LACTATE-PRODUCING BACTERIUM-*LACTOBACILLUS ZEAE* TO CONTROL ENTEROTOXIGENIC *ESCHERICHIA COLI* F4 INFECTION IN AN *IN VITRO* PORCINE INTESTINAL EPITHELIAL CELL MODEL

by

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ABSTRACT

Antibiotics have been widely included in feeds to prevent post-weaning diarrhea (PWD) and increase the overall growth performance of pigs. However, there is a trend to minimize or eliminate the use of in-feed antibiotics. The development of effective alternatives to in-feed antibiotics (e.g., probiotics) is crucial for maintaining the sustainability of swine production. For young piglets, an effective probiotic is expected to deliver at least one of the following functions to the gut: 1) stimulating the development of a healthy microbiota - predominated by beneficial bacteria, 2) preventing enteric pathogens from colonization, 3) increasing digestive capacity and lowering the pH, 4) improving mucosal immunity, or 5) enhancing gut tissue maturation and integrity. Our previously isolated lactate-producing bacteria (e.g. *Lactobacillus zeae* LB1) have shown to prevent *Caenorhabditis elegans* (*C. elegans*) from enterotoxigenic *Escherichia coli* F4 (ETEC F4)-related death by inhibiting enterotoxin gene expression of the pathogen rather than interfering with its intestinal colonization. However, the protective effects of *L. zeae* LB1 at cellular and molecular levels have not been investigated yet in pigs. In the present study, porcine intestinal cells (IPEC-J2) were used to investigate the potential of *L. zeae* LB1 on modulating intestinal barrier and innate immune functions and protecting against intestinal injuries and inflammatory reactions induced by ETEC F4 infection. The results suggested that probiotic *L. zeae* LB1 effectively protected the intestinal cells from ETEC F4 infection by inhibiting inflammation and maintaining barrier integrity via downregulating TLR4, TLR5, and ETEC F4 virulence-related factors expressions. In conclusion, our data provide further evidence on the mechanisms at the cellular and molecular levels that probiotic *L. zeae* LB1 may improve gut health by enhancing barrier function and reducing inflammatory cytokines secretion in pigs under physiological challenges.

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DEDICATION

This thesis is dedicated to my grandparents and parents, who nurtured me to be what I am today.

FOREWORD

Part of this thesis has been presented as an oral presentation at the American Society of Animal Science-Canadian Society of Animal Science-Western Section American Society of Animal Science (ASAS-CSAS-WSASAS) Virtual Annual Meeting on July 19-23, 2020 and also been presented as a poster at the 2020 Animal Nutrition Conference of Canada, Webinar Series, May 26-June 11, 2020. This thesis was written in manuscript format. The manuscript and abstracts have been listed as follows:

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LIST OF ABBREVIATIONS

ADG	Average Daily Gain
ADFI	Average Daily Feed Intake
AGP	Antibiotic Growth Promoters
AHL	Acyl-Homoserine Lactones
AI-2	Auto Inducer 2
Akt	Protein Kinase B
APCs	Antigen-Presenting Cells
API	Analytical Profile Index
BCA	Bicinchoninic Acid
Bcl3	B-cell Chronic lymphocytic leukemia/lymphoma
bp	Base Pairs
BSA	Bovine Serum Albumin
Caco-2	Cancer coli-2
CAI	Cholerae Autoinducer
cAMP	3',5'-cyclic Adenosine Monophosphate
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
C. elegans	*Caenorhabditis elegans*
CFU	Colony of Units
cGMP	3',5'-cyclic Guanosine Monophosphate
CO_2	Carbon Dioxide
CqsA	Cholerae Autoinducer Synthase

CycA	Cyclophilin-A
Da	Dalton(s)
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DCs	Dendritic Cells
DDH_2O	Double Distilled Water
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture Ham’s F-12
DPD	4,5-Dihydroxy-2,3-Pentanedione
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
EHEC	Enterohemorrhagic *Escherichia coli*
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular-signal-Regulated Kinase
ESR	Millicell Electrical Resistance System
ETEC	Enterotoxigenic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
F-Actin	Filamentous Actin
FAO	Food and Agriculture Organization of the United Nations
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
gapA	Glyceraldehyde-3-Phosphate Dehydrogenase A
GIT	Gastrointestinal Tract

GPCR	G Protein-Coupled Receptor
GRAS	Generally Recognized as Safe
HAI	Harveyi Autoinducer
H_2O_2	Hydrogen Peroxide
HRP	Horseradish Peroxidase
IEC	Intestinal Epithelial Cells
IFN	Interferon
IGF	Insulin-like Growth Factor
IL	Interleukin
IPI-2I	Immortal Pig Intestinal-2I
IRAK-M	Interleukin-1 Receptor-Associated Kinase M
JNK	c-Jun N-terminal Kinase
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IPEC	Intestinal Porcine Epithelial Cells
LAB	Lactic Acid Bacteria
LDH	Lactase Dehydrogenase
LPS	Lipopolysaccharide
LT	Heat-labile Enterotoxin
LuxS	*S*-ribosylhomocysteinase
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time of Flight
MAPK	Mitogen-Activated Protein Kinase

MCP	Monocyte Chemoattractant Protein
MHC	Major Histocompatibility Complex
MKP	Mitogen-activated protein Kinase Phosphatase
MLCK	Myosin Light-Chain Kinase
MntR	Metalloregulator
mRNA	Messenger Ribonucleic Acid
MRS	Man, Rogosa, and Sharpe broth
MUC4	Mucin 4
NADH	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
NLRs	NOD-like Receptors
NOD	Nucleotide-binding Oligomerization Domain
OCLN	Occludin
OD	Optical Density
OIE	Office International des Epizooties
pBD	Porcine Beta-Defensins
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PFGE	Pulsed-Field Gel Electrophoresis
PIE	Porcine Intestinal Epitheliocyte
PPAR	Peroxisome Proliferator-Activated Receptor

PVDF	Polyvinylidene Difluoride
PWD	Post-Weaning Diarrhea
QS	Quorum Sensing
RFLP	Restriction Fragment Length Polymorphism
RIPA	Radioimmunoprecipitation
ROS	Reactive Oxygen Species
rRNA	Ribosomal Ribonucleic Acid
R-THMF	(2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran
SAM	*S*-adenosylmethionine
SCFA	Short Chain Fatty Acid
SEM	Standard Error Mean
SDS-PAGE	Sodium Dodecyl Sulfate—Poly Acrylamide Gel Electrophoresis
SIgA	Secretory Immunoglobulin A
SIGIRR	Single Ig IL-1-Related Receptor
SOD	Superoxide Dismutase
ST	Heat-stable Enterotoxin
TBST	Tris-Buffered Saline containing Tween 20
Tc	Cytotoxic T cells
TEER	Transepithelial Electrical Resistance
Th	Helper T cells
TIR	Toll/IL-1 receptor
TIRAP/Mal	TIR Adaptor Protein
TJ	Tight Junction

TLRs	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor – Alpha
TRAM	Translocating Chain-Associated Membrane Protein
Treg	Regulatory T cells
TRIF	Toll/IL-1 Receptor Domain-containing Adapter inducing IFN-β
Ts	Suppressor T cells
TSB	Tryptic Soy Broth
TTGE	Temporal Temperature Gradient Gel Electrophoresis
VFA	Volatile Fatty Acid
VTEC	Verotoxin-producing *Escherichia coli*
WHO	Worldwide Health Organization
ZO-1	Zonula Occludens-1

CHAPTER 1 INTRODUCTION

As a powerful disease-control tool and growth promoter in improving animal production and performance, antibiotics which were first discovered by Alexander Fleming in the 1920s, are prevalently used in the animal industry for a few decades (Reardon, 2015). Actions of antibiotics primarily aim attention at stimulating metabolic processes, improving nutrient absorption, and interfering with microorganisms causing non-specific subclinical diseases. Subsequently, the inclusion of antibiotics has been greatly extended into the animal feed industry promptly due to its high efficacy and considerably economic effectiveness (Vieco-Saiz et al., 2019). Notwithstanding the huge contribution to the animal industry, many issues regarding the indiscriminate use of antimicrobial growth promoters (AGP) have emerged. For instance, the development of bacteria with antibiotic resistance (e.g., *Streptococcus pneumoniae*, *Streptococcus pyogenes*, staphylococci, members of the *Enterobacteriaceae* and *Pseudomonas* families) and meat products with antibiotic residues has become a crisis to human health and the environment (Neu, 1992). Due to the improper use of antibiotics for animals contributes to the emergence of antibiotic-resistance bacteria, the prohibition of in-feed antibiotics had been put into practice in 2006 in the European Union and Canada took the corresponding action by only allowing the critically important antimicrobials used for therapeutic treatments in livestock as of December 2018 (European Union, 2006; Government of Canada, 2018). So as to mitigate the consumption of antibiotics especially to overcome the most challenging period in young animals, a large body of antibiotic alternatives, such as the immunity modulating agents, bacteriophages and their lysins, antimicrobial peptides, probiotics, prebiotics, and synbiotics, phytogenic substances, inhibitors targeting pathogenicity (bacterial quorum sensing, biofilm, and virulence), and feeding enzymes has been developed (Cheng et al., 2014). Amongst these alternatives to antibiotics, probiotics, which have potent in

antimicrobial activities and have capacities to enhance nutrient digestibility, exhibit promising alternatives to antibiotics when administered to young piglets without a well-developed digestive system and readily suffering from enteric diseases.

CHAPTER 2 LITERATURE REVIEW

2.1 PROBIOTICS IN SWINE INDUSTRY

Probiotic, a Greek language derivation meaning “for life”, is defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hotel and Cordoba, Food and Agriculture Organization of the United Nations (FAO) / Worldwide Health Organization (WHO), 2001). Probiotics are extensively present in dairy cheese and yogurt, which were initially recognized as beneficial microorganisms. The probiotic stains with well recognized beneficial effects on swine gut health and nutrition include *Lactobacillus rhamnosus* (*L. rhamnosus*), *L. reuteri*, bifidobacteria and certain strains of *L. casei*, *L. acidophilus*, *Bacillus coagulans* (*B. coagulans*), *Escherichia coli* strain Nissle 1917 (*E. coli* Nissle 1917), certain enterococci, especially *Enterococcus faecium* SF68, and the yeast *Saccharomyces boulardii*, most of which originate from lactic acid bacteria (LAB) genera (Pandey et al., 2015). LAB genera, which produce lactic acid as their major metabolic end-product of carbohydrate fermentation, include *Lactobacillus*, *Lactococcus, Leuconostoc*, *Pediococcus*, *Streptococcus*, *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Mokoena, 2017). This LAB group comprises gram-positive, acid-tolerant, generally non-sporulating, non-respiring rod (bacillus) or spherical (coccus) shaped bacteria (Yang et al., 2015). Besides, more probiotic candidates constantly emerge with more refined and specific research efforts. Significant health benefits of probiotics are substantially credited to their characterized properties of maintaining microbiota equilibrium along the digestive tract and stimulating a range of host defense mechanisms against the risk of some types of pathogenic invasion in young livestock (Dong et al., 2014; Liu et al., 2014; Zhang et al., 2019). *Lactobacillus* and *Bifidobacterium* are two major genera of healthy gastrointestinal microbes,

commonly used and marketed in feeds worldwide. Most of these microbes will be excreted out of animal bodies along with defecating. Consequently, constant supplementations of these probiotics in animal feed are capable to maximize the opportunities of neither suffering from enteric diseases nor fecal pathogen shedding (Liao and Nyachoti, 2017).

Potential probiotic candidates should be generally recognized as safe (GRAS) and devoid of antagonistic action (cytotoxicity, antimicrobial resistance, and hemolyzable effect) (Lee and Salminen, 1995). Probiotics for human consumption are restricted to be of human origin but not for animal consumption even though host-species specific strains are commonly recommended (Dowarah et al., 2017). Proper identification and characterization of cultures of probiotics before use are quite important. Initial screening of potential probiotic candidates usually includes the determination of biochemical indexes associated with resistance to the digestive stress (e.g., low pH, bile salt, gastric juice, hydrolytic enzymes, etc.), which is to guarantee the minimum probiotic loss and successfully reaching the target sites when passing through the alimentary canal. For the probiotic production and processing, there are several criteria mentioned by Plessas et al. (2012), such as phage resistance, viability and stability during processing and storage, and good sensory performance as well. The bottom line of feeding probiotics as substitutes to antimicrobials lays on the enhancement of animal growth performance and production through improving nutrient digestion and absorption, increasing the survival rate of young animals and reducing pathogenic infection (e.g., necrotic enteritis in chickens or diarrhea in pigs) and fecal pathogen shedding (Murphy et al., 2017).

General feeding strategies of probiotics for animal consumption are in the forms of live cells, dried products (freeze-dried or lyophilized, and spray-dried), fermentation, encapsulation, or in a multi-strain regimen (Bajagai et al., 2016; Piyadeatsoontorn et al., 2019). Encapsulation is one of

the most stable methods to maintaining probiotic biological activity either during storage or ingestion due to the employment of materials with high tolerance to temperature, acid and bile salts. Meanwhile, the optimal rate of gastrointestinal release of encapsulated probiotics is assured. Several heat susceptible probiotics such as *Lactobacillus* are usually present as lyophilized or encapsulated forms in animal feeds. It should be of note that lyophilized probiotics recommended by Dowarah et al. (2017) would lose their viability during lyophilization and also need time to recover from the cold temperature. Piyadeatsoontorn et al. (2019) indicated that probiotic strains (*L. plantarum* and *L. paraplantarum*) prepared in encapsulated form had greater cell viability than lyophilized powder when stored at 25 ℃ ~ 30 ℃ for 2 days.

2.2 ANTIMICROBIAL EFFECTS OF PROBIOTICS

In connection with the favorable claims of probiotics, they should be able to suppress the colonization of pathogens by competitive exclusions, which has been deemed as a preliminarily prophylactic way of protecting intestinal epithelial cells from pathogenic invasion (Vieco-Saiz et al., 2019; generalized in Figure 2.1). Other potential preventive mechanisms are via the regulation of inflammatory and immune responses, and the underlying mechanisms of which have been studied by many research groups with inconsistent results (Table 2.1).

Table 2.1 List of probiotics currently studied for underlying mechanisms in porcine-related systems.

Probiotics	System	Challenge	Signaling cascades	Summary	Ref.
***L. rhamnosus* GG**	IPEC-J2 cells	LPS	NF-κB and MAPK (i.e., p65, p38, ERK1/2)	Alleviated LPS-induced inflammatory cytokines (IL-6 and TNF-α) and TLRs-2/4/9 activation at messenger ribonucleic acid mRNA level, consequently inhibited MAPK and NF-κB signaling activations	(Gao et al., 2017)
***L. rhamnosus* ATCC 7469**	IPEC-J2 cells	ETEC F4 strain (serotype O149:K91, F4ac)	EGFR-independent Akt phosphorylation	Suppressed the elevation of TNF-α, TLR4 and NOD2	(Zhang et al., 2015)
***L. plantarum* CGMCC1258**	IPEC-J2 cells	ETEC F4 strain (serotype O149:K91, F4ac)	NF-κB (IκBα) and MAPK (p38)	Decreased IL-8 and TNF-α, higher mRNA levels of negative TLR regulators	(Wu et al., 2016)
			JNK	Increased JNK activation	(Zhu et al., 2020)
***Pediocccus pentosaceus* L1**	IPEC-J2 cells	ETEC F4 strain C83902 (serotype O8:K87, F4ac)	NF-κB signaling	Antagonized growth and adherence of ETEC F4, reduced responses of IL-6, IL8, and TNF-α probably via down-modulating gene expressions of *RELA* and *NFKB1* involved in NF-κB activation.	(Yin et al., 2020)
***L. plantarum* N14**	PIE	ETEC 987P	TLR signaling	Upregulated the negative regulators of the TLR signaling IRAK-M and MKP-1.	(Murofushi et al., 2015)
***L. reuteri* LR1**	IPEC-1 cells	ETEC F4 strain (serotype O149:K91, F4ac)	MLCK	Improved content of TJ proteins (ZO-1 and occludin) in a MLCK-dependent manner	(Yi et al., 2018)

L. acidophilus	Weaned piglets	LPS	NF-κB and MAPK (p65, p38)	Suppressed ETEC-induced upregulation of pro-inflammatory cytokines, increased the expression of the negative regulators of TLRs signaling, including Tollip, IRAK-M, A20 and Bcl-3 in spleen of ETEC-challenged piglets.	(Li et al., 2016)
***Bifidobacterium animalis* ssp. *lactis* Bb-12**	Swine monocytes and mesenteric cells	Not determined	TLR signaling	Participated in IL-10 production	(Arenas-Padilla et al., 2018)

Note: **IPEC-J2**: Porcine intestinal epithelial cells; **LPS**: Lipopolysaccharide; **ETEC**: Enterotoxigenic *Escherichia coli*; **PIE**: Porcine intestinal epitheliocyte; **NF-κB**: Nuclear factor kappa-light-chain-enhancer of activated B cells; **MAPK**: Mitogen-activated protein kinase; **EGFR**: Epidermal growth factor receptor; **ERK**: extracellular-signal-regulated kinase; **Akt**: Protein kinase B; **JNK**: c-Jun N-terminal kinase; **MLCK**: Myosin light-chain kinase; **TNF-α**: tumor necrosis factor – alpha; **TLRs**: Toll-like receptors; **NOD**: nucleotide-binding oligomerization domain; **IRAK-M**: interleukin-1 receptor-associated kinase M; **MKP**: mitogen-activated protein kinase phosphatase; **TJ**: tight junction; **ZO-1**: Zonula Occludens-1; **Bcl3**: B-cell Chronic lymphocytic leukemia/lymphoma.

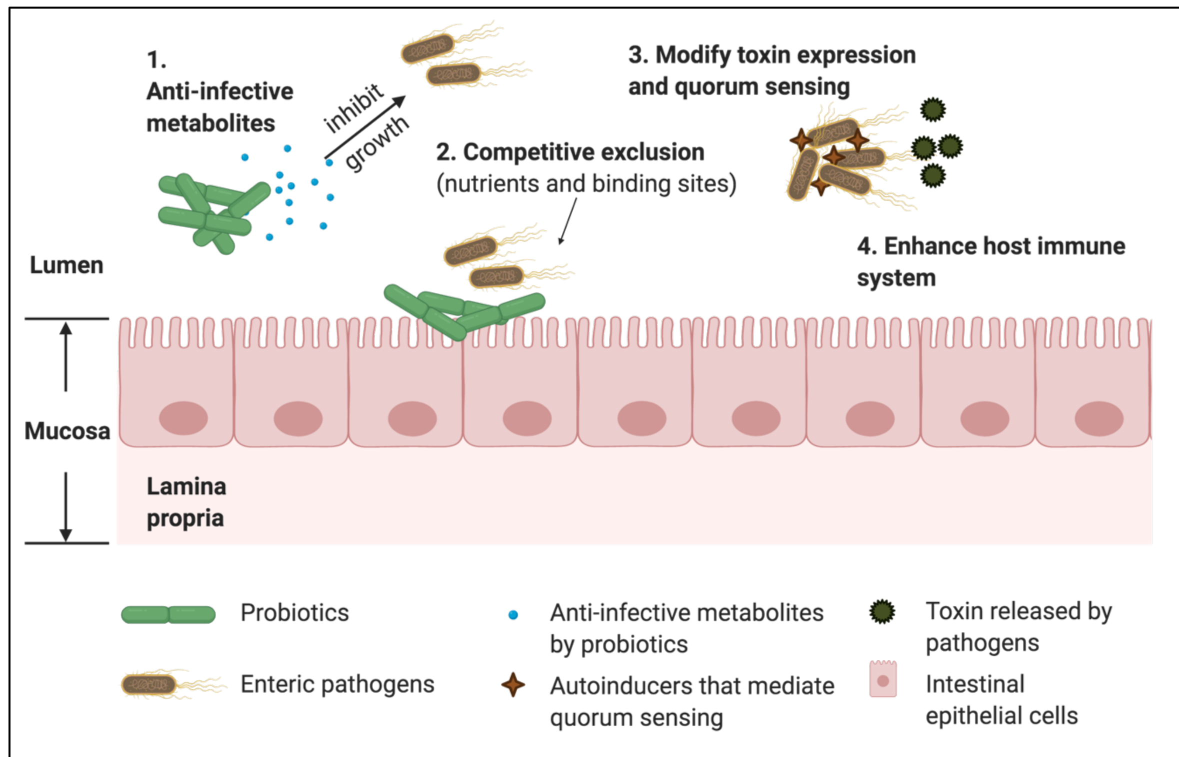


Figure 2.1 Mechanisms of pathogen inhibition by LAB-probiotics (Adapted from Vieco-Saiz et al., 2019 with modifications). This diagram summarizes the main mechanisms of pathogen inhibition by LAB probiotics. These include the production of anti-infective metabolites, competitive exclusion and modification of toxin expression and quorum sensing.

2.2.1 Anti-infective metabolites

Data from Office International des Epizooties (OIE)-World Organization for Animal Health (2020) showed that the most common pathogenic infections in swine production are due to *Salmonella enterica* (*S. enterica*), *E. coli*, *Streptococcus suis*, and *Pasteurella multocida*. A large number of studies demonstrated that the application of probiotics enables protection from pathogenic invasion through the generation of antimicrobial compounds including bacteriocins (with antibiotic potent), organic acids, and hydrogen peroxide (Liao and Nyachoti, 2017).

Bacteriocins characterized by bacteriostatic or bactericidal proprieties and generated by LAB bacteria, target specific microorganisms without harboring cytotoxic traits or causing bacteria population disorders (Belguesmia et al., 2010). There are two main categories of bacteriocins, including high molecular mass proteins (25-80 k Daltons/Da) chiefly from *Gracilicutes* (mostly *enterobacteriaceae*) and low molecular mass peptides (<10 kDa) mainly by *Firmicutes* (mostly LAB), which are emerging as credible antimicrobial alternatives due to well-established efficacy in pathogen mitigation strategies (especially for those likely to be already resistant to critical antibiotics) by either single or cocktail regimens (Cameron et al., 2019). Bacteriocins generated by gram-positive bacteria comprise four classes: Class I, known as lantibiotics, consisting of atypical post-translationally, enzymatically modified amino acids, exhibit resistance to heat, extreme pHs and several enzymes; class II includes thermostable and unmodified bacteriocins; class III includes larger (>10 kDa), thermolabile bacteriocins; and class IV includes thermostable and post-translationally modified circular peptides with resistance to extreme pHs and proteolytic enzymes, which might also require binding to lipids or carbohydrates for activity (Gillor et al., 2008; Lagha et al., 2017). To date, compelling bacteriocins produced by the LAB group mostly comprise classes I and II. Herein, these two categories are mainly stressed in this thesis.

