statistics		
		Intercept	SE_Intercept	Slope	SE_Slope	RMSE	R^2	*p*-Value
Duodenum, Week 5								
Villus Height	15	1.01	0.026	0.013	0.003	0.046	0.53	0.002
Crypt Depth	15	0.99	0.037	0.008	0.005	0.063	0.18	0.117
Villus Height/Crypt Depth	15	1.01	0.042	0.002	0.005	0.073	0.01	0.776
Jejunum, Week 2								
Villus Height	11	0.98	0.050	0.012	0.007	0.113	0.23	0.132
Crypt Depth	11	1.00	0.020	−0.014	0.003	0.046	0.71	0.001
Villus Height/Crypt Depth	11	0.98	0.088	0.029	0.013	0.197	0.38	0.044
Jejunum, Week 3								
Villus Height	17	0.99	0.040	0.019	0.006	0.106	0.42	0.005
Crypt Depth	17	1.01	0.035	−0.012	0.005	0.093	0.29	0.027
Villus Height/Crypt Depth	17	0.97	0.085	0.038	0.012	0.226	0.40	0.007
Jejunum, Week 5								
Villus Height	17	1.00	0.026	0.005	0.003	0.052	0.13	0.148
Crypt Depth	17	0.99	0.024	−0.007	0.003	0.049	0.28	0.029
Villus Height/Crypt Depth	17	1.01	0.030	0.009	0.004	0.059	0.29	0.025
Ileum, Week 3								
Villus Height	13	1.01	0.020	0.006	0.003	0.046	0.30	0.052
Crypt Depth	13	1.00	0.057	0.007	0.008	0.127	0.06	0.434
Villus Height/Crypt Depth	13	1.00	0.035	−0.001	0.005	0.077	0	0.873
Ileum, Week 5								
Villus Height	15	0.99	0.055	0.000	0.007	0.095	0	0.962
Crypt Depth	15	0.99	0.030	0.010	0.004	0.052	0.37	0.016
Villus Height/Crypt Depth	15	1.00	0.025	−0.009	0.003	0.043	0.41	0.011

n_Treat, number of treatments means; SE, standard error; RMSE, root mean square error. [1] Probiotic genera included for these response variables were *Bacillus*, *Lactobacillus*, *Enterococcus*, and *Saccharomyces*. [2] Pathogens included for these response variables were *E. coli*, *C. perfringens*, *S. enteritidis*, *E. maxima*, *E. tenella*, *E. acervulina*, *E. mitis*, *E. praecox*, and *F. graminearum*. [3] Means of days post-infection ranged from 5.2 to 32.0 days for various ages and gut segments. [4] Data were calculated as log$_2$ fold change between probiotic and control treatments and expressed in fold change using a logarithmic scale to base 2.

3.4. Probiotic Effects on Growth Performance

The meta-regression results for the growth performance in broiler chickens without and with pathogen challenges are presented in Tables 5 and 6, respectively. Both under pathogen and non-pathogen challenges, dietary probiotics did not affect the ADFI, ADG, and FCR of broilers either in the starter, finisher, or overall phases.

Table 5. Prediction of growth performance of broiler chickens at starter (weeks 1–3), finisher (weeks 4–6), and overall (weeks 1–6) periods without pathogen challenge.

Response Variable (Y) [1]	n_{Treat}	Parameter Estimates				Model Statistics		
		Intercept	$SE_{Intercept}$	Slope	SE_{Slope}	RMSE	R^2	*p*-Value
Starter, Week 1–3								
ADFI (g)	33	48.79	2.405	0.253	0.334	8.736	0.02	0.455
ADG (g)	30	32.52	1.267	0.215	0.176	4.425	0.05	0.234
FCR	33	1.46	0.050	−0.002	0.007	0.180	0	0.741
Finisher, Week 4–6								
ADFI (g)	29	148.29	7.481	0.600	1.025	25.025	0.01	0.563
ADG (g)	26	73.80	4.703	0.661	0.644	15.012	0.04	0.315
FCR	29	1.99	0.057	−0.006	0.008	0.189	0.02	0.422
Overall, Week 1–6								
ADFI (g)	32	97.98	4.503	0.443	0.624	15.736	0.02	0.483
ADG (g)	26	53.54	2.414	0.514	0.333	7.705	0.09	0.135
FCR	32	1.77	0.030	−0.007	0.004	0.105	0.07	0.132

n_{Treat}, number of treatments means; SE, standard error; RMSE, root mean square error; ADFI, average daily feed intake; ADG, average daily weight gain; FCR, feed conversion ratio. [1] Probiotic genera included for these response variables were *Bacillus*, *Bifidobacterium*, *Lactobacillus*, *Clostridium*, *Enterococcus*, and *Saccharomyces*.

Table 6. Prediction of growth performance of broiler chickens at starter (week 1–3), finisher (week 4–6), and overall (week 1–6) periods with pathogen challenge.

Response Variable (Y) [1,2,3]	n_{Treat}	Parameter Estimates				Model Statistics		
		Intercept	$SE_{Intercept}$	Slope	SE_{Slope}	RMSE	R^2	*p*-Value
Starter, Week 1–3								
ADFI (g)	22	51.82	2.483	0.132	0.347	7.456	0.01	0.709
ADG (g)	22	34.72	2.260	0.270	0.316	6.787	0.04	0.403
FCR	22	1.49	0.066	−0.007	0.009	0.198	0.03	0.464
Finisher, Week 4–6								
ADFI (g)	13	142.90	16.708	1.388	2.474	40.945	0.03	0.586
ADG (g)	13	68.16	11.278	0.758	1.670	27.638	0.02	0.659
FCR	13	2.04	0.135	−0.003	0.020	0.330	0	0.879
Overall, Week 1–6								
ADFI (g)	17	95.08	4.156	0.102	0.634	11.776	0	0.874
ADG (g)	17	55.76	4.857	0.259	0.741	13.760	0.01	0.731
FCR	17	1.75	0.091	−0.007	0.014	0.257	0.02	0.626

n_{Treat}, number of treatment means; SE, standard error; RMSE, root mean square error; ADFI, average daily feed intake; ADG, average daily weight gain; FCR, feed conversion ratio. [1] Probiotic genera included for these response variables were *Bacillus*, *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, and *Saccharomyces*. [2] Pathogens included for these response variables were *E. coli*, *C. perfringens*, *S. enteritidis*, *E. maxima*, *E. tenella*, *E. acervulina*, *E. mivati*, *E. mitis*, and *E. praecox*. [3] Means of days post-infection for the starter, finisher, and overall periods were 10.8, 33.2, and 34.9 days, respectively.

3.5. Backward Elimination Analysis

The backward elimination analysis was conducted separately for data without (Table 7) and with pathogen challenge (Tables 8 and 9). For the data of chickens without pathogen challenge, backward elimination analysis showed that dietary probiotic concentration was the main factor influencing the expression of *MUC2*, *ZO1*, and *OCLN* in jejunum and ileum and *CLDN1* in jejunum at week 3 of life (R^2 = 0.36–0.57; *p* < 0.05). Moreover, increasing dietary ME levels counteracted the positive relationship between dietary probiotic con-

centration and jejunal *MUC2* expression at week 6 of life ($R^2 = 0.70$; $p < 0.05$). In contrast, an increasing dietary CP level potentiated the increase in jejunal *OCLN* expression with increasing dietary probiotic concentrations at week 6 of life ($R^2 = 0.62$; $p < 0.05$). The positive relationship between dietary probiotic concentration and jejunal *ZO1* expression was potentiated by dietary ME but counteracted by dietary CP at week 6 of life ($R^2 = 0.70$; $p < 0.05$). Both dietary ME and CP levels counteracted the increase in ileal *ZO1* expression with increasing dietary probiotic concentrations at week 6 of life ($R^2 = 0.76$; $p < 0.05$). For the gut histomorphology, backward elimination analysis showed that dietary probiotics were the only factor influencing the jejunal villus height at week 6 of life ($R^2 = 0.28$; $p = 0.02$). A higher dietary ME level potentiated the increase in jejunal and ileal villus height/crypt depth ratio at week 3 of life ($R^2 = 0.68$–0.72; $p < 0.05$) but counteracted the increase in ileal villus height/crypt depth ratio at week 6 of life ($R^2 = 0.76$; $p < 0.05$) with higher concentrations of dietary probiotics. In addition, an increasing dietary CP level potentiated the positive relationship between dietary probiotic concentration and ileal villus height at week 6 of life ($R^2 = 0.72$; $p < 0.05$).

Table 7. Best-fit equations showing the response variables of gut barrier function-related gene expression and histomorphology (fold change) in relation to increasing dietary probiotics, metabolizable energy, and crude protein level in broiler chickens without pathogen challenge using backward elimination technique.

Response Variable (Y) [1,2]	Predictor (X)	n_{Treat}	Parameter Estimates				Model Statistics			
			Intercept	$SE_{Intercept}$	Slope	SE_{Slope}	RMSE	R^2	VIF	*p*-Value
Jejunum, Week 3										
MUC2		10	0.95	0.149			0.336	0.40		
	Probiotic (CFU/kg)				0.053	0.023			1.00	0.050
ZO1		11	0.99	0.046			0.101	0.49		
	Probiotic (CFU/kg)				0.019	0.007			1.00	0.016
OCLN		13	0.95	0.233			0.594	0.36		
	Probiotic (CFU/kg)				0.009	0.004			1.00	0.030
CLDN1		11	7.37	2.871			0.209	0.62		
	Dietary ME (MJ/kg)				−0.514	0.231			1.00	0.057
	Probiotic (CFU/kg)				0.039	0.014			1.00	0.026
Villus Height		15	−0.83	0.946			0.073	0.74		
	Dietary ME (MJ/kg)				0.146	0.076			1.00	0.077
	Probiotic (CFU/kg)				0.022	0.004			1.00	0.000
Villus Height/Crypt Depth		15	−4.69	1.627			0.125	0.72		
	Dietary ME (MJ/kg)				0.456	0.130			1.00	0.004
	Probiotic (CFU/kg)				0.029	0.007			1.00	0.001
Jejunum, Week 6										
MUC2		10	26.72	9.897			0.688	0.70		
	Dietary ME (MJ/kg)				−1.961	0.754			1.00	0.035
	Probiotic (CFU/kg)				0.152	0.050			1.00	0.018
ZO1		14	0.34	4.308			0.309	0.70		
	Dietary ME (MJ/kg)				0.749	0.325			1.20	0.044
	Dietary CP (%)				−0.460	0.182			1.19	0.030
	Probiotic (CFU/kg)				0.071	0.018			1.01	0.003
OCLN		16	−13.01	3.829			0.440	0.62		
	Dietary CP (%)				0.702	0.193			1.00	0.003
	Probiotic (CFU/kg)				0.072	0.023			1.00	0.009
Villus Height		19	1.00	0.032			0.084	0.28		
	Probiotic (CFU/kg)				0.011	0.004			1.00	0.020
Ileum, Week 3										
MUC2		10	0.88	0.217			0.439	0.57		
	Probiotic (CFU/kg)				0.095	0.030			1.00	0.012
OCLN		13	0.85	0.150			0.338	0.47		
	Probiotic (CFU/kg)				0.064	0.020			1.00	0.009
CLDN1		10	0.96	0.115			0.233	0.39		
	Probiotic (CFU/kg)				0.036	0.016			1.00	0.054

Table 7. *Cont.*

Response Variable (Y) [1,2]	Predictor (X)	n_Treat	Parameter Estimates				Model Statistics			
			Intercept	SE_Intercept	Slope	SE_Slope	RMSE	R²	VIF	*p*-Value
Crypt Depth		11	1.00	0.058			0.130	0.29		
	Probiotic (CFU/kg)				−0.016	0.008			1.00	0.088
Villus Height/Crypt Depth		11	−4.09	2.538			0.122	0.68		
	Dietary ME (MJ/kg)				0.856	0.348			3.20	0.044
	Dietary CP (%)				−0.256	0.129			3.20	0.087
	Probiotic (CFU/kg)				0.023	0.008			1.00	0.021
Ileum, Week 6 *ZO1*		14	57.76	14.077			0.582	0.76		
	Dietary ME (MJ/kg)				−3.390	0.755			1.71	0.001
	Dietary CP (%)				−0.695	0.299			1.72	0.043
	Probiotic (CFU/kg)				0.116	0.039			1.02	0.014
OCLN		16	7.27	2.943			0.231	0.45		
	Dietary ME (MJ/kg)				−0.489	0.228			1.02	0.052
	Probiotic (CFU/kg)				0.031	0.014			1.02	0.045
Villus Height		17	−0.02	0.382			0.049	0.72		
	Dietary CP (%)				0.052	0.019			1.00	0.019
	Probiotic (CFU/kg)				0.014	0.003			1.00	<0.001
Villus Height/Crypt Depth		17	2.49	0.603			0.048	0.76		
	Dietary ME (MJ/kg)				−0.114	0.046			1.01	0.028
	Probiotic (CFU/kg)				0.015	0.003			1.01	<0.001

n_Treat, number of treatment means; SE, standard error; RMSE, root mean square error; VIF, variance inflation factor; ME, metabolizable energy; CP, crude protein; *MUC2*, mucin-2; *ZO1*, zonula occludens-1; *OCLN*, occludin; *CLDN1*, claudin-1. [1] Probiotic genera included for these response variables were *Bacillus*, *Bifidobacterium*, *Lactobacillus*, *Clostridium*, *Enterococcus*, *Pediococcus*, *Paenibacillus*, and *Saccharomyces*. [2] Data were calculated as log₂fold change between probiotic and control treatments and expressed in fold change using a logarithmic scale to base 2.

Table 8. Best-fit equations showing the gut barrier and immune-related gene expression (fold change) in relation to increasing levels of dietary probiotics, metabolizable energy, and crude protein, as well as days post-infection in broiler chickens with pathogen challenge using backward elimination technique.

Response Variable (Y) [1,2,3,4]	Predictor (X)	n_Treat	Parameter Estimates				Model Statistics			
			Intercept	SE_Intercept	Slope	SE_Slope	RMSE	R²	VIF	*p*-Value
Jejunum, Week 2 *ZO1*		14	6.23	1.644			0.048	0.81		
	Dietary ME (MJ/kg)				−0.512	0.145			1.49	0.005
	Dietary CP (%)				0.054	0.016			1.50	0.006
	Probiotic (CFU/kg)				0.015	0.003			1.00	0.000
CLDN3		10	1.00	0.040			0.089	0.97		
	Probiotic (CFU/kg)				0.103	0.007			1.00	<0.001
IL1B		10	1.00	0.015			0.035	0.63		
	Probiotic (CFU/kg)				−0.009	0.003			1.00	0.006
IFNG		10	1.00	0.037			0.083	0.82		
	Probiotic (CFU/kg)				−0.037	0.006			1.00	0.000
Jejunum, Week 3 *ZO1*		17	14.06	3.798			0.201	0.64		
	Dietary ME (MJ/kg)				−0.517	0.190			1.26	0.018
	Dietary CP (%)				−0.316	0.099			1.26	0.007
	Probiotic (CFU/kg)				0.032	0.011			1.01	0.012
OCLN		17	9.41	3.919			0.424	0.46		
	Dietary CP (%)				−0.404	0.187			1.00	0.048
	Probiotic (CFU/kg)				0.060	0.023			1.00	0.020
IL1B		17	−2.10	1.215			0.169	0.68		
	Dietary CP (%)				0.146	0.057			1.01	0.023
	Probiotic (CFU/kg)				−0.040	0.009			1.01	0.001
IL6		12	−4.66	1.799			0.145	0.77		
	Days post-infection				−0.022	0.008			1.12	0.021

Table 8. *Cont.*

Response Variable (Y) [1,2,3,4]	Predictor (X)	n_{Treat}	Parameter Estimates				Model Statistics			
			Intercept	$SE_{Intercept}$	Slope	SE_{Slope}	RMSE	R^2	VIF	*p*-Value
	Dietary CP (%)				0.285	0.088			1.11	0.012
	Probiotic (CFU/kg)				−0.034	0.009			1.01	0.005
IL10		13	0.35	0.382			0.637	0.67		
	Days post-infection				0.066	0.033			1.01	0.072
	Probiotic (CFU/kg)				0.057	0.038			1.01	0.002
TNFA		10	1.01	0.066			0.150	0.46		
	Probiotic (CFU/kg)				−0.026	0.010			1.00	0.033
Jejunum, Week 4										
ZO1		12	11.29	2.383			0.113	0.83		
	Days post-infection				0.018	0.008			2.07	0.066
	Dietary ME (MJ/kg)				−0.828	0.194			2.05	0.003
	Probiotic (CFU/kg)				0.032	0.008			1.02	0.003
IL1B		10	5.03	1.414			0.164	0.87		
	Dietary CP (%)				−0.204	0.071			1.00	0.024
	Probiotic (CFU/kg)				0.076	0.012			1.00	0.000
Ileum, Week 2										
TLR4		10	−2.07	0.604			0.052	0.95		
	Dietary CP (%)				0.143	0.028			1.00	0.001
	Probiotic (CFU/kg)				−0.035	0.004			1.00	<0.001
Ileum, Week 3										
ZO1		16	11.94	4.302			0.409	0.46		
	Dietary CP (%)				−0.514	0.201			1.00	0.024
	Probiotic (CFU/kg)				0.048	0.024			1.00	0.063
OCLN		16	12.02	4.638			0.441	0.45		
	Dietary CP (%)				−0.517	0.217			1.00	0.033
	Probiotic (CFU/kg)				0.054	0.025			1.00	0.052
IFNG		12	1.01	0.043			0.097	0.71		
	Probiotic (CFU/kg)				−0.032	0.006			1.00	0.001
Ileum, Week 4										
ZO1		11	3.67	0.826			0.098	0.87		
	Dietary ME (MJ/kg)				−0.208	0.064			1.08	0.012
	Probiotic (CFU/kg)				0.036	0.006			1.08	0.001
OCLN		11	7.67	1.803			0.208	0.84		
	Dietary CP (%)				−0.335	0.089			1.06	0.006
	Probiotic (CFU/kg)				0.058	0.014			1.06	0.003
CLDN1		11	12.08	3.798			0.438	0.69		
	Dietary CP (%)				−0.557	0.189			1.06	0.018
	Probiotic (CFU/kg)				0.062	0.029			1.06	0.061
Ceca, Week 2										
IL6		18	0.96	0.093			0.270	0.31		
	Probiotic (CFU/kg)				−0.034	0.013			1.00	0.017
IL10		10	46.42	16.880			0.735	0.79		
	Days post-infection				0.506	0.177			1.96	0.029
	Dietary ME (MJ/kg)				−3.868	1.412			1.97	0.034
	Probiotic (CFU/kg)				0.200	0.059			1.01	0.015
Ceca, Week 4										
ZO1		10	−4.26	0.721			0.147	0.96		
	Days post-infection				0.727	0.095			1.00	0.0001
	Probiotic (CFU/kg)				0.014	0.001			1.00	<0.001

n_{Treat}, number of treatment means; SE, standard error; RMSE, root mean square error; VIF, variance inflation factor; ME, metabolizable energy; CP, crude protein; *MUC2*, mucin-2; *ZO1*, zonula occludens-1; *OCLN*, Occludin; *CLDN1,-3*, claudin-1,-3; *IL6, -10, -1B*, interleukin-6, -10, -1beta; *TLR4*, Toll-like receptor-4; *IFNG*, interferon-gamma; *TNFA*, tumor necrosis factor-alpha. [1] Probiotic genera included for these response variables were *Bacillus*, *Bifidobacterium*, *Lactobacillus*, *Paenibacillus*, *Clostridium*, *Enterococcus*, *Pediococcus*, *Streptococcus*, and *Saccharomyces*. [2] Pathogens included for these response variables were *E. coli*, *C. perfringens*, *S. enteritidis*, *E. maxima*, *E. tenella*, *E. acervulina*, *E. mivati*, *E. brunetti*, *E. mitis*, *E. praecox*, *F. graminearum*, *S. pullorum*, *S. minnesota*, *L. monocytogenes*, and Aflatoxin B1. [3] Means of days post-infection ranged from 3.4 to 17.3 days for various ages and gut segments. [4] Data were calculated as $\log_2$fold change between probiotic and control treatments and expressed in fold change using a logarithmic scale to base 2.

Table 9. Best-fit equations showing the gut histomorphology response variables (fold change) in relation to increasing levels of dietary probiotics, metabolizable energy, and crude protein, as well as days post-infection in broiler chickens with pathogen challenge using backward elimination technique.

