Potential of Egg White Ovomucin and Its Hydrolysates in Mitigating Enteric Infections as an Antimicrobial Alternative

by

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ABSTRACT

Ovomucin, accounting for ~3.5% of egg white, is a mucin-type glycoprotein and composed of ~ 33% carbohydrate content with 2.6-8% sialic acid. Ovomucin and its derived hydrolysates possess various biological activities, including anti-adhesive, anti-oxidative, and immunomodulatory activities. The overall objectives of this thesis were to investigate the anti-adhesive effect of ovomucin hydrolysates against bacterial adhesion to intestinal epithelia and their beneficial effect on intestinal barrier function against chemical and pathogenic challenges using *in vitro* and *in vivo* models.

Using lipopolysaccharide (LPS)-treated differentiated human colorectal adenocarcinoma cells (Caco-2), ovomucin-protex 26L hydrolysate (OP) at concentrations of 0.1 mg/mL, 0.5 mg/mL, and 1.0 mg/mL significantly restored the transepithelial electrical resistance (TEER) values and decreased the paracellular FITC-dextran (4 kDa and 40 kDa) flux permeability. OP also significantly maintained the expression levels of tight junction proteins, including occludin and ZO-1, and preserved their structures and cell surface localization. Additionally, OP dramatically inhibited the phosphorylation of mitogen-activated protein kinase (MAPK) p38, extracellular signal-regulated kinase ½ (ERK1/2), and the activation and nucleus-translocation of nuclear factor κB (NF-κB) p65.

The anti-adhesive and anti-inflammatory effects of OP, along with ovomucin-pepsin/pancreatin hydrolysate (OPP), were also investigated in enterotoxigenic *Escherichia coli* (ETEC) K88-challenged porcine small intestinal epithelial cells (IPEC-J2) and Caco-2 cells. OP and OPP both effectively inhibited ETEC K88 adhesion to the surfaces of intestinal epithelial cells

and removal of sialic acids impaired their anti-adhesive capacities. Besides, OP restored epithelial permeability as indicated by elevated TEER values in IPEC-J2 cells. However, restored expression of tight junction proteins, including claudin-3, occludin, and ZO-1, were only observed in Caco-2 cells. OP and OPP suppressed activation of MAPK and NF-κB p65 signaling pathways in both cell lines. ETEC K88-associated virulence in activating calcium/calmodulin dependent protein kinase 2 (CaMK II), elevating intracellular Ca^{2+} concentration, and inducing oxidative stress were attenuated by OP and OPP.

To study the *in vivo* potential of ovomucin (OVM), OP, and OPP in preventing bacterial adhesion, colonization, and disease severity, a *Citrobacter rodentium*-induced colitis model was used. Supplementation of OVM, OP, and OPP in a basal diet did not impact body weight throughout the 4-week experimental period. At 7-d post-infection (7 dpi), OP treatment significantly reduced *C. rodentium* load in the gut of mice. OP and OVM both attenuated colitis severity evidenced by lower levels of colonic pathology and inflammatory cytokines/chemokines. However, the OPP group showed increased pathological severity with significantly decreased microbial diversity in the gut microbiota. *In vitro* culture of *C. rodentium* demonstrated that OPP better promoted bacterial growth in the minimal medium and showed numerically higher *C. rodentium* adhesion to mouse rectal epithelial cells (CMT-93) compared to OP.

To study the potential of OP as an antimicrobial alternative in preventing enteric infection, ETEC K88-infected 21-day weaned piglets were used. Ovalbumin is the most abundant protein in chicken egg white, ovalbumin-protex 26L hydrolysate (OVAP) was also included in this study. ETEC K88 infection induced diarrhea in piglets, triggered inflammatory responses, and disrupted intestinal integrity in intestinal mucosa. OP supplementation changed the diarrhea pattern of piglets after ETEC K88 inoculation, improved the morphology of jejunum, and mediated the

production of pro-inflammatory cytokines in jejunal and colonic mucosa. Notably, OVAP supplementation significantly decreased ETEC K88 abundance in the piglet colon.

In summary, this thesis demonstrated the potential of ovomucin and its hydrolysate, particularly OP, as an anti-adhesive substance to inhibit pathogenic infection and benefit intestinal function and health.

PREFACE

This thesis is an original work conducted by Xiaoyu Bao and written according to the guidelines for formatting thesis of the Faculty of Graduate Studies and Research at the University of Alberta. The concept of this thesis originated from my supervisor Dr. Jianping Wu and the research was funded by the grants from Alberta Ministry of Technology & Innovation, the AMR – One Health Consortium, the Major Innovation Fund program of the Ministry of Jobs, Economy and Innovation, Government of Alberta. The experiment protocol for the mouse study was approved by the Animal Care and Use Committee at the University of Alberta (Protocol AUP 00000676) and the protocol for the piglet study was approved by the Animal Care Committee of the University of Manitoba (F23-009, AC11812) following the guidelines issued by the Canadian Council on Animal Care.

This thesis is consisted of seven chapters: Chapter 1 provides a general introduction on the research objectives; Chapter 2 is a literature review on several key topics related to this thesis, including antimicrobial resistance and alternatives in swine industry, food-derived anti-adhesive components, ovomucin/its hydrolysates and their potential in preventing infection as an anti-adhesive and anti-inflammatory substance. Parts of Chapter 2 have been published as "Impact of food-derived bioactive peptides on gut function and health" in *Food Research International* and "Bioactives of Egg White Proteins and Peptides" in *Handbook of Egg Science and Technology*. Another section has been submitted to *Critical Reviews in Food Science & Nutrition* as "Natural anti-adhesive components against pathogenic bacterial adhesion and infection in gastrointestinal tract: Case studies of *Helicobacter pylori*, *Salmonella*, *Clostridium difficile*, and diarrheagenic *Escherichia coli*". Chapter 3 has been published as Xiaoyu Bao and Jianping Wu, "Egg white ovomucin hydrolysate inhibits intestinal integrity damage in LPS-treated Caco-2 cells" in *Journal of Functional Foods*; Chapter 4 has been published as Xiaoyu Bao, Michael Gänzle, and Jianping

Wu, “Ovomucin hydrolysates reduce bacterial adhesion and inflammation in enterotoxigenic *Escherichia coli* (ETEC) K88-challenged intestinal epithelial cells” in *Journal of agricultural and food chemistry*; Chapter 5 has been published as Xiaoyu Bao, Tingting Ju, Stephanie Tollenaar, Consolato Sergi, Benjamin P. Willing, and Jianping Wu, “Ovomucin and its hydrolysates differentially influenced colitis severity in *Citrobacter rodentium*-infected mice” in *Food & Function*. Chapter 6 entitled as “Ovomucin and ovalbumin hydrolysates protect early weaned piglets against enterotoxigenic *Escherichia coli* K88” contains the work on enterotoxigenic *Escherichia coli* K88-challenged piglet and is in preparation for submission; Chapter 7 provides concluding remarks and outlines potential directions for future research.