Nisin is the most broadly recognized product in class I bacteriocins and it is found from the metabolites of *Lactococcus lactis*, which has been proved to prevent infections successfully (Lagha et al., 2017). Combined treatments of several LAB-derived bacteriocins (i.e., nisin, colistin, and enterocin) enable to eradicate the planktonic and biofilm of few colistin-resistant swine-origin *E. coli* strains (Al Atya et al., 2016). Consistent results were reported by Field et al. (2017) who highlighted that the concomitant use of nisin, trans-cinnamaldehyde essential oils, and ethylenediaminetetraacetic acid (EDTA) could be used as a new solution to swine-origin *E. coli*

strains mitigation via extending their lag-phases of growth (Field et al., 2017). Additionally, nisin has also been shown to be capable of regulating immunoreactivity on porcine peripheral blood leucocytes *in-vitro*, by which suppressed the production of interleukin (IL)-6 and phagocytic activity stimulated by *E. coli* strains (Małaczewska et al., 2019). Two consistent outcomes were demonstrated by Singh et al. (2014) and Rishi et al. (2014), who put forth novel adjunct formulations of β-lactam with nisin. These authors suggested these formulations to confer anti-infective activity against *S. enterica* serovar Typhimurium involving disrupting membrane, suppressing the synthesis of deoxyribonucleic acid (DNA), RNA, and protein, as well as modifying immune responses in the host (Singh et al., 2014; Rishi et al., 2014).

Reuterin (β-hydroxypropionaldehyde, class II bacteriocins), produced from glycerol metabolism by *L. reuteri*, exerts an antibiotic effect by interfering with DNA synthesis and it is resistant to proteolysis and lipolysis (Singh, 2018). Reuterin yields are frequently found among swine-origin *L. reuteri* isolates which were likely to be encountered throughout the gastrointestinal tract (Rodriguez et al., 2003). The antimicrobial effect of reuterin against *E. coli* was discovered to be probably pertinent to cause gene expression alterations in response to oxidative stress by modifying thiol groups in *E. coli* cells (Schaefer et al., 2010). A critical synergistic bactericidal action against food-borne Gram-negative pathogens (i.e., *E. coli* O157:H7 and *S. enterica*) was found with the co-administration of reuterin with lactoperoxidase but not with nisin in a refrigerated milk assay (Arqués et al., 2008).

Bacteriocins might act at colonizing peptides with evidence that only bacteriocin-producing *L. salivarus* DPC6005 was mostly dominated in ileal digesta and mucosa of weaned piglets when co-administered with other probiotic strains (Walsh et al., 2008). This may suggest their bacteriocins engaging in immunomodulation effects via downregulation of cluster of

differentiation (CD)-25 T cells and monocytes but initiating the levels of $CD4^+$ $CD8^+$ T cells production and IL-8 mRNA (Walsh et al., 2008). Moreover, another thermostable class II bacteriocin produced by *L. salivarius* UCC118—ABP-118, was confirmed as the primary mediator of protection against *Listeria monocytogenes* in mice (Corr et al., 2007). This is coincident with findings in *L. salivarius* UCC118 fed pigs that Riboulet-Bisson et al. (2012) speculated that ABP-118 bacteriocin may contribute to the effect on Gram-negative microorganisms, which collectively suggests a decisive and favorable influence of bacteriocin-producing LAB on pathogen mitigation. Meanwhile, however, they pointed out that there is no unambiguous correlation between colonization and bacteriocin-producing property as no significant colonization activity observed between the wild type of *L. salivarius* UCC118 and its bacteriocin-lacking derivatives.

Organic acids are the main end products of LAB fermentation. They play an important role in maintaining an acidic environment and creating a selective barrier against non-acidophiles (Singh, 2018). These include acetate, propionate and butyrate and also are well-known as short-chain fatty acids (SCFA). Organic acids as antibiotic alternatives have been thoroughly demonstrated by Ricke (2003). According to this author, organic acids act by increasing bacterial intracellular protons once organic acids ionize at the bacterial internal site, whereupon accelerating the depletion of bacterial energy to pump protons out of cells. Acetic acid as a heterofermentative metabolite of LAB is established to provide energy for colon mucosa, which concomitantly is of advantage to gut barrier function (Hijova and Chmelarova, 2007). Likewise, lactic acid excreted by LAB is capable of suppressing the dissemination of *Salmonella spp*., *E. coli*, or *Listeria monocytogenes*, which is achieved by its antimicrobial action through destructing the cytoplasmic membrane and hindering membrane potential of bacterial cells (Wang et al., 2015; Singh, 2018). In addition to pH reduction within ecological niches, necessary for the elucidation of the precise

role of unionized organic acids in the disruption of pathogen microorganisms is that they have a shorter chain in contrast to long-chain fatty acids, making them an effective and non-specific permeabilizer to entering Gram-negative outer cell membranes (Alakomi et al., 2000). Impairment of acid secretion due to the not well-developed digestive tract of newly weaned piglets and subsequent higher acid-binding capacity of minerals in creep feed mutually lead to a more alkaline stomach environment and thus disrupt the first defense line that filters most outer pathogens (Roselli et al., 2005). As a result, dietary organic acid inclusion for piglet diets becomes a feasible method of maintaining the gut ecosystem. But an emerging issue of acid-tolerant pathogens has been observed, perhaps being related to increased virulence when exposed to an acidic environment (Ricke, 2003). Supplementations of probiotics (i.e., *Lactobacillus*, *Bacillus*) in post-weaning piglet diets had a positive effect on the augmentation of ileal propionic acid production as well as lactic and acetic acid concentrations in ileum and colon, perhaps resulting in a reduction in ileal *E. coli* counts and risks of pathogen-related diarrhea (Giang et al., 2010; Prieto et al., 2014). The dietary probiotic blend is also provided as a mitigation strategy of fecal noxious gas content, which had been verified by numerous reports where probiotic supplementations were implicated in diminishing fecal NH_3-N and butyric acid contents in growing-finishing pigs without effect on acetic and propionic acid concentrations (Chen et al., 2006; Tufarelli et al., 2017; Yan and Kim, 2011). However, it should be noted that only undissociated short-chain fatty acids can exert their antimicrobial functions on inhibiting undesirable microorganisms, which thus cause copious encapsulation technological methods to enhance probiotic resistance to passage environment.

Hydrogen peroxide (H_2O_2) produced by probiotics in the presence of oxygen, whereby the flavoprotein-containing oxidases (e.g. nicotinamide adenine dinucleotide phosphate (NADH) oxidase) consume oxygen to give rise in the levels of superoxide anions (O_2^-) and H_2O_2 appears to

possess antagonistic activity by elevating oxidative damage in several bacterial strains (Kullisaar et al., 2002; Singh, 2018). The interaction of different oxygen levels and probiotics (*L. acidophilus* and *Bifidobacterium spp.*) did not affect the optimum pH value (pH = 5), the response of superoxide dismutase (SOD), and probiotic sensitivity to H_2O_2 (Talwalkar and Kailasapathy, 2003). On the other hand, the catalytic decomposition rate of H_2O_2 significantly increased at 21% oxygen concentration with a simultaneous increase in specific activities of NADH oxidase and NADH peroxidase (Talwalkar and Kailasapathy, 2003). The majority of reports from H_2O_2-producing probiotics emphasized on their protective role against bacterial vaginosis a few decades ago, which is reasoned to be of importance in healthy vaginal colonization. A previous study had revealed the major predominance of H_2O_2 producers in normal vaginal flora and postulated the H_2O_2-producing potent in the nonspecific inhibition of catalase-negative organism's growth (Eschenbach et al., 1989). Nonetheless, the most favorable conditions to obtain the highest biomass of H_2O_2 among investigated vaginal *Lactobacillus* strain in a short duration were under agitated cultures at 37 °C and pH 6.5 (Tomás et al., 2003). Besides, Reid (2001) found there were other non-H_2O_2 producers (i.e., *L. rhamnosus* GR-1) present in the vaginal isolates, which may indicate that not only H_2O_2 but the interaction with other antimicrobial compounds from probiotics could be responsible for pathogenic inhibition. A non-H_2O_2 producer was found to be capable of significantly reducing glutathione activity in packed red blood cells, while increasing concentrations of catalase and SOD in erythrocytes in weaned piglets fed with *Pediococcus acidilactici*-supplemented diets (Dowarah et al., 2018). It is known that SOD and catalases are regarded as powerful indicators of antioxidative activity. SOD catalyses the dismutation of O_2^- to O_2 and H_2O_2, providing cellular defense against reactive oxygen species (ROS) and catalases are highly specific enzyme active in reducing H_2O_2 to water (Fukai and Ushio-Fukai, 2011). However,

the antibacterial activity porcine-related studies, based on only H_2O_2 produced by probiotics, is questionable. Comparatively low concentration of H_2O_2 generated by probiotic strains may not be likely detrimental to intestinal epithelium as one study had demonstrated that concentration of H_2O_2 up to 0.1 mM would significantly decrease 20% of cell viability in IPEC-J2 cells (Yin et al., 2017). The maximum H_2O_2 content (5 μg/mL ~ 309 μg/mL) observed in three *L. fermentum* strains was far less to cause damage (Kullisaar et al., 2002). Additionally, exposure to non-lethal doses of H_2O_2 might likely protect *E. coli* against subsequent H_2O_2-induced killing effect, which would be intractable when bacteria experience such oxidative stress in gradients along the dynamic alimentary canal in animals (Rodríguez-Rojas et al., 2020). Thus, it is critical to ensure a constant concentration of H_2O_2 directed against pathogens. However, it has been found that bacteria evolve the ability of scavenge toxic oxygen radicals when exposed to ROS through eliciting several scavenging enzymes generated by pathogens or through the metalloregulator MntR in controlling bacterial resistance to oxidative stresses (Chen et al., 2017; Rodríguez-Rojas et al., 2020). Therefore, it is of importance to use H_2O_2 combined with other agents (i.e., lactoperoxidase, thiocyanate ion) to enhance the bacteriostatic activities in inhibiting bacterial metabolism and growth (i.e., oral streptococci) (Thomas et al., 1994).

Anti-infective property of other metabolised by-products of probiotics is relatively less studied. Still, they exert additive antimicrobial effects via inhibiting colonization of conditionally pathogenic microorganisms (i.e., ethanol produced by heterofermentative LAB), interfering with arginine metabolism of Gram-negative bacteria (i.e., diacetyl), as well as building up an anaerobic milieu with carbon dioxide liberation (Vieco-Saiz et al., 2019). Taken together, specific probiotic-derived inhibitory ingredients and subsequent involvement in a series of metabolism pathways

also account for the modulatory effects of intestinal epithelial cells and immune systems which are probiotic gene-specific (Yan and Polk, 2011).

2.2.2 Competitive exclusion

The competitive exclusion, firstly proposed by Hardin (1960), refers to complete compete cannot coexist, by which probiotics could competitively exclude pathogens, by fighting for adherent receptors (i.e., glyco-conjugates) and nutrients over intestinal mucosa and enhancing biofilm formation, whereby establishing persistent resistance to pathogenic and other non-indigenous microbial colonization in the host (Dowarah et al., 2017; Miyazaki et al., 2010). Interestingly, some yeasts (i.e., *Saccharomyces boulardii* and *Saccharomyces cerevisiae*) are rich in mannose on the surface, which might act as receptors of type I fimbriae-encoding enteropathogenic bacteria (i.e., *E. coli* and *Salmonella*), therefore effectively capturing pathogens and minimizing the risks of pathogens' attachments to the mucosal surface (Tiago et al., 2012). Regardless of different *Lactobacillus* strains (*Lactobacillus rhamnosus* GG (LGG), *L. reuteri* ATCC 55730 and DSM 12246, *L. johnsonii* NCC 533) with variable binding efficiencies (38%-16%) to IPEC-J2 cells, all of them decreased the adhesion of enteropathogenic *Escherichia coli* (EPEC) O138 by more than 2-fold (Larsen et al., 2007). Similarly, the attachment of ETEC to immortal pig intestinal-2I (IPI-2I) cells was lowered by 80% with the presence of probiotic *Saccharomyces cerevisiae* var. Boulardii (Badia et al., 2012). Indeed, these results are consistent with a report indicating that probiotic *Bacillus* administration had the similar effects as antibiotic-mediated diet, which was able to reduce ileal *E. coli* counts dramatically in post-weaning piglets (Prieto et al., 2014).

2.2.3 Modification of toxin and autoinducer expressions of pathogens

Enterotoxigenic pathogens (e.g., ETEC) release toxins after internalization upon the intestinal mucosa. Research insights on the effects of probiotic administration on the expressions of toxins and other virulence factors reported that the amalgamation of *L. acidophilus*, *L. casei* and *L. rhamnosus* could antagonize the cytotoxicity of *Clostridioides difficile* via impeding toxin A/B generation and counteracting toxic effect in patients (Gunaratnam et al., 2019). Exopolysaccharides obtained from *Lactobacillus* and *Bifidobacterium* strains were able to retard the cytotoxic effect of toxins of *Bacillus cereus* in cancer coli-2 (Caco-2) colonocytes (Ruas-Madiedo et al., 2010). The symbiotic combination of probiotics with other additives such as prebiotics may exert better functionality on the host. For instance, co-culture of ETEC with *L. rhamnosus* and short-chain fructo-oligosaccharides was shown to significantly decrease heat-labile toxin (LT) production, which potentially illustrated that carbohydrates play a prebiotic role and participate in optimizing the establishment and prevalence of probiotics against pathogenic invasion within GIT (Anand et al., 2019). Some case studies reported that probiotics have the capability of inhibiting biofilm generation of enteric pathogens. For example, *L. acidophilus* A4 produced exopolysaccharides to interfere with gene expression of curli generation and chemotaxis in *E. coli*, and thereafter inhibiting *E. coli* biofilm formation (Kim, Y. and Kim, S. H, 2009). *L. acidophilus*, *L. plantarum*, *Bifidobacterium longum* and *B. lactis* also showed the capacity of suppressing biofilm generation of enteric pathogens (i.e., *S. typhimurium* and *E. coli*) in Caco-2 cells (Candela et al., 2008). Similarly, Medellin-Peña et al. (2007) highlighted that supplementation with *L. acidophilus* exhibited strong inhibitory activity on autoinducer-2 (AI-2) production of human-origin enterohemorrhagic *E. coli* (EHEC) O157: H7, whereby the locus of enterocyte effacement was inactivated and thus inhibited EHEC adhesion. However, the

interaction between AI-2 and enterotoxin expressions remains elusive. It had been illustrated that both *L. acidophilus* and *L. sakei* NR28 could reduce virulence expression of EHEC O157: H7 via suppressing AI-2 associated activity (Kim et al., 2008; Park et al., 2014). A report from Zhu et al. (2011) suggested that there might be a negative involvement between AI-2 activity and ETEC F4 JG280 enterotoxin expressions. They put forth that AI-2 producing ETEC F4, which lacked the enterotoxin genes did not cause cell death; besides, overexpression of AI-2 genes mitigated ETEC F4-induced IPEC-J2 cell death. Except for antimicrobial property, Liu et al. (2010) also found that pre-treatment of *L. acidophilus* plays an antiviral role in augmenting the viral titers presumably as a consequence of suppressing enterotoxin expressions by probiotic supplementations.

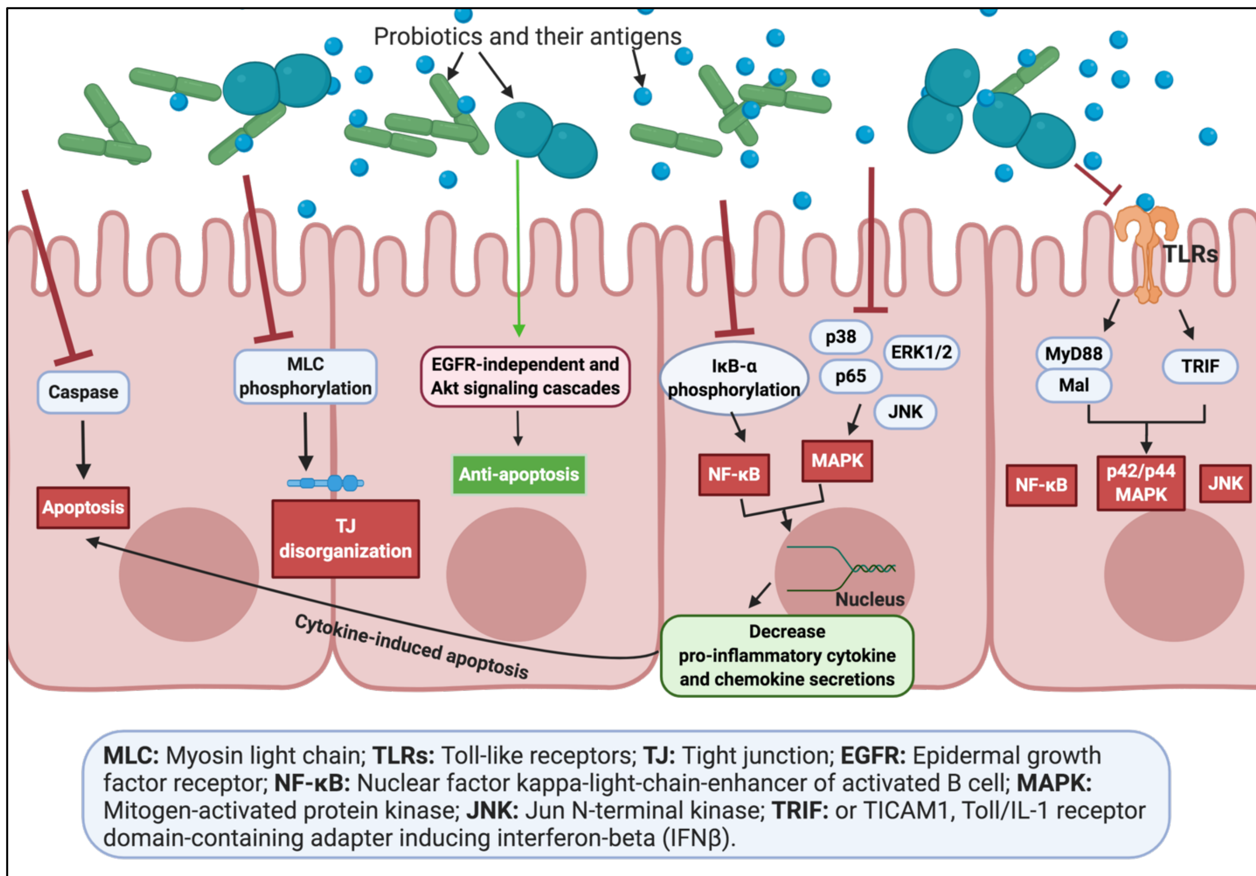


Figure 2.2 Schematic diagram of LAB involved modes of action resistant to pathogen infection in the host (as the purpose of displaying the underlying modes of action summarized

in Table 2.1).

2.2.4 Enhancement of host immune responses

Probiotics play a crucial part in the gut mucosal defense barrier, as demonstrated by its implication on specific immunomodulation in innate and adaptive manners. Inflammatory reactions are firstly triggered in response to the gut epithelium infection and damage, and meanwhile, the innate immune systems will be initiated to recruit innate immune cells (e.g., phagocytic cells, dendritic cells/DCs, natural killer/NK cells, mast cells, macrophages) to control damaged cells, thereafter effector cells (T and B lymphocytes) mobilized exclusively in response to humoral and intracellular infection in the antigen-specific immune system (Isolauri et al., 2001).

2.2.4.1 Innate immune responses

The innate immune system includes physical and anatomical barriers as well as effector cells, antimicrobial peptides, soluble mediators, and cell receptors (Anaya et al., 2013). In response to the recognition of external pathogenic signaling molecules by cellular receptors (e.g., TLRs, NOD-like receptors (NLRs), etc.) over intestinal epithelial cells and sub-epithelial immune cells (e.g., mast cells, macrophages, and DCs), the innate immunomodulatory system is activated concomitantly with a rapid burst of soluble mediators (e.g., cytokines, chemokines) into the surrounding tissue and circulation (Anaya et al., 2013; Luo et al., 2015). These mediators recruit effector cells (e.g., granulocytes, monocytes, innate lymphoid cells) to the site of infection where, via multiple mechanisms, they aid in containing and eventually clearing the pathogens (Anaya et al., 2013). The schematic pathways of host immune responses interacting with bacteria are roughly described in Figure 2.3.

Gut mucosal barrier acts as the first line of host defense against gastrointestinal tract disorders. Enterotoxins produced by ETEC often elicit intracellular accumulation of 3',5'-cyclic adenosine monophosphate (cAMP) and 3',5'-cyclic guanosine monophosphate (cGMP) concurrently with an efflux of chloride ions from the cells, which leads to severe watery diarrhea and death in newly-weaned piglets (Read et al., 2014). Under cellular mechanisms, ETEC-infected epithelial cells display a significant decline in the trans-epithelial electrical resistance (TEER) with concomitantly higher monolayer permeability, which is a direct consequence to a reduction in the expression levels of TJ proteins (Brosnahan and Brown, 2012). Several reports have established protective effects of probiotic supplementations on barrier integrity in *in-vitro* or *in-vivo* experiments against enteropathogenic disruption (Karimi et al., 2018; Wu et., 2016; Yang et al., 2015; Zhang et al., 2015).

The role played by probiotics at enhancing resistance to pathogenic infection is often associated with their regulation in the secretions of antimicrobial peptides and inflammatory cytokines. Liu et al. (2017) suggested that *L. reuteri* supplementation had an evident increase in the expression of porcine intestinal beta-defensin peptides (pBD2, pBD3, pBD114, and pBD129) in the model IPEC-J2 cells and neonatal piglets. Similar results were also observed by Zhou et al. (2014) who pointed out that ETEC-induced pathogenicity is able to be blocked through the upregulation of antimicrobial peptides with the addition of *L. reuteri* in IPEC-J2 cell and *C. elegans* models. Moreover, probiotic-derived antimicrobial peptides might also be involved in the expressions of ZO-1, occludin and claudin proteins *in vivo* and *in vitro* (Han et al., 2015; Zhang et al., 2015). Whilst, Yu et al. (2018) observed no significant improvement in the protein abundance of ZO-1 in IPEC-J2 cells supplemented with antimicrobial peptide against ETEC infection, which may indicate a variation among peptides with different sources. It is of note that the innate immune

system is activated concomitantly with a rapid burst of soluble mediators (e.g., cytokines, chemokines) into the surrounding tissue and circulation, which is commonly known as inflammatory reaction (Anaya et al., 2013; Luo et al., 2015). These explain the rationale for the crosstalk between cytokines and mucosal disruption in response to enteric pathogen-implicated damage. There is a stunning array of evidence that illustrate a robust suppression of enteric pathogen-induced pro-inflammatory cytokine and chemokine secretions in the host treated with various probiotic strains (Badia et al., 2012; Liu et al, 2010; Skjolaas et al., 2007). Additionally, data presented by Zhou et al. (2014) and Qiu et al. (2017) demonstrated that the addition of *Lactobacillus* probiotics can inhibit ETEC infection through the upregulation of host expression of anti-inflammatory cytokines. It is of note that cocktails of probiotic with other compounds and probiotic-derived components can also exert strong regulation of cytokine secretions in infected hosts (Chytilová et al., 2013; Gao et al., 2017; Yan and Polk, 2002; Yan et al., 2007). The extent of cytokine modulation by probiotics is likely to be target-specific, namely, their effects against pathogen infections are not of broad-spectrum, thus leading to inconsistent results observed in many probiotic studies (Liu et al., 2010; Skjolaas et al., 2007).