Response Variable (Y) [1,2,3,4]	Predictor (X)	n_{Treat}	Parameter Estimates				Model Statistics			
			Intercept	$SE_{Intercept}$	Slope	SE_{Slope}	RMSE	R^2	VIF	*p*-Value
Duodenum, Week 5										
Villus Height		15	0.78	0.073			0.035	0.75		
	Days post-infection				0.007	0.002			1.02	0.007
	Probiotic (CFU/kg)				0.012	0.003			1.02	0.001
Jejunum, Week 2										
Crypt Depth		11	0.14	0.261			0.032	0.88		
	Dietary CP (%)				0.040	0.012			1.00	0.011
	Probiotic (CFU/kg)				−0.013	0.002			1.00	0.000
Villus Height/Crypt Depth		11	0.98	0.088			0.197	0.38		
	Probiotic (CFU/kg)				0.029	0.013			1.00	0.044
Jejunum, Week 3										
Villus Height		17	−3.67	1.782			0.072	0.77		
	Days post-infection				−0.007	0.004			1.26	0.070
	Dietary ME (MJ/kg)				0.385	0.144			1.25	0.019
	Probiotic (CFU/kg)				0.019	0.004			1.02	0.000
Crypt Depth		17	−1.06	0.828			0.080	0.51		
	Dietary CP (%)				0.098	0.039			1.01	0.026
	Probiotic (CFU/kg)				−0.011	0.004			1.01	0.020
Villus Height/Crypt Depth		17	6.18	1.979			0.191	0.60		
	Dietary CP (%)				−0.247	0.094			1.01	0.020
	Probiotic (CFU/kg)				0.036	0.010			1.01	0.004
Jejunum, Week 5										
Crypt Depth		17	−2.85	1.084			0.037	0.62		
	Dietary ME (MJ/kg)				0.301	0.085			1.00	0.003
	Probiotic (CFU/kg)				−0.008	0.002			1.00	0.005
Villus Height/Crypt Depth		17	1.20	0.067			0.048	0.57		
	Days post-infection				−0.007	0.002			1.07	0.010
	Probiotic (CFU/kg)				0.012	0.003			1.07	0.002
Ileum, Week 3										
Villus Height		13	0.95	0.019			0.029	0.74		
	Days post-infection				0.006	0.001			1.00	0.002
	Probiotic (CFU/kg)				0.006	0.002			1.00	0.010
Ileum, Week 5										
Crypt Depth		15	1.28	0.072			0.034	0.75		
	Days post-infection				−0.009	0.002			1.02	0.001
	Probiotic (CFU/kg)				0.012	0.002			1.02	0.001
Villus Height/Crypt Depth		15	1.00	0.025			0.043	0.41		
	Probiotic (CFU/kg)				−0.009	0.003			1.00	0.011

n_{Treat}, number of treatment means; SE, standard error; RMSE, root mean square error; VIF, variance inflation factor; ME, metabolizable energy; CP, crude protein. [1] Probiotic genera included for these response variables were *Bacillus*, *Lactobacillus*, *Enterococcus*, and *Saccharomyces*. [2] Pathogens included for these response variables were *E. coli*, *C. perfringens*, *S. enteritidis*, *E. maxima*, *e. tenella*, *E. acervulina*, *E. mitis*, *E. praecox*, and *F. graminearum*. [3] Means of days post-infection ranged from 5.2 to 32.0 days for various ages and gut segments. [4] Data were calculated as log_2fold change between probiotic and control treatments and expressed in fold change using a logarithmic scale to base 2.

Backward elimination analysis for data from studies with pathogen challenge showed that the dietary probiotics concentration was a major factor influencing the expressions of *CLDN3*, *IL6*, *IL10*, *IL1B*, *TNFA*, and *IFNG* either in the jejunum, ileum, or ceca at week 2 and 3 of life (R^2 = 0.31–0.97; $p < 0.05$; Table 8). In addition, an increasing dietary ME level counteracted the positive relationship between dietary probiotic concentration and *ZO1* and *IL10* expression either in the jejunum, ileum, or ceca at weeks 2 and 4 of life (R^2 = 0.79–0.87; $p < 0.05$). Further results showed that dietary CP level counteracted the

increased expression of *ZO1*, *OCLN*, and *CLDN1* ($R^2 = 0.45$–0.84; $p < 0.05$) as well as the decreased expression of *IL6*, *IL1B*, and *TLR4* ($R^2 = 0.68$–0.95; $p < 0.05$) with higher dietary probiotic concentrations in both the jejunum and ileum from weeks 2 to 4 of life. The positive relationship between dietary probiotic concentration and jejunal *ZO1* expression was counteracted by a higher dietary ME but potentiated by increasing dietary CP at week 2 of life ($R^2 = 0.81$; $p < 0.05$). Both dietary ME and CP levels counteracted the increase in jejunal *ZO1* expression with higher concentrations of dietary probiotics at week 3 of life ($R^2 = 0.76$; $p < 0.05$). Increasing DPI potentiated increased cecal *ZO1* and *IL10* expression ($R^2 = 0.79$–0.96; $p < 0.05$) and decreased jejunal *IL6* expression ($R^2 = 0.77$; $p < 0.05$) with increasing dietary probiotic concentrations at weeks 2 and 4 of life.

For the gut histomorphology (Table 9), backward elimination analysis indicated that dietary probiotic concentration was the only factor influencing the villus height/crypt depth ratio in the jejunum and ileum at weeks 2 and 5 of life ($R^2 = 0.38$–0.41; $p < 0.05$). A higher dietary ME level potentiated an increase in the jejunal villus height ($R^2 = 0.77$; $p < 0.05$) but counteracted the decrease in jejunal crypt depth ($R^2 = 0.62$; $p < 0.05$), with higher concentrations of dietary probiotics at week 3 or 5 of life. In contrast, increasing dietary CP levels counteracted the increase in jejunal villus height/crypt depth ratio ($R^2 = 0.60$; $p < 0.05$) but potentiated the decrease in jejunal crypt depth ($R^2 = 0.51$–88; $p < 0.05$) with increasing dietary probiotic concentrations at weeks 2 and 3 of life. Increasing DPI potentiated the increase in villus height in the duodenum and ileum at weeks 3 and 5 of life ($R^2 = 0.75$–0.77; $p < 0.05$) but counteracted the increase in the jejunal villus height/crypt depth ratio and ileal crypt depth at week 5 of life ($R^2 = 0.57$–0.75; $p < 0.05$) with increasing dietary probiotic concentrations.

4. Discussion

Factors such as type and dosage, chicken breed, rearing stage, the composition of the basal diet, and the health status of the bird can influence the physiological effects of probiotics in chickens, adding to the variation among individual studies. Due to that, literature results on the ability of dietary probiotics to modulate the expression of genes related to immune response and barrier function in the gastrointestinal tract of broiler chickens are inconsistent [5,13,14]. Likewise, the effects of dietary probiotics on changes in histo-morphological parameters of the small intestine and performance in chickens also vary [15–18]. The original research included in this meta-analysis covers a wide scope of experimental settings, which should enable inferring predictions for the effect of probiotics on the target variables. However, it needs to be noted that the present meta-regressions only provide general trends for probiotic use in chicken diets. The data available for the individual probiotics did not meet the minimum requirements. Therefore, the data for the single and multi-species probiotics from the individual studies were combined to perform the meta-regression analysis. A similar limitation existed for the pathogens and aflatoxins administrated in the challenge studies. It also needs to be kept in mind that there is a chance that studies with no or adverse effects of probiotics were not published. From the parameters that met the minimum selection criteria, meta-regressions support the effectiveness of probiotics in sustaining small intestinal and cecal barrier function as well as structural components under non-challenged and challenged conditions while also controlling pro-inflammatory signaling under challenged conditions. The meta-regressions also supported that probiotics may effectively counteract potential damage caused by pathogens or mycotoxins in the lower part of the small intestine, such as oxidative stress and compromised barrier function. Regressions further indicated a beneficial effect of probiotics on absorptive and secretory functions by increasing villus height and decreasing crypt depth in the small intestine, especially under pathogen-challenged conditions. Our results also provided evidence for the gut segment- and age-specific effects. However, it needs to be kept in mind that sufficient data were not always available for the same parameters at the various ages of the birds. Consequently, our results provide a general

idea about target variables that were modified by the addition of probiotics in the grower-finisher phase.

Mechanistically, there are several potential modes of action on how the probiotics can influence mucosal gene expression, depending on the actual species and strain of probiotics used. The administrated probiotics across the included non-challenge and challenge studies were *Bacillus*, *Lactobacillus*, *Clostridium*, *Pediococcus*, *Bifidobacterium*, *Streptococcus*, *Paenibacillus*, *Enterococcus*, and *Saccharomyces*. Bacteria interact with the host via microbial metabolites and microbe-associated molecular patterns, which represent specific cell surface structures [19,20]. Consequently, we can assume that parts of the mucosal signaling may have been mediated via the activation of G protein-coupled receptors, pattern recognition receptors, and microbe–microbe interactions, including the production of antimicrobial and fermentation metabolites [21–23]. Across the various species, the present meta-regressions supported the anti-inflammatory effects of probiotics under challenged conditions, which may have subsequently contributed to the upregulation of the mucosal barrier, including the expression of tight junction proteins and other first line of defense genes. Certain G protein-coupled receptors sense fermentation end products, such as short-chain and medium-chain fatty acids [24,25]. Due to the lack of data from the original studies, we can only speculate about the fermentation acids that changed locally in the gut due to the probiotic supplementation. *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Streptococcus*, *Paenibacillus*, *Bifidobacterium*, and *Bacillus* produce lactic acid as a major fermentation product, but depending on the strain, they also produce short-chain fatty acids [26,27]. *Clostridium* is probably mainly signaled via short-chain fatty acids [28,29]. Short-chain fatty acid-induced G protein-coupled receptor activation may decrease the expression of pro-inflammatory cytokines via the inhibition of *NFKB* expression [21]. Unfortunately, we could not extract sufficient data to assess the probiotic effect on *NFKB* expression under un-challenged and challenged conditions as well as on cytokine expression in non-challenged chickens. Nevertheless, moderation of the activation of the pro-inflammatory NF-kB signaling pathway may be behind the present findings for negative effects of probiotics on the expression of *IL1B* and *INFG* at the jejunal mucosa in week 2 of age and expression of *IL1B*, *IL6*, and *TNFA* in the challenged birds at week 3 of age. Moreover, based on the coefficient of determination for the cytokine expression under challenged conditions, probiotics seemed to be very efficient in the jejunum at week 2 of age and in the ileum at week 3 of age in the challenged chickens. Simultaneously, probiotics may act as anti-inflammatory agent by upregulating the expression of *IL10* in innate and adaptive immune cells [30], as indicated by the present results for the jejunum at week 3 and ceca at week 2 of age. Moreover, *Bacillus*-based probiotics may not only act as an anti-inflammatory agent via fermentation acids but by producing quorum-sensing peptides, such as competence and sporulation factor, which signals via the Akt and p38 MAPK pathways [31,32]. *Saccharomyces*-based probiotics, especially *Saccharomyces cereviceae*, have been shown to effectively suppress inflammation by binding certain pathogens and toxins via mannose residues on their cell surface. This may be behind the efficacy of *Sacharomyces* to control *Escherichia coli* and *Salmonella* spp. as well as and mitigate the effects of *Fusarium*-produced mycotoxins [33–35], which were the harmful agent used in the respective challenge studies.

Another mode of action in how fermentation metabolites (especially butyrate) can modulate pro-inflammatory signaling pathways is via inhibition of histone deacetylases in macrophages and dendritic cells [22,36]. From the included probiotics, mainly *Clostridium butyricum* produces butyrate [37–39]. The other genera as lactic acid-producing bacteria may have increased the intestinal butyrate levels via cross-feeding [40,41] and hence indirectly affected the activity of histone deacetylases and modified the expression of pro- and anti-inflammatory cytokines as well as of genes related to the barrier function and host secretions. In the absence of actual data for intestinal butyrate levels, however, we can only speculate whether the presence of the probiotics led to physiologically relevant changes in intestinal butyrate production. Aside from interacting directly with the host, it can be assumed that part of the observed effects was mediated via the interaction of the probiotics with the

commensal microbiota through fermentation acids and antimicrobials [42,43]. The latter metabolites can help shape the overall microbiota composition and inhibit the proliferation of pathogens and/or the expression of virulence factors [44,45]. For instance, reuterin produced by *Limosilactobacillus reuteri* is effective to control dysbiosis [46,47]. Similarly, antimicrobial compounds produced by certain *Bacillus*-based probiotics, such as surfactin, iturin, and fengycin, have also been reported to be effective against pathogenic bacteria [48]. Any alteration in the microbial composition automatically changes the composition of the microbial cell surface structures, which are recognized by pattern-recognition receptors at the gut mucosa and immune cells [49]. Unfortunately, not much data were available for the expression of pattern-recognition receptors in the included studies. In pathogen-challenged birds, our meta-regressions support a downregulating effect of the probiotics on *TLR4* expression in the ileum at week 2 of age. In the respective original studies, the pathogens that were administrated were Gram-negative bacteria, such as *Escherichia coli* and *Salmonella* spp. [50,51], which comprise highly immune-reactive lipopolysaccharide recognized by TLR-4 [20]. This finding may indicate that probiotics effectively inhibited the proliferation of the administrated pathogens and/or moderated the TLR-4 activation. Harmful agents, such as *Eimeria*, fungi, and mycotoxins, likely signaled via different pattern recognition receptors than TLR-4. In general, it is thinkable that probiotics mediated their anti-inflammatory effect via lower ligand-specific activation of the respective pattern recognition receptors. This, in turn, probably led to a lower *NFkB* expression and/or gene expression within the AMP-activated protein kinase, MAPK, or Akt-signaling pathways [52,53], and ultimately to a downregulation in expression levels of pro-inflammatory cytokines (e.g., *IL1B*, *IL6*, *INFG*, and *TNFA*) at the investigated gut sites.

The literature results suggested a protective effect of probiotics on intestinal integrity due to increased mucus production [54,55] and by stimulating the expression of tight junction proteins [55–57]. The present meta-regressions confirmed this assumption. However, fewer data were available for *MUC2* expression from the challenge studies; therefore, the present findings mainly support the beneficial effects of probiotics in non-challenged birds. Moreover, the stimulating effect of probiotics on the *MUC2* expression seemed to last longer in the jejunum than in the ileum of chickens under non-challenged conditions, which may be related to the length of the small intestine and age-related maturation of the immune system in the older chicken [42,58]. The aforementioned effects of probiotics on lower pro-inflammatory cytokine expression may explain their stimulatory effect on the expressions of *CLDN3*, *OCLN*, and *ZO1* in the jejunum, *OCLN* and *ZO1* in the ileum, and *ZO1* in the ceca at week 2, 3, or 4 of age. However, the stimulatory effect was not consistent for all available tight junction protein genes, especially for the claudin genes, which might be related to developmental changes in the gut epithelial functioning and the actual role of the tight junction protein, which needs further investigation. When comparing the non-challenged with the challenged conditions, our meta-regressions indicated an upregulation of the expression of *CLDN1* by the probiotics in non-challenged birds at week 3 of age. Under challenged conditions, however, probiotics did not modify the transcription of *CLDN1* but that of *CLDN3* at week 2 of age.

In individual studies, probiotics were shown to modulate gut histo-morphological parameters [37,59,60]. Our meta-regressions confirm that probiotics can effectively increase villus height and villus height/crypt ratio in non-pathogen- and pathogen-challenged conditions. Probiotics may increase villus height by inducing mitotic cell division and promoting epithelial cell proliferation [61]. Longer villi are associated with improved digestive and absorptive capabilities at the small intestinal mucosa [61]. In addition, probiotics seemed to have a stronger effect on crypt depth under pathogen-challenged conditions in both jejunum and ileum. A shallower crypt is associated with slower cell turnover [62], potentially indicating that the probiotics prevented the disruption of epithelial cells due to the administrated pathogens.

The backward elimination analysis was helpful in the assessment of the impact of certain dietary effects on the target variables. According to the best-fit model, higher dietary

ME and CP levels were important influential factors that counteracted the efficacy of probiotics to increase the expression of *MUC2*, tight junction proteins, and anti-inflammatory cytokines and decrease pro-inflammatory cytokines in the small intestine and ceca. For instance, higher dietary CP may act pro-inflammatory in birds under challenged conditions by stimulating the proliferation of proteolytic taxa in the gut, which could lead to the activation of *TLR4* expression. Of the administrated pathogens and toxins, *Salmonella* and *Escherichia coli* as Gram-negative and proteolytic bacteria, for instance, may have benefited from increased dietary CP levels. Higher dietary ME, most often caused by a higher starch content of the diet, has been shown to reduce the number of butyric acid-producing bacteria and increase Gram-negative bacteria [63,64], which may act as a pro-inflammatory agent. In contrast to the finding at the gene expression level, the best-fit model also indicated that higher levels of dietary ME and CP could enhance the effect of probiotics on intestinal villus height, which may be related to the stimulation of growth and proliferation of intestinal epithelial cells due to greater nutrient availability [65]. The backward elimination analysis also suggested a certain recovery of the gut mucosa after the pathogen challenge that was independent of the probiotics. Accordingly, with increasing time post-infection, the expression levels of genes for pro-inflammatory cytokines decreased, whereas those of genes coding for anti-inflammatory cytokines and tight junction proteins increased.

5. Conclusions

This present meta-analysis confirmed the results from individual studies at the gene expression level that probiotics can support intestinal barrier function in the small intestine under non-pathogen-challenged conditions in broiler chickens. From the available data that were used in this present analysis, it can be further deduced that under challenged conditions with pathogens and mycotoxins, probiotics do not only increase the expression of barrier function genes, but they mediate anti-inflammatory effects via modulation of cytokine expression in the small intestine and ceca. The effect of probiotics was not limited to the changes in gene expression but was also detectable at the structural level, where they improved villus height and crypt depth and hence influenced absorptive and secretory processes at the small intestinal epithelium. However, the present meta-regressions did not support the effect of probiotics on growth performance. Other sources of variation that could potentially influence or counteract the effects of probiotics in the diet included the dietary levels of ME and CP as well as the DPI in the challenge studies. Limitations of this present meta-analysis were that insufficient data were available from individual studies for the various probiotics and administrated pathogens and mycotoxins. Therefore, the present meta-regressions provide general trends that should be verified in the future when more data for the various single and multi-strain probiotics are available.

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4.3. Manuscript 3

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Lactation-related dynamics of bacterial and fungal microbiomes in feces of sows and gut colonization in suckling and newly weaned piglets

SCHOLARONE™
Manuscripts

1   **Lactation-related dynamics of bacterial and fungal microbiomes in feces of sows and**

2   **gut colonization in suckling and newly weaned piglets**

3

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1

LAY SUMMARY

During the suckling phase, piglets are commonly housed in the same pen as their mother sows until weaning. Consequently, mother's feces are a rich source of microbes for the colonization of the neonatal gut. In the past, mainly maturational changes in the bacterial community of piglet's gut have been investigated, whereas other microbial groups, such as fungi, received less attention. In the present study, we could show that the bacterial and fungal communities in sow feces changed with progressing lactation and that bacterial and fungal taxa present in sow feces were also present in gastric and cecal digesta of the suckling piglets. These findings support the importance of maternal microbes for the gut colonization of neonatal piglets. In both, bacterial and fungal communities, weaning left a characteristic mark in the overall community structures and taxa abundances, which can be explained by the withdrawal of sow milk and low feed intake. Correlation analysis also revealed potential bacterial and fungal marker candidates for softer and firm feces.

TEASER TEXT

We investigated the bacterial and fungal communities in the feces of sows during lactation which appeared to have contributed to the gut colonization of suckling piglets. Postweaning, we could identify taxa that were linked to firm and softer feces and may serve as bacterial and fungal marker for the gut homeostatic condition.

2

ABSTRACT

Changes in the gut microbial composition of the sow during lactation may influence the gut microbial colonization in their offspring, for which less information was available in the literature. This study aimed to assess: 1) the changes that occur in the bacterial and fungal communities in sow feces during the 28-day lactation period as well as in gastric and cecal digesta of piglets until one week after weaning, and 2) bacterial and fungal taxa in cecal digesta of the piglets postweaning that associate with fecal consistency. Fecal samples from sows for microbial analysis were collected (n=20) on days postpartum (**DPP**) 1, 6, 13, 20 and 27, as well as from weaned piglets for fecal scoring on day of life (**DoL**) 30 and 34. Gastric and cecal digesta of piglets was collected on DoL3, 7, 14, 21, 28, 31 and 35 (n=5/sex/DoL). Progressing lactation affected bacterial and fungal communities in sow feces. Great alterations were observed within the dominant taxa in sow feces, including 10.3- and 3.0-fold increases in *Lactobacillus* from DPP1 to 6 and *Kazachstania* from DPP1 to 13, respectively ($P < 0.001$). Although time- and gut site-related differences existed, bacterial and fungal taxa found in sow feces were also present in gastric and cecal digesta of piglets, which supports their role for gut colonization in neonatal piglets. In piglets, bacterial and fungal alpha-diversities showed certain fluctuations during the suckling period, whereby weaning affected more the fungal than bacterial diversity at both gut sites ($P < 0.05$). At both gut sites, *Lactobacillus* largely increased from DoL3 to 7 and remained a dominating taxon until DoL35 ($P < 0.05$). Postweaning, plant-glycan fermenters (e.g., *Prevotella*-9) seemed to replace milk-glycan fermenting *Fusobacterium* and *Bacteroides* ($P < 0.05$). In gastric and cecal digesta, *Kazachstania*, *Tausonia*, *Candida* and *Blumeria* were dominating fungi from DoL3 to 35, with *Kazachstania* becoming even more dominant postweaning ($P < 0.001$). Fecal consistency was softer on DoL34 than 30 ($P < 0.05$). Correlation analysis identified that softer feces were linked to cecal abundances of plant-glycan and proteolytic bacterial taxa including pathobionts (e.g.,

3

68   *Clostridium* sensu stricto) on DoL34. However, the potential association between cecal mold

69   and plant-pathogenic fungi *Talaromyces*, *Mrakia*, and *Blumeria* and softer feces are worth to

70   investigate in the future in relation to (gut) health of piglets.

71

72   **KEY WORDS**

73   Bacteriome, Lactation, Mycobiome, Piglet, Sow, Weaning

4

75 **LIST OF ABBREVIATIONS**

76 DoL, day of life

77 DPP, day post-partum

INTRODUCTION

80 The postnatal microbial colonization is a substantial contributor to gut maturation, including

81 immune and barrier functions (Everaert et al., 2017). Microbes that colonize the piglet's

82 gastrointestinal tract after birth originate from the sow (i.e., vagina, skin/nipple surface,

83 colostrum/milk, and feces), environment, and handling farm staff (Jost et al., 2014; Chen et al.,

84 2018). During the suckling period, piglets are commonly housed in the farrowing pens with

85 their sows until weaning (Nowland et al., 2019). Hence, changes in the gut microbial

86 composition of the sow that occur during lactation may influence the gut microbial colonization

87 in their offspring. Despite increasing knowledge on the gut microbial development in piglets,

88 alterations in the gut microbial composition of the dam over the course of lactation were rarely

89 investigated so far.

90 Moreover, knowledge on the postnatal fungal colonization of the piglet's gut and whether this

91 development is driven by the fecal mycobiome of the sow is still scarce. The stomach of

92 suckling and weaned piglets contains a diverse and complex microbiome (Mann et al., 2014;

93 Lerch et al., 2023), which contributes to the degradation of nutrients and production of

94 fermentation metabolites. Although some data for the postnatal development of bacteria in the

95 stomach exist (Lerch et al., 2023), the development of gastric fungal communities has been

96 poorly described. Evidence from our group supports the importance to investigate fungal

97 communities in the neonatal period (Lerch et al., 2023), as some of the fungi found in cecal

98 digesta represented plant pathogens and mold fungi (Yeh et al., 2021), which may compromise

99 the development of the immune system (Wang et al., 2023). This rises the need to monitor the

100 development of the gastrointestinal microbial communities in healthy piglets from pre- and

101 postweaning more closely.

102 Scoring the fecal consistency in piglets is also helpful for identifying eubiotic and dysbiotic

103 gut conditions. Recently, we could show that feces of different colors and consistencies differ

6

104   in their bacterial composition in suckling and newly weaned piglets (Metzler-Zebeli et al.,

105   2023). However, feces are mostly representative for the distal large intestine of piglets;

106   therefore, targeting the microbial composition in more proximal parts of the gut (e.g., cecum)

107   and link them to the fecal score may provide further marker taxa for eubiotic and dysbiotic gut

108   conditions. Especially, the relation between intestinal fungi and fecal score have been little

109   investigated so far.

110   The objectives of this study were to assess 1) the changes that occur in the bacterial and fungal

111   communities in sow feces during the lactation period as well as in gastric and cecal digesta of

112   piglets from day of life (**DoL**) 3 until one week after weaning; and 2) bacterial and fungal taxa

113   in cecal digesta of piglets postweaning that associate with fecal consistency. Our research was

114   based on the following hypothesis: 1) microbial communities in piglet's stomach and cecum

115   would comprise similar bacterial and fungal taxa as sow feces during the suckling phase but

116   would diverge postweaning; and 2) the association of fecal scores with microbial taxa in cecal

117   digesta would allow the identification of taxa linked to a lower (dysbiosis) and higher

118   (eubiotics) gut homeostatic state postweaning.

119

120   <p align="center">**MATERIALS AND METHODS**</p>

121   *Ethical statement*

122   All procedures requiring animal handling and treatment have been approved by the institutional

123   ethics committee of the University of Veterinary Medicine Vienna and National authority in

124   accordance with the Law for Animal Experiments in Austria (GZ 2020-0.437.208).

125

126   *Animals, housing, and experimental procedures*

127   The pig experiment was conducted at the pig facility of the University of Veterinary Medicine

128   Vienna (VetFarm) under production conditions, consisting of two consecutive replicate

129   batches. In each replicate batch, 10 sows (Large White) and their litters (Large White ×

130   Piétrain) were used. The experiment lasted from 26 days prior to farrowing, throughout the 28-

131   day lactation period to 7 days after weaning. At the pig facility, sows and their litters were

132   handled according to standard procedures at the pig facility. Sows were group-housed in pens

133   during gestation but had access to individual feeders. Five days before farrowing, sows were

134   transferred to separate farrowing pens (BeFree, Schauer Agrotonic, Prambachkirchen, Austria;

135   2.3 × 2.6 m in size), which were equipped with a feeder, bowl drinker, and hayrack for the sow

136   (for environmental enrichment and nesting behavior), as well as a round feeder, small bowl

137   drinker, and nest with heated flooring for the piglets. All sows gave birth within 48 hours and

138   were not restrained during the farrowing process and the whole lactation. Piglets were

139   supplemented with iron by injection on DoL4 (2 mL of Ferriphor 100 mg/mL, OGRIS Pharma

140   Vertriebs-GmbH, Wels, Austria), followed by castration of male piglets on DoL11 (general

141   sedation with Stresnil 40 mg/mL, 0.025 mL/kg body weight, Elanco Tiergesundheit AG, Basel,

142   Switzerland and Narketan 100 mg/mL, 0.1 mL/kg body weight, Vetoquinol Österreich GmbH,

143   Vienna, Austria). On DoL17, piglets were vaccinated (1 mL Ingelvac CircoFLEX and 1 mL

144   Ingelvac MycoFLEX, both from Boehringer Ingelheim GmbH, Ingelheim/Rhein, Germany).

145   On DoL28, sows were removed from the farrowing pens and piglets were transferred to rearing

146   pens in an outdoor climate house with a heated lying area. Each rearing pen had a size of 3.3 ×

147   4.6 m each and was equipped with one round feeder, nipple and bowl drinkers, and a heated

148   nest. The animal's health was monitored daily throughout the experiment. Water was freely

149   available to sows and piglets throughout the experiment.

150

151   ***Feed and feeding***

152   The feeding protocol of gestating and lactating sows as well as of suckling and weaned piglets

153   corresponded to the standard protocol at the pig facility. From 26 to 5 days prior to farrowing,

154   sows were provided with gestation diet in the morning (08:00 hour) and afternoon (14:30 hour)

155   (3-4 kg/meal; Königshofer GmbH, Ebergassing, Austria; Supplementary Table S1 and S2).

156   After moving the sows to the farrowing pens 5 days before the farrowing date, the sows were

157   offered lactation diet (approx. 3 kg/meal; Königshofer GmbH, Ebergassing, Austria;

158   Supplementary Table S1 and S2) in the morning and afternoon (08:00 and 14:30 hours).

159   Additionally, sows received 500 g of linseeds soaked in water once a day for 5 days prior to

160   farrowing to avoid constipation. After farrowing, the feed amount of the sows was gradually

161   increased (approx. 4–9 kg/meal) according to the regular feeding protocol. Litters received

162   creep feed that was manually prepared at least twice daily (08:00 and 15:00 hours) from DoL3

163   to 35. The creep feed was a commercial milk replacer (NuriStart Sweet, BIOMIN Holding

164   GmbH, Part of dsm-firmenich, Getzersdorf, Austria; Supplementary Table S2), which was

165   prepared according to the manufacturer's instructions. The milk replacer was offered in liquid

166   form, by mixing the powder with warm water (45°C) at a ratio of 500 g/L (w/v). From DoL3

167   to 23, the piglets were given 100% of milk replacer. Then, the milk replacer was gradually

168   mixed with the prestarter diet from DoL24 to 26 (Königshofer GmbH, Ebergassing, Austria;

169   Supplementary Table S1 and S2), starting with a ratio of 70:30 (w/w) on DoL24, 50:50 (w/w)

170   on DoL25, and 30:70 (w/w) on DoL26, respectively, and provided in mash form. After that,

171   the litters were fed 100% of the prestarter diet as mash on DoL27 and in dry form after weaning

172   from DoL28 to 35. All diets used in the study met or exceeded the current recommendations

173   for nutrient requirements (NRC, 2012). Leftover creep feed and spills were collected to

174   estimate the creep feed intake.

175

176   ***Body weight measurement, fecal score, and collection***

177   Piglets were weighed at 8 time points, immediately after birth, DoL4, 6, 13, 20, 27, 30, and 34.

178   Freshly defecated feces of sows or feces after rectal stimulation were collected on days

179 postpartum (**DPP**) 1, 6, 13, 20, and 27 for microbiome analysis. To avoid contamination from

180 the floor, only the inside part of the defecated feces was used and homogenized with a sterile

181 spatula before placing them into cryo tubes. The tubes were kept on ice before storage at -80°C

182 until further analysis. Fecal samples from weaned piglets were collected by means of rectal

183 stimulation on DoL30 and DoL34 to assess the fecal consistency. To obtain the samples, the

184 inner anal sphincter was stimulated by inserting a sterile cotton tip and gently rotating it. The

185 consistency was scored according to: 0 (balls normal), 1 (shaped soft), 2 (pasty), 3 (shaped

186 very soft), 4 (mild diarrhea), and 5 (watery diarrhea).

187

188 ***Gut sampling***

189 In each of the two replicate batches, gut samplings took place on DoL3, 7, 14, 21, 28, 31, and

190 35. On each sampling day, 5 female and 5 male piglets were used for invasive sampling in each

191 replicate batch. From each litter, one piglet (with alternating sexes on the consecutive sampling

192 days) was selected per sampling day based on having average body weight within the litter.

193 Prior to slaughter, piglets were weighed and anesthetized in the ear vein with azaperone

194 (Stresnil 40 mg/mL, 0.025 mL/kg body weight, Elanco Tiergesundheit AG, Bad Homburg,

195 Germany) and ketamine (Narketan 100 mg/mL, 0.1 mL/kg body weight, Vetoquinol Österreich

196 GmbH, Vienna, Austria). Afterwards, piglets were euthanized with embutramide (T61, 0.1

197 mL/kg body weight, Intervet GesmbH, Vienna, Austria) via intracardiac injection. Piglets were

198 bled by cutting the neck. Then, the abdomen was opened and the entire gut was removed

199 aseptically. The stomach and cecum were identified, clamped, and separated. Digesta samples

200 were then collected from both gut segments. Homogenized digesta samples for microbiome

201 analysis were snap-frozen in liquid nitrogen and stored at -80°C.

202

203 ***DNA extraction, 16S rRNA and ITS sequencing and bioinformatics***

10

204 For DNA extraction, the protocol as described in Lerch et al. (2023) was used (DNeasy

205 PowerSoil Pro Kit; Qiagen, Hilden, Germany) with the same modifications including a heating

206 step and mechanical lysis. The concentration of DNA in each extract was measured with the

207 Qubit fluorometer (Qubit 4 Fluorometer, Thermo Fisher Scientific, USA) using the Qubit

208 dsDNA HS Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Targeted 16S

209 rRNA (V3-V4 hypervariable region) and ITS2 amplicon sequencing was performed in an

210 external laboratory (Novogene, Cambridge, UK). In order to do so, aliquots of the DNA

211 extracts were sent for library preparation (NEBNext Ultra II DNA Library Prep Kit, Illumina,

212 San Diego, CA, USA). The 16S rRNA amplicon was amplified using primers 341F-ill (5′-

213 CCTACGGGNGGCWGCAG-3′) and 802R-ill (5′-GACTACHVGGGTATCTAATCC-3′),

214 and the ITS2 region was amplified using primers ITS3 (5′-GCATCGATGAAGAACGCAGC-

215 3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). Equimolar pools of samples were

216 sequenced to generate 250bp paired-end raw reads in the Novaseq 6000 platform (Illumina).

217 Demultiplexing and trimming of the raw sequences was performed by Novogene.

218 Raw sequencing reads (Fastq files) for the 16S rRNA and fungal ITS amplicons were

219 processed, aligned, and classified independently using the Divisive Amplicon Denoising

220 Algorithm 2 (DADA2; version 1.26.0) in R studio (version 1.4.1106) (Callahan et al., 2016).

221 The 'file.path' function was used to pre-filter sequences in order to remove reads with

222 ambiguous bases for both bacterial and fungal sequences. Afterwards, the forward and reverse

223 read quality profiles were separately examined using the 'filterAndTrim' function (truncQ=5).

224 To account for the decrease in quality score of the subsequent nucleotides, the total length of

225 forward and reverse reads was truncated to 220 nucleotides with a maximum error rate of 5 for

226 both forward and reverse reads. For the fungal ITS amplicons, the first 10 nucleotides of each

227 read were trimmed to account for the decrease in quality score of the following nucleotides,

228 and a minimum length of 50 nucleotides was enforced to eliminate very low-length sequences.

11

229   Reads with ambiguities were removed from both amplicon sets, as were reads that exceeded

230   the probabilistic estimated error of two nucleotides for the ITS reads. For both the 16S rRNA

231   and ITS amplicons, amplicon sequence variants were inferred after de-replication of the filtered

232   data and estimation of error rates (Callahan et al., 2016). The inferred forward and reverse

233   sequences were then merged, with paired sequences that did not perfectly match removed to

234   control for residual errors, and a sequence table was constructed. The removeBimeraDenovo

235   () function was used to remove chimeras, and taxonomy was assigned using the SILVA 138.1

236   ribosomal RNA database for bacteria (Quast et al., 2012) and the UNITE ITS database (version

237   9.0) for fungi (Nilsson et al., 2019) with a 3% dissimilarity threshold. Alpha-diversity

238   (Shannon, Simpson, Chao1) analysis was performed using phyloseq (version 1.42.0). For beta-

239   diversity analysis, statistical assessment of dissimilarity matrices (Bray-Curtis) was performed

240   using the 'adonis2' function in the R package 'vegan' (version 2.6.4) (Oksanen et al., 2022),

241   separately for the bacterial and fungal composition. The permutational multivariate analysis of

242   variance (PERMANOVA) was used on the Bray-Curtis distance matrices to assess the

243   dissimilarities between the bacterial and fungal community structures in sows' feces post-

244   partum and in the gastric and cecal digesta of piglets on the various sampling days during the

245   suckling period. The statistical significance was determined after 999 random permutations.

246   The two-dimensional non-metric multidimensional scaling (NMDS) ordination plots generated

247   by the ggplot2 package using the 'metaMDS' function were used to visualize the clustering of

248   bacteriomes and mycobiomes in gastric and cecal digesta according to sample type and age as

249   well as between sow feces and digesta samples of piglets.

250   The datasets generated for this study were deposited into the NCBI Bioproject databank under

251   accession number PRJNA1103974.

252

253   ***Statistical analyses***

254   The Shapiro-Wilk test with the UNIVARIATE procedure in SAS (version 9.4; SAS Institute,

255   Inc., Cary, NC) was used to test the normal distribution of the residuals of all variables. The

256   residuals were transformed using the Boxcox method and the Transreg procedure in SAS if

257   they were not normally distributed. All data, both from sows, i.e., gut microbiome, and from

258   piglets, i.e., body weight, gut microbiome, and fecal score, were subjected to ANOVA using

259   the MIXED procedure in SAS. Across datasets for sow feces and digesta samples from piglets,

260   bacterial (> 0.2% of all reads) and fungal taxa (> 0.05% of all reads) were taken into

261   consideration. The ranked relative abundances were analyzed in SAS. Repeated measures were

262   used to investigate effects with progressing lactation of the sows (DPP) and increasing age of

263   the piglets (DoL). For data of the sow, a random model was used that included the fixed effects

264   of DPP, replicate batch, litter, and the respective two- and three-way interactions. The random

265   model for data of the piglets included the fixed effects of sex, replicate batch, DoL, litter, and

266   the respective two- and three-way interactions. For most parameters in piglets, differences

267   between sexes were not detected and were excluded from the final model. For the growth

268   performance of the piglets, a separate random model, consisting of the fixed effects replicate

269   batch, sex, and their interaction, as well as litter size at birth and date of birth as covariates,

270   was applied. The sow and piglet represented the experimental unit. Degrees of freedom were

271   approximated by the Kenward-Rogers method (ddfm = kr). Data were reported as the least-

272   square means ± standard errors of the mean (SEM). Multiple pairwise comparisons among

273   least-square means were performed using the pdiff statement. A significant difference was

274   defined at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$. Descriptive statistics using PROC MEANS

275   procedure in SAS were applied to calculate feed intake of sows during late gestation and

276   lactation, as well as creep feed intake of piglets during suckling period. PROC CORR in SAS

277   was used to calculate Pearson correlation coefficients between cecal bacterial and fungal taxa

278   and postweaning fecal scores on DoL30 and 34. To visualize the obtained correlations, heat

13

279   maps were generated using the 'levelplot()' function in the lattice package in R Studio (version

280   2023.06.0).

281

282   **RESULTS**

283   *Feed intake of sows and piglets and growth performance of piglets*

284   The average feed intake of sows during late gestation and lactation period was 3.5 and 6.6

285   kg/day and sow, respectively (Supplementary Table S3). The average litter size was 13.9 ± 1.7

286   (SD). The creep feed intake was on average 19.6 g/day and piglet during the suckling period

287   (Supplementary Table S4). Except for the higher birth weight of male piglets compared to

288   female piglets ($P = 0.014$; Supplementary Table S5), body weight and average daily weight

289   gain was similar during the suckling and early postweaning period.

290

291   *Changes in bacterial and fungal abundance and composition in the feces of lactating sows*

292   The PERMANOVA based on Bray-Curtis dissimilarities showed significant separation

293   between animal (i.e., sows and piglets), age (i.e., DoL and DPP), and gut segment (i.e., feces,

294   stomach, and cecum) for the bacterial and fungal communities ($P < 0.001$; Supplementary

295   Table S6). The non-metric multidimensional scaling (Bray–Curtis distance) demonstrated

296   separate clustering for the bacterial and fungal communities in sow feces, gastric and cecal

297   digesta. Time-related clustering of the microbial communities was visible, especially for the

298   communities in gastric and cecal digesta of the piglets but also for the fungal communities in

299   sow feces with increasing DPP (Fig. 1A and 1B).

300   Day postpartum did not influence the bacterial species richness (Chao1) in the feces of sows

301   (Table 1), whereas the bacterial alpha-diversity (Shannon and Simpson) increased from DPP1

302   to 6 and decreased thereafter until DPP27 ($P < 0.05$). The DPP affected the fungal species

303   richness (Chao1) and alpha-diversity (Shannon and Simpson; $P < 0.05$; Table 1) but in an

                                                                                                                14

304 opposite manner compared to the bacterial community. The fungal species richness and alpha-

305 diversity decreased from DPP1 to 13 but increased thereafter until DPP27 ($P < 0.05$). These

306 observations were supported by the results from 16S rRNA and ITS2 amplicon sequencing

307 (Fig. 2A and 2B). As an example, the most abundant genera *Lactobacillus* increased by 10.3-

308 fold from DPP1 to 6 ($P < 0.001$), whereas *Streptococcus* decreased by 3.5-fold from DPP1 to

309 13 and increased thereafter until DPP27 ($P < 0.001$). *Rikenellaceae* RC9 gut group increased

310 by 1.6-fold from DPP1 to 6 but decreased afterwards on DPP13 by 1.5-fold ($P < 0.001$),

311 whereas *Terrisporobacter* increased from DPP1 to 20 by up to 1.4-fold ($P = 0.034$). As

312 examples for fungal taxa, *Kazachstania* as the most dominating fungi in sow feces increased

313 by 3.0-fold from DPP1 to 13 but decreased thereafter until DPP27 by up to 1.9-fold ($P < 0.001$).

314 The abundance of *Tausonia*, which was the most dominant taxon on DPP1, declined by 28.9-

315 fold from DPP1 to 13 but remained stable thereafter until DPP27 ($P < 0.001$). The abundance

316 of *Geotrichum* was low on DPP1 and 6, whereas it increased to DPP27 by up to 20.4-fold,

317 becoming the second dominant fungal genus.

318

319 ***Age-related changes in bacterial and fungal communities in gastric and cecal digesta of***

320 ***piglets***

321 Day of life did not affect bacterial and fungal species richness (Chao1) but influenced bacterial

322 (Simpson) and fungal (Shannon, Simpson) alpha-diversity ($P < 0.05$) in the gastric digesta

323 (Table 2). Accordingly, the bacterial alpha-diversity decreased from DoL14 to 31, whereas

324 fungal alpha-diversity decreased from DoL28 to 35 in gastric digesta ($P < 0.05$). In cecal

325 digesta, DoL differently affected bacterial and fungal species richness (Chao1) and alpha-

326 diversity (Shannon, Simpson; $P < 0.05$; Table 2). In terms of relative abundances in gastric

327 digesta (Fig. 3A), the predominant *Lactobacillaceae* genera, including *Lactobacillus*,

328 *Limosilactobacillus*, HT002 and *Ligilactobacillus*, together with *Streptococcus* largely altered

329 their abundances from DoL3 to 7 and/or from DoL28 to 31 and 35 ($P < 0.05$). Alterations in

330 relative abundances of fungal genera in gastric digesta were similarly visible (Fig. 3B). For

331 instance, gastric abundances of *Candida* and *Blumeria* largely increased from DoL7 to 14,

332 whereas those of an unclassified genus Incertae sedis and *Kazachstania* greatly increased from

333 DoL28 to 31 ($P < 0.05$).

334 In cecal digesta, *Fusobacterium* and *Bacteroides* were the predominant genera on DoL3,

335 whereas *Lactobacillus* became the dominant taxon from DoL7 to 31 (Fig. 3C). Postweaning,

336 *Prevotella*-9 became dominant, whereas the abundances of *Fusobacterium* and *Bacteroides*

337 largely dropped compared to the time points during the suckling phase ($P < 0.05$). The

338 abundance of Fungi genus Incertae sedis greatly increased from DoL3 to 7 but decreased

339 thereafter until DoL35 ($P < 0.001$; Fig. 3D). The abundance of *Tausonia* decreased

340 postweaning compared to the suckling phase, whereas the abundance of *Kazachstania* largely

341 increased from DoL28 to 35 ($P < 0.001$).

342

343 ***Postweaning fecal scores***

344 The fecal score was 0.67 on DoL30 and increased by 3.1-fold on DoL34 ($P < 0.001$; Fig. 4A).

345 The fecal score correlated positively ($P < 0.05$) with *Holdemanella,* ($r = 0.55$) and negatively

346 with *Turicibacter (r = -0.54)* and *Odoribacter (r = -0.52)* but not with fungal genera on DoL30

347 (Fig. 4B). On DoL34, the fecal score positively correlated with three fungal genera

348 (*Talaromyces*, *Mrakia*, and *Blumeria* ($r > 0.5$; $P < 0.05$; Fig. 4C), as well as with nine bacterial

349 genera (*Clostridium* sensu stricto 1, *Prevotellaceae* NK3B31 group, *Parabacteroides*,

350 *Terrisporobacter*, *Romboutsia*, *Turicibacter*, dgA-11 gut group, *Prevotellaceae* UCG-001, and

351 *Elusimicrobium* ($r > 0.5$; $P < 0.05$; Fig. 4D).

352

353   **DISCUSSION**

354   The present study provides novel information about the alterations of the bacterial and fungal

355   communities in sow feces. Moreover, our results showed that there was a large overlap in

356   bacterial and fungal taxa between sow feces and digesta of piglets. These results support the

357   role that the microbial community in sow feces plays for the neonatal gut colonization of

358   piglets. For instance, the abundances of *Lactobacillus, Streptococcus* and *Tausonia* in gastric

359   and cecal digesta of piglets corresponded to their abundance in sow feces, especially in the

360   early suckling phase. Nevertheless, the differences in relative abundances indicated that the

361   establishment of the microbes in the gut segments depended on the 'local' conditions (e.g.,

362   lower stomach pH in stomach and nutrient availability) and other sources of microbes (e.g.,

363   sow colostrum/transient milk and skin). This assumption was supported by the beta-diversity,

364   demonstrating differently clustering bacterial and fungal communities for sow feces, and

365   gastric and cecal digesta of piglets. Moreover, our results provide novel data about the fungal

366   diversity that establishes in the piglet's gastrointestinal tract from early in life (DoL3) and their

367   changes in composition after weaning. Especially the large variety of fungal taxa that were

368   present in gastric and cecal digesta of the piglets on DoL3 are worth to highlight; with

369   abundance patterns (more in gastric digesta) that resembled to a certain degree the fungal

370   abundances in sow feces on DPP1. The uptake of feces by the piglets is similar to the concept

371   of 'fecal transplants' (McCormack et al., 2018). Therefore, our results may be useful for future

372   formulation of transition and lactation diets for sows to modulate their fecal microbiome in

373   order to target specific developmental stages in the gut microbiome of suckling piglets.

374   Different types of fungi were present in sow feces and piglets' digesta including common gut

375   inhabitants (e.g., *Kazachstania*; Harlow et al., 2024), plant-related fungi (Pietrusińska and

376   Tratwal, 2020), yeasts (e.g., *Candida*; Pérez, 2021) and mold fungi (Liew and Mohd-Redzwan,

377   2018). Metabolically, these fungi probably filled different niches, breaking down residual

378   dietary carbohydrates (Luo et al., 2021) as well as being involved in epithelial glucose turnover

379 (Hu et al., 2023). Some plant-related fungi – some of them plant pathogens (e.g., *Blumeria*;

380 Mapuranga et al., 2022) – may have been transient, colonizing the plant feed particles. Their

381 role in the gut microbe-microbe/host interactions needs further research as some of them are

382 categorized as plant pathogens. Furthermore, the abundance of *Cladosporium, Alternaria,*

383 *Fusarium,* and *Aspergillus* in sow feces and piglet's digesta showed that the animals were

384 exposed to mold and ergot fungi from the environment and feed and hence potentially to

385 mycotoxins and ergot alkaloids which may compromise their health (Coufal-Majewski et al.,

386 2016; Deligeorgakis et al., 2023).

387 Results for the beta-diversity and ANOVA demonstrated that the bacterial and fungal

388 communities in sow feces were not stable throughout lactation but continuously change in

389 relative abundances and diversity. This is probably a result of stress and dietary changes around

390 farrowing as well as the gradual increase in feed intake level during lactation (Jašarević et al.,

391 2017; Lu et al., 2022). More specifically, the bacterial community changed more between

392 DPP1 and 6, whereas the four dominant fungal taxa showed large changes in their abundances

393 throughout the lactation phase. Obviously, the fungal community was more susceptible to

394 changes in fermentable substrate quality and quantity that reached the distal parts of the hindgut

395 and/or substrate-related microbial interactions with progressing lactation than the bacterial

396 community. Sows had access to hay as environmental enrichment; as a slowly fermentable

397 fiber it likely acted as substrate for fibrolytic bacterial (e.g., *Treponema* and *Fibrobacter*; Xie

398 et al., 2018) and fungal taxa (e.g., *Mucor;* Karimi and Zamani, 2013) in feces. Unfortunately,

399 we do not have exact data for the hay intake of sows. Of note, the species richness and diversity

400 of the fungi were greater in cecal digesta of piglets than in sow feces in the first two weeks of

401 life, which may be linked to the immature immune system and microbe-to-microbe

402 interactions.

403   The most drastic changes in the communities in sow feces were within the dominating lactic

404   acid bacteria and *Tausonia* from DPP1 and 6. *Streptococcus, Lactobacillus* and *Tausonia*

405   utilize dietary or host-related glycans (Zúñiga et al., 2018; Ma et al., 2020). As the difference

406   in the pre-farrowing to post-farrowing diet was mainly the discontinuation of feeding soaked

407   linseeds, the mucilage of the seed coat (Kajla et al., 2015; Trochine et al., 2022) may have

408   promoted the growth of *Streptococcus* and *Tausonia*. Without linseeds, this may have given a

409   growth advantage to *Lactobacillaceae* genera. The piglets showed a similar increase in

410   *Lactobacillaceae* in gastric and cecal digesta from DoL3 to 7, which may be advantageous due

411   to their multiple beneficial effects on the control of gut homeostasis and immune development

412   (Valeriano et al., 2017; Zhang et al., 2022). From the abundance patterns, it can be assumed

413   that sow feces were not the only source of *Lactobacillaceae* for the colonization of the piglet's

414   stomach and distal gut. However, their predominance throughout the suckling phase was likely

415   supported by the milk glycans.

416   The beta-diversity analysis supported the commonly observed age-related alterations of the gut

417   bacterial and fungal community in the suckling piglets. The diversity differed for the stomach

418   and cecum, showing the dependence of the microbial community on the available substrate at

419   the two gut sites. Despite the full availability of nutrients in the colostrum and milk to gastric

420   microbes, the abundance patterns of some milk glycan utilizers, such as *Lactobacillus,* were

421   similar between the two gut sites. However, the diverging abundance patterns of other milk

422   glycan fermenters, such as *Limosilactobacillus*, HT002, and *Ligilactobacillus, Actinobacillus,*

423   *Fusobacterium* and *Bacteroides* (Garcia-Alija et al., 2022), between gastric and cecal digesta

424   indicated different substrate availabilities and subsequent cross-feeding relationships, which

425   may have contributed to the higher diversity in cecal digesta. Moreover, the bacterial and

426   fungal composition probably reflected the slowly increasing amounts of creep feed as well as

427   the change in the type of creep feed from the milk replacer to the prestarter from DoL21 to 28.

19

428 Even in very small amounts plant glycans, such as starch, probably supported the growth of

429 starch-degrading taxa such as *Turicibacter*, *Terrisporobacter*, *Porphyromonas* and *Prevotella*

430 in digesta (Umu et al., 2015; Sun et al., 2015; Trachsel et al., 2019). As an example, the starch

431 component may have proportionally lowered the abundance of milk glycan-fermenting bacteria

432 in gastric digesta on DoL21. Other bacteria, such as *Clostridium* sensu stricto, may have thrived

433 on milk peptides, either from sow milk or milk replacer.

434 *Kazachstania* is a commensal yeast in the porcine gut (Summers et al., 2021), which per se

435 may explain its high presence in sow feces and gastric and cecal digesta of piglets. They feed

436 on dietary or host-derived sugars, such as glucose and galactose (Kondybayev et al., 2023).

437 Previously, *Kazachstania slooffiae* has been linked to the provision of amino acids and energy

438 to other bacteria, such as *Lactobacillus* and *Prevotella*, as well as the host piglet (Summers et

439 al., 2021; Hu et al., 2023). From the abundance patterns, it is unfortunate that a great part of

440 the fungal sequences belonged to unclassified fungi in gastric and cecal digesta of piglets for

441 which we can only assume that they probably utilized milk components. The yeast *Candida*

442 increased in gastric more than in cecal digesta of piglets during the suckling phase, which may

443 be linked to the glycan intake from milk and creep feed. Another high abundant fungus in sow

444 feces, *Geotrichum*, which was abundant in < 0.05% of fungal communities in the digesta of the

445 piglets may exert some positive effects on the host as it has been used as probiotics in ruminant

446 nutrition to promote productivity and bacterial diversity in dairy cattle (Zaman et al., 2022),

447 whereas very little is known for pigs. According to its abundance pattern in feces throughout

448 lactation, the abundance of *Geotrichum* seemed to be related to the feed intake level of the

449 sows.

450 The diversity abundance patterns of the bacterial and fungal communities allowed

451 distinguishing the time point of weaning. Similar to previous observations in neonatal piglets

452 (Lerch et al., 2023), the bacterial and fungal diversity in gastric and cecal digesta were

20

453   differently affected by weaning. In the present study, it can be assumed that the weaning-related

454   removal of sow milk, the mostly plant-based diet and low feed intake as well as changes in the

455   bacterial crosstalk caused decreasing fungal diversity in cecal digesta from DoL28

456   (preweaning) to DoL35 (postweaning). It is difficult to relate the changes in the fungal

457   community postweaning to the action of certain bacteria as our understanding is only

458   advancing. Weaning changed the bacterial and fungal communities in the sense of milk glycan

459   fermenters being replaced by plant glycan utilizers. For instance, the removal of the milk

460   glycans reduced the abundance of *Limosilactobacillus* and HT002 in gastric digesta. By

461   contrast, other *Lactobacillaceae*, such as *Lactobacillus* and *Ligilactobacillus,* in gastric and

462   cecal digesta remained stable or increased in their abundance postweaning, indicating that they

463   were capable to utilize the glycans in the prestarter diet (i.e., starch and lactose) and derived

464   from the host. Of note, *Bifidobacterium*, another genus known for its milk glycan-fermenting

465   capabilities (Jang and Kim, 2022), only raised in its abundance in gastric digesta on DoL31.

466   This taxon possibly benefited from the opening niches of *Lactobacillaceae* but seemed to be

467   outcompeted again on DoL35, e.g., by HT002 and *Ligilactobacillus*. In cecal digesta, typical

468   plant glycan-utilizing taxa, like *Prevotellaceae* genera and *Succinivibro* (Tan and Nie, 2020),

469   became dominating on DoL31 and 35. Regarding the fungal community, especially

470   *Kazachstania* seemed to be promoted by postweaning gastric and cecal conditions, potentially

471   benefiting from increased glucose release from starch fermentation or mucosal glycolysis.

472   From the abundance of pathobionts, the increase in *Campylobacter* in cecal digesta

473   postweaning may be worth mentioning. By contrast, the gut conditions postweaning seemed to

474   lower the abundance of classified mold and ergot fungi in gastric and cecal digesta. The piglets

475   from which the gut samples were collected were not diarrhetic. This may be the reason for the

476   few relationships between cecal microbial genera and fecal consistency on DoL30. Piglets had

477   firm feces on DoL30; accordingly, they only moderately correlated with three starch-

21

478   fermenting and short-chain fatty acid-producing bacteria which included *Holdemanella*,

479   *Turicibacter* and *Odoribacter* but not *Campylobacter*. On DoL34, only the positive association

480   between fecal score and *Clostridium* sensu stricto 1 may relate softer feces to bacterial toxins.

481   The other positive correlations with bacterial taxa (e.g., *Prevotellaceae* NK3B31 group,

482   *Prevotellaceae* UCG-001 and *Terrisporobacter*) may have been indicative for increased

483   complex carbohydrate fermentation and short-chain fatty acid production in the large intestine,

484   which are osmotically active and increase the fecal water content (Amat et al., 2020; Niu et al.,

485   2023). The three identified positive correlations of the fecal score with fungi were only with

486   low abundant genera. Nevertheless, as these taxa were mold and plant pathogenic fungi, an

487   activation of secretory functions in the large intestine may be thinkable.

488   In conclusion, this study demonstrated that progressing lactation affected bacterial and fungal

489   community structure and bacterial richness in sows. Our results also support that sow feces

490   were a contributing source of microbes for the gut colonization of piglets, as indicated by the

491   shared bacterial and fungal taxa in sow feces and in the gastric and cecal digesta of piglets. We

492   could identify microbial taxa in cecal digesta that associated with the fecal scores on DoL30

493   and DoL34. However, the feces were firm to soft but not liquid diarrhea. Therefore, the

494   relationships miss information for a low gut homeostatic state postweaning.

495

496   **DISCLOSURES**

500

501   **ACKNOWLEDGEMENTS**

513

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662 **Figure legends**

663 **Figure 1**. Non-metric multidimensional scaling (NMDS) plot of pairwise Bray–Curtis

664 dissimilarities between communities of bacteriome (A) and mycobiome (B) in sow feces across

665 days postpartum (DPP) and in the gastric and cecal digesta of piglets across days of life (DoL).

666 Ellipses represent the standard deviation. Feces on DPP3 (dark blue), 7 (yellow), 14 (green),

667 21 (purple), and 28 (dark gray). Gastric digesta on DoL3 (green), 7 (purple), 14 (red), 21 (blue),

668 and 28 (gray). Cecal digesta on DoL3 (red), 7 (blue), 14 (dark gray), 21 (dark blue), and 28

669 (yellow). Weaning took place on DoL28. Number 1 indicates sow feces, 2 is piglet's gastric

670 digesta, and 3 is piglet's cecal digesta.

671 **Figure 2.** Differences in relative abundance (%) of bacterial (> 0.2% of all reads; A) and fungal

672 (> 0.05% of all reads; B) genera in sow feces across days postpartum. Weaning took place on

673 day 28 postpartum. Effect ($P < 0.05$) of day postpartum is indicated by '*'.

674 **Figure 3.** Differences in the relative abundance (%) of bacterial genera (> 0.2% of all reads)

675 in gastric (A) and cecal (C) digesta as well as fungal genera (> 0.05% of all reads) in gastric

676 (B) and cecal (D) digesta of suckling and newly weaned piglets across days of life. Weaning

677 took place on day 28 of life. Effect ($P < 0.05$) of day of life is indicated by '*'.

678 **Figure 4**. Differences in fecal scores of weaned piglets between days 30 and 34 of life (A) and

679 Pearson's correlation heat map showing significant associations ($P < 0.05$) of fecal scores with

680 cecal bacterial genera (B) of weaned piglets on day 30 of life, as well as with cecal fungal (C)

681 and bacterial (D) genera on day 34 of life. The fecal scoring system consisted of 0 (balls

682 normal), 1 (shaped soft), 2 (pasty), 3 (shaped very soft), 4 (mild diarrhea), and 5 (watery

683 diarrhea). Piglets were weaned on day 28 of life. Fecal score values are least squares means

684 and standard error of the mean.

685 **Table 1**. Development in species richness (Chao1) and alpha diversity (Shannon and Simpson) indices for the bacterial and fungal community in

686 sow feces after farrowing

Day postpartum	1	6	13	20	27	SEM	P-value
Bacterial community							
Chao1	1220	1266	1286	1269	1249	38.654	0.858
Shannon	4.96[bc]	5.24[a]	5.14[ab]	5.05[abc]	4.92[c]	0.079	0.020
Simpson	0.957[b]	0.976[a]	0.973[a]	0.969[ab]	0.961[b]	0.004	0.007
Fungal community							
Chao1	178[a]	99[b]	95[b]	211[a]	174[a]	23.292	0.001
Shannon	1.65[a]	1.06[bc]	0.83[c]	1.38[ab]	1.67[a]	0.195	0.007
Simpson	0.546[a]	0.393[ab]	0.265[b]	0.401[ab]	0.549[a]	0.059	0.005

687 Values are presented as least squares means ± standard error of the mean (SEM). Weaning took place on day 28 postpartum.

688 [a,b,c,d]Means without a common superscript in the same row differ ($P < 0.05$).

Table 2. Age-related development of species richness (Chao1) and alpha diversity (Shannon and Simpson) for the bacterial and fungal communities in the gastric and cecal digesta of suckling and newly weaned piglets

Day of life	3	7	14	21	28	31	35	SEM	P-value
Bacterial community in gastric digesta									
Chao1	475	441	498	491	912	574	596	134.72	0.178
Shannon	3.54	3.49	3.61	3.38	3.57	3.12	3.08	0.150	0.070
Simpson	0.913[a]	0.907[a]	0.923[a]	0.893[ab]	0.887[ab]	0.860[b]	0.855[b]	0.013	0.002
Fungal community in gastric digesta									
Chao1	68	71	58	71	71	68	54	6.239	0.309
Shannon	2.23[ab]	2.47[a]	2.02[b]	2.25[ab]	2.18[ab]	1.88[bc]	1.53[c]	0.135	<0.001
Simpson	0.772[ab]	0.825[a]	0.740[ab]	0.763[ab]	0.758[ab]	0.718[bc]	0.622[c]	0.038	0.020
Bacterial community in cecal digesta									
Chao1	767[c]	854[ab]	838[abc]	816[abc]	896[a]	888[ab]	803[bc]	28.92	0.039
Shannon	4.21[b]	4.88[a]	4.87[a]	4.81[a]	4.93[a]	4.72[a]	4.71[a]	0.095	<0.001
Simpson	0.939[b]	0.971[a]	0.974[a]	0.970[a]	0.974[a]	0.962[a]	0.964[a]	0.005	<0.001
Fungal community in cecal digesta									
Chao1	240[a]	136[cd]	213[ab]	173[bc]	183[abc]	161[bc]	107[d]	19.84	<0.001
Shannon	3.08[a]	2.74[a]	3.02[a]	2.51[a]	2.54[a]	1.77[b]	0.66[c]	0.201	<0.001
Simpson	0.816[a]	0.866[a]	0.865[a]	0.788[a]	0.790[a]	0.530[b]	0.207[c]	0.048	<0.001

[a,b,c,d]Means without a common superscript in the same row differ ($P < 0.05$).

Values are presented as least squares means ± standard error of the mean (SEM). Weaning took place on day 28 of life.

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Figure 1

A

B
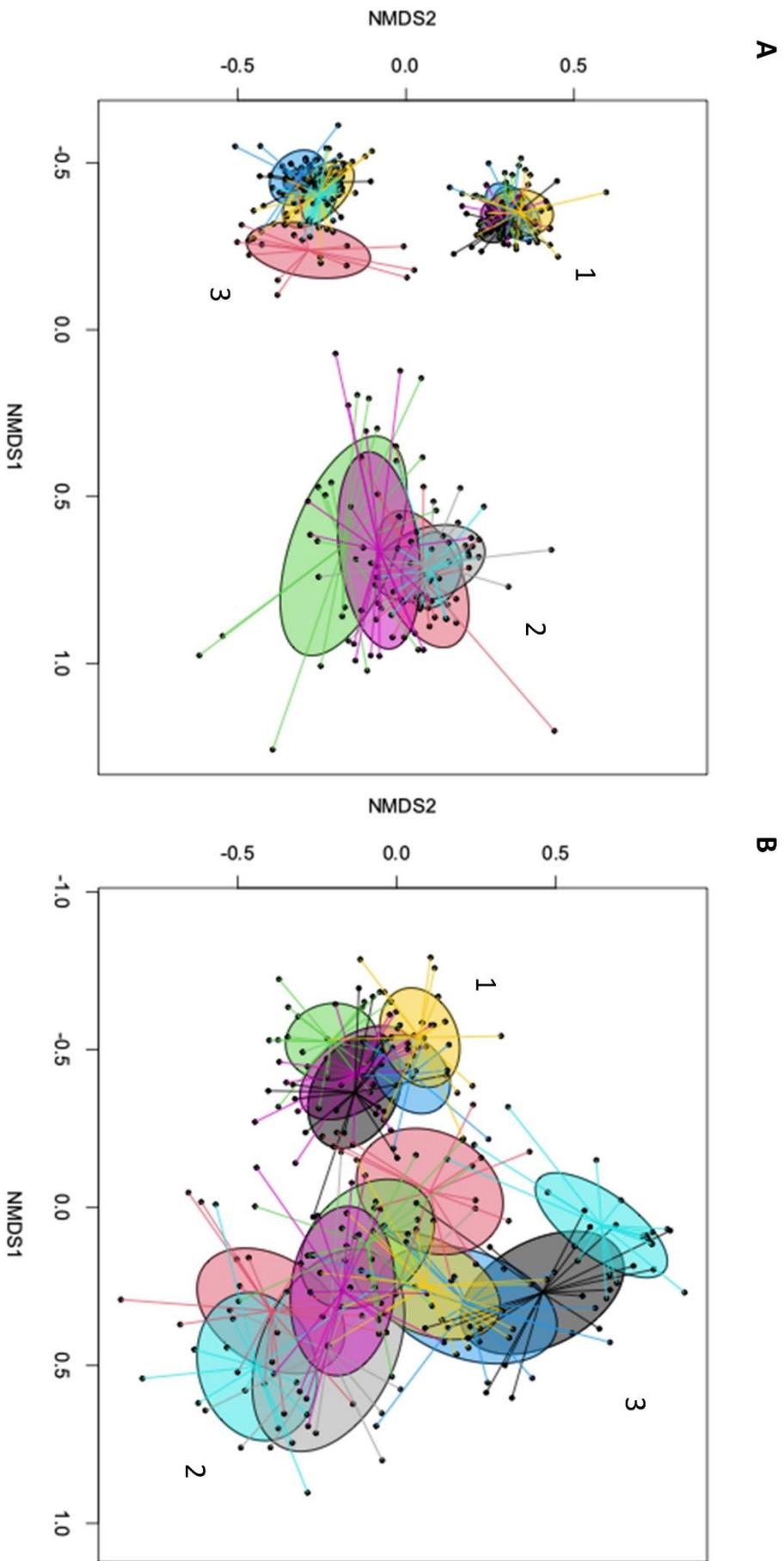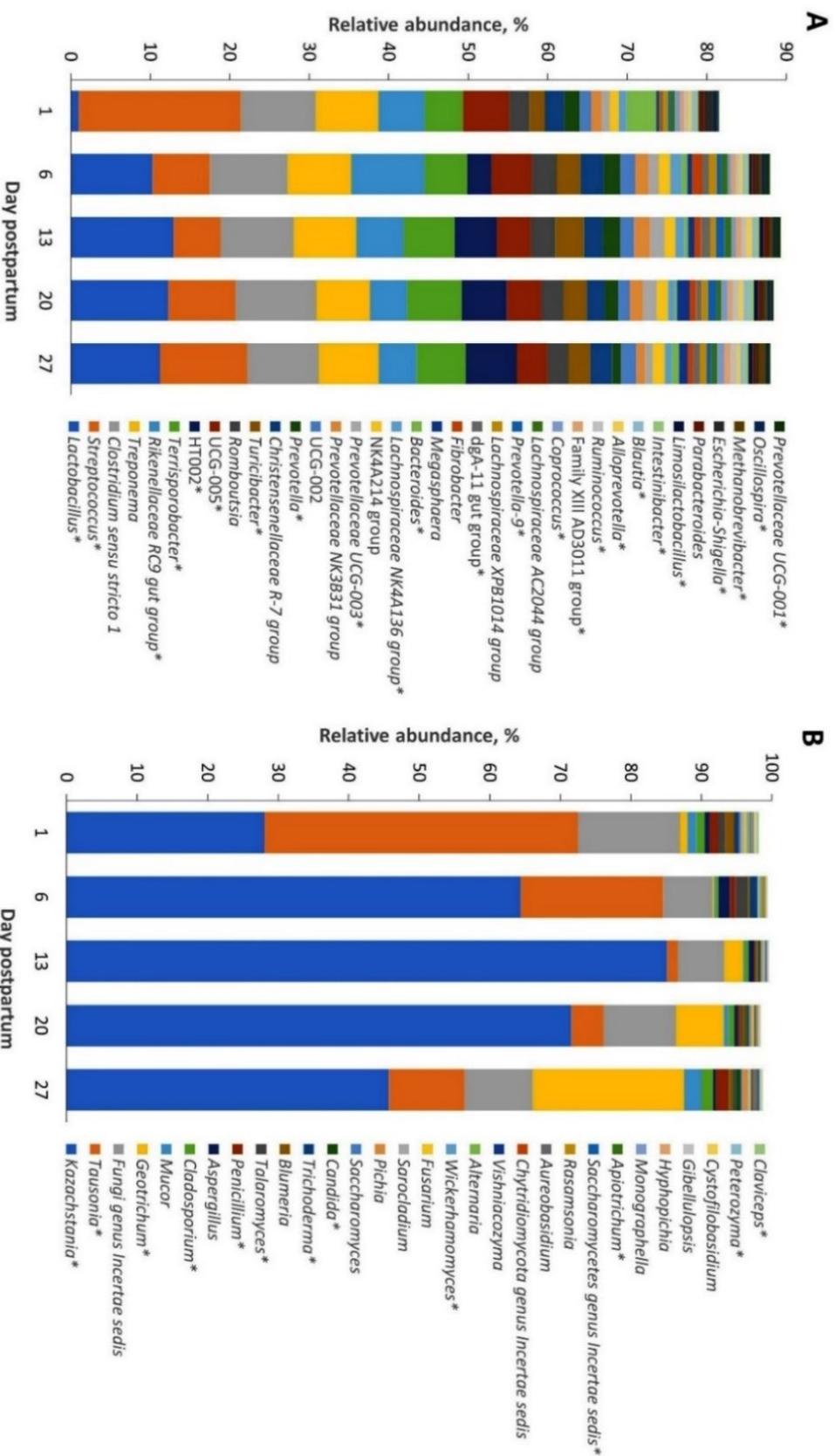
Figure 2

697   **Figure 3**
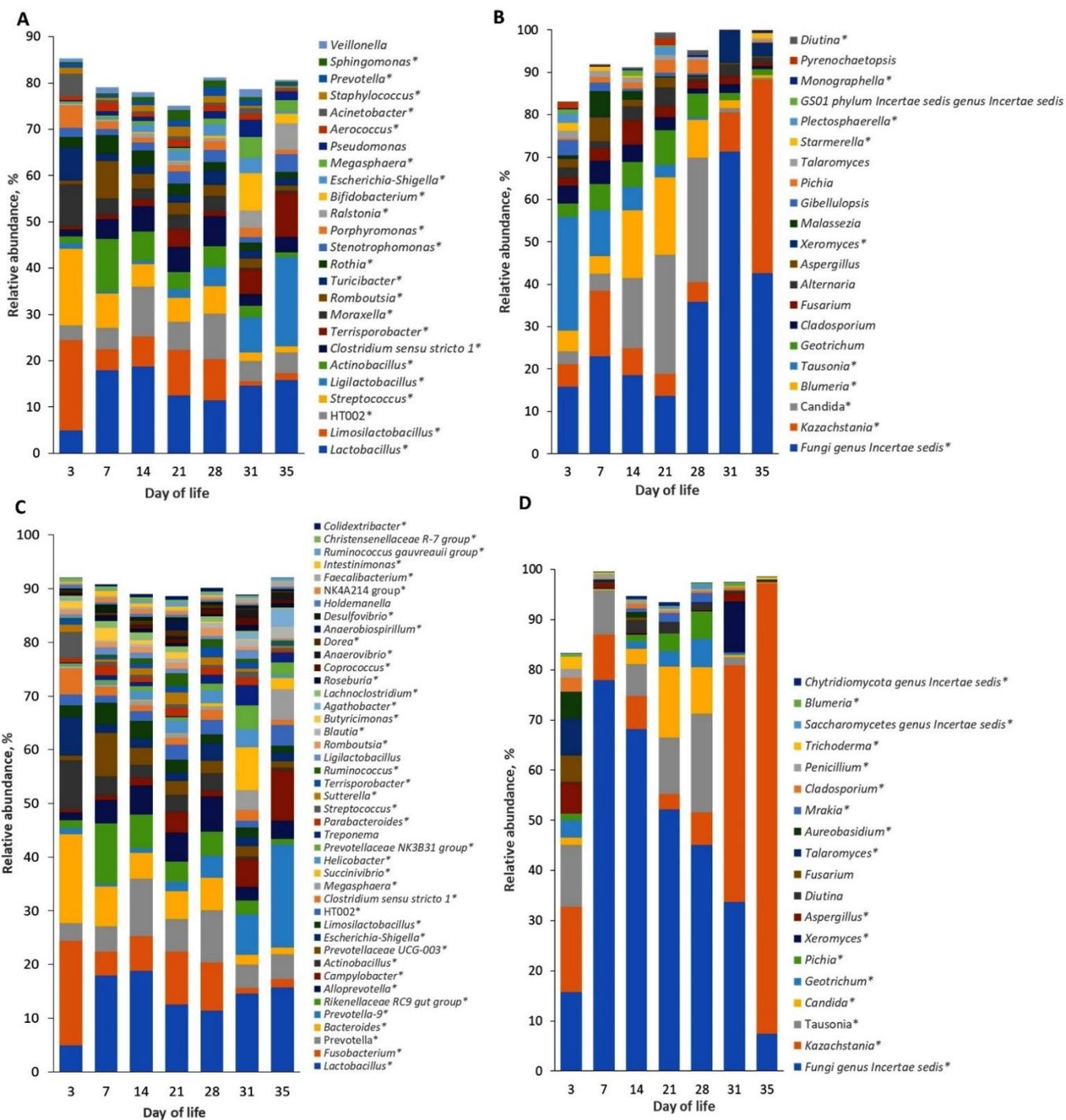
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Figure 4
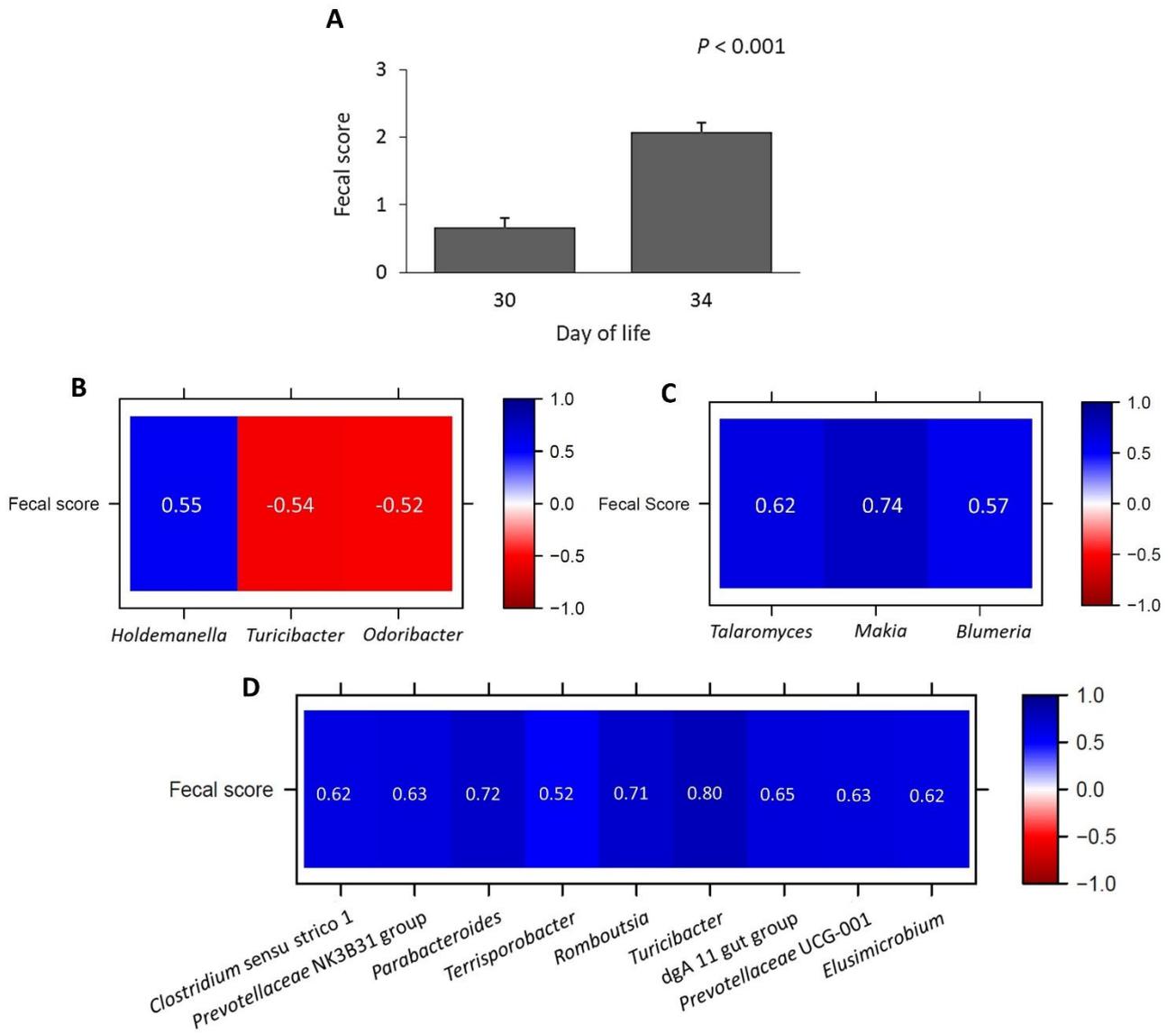
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5. GENERAL DISCUSSION

5.1. Advantages of using multiple experimental models in this PhD thesis

In order to gain a comprehensive understanding of the complex interactions between gut microbes and the host in monogastric farm animals, in particular in chickens and pigs, three complementary experimental models were used in this thesis: *ex vivo* (manuscript 1), *in vivo* (manuscript 3) and meta-analytical (manuscript 2) models.

In recent years, various techniques and gut models have been developed to simulate the *in vivo* environment of animals. This is done to gain a better understanding of animal gut physiology and to find ways to improve animal welfare, gut health, and production outcomes. Among the models available, the *ex vivo* model has been widely adopted. This model refers to experiments performed directly on animal-derived tissues (Shi et al., 2019). *Ex vivo* models provide a more detailed description of the mechanisms and interactions between gut microbes and hosts in the gastrointestinal tract (Ghiselli et al., 2021). *Ex vivo* models offer advantages as "screening" devices or techniques to investigate mechanisms that may help explain *in vivo* results. Thus, *ex vivo* techniques can provide a simplified and controlled way to elucidate specific interactions involving gut tissues, microbes, and their metabolites (Ghiselli et al., 2021). By implementing Ussing chamber and organ bath techniques, the primary objective of the current *ex vivo* experiments were to assess whether short chain fatty acids (SCFA) at different acetate: butyrate ratios and concentrations can improve intestinal barrier function in chickens. In addition, this *ex vivo* study is also useful to confirm whether SCFA exhibit the same mechanism as demonstrated in previous studies in other animals to modify intestinal contractibility, particularly in the jejunum and cecum using laying hens as an animal model.

In addition to *ex vivo* models, *in vivo* experiments were also conducted for this thesis. *In vivo* experiments are preferred because, unlike *ex vivo* models, they represent a complete living biological system (Xu et al., 2021). *In vivo* studies are also crucial because they allow experiments on intact organisms that embody complex interactions between different physiological processes (Xu et al., 2021). Therefore, this model can provide relevant information on gut microbe-mucosa interactions, mechanisms of action, and interactions with organ systems. In the current *in vivo* study, sows and suckling and weaned piglets were used as animal models. This *in vivo* study had several objectives. The first was to obtain detailed information on the evolution of bacteria and fungi in sow feces during lactation, which has been little described before. The second was to obtain information on the development of gastric and cecal microbes, especially fungi, in suckling and newly weaned piglets, which has not been studied in detail, and then to assess whether this development was driven by maternal fecal

microbes. The third was to assess bacterial and fungal taxa in cecal digesta of the piglets postweaning that were associated with the fecal consistency. This could identify potential candidate bacterial and fungal markers for soft and firm feces as indicators of lower and higher gut homeostatic conditions after weaning. The last model included in the thesis is a meta-analysis. The main target of this meta-analysis was to obtain valid conclusions on the different results of direct-fed microbial (probiotic) studies, including parameters such as expression of genes related to gut barrier and immune functions, histomorphological measurements and growth performance in broiler chickens. To gain a better understanding of probiotic efficacy, the analysis separated studies with and without pathogen challenge. Additionally, it is important to consider other factors, such as dietary metabolizable energy (ME), crude protein (CP), and days post-pathogen infection (DPI) when evaluating the efficacy of probiotics.

Altogether, by implementing three complementary study models, i.e., *ex vivo*, *in vivo*, and meta-analysis in this PhD thesis, a more comprehensive overview of the role of SCFA, maternal microbes and probiotics on the development of gut microbes and their interactions with the host, particularly chickens and piglets, was obtained.

5.2. The present *ex-vivo* study supports the modulating effect of short-chain fatty acids on intestinal barrier function and contractibility in chickens

Gut microbes and their metabolites have been shown to affect chicken gut health *in vivo*, including modulating intestinal barrier function (Ali et al., 2022). This is indicated, for example, by a change in the expression of genes related to metabolite transporters, such as sodium monocarboxylate transporters, which function to transport SCFA into epithelial cells (Ali et al., 2022). Unfortunately, studies to clarify the transport mechanism of SCFA in the chicken gastrointestinal tract are still limited. In other animals, such as mice, *ex vivo* studies using the Ussing chamber have been performed and have shown that SCFA is able to modify net ion flux and intestinal permeability (Larraufie et al. 2017; Blakeney et al. 2019). However, the modulating effects of these factors on the chicken intestine, whether they are the same or not, have not been investigated. The present Ussing chamber experiment showed that both more butyrate and higher SCFA concentrations led to an increase in mucosal-serosal cation flux and improved barrier function in the jejunum (manuscript 1). The increase in cation flux is likely related to the activation of sodium monocarboxylate transporters expressed at the apical membrane of the small intestine (Sivaprakasam et al. 2017; Metzler-Zebeli et al. 2019b). In addition to intestinal barrier function, intestinal contractibility (i.e., jejunal and cecal segments) was also investigated using organ bath. The peristaltic motility in the intestinal tract depends

on the contraction and relaxation of intestinal smooth muscle (Röhm et al., 2021). The present study confirmed that higher butyrate and SCFA concentrations promoted cecal contractibility (manuscript 1), which might help in mixing and emptying the cecal contents *in vivo.* Additionally, another *ex vivo* study on porcine fetal intestine showed that the addition of SCFA can reduce jejunal permeability and cause muscle relaxation in fetal jejunal tissue (Metzler-Zebeli et al., 2022). In conclusion, the present *ex vivo* study confirmed the modulatory effects of short-chain fatty acids on intestinal barrier function and contractability, whereby increasing butyrate proportion and SCFA concentration improved jejunal barrier function and stimulated cecal muscle contractibility in laying hens.

5.3. Microbial dynamics of sow feces during lactation and its contribution to the establishment of intestinal colonization in neonatal piglets

Sow feces are one of the sources of microbial colonization of the postnatal piglet gut (Li et al., 2022). A recent study showed that access to maternal feces could improve piglet immune competence and growth performance (Aviles-Rosa et al., 2019). To date, some studies have investigated the influence of maternal microbes, including feces, on microbial development in piglets (Chen et al., 2022b; Lim et al., 2023). However, they focused more on the dynamics of bacterial communities in fecal samples. Information is still lacking on the development of the other microbial communities, especially fungi, in sows during lactation and their contribution to the development of microbial communities in neonatal piglets. The present study showed that bacterial and fungal communities were separately clustered between sows and piglets, as well as between ages (i.e., day postpartum/DPP and day of life/DoL) and gut segments (i.e., feces, stomach, and cecum) (manuscript 3). In sow feces, bacterial diversity increased from DPP1 to 6 but then decreased to DPP27, whereas fungal abundance and diversity decreased from DPP1 to 13 but increased thereafter until the last day of lactation. In piglets, bacterial and fungal diversity in the gastric digesta decreased from DoL14 to 31 and from DoL28 to 35, respectively. In the cecal digesta, richness and diversity of the bacterial and fungal communities were affected differently by age. This is consistent with a previous study describing age to be the dominant factor leading to variation in the porcine gut microbiome (Gaire et al., 2023). Furthermore, shared bacterial and fungal taxa were found in the feces of lactating sows and in the gastric and cecal digesta of suckling piglets, suggesting that microbes in sow feces contribute to the gut colonization of newborn piglets.

In terms of bacterial communities, *Lactobacillus* and *Streptococcus* were the predominant genera in sow feces during lactation. In piglets, the gastric digesta was dominated by

Streptococcus and genera from the family *Lactobacillaceae*, such as *Lactobacillus, Limosilactobacillus,* HT002*,* and *Ligilactobacillus.* The dominant bacteria in the cecal digesta were more diverse, such as *Lactobacillus, Fusobacterium, Prevotella,* and *Bacteroides*, which belonged to several families. The high abundance of *Lactobacillaceae* genera is beneficial to gut health as they regulate the immune system, increase metabolic capacity, and enhance barrier function (Chen et al., 2018; Zhang et al., 2022). The increased lactic acid-producing bacteria, e.g., *Lactobacillus*, are also beneficial to eliminate harmful microbes by lowering the intestinal pH and produce SCFA through a cross-feeding mechanism (Foushe et al., 2016; Vasquez et al., 2022). The increased *Ligilactobacillus* after weaning may be useful to modulate innate immune responses in piglets (Indo et al., 2021). The higher abundance of *Streptococcus, Bacteroides,* and *Prevotella* increased utilization of dietary or host-related glycans (Guevarra et al., 2018; Ma et al., 2020).

For the fungal communities, *Kazachstania* and *Tausonia* were the most abundant genera in sow feces during lactation. In piglets, both gastric and cecal digesta were dominated by *Kazachstania, Tausonia, Candida*, and Fungi genus Incertae sedis. *Kazachstania* is a commensal yeast in the pig intestine (Summers et al., 2021) and, together with *Tausonia*, utilizes dietary or host-derived sugars (Kondybayev et al., 2023). Previous research has shown that *Kazachstania* is symbiotic with beneficial bacteria, such as *Lactobacillus* and *Prevotella*, and provides amino acids as an energy source for microbial and piglet growth (Summers et al., 2021). It is possible that other fungal genera may also have a symbiotic relationship with other beneficial bacteria, but more research is needed to confirm this assumption. As opportunistic fungi (Pérez, 2021), the presence of *Candida* in the intestinal digesta of piglets should be monitored as they may act as pathogens under certain circumstances. After evaluating the postweaning feces, we found that the firm to soft feces correlated with certain bacteria and fungi in the cecal digesta, including starch-, fiber-, and protein-degrading bacterial genera as well as plant-pathogenic and mold fungi.

In conclusion, the present *in vivo* study demonstrated that the bacterial and fungal communities in sow feces changed during lactation and contributed to gut colonization of the neonatal piglets. Therefore, it is important to monitor the microbial composition of sow feces when conducting gut microbiome studies in suckling piglets. It is also crucial to consider the role of fungi, not only bacteria, and their potential symbiosis when studying the gut microbiome in newborn piglets.

5.4. A meta-analysis confirms the efficacy of dietary probiotics in improving gut health in chickens at the gene and structural levels

Data published in the literature over the past few decades regarding the beneficial effects of dietary probiotics on gut health and growth performance in broiler chickens have been inconsistent. The present meta-analysis was conducted in two steps, including meta-regression and backward elimination analysis. This procedure was advantageous in that we could first identify parameters that were positively or negatively influenced by probiotics. In the second step, we used backward elimination analysis to assess whether other factors influenced the effect of probiotics. After the initial process, we selected studies that investigated the effect of dietary probiotics on the expression of genes related to intestinal barrier and immune function as the primary parameters for this meta-analysis. From the selected studies, we also extracted data related to histomorphological measures and growth performance as additional parameters. To better understand the effects of probiotics on the observed parameters, we categorized studies into those with and without pathogen challenge. The study included a total of 9 probiotic genera: *Bacillus, Lactobacillus, Enterococcus, Clostridium, Pediococcus, Bifidobacterium, Paenibacillus, Streptococcus,* and *Saccharomyces.*

The present meta-regression showed that dietary probiotics may effectively improve intestinal barrier function by upregulating the expression of genes for mucins and tight junction proteins in the small intestine under non-pathogen-challenged conditions (manuscript 2). The increase in mucin expression indicates an increased mucus layer produced by goblet cells, which serves as a physical barrier to protect the intestinal epithelium from harmful microbes (Duangnumsawang et al., 2021). Likewise, the upregulation of tight junction proteins indicates improved regulation of paracellular permeability across the intestinal epithelium (Awad et al., 2017). Under conditions of pathogen and mycotoxin challenge, probiotics not only enhanced intestinal barrier function but also exerted anti-inflammatory effects by modulating cytokine expression in the small intestine and ceca. Enhancing immune function during pathogen infection is desirable because it can help fight disease and reduce the risk of death (Song et al., 2021). Additionally, dietary probiotics have been demonstrated to improve histomorphological measures of the small intestine at certain ages, which may improve the absorption and secretion processes of the small intestinal epithelium (Fathima et al., 2022). Unfortunately, the current meta-analysis did not find evidence supporting the effect of probiotics on growth performance in broiler chickens, such as feed intake, body weight gain, and feed conversion.

The backward elimination analysis included three additional predictors: levels of dietary metabolizable energy (ME) and crude protein (CP), and days post-infection (DPI), particularly in challenge studies. Based on our analysis, we discovered that feeding probiotics to diets with higher ME and CP reduced their efficacy in promoting gut immune and barrier function-related gene expression. However, it has a positive impact on histomorphological parameters. Higher dietary levels of ME and CP have been shown to decrease the number of butyric acid-producing bacteria while increasing the number of gram-negative and proteolytic bacteria (Zhang et al., 2020b; Xi et al., 2022). This shift in bacterial populations may lead to inflammation and ultimately affect gene expression. Additionally, the observed histomorphological changes may be attributed to the potential effect of probiotics on the growth and proliferation of intestinal epithelial cells. The results further showed that the longer the DPI, the higher the expression of genes that improved intestinal immune and barrier function, possibly due to the sufficient time needed for cells to recover after pathogen infection.

In conclusion, this meta-analysis supports the idea that dietary probiotics have a beneficial effect on gut barrier function and structure in broiler chickens. Additionally, probiotics also effectively improved immune function during pathogen challenge. When investigating probiotics, it is important to consider the levels of dietary metabolizable energy and crude protein, as they greatly affect the efficacy of probiotics.

5.5. Limitations of each experimental model used in this PhD thesis

5.5.1. *Ex vivo* model

The *ex vivo* models offer the advantage of serving as a screening tool to investigate mechanisms to reduce the number of *in vivo* trials. Nevertheless, there are limitations to the *ex vivo* studies, both in the Ussing chamber and organ bath, which are described below:

In the present *ex vivo* studies, only the jejunum and cecum were examined due to their important role in birds. The jejunum, as the longest part of the intestine, is critical for nutrient assimilation, while the cecum has the highest microbial activity (Oakley et al. 2014; Metzler-Zebeli et al. 2019). In addition, these segments are less studied than others. For a more comprehensive overview, it is recommended that future measurements be performed in other parts of the gastrointestinal tract, including the stomach. This is because tissues from different segments of the gut may elicit different physiological responses (Metzler-Zebeli et al. 2017b). In addition, we did not have the opportunity to examine the signaling pathways involved in muscle contraction in more detail, which should be done in a future study. Theoretically, there are several activation pathways that could mediate the effects of SCFA, including activation of

transcription factors or G protein receptors that have different affinities for different SCFA (Kumar et al., 2020). Therefore, these activation routes should be followed at the molecular level. Another limitation of the current *ex vivo* experiment is that it was not possible to determine the extent to which SCFA affect longitudinal and circular smooth muscle contractions in the jejunum and cecum, both of which play an important role in controlling intestinal peristaltic motility (Röhm et al., 2021). A previous study examined the effects of SCFA on circular and longitudinal smooth muscle contractions in the canine colon (McManus et al., 2002), which may serve as a reference for further research.

In conclusion, to improve our understanding of the modulatory effects of SCFA on gastrointestinal barrier function and contractibility, it is recommended that results from Ussing chamber and organ bath experiments be supported by additional experiments and gene-level laboratory analyses, especially for receptors or transporters associated with SCFA.

5.5.2. *In vivo* model

As previously stated, *in vivo* studies have confirmed that the microbial community in sow feces undergoes changes during lactation. Additionally, maternal fecal microbes contribute to the age-specific gut colonization of suckling piglets. This is evidenced by the similarity of certain bacterial and fungal taxa in sow feces and piglet gastric and cecal contents (manuscript 3). Similar to the *ex vivo* experiments, the current *in vivo* study of the mother-offspring axis in establishing gut microbial colonization in newborn piglets also has limitations. These limitations are discussed below:

The present *in vivo* study did not examine whether the development of microbial communities from other sources in sows, such as vagina, skin surface, and colostrum/milk, followed the same pattern as feces. As these maternal sources also contribute to the postnatal colonization of the piglet gut (Jost et al., 2014), it is recommended that this study be conducted in the future. Apart from sows, other microbial sources that may influence piglet's gut colonization, such as environmental aspects and handling by farm personnel (Chen et al., 2018b), are also worth investigating to gain a holistic understanding of piglet microbial development during the suckling and early postweaning periods. In addition, the study did not investigate how often piglets consumed sow feces or whether this behavior occurred daily during the suckling period or was limited to certain ages. As mentioned above, the consistency of piglet feces in the early postweaning period ranges from hard to soft. Since watery fecal consistency as an indicator of diarrhea was not found in the current study (Pedersen and Toft, 2011), we could not identify microbial biomarkers for dysbiotic conditions. Another limitation of the current research is the

lack of exploration of the impact of changes in piglet gut microbial composition, especially those driven by sow microbes, on piglet gut function and development. This includes aspects such as barrier and immune function as well as histomorphological development. Further studies are needed to investigate these aspects.

In conclusion, sow feces have been shown to play an important role in postnatal colonization of the piglet gut, but other maternal sources and external factors need to be considered to get a complete overview of the mother-offspring axis. Additionally, further studies on gut structure and genes are necessary to better understand gut microbe-mucosal interactions in piglets.

5.5.3. Meta-analysis

A meta-analysis is a statistical technique that combines and synthesizes the results of multiple studies (Haidich, 2010; Lee, 2019). This method increases the sample size, and thus the power, to examine the effects of interest by combining the primary studies and providing precise estimates of those effects. Meta-analyses provide more reliable data than narrative reviews (Yuan and Hunt, 2009; Lee, 2019). They provide transparent decision making and objective measures of quantitative evidence through statistical analysis. Meta-analyses can limit or overcome the biases of narrative reviews (Lee, 2019). The systematic approach and transparency of meta-analyses help to resolve conflicts and uncertainties between studies, leading to powerful conclusions (Lee, 2019, Paul and Barari, 2022). However, meta-analyses have limitations, which are outlined below.

The present meta-analysis only shows general trends for the use of probiotics in chicken feed (manuscript 2). This is due to the fact that the available data for individual probiotics did not meet the minimum requirements, therefore single and multi-species probiotic data from individual studies were combined for the meta-analysis. The same limitation applies to pathogens and aflatoxins in challenge studies. It is important to remember that meta-analysis should be avoided when studies are too heterogeneous to be comparable (Lee, 2019), as the results of meta-analysis may be meaningless, and the true effect may be obscured. Therefore, it is recommended that this study is re-evaluated in the future as more data become available for both single-strain and multi-strain probiotics as well as for the specific pathogen.

Before conducting a meta-analysis, it is important to consider the comparability of data to ensure valid conclusions (Jones et al., 2008; Lee, 2019). To do this, the data must be grouped accordingly. For example, in this meta-analysis, studies were categorized into pathogen-challenged and unchallenged groups. Gene expression and histomorphology were analyzed by intestinal segment and age, while growth performance was separated by stage of rearing.

Unfortunately, due to data limitations in the included literature, not all histomorphology and gene expression parameters could be regressed for all intestinal segments and ages of chickens in both pathogen and non-pathogen challenge groups. Additionally, SCFA, which are the most important parameter to explain probiotic-host mucosal interaction, could not be included in the analysis due to insufficient data. It is also important to note that the dependent variable may be influenced by multiple predictors, not just one. For this reason, to obtain more accurate prediction, this meta-analysis evaluated other potential predictors in addition to probiotics, using backward elimination procedures to evaluate dietary metabolizable energy, crude protein, and days post infection specifically for the pathogen challenge dataset. Unfortunately, only these three additional predictors could be included due to the lack of data provided in the literature.

In conclusion, this meta-analysis provides a general trend for the use of different probiotics and therefore should be verified in the future when more data from the literature become available. Due to insufficient SCFA data, this study could not verify the mechanism of action of probiotics in modulating the observed parameters. This study was also less comprehensive because the datasets did not have complete information on all parts of the intestine and the ages of the chickens. Therefore, to support future meta-analysis studies, it is important to provide more information about the experimental design and set-ups in original articles.

5.6. Transferability of the obtained results to the field situation

This thesis uses various methodological approaches to provide a comprehensive understanding of the role of gut microbes and their metabolites in monogastric farm animals, specifically chickens and pigs. The present results reinforce previous research and offer potential practical applications in the field of poultry and swine production. For example, when SCFA are used as supplemental feed for poultry (e.g., laying hens), it is recommended that SCFA are provided as a mixture rather than as single SCFA, reflecting their combined production during microbial fermentation in the gastrointestinal tract. Based on the general trend of our meta-analysis, dietary supplementation with probiotics may improve intestinal function and health in broiler chickens, but not growth performance. However, these findings need to be re-evaluated when more literature is available. The backward elimination analysis showed that when administering probiotics, farmers should also consider balancing the nutrients in the diet, including levels of metabolizable energy and crude protein. Excessive levels of these nutrients may interfere with the effectiveness of probiotics in improving the function and health of the chicken gut. The information obtained about changes in the maternal

microbiome during lactation is useful for formulating diets to specifically increase certain bacterial and fungal taxa in sow feces to modulate gastrointestinal colonization in neonatal piglets. It is important for the farmer to monitor the feeding behavior of the piglets, as the development of gut microbes from suckling to weaning is a dynamic process that is susceptible to environmental factors. Considering that the study on pigs was conducted under production conditions, it is more applicable in the field.

5.7. Synopsis and outlook

In general, this PhD thesis focuses on three different topics related to the gut microbiome-host interplay. First, investigating the local effect of SCFA on gut barrier function and contractibility in laying hens using *ex vivo* models. Second, assessing the mother-offspring axis in establishing gut microbial colonization in suckling and newly weaned piglets using *in vivo* models. Third, assessing the modulatory effect of dietary probiotics on gut barrier and immune response, histomorphology, and growth performance in broiler chickens using a meta-analytical model. The *ex vivo* study found that higher proportions of butyrate and SCFA concentrations could improve jejunal barrier function and enhance cecal muscle contractibility, which may promote fermentative processes in the cecum, if true *in vivo.* In the present *in vivo* study, there were significant changes in the bacterial and fungal communities in sow feces during lactation. As the microbes from sow feces were also present in the gastric and cecal digesta of the suckling piglets, it can be assumed that they contributed to the intestinal colonization of the piglets. A meta-analysis study confirmed that probiotics may have beneficial effects on gut integrity and structure in chickens. The effects of probiotics were found to be influenced by dietary metabolizable energy, crude protein, and days post-infection. However, it is important to note that this study has limitations and requires further research. For instance, the current meta-analysis provides only general trends. Therefore, it should be verified in the future when more data are available for different single and multi-strain probiotics. In addition, further research is needed toelucidate the signaling pathways of SCFA in modulating intestinal contractibility. Furthermore, it is necessary to study the effects of changes in the gut bacterial and fungal communities of neonatal piglets in relation to gut development and function. This will provide a comprehensive understanding of the interaction between host and gut microbes. Overall, results from this PhD thesis are useful for dietary strategies to improve gut health in chickens and suckling piglets.

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7. SUPPLEMENTARY MATERIALS

7.1. Supplementary material for manuscript 2

Yosi, F.; Metzler-Zebeli, B.U. 2023. Dietary probiotics modulate gut barrier and immune-related gene expression and histomorphology in broiler chickens under non- and pathogen-challenged conditions: A meta-analysis. Animals, 13, 1970. https://doi.org/10.3390/ani13121970

Article

Dietary Probiotics Modulate Gut Barrier and Immune-Related Gene Expression and Histomorphology in Broiler Chickens under Non- and Pathogen-Challenged Conditions: A Meta-Analysis

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Table S1. List of references and the respective experimental variables included in the meta-analysis.

Study	Reference	Basal Diet³	Dietary Probiotics¹	Pathogen				Gut Segments				Variables			
				No	Yes	Types²	Route	D	J	I	C	BAR	IMU	HIS	GRO
1	Zhen et al. (2018)	corn, soybean meal	B. coagulans	×	×	S. enteritidis	oral	×				×	×	×	×
2	Kan et al. (2021)	corn, wheat, soybean meal	B. licheniformis	×	×	E. maxima, C. perfringens	oral			×		×	×	×	×
3	Zhang et al. (2021)	corn, soybean meal, fish meal	B. coagulans	×							×	×		×	×
4	Li et al. (2015)	corn, soybean meal, corn gluten meal	B. amyloliquefaciens		×	Escherichia coli	injection	×	×			×	×	×	×
5	Liu et al. (2021)	corn, soybean meal	B. subtilis		×	C. perfringens	oral	×				×	×	×	×
6	Wang et al. (2021)	corn, soybean meal, corn gluten meal	B. subtilis	×	×	C. perfringens	oral	×				×	×	×	×
7	Wu et al. (2018)	corn, soybean meal	B. coagulans	×	×	E. maxima, E. tenella, C. perfringens	oral	×				×	×	×	×
8	Musa et al. (2019)	corn, wheat, distiller grain, soybean meal, corn protein powder, peanut meal,	B. subtilis, B. licheniformis		×	C. perfringens	oral	×	×	×		×	×	×	×
9	Bilal et al. (2021)	corn, soybean meal	B. subtilis, B. pumilus	×		E. maxima, E. tenella, E. acervulina	oral	×	×	×		×	×	×	×
10	Chaudhari et al. (2020)	corn, soybean meal	B. licheniformis, B. amyloliquefaciens		×	Escherichia coli	oral		×	×	×	×	×	×	×
11	Wu et al. (2021)	corn, soybean meal	L. acidophilus		×	Escherichia coli	oral		×	×		×	×	×	×
12	Xu et al. (2021)	corn, bran, soybean meal, cottonseed	C. butyricum		×	E. maxima, C. perfringens	oral		×	×		×	×	×	×

No.	Reference	Ingredients	Probiotics	Challenge	Route							
13	Wu et al. (2019)	meal, corn gluten meal / corn, soybean meal	E. faecium	C. perfringens	oral	×	×		×	×	×	×
14	He et al. (2019)	fish meal, corn gluten meal	B. subtilis, B. licheniformis, S. cerevisiae			×		×	×	×	×	×
15	Memon et al. (2021)	corn, soybean meal	B. subtilis	E. tenella	oral	×		×	×	×	×	×
16	Mountzouris et al. (2019)	corn, soybean meal	L. reuteri, L. salivarius, E. faecium, Bi. animalis, P. acidilactici			×		×	×	×	×	×
17	Wu et al. (2019)	corn, wheat, soybean meal, fish meal	L. plantarum, Pa. polymyxa			×		×	×	×	×	
18	Huang et al. (2019)	corn, wheat, soybean meal	E. faecium	Escherichia coli	injection	×		×	×	×	×	×
19	Bai et al. (2013)	corn, soybean meal, rapeseed meal, cottonseed meal	L. fermentum, S. cerevisiae					×	×	×	×	×
20	Yang et al (2021)	corn, bran, soybean meal, fish meal	B. subtilis	E. tenella	oral	×	×	×	×	×	×	
21	Wang et al. (2016)	corn, soybean meal	S. cerevisiae	Escherichia coli	oral	×	×	×	×	×	×	×
22	Chang et al. (2020)	corn, soybean meal	L. acidophilus, L. fermentum, L. casei, P. acidilactici	S. enterica	oral	×	×	×	×	×	×	×
23	Lan et al. (2020)	corn, bran, soybean meal, rapeseed meal	P. pentosaceus	S. enteritidis	oral	×	×	×	×	×	×	×
24	Wang et al. (2017)	corn, soybean meal	L. johnsonii	E. maxima, E. acervulina, C. perfringens	oral	×	×	×	×	×	×	×

No.	Reference	Diet	Probiotic		Challenge organism	Route						
25	Li et al. (2018)	corn, soybean meal	L. acidophilus	×	C. perfringens	oral	×	×	×	×		
26	Wang et al. (2017)	corn, soybean meal, fish meal	L. plantarum	×			×		×	×		
27	Chen et al (2022)	corn, soybean meal	L. salivarius	×	S. pullorum, Aflatoxin B1	oral, diet	×	×	×	×		
28	Calik et al. (2019)	corn, soybean meal	B. amyloliquefaciens	×	acervulina	oral	×	×	×	×	×	
29	Qiu et al. (2021)	corn, soybean meal	B. subtilis	×			×	×	×	×	×	
30	Cao et al. (2019)	corn, soybean meal	L. plantarum	×	C. perfringens	oral	×	×	×	×	×	
31	Li et al. (2017)	wheat, soybean meal	B. subtilis	×	C. perfringens	oral	×	×	×	×	×	
32	Aliakbarpour e al. (2012)	corn, soybean meal	B. subtilis, L. casei, L. acidophilus, Bi. Thermophilum, E. faecium	×			×	×	×	×	×	
33	Gao et al. (2022)	fish meal	L. plantarum	×			×	×	×	×	×	
34	Hosseini et al. (2019)	corn, soybean meal	B. subtilis, B. tequilensis	×			×	×	×	×	×	
35	Konieczka et al (2022)	triticale, soybean bean meal	B. licheniformis, B. amyloliquefaciens, B. subtilis	×	E. tenella, E. acervulina, E. mitis, E. praecox	oral	×	×	×	×	×	
36	Hosseini et al. (2018)	meal, corn gluten meal	B subtilis, B. licheniformis	×			×	×	×	×		
37	Gong et al. (2021)	corn, wheat, soybean meal, fish meal	L. plantarum, Pa. polymyxa		C. perfringens	oral	×	×	×	×		
38	Zhao et al (2022)	corn, soybean meal	B. licheniformis	×	C. perfringens	oral	×	×	×	×	×	
39	Liu et al. (2022)	fish meal, rapeseed meal	C. butyricum	×			×	×	×	×	×	

No.	Reference	Diet	Probiotics		Challenge	Route					
40	Guo et al. (2021)	barley, corn, soy-bean meal, fish meal	L. fermentum, B. coagulans	x	C. perfringens	oral			x	x	x
41	Emami et al. (2019)	corn, soybean meal	L. acidophilus, L. casei, E. faecium, Bi. Bifidum	x	C. perfringens, E. maxima, E. tenella, E. acervulina, E. mivati	spray	x	x	x	x	x
42	Mohammed et al. (2021)	corn	E. faecium, Bi. Bifidum, St. faecalis, C. butyricum, B. mesentericus	x	S. minnesota	oral		x	x	x	x
43	Mohsin et al. (2022)	corn, soybean meal,	L. plantarum	x	E. tenella	oral		x	x	x	x
44	Wu et al. (2018)	corn gluten meal	L. plantarum	x	E. graminearum	diet	x	x	x	x	x
45	Wang et al. (2022)	corn, soybean meal	B. subtilis	x	S. pullorum	oral		x	x	x	x
46	Deng et al. (2020)	corn gluten meal, soybean meal,	L. acidophilus, L. planta-rum	x	L. monocytogenes	oral		x		x	x
47	Ateya et al. (2019)	corn gluten meal, soybean meal,	P. acidilactici	x	Escherichia coli	oral	x		x	x	x
48	Zhang et al. (2022)	corn gluten meal, soybean meal,	B. amyloliquefaciens	x	C. perfringens	spray	x	x	x	x	x
49	Dong et al. (2016)	cottonseed meal	E. fecalis	x			x	x	x	x	x
50	Xiao et al. (2022)	corn, soybean meal	B. methylotrophicus	x			x	x	x	x	x
51	Azizi et al. (2021)	corn, rice, soybean meal	L. acidophilus, L. ani-malis, L. reuteri, L. fer-mentum, L. gallinarum, S. cerevisiae	x	E. graminearum	diet	x	x	x	x	x
52	Emami et al. (2020)	corn, soybean meal	B. licheniformis	x	C. perfringens, E. maxima, E. tenella, E. acervulina, E. mivati	spray	x	x	x	x	x

	Author	Diet[3]	Probiotic[1]			Challenge[2]	Route					
53	Gharib-Naseri et al. (2020)	wheat, soybean meal, sorghum	B. amyloliquefaciens	×	×	C. perfringens, E. maxima, E. acervulina, E. brunetti	oral		×	×	×	×
54	Bodinga et al. (2020)	corn, soybean meal	B. subtilis		×	C. perfringens	oral	×	×	×	×	×

[1] B, *Bacillus*; Bi, *Bifidobacterium*; C, *Clostridium*; E, *Enterococcus*; L, *Lactobacillus*; Pa, *Paenibacillus*; P, *Pediococcus*; S, *Saccharomyces*; St, *Streptococcus*.
[2] C, *Clostridium*; E, *Eimeria*; F, *Fusarium*; L, *Listeria*; S, *Salmonella*.
[3] Main energy and protein feedstuffs of basal diet.
D, duodenum; J, jejunum; I, ileum; C, caeca; BAR, gut barrier-related gene expression; IMU, gut immunity-related gene expression; HIS, histomorphology; GRO, growth performance.

Table S2. Descriptive statistics for predictive and response variables of jejunal and ileal gene expression (fold-change) related to barrier function and immune response in broiler chickens at week 3 and 6 of life without pathogen challenge.

Variable[1,2]	nTreat	Mean	SEM	Min.	Max.	Median
Jejunum, Week 3						
Dietary probiotics (log$_{10}$ CFU/kg)	17	4.8	1.16	0	11.0	6.6
Dietary ME (MJ/kg)	17	12.4	0.06	12.1	12.9	12.4
Dietary CP (%)	17	21.3	0.14	20.5	22.1	21.0
MUC2	10	1.2	0.13	0.9	2.3	1.0
ZO1	11	1.1	0.04	0.9	1.4	1.0
OCLN	13	1.4	0.20	0.9	3.5	1.0
CLDN1	11	1.2	0.10	0.9	1.9	1.0
Jejunum, Week 3						
Dietary probiotics (log$_{10}$ CFU/kg)	22	4.7	0.96	0	11.0	6.8
Dietary ME (MJ/kg)	22	12.4	0.05	12.1	12.9	12.3
Dietary CP (%)	22	21.3	0.11	20.5	22.0	21.0
IL1B	11	1.0	0.04	0.6	1.1	1.0
IFNG	14	1.0	0.04	0.8	1.4	1.0
TLR2	11	1.1	0.12	0.7	2.1	1.0
Jejunum, Week 6						
Dietary probiotics (log$_{10}$ CFU/kg)	24	5.8	0.95	0	11.0	8.7
Dietary ME (MJ/kg)	24	13.0	0.06	12.6	13.4	13.2
Dietary CP (%)	24	19.5	0.15	18.2	20.7	19.3
MUC2	10	1.8	0.37	1.0	4.5	1.3
ZO1	14	1.7	0.33	1.0	5.6	1.2
OCLN	16	1.4	0.18	0.6	3.1	1.0
CLDN1	12	1.4	0.18	0.5	2.6	1.3
Ileum, Week 3						
Dietary probiotics (log$_{10}$ CFU/kg)	13	5.7	1.32	0	11.3	8.0
Dietary ME (MJ/kg)	13	12.3	0.05	12.1	12.6	12.2
Dietary CP (%)	13	21.5	0.18	21.0	22.4	21.0
MUC2	10	2.0	0.64	0.7	7.5	1.0
ZO1	11	1.2	0.19	0.1	2.3	1.0
OCLN	13	1.8	0.62	0.7	9.0	1.0
CLDN1	10	1.3	0.15	0.7	2.2	1.0
Ileum, Week 6						
Dietary probiotics (log$_{10}$ CFU/kg)	19	6.2	0.99	0	10.4	8.5
Dietary ME (MJ/kg)	19	12.9	0.06	12.6	13.3	12.8
Dietary CP (%)	19	19.4	0.18	18.1	20.7	19.2
MUC2	15	1.9	0.39	0.7	5.2	1.0
ZO1	14	2.3	0.63	0.5	8.6	1.0
OCLN	16	1.3	0.17	0.7	3.4	1.1

CLDN1	11	1.5	0.26	0.6	3.1	1.0

nTreat, number of treatments mean; SEM, standard error of means; Min, minimum; Max, maximum; ME, metabolizable energy; CP, crude protein; CFU, colony-forming unit; *MUC2*, mucin-2; *ZO1*, zonula occludens-1; *OCLN*, occludin; *CLDN1*, claudin-1; *IL1B*, interleukin-1beta; *TLR2*, toll-like receptor-2; *IFNG*, interferon-gamma.

[1]Probiotic genera included for these response variables were *Bacillus, Bifidobacterium, Lactobacillus, Clostridium, Enterococcus, Pediococcus, Paenibacillus,* and *Saccharomyces.*

[2]Data were calculated as log2fold change between probiotic and control treatments and then expressed in fold-change using a logarithmic scale to base 2.

Table S3. Descriptive statistics for predictive and response variables of jejunal and ileal histomorphology (fold-change) in broiler chickens at week 3 and 6 of life without pathogen challenge.

Variable[1,2]	nTreat	Mean	SEM	Min.	Max.	Median
Jejunum, Week 3						
Dietary probiotics (log$_{10}$ CFU/kg)	15	5.0	1.27	0	11.0	6.6
Dietary ME (MJ/kg)	15	12.5	0.07	12.1	12.9	12.5
Dietary CP (%)	15	21.3	0.19	19.9	22.1	21.5
Villus Height	15	1.1	0.04	1.0	1.5	1.0
Crypt Depth	15	1.0	0.04	0.7	1.3	1.0
Villus Height/Crypt Depth	15	1.2	0.08	0.9	1.9	1.0
Jejunum, Week 6						
Dietary probiotics (log$_{10}$ CFU/kg)	19	5.9	1.08	0	11.0	9.0
Dietary ME (MJ/kg)	19	12.9	0.06	12.5	13.2	12.9
Dietary CP (%)	19	19.4	0.16	18.2	20.4	19.2
Villus Height	19	1.1	0.03	0.9	1.3	1.0
Crypt Depth	19	1.1	0.04	0.7	1.5	1.0
Villus Height/Crypt Depth	19	1.0	0.04	0.8	1.5	1.0
Ileum, Week 3						
Dietary probiotics (log$_{10}$ CFU/kg)	11	5.1	1.50	0	11.0	8.5
Dietary ME (MJ/kg)	11	12.4	0.06	12.1	12.7	12.4
Dietary CP (%)	11	21.6	0.16	21.0	22.1	22.0
Villus Height	11	1.0	0.03	0.9	1.3	1.0
Crypt Depth	11	0.9	0.04	0.6	1.1	1.0
Villus Height/Crypt Depth	11	1.1	0.06	0.9	1.7	1.0
Ileum, Week 6						
Dietary probiotics (log$_{10}$ CFU/kg)	17	6.1	1.14	0	11.0	9.0
Dietary ME (MJ/kg)	17	13.0	0.06	12.6	13.2	13.0
Dietary CP (%)	17	19.6	0.15	18.9	20.7	19.2
Villus Height	17	1.1	0.04	0.9	1.5	1.0
Crypt Depth	17	1.0	0.03	0.7	1.2	1.0
Villus Height/Crypt Depth	17	1.1	0.04	0.8	1.4	1.1

nTreat**,** number of treatments means; SEM, standard error of means; Min, minimum; Max, maximum; ME, metabolizable energy; CP, crude protein; CFU, colony-forming unit.

[1]Probiotic genera included for these response variables were *Bacillus, Bifidobacterium, Lactobacillus, Clostridium, Enterococcus*, and *Saccharomyces.*

[2]Data were calculated as log$_2$fold change between probiotic and control treatments and then expressed in fold-change using a logarithmic scale to base 2.

Table S4. Descriptive statistics for predictors and response variables of growth performance in broiler chickens at starter, finisher, and overall periods without pathogen challenge.

Variable[1]	nTreat	Mean	SEM	Min.	Max.	Median
Starter (1-3 Weeks)						
Dietary probiotics (log$_{10}$ CFU/kg)	33	5.6	0.80	0	11.0	8.5
Dietary ME (MJ/kg)	33	12.4	0.03	12.1	12.8	12.5
Dietary CP (%)	33	21.5	0.13	21.0	23.0	21.1
ADFI (g)	33	50.2	1.52	35.9	65.3	49.6
ADG (g)	30	33.7	0.82	25.8	44.6	33.7
FCR	33	1.5	0.03	1.1	1.8	1.4
Finisher (4-6 Weeks)						
Dietary probiotics (log$_{10}$ CFU/kg)	29	5.7	0.86	0	11.0	8.5
Dietary ME (MJ/kg)	29	12.8	0.04	12.5	13.2	12.8
Dietary CP (%)	29	19.5	0.11	18.9	20.7	19.2
ADFI (g)	29	151.8	4.69	105.9	199.7	153.0
ADG (g)	26	77.6	2.98	51.0	107.2	77.2
FCR	29	2.0	0.04	1.5	2.3	1.9
Overall (1-6 Weeks)						
Dietary probiotics (log$_{10}$ CFU/kg)	32	5.7	0.80	0	11.0	8.5
Dietary ME (MJ/kg)	32	12.7	0.03	12.3	13.0	12.6
Dietary CP (%)	32	20.6	0.11	19.7	21.5	20.3
ADFI (g)	32	100.5	2.79	71.4	132.0	99.5
ADG (g)	26	56.5	1.57	42.1	71.3	56.7
FCR	32	1.7	0.02	1.4	2.0	1.7

nTreat, number of treatments means included; SEM, standard error of means; Min, minimum; Max, maximum; ME, metabolizable energy; CP, crude protein; CFU, colony-forming unit; ADFI, average daily feed intake; ADG, average daily weight gain; FCR, feed conversion ratio.

[1] Probiotic genera included for these response variables were *Bacillus*, *Bifidobacterium*, *Lactobacillus*, *Clostridium*, *Enterococcus*, and *Saccharomyces*.

Table S5. Descriptive statistics for predictive and response variables of jejunal, ileal, and caecal gene expression (fold-change) related to barrier function in broiler chickens from week 2 to 5 with pathogen challenge.

Variable[1-3]	nTreat	Mean	SEM	Min.	Max.	Median
Jejunum, Week 2						
Dietary probiotics (log$_{10}$ CFU/kg)	18	4.9	1.07	0	10.7	8.2
Days post-infection	18	6.6	0.78	3.0	12.0	6.0
Dietary ME (MJ/kg)	18	12.5	0.05	12.1	12.8	12.6
Dietary CP (%)	18	21.8	0.24	20.0	23.0	21.8
ZO1	14	1.1	0.04	0.7	1.3	1.0
OCLN	16	1.6	0.62	0.6	10.8	1.0
CLDN1	14	1.1	0.17	0.6	3.3	1.0
CLDN3	10	1.5	0.17	1.0	2.3	1.2
Jejunum, Week 3						
Dietary probiotics (log$_{10}$ CFU/kg)	19	5.3	1.07	0	10.7	8.7
Days post-infection	19	7.0	0.59	4.0	12.0	7.0
Dietary ME (MJ/kg)	19	12.5	0.06	12.1	12.9	12.4
Dietary CP (%)	19	20.9	0.12	20.0	22.0	21.0
MUC2	10	1.2	0.19	0.7	2.8	1.0
ZO1	17	1.2	0.08	0.9	2.0	1.0
OCLN	17	1.3	0.14	0.7	3.1	1.0
CLDN1	14	1.6	0.45	0.4	7.3	1.0
Jejunum, Week 4						
Dietary probiotics (log$_{10}$ CFU/kg)	12	5.0	1.28	0	9.2	7.7
Days post-infection	12	12.0	1.69	7.0	21.0	10.0
Dietary ME (MJ/kg)	12	12.6	0.07	12.4	13.0	12.5
Dietary CP (%)	12	20.0	0.28	19.0	21.2	19.8
ZO1	12	1.3	0.10	0.9	2.0	1.1
OCLN	12	1.3	0.20	0.6	2.8	1.0
Jejunum, Week 5						
Dietary probiotics (log$_{10}$ CFU/kg)	13	6.9	1.10	0	9.6	9.0
Days post-infection	13	28.8	2.26	17.0	34.0	34.0
Dietary ME (MJ/kg)	13	12.7	0.03	12.5	12.8	12.8
Dietary CP (%)	13	19.0	0.02	19.0	19.2	19.0
MUC2	13	1.1	0.20	0.2	2.7	1.0
Ileum, Week 3						
Dietary probiotics (log$_{10}$ CFU/kg)	16	5.6	1.12	0	9.7	8.4
Days post-infection	16	14.3	1.62	4.0	20.0	16.0
Dietary ME (MJ/Kg)	16	12.3	0.04	12.1	12.6	12.2
Dietary CP (%)	16	21.4	0.13	21.0	22.4	21.2
ZO1	16	1.3	0.15	0.4	2.7	1.0
OCLN	16	1.3	0.16	0.4	2.7	1.0
CLDN1	11	1.0	0.14	0.5	2.1	1.0

Ileum, Week 4						
Dietary probiotics (log$_{10}$ CFU/kg)	11	5.1	1.50	0	11.3	8.0
Days post-infection	11	11.6	0.85	8.0	14.0	14.0
Dietary ME (MJ/kg)	11	12.8	0.15	12.1	13.2	13.2
Dietary CP (%)	11	20.5	0.17	20.0	21.2	20.0
ZO1	11	1.2	0.11	1.0	2.2	1.1
OCLN	11	1.3	0.18	0.8	2.9	1.0
CLDN1	11	1.3	0.21	0.5	2.7	1.0
Caeca, Week 4						
Dietary probiotics (log$_{10}$ CFU/kg)	10	4.6	1.29	0	8.7	6.5
Days post-infection	10	7.6	0.16	7.0	8.0	8.0
Dietary ME (MJ/Kg)	10	12.7	0.08	12.6	13.2	12.6
Dietary CP (%)	10	19.2	0.13	19.0	20.0	19.0
ZO1	10	2.6	1.05	1.0	11.9	1.5

nTreat**,** number of treatments means; SEM, standard error of means; Min, minimum; Max, maximum; ME, metabolizable energy; CP, crude protein; CFU, colony-forming unit; *MUC2*, mucin-2; *ZO1*, zonula occludens-1; *OCLN*, occludin; *CLDN1, -3*, claudin-1, -3.

[1]Probiotic genera included for these response variables were *Bacillus, Bifidobacterium, Lactobacillus, Paenibacillus, Clostridium, Enterococcus, Pediococcus*, and *Saccharomyces.*

[2]Pathogens included for these response variables were *E. coli, C. perfringens, S. enteritidis, E. maxima, E. tenella, E. acervulina, E. mivati, E. brunetti, E. mitis, E. praecox, F. graminearum, S. pullorum*, and Aflatoxin B1.

[3]Data were calculated as log$_2$fold change between probiotic and control treatments and then expressed in fold-change using a logarithmic scale to base 2.

Table S6. Descriptive statistics for predictive and response variables of jejunal, ileal, and caecal gene expression (fold-change) related to immune response in broiler chickens from week 2 to 4 of life with pathogen challenge.

Variable[1-3]	nTreat	Mean	SEM	Min.	Max.	Median
Jejunum, Week 2						
Dietary probiotics (log$_{10}$ CFU/kg)	14	4.8	1.15	0	8.9	8.2
Days post-infection	14	7.0	0.96	3.0	12.0	6.0
Dietary ME (MJ/kg)	14	12.4	0.05	12.1	12.6	12.5
Dietary CP (%)	14	21.9	0.21	21.0	23.0	21.8
IL1B	10	1.0	0.03	0.7	1.2	1.0
IL10	14	1.2	0.20	0.2	2.9	1.0
IFNG	10	0.9	0.06	0.5	1.0	0.9
Jejunum, Week 3						
Dietary probiotics (log$_{10}$ CFU/kg)	25	5.2	0.95	0	10.7	8.5
Days post-infection	25	8.2	0.85	4.0	20.0	7.0
Dietary ME (MJ/kg)	25	12.4	0.05	12.1	12.9	12.4
Dietary CP (%)	25	21.0	0.13	20.0	22.0	21.0
IL1B	17	0.8	0.07	0.2	1.3	1.0
IL6	12	0.9	0.08	0.4	1.1	1.0
IL10	13	1.7	0.33	0.5	4.6	1.0
IFNG	18	0.9	0.07	0.4	1.5	1.0
TNFA	10	0.9	0.06	0.6	1.2	1.0
Jejunum, Week 4						
Dietary probiotics (log$_{10}$ CFU/kg)	14	5.1	1.23	0	10.0	7.7
Days post-infection	14	11.8	1.04	7.0	17.0	13.5
Dietary ME (MJ/kg)	14	12.7	0.09	12.4	13.2	12.6
Dietary CP (%)	14	19.7	0.21	19.0	21.0	19.5
IL1B	10	1.4	0.14	1.0	2.2	1.2
IFNG	14	1.2	0.13	0.6	2.4	1.0
Ileum, Week 2						
Dietary probiotics (log$_{10}$ CFU/kg)	12	4.6	1.39	0	9.7	4.1
Days post-infection	12	3.5	0.77	1.0	7.0	2.5
Dietary ME (MJ/kg)	12	12.6	0.12	12.1	13.2	12.8
Dietary CP (%)	12	21.6	0.18	20.9	22.1	21.6
IFNG	10	1.0	0.16	0.4	2.2	1.0
TLR4	10	0.9	0.10	0.5	1.6	1.0
Ileum, Week 3						
Dietary probiotics (log$_{10}$ CFU/kg)	12	5.1	1.32	0	9.7	8.0
Days post-infection	12	13.7	2.27	4.0	20.0	20.0
Dietary ME (MJ/kg)	12	12.3	0.05	12.1	12.5	12.2
Dietary CP (%)	12	21.4	0.17	21.0	22.4	21.1
IL10	10	1.1	0.11	0.7	2.0	1.0
IFNG	12	0.9	0.05	0.6	1.1	0.9

Ileum, Week 4						
Dietary probiotics (log$_{10}$ CFU/kg)	10	5.9	1.63	0	11.3	9.0
Days post-infection	10	17.3	2.60	8.0	27.0	17.0
Dietary ME (MJ/kg)	10	12.4	0.11	12.1	12.8	12.1
Dietary CP (%)	10	19.8	0.41	19.0	22.1	19.0
TNFA	10	0.8	0.09	0.2	1.0	1.0
Caeca, Week 2						
Dietary probiotics (log$_{10}$ CFU/kg)	22	5.5	1.04	0	12.4	8.0
Days post-infection	22	3.4	0.60	0.2	7.0	3.0
Dietary ME (MJ/kg)	22	12.0	0.10	11.4	12.8	12.1
Dietary CP (%)	22	20.8	0.25	19.2	23.0	21.0
IL6	18	0.9	0.07	0.1	1.5	1.0
IL8	10	0.9	0.19	0.1	2.1	1.0
IL10	10	2.4	0.42	1.0	4.7	2.5

nTreat**,** number of treatments means; SEM, standard error of means; Min, minimum; Max, maximum; ME, metabolizable energy; CP, crude protein; CFU, colony-forming unit; *IL6, -8, -10, -1B*, interleukin-6, -8, -10, -1B; *TLR4*, toll-like receptor -4; *IFNG*, interferon-gamma; *TNFA*, tumor necrosis factor-alpha.

[1]Probiotic genera included for these response variables were *Bacillus, Bifidobacterium, Lactobacillus, Paenibacillus, Clostridium, Enterococcus, Pediococcus, Streptococcus,* and *Saccharomyces.*

[2]Pathogens included for these response variables were *E. coli, C. perfringens, S. enteritidis, E. maxima, E. tenella, E. acervulina, E. mivati, E. brunetti, F. graminearum, S. minnesota,* and *L. monocytogenes.*

[3]Data were calculated as log$_2$fold change between probiotic and control treatments and then expressed in fold-change using a logarithmic scale to base 2.

Table S7. Descriptive statistics for predictive and response variables of duodenal, jejunal and ileal histomorphology (fold-change) in broiler chickens at week 2, 3 and 5 of life with pathogen challenge.

Variable[1-3]	nTreat	Mean	SEM	Min.	Max.	Median
Duodenum, Week 5						
Dietary probiotics ($\log_{10}$ CFU/kg)	15	7.2	0.97	0	9.4	9.0
Days post-infection	15	32.0	1.07	24.0	34.0	34.0
Dietary ME (MJ/kg)	15	12.8	0.02	12.8	13.0	12.8
Dietary CP (%)	15	19.2	0.11	19.0	20.0	19.0
Villus Height	15	1.1	0.02	1.0	1.3	1.1
Crypt Depth	15	1.1	0.03	0.8	1.2	1.0
Villus Height/Crypt Depth	15	1.0	0.03	0.9	1.3	1.0
Jejunum, Week 2						
Dietary probiotics ($\log_{10}$ CFU/kg)	11	5.2	1.50	0	10.7	8.7
Days post-infection	11	5.2	0.48	3.0	7.0	5.0
Dietary ME (MJ/kg)	11	12.4	0.09	12.1	12.8	12.4
Dietary CP (%)	11	21.2	0.25	20.0	22.1	21.0
Villus Height	11	1.0	0.04	0.9	1.4	1.0
Crypt Depth	11	0.9	0.03	0.7	1.0	1.0
Villus Height/Crypt Depth	11	1.1	0.08	1.0	1.8	1.0
Jejunum, Week 3						
Dietary probiotics ($\log_{10}$ CFU/kg)	17	5.3	1.12	0	10.7	8.2
Days post-infection	17	9.8	1.34	4.0	20.0	7.0
Dietary ME (MJ/kg)	17	12.3	0.03	12.1	12.5	12.3
Dietary CP (%)	17	21.1	0.12	20.0	22.0	21.0
Villus Height	17	1.1	0.04	0.9	1.5	1.0
Crypt Depth	17	1.0	0.03	0.7	1.1	1.0
Villus Height/Crypt Depth	17	1.2	0.07	1.0	2.2	1.1
Jejunum, Week 5						
Dietary probiotics ($\log_{10}$ CFU/kg)	17	6.9	0.96	0	9.4	9.0
Days post-infection	17	30.6	1.35	20.0	34.0	34.0
Dietary ME (MJ/kg)	17	12.8	0.03	12.6	13.0	12.8
Dietary CP (%)	17	19.2	0.09	19.0	20.0	19.0
Villus Height	17	1.0	0.02	0.9	1.2	1.0
Crypt Depth	17	1.0	0.03	0.7	1.1	1.0
Villus Height/Crypt Depth	17	1.1	0.04	0.8	1.4	1.1
Ileum, Week 3						
Dietary probiotics ($\log_{10}$ CFU/kg)	13	5.5	1.26	0	9.7	8.5
Days post-infection	13	10.3	1.73	4.0	20.0	7.0
Dietary ME (MJ/kg)	13	12.3	0.04	12.1	12.5	12.3
Dietary CP (%)	13	21.2	0.10	21.0	22.0	21.1

Villus Height	13	1.0	0.02	1.0	1.2	1.0
Crypt Depth	13	1.0	0.04	0.9	1.4	1.0
Villus Height/Crypt Depth	13	1.0	0.02	0.9	1.2	1.0
Ileum, Week 5						
Dietary probiotics ($\log_{10}$ CFU/kg)	15	7.2	0.97	0	9.4	9.0
Days post-infection	15	32.0	1.07	24.0	34.0	34.0
Dietary ME (MJ/kg)	15	12.8	0.02	12.8	13.0	12.8
Dietary CP (%)	15	19.2	0.11	19.0	20.0	19.0
Villus Height	15	1.0	0.03	0.8	1.2	1.0
Crypt Depth	15	1.1	0.03	0.9	1.3	1.1
Villus Height/Crypt Depth	15	0.9	0.03	0.7	1.1	1.0

nTreat, number of treatments means; SEM, standard error of means; Min, minimum; Max, maximum; ME, metabolizable energy; CP, crude protein; CFU, colony-forming unit.

[1]Probiotic genera included for these response variables were *Bacillus*, *Lactobacillus*, *Enterococcus*, and *Saccharomyces.*

[2]Pathogens included for these response variables were *E. coli, C. perfringens, S. enteritidis, E. maxima, E. tenella, E. acervulina, E. mitis, E. praecox*, and *F. graminearum.*

[3]Data were calculated as $\log_2$ fold change between probiotic and control treatments and then expressed in fold-change using a logarithmic scale to base 2.

Table S8. Descriptive statistics for predictors and response variables of growth performance in broiler chickens at starter, finisher, and overall periods with pathogen challenge.

Variable[1,2]	nTreat	Mean	SEM	Min.	Max.	Median
Starter (1-3 Weeks)						
Dietary probiotics (log$_{10}$ CFU/kg)	22	5.5	1.00	0	9.7	9.0
Days post-infection	22	10.8	1.00	5.0	20.0	10.0
Dietary ME (MJ/kg)	22	12.4	0.04	12.1	12.7	12.4
Dietary CP (%)	22	21.1	0.05	21.0	21.8	21.0
ADFI (g)	22	52.6	1.57	40.6	63.3	52.3
ADG (g)	22	36.2	1.46	28.4	50.2	33.2
FCR	22	1.5	0.04	1.1	1.9	1.4
Finisher (4-6 Weeks)						
Dietary probiotics (log$_{10}$ CFU/kg)	13	5.0	1.33	0	9.6	9.0
Days post-infection	13	33.2	1.50	27.0	41.0	33.0
Dietary ME (MJ/kg)	13	12.7	0.05	12.5	13.0	12.8
Dietary CP (%)	13	19.3	0.10	19.0	19.8	19.0
ADFI (g)	13	149.8	11.04	108.2	219.4	142.7
ADG (g)	13	72.1	7.54	31.7	114.3	80.7
FCR	13	2.0	0.09	1.7	2.8	1.9
Overall (1-6 Weeks)						
Dietary probiotics (log$_{10}$ CFU/kg)	17	4.8	1.13	0	9.6	8.1
Days post-infection	17	34.9	1.38	27.0	41.0	36.0
Dietary ME (MJ/kg)	17	12.7	0.06	12.4	13.0	12.5
Dietary CP (%)	17	20.3	0.08	19.9	20.8	20.1
ADFI (g)	17	95.6	2.78	75.6	119.5	93.8
ADG (g)	17	57.0	3.26	31.7	75.7	55.2
FCR	17	1.7	0.06	1.4	2.3	1.7

nTreat**,** number of treatments means; SEM, standard error of means; Min, minimum; Max, maximum; ME, metabolizable energy; CP, crude protein; CFU, colony-forming unit; ADFI, average daily feed intake; ADG, average daily weight gain; FCR, feed conversion ratio.

[1]Probiotic genera included for these response variables were *Bacillus, Bifidobacterium, Lactobacillus, Enterococcus,* and *Saccharomyces.*

[2]Pathogens included for these response variables were *E. coli, C. perfringens, S. enteritidis, E. maxima, E. tenella, E. acervulina, E. mivati, E. mitis,* and *E. praecox.*

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7.2. Supplementary material for manuscript 3

Yosi F, Lerch F, Vötterl JC, Koger S, Verhovsek D, Metzler-Zebeli BU. Lactation-related dynamics of bacterial and fungal microbiomes in feces of sows and gut colonization in suckling and newly weaned piglets (has been submitted and is peer-reviewed by Journal of Animal Science)

1   **Lactation-related dynamics of bacterial and fungal microbiomes in feces of sows and**

2   **gut colonization in suckling and newly weaned piglets**

3

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22   **Supplementary Materials:**

23   **Table S1.** Ingredient composition of gestation, lactation and prestarter diets

Ingredients, %	Gestation diet[1]	Lactation diet[2]	Prestarter[3]
Barley meal	50.0	20.0	29.7
Wheat bran	15.8	6.4	–
Corn meal	10.0	29.0	10.8
Wheat meal	8.1	10.0	10.0
Soybean meal	4.2	9.3	–
Fullfat soy	–	–	14.1
Wheat pressure cooked	–	–	9.9
Sweet whey powder	–	–	3.0
Potato protein	–	–	5.0
Rapeseed meal	–	5.0	–
Dried pulp	4.0	1.5	–
Commercial breeding premix	2.8	3.5	–
Lignocellulose	–	0.5	0.9
Palm kernel	–	–	2.0
Dried vinasse[4]	1.5	–	1.9
Dextrose	–	–	5.0
Lactose	–	–	3.0
Apple pomace	1.5	1.0	–
Bakery products	1.2	12.8	–
Hay cobs	0.5	–	–
Oil	0.3	0.5	–
Rapeseed oil	–	–	0.5
Lysine HCl	–	0.2	0.7
Threonine	–	0.06	0.3
Methionine	–	0.02	0.3
Tryptophane	–	–	0.1
Limestone (calcium carbonate)	–	0.2	0.6
Sodium chloride	0.03	–	0.5
Mono calcium phosphate	–	–	1.4
Magnesium phosphate	–	–	0.2
Vitamin E	0.02	0.02	–
Vitamin/trace element premix	–	–	0.4

24   [1]Vitamin and mineral composition per kg feed: 9,600 IE of vitamin A, 1,600 IE of vitamin D3,

25   156 mg of vitamin E, 82 mg of Fe as iron(II) sulfat monohydrate, 12 mg of Cu as copper(II)

26   sulfat pentahydrate, 90 mg of Zn as zinc oxide, 2.6 mg of Mn as manganese(II) oxide.

27   Technological additives: 700 FTU of 6-phytase, 60 mg of butylated hydroxytoluene.

28   [2]Vitamin and mineral composition per kg feed: 12,000 IE of vitamin A, 2,000 IE of vitamin

29   D3, 170 mg of vitamin E, 100 mg of Fe as iron(II) sulfat monohydrate, 15.1 mg of Cu as

30   copper(II) sulfat pentahydrate, 110 mg of Zn as zinc oxide, 0.7 mg of Mn as manganese(II)

31   oxide. Technological additives: 880 FTU of 6-phytase, 70 mg of butylated hydroxytoluene.

32   [3]Vitamin and mineral composition per kg feed: 16,000 IE of vitamin A, 2,000 IE of vitamin D3,

33   150 mg of vitamin E, 4.0 mg of vitamin K3, 2.8 mg of vitamin B1, 8.2 mg of vitamin B2,

34   5.0 mg of vitamin B6, 50 mg of vitamin B12, 60 mg of nicotinic acid, 20 mg of panthothenic

35   acid, 500 mg of cholin chloride, 1,050 mcg of folic acid, 150 mcg of biotin, 124 mg of Fe as

36   iron(II) sulfat monohydrate, 80 mg of Mn as manganese(II) oxide, 3.1 mg of I as calcium iodate,

37   121 mg of Zn as zinc oxide, 0.45 mg of Se as sodium selenite, 124 mg of Cu as copper(II) sulfat

38   pentahydrate. Technological additives: 250 FTU of phytase (4a16), 100 mg of butylated

39   hydroxytoluene.

40   [4]CITROFEED, dried residues from citric acid production.

3

41 **Table S2**. Analyzed nutrient composition of gestation and lactation diets for sows as well as

42 the milk replacer and prestarter diet for piglets

Chemical composition, % dry matter	Gestation diet	Lactation diet	Milk replacer[1,2]	Prestarter diet[2]
Dry matter, %	90.4	89.3	91.6	89.8
Crude protein	14.7	17.8	23.3	18.2
Crude fiber	6.5	4.6	1.9	4.9
Ether extract	5.1	4.9	10.3	7.3
Crude ash	5.7	6.6	6.8	5.1
Nitrogen-free extract	68.2	66.2	57.9	65.0
Starch	44.3	46.7	25.4	42.5
Metabolizable energy, MJ/kg	14.2	14.9	16.9	15.4
Macro minerals, %				
Calcium	0.89	1.20	0.80	0.73
Phosphorus	0.63	0.67	0.80	0.72
Magnesium	0.23	0.26	0.20	0.21
Potassium	0.78	0.78	1.09	0.67
Sodium	0.28	0.36	0.50	0.24
Trace minerals, ppm				
Iron	264.0	324.7	264.2	333.2
Manganese	95.2	96.9	87.3	118.1
Zinc	142.2	166.9	128.8	181.7
Copper	18.3	24.1	146.3	152.1

43 [1]NuriStart Sweet, BIOMIN Holding GmbH, Part of dsm-firmenich, Getzersdorf, Austria

44 [2]Piglets were fed with 100% milk replacer from day of life (DoL) 3 to 23; combinations milk

45 replacer and prestarter diet with a ratio of 70:30 (w/w) on DoL24, 50:50 (w/w) on DoL25, and

46 30:70 (w/w) on DoL26, respectively; and 100% prestarter diet on DoL27 and 28.

4

47 **Table S3.** Descriptive statistics for average daily feed intake of sows during the gestation and

48 lactation period

Average daily feed intake, kg/DM[1]	Mean	SD
Pre-farrowing (gestation period)		
26 to 5 days before farrowing	3.5	1.30
5 days before farrowing	2.5	0.43
Post-farrowing (lactation period)[2]		
Days 1 to 7	3.8	0.95
Days 8 to 14	6.3	1.68
Days 15 to 21	8.2	1.22
Days 22 to 28	8.3	1.93

49 DM, dry matter; SD, standard deviation.

50 [1]Sows were fed with gestation diet from 26 to 5 days prior to farrowing, and the lactation diet

51 from 5 days before farrowing until 28 days postfarrowing.

52 [2]Mean values for the feed intake postfarrowing are provided. The feed intake that was offered

53 to the sows was gradually increased in the first three weeks of lactation.

5

54   **Table S4.** Descriptive statistics for average daily creep feed intake of piglets during the

55   suckling period

Average daily feed intake, g/DM[1,2]	Mean	SE
DoL3 to 9	19.7	1.14
DoL10 to 16	16.5	0.77
DoL17 to 23	20.9	0.88
DoL24 to 26	23.5	1.79
DoL27 to 28	17.4	1.92

56   DM, dry matter; DoL, day of life; SE, standard error.

57   [1]Creep feed intake was estimated on litter basis.

58   [2]Piglets were fed with 100% milk replacer from DoL3 to 23; combinations of milk replacer

59   and prestarter diet with a ratio of 70:30 (w/w) on DoL24, 50:50 (w/w) on DoL25, and 30:70

60   (w/w) on DoL26, respectively; and 100% prestarter diet on DoL27 and 28.

6

61   **Table S5.** Body weight and average daily gain of suckling and newly weaned piglets

Item	Female	Male	SEM	*P*-value
Body weight, kg				
Birth	1.4[b]	1.5[a]	0.032	0.014
DoL4	1.7	1.7	0.016	0.678
DoL6	2.1	2.1	0.015	0.140
DoL13	3.7	3.7	0.048	0.861
DoL20	5.5	5.4	0.055	0.207
DoL27	7.2	7.3	0.065	0.619
DoL30	7.1	7.2	0.041	0.289
DoL34	7.4	7.3	0.038	0.434
Average daily gain, kg				
DoL1 to 4	0.13	0.13	0.006	0.902
DoL4 to 6	0.19	0.20	0.007	0.186
DoL1 to 6	0.16	0.16	0.006	0.210
DoL6 to 13	0.23	0.23	0.007	0.823
DoL13 to 20	0.25	0.24	0.008	0.255
DoL20 to 27	0.27	0.27	0.010	0.588
DoL27 to 30	0	0	0.026	0.782
DoL30 to 34	0.05	0.03	0.031	0.630
Average daily gain: birth to day 34 of life, kg				
DoL1 to 27	0.24	0.24	0.008	0.795
DoL28 to 34	0.02	0.01	0.021	0.647
DoL1 to 34	0.18	0.18	0.007	0.986

62   Values are presented as least squares means ± standard error of the mean (SEM). DoL, day of

63   life. Piglets were weaned on DoL28.

64   [a,b]Means without a common superscript in the same row differ (*P* < 0.05).

65 **Table S6.** Permutational multivariate analysis of variance (PERMANOVA) results for dissimilarities in the bacterial and fungal communities in

66 sow feces and gastric and cecal digesta of piglets during the suckling phase

Source of variation	df	Sum of squares	R²	F	P-value
Bacterial community					
Day × animal × gut segment[1]	14	36.93	0.651	36.013	0.001
Residual	270	19.78	0.349		
Total	284	56.71	1.000		
Fungal community					
Day × animal × gut segment[1]	14	30.38	0.444	13.245	0.001
Residual	232	38.01	0.556		
Total	246	68.39	1.000		

67 The analysis based on pairwise distance of a multivariate data set and values were obtained using type III sums of squares with 999 permutations

68 of residuals, considering significant difference at $P < 0.05$; df, degrees of freedom; F, F-value by permutation.

69 [1]Day, days of life for piglets and days postpartum for sows; animal, piglet and sow; gut segment, sow's feces and gastric and cecal digesta of

70 piglets.