Dr. Jianping Wu (for chapters 3-6), Dr. Michael Gänzle (for chapters 4 and 6), Drs. Benjamin P. Willing and Tingting Ju (Chapter 5), Dr. Ruurd Zijlstra and Dr. Chengbo Yang (Chapter 6) contributed to the experimental design, data interpretation, manuscript preparation and edits. Dr. Consolato Sergi and Mrs. Stephanie Tollenaar contributed to performing pathological scoring and preparing intestinal tissue sections, respectively, in the mouse study in Chapter 5. Drs. Hooman Derakhshani, Haoxiang Xu, Jieyuan Jiang, Shunshun Jin, and Shengnan Li, Mr. Robert Stuski, Freddy Pezas, and Zhen Cai, Ms. Jasmine Haridy and Wajiha Shahzad provided training and technical assistance in piglet study at the University of Manitoba including feed preparation, animal husbandry, and sampling upon termination. I was responsible for literature search relevant to the studies, experimental design and conduction, data collection and analysis, and drafting the thesis.

DEDICATION

Dedicated to my beloved parents,

Yishu Bao and Youju Zhou

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2′-FL: α1,2-fucosyllactose

3-FL: 3-Fucosyllactose

3LL: Lewis lung cancer cells

3′S3-FL: 3′-sialyl-3-fucosyllactose

6′-SL: 6′-sialyllactose

ABTS: 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)

ACE: Angiotensin-converting enzyme

AMR: Antimicrobial resistance

AMU: Antimicrobial use

AVI: Avilamycin

bLf: Bovine milk lactoferrin

BW: Body weight

Caco-2: Human colorectal adenocarcinoma cells

CaMK II: Calcium/calmodulin-dependent protein kinase 2

cCMP: Cyclic cytosine monophosphate

CD: Crypt depth

CFAs: Colonization factor antigens

CFs: Colonization factors

CFUs: Colony forming units

cGMP: Cyclic guanosine monophosphate

CLDN3: Gene encoding protein Claudin-3

CMP: Casein glycomacropeptide

C. rodentium: *Citrobacter rodentium*

DAPI: Diamidino-2-phenylindole dihydrochloride

DHE: Dihydroethidium

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DMEM: Dulbecco modified Eagle's medium

DMSO: Dimethyl sulfoxide

DSLNT: disialyllacto-N-tetraose

DSS: Dextran sulfate sodium

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

EGF: Epidermal growth factor

EHEC: Enterohemorrhagic *Escherichia coli*

EPEC: Enteropathogenic *Escherichia coli*

ERK1/2: Extracellular signal-regulated kinases 1/2

EspA: *E. coli* secreted protein A

EspB: *E. coli* secreted protein B

ETEC: Enterotoxigenic *Escherichia coli*

FaeG: K88 fimbrial protein AB

FBS: Fetal bovine serum

FDR: False discovery rate

FITC: Fluorescein Isothiocyanate

Fluo-4 AM: Labeled calcium indicator acetoxymethyl ester

FOS: Fructooligosaccharides

fSC: Free secretory component

HEK-293: Human embryonic kidney 293 cells

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

hLf: Human milk lactoferrin

IBD: Inflammatory Bowel Disease

ICAM-1: Intercellular cell adhesion molecule-1

IFN-γ: Interferon γ

IgY: Egg yolk antibodies

IκBα: Nuclear factor κB inhibitor α

IL-1β: Interleukin 1β

IL-2: Interleukin 2

IL-4: Interleukin 4

IL-6: Interleukin 6

IL-10: Interleukin 6

IPEC-J2: Porcine small intestinal epithelial cells

ITS: Insulin–transferrin–selenium

KC/GRO: Keratinocyte chemoattractant/human growth-regulated oncogene chemokine

LAL: Limulus amebocyte lysate

LNF II lacto-N-fucopentaose II

LNF I, lacto-N-fucopentaose I

LPS: Lipopolysaccharides

LSTa: sialyllacto-N-tetraose a

LT: Heat-labile toxin

MAPK: Mitogen-activated protein kinase phosphatase

MCP-1: Monocyte chemoattractant protein-1

MFGM: Milk fat globule membrane

MIC: Minimal inhibitory concentrations

MOI: Multiplicity of infection

MUC2: Gene encoding protein Mucin 2

MUC4: Gene encoding protein Mucin 4

NEAA: Nonessential amino acids

NF-κB: Nuclear factor κB; Nuclear factor kappa-light-chain-enhancer of activated B cells

OCLN: Gene encoding protein Occludin

OP: Ovomucin-protex 26L hydrolysate

OPP: Ovomucin-pepsin/pancreatin hydrolysate

OVAP: Ovalbumin-protex 26L hydrolysate

OVM: Ovomucin

PBS: Phosphate-buffered saline

PCoA: Principal coordinate analysis

PCR: Polymerase chain reaction

qPCR: Quantitative polymerase chain reaction

RT: Room temperature

SCFA: Short-chain fatty acid

SEKI: Human melanoma cells

SEM: Standard error of the mean

Sta/STb: Heat-stable toxins

TEER: Transepithelial electrical resistance

Tir: Translocated intimin receptor

TLRs: Toll-like receptors

TNF-α: Tumor necrosis factor-α

VH: Villus height

ZO-1: Gene encoding protein Zonula occludens-1

CHAPTER 1 – General Introduction and Thesis Objectives

1.1 General introduction

Antimicrobial resistance (AMR) is rapidly emerging as a critical threat to public health. If no proactive solutions are implemented to slow down the rise of resistance, AMR is predicted to cause nearly 10 million deaths annually and incur a cumulative cost of 100 trillion USD by 2050, among which resistant *Escherichia coli* is anticipated to account for over 3 million annual deaths and more than 40 trillion USD in loss (O'Neill, 2016). Bacterial adhesion is the initial step for colonization and pathogenesis. Generally, proteinous cell appendages, such as flagella and fimbriae, function as adhesins that are essential for bridging bacterial cells and a substratum (Hori & Matsumoto, 2010). Adhesion of pathogens and exposure of microbial components, such as lipopolysaccharides (LPS) and toxins, can impair intestinal barrier function and cause intracellular signaling cascades that are involved in inflammatory responses.

Recognizing the critical role of bacterial adhesion in pathogenesis, the anti-adhesive therapy to interfere with the initial attachment of microbes and their toxins to cell surfaces is a promising approach to preventing infection and reducing antimicrobial use (Pecoraro et al., 2023; Shoaf-Sweeney & Hutkins, 2008). For example, the fimH antagonist D-mannose inhibiting pili formation of uropathogenic *Escherichia coli*, the synthetic antibody scFv-Fc KP3 targeting the type 3 fimbrial subunit of *Klebsiella pneumoniae*, and mucins functioning as decoy receptors for *E. coli*, *Helicobacter pylori*, *Salmonella* spp., and *Staphylococcus aureus*, have shown significant potential for infection control and resistance prevention (Vila et al., 2020; National Library Medicine, n.d.). Notably, foodstuffs and derived anti-adhesive compounds, including fucoidans from algae, phenolics from cranberry, oligosaccharides and glycoproteins from human and bovine milk, have

shown efficacies in inhibiting colonization and promoting eradication of several model enteric pathogens like *H. pylori*, *Clostridioides difficile*, and diarrheagenic *E. coli*, in clinical trials (Gao et al., 2021; Howell, 2020a, 2020b; Li et al., 2021; El Gendy et al. 2018; Sun & Wu, 2017; Zou et al., 2009). These anti-adhesive substances are generally glycoconjugates that share similar glycan structures with the bacteria and/or bacterial virulence factors, or host receptors, thus competing out their adhesion to host receptors or serving as decoy receptors for bacteria/virulence factors to facilitate their shedding and clearance from the mucosa. Additionally, anti-adhesive components demonstrate potential to counteract the adverse effects and improve the efficacies of traditional antimicrobial therapies. Therefore, exploring the anti-adhesive properties of diverse natural components offers a promising avenue for developing interventions inhibiting pathogenic bacterial adhesion, diminishing bacterial infections, decreasing or replacing antimicrobial use, reducing treatment course, and/or preventing treatment failures.

Ovomucin is a member of mucin family responsible for the viscous property and gel structure of fresh egg white (Kato et al., 1985). It is composed of α- and β-ovomucin subunits, with roughly 15% and 60% carbohydrate contents, respectively (Hiidenhovi, 2007). On average, the carbohydrate content of ovomucin is around 33%, of which 2.6-8% is sialic acid (Mine, 1995). Carbohydrates identified in ovomucin include mannose (Man), GalNAc, Gal, GlcNAc, NeuAc, Fuc, and sulfated saccharides (Donovan et al., 1970; Kato & Sato, 1971). Ovomucin is vital for the protection of egg white against bacterial infection and the breakdown of the physical structure of egg white and/or precipitation of ovomucin can render egg white susceptible for bacterial infection (Guyot et al., 2016; Yadav & Vadehra, 1977). Ovomucin and its hydrolysates display multiple bioactivities, including but not limited to anti-adhesive, anti-inflammatory, and anti-oxidative activities (Tu et al., 2020). Hence, ovomucin stands as a promising source of bioactive

glycopeptides, facilitating the assessment of their anti-adhesive properties against various pathogenic bacteria, with the potential application in preventing bacterial infections and mitigating AMR.

1.2 Hypotheses and objectives

Based on the general background information, we hypothesized that ovomucin hydrolysates could inhibit bacterial adhesion and colonization, protect intestinal epithelial barrier function, modulate the homeostasis of gut microbiome, and thus function as a promising antimicrobial alternative. The overall objectives of this research are to investigate the anti-adhesive properties of ovomucin hydrolysates in cells and animals, with specific objectives of this project were addressed:

1) To investigate the anti-adhesive activity of ovomucin hydrolysates and their effects on the epithelial barrier integrity and inflammatory responses caused by *E. coli* and its virulence factor (i.e. LPS), using porcine and human intestinal epithelial cells, IPEC-J2 and Caco-2, respectively (Chapter 3 and 4);

2) To study the anti-adhesive and anti-inflammatory activities of ovomucin and ovomucin hydrolysates using *Citrobacter rodentium*-infected mice (Chapter 5);

3) To determine the efficacy of ovomucin hydrolysate in decreasing enterotoxigenic *E. coli* colonization and reducing diarrhea incidence in post-weaning piglets (Chapter 6).

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CHAPTER 2 – Literature Review

2.1 Overview of antimicrobial resistance (AMR)

2.1.1 AMR and antimicrobial use (AMU) in swine industry

Antimicrobial resistance (AMR) is a global public health threat. AMR is responsible for about 33,000 deaths and costs about 1.1 billion Euros to the healthcare systems of EU/EEA countries each year (OECD, 2019). According to the latest report from the Centers for Disease Control and Prevention (CDC), more than 2.8 million antibiotic-resistant infections occur in the US each year, contributing to more than 35,000 annual deaths (CDC, 2019). Antimicrobial resistance was predicted to claim nearly 10 million lives a year and cost a cumulative 100 trillion USD by 2050 if proactive solutions are not taken to slow down the rise of drug resistance (O'neill, 2014). Epidemiological surveillance networks in Europe documented that more than half of the clinical *E. coli* isolates were resistant to at least one of the antimicrobial groups (i.e. aminopenicillins, fluoroquinolones, third-generation cephalosporins, aminoglycosides and carbapenem) (ECDPC, 2020). Antimicrobials are the most commonly prescribed drugs in the food-producing animal industry; in some countries approximately 80% of total consumption of medically important antimicrobials is in the animal sector (Lekagul et al., 2019). Overuse and misuse of antimicrobials in animals are recognized as the major contributors to the development of resistant organisms (Landers et al., 2012). WHO calls for restrictions on use of antibiotics in food animal production (WHO, 2017). The European Union and other countries have banned the use of antimicrobials as growth promotors in food animal production (Bond, 2018; Canada, 2018; EU, 2003; FDA, 2013). Minimizing the use of antibiotics in all sectors and developing effective infection control are urgently needed to combat the AMR crisis.

The global use of antimicrobials in food-producing animals is estimated to increase by 67%, from 63,151 tons to 105,596 tons between 2010 and 2030 (Van Boeckel et al., 2015). The common antimicrobials used in food-producing animals include aminoglycoside, beta-lactams, lincosamide, macrolide, quinolone, sulphonamide, and tetracycline (Cutler et al., 2020; Torres et al., 2021). Quinolones are one of the "Prioritization of Critically Important Antimicrobials" (WHO, 2018). Quinolones and beta-lactams are also the first-line choice antimicrobials for human infections due to their broad-spectrum action and low toxicity; however, resistance to quinolones and extended-spectrum beta-lactams have been widely reported (Bush & Bradford, 2020; WHO, 2014). In the swine industry, penicillin and tetracyclines are the most commonly used antimicrobial classes. Generally, more than 90% of antimicrobial substances are administrated orally for prophylactic purpose via both feed and water, and 90% of them is administered between birth and ten weeks of age (Lekagul et al., 2019). First-line treatment of severe diarrhea in neonatal and weaned piglets, mainly caused by enterotoxigenic *Escherichia coli* (ETEC), include neomycin, apramycin, and narrow-spectrum penicillin (Cutler et al., 2020; Nagy & Fekete, 2005). ETEC causes significant economic losses due to piglet death, morbidity, cost of medication, and decreased growth performance (Hampson, 1994; Fairbrother et al., 2005). Depending on the severity of the disease, the cost of post-weaning diarrhea excluding expenditure on antimicrobial drugs has been estimated around $44 per year for each sow (Sjölund et al., 2014).

2.1.2 Mechanisms of ETEC adhesion and infection

ETEC is also the predominant cause of diarrhea in children and travelers in developing countries (Khalil et al., 2021; Qadri et al., 2005). ETEC employs an array of fimbrial, afimbrial, helical, and fibrillar appendages termed colonization factors (CFs) on its cell surface to initial adhesion to intestinal epithelial cells. To date, at least 29 CFs have been identified. Fimbrial CFs, also known

as colonization factor antigens (CFAs), including CFA/I (rigid rod shaped), CFA/II (flexible fimbrial; including CS1/2/3), and CFA/IV (afimbrial; including CS4/5/6) stand out as the most prevalent types (Bhunia, 2018; Turner et al., 2006). An exoprotein adhesin EtpA has been proven to engage with the major subunit of flagella (i.e. flagellin) in facilitating ETEC adhesion and intestinal colonization (Roy et al., 2009). Human ETEC CFs primarily interact with glycoconjugate receptors, encompassing sialic acid-containing glycoconjugates (such as GM2 sialoglycolipids, sialoglycoproteins (26 kDa), and sialogangliosides), asialogangliosides (asialo-GM1), GalNac(β1-4)Gal-containing glycoconjugates, and a range of non-acid glycosphingolipids that predominantly mediate CFA/I binding (Gaastra & Svennerholm, 1996; Jansson et al., 2006). Notably, an acid glycosphingolipid sulfatide (SO3-3Galβ1-ceramide) (Jansson et al., 2009) and the extracellular matrix protein fibronectin (Roy et al., 2012) have been identified to interact with CFA/IV (i.e. coli surface antigen 6; CS6). Other receptors, including Lewis a (Lea) antigen, globotriaosylceramide (Gb3), and lactosylceramide, are also reported and comprehensively reviewed in the excellent review (von Mentzer & Svennerholm, 2023).

Animal ETEC exhibit distinct and less heterogeneous CFs compared to human ETEC. The prevalent CFs include F4 (K88), F5 (K99), F6 (987P), F17, F18, and F41 fimbriae, among which F4 and F18 ETEC strains are particularly associated with post-weaning diarrhea in piglets (Luppi et al., 2016). In general, the receptors for ETEC K88 fimbriae are primarily glycoproteins and glycolipids on the surface of intestinal epithelial cells in piglets, as discussed in several excellent reviews (Jin & Zhao, 2000; Xia et al., 2015; Dubreuil et al., 2016). The K88 fimbriae interacts with specific sugar residues, for example β-linked galactose, present in the glycan chains of the receptors on the host cell surface (Grange et al., 1998, 1999, & 2002). K88 variants also show preference for sialic acid or neutral glycan structures (Grange et al., 1996; Melkebeek et al., 2012).

Additional proteins that support ETEC adhesion to epithelial cells include but not limited to the outer-membrane proteins Tia (25 kDa) (Fleckenstein et al., 1996; Mammarappallil & Elsinghorst, 2000) and TibA (104 kDa) (Dubreuil et al., 2016; Lindenthal & Elsinghorst, 2001; Turner et al., 2006). ETEC produces two main enterotoxins, heat-stable toxins (STa, STb) and heat-labile toxin (LT), which alter the absorption of intestinal epithelium and induce hypersecretion of fluid and electrolytes, provoking diarrhea. STa provokes intracellular signaling cascades through binding to its receptor guanylate cyclase C that is prevalently expressed on the intestinal epithelial cells, while STb binds to an acidic glycosphingolipid sulfatide and induces secretion and accumulation of electrolytes in the intestinal lumen (Bhunia, 2018; Dubreuil et al., 2016). LT shares structural and functional characteristics with cholera toxin from *Vibrio cholerae*, consisting of an AB5 oligomeric structure where subunit A primarily accounts for the toxic and enzymatic activity of the toxin, while B subunit mediates toxin binding with the receptor GM1 ganglioside expressed by various cell types (Griffiths et al., 1986). Notably, LT is enriched in bacterial outer membrane vesicles and associated with lipopolysaccharide via a GM1-independent binding region in the B subunit, promoting bacterial adhesion to host cells as an adhesin (Fig. 2.1) (Horstman & Kuehn, 2002; Johnson et al., 2009). ETEC virulence has also been demonstrated to involve impaired intestinal integrity (Roselli et al., 2007; Roselli et al., 2003; Yu et al., 2015), enterocyte cytotoxicity (Xia et al., 2018), autophagy (Tang et al., 2014), exacerbated inflammatory responses via toll-like receptors (TLRs), nuclear factor κB (NF-κB), and mitogen-activated protein kinase phosphatase (MAPK) pathways (Wan et al., 2019; Wu et al., 2016; Yu et al., 2015), and the disruption of intestinal histomorphology and dysbiosis of gut microbiota (Wang et al., 2018).

The gastrointestinal mucosa comprises residential microorganisms, a mucus layer secreted mainly by goblet cells, a single layer of epithelial cells, and the underlying lamina propria, where

numerous immune cells and lymphatic vessels exist (Mowat & Agace, 2014). These four parts are closely linked and constitute a community to modulate each other directly or indirectly in the case of intestinal homeostasis and pathogenesis of the gut-related diseases (Deplancke and Gaskins, 2001, Kamada and Núñez, 2014). Intestinal barrier defects are associated with a broad range of diseases and disorders ranging from localized gastroenterological disorders, including ETEC infection and related diarrhea, to neurologic, respiratory, metabolic, hepatic, and cardiovascular illnesses. Diarrhea is a leading cause of morbidity and mortality in children and piglets (WHO, 2024) and generally resulted from the consumption of contaminated food and water. The imbalance in the absorption and secretion of electrolytes and water during diarrhea leads to dehydration, the most dangerous and potentially life-threatening consequence. However, diarrhea also plays a protective role in clearing pathogens from the intestine, thereby limiting the severity and duration of the infection (Tsai et al., 2017). Notably, ETEC shedding from feces during diarrhea and presence in the environment facilitate the expansion, transmission, and persistence of the pathogen (Dubreuil et al., 2016; Kempf et al., 2022).

2.1.3 Antimicrobial alternatives investigated in swine production

Various novel ingredients, such as organic acids, zinc oxide, prebiotics, probiotics, synbiotics, dehydrated porcine plasma, antimicrobial peptides, chitosan, lysozyme, plant extracts, and bacteriophages, have been studied as alternatives to antimicrobials (Barba-Vidal et al., 2018; Lekagul et al., 2019). Organic acids have been used for a long time in weaning pigs to support pig growth performance as acidifiers and replace antibiotic growth promotors because they can inhibit undesirable mold and bacterial contamination, lower the pH of feed, and promote gastric digestion and intestinal absorption. They can also control the dysbiosis characterized by coliforms overgrowth and *Lactobacilli* depression, typical of weaning, and modulate intestinal fermentation

patterns (Hermes et al., 2013; Ofek et al., 2003). Nevertheless, the outcome was not always consistent and highly influenced by various factors such as the type and dose of organic acids used, supplementation duration, type of the diet and buffering capacity, hygiene and welfare standard, health status, and age of piglets (González-Ortiz et al., 2014). Zinc oxide was widely used to inhibit pathogenic infection, stimulate growth rate, and improve animal intestinal integrity and immune function, but was reported to cause heavy metal contamination in environment, induce antimicrobial resistance, and generate bacterial strains with increased pathogenicity to humans (Kiarie et al., 2010). Probiotics (especially *Lactobacillus*) or synbiotics are gaining interest in recent years that they can benefit intestinal health, improve piglet growth performance, reduce ETEC attachment to the ileal mucosa and severity of diarrhea (Cutler et al., 2020). However, their feasibility needs further exploration due to the contradictory studies on their effectiveness, potential risks in animals with damaged gut health or pathogen pressure, and uncertainties around their effects and underlying mechanisms (Barba-Vidal et al., 2018). Collectively, various attractive approaches to substitute for antibiotics, including direct-acting small molecules representing new mechanisms of action and non-conventional strategies combining biotechnology, genetic engineering, and synthetic chemistry, have been summarized in excellent reviews (Ghosh et al., 2019; Theuretzbacher et al., 2020), detailed researches are needed to investigate practical alternatives to improve gut health and combat enteric infection without promoting AMR and economic losses from AMU restrictions.

2.2 Anti-adhesive strategy in combating AMR

2.2.1 Anti-adhesive strategy

The anti-adhesive strategy to interfere with pathogens' initial attachment and toxins to cell surfaces is a promising approach against colonization and infection of pathogenic bacteria (Shoaf-Sweeney

& Hutkins, 2008). Various dietary carbohydrates in the form of glycoconjugates can act as decoy receptors of pathogenic cells as bacterial adhesion is commonly mediated by carbohydrate-protein interactions, thus preventing adhesion of pathogens to host cells and reducing the incidence of infectious diseases (Lane et al., 2010). Compared with present chemotherapies or antimicrobials, anti-adhesion molecules, especially those naturally derived from food sources, are ecologically safe as they are not bactericidal and unlikely to induce selection pressure on resistant strains (Lane et al., 2010; Sharon & Ofek, 2000; Sun & Wu, 2017). With the increasing global concern of AMR, the anti-adhesion strategy represents a promising approach to combating bacterial infections and reducing resistant strain development.

2.2.2 Natural anti-adhesive components against ETEC

2.2.2.1 Human milk oligosaccharides and glycoconjugates

Human milk and colostrum, along with their non-immunoglobulin fractions, inhibited hemagglutination mediated by CFA/I, CFA/II, and K88 fimbriae (excluding type I pilus) (Holmgren et al., 1981). In this study, both unfractionated human milk and colostrum were observed to block the binding of enterotoxin LT to its receptor GM1 ganglioside *in vitro*. The inhibitory activity resided in both the immunoglobulin and non-immunoglobulin fractions, suggesting the presence of components functioning as structural analogs of the receptors for adhesins and enterotoxins of different ETEC strains isolated form human and piglet sources (Holmgren et al., 1981). Subsequently, non-immunoglobulin fractions of human milk and colostrum were confirmed to suppress CFA/I- and CFA/II-mediated ETEC adhesion (excluding type I pilus) to guinea pig intestinal epithelial cells and mucosa (Ashkenazi & Mirelman, 1987). Receptor-like glycoconjugates in human milk and colostrum, responsible for inhibiting ETEC adhesion to intestinal mucosa, were identified through processes such as protein denaturation via

heating, peptide backbone breakdown with trypsin digestion, and glycan moiety alteration by periodate treatment (Ashkenazi & Mirelman, 1987). Unfractionated human milk efficiently inhibited the adhesion of different ETEC strains carrying CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS8, and their combinations to Caco-2 cells, wherein the immunoglobulin and non-immunoglobulin fractions demonstrated different efficacies and the free secretory component (fSC) exhibited a binding profile similar to the immunoglobulin fraction for six colonization factors (excluding CS6 from CS5/6 and CS6 strains) and other bacterial surface proteins with a molecular weight of ~40 kDa (Oliveira et al., 2007). This observation aligned with an immunogold labeling assay that reported the interaction of fSC with ETEC CFA/II fimbrial adhesin (de Oliveira et al., 2001). Both fSC and human milk lactoferrin (hLf) were reported to inhibit hemagglutination induced by ETEC CFA/I positive strains with the minimal inhibitory concentrations (MIC) of 0.06 mg/mL and 0.1 mg/mL, respectively (Giugliano et al., 1995). Bovine milk lactoferrin (bLf), similar with hLf, were found to bind to various diarrheagenic *E. coli*, including ETEC, independent of iron-associated virulence factors, where ETEC strains exhibited higher hLf binding than enteropathogenic *E. coli* (*EPEC*), and enterohemorrhagic *E. coli* (EHEC) (Naidu et al., 1991).

The inhibitory effect of human milk gangliosides, particularly monosialoganglioside 1 (GMl), on ETEC enterotoxin LT has been well studied as receptor analogs (Lægreid & Otnæss, 1987). Ganglioside contents in human colostrum and milk are higher than those of bovine source and have proven more effective against ETEC adhesion to Caco-2 cells (Idota & Kawakami, 1995). Specifically, GM1and GM3 served as effective inhibitors in ETEC adhesion, and GM3 harboring a carbohydrate moiety corresponding to N-acetylneulaminyl-lactose (NeuAc(α2-3)Gal(β1-4)G1c) also hindered EPEC adhesion to Caco-2 cells (Idota & Kawakami, 1995). Notably, gangliosides GM1, GM3, and GD3, and sialic acid at physiologic concentrations were found to interfere with

the adhesion of pathogenic bacteria associated with neonatal diarrheic episodes, including *Helicobacter pylori*, *Salmonella typhi*, *Campylobacter jejuni*, EPEC O55:K99, ETEC O25:H42, *Listeria monocytogenes*, and *Shigella sonnei*. These milk components competed with bacteria and displaced adherent bacteria from Caco-2 cells (Salcedo et al., 2013). Moreover, supplementing gangliosides to an adapted milk formula reduced relative abundance of *E. coli* while promoted the growth of bifidobacteria in preterm infants, hence diminishing pathogenic *E. coli* infections (Rueda et al., 1998).

Human milk oligosaccharides, both free and conjugated, have undergone extensive investigation for their role in protecting breast-feeding infants from enteric infections. In the context of ETEC adhesion and pathogenesis, fucosyloligosaccharides were among the diverse carbohydrates initially fractionated from human milk displaying inhibitory effect on STa toxicity through binding to the extracellular domain of STa receptor guanylate cyclase and competing out STa adhesion (Newburg, 1999; Newburg et al., 1990). Being part of the innate immune system for breastfed infants, fucosyloligosaccharide expression in human milk has been linked to maternal genotypic polymorphisms of Lewis blood group types, with Le^{a-b+} mothers providing more α1,3- or α1,4-linked fucosyloligosaccharides (e.g. LNF II lacto-N-fucopentaose 2 harboring a Le^a epitope and 3-FL with a Le^x epitope) than Le^{a-b-} mothers whose milk contains more α1,2-linked fucosyloligosaccharides (e. g. LNF I, lacto-N-fucopentaose I having a H-1 epitope and 2′-FL with H-2) (Newburg et al., 2004). Notably, higher contents of α1,2-linked fucosyloligosaccharides offered protection against ETEC-associated diarrhea in nursing infants (Newburg et al., 2004). Oligosaccharides from human colostrum and milk exhibited stronger anti-hemagglutination activity induced by CFA/I- and CFA/II-positive ETEC human strains than those from bovine source, and sialic acid-containing oligosaccharides possibly participated in the recognition and

interaction process, including sialyllacto-N-tetraose a (LSTa), disialyllacto-N-tetraose (DSLNT), 3′-sialyl-3-fucosyllactose (3′S3-FL), and 6′-sialyllactose (6′-SL) (Martín-Sosa et al., 2002). *In vitro* adhesion study showed that α1,2-fucosyllactose (2′-FL), a dominant human milk oligosaccharide, could effectively decrease ETEC F18 adhesion to the F18-susceptible intestinal epithelial cells originated from the jejunum of an adult pig, but failed to limit ETEC F18-induced diarrhea and mucosal damage and dysfunction in newborn pigs (Cilieborg et al., 2017). Intriguingly, enzymatically synthesized analogs of human milk oligosaccharides, such as the di-fucosylated β-cyclodextrin containing α-1,3-linked α-L-fucose moieties to glucose, have been developed based on advanced understanding of their structure-function relationship; these analogs, mimicking the structure of 3-FL, blocked ETEC adhesion to Caco-2 cells by acting as decoy receptors (Verkhnyatskaya et al., 2021). These data underscore the vital role of glycoconjugates, particularly sialylated and fucosylated glycans, in human milk and inspire the development of biological analogs to prevent pathogenic adhesion and infections (Table 2.1).

2.2.2.2 Bovine milk oligosaccharides and glycoconjugates

Bovine colostrum, when fed to piglets, effectively reduced ETEC colonization and decreased the frequency of diarrhea compared to a milk formula (Sugiharto et al., 2015). Casein glycomacropeptide (CMP) from human milk whey fraction was initially evaluated without anti-hemagglutination activity against CFA/I and CFA/II expressing human ETEC strains (Neeser et al., 1988). However, bovine milk CMP efficiently inhibited ETEC K88ac adhesion to porcine small intestinal epithelial (IPEC-J2) cells, ileal mucosa tissues, and gut colonization, partially through K88 fimbriae binding, with the terminal carbohydrates such as Galb(1-3)GalNAc and NeuAc(2-6)Gal functioning as receptor analogues (Hermes et al., 2011, 2013). Similar action was

found for camel milk CMP against ETEC K88-induced hemagglutination of porcine erythrocytes (Althnaibat et al., 2022).

Bovine milk fat globule membrane (MFGM), along with purified MFGM gangliosides (sialylated-sphingolipids) GM3 and GD3, and lactosylceramide, were found to bind to K99-, F41-, F17-bearing ETEC strains isolated from diarrheic calves and inhibit bacterial hemagglutination, with the binding affinity varying among different fimbriae/adhesins. This suggested that the composition and structure of ceramides could modulate sugar conformation and receptor recognition (Martín et al., 2003; Sanchez-Juanes et al., 2009). Recognizing the effectiveness of MFGM in impeding pathogenic ETEC and EHEC adhesion and colonization evidenced by *in vitro* and *in vivo* bioassays (Bagel et al., 2023; Bagel et al., 2022; Douëllou et al., 2018; Ross et al., 2016), MFGM is acknowledged as a source for developing anti-adhesive strategies to control bacteria infections. Nevertheless, it is crucial to consider specific (glyco)-constituents derived from MFGM, understand their detailed mechanisms of action, assess their impact on commensal microbes, and evaluate potential undesired outcomes for therapeutic development (Bagel & Sergentet, 2022). Bovine milk contains a lower content of free oligosaccharides than human milk, with sialylated oligosaccharides, particularly sialyllactose, being a more abundant fraction than neutral oligosaccharides that primarily consist of N-Acetyl-lactosamine (>70%) (Gopal & Gill, 2000). Oligosaccharides and glycoconjugates from bovine colostrum and milk have been extensively examined for their impact on the adhesion of pathogenic *E. coli*. Bovine colostrum, milk, and α2,6-sialylated oligosaccharides were found to effectively inhibit hemagglutination induced by diverse ETEC strains isolated from diarrheic calves that bear different adhesins, including K99, F41, and F17 fimbriae (Martín et al., 2002). These findings collectively emphasize

a consistent trend for milk oligosaccharides in safeguarding newborn mammals from pathogenic infections.

2.2.2.3 Plant extracts

Extracts of tempe, a traditional food made from mold fermentation of soaked and cooked soya beans, notably inhibited ETEC K88-induced hemagglutination and adhesion to porcine brush border membranes, in turn leading to the control of diarrhea severity in ETEC K88-challenged piglets (Kiers et al., 2002, 2003). Extracts from different processing stages (soaking, cooking, and fermentation) were compared, revealing that those from fermented products (i.e. Tempe) exhibited the highest inhibitory impact on ETEC adhesion to Caco-2 cells, particularly through interacting with bacterial cells and impairing bacterial capacity to adhere to intestinal cells (Roubos‐van den Hil et al., 2009). The bioactive component responsible for the inhibitory activity of Tempe has been identified to have a high arabinose content derived from the arabinan or arabinogalactan side chain of the pectic cell wall polysaccharides of soybeans, released or produced during fermentation (Roubos-van den Hil et al., 2010). Similarly, extracts of Tofu, another significant soya bean product, demonstrated substantial inhibitory activity against ETEC K88 adhesion, which was further stimulated during digestion through stomach and small intestine (Mo et al., 2012). Pea hull and meal extracts, whether digested *in vitro* by pepsin and pancreatin or not, strongly attached to ETEC K88, potentially through providing alternative binding sites for the bacterial cells, while faba bean hull extracts more effectively disrupted enterotoxin LT binding to GM1 receptor on epithelial cells. Each hull extracts improved net fluid absorption in ETEC K88-challenged weaning piglets in the small intestinal segment perfusion model (Becker et al., 2012; Van der Meulen & Jansman, 2010). In a comprehensive screening of plant ingredients interfering with ETEC K88 adhesion, extracts from pumpkin, sesame seed, and tomato demonstrated binding capacities to

ETEC K88 and K99, assessed by calculating the growth parameters of adherent bacteria (Becker et al., 2007; Becker & Galletti, 2008). Subsequent studies showed that extracts from wheat bran, locust bean, exopolysaccharides from olive fermentation, along with bovine CMP, significantly inhibited ETEC K88 adhesion to IPEC-J2 cells and porcine ileal mucus. Specifically, protein/glycoprotein components in wheat bran extracts, and galactomannans/phenolics in locust bean extracts were identified responsible for their anti-adhesive properties (González-Ortiz et al., 2013, 2014; Hermes et al., 2011).

Fiber-containing products derived from wheat, lentils, oat bran, and guar inhibited adhesion of human ETEC strain H10407 to type III mucin, Caco-2 and HT29-MTX cells, among which lentil fiber exhibited a remarkable suppression of enterotoxin LT production (Sauvaitre et al., 2021). In addition to their established role in reducing *C. difficile* adhesion and colonization, inulin and fructooligosaccharides (FOS) were shown to impede ETEC (CFA/I-, enterotoxins-positive strain) adhesion to HT-29 cells and diminish the toxicity of ST and LT as evidenced by decreased intracellular levels of cGMP and cCMP. These effects were observed both individually and in combination with the probiotic *Lactobacillus rhamnosus* NCDC 298 (Anand et al., 2018).

Phenolics from tea were found to decrease ETEC K88 adhesion to IPEC-J2 cells (Ma et al., 2021). Phenolics from the medicinal plant *Galla Chinensis*, containing gallic acid as the major component, and terpenoids like oleanolic acid, ursolic acid, and betulinic acid from fruit Chaenomeles inhibited the interaction of enterotoxin LT with its receptor GM1 and decreased LT-induced fluid accumulation in mouse gut, indicating their potential in attenuating ETEC pathogenesis (Chen et al., 2007; Chen et al., 2006). Phenolics found in the berry fruit jaboticaba (*Myrciaria jaboticaba* (Vell.) Berg) showed bactericidal activity against ETEC strains and decreased the biofilm formation, cell surface hydrophobicity, motility, and mucin adhesion of

ETEC (Lima et al., 2023). Notably, jaboticaba phenolics impaired the enzymatic and efflux pump activities, increasing the susceptibility of ETEC strains to a range of clinically important antimicrobial agents (Lima et al., 2023). These findings indicate the potential of anti-adhesive components derived from diverse plant sources in preventing enteric infection caused by pathogenic *E. coli*, providing insight for future research on their application to reduce antibiotic use for disease management. However, some pure and commercially available natural polyphenols have been studied that their modulation of bacterial virulence factors might be non-specific and potentially cause adverse effects (Dávila-Aviña et al., 2020; Verhelst et al., 2010). Therefore, further understanding the underlying mechanisms and accurate concentration application are essential to avoid undesirable virulence induction.

2.2.2.4 Probiotics and microbial exopolysaccharides

Probiotics typically composed of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* species, have shown promise as anti-adhesive strategies for reducing enteric infections, including those caused by ETEC (Bermudez-Brito et al., 2012). Probiotics can inhibit pathogen adhesion through various mechanisms. A key approach is the competition for binding sites on intestinal epithelial cells. By occupying the receptors for ETEC adhesins, probiotics can effectively prevent pathogens from accessing those sites. For instance, some *Lactobacillus* strains like *L. fermentum,* and *L. paracasei* have been demonstrated to adhere to intestinal epithelial cells, thus inhibiting ETEC adhesion (Pazhoohan et al., 2020). Probiotics can also act synergistically with bioactive compounds to enhance their anti-adhesive effects. For example, *L. rhamnosus* and *Streptococcus thermophilus* combined with B-type lotus seedpod oligomeric procyanidin, as well as *L. rhamnosus* with FOS, have been reported to reduce ETEC adhesion (Anand et al., 2018; Tang et al., 2024).

Additionally, exopolysaccharides produced by microorganisms are also capable of inhibiting ETEC adhesion to cultured cells, including levan-type exopolysaccharide from *Bacillus amyloliquefaciens* and d-glucan polysaccharides from *L. reuteri* (Kšonžeková et al., 2016; Cai et al., 2019). Notably, reuteran by *L. reuteri* has shown anti-adhesive effect against ETEC binding to porcine erythrocytes and the jejunal mucosa of piglets (Wang et al., 2010; Chen et al., 2014). Fermented diets by reuteran- and levan-producing *L. reuteri* have been demonstrated to decrease ETEC K88 colonization in weaned piglets and repress the production of STb (Yang et al., 2015). Furthermore, as aforementioned that tempe extracts containing soybean-derived polysaccharide structures showed anti-adhesive activity, tempe also contains a diverse population of *Lactobacillales* strains that are known for their capacities to produce exopolysaccharides (Khalil et al., 2018). Dextran and levan from tempe-associated *Lactobacillales* strains have been reported to interact with both human and pig-associated ETEC strains and inhibit bacterial auto-aggregation, suggesting their potential in alleviating ETEC-mediated diarrhea (Pramudito et al., 2024).

2.2.2.5 Marine sources and eggs

Algae-derived compounds are sources of glycoconjugates with anti-adhesive activities against diverse pathogens. Depolymerized sulfated galactans from red algae *Eucheuma serra* and *Gracilaria verrucosa* with molecular weight below 20 kDa exhibited bactericidal activity against ETEC K88. These galactans bound to bacterial surface and disrupted cell permeability and morphology, preserving intestinal histomorphology and leading to a reduction in ETEC K88-induced diarrhea of mice (Liu et al., 2019; Ma et al., 2021).

Ovomucoid in chicken egg white carries short oligomannoside-type and primarily hybrid-type N-linked carbohydrate chains. Ovomucoid-derived glycopeptides are the first example among the chicken egg proteins investigated for their interaction with pathogenic bacteria, demonstrating the

inhibition of bacteria adhesion and hemagglutination of bovine erythrocytes induced by human CFA/II-positive ETEC strains (Neeser et al., 1988). Ovalbumin, while lacking anti-hemagglutination activity against human CFA/I and CFA/II-positive ETEC strains, exhibited binding capacities to *S. typhi* and Shiga toxin-producing *E. coli* O84 (Leusch, Drzeniek, Hefta, et al., 1991; Leusch, Drzeniek, Markos-Pusztai, et al., 1991). Notably, with high-mannose-type carbohydrate side chains, ovalbumin-generated short Man5GlcNAc2-contaning glycopeptides acted as decoy receptors for type I pilus-mediated adhesion of EPEC strains (Neeser et al., 1986; Tai et al., 1975). Ovomucin from chicken egg white also exhibits anti-adhesive activity against diverse pathogens, including viruses and pathogenic bacteria, which will be further covered in the next section. These findings collectively inspire future research aimed at expanding repertoires of naturally occurred anti-adhesive substances for their utilization as nutraceuticals and therapeutics targeting infectious disease controlling and AMR mitigation.

2.3 Ovomucin and its hydrolysates

Ovomucin is a glycoprotein of high molecular weight in egg white. Molecular weight of different forms of ovomucin varies dramatically as summarized in previous review (Omana et al., 2010). Ovomucin consists of a carbohydrate rich β-subunit (~400 kDa) and a carbohydrate poor α-subunit (~220 kDa) with roughly 60% and 15% carbohydrate contents, respectively (Itoh et al., 1987; Kato & Sato, 1971; Robinson & Monsey, 1971). On average, it consists of 33% of carbohydrate content and unfractionated form contains 10-12% hexosamine, 15% hexose and 2.6-8% sialic acid (Mine, 1995). The carbohydrate structures are mainly composed of 3-5 units of N-acetylgalactosamine, galactose, N-acetylglucosamine, and sialic acid (Kato et al., 1978; Gérard Strecker et al., 1987, 1989; Strecker et al., 1992). Ovomucin has little α-helix and appears to contain about 70% β-structure and 30% random coil at neutral pH in 1 M KCl (Donovan et al., 1970). It is inherently

heterogeneous and connected by disulfide bonds, reduction of which contributes to depolymerization of ovomucin, decrease in foaming property, and egg white thinning (Donovan et al., 1970; Donovan et al., 1972; Kato et al., 1985). However, as dissociation proceeds, the emulsifying property of ovomucin increases due to greater surface hydrophobicity (Kato et al., 1985). Previous research has well summarized its physiochemical properties and extraction methods (Omana et al., 2010). To date, studies have demonstrated that ovomucin and its hydrolysates show various bioactivities, including anti-adhesive, anti-inflammatory, anti-oxidative, anti-tumor, bifidogenic, and angiotensin-converting enzyme (ACE) inhibitory activities.

2.3.1 Anti-adhesive activity

Ovomucin was initially reported to be a competitive inhibitor for hemagglutination induced by influenza virus (Gottschalk & Lind, 1949). Later investigations also demonstrated its binding activity to bovine rotavirus, hen Newcastle disease virus, and human influenza virus (Tsuge et al., 1996a, 1996b, 1997a; Wang et al., 2018). The α-subunit and β-subunit showed binding differences and the sialic acid residues in the β-subunit greatly contributed to the binding of ovomucin to Newcastle disease virus (Tsuge et al., 1996b, 1997a, 1997b). Ovomucin could bind to avian influenza viruses H_5N_1 and H_1N_1, and sialic acid was involved (Xu et al., 2018). This is consistent with the acknowledgement that sialic acid moiety of the sugar chains is the most frequent recognition ligand of microorganisms (Wasik et al., 2016). Besides, interaction with Mg^{2+} can affect the adhesion capacity of ovomucin to Newcastle disease virus (Shan et al., 2014).

Ovomucin also showed anti-adhesive activity to bacteria. Urease is a surface adhesin of *Helicobacter pylori* that causes gastric infection and peptic ulcers. Study revealed that ovomucin could inhibit urease adhesion to porcine gastric mucin and eliminate pathogen colonization from the stomach of *H. pylori*-infected mice (Kodama & Kimura, 2001). Ovomucin glycopeptides (~40

to ~100 kDa), prepared and separated after pronase digestion, bound to EHEC O157:H7, with sialylated carbohydrate moieties providing specific binding sites for the bacterium (Kobayashi et al., 2004). Later research unraveled the anti-adhesive property of ovomucin hydrolysates digested by different enzymes including protease P, protease N, acid protease II, pronase, protex 26L, and pepsin/pancreatin, against ETEC K88 by a hemagglutination assay (Sun et al., 2017). Based on this assay, glycopeptides from ovomucin by protease P, protex 26L, pepsin/pancreatin, and pronase digestion inhibited ETEC K88 strains adhesion to porcine small intestinal epithelial cells (Sun et al., 2019). And consistent with that reported from intact ovomucin, the carbohydrate moieties in glycopeptides through binding K88 fimbriae as decoy receptors contributed to its anti-adhesive ability (Sun et al., 2019).

2.3.2 Anti-inflammatory activity

The latest study showed that intact ovomucin could attenuate dextran sulfate sodium (DSS)-induced colitis in mice and reduce mRNA expression and protein levels of inflammatory cytokines, including TNF-α, IL-6, IL-1 β, IFN-γ, IL-8, and IL-10 in colon tissues, as well as TNF-α levels in serum (Tu et al., 2021). Ovomucin hydrolysate prepared by Alcalase could inhibit tumor necrosis factor-mediated nuclear factor-κB (NF-κB) activation and decrease the expression of the proinflammatory protein intercellular cell adhesion molecule-1 (ICAM-1) in human dermal fibroblasts (Sun, Chakrabarti, et al., 2016). These studies indicate the ability of intact ovomucin and its derived peptides to prevent inflammatory response and further potential application in managing chronic and/or acute inflammation that is associated with various diseases.

2.3.3 Anti-oxidative activity

Ovomucin hydrolysates prepared by protamex, flavourzyme, and Alcalase showed free radical scavenging activity at 90.4, 89.4, and 88.6%, respectively, by the 2,2′-azinobis (3-

ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay. Also, peptides LDEPDPL (f686−692) and NIQTDDFRT (f539−547) from the ovomucin α-subunit were identified from the hydrolysate by enzyme protamex and exhibited anti-oxidative activity of 51.8% and 24.7%, respectively, as determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Chang et al., 2013). Ovomucin hydrolysate obtained by heating at 100 ºC for 15 min at pH 12.0 also showed a strong anti-oxidative activity (Abeyrathne et al., 2016). Further biological assays are needed to validate their anti-oxidative activity as these chemical assays are not based on living organisms. A pentapeptide (WNWAD) derived from egg white ovomucin-pepsin hydrolysate was reported to protect HEK-293 cells against H_2O_2-induced oxidative stress by reducing intracellular ROS accumulation and inhibiting the mitochondria-mediated cell apoptosis pathways (Liu et al., 2014).

2.3.4 Anti-tumor activity

The β-subunit of ovomucin was firstly proved to exert cytotoxicity on cultured human melanoma (SEKI) and Lewis lung cancer cells (3LL) (Ohami, 1993). Later, Yokota et al. reported that β-subunit of ovomucin significantly reduced the proliferation of sarcoma-180 cells and promoted the swelling and bleb formation of microvilli and abnormal chromatin clumping (Yokota et al., 1999b). The shape and structure of the nuclear and organelles also showed obvious changes associated with cell necrosis. And then they reported that a highly glycosylated fragment (120 kDa) in β-subunit from the pronase-digested ovomucin could interact with the basic fibroblast growth factor receptor on the surface of sarcoma-180 cells to inhibit cell growth and induce cell necrosis (Yokota et al., 2000). *In vivo* studies also demonstrated its anti-tumor activity. By inoculating Meth-A fibrosarcoma cells to establish a double-grafted tumor system in mice, highly glycosylated fragments (220 kDa and 120 kDa) from pronase-treated ovomucin were reported to modulate the immune system and cure the right (treated) tumor and inhibit the growth of the left (distant) one

(Watanabe et al., 1998). In the same study, they found that the sialic acid residues in the 120 kDa fragment might play a crucial role in regressing the distant tumors (Watanabe et al., 1998). This was further evidenced that injection of ovomucin β-subunit recruited neutrophils, macrophages, and lymphocytes on the margin of the degenerated and necrotic tumor tissue in the sarcoma-180 cell-xenografted mice (Yokota et al., 1999a). A 70 kDa highly glycosylated fragment in the α-subunit separated from pronase-treated ovomucin was also illustrated to exhibit similar anti-tumor effects as the β-subunit and inhibit angiogenesis (Oguro et al., 2000).

2.3.5 Other bioactivities

The latest *in vivo* study demonstrated that ovomucin could increase the relative abundance of intestinal beneficial bacteria like *Lactobacilli*, *Faecalibaculum*, *Ruminococcus*, etc. and elevate the production of short chain fatty acids (Tu et al., 2021). In this study, ovomucin was shown to mitigate colitis severity, improve intestinal morphology, boost the intestinal barrier function by restoring the expression of *MUC2* and tight junction proteins, and regulate the release of inflammatory cytokines (Tu et al., 2021). Ovomucin hydrolysate by pepsin digestion for 3 h followed by pancreatin for 3 h showed bifidogenic activity as it could promote the *in vitro* growth of *Bifidobacterium infantis* and increase the production of lactate after 24 h incubation as a sole carbon source (Sun, Gänzle, et al., 2016). *O*-glycans on ovomucin could be degraded by *B. bifidum* and *Akkermansia muciniphila* and serve as a carbon source to support the *in vitro* growth of *A. muciniphila*, indicating the potential of ovomucin to modulate beneficial microbes and improve gut health (Takada et al., 2020). These findings collectively imply the great potential of ovomucin and its hydrolysates in modulating intestinal function and health (Tu et al., 2020).

In addition to those mentioned above that ovomucin/fragments showed immunomodulatory activity in tumor cell xenografted mice and colitis mice, ovomucin could enhance the proliferation

of mouse spleen lymphocytes stimulated by lipopolysaccharide (Otani & Maenishi, 1994). Pronase E/papain-digested ovomucin hydrolysate could promote the differentiation of cultured macrophage-like cells J774.1 and increase H_2O_2 generation and IL-1 production (Tanizaki et al., 1997). The activation of macrophage cells was attributed to the *O*-linked sulfated carbohydrate chains, among which N-acetyl-neuraminyl-(2,3)-galactosy1-(1,3)-N-acetylgalactosamine-6-sulfate was identified as the main sulfated oligosaccharide structure in ovomucin (