So, how the innate immune responses are activated? There are many cell surface receptors in response to the recognition of external pathogenic signaling molecules, such as G protein-coupled receptor (GPR), TLRs, NLRs, etc. TLRs are a significant class of pattern recognition receptors that are present on intestinal epithelial cells and immune cells, which participate in the induction of both tolerance and inflammation. Many enlightening works of literature have been published providing new insight into the immunoregulation of probiotics for several intestinal pathogen-associated inflammations in which TLRs exert a significant role. There is also a shred of evidence showing positively immunoregulatory effects of probiotics or probiotic-derived components by

modulating TLRs and NODs expressions, subsequently impeding (p38) MAPK and NF-κB pathways or initiating epidermal growth factor receptor (EGFR)-independent and Akt signaling cascades (Gao et al., 2017; Wu et al., 2016; Yan and Polk, 2002; Yan et al., 2007; Zhang et al., 2015; Zhu et al., 2020). Moreover, probiotic administrations appear to participate in the activation of peroxisome proliferator-activated receptor (PPAR)-γ and GPR 41, which therefore triggers elevated defensin production in intestinal epithelial cells (Liu et al., 2017).

The release of cytokines and chemokines in response to pathogen-associated inflammation recruits effector cells (e.g., granulocytes, monocytes, innate lymphoid cells) to the site of infection where, via multiple mechanisms, they aid in containing and eventually clearing the pathogens (Anaya et al., 2013). Chytilová et al. (2013) reported that the pre-treatment of *L. plantarum* can potentially modulate the phagocytosis in piglets challenged with ETEC F4 mainly via triggering helper T cells (Th1)-mediated cell immunity in porcine intraepithelial part. While *Saccharomyces* treatment-triggered modulation in porcine monocyte-derived DCs, the same was not found in DCs co-cultured with ETEC and *Saccharomyces* (Badia et al., 2012). What is intriguing is that healthy pigs supplemented with *Bacillus* showed higher granulocyte percentage (and lower lymphocyte) without adverse impact on histopathological examination of organ tissues, which might be due to local recognition and adaptation to in-feed probiotics but would not induce inflammation (Prieto et al., 2014). A corollary, which complies with the characteristic of the adjuvant effect that probiotics supplementations tend to elevate the levels of effector cells even without bacterial invasion, which was in parallel with data from a pig trial with multi-microbial preparations that had increased phagocytic activity in monocytes with a surging percentage of phagocytic cells as well (Laskowska et al., 2019). However, the underlying mechanisms of probiotics on the relief of pathogen infections in terms of the involvement of innate lymphoid cells in immunomodulation

remain elusive and require more research efforts. Additionally, in light with outcomes from human and murine studies, the activities of DCs concerning their maturation and cytokine stimulation are quite different in response to various probiotic and pathogenic strains (Christensen et al., 2002; Hart et al., 2004; O'Mahony et al., 2006).

2.2.4.2 Adaptive immune responses

In contrast to innate immunity, the adaptive or acquired immune system establishes long-term memory, which aims to identify and destroy the specific pathogenic antigens or any harmful extracellular particles. T and B lymphocytes constitute the specific immune system. T cells mainly encompass cytotoxic T cells (Tc), helper T cells (Th), and suppressor T cells (Ts), while memory cells and plasma cells are the two types of B cells. The activation of B cells by extracellular protein antigens relies on Th cells for optimum functions only when B cells act as antigen-presenting cells (APCs), which present the processed pathogen antigens with major histocompatibility complex (MHC II) to the cluster of differentiation $CD4^+$ Th cells, after that, stimulating the differentiation of activated B cell clones into memory and plasma B cells. Currently, at least four types of $CD4^+$ Th cells exist: Th1, Th2, Th17 and regulatory T cells (Tregs). While foreign molecules originating from intracellular pathogens are presented to $CD8^+$cytotoxic T cells via MHC I (Anaya et al., 2013).

Discrimination between pathogens and healthy microbiota directly by DCs is in connection with the maintenance of Th and Treg cells in the intestinal epithelial layers. Some strains of *L. gasseri*, *L. johnsonii*, and *L. reuteri* elicited the potential regulatory roles in clearly adjusting $CD4^+$ and $CD8^+$ T cell responses toward Th1 and Tc1 pathways with interferon-gamma (IFN-γ) secretion by stimulation of human myeloid DCs (Mohamadzadeh et al., 2005). Likewise, initiation of monocyte-derived DCs exposed to *Lactobacillus* (strains of *L. reuteri* and *L. casei*) is ready to activate the proliferation of Treg cells with mounting levels of IL-10 while interfering with the

development of other effector T cells (Smits et al., 2005). Likewise, Treg cells are also able to be stimulated by the oral inoculation of Bacillus to weaned pigs negative to F4ab/ac receptor after ETEC F4/ verotoxin-producing *E. coli* (VTEC)/ EPEC infection (Zhou et al., 2015). Besides, piglets fed with the administration of Bacillus also had a substantial increase in the populations of intraepithelial $CD8^+$ T cells in the jejunal epithelium and $CD25^+$ lymphocytes in the lamina propria with high expression of γδT cells at the post-weaning period (35-day age) (Scharek et al., 2007). By contrast, minor effects of probiotic *E. coli* strain Nissle 1917 on increasing the distribution of $CD8^+$ cells in colonic mucosa were revealed when supplemented with probiotics in healthy young pigs (Duncker et al., 2006). One needs to bear in mind that additional responses to changes in microflora antigenic stimulation should be curtailed to decrease the loss of microflora-specific immunoglobulins, most of which target the intestinal microorganisms and dietary antigens (Richards et al., 2005). Consequently, minimum probiotic-administered quantity requires to be regulated in the animal feed to avoid the inefficiency of probiotic bacteria initiating immunomodulation. Few studies are investigating probiotic presenting alterations in the expansion of T cells in the peripheral blood and intestinal tissues upon ETEC infection. Work from Luo et al. (2015) enlightened that IL-17B- and IL-17F-producing Th17 cells were pertinent to the host immunomodulation against ETEC F4-induced diarrhea in piglets. The potential roles of Th17 in participating in the mucosal immunity to eliminate pathogens, such as by triggering pathogen-specific secretory immunoglobulin A (SIgA), were summarized by several studies (Cao et al., 2012; Hirota et al., 2013; Jaffar et al., 2009). Wang et al. (2009) observed that the concentrations of pro-inflammatory cytokines and $CD4^+$ lymphocytes increased with the addition of *L. fermentum* in ETEC challenged piglets. *Bacillus* pre-treatment at low- and high-dose, respectively, acts as a promising regimen by inducing an increase in the proportion of peripheral blood $CD4^-CD8^-$ T-

cell subpopulations and the expansion of $CD4^{-}CD8^{-}$ T cells in the inflamed intestine in weaned Mucin 4 resistant pigs following ETEC challenge (Yang et al., 2016). On the other hand, studies of probiotic-triggered adjustment in the activation of adaptive immune responses have been conducted under other enteric pathogen infections. One study where piglets were supplemented with the same strain of *Enterococcus faecium* to demonstrate probiotic-host interaction under *S. Typhimurium* infection model, it did not detect the probiotic-mediated potential to strengthen the portions of CD8αβ T lymphocytes in either peripheral blood samples or piglet jejunal epithelium (Mafamane et al., 2011). Another study conducted with newly weaned piglets found that exclusive administration of LGG would slightly render the augmentation of intraepithelial $CD3^{-}CD19^{-}$T-$bet^{+}IFN\gamma^{+/-}$cell subsets in the peripheral blood without presenting inflammatory reactions (Zhang et al., 2019). Instead, the supplementation of LGG could also attenuate this risk evoked by *S. enterica* serovar infection and maintain gut microbiota equilibrium (Zhang et al., 2019). Apart from modulation of T cell-mediated responses, the ability of probiotics to trigger antimicrobial antibodies (e.g., IgA, IgG) produced from effector B/plasma cells explains their critical function in the host defense against extracellular pathogens. The results of a study that determined a shift in the natural shedding of enteric viruses established that pigs supplemented with probiotic *Enterococcus faecium* did not show the advancement of IgA and IgG levels compared with that of control diet (Kreuzer et al., 2012). While they found that populations of CD8β T cells, $CD21^{+}$/MHC II^{+} and membrane-IgM^{+} B cells depicted higher in probiotic-supplemented piglets at 12-, 26- and 54-day age, respectively. Given that administration of different probiotic strains does intimately correlate to communicating with host immune regulation, more related research is required to elaborate on how the underlying immunoregulatory mechanism work between probiotic-host under pathogen stimulation.

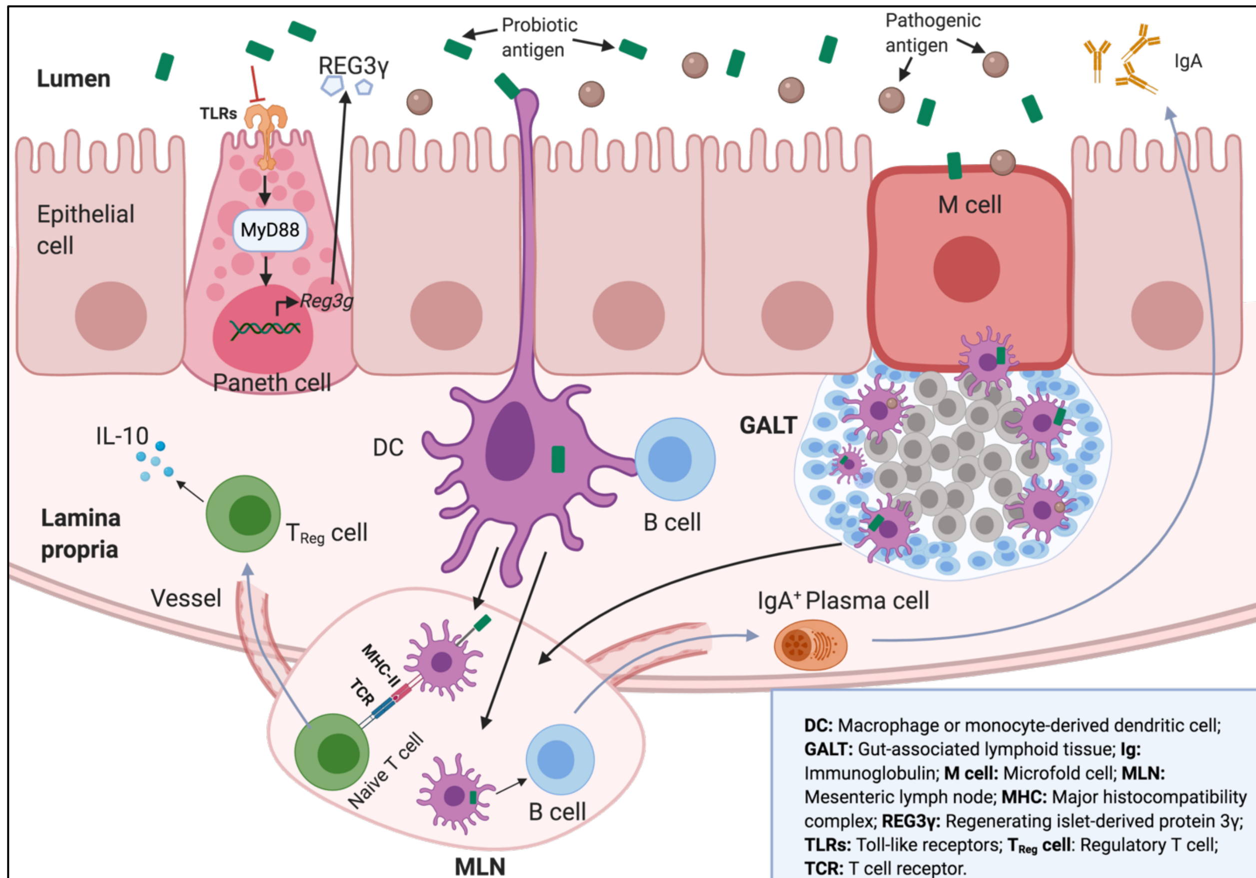


Figure 2.3 Commensal microorganisms modulate intestinal immunity (Adapted from Kraehenbuhl and Corbett, 2004; Perez-Lopez et al., 2016 with modifications). Commensal bacteria in the gut lumen are continuously sampled by cells of the immune systems. In Paneth cells, bacteria antigens are recognized by TLRs which accordingly induce the expression of the antimicrobial lectin REG3γ. M cells of the gut epithelium can also import bacteria into the dome region of the GALT where DCs engulf the microbes. Sub-epithelial CX3C-chemokine receptor 1 $(CX3CR1)^+$ DCs are able to sample commensals directly and present antigenic peptides from captured bacteria to B and T lymphocytes either locally in the GALT, or within the MLNs that drain the gut submucosa. Presentation of microbial antigens to T cells triggers Treg cells to migrate into the gut lumen and meanwhile induce the anti-inflammatory cytokine IL-10 production. Likewise, activation of B cells induces a commensal-specific IgA response from plasma cells that

prevents the commensals from straying beyond the gut mucosa where they could elicit a systemic inflammatory response and simultaneously modulate the composition of gut microflora (Kraehenbuhl and Corbett, 2004; Perez-Lopez et al., 2016).

2.3 POST-WEANING DIARRHEA IN PIGLETS

2.3.1 ETEC-related diarrhea

Weaning and post-weaning periods are the most stressful conditions in commercial swine production (Ahasan et al., 2015). ETEC bacteria is studied as a principal pathogen, which would induce severe watery diarrhea and quick dehydration typically in newly-weaned piglets, and thereafter lead to acute death, causing huge losses in swine farming (Nagy and Fekete, 1999). The cell density of ETEC at 1×10^5 colony of units (CFU)/mL caused phosphatidylserine expression and disrupted metabolism, signs indicative of the early stages of apoptosis (Johnson et al., 2009). The binding of the ETEC colonization factor (CF) to the specific CF receptor in the intestinal brush border, which allows for ETEC adhesions to the porcine intestinal epithelium, is considered a prerequisite step in the subsequent pathogenesis of diarrhea (Jin and Zhao, 2000; Nagy and Fekete, 1999). The most common adhesins of ETEC in animals are the fimbriae (or pili) on the surface: F4 (K88), F5 (K99), F6 (987p), F17 and F18 fimbriae, amongst which ETEC F4-related diarrhea is one of the most frequently seen in clinical cases (more than 90%) in weaning piglets (Nagy and Fekete, 1999). On the other hand, enterotoxins released by ETEC are responsible for fluid secretion that have crucial impacts on the piglets' diarrhea once ETEC fimbriae implementing well-adhesion and amplification over the intestinal brush border (Dubreuil, 2013). LT and heat-stable toxin (STa and STb) are two primary categories of enterotoxins generated from ETEC bacteria. Pathogenesis of LT aims to trigger an imbalance in the osmotic state of the intestinal epithelium via exaggerating

chloride secretion into the lumen and with respect to ST, which interrupts intestinal fluid secretion through stimulating the production of cGMP by STa as well as inducing chloride and sodium secretions to disrupt the intestinal osmotic state by STb (Dubreuil, 2013). Additionally, a decline in the trans-epithelial electrical resistance of IPEC-J2 cells is a consequence of the damaged membrane integrity in occludin, ZO-1, and claudin-1 after challenged with ETEC (Brosnahan and Brown, 2012).

2.3.2 Quorum sensing in ETEC bacteria

The process that bacteria employ to communicate intercellularly about cell density is called quorum sensing, which relies on the self-generation and perception of autoinducers to modify their physiological behaviors (typically at high cell density), such as symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation (Abisado et al., 2018; Ng and Bassler, 2009; Rutherford and Bassler, 2012). To date, three distinct documented autoinducers used in *Vibrio spp*. bacteria are 3-hydroxy-C4-N-(3-hydroxybutanoyl)-l-homoserine lactone (AHL or harveyi autoinducer-1, HAI-1), 4,5-dihydroxy-2,3-pentanedione (DPD or AI-2), and (S)-3-hydroxytridecan-4-one (cholerae autoinducer-1 or CAI-1), which are synthesized by LuxM, LuxS, CqsA enzymes, respectively (Karnjana et al., 2020). From the light organ of the Hawaiian Bobtail Squid *Euprymna scolopes*, Ruby (1996) firstly discovered the AHL-associated cognate regulatory circuit in the bioluminescent marine bacterium *Vibrio fischeri* (Gram-negative bacteria). Current advances in the quorum sensing mechanisms of *Vibrio spp*. indicate a wide array of quorum sensing components that Gram-negative bacteria possess to display cell-cell communication both within and between bacterial species. Here, we concentrate on recapitulating how the quorum sensing flow works in the ETEC bacteria. In enteric pathogens such as *E. coli*,

they produce and detect AI-2 signal molecules to organize their quorum sensing milieu and regulate gene expression in response to cell density (Ng and Bassler, 2009). AI-2 molecules are derived from the shared precursor DPD, which is encoded by *luxS* gene and is generated from *S*-adenosylmethionine (SAM) in three enzymatic reactions (Ma et al., 2017). The distinct form of AI-2 used in *E. coli* is an unborated rearranged DPD moiety [(2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (R-THMF)], which binds to the periplasmic transporter—Lsr (LuxS-regulated) to enter *E. coli* cell and consequently activating Quorum sensing (QS)-controlling target genes (Ma et al., 2017; Ng and Bassler, 2009). Due to homologs of LuxS synthase exist in a wide range of bacterial genomes (including *E. coli*, and *S. typhimurium*), it is proposed AI-2 as an extensive signal molecule to detect cell-to-cell communication (Wang et al., 2019). However, it should be bear in mind that without cognate receptors, DPD would be unstructured and spontaneously transformed into different derivatives in different bacterial species and under various growth conditions, which could be responsible for AI-activity (Ng and Bassler, 2009). Additionally, based on *V. harveyi* bioassay, only extracellular AI-2 production or the accumulated bioluminescence can be identified (Winzer and Hardie, 2003).

However, the interaction between bacterial quorum sensing and virulence factors is intricate. A research group suggested that there might be a negative correlation between gene expression of *luxS* and *estA* of ETEC F4 in the IPEC-J2 cell model and bacterial quorum sensing (Zhu et al., 2011). They demonstrated that the deletion of enterotoxin genes did not impact the regular AI-2 production though, and that overexpressed *luxS* genes could either inhibit the *estA* gene expression or reduce IPEC-J2 cell death challenged with 1×10^8 CFU/mL of ETEC JG280. Intriguingly, Zhou et al. (2014) indicated that both quorum sensing-related gene expressions (*luxS* and *pfs*) and biofilm formation decreased in the *flic* mutant (encoding the major flagellin protein) but increased

in the *faeG* mutant of ETEC (encoding the major subunit of F4 fimbriae). Moreover, they also pointed out that the deletion of *flic* and/or *faeG* genes remarkably downregulated the AI-2 activity in the exponential-phase bacterial culture supernatants. There are a few pieces of research concentrated on investigating the effects of live probiotic strains or their secreted compounds on mitigating ETEC pathogenesis, presumably via blocking the quorum sensing mechanisms but it remains to be far eluded thoroughly. Evidence indicating the inhibitory effects of *L. acidophilus* on quorum sensing signals of EHEC O157:H7 had been determined with a notable reduction of extracellular AI-2 generation and virulence-related gene expressions (*luxS*, *qseA*, *ler*, *espA*, *espD*, *tir*, *eaeA*, *flic*, and *hlyB*) (Medellin-Peña et al., 2007). Consistent outcomes were obtained in an *in vivo C. elegans* study which determined an outstanding downregulation of AI-2 activity and virulence factors of EHEC O157:H7 supplemented with 1.0% (w/v) *L. acidophilus* A4 cell extract (Kim et al., 2008). Advances in the quorum sensing signaling pathways that a multitude of bacteria utilize to regulate virulence factor expressions provide more pieces of evidence for antimicrobial therapies by interfering or inhibiting bacterial cell-to-cell communication. There are various ways of disrupting usual quorum sensing systems in target bacteria, which supports the potential anti-quorum sensing therapies for bacteria-related diseases as reviewed by Jiang et al. (2019). Briefly, these strategies for interfering with quorum sensing include, inactivating quorum sensing receptors, inhibiting synthesis or initiating degradation of signaling molecules or autoinducers, and combing anti-quorum sensing agents with antibiotics (Jiang et al., 2019).

2.4 BENEFITS OF PROBIOTICS IN PIGLET GROWTH PERFORMANCE

Young piglets, especially during the weaning period, are in a state of stress, which is involved with changes in feed formula and new environment. Additionally, the digestive systems of

weaning piglets are not well developed and readily suffering from pathogen-incurred enteric diseases (i.e., diarrhea, edema, enterohemorrhage). Disorders in gut functions eventually cause poor feed intake and body weight loss and eventually lead to huge economic loss in swine industry. The plethora of reports from various probiotic strains on pig-related studies provide critical evidence indicating that dietary probiotic addition is in favor for establishing a stable and healthy gut microflora milieu, and as well as improving intestinal barrier functions, therefore enhancing host resistance to post-weaning stress.

In most cases, the effects of combined probiotic strains are explained by the additive contribution of each strain to the alteration of indigenous bacterial growth. Not only the population but the abundance of total indigenous lactobacilli was elevated by the application of different lactobacilli strains with various origins in pigs (Ohashi et al., 2007; Takahashi et al., 2007). Stimulation of indigenous desirable microbiota manifests higher exposure to an increment in SCFA production by probiotic fermentation. For instance, accessions of *L. fermentum* to the newborn piglet diet predominantly elevated the concentrations of butyrate and branched-chain fatty acids, and equally scaled down the amount of *Clostridium sporogenes* (Liu et al., 2014). Generation of organic acids from probiotic fermentation in the lower gut provides the primary energy source such as butyric acid for colonic epithelial cells, which facilitates to build of a suitable fermentation environment that accelerates the breakdown of undigestible nutrients (Hijova and Chmelarova, 2007). In addition, organic acids are aforementioned to deplete pathogen energy with the capability of marking down intracellular pH value (Ricke, 2003). This also triggers good results of alleviating post-weaning diarrhea (PWD). Ohashi and Ushida (2009) reviewed that SCFA production by probiotic fermentation might participate in mitigating incidences of PWD through altering the colon motility, which could be postulated from their report suggesting that increase

terminal colon motility without promoting defecation may be related to the stimulation of colonic fermentation as a result of fecal pH decline in pigs fed with *L. casei* Shirota (Ohashi et al., 2001). The hold of digesta in the large intestine may increase the water absorption from the digesta, presumably explaining the recovery from diarrhea when probiotics are consumed. Many enlightening reports have been published substantiating the reduced diarrhea frequency in weaned piglets with probiotic administrations (Hayakawa et al., 2016; He et al., 2018; He et al., 2019; Lu et al., 2018; Sayan et al., 2018).

On the other hand, healthy gut ecology is equipped with favorable intestinal morphology, potentially enhanced by a desirable microbiota population. Significant improvements of villus height and the ratio of villus height to crypt depth have been observed by different research groups when probiotics were administered to weaned piglets against enteric viruses and ETEC bacteria (Hayakawa et al., 2016; Mao et al., 2016; Sayan et al., 2018). Elongated villus extends the accessible area for nutrient absorption upon the mucosal layer, critically in favor of enhancing nutrient digestibility for diet-adapting newly-weaned piglets. Improved intestinal morphology is concomitant with significant increases in average daily gain (ADG) and feed intake (ADFI) (Hayakawa et al., 2016; Sayan et al., 2018). However, Huang et al. (2004) suggested that neither ADG nor ADFI was remarkably enhanced by 21-day lactobacilli administration in newly weaned piglets. However, data from Ahmed indicating that *L. reuteri*-based diet was exclusively able to enhance ADG and ADFI in weaned pigs from day 1 to day 14 in contrast to that with antibiotic-mediated and *Bacillus*-added diets (Ahmed et al., 2014). Collectively, the efficacy of probiotic administrations is likely associated with feeding schedule limitations. Results from the effects of probiotics on fecal bacteria composition display a similar issue. For example, *Lactobacillus*-based treatment significantly diminished fecal *E. coli* concentration on day 28; nonetheless, pigs fed with

the *Bacillus*-based diet had a considerably lower fecal concentration of *E. coli* on day 21 compared with *Lactobacillus*-treated pigs (Ahmed et al., 2014). This may depend on the morphology and physiological status of animal intestines, which would vary with animal age, and therefore elder animals with better gut health have no or weaker responses to probiotic supplementations.

2.5 RESEARCH ABOUT *LACTOBACILLUS ZEAE*

Lactobacillus zeae (*L. zeae*), an autochthonous and facultatively heterofermentative microorganism, is one species of the genus *Lactobacillus* normally present as an indigenous native of swine and poultry GI tract (Bajagai et al., 2016). Traditional isolation and characterization of gut microbiota (e.g., *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*) were based on culture-dependent techniques through selective culturing, morphological, biochemical, and physiological assays. Briefly, *Lactobacillus* mixtures were collected by using antiseptic swabs and placed into sterile Man, Rogosa, and Sharpe (MRS) broth incubated for 24 hours at an optimal temperature of 37°C. Then, *Lactobacillus* selection agar was used to selectively isolate lactobacilli bacteria and Gram stains or the Analytical Profile Index (API) carbohydrate fermentation pattern was done to establish Gram-positive or -negative bacteria (McCoy and Gilliland, 2007; Gong and Yang, 2012).

In several literature studies, the taxonomy of the *L. casei* was a subject of controversy as *L. casei*, *L. zeae*, and *L. rhamnosus* are closely related taxa, making it impossible to employ DNA-DNA based hybridization as the only method to determine these closely related microbial species (Huang et al., 2018). Thus, considerable reports using various developed technological and integration techniques were preferred over the DNA-DNA hybridization techniques as they enable immediate species identification of *L. casei*, *L. zeae*, and *L. rhamnosus* isolates. Such techniques include sequence signature analysis and immunoblotting (Dobson et al., 2004), group-specific

polymerase chain reaction (PCR) combined with SNaPshot minisequencing using heat shock protein 70 (*dnaK* gene) or species-specific restriction fragment length polymorphism (RFLP) profiles of the *Lactobacillus* strains (Huang et al., 2011; Huang and Lee, 2011), PCR-amplification and temporal temperature gradient gel electrophoresis (TTGE) (Vasquez et al., 2001), and combination of phenotypic (MALDI-TOF) and molecular (16S ribosomal RNA (rRNA), *pheS*, *rpoA* genes sequencing) methods (Delavenne et al., 2013). On the other hand, sequence comparison between the 16S rRNA gene copies of closely related *Lactobacillus* species was not reliable enough to distinguish the correct phylogenetic relationships of strains in a single *Lactobacillus* genus due to some species sharing almost the same sequence in some copies (i.e., *L. casei* CCUG 21451^T and *L. zeae* CCUG 35515^T) (Vásquez et al., 2005). Accordingly, a phylogenetic study of *Lactobacillus* strains displayed by the *tuf* signature sequence proposed that the utilization of partial *tuf* genes sometimes allowed to identify the inferring strains within a species in terms of slight variability of *tuf*-nt sequences (Chavagnat et al., 2002). While Delavenne et al. (2013) recommended Pulsed-Field Gel Electrophoresis (PFGE) typing of *L. zeae* isolates, and they referred to the comparative *pheS* gene sequence analysis which acts as the most useful tool for the classification of strains.

However, unlike *L. casei* and *L. rhamnosus*, *L. zeae* has yet been used as probiotics in human and animal, which indeed requires more findings based on the biochemical and biophysical properties and some general characteristics of responses of all *L. zeae* strains (Björneholm et al., 2002). In terms of carbohydrate fermentation, *L. zeae* strains produce acid from glucose, galactose, maltose, sucrose, trehalose, mannitol and cellobiose, but they do not ferment raffinose or L-arabinose, which could be used as a promising milk starter culture (Gueimonde et al., 2004). *L. zeae* RMK354 strain isolated from raw milk showed strong angiotensin-converting enzyme

inhibitory activity and higher esterase and leucine arylamidase activities (Lim et al., 2008). Also, *L. zeae* RMK354 indicated variable susceptibility to tested antibiotics (more sensitive to penicillin-G, bacitracin, novobiocin, but most resistance to polymyxin B and vancomycin) and comparatively tolerant to low pH and bile juice and appeared to have medium inhibitory activity against *S. Typhimurium* with the rate of 60% (Lim et al., 2008). *L. zeae* ATCC 15820 utilized citrate as a sole energy source and displayed a citrate transport system inducible by citrate without the presence of an equivalent plasmid coding for citrate permease (de Figueroa et al., 2000). Based on the results from the resistance trials, *L. zeae* LB1 strain isolated from chicken feces had been reported by Yang et al. (2014) that indicated good tolerance to low pH, bile salt (from 0.3% to 1.5% (v/v)), and high susceptibility to all tested antibiotics (more sensitive to ciprofloxacin, tetracycline, erythromycin, ampicillin, gentamicin, but most resistant to penicillin-G, chloramphenicol, and lincomycin).

There are only several pieces of evidence of the underlying mechanisms that *L. zeae* use in the host under pathogenic microorganisms' infection. The regulation in the gene expression of both ETEC toxins and antimicrobial peptides/defense molecules presumably mediated by *L. zeae* LB1 through the p38 MAPK and insulin/insulin-like growth factor (DAF/IGF) signaling pathways in the nematode was identified to be responsible for the protective effects (Zhou et al., 2014; Zhou et al., 2018). IL-12 induction by *L. zeae* YIT 0278^T (DSM 20178) is highly correlated with its resistance to the intracellular digestion by N-acetylmuramidase (lysozyme and M-1 enzyme) in macrophages after phagocytosis, which indicates that comparative intact phagocytosed bacteria readily tend to induce IL-12 production throughout 24-hour phagocytosis period (Shida et al., 2006).

Lactobacillus and *Bifidobacterium* are sensitive to room temperature and acidic environments such as stomach acid, and this becomes the major challenge to greatly influence the consistent and reproducible results obtained from probiotic-related studies. More attention has been paid to encapsulation techniques, to maintain probiotic viability during processing, storage and transition through the gastrointestinal tract. Also, this new technology advances the development of commercial probiotic products. Though with good tolerance to an acidic environment, selected *L. zeae* LB1 encapsulated with sodium alginate and sodium caseinate in a spray-dried manner had demonstrated good protection of probiotic bacterial cells when stored at the water activity range from 0.11 to 0.76, while encapsulation with sodium alginate and soy protein isolate presented the best resistance to gastric fluid (Liu et al., 2018).

2.6 DISCREPANCIES AMONG DIFFERENT STUDY SYSTEMS FOR SELECTING PROBIOTICS

Cell lines used for *in-vitro* preselecting probiotics for swine include IPEC-J2, IPEC-J1, and IPI-2I which all derive from porcine intestinal cells. Cell systems are usually built up to study underlying microbe-host interaction mechanisms of probiotics at the intestinal level before applying probiotics into the animal diet to corroborate their effects. In general, direct *in-vivo* study systems involve the use of *C. elegans* or carrying out animal trials to distinctly observe animal performance and production via evaluations of various phenotypic and biological measurements. Both IPEC-J2 and IPEC-J1 are non-transformed (compared to IPI-2I cell line) and intestinal porcine epithelial cells derived from jejunum in 12-hour old piglets while their differences are mainly implicated in the comparisons of morphological differentiation, function, and metabolisms explicitly ascribed by Nossol et al. (2015). They conducted a wide array of genome-wide gene

expression analysis, regulation pathways, as well as functional measurements and eventually they concluded that IPEC-J2 serves as a preferential tool to study metabolism pathways, with the nature of superiorly morphological differentiation and functional differentiation. It is in accordance with the statement by Paszti-Gere et al. (2012) who consented to the application of IPEC-J2 cells as a specific model for porcine-originated infection researches on screening probiotic candidates.

Despite IPEC-J2 cells provide specificity for studying porcine-derived infections, they are restricted as unicellular organisms with different immune systems in comparison to multicellular *C. elegans*. *C. elegans* has been extensively harnessed as an *in-vivo* model focusing on micro-host interactions, thanks to its small size, short generation time, and suitability for genetic analysis and innate immunity studies (Riddle et al. 1997). Additionally, *C. elegans* can be infected and killed by a few animal pathogens, e.g., *Salmonella* (Aballay et al., 2000; Ikeda et al., 2007) and *E. coli* (Irazoqui et al., 2010) and have an inducible immune system which can mimic part of nonspecific immunomodulation in mammals and represent similarity in the immune response (Wang et al., 2011). Hence, *C. elegans* with these unique characteristics provide an advanced and ideal tool for quick preselecting potential probiotics and also can be used as a reference in pig studies. Despite that, *C. elegans* are unable to evaluate a large number of probiotic isolates and besides they possess their own digestive microbiota, which presumably impacts the results of selecting probiotics (Zhou et al., 2014). Zhou et al. (2014) performed a study using both *C. elegans* and IPEC-J2 cells to screen probiotics against ETEC infection, confirming that both assays had defined the same *L. reuteri* CL9 strain with the highest protection to cells and worms. It becomes clear that the two assays are complementary to each other, and their combination can improve the efficiency and reliability in the process of selecting probiotics for the control of bacterial pathogens. Further

animal trials are required to verify these probiotic candidatures against porcine pathogenic infections.

2.7 CHALLENGES/LIMITATIONS ASSOCIATED WITH PROBIOTIC STUDIES

Many research groups gained inconsistent results about the efficacies of various probiotics. Most studies reported positive effects on the growth performance of newly-weaned piglets (Stein and Kil, 2006), while some reports described no effect or even an adverse effect on pig growth performance (Lallès et al., 2007). The inconsistency indicates the relative complexity in the development and application of probiotics in the swine feed industry. Varying outcomes from researches on studying the effects of probiotics have been implicated in the establishment of an experimental process, study on molecular mechanisms, analytical techniques in the molecular and genomic aspects, as well as practical applications.

On one hand, host responses from probiotic administration and harmful stimulation are dependent on the methodology and parameter of experiments, specifically such as experimental duration, dosages, forms of probiotics and infection, the combination of multiple strains or other supplementary chemicals, and tested subjects. Experimental duration should be restricted to exclude variants in rapid pH changes leading to cell damage especially under *in-vitro* cell models. Most probiotic genera tested originate from LAB including *Streptococcus*, *Lactobacillus*, *Lactococcus*, and *Leuconostoc*. These LAB generate organic lactic acid as the primary metabolite which would bring about cell medium color alteration in few hours especially added at a relatively higher concentration (data not shown). Likewise, color change in the cell medium is also regarded as one of the properties that harmful bacterial invasion and nutrient depletion have shown. Variable doses of probiotics and pathogens or stimulants would have been adapted depending on the

respective levels of their protection and infection to the host. Moreover, synergistic effects from the cocktail of multi-strain probiotics or with other bioactive chemicals (such as prebiotics, phytogenic compounds, etc.) provide research and market prospect with broad applications of combined products and the potential benefits of additive products in the animal feed industry. The selection of probiotic forms can directly impact the effectiveness and subsequent functions of them on ameliorating cell damage. Different forms of probiotics have variable efficacies as previously described (Bajagai et a., 2016; Dowarah et al., 2017; Piyadeatsoontorn et al., 2019). Furthermore, the study on probiotics should foremost choose homologous cell lines, autochthonous probiotics and pathogens to be tested. It is of significance that each probiotic strain more likely does work for resistance to specific pathogenic infection under specific duration even exerts the best synergic effects in combination with other supportive components.

The aforementioned considerations in setting up experiments can also induce the alteration of molecular mechanism pathway and gene expression triggered by probiotic additions or pathogen infection. Notwithstanding that, it is elusive to precisely demonstrate all these microbe-host interactions under immune response. As Dowarah et al. (2017) proposed, being difficult to demonstrate the immunomodulation by probiotics is predominantly credited to their protective characteristic rather than wiping out the pathogenic invasion in the alimentary tract, and often affected by the animal's immune status and the various applied situations. Not only that, all improvements or positive effects observed from probiotic supplementations on intestinal epithelial integrity, anti-inflammatory reactions, antioxidant status and so on are the basis for safeguarding immune defense and any imbalance of these will cause immune responses eventually. This is consistent with the summary by Thaiss et al. (2014) that host innate immunity and gut microbiota persist an interdependent relation upon which microbial activity would impact the innate

immunomodulation and simultaneously their stable ecosystem relies on the proper function of innate immune effector cells. Knockout of genes involved in a few main metabolic pathways or utilizing clones and mutants have been employed as common ways processed by many research groups to find out which metabolic pathway that probiotics may be involved in to regulate the cell signaling in the host (Zhou et al., 2018). Additionally, it highlights the need for scientific research to better understand the mode of action and particularly molecular mechanisms underlying the probiotic effects. Clearly, easy measurement(s) of the characteristics relating to probiotic effects would assist in the effective development and application of novel probiotics, but this is often lacking. Whereas, the recent development of technologies for molecular and genomic studies has made it possible to identify the molecular mechanisms and key biomarkers associated with beneficial microbiota compositions (Gong and Yang, 2012). There is evidence from work performed on the precision microbiome retrieval for the successful control of *Clostridium difficile* using various “omic” technologies (Buffie et al., 2015).

Despite several potential probiotic candidates have been thoroughly demonstrated to act highly efficiently with respect to experimental outcomes, there remain extensively questionable on probiotic viability during feed processing, storage, and ingestion. Given that, the advent of various processing tools has provided feasible protection on probiotics (especially for heat-sensitive bacteria) from inactivation during handling and GIT transition. For instance, microencapsulation, freeze-dried, and spray-drying forms of probiotics are commonly used in the practical employment of animal feed to overcome these frequent issues. Whereas it should be of note that the probiotics may encounter the difficulty of selecting encapsulation materials and a few stress conditions (such as heat stress, dehydration, oxygen exposure, and osmotic stress), thereby incurring probiotic disruption as indicated by Liu et al. (2015).

Characteristics of various probiotic strains make them challenging the comparison of probiotic impact results either on the *in-vitro* or *in-vivo* animal immune systems. Therefore, it still requires more refined and specific research efforts on eliciting cardinal probiotic mechanisms for better comprehension and application of probiotics.

CHAPTER 3 HYPOTHESES AND OBJECTIVES

3.1 HYPOTHESES

The following hypotheses were tested in this thesis:

1. *Lactobacillus zeae* (*L. zeae* LB1 strain) can protect porcine intestinal epithelial cells from enterotoxigenic *Escherichia coli* (ETEC) F4 infection; and
2. *L. zeae* LB1 can reduce the expression of toxins and virulence-related factors in ETEC F4.

3.2 OBJECTIVES

The overall objective was to evaluate the effectiveness of *L. zeae* LB1 against ETEC F4 infection with an *in vitro* porcine intestinal epithelial cell model (IPEC-J2). Specific objectives were to:

1. Investigate the potential of *L. zeae* LB1 on modulating intestinal barrier and innate immune functions and protecting against intestinal injuries and inflammatory reactions induced by ETEC F4-infected IPEC-J2 cells; and
2. Evaluate the effects of *L. zeae* LB1 on the expression of enterotoxins and virulence-related factors in ETEC F4.

CHAPTER 4 EVALUATING THE EFFECTIVENESS OF *LACTOBACILLUS ZEAE* AGAINST ENTEROTOXIGENIC *ESCHERICHIA COLI* F4 INFECTION IN AN *IN VITRO* PORCINE INTESTINAL EPITHELIAL CELL MODEL

4.1 ABSTRACT

Lactobacillus zeae (*L. zeae* LB1 strain) has been shown to prevent *Caenorhabditis elegans* (*C. elegans*) from death caused by enterotoxigenic *Escherichia coli* (ETEC) infection through downregulating the gene expression of ETEC toxins and mediating antimicrobial peptides/defense molecules under the p38 mitogen-activated protein kinase (MAPK) and insulin/insulin-like growth factor (DAF/IGF) signaling pathways in the nematode. However, the protective effects of *L. zeae* LB1 at cellular and molecular levels have not yet been investigated in pigs. In the present study, porcine intestinal cells (IPEC-J2) were used to investigate the potential of *L. zeae* LB1 on modulating intestinal barrier and innate immune functions and protecting against intestinal injuries and inflammatory reactions induced by ETEC F4 infection. The results showed that the pre-treatment of IPEC-J2 cells with *L. zeae* LB1 significantly alleviated the cytotoxicity and cell death induced by ETEC F4 ($P < 0.05$) and reduced the mRNA abundance of interleukin (IL)-8 and IL-6, and the secretion of IL-8 induced by ETEC F4 ($P < 0.05$). The *L. zeae* LB1 pre-treatment maintained significantly higher values of trans-epithelial electrical resistance (TEER) and a concomitant lower dextran-fluorescein fluxes from the apical side to the basolateral side when compared with the cells challenged by ETEC F4 ($P < 0.05$). *L. zeae* LB1 pre-treatment also prevented morphological damage of tight junctions and cytoskeleton caused by the ETEC F4 challenge, indicating that *L. zeae* LB1 pre-treatment maintained the structural integrity of tight junctions. However, *L. zeae* LB1 inclusion showed no significant effect on Zonula Occludens-1

(ZO-1) expression at both mRNA and protein levels ($P > 0.05$) from the ETEC F4 challenge but a significant increase in the protein expression of occludin ($P < 0.05$). The *L. zeae* LB1 administration had no effect ($P > 0.05$) on the ETEC F4 adhesion and the mRNA abundance of antimicrobial defensins (pBD2, pBD3), while it reduced the mRNA abundance of toll-like receptor 4 and 5 (TLR4 and 5) and virulence-related factors of ETEC F4 ($P < 0.05$). Moreover, a DNA marker-based test targeting the mucin 4 gene that encodes the F4 fimbria receptor indicated that the IPEC-J2 cell line was from the pig that was identified as resistant to developing ETEC-F4 diarrhea. These results suggested that probiotic *L. zeae* LB1 effectively protected the intestinal cells from ETEC F4 infection by inhibiting inflammation and maintaining barrier integrity via downregulating TLR4, TLR5, and ETEC F4 virulence-related factors expressions instead of preventing ETEC F4 adhesion. Our data provide further evidence on the mechanisms at the cellular and molecular levels that probiotic *L. zeae* LB1 could improve gut health in pigs.

Keywords: *Lactobacillus zeae*, Enterotoxigenic *Escherichia coli*, IPEC-J2 cells, Tight Junction, Cytokines

4.2 INTRODUCTION

As a powerful disease-control tool and growth promoter in improving animal production and performance, antibiotics have been prevalently used in the animal industry for a few decades mainly as growth promoters (Kumar et al., 2018; Reardon et al., 2015). Actions of antibiotics mainly aim attention at stimulating metabolic processes, improving nutrient absorption, and interfering with microorganisms causing non-specific subclinical diseases. Consequently, the inclusion of antibiotics has been promptly extended into the animal feed industry due to its high efficacy and considerably economic effectiveness. Notwithstanding the huge contribution that it

did to the animal industry, many issues regarding the indiscriminate use of antimicrobial growth promoters (AGP) have emerged. For instance, the development of bacteria with antibiotic resistance (e.g., *Streptococcus pneumoniae, Streptococcus pyogenes*, staphylococci, members of the *Enterobacteriaceae* and *Pseudomonas families*) and meat products with antibiotic residues has become a crisis to human health and the environment (Neu, 1992). Because of this impact, the prohibition of in-feed antibiotics had been put into practice in 2006 by the European Union (European Union, 2006). Canada also took the corresponding action by allowing to only use the critically important antimicrobials for therapeutic treatments in livestock as of December 2018 (Government of Canada, 2018). There has been a wide range of antibiotic alternatives emerging in response to phasing out in-feed antibiotics. Alternatives including immunity modulating agents, bacteriophages and their lysins, antimicrobial peptides, probiotics, prebiotics, and synbiotics, phytogenic substances, inhibitors targeting pathogenicity (bacterial quorum sensing, biofilm, and virulence), and feeding enzymes (Cheng et al., 2014) have since been developed. Among these, a potent antimicrobial activity and capacity to enhance nutrient digestibility make emerging probiotics a promising candidate for substituting in-feed antibiotics, especially for young piglets without a well-developed digestive system and readily suffering from enteric diseases.

Probiotics with beneficial effects on swine gut health and nutrition include several bacteria like, *L. rhamnosus*, *L. reuteri*, bifidobacteria and certain strains of *L. casei*, *L. acidophilus*-group, *Bacillus coagulans* (*B. coagulans*), and the yeast *Saccharomyces boulardii* (Pandey et al., 2015; Yin et al., 2020; Zhou et al., 2018). Lactic acid-producing bacteria (LAB) are commonly known with probiotic properties, which are gram-positive, acid-tolerant, non-sporulating, non-respiring rod (bacillus) or spherical (coccus) shaped (Liao and Nyachoti, 2017). Several LAB strains have been considered as beneficial tools on improving swine production, which compromise

Lactobacillus, *Bifidobacterium*, and *Lactococcus*, etc (Liao and Nyachoti, 2017). Benefits of LAB probiotics are primarily credited to their potency in maintaining intestinal microbiota equilibrium as well as stimulating a range of the host defensive mechanisms against pathogenic invasion in young livestock (Ho et al., 2020; Yin et al., 2020).

Potential probiotic candidates should be generally recognized as safe (GRAS) and devoid of antagonistic action (cytotoxicity, antimicrobial resistance, and hemolyzable effect) (Lee and Salminen, 1995). Probiotics for human consumption are restricted to be of human origin but not in animal consumption even though host-species specific strains are commonly recommended (Dowarah et al., 2017). While properly identification and characterization of cultures of probiotics before use are quite important, initial screening of potential probiotics candidates by determination of biochemical indexes about resistance to the digestive stress (e.g., low pH, bile salt, gastric juice, hydrolytic enzymes, etc.) is needed. This is so, to guarantee the minimum probiotic loss and granting successful reaching of target sites when passing through the gastrointestinal tract. Probiotics candidates should also be able to suppress the colonization of pathogens by competitive exclusion, which has been deemed as a preliminarily prophylactic way of protecting intestinal epithelial cells from pathogenic invasion (Vieco-Saiz et al., 2019). Other preventive methods of these probiotics are via the regulation of inflammatory and immune responses, the underlying mechanisms of which have been studied by many research groups with inconsistent results (Yang et al., 2015; Yin et al., 2020; Zhou et al., 2014).

In the swine industry, ETEC infection is the main cause of enteric diarrhea concomitantly with poor performance and economical losses to swine production (Luppi et al., 2016). LAB bacteria were shown to have protective responses against ETEC infections (e.g., Zhou et al., 2014; Zhou et al., 2018). These studies were based on either *in vivo* trials (Zhou et al., 2014) or *in vitro*

studies involving the use of cell-line models such as IPEC-J2 cell line (Zhou et al., 2014; Wang et al., 2016; Yin et al., 2020). One *in vitro* study had looked at the protective responses of *L. zeae* LB1 against ETEC F4 by monitoring cell signaling and gene expression of ETEC F4 toxins (Zhou et al., 2018). In this recent study, *L. zeae* LB1 was shown to prevent *C. elegans* from death caused by ETEC F4 infection through downregulating the gene expression of ETEC F4 toxins and mediating antimicrobial peptides/defense molecules under the p38 MAPK and DAF/IGF signaling pathways in the nematode (Zhou et al., 2018). However, the protective effects of *L. zeae* LB1 at a cellular level have not been investigated yet. Furthermore, prophylactic advantages of *L. zeae* LB1 in terms of protecting intestinal epithelial cells from pathogenic invasion via the regulation of inflammatory and immune responses is not known. In a recent study by Yin et al. (2020), downregulation of expression of ETEC F4 induced pro-inflammatory genes encoding IL-6, tumor necrosis factor-alpha (TNF-α) and IL-8 in IPEC-J2 cells was observed. These authors did not use *L. zeae* LB1 in their study; they used another LAB bacterium, *Pediocccus pentosaceus*. It is not known if similar downregulation of inflammatory responses would be obtained with the *L. zeae* LB1 and this needs further investigation.

In the present study, we hypothesize that *L. zeae* LB1 can protect intestinal cells from ETEC F4 infection. IPEC-J2 cells were used to investigate the potential of *L. zeae* LB1 on modulating intestinal barrier and innate immune functions against intestinal injuries and inflammatory reactions induced by ETEC F4 infection.

4.3 MATERIALS AND METHODS

4.3.1 Cell culture

The non-transformed neonatal jejunal epithelial cell line IPEC-J2 was a kind gift of Dr. Joshua

Gong, Guelph Food Research Centre, Agriculture and Agri-Food Canada. Cells were cultured in 25 cm^2 or 75 cm^2 flasks in Dulbecco's Modified Eagle Medium: Nutrient Mixture Ham's F-12 (DMEM:Ham's F-12 at 1:1) (Gibco^{TM}, Thermo Scientific, Ottawa, ON, Canada) supplemented with 5 ~ 10% fetal bovine serum (FBS, Gibco), penicillin (100 IU/mL, Sigma-Aldrich, Oakville, Ontario, Canada), streptomycin (100 μg/mL, Sigma-Aldrich) and 3 ng/mL of epidermal growth factor (Gibco) and maintained in a Steri-Cycle CO_2 incubator (Thermo Electron Corporation, Thermo Scientific, Ottawa, ON, Canada) with 5% CO_2 at 37 °C. The medium was changed every other day. Before being transferred into the assay plates, the fully confluent cells were washed with 1 × phosphate-buffered saline (PBS) and digested with 0.25% trypsin-EDTA (Gibco) to be harvested. Harvested cells were resuspended with a suitable amount of medium to obtain the concentration of 1×10^5 cells/mL, approximately. The desired confluent monolayers in the plates were washed with 1 × PBS twice and the cell medium was replaced with 1% FBS in DMEM or DMEM/F12 at least 1 hour before treatments.

4.3.2 Bacteria culture

The strain was originally isolated by the Department of Animal Science of the University of Manitoba in July 2010. It was isolated from feces of piglets infected with post-weaning diarrhea (PWD) at the Veterinary Diagnostic Services Laboratory, Government of Manitoba, Canada. The isolate was confirmed using ETEC F4 antiserum-slide agglutination test. All 4 virulence genes (*luxS, estA*, *estB*, *faeG* and *elt*) were expressed in the ETEC F4 strain used in the study. The isolate was preserved in the CRYOINSTANT (consisting of 2 mL cryovials containing 25 porous beads and cryopreservative-added broth, Deltalab, Rubí, Barcelona, Spain) at -80 °C. ETEC F4 was first cultured in a 50 mL conical polypropylene tube with 10 mL Tryptic soy broth (TSB, Millipore Sigma) at 37 °C overnight (16 ~ 18 hours) with shaking speed at 150 rpm. On the following day

before challenging, an aliquot of 100 μL overnight bacterial suspension was transferred into 10 mL of fresh TSB and inoculated for another 1 ~ 2 hours until reaching the log phase. The optical density (OD) value or absorbance was checked by a spectrophotometer (Biochrom™, Fisher Scientific, Ottawa, ON, Canada) at 600 nm (calibrating with the same new broth). The OD values around 0.3 ~ 0.5 were used for subsequent challenges and the bacterial suspension was diluted into the expected concentrations with 1% FBS in DMEM or DMEM/F12 medium.

Newly isolated *L. zeae* LB1 strain was obtained from the Guelph Food Research Centre, Agriculture and Agri-Food Canada and stored at -80 °C in sterile 15 ~ 20% v/v glycerol. *L. zeae* LB1 were routinely cultured in De Man, Rogosa & Sharpe (MRS) broth (Millipore, Sigma) and placed into a 2.5 L Anaerobic jar (Millipore, Sigma) with an anaerobic atmosphere generation bag (Millipore, Sigma) at 37 °C overnight (16 ~ 18 hours). The OD value was checked by a spectrophotometer (Biochrom™, Fisher Scientific) at 600 nm (calibrating with the same new broth). The OD values around 0.8 ~ 1.0 were used for subsequent challenges. The bacterial suspension was centrifuged at 21,000 × g for 5 minutes and the inoculum was washed with 1 × PBS twice then diluted to the expected concentrations with 1% FBS in DMEM or DMEM/F12 medium. Each following experiment was conducted independently at least three times and each treatment had at least three replicates.

4.3.3 Cell viability assay

IPEC-J2 cells were split into 24-well plates (Thermo Scientific). When reaching 70 ~ 80% confluency (around 3×10^5 cells/well), IPEC-J2 cells were added with 0.5 mL of cell medium or bacterial suspensions per well as following: 1) CON (control): DMEM with 1% FBS (as described previously by Zhu et al., 2011); 2) F4: enterotoxigenic *Escherichia coli* (*E. coli*) F4 infection at the concentration of 1×10^6 CFU/mL for 3 hours (the bacterial concentration and time of incubation

based on preliminary experiments to allow for membrane damage) (Karimi et al., 2018); 3) F4 + LB1: 2-hour pre-treatment of *L. zeae* LB1 (at the concentrations of 1 × 10^7 CFU/mL, 5 × 10^7 CFU/mL, 1 × 10^8 CFU/mL) followed by mixed treatment of ETEC F4 (1 × 10^6 CFU/mL) with *L. zeae* LB1 strain (at the concentrations of 1 × 10^7 CFU/mL (1:10), 5 × 10^7 CFU/mL (1:50), and 1 × 10^8 CFU/mL (1:100)) for another 3 hours (the ratios between F4 and *L. zeae* LB1 at 1:1, 1:2, 1:5 were determined by our preliminary experiments, which did not show significantly reduction of cytotoxicity in infected IPEC-J2 cells with *L. zeae* LB1 pre-treatments); 4) LB1: 2-hour pre-treatment of *L. zeae* LB1 (at the concentration of 1 × 10^8 CFU/mL) and changed with new *L. zeae* LB1 suspension at the same concentration for another 3 hours. After treatments, the supernatants were collected for quantifying the lactate dehydrogenase (LDH) amount. LDH quantity was measured by an LDH detection kit (CyQUANT™, Thermo Fisher Scientific, Ottawa, ON, Canada) following the manufacturer's instructions (Fotakis and Timbrell, 2006). Results were presented as relative fold change to the control. IPEC-J2 cells were washed with 1 × PBS twice and harvested with 0.25% Trypsin-EDTA (Gibco). Harvested cells were pooled together and stained by 0.4% trypan blue exclusion (Thermo Scientific, Ottawa, ON, Canada) and an aliquot of 10 µL was added upon a hemacytometer (Bright-Line, USA). Dyed cells were observed under EVOS XL Core Imaging System (Invitrogen™, Fisher Scientific, Ottawa, ON, Canada) and the results were presented as a percentage of cell death. In light of cell viability results, the ratio of ETEC F4 to *L. zeae* LB1 at 1:10 was used for the following experiments.

4.3.4 Real-time polymerase chain reaction (PCR) and Enzyme-linked immunosorbent (ELISA) assays

For detecting messenger ribonucleic acid (mRNA) expression of tight junction proteins and cytokines, IPEC-J2 cells were split into 12-well plates. When reaching 100% confluency, cells

were treated with 1 mL of cell medium or bacterial suspension per well as following: 1) CON: DMEM/F12 with 1% FBS; 2) F4: ETEC F4 infection at the concentration of 1×10^6 CFU/mL for 3 hours; 3) F4 + LB1: 2-hour pre-treatment of *L. zeae* LB1 (at the concentration of 1×10^7 CFU/mL) followed by mixed treatment of ETEC F4 (1×10^6 CFU/mL) with *L. zeae* LB1 strain (at the concentration of 1×10^7 CFU/mL) for another 3 hours; 4) LB1: 2-hour pre-treatment of *L. zeae* LB1 (at the concentration of 1×10^7 CFU/mL) and changed with new *L. zeae* LB1 suspension at the same concentration for another 3 hours. After treatments, the cells were washed with 1 × PBS twice and 0.5 mL of TRIzol reagent (Invitrogen, stored at 4 °C) were added for 5 minutes at room temperature to extract the total RNA following the manufacturer's instructions (Rio et al., 2010). Concentrations of solubilized RNA samples were determined using NanoDrop 2000 (Thermo Scientific). RNA integrity was checked by agarose gel electrophoresis. For complementary deoxy ribonucleic acid (cDNA) synthesis, one µg of the total RNA was reversely transcribed into cDNA using an iScript™ cDNA Synthesis Kit (Bio-Rad, Mississauga, ON, Canada). Primers for Real-time PCR analysis were designed with Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The sequences of primers were listed in Table 2.2.

Cyclophilin-A (CYCA) was used as a reference gene. All the primers spanned at least two exons and were synthesized by Integrated DNA Technologies, Inc. Gene expression of target tight junction proteins (Zonula Occludens/ZO-1, occludin, and claudin), cellular receptors (Toll-like receptors (TLRs)-2/4/5/7) and cytokines (IL-8, IL-6 and IL-10, etc.) was determined by Real-time PCR using SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad) on a CFX Connect™ Real-time PCR Detection System (Bio-Rad) with the following program: 95 °C for 5 minutes, denature at 95 °C for 15 seconds, annealing at 58 °C for 15 seconds, and extension at 72 °C for 30 seconds with 40 cycles. Data were anayzed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Quantification of IL-8 in harvested supernatants was performed by a swine IL-8 ELISA kit following manufacturer's instructions (Invitrogen) and IL-8 level was expressed as pg/mL.

4.3.5 Barrier integrity

Trans-epithelial electrical resistance (TEER) values were measured to assess the IPEC-J2 monolayer resistance in response to ETEC F4 infection with or without pre-treatments of *L. zeae* LB1. The bacterial effects on the epithelial monolayer were evaluated by measuring TEER values under the Millicell Electrical Resistance System (ESR-2, Millipore, Sigma). To obtain polarized monolayers, IPEC-J2 cells were split onto Trans-well filter inserts (0.4 mL of medium volume in the apical compartment and 0.6 mL in the basolateral compartment; 0.4 μm pore size, 12 mm insert size, 0.6 cm^2 filtration area; hydrophilic polytetrafluoroethylene; Millipore, Sigma) at a concentration of $5 \times 10^5 \sim 10^6$ cells/mL and TEER values were monitored every other day until reaching around 1000 ~ 2000 Ω. IPEC-J2 cells were treated with 0.4 mL of cell medium or bacterial suspension in the apical compartment as described in the Real-time PCR assay (0.6 mL of DMEM/F12 with 1% FBS in the basolateral compartment). Initial TEER values were measured before treatments (0 hour) and then at various time intervals after treatments (1, 2, 3, 4, 5, 6 hours) and expressed as the ratio of TEER values at a time in relation to the initial value for each series. The net value of the TEER values was corrected for background resistance by subtracting the contribution of a cell-free filter and the medium (200 Ω). The TEER values of monolayers without added bacteria represented the control for each experiment. Quantification of cell permeability was conducted as described previously (Omonijo et al., 2018). Experiments were performed with triplicate determinations and repeated twice independently.

4.3.6 Immunofluorescence staining

IPEC-J2 cells were cultured onto coverslips (Fisher Scientific) in 24-well plates till 100%

confluency and treated with 0.5 mL of cell medium or bacterial suspension per well as described in the Real-time PCR assay. After treatments, the cells were washed with 1 × PBS twice and fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) for 15 minutes at room temperature. Fixed cells were then washed, and cell membranes were permeabilized using 0.3% Triton X-100 in PBS (PBS/Triton) for 15 minutes at room temperature. After membrane permeabilization, IPEC-J2 cells were blocked with 5% goat serum (Sigma-Aldrich) in PBS/Triton for 1 hour. After blocking, cells for detecting ZO-1 morphology were then incubated with an anti-rabbit ZO-1 polyclonal antibody (dilution 1:100 in 1% Bovine serum albumin/BSA, Thermo Fisher Scientific) at 4 ˚C overnight, while cells used for detecting actin filaments were incubated with CF® 594 Phalloidin (dilution 1:40 in 1% PBS, Biotium, San Francisco, US) for 1 hour at room temperature in the dark. Cells incubated with primary ZO-1 antibody overnight were then washed 3 times and incubated with an Alexa Fluor™ 488 goat anti-rabbit antibody (dilution 1:1000 in 1% BSA, Thermo Fisher Scientific) for 1 hour at room temperature in the dark. Rinsed cells were counter-stained with 4,6-Diamidino-2-phenylindole (DAPI, Sigma-Aldrich), mounted with ProLong™ Glass Antifade Mountant (Invitrogen) and micrographs were taken by ZEISS Apotome.2 Microscopy (Carl-Zesis Ltd, Toronto, ON, Canada).

4.3.7 Western blotting

For assays concerning the expressions of tight junction (TJ) proteins, IPEC-J2 cells were split into 6-well plates for complete confluence and treated with 2 mL of cell medium or bacterial suspension per well as described in the Real-time PCR assay. After treatments, all the following procedures were conducted over ice. IPEC-J2 cells were washed with cold 1 × PBS twice then detached in 1 mL of 1 × PBS using an aseptic cell scraper. Detached cells were transferred into Eppendorf tubes and harvested by centrifuging at 1,000 × g for 5 minutes at 4 ℃. Harvested cells

were lysed by pipetting with 70 ~ 80 μL of radioimmunoprecipitation (RIPA) lysis buffer (Sigma-Aldrich) containing a complete cocktail of proteinase inhibitors and incubated on ice for 10 minutes. Cells were further lysed by vortex for two to three times (40 seconds each time) then centrifuging at the maximum speed for 10 minutes at 4 °C to obtain the supernatant (total protein amount). Total protein concentration was analyzed by a bicinchoninic acid (BCA) protein detection kit (Thermo Fisher Scientific) following the manufacturer's instructions. Briefly, protein detection of each protein sample was triplicated plus three blanks in a 96-well plate were used. The working solution was prepared by combining 50 parts of Reagent A and 1 part of Reagent B. Each well contained 200 μL of working solution, 2 μL of protein sample except for blanks, and 23 μL (25 μL for the blanks) of double distilled water (ddH_2O). Then, gently tapped the plate to allow well mixing and incubated the plate at 37 °C for 30 minutes. The absorbance of protein samples was determined at 562 nm under a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT). All protein samples were adjusted into the same concentration (1 ~ 1.5 μg/μL) with RIPA buffer and subsequently denatured by adding loading buffer (mixing approximately 9 parts of 4 × Laemmli Sample Buffer and 1 part of 2-mercaptoethanol, Bio-Rad and Gibco, respectively) at 95 °C for 5 ~ 10 minutes. Equal amounts of protein (10 ~ 20 μg) and protein standard marker (Bio-Rad) were loaded on 4 ~ 15% precast gels, and subsequently undergoing sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) using a mini-gel apparatus (Bio-Rad). The proteins were then transferred to Immune-Blot polyvinylidene difluoride (PVDF, Bio-Rad) membranes (pre-hydrated in 100% methanol) using a Trans-Blot® Turbo™ transfer system (Bio-Rad). The membrane was blocked in 5% skim milk (5 grams of skim milk powder dissolved in 100 mL of 1 × Tris-buffered saline/TBS containing 0.1% Tween 20 (TBST)) at room temperature for 1 hour and incubated overnight at 4 °C with the following primary

antibodies (all purchased from Invitrogen): rabbit polyclonal anti-ZO-1, 1/1000; anti-occludin, 1/500; anti-claudin-3, 1/500; and anti-beta-actin (anti-β-actin), 1/5000. The reference protein β-actin was used as a loading control. The membranes were then washed with TBST five times for 5 minutes and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit sera, 1/4000, or with HRP-conjugated goat anti-mouse sera, 1/250 for 1 hour at room temperature. The immunoblots were washed five times for 5 minutes at room temperature. The protein bands were developed using Clarity Max ECL Western Blotting Substrates (Bio-Rad) according to the manufacturer's recommendation and visualized by a ChemiDoc^{TM} MP imaging system (2.4.0.03, Bio-Rad). Band densities were quantified using Image Lab 6.0 software (Bio-Rad) and adjusted by subtracting background volume. Data were presented as the ratio of target band intensities relative to the β-actin band intensities.

4.3.8 Bacterial adhesion assay

IPEC-J2 cells were cultivated onto 6-well plates and used for the following experiments when reaching 100% confluence. After incubation, cells were washed with PBS and lysed with 1% (v/v) Triton X-100 (Sigma-Aldrich) in ddH$_2$O for 10 minutes at room temperature. Then lysates were serially diluted in TBS ($1 \times 10^{-1} \sim 1 \times 10^{-5}$) and each aliquot of 40 μL was plated in Tryptic soy agar Petri dishes overnight at 37 °C to enumerate CFU and the data were calculated as the ratio relative to the average CFU of the control (ETEC F4-treated group as the control).

4.3.9 Genetic susceptibility screening of IPEC-J2 cells

IPEC-J2 cells were cultured in a 25 cm^2 flask and once reaching full confluence, cells were harvested with 0.25% Trypsin followed by centrifugation to obtain cell pellet. Positive control employed the minced tail sample from piglets susceptible to ETEC F4 adhesion. The preparation of DNA from cell and tissue lysates was conducted by PureLink^{TM} Genomic DNA Mini Kit

(Invitrogen) following the manufacturer's instructions. Briefly, the cell pellet and minced pig tail were firstly lysed with Digestion Buffer followed by binding DNA, washing DNA, and eluting DNA. The PCR of mucin 4 (MUC4) gene was performed using DreamTaq DNA polymerase (Thermo Fisher Scientific) with 2 mM $MgCl_2$, 200 μM of each dNTP, 400 μM of each primer in a total volume of 25 μL. Thermocycling was performed at 95 ºC for 5 minutes for initial denaturation and further processed at 95 ºC for 30 seconds, following by annealing at 65 ºC for 30 seconds and undergoing extension at 72 ºC for 1 minute with 35 cycles. The size of the PCR product obtained from porcine genomic DNA was 367 base pairs (bp) and 5 μL of the PCR products were digested with FastDigest XbaI (Thermo Fisher Scientific) at 37 ºC for 5 minutes following the manufacturer's instructions. All digested PCR products were electrophoresed on a 2% agarose gel in a Tris-borate-ethylenediaminetetraacetic acid (EDTA) buffer and visualized by staining with SYBR Green (Invitrogen). The resistant allele (R) was indigestible by XbaI, whereas the susceptible allele (S) was digested into 151 bp and 216 bp fragments.

4.3.10 Statistical analysis

Data were presented as means ± standard error of mean. The statistical analyses were performed with the GraphPad Prism 8 (GraphPad Software, La Jolla, USA). Differences between means were evaluated by one-way ANOVA. Multiple comparisons were done using Tukey's multiple comparisons test. The level of significance was set at $P < 0.05$.

Table 2.2 The sequences of primers used in this study.

Gene symbol	Primer sequences (5'-3')	Product size (bp)	Genbank ID	References
pBD2	F: TGTCTGCCTCCTCTCTTCC	149		Wang et al., 2019
	R: AACAGGTCCCTTCAATCCTG			
pBD3	F: CCTTCTCTTTGCCTTGCTCTT	163		
	R: GCCACTCACAGAACAGCTACC			
ETEC F4 *elt*	F: TCTCTATGTGCATACGGAGC	322		Reischl et al., 2002
	R: CCATACTGATTGCCGCAAT			
ETEC F4 *est*A	F: CAACTGAATCACTTGACTCTT	158		Noamani et al., 2003
	R: TTAATAACATCCAGCACAGG			
ETEC F4 *est*B	F: TGCCTATGCATCTACACAAT	113		
	R: CTCCAGCAGTACCATCTCTA			
ETEC F4 *faeG*	F: ACTGGTGATTTCAATGGTTCG	215		Zhu et al., 2011
	R: GTTACTGGCGTAGCAAATGC			
ETEC F4 *gapA*	F: TCCGTGCTGCTCAGAAACG	299		
	R: CACTTTCTTCGCACCAGCG			
ETEC F4 *luxS*	F: ATGCCGTTGTTAGATAGCTTCAC			Yang et al., 2014
	R: CTAGATGTGCAGTTCCTGCAACT			
pIL-8	F: AGAGGTCTGCCTGGACCCCA	126	NM_213867	Paszti-Gere et al., 2012
	R: GGGAGCCACGGAGAATGGGT			
pIL-6	F: AAGGTGATGCCACCTCAGAC	151	M86722	Kim et al., 2010
	R: TCTGCCAGTACCTCCTTGCT			
pIL-10	F: CATCCACTTCCCAACCAGCC	220	NM_214041	Lee and Kang, 2017
	R: CTCCCCATCACTCTCTGCCTTC			
pTLR2	F: ACATGAAGATGATGTGGGCC			Tohno et al., 2005
	R: TAGGAGTCCTGCTCACTGTA			
pTLR4	F: GCCATCGCTGCTAACATCATC	108		Zhang et al., 2015
	R: CTCATACTCAAAGATACACCATCGG			
pZO-1	F :GATCCTGACCCGGTGTCTGA	200	XM_021098856	Koo et al., 2020
	R: TTGGTGGGTTTGGTGGGTTG			
pOCLN	F: GAGAGAGTGGACAGCCCCAT	163		

	R: TGCTGCTGTAATGAGGCTGC		NM_001163 647	
pCycA	F: GCGTCTCCTTCGAGCTGTT	160	NM_ 214353	Farkas et. al., 2015
	R: CCATTATGGCGTGTGAAGTC			
ETEC F4 MUC4[1]	F: GTGCCTTGGGTGAGAGGTTA	367		Rasschaert et al., 2007
	R: CACTCTGCCGTTCTCTTTCC			

Note: Prefix "***p***" indicates porcine origin. [1] indicates the sequences of primer prepared for DNA genotyping. **BD**: Beta-defensin; **IL**: Interleukin; **TLR**: Toll-like receptor; **ZO-1**: Zonula Occludens-1; **CycA**: Cyclophilin-A, used as a housekeeping gene; ***gapA***: Glyceraldehyde-3-phosphate dehydrogenase A, used as a housekeeping gene for *E. coli* mRNA quantification. Annealing temperatures for Real-time PCR and MUC4 genotyping were 58 ℃ and 65 ℃, respectively.

4.4 RESULTS

4.4.1 Effects of *L. zeae* LB1 on the cytotoxicity of ETEC F4 in IPEC-J2 cells

As shown in Figure 4.1 A, the ETEC F4 at 1×10^6 CFU/mL significantly induced cytotoxicity when compared to the control cells ($P < 0.05$). This adverse effect was significantly attenuated by simultaneously adding ETEC F4 plus *L. zeae* LB1 at the ratio of 1:10, 1:50, and 1:100 ($P < 0.05$). There was no difference observed in the cytotoxicity between the control cells and the cells treated with *L. zeae* LB1 alone ($P > 0.05$). As shown in Figure 4.1 B, the ETEC F4 at 1×10^6 CFU/mL induced significant cell death and this effect was significantly attenuated by simultaneously adding F4 with *L. zeae* LB1 at a ratio of 1:10 ($P < 0.05$). There was no difference observed in the cell death between the control cells and the cells treated with *L. zeae* LB1 alone ($P > 0.05$).

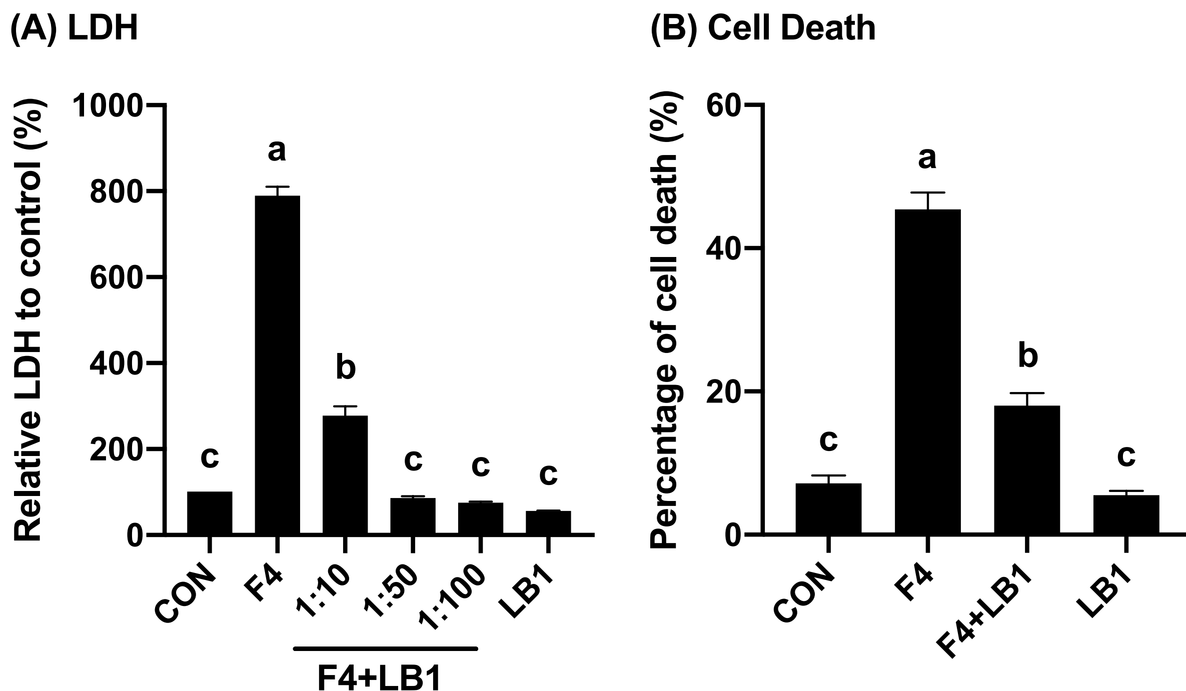
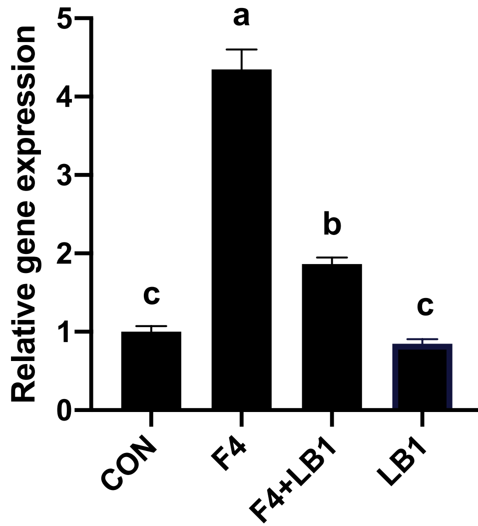


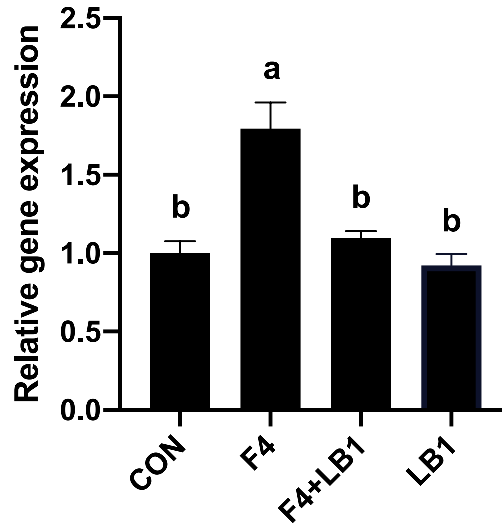
Figure 4.1 Effects of *Lactobacillus zeae* (*L. zeae*) LB1 on the cytotoxicity (a) and cell viability (b) of enterotoxigenic *Escherichia coli* F4 (ETEC F4) in intestinal porcine epithelial cells

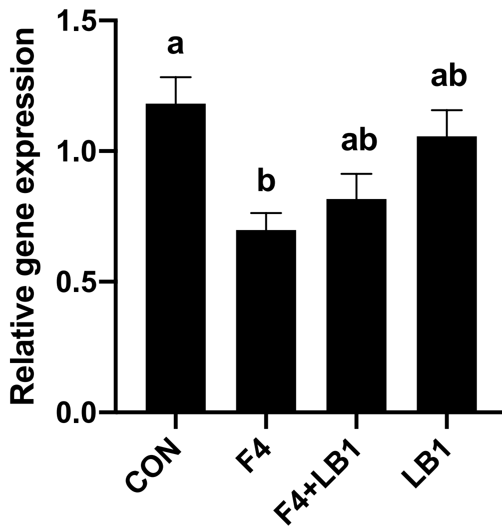
(IPEC-J2 cells). The results were presented as mean ± SEM, n = 4. Different letter indicates significantly different from each other, $p < 0.05$. LDH: Lactate dehydrogenase; CON: control; F4: ETEC F4; F4 + LB1 (**a**): ETEC F4 combined with various ratios of *L. zeae* LB1; F4 + LB1 (**b**): ETEC F4 combined with *L. zeae* LB1 at a ratio of 1:10; LB1: *L. zeae* LB1.

4.4.2 Effects of *L. zeae* LB1 on cytokines in IPEC-J2 cells

The ETEC F4 challenge at 1×10^6 CFU/mL/well significantly increased the mRNA abundance of IL-8 and IL-6 ($P < 0.05$) (Figure 4.2 A and 4.2 B). In contrast, the combination of ETEC F4 with *L. zeae* LB1 at a ratio of 1:10 slightly elevated the mRNA abundance of IL-8 compared with the control cells ($P < 0.05$) but significantly diminished the ETEC F4-triggered gene expression of IL-8 ($P < 0.05$). There were no significant differences in the mRNA expression of IL-6 among treatments other than only ETEC F4-challenged cells ($P > 0.05$). Treatment of *L. zeae* LB1 alone had no significant effects on the mRNA abundance of both IL-8 and IL-6 when compared to the control cells ($P > 0.05$). Nevertheless, the ETEC F4-triggered prominent decline in the mRNA abundance of anti-inflammatory cytokine IL-10 was not significantly counteracted by adding *L. zeae* LB1 ($P > 0.05$) (Figure 4.2 C). As shown in Figure 4.2 D, IL-8 concentration in the culture supernatant was significantly elevated in the ETEC F4-treated cells ($P < 0.05$), which was counteracted by adding *L. zeae* LB1 ($P < 0.05$). However, there was no significant difference observed in the IL-8 concentration in the control cells and the cells treated with *L. zeae* LB1 alone ($P > 0.05$).







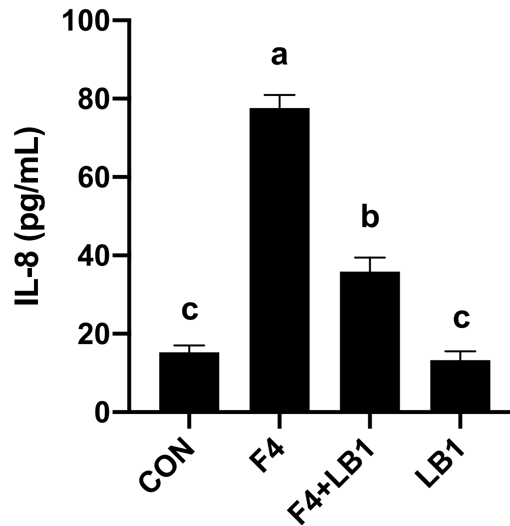


Figure 4.2 Effects of *Lactobacillus zeae* (*L. zeae*) LB1 on pro-inflammatory (a, b, d) and anti-inflammatory cytokines (c) in intestinal porcine epithelial cells (IPEC-J2 cells). Data were analyzed using $2^{-\Delta\Delta CT}$ method and the results were presented as mean ± SEM, n = 4. Different letter

indicates significantly different from each other, $p < 0.05$. IL: Interleukin; CON: control; F4: ETEC F4; F4 + LB1: ETEC F4 combined with *L. zeae* LB1 at a ratio of 1:10; LB1*:* *L. zeae* LB1.

4.4.3 Effects of *L. zeae* LB1 on barrier integrity in IPEC-J2 cells

The control cells (with DMEM/F12 with 1% FBS) maintained normal TEER values for 6 hours (Figure 4.3 A). While the ETEC F4 challenge at 1×10^6 CFU/mL significantly decreased the TEER values in the cells from 5-hour to 6-hour treatment with approximately 50% initial TEER values ($P < 0.05$). Though in comparison with the control treatment, there was a significant reduction in TEER value of the cells after 6-hour treatment with ETEC F4 and *L. zeae* LB1 ($P < 0.05$), it still remained more than 70% of electrical resistance compared to that only ETEC F4 treatment did ($P < 0.05$). The cells treated with *L. zeae* LB1 alone retained the electrical resistance when compared to the control cells ($P > 0.05$). Similarly, a surge of fluorescence intensity was observed in the ETEC F4 exposure treatment, which was significantly attenuated after *L. zeae* LB1 supplementation ($P < 0.05$) with no difference from the control diet ($P > 0.05$) (Figure 4.3 B).

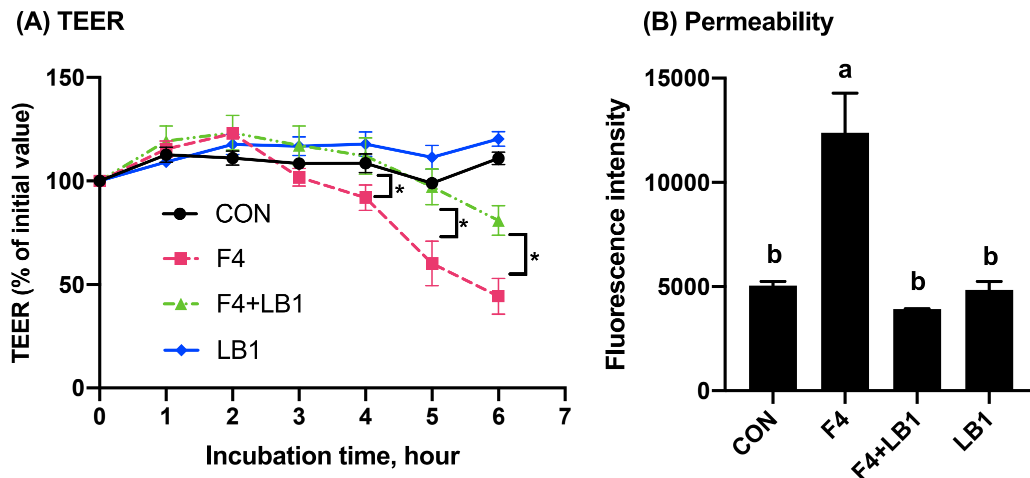


Figure 4.3 Effects of *Lactobacillus zeae* (*L. zeae*) LB1 on barrier integrity in intestinal porcine epithelial cells (IPEC-J2 cells). The results were presented as mean ± SEM, n = 3. Different letter indicates significantly different from each other, $p < 0.05$. TEER: Trans-epithelial electrical resistance; CON: control; F4: ETEC F4; F4 + LB1: ETEC F4 combined with *L. zeae* LB1 at a ratio of 1:10; LB1: *L. zeae* LB1.

4.4.4 Effects of *L. zeae* LB1 on the morphological changes of tight junction and cytoskeleton in IPEC-J2 cells

In the control and only *L. zeae* LB1-included treatments, ZO-1 proteins were linearly distributed as z-series intercellularly or spot-like at the nuclei. However, ZO-1 proteins were prone to be curved at the cell boundaries and shallower in the cytoplasmic in cells challenged with only ETEC F4 at a concentration of 1×10^6 CFU/mL. Meanwhile, a combination of ETEC F4 and *L. zeae* LB1 was not able to tell the protection of *L. zeae* LB1 on the cells in light of the ZO-1 staining results. On the other hand, ETEC F4-infected cells displayed decreased filamentous actin (F-actin) staining in the region of the cytoplasm. In comparison, the *L. zeae* LB1 treatment maintained a regular F-actin network with strong red-color staining beneath the plasma membrane and in the cytoplasm against ETEC F4 infection (Figure 4.4).

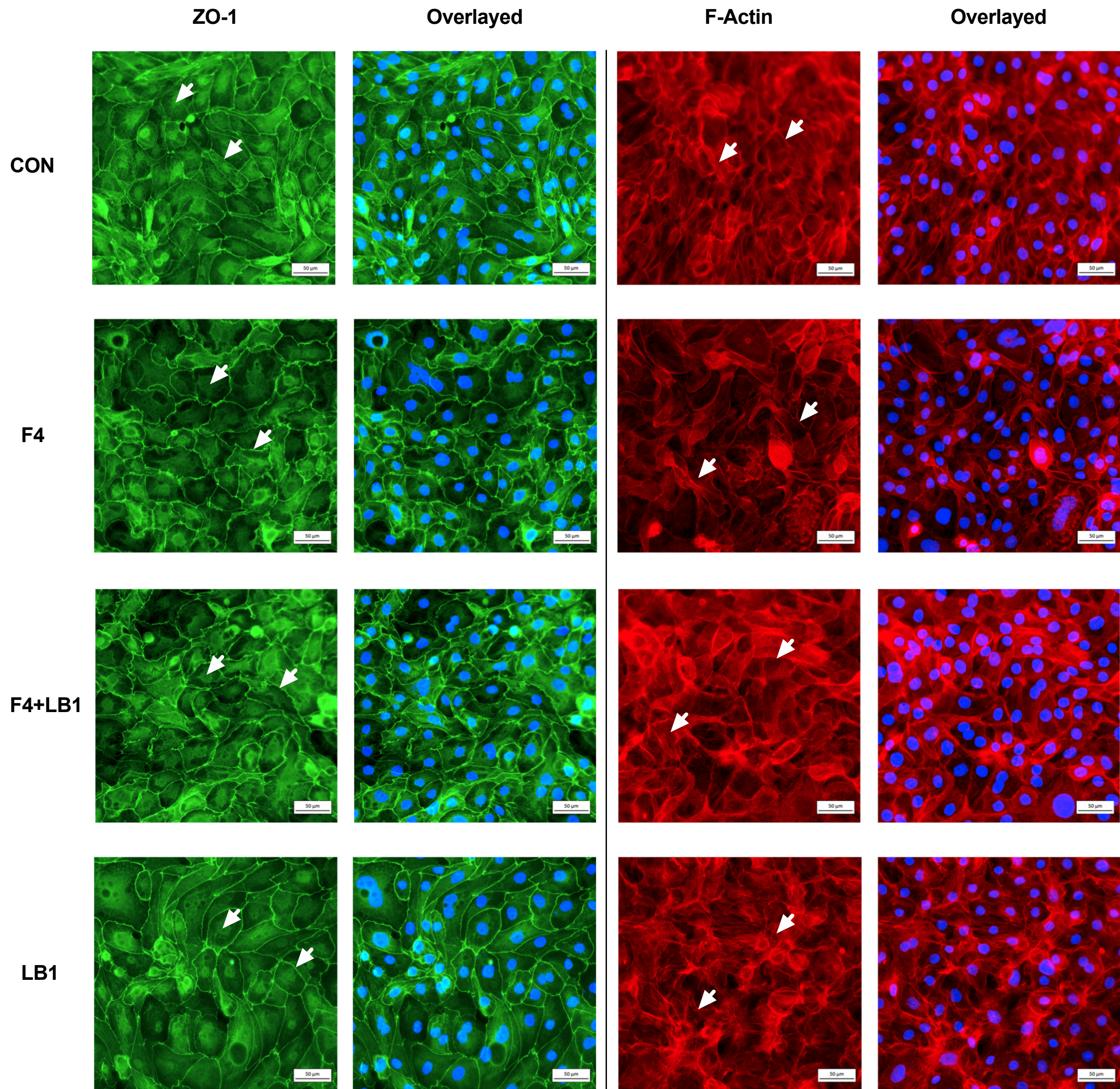
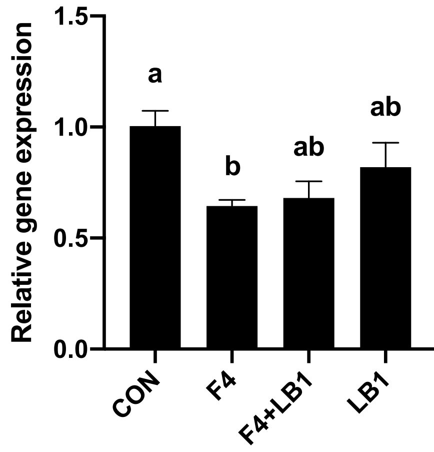
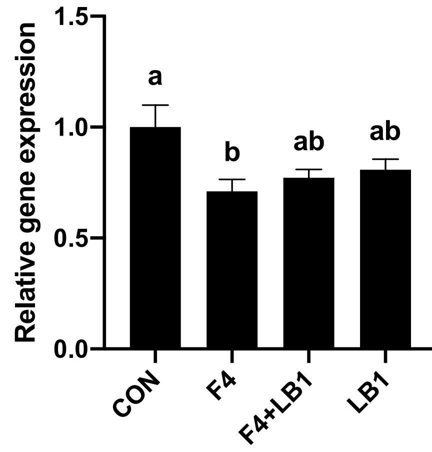


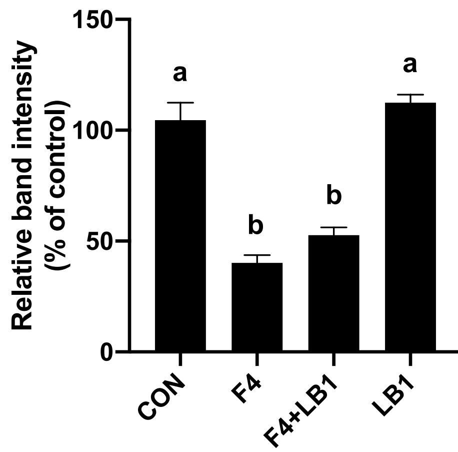
Figure 4.4 Effects of *Lactobacillus zeae* (*L. zeae*) LB1 on the morphological changes of tight junction and cytoskeleton in intestinal porcine epithelial cells (IPEC-J2 cells). Original magnification, 400 ×. ZO-1 staining (Green): linear at the cellular boundaries and at the nuclei; F-actin staining (Red, filamentous actin): extends from cell membrane to cell nucleus; Nucleus staining (Blue). ZO-1: Zonula occludens-1; F-Actin: Filamentous actin; CON: control; F4: ETEC F4; F4 + LB1: ETEC F4 combined with *L. zeae* LB1 at a ratio of 1:10; LB1: *L. zeae* LB1.

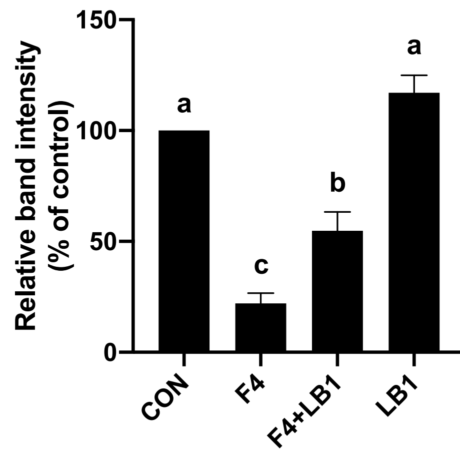
4.4.5 Effects of *L. zeae* LB1 on tight junction proteins in IPEC-J2 cells

The mRNA abundance of ZO-1 and OCLN was downregulated after 3 hours of ETEC F4 exposure compared to the control cells ($P < 0.05$), which, however, could not be compensated even by the *L. zeae* LB1 inclusion ($P > 0.05$) (Figure 4.5 A and 4.5 B). The relative protein abundance of ZO-1 and OCLN was lower in the cells treated with ETEC alone than in the control cells ($P < 0.05$) (Figure 4.5 C-E). The relative protein abundance of ZO-1 in the cells treated with ETEC F4 and *L. zeae* LB1 was not different with the relative protein abundance of ZO-1 in the cells treated with ETEC alone ($P > 0.05$). However, the relative protein abundance of OCLN was higher in the cells treated with ETEC F4 and *L. zeae* LB1 than in the cells treated with ETEC alone ($P < 0.05$). Moreover, the relative protein abundance of ZO-1 and OCLN was not different from the control cells and the cells treated with *L. zeae* LB1 alone ($P > 0.05$).


(A) ZO-1 mRNA
Relative gene expression
1.5
1.0
0.5
0.0
a
b
ab
ab
CON
F4
F4+LB1
LB1


(B) OCLN mRNA
Relative gene expression
1.5
1.0
0.5
0.0
a
b
ab
ab
CON
F4
F4+LB1
LB1


(C) ZO-1 protein
Relative band intensity
(% of control)
150
100
50
0
a
b
b
a
CON
F4
F4+LB1
LB1


(D) OCLN protein
Relative band intensity
(% of control)
150
100
50
0
a
c
b
a
CON
F4
F4+LB1
LB1

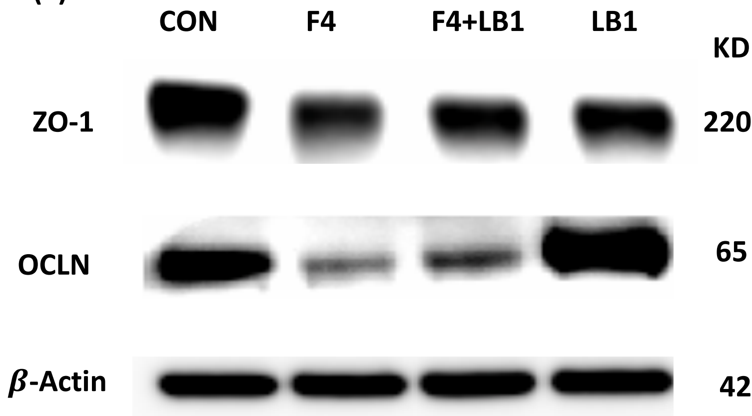
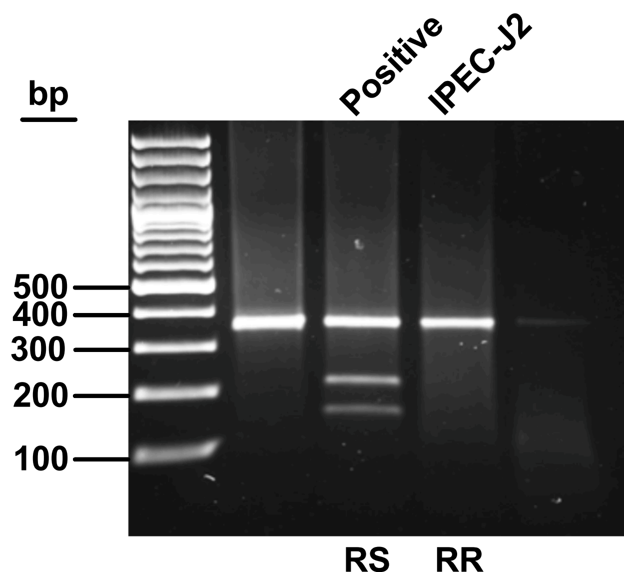

(E)
CON
F4
F4+LB1
LB1
KD
ZO-1
220
OCLN
65
β-Actin
42

Figure 4.5 Effects of *Lactobacillus zeae* (*L. zeae*) LB1 on tight junction proteins in intestinal porcine epithelial cells (IPEC-J2 cells). Data of gene expression were analyzed using $2^{-\Delta\Delta CT}$ method and the results were presented as mean ± SEM, n = 3. Different letter indicates significantly different from each other, $p < 0.05$. ZO-1: Zonula occludens-1; OCLN: Occludin; CON: control; F4: ETEC F4; F4 + LB1: ETEC F4 combined with *L. zeae* LB1 at a ratio of 1:10; LB1: *L. zeae* LB1.

4.4.6 Mucin 4 genotype of IPEC-J2 and ETEC F4 adhesion on IPEC-J2 cells

A DNA marker-based test targeting the mucin 4 gene that encodes the F4 fimbria receptor indicated the IPEC-J2 cell line had come from a pig identified as resistant to developing ETEC-F4 diarrhea (Figure 4.6A). As shown in Figure 4.6B, there was no significant difference ($P > 0.05$) observed in the ETEC F4 adhesion in the IPEC-J2 cells treated with ETEC F4 alone or with ETEC F4 and *L. zeae* LB1.



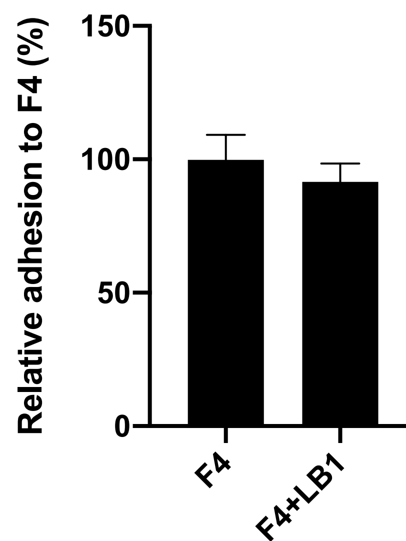
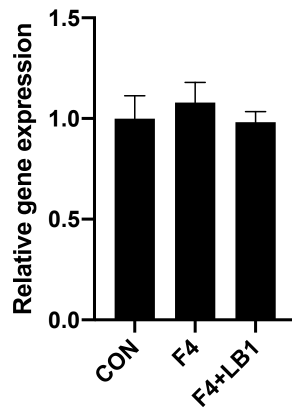


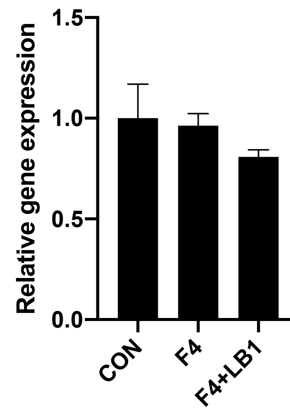
Figure 4.6 Mucin 4 genotype of intestinal porcine epithelial cells (IPEC-J2 cells) and

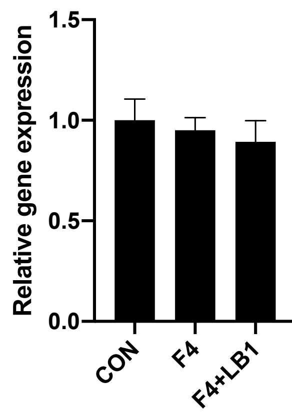
enterotoxigenic *Escherichia coli* F4 (ETEC F4) adhesion on IPEC-J2 cells. The results were presented as mean ± SEM, n = 4. No letter indicates insignificantly different from each other, $p > 0.05$. F4: ETEC F4; F4 + LB1: ETEC F4 combined with *L. zeae* LB1 at a ratio of 1:10.

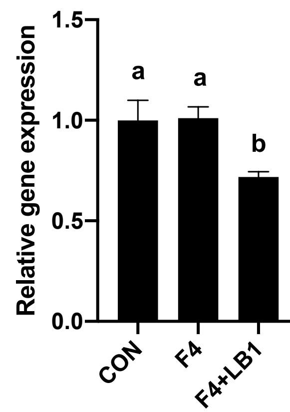
4.4.7 Effects of *L. zeae* LB1 on the mRNA abundance of β-defensins and toll-like receptors

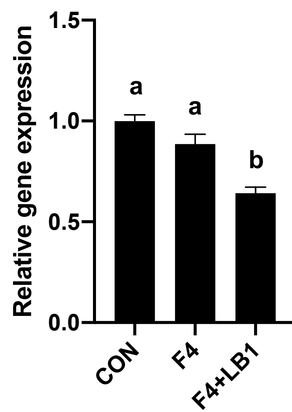
There was no significant difference ($P > 0.05$) observed in the mRNA abundance of defensins (BD2 and BD3) and TLR2 in the IPEC-J2 cells among the treatment groups (Figure 4.7 A-C). The mRNA abundance of TLR4 and TLR5 was lower ($P < 0.05$) in the IPEC-J2 cells treated with ETEC F4 and *L. zeae* LB1 than in the IPEC-J2 cells treated with ETEC F4 along or without ETEC treatment (Figure 4.7 D and 4.7 E). However, there was no difference ($P > 0.05$) observed in the mRNA abundance of TLR4 and TLR5 in the IPEC-J2 cells treated with ETEC F4 alone or without ETEC treatment (Figure 4.7 D and 4.7 E). The mRNA abundance of TLR7 was lower ($P < 0.05$) in the IPEC-J2 cells treated with ETEC F4 and *L. zeae* LB1 or ETEC F4 alone when compared to the control cells (Figure 4.7 F). However, there was no difference ($P > 0.05$) in the mRNA abundance of TLR7 in the IPEC-J2 cells treated with ETEC F4 and *L. zeae* LB1 or ETEC F4 alone (Figure 4.7 F).


(A) BD2 mRNA
Relative gene expression
1.5
1.0
0.5
0.0
CON
F4
F4+LB1


(B) BD3 mRNA
Relative gene expression
1.5
1.0
0.5
0.0
CON
F4
F4+LB1


(C) TLR2 mRNA
Relative gene expression
1.5
1.0
0.5
0.0
CON
F4
F4+LB1


(D) TLR4 mRNA
Relative gene expression
1.5
1.0
0.5
0.0
a
a
b
CON
F4
F4+LB1


(E) TLR5 mRNA
Relative gene expression
1.5
1.0
0.5
0.0
a
a
b
CON
F4
F4+LB1

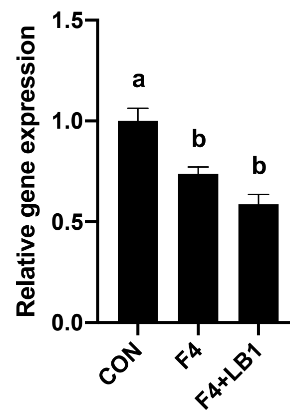

(F) TLR7 mRNA
Relative gene expression
1.5
1.0
0.5
0.0
a
b
b
CON
F4
F4+LB1

Figure 4.7 Effects of *Lactobacillus zeae* (*L. zeae*) LB1 on the mRNA abundance of β-defensins (a, b) and toll-like receptors (c-f). Data were analyzed using $2^{-\Delta\Delta CT}$ method and the results were presented as mean ± SEM, n = 5. Different letter indicates significantly different from each other, $p < 0.05$. BD: β-defensins; TLR: Toll-like receptors; CON: control; F4: ETEC F4; F4 + LB1: ETEC F4 combined with *L. zeae* LB1 at a ratio of 1:10.

4.4.8 Effects of *L. zeae* LB1 on the mRNA abundance of virulence-related factors of ETEC F4

As shown in Figure 4.8, the mRNA abundance of virulence-related factors including *luxS*, *faeG*, *elt*, *estA*, and *estB* was significantly lower ($P < 0.05$) in the ETEC F4 treated with *L. zeae* LB1 than in the ETEC F4 alone.

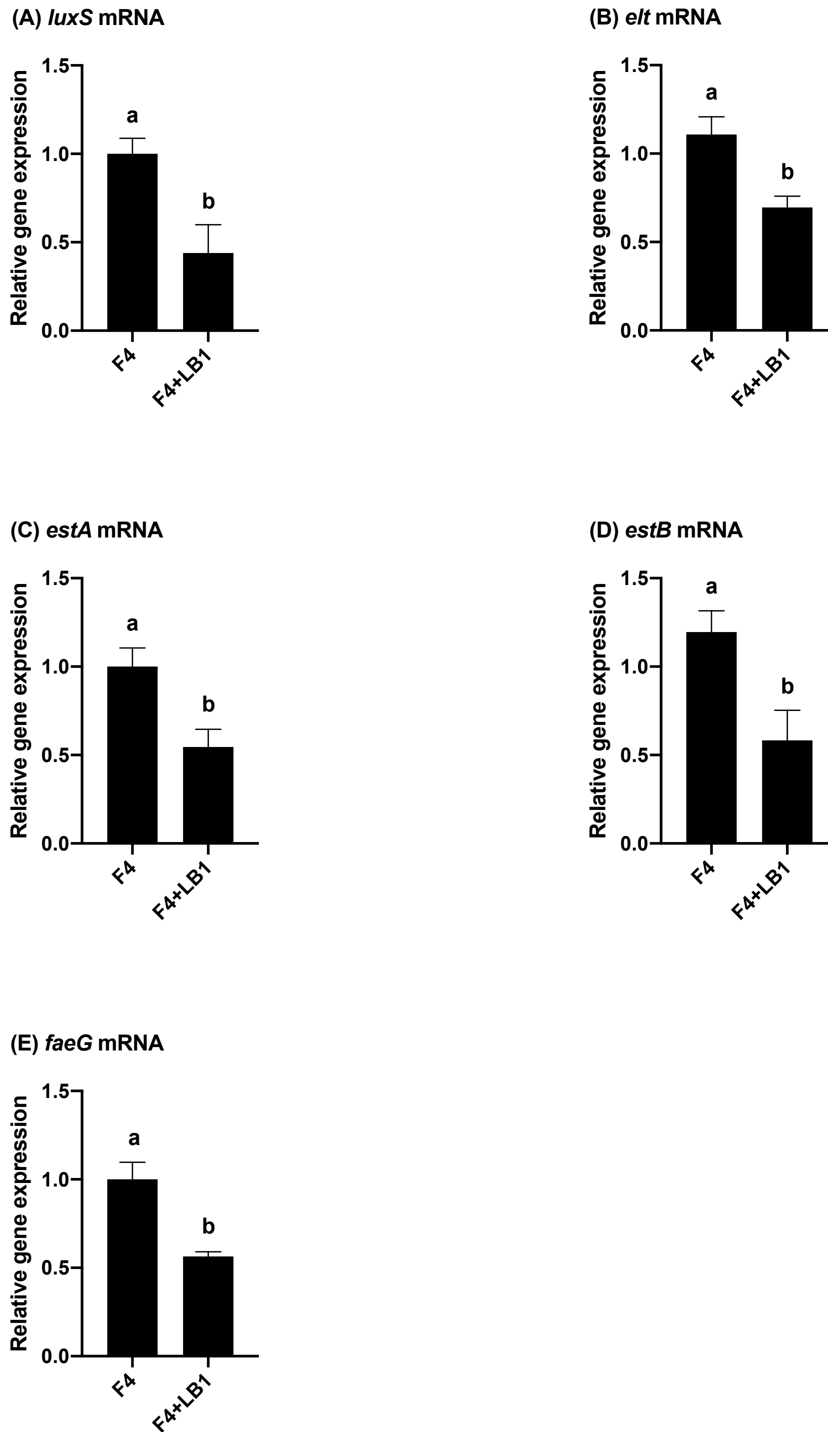


Figure 4.8 Effect of *Lactobacillus zeae* (*L. zeae*) LB1 on the mRNA abundance of virulence-related factors of enterotoxigenic *Escherichia coli* F4 (ETEC F4). Data were analyzed using 2-

$^{\Delta\Delta CT}$ method and the results were presented as mean ± SEM, n = 5. Different letter indicates significantly different from each other, $p < 0.05$. *luxS* genes encode autoinducers AI-2 molecules; *faeG* genes encode fimbrial adhesins; *elt* genes encode heat-labile enterotoxin LT; *estA* and *estB* genes encode heat-stable enterotoxins STa and STb, respectively; F4: ETEC F4; F4 + LB1: ETEC F4 combined with *L. zeae* LB1 at a ratio of 1:10.

4.5 DISCUSSION

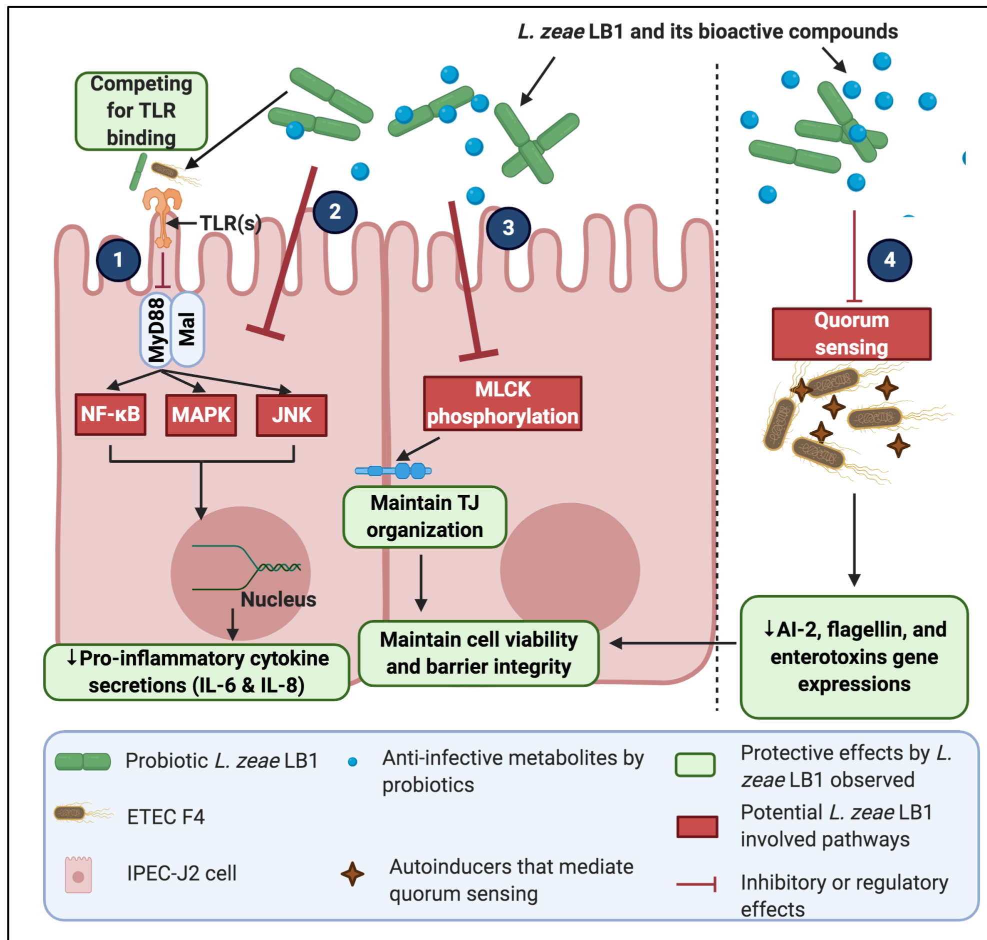


Figure 4.9 Schematic graph of potential *L. zeae* LB1-involved signaling cascades against ETEC F4 infection on IPEC-J2 cells in light of results from this study. The potential signaling pathways that *L. zeae* LB1 may implicate in the host-bacteria (or IPEC-J2 cells to ETEC F4) interplay including nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB), MAPK, c-Jun N-terminal kinase (JNK), myosin light chain kinase (MLCK), and quorum sensing. Namely, *L. zeae* LB1 may mitigate ETEC F4-induced barrier damage and immune system disorders through regulating bindings (i.e., through TLRs) upon the mucosal layers and interrupting *E. coli* communication (i.e., through AI molecules) as well as their virulence-related factor expressions (i.e., fimbriae and enterotoxins).

In the previous studies, *C. elegans* was utilized to evaluate the effects of *L. zeae* LB1 on the longevity of the nematode and the host defense against ETEC F4 JG280 (Zhou et al., 2014; Zhou et al., 2018). These authors concluded that the tested *L. zeae* LB1 provided adequate protection against the nematode death from the ETEC F4 invasion, which indicated that *L. zeae* LB1 can conceivably mitigate ETEC F4 infectious disease in pathogen-infected and inflamed animals. However, the protective effects of *L. zeae* LB1 at the cellular and molecular levels have not been investigated yet in pigs. Although animal trials or a combination of animal trials with *in vitro* cell models would be ideal for a more complete characterization of probiotic candidates for the swine industry, studies with cell cultures are relatively cost-effective and simple to handle than animal trials. High morphological and functional differentiation of this cell line made these cells a perfect specific model for porcine-originated infection studies on screening probiotic candidates (Nossol et al., 2015; Zhou et al., 2014; Paszti-Gere et al., 2012). Moreover, most parameters measured under porcine serum conditions were much closer to those of typical pig jejunocytes than ever reported since the cell line initial establishment in 1989 (Zakrzewki et al., 2013). Furthermore, this cell line has been the choice as a model for the study of probiotics in the innate immune responses of pigs (Liu et al., 2010) and for studying adherence and pathogenesis of enterotoxigenic *Escherichia coli* (Koh et al., 2008). Therefore, an in vitro IPEC-J2 cell model was used to evaluate the effectiveness of *L. zeae* LB1 against ETEC F4 infection at a cellular level in our study.

Our results indicated that ETEC F4 caused significant cytotoxicity and cell death in the IPEC-J2 cells based on the LDH and cell death assays and the *L. zeae* LB1 administration significantly attenuated cytotoxicity and cell death in the IPEC-J2 cells treated with ETEC F4. These results suggest that *L. zeae* LB1 can also effectively protect intestinal cells against ETEC F4 infection. These are consistent with a previous study in which, the initial inocula of ETEC F4 from 1×10^6

to 1 × 10^8 CFU/mL caused significantly IPEC-J2 cell death (Zhu et al., 2011) while the *L. zeae* LB1 inclusion (1 × 10^8 CFU/mL) effectively protected the nematode against ETEC F4 JG280 exposure.

Three types of gut inflammation have been observed in pigs: pathogen infection-associated, diet allergen-associated and weaning-associated gut inflammation (Omonijo et al., 2018). Although the inflammation may not cause full-blown clinical symptoms, it can compromise growth performance, decrease bacterial diversity, and cause considerable economic loss in pig production (Gresse et al., 2017). Therefore, non-antibiotic strategies to reduce inflammation is key to prevent pathogen infection and improve gut health in weaned pigs. Our results demonstrated ETEC F4 increased the mRNA abundance of IL-8 and IL-6 and the protein abundance of IL-8 in the intestinal cells, suggesting that ETEC F4 infection can cause inflammation in the intestine. The inflammation was significantly attenuated by the *L. zeae* LB1 administration, which explained that *L. zeae* LB1 can also effectively protect intestinal cells against ETEC F4 infection.

One of the negative consequences of intestinal inflammation is increased intestinal permeability, or "leaky gut," associated with impaired nutrient absorption and increased diarrhea incidence (Hui et al., 2020). The gut mucosal barrier acts as the first line of host defense against pathogens. A decline in the TEER values of IPEC-J2 cells has been observed in the *in-vitro* experiments because of the destruction of barrier integrity under the ETEC F4 challenge (Brosnahan and Brown, 2012). Our results indicated that ETEC F4 decreased the TEER values of IPEC-J2 cells and the *L. zeae* LB1 administration significantly attenuated the reduction of the TEER values induced by ETEC F4. Our findings were in agreement with the positive effects of LAB strains (i.e., *L. reuteri*, *L. plantarum*) on retaining TEER values subsequently against ETEC infection in IPEC-J2 cells (Wu et al., 2016; Yang et al., 2015). Probiotic-enhanced TEER values

meanwhile guarantee lower monolayer permeability, which was demonstrated by Karimi et al. (2018) reporting that the pre-treatment with *L. reuteri* was able to maintain relatively higher permeability against ETEC infection by decreasing more than 50% of FITC-dextran leakage. Likewise, our results demonstrated that ETEC F4-induced monolayer leakage was significantly counteracted by approximately 50% in the cells pre-treated with *L. zeae* LB1.

In the present study, the protection of the *L. zeae* LB1 on the barrier integrity was observed by the confocal immunofluorescence staining assay. The *L. zeae* LB1 pre-incubation notably maintained normal cell shape against pathogenic infection with the concomitant enhancement in tight junction ZO-1 morphology (or preventing ZO-1 delocalization) and the cytoskeleton of F-actin. Similarly, the protection of *L. Plantarum* addition on morphology and cytoskeleton in IPEC-J2 cells was also confirmed via confocal immunohistochemistry by Wu et al (2016). Previous studies also suggested that ETEC F4-induced reduction in the mRNA and protein expressions of TJs (claudin-1, occludin and ZO-1) was incredibly inhibited by the *Lactobacillus* supplementations either observed in an IPEC-J2 cell model or from sampled jejunum in a newborn piglet diarrhea model (Yang et al., 2015; Wu et al., 2016; Zhang et al., 2015). In line with their findings, we observed a significant decrease both in the mRNA and protein expressions of ZO-1 and occludin (but not claudin-1 and claudin-3, data not shown) in the ETEC F4-challenged cells. However, the *L. zeae* LB1 administration did not prevent the reduction of ZO-1 mRNA and protein abundance induced by ETEC F4. However, the protein abundance of occludin was prone to be higher in all *L. zeae* LB1-treated cells. Other TJ related proteins are also important in maintaining cell integrity. A recent study suggested that the oral administration of *L. paraplantarum* increased E-cadherin protein abundance (Mihailović et al., 2017), which suggests that it is also necessary to investigate the effects of *L. zeae* LB1 on the expression of other TJ proteins.

We also observed that the *L. zeae* LB1 pre-treatment maintained the distribution of actin filaments in the ETEC F4-infected cells. Due to actomyosin disorganization, via activating an MLCK pathway and clipping of occludin by protease (monocyte chemotactic protein/MCP), which allows greater amounts of pathogenic antigens entering paracellularly and contributes towards further inflamed reaction (Perrier and Corthesy, 2011). Moue et al. (2008) reported that ETEC-stimulated porcine intestinal epithelial cells significantly increased the levels of MCP-1. Herein, we speculate that *L. zeae* LB1 may be implicated in regulating the MLCK pathway to enhance occludin protein abundance (or maintain the localization of tight junction proteins. Besides, the *L. zeae* LB1 presumably enables to inhibit the process of occludin clipped by bacteria-related or -triggered MCP.

It was suggested that *L. zeae* LB1 regulated *C. elegans* signalling through the p38 MAPK and DAF/IGF pathways to control the production of antimicrobial peptides and defense molecules against ETEC F4 infection (Zhou et al., 2014; Zhou et al., 2018). However, the mRNA abundance of defensins BD2 and BD3 in the IPEC-J2 cells was not affected by ETEC F4 alone or a combination of ETEC F4 and *L. zeae* LB1 in the present study. It was reported that probiotic-induced defensin enhancement on intestinal epithelial cells was presumably through upregulation of short-chain fatty acids (e.g., butyric acid) as well as the initiation of peroxisome proliferator-activated receptor (PPAR)-γ and G protein-coupled receptor (GPR) 41 (Liu et al., 2017). Although it may be necessary to investigate the effects of *L. zeae* LB1 on other antimicrobial peptides (i.e., BD1, BD114, BD129), the protection of *L. zeae* LB1 on the ETEC F4 infection is not likely related to stimulating defensin secretion in the IPEC-J2 cells.

The development of an ETEC-F4 infection partially depends on the presence and amount of F4 receptors found in the brush border membrane of the small intestine (Sterndale et al., 2019).

Piglets identified as susceptible have a sufficient amount of F4 receptors in the small intestine and this susceptibility has been identified as the dominant autosomal allele and mapped at the mucin 4 (MUC4) gene on the chromosome (Fontanesi et al., 2012). Our results indicated the IPEC-J2 cell line was originated from a pig identified as resistant to developing ETEC-F4 diarrhea. Moreover, the *L. zeae* LB1 did not affect the ETEC F4 adhesion on the IPEC-J2 cells. Therefore, the protection of *L. zeae* LB1 on the ETEC F4 infection is not likely related to interrupting the ETEC F4 adhesion on the intestinal cells.

Enterotoxins (ST and LT) production have been considered as the main causative factor in ETEC related PWD cases as they target at interfering with electrolyte and 3',5'-cyclic guanosine monophosphate (cGMP) levels (Zhou et al., 2014). Cell death was likely coincident with the enterotoxin expressions as resistant MUC4 genotype was observed in the IPEC-J2 cell line based on the DNA genotyping data. That is, the IPEC-J2 cell line we handled was not susceptible to ETEC F4 fimbrial adhesins, however, ETEC F4-induced cell death was effectively reduced with *L. zeae* LB1 pre-incubation, indicating that *L. zeae* LB1 did not affect ETEC F4 adhesion practically but decreased by approximately 50% of virulence-related gene expressions of ETEC F4. Indeed, it has been previously reported that *L. zeae* LB1 was primarily responsible for minimizing *C. elegans* death caused by individual enterotoxin clones from ETEC F4 JG280 through inhibition of the enterotoxin gene expression rather than retardation of ETEC F4 intestinal colonization (Zhou et al., 2014).

It has been estimated that there likely exists an interplay between bacterial quorum sensing and virulence factors which however is intricate. Quorum sensing is the process whereby bacteria communicate with each other by monitoring cell density, which relies on generating and detecting autoinducers to modify their physiological behaviors including virulence related factors, biofilm

formation, locomotion, etc. (Abisado et al., 2018; Ng and Bassler, 2009; Rutherford and Bassler, 2012). In enteric pathogens such as *Escherichia coli*, they produce and detect AI-2 signal molecules to organize their quorum sensing milieu and regulate gene expression in response to cell density (Ng and Bassler, 2009). AI-2 molecules derive from the shared precursor 4,5-dihydroxy-2,3-pentanedione (DPD), which is encoded by *luxS* gene and is generated from *S*-adenosylmethionine (SAM) in three enzymatic reactions (Ma et al., 2017). Due to homologs of LuxS synthase exist in a wide range of bacterial genomes (including *E. coli*, and *Salmonella typhimurium* (*S. typhimurium*)), it is proposed extracellular AI-2 production as an extensive target to detect cell-to-cell communication (Wang et al., 2019). A research group suggested that there might be a negative correlation between gene expressions of *luxS* and *estA* of ETEC F4 in the IPEC-J2 cell model and extracellular AI-2 production (Zhu et al., 2011). They demonstrated that the deletion of enterotoxin genes did not impact the regular AI-2 production though, and that overexpression of *luxS* genes could either inhibit the *estA* gene expression or reduce IPEC-J2 cell death challenged with 1×10^8 CFU/mL of ETEC F4 JG280. Evidence indicating the inhibitory effects of *L. acidophilus* on quorum sensing signals of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 had been determined with a notable reduction of extracellular AI-2 generation and virulence-related gene expressions (*luxS*, *qseA*, *ler*, *espA*, *espD*, *tir*, *eaeA*, *flic*, and *hlyB*) (Medellin-Peña et al., 2007). Consistent outcomes were obtained in an *in vivo C. elegans* study which determined a significant downregulation of AI-2 activity and virulence factors of EHEC O157:H7 supplemented with 1.0% (w/v) *L. acidophilus* A4 cell extract (Kim et al., 2008). Therefore, it is necessary to further investigate the effects of *L. zeae* LB1 on the quorum-sensing systems of ETEC F4.

ETEC F4-induced intestinal barrier damage is often related to the upregulation of pro-inflammatory cytokines (i.e., IL-8, IL-6, IL-1β, and TNF-α) (Wu et al., 2016; Yu et al., 2018; Zhang et al., 2015; Zhou et al., 2014) and the downregulation of anti-inflammatory cytokine IL-10 (Zhang et al., 2015). Indeed, we observed pronounced elevation in the mRNA levels of pro-inflammatory cytokines (IL-8 and IL-6) and IL-8 protein concentration in the infected cell culture supernatant whilst the impetus was prominently marked down with prior exposure to *L. zeae* LB1. Similar enhanced resistance to pathogens have also been demonstrated by other probiotic administrations (e.g., *Pediococcus pentosaceus*, *B. licheniformis*, *L. reuteri*, *Saccharomyces*) via inhibiting the secretion of pro-inflammatory cytokines against enteric pathogens or their related LPS stimulation (Yin et al., 2020; Badia et al., 2012; Liu et al., 2010; Skjolaas et al., 2007). A subtle difference but not significantly in compensating ETEC F4-triggered IL-10 reduction in *L. zeae* LB1-pretreated cells were observed in our study. Results from Zhou et al. (2014) suggested that ETEC-induced pathogenicity is able to be blocked through the upregulation of anti-inflammatory cytokine IL-10 and antimicrobial peptides with the addition of *L. reuteri* in the IPEC-J2 cells and *C. elegans.* Critically, a multitude of reports accounts for the interaction between cytokines and intestinal barrier damage, whereby various probiotic strains are involved in non-specific immunomodulation (Yang et al., 2015; Wu et al., 2016). It is of note that the innate immunomodulatory system is activated concomitantly with a rapid burst of soluble mediators (e.g., cytokines, chemokines) into the surrounding tissue and circulation, which is commonly known as an inflammatory reaction (Aristizábal and González; Luo et al., 2015). These explain the rationale for the crosstalk between cytokines and mucosal disruption in response to enteric pathogen-implicated damage.

There are many cellular receptors in response to the recognition of external pathogenic signaling molecules, such as TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors/NLRs, etc. TLRs are a significant class of pattern recognition receptors that are present on intestinal epithelial cells and immune cells, which participate in the induction of both tolerance and inflammation. Many enlightening works of literature have been published providing new insight into the immunoregulation of probiotics for several intestinal pathogen-associated inflammations in which TLRs exert a significant role (Gao et al., 2017; Wu et al., 2016; Yan and Polk, 2002; Yan et al., 2007; Zhang et al., 2015; Zhu et al., 2020). Herein, we examined the gene expressions of TLR2, TLR4, and TLR5 and concluded that ETEC F4 did not impact their expressions other than reduced the gene expression of TLR7. Simultaneously, infected cells with *L. zeae* LB1 inclusion showed a dramatic decrease in the mRNA level of TLR4 and TLR5 in contrast to only ETEC F4-treated and non-infected cells. TLR4-mediated signaling cascades activate both the MyD88-dependent and the Toll/IL-1 receptor domain-containing adapter inducing IFN-β (Trif)-dependent pathways and enable to elicit the production of inflammatory cytokines, while TLR4-mediated Trif-dependent pathway can concomitantly induce type- I interferon (IFN) secretion (Kawai and Akira, 2006). Additionally, TLR5 can recognize ETEC flagellin and only requires MyD88 as essential adapters to initiate MyD88-dependent cascades (Kawai and Akira, 2006). In the present study, we nearly did not observe any reliable data of IFN (cycle quantities more than 35 and other samples cannot detect IFN expressions, data not shown) among any treatments in Real-time PCR analysis. We suggested that *L. zeae* LB1 administration was primarily implicated in competing for bindings of TLR4 and TLR5, subsequently inhibiting the MyD88-dependent downstream pathway to downregulate the ETEC F4-induced production of pro-inflammatory cytokines. It had been reported from Chytilová et al. (2014) that the pre-

treatment of *L. plantarum* significantly reduced the gene expressions of TLR2 and TLR5 (but not TLR4) in the jejunum from ETEC F4 challenged piglets (O8:K88ab:H9:F4ab, without enterotoxin production). Here, we speculated that the potentiation of barrier integrity and amelioration of pro-inflammatory cytokines are likely attributed to the significant downregulation of TLR4 and TLR5 gene expressions in *L. zeae* LB1-preincubated cells challenged with ETEC F4 bacteria. Whereas, in addition to inhibiting TLR4 and TLR5 levels over the cell surface, whether *L. zeae* LB1 interfere with the activity of the Toll/IL-1 receptor (TIR)-domain-containing cytosolic adapters (i.e., MyD88, TIR adaptor protein (TIRAP/Mal), Trif, and translocating chain-associated membrane protein (TRAM)) and transcription factors (i.e., NF-κB and 2 MAP kinases-p38 and c-Jun N-terminal kinase) are not thoroughly investigated yet (Kawai and Akira, 2006; Lu et al., 2008; O'Neill et al., 2003). Yin et al. (2020) currently reported that the expression of genes involved in NF-κB pathway, including RELA and NFKB1 were repressed when *Pediococus pentosaceus* was added to ETEC F4 infected cells. The same is not known when *L. zaea* is used as a probiotic against ETEC F4 infection and this needs to be investigated. Moreover, negative regulators (i.e., single Ig IL-1-related receptor/SIGIRR, B-cell CLL/lymphoma 3 (Bcl3)), and mitogen-activated protein kinase phosphatase/MKP-1) of TLRs appeared to be highly elevated in ETEC F4-infected IPEC-J2 cells provided with *L. plantarum*, which consequently regulates NF-κB and MAPK signaling cascades (Wu et al., 2016). A recent study reporting a critical reversion in the ETEC F4-reduced expression of a mitochondrial transporter for basic amino acids in IPEC-J2 cells with *L. plantarum* pre-treatment, which may highlight involvement of *L. plantarum* in the JNK activation (Zhu et al., 2020). The potential signaling pathways that *L. zeae* LB1 is likely involved to modulate the cellular responses against *E. coli* infection are generalized in Figure 4.9.

4.6 CONCLUSIONS

In summary, this study suggested that the *L. zeae* LB1 strain effectively protected the intestinal cells from ETEC F4 infection by inhibiting inflammation and maintaining barrier integrity via downregulating TLR4, TLR5, and ETEC F4 virulence-related factors expressions instead of preventing ETEC F4 adhesion. Our data provide further evidence on the mechanisms at the cellular and molecular levels that probiotic *L. zeae* LB1 could improve gut health in pigs. However, our study was limited by the *in-vitro* cell model and specifically focus on the inflammatory responses as well as the cell monolayer integrity, the evidence of which was not adequate to demonstrate the effects of *L. zeae* LB1 on the *in-vivo* animal performance against ETEC F4 invasion. Therefore, future efforts would not only emphasize evaluating the downstream cascades that microbial interactions have on cells but also confirming probiotic effects on pathogenic prevention in animal studies.

CHAPTER 5 GENERAL DISCUSSION AND CONCLUSION

5.1 GENERAL DISCUSSION

Enterotoxigenic *Escherichia coli* (ETEC) associated diarrhea during the post-weaning period is the main cause of mortality and it is considered the most stressful condition in commercial porcine production (Ahasan et al., 2015) with major economic losses. Due to the efforts made on reducing the use of antibiotics in animal feeds, probiotic strains with antimicrobial potency have emerged steadily from more refined and specific research directions and accomplishments. Mechanisms of pathogen inhibition by probiotics (especially referring to lactic acid bacteria) include inhibition of pathogenic growth by secreting their metabolites and lowering gut pH, and this way suppressing the colonization of pathogens via competitive exclusions, as well as modifying virulence-related factors gene expression of ETEC (Vieco-Saiz et al., 2019). Other potential preventive actions are via the regulation of inflammatory and immune responses, which have been studied by many research groups but coming up with various inconsistent results (Wu et al., 2016; Yin et al., 2020). Differences among studies may be due to various factors. The type of experimental trial, i.e., if *in vitro* studies with cell lines or *in vivo* studies with model organisms (e.g. *Caenorhabditis elegans* (*C.elegans*)) or the use of pigs in the trials or a combination of the various trials were used seems to be a major factor behind the above inconsistency of results.

Among 13 lactic acid-producing bacteria (LAB) isolates that varied in their ability to protect the nematode *C. elegans* from death induced by ETEC F4 JG280 strain infection, *Lactobacillus zeae* (*L. zeae*) LB1 offered the highest level of protection (Zhou et al., 2014). Meanwhile, *L. zeae* LB1 was able to inhibit the gene expressions of ETEC F4 enterotoxins but enhance that of antimicrobial peptides possibly via the p38 mitogen-activated protein kinase (MAPK) and insulin/insulin growth factor (DAF/IGF) signaling pathways (Zhou et al., 2018). Although the

screening of various probiotics candidates against ETEC F4 was based on an *in-vivo* animal trial, i.e., with the nematode *C. elegans* (Zhou et al., 2014; Zhou et al., 2018) and despite the valuable insights obtained from these trials, it is important to recognize limitations of the use of *C. elegans* when preselecting probiotics against ETEC F4. For instance, *C. elegans* possess its digestive microbiota, which presumably impacts the results when selecting probiotics (Zhou et al., 2014). Moreover, for investigating porcine intestinal transport and barrier proprieties on a cellular and molecular level, rather than *C. elegans*, cell lines with the closest match to the source epithelium are of extreme importance. Porcine intestinal epithelial cell line (IPEC-J2) has been concluded to serve as a preferential tool to study the metabolism pathway, with the nature of superiorly morphological and functional differentiation compared to other cell lines (Nossol et al., 2015). This is following Zhakrzwski et al. (2013) and Paszti-Gere et al. (2012) who recognized IPEC-J2 cells as the best specific unicellular model for porcine-originated infection researches on screening probiotic candidates. Despite that, it has been pointed as a concern, that this cell line exhibits atypically higher trans-epithelial electrical resistance (TEER) and therefore lower active transport rates under conventional fetal bovine serum (FBS) conditions (compared with porcine serum culture) (Zakrezewski et al., 2013). This can be beneficial to use when investigating compounds harming the barrier integrity (Vergauwen, 2015). While investigating the effects of potentially protective compounds on the TEER values need to be validated in IPEC-J2 cells cultured with FBS or porcine serum. In the present study, TEER values obtained before treatments were approximately 1000 ~ 2000 $\Omega/0.6\ cm^2$, which was consistent with high resistance manifested by Brosnahan and Brown (2012). Although under relatively larger resistance values which would likely impact the investigations of probiotic *L. zeae* LB1 on IPEC-J2 cell membrane transport, we can still conclude that the *L. zeae* LB1 attenuated TEER changes. To a solid extent, our results

also corroborated the protective and regulatory effects of *L. zeae* LB1 on ETEC F4-triggered pro-inflammatory cytokine production and virulence factor expressions, all of which potentially support the maintenance of *L. zeae* LB1 on normal cell viability (Figure 4.1). Our outcomes provide a gateway to *L. zeae* LB1 as a promising probiotic strain for future *in vivo* animal studies. In addition, to the aforementioned possible mechanisms by which *L. zeae* LB1 supplementations implicated in preventing ETEC F4 infection on IPEC-J2 cells, further mechanisms are still required to be evaluated in terms of oxidative stress, apoptosis, quorum sensing, and innate immune responses, etc.

Despite cost-effective and a reliable replacer of animal trials, *in vitro* probiotic trials should not replace *in vivo* trials. Any *in vitro* trial with cell lines should eventually be combined and supported by *in-vivo* probiotic trials. Outcomes obtained from *in-vivo* probiotic trials have been very helpful in showing the probiotic beneficial effects on swine body weight gain, production quality, gut microbiota equilibrium, inhibiting pathogen toxin bioavailability and infections (Vieco-Saiz et al., 2019). Significant effects of probiotics on enhancing animal performance are substantially credited to their characterized properties of maintaining microbiota equilibrium against pathogen invasion along the alimentary tract and by stimulating a range of the host immunomodulation in post-weaning piglets (Dong et al., 2014; Liu et al., 2014; Zhang et al., 2019). As most of these microbes will be excreted along with defecating, constant supplementations of probiotics in animal feed are recommended to maximize the opportunities of neither suffering from the enteric diseases nor fecal pathogen shedding and without negative impacts on animal performance (Liao and Nyachoti, 2017). Hence, the above parameters including disease resistance, gut integrity and nutrient absorptivity should be considered when conducting *in-vivo* animal trials. On the other hand, *in-vitro* IPEC-J2 trials are mainly relevant to only piglets and therefore it still

remains extensively questionable to confirm probiotic effects on different stages of swine (i.e., sows and growing-finishing pigs) and this should be investigated further.

It has come to one attention that inconsistent results were gained by massive research groups about probiotic studies, which indicates the relative complexity in the development and application of different probiotic strains in the swine feed industry. Moreover, there is the inconsistency of results and selection of the immune response parameters when evaluating probiotic candidates. For example, the cytokines whose transcript and protein expression were modulated varied among studies (summarized in Table 2.1 and schematic flows shown as Figure 2.2). Some studies only considered the ETEC F4-induced expression of proinflammatory transcripts (e.g., interleukin (IL)-6 and tumor necrosis factor (TNF)-α) and tight junction proteins (e.g., Zonula Occludens/ZO-1 and occludin), while other also looked at the impact on anti-inflammatory cytokines (e.g., IL-10) (Table 2.1). This inconsistency makes it challenging the comparison of probiotic impact results on the animal immune system. Furthermore, when animals are used in *in vivo* studies, the immune status of the animals may be initially different among studies and during the trials and this would also explain/cause discrepancy among studies.

It should be noted that different probiotic strains are designated for specific pathogen infections and host immunoregulation. Besides, slight alterations in experimental conditions among studies might directly affect the molecular mechanism pathways. As Dowarah et al. (2017) proposed, the difficultly to demonstrate the immunomodulation by probiotics is predominantly credited to their protective characteristic rather than wiping out the pathogenic invasion in the alimentary tract and often affected by the animal's immune status in the various applied situations. Not only that, all improvements or positive effects observed from probiotic supplementations on intestinal epithelial integrity, anti-inflammatory reactions, and antioxidant status are the basis for

safeguarding immune defense. Any imbalance of these will cause immune responses eventually and these responses need to be taken into account on any *in vitro* and/or *in vivo* probiotics trials.

With respect to the probiotic market, efforts on investigating probiotic candidates (i.e., *L. zeae* LB1) provide more opportunities for reducing antibiotic use in animal feed and may have the potential as health promoters to modulate intestinal microflora. Whereas, they are still required to satisfy sorts of stringent regulatory requirements and need as much research efforts as to be considered into the design of probiotic supplementations. Furthermore, most probiotic species especially *Lactobacillus* are susceptible to temperature, gastric acid, and bile salt. These factors would influence the probiotic viability during feed processing and storage, and gut passage. This will also cause variations among studies as different susceptibilities are expected for different probiotics (Wang et al., 2016). Accordingly, various feeding strategies of probiotics should be taken into account for animal consumption, such as in the forms of live, freeze-dried, spray-dried, fermentation, encapsulation, or multi-strain probiotics to maintain probiotic biological activity.

5.2 GENERAL CONCLUSION

The probiotic *L. zeae* LB1 strain can be administrated to piglets to prevent ETEC F4 infection because the probiotic *L. zeae* LB1 can inhibit inflammation and maintaining barrier integrity via downregulating TLR4, TLR5, and ETEC F4 virulence-related factors expressions instead of preventing ETEC F4 adhesion. Future efforts would emphasize further evaluating the downstream cascades that microbial interactions have an impact on cells (e.g., quorum sensing) and also confirming probiotic effects on pathogenic prevention in pig studies.

CHAPTER 6 FUTURE DIRECTIONS

The specific protective factors and mechanisms of individual probiotics species can be different, including the development of a healthy microbiota, preventing enteric pathogens from colonization, increasing digestive capacity and lowering the pH, improving mucosal immunity, and enhancing gut tissue maturation and integrity. Moreover, one single probiotic stain can't have all of the above benefits. Therefore, we will investigate the potential of individual isolated probiotic bacteria and their cocktails as alternatives to antibiotics in an enterotoxigenic *Escherichia coli* (ETEC) F4-induced disease model in weaned piglets, including mechanistic studies on the mode of action in modulating cross-talks among immunity, disease resistance, oxidative stress and the gut microbiota. Specific future research directions are:

- To further screen individual newly isolated probiotic bacteria and evaluate the molecular mechanisms of probiotic effects;
- To determine the effects of the *L. zeae* LB1 alone or in combination with other potential probiotics on the innate immunity, disease resistance, gut integrity, oxidative stress, nutrient absorption, and gut microbiome in post-weaning piglets challenged with or without ETEC F4; and
- To determine the effects of *L. zeae* LB1 alone or in combination with other potential probiotics on the growth performance of pigs under the conditions mimicking commercial swine production.

CHAPTER 7 LITERATURE CITED

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