USE OF NEWLY SELECTED BACILLUS ISOLATES TO CONTROL ENTEROTOXIGENIC *ESCHERICHIA COLI* INFECTIONS AND IMPROVE GUT HEALTH AND PERFORMANCE OF PIGLETS

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ABSTRACT

Post-weaning diarrhea (**PWD**) represents a prevalent disease resulting in substantial economic detriment to the worldwide pig farming sector. Enterotoxigenic *Escherichia coli* (**ETEC**) is the primary pathogen responsible for PWD. The ETEC F4 typically targets the small intestine, through its pili for adhesion to the pig gut and released enterotoxins that impair intestinal barrier and trigger immune responses (e.g., inflammation). We hypothesized that *Bacillus licheniformis* (***B. licheniformis***) would have beneficial effects on enhancing gut health of piglets. Two *B. licheniformis* isolates, PF9 and HG76, were selected and subsequently characterized, and evaluated in the *in vitro* and *in vivo* studies. The isolate PF9 demonstrated tolerance to low pH and high bile salts and the ability to extend the lifespan of *C. elegans* infected with ETEC ($P < 0.05$). In the ETEC F4 challenged weaned piglet studies, villus height (**VH**) of jejunum and ileum in the pigs supplemented with *B. licheniformis* HG76 were higher than other pigs ($P < 0.05$). There was an increase in jejunal and ileal crypt depth (**CD**) in the HG76 group when compared to the non-challenged group ($P < 0.05$). The *Vmax* value of sucrase in the HG76 group was higher than the non-challenged group ($P < 0.05$). Piglets supplemented with *B. licheniformis* PF9 showed lower relative abundance of Bacteroidetes in the colon than piglets from the non-challenged group ($P < 0.05$). The supplementation of *B. licheniformis* PF9 reduced the diarrhea score and increased the relative mRNA levels of aminopeptidase N (**APN**), occludin (**OCLN**), zonula occludens-1 (**ZO-1**), excitatory amino acid transporter 1 (**EAAC1**), and Na^+-glucose cotransporter 1 (**SGLT1**) ($P < 0.05$).

Both *B. licheniformis* PF9 and HG76 were supplemented for the feeding trial. No significant differences ($P > 0.05$) in the growth performance were found among all groups in the feeding trial. Compared with the control group, dietary *B. licheniformis* HG76 significantly ($P <$

0.05) reduced the diarrhea index score on days 4, 5, 7, 9, 10, 11 and 12. The ETEC F4 infection significantly induced the IPEC-J2 cell death, and this effect was significantly attenuated by *B. licheniformis* HG76 treatment ($P < 0.05$). The gene expression of ZO-1 was significantly increased during ETEC F4 infection with *B. licheniformis* HG76 treatment ($P < 0.05$). Moreover, the treatment of IPEC-J2 cells with *B. licheniformis* HG76 significantly alleviated the ETEC F4-induced elevation of the mRNA abundance of tumor necrosis factor alpha (**TNF-α**) ($P < 0.05$).

In conclusion, the supplementation of *B. licheniformis* PF9 relieved diarrhea, increased the relative mRNA level of several nutrient transporters, the gut barrier associated proteins, and digestive enzymes, and altered the microbiota composition in the ETEC F4 challenged weaned piglets. *B. licheniformis* HG76 could protect the intestinal cells from ETEC F4 infection by inhibiting inflammation and maintaining barrier integrity. The supplementation of *B. licheniformis* HG76 improved the intestinal morphology and increased digestive enzyme activity. Dietary supplementation with *B. licheniformis* PF9 and HG76 could relieve diarrhea severity after weaning. Therefore, *B. licheniformis* PF9 and HG76 have potential for improving piglets' gut health.

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CONTRIBUTIONS OF AUTHORS

This thesis was prepared by Haoxiang Xu and reviewed, revised and approved by internal examiners including Drs. Chengbo Yang, Martin Nyachoti, Joshua Gong and Kangmin Duan.

All manuscripts (Chapter Four to Chapter Seven) had been revised and approved by the co-authors, and the contributions of all authors were described below.

Chapter Four: Manuscript I. Chengbo Yang and Joshua Gong conceived the study. Joshua Gong and Hai Yu designed the *in vitro* and *C. elegans* experiments. Haoxiang Xu and Chengbo Yang designed the pig experiments. Haoxiang Xu, Peng Lu and Chengbo Yang performed the pig experiments and analyses. Linyan Li performed the lifespan assay of *C. elegans*. Hai Yu was in charge of the *in vitro* experiments for bacterial isolation and identification as well as bacterial tolerance. Paula Azevedo conducted the analysis of the microbiota data. Haoxiang Xu, Joshua Gong and Chengbo Yang prepared the manuscript. All authors reviewed the manuscript.

Chapters Five and Six: Manuscript II. Chengbo Yang, Joshua Gong, Martin Nyachoti and Karmin O conceived the study. Haoxiang Xu and Chengbo Yang designed the experiments. Haoxiang Xu, Changning Yu and Shunshun Jin performed the experiments. Hai Yu conducted the isolation and characterization of HG76 and helped the analysis of the data. Paula Azevedo revised the manuscript. Haoxiang Xu prepared the manuscript. All authors reviewed the manuscript.

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LIST OF ABBREVIATIONS

AB/PAS	Alcian blue/Periodic acid–Schiff
ADG	Average daily gain
ADP	Adenosine diphosphate
AGP	Antibiotic growth promoters
AHL	Acyl homoserine lactone
AI	Autoinducers
AKP	Alkaline phosphatase
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
APC	Antigen-presenting cells
APN	Aminopeptidase N
ASCT2	Neutral amino acid transporter 2
ATP	Adenosine triphosphate
B^0AT1	Neutral amino acid transporter

B. licheniformis	*Bacillus licheniformis*
BW	Body weight
CD	Crypt depth
CE	Competitive exclusion
C. elegans	*Caenorhabditis elegans*
CLDN3	Claudin 3
Ct	Cycle threshold
CycA	Cyclophilin-A
DC	Dendritic cells
DMEM	Dulbecco’s modified Eagle’s medium
DFM	Direct-fed microbials
Dpi	Days post-inoculum
EAAC1	Excitatory amino acid transporter 1
EGF	Epidermal growth factor
EHEC	Enterohemorrhagic *Escherichia coli*

EO	Essential oils
EPS	Exopolysaccharides
ETEC	Enterotoxigenic *Escherichia coli*
EU	European Union
FAO	Food and Agriculture Organization
FBS	Fetal bovine serum
FCR	Feed conversion ratio
FITC-D	Fluorescein isothiocyanate-dextran
GALT	Gut-associated lymphoid tissue
GIT	Gastrointestinal tract
GSH	Glutathione
GSSG	Oxidized glutathione
Hpi	Hours post-inoculum
IAP	Intestinal alkaline phosphatase
IEC	Intestinal epithelial cells

IgA	Immunoglobulin A
IκBα	Inhibitor of NF-κB
IL	Interleukin
IPEC-J2	Intestinal porcine epithelial cell line
KRB	Krebs ringer bicarbonate
LB	Lysogeny broth
LGG	*Lacticaseibacillus rhamnosus* GG
LPS	Lipopolysaccharide
LT	Heat labile toxins
MAPK	Mitogen-activated protein kinase
MGA	Maltase-glucoamylase
MIA	Medically important antimicrobials
MUC2	Mucin 2
MUC4	Mucin 4
NC	Negative control

NF-κB	Nuclear factor kappa B
NGM	Nematode growth medium
NLR	NOD like receptors
NOD	Nucleotide binding oligomerization domain
OCLN	Occludin
OD	Optical density
OTU	Operational taxonomic units
PBS	Phosphate-buffered saline
PEDV	Porcine epidemic diarrhea virus
PepT1	Peptide transporter 1
PPAR-γ	Peroxisome proliferator-activated receptor γ
PRR	Pattern recognition receptors
PWD	Post-weaning diarrhea
QS	Quorum sensing
rRNA	Ribosomal RNA

SAS	Statistical analysis system
SCFA	Short-chain fatty acids
SEM	Standard errors of the means
SGLT1	Na^+-glucose cotransporter 1
SID	Standardized ileal digestibility
SNP	Single nucleotide polymorphisms
ST	Heat-stable toxin
TAC	Total antioxidant capacity
TBST	Tris-buffered saline with Tween 20
TEER	Transepithelial electrical resistance
TGE	Transmissible gastroenteritis
TJ	Tight junctions
TLR	Toll like receptor
TNF-α	Tumor necrosis factor α
TSA	Tryptic soy agar

TSB	Tryptic soy broth
VH	Villus height
VH:CD	Ratio of villus height and crypt depth
V_{max}	The maximal enzyme activity
WHO	World Health Organization
WST-1	Water-soluble tetrazolium salts
ZO-1	Zonula occludens-1

CHAPTER ONE : GENERAL INTRODUCTION

Background

The pork industry ranks the 4th largest farming industry in Canada, which provides sufficient supplies of affordable and high-quality protein for Canadians and people around the world. Swine operations in Canada are concentrated in the Mideastern provinces (e.g. Quebec, Ontaria and Manitoba), which account for 81% of Canada's inventory. As of January 1, 2023, there were 14.17 million hogs on 7,330 farms in Canada (AAFC, 2023). However, the pork industry in Canada today is still faced with challenges, such as post-weaning diarrhea (**PWD**), which is a commonly occurring and economically important disease and threatens producers, animal health and the sustainability of the swine industry.

There are different bacterial and viral agents that can cause PWD. However, enterotoxigenic *Escherichia coli* (**ETEC**) is the most predominant cause of PWD (Luppi, 2017). Over the past 70 years, dietary antibiotics have been commonly used in swine and poultry production due to their effectiveness in prevention and treatment of animal diseases (Teillant and Laxminarayan, 2015). However, the extensive use of antibiotics has been suggested to contribute to the emergence of antibiotic resistant bacteria, which presents a serious challenge to public health (Serwecińska, 2020). In order to keep animal health and maintain sustainability of pork production, the development of antibiotics alternatives to reduce the use of antibiotics is becoming increasingly important in the livestock industry (Scott et al., 2019).

Probiotics are one of the potential antibiotic alternatives associated with health benefits for animals. The most commonly used probiotics in the swine industry are bacteria, such as *Bacillus*, *Bifidobacterium*, and *Lactobacillus*. *Bacillus* spp. are capable of forming endospores to endure extreme environmental conditions, which makes them a suitable candidate as feed additives

due to their resistance to heat processing, stomach acid, and bile salts (Bahaddad et al., 2023). Therefore, our studies focused on investigating the effects of *Bacillus* isolates to control enteric infections in piglets.

1.2 Thesis structure

Chapters of this thesis are structured as follows:

- Chapter Two provides information about PWD causes and changes, gut health related systems, definition, mode of action, and the application of probiotics in animal nutrition.
- Chapter Three outlines the research gap, hypotheses and objectives.
- Chapters Four to Seven present the studies on the effects of selected probiotics on gut health and performance of weaned piglets.
- Chapters Eight to Nine discuss and summarize the thesis.

CHAPTER TWO : LITERATURE REVIEW

2.1 Background

It is estimated by the Food and Agriculture Organization of the United Nations that the global human population will exceed 9 billion by 2050, leading to an increase in meat consumption (Tilman and Clark, 2015). As an important source of meat, the demand of pork will consequently rise. To meet this growing demand, pork production systems must become more efficient and sustainable, promoting the expansion of intensive farming worldwide (Henchion et al., 2017). However, intensive animal farming systems favorite the transmission of animal diseases, adversely, which affects animal health and reduces productivity. Besides the intensive farming systems, other factors also impact animal health and disease incidences.

One of the common diseases found in swine is the postweaning diarrhea (**PWD**), mainly affecting piglets during the first two weeks following weaning (Canibe et al., 2022). Early and abrupt weaning is a common practice. However, due to the dietary transition, social and environmental stressors, the weaned piglets experience numerous physiological, structural, microbiological and functional changes in the gastrointestinal tract (**GIT**). These changes increase the susceptibility of piglets to GIT infections leading to PWD. The presence and proliferation of enterotoxigenic *Escherichia coli* (**ETEC**) has been identified as the major cause of PWD (Eriksen et al., 2021).

To prevent the transmission of PWD among pigs and reduce the economical losses, swine producers have traditionally used antibiotics. The use of antibiotic growth promoters (**AGP**) in animal diets has been found to significantly improve the production performance, minimize the occurrence of diseases, reduce the morbidity and mortality, and decrease the feeding costs (Low et al., 2021). However, the overuse or misuse of antibiotics has been suggested to lead to an

increase of antimicrobial resistance (**AMR**) in zoonotic pathogens, posing a threat to public health (Salam et al., 2023). In response, the European Union (**EU**) banned the use of all AGP in animal feeds in January 2006. Since then, many countries, including Canada, USA, Mexico, China, Japan and India, have also restricted the use of AGP (Eriksen et al., 2021).

The increased restriction of using AGP in pig diets has led to a growing interest in developing highly efficient AGP alternatives. Several AGP alternative products are available on the market, including probiotics, prebiotics, minerals, acidifiers, plant extracts, and nucleotides. These alternatives aim to improve growth performance, regulate the gut microbiota, boost the immune system, and mitigate the side effects of weaning challenges (Liu et al., 2018). Among these products, probiotics have generated significant attention due to their beneficial effects on swine health through various mechanisms, such as antibacterial activity, competitive exclusion, and immunomodulatory properties (Adhikari and Kim, 2017). Therefore, it is crucial to evaluate the efficacy of these AGP alternatives to ensure that they meet the needs of modern pig production systems.

2.2 Post-weaning diarrhea (PWD)

Weaning is one of the most challenging stages with significant influence on piglets' health due to the sudden separation from the sows and major changes in diets, physical and social environments. This stress can impair the gut microbiota, physiological and biochemical functions, digestion and absorption, and immune functions. Consequently, weaned piglets under multiple stresses may suffer from PWD, gut damage and even death (St-Pierre et al., 2023).

2.2.1 Intestine changes after weaning

The intestines play vital roles in maintaining host health, including function to serve as the primary site for nutrients digestion and absorption, and act as a physical barrier to prevent

exogenous harmful substances from entering the circulation system (Wang et al., 2021). However, changes in piglets occur after weaning that can impact these functions.

2.2.1.1 Changes in gut morphology and enzyme activity

The integrity of the intestinal structure is crucial for nutrients digestion and absorption in piglets. Gut morphology, including villus height (**VH**), crypt depth (**CD**), and the ratio of villus height and crypt depth (**VH:CD**) indicates the health status of the gut ligning (Zhu et al., 2022).

Weaning of piglets usually takes place between 3 and 4 weeks after birth. After weaning, the intestinal environment changes dramatically due to the shift from highly digestible sow milk to plant-based solid feed, which may lead to temporary anorexia. Significant changes in histological parameters, such as villous atrophy, crypts hyperplasia and enzymatic activity changes of the small intestines, may occur after weaning. Increased rate of cell loss leading to a higher rate of mitosis in crypts and their hyperplasia combined with a slower rate of cell renewal due to reduced cell divisions, are the two main factors contributing to villous atrophy (Barszcz and Skomiał, 2011).

Digestive enzymes play a crucial role in the growth and development of pigs by improving feed efficiency and regulating metabolic process (Tan et al., 2021). Morphological changes in the small intestine of weaned piglets are accompanied by reduced activity of brush border enzymes, such as lactase, sucrase and maltase, which are the key enzymes of carbohydrate digestion and absorption in piglets (Pácha, 2000). The activity of these disaccharidases decreased dramatically after weaning, which is considered to be a vital cause of diarrhea in weaned piglets (Zou et al., 2021). Moreover, alkaline phosphatase (**AKP**) in the gut villus epithelium also plays a critical role in gut digestion and absorption, facilitating nutrient uptake and transport, and converting adenosine diphosphate (**ADP**) into adenosine triphosphate (**ATP**). A study by Tao et al. (2016) showed that

weaning significantly decreased AKP activity in piglets, indicating that weaning stress adversely affects gut digestion and absorption functions.

During the weaning period, nutrient malabsorption in the small intestine is aggravated by the poor electrolytes' absorption and secretion (Nabuurs et al., 1994). The epithelial cells and the mucin layer in the gut serves as the first line of defense, protecting weaned piglets from harmful bacteria, toxins and antigens (Campbell et al., 2013). Disrupted epithelial barrier function, decreased tight junction protein expression, reduced mucin production and goblet cells numbers, increased intestinal permeability and disease susceptibility have all been reported in weaned piglets (Peace et al., 2011; Hu et al., 2013; McLamb et al., 2013). Overall, weaning in pigs induces significant changes on gut morphology and enzyme activities, impacting nutrient absorption and overall health.

2.2.1.2 Changes in immunity

The gut has a highly complex local immune system, comprising immune organs, immune cells (such as macrophages, neutrophils, lymphocytes, lamina propria, and intraepithelial lymphocytes), and immune related molecules, such as immunoglobulins, antibacterial peptides, and cytokines (Zhang et al., 2019).

Intestinal epithelial cells play important roles in the absorption of nutrients, secretions of fluid, as well as providing a barrier against pathogens in the lumen. The cross-talk that occurs between intestinal epithelial cells and underlying lamina propria cells serves to transfer immune-related signals to the local adaptive immunity to maintain gut immune homeostasis (Rimoldi et al., 2005). Normally, intestinal epithelial cells can recognize beneficial and pathogenic substances through pattern recognition receptors (**PRR**), such as nucleotide binding oligomerization domain like receptors (**NLR**) and toll-like receptors (**TLR**) (Wicherska-pawłowska et al., 2021). This

recognition process activates the immune response and inhibits inflammation by regulating the signaling pathways, including peroxisome proliferator-activated receptor γ (**PPAR-γ**), Mitogen-activated protein kinase (**MAPK**), and nuclear factor kappa B (**NF-κB**) (Dana et al., 2019).

Weaned piglets may face several pathogenic and environmental challenges that disrupt the immune barrier function, leading to the dysfunction of intestinal immune cells, such as T lymphocytes, and intestinal mast cell (Upadhaya and Kim, 2021). Weaning stress would activate the gut immune system, producing pro-inflammatory cytokines, such as TNF-α, IFN-γ, IL-1β, IL-6, and IL-8. Overproduction of these pro-inflammatory cytokines can result in gut damages and dysfunctions (Guan et al., 2021). Intestinal $CD4^+$ and $CD8^+$ T lymphocytes in weaned piglets are dramatically increased and proinflammatory cytokines are enhanced, indicating a robust activation of the gut immune system (McCracken et al., 1999; Pié et al., 2004; Hu et al., 2013). Consequently, weaning triggers changes in immunity, often leading to temporary immune suppression and increased susceptibility to diseases.

2.2.1.3 Changes in microbial ecology

It has been reported that weaning reduces the relative abundance of *Lactobacillus* group and obligate anaerobic bacteria (e.g., *Clostridia* and *Bateroidia*), while increasing *E. coli*, *Prevotella* spp., *Clostridium* spp., and facultative anaerobic bacteria (e.g., *Proteobacteriaceae* and *Enterobacteriaceae*) in the gut (Konstantinov et al., 2006; Petri et al., 2010; Tao et al., 2015; Gresse et al., 2017). The abrupt decrease in *Lactobacillus* spp., which plays a major role in disease prevention, during the weaning transition can significantly increase the risk of disease (Konstantinov et al., 2006).

The inclusion of plant ingredients in the nursery diets for weaned piglets, profoundly influences the development of bacterial species that are preferred in the gut ecosystem (Luo et al., 2022). In response to the diet change, the abundance of *Prevotellaceae* increases to metabolize the glycans and polysaccharides present in the plant ingredients (Kiernan et al., 2023). Additionally, to metabolize other components in the diet, such as tagatose, there is an increase in the abundance of *Ruminococcaceae*, *Oscillospiraceae*, *Veillonellaceae,* and *Lachnospiraceae* (St-Pierre et al., 2023). Moreover, the increased abundance of *Fusobacteriaceae*, *Campylobacter*, and *Sutterella* has been found in piglets suffering from PWD (Kingkan et al., 2023). Therefore, changes in microbial ecology during weaning involve significant shifts in the gut microbiota composition, impacting digestive and immune functions.

2.2.2 PWD factors/reasons

The occurrence of PWD is caused by pathogenic bacteria and viruses including ETEC, corona viruses (e.g., Porcine epidemic diarrhea virus (**PEDV**) and transmissible gastroenteritis virus (**TGE**)) and rotaviruses. Among these, ETEC strains are the main infectious agents of PWD (Luppi, 2017). Besides the pathogens, several other factors including the sows, piglets, environmental conditions, and management practices will lead to PWD as well (Rhouma et al., 2017).

2.2.2.1 Weaning age

The age at which piglets are weaned, along with their health status before weaning, are critical factors influencing the development of PWD (Eriksen et al., 2022). Furthermore, the period after weaning is a crucial stage in the pig's development, during which the gut immune system is not fully developed. The cessation of sow milk consumption, which includes the vital antibody IgA, heightens the vulnerability of pigs to pathogenic infections (Maciag et al., 2022). Research

examining the outcomes of piglets weaning before 21 days has increasingly supported the shift towards weaning not before 26 days of age, as a means to diminish the incidence of PWD (Eriksen et al., 2021). Additionally, delaying the weaning age of pigs can lessen the stress experienced during this phase, which helps to develop a more mature gut system and allows for a smoother adaption to solid feed. Consequently, it can result in improved growth performance and enhanced immune response (Huting et al., 2021).

2.2.2.2 Feed intake

Piglets typically experience a decrease in feed consumption after weaning. The duration and severity of this reduced appetite can vary among farms, influenced by factors such as livestock management practices and the type of feed provided (Middelkoop et al., 2019). Reduced feed consumption in the initial week following weaning is associated with an increased likelihood of intestinal disease development during the post-weaning phase, and plays a role in causing gut inflammation, negatively impacting both the crypt depth and villus height (Wei, 2021). The alteration in the structure of the gut facilitates the development of a favorable environment for the growth of pathogens like *E. coli*. This change also enables pathogens and toxins to penetrate through the epithelial layer, a consequence of the associated inflammation (Potrykus et al., 2021).

2.2.2.3 ETEC

The gram-negative bacterium *E. coli* belongs to the family *Enterobateriaceae*. ETEC pathotype is the most common and important cause of PWD in pigs, which is characterized by two types of virulence factors: 1) adhesins mediating ETEC binding to host receptors and subsequent colonization of the intestinal epithelium and 2) enterotoxins causing fluid and electrolyte hyper-secretion that leads to diarrhea, both essential for disease development (Nagy and Fekete, 2005).

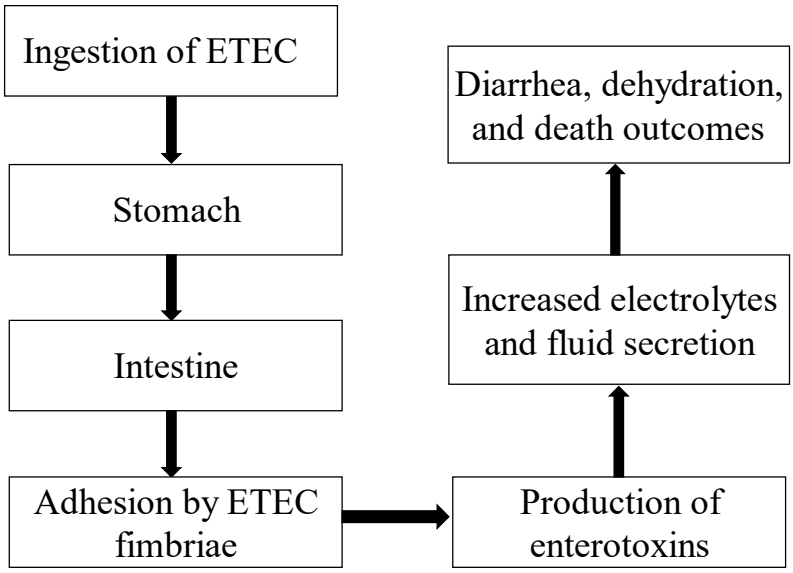
The fimbrial adhesins of ETEC, which are fimbrial bio polymeric filaments located at the external surface, play a critical role in initiating PWD. A typical ETEC fimbriae contain hundreds of copies of fimbrial major structural subunits and a few copies of minor structural subunits. They attach to host cell receptors and facilitate adherence between ETEC and the porcine small intestinal epithelium (Zhang, 2014). The fimbrial adhesins most commonly associated with ETEC are K88 (F4), K99 (F5), 987P (F6), F41, and F18. The occurrence of PWD typically correlates with the growth of one or more strains of β-hemolytic ETEC in the small intestines of pigs. The most predominant and important ETEC fimbrial adhesins in PWD are F4 and F18 (Luppi et al., 2016). The F4 fimbriae are adaptable structures, existing in various variants, such as F4ab, F4ac, or F4ad (Dierick et al., 2020).

The adhesion and colonization of the small intestinal epithelial cells by ETEC rely on the presence of specific receptors, known as F4 receptors, which are present on the brush borders of villous enterocytes (Xia et al., 2016). The existence and functionality of these receptors are vital in determining how susceptible pigs are to ETEC infection (Luise et al., 2019a). The binding of ETEC to the intestinal mucosa of pigs is a key step in the development of PWD (Sun and Kim, 2017). For ETEC F4, single nucleotide polymorphisms (**SNP**) located on Mucin 4 gene (**MUC4**) have been proposed as the most frequently used genetic markers for pig ETEC resistance/susceptibility (Jørgensen et al., 2003).

Once the ETEC have adhered and colonized the small intestine of pigs, the enterotoxins can be produced. The genes responsible for producing enterotoxins in ETEC bacteria are located on plasmids and affect the gut of pigs (Hu et al., 2023). Both ETEC F4 and F18 can produce two types of enterotoxins, heat stable (enteroaggregative *E. coli* heat-stable toxin 1, STa, and STb) and heat labile (**LT**) enterotoxins, which leads to an increase of electrolytes and fluid secretion into the

gut lumen and therefore, changing the proper gut functions (Luppi, 2017). Overproduction of electrolytes and fluid can result in diarrhea, metabolic acidosis, dehydration, and potentially fatal outcomes (Gałęska et al., 2022). Challenges with ETEC in pigs have been linked to significant villous atrophy and a substantial reduction in crypt depth across all gut segments of the infected animals (Blirup-Plum et al., 2023). Consequently, ETEC causes PWD in piglets by producing toxins that disrupt the intestinal lining, leading to severe watery diarrhea.

Figure 2.1 Overview of the pathogenesis of ETEC leading to the postweaning diarrhea in piglets.



The death of piglets is generally due to the dehydration, while the pathogenesis of ETEC leading to PWD in piglets involves a multifaceted process. After weaning, piglets experience stress and dietary changes, weakening their immune defenses.

2.2.2.4 Quorum Sensing (QS)

The concept of QS is wherein bacteria employ complex cell-to-cell communication systems to coordinate responses to the environmental stimuli (Bassler and Losick, 2006), and coordinate responses within a bacterial population through chemical molecules (Fuqua et al., 2001). One of the autoinducers, AI-3, is involved in enterohemorrhagic *E. coli* (**EHEC**) cross talk with the epinephrine-norepinephrine host signaling system (Freestone et al., 2002). Moreover, EHEC senses the host hormones epinephrine and norepinephrine through the membrane spanning sensor QseC (Walters et al., 2006). A two-component system named QseBC is made up of a QseC, and a RR QseB, in which QseC phosphorylate on binding of its ligand and transfer the phosphate moiety to QseB transcriptional regulators (Sperandio et al., 2002). Given that AI-3 production is not limited to EHEC strains, ETEC may use host molecules (epinephrine), as well as conditioned medium (e.g., AI-3 molecules) as an environmental signal to regulate the expression of important virulence factors critical for its pathogenic process (Sturbelle et al., 2015).

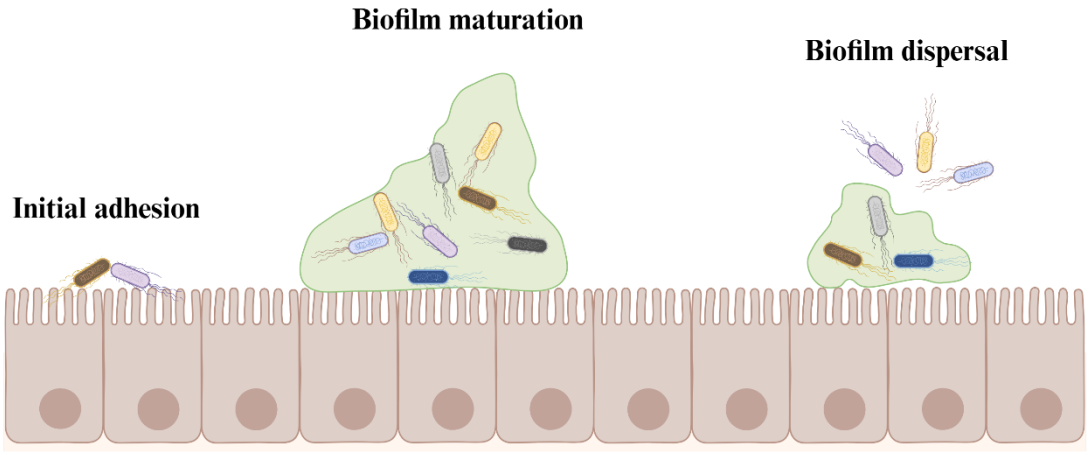
The process where *E. coli* colonizes the small intestine involves its ingestion by the animals and its swift multiplication (Cheng et al., 2014). Sturbelle et al. (2015) discovered that incorporating conditioned medium into *E. coli* culture, notably enhanced its growth rate, which could potentially happen as a result of certain elements that are present from the culture. These molecules may influence genes that regulate bacterial growth. Consequently, the emerging bacterial population may also produce these same molecules.

It has been known that *E. coli* lacks the ability to independently produce the acyl homoserine lactone (**AHL**) molecule. However, it possesses SdiA, a molecular receptor for AHL, which enables *E. coli* to detect AHL signals emitted by nearby flora in the infection pathway. Through a process known as QS eavesdropping, *E. coli* can monitor these signals and subsequently

modulate its own virulence in response (Pacheco et al., 2021). The ability of ETEC strains to adhere was markedly reduced when exposed to various sources or types of AHL. This reduction in adhesion capacity could be due to AHL's suppression of the expression of adhesion factors, such as flagella (Yang et al., 2022).

Bacteria naturally occur as freely drifting plankton or as colonies of microorganisms creating biofilms (Wheeler et al., 2019). Biofilm is a structured community of microorganisms that are encased within a complex structure of bacterial polysaccharides (Hobley et al., 2015). The typical procedure for the development of biofilms begins with free-floating, bacteria adhering to a surface. Following initial attachment, bacteria are enveloped in exopolysaccharides, which leads to the formation of microcolonies. These microcolonies then cluster together, creating a mature biofilm. The final stage in the life cycle of a biofilm is the dispersion (Alotaibi, 2021). The formation of biofilms has been verified to be regulated by the LuxS/AI-2 QS system. *E. coli* forms biofilms in response to the small molecule autoinducer-2 (AI-2). LuxS functions as an AI-2 synthase, facilitating the production of AI-2 from S-adenosylmethionine through enzymatic procedures. The substrate for LuxS is S-ribosylhomocysteine, which generates two products: homocysteine and AI-2. As a component of the activated methyl cycle, LuxS might also influence biofilm formation through AI-2-independent metabolic effects. The biochemical intermediates and biosynthetic pathways involved in AI-2 biosynthesis have been found to be identical in different gram-negative bacteria, including *E. coli* and *Salmonella* (Sun et al., 2020). Consequently, QS in bacterial populations influences PWD in piglets by regulating virulence factor expression and biofilm formation, leading to enhanced bacterial colonization and infection.

Figure 2.2 Biofilm development occurs in four distinct phases: initial adhesion to a surface, formation of microcolonies, maturation and diversification of the biofilm, and eventual detachment from the surface (Adapted from Alotaibi, 2021).



2.3 Gut health

The gut of a pig is a multifaceted system. A healthy gut plays an important role in enhancing feed efficiency by improving feed digestion and nutrient absorption, therefore, ensuring a healthy gut is key in swine production (Willing et al., 2012). During the weaning period in particular, the structure of the pigs' gut undergoes significant modifications in size, witnesses swift shifts in its microbial population, experiences elevated rates of protein turnover, and rapidly adjusts its digestive and immune capabilities (St-Pierre et al., 2023).

The gut health refers to the state and efficient functioning of the intestines, which play a critical role in the digestive system. There are four major interconnected pillars of gut health: (1) barrier function, (2) microbiota balance, (3) oxidative homeostasis, and (4) effective gut immune function. It is imperative to regulate these aspects for maintaining gut health status (Chalvon-Demersay et al., 2021). Gut health in piglets is crucial for their overall growth and development, influencing nutrient absorption, immune function, and resistance to diseases like diarrhea.

2.3.1 Defense system of swine

The epithelium of the small intestine in pigs serves three important roles: 1) the secretion and uptake of electrolytes and water, ensuring the luminal contents maintain a appropriate viscosity and facilitating the expulsion of harmful substances; 2) the digestion and absorption of nutrients; 3) acting as a protective barrier against pathogens (Berg, 2000).

2.3.1.1 Barrier function

Dysfunction of the gut barrier and increased gut permeability would facilitate the movement of bacteria and the entry of pathogens from the gut lumen into the body of animals, which leads to further infections and immune responses (Peng et al., 2021). Several factors have been identified that can significantly impact the barrier function of piglets after weaning, including

weaning stress, weaning age, diet composition, and feed intake (Modina et al., 2019). Maintaining an adequate level of feed intake after weaning prevents the loss of paracellular barrier function by ensuring that villous height is preserved, which indicates that insufficient feed intake plays a crucial role in the compromised barrier function observed in the post-weaning period (Ribeiro et al., 2023).

To enhance the gut barrier function after weaning, there are three distinct strategies that can be employed through dietary modifications: 1) enhancing the diet palatability to boost feed intake of pigs; 2) key nutrients (such as some essential amino acids) that can be administered to pigs to maintain gut barrier integrity; 3) supplementation of specific bioactive elements into the diet aiming to regulate stress and immune responses, thereby safeguarding the barrier function (Zheng et al., 2021). These strategies aim to strengthen the gut barrier, which plays a crucial role in defending against pathogens and toxins, as well as in regulating nutrient absorption and immune responses in piglets.

2.3.1.2 Epithelial transport

The transport of substances across the epithelium of the small intestine can be categorized into two types: paracellular and transcellular transports. Paracellular transport involves the movement of substances through the spaces between epithelial cells, which is facilitated by a complex network of proteins known as tight junctions (**TJ**). The function of TJ is to link the epithelial cells together and effectively close the gaps between them, allowing specific passage of water, small molecules, and ions, selectively controlling the permeability (Tervonen et al., 2019). Transcellular transport refers to the process where nutrients are up-taken via either active or passive transport through enterocytes and endocytosis.

Ussing chambers are commonly used in *ex vivo* studies to assess the gut barrier function and epithelia transport. The transepithelial electrical resistance (**TEER**), which indicates the loosening of the TJ among epithelial cells, could be measured in Ussing chambers. Higher TEER values indicate reduced paracellular permeability, while lower TEER values suggest increased paracellular permeability (Srinivasan et al., 2015).

2.3.1.3 Absorption

The process of nutrients absorption through the small intestine involves two main ways: active and passive absorptions. Active absorption occurs through specialized transporters (such as sodium-glucose cotransporter 1, peptide transporter 1, excitatory amino acid transporter) to transport nutrients (i.e. glucose or amino acids) simultaneously with sodium transport across the intestinal epithelium (Patra, 2018).

The active absorption during the post-weaning period is affected by three key factors: age at weaning, level of feed intake, and composition of the diet (Wensley et al., 2021). Generally, the ability of the small intestine to absorb nutrients diminishes after weaning, especially when piglets are weaned at 3 weeks of age or before (Huting et al., 2021). Meanwhile, the passive transcellular absorption after weaning is also reduced due to a defence mechanism, which prevents the pathogens from penetrating into the animals' blood circulation (Broom and Kogut, 2018). Therefore, absorption involving the uptake of essential nutrients from the feed, is crucial for growth, immunity, and overall health of pigs.

2.3.2 Gut microbiota

Adjusting the composition of the gut microbiota can have instant and lasting impacts on the gut health of pigs (Choudhury et al., 2021). Diet and nutritional components significantly influence the composition and stability of the microbiota in pigs' gut (Bergamaschi et al., 2020).

The gut of pigs is inhabited by a rich, ever-changing, and intricately diverse population of microorganisms, predominantly consisting of bacteria (Patil et al., 2020).

The gut microbiota closely engages with immune cells in the gut, enhancing their ability to regulate the immune response, and serving as the primary defense against pathogens through competitive exclusion (Yoo et al., 2020). Without the gut microbiota, the intestinal mucosal immune system fails to fully develop, leading to a decrease in the number of operational regulatory T cells. This reduction hampers the capacity to fight off pathogenic bacteria (Raheem et al., 2021). Microbiota-induced production of immunoglobulin A (**IgA**) regulates bacterial colonization and prevents bacteria from crossing the epithelial layer (Weis and Round, 2021). Additionally, the microbiota has the capability to metabolize specific toxins present in the diets and synthesize some vitamins, which can be utilized by the intestinal epithelium of the animals (Uebanso et al., 2020). Consequently, the gut microbiota plays a key role in the gut health of piglets by aiding digestion, enhancing nutrient absorption, and protecting against pathogens.

2.3.2.1 Composition of gut microbiota

The large intestine serves as the primary site for microbial fermentation in pigs, hosting a more diverse microbial population than the small intestine (Gardiner et al., 2020). In the initial week after weaning, there is a significant transformation in the gut microbiota of pigs (Cremonesi et al., 2022). The colonization by bacteria like *Blautia*, *Roseburia*, and *Prevotella* in the gut at early age could enhance piglets' capacity to digest glycan and other carbohydrates in the nursery diets (Saladrigas-García et al., 2022). Healthy piglets exhibit a greater abundance of bacteria from the *Lactobacillaceae, Prevotellaceae, Ruminococcaceae*, and *Lachnospiraceae* families compared to piglets suffering from PWD (Nowland et al., 2022). Species within the *Prevotella* and *Roseburia*

genera play a key role in producing short-chain fatty acids, which influence the host's energy metabolism and gut health, inversely related to pathogenic infections in the gut (Amat et al., 2020).

The timely feed intake after weaning has a notable and significant correlation with the abundance of *Bacteroides* and *Prevotella* (Gardiner et al., 2020). In piglets experiencing weaning stress and showing reluctance to consume diets, there is often a decrease in *Prevotella* levels accompanied by an increase in *Bacteroides* (Saladrigas-García et al., 2021). Conversely, upon the initiation of diets consumption post-weaning, there is a notable increase in *Prevotella* levels, rendering it the predominant component in pig feces thereafter (Qi et al., 2021).

The *Enterobacteriaceae* family comprises a vast group of Gram-negative bacteria with similar biological traits and resides in the gut of pigs, and some pathogens (such as *Enterobacter, Citrobacter, Salmonella, Escherichia coli*) causing infectious gut diseases belong to this family (Poulin-Laprade et al., 2021). One week before the onset of diarrhea symptoms, there is a noticeable increase in *Enterobacteriaceae* bacteria within the intestines of pigs at day 30, suggesting that these bacteria may serve as an early indicator of PWD in piglets (René et al., 2023).

2.3.2.2 Interaction between the intestinal epithelial cells and gut microbiota

Intestinal epithelial cells (**IEC**) detect signals from microbes in the gut, adjusting to the changes in the microenvironment of the gut through altering gut barriers and communicating signals to immune cells (Iyer et al., 2023). The interactions between the IEC and gut microbiota could be categorized into three distinct types: metabolites produced by bacteria, components of bacterial cells, and the microbes (Mayorgas et al., 2020).

Metabolites produced through microbial fermentation and growth significantly influence the gut microbiota and impact the immune system (Yoo et al., 2020). Short-chain fatty acids (**SCFA**), tryptophan derivatives, and secondary bile acids are three key bacterial metabolites,

recognized for their critical contributions to sustaining the integrity of the gut epithelium (Masse and Lu, 2023).

Bacteria belonging to the Bacteroidetes phylum are widely recognized for the capacity to produce propionate and acetate, while bacteria within the Firmicutes phylum are known for their effective production of butyrate (Stojanov and Berlec, 2020). The roles of SCFA include not only providing energy to the host, but they also modulate the physiological functions of immune cells and IEC (Martin-gallausiaux et al., 2021). The activation of G protein–coupled receptors, driven by acetate, propionate, and butyrate, enhances the colonic epithelium's secretion of chemokines and cytokines, aiding in the elimination of pathogens (Ney et al., 2023). Additionally, SCFA stimulate the production and secretion of mucus by goblet cells (Yang, 2021).

The epithelial cells absorb bile acids, which are then released into the ileum's lamina propria. The bile acids that are not absorbed undergo conversion into secondary bile acids by the microbiota (Sun et al., 2021). Deoxycholic acid is one of the secondary bile acids, which can stimulate G protein–coupled receptor Takeda G protein receptor 5 on endocrine cells to enhance the peristalsis of colon (Gou et al., 2023). Therefore, the secondary bile acids produced by the gut bacteria affect the growth and function of IEC.

Dietary tryptophan is metabolized into indole by tryptophanase secreted from gut microbiota (Johnston and Zhang, 2020). Indole enhances the expression of molecules associated with cell tight junctions, such as claudins and occludin, by stimulating the pregnane X receptor (Ghosh et al., 2021). Collectively, indole, a tryptophan metabolite that depends on microbiota, is crucial for sustaining the integrity of the epithelial barrier (Li et al., 2021).

The pattern recognition receptors on IEC, such as TLR and NLR, play a crucial role in immune surveillance (Layunta et al., 2021). These receptors are adept at detecting components of

gut microbiota, like flagellin and lipopolysaccharide (**LPS**) (Eshleman and Alenghat, 2021). Upon recognition of these bacterial elements, IEC initiate a signaling cascade that promotes epithelial cell proliferation and stimulates the secretion of mucus, antimicrobial agents, and cytokines, thereby enhancing the gut barrier functions (Soderholm and Pedicord, 2019). Overall, the interaction between IEC and gut microbiota of piglets involves a symbiotic relationship where the microbiota aids in nutrient absorption and immune function, while IEC provide a protective barrier and create a suitable environment for beneficial microbes.

2.3.3 Immune system

The mucosal immune system is present in multiple anatomical sites within the body, including the reproductive, respiratory, and digestive systems, which performs a multifaceted function in protecting the host (Silva et al., 2015). The mucosal immune system within the gut is crucial, because it not only aids in the digestion and absorption of nutrients, but also acts as a vital defense mechanism to protect the body from pathogens (Guo et al., 2018). The immune response within the intestinal mucosa is initiated by the mucosal tissues, where cells capable of reacting to immune challenges are activated by various antigens and pathogens (Kogut et al., 2016). A defining feature of mucosal immunity is the generation of secretory immunoglobulin A (sIgA) by plasma cells (Li et al., 2020). The immune system plays a crucial role in maintaining gut health of piglets by defending against pathogens and promoting a balanced microbial environment.

2.3.3.1 Immune cells

The immune system of the gut mucosa can be classified into induction and effector sites based on the structure and function of tissues (Kurashima and Kiyono, 2017). The induction site primarily pertains to the gut-associated lymphoid tissue (**GALT**), consisting of well-structured Peyer’s patches, isolated lymphoid follicles, and mesenteric lymph nodes (Portilla et al., 2021).

Within these induction sites, antigen-presenting cells (**APC**), including macrophages, dendritic cells, and B cells, are responsible for capturing and presenting pathogens to the immune effector cells (Gaudino and Kumar, 2019).

Microfold cells are distinguished by limited number of lysosomes, tiny cytoplasmic vesicles, and short microvilli, which have the ability to engulf pathogens (Miller et al., 2007). The intraluminal pathogens, including proteins, parasites, viruses, and microspheres could be ingested and transported by Microfold cells to APC located in the underlying lymphoid tissue (Zhang et al., 2023). Subsequently, the pathogens are introduced to the receptor molecules and ultimately to the helper T lymphocytes, which release various lymphokines to activate B lymphocytes (Broere et al., 2011). The activated B lymphocytes then undergo differentiation and proliferation, leading to the production of significant amounts of sIgA. The sIgA is then secreted into the gut lumen, altering immunological characteristics (Seikrit and Pabst, 2021).

Effector sites consist of a widespread dispersion of lymphocytes within the epithelial layer and the underlying lamina propria, which includes lamina propria lymphocytes and intraepithelial lymphocytes (Farré et al., 2020). Once the APC present pathogens to these lymphocytes, the generation of antibodies and the secretion of diverse immunomodulatory cytokines will be triggered (Kasper and Reder, 2014).

2.3.3.2 Innate immune response

The innate response of the immune system possesses essential mechanisms for eradicating pathogens and limiting systemic adaptive responses to the microbiota to preserve a balanced microenvironment. (Kumar et al., 2011). It has been reported that sIgA plays a crucial role in conveying the microbiota to the immune system (Macpherson et al., 2018). After binding to receptor on Microfold cells, sIgA selectively exposes bacterial elements to dendritic cells (**DC**) to

secrete interleukin-10 (**IL-10**) (Abokor et al., 2021). The presentation of commensal species to DC aligns with the anti-inflammatory properties of sIgA, which helps in reducing inflammation that might occur due to the large number of bacteria present in the lumen (Luciani et al., 2022).

Additionally, sIgA also plays a vital role in the initial defense mechanism to fight against pathogens. It prevents pathogens and toxins from attaching to the dense mucus layer covering the epithelial cells by widely recognizing pathogenic markers on their surface, which then binds pathogens together within the gut lumen to prevent the pathogens to colonize (León and Francino, 2022). Inflammasomes play an important role as innate immune system mediators in the interaction between the microbiota and host, detecting both damage-associated molecular patterns and pathogen-associated molecular patterns (Liwinski et al., 2020).

2.3.3.3 Adaptive immune response

Inadequate adaptive immune responses can lead to insufficient reactions to the microbiota and alterations in the composition of bacteria (Zheng et al., 2020). In animals that lack adaptive immunity with a deficiency in T-box family of transcription factors can lead to dysfunction of the epithelial layer and spontaneous colitis (Wu and Wu, 2012). The colitis is characterized by a heightened production of TNF-α from DC in the colon, which can be effectively regulated through the $CD4^+$ and $CD25^+$ regulatory T cells (Pedros et al., 2016).

Stimulation of both the innate and adaptive immune systems by microbes has proven to be essential for maintaining gut health (Geremia et al., 2014). Furthermore, research has emphasized the critical role of the microbiota in promoting the development of specific T-cell subsets, such as regulatory T cells and Th17 cells (Brown et al., 2019). Studies have also shown that about half of the genes influenced by the microbial colonization are involved in pathways related to immune responses (Schirmer et al., 2020). The adaptive immune response in piglets plays a crucial role in

gut health by specifically targeting and eliminating pathogens, thereby reducing infection and promoting intestinal homeostasis.

2.4 Antibiotics

Due to the high animal density conditions prevalent in intensive animal farming, the risk of infection transmission is heightened, making the animals more susceptible to diseases (Maes et al., 2020). Many of these diseases not only compromise animal health and productivity but also pose risks for transmission to humans (Rahman et al., 2020). To mitigate these risks, producers often resort to administering antibiotics to animals. However, the indiscriminate or excessive use of antibiotics can promote the development of antibiotic-resistant bacteria and the emergence of resilient pathogens over time (Caneschi et al., 2023). The overuse of antibiotics disrupts the gut microbiota balance, potentially causing long-term health issues for the animals and humans.

2.4.1 Antibiotics application as growth promoters

Studies have indicated that incorporating low doses of antibiotics into animal diets can markedly enhance the production of animals (Low et al., 2021). The positive impacts of using sub-therapeutic antibiotics for growth promotion purpose encompass a lowered incidence of subclinical illnesses, a decrease in sickness and death rates, an enhancement in growth rates, and a reduction in feed cost (Barton, 2014). Moreover, these antibiotics facilitate the optimal conversion of feed into animal products (Li, 2017). Some antibiotics, such as penicillins, tetracyclines, and aminoglycosides, are not only employed as growth promoters in animals but also widely used to combat bacterial infections in humans (Dutta et al., 2019).

2.4.2 Modes of action

Various mechanisms contribute to regulating animal health and growth via antibiotic use, with several aspects yet to be fully comprehended (Palma et al., 2020). Despite numerous

physiological, metabolic, nutritional, and immunological responses to the use of antibiotics, consistent outcomes have been reported with enhanced feed efficiency and growth rates (Gaskins et al., 2002).

Four major mechanisms have been suggested to explain how antibiotics enhance animal growth: (a) enhanced absorption and utilization of nutrients via thinner polarized epithelial layers (b) improved nutrients availability due to the less competition of pathogens with the host for nutrients; (c) the decrease in the production of microbial metabolites that hinder growth in the animals; and (d) prevention of subclinical infections (Looft et al., 2012).

The ability of antibiotics to kill or inhibit bacteria can decrease the number and diversity of microorganisms in the gut, which lessens the competition for nutritional resources and the metabolites that play a role in the metabolism of nutrients (Yang et al., 2020). Antibiotics could also reduce inflammation and the production of cytokines that promote inflammation (Knoop et al., 2016). Antibiotic growth promoters play a dual role by not only promoting growth but also serving an anti-inflammatory function, thereby conserving energy that would otherwise be expended, and redirecting it towards enhancing animal production processes (Menconi et al., 2014). The supplementation of antibiotics in diets induces noticeable shifts in the microbiota composition by reducing the amount of pathogenic bacteria, ultimately fostering homeostasis (Pi et al., 2019). Understanding these modes of actions is essential for optimizing growth performance, ensuring animal health, and developing effective alternatives to antibiotics.

2.4.3 Antimicrobial resistance

Even in the absence of antibiotics, the persistence of antimicrobial resistance in bacteria can be perpetuated through mechanisms such as cross-resistance and co-resistance (Vats et al., 2022). Cross-resistance indicates resistance against a broad category of antibiotics through

generating efflux pumps or enzymes that alter antibiotics structures (Amsalu et al., 2020). The regulatory protein CzcR regulates the activity of the CzcCBA efflux pump in *Pseudomonas aeruginosa*, and plays a role in regulating resistance to carbapenems by inhibiting the production of OprD porin (Ducret et al., 2023).

Co-resistance occurs when the same bacterial strain exhibits resistance to multiple classes of antibiotics (Zavaryani et al., 2020). This phenomenon involves the harboring of numerous resistance genes within the same genetic element, such as transposon or plasmid, within the bacteria (Partridge et al., 2018). The co-resistance between amoxicillin and ciprofloxacin in *E. coli* suggests that the administration of either antibiotic will simultaneously elevate resistance to both antibiotics (Holmer et al., 2019). Consequently, antimicrobial resistance poses a significant threat to global health by rendering standard treatments ineffective, leading to persistent infections and increased mortality.

2.5 Antibiotics alternatives - Probiotics

There are various concerns associated with using antibiotics as growth promoters in animals, include promoting the emergence and dispersion of antimicrobial resistant strains, and the spread of antimicrobial resistance genes to susceptible bacteria in the environment, which threatens animal's and human's health (Al-Dobaib and Mousa, 2009). In response to the escalating global threat of antimicrobial resistance, several countries have implemented policies to eliminate the use of antibiotic growth promoters in food animal production, which raises the urgency of finding effective antibiotic alternatives (Patel et al., 2020).

Among the various strategies devised to substitute antibiotics for growth promoters, the most recognized and thoroughly investigated antibiotic alternatives include probiotics, prebiotics, essential oils, and acidifiers (Rahman et al., 2022). This thesis focuses on probiotics. These

beneficial microorganisms enhance gut health of animals, making them a promising solution for sustainable swine production.

2.5.1 Introduction of probiotics

The definition of probiotics experienced some redefinitions. The first definition of probiotics came as ‘organisms and substances which contribute to intestinal microbial balance’ (Parker, 1974). Fifteen years later, a scientist redefined this term by emphasising only live organisms as ‘a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance’ (R, 1989). The latest definition of probiotics term is ‘live microorganisms which, when administered in adequate amounts, confer a health benefit on the host’ , which was defined by the Food and Agriculture Organization (**FAO**) of the United Nations and World Health Organization (**WHO**) (WHO, 2001). Direct-fed microbials (**DFM**) is another term used in the feed industry, often used interchangeably with probiotics, both in animal feed and human consumption. The term DFM was defined by the Association of American Feed Control Officials (**AAFCO**) as ‘feed additive which is a source of living (viable) naturally occurring microorganisms’ (AAFCO, 2017).

Probiotics can be categorized into three key groups: Lactic acid producing bacteria, *Bacillus*, and yeast (Cissé et al., 2019). *Saccharomyces cerevisiae* is a species of yeast, tolerant to low pH and high bile salts, that has been commonly used as a supplement in the feeding of swine by exerting beneficial effects on modulating the immune system and competitive exclusion of pathogens (Elghandour et al., 2020). Lactic acid producing bacteria are known to positively impact health, such as *Lactobacillus* spp, *Bifidobacterium* spp, *Melissococcus* spp, *Enterococcus* spp, *Pediococcus* spp, *Streptococcus* spp, and *Oenococcus* spp. These bacteria are gram-positive, non-sporulating, non-respiring cocci or rods, producing lactic acid as their major end products by

fermentation of carbohydrates via glycolysis pathway under anaerobic conditions. The benefits of lactic acid producing bacteria are associated with enhanced gut barrier integrity, improved immune system regulation, and increased production of beneficial metabolites (Singh et al., 2016). The probiotic strain *Lacticaseibacillus rhamnosus* GG (**LGG**), formerly known as *Lactobacillus rhamnosus*, is extensively researched for its potential advantages in treating GIT disorders (Mahalak et al., 2022). *Lactobacillus reuteri* is one of the dominant species in GIT of swine and has been widely used in swine diets (Yang et al., 2022).

Of the various available probiotics in the global feed additives market, many strains of *Bacillus* spp. are being commonly utilized as probiotics for swine. The genus *Bacillus* consist of a group of gram positive, catalase-positive, facultative anerobic, spore-forming, and rod-shaped bacteria, which is different from *Sporolactobacilli* and *Clostridia* (Lorenzo et al., 2018). There are more than 2,500 species of *Bacillus* spp. being recognized so far. *B. subtilis*, *B. licheniformis*, *B. coagulants*, *B. cereus*, *B. velezensis* and *B. amyloliquefaeciens* are the most common *Bacillus* species, which are normally isolated from soil and the GIT of animals (Luise et al., 2022). *B. licheniformis* has been shown to have strong abilities to produce a wide range of antimicrobial agents, such as bacteriocins, to inhibit pathogen growth, and excrete digestive enzymes to improve nutrient utilization (Kuebutornye et al., 2020).

2.5.2 Isolation and characterization of probiotics

Probiotic strains can be derived from a wide range of sources including the environment, plants, animals, and humans. However, it is advisable to isolate strains from the gut microbiota of animals living under relevant environments when selecting probiotics for animal use, which makes colonization easier and guarantees a more precise application (Al-Shawi et al., 2020).

Both phenotypic and genotypic methods have been used to identify each potential probiotic strain. Methods for identifying genotypes have become increasingly important for distinguishing species and differentiating between strains of probiotics. Several molecular techniques, including ribotyping, pulse field gel electrophoresis, rRNA gene sequencing, and protein profiling, are available for this purpose (Arora and Baldi, 2017). Among the different methods for genotypic identification, 16S rRNA gene sequencing is often preferred for identifying microbes, because of its high precision and the capacity to establish taxonomic connections between microbial strains (Church et al., 2020). For a probiotic to get approval for regulatory purpose, it is better to use whole-genome sequencing (**WGS**) for such precise bacterial identification, which enables the detailed characterization of both existing and newly identified strains, leveraging Genbank databases for comparison, and the prediction of phenotypes (Shridhar et al., 2022).

For a bacterial strain to be classified as a probiotic, it must exhibit several essential traits, such as retaining its viability and activity during production, storage of the product, and transit through the GIT. Assessing the tolerance of new probiotics strains to highly acidic and bile salts in GIT environments is essential for confirming their effectiveness and broadening the diversity of microbial species and products that can be used as probiotics (Wendel, 2022). Therefore, isolation and characterization of probiotics are crucial for identifying beneficial strains without antibiotic resistant genes that can be safe to use to enhance gut health and prevent diseases by balancing the microbial ecosystem.

2.5.3 The metabolites of probiotics

Probiotics can produce numerous metabolites, such as bacteriocins, SCFA, extracellular proteins, extracellular vesicles and indole, which safeguard the gut epithelial barrier by directly eliminating the pathogens, binding with specific receptors, stimulating mucus production by goblet

cells, boosting the expression of tight junction proteins, and increasing the secretion of antimicrobial peptides (Senchukova, 2023).

Probiotics can suppress inflammation through various mechanisms that influence the gut microbiota. One of the mechanisms is that probiotics can influence epithelial cells to produce cytokines, thereby exerting anti-inflammatory effects. A study conducted by Buccigrossi et al. (2022) demonstrated that LGG mitigates diarrhea caused by rotavirus by blocking both cytotoxic and enterotoxic pathways. Specifically, LGG hindered the process of apoptosis and the activation of p65, as well as its movement from the cytoplasm to the nucleus, thus impeding the harmful effects of rotavirus. Probiotics can also regulate the intestinal crypts to produce more defensin, which in turn helps control the growth of the beneficial microorganisms within these crypts and plays a role in reducing the occurrence of inflammatory bowel disease (Chen et al., 2021). Bacteriocins, serving as antibacterial peptides, have the capability to elevate the status of their producing bacteria within their niche, thus ensuring a competitive edge for probiotics (Pircalabioru et al., 2021).

The production of SCFA represent the primary metabolites generated through the fermentation processes of probiotics, arising specifically from the breakdown of non-digestible carbohydrates (Fusco et al., 2023). It has been found that homo-fermentative bacteria generate SCFA from the fermentation of carbohydrates, converting these into pyruvate through the glycolytic pathway. On the other hand, heterofermentative bacteria, including bifidobacteria, produce SCFA via a different process known as the phosphoketolase pathway (Koduru et al., 2022). Enzymes produced by the probiotics perform a range of biochemical, physiological, and regulatory roles. The genus *Bacillus* are likely the most significant bacterial producers of proteases (Sharma et al., 2019). The proteases secreted from *B. pumilus* have demonstrated effectiveness in breaking

down polymeric extracellular substances, leading to a significant reduction of biofilm produced by *Serratia marcescens* (Tkachuk and Zelena, 2021).

Exopolysaccharides (**EPS**) are biopolymers produced by probiotics during growth. The EPS extracted from *L. fermentum* S1, when applied in doses ranging from 1–4 $mg \cdot mL^{-1}$, demonstrated strong antioxidant capabilities. Additionally, these EPS were effective in inhibiting the development of biofilms by *E. coli* and *S. aureus* (Alotaibi, 2021).

2.5.4 Modes of action of probiotics against pathogens

There are at least three proposed modes of actions by which probiotics could combat pathogenic microorganisms, including production of inhibitory compounds, modification of immune system, and competitive exclusion (Chouraddi et al., 2023).

The critical step of pathogens to exert their harmful effects on animals involves adhesion to the intestinal wall. This process begins with nonspecific physical contact, followed by adhesion through specific cell wall components (Haddaji et al., 2015). The role of probiotics is to inhibit the adhesion of pathogens to the intestinal wall, which can compete with harmful bacteria for the receptors (Monteagudo-Mera et al., 2019). Probiotics can synthesize adhesive molecules (such as pyruvate dehydrogenase, glyceraldehyde-3-phosphate, and enolases) which allow them to attach to the host's extracellular matrix (Muscariello et al., 2020). For example, it has been reported that *Bacillus*-based probiotics effectively adhere to the intestinal mucosa of shrimp, showcasing the capability to outcompete and displace *Vibrio parahaemolyticus* (Van Doan, 2021).

The GIT of animals is occupied by trillions of dense and diverse microbes. Competitive exclusion (**CE**) is a gut lumen phenomenon where bacterial species in the same environment compete for limited nutrients and space resources. There are two competitive strategies of CE: exploitation and interference competition. Exploitation competition involves the rapid

consumption of limited and essential survival resources without direct contact, while interference competition occurs when probiotics directly harm pathogens through the production of antimicrobial agents (Knipe et al., 2021).

Exploitation competition for nutrient involves increasing nutrient capture speed through the extracellular secretion of molecules, such as protease, which break down large nutrients into smaller and more accessible molecules (Fritts et al., 2021). On the other hand, the exploitation competition for space involves the rapid colonisation of unoccupied areas in the GIT, or competing for already occupied space through the production of adhesins (Umu et al., 2017). Interference competition involves the production of antimicrobials, including broad-spectrum antibiotics like quinolones, and strain-specific bacteriocins (Hanchi et al., 2018). Nevertheless, some probiotics species have evolved to produce specific antimicrobials to combat pathogens, while others employ a broader range of competitive strategies. Interference competition relies on specific contact dependent systems, such as Type V and VI secretion systems, which recognise pathogens and deliver them into target cells to mediate their destruction (Storey et al., 2020). Consequently, the ability of probiotics to competitively exclude harmful microbes offers several benefits for host health. These advantages include decreasing the attachment and colonization of pathogens, preventing their further spread, and reducing their presence within the host. Moreover, CE enables probiotics to colonize the GIT, which is essential for their sustained positive effects on the host.

The integrity and full functionality of the intestinal epithelial barrier are crucial, as it serves both physical and biochemical functions, preventing unwanted compounds from entering the system (Gieryńska et al., 2022). Probiotics operate through various mechanisms to enhance the function of the intestinal barrier and maintain the homoeostasis. Li et al. (2022) showed that *B. licheniformis* PF9 conferred protective effects on the intestinal barrier by upregulating the

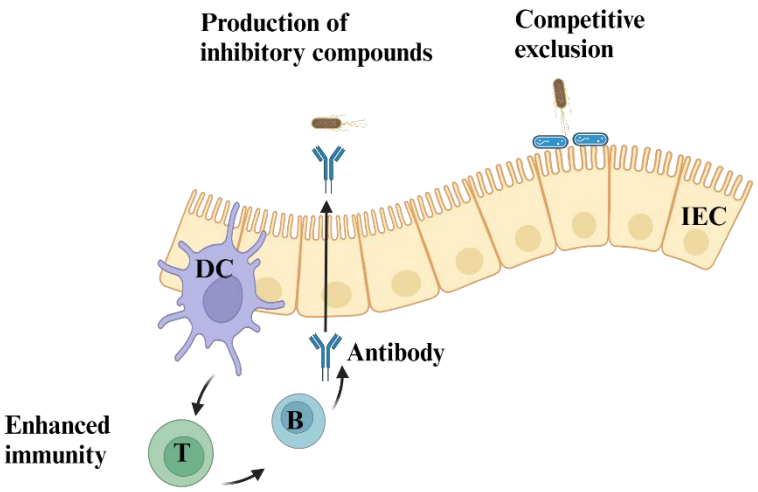
expression of tight junction proteins in IPEC-J2 cells infected with ETEC F4. Similarly, Chen et al. (2021) found that the pretreatment of IPEC-J2 cells with *L. zeae* LB1 alleviated the ETEC F4-induced damage in the cell monolayer.

Another beneficial action of probiotics is their ability to modulate the host's immune system, thereby enhancing the integrity of the gut mucosal barrier and functioning as immune modulators (Liu et al., 2022). Probiotics can recognise pattern recognition receptors (**PRRs**) expressed on immune cells, such as TLR, NLR, and C-type lectin receptors. This recognition is followed by the regulation of several signalling pathways, including the production of NF-κB and mitogen-activated protein kinases, thereby facilitating communication with the host (Choi et al., 2023). The NF-κB pathway regulates the expression of various cytokines genes related to inflammation, which closely participate in the immune responses (Bhatt and Ghosh, 2014). Nevertheless, probiotics might also interact with dendritic cells (**DC**), which are crucial in the innate immune response and serve as a link between the innate and adaptive immune responses. Via their PRRs, both intestinal epithelial cells and DC are capable of communicating with and responding to the microorganisms in the gut (Ghavami et al., 2020). Probiotics can stimulate the activation of macrophages to phagocytose various pathogens and induce the production of pro-inflammatory cytokine, such as IL-1, IL-6, TNF-a, and IFN-c (Elkhawaga et al., 2023). In chickens, treatment with *Lactobacillus* isolates has shown clear effectiveness in mitigating the alterations in pro-inflammatory cytokines gene expression induced by *Salmonella*. The beneficial impact extends to genes responsible for encoding pro-inflammatory cytokines, as well as T helper 1 and 2 cytokines (Hu et al., 2015). Moreover, *L. rhamnosus* LB1 had positive impact on enhancing the gut health and functionality of piglets, which was linked to its ability to modulate proteins and genes associated with inflammatory function (Wu et al., 2021). In fact, some spore-forming

probiotics, such as *Bacillus* strains, have been shown to traverse microfold cells and migrate into Peyer's patches. There, they stimulate the DC and macrophages, ultimately leading to the activation of B cells for the production and secretion of immunoglobulin A (Szydłowska and Sionek, 2023). Therefore, the critical role of probiotics in immune system regulation relies not only in activating immune signalling pathways, but also in modulating the activity of immune cells (Mazziotta et al., 2023).

Probiotics species, such as *Lactobacilli* and *Bifidobacteria*, can produce polypeptides or low molecular weight proteins known as bacteriocins, which form pores in the membranes of the target cells. This mechanism aids in reducing the number of pathogens present in the GIT (Anjana and Tiwari, 2022). On the other hand, these species can also produce some other kinds of thermostable bacteriocins with specific inhibitor activity, exerting antimicrobial functions to combat various types of pathogens (Mokoena et al., 2021). For instance, *L. plantarum* demonstrates antimicrobial properties through the production of various bacteriocins aimed at impeding pathogen growth (Dinev et al., 2018). In addition to bacteriocins, probiotics can generate other inhibitory compounds, such as H_2O_2 and organic acids, like lactate, butyrate, acetate, and propionate. These compounds have been shown to exhibit inhibitory effects on both Gram-positive and Gram-negative bacteria (Rafique et al., 2023). Overall, understanding the modes of action of probiotics is crucial for effectively leveraging their benefits in promoting gut health and preventing diseases.

Figure 2.3 Schematic diagram illustrating potential modes of actions whereby probiotic bacteria have beneficial effects on the host (Adapted from Chouraddi et al., 2023).



These mechanisms include: (1) competitive exclusion for binding sites, (2) production of inhibitory compounds, and (3) stimulation of immune response. IEC: intestinal epithelial cells, DC: dendritic cells, T: T-cells, B: B-cells.

2.5.5 Colonization by probiotics

Screening potential probiotic strains often involves assessing their adhesive qualities, as it is believed that strains capable of adherence are more likely to colonize the intestine effectively (Byakika et al., 2019). While adhesion alone does not guarantee health benefits, it can help probiotic bacteria compete with pathogens by binding to the intestinal mucosa, thus blocking sites on host cells. Furthermore, probiotics' adhesive properties may trigger signaling in the host, potentially leading to temporary colonization, extending their presence in the gut, thereby maximizing the time for probiotics to deliver beneficial effects (Van et al., 2020).

Adhesion of probiotics to gut epithelial cells is probably facilitated by a combination of electrostatic and hydrophobic interactions, passive or steric forces, or the compatible linking of receptors and adhesives (both protein-based and non-protein surface elements), which enables multiple connections between the bacterium and the host cells (Javanshir et al., 2021). The probiotics use various components such as pili, proteins, exopolysaccharides, and their naturally hydrophobic cell surfaces to enhance their ability to adhere and to outperform pathogens in colonization on the intestinal cells (Bhowmik et al., 2021). Studies have demonstrated that the SpaCBA pilus found in *L. rhamnosus* GG is crucial for its effective adhesion to the Caco-2 intestinal epithelial cell line and for biofilm formation.

Although there is no definitive evidence to suggest that *Bacillus* species can establish permanent colonization in the gut, certain species of *Bacillus* have the ability to remain viable in the gut for extended durations following administration. As an illustration, the number of *B. subtilis* PY79 from chickens increased over the course of a 7-week experimental period (Lu et al., 2024).

Numerous elements can impact the adhesion capability of probiotics. Some factors are associated with the *in vitro* culture methods used, including bacterial concentration, composition of the buffer, duration of incubation, and the type of growth medium. Additionally, adhesion is influenced by *in vivo* conditions such as the composition of the normal intestinal microbiota, digestion processes, and the characteristics of the feed matrix (Gorreja and Walker, 2022). Therefore, to determine the transient colonization and adhesion of probiotics, both *in vitro* and *in vivo* methods are needed.

2.5.6 Safety of probiotics

Safety is a significant consideration in the use of probiotics. Presently, a considerable portion of probiotics research is focused on ensuring their safe application. Before a probiotic can be employed in animal production, it must have a thoroughly established safety record, a process rigorously overseen by regulatory bodies such as the Food and Drug Administration and the European Food Safety Authority (Roe et al., 2022).

The growing concern over the transfer of resistance genes to the host microbiota could lead to a decline in commercial interest. The development of resistance in probiotics has been documented and requires further investigation (Meroni et al., 2021).

From a toxicological standpoint, probiotics present unique challenges unlike those associated with chemicals. The safety assessment of a probiotic strain starts with its taxonomic classification and strain identification by WGS. The genetic information from the genome of a probiotic strain is vital for genotypic analysis, which, when combined with phenotype, offers a comprehensive understanding of the significance of various genes associated with adhesion and colonization (Peng et al., 2023).

After verifying the probiotic, the next step of evaluation involves determining if the probiotic strain has a significant track record of safe usage. This involves examination of phylogenomic databases alongside WGS to identify the presence of various virulence factors, toxins and antibiotic resistance genes. Additionally phenotypic assessments for antibiotic resistance are conducted using conventional antibiotic screening methods (Jiang et al., 2023).

The duration of dosing in the safety studies is crucially tied to identifying relevant toxicity endpoints for evaluating probiotics safety. A critical safety consideration, especially in the *in vivo* administration of probiotics, is translocation, which refers to the movement of live microbes from the gut to different parts of the body (Vanuytsel et al., 2021).

In addition to assessing potential toxic effects of the strain, it is important to evaluate various other aspects associated with the finished probiotic product. This evaluation should cover the raw materials utilized in manufacturing and processing probiotics (Anadón et al., 2021). Furthermore, specific safety details may need to be selectively considered based on the specific probiotic under evaluation.

2.5.7 Application of probiotics in piglets

The post weaning phase for piglets is a critical period during which their gut and immune systems are not fully mature, rendering them vulnerable to pathogenic microorganisms. Moreover, this phase significantly influences the subsequent performance of the pigs until they are ready for market. Consequently, there is a growing interest in the swine industry regarding the application of probiotics during this post weaning period (Nowland et al., 2019).

The method of administration of probiotics to piglets is also a critical factor to take into consideration, as the viability and stability of probiotic cells can significantly impact their efficacy. For instance, some probiotics species are extremely sensitive to heat processing during feed

manufacturing, while other species may not be highly tolerant to the low pH in the stomach of piglets. For this case, the development of coating technology such as microencapsulation or acclimatization procedures could be useful strategies to broaden the application of probiotics as feed additives (Guimarães et al., 2018).

Although there are many benefits of dietary supplementation of probiotics in swine diets, the challenges to the application of probiotics in feed also exist. One of the greatest challenges associated with probiotics used in feed which needs to be overcome is the probiotic effectiveness and reliability when compared to AGP. Table 2.1 presents recent study findings of the impact of probiotics on the performance of piglets. The table also provides key information on the probiotic strains, dosage, and duration period of supplementation, which is critical for determining the effectiveness of probiotics.

Table 2.1 The effects of probiotics on performance of piglets

Pigs age	Strain	Concentration	Dosage	Challenge	Duration	Outcomes	Reference
Piglets (25 d)	*B. halotolerans*	1×10^6 CFU/g	NA	ETEC F4	18 days	↑ TJ proteins ↓endotoxin, D-lactic acid	(Li et al., 2024)
Piglets (21 d)	*B. amyloliquefaciens*	1×10^5 CFU/g	NA	LPS	21 days	↑AhR/STAT3 pathway	(Wang et al., 2024)
Piglets (21 d)	*L. rhamnosus*	1.4×10^9 CFU/g	3.5×10^8 CFU/pig/d	NA	11 days	↑ BWG, *Lactobacillus* relative abundance	(Li et al., 2023)
Piglets (21 d)	*B. licheniformis*	1×10^{11} CFU/g	1000 g/t	NA	14 days	↑ BW, ADG, SOD, VH, VH:CD, occludin, ZO-1, IgA ↓ MDA	(Sun et al., 2023)
Piglets (28 d)	*B. licheniformis*	1×10^9 CFU	500 and 1000 mg/kg	NA	28 days	↑ ADG, IgA, IgM, IL-10, T-AOC and SOD ↓ diarrhea incidence, MDA, IL-1β and IL-6	(Yu et al., 2022)

Piglets (28 d)	*L. plantarum*	1.3×10^7 CFU/g	0.1% and 0.2%	NA	42 days	↑ ADG, *Lactobacillus* ↓ *E. coli*	(Wang and Kim, 2021)
Piglets (21 d)	*L. acidophilus*, *L. casei*, *B. thermophilum* and *E. faecium*	2.5×10^7 CFU/g	0.15% and 0.10%	ETEC F18	25 days	↑ ADG, ADFI, VH ↓TNFα	(Sun et al., 2021)
Piglets (28 d)	*B. subtilis* and *B. licheniformis*	NG	0.01%	NA	2 weeks	↑ ADG	(Mun et al., 2021)
Piglets (28 d)	*B. subtilis*, *B. licheniformis* and *S. cerevisiae*	1.5×10^9 CFU/g	0.05% and 0.1%	NA	6 weeks	↑ BW, ADG, *Lactobacillus* ↓ Ammonium gas	(Mun et al., 2021)
Piglets (28 d)	*L. plantarum*	1.2×10^9 CFU/kg	0.06%	NA	6 weeks	↑ BW, ADG, G: F	(Yang et al., 2020)
Piglets (21 d)	*B. subtilis*, *E. faecium*	2×10^8 CFU/g	300 mg/kg	NA	25 days	↑ ADG, ADFI, G: F	(Zhang et al., 2020)

TJ: tight junction; BWG: body weight gain; BW: body weight; ADG: average daily gain; SOD: superoxide dismutase; VH: villus height; CD: crypt depth; ZO-1: zonula occludens-1; ADFI: average daily feed intake; MDA: malondialdehyde; IL: interleukin; G: F: gain to feed ratio; NA: not applicable.

As depicted in the table, probiotics exhibit diverse potential effects, including enhancing growth performance, reducing the incidence of diarrhea, restoring gut microbiota balance by increasing beneficial bacteria and decreasing harmful bacteria counts, enhancing gut barrier function, and stimulating immunity. Li et al. (2024) reported that *B. halotolerans* SW207 increased the production of tight junction proteins through both gene expression and protein levels. Additionally, it decreased levels of oxidative stress indicators and inhibited the activation of the TLR4/MyD88/NF-κB signaling pathway, thereby improving the mechanical barrier function in piglets. The supplementation with *B. amyloliquefaciens* SC06 was found to not only enhance the regeneration of intestinal stem cells but also led to the increased expression of the aryl hydrocarbon receptor in piglets challenged with LPS (Wang et al., 2024). In addition, Li et al. (2023) showed that dietary treatments with microencapsulated *L. rhamnosus* LB1, particularly in combination with bovine colostrum, could improve body weight gain of weaned piglets, and modulate gut microbiota by increasing *Lactobacillus* relative abundance. Moreover, Wu et al. (2021) reported that dietary supplementation with *L. rhamnosus* LB1 reduced oxidative stress, alleviated diarrhea, and enhanced immune function and gut integrity of piglets, which mediated through alterations in gene and protein expression of related functional molecules.

Recently, Li et al. (2022) reported that *Bacillus licheniformi*s PF9 can reduce inflammation-related cytokines by blocking NF-κB signaling pathways. Additionally, it significantly enhances the integrity of the intestinal porcine epithelial cell line (IPEC-J2) when infected with ETEC F4. This cell model study highlights the promise of this bacterium as a probiotic, suggesting its potential to improve gut health and performance in weaned piglets following an ETEC F4 challenge, warranting further investigation. For another new isolate, HG76, it is also worthy to investigate its benefits on weaned piglets through *in vivo* and *in vitro* trials.

CHAPTER THREE : HYPOTHESES AND OBJECTIVES

3.1 Research gap

The application of probiotics in swine production has been practiced for a long time, demonstrating beneficial effects on swine health and performance. The interest in probiotics has increased significantly as many countries around the world have decided to restrict or ban the use of antibiotics as growth promoters (**AGP**). Key areas for further research include understanding the molecular mechanisms underlying the effects of probiotics, utilizing advanced genomic techniques to analyse gut microbiota profiles and identify effective strains, and developing appropriate *in vivo* and *in vitro* models to enhance the efficiency of probiotics selection and evaluation (Gong et al., 2020). Although various types of probiotics have been studied, more novel and specialized potential probiotics, such as *B. licheniformis* PF9 and HG76 are still expected to be investigated.

3.2 Hypothesis and objectives

3.2.1 Hypothesis

The hypothesis was that evaluation of probiotic candidates through both effective *in vitro* and lab animal models would lead to the discovery of novel and specialized probiotic candidates that can improve the gut health and growth performance of piglets.

3.2.2 Objectives

The overall objective of this project was to select two novel probiotics candidates for dietary supplementation in piglets, with the aim of establishing a healthy gut microbiota and reducing susceptibility to ETEC induced PWD.

The specific objectives were as follows:

1. To select, identify, and characterize two isolates PF9 and HG76.

2. To investigate effects of *B. licheniformis* PF9 on gut health and performance of weaned piglets under ETEC F4 challenge conditions.

3. To investigate whether *B. licheniformis* HG76 can enhance defense mechanisms and mitigate inflammation in IPEC-J2 cells during ETEC F4 infection.

4. To explore if *B. licheniformis* HG76 could improve gut health and growth performance of weaned piglets under ETEC F4 challenge conditions.

5. To evaluate the effects of combining *B. licheniformis* PF9 and HG76 on the growth performance, diarrhea index score, enzyme activity and tight junction proteins of nursery piglets.

CHAPTER FOUR : MANUSCRIPT I

Functional evaluation of *Bacillus licheniformis* PF9 for its potential in controlling enterotoxigenic *Escherichia coli* in weaned piglets[1]

[1]**Xu, H.**, Gong, J., Lu, P., Azevedo, P., Li, L., Yu, H., Yang, C. 2024. Functional evaluation of *Bacillus licheniformis* PF9 for its potential in controlling enterotoxigenic *Escherichia coli* in weaned piglets. Transl. Anim. Sci. txae050. doi:10.1093/tas/txae050.

4.1 Abstract

During the bacterial selection, isolate PF9 demonstrated tolerance to low pH and high bile salts and an ability to extend the lifespan of *C. elegans* infected with enterotoxigenic *Escherichia coli* (**ETEC**) ($P < 0.05$). Thirty-two weaned piglets susceptible to ETEC F4 were randomly allocated to 4 treatments as follows: 1) non-challenged negative control group (**NNC**; basal diet and piglets gavaged with phosphate-buffered saline (**PBS**)); 2) negative control group (**NC**; basal diet and piglets challenged with ETEC F4, 3×10^7 CFU per pig); 3) positive control (**PC**; basal diet + 80 mg·kg^{-1} of avilamycin and piglets challenged with ETEC F4); and 4) probiotic candidate (PF9; control basal diet + 2.5×10^9 CFU·kg^{-1} diet of *B. licheniformis* PF9 and piglets challenged with ETEC F4). The infection of ETEC F4 decreased average daily gain (**ADG**) and gain:feed in the NC group when compared to the NNC group ($P < 0.05$). The inoculation of ETEC F4 induced severe diarrhea at 3 h post-inoculum (**hpi**), 36 hpi, 40 hpi in the NC group when compared to the NNC group ($P < 0.05$). The supplementation of *B. licheniformis* PF9 significantly relieved diarrhea severity at 3 hpi when compared to the NC group ($P < 0.05$). The inoculation of ETEC F4 reduced duodenal, jejunal and ileal villus height (**VH**) in the NC group when compared to the NNC group. A significant ($P < 0.05$) decrease was detected in the duodenal VH in the PC and NNC groups. Moreover, the NNC group had a reduced relative mRNA level of Na^+-glucose cotransporter 1 (**SGLT1**) when compared to the NC group ($P < 0.05$). Compared to the NC and NNC groups, the supplementation of *B. licheniformis* PF9 increased the relative mRNA levels of aminopeptidase N (**APN**), occludin (**OCLN**), zonula occludens-1 (**ZO-1**), and SGLT1 ($P < 0.05$). The supplementation of *B. licheniformis* PF9 also significantly increased the relative mRNA level of excitatory amino acid transporter 1 (**EAAC1**) when compared to the NC group ($P < 0.05$). Piglets supplemented with *B. licheniformis* PF9 showed lower relative abundance of Bacteroidetes in the

colon than piglets from the NNC group ($P < 0.05$). The NNC group had a higher relative abundance of Firmicutes in the ileum than all the challenged piglets ($P < 0.05$); however, a lower relative abundance of Proteobacteria in the ileum and colon was observed in the NC group ($P < 0.05$). This study provides evidence that *B. licheniformis* PF9 has the potential to improve the gut health of piglets under disease challenge conditions.

Key words: *Bacillus licheniformis* PF9, diarrhea, enterotoxigenic *E. coli*, gut health, weaned piglets

4.2 Introduction

Post-weaning diarrhea (**PWD**) is a common disease that causes a significant economic loss in the global swine industry (Rhouma et al., 2017). Enterotoxigenic *Escherichia coli* (**ETEC**) is the main infectious agent of PWD (Luppi, 2017). Antibiotic growth promoters (**AGP**) have been commonly added to feed to improve animal growth performance and prevent infectious diseases such as PWD (Thoms, 2012). However, the use of AGP in livestock production has been suggested to contribute to the development of antibiotics resistance, which threatens human health (Martin et al., 2015). The restriction of AGP use in feed was enforced in European Union countries since 2006; in 2017, according to rules of the Food and Drug Administration of United States, animal producers required a veterinary feed directive for feed applications of antibiotics (Ekakoro et al., 2019); in Canada, prescriptions issued by veterinarians are needed for the use of all medically important antimicrobials (**MIA**) as defined by the World Health Organization, and growth promotion claims on the MIA product labels have been removed according to Health Canada regulations and policies implemented in 2018 (Lardé et al., 2021).

To overcome the potentially negative effects of removing AGP, several AGP alternatives, including pro- and pre-biotics, zinc, copper, acidifiers, and plant extracts, have been tested (Liu et al., 2018). Among them, dietary supplementation of probiotics has been reported to have positive effects on improving gut health and immunity, nutrient digestibility, and suppressing pathogens, hence enhancing growth performance and preventing enteric diseases (Liao and Nyachoti, 2017). Various dietary probiotics have been studied in the past few decades. *Bacillus licheniformis* (***B. licheniformis***) has been reported to have the function of secreting digestive enzymes to enhance nutrient digestion and absorption and antimicrobial peptides to inhibit growth of *Brachyspira hyodysenteriae* and *Clostridium perfringens* (Rozs et al., 2001; Horng et al., 2019). Recently, our group reported that *B. licheniformis* PF9 was capable of reducing inflammation-related cytokines through blocking the NF-κB signaling pathways, while, significantly enhancing the intestinal porcine epithelial cell line integrity when infected with ETEC F4 (Li et al., 2022). It is hypothesized that dietary *B. licheniformis* PF9 will improve gut health and growth performance of piglets. Therefore, the objective of the current report is to describe how *B. licheniformis* PF9 was selected and investigate its potential in application to improve the gut health and performance of weaned piglets including growth performance, digestive enzymes, gut morphology and integrity, nutrient transport, oxidative stress, innate immune responses and gut microbiota after the ETEC F4 challenge.

4.3 Materials and methods

The pig trial and animal care protocols (F19-020, AC11516) were approved by the Animal Care Committee of the University of Manitoba. The piglets used in this trial were cared for under the guidelines of the Canadian Council on Animal Care (CCAC, 2009).

4.3.1 *B. licheniformis* PF9 and its culture preparation

B. licheniformis PF9 was recently shown through an *in vitro* study that it can improve barrier function and alleviate inflammatory responses against ETEC infection (Li et al., 2022). The bacterium was isolated from the feces of pigs raised on a free-range farm in Ontario, Canada, where antibiotics were not used in raising the pigs. It is heat resistant. The selection of a heat resistant bacterium was conducted through a screening of multiple feces samples by suspending 0.5 g of pig feces in 4.5 mL of phosphate-buffered saline (**PBS**, pH 7.0) and heating the mixtures at 80ºC for 10 min followed by a series of dilutions of each sample. The diluted samples were plated on the tryptic soy agar (**TSA**; Millipore Sigma, Oakville, ON, Canada) and incubated at 37ºC until colonies became visible. Finally, a single colony was selected for a pure bacterial culture.

To prepare a PF9 culture for the pig trial, a single colony of *B. licheniformis* PF9 from an agar plate was inoculated into 6 mL of tryptic soy broth (**TSB**; Millipore Sigma, Oakville, ON, Canada) medium in a 12 mL culture tube followed by the incubation at 37ºC for 16 h without shaking to get an initial culture. The initial culture was mixed with 250 mL of TSB in a 500 mL flask and incubated at 37ºC for 16 h without shaking. All the incubations were carried out aerobically. The culture (500 mL) with more than 99% of the cells in a vegetative form was evenly mixed with 50 kg of feed which included 2.5×10^9 CFU·kg^{-1} of *B. licheniformis* PF9 in the diet.

4.3.2 *In vitro* characterization of *B. licheniformis* PF9

To determine the species identity of isolate PF9, its 16S ribosomal RNA (**rRNA**) gene was sequenced and then blasted against the database sequences available on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). Further determination of isolate PF9 to be *B. licheniformis* or *B. paralicheniformis* was carried out using the method of particular molecular markers developed by Olajide et al., (2021). Both 16S rRNA gene sequencing

and the marker detection to distinguish *B. licheniformis* from *B. paralicheniformis* were conducted by the Agriculture and Food Laboratory (Laboratory Service Division, University of Guelph, Guelph, Ontario, Canada), an ISO/IEC 17025 accredited laboratory (https://afl.uoguelph.ca/accreditations).

To determine the antimicrobial activity of *B. licheniformis* PF9, an inhibitory assay was carried out by measuring the culture optical density at 600 nm (**OD_{600}**) of a target pathogen at 30 min intervals during an 18-h incubation at 37ºC with a Bioscreen C MBR (Oy Growth Curves Ab Ltd., Helsinki, Vuorimiehenkatu, Finland). The assay mixture (300 µL each) was constituted of 150 µL of TSB containing 1% inoculum of ETEC JG280 or *Salmonella* Typhimurium DT104 and 150 µL of filter-sterilized extracellular culture fluid of PF9. The control in the assay was the replacement of the extracellular culture fluid of PF9 with 150 µL of TSB without the bacterial inoculum, which was conducted in parallel.

To determine the tolerance of *B. licheniformis* PF9 to low pH and high bile salts, the isolate was tested based on the method previously reported (Yang et al., 2014) using the Bioscreen C MBR (Oy Growth Curves Ab Ltd., Helsinki, Vuorimiehenkatu, Finland) to monitor the bacterial growth. Tolerance to low pH was evaluated by incubating bacterial cells in Lysogeny broth (**LB**; Millipore Sigma, Oakville, ON, Canada) at 37ºC, which had been treated with simulated gastric fluid (Wang et al., 2009) for 2 h with the pH values of 2.0 and 5.6, respectively. Tolerance to bile salts was investigated by incubating the isolate in the LB containing various concentrations (0, 0.1, 0.5, 1.0, and 1.5%) of bile salts (Catalog No. LP005, Oxoid, Nepean, ON, Canada) at 37ºC. The OD value of a sample was determined at a wavelength of 600 nm. The selected range of bile salts was based on the physiological concentration in the small intestine (Weinman et al., 1998).

4.3.3 Culturing and lifespan assay of *C. elegans*

The SS104 strain of *Caenorhabditis elegans* (***C. elegans***) harboring a temperature-sensitive allele of *glp-4* (*bn2*) was obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN, USA). *C. elegans* was maintained on nematode growth medium (**NGM**) plates seeded with *E. coli* OP50 (1×10^8 CFU·mL^{-1}) that was used as food for the nematode according to the procedures described by Breger et al., (2007). The ETEC strain JG280 is a hemolytic *E. coli* of serotype O149: K88 (F4), a porcine isolate possessing toxin genes and resistant to some antibiotics (Noamani et al., 2003).

The lifespan assays of *C. elegans* were conducted based on the previously published method (Zhou et al., 2014). Briefly, gravid adult worms were treated with sterile water (containing 0.5% NaClO solution and 0.5 M NaOH) to synchronize worms to the same age and stage. The eggs were released and isolated by centrifugation (1 min at 1,300 × g) and resuspended in M9 buffer, and then hatched for 16 h at 20°C. The L1 larvae of *C. elegans* were transferred to NGM agar with *E. coli* OP50 (1×10^8 CFU·mL^{-1}) as food for the worms at 25°C for 48–60 h until the worms reached the L4 stage. The worms collected from the NGM plate with M9 buffer were washed three times in the S medium, 18-26 worms were then transferred to each well containing 2 mL of S medium on a 24-well plate and incubated at 25°C.

The treatments of assay included 1) Control (treated with *E. coli* OP50 only), 2) ETEC infection (incubated with *E. coli* OP50 for 18 h followed by incubation of ETEC JG280 for 12 days), and 3) *B. licheniformis* PF9 pretreatment (incubated with *B. licheniformis* PF9 for 18 h followed by incubation of ETEC JG280 for 12 days). Each assay was designated as d 0 when the worms were fed *E. coli* OP50 or *B. licheniformis* PF9. The worms were collected after the 18 h

incubation period via centrifugation and suspension and washed three times in the S medium. The assays were designated as d 1 when the washed worms were mixed with ETEC JG280 (2×10^8 CFU mL^{-1}) in a 24-well plate. The lifespan assay lasted for 12 days. The number of live worms was recorded daily, and the survival rate of worms was calculated by the formula: survival rate (%) = (live worms/total worms used) × 100. A worm was considered dead when it failed to respond to touching.

4.3.4 ETEC F4 culture preparation

The ETEC F4 from -80°C stock was streaked on the media of TSA to aerobically grow for 16 h at 37°C. A single colony of ETEC F4 was used to inoculate 10 mL of TSB in a 50 mL sterile tube followed by aerobic incubation at 37°C for 16-18 h with shaking (150 rpm). The culture was further subcultured by inoculating 10 mL TSB with 1% inoculum and incubated for 2.5 h at 37°C with shaking (150 rpm) until its OD_{600} value reached 0.3. The ETEC F4 culture was diluted by PBS to obtain a cell suspension at approximately 1×10^7 CFU·mL^{-1}.

4.3.5 Selection of piglets

The piglets susceptible to ETEC F4 were selected for the ETEC challenge trial following the method described by Jensen et al. (2006). Briefly, the tails of piglets were collected on 3 days after farrowing when docked. DNA was extracted according to previous publication (Truett et al., 2000). The mucin 4 (**MUC4**) gene was detected by a PCR assay. The assay mixture (25 μL) was composed of DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 200 μmol·L^{-1} of dNTP, 2 mmol·L^{-1} $MgCl_2$, 400 μmol·L^{-1} of forward primer (5′-GTGCCTTGGGTGAGAGGTTA-3′) and reverse primer (5′-CACTCTGCCGTTCTCTTTCC-3′). Thermocycling was performed with initial denaturation at 95℃ for 5 min and subsequently by 35 cycles with each cycle having denaturing at 95℃ for 30 s, annealing at 65℃ for 30 s and extension at 72℃ for 1 min. The PCR

product size from the tail genomic DNA was 367 bp. The product (5 µL) was digested by XbaI (Thermo Fisher Scientific, Waltham, MA, USA) for 5 min at 37℃ according to the protocols provided by the supplier. After digestion, the PCR products stained by SYBR Green (Invitrogen, Carlsbad, CA, USA) were electrophoresed on an agarose gel (2%) in a Tris-borate-EDTA buffer. The susceptible allele (S) was digested by FastDigest XbaI into two fragments with 151 bp and 216 bp, respectively, whereas the resistant allele (R) was indigestible. Similar body weight piglets contained both the SS-genotype (homozygote) and RS-genotype (heterozygote) were chosen for the experiment, as Matsumoto et al. (2020) revealed that RS-genotyped pigs showed no significant differences in fecal score, incidence of diarrhea and diarrhea duration compared to SS-genotyped pigs.

4.3.6 Experimental design

Thirty-two weaned pigs susceptible to ETEC F4 (TN Tempo × TN70; half castrated male and half female piglets; average body weight of 8.15 ± 0.18 kg) were transferred to T.K. Cheung Centre for Animal Science Research from the Glenlea Swine Research Unit of the University of Manitoba at the age of 28 d (weaning at 21 d). All piglets were housed in individual pens in a temperature-controlled room with the room temperature kept at 29 ± 1ºC within the pre-challenge period (0-7 d), and after that decreased by 1.5ºC during the post-challenge period (8-10 d). All piglets for this trial were randomly allocated to 4 treatments (8 replicates per treatment, half castrated male and half female). The basal diet (**Table 4.1**) based on corn-soy was made to meet or exceed the recommendations for 7-11 kg piglets from the NRC (2012). The treatments were designed as follows: 1) non-challenged negative control group (**NNC**; basal diet and piglets gavaged with PBS); 2) negative control group (**NC**; basal diet and piglets challenged with ETEC F4, 3×10^7 CFU per pig); 3) positive control (**PC**; basal diet + 80 $mg \cdot kg^{-1}$ of avilamycin and

piglets challenged with ETEC F4, 3×10^7 CFU per pig); and 4) probiotic candidate (PF9; control basal diet + 2.5×10^9 CFU·kg^{-1} diet of *B. licheniformis* PF9 and piglets challenged with ETEC F4, 3×10^7 CFU per pig). The whole trial lasted for 10 days. Piglets had *ad libitum* access to feed and water throughout the whole trial. Individual piglet's body weight and pen feed disappearance were recorded to calculate average daily feed intake (**ADFI**), average daily gain (**ADG**) and gain:feed within the pre-challenge period and post-challenge period, respectively. For the ETEC F4 challenge, each piglet in the NC, PC and PF9 groups was administered with 3 mL of ETEC F4 (1×10^7 CFU·mL^{-1}) on d 7 (Koo et al., 2020). The rectal temperature was measured by digital thermometer before the inoculation, 3, 24, and 48 h post-inoculum (**hpi**). The diarrhea index score was recorded at 3, 8, 12, 16, 20, 24, 28, 32, 36, 40, and 48 hpi following the method of Marquardt et al. (1999).

Table 4.1 Composition and nutrient level of the basal diet[1] (kg, as-fed basis)

Item	Basal diet
Ingredients, %	
Corn grain	48.4
Soybean meal (480 g crude protein·kg^{-1})	16.0
Fish meal	6.6
X-SOY600[2] (600 g crude protein·kg^{-1})	11.0
Whey permeate	12.4
Soybean oil	1.5
Limestone	1.42
Salt	0.5
Biofos[3]	0.57
L-lysine	0.28
Threonine	0.13
DL-methionine	0.15
L-tryptophan	0.05
Vitamin-mineral premix[4]	1.0

Calculated nutrient levels (%)	
Crude protein	22.35
SID[5] Lysine	1.34
SID Methionine	0.5
SID Threonine	0.87
SID Tryptophan	0.27
Metabolizable energy (kcal·kg^{-1})	3,390
Net energy (kcal·kg^{-1})	2,475

[1]The diet for PC was additionally added with 80 mg·kg^{-1} of avilamycin (Surmax 100 Premix, avilamycin premix 100 g·kg^{-1}, Elanco Canada Limited, Guelph, Ontario, Canada) based on the basal diet. The PF9 diet was added with 2.5×10^{9} CFU·kg^{-1} of *B. licheniformis*.

[2]Soy protein concentration (CJ Selecta, Goiania, State of Goiás, Brazil).

[3]Monocalcium phosphate with 21% Ca and 17% P (Mosaic Co., Plymouth, MN, USA).

[4]Supplied per kilogram of diet as follows: 2,200 IU vitamin A, 1.5 mg vitamin B1, 0.02 mg vitamin B12, 0.2 mg biotin, 4 mg vitamin B2, 220 IU vitamin D3, 16 IU vitamin E, 0.5 mg vitamin K, 600 mg choline chloride, 30 mg niacin, 7 mg pyridoxine, 0.3 mg folic acid, 12 mg calcium pantothenate, 0.14 mg calcium iodate, 0.3 mg sodium selenite, 6 mg copper sulphate, 100 mg ferrous sulfate, 4 mg manganese oxide, and 100 mg zinc oxide.

[5]Standardized ileal digestibility.

4.3.7 Collection of tissue and digesta samples

All piglets from this trial were anesthetized with ketamine:xylazine and euthanized by a captive bolt gun at the end of the trial (on d 11). First, the abdomen of pig was quickly opened up, and the gut was quickly removed for sample collection. A 10 cm of jejunum (400 cm from the stomach-duodenum junction) was obtained, put in a tube containing cold Krebs ringer bicarbonate (**KRB**) buffer, and immediately transferred to the laboratory to perform the permeability assay using a Ussing chamber. The other 15 cm of the jejunum was collected and quickly frozen in liquid nitrogen prior to storage at –80°C for follow-up analyses. A section of 3 cm each of the duodenum (8 cm from the stomach), mid jejunum (4 m from the stomach-duodenum junction), and mid ileum (10 cm from the ileum-cecum junction) was removed and fixed in 10% formaldehyde solution for histological analysis of gut morphology. The digesta from the ileum (5 cm of ileum from the middle) and colon (2 cm of colon from the middle) were also obtained and quickly frozen in liquid nitrogen before storage at –80°C for the follow-up analyses (Choi et al., 2020).

4.3.8 *In vivo* gut permeability

Each piglet received by oral gavage 10 mg in 5 mL PBS buffer of fluorescein isothiocyanate-dextran 70 kDa (**FITC-D70**; Sigma-Aldrich Co., St. Louis, MO, USA) on 3 dpi. Four hours after gavage, blood samples from the jugular vein of each piglet were collected into vacutainer tubes (Becton Dickinson, Rutherford, NJ, USA) covered by aluminum foil for light blocking, and allowed to clot for 3 h at 22ºC. After centrifuging for 15 min at 750 × g, the obtained serum samples were stored at – 80°C for further analyses. The FITC-D70 content in the serum was measured by a Bio-Tek PowerWave^{TM} HT Microplate Scanning Spectrophotometer (BIO-TEK Instruments Inc., Winooski, VT, USA) under the excitation wavelength of 485 nm and emission wavelength of 528 nm and calculated through the standard curve.

4.3.9 Analysis of transepithelial electrical resistance

Transepithelial electrical resistance (**TEER**) was determined as the electrophysiological property by a Ussing chamber (Physiologic Instruments Inc., San Diego, CA, USA) with voltage and current electrodes filled with KRB buffer and housed in 3% agar bridges. Five mL KRB buffer solution was filled in the mucosal chambers and the serosal chambers with 10 $mmol \cdot L^{-1}$ D-mannitol and D-glucose, respectively. The chambers were kept at temperature of 37°C through a water-jacketed reservoir and continued pumping of the 95% O_2 and 5% CO_2 mixed gas. The tissue without serosal and longitudinal muscle layers was mounted in Ussing chambers, equilibrated for 10 min, and then the TEER was recorded after mounting for 10 min (Choi et al., 2020).

4.3.10 Gut morphology

The gut tissue samples were embedded in paraffin after being fixed in a 10% formaldehyde solution, sliced into samples with 5 μm thickness, and then transferred onto glass slides. The dewaxed parts were soaked in xylene, 100% and 95% ethanol for 5 min each for two cycles. After that, the gut tissues were left in Alcian blue at 22ºC for 15 min and washed with pure water for 2 min and in Schiff for 10 min followed by another washing in pure water for 10 min. Finally, hematoxylin was used for a second staining by 10 s, and then the samples were washed with water and dehydrated. Each slide was photographed and visualized using a digital camera (Lumenera Corp., Ottawa, ON, Canada) and microscope equipped with 10× lenses (Carl Zeiss Microscopy Deutschland GmbH, Göttingen, Germany) for quantification of Alcian blue/ Periodic acid–Schiff (**AB/PAS**) staining. Fifteen villi and associated crypts of each sample were selected for measuring villus height (**VH**), crypt depth (**CD**), and VH:CD using a Infinity Analyze software (Lumenera Corp., Ottawa, ON, Canada) (Choi et al., 2020).

4.3.11 Analysis of intestinal digestive enzyme activities

Maximal enzyme activity ($\mathbf{V}_{max}$) of intestinal digestive enzymes in the mid-jejunum including sucrase, maltase, intestinal alkaline phosphatase (**IAP**), aminopeptidase N (**APN**), and maltase-glucoamylase (**MGA**) were all measured. Specifically, around 200 mg of frozen mid-jejunum tissues were homogenized on ice in cold homogenizing buffer (Phenylmethylsulfonyl fluoride and D- mannitol, pH 7.4) with a polytron homogenizer. The original tissue homogenate sample suspension was transferred into the tube to determine the protein content by BCA Protein Assay Kit (Thermo Fisher Scientific, Ottawa, ON, Canada). The activities of sucrase and maltase were measured following the method written by Dahlqvist (1964). IAP activity was analyzed following the method of Hübscher and West (1965), and APN activity was measured using the procedure of Maroux et al. (1973). MGA activity was determined using the procedure of Lackeyram et al., (2012).

4.3.12 Assays of total GSH, GSH/GSSG, and antioxidant capacity

Total antioxidant capacity (**TAC**) in the mid-jejunum was analysed using the Total Antioxidant Capacity Kits of Colorimetric Microplate Assay (Oxford Biomedical Research Inc., Oxford, MI, USA) (Yang, 2011). Specifically, about 200 mg of mid-jejunal tissues were homogenized with 1 mL of cold PBS for 30 s on ice and centrifuged for 12 min at 3,600 × g at 4ºC. Firstly, 25 µL supernatant was used for protein level analysis. Secondly, the TAC in aliquot of supernatant was determined by all antioxidants capacity to convert Cu^{2+} to Cu^{+} following the manufacturer's protocol. Cu^{+} was complexed with bathocuproine to create a stable form which was detected by Bio-Tek PowerWave^{TM} HT Microplate Scanning Spectrophotometer (BIO-TEK Instruments Inc. Winooski, VT, USA) with a 96-well plate reader at a wavelength of 450 nm. The values were based on the standard curve (Choi et al., 2020).

Glutathione (**GSH**) and oxidized glutathione (**GSSG**) levels of the mid-jejunum were analyzed using the detection kit for glutathione colorimetric according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Specifically, around 30 mg of tissues were homogenized for 30 s on ice in 750 µL of ice-cold PBS followed by centrifugation for 10 min at 3,600 × g at 4ºC. After determining the protein concentration, 5-sulfosalicylic acid dihydrate used for precipitating protein was added into the supernatant followed by another centrifugation for 10 min at 3,600 × g at 4ºC. The protein level of all homogenized tissues was measured by a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The total GSH and GSSG levels were then measured using this equation: Reduced GSH = Total GSH – 2 × GSSG (Choi et al., 2020).

4.3.13 RNA extraction and gene expression analysis

Total RNA in 50 mg mid-jejunal tissues was extracted by the total RNA isolation kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). The RNA concentration and OD_{260}:OD_{280} ratios were measured by a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Ottawa, ON, Canada). One µg RNA was used for synthesizing cDNA using an iScript^{TM} cDNA Synthesis Kit (Bio-Rad Laboratories, Mississauga, ON, Canada) following the protocol provided by the manufacturer. Primer-Blast was used for designing the primers (**Table 4.2**) and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA). A total volume of 20 µL containing 1 µL cDNA, 300 nmol·L^{-1} of each forward and reverse primers and 10 µL SYBR Green supermix (Biorad, Ontario, Canada) was used for Real-time PCR assays, which were performed on a Real-Time PCR Detection System (Bio-Rad Laboratories, Ontario, Canada) (Omonijo et al., 2019). All reactions conditions were, denaturation at 95°C for 3 min, 40 cycles of 20 s at 95°C, 30 s at 60°C and then 30 s at 72°C. The target mRNA abundance was normalized with GAPDH and relative

mRNA levels were determined by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The values of the cycle threshold (**Ct**) were acquired when targeted genes were amplified above a threshold of 30 fluorescence units. Duplication of each gene was performed for each sample (Choi et al., 2020).

Table 4.2 Primer sequences of ETEC F4 receptor, nutrient transporters, tight junction proteins, digestive enzymes, and inflammatory cytokines of piglets[1]

Genes	Amplicon (bp)	Sequence (5' to 3')	References
MUC2	90	CCAGGTCGAGTACATCCTGC GTGCTGACCATGGCCCC	(Choi et al., 2020)
MUC4	367	GTGCCTTGGGTGAGAGGTTA CACTCTGCCGTTCTCTTTCC	(Jensen et al., 2006)
ZO-1	200	GATCCTGACCCGGTGTCTGA TTGGTGGGTTTGGTGGGTT	(Omonijo et al., 2018)
OCLN	93	CTGTGGATGTCCTGCGTGT GGTTGCTTGCAAAGTGGTGTT	(Lee and Kang, 2017)
CLDN3	123	CTACGACCGCAAGGACTACG TAGCATCTGGGTGGACTGGT	(Omonijo et al., 2018)
B^0AT1	102	AGGCCCAGTACATGCTCAC CATAAATGCCCCTCCACCGT	(C. Yang et al., 2016)
SGLT1	153	GGCTGGACGAAGTATGGTGT GAGCTGGATGAGGTTCCAAA	(Yang et al., 2011)
PepT1	143	ATCGCCATACCCTTCTG TTCCCATCCATCGTGACATT	(Omonijo et al., 2018)

ASCT2	206	GCCAGCAAGATTGTGGAGAT GAGCTGGATGAGGTTCCAAA	(C. Yang et al., 2016)
EAAC1	168	CCAAGGTCCAGGTTTTGGGT GGGCAGCAACACCTGTAATC	(Omonijo et al., 2018)
MGA	118	GCCCCTTCTGCATGAGTTCT CGTCACTTTCTCTGCACCCT	(Choi et al., 2020)
APN	114	GGACGATTGGGTCTTGCTGA GGGATGACCGACAGGTTTGT	(Choi et al., 2020)
IL1β	91	TGGCTAACTACGGTGACAACA CCAAGGTCCAGGTTTTGGGT	(Choi et al., 2020)
IL8	126	CACCTGTCTGTCCACGTTGT AGAGGTCTGCCTGGACCCCA	(Omonijo et al., 2018)
IL10	220	CATCCACTTCCCAACCAGCC CTCCCCATCACTCTCTGCCTTC	(Lee and Kang, 2017)
TLR2	109	ACATGAAGATGATGTGGGCC TAGGAGTCCTGCTCACTGTA	(Tohno et al., 2005)
TLR5	86	GTTCTTTATCCGGGTGACTT AATAAGTCAGGATCGGGAGA	(Choi et al., 2020)

TLR7	107	GCTGTTCCCACTGTTTTGCC GAGCTGGATGAGGTTCCAAA	(Choi et al., 2020)

[1]MUC2: mucin 2; MUC4: mucin 4; OCLN: occludin; ZO-1: zonula occludens-1; CLDN3: claudin 3; EAAC1: excitatory amino acid transporter 1; ASCT2: neutral amino acid transporter 2; B^0AT1: neutral amino acid transporter; PepT1: peptide transporter 1; SGLT1: Na^+-glucose cotransporter 1; MGA: maltase-glucoamylase; APN: aminopeptidase N; IL1β: interleukin 1β; IL8: interleukin 8; IL10: interleukin 10; TLR2: toll like receptor 2; TLR5: toll like receptor 5; TLR7: toll like receptor 7.

4.3.14 Microbiota analysis

The PCR amplification and 16S rRNA sequencing were performed by Genome Quebec. Bacterial DNA from ileal and colonic digesta was extracted using a Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Extracted DNA from the digesta was amplified by PCR, targeting the V3-V4 hypervariable region of the 16S rRNA gene with a forward primer 341 F (5′- CCTACGGGNGGCWGCAG-3′) and a reverse primer 805 R (5′-GACTACHVGGGTATCTAATCC-3′) (Caporaso et al., 2012).

The PCR reaction system was 25 µL, containing 1×reaction buffer (Q5^{TM}), 5% DMSO, l µL DNA template, 0.2 mM dNTP, 0.5 U Q5^{TM} DNA polymerase, and 0.6 µM of forward and reverse primers. The PCR amplifications were performed according to the following procedures: 30 s of 98°C denaturation, followed by 25 cycles of 10 s at 98°C, annealing at 60°C for 15 s, and elongation at 72°C for 30 s, with a final extension at 72°C for 2 min. After verification of the amplificons on 2% agarose gel, the barcoding step was conducted with a total volume of 20 µL, containing 1 × reaction buffer, 5% DMSO, 0.2 mM dNTP, 1.8 mM Mg^{2+}, 0.2 µM primers, 0.5 U polymerase and 1 µL DNA template. Initially, the PCR program started with 95°C for 10 min; 95°C for 15 s, 60°C for 30 s and 72°C for 1 min, repeat for 15 cycles; 72°C for 3 min. The verification of barcode incorporation for each sample was done on 2% agarose gel. Each amplicon was quantified with the QuantiT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The library was then generated by pooling the same quantity of each amplicon and cleaning-up the pool with sparQ PureMag Beads (Quantabio, Beverly, MA, USA). Afterward, the library was quantified using Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Sigma-Aldrich Canada Co., Oakville, ON, Canada). Average fragment size was determined using a LabChip GX (PerkinElmer, Waltham, MA, USA) instrument. Before

sequencing, 5% of the PhiX control library was added to the amplicon pool (loaded at a final concentration of 10 pM). The PhiX control library helps to improve the unbalanced base composition on the flowcell. Illumina MiSeq sequencing was carried out by Genome Quebec. Sequences were inputted into Quantitative Insights Into Microbial Ecology 2 (QIIME2; version 2021.8) for bioinformatics analysis (Caporaso et al., 2010; Bolyen et al., 2019).

Plugin q2-dada2 was applied for features construction and quality control (Callahan et al., 2016). Taxonomic classification was then conducted by the feature-classifier plugin (Quast et al., 2013; Bokulich et al., 2018).

4.3.15 Statistical analysis

Survival curves for *C. elegans* were compared using the Kaplan-Meier survival analysis followed by a log-rank test. One-way analysis of variance (**ANOVA**) and the Tukey's multiple comparisons were used to test for significant differences between the means. Tolerance to low pH and high bile salts data were analyzed by the SAS (V.3.8; SAS Institute, Cary, NC, USA) GLM procedure and Tukey multiple comparisons. Means with $P < 0.05$ were considered a significant difference.

PROC MIXED of SAS with each piglet as the experimental unit in a randomized complete block design were used for analyzing the data. The statistical analyses included sex (barrows and gilts) and treatment as main effects, and the interaction sex by treatment. The least-square means with the option of Tukey-adjusted PDIFF was applied to split and calculate the treatment mean value. The NC group was compared with either the NNC group by preplanned contrasts to determine the effects of ETEC F4, or the PC group or the PF9 group to determine the avilamycin or the *B. licheniformis* effects, respectively. Results shown in tables are indicated as LSMEANS

and pooled SEM, while results shown in figures are indicated as mean ± SEM. $P < 0.05$ were considered a significant difference.

Statistical analysis and data visualization for ileal and colonic microbiota were performed by the R program (version 4.0.2). Chao1, Shannon and Simpson alpha diversity indices and relative abundance were performed by the phyloseq package. Ggplot2 package in R program was applied to visualize the relative abundance, which aggregated at different taxonomical levels. Significant relative abundance was observed by the Kruskal-Wallis rank sum test. The conover test with the agricolae package was used for multiple pairwise comparison. Beta diversity was performed by the Bray-Curtis dissimilarity.

4.4 Results

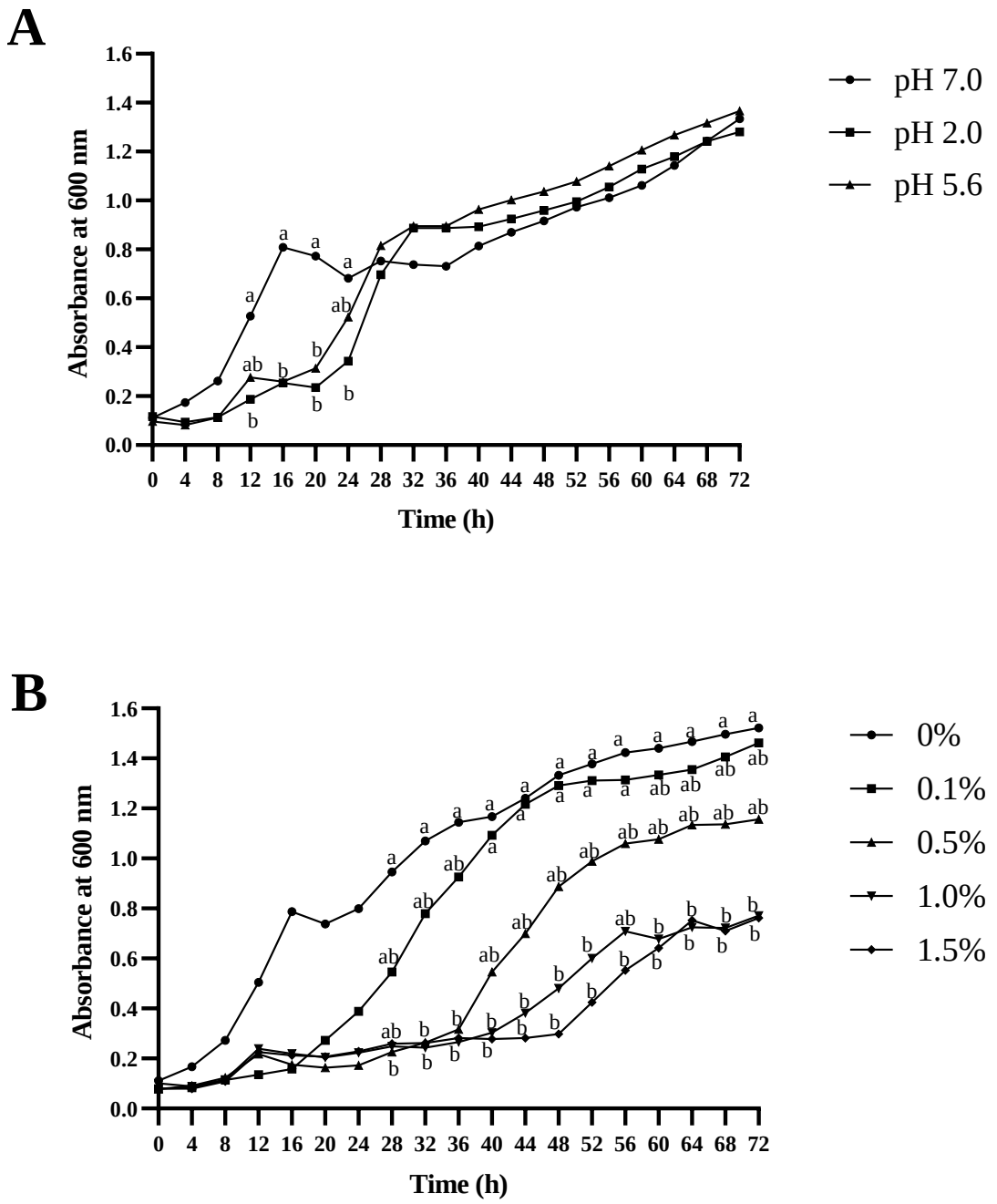
4.4.1 Species identification of isolate PF9 and its tolerance to low pH and high bile salts

According to the final test report from the Agriculture and Food Laboratory, University of Guelph, sequencing of the 16S rRNA gene (1,485 bp) of isolate PF9 revealed > 99.8% similarity to *B. licheniformis* or *B. paralicheniformis* compared with known 16S rRNA gene sequences in the NCBI GenBank database. In contrast to a *B. paralicheniformis* strain that served as a positive control in the assay, isolate PF9 showed no amplicons of *fenC* and *fenD*, the two markers from the fengycin operon of *B. paralicheniformis*, confirming that the isolate PF9 belongs to *B. licheniformis*. In the antimicrobial assays, the isolate partially inhibited the growth of ETEC JG280 and *Salmonella* Typhimurium DT104 (18 – 30%).

In the tolerance assay to low pH, *B. licheniformis* PF9 had similar optical OD_{600} values after 28 h growth at pH 7.0 regardless of the culture being pretreated at pH 2.0 or 5.6, indicating its tolerance to low pH (**Figure 4.1 A**). In the tolerance assay to high bile salts, *B. licheniformis* PF9 at 0.1% (v/v) concentration of bile salts showed similar growth with the control (0%) group

(**Figure 4.1 B**). At 0.5% (v/v), the isolate had poorer growth than the control group before 36 h incubation. However, after 72 h the growth of the isolate reached more than 1.0 at OD_{600} without a significant difference ($P > 0.05$) compared to the control (0%). At 1.0% and 1.5% (v/v), the growth of the isolate was suppressed until 48 h incubation. After 72 h, the same cultures had about half of the OD_{600} values of the control ($P < 0.05$). These results suggest that *B. licheniformis* PF9 has a certain degree of tolerance to bile salts.

Figure 4.1 Tolerance of *B. licheniformis* PF9 to low pH (A) and high bile salts (B).

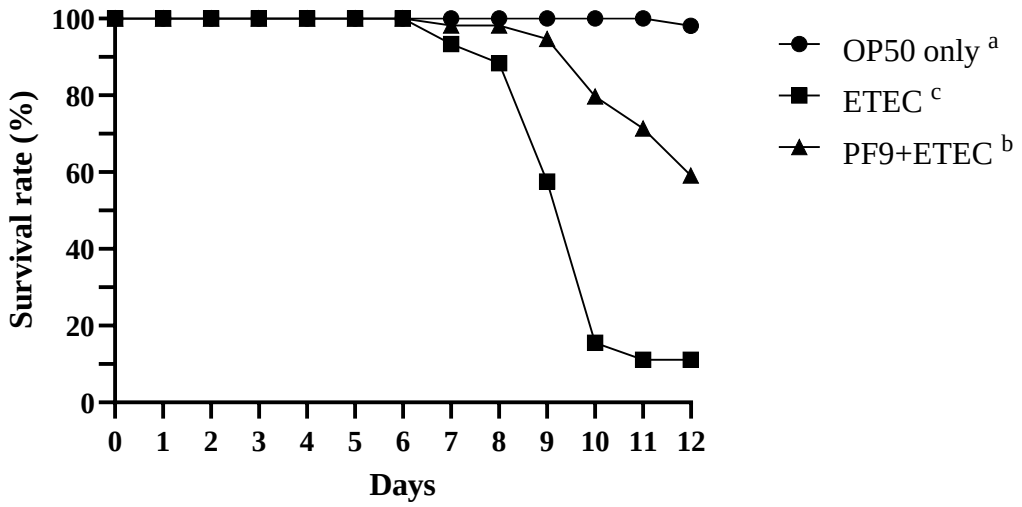


[a,b]Labels with different letters represent a significant difference ($P < 0.05$).

4.4.2 Protection to *C. elegans* infected with ETEC F4

Figure 4.2 shows the effects of *B. licheniformis* PF9 on the lifespan of *C. elegans* infected with ETEC JG280. The lifespan of worms infected with ETEC JG280 was dramatically decreased after eight days of incubation. However, the nematode pretreated with *B. licheniformis* PF9 had a significantly extended lifespan ($P < 0.05$) compared to those infected with ETEC JG280 only. On d 12, only about 10% of the worms survived the ETEC infection, while the survival rate was increased to nearly 60% by the *B. licheniformis* PF9 pretreatment.

Figure 4.2 Lifespan of *C. elegans* with or without the pretreatment of *B. licheniformis* isolate PF9 after infection with ETEC.



Treatments: ●, treated with *E. coli* OP50 only; ■, treated with ETEC JG280; ▲, treated with *B. licheniformis* isolate PF9 and then ETEC JG280. All the groups showing different letters were significantly different ($P < 0.05$) in their survival curves.

4.4.3 Growth performance, diarrhea index score, and rectal temperature

The statistical analysis demonstrated no significant interactions ($P > 0.05$) of sex and treatments for the variables studied (data not shown). Therefore, both male and female pigs were combined for the following analyses. There was no significant difference ($P > 0.05$) in the ADG, ADFI and gain:feed found among all groups during the pre-challenge period (0-7 d) and whole period (0-10 d) (**Table 4.3**). The ADFI among all treatment groups during the post-challenge period did not show any significant differences ($P > 0.05$). Compared to the NNC group, the inoculation of ETEC F4 significantly decreased ADG and gain:feed in the NC group ($P < 0.05$) and there was a significant increase in FCR observed between the PC and NNC groups ($P < 0.05$). As shown in **Figure 4.3**, no significant difference ($P > 0.05$) was found in the rectal temperature observed among all treatments during 48 hpi. Compared to the NNC group, the ETEC F4 challenge significantly ($P < 0.05$) increased the severity of diarrhea at 3, 36, and 40 hpi in the NC group. The supplementation of *B. licheniformis* significantly ($P < 0.05$) relieved diarrhea at 3 hpi compared to the NC group (**Figure 4.4**).

Table 4.3 Effects of *B. licheniformis* PF9 on the growth performance of weaned piglets during the pre-challenge period (0-7 d), post-challenge period (8-10 d) and whole period

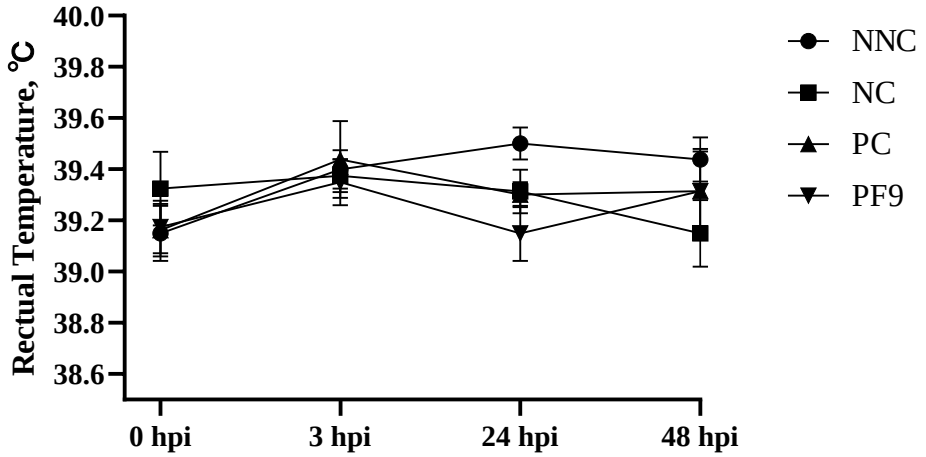
		Challenged groups				*P* value			
Items[1]	NNC	NC	PC	PF9	SEM	TRT	NNC vs NC	NC vs PC	NC vs PF9
Pre-challenge period									
ADG ($g \cdot d^{-1}$)	265	270	241	274	17.57	0.91	0.99	0.93	0.99
ADFI ($g \cdot d^{-1}$)	348	347	348	352	19.32	0.99	1.00	1.00	0.99
Gain:Feed	0.767	0.759	0.747	0.775	0.019	0.96	1.00	0.99	0.99
Post-challenge period									
ADG ($g \cdot d^{-1}$)	389^{a}	208^{b}	327^{ab}	298^{ab}	20.30	0.01	0.01	0.06	0.29
ADFI ($g \cdot d^{-1}$)	607	499	560	572	20.35	0.20	0.35	0.42	0.20
Gain:Feed	0.709^{a}	0.481^{b}	0.556^{ab}	0.576^{ab}	0.026	0.01	0.01	0.08	0.27

Whole period									
ADG ($g \cdot d^{-1}$)	331	232	261	295	17.26	0.17	0.19	0.89	0.32
ADFI ($g \cdot d^{-1}$)	469	418	365	405	17.71	0.36	0.76	0.67	0.99
Gain:Feed	0.749	0.573	0.600	0.721	0.027	0.04	0.10	0.94	0.21

[1]NNC: non-challenged negative control group with basal diet and piglets gavaged with phosphate buffered saline; NC: negative control group with basal diet and piglets challenged with ETEC F4; PC: positive control group with basal diet + 80 $mg \cdot kg^{-1}$ of avilamycin and piglets challenged with ETEC F4; PF9: isolate PF9 group with basal diet + 2.5×10^9 $CFU \cdot kg^{-1}$ diet of *B. licheniformis* and piglets challenged with ETEC F4; ADG: average daily gain; ADFI: average daily feed intake.

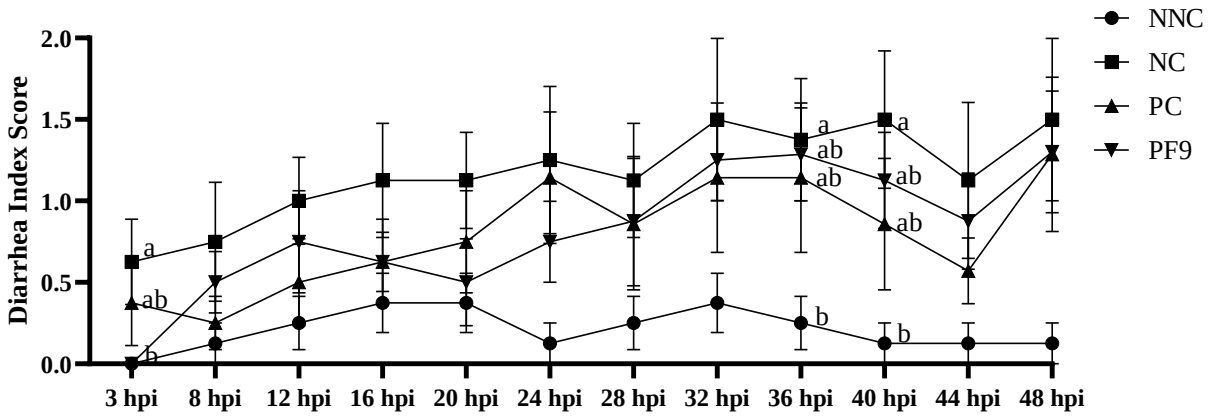
[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

Figure 4.3 Effects of *B. licheniformis* PF9 on rectal temperature in piglets.



Rectal temperature was measured in the non-challenged negative control (NNC) group with basal diet and piglets gavaged with phosphate buffered saline; negative control (NC) group with basal diet and piglets challenged with ETEC F4; positive control (PC) group with basal diet + 80 mg·kg^{-1} of avilamycin and piglets challenged with ETEC F4; PF9 group with basal diet + 2.5×10^9 CFU·kg^{-1} diet of *B. licheniformis* and piglets challenged with ETEC F4. Each value is represented by the mean ± SEM.

Figure 4.4 Effects of *B. licheniformis* PF9 on diarrhea index score in weaned piglets.



The diarrhea index score was measured in the non-challenged negative control (NNC) group with basal diet and piglets gavaged with phosphate buffered saline; negative control (NC) group with basal diet and piglets challenged with ETEC F4; positive control (PC) group with basal diet + 80 $mg \cdot kg^{-1}$ of avilamycin and piglets challenged with ETEC F4; PF9 group with basal diet + 2.5×10^9 $CFU \cdot kg^{-1}$ diet of *B. licheniformis* and piglets challenged with ETEC F4. Diarrhea index score 0 represent normal feces; 1 represent soft feces; 2 represent mild diarrhea; and 3 represent severe diarrhea. Each value is represented by the mean ± SEM. Different letters shown in the bars indicate significant difference ($P < 0.05$).

4.4.4 Gut morphology

The inoculation of ETEC F4 significantly ($P < 0.05$) decreased duodenal, jejunal and ileal VH in the NC group compared to the NNC group, and there was a significant difference ($P < 0.05$) in duodenal VH between the PC and NNC group (**Table 4.4**). However, there was no significant difference ($P > 0.05$) in all small intestinal CD and VH:CD among all treatments.

4.4.5 Gut permeability

As shown in **Table 4.5**, there was no significant difference ($P > 0.05$) observed in the TEER measured by the Ussing chamber and *in vivo* gut permeability among all treatments.

4.4.6 Intestinal digestive enzyme activities, total GSH, GSH/GSSG and TAC

There was no significant difference ($P > 0.05$) in the V_{max} values of APN, IAP, MGA, sucrase and maltase among all treatments (**Table 4.6**). No significant difference ($P > 0.05$) was found in the GSH, GSSG, reduced GSH, reduced GSH:GSSG ratio and TAC among all treatments (**Table 4.6**).

Table 4.4 Effects of *B. licheniformis* PF9 on gut morphology with villus height (VH, μm), crypt depth (CD, μm) and VH:CD in the duodenum, jejunum, and ileum of weaned piglets

		Challenged groups				*P* value			
Items[1]	NNC	NC	PC	PF9	SEM	TRT	NNC vs NC	NC vs PC	NC vs PF9
Duodenum									
VH	555[a]	467[b]	472[b]	514[ab]	10.33	0.01	0.04	0.99	0.25
CD	158	156	157	158	2.54	0.99	0.99	0.99	0.99
VH:CD	3.32	3.03	3.00	3.18	0.06	0.21	0.42	0.99	0.86
Jejunum									
VH	519[a]	445[b]	465[ab]	470[ab]	6.72	0.02	0.01	0.65	0.34
CD	148	145	144	147	1.93	0.92	0.98	0.99	0.99
VH:CD	3.20	3.14	3.26	3.26	0.05	0.81	0.97	0.86	0.82

Ileum									
VH	411[a]	361[b]	386[ab]	401[ab]	5.69	0.03	0.03	0.44	0.11
CD	140	149	140	143	2.15	0.49	0.54	0.51	0.74
VH:CD	3.02	2.66	2.71	2.79	0.05	0.08	0.11	0.99	0.77

[1]NNC: non-challenged negative control group with basal diet and piglets gavaged with phosphate buffered saline; NC: negative control group with basal diet and piglets challenged with ETEC F4; PC: positive control group with basal diet + 80 mg·kg^{-1} of avilamycin and piglets challenged with ETEC F4; PF9: isolate PF9 group with basal diet + 2.5 × 10^{9} CFU·kg^{-1} diet of *B. licheniformis* and piglets challenged with ETEC F4.

[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

Table 4.5 Effects of *B. licheniformis* PF9 on electrophysiological properties with transepithelial electrical resistance (TEER, $\Omega \cdot cm^2$) in jejunum mounted in Ussing chambers and flux of fluorescein isothiocyanate–dextran 70 kDa (FITC-D70, $\mu g \cdot mL^{-1}$) in weaned piglets

		Challenged groups				*P* value			
Items[1]	NNC	NC	PC	PF9	SEM	TRT	NNC vs NC	NC vs PC	NC vs PF9
Ex vivo									
TEER	51.62	52.13	56.42	55.64	3.16	0.91	0.99	0.97	0.99
In vivo									
FITC-D70 flux	0.77	0.92	0.87	0.83	0.03	0.13	0.10	0.92	0.49

[1]NNC: non-challenged negative control group with basal diet and piglets gavaged with phosphate buffered saline; NC: negative control group with basal diet and piglets challenged with ETEC F4; PC: positive control group with basal diet + 80 $mg \cdot kg^{-1}$ of avilamycin and piglets challenged with ETEC F4; PF9: isolate PF9 group with basal diet + 2.5×10^9 $CFU \cdot kg^{-1}$ diet of *B. licheniformis* and challenged with ETEC F4

Table 4.6 Effects of *B. licheniformis* PF9 on the digestive enzymes' activities (nmol·L^{-1}·mg $protein^{-1}$·min^{-1}), the total glutathione (GSH, nmol·L^{-1}·mg $protein^{-1}$), oxidized glutathione (GSSG, nmol·L^{-1}·mg $protein^{-1}$), reduced GSH:GSSG, and total antioxidant capacity (TAC, mmol·L^{-1}·mg $protein^{-1}$) in the mid-jejunum of weaned piglets

		Challenged groups				*P* value			
Items[1]	NNC	NC	PC	PF9	SEM	TRT	NNC vs NC	NC vs PC	NC vs PF9
IAP	0.35	0.49	0.35	0.32	0.04	0.34	0.60	0.38	0.28
APN	0.15	0.13	0.14	0.14	0.01	0.98	0.98	0.99	0.99
Maltase	105.09	101.88	109.86	117.94	10.32	0.96	0.99	0.99	0.96
MGA	5.24	5.98	7.61	7.95	0.99	0.75	0.99	0.94	0.94
Sucrase	9.73	10.67	11.71	12.93	1.15	0.80	0.96	0.99	0.87
Total GSH	2.62	2.19	2.26	2.54	0.28	0.94	0.97	0.99	0.95
GSSG	0.51	0.39	0.37	0.54	0.08	0.88	0.96	0.99	0.92

Reduced GSH[2]	1.68	1.56	1.62	1.33	0.17	0.73	0.99	0.99	0.84
Reduced GSH:GSSG	3.67	4.77	4.02	3.66	0.68	0.93	0.97	0.96	0.93
TAC	69.06	64.04	68.91	66.24	2.47	0.94	0.96	0.96	0.99

[1]IAP: intestinal alkaline phosphatase; APN: aminopeptidase N; MGA: maltase-glucoamylase; NNC: non-challenged negative control group with basal diet and piglets gavaged with phosphate buffered saline; NC: negative control group with basal diet and piglets challenged with ETEC F4; PC: positive control group with basal diet + 80 $mg \cdot kg^{-1}$ of avilamycin and piglets challenged with ETEC F4; PF9: isolate PF9 group with basal diet + 2.5×10^9 $CFU \cdot kg^{-1}$ diet of *B. licheniformis* and piglets challenged with ETEC F4.

[2]Reduced GSH = Total GSH – 2 × GSSG.

4.4.7 Relative mRNA abundance in the jejunum

The relative mRNA levels of genes in the mid jejunum related to gut barrier function, nutrient transportation, digestive enzymes, and immunity were determined by Real-time PCR assays. As shown in **Table 4.7**, no significant difference ($P > 0.05$) was observed in the relative mRNA levels of claudin 3 (**CLDN3**), mucin 2 (**MUC2**), maltase-glucoamylase (**MGA**), peptide transporter 1 (**PepT1**), neutral amino acid transporter 2 (**ASCT2**), neutral amino acid transporter (**B^0AT1**), interleukin-1β (**IL-1β**), interleukin-6 (**IL-6**), interleukin-8 (**IL-8**), interleukin-10 (**IL-10**), toll like receptor 5 (**TLR5**), and toll like receptor 7 (**TLR7**) among all treatments. Compared to the NNC group, the inoculation of ETEC F4 significantly ($P < 0.05$) decreased the relative mRNA levels of Na^+-glucose cotransporter 1 (**SGLT1**) in the NC group. When compared to the NC and NNC groups, the group supplemented with *B. licheniformis* PF9 significantly ($P < 0.05$) increased the relative mRNA levels of SGLT1, occludin (**OCLN**), zonula occludens-1 (**ZO-1**), and aminopeptidase N (**APN**). Moreover, the supplementation of *B. licheniformis* significantly ($P < 0.05$) increased the relative mRNA level of excitatory amino acid transporter 1 (**EAAC1**) when compared to the NC group.

Table 4.7 Effects of *B. licheniformis* PF9 on the relative mRNA levels of genes related to gut barrier function, nutrient transportation, immunity, and digestive enzymes in weaned piglets[1]

		Challenged groups				*P* value			
Items[1]	NNC	NC	PC	PF9	SEM	TRT	NNC vs NC	NC vs PC	NC vs PF9
Gut barrier function									
ZO-1	1.08[b]	1.02[b]	1.64[ab]	2.38[a]	0.20	<0.01	0.99	0.77	<0.01
CLDN3	1.01	0.84	1.05	1.31	0.09	0.47	0.75	0.55	0.51
OCLN	1.01[b]	1.08[b]	1.71[ab]	2.76[a]	0.21	<0.01	0.98	0.49	<0.01
MUC2	1.02	0.97	1.00	0.85	0.05	0.83	0.99	0.99	0.89
Nutrient transportation									
SGLT1	1.00[b]	0.57[c]	0.76[abc]	1.72[a]	0.13	<0.01	0.04	0.92	<0.01
PepT1	1.04	0.92	1.05	1.37	0.09	0.33	0.89	0.96	0.28

B^0AT1	1.00	0.74	0.90	1.25	0.12	0.53	0.57	0.96	0.61
EAAC1	1.08^{ab}	0.42^{b}	0.73^{ab}	1.23^{a}	0.13	0.03	0.09	0.83	0.04
ASCT2	1.05	0.91	1.41	1.12	0.10	0.35	0.95	0.32	0.87
Digestive enzymes									
MGA	1.01	0.75	0.74	1.33	0.10	0.08	0.32	1.00	0.07
APN	1.03^{b}	0.96^{b}	1.23^{ab}	1.90^{a}	0.11	<0.01	0.99	0.77	<0.01
Immunity									
IL-6	1.08	1.04	0.91	1.05	0.11	0.98	0.99	0.99	1.00
IL-8	1.04	1.09	0.82	0.76	0.08	0.12	0.99	0.63	0.12
IL-10	1.09	0.82	1.26	1.32	0.15	0.54	0.92	0.82	0.50
IL-1β	1.03	1.11	0.51	0.51	0.06	0.12	0.99	0.38	0.37

TLR5	1.05	0.97	1.29	1.68	0.13	0.24	0.98	0.83	0.21
TLR7	1.07	0.98	1.03	1.01	0.10	0.99	0.99	0.99	0.99

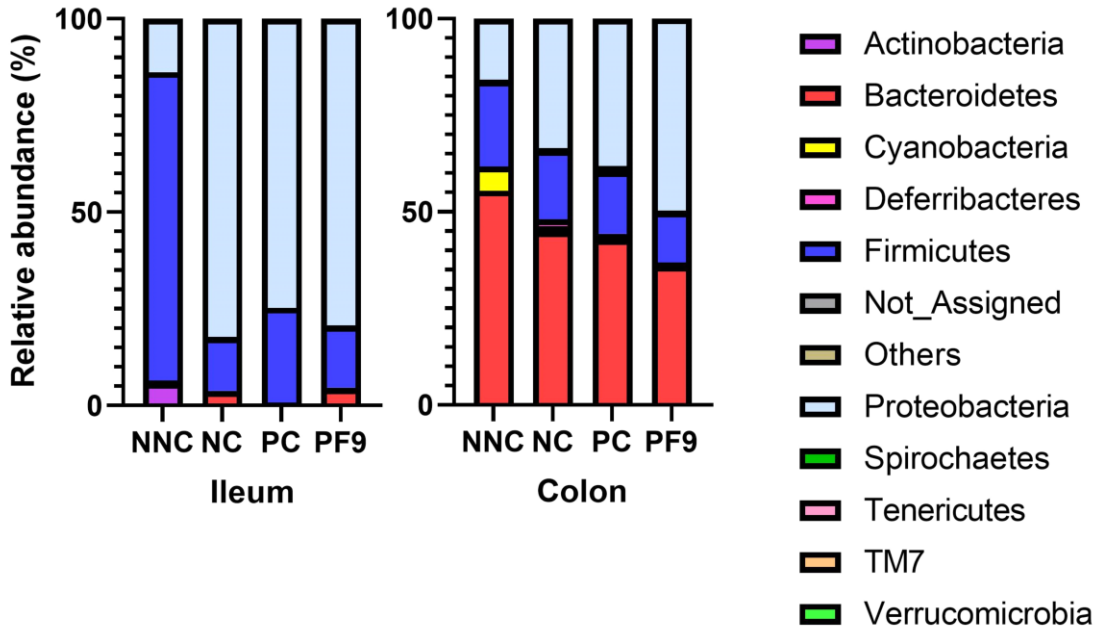
[1]MUC2: mucin 2; ZO-1: zonula occludens-1; OCLN: occludin; CLDN3: claudin 3; PepT1: peptide transporter 1; SGLT1: Na^+-glucose cotransporter 1; ASCT2: neutral amino acid transporter 2; B^0AT1: neutral amino acid transporter; EAAC1: excitatory amino acid transporter 1; MGA: maltase- glucoamylase; APN: aminopeptidase N; IL-1β: interleukin-1β; IL-6: interleukin-6; IL-8: interleukin-8; IL-10: interleukin-10; TLR5: toll like receptor 5; TLR7: toll like receptor 7.

[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

4.4.8 Gut microbiota

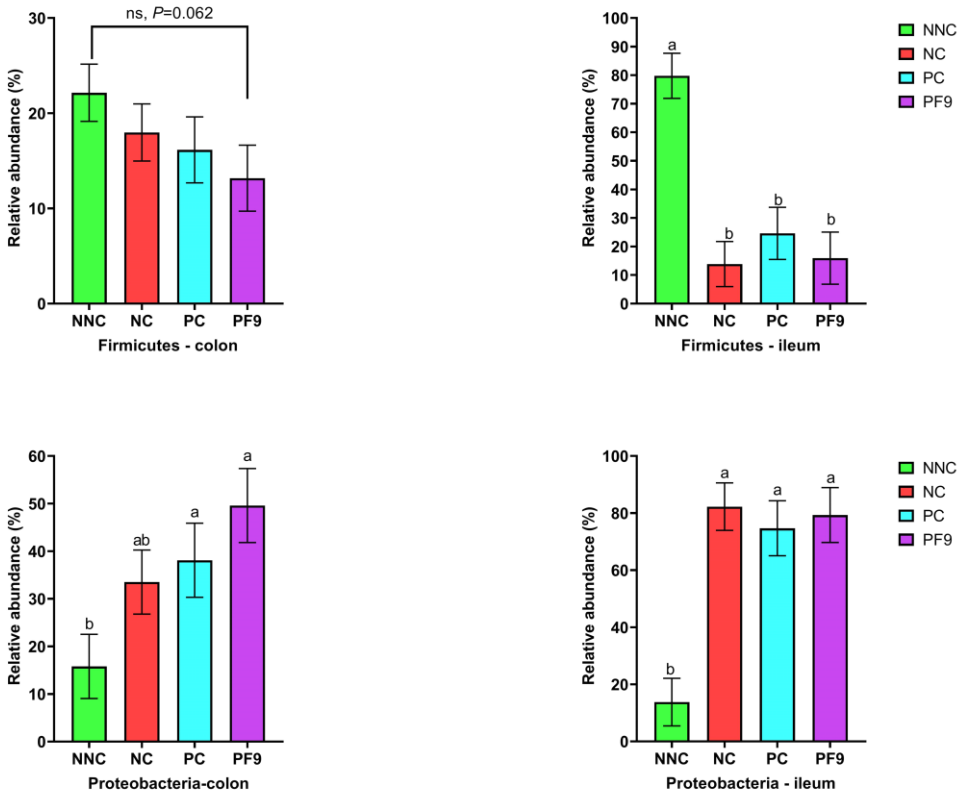
In the current study, 3,906,109 qualified reads in total were obtained. A mean of 69,751 reads per sample and 2,862 operational taxonomic units (**OTU**) in total were identified. The top phyla in the ileal digesta of non-challenged pigs was Firmicutes, which was replaced by Proteobacteria in challenged pigs. The colonic digesta was dominated by Bacteroidetes, Firmicutes, and Proteobacteria (**Figure 4.5**). Piglets supplemented with *B. licheniformis* PF9 had lower relative abundance of Bacteroidetes in the colon than piglets in the NNC group ($P < 0.05$). Non-challenged piglets had higher relative abundance of Firmicutes in the ileum than the challenged piglets ($P < 0.05$); however, lower relative abundance of Proteobacteria ($P < 0.05$) in the ileum and colon was observed in the NC group only (**Figure 4.6**). For alpha diversity (Chao1, Shannon and Simpson indices), there was no significant difference ($P > 0.05$) in the ileum and colon among all treatments (**Figure 4.7**). No significant difference ($P > 0.05$) was found in the beta diversity (Bray-Curtis distance) of ileal and colon digesta among treatments (**Figure 4.8**).

Figure 4.5 Stacked bar plot showing the relative abundance of bacterial phyla in the ileum and colon.



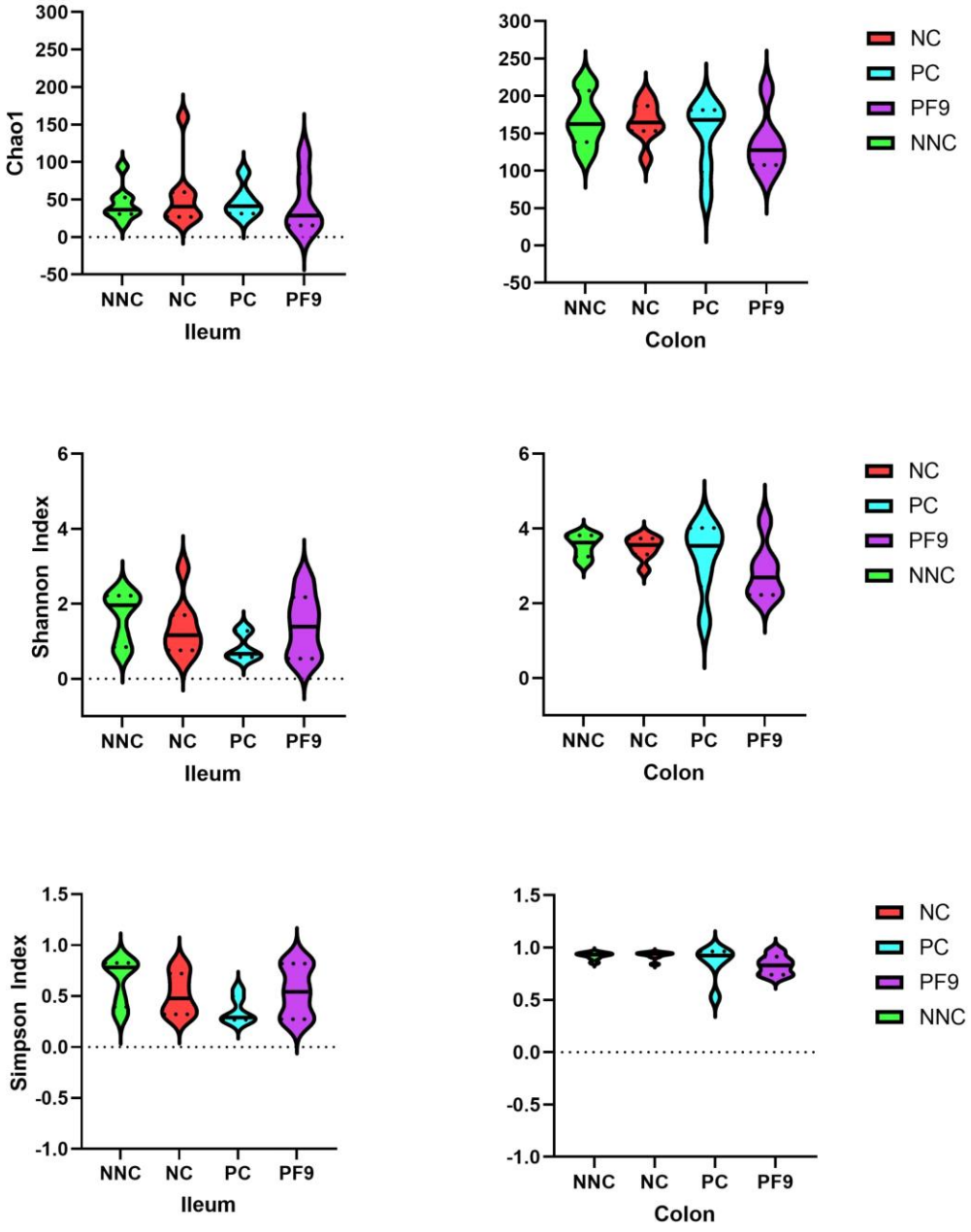
Non-challenged negative control (NNC) group with basal diet and piglets gavaged with phosphate buffered saline; negative control (NC) group with basal diet and piglets challenged with ETEC F4; positive control (PC) group with basal diet + 80 $mg \cdot kg^{-1}$ of avilamycin and piglets challenged with ETEC F4; PF9 group with basal diet + 2.5×10^9 $CFU \cdot kg^{-1}$ diet of *B. licheniformis* and piglets challenged with ETEC F4.

Figure 4.6 Bar plot showing the relative abundance of bacterial phylum in ileum and colon.



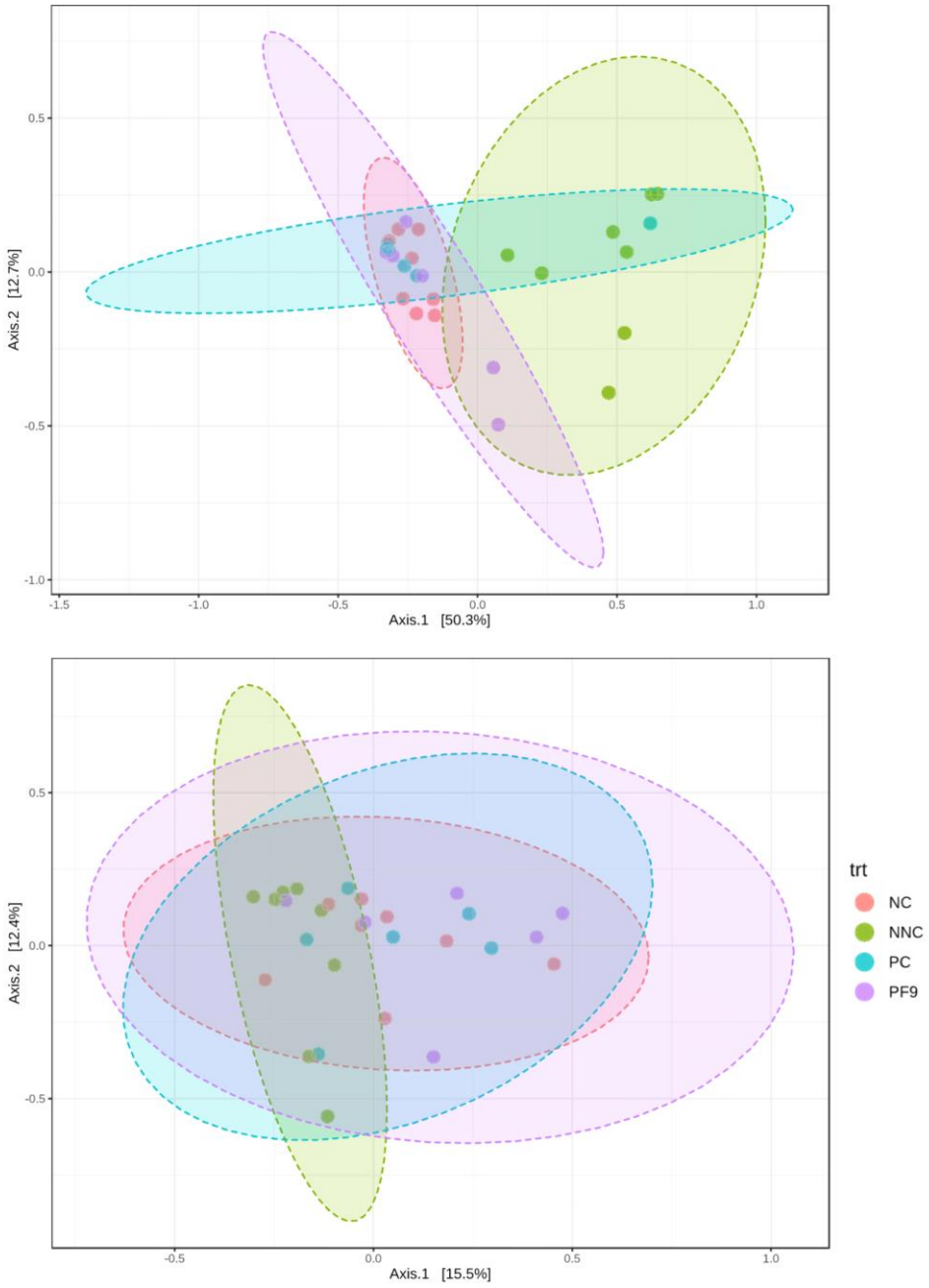
[a,b]Means without a common superscript are different ($P < 0.05$). Non-challenged negative control (NNC) group with basal diet and piglets gavaged with phosphate buffered saline; negative control (NC) group with basal diet and piglets challenged with ETEC F4; positive control (PC) group with basal diet + 80 mg·kg^{-1} of avilamycin and piglets challenged with ETEC F4; PF9 group with basal diet + 2.5 × 10^{9} CFU·kg^{-1} diet of *B. licheniformis* and piglets challenged with ETEC F4.

Figure 4.7 Alpha diversity as indicated by Chao1, Shannon and Simpson index in digesta of ileum and colon.



Non-challenged negative control (NNC) group with basal diet and piglets gavaged with phosphate buffered saline; negative control (NC) group with basal diet and piglets challenged with ETEC F4; positive control (PC) group with basal diet + 80 $mg \cdot kg^{-1}$ of avilamycin and piglets challenged with ETEC F4; PF9 group with basal diet + 2.5×10^9 $CFU \cdot kg^{-1}$ diet of *B. licheniformis* and piglets challenged with ETEC F4.

Figure 4.8 PCA plot of beta diversity of microbiota in digesta of ileum and colon based on Bray–Curtis dissimilarities.



Non-challenged negative control (NNC) group with basal diet and piglets gavaged with phosphate buffered saline; negative control (NC) group with basal diet and piglets challenged with ETEC F4; positive control (PC) group with basal diet + 80 $mg \cdot kg^{-1}$ of avilamycin and piglets challenged with ETEC F4; PF9 group with basal diet + 2.5×10^9 $CFU \cdot kg^{-1}$ diet of *B. licheniformis* and piglets challenged with ETEC F4.

4.5 Discussion

The aim of this study was to characterize *B. licheniformis* PF9 and investigate its potential to relieve the negative effects of ETEC infection on weaned piglets, which impairs gut health including diarrhea and inflammation, by conducting both *in vitro* and *in vivo* studies.

The *B. licheniformis* PF9 showed a tolerance to low pH and high bile salts *in vitro*, which provided the basic physiological foundation for the following *in vivo* animal trial. The same justification was also offered by the lifespan assay of *C. elegans*, in which *B. licheniformis* PF9 partially prevented the nematode from death caused by ETEC infection. Our previous studies have demonstrated that *Lactobacillus* was able to protect *C. elegans* against ETEC infection by suppressing the gene expression of STa and STb enterotoxins in ETEC (Zhou et al., 2014). In addition, the same *Lactobacillus* isolate could regulate the cell signaling of *C. elegans* to increase the production of defence molecules to combat ETEC infection (Zhou et al., 2018). It is unknown at present, if *B. licheniformis* PF9 has the same molecular mechanisms as the *Lactobacillus* isolate in the protection of *C. elegans* and this warrants further investigation.

The ETEC F4 challenge model in weaned piglets was established for inducing the enteric infection (Opapeju et al., 2015). The ETEC F4 virulence and F4 fimbriae receptors in piglets are the two main factors leading to the pathogenesis of ETEC F4 (Luppi et al., 2016). Single nucleotide polymorphisms (SNP) on F4 receptors MUC4 have been frequently used as the genetic markers for ETEC F4 susceptibility or resistance in pigs; therefore, the ETEC F4 susceptible piglets were chosen in the current study after gene screening of the susceptible alleles of MUC4 (Jørgensen et al., 2003).

In the present study, the supplementation of *B. licheniformis* PF9 did not affect the growth performance of piglets during the pre-challenge period. The inoculation of ETEC F4 significantly

decreased the ADG and gain:feed, which was similar to the findings of Ren et al. (2014). The potential mechanisms related to decreased ADG and gain:feed of piglets challenged with ETEC F4 have been attributed to reduced nutrient utilization efficiency (Choi et al., 2020); induced diarrhea (Sayan et al., 2018) and inflammatory responses (Xu et al., 2020); and reduced nutrients available for host growth because of ETEC F4 competition for available nutrients with the piglets (Mingmongkolchai and Panbangred, 2018). Inconsistent effects of the supplementation of *Bacillus* on pig growth performance have been reported. Pan et al. (2017) reported that the supplementation of probiotics containing *B. licheniformis* and *Saccharomyces cerevisiae* had positive effects on ADG and gain:feed in weaned piglets challenged with ETEC K88. However, Luise et al. (2019a) reported that *B. amyloliquefaciens* and *B. subtilis* did not show improvement in growth performance. These inconsistent results may be due to the differences of tested *Bacillus* isolates, their physiological status, ETEC strains (e.g., F4 and F18), and tested doses (de Lange et al., 2010) or experimental designs (e.g. the experimental period including pre-treatment with the probiotic bacterium).

The higher diarrhea index score due to the ETEC challenge was an indicator showing the success of ETEC F4 infection in piglets (Luise et al., 2019b). In the present study, the supplementation of *B. licheniformis* PF9 reduced the severity of diarrhea to score values similar to those obtained with the antibiotic diet. It showed that both *B. licheniformis* PF9 and avilamycin mitigated the early stages of diarrhea caused by the ETEC F4 infection. Thus, *B. licheniformis* PF9 could be a promising alternative to antibiotics. This observation is consistent with a previous study described by Chen et al. (2021), which may in part be explained by enhanced gut barrier function with increased mRNA expression of the tight junction proteins ZO-1 and OCLN in the jejunum detected in the present study. This speculation was supported by the observation that the same

isolate (*B. licheniformis* PF9) increased the expression levels of ZO-1 and OCLN in the ETEC F4-infected IPEC-J2 cells (Li et al., 2022). It has been well reported that the supplementation of probiotics can improve the gut barrier function of piglets (Wang et al., 2018; Yi et al., 2018).

To study gut permeability of weaned piglets, 2 $mg \cdot mL^{-1}$ FITC-D70 in PBS was orally delivered to the piglets by gavage. In brief, FITC-D70 cannot pass through the epithelial barrier in the gut and be digested by gut digestive enzymes during normal healthy conditions; however, once the tight junction proteins have been affected by a pathogen, toxin or inflammation, the FITC-D70 molecule could enter blood circulation and could be detected in serum samples (Choi et al., 2020). The impairment of gut barrier function by the ETEC F4 challenge could increase the gut permeability with increased FITC-D70 flux, which affects several alternations in the morphology of gut. It has been reported that the supplementation of *Bacillus subtilis* reduced paracellular permeability determined by measuring the flux of FITC-D4 across the jejunal mucosa (Kim et al., 2019). The inconsistent results with our current study may be due to the different probiotics strains.

The higher VH could indicate better nutrient digestion and absorption as the enterocytes located in the villus of piglets was critical in nutrient utilization (Chen et al., 2018). In the present study, the inoculation of ETEC F4 decreased the VH of the small intestine, which is consistent with a report by Choi et al. (2020). ETEC F4 adhering to the enterocyte brush border membrane of gut mucosa causing villous atrophy may be the possible reason for decreasing VH (Luppi, 2017). In the current study, the supplementation of *B. licheniformis* demonstrated a tendency to increase VH in the ileum when compared with the NC group, which is similar with a study from Zong et al. (2019). This may partially explain the increased relative mRNA abundance of the brush border digestive enzymes, APN and MGA, in *B. licheniformis* PF9 treated piglets.

In this study, the ETEC F4 infection significantly decreased the relative mRNA level of SGLT1 in the mid-jejunum, which was similar to results found in a study by Choi et al. (2020). The SGLT1 plays an important role in the gut glucose transport system in pigs (Yang et al., 2011). The decreased SGLT1 may be associated with secreted toxins and villous atrophy from ETEC F4 (Wu et al., 2015). In the present study, supplementation with *Bacillus licheniformis* PF9 resulted in increased relative mRNA expression of SGLT1 and EAAC1. This finding is consistent with the results reported by Cao et al. (2020), which indicate that enhanced expression of nutrient transporters in the intestine reflects improved absorption capabilities of epithelial cells in the small intestine, a benefit associated with probiotic supplementation.

The diversity and composition of gut microbiota in piglets is strongly related to animal health conditions and nutrient compositions that are provided by animal diets (Guevarra et al., 2018). In the present study, the supplementation of *B. licheniformis* PF9 showed no impact on the microbiota diversity in the digesta of ileum and colon, which were similar results to those found in a study by He et al. (2020). Nonetheless, with findings that were inconsistent with those previously published by Duarte et al. (2020), the ETEC infection significantly increased the relative abundance of Proteobacteria and reduced the relative abundance of Firmicutes. This may be partially explained by the production of enterotoxins due to ETEC infection and secretion of fluid to the gut lumen (Wang et al., 2019), which creates an ideal environment for the growth of Proteobacteria (Gresse et al., 2017). The results from the current study indicated that the supplementation of *B. licheniformis* PF9 decreased the relative abundance of Bacteroidetes, which is consistent with the study reported by Cui et al. (2013).

In conclusion, results from the current study demonstrated that the heat-resistant *B. licheniformis* PF9 was tolerant to low pH and high bile salts. It provided protection to *C. elegans*

against ETEC infection. The infection of ETEC F4 impaired growth performance, damaged gut morphology, induced diarrhea, and changed the gut bacterial population in weaned piglets. The supplementation of *B. licheniformis* PF9 reduced diarrhea score, increased the relative mRNA level of several nutrient transporters, the gut barrier associated proteins, and digestive enzymes, and lowered the relative abundance of Bacteroidetes in the ETEC F4 challenged weaned piglets. These results suggest that *B. licheniformis* PF9 has potential for improving pig gut health. Further studies are needed to evaluate different doses of this isolate and a cocktail of probiotics containing at least two different probiotic candidates for their potentials in controlling enteric infections and improving gut health and performance of pigs.

CHAPTER FIVE : MANUSCRIPT II

Effects of *Bacillus licheniformis* HG76 on barrier function and inflammatory responses against enterotoxigenic *Escherichia coli* F4 infection in the porcine intestinal epithelia cells[2]

5.1 Abstract

Enterotoxigenic *Escherichia coli* (**ETEC**) F4 normally invades the small intestine and secretes enterotoxins, which damage the gut barrier function and stimulate the immune reactions characterized by inflammation. *Bacillus licheniformis* (***B. licheniformis***) has been shown in many studies to improve gut health in animals. However, further elucidation of the molecular mechanisms underlying the probiotic effects is still required. The intestinal porcine epithelial cell line (**IPEC-J2**) has often been used for mechanistic studies at the cellular level and was thus employed in the current study to investigate the effects of *B. licheniformis* HG76 on the cell viability, expression of tight junction proteins, pro-inflammatory cytokines, and toll-like receptors when IPEC-J2 cells were challenged with ETEC F4. The results showed that ETEC F4 infection significantly induced IPEC-J2 cell death and this effect was attenuated by *B. licheniformis* HG76 treatment ($P < 0.05$). Whereas *B. licheniformis* HG76 administration had no significant effects on the mRNA abundance of occludin (**OCLN**), claudin 1 (**CLDN1**), and claudin 3 (**CLDN3**) ($P > 0.05$), the gene and protein expressions of zonula occludens-1 (**ZO-1**) in the ETEC F4 infected group were the lowest among all the groups ($P < 0.05$). There was a significant increase of fluorescein isothiocyanate-dextran after the ETEC F4 challenge ($P < 0.05$). Meanwhile, ETEC F4 infection significantly increased the mRNA abundance of toll-like receptors (**TLR**2, TLR4, and TLR5) ($P < 0.05$) and tumor necrosis factor-α (**TNF-α**), but the *B. licheniformis* HG76 treatment alleviated these increases. In summary, these results suggested that *B. licheniformis* HG76 could protect the intestinal cells from ETEC F4 infection by inhibiting inflammation and maintaining barrier integrity.

Key words: *Bacillus licheniformis* HG76, porcine intestinal epithelial cell, enterotoxigenic *Escherichia coli* F4, barrier function, inflammatory response

5.2 Introduction

The intestine serves as the defense barrier against the invasion of harmful substances, as well as the site where nutrients are digested, absorbed and metabolized. Intestinal epithelial cells are categorized as either absorptive cells or secretory cells, which contribute to the barrier function (Wiarda et al., 2023). Porcine intestinal epithelial cell J2 (**IPEC-J2**) from the jejunum of piglets is a well-characterized and non-carcinogenic cell line, which makes it an appropriate cell model for studying intestinal inflammation, barrier function, and host–microbe interactions (Pézsa et al., 2022).

Tight junctions (**TJ**) of intestinal epithelial cells are responsible for controlling paracellular permeability to water, ions, and macromolecules (Suzuki, 2020). Some of the infectious intestinal pathogens can specifically target the TJ of the host, leading to increased epithelial permeability, consequently allowing the invasion of harmful substances and pathogens to the bloodstream leading to inflammatory reactions. The pathophysiology of some gut diseases, such as diarrhea and inflammatory bowel diseases, is associated with the dysfunction of intestinal barrier (König et al., 2016). Gut homeostasis is vital in maintaining the intestinal epithelial barrier function, which safeguards the growth and health of the host.

The major bacterial agent causing post-weaning diarrhea (**PWD**) in swine is enterotoxigenic *Escherichia coli* (**ETEC**), with clinical signs of watery diarrhea, associated depression, inappetence and dehydration in weaned piglets. The occurrence of PWD results in significant economic costs to the global swine industry because of the high morbidity and mortality, costs for treatment and labor, as well as growth retardation (Laird et al., 2021). In practice, various antibiotics are commonly used prophylactically to control the enteric infections in animals (Girard and Bee, 2020). However, the use of antibiotics not only affect pathogens but also the commensal

microbiota in the intestine, which results in dysbiosis. Moreover, the extensive and uncontrolled usage of antibiotics as growth promoters (**AGP**) worldwide has promoted the spread of antibiotic resistant bacteria and environmental resistance genes, which endangers human and animal health (Low et al., 2021). As a consequence, many countries have already banned the use of AGP (Wilhelm et al., 2023). However, the restrictions on the use of AGP had economic consequences for animal production, including increased morbidity and mortality, decreased growth and feed efficiency, and raised veterinary costs (Hodak et al., 2023). Thus, there is an urgent need to identify and develop effective alternatives to AGP.

Probiotics, prebiotics, phytogenics, enzymes, organic acids, antimicrobial peptides, minerals, and bacteriophages are being considered as AGP alternatives. Several studies have shown that probiotics may be one of the most promising choices among alternatives to AGP, and their beneficial effects in several animal species have been demonstrated recently (Quan et al., 2023; Konieczka et al., 2023; Derakhshan et al., 2023; Xu et al., 2023; Ruvalcaba et al., 2023; Veisi et al., 2023). The most commonly used probiotics belong to the genera *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, and *Bacillus* (Hanchi et al., 2018). *Bacillus licheniformis* (***B. licheniformis***), a gram-positive and endospore-forming bacterium belonging to the family *Bacillaceae*, has been demonstrated to possess a robust capability to produce digestive enzymes and antimicrobial peptides, reduce the morbidity and mortality rates, enhance the growth performance, and alleviate the diarrhea incidence in weaned piglets (Lin and Yu, 2020). Our group demonstrated that *B. licheniformis* PF9 might decrease inflammation-related cytokines by blocking the nuclear factor kappa B (**NF-κB**) signaling pathways and enhance the integrity of IPEC-J2 cells (Li et al., 2022). Pézsa et al. (2022) found that the treatment of IPEC-J2 cells with *B. licheniformis* and *B. subtilis* showed effective antioxidant, anti-inflammatory, and inhibition

effects upon *S.* Typhimurium and *E. coli* challenge. Another study indicated that oral administration of *B. licheniformis* and *B. subtilis* spore mixture partially improves *E. coli*-induced diarrhea via upregulating IL-22 and the inhibitor of NF-κB (**IκBα**) expression, and protects gut epithelial barrier integrity by increasing zonula occludens-1 (**ZO-1**) expression in weaned mucin 4 (**MUC4**) resistant piglets (Yang et al., 2016). Thus, it appears that *B. licheniformis* could be a good candidate of AGP alternatives to prevent pathogen infections in the gut of animals.

In our study, we hypothesized that *B. licheniformis* HG76 could alleviate inflammatory reactions and tight junction damage in IPEC-J2 cells treated with ETEC F4. Therefore, our research focused on investigating how *B. licheniformis* HG76 could enhance defense mechanisms and mitigate inflammation in IPEC-J2 cells during pathogenic attacks, particularly by strengthening barrier functions and modulating immune responses.

5.3 Materials and methods

5.3.1 Materials

Dulbecco's modified Eagle's medium nutrient/Ham's F12 (**DMEM/F12** 1:1), streptomycin (100 $U \cdot mL^{-1}$), penicillin (100 $U \cdot mL^{-1}$), fetal bovine serum (**FBS**), and epidermal growth factor (**EGF**) were obtained from Thermo Fisher Scientific Inc., Ottawa, ON, Canada.

5.3.2 Cell culture

The IPEC-J2 cell line (ACC 701, RRID: CVCL_2246) was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Culture GmbH, Braunschweig, Germany. The cells were cultured in a 75 cm^2 flask in DMEM/F12 medium supplemented with 10% FBS, 1% penicillin (100 $U \cdot mL^{-1}$) and treptomycin (100 $U \cdot mL^{-1}$), and 3 $ng \cdot mL^{-1}$ EGF at 37°C in a humidified incubator (Thermo Electron Corp., Burlington, ON, Canada) with 95% O_2 and 5% CO_2. The culture medium was replaced every two to three days. After reaching 80-90% confluency,

the cells were digested with 0.25% trypsin and then split into 96 and 12 well plates (Corning Inc., New York City, NY, USA), respectively, for further viability, immunity and barrier function experiments.

5.3.3 Bacterial culture

The ETEC F4 strain was stored in 2 mL cryovials containing cryopreservative-infused broth and firstly incubated in tryptic soy broth (**TSB**, Sigma-Aldrich, Oakville, ON, Canada) at 37ºC for 16-18 h. Followed by dilution with fresh TSB medium, the cultures were sub cultured under the identical incubation conditions until reaching the logarithmic growth phase. When the optical density value at 600 nm (**OD_{600}**) reached approximately 0.3-0.5, measured by spectrophotometer (Thermo Fisher Scientific Inc., Ottawa, ON, Canada), the final ETEC F4 cultures were used for the following experiments.

5.3.4 Isolation of *B. licheniformis* HG76 and its culture preparation

The *B. licheniformis* HG76 was isolated from cecal contents of Rhode Island Red laying hens raised in antibiotic-free farms from Ontario, Canada. The cecal samples of hens were collected at day of sacrifice, then suspended in phosphate-buffered saline (**PBS**, pH 7.0) at 0.1 $g \cdot mL^{-1}$, and heat treated at 80°C for 10 mins, plated on tryptic soy agar (**TSA**, Sigma-Aldrich, Oakville, ON, Canada) at 1:100 and 1:1000 dilutions. After aerobic incubation at 37°C for 18 h, single colonies were selected.

To prepare a *B. licheniformis* HG76 culture for the IPEC-J2 cells trial, a single colony of *B. licheniformis* HG76 from the plate was incubated in TSB for 24 h with shaking (160-180 rpm) at 37ºC. The cultures were used for the following experiment when the OD_{600} values reached around 0.8-1.0.

5.3.5 Experimental design

The IPEC-J2 cells were detached using trypsin (0.25%, Thermo Fisher Scientific Inc., Ottawa, ON, Canada) and diluted to 5×10^5 cells·mL^{-1}. To reach 80-90% confluency, IPEC-J2 cells were subsequently inoculated into various well plates and cultured in DMEM/F12 medium for about 2-3 d. The treatments of each well were as follows: 1) CON (the control group, cells cultured in DMEM/F12 medium without treatment); 2) ETEC (the infection group, cells infected with ETEC F4 at a final concentration of 1×10^6 CFU·mL^{-1} in DMEM/F12 medium for 3 h); 3) E+H (The ETEC + HG76 group, cells pretreated with the selected concentration (5×10^6, 1×10^7, 2.5×10^7, 5×10^7, 1×10^8 CFU·mL^{-1}, respectively) of *B. licheniformis* HG76 for 2 h, after rinsing in PBS three times, the medium was then replaced with both ETEC F4 (1×10^6 CFU·mL^{-1}) and the selected concentration of *B. licheniformis* HG76 for another 3 h.); and 4) HG76 group (cells pretreated with $1x10^8$ CFU/mL of *B. licheniformis* HG76 for 2 h and changed them with new *B. licheniformis* HG76 at the same concentration for another 3 h).

5.3.6 Cell viability assay

The assessment of cell viability was performed utilizing water-soluble tetrazolium salts (**WST-1**), a reagent designed for cell proliferation analysis (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's manuals. In brief, IPEC-J2 cells were cultured in 96-well plates and then incubated with the treatments as follows: 1) CON (the control group, cells cultured in DMEM/F12 medium without treatment); 2) ETEC (the infection group, cells infected with ETEC F4 at a final concentration of 1×10^6 CFU·mL^{-1} in DMEM/F12 medium for 3 h); 3) T1-T5 (the ETEC + HG76 group, cells pretreated with *B. licheniformis* HG76 at 5×10^6 CFU·mL^{-1}, 1×10^7 CFU·mL^{-1}, 2.5×10^7 CFU·mL^{-1}, 5×10^7 CFU·mL^{-1}, 1×10^8 CFU·mL^{-1}, respectively for 2 h, after rinsing in PBS three times, the medium was then replaced with both ETEC F4 (1×10^6 CFU·mL^{-}

[1]) and *B. licheniformis* HG76 [5×10^6 CFU·mL^{-1} (1:5), 1×10^7 CFU·mL^{-1} (1:10), 2.5×10^7 CFU·mL^{-1} (1: 25), 5×10^7 CFU·mL^{-1} (1:50), 1×10^8 CFU·mL^{-1} (1:100)] for another 3 h.); and 4) HG76 group (cells pretreated with 1×10^8 CFU·mL^{-1} of *B. licheniformis* HG76 for 2 h and changed with new HG76 suspension at the same concentration for another 3 h). Subsequently, the cells underwent two washes using PBS, followed by the addition of 100 μL of fresh culture medium with a 10% concentration of WTS-1 to each well. Following one hour incubation, the absorbance at 450 nm was determined using a multimode microplate reader (Synergy H4, BioTek Instruments Inc., Winooski, VT, USA). The data on cell viability was expressed in terms of the percentage relative to the untreated control cells.

5.3.7 RNA extraction and Real-Time PCR

To detect mRNA expression of toll-like receptors, tight junction proteins and cytokines, IPEC-J2 cells were cultured in 12-well culture plate. The cells were treated with four different treatments as indicated in the experimental design. The cells were washed twice with 1 × PBS, and the total RNA was isolated by using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The RNA concentration and purity were determined by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Ottawa, ON, Canada) with purity of all RNA samples ascertained (OD_{260}:OD_{280} ratios) between 1.9 and 2.1. Reverse transcription was conducted with about 1 μg of total RNA from each sample by using the iScript cDNA Synthesis kit (Bio-Rad Ltd., Mississauga, ON, Canada) to synthesize cDNA. The primers for real-time PCR analysis were designed by using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and synthesized by Integrated DNA Technologies Inc. The sequences of primers are listed in **Table 5.1**. The Real-time PCR was performed by using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Ltd., Mississauga, ON, Canada), and the thermal programs of PCR reactions were as

follows: 3min at 95ºC, followed by 40 cycles of 20 s denaturing at 95ºC, 30 s annealing at 60ºC, and 30 s extension at 72ºC. Real-time PCR data were analyzed to determine the relative fold changes in gene expression levels by using the $2^{-\Delta\Delta CT}$ method, and the cyclophilin-A (**CycA**) gene was chosen as the housekeeping gene in IPEC-J2 cells (Zhao et al., 2021).

5.3.8 Western blotting

Proteins were extracted from IPEC-J2 cells using RIPA Lysate Buffer (MilliporeSigma Canada Ltd., Oakville, ON, Canada), and the concentrations of proteins were measured with the BCA Protein Assay Kit, following the instructions provided by the manufacturer (Thermo Fisher Scientific, Ottawa, ON, Canada). Proteins were separated using Mini-PROTEAN TGX Stain-Free™ Protein Gels (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) and then transferred onto the nitrocellulose membrane (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) by a Trans-Blot Turbo transfer system (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). The membranes were then blocked with 5% skim milk in tris-buffered saline with 0.1% Tween 20 (**TBST**) at 22 ℃ for 1.5 h, and incubated in primary antibodies from Thermo Fisher Scientific, Ottawa, ON, Canada: rabbit anti-ZO-1 (1:1,000), rabbit anti-occludin (1:1,000), and mouse anti-β-actin (1:4,000) that were diluted in 5% skim milk in TBST at 4◦C overnight. After washing the membranes for three times with TBST, the membranes were incubated with secondary antibodies goat anti-mouse IgG (1:500) for β-actin and goat anti-rabbit IgG (1:4,000) for ZO-1 and OCLN at 22 °C for 1 h. The Clarity Max ECL Western Blotting Substrate and ChemiDoc™ MP imaging system (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) were used for visualization of the proteins. Image Lab 6.0 software was utilized to analyze the protein bands. β-actin served as the internal standard. The levels of the target proteins were expressed as the ratio of the optical density of the protein bands to the optical density of the corresponding β-actin band.

5.3.9 Transepithelial electric resistance (TEER) and cell permeability measurement

The IPEC-J2 cells, at a density of 5×10^5 cells per well, were seeded into Millicell membrane cell inserts (12 wells, Corning Costar, New York, NY, USA). The TEER measured by a Millicell Electrical Resistance System (ESR-2, Sigma-Aldrich, Oakville, ON, Canada) was monitored every two days until the cell monolayer was fully differentiated. The TEER of four treatment groups: Control (CON), ETEC F4 alone (ETEC), ETEC F4 combined with *B. licheniformis* HG76 (E+H), and *B. licheniformis* HG76 alone (HG76) was measured before (0 h) and after ETEC F4 treatments (1-6 h), respectively.

To measure the paracellular permeability of cell monolayers, 0.5 $mg\cdot mL^{-1}$ of 4 kDa fluorescein isothiocyanate-dextran (**FITC-D4**, Sigma-Aldrich, Oakville, ON, Canada) was added to the inserts. After 48 h of incubation, aliquots of the basolateral medium were collected. The diffused FITC-D4 was measured using fluorometry (485 nm, 528 nm) with a microplate reader (BioTek, Winooski, VT, USA).

5.3.10 Statistical analysis

Data were displayed as means ± standard errors of the means (**SEM**). Statistical analysis was conducted using GraphPad Prism 9.5.1 (GraphPad Software Inc., La Jolla, CA, USA). Variations between average values were assessed using one-way analysis of variance (**ANOVA**). Tukey's multiple comparisons test was employed. Significance was determined at a *P*-value threshold of less than 0.05.

Table 5.1 Primer sequences of tight junction proteins, inflammatory cytokines, and toll-like receptors of piglets[1]

Genes	Amplicon (bp)	Sequence (5’ to 3’)	References
ZO-1	200	GATCCTGACCCGGTGTCTGA TTGGTGGGTTTGGTGGGTT	(Omonijo et al., 2018)
OCLN	163	GAGAGAGTGGACAGCCCCAT TGCTGCTGTAATGAGGCTGC	(Li et al., 2022)
CLDN1	93	CTGTGGATGTCCTGCGTGT GGTTGCTTGCAAAGTGGTGTT	(Lee and Kang, 2017)
CLDN3	123	CTACGACCGCAAGGACTACG TAGCATCTGGGTGGACTGGT	(Omonijo et al., 2018)
TNF-α	146	TTCCAGCTGGCCCCTTGAGC GAGGGCATTGGCATACCCAC	(Li et al., 2022)
IL8	126	CACCTGTCTGTCCACGTTGT AGAGGTCTGCCTGGACCCCA	(Omonijo et al., 2018)
TLR2	109	ACATGAAGATGATGTGGGCC TAGGAGTCCTGCTCACTGTA	(Tohno et al., 2005)

TLR4	108	GCCATCGCTGCTAACATCATC CTCATACTCAAAGATACACCA TCGG	(Li et al., 2022)
TLR5	86	GTTCTTTATCCGGGTGACTT AATAAGTCAGGATCGGGAGA	(Choi et al., 2020)
TLR7	107	GCTGTTCCCACTGTTTTGCC GAGCTGGATGAGGTTCCAAA	(Choi et al., 2020)
CycA	160	GCGTCTCCTTCGAGCTGTT CCATTATGGCGTGTGAAGTC	(Li et al., 2022)

[1]Note: OCLN: occludin; ZO-1: zonula occludens-1; CLDN1: claudin 1; CLDN3: claudin 3; TNF-α: tumor necrosis factor α; IL8: interleukin 8; TLR2: toll like receptor 2; TLR4: toll like receptor 4; TLR5: toll like receptor 5; TLR7: toll like receptor 7; CycA: cyclophilin-A.

5.4 Results

5.4.1 Effects of *B. licheniformis* HG76 on the viability of IPEC-J2 cells

As shown in **Figure 5.1**, ETEC F4 at 1×10^6 CFU·mL^{-1} induced significant cell death and this effect was significantly attenuated by simultaneously adding ETEC F4 with *B. licheniformis* HG76 ($P < 0.05$). However, there was no difference observed in the cell viability between the control cells and the cells treated with *B. licheniformis* HG76 alone ($P > 0.05$). Therefore, the concentration ratio of ETEC F4 and *B. licheniformis* HG76 at 1:5 (with 1×10^6 CFU·mL^{-1} ETEC F4: 5×10^6 CFU·mL^{-1} *B. licheniformis* HG76) was chosen in the following trial to investigate the effects of *B. licheniformis* HG76 on barrier function and inflammatory responses induced by ETEC F4 in the IPEC-J2 cells.

5.4.2 Effects of *B. licheniformis* HG76 on ETEC F4 induced gene expression of tight junctions in the IPEC-J2 cells

In order to explore the effects of *B. licheniformis* HG76 on gene expression of TJs in the IPEC-J2 cells infected with ETEC F4, the mRNA expressions of TJs (**OCLN**: occludin; **ZO-1**: zonula occludens-1; **CLDN1**: claudin 1; **CLDN3**: claudin 3) were measured by real time PCR. As shown in **Figure 5.2**, it was demonstrated that *B. licheniformis* HG76 didn't show significant differences in the mRNA expression of OCLN, CLDN1, and CLDN3 when compared with other groups ($P > 0.05$). However, the gene expressions of ZO-1 in the E + H group were significantly higher than those in the CON and ETEC groups ($P < 0.05$).

5.4.3 Effects of *B. licheniformis* HG76 on ETEC F4 induced gene expression of pro-inflammatory cytokines in the IPEC-J2 cells

As shown in **Figure 5.3**, there were no significant differences in the relative mRNA abundance of IL-8 in the four different treatments ($P > 0.05$). However, the expression of mRNA

TNF-α was significantly increased after challenging cells with ETEC F4 ($P < 0.05$), and the treatment with *B. licheniformis* HG76 could reverse this change.

5.4.4 Effects of *B. licheniformis* HG76 on ETEC F4 induced gene expression of toll-like receptors in the IPEC-J2 cells

As shown in **Figure 5.4**, the mRNA expression of TLR2, TLR4, and TLR5 were significantly higher in the ETEC F4 group ($P < 0.05$), but this increase in the mRNA expression could be attenuated by the *B. licheniformis* HG76 treatment. On the other hand, the mRNA level of TLR7 did not show any significant changes in the four different groups ($P > 0.05$).

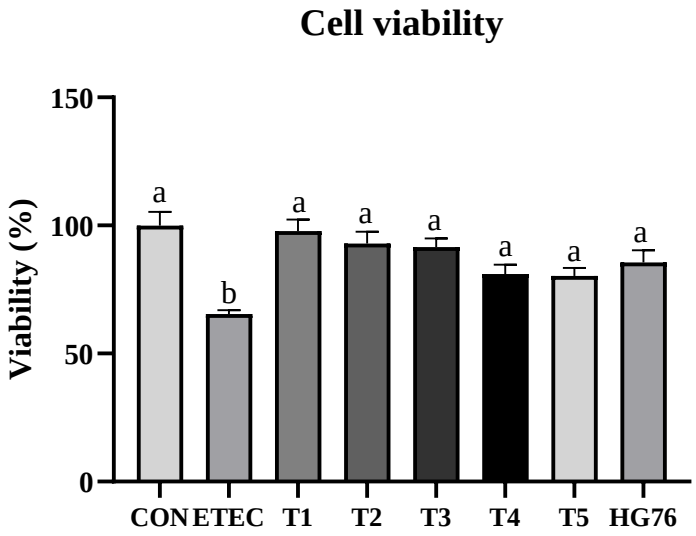
5.4.5 Effects of *B. licheniformis* HG76 on ETEC F4 induced protein expressions of tight junctions in IPEC-J2 cells

As the results show in **Figure 5.5**, *B. licheniformis* HG76 did not influence the proteins expressions of OCLN ($P > 0.05$), which was in agreement with the mRNA expression of OCLN. However, ETEC F4 infection significantly decrease protein expression levels of ZO-1 in IPEC-J2 cells ($P < 0.05$) when compared to the CON and HG76 groups.

5.4.6 Effects of *B. licheniformis* HG76 on TEER and cell permeability in IPEC-J2 cells

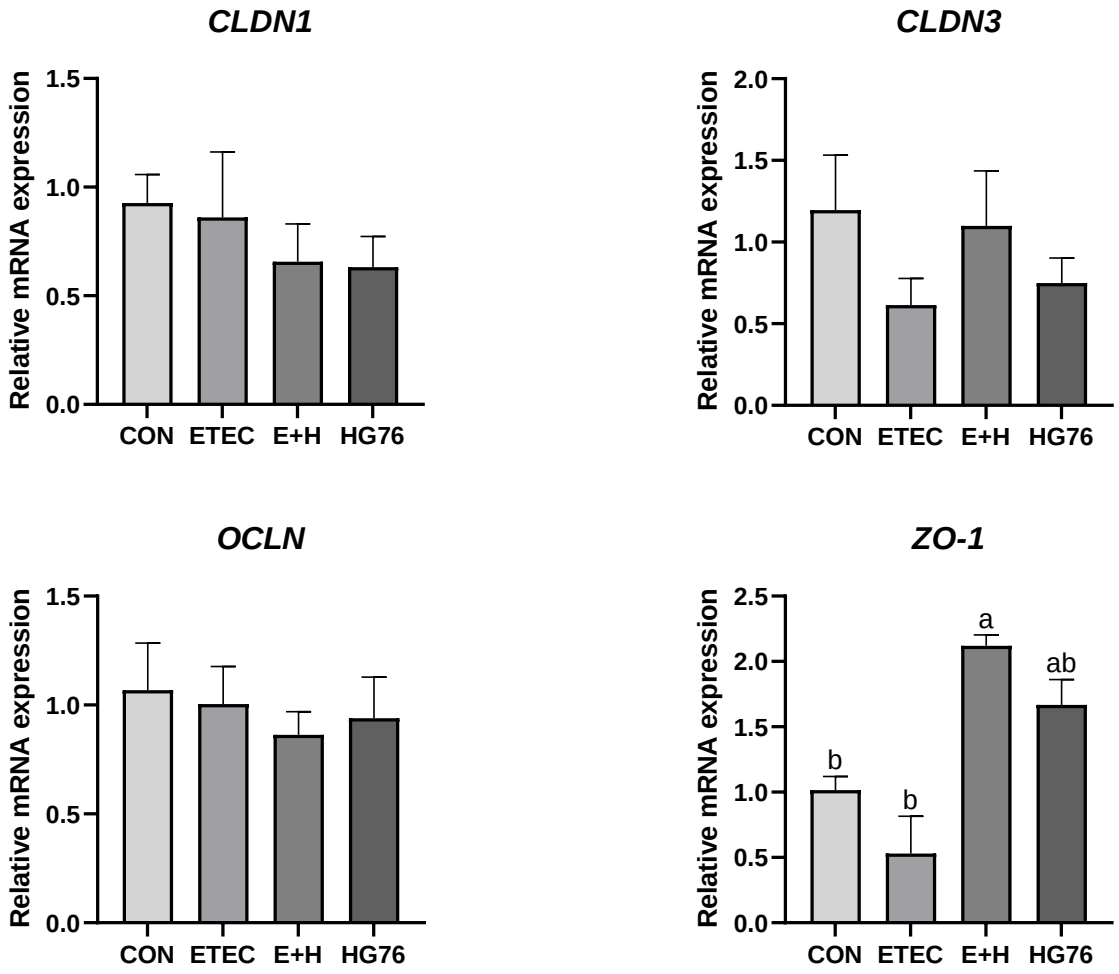
As shown in **Figure 5.6A**, there was no significant difference ($P > 0.05$) on the TEER values among all treatments. However, there was a significant increase (**Figure 5.6B**, $P < 0.05$) of fluorescence intensity after ETEC F4 challenge, while the *B. licheniformis* HG76 supplementation numerically decreased ($P > 0.05$) the fluorescence intensity in the E+H group.

Figure 5.1 Effects of *B. licheniformis* HG76 on the viability of enterotoxigenic *E. coli* F4 (ETEC F4) infection in the intestinal porcine epithelial cells (IPEC-J2 cells).



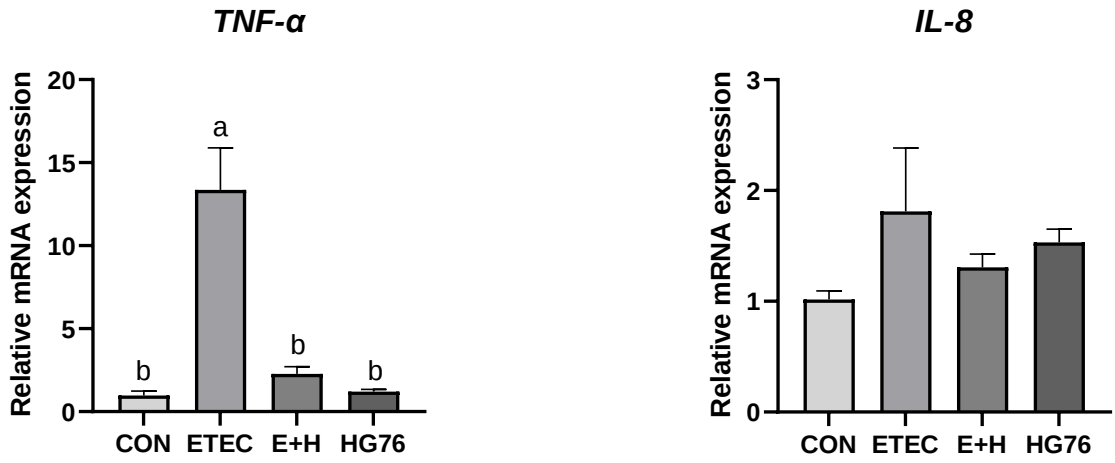
IPEC-J2 cells were treated with different groups. The CON group was without treatment. The ETEC group was treated with ETEC F4 at 1×10^6 CFU·mL^{-1}. The ETEC + HG76 groups (T1-T5) were pretreated with *B. licheniformis* HG76 (5×10^6, 1×10^7, 2.5×10^7, 5×10^7, 1×10^8 CFU·mL^{-1}, respectively) for 2 h, and then treated both ETEC F4 and *B. licheniformis* HG76 with various ratios (1:5, 1:10, 1: 25, 1:50, or 1:100) for another 3 h. The HG76 group were treated with 1×10^8 CFU·mL^{-1} of *B. licheniformis* HG76. Cell viability results were expressed as a proportion of the control group. The results are displayed as means ± SEM. Different superscript letters on bars indicate significant differences ($P < 0.05$).

Figure 5.2 Effects of *B. licheniformis* HG76 on enterotoxigenic *E. coli* F4 (ETEC F4) induced gene expression of tight junctions (TJ) in the intestinal porcine epithelial cells (IPEC-J2 cells).



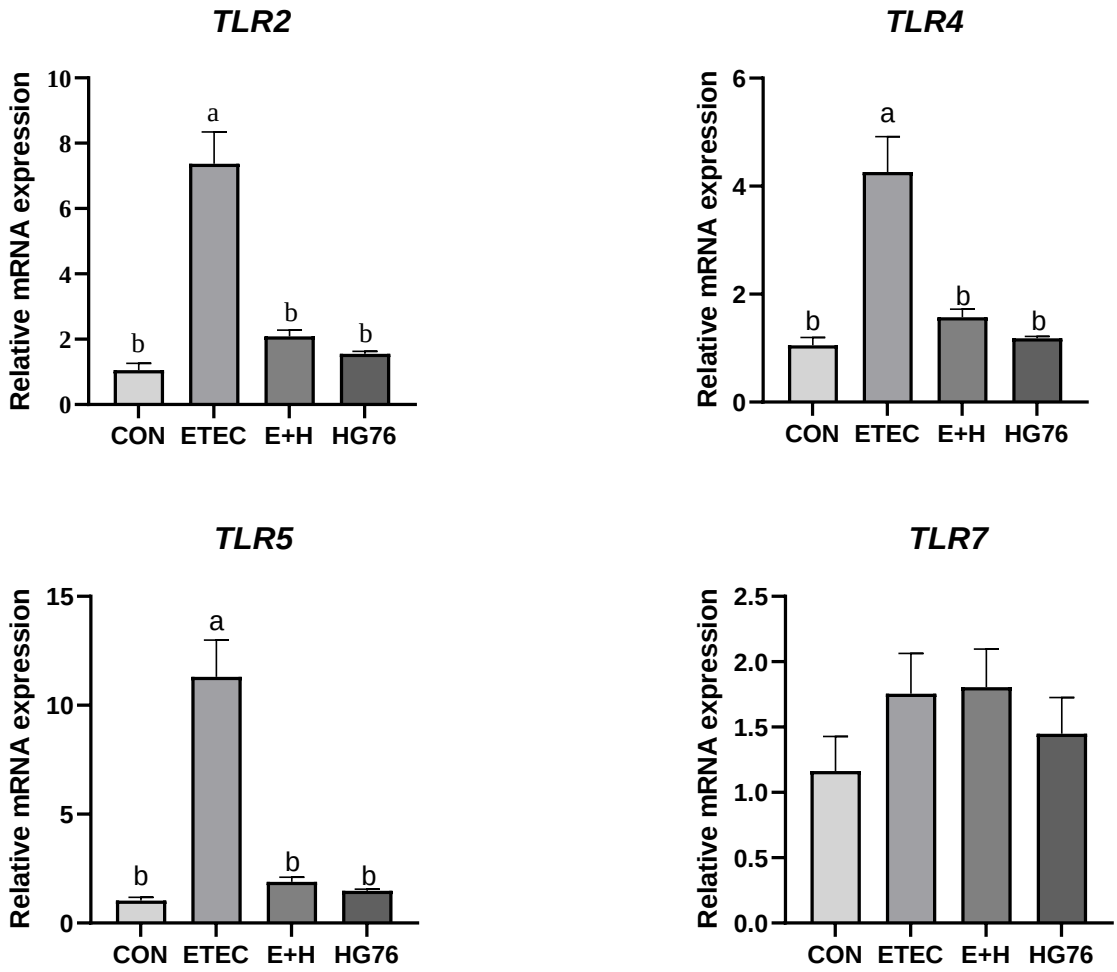
The CON group was with no treatment. The groups of ETEC, E+H, and HG76 were treated with 1×10^6 CFU/mL ETEC F4 alone, both ETEC F4 (1×10^6 CFU/mL) and *B. licheniformis* HG76 (5×10^6 CFU/mL), and 5×10^6 CFU/mL *B. licheniformis* HG76 alone, respectively. Total RNA was extracted from IPEC-J2 cells and the gene expressions of CLDN1, CLDN3, OCLN, and ZO-1 were detected by Real-Time PCR. The data were displayed as means ± SEM. Different superscript letters on bars indicate significant differences ($P < 0.05$).

Figure 5.3 Effects of *B. licheniformis* HG76 on enterotoxigenic *E. coli* F4 (ETEC F4) induced gene expression of pro-inflammatory cytokines in the intestinal porcine epithelial cells (IPEC-J2 cells).



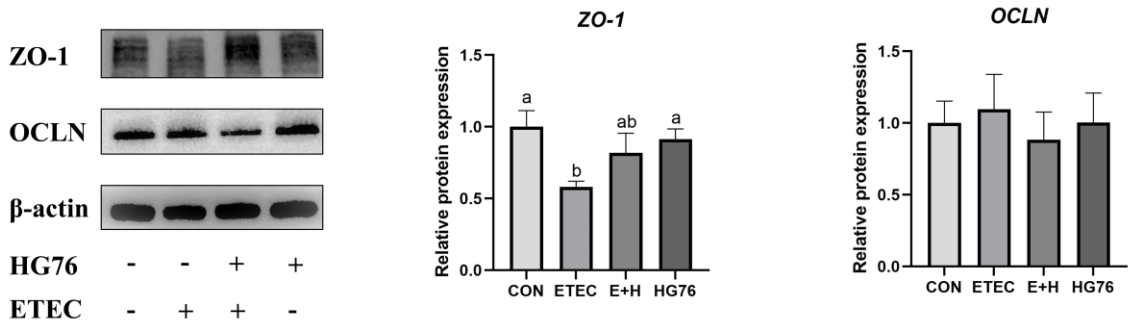
The CON group was with no treatment. The groups of ETEC, E+H, and HG76 were treated with 1×10^6 CFU/mL ETEC F4 alone, both ETEC F4 (1×10^6 CFU/mL) and *B. licheniformis* HG76 (5×10^6 CFU/mL), and 5×10^6 CFU/mL *B. licheniformis* HG76 alone, respectively. The data were analyzed using the $2^{-\Delta\Delta CT}$ method and displayed as means ± SEM. Different superscript letters on bars indicate significant differences ($P < 0.05$).

Figure 5.4 Effects of *B. licheniformis* HG76 on enterotoxigenic *E. coli* F4 (ETEC F4) induced gene expression of toll-like receptors in the intestinal porcine epithelial cells (IPEC-J2 cells).



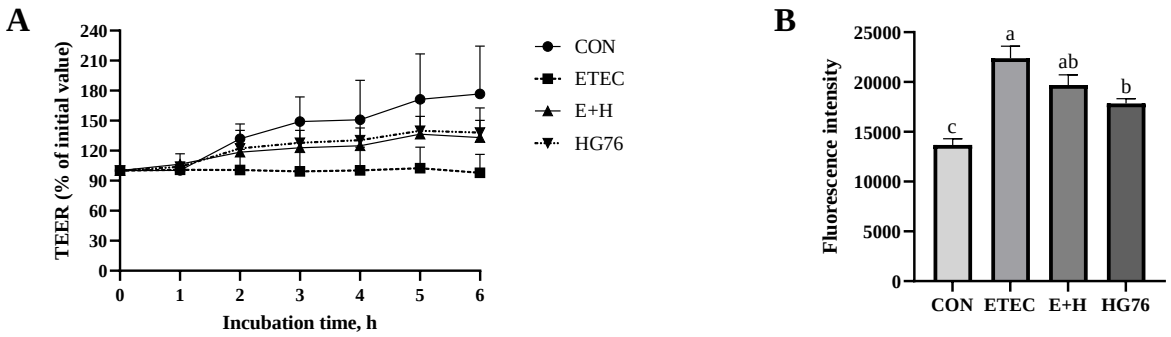
The CON group was with no treatment. The groups of ETEC, E+H, and HG76 were treated with 1×10^6 CFU/mL ETEC F4 alone, both ETEC F4 (1×10^6 CFU/mL) and *B. licheniformis* HG76 (5×10^6 CFU/mL), and 5×10^6 CFU/mL *B. licheniformis* HG76 alone, respectively. The data were analyzed using the $2^{-\Delta\Delta CT}$ method and displayed as means ± SEM. Different superscript letters on bars indicate significant differences ($P < 0.05$).

Figure 5.5 Effect of *B. licheniformis* HG76 on ETEC F4-induced expressions of tight junctions (TJ) protein in intestinal porcine epithelial cells (IPEC-J2 cells).



The CON group was with no treatment. The groups of ETEC, E+H, and HG76 were treated with 1×10^6 CFU/mL ETEC F4 alone, both ETEC F4 (1×10^6 CFU/mL) and *B. licheniformis* HG76 (5×10^6 CFU/mL), and 5×10^6 CFU/mL *B. licheniformis* HG76 alone, respectively. Total protein was extracted from IPEC-J2 cells and the protein expressions of ZO-1 and OCLN were detected by Western blotting. The data were presented as mean ± SEM. Different letters represent a significant difference ($P < 0.05$).

Figure 5.6 Effect of *B. licheniformis* HG76 on transepithelial electric resistance (TEER) and permeability in enterotoxigenic *E. coli* F4 (ETEC F4)-challenged intestinal porcine epithelial cells (IPEC-J2 cells).



(A) TEER values were measured before (0 h) and after ETEC F4 treatments (1-6 h). (B) Cell permeability was tested by the fluorescent intensity of FITC-D4. The CON group was with no treatment. The groups of ETEC, E+H, and HG76 were treated with 1×10^6 CFU/mL ETEC F4 alone, both ETEC F4 (1×10^6 CFU/mL) and *B. licheniformis* HG76 (5×10^6 CFU/mL), and 5×10^6 CFU/mL *B. licheniformis* HG76 alone, respectively. The data were displayed as mean ± SEM. Different superscript letters on bars indicate significant differences ($P < 0.05$).

5.5 Discussion

The intestine is not only proficient in digesting and absorbing nutrients, but it also houses a substantial number of immune cells. These cells act promptly when harmful bacteria penetrate the intestinal epithelium (Song et al., 2023). Diseases and malfunctions of the gut can lead to diarrhea and inflammatory bowel diseases, which negatively impact the health and growth of animals (Yeshi et al., 2020). The proper barrier function of intestinal epithelial cells (IPEC), which is crucial in providing physical defense, plays a significant role in maintaining gut health (Kocot et al., 2022). As a result, there is growing interest in nutritional strategies to strengthen the gut barrier for the health and growth of animals.

The widely recognized intestinal pathogen ETEC F4 is responsible for causing PWD and intestinal dysfunctions, which presents a significant challenge not only to human health but also to various species of farm animals (Augustino et al., 2022). In the livestock sector, where preventive antibiotic use for ETEC infections has been increasingly limited or banned in many countries, there is a growing development of AGP alternatives. These alternatives aim to reduce intestinal inflammation and maintain the digestive health of animals (Guevarra et al., 2019). Probiotics, a promising category among these alternatives, offer advantageous health effects to the host. Probiotics could achieve these by preserving the integrity of the gut barrier, modulating the immune response, inhibiting pathogenic bacteria, and generating bactericidal substances (Liao and Nyachoti, 2017; Iqbal et al., 2021).

As a type of gram-positive bacteria, *B. licheniformis* is widely recognized as a beneficial probiotic microorganism (Romo-Barrera et al., 2021). A recent study showed that *B. licheniformis* enhanced the growth performance of piglets, reduced damage to the intestinal mucosa caused by LPS challenge, enhanced immune function and mitigated inflammatory response through

increased levels of IgA and IgG and reduced concentrations of IL-6 and IL-1β, and protected against intestinal injury caused by LPS through the regulation of the NLR family pyrin domain containing 3 inflammasome (Yu et al., 2023). A study from our group reported that *B. licheniformis* PF9 might decrease pro-inflammation cytokines by inhibiting the NF-κB signaling pathways and plays a significant role in improving the integrity of IPEC-J2 cells (Li et al., 2022). Moreover, the inclusion of another probiotic of the same species, i.e., *B. licheniformis* S6 in the diet of weaned piglets enhanced intestinal integrity, partly due to its role in modulating intestinal barrier function and microbial diversity (Sun et al., 2023). Furthermore, feeding growing pigs with a diet supplemented with *Bacillus licheniformis* and *Bacillus subtilis* combination has been shown to notably enhance the digestibility of crude protein and reduce the counts of fecal *E. coli*, as well as decrease emissions of ammonia and hydrogen sulfide (Kim et al., 2022). While the *B. licheniformis* is commonly used as a feed additive, the specific mechanism by which it protects gut health from disruption caused by ETEC remains to be further determined. In the current study, the ETEC F4 was employed to create an *in vitro* model simulating the invasion of harmful bacteria. The present study hypothesized that *B. licheniformis* HG76 could play a role in mitigating the changes in gut barrier function and the inflammatory responses of IPEC-J2 and piglets caused by ETEC F4.

In an *in vitro* IPEC-J2 study, measuring cell viability and proliferation is crucial, because the WST-1 cell proliferation and viability assay serves as an effective method to assess cells that are active both mitochondrially and metabolically (Wendner et al., 2023; Rok et al., 2023). Findings of the present study showed that ETEC F4 led to considerable IPEC-J2 cell death. Furthermore, the treatment with *B. licheniformis* HG76 notably reduced the cell death in IPEC-J2 cells that were challenged with ETEC F4. These findings indicate that *B. licheniformis* HG76 is

effective in safeguarding IPEC-J2 from ETEC F4 infection, aligning with the outcomes of a previous study that reported a beneficial impact of another strain of the same species, i.e., *B. licheniformis* PF9, in preventing cell death and maintaining the cellular membrane integrity of IPEC-J2 cells (Li et al., 2022).

Gut barrier function is crucial for intestinal development and the progression of diseases. The proper functions of gut barrier are closely linked with functional TJ, which are situated between epithelial cells and serve a critical role in forming the complex structures for paracellular permeability (Di Sabatino et al., 2023). Therefore, changes in TJ can disrupt the normal paracellular permeability, affecting the transport of nutrients, and compromising the integrity of the epithelium (Aleman et al., 2023). The critical transmembrane protein ZO-1 is essential for maintaining the physiological functions of TJ. Increased expression of ZO-1 may lead to decreased gut permeability in IPEC-J2 cells or in weaned piglets (Li et al., 2023). The present study showed that ETEC F4 decreased the protein levels of ZO-1 and that treatment with *B. licheniformis* HG76 during ETEC F4 challenge could increase the mRNA expression of ZO-1 in IPEC-J2 cells. Similar results have also been reported by Wu et al. (2016) with another probiotic, i.e., *Lactobacillus plantarum* in a very similar ETEC challenge model. These authors found that *Lactobacillus plantarum* inhibited the reduction of TJ mRNA expression caused by ETEC K88. Moreover, the TEER and FITC-D4 are another useful parameters for evaluating the epithelial barrier function. The results showed that the treatment with *B. licheniformis* HG76 could enhance barrier functions in the IPEC-J2 cells.

The production of cytokines is viewed as a significant indicator of immune response and inflammation to ETEC F4 (Brubaker et al., 2021). A recent study found that the infection with ETEC notably raised the levels of TNF-α, but this increase was significantly diminished when

Bacillus subtilis CP9 was co-incubated with ETEC (Sudan et al., 2022). This corroborating evidence aligns with the data we have presented, showing that the mRNA levels of TNF-α significantly rose following exposure to ETEC F4, and treatment with *B. licheniformis* HG76 was able to reverse this change. These findings indicate that *B. licheniformis* HG76 may reduce pro-inflammatory cytokine production, potentially slowing cell damage and somewhat mitigating inflammation.

Cells possess numerous receptors that respond to external pathogenic signaling molecules, such as TLRs, and nucleotide-binding oligomerization domain (NOD)-like receptors (**NLR**) (El-Zayat et al., 2019). The key category of pattern recognition receptors, i.e., TLRs, found on immune cells and IPEC cells play an important role in triggering both inflammation and tolerance (Pardo-Camacho et al., 2018). The results showed that *B. licheniformis* HG76 treatment could attenuate the increased mRNA expression of TLR2, TLR4, and TLR5 due to the ETEC F4 infection, which is similar to the results reported by Sudan et al. (2022). We suggested that *B. licheniformis* HG76 administration was primarily implicated in competing for bindings sites of TLRs.

In conclusion, our study indicated that *B. licheniformis* HG76 alleviated tight junction damage and inflammatory reactions by increasing expression of tight junction proteins, inhibiting pro-inflammatory cytokines and toll-like receptors at the cellular level during ETEC F4 challenge.

CHAPTER SIX : MANUSCRIPT III

Dietary supplementation of *Bacillus licheniformis* HG76 influenced gut health of weaned piglets under enterotoxigenic *Escherichia coli* F4 infection[3]

6.1 Abstract

This study aimed to explore the potential of *Bacillus licheniformis* HG76 in improving gut health and performance of weaned piglets under enterotoxigenic *Escherichia coli* (**ETEC**) F4 challenge conditions. Twenty-eight weaned piglets susceptible to ETEC F4 at 28 d of age were individually allotted to four experimental groups: unchallenged control group (NNC, control diet), challenged control group (NC, control diet and pigs challenged with ETEC F4, 2.5×10^7 CFU per pig), antibiotic group (PC, control diet supplemented with avilamycin at 80 $mg \cdot kg^{-1}$ and pigs challenged with ETEC F4, 2.5×10^7 CFU per pig), and *Bacillus* group (HG76, control diet supplemented with *B. licheniformis* HG76 at 1.0×10^9 CFU kg^{-1} diet and pigs challenged with ETEC F4, 2.5×10^7 CFU per pig). During the post-challenge phase, ETEC F4 infection decreased average daily gain (**ADG**) in the NC group when compared to the NNC group ($P < 0.05$). Compared to the NNC group, the inoculation with ETEC F4 increased the severity of diarrhea at 44 h post-inoculum (**hpi**) in the NC group ($P < 0.05$). In addition, ETEC F4 infection decreased duodenal villus height (**VH**) in the NC, PC and HG76 groups when compared to the NNC group. The jejunal and ileal VH in the HG76 group were higher than other groups ($P < 0.05$). There was an increase ($P < 0.05$) in jejunal and ileal crypt depth (**CD**) in the HG76 group when compared to the NNC group. The pigs in the NNC group had the highest duodenal VH:CD ratio ($P < 0.05$). The V*max* value of sucrase in the HG76 group was higher than the NNC group ($P < 0.05$). When compared to the NC group, the relative mRNA level of interleukin 6 (**IL-6**) was increased in the PC group ($P < 0.05$). This study reveals that although the supplementation of *Bacillus licheniformis* HG76 improved intestinal morphology and increased digestive enzyme activity, it had limited impacts on growth performance and severity of diarrhea in ETEC F4 challenged piglets.

Therefore, further studies under commercial practice conditions are required to determine its potential in improving performance and gut health of piglets.

Key words: Post-weaning diarrhea, *Bacillus licheniformis* HG76, enterotoxigenic *E. coli* F4, gut health, weaned piglets

6.2 Introduction

Weaning is a critical period for piglets as they are subjected to diets, environmental, and social changes. The immature immune system of newly weaned piglets increases the susceptibility to pathogens due to such changes. Post-weaning diarrhea (**PWD**), commonly caused by enterotoxigenic *Escherichia coli* (**ETEC**), is one of the most serious and economically important diseases in global swine production systems (Rhouma et al., 2017; García et al., 2020). In-feed antibiotic growth promoters (**AGP**) have been widely used as a tool for piglets smooth transition during the post weaning period, effectively preventing PWD and improving growth performance (Han et al., 2021). However, the prophylactic use of AGP has been suggested to markedly increase the risk of antimicrobial resistance, which becomes a threat to global public health systems and as a consequence their use has been banned in many countries (Hernandez et al., 2022; Lardé et al., 2021). The quest for AGP alternatives has intensified with special focus in recent years being placed on organic acids, enzymes, probiotics, prebiotics, synbiotics, minerals, phytogenics, antimicrobial peptides, bacteriophages, and egg yolk antibodies (Liu et al., 2018). Of these feed additives classes, probiotics have gained recognition for maintaining intestinal health by improving the digestion and absorption of nutrients, enhancing immune response, altering microbiome composition, and improving growth performance (Ding et al., 2021). Some probiotics, such as *Lactobacillus*, *Bifidobacterium*, and *Bacillus*, show antimicrobial activity against some pathogens. For example, it has been reported that *Bacillus* spp. had ability to produce a number of

digestive enzymes (e.g., carbohydrase and proteases), increase nutrient digestibility, deliver antimicrobial peptides, and inhibit the growth of pathogenic bacteria (Horng et al., 2019; Bahaddad et al., 2023). Our group found that *B. licheniformis* PF9 from swine feces could attenuate the expression levels of inflammation-related cytokines, and alleviate the injury of epithelial barrier function caused by ETEC F4 infection (Li et al., 2022). Furthermore, pigs supplemented with *B. licheniformis* PF9 had better health as indicated by improved nutrients' absorption, gut barrier integrity, immunity, and digesta microbiota (Xu et al., 2024). Therefore, the current study was to explore the potential of *B. licheniformis* strain HG76 to improve gut health and growth performance of weaned piglets under ETEC F4 challenge conditions.

6.3 Materials and methods

6.3.1 Bacterial preparation

An aliquot from the stock ETEC F4 stored at -80°C freezer was aerobically grown on tryptic soy agar (**TSA**) plate for 16 h at 37°C. A single colony with clear hemolysis was inoculated to 10 mL of sterile tryptic soy broth (**TSB**) and aerobically incubated for 16-18 h with shaking (150 rpm) at 37°C. The culture was further sub-cultured by inoculating 10 mL of TSB with 1% inoculum and further incubated for 2.5 h at 37°C with orbital shaking at 150 rpm until the OD_{600} value reached 0.3. Following dilution by sterile phosphate-buffered saline (**PBS**), the final ETEC F4 inoculum was approximately 5×10^6 CFU·mL^{-1}.

To prepare a HG76 culture for the animal trial, a single colony of *B. licheniformis* HG76 from an agar plate was inoculated into 6 mL of TSB medium and incubated without shaking for 24 h at 37ºC to get an initial culture. The initial culture was mixed with 250 mL of TSB medium in a 500 mL flask without shaking for another 24 h at 37ºC. All the incubation processes were

conducted in aerobic condition. The culture (500 mL) was mixed with 50 kg of basal diet in a mixer for 20 mins, with final inclusion of 1.0×10^9 CFU kg^{-1} of *B. licheniformis* HG76 in the diet.

6.3.2 Animals selection

The weaned piglets susceptible to ETEC F4 were selected according to the method reported by Jensen et al. (2006). In brief, the tails of piglets were collected when docked on 3 days after birth. DNA of tails were extracted following the method described by Truett et al., (2000). The mucin 4 (**MUC4**) gene, encoded for the F4 fimbriae receptor, was detected by a PCR assay. The assay was completed on genomic DNA in a total mixture volume of 25 µL, containing 2.5 µL of reaction buffer, 0.5 µL of dNTP, 0.2 µL of DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 1.0 µL of each primer: forward (5′-GTGCCTTGGGTGAGAGGTTA-3′) and reverse (5′-CACTCTGCCGTTCTCTTTCC-3′). Thermocycling was subjected to initial denaturation at 95℃ for 5 min and followed by 35 cycles of amplification consisting of a 30 s denaturing at 95℃, 30 s annealing at 65℃, 1 min extension at 72℃, and final extension for 10 min at 72℃. Enzyme digestion by XbaI (Thermo Fisher Scientific, Waltham, MA, USA) to identify polymorphism on 5 µL PCR product was performed at 37℃ for 5 min following the supplier's protocol. The PCR products after digestion were stained by SYBR Green (Invitrogen, Carlsbad, CA, USA) and run on a 2% agarose gel using 100 bp gene ladder by electrophoresis for 40 min at 80 V. Bands were visualised on ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA) with resistant allele (R) viewed as one single band at 367 bp, whereas the susceptible allele (S) viewed as two bands at 151 bp and 216 bp (Choi et al., 2020).

6.3.3 Experimental design

A total of 28 TN Tempo × TN70 piglets (weaned on d 21, initial body weight 7.80 ± 0.14 kg) were obtained at the age of 28 d from the Glenlea Swine Research Unit and transferred to T.K.

Cheung Centre for Animal Science Research of the University of Manitoba. All piglets were randomly assigned to 4 experimental groups (7 replicates per treatment, 4 gilts and 3 barrows): 1) unchallenged control group (NNC, control diet), 2) challenged control group (NC, control diet and pigs challenged with ETEC F4, 2.5×10^7 CFU per pig), 3) antibiotic group (PC, control diet supplemented with avilamycin at 80 mg·kg^{-1} and pigs challenged with ETEC F4, 2.5×10^7 CFU per pig), and 4) *Bacillus* group (HG76, control diet supplemented with *B. licheniformis* HG76 at 1.0×10^9 CFU kg^{-1} diet and pigs challenged with ETEC F4, 2.5×10^7 CFU per pig). All pigs were housed in individual pens with free access to water and feed throughout the trial period (11 days). Animal rooms were equipped with air conditioners, heaters, and fans to achieve controlled temperature (29 ± 1ºC before challenging (0-7 d), and decreased by 1.5ºC after challenging (8-11 d)). The corn-soybean meal-fish meal basal diet (**Table 6.1**) was formulated to meet or exceed the NRC (2012) requirements for 7-11 kg piglets. Pigs and feeders were weighed at the beginning of the trial, before inoculation and at the end of the trial. Average daily gain (**ADG**), average daily feed intake (**ADFI**), and gain:feed were calculated for pre-challenge, post-challenge, and the whole experimental period. Each piglet in the NC, PC and HG76 groups was orally inoculated with 5 mL of ETEC F4 (5×10^6 CFU·mL^{-1}) on d 7, whereas the piglet in the NNC group received the same volume of PBS (Koo et al., 2020). The fecal scores ranging from 0 to 3 (0 = normal feces, 1 = soft feces, 2 = mild diarrhea, and 3 = severe diarrhea.) were graded every 4 h until 48 h post-inoculum (hpi) by the method described by Marquardt et al. (1999). The rectal temperature was measured before the inoculation, 4, 24, 48, and 72 hpi. The protocols (F19-020/2, AC11516) describing the management and care of animals were reviewed and approved by the Animal Care Committee of the University of Manitoba, and the piglets were cared for under the guidelines of Canadian Council on Animal Care (CCAC, 2009).

6.3.4 Intestinal tissue sampling

At the end of the trial (d 12), all piglets were sedated by intramuscular injection of ketamine:xylazine and sacrificed. The abdominal cavity was opened up to expose the gastrointestinal tract (**GIT**) without damaging its wall. The mid points of the duodenum (8 cm from the stomach), jejunum (4 m from stomach-duodenum junction), and ileum (10 cm from the ileum-cecum junction) of each piglet were obtained and fixed in 10% formaldehyde solution for morphological analyses. A 10 cm of mid-jejunum segment was collected in cold Krebs-ringer bicarbonate (**KRB**) buffer, and immediately delivered to the laboratory for the Ussing chamber assay. The other 15 cm of mid-jejunum sample was obtained from each pig and storage in –80°C freezer for enzyme and gene expression analyses (Choi et al., 2020).

6.3.5 *In vivo* gut permeability

Each piglet was orally administrated fluorescein isothiocyanate-dextran 70 kDa (**FITC-D70**; Sigma-Aldrich Co., St. Louis, MO, USA) at the dose of 10 mg in 5 mL of PBS buffer on 3 dpi. At 4 h after oral administration, blood samples (6 mL per pig) were collected via jugular vein puncture into vacutainer tubes (Becton Dickinson, Rutherford, NJ, USA). The tubes were covered by aluminum foil for light blocking and clotting at 22ºC for 3 h, and then centrifuged at 2,000 × g at 4 ºC for 15 min to separate the serum from the red blood cells. The FITC-D70 levels in the obtained serum were measured at the excitation wavelength of 485 nm and emission wavelength of 528 nm using a Bio-Tek PowerWave^{TM} HT Microplate Scanning Spectrophotometer (BIO-TEK Instruments Inc., Winooski, VT, USA) and calculated via standard curve (Choi et al., 2020).

6.3.6 *Ex vivo* Ussing chamber to measure gut permeability

The gut epithelia tight junction permeability was measured by transepithelial electrical resistance (**TEER**) in Ussing chambers (Physiologic Instruments Inc., San Diego, CA, USA).

Tunica mucosa and *tela submucosa* of fresh mid-jejunum samples were stripped of underlying *tunica muscularis* and *tunica serosa* by scissors and tweezers, and mounted in Ussing chambers to equilibrate for 10 min. Mucosal and serosal chambers were filled with 5 mL warm (37°C) KRB solutions containing 10 mM D-mannitol and D-glucose respectively to maintain osmotic balance. The chambers were kept by pumping mixed gas (O_2/CO_2, 95%/5%) and maintained at 37°C by water-jacketed reservoirs. The voltage and current electrodes were connected to each chamber by 3% KCl-agar bridges filled with KRB buffer. Tissue TEER values were monitored constantly after mounting for 10 min as measures of tissue integrity with the tissue unclamped in open circuit mode, and determined from voltage deflections induced by bipolar constant-current pulses (Choi et al., 2020).

6.3.7 Gut morphology

Each intestinal sample embedded in paraffin was cut into 5 μm thick slices and laid flat onto glass slide. The dewaxed parts were soaked twice in xylene, 100% and 95% ethanol for 5 min each. The samples were immersed by Alcian blue at 22ºC for 15 min, cleaned with double-distilled water for 2 min, followed by staining with Schiff for 10 min and a wash in double-distilled water for 10 min. After second staining with hematoxylin for 10 seconds, the stained slides were then cleared and dehydrated. Each slide was photographed and visualized by the digital camera (Lumenera Corp., Ottawa, ON, Canada) and the microscope (Carl Zeiss Microscopy Deutschland GmbH, Göttingen, Germany) for quantification of Alcian blue/ Periodic acid–Schiff (**AB/PAS**) staining. A minimum of 15 intact, well-oriented crypt-villus parts of each sample were assessed and villus height (**VH**), crypt depth (**CD**) and VH:CD were calculated using the *ImageJ* software (Choi et al., 2020). Samples' VH was measured from the tip of the villi to the villus-crypt junction, while CD was calculated based on the invaginated depth between adjacent villi.

6.3.8 Intestinal digestive enzyme activities

Maximal enzyme activity ($\mathbf{V}_{max}$) of some intestinal digestive enzymes, including, intestinal alkaline phosphatase (**IAP**), sucrase, and maltase in the mid-jejunum were measured. In brief, after thawing, around 200 mg of mid-jejunum sample was homogenized in ice-cold homogenizing buffer (50 mM D- mannitol and 0.1 mM Phenylmethylsulfonyl fluoride at pH 7.4) using a polytron homogenizer at max speed for 90 s. After centrifuging at 3,000 × g for 15 min, the supernatant was assayed for the protein content by BCA Protein Assay Kit (Thermo Fisher Scientific, Ottawa, ON, Canada), followed by the measurement of the sucrase, maltase, and IAP activities, in accordance with the method described by Dahlqvist (1964) and Hübscher and West (1965), respectively.

6.3.9 Quantitative real-time PCR

The total RNA was isolated from mid-jejunum using the total RNA isolation kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Quantification and quality control of the RNA samples were assessed by the ND spectrophotometer (Thermo Fisher Scientific Inc., Ottawa, ON, Canada) with an optimal ratio OD_{260}:OD_{280} values between 1.8 and 2.0, and confirmed by 1.5% of agarose gel electrophoresis. cDNA was synthesized using iScript^{TM} cDNA Synthesis Kit (Bio-Rad Laboratories, Mississauga, ON, Canada) with 1,000 ng of RNA as template. Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA), the sequence and amplified fragments' length were shown in **Table 6.2**. The amplification was carried out in a overall volume of 20 μL containing 1 μL of cDNA, 300 nmol·L^{-1} of each primer, and 10 μL of SYBR Green supermix (Biorad, Ontario, Canada) on a Real-Time PCR Detection System (Bio-Rad Laboratories, Ontario, Canada) (Omonijo et al., 2019). The PCR reaction cycling conditions were initial denaturation at 95°C for 3 min, followed by 40 cycles of 20 s at 95°C, 30 s at 60°C

and then 30 s at 72°C. The housekeeping gene GAPDH was used as an internal control for normalization. The target gene relative mRNA expression levels were calculated using the $2^{-\Delta\Delta}$CT method described by Livak and Schmittgen (2001).

6.3.10 Statistical analysis

The design was a randomized complete block design, with each individual animal as the experimental unit and dietary treatments as the main effect with no random effects. All data were analyzed using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC, USA). The least-square means statement with the Tukey-adjusted PDIFF option was applied to separate and calculate the mean value of each treatment. Results are expressed as the mean ± SEM. Differences were considered statistically significant at $P < 0.05$, and trends ($0.05 \leqslant P \leqslant 0.10$) were also presented.

Table 6.1 Composition and nutrient level of the basal diet[1] (kg, as-fed basis)

Item	Basal diet
Ingredients, %	
Corn	48.4
Soybean meal (48% crude protein)	16.0
Whey permeate	12.4
X-SOY600[2]	11.0
Fish meal	6.6
Soybean oil	1.5
Limestone	1.42
Vitamin-mineral premix[3]	1.0
Biofos[4]	0.57
Salt	0.5
L-lysine	0.28
Threonine	0.13
DL-methionine	0.15

L-tryptophan	0.05
Calculated nutrient levels (%)	
Crude protein	22.35
SID[5] Lysine	1.34
SID Threonine	0.87
SID Methionine	0.5
SID Tryptophan	0.27
Net energy (kcal·kg^{-1})	2,475
Metabolizable energy (kcal·kg^{-1})	3,390
Analyzed nutrient levels (%)	
Crude protein	21.60
Dry matter	90.81
NDF[7]	5.86
ADF[8]	2.72

Note: [1]The diet for PC was based on the basal diet supplemented with 80 mg·kg^{-1} of avilamycin (Surmax 100 Premix, avilamycin premix 100 g·kg^{-1}, Elanco Canada Limited, Guelph, Ontario, Canada). The HG76 diet was supplemented with 1×10^9 CFU·kg^{-1} of *B. licheniformis*.

[2]Soy protein concentration (600 g crude protein·kg^{-1}, CJ Selecta, Goiania, State of Goiás, Brazil).

[3]Supplied per kilogram of diet as follows: 2,200 IU vitamin A, 1.5 mg vitamin B1, 0.02 mg vitamin B12, 4 mg vitamin B2, 0.2 mg biotin, 220 IU vitamin D3, 30 mg niacin, 600 mg choline chloride, 16 IU vitamin E, 0.5 mg vitamin K, 0.3 mg folic acid, 7 mg pyridoxine, 12 mg calcium pantothenate, 6 mg Cu as copper sulphate, 0.3 mg Se as sodium selenite, 0.14 mg I as calcium iodate, 100 mg Zn as zinc oxide, 4 mg Mn as manganese oxide, and 100 mg Fe as ferrous sulfate.

[4]Monocalcium phosphate (21% Ca and 17% P, Mosaic Co., Plymouth, MN, USA).

[5]Standardized ileal digestibility.

Table 6.2 Primer sequences of ETEC F4 receptor, nutrient transporters, tight junction proteins, and inflammatory cytokines of piglets[1]

Genes	Amplicon (bp)	Sequence (5' to 3')	References
MUC4	367	GTGCCTTGGGTGAGAGGTTA CACTCTGCCGTTCTCTTTCC	(Jensen et al., 2006)
OCLN	93	CTGTGGATGTCCTGCGTGT GGTTGCTTGCAAAGTGGTGTT	(Lee and Kang, 2017)
CLDN3	123	CTACGACCGCAAGGACTACG TAGCATCTGGGTGGACTGGT	(Omonijo et al., 2018)
B^0AT1	102	AGGCCCAGTACATGCTCAC CATAAATGCCCCTCCACCGT	(C. Yang et al., 2016)
SGLT1	153	GGCTGGACGAAGTATGGTGT GAGCTGGATGAGGTTCCAAA	(Yang et al., 2010)
PepT1	143	ATCGCCATACCCTTCTG TTCCCATCCATCGTGACATT	(Omonijo et al., 2018)
ASCT2	206	GCCAGCAAGATTGTGGAGAT GAGCTGGATGAGGTTCCAAA	(C. Yang et al., 2016)
EAAC1	168	CCAAGGTCCAGGTTTTGGGT GGGCAGCAACACCTGTAATC	(Omonijo et al., 2018)

IL-6	69	GACAAAGCCACCACCCCTAA CTCGTTCTGTGACTGCAGCTT ATC	(Dowley et al., 2022)

Note: MUC4: mucin 4; OCLN: occludin; CLDN3: claudin 3; EAAC1: excitatory amino acid transporter 1; ASCT2: neutral amino acid transporter 2; B^0AT1: neutral amino acid transporter; PepT1: peptide transporter 1; SGLT1: Na^+-glucose cotransporter 1; IL-6: interleukin-6.

6.4 Results

6.4.1 Growth performance, fecal score, and rectal temperature

There was no significant difference ($P > 0.05$) in the ADFI, ADG, and Gain:Feed found during the pre-challenge (0-7 d) and whole phase (0-11 d) among all treatments (**Table 6.3**), but there was a trend ($P = 0.078$) of increase in ADFI in the HG76 group when compared to the NNC group. During the post-challenge phase, ETEC F4 infection significantly decreased ADG in the NC group when compared to the NNC group ($P < 0.05$). There was no significant difference in the rectal temperature observed among all treatment groups during 72 hpi ($P > 0.05$) (**Figure 6.1**). As shown in **Figure 6.2**, compared to the NNC group, the inoculation with ETEC F4 significantly increased the severity of diarrhea at 44 hpi in the NC group ($P < 0.05$).

6.4.2 Gut morphology

The inoculation with ETEC F4 significantly ($P < 0.05$) decreased duodenal VH in the NC, PC and HG76 group when compared to the NNC group, and VH of jejunum and ileum in HG76 group were significantly higher than other groups. There was a significant increase ($P < 0.05$) in jejunal and ileal CD in the HG76 group when compared to the NNC group. The duodenal VH:CD ratio in the NNC group was the highest ($P < 0.05$) among treatments (**Figure 6.3**).

6.4.3 Gut permeability

There was no significant difference ($P > 0.05$) found in TEER measured using the Ussing chamber and *in vivo* gut permeability by means of FITC-D70 flux, among all treatments (**Table 6.4**).

6.4.4 Intestinal digestive enzyme activities

There was no significant difference ($P > 0.05$) in the V*max* values of IAP and maltase among all treatments. However, the V*max* value of sucrase in the HG76 group was significantly ($P < 0.05$) higher than the V*max* in the NNC group (**Figure 6.4**).

6.4.5 Relative mRNA abundance of tight junctions, nutrient transporters and immunity markers in the jejunum

The relative mRNA levels of genes in the mid-jejunum related to intestinal barrier function and immunity are shown in **Figure 6.5**, while those related to nutrient transportation are indicated in **Figure 6.6**. There was no significant difference ($P > 0.10$) observed in the relative mRNA levels of claudin 3 (**CLDN3**), occludin (**OCLN**), neutral amino acid transporter 2 (**ASCT2**), excitatory amino acid transporter 1 (**EAAC1**), peptide transporter 1 (**PepT1**), Na^+-glucose cotransporter 1 (**SGLT1**), and neutral amino acid transporter (**B^0AT1**) among all treatments. When compared to the NC group, the relative mRNA level of interleukin 6 (**IL-6**) was significantly ($P < 0.05$) increased in the PC group.

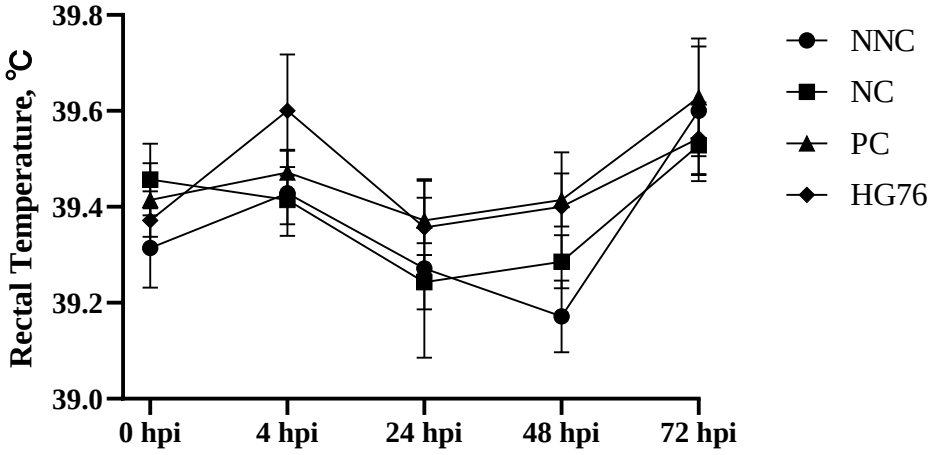
Table 6.3 Effects of *B. licheniformis* HG76 on the growth performance of weaned piglets during the pre-challenge phase (0-7 d), post-challenge phase (7-11 d) and whole phase (0-11 d)

		ETEC F4-challenged				
Items	NNC	NC	PC	HG76	SEM	*P* value
Pre-challenge phase						
ADG ($g \cdot d^{-1}$)	209	223	257	261	12.39	0.336
ADFI ($g \cdot d^{-1}$)	253	290	304	309	9.84	0.078
Gain:Feed	0.747	0.754	0.885	0.871	0.031	0.248
Post-challenge phase						
ADG ($g \cdot d^{-1}$)	460[a]	303[b]	313[ab]	328[ab]	23.31	0.013
ADFI ($g \cdot d^{-1}$)	482	419	450	452	14.86	0.345
Gain:Feed	0.842	0.717	0.720	0.801	0.038	0.437
Whole phase						
ADG ($g \cdot d^{-1}$)	263	244	287	272	13.37	0.705
ADFI ($g \cdot d^{-1}$)	315	349	361	346	9.79	0.203
Gain:Feed	0.826	0.719	0.782	0.778	0.024	0.292

Note: NNC (unchallenged control): pigs fed a control diet and gavage with phosphate buffered saline; NC (challenged control): pigs fed a control diet and challenged with ETEC F4, 2.5×10^7 CFU per pig; PC (antibiotic): NC + 80 $mg \cdot kg^{-1}$ of avilamycin and pigs challenged with ETEC F4; HG76: NC + 1×10^9 CFU kg^{-1} feed of *B. licheniformis* HG76 and pigs challenged with ETEC F4; ADG: average daily gain; ADFI: average daily feed intake.

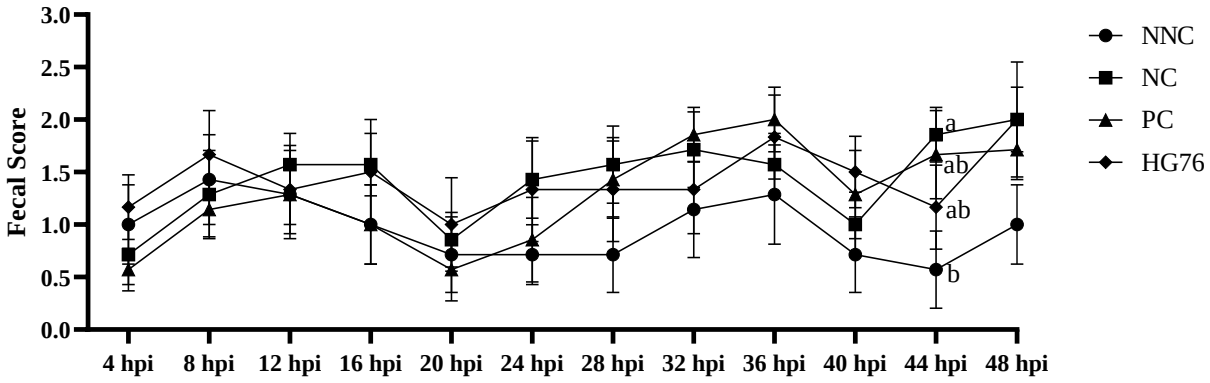
[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

Figure 6.1 Effects of *B. licheniformis* HG76 on rectal temperature in weaned piglets.



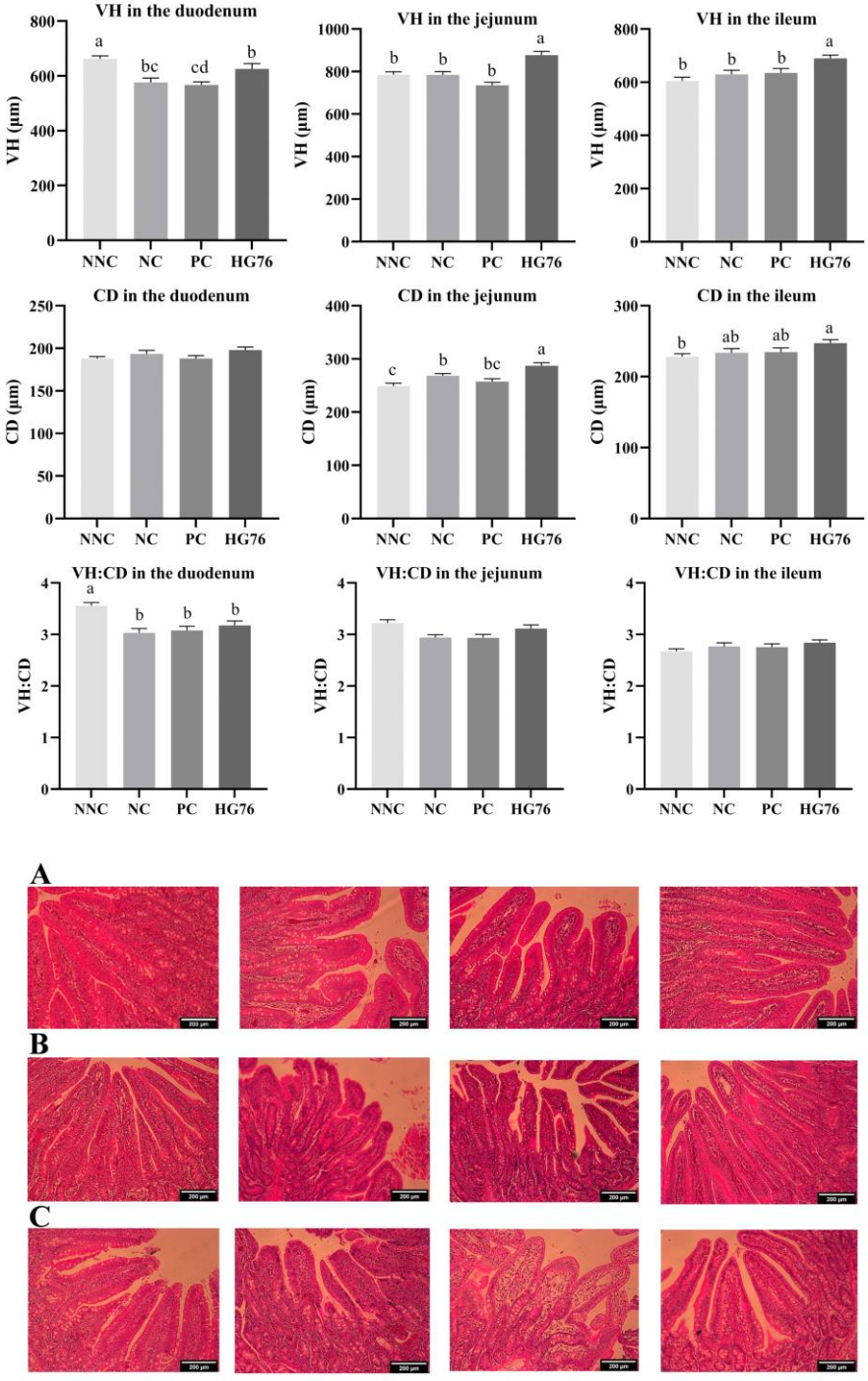
Rectal temperature of weaned piglets was measured during 72 hpi (hour post-inoculation) in the NNC (unchallenged control): pigs fed a control diet and gavage with phosphate buffered saline; NC (challenged control): pigs fed a control diet and pigs challenged with ETEC F4, 2.5×10^7 CFU per pig; PC (antibiotic): NC + 80 mg·kg^{-1} of avilamycin and pigs challenged with ETEC F4; HG76: NC + 1×10^9 CFU kg^{-1} feed of *B. licheniformis* HG76 and pigs challenged with ETEC F4. Each value represents the mean ± SEM.

Figure 6.2 Effects of *B. licheniformis* HG76 on fecal score in weaned piglets.



Fecal score of weaned piglets was measured during 48 hpi in the NNC (unchallenged control): pigs fed a control diet and gavage with phosphate buffered saline; NC (challenged control): pigs fed a control diet and challenged with ETEC F4, 2.5×10^7 CFU per pig; PC (antibiotic): NC + 80 mg·kg^{-1} of avilamycin and pigs challenged with ETEC F4; HG76: NC + 1×10^9 CFU kg^{-1} feed of *B. licheniformis* HG76 and pigs challenged with ETEC F4. Fecal score = 0, normal feces; 1, soft feces; 2, mild diarrhea; and 3, severe diarrhea. Each value represents the mean ± SEM. Bars with different letters are significantly different ($P < 0.05$).

Figure 6.3 Effects of *B. licheniformis* HG76 on gut morphology including villus height, crypt depth and their ratio in the small intestine of weaned piglets.

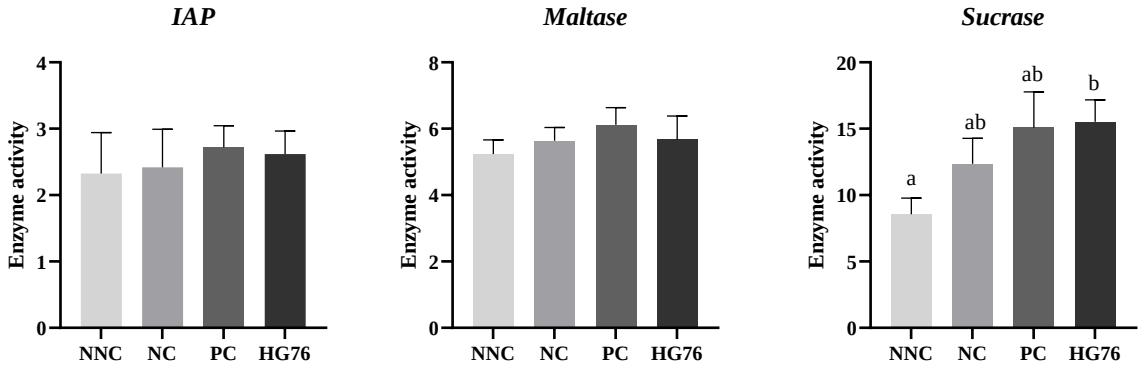


Note: NNC (unchallenged control): pigs fed a control diet and gavage with phosphate buffered saline; NC (challenged control): pigs fed a control diet and challenged with ETEC F4, 2.5×10^7 CFU per pig; PC (antibiotic): NC + 80 $mg \cdot kg^{-1}$ of avilamycin and pigs challenged with ETEC F4; HG76: NC + 1×10^9 CFU kg^{-1} feed of *B. licheniformis* HG76 and pigs challenged with ETEC F4. (A), (B), and (C) represent the morphology of duodenum, jejunum, and ileum, respectively.

Table 6.4 Effects of *B. licheniformis* HG76 on electrophysiological properties including transepithelial electrical resistance (TEER, $\Omega \cdot cm^2$) of jejunum mounted in Ussing chambers (*Ex vivo*) and flux of fluorescein isothiocyanate–dextran 70 kDa (FITC-D70, $\mu g \cdot mL^{-1}$) in weaned piglets (*In vivo*).

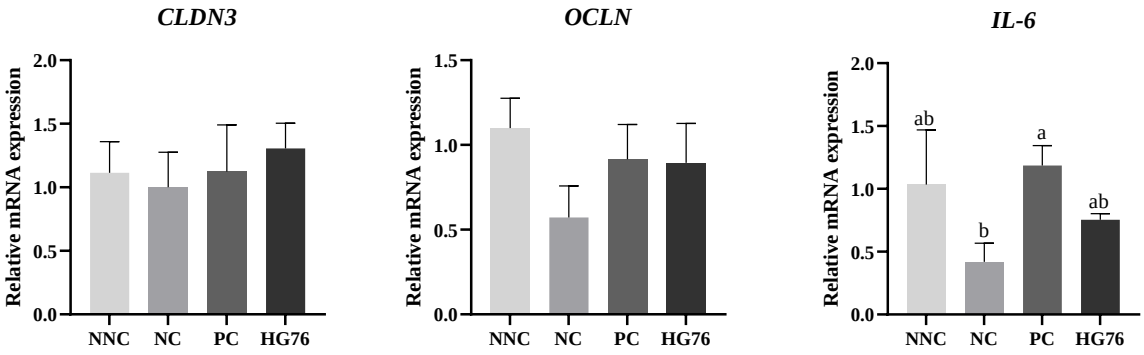
		ETEC F4-challenged				
Items	NNC	NC	PC	HG76	SEM	*P* value
Ex vivo						
TEER	74.92	110.86	93.33	75.03	10.68	0.681
In vivo						
FITC-D70 flux	0.86	1.07	1.04	0.87	0.05	0.236

Figure 6.4 Effects of *B. licheniformis* HG76 on the digestive enzymes' activities (nmol·L^{-1}·mg protein^{-1}·min^{-1}) in the mid-jejunum of weaned piglets.



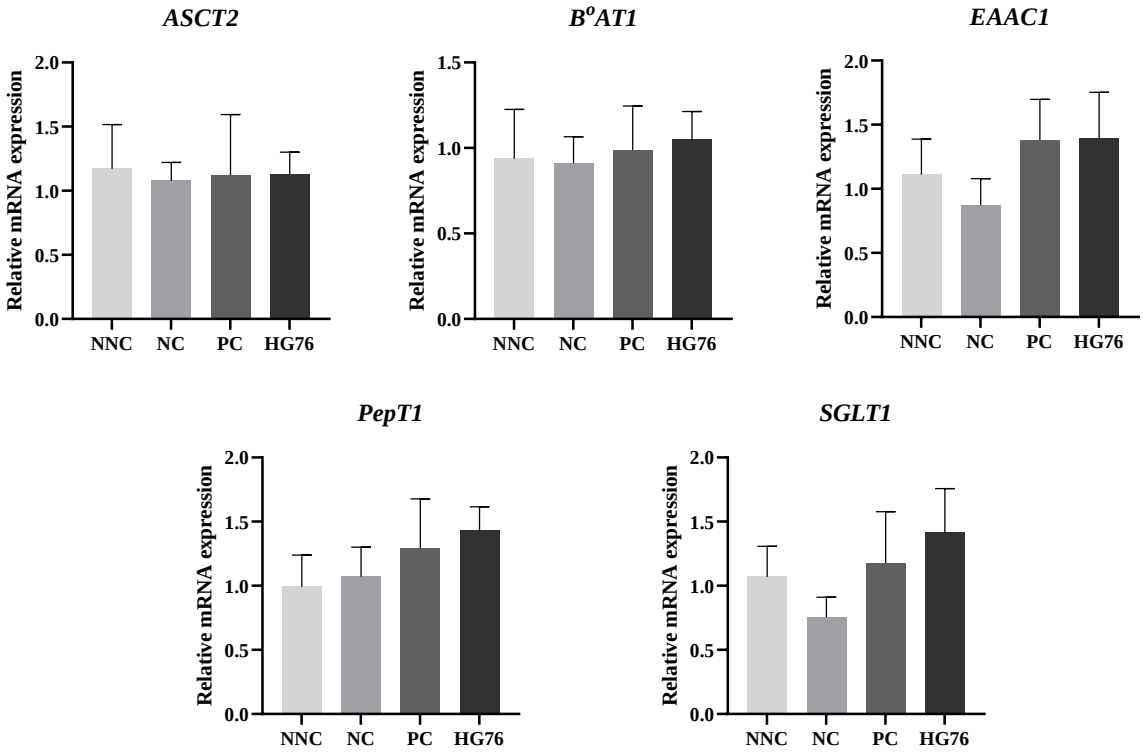
Note: IAP: Intestinal alkaline phosphatase; NNC (unchallenged control): pigs fed a control diet and gavage with phosphate buffered saline; NC (challenged control): pigs fed a control diet and challenged with ETEC F4, 2.5 × 10^7 CFU per pig; PC (antibiotic): NC + 80 mg·kg^{-1} of avilamycin and pigs challenged with ETEC F4; HG76: NC + 1 × 10^9 CFU kg^{-1} feed of *B. licheniformis* HG76 and pigs challenged with ETEC F4.

Figure 6.5 Effects of *B. licheniformis* HG76 on the relative mRNA levels of genes related to intestinal barrier function and immunity in weaned piglets.



Note: CLDN3: claudin 3; OCLN: occludin; IL-6: interleukin-6. NNC (unchallenged control): pigs fed a control diet and gavage with phosphate buffered saline; NC (challenged control): pigs fed a control diet and challenged with ETEC F4, 2.5×10^7 CFU per pig; PC (antibiotic): NC + 80 mg·kg^{-1} of avilamycin and pigs challenged with ETEC F4; HG76: NC + 1×10^9 CFU kg^{-1} feed of *B. licheniformis* HG76 and pigs challenged with ETEC F4.

Figure 6.6 Effects of *B. licheniformis* HG76 on the relative mRNA levels of genes related to nutrient transportation in weaned piglets.



Note: ASCT2: Neutral amino acid transporter 2; EAAC1: Excitatory amino acid transporter 1; PepT1: Peptide transporter 1; SGLT1: Na^+-glucose cotransporter 1; B^0AT1: Neutral amino acid transporter. NNC (unchallenged control): pigs fed a control diet and gavage with phosphate buffered saline; NC (challenged control): pigs fed a control diet and challenged with ETEC F4, 2.5×10^7 CFU per pig; PC (antibiotic): NC + 80 mg·kg^{-1} of avilamycin and pigs challenged with ETEC F4; HG76: NC + 1×10^9 CFU kg^{-1} feed of *B. licheniformis* HG76 and pigs challenged with ETEC F4.

6.5 Discussion

The objective of the present study was to determine the potential of *B. licheniformis* HG76 to mitigate the severity of diarrhea, intestinal health and immunity of weaned piglets caused by ETEC F4 infection. The ETEC F4 challenge model in weaned piglets has been widely applied in determining the efficacy of feed additives to counteract PWD (Adewole et al., 2016; Canibe et al., 2022). Genetic differences with different phenotypes are found in piglets' responses for susceptibility towards fimbriae to ETEC infection. The differential expression of specific fimbriae receptors present on intestinal epithelium is genetically determined to be resistant or susceptible to fimbriae (Hancock et al., 2022). Some single nucleotide polymorphisms (SNP) have been found in mucin gene family. MUC4 is the most extensively studied genetic marker related to ETEC F4 susceptibility (Sinha et al., 2019). Therefore, the weaned piglets with susceptible alleles of MUC4 were chosen for the current ETEC F4 challenge study.

In the current study, during the post-challenge phase, ETEC F4 infection had remarkably increased the severity of diarrhea, which was consistent with the decreased ADG and VH in the NC pigs. Gut health is critically important for swine disease resistance and growth performance of weaned piglets. It has been reported that ETEC induced PWD is highly related to disrupted intestinal morphology and functions as physical barrier (Szabó et al., 2023) and negatively impacted growth performance. The toxins secreted by ETEC could be behind the villus atrophy observed, which would affect the digestive and absorptive functions of weaned piglets and lead to decreased performance (Kim et al., 2022).

Gut morphology could indicate the health status of GIT and its response to certain diets, feed additives and pathogens. The small intestinal epithelium is structured by unique structures of crypt-villus units, including the villus as finger-like protrusion of the intestinal wall and the crypts

as invaginations in the epithelium lining (Bayer et al., 2021). The villus is involved in the absorption of nutrients and plays crucial roles as physical barrier to protect the animals. The crypts contain four to six independent stem cells, which quickly proliferate and differentiate into absorptive enterocytes and secretory cells, e.g., goblet cells, enteroendocrine cells and Paneth cells (Chen et al., 2018). Up to ninety percent of the epithelial cells consists, however, of absorptive enterocytes in the intestinal epithelium, which are responsible for the absorption of nutrients (Farré et al., 2020). The supplementation of *B. licheniformis* HG76 increased jejunal and ileal VH, which was also reported in other studies where the *B. licheniformis* was used in pigs and poultry (Sun et al., 2023; Pan et al., 2022). The improvement of the intestinal morphology, including increased VH and VH:CD ratio, enhances the pigs' performance by increasing the digestion and absorption of nutrients. In the present study, the increase in CD in the jejunum and ileum of HG76 group was not excessive as it would happen in cases of inflammation. The slight, yet, significant, increase in CD may be related to energy expenditure, because the deeper CD suggests an increased intestinal cell proliferation and turnover rate in the intestinal epithelium and may indicate an energy expenditure mechanism (Metzler-Zebeli et al., 2017). On the other hand, the energy expenditure may be compensated by the increased VH in the HG76 piglets, as there were no adverse effects on the growth performance of piglets in the HG76 group.

Carbohydrates are one of the most critical components in pig diets. In the GIT, carbohydrates are digested by amylases and disaccharidases (sucrase, lactase and maltase), and then absorbed in the intestine (Gao et al., 2022). Previously published research has indicated that the supplementation of *Bacillus* in pig diets increased the activity of sucrase (Hu et al., 2018), which is consistent with the observation in the current study. We speculate that the increase in the

digestive enzyme activity could be attributed to the sucrase secretion and stimulation of endogenous sucrase synthesis by the *Bacillus*.

Cytokines are small secreted peptides that show immuno-regulatory functions and aid cell-to-cell communication during immune responses (Wang et al., 2021). The cytokine IL-6 is produced by macrophages, monocytes, and T cells, and serves both pro-inflammatory and anti-inflammatory functions and helps in Th17 cells differentiation (Chonov et al., 2019). Supplementation with avilamycin, in the present study, increased IL-6 mRNA expression. Similar results were reported by Gadde et al. (2017) in broiler chickens given another antibiotic. Park et al. (2020) also reported an increased IL-6 mRNA expression in broiler chickens given another antibiotic. These authors proposed that this increase in IL-6 by the antibiotic could help in the production of more heterophils by the birds, in order to respond and eliminate pathogens. We speculate that avilamycin would also increase the production of IL-6 associated with a higher production of neutrophils in pigs. The above findings indicate that *B. licheniformis* HG76 may reduce pro-inflammatory cytokine production, potentially slowing cell damage and mitigating inflammation.

Although *Bacillus licheniformis* HG76 was able to ameliorate the intestinal morphology and increase the activity of digestive enzymes, limited improvements were observed on growth performance and severity of diarrhea in ETEC F4 challenged piglets. Commercial-scale pig trials are recommended to further evaluate the effects of supplementation of *Bacillus licheniformis* HG76 on pig performance.

CHAPTER SEVEN : MANUSCRIPT IV

Effects of dietary *Bacillus licheniformis* PF9 and HG76 on growth performance, diarrhea index score, enzyme activity and expression of tight junction proteins in nursery piglets

7.1 Abstract

The study was conducted to investigate the effects of dietary *Bacillus licheniformis* PF9 and HG76 on growth performance, diarrhea index score, enzyme activity and expression of tight junction proteins in nursery piglets. Eighty piglets were allocated to five groups, fed basal diet (NC), avilamycin (PC), *B. licheniformis* PF9 (2.5×10^9 CFU·kg^{-1} of basal diet), *B. licheniformis* HG76 (1×10^9 CFU·kg^{-1} of basal diet), and combination of *B. licheniformis* PF9 and HG76 (P+H, 2.5×10^9 CFU·kg^{-1} of PF9 and 1×10^9 CFU·kg^{-1} of HG76), respectively. Each group included eight replicates with two piglets per replicate for a 28-day trial. No significant difference ($P > 0.10$) in growth performance were found among groups. Compared with the NC group, dietary *B. licheniformis* PF9 and HG76 and avilamycin significantly ($P < 0.05$) reduced the diarrhea index score on days 4, 5, 7, 9, 10, 11 and 12. These findings suggested that dietary supplementation with *B. licheniformis* PF9 and HG76 could relieve diarrhea severity after weaning without compromising piglets' performance.

Key words: *Bacillus licheniformis* PF9 and HG76, diarrhea, growth performance, nursery piglets

7.2 Introduction

Post-weaning diarrhea (**PWD**) in the swine industry is a globally prevalent disease characterized by high rates of illness. The occurrence of PWD significantly impacts productivity by causing both losses in production efficiency and increased mortality rates of piglets (Serbessa et al., 2023). Administering antibiotics as growth promoters (**AGP**) has been proven successful in diminishing PWD and fostering immunomodulatory and anti-inflammatory reactions, which contributes to the improved growth and development of weaned piglets (Uddin et al., 2021). Nevertheless, excessive use of AGP in animal production industry is leading to a rise in multi-drug resistant pathogens and antibiotic residues in animal products, posing a growing concern for global health (Vidovic and Vidovic, 2020). A significant number of countries have implemented bans on the use of AGP for enhancing animal growth, for instance, EU banned AGP in 2006, and numerous countries around the globe have followed (Niranjan et al., 2023). Therefore, it is crucial to identify suitable alternatives for AGP in preventing diarrhea in weaned piglets.

In recent years, various AGP alternatives have been explored, with probiotics emerging as a notable option (Niranjan et al., 2023). Research has also delved into preventing PWD in piglets through the use of probiotics or functional metabolites derived from probiotics (Arsène et al., 2021). Of the various probiotic strains examined, *Bacillus licheniformis* (***B. licheniformis***) has been noted for its potent capacity to produce digestive enzymes and antimicrobial peptides, which aid in improving nutrient absorption and suppressing the growth of pathogens (Shleeva et al., 2023). Our recent study had found that pigs supplemented with *B. licheniformis* PF9 had better gut health through enhancing gut barrier integrity, nutrient absorption, gut microbiota, and immunity in the enterotoxigenic *Escherichia coli* (**ETEC**) F4 challenge conditions (Xu et al., 2024). The treatment with *B. licheniformis* also displayed a comparable beneficial impact on reducing

Salmonella, evidenced by a greater number of pigs that tested negative for *Salmonella* in feces relative to the control group (Barba-Vidal et al., 2017). Moreover, the probiotic *B. licheniformis* produced substances with antimicrobial properties and demonstrated a robust capacity for combating pathogenic bacteria (Pahumunto et al., 2023).

The *in vitro C. elegans*, intestinal porcine epithelial cell line (**IPEC-J2**) trials and *in vivo* ETEC F4 challenge trials related to the treatments of *B. licheniformis* PF9 and HG76 have been evaluated in our previous studies (Xu et al., 2024; Li et al., 2022). It is important to evaluate the beneficial effects of *B. licheniformis* PF9 and HG76 for practical application and also explore if synergetic beneficial effects can be seen when combining PF9 and HG76. We hypothesized that combination of *B. licheniformis* PF9 and HG76 would reduce the severity of diarrhea after weaning, thereby improving the growth performance of nursery piglets. Therefore, this study aims to investigate the effects of combination of *B. licheniformis* PF9 and HG76 on the growth performance, diarrhea index score, enzyme activity and expression of tight junction proteins of nursery piglets.

7.3 Materials and methods

The experimental protocols (F19-020/1/2, AC11516) were approved by the University of Manitoba's Animal Care Committee. The piglets involved in the study were maintained according to the standards set by the Canadian Council on Animal Care (CCAC, 2009).

7.3.1 *B. licheniformis* PF9 and HG76 culture preparation

To prepare a *B. licheniformis* culture, a single colony of this bacterium was taken from an agar plate and introduced into 6 mL of Luria Bertani broth (**LB**, Millipore Sigma Canada Ltd., Oakville, ON, Canada) in a 12 mL culture tube. The process was done under aerobic conditions, maintaining a temperature of 37ºC to incubate for 24 h with continuous shaking (180 rpm).

Following the initial step, 5 mL of the initial culture was transferred into a 1 L flask containing 300 mL of LB broth and then incubated in the flask with shaking (180 rpm) under aerobic conditions at 37ºC for another 24 h (Xu et al., 2024). After incubation, 500 mL of this culture was mixed uniformly into 50 kg of feed with the final mixture contained 2.5×10^{9} CFU·kg^{-1} of *B. licheniformis* PF9 and 1×10^{9} CFU·kg^{-1} of *B. licheniformis* HG76 in the diet, respectively.

7.3.2 Animals and diets

A total of eighty weaned piglets (TN Tempo × TN70; 40 castrated male and 40 female piglets) at 28 days old (weaning at day 21) with average body weight (**BW**) of 6.90 ± 0.06 kg were selected from the Glenlea Swine Research Unit of the University of Manitoba and then transferred to the T.K. Cheung Centre for Animal Science Research. All piglets were randomly divided into five groups (8 replicates per group with 2 piglets per replicate) in a completely randomized design. The dietary treatments comprised a corn and soybean meal-based basal diet as negative control (NC), a basal diet supplemented with 80 mg·kg^{-1} of avilamycin for three weeks as positive control (PC), a basal diet supplemented with 2.5×10^{9} CFU·kg^{-1} diet of *B. licheniformis* PF9 (PF9), a basal diet supplemented with 1×10^{9} CFU·kg^{-1} diet of *B. licheniformis* HG76 (HG76), and a basal diet supplemented with combination of *B. licheniformis* PF9 and HG76 (P+H; 2.5×10^{9} CFU·kg^{-1} of PF9 and 1×10^{9} CFU·kg^{-1} of HG76). Diets were formulated (**Table 7.1**) to meet or exceed the nutrient requirements recommendation for 7-11 kg and 11-25 kg pigs by the National Research Council (NRC, 2012).

The whole period of this feeding trial was 28 days. All piglets were housed in pens (two pigs per pen) equipped with separated stainless-steel feeders and nipple drinkers. Throughout the whole experimental period, piglets had *ad libitum* access to feed and water. Room temperature was maintained at 29 ± 1ºC during week 1 and decreased by 1ºC each week controlled by

temperature-controlled system. Piglets were checked for health status at least two times daily, and reported to veterinary service if any illnesses were detected.

7.3.3 Growth performance and fecal score

Piglet's BW and pen feed consumption were measured on day 14 and 28 to determine the average daily gain (**ADG**), average daily feed intake (**ADFI**), and gain:feed within phase 1 and phase 2, respectively.

The diarrhea index score was recorded at 9 am every day of the first two weeks following the method of Marquardt et al., (1999), as score 0 indicates normal, 1 indicates soft feces, 2 indicates mild diarrhea, and 3 indicates severe diarrhea.

7.3.4 Analysis of enzyme activity

Maximal enzyme activity ($\mathbf{V}_{max}$) of intestinal alkaline phosphatase (**IAP**) in the mid-jejunum was measured. Approximately 200 mg of frozen mid-jejunum tissues were homogenized on ice using a polytron homogenizer in cold homogenizing buffer containing phenylmethylsulfonyl fluoride and D-mannitol at pH 7.4. The homogenate was then transferred into a tube for protein content determination by BCA Protein Assay Kit (Thermo Fisher Scientific, Ottawa, ON, Canada). The IAP activity was analyzed according to the method described by Hübscher and West (1965).

7.3.5 RNA extraction and Real-Time PCR

To detect mRNA expression of tight junction proteins, total RNA was isolated from the mid-jejunum using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration and purity were measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Ottawa, ON, Canada), ensuring all samples had OD_{260}:OD_{280} ratios between 1.9 and 2.1. Reverse transcription was performed with approximately

1 μg of total RNA from each sample using the iScript cDNA Synthesis Kit (Bio-Rad Ltd., Mississauga, ON, Canada) to synthesize cDNA. Primers for real-time PCR analysis were designed using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and synthesized by Integrated DNA Technologies Inc., with their sequences listed in **Table 7.2**. Real-time PCR was conducted using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Ltd., Mississauga, ON, Canada). The PCR thermal cycling conditions were: 3 minutes at 95ºC, followed by 40 cycles of 20 seconds denaturation at 95ºC, 30 seconds annealing at 60ºC, and 30 seconds extension at 72ºC. GAPDH gene was used for normalization. Relative fold changes in gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

7.3.6 Western blotting

Proteins were extracted from the mid-jejunum using RIPA Lysate Buffer (Millipore Sigma Canada Ltd., Oakville, ON, Canada). The protein concentrations were measured with the BCA Protein Assay Kit according to the manufacturer's instructions (Thermo Fisher Scientific, Ottawa, ON, Canada). Proteins were then separated using Mini-PROTEAN TGX Stain-Free™ Protein Gels (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) using the Trans-Blot Turbo transfer system (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada).

The membranes were blocked with 5% skim milk in tris-buffered saline with 0.1% Tween 20 (**TBST**) at 22°C for 1.5 hours and incubated overnight at 4°C with primary antibodies from Thermo Fisher Scientific (Ottawa, ON, Canada): mouse anti-β-actin (1:4,000) and rabbit anti-occludin (1:1,000), both diluted in 5% skim milk in TBST. After three washes with TBST, the membranes were incubated for 1 hour at 22°C with secondary antibodies: goat anti-mouse IgG (1:500) for β-actin and goat anti-rabbit IgG (1:4,000) for occludin.

Protein visualization was performed using the Clarity Max ECL Western Blotting Substrate and the ChemiDoc™ MP imaging system (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). Image Lab 6.0 software was used to analyze the protein bands. β-actin was used as the internal standard, and the target protein levels were expressed as the ratio of the optical density of the protein bands to that of the corresponding β-actin bands.

7.3.7 Statistical analysis

The data were analyzed using a one-way ANOVA via the general linear model method of Statistical Analysis System (SAS) software (V.3.8; SAS Institute, Cary, NC, USA). The design was a randomized complete block design, with pen as the experimental unit. In this experiment, replicates served as the unit of analysis. Mean values were compared using Tukey adjusted PDIFF significant difference test. The results presented in the table are displayed as Least Squares Means and the combined standard error of the mean (SEM). The *P*-value less than 0.05 was deemed statistically significant.

Table 7.1 Composition of basal diets

Ingredient, g/kg	Phase 1 (0-14 d)	Phase 2 (15-28 d)
Corn	486	493.4
Wheat	50	100
Barley	50	100
Soybean meal	220	220
Fish meal	50	30
Dried whey powder	100	20
Vegetable oil	13	2.3
Limestone	7.4	9.2
Dicalcium phosphate	6.6	8
Salt	4	4
Vitamin and mineral premix[1]	10	10
L-Lysine-HCl	3	3.1
Calculated composition		
Dry matter %	90.67	89.67
Net energy kcal/kg	2,448	2,412
Metabolizable energy kcal/kg	3,412	3,317
Crude protein %	19.5	18.7
SID Lys %	1.35	1.23
Total calcium %	0.74	0.70
Available phosphorus %	0.65	0.60

Note: [1]Vitamin and mineral premix, provided per kg of diet: vitamin A, 2,200 international unit (IU); vitamin B1, 1.5 mg; vitamin B2, 4 mg; vitamin B12, 0.02 mg; biotin, 0.2 mg; vitamin D3, 220 IU; vitamin E, 16 IU; vitamin K, 0.5 mg; niacin, 30 mg; folic acid, 0.3 mg; choline chloride, 600 mg; pyridoxine, 7 mg; I as calcium iodate, 0.14 mg; calcium pantothenate, 12 mg; Fe as ferrous sulfate, 100 mg; Zn as zinc oxide, 100 mg; Se as sodium selenite, 0.3 mg; Mn as manganese oxide, 4 mg; and Cu as copper sulphate, 6 mg.

Table 7.2 Primer sequences of tight junction proteins of piglets[1]

Genes	Amplicon (bp)	Sequence (5’ to 3’)	References
ZO-1	200	GATCCTGACCCGGTGTCTGA TTGGTGGGTTTGGTGGGTT	(Omonijo et al., 2018)
OCLN	93	CTGTGGATGTCCTGCGTGT GGTTGCTTGCAAAGTGGTGTT	(Lee and Kang, 2017)
CLDN3	123	CTACGACCGCAAGGACTACG TAGCATCTGGGTGGACTGGT	(Omonijo et al., 2018)

[1]Note: OCLN: occludin; ZO-1: zonula occludens 1; CLDN3: claudin 3.

7.4 Results

7.4.1 Growth performance

As shown in **Table 7.3**, although there was no significant difference ($P > 0.10$) in the ADG, ADFI and gain:feed found among all the groups during phase 1 (1-14 d), phase 2 (15-28 d) and overall period (1-28 d), gain:feed of the PF9 group and P+H group in phase 1 was 7.1% and 6.3% numerically higher than the NC group, respectively.

7.4.2 Diarrhea index score

As shown in **Figure 7.1**, the supplementation of *B. licheniformis* and antibiotic significantly ($P < 0.05$) relieved diarrhea severity on days 4, 5, 7, 9, 10, 11, 12 when compared to the NC group.

7.4.3 Enzyme activity

There was no significant difference ($P > 0.10$) in the V*max* values of IAP among all the treatments (**Figure 7.2**).

7.4.4 Relative mRNA abundance of tight junctions in the jejunum

The relative mRNA levels of genes in the mid-jejunum related to tight junctions areshown in **Figure 7.3**. There was no significant difference ($P > 0.10$) observed in the relative mRNA levels of ZO-1, OCLN, and CLDN3 among all the treatments.

7.4.5 Protein expression of tight junctions in the jejunum

The protein expression of OCLN in the jejunum was assessed using western blotting. As illustrated in **Figure 7.4**, consistent with the mRNA expression levels of OCLN, there were no significant differences in the protein expression of OCLN across all the groups ($P > 0.10$).

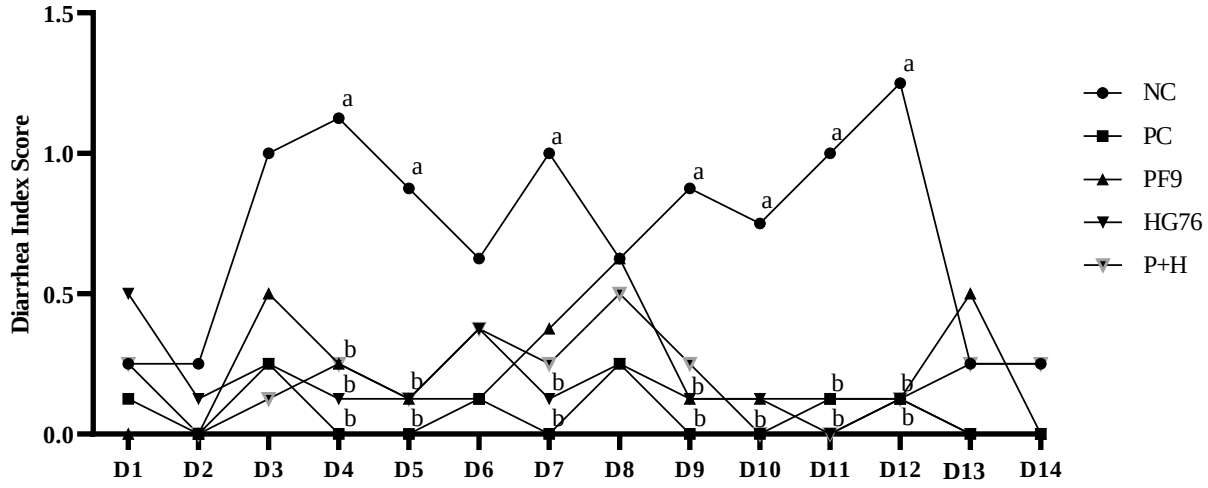
Table 7.3 Effects of *B. licheniformis* PF9 and HG76 on the growth performance of nursery piglets

Items	Treatments[1]					SEM	*P*-value
	NC	PC	PF9	HG76	P+H		
Phase 1 (1-14 d)							
ADG, g/d	272	276	286	267	269	7.12	0.799
ADFI, g/d	340	332	338	323	342	6.53	0.883
Gain:Feed	0.799	0.827	0.846	0.827	0.836	0.013	0.737
Phase 2 (15-28 d)							
ADG, g/d	527	589	531	526	535	2.78	0.522
ADFI, g/d	850	916	870	864	841	9.81	0.761
Gain:Feed	0.624	0.644	0.615	0.610	0.636	0.008	0.591
Overall (1-28 d)							
ADG, g/d	391	435	404	394	416	8.31	0.562
ADFI, g/d	582	624	616	601	613	10.63	0.845
Gain:Feed	0.694	0.724	0.693	0.685	0.710	0.009	0.463

Note: NC: negative control group with basal diet; PC: positive control group with basal diet + 80 $mg \cdot kg^{-1}$ of avilamycin for 3 weeks; PF9: basal diet + 2.5×10^9 $CFU \cdot kg^{-1}$ feed of *Bacillus*

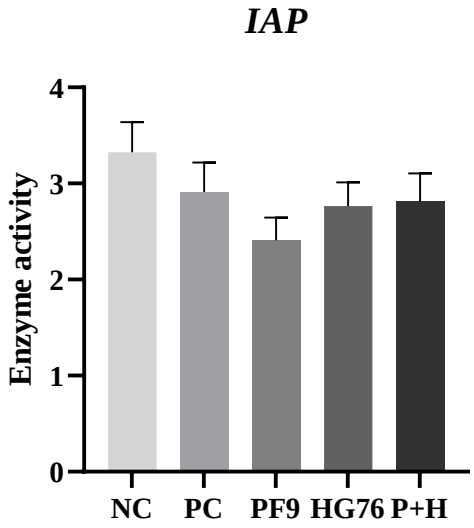
licheniformis PF9; HG76: basal diet + 1×10^9 CFU·kg^{-1} feed of *Bacillus licheniformis* HG76; P+H: basal diet + 2.5×10^9 CFU·kg^{-1} of *Bacillus licheniformis* PF9 + 1×10^9 CFU·kg^{-1} of *Bacillus licheniformis* HG76; ADG: average daily gain; ADFI: average daily feed intake.

Figure 7.1 Effects of *B. licheniformis* PF9 and HG76 on diarrhea index score in weaned piglets



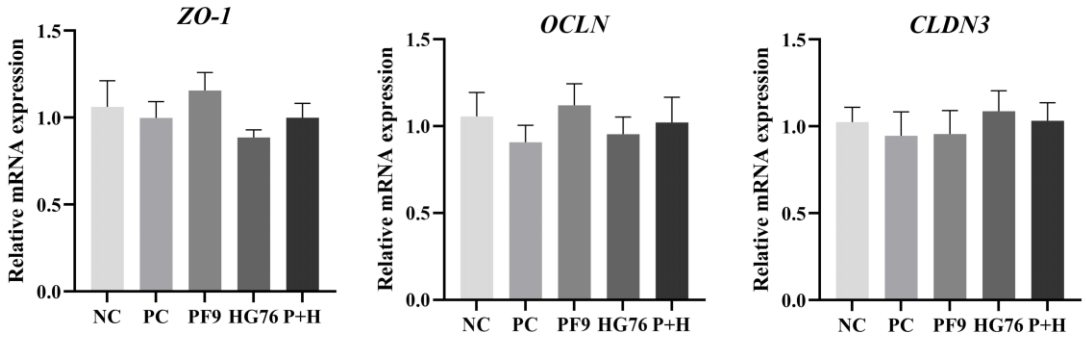
Note: The diarrhea index score was measured in the NC: negative control group with basal diet; PC: positive control group with basal diet + 80 mg·kg^{-1} of avilamycin for 3 weeks; PF9: basal diet + 2.5×10^{9} CFU·kg^{-1} feed of *B. licheniformis* PF9; HG76: basal diet + 1×10^{9} CFU·kg^{-1} feed of *B. licheniformis* HG76; P+H: basal diet + 2.5×10^{9} CFU·kg^{-1} of *B. licheniformis* PF9 + 1×10^{9} CFU·kg^{-1} feed of *B. licheniformis* HG76 during phase 1 (Day 1-14) period. Diarrhea index score 0 represents normal feces; 1 represents soft feces; 2 represents mild diarrhea; and 3 represents severe diarrhea. Each value is represented by the mean. Different letters shown in the bars indicate significantly difference ($P < 0.05$).

Figure 7.2 Effects of *B. licheniformis* PF9 and HG76 on the intestinal alkaline phosphatase activities (nmol·L^{-1}·mg $protein^{-1}$·min^{-1}) in the mid-jejunum of weaned piglets



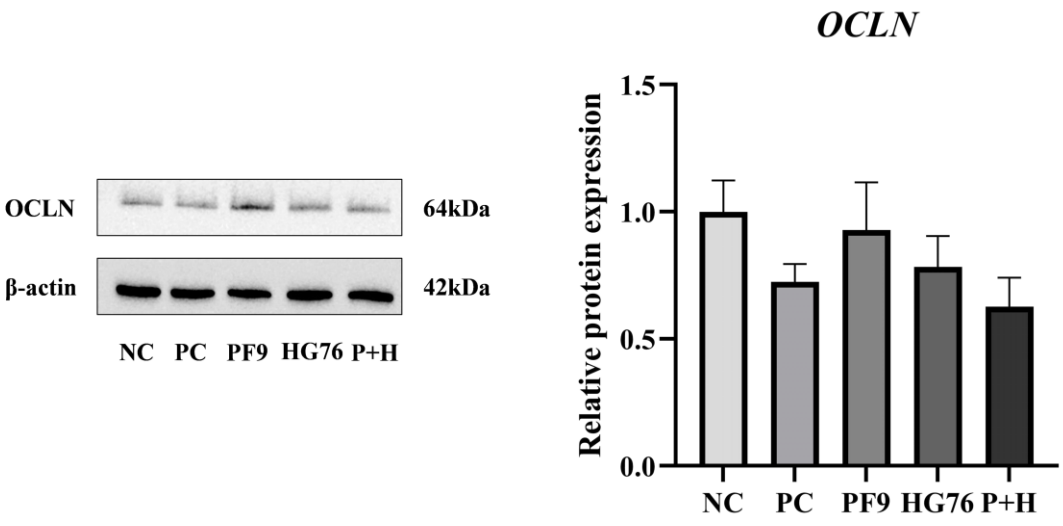
Note: NC: negative control group with basal diet; PC: positive control group with basal diet + 80 mg·kg^{-1} of avilamycin for 3 weeks; PF9: basal diet + 2.5×10^9 CFU·kg^{-1} feed of *B. licheniformis* PF9; HG76: basal diet + 1×10^9 CFU·kg^{-1} feed of *B. licheniformis* HG76; P+H: basal diet + 2.5×10^9 CFU·kg^{-1} of *B. licheniformis* PF9 + 1×10^9 CFU·kg^{-1} feed of *B. licheniformis* HG76.

Figure 7.3 Effects of *B. licheniformis* PF9 and HG76 on the relative mRNA levels of genes related to tight junctions in the mid-jejunum of weaned piglets



Note: ZO-1: zonula occludens 1; OCLN: occludin; CLDN3: claudin 3. NC: negative control group with basal diet; PC: positive control group with basal diet + 80 mg·kg^{-1} of avilamycin for 3 weeks; PF9: basal diet + 2.5×10^{9} CFU·kg^{-1} feed of *B. licheniformis* PF9; HG76: basal diet + 1×10^{9} CFU·kg^{-1} feed of *B. licheniformis* HG76; P+H: basal diet + 2.5×10^{9} CFU·kg^{-1} of *B. licheniformis* PF9 + 1×10^{9} CFU·kg^{-1} feed of *B. licheniformis* HG76.

Figure 7.4 Effects of *B. licheniformis* PF9 and HG76 on the protein expression of occludin in the mid-jejunum of weaned piglets



Note: OCLN: occludin. NC: negative control group with basal diet; PC: positive control group with basal diet + 80 mg·kg^{-1} of avilamycin for 3 weeks; PF9: basal diet + 2.5×10^{9} CFU·kg^{-1} feed of *B. licheniformis* PF9; HG76: basal diet + 1×10^{9} CFU·kg^{-1} feed of *B. licheniformis* HG76; P+H: basal diet + 2.5×10^{9} CFU·kg^{-1} of *B. licheniformis* PF9 + 1×10^{9} CFU·kg^{-1} feed of *B. licheniformis* HG76.

7.5 Discussion

In general, *Bacillus* is commonly used in animal feeds as one of the most effective AGP alternatives. In the present study, we found that neither antibiotics nor *B. licheniformis* supplementation showed significant effects on growth performance of nursery piglets during the whole four-week period. Similarly, Kritas and Morrison (2005) did not show any significant impact of supplementation of *B. subtilis* and *B. licheniformis* on the growth performance of piglets. Moreover, the study conducted by Hung et al. (2019) showed that antibiotics and products fermented with *B. licheniformis* did not influence the BW, ADFI, ADG, or FCR in weaned piglets compared to the control group. However, varying outcomes in livestock production were found according to previous research. In particular, supplementation of *Bacillus* to weaned piglets caused an improvement in FCR and BWG (Mingmongkolchai and Panbangred, 2018).

Yu et al. (2022) found that adding 500 $mg \cdot kg^{-1}$ of *B. licheniformis* significantly increased the ADG compared with the control group in weaned piglets, which may be due to the production of beneficial metabolites (such as lysozymes and digestive enzymes) from *B. licheniformis*. In a recent study, Sun et al. (2023) reported that dietary supplementation with *B. licheniformis* BL−S6 promoted BW gain and ADG of weaned piglets. The inconsistency may stem from the use of different strains of *Bacillus,* the dosage of administration, the animal age, health status of the host, and the duration of probiotic application (Gaggìa et al., 2010).

Weaning, a common challenge to young piglets, is often marked by dietary shifts, alterations in the gut environment and morphology. The weaning stress and ETEC can lead to reduced growth rates, increased occurrences of PWD, disruptions in the balance of intestinal microecology and intestinal inflammation (Pluske et al., 2018). Some *Bacillus* strains could be effective in alleviating infectious enteritis or diarrhea in weaned piglets (Luise et al., 2022). The

spore or live form of *B. licheniformis* is one of the most commonly used probiotics in the weaned piglets' diet to prevent PWD (Canibe et al., 2022). In our previous ETEC F4 challenge studies (Xu et al., 2024), we found that *B. licheniformis* PF9 and HG76 significantly relieved diarrhea severity, which was aligned with findings of the present study. Diarrhea incidence may be linked to pigs' growth performance. Therefore, diarrhea incidence of weaned piglets was recorded daily in phase 1. In the current study, we observed that the piglets with supplementary antibiotic and *B. licheniformis* had significantly lower diarrhea scores than the piglets from the control group. Although there were no significant effects of dietary *B. licheniformis* on the growth performance, the supplementation of *B. licheniformis* PF9 alone and combination of PF9 and HG76 numerically improved the gain:feed, which can be partially explained by the reduced incidence of diarrhea in *B. licheniformis* supplemented piglets.

It has been reported that IAP offers several benefits for mammalian intestinal health, including preventing and reducing intestinal inflammation and bacterial translocation, regulating calcium absorption, and modulating intestinal bacterial growth and local intestinal pH (Ghosh et al., 2020). Moreover, the tight junctions (**TJ**) are crucial for maintaining the intestinal barrier's function. Comprising various junctional molecules, such as zonula occludens 1, claudin and occludin, TJ regulate the paracellular permeability of water, ions, and macromolecules. One of the key functions of TJ is to serve as a physical barrier against luminal inflammatory molecules. When the integrity and structure of the TJ barrier are compromised, it can lead to the activation of immune cells and chronic inflammation in various tissues (Suzuki, 2020). Therefore, IAP activity, the gene and protein expression of TJ were measured in this study to evaluate the effects of probiotics candidates on the gut health of piglets. However, there were no significant effects found in these parameters, which maybe due to the highly controlled experimental conditions in our

animal research centre. Therefore, further research will need to be conducted in commercial barns where relatively complicated factors are present.

In conclusion, the present study indicated that dietary *B. licheniformis* PF9 and HG76 could exhibit a beneficial role in improving piglets' performance via improving gain:feed and relieving diarrhea severity after weaning.

CHAPTER EIGHT : GENERAL DISCUSSION

Post-weaning diarrhea (**PWD**) is a prevalent and economically significant condition in the swine industry, primarily affecting pigs during the first one to two weeks after weaning (Eriksen et al., 2022). Enterotoxigenic *Escherichia coli* (**ETEC**) is identified as the leading cause of PWD in pigs (Ibragimov et al., 2024). After adhering to and colonizing the gut, ETEC releases heat labile and heat stable enterotoxins, which increases the flow of water and electrolytes into the lumen of small intestine. This leads to clinical manifestations such as of dehydration, diarrhea, and even death (Aminian et al., 2023). The recommended therapy for PWD includes the provision of electrolytes and antibiotics. However, increasing concerns about antimicrobial resistance have led many countries to phase out the use of antibiotics as growth promoters (**AGP**) (Abreu et al., 2023). As swine producers continue to phase out or restrict the use of AGP in their production systems, it becomes imperative to identify effective AGP alternatives to maintain herd health and productivity.

One promising strategy involves incorporating probiotics into swine diets, which has been proposed to improve gut function and health, thus mitigating the negative impacts of PWD on the growth performance (Su et al., 2022). Despite the extensive research into various probiotics strains and the availability of commercial probiotic products, there is still a need to fill gaps in their development and application. Consequently, the objectives of this thesis aimed at evaluating the beneficial effects of selected probiotics candidates, namely, *B. licheniformis* PF9 and HG76 on piglet health and growth under both normal physiological conditions and ETEC F4 challenge scenarios, employing both *in vitro* and *in vivo* methodologies.

The initial phase in the selection of probiotics candidates comprises a sequence of *in vitro* experiments. These experiments evaluate their capacity to withstand harsh conditions such as low

pH and high bile salts concentrations, adhere to epithelial cells, and exhibit antagonistic effects against specific pathogens (Byakika et al., 2019). In Chapter Four, we reported that *B. licheniformis* PF9 exhibited notable tolerance to low pH and high bile salts concentration *in vitro*, a crucial attribute for probiotic functionality. This resilience is imperative for probiotic cells to survive the acidic gastric environment and endure the presence of bile acids in the small intestine, facilitating their effectiveness within the gut ecosystem (Koga, 2022). Bile salts synthesized in the liver from cholesterol and subsequently released into the duodenum in conjugated forms with either glycine or taurine, play pivotal roles in fat absorption and enterohepatic recirculation (Di Ciaula et al., 2017). Furthermore, bile salts possess the ability to disrupt and destroy bacterial membranes, rendering them susceptible to destruction (Urdaneta and Casadesús, 2017). Therefore, to survive in the gut, the probiotic strains must be able to withstand high concentrations of bile salts.

The lifespan assay of *C. elegans* in Chapter Four also showed that *B. licheniformis* PF9 partially increased the survival rate of *C. elegans* under ETEC infection. The insulin-like pathway influences both lifespan and the innate immunity of *C. elegans* under exposure to pathogens (Prithika et al., 2016). For instance, *L. zeae* LB1 influenced the transcriptional regulator of insulin-like receptor (daf) signaling pathways, leading to increased susceptibility to ETEC infection in mutants with defective daf-16 (Zhou et al., 2018). Moreover, *L. zeae* LB1 notably inhibited the expression of ETEC toxin genes (Zhou et al., 2014). The protective mechanisms of *B. licheniformis* PF9 in *C. elegans* are currently unknown and require further investigations.

Based on the ETEC challenge study results in Chapters Four and Six, pigs supplemented with *B. licheniformis* PF9 and HG76 experienced less severe diarrhea as indicated by lower fecal scores compared to the negative control. Fecal scores are non-parametric data and analyzed using

the Mann-Whitney U-test and the Kruskal-Wallis test with Dunn's multiple comparison test in SAS (Werner et al., 2020). Although the fecal scores serve as an important indicator of ETEC F4 infection severity in piglets, evaluating the receptors on the intestinal villi could provide additional critical insights. The analysis of various genetic markers linked to susceptibility to ETEC improves the ability to identify piglets susceptible to ETEC F4. However, the variability in the phenotypic expression of F4 receptors is thought to be influenced by gene epistasis (Van Den Broeck et al., 2000). Hence, verifying the susceptibility of piglets to ETEC requires evaluating the presence of F4 receptors on the intestinal brush border (Luise et al., 2019b). It is recommended for the future ETEC challenge studies to evaluate the presence of ETEC receptors via quantifying ETEC-adhering bacteria on the brush border of the jejunal villi, following the methods described by Van Den Broeck et al. (1999).

The adhesion of ETEC to the epithelial cells in the small intestine is a crucial initial step in ETEC infection and subsequent diarrhea development. This adhesion is facilitated by the interaction between adhesins (pili or fimbriae) on the bacterial surface and receptors on the microvilli of the host's small intestine (Pakbin et al., 2021). The most prevalent fimbriae in ETEC infected pigs are F4 and F18, classified according to antigen types. It has been found that the binding of ETEC F4 and F18 to the small intestine of pigs varies with age, which is attributed to changes in the expression levels of receptors at different stages of development. F4 fimbriae are found in both nursery and weaned pigs, while F18 fimbriae are generally observed in weaned pigs (García et al., 2020). Although my studies focused on ETEC F4, ongoing research and control measures are also needed for ETEC F18 due to its prevalence, which ranges from 12% to 44% in various regions worldwide (Duarte et al., 2023).

In terms of microbiome analysis, the dominant phyla *Firmicutes* and *Bacteroidetes*, as well as the genera *Enterobacteriaceae* and *Lactobacillus* are the major microbiota communities in pig feces after weaning. For those pigs with PWD, *Bacteroidetes* abundance was lower, while *Enterobacteriaceae* abundance was higher than healthy piglets. In addition, *Bacteroides* and *Prevotella* are the predominant genera in the *Bacteroidetes* phylum in weaned piglets, which is highly associated with their ability to produce enzymes that degrade plant polysaccharides (Dou et al., 2017). Moreover, metabolomic analysis has been used to understand the molecular mechanisms of host responses to ETEC infection; it is of interest to further investigate the metabolic changes in the ETEC-challenged piglets in the future studies.

For the animal trials, avilamycin was used as the antibiotic positive control. This choice was based on its approval by the Canadian Food Inspection Agency as a medicating ingredient to reduce the incidence and severity of post-weaning diarrhea in swine. Although avilamycin does not specifically target ETEC, its use could potentially decrease the formation of pili on the surface of ETEC (Choi et al., 2020). This reduction in adhesive pili may hinder the bacteria's ability to attach to the intestinal mucosa, thereby preventing the gut damage that leads to diarrhea in weaned piglets.

In numerous strains of probiotic bacteria, the ability to successfully colonize the gut is assessed based on their adherence to the mucosal surface of epithelial cells, which is considered as a significant characteristic for their activity (Mendonça et al., 2023). In the study conducted by Rohith and Halami (2021), the ability of probiotic bacteria to adhere was assessed through measurements of adhesion scores and adhesion percentages. *B. licheniformis* MCC 2514 demonstrated a high level of adhesion on fibronectin based on these metrics. Although we did not

conduct the *in vitro* adhesion assessments of the two isolates in the current project, such evaluations will be a valuable option in future studies.

To ensure the safety of probiotics, several steps and parameters are required for the comprehensive assessments, which includes identification of the strain, toxigenicity and virulence assessment, a well-documented history of safe use, assessing antibiotic resistance, and toxicology studies (Roe et al., 2022). The concern with antibiotic resistance in probiotic strains lies in the possibility that the resistance could be directly transferred or indirectly passed on via intermediary microbes to pathogenic organisms within the microbiota. This could result in the development of resistance to antibiotics in pathogenic bacteria, making them difficult to treat (Ding et al., 2023). Consequently, only probiotic strains that do not promote the spread of antibiotic resistance should be selected for application. It is essential to assess the antibiotic resistance profile and perform taxonomic identification to the species level using molecular techniques (Ahmed et al., 2023). Employing whole-genome sequencing facilitates rapid screening for genes that confer antibiotic resistance and those that express virulence. The analysis of the entire genome is regarded as a reliable method for predicting the risk factors (Muraya et al., 2022). For safety assessment purpose, our collaborators are currently testing the whole-genome sequences of the isolates used in this project.

Based on the results of the current four studies, both *B. licheniformis* PF9 and HG76 could enhance gut health. However, there were some disparities among studies, including growth performance, severity of diarrhea, and expressions of genes and proteins. These disparities could be caused by the difference between these two isolates in terms of tolerance to low pH and high bile salts, pathogen inhibition properties, and supplemental dose.

CHAPTER NINE : SUMMARY AND FUTURE STUDIES

9.1 Summary

The major findings drawn from the present research (Chapters Four - Seven) are as follows:

1. The heat-resistant *B. licheniformis* PF9 exhibited tolerance to low pH and high bile salts and offered protective benefits to *C. elegans* against ETEC infection.

2. Dietary *B. licheniformis* PF9 alleviated diarrhea, improved the relative mRNA expressions of various nutrient transporters, digestive enzymes, and proteins associated with gut barrier function in ETEC F4 challenged piglets.

3. Dietary *B. licheniformis* PF9 reduced the relative abundance of Bacteroidetes in weaned piglets challenged with ETEC F4.

4. Heat-resistant *B. licheniformis* HG76 could potentially enhance gut health by improving the relative mRNA expressions of tight junction proteins, reducing the expressions of toll-like receptors and pro-inflammatory cytokines in response to ETEC F4 exposure in IPEC-J2 cells.

5. Dietary *B. licheniformis* HG76 improved gut development and enhanced the activity of digestive enzymes in weaned piglets challenged with ETEC F4.

6. The supplementation of *B. licheniformis* HG76 had limited improvement on the severity of diarrhea and growth performance of ETEC F4 challenged piglets.

7. Dietary supplementation with *B. licheniformis* PF9 and HG76 may play a positive role in enhancing piglets' performance and mitigating the severity of post-weaning diarrhea under unchallenged conditions.

9.2 Future studies

The present research demonstrated the potential of *B. licheniformis* PF9 and HG76 as antibiotic alternatives in pigs. Nonetheless, further studies are required before these strains can be applied in swine production. In my opinion, the future studies directions are as follows:

1. Conduct large-scale feeding trials in commercial farms to investigate the effect of *B. licheniformis* PF9 and HG76 on growth performance of piglets;

2. Evaluate a cocktail of probiotics containing at least two probiotic candidates for their potentials in controlling enteric infections and improving gut health and performance of pigs through *in vivo* studies; and

3. Determine the safety of probiotic candidates through comprehensive assessments.

CHAPTER TEN : LITERATURE CITED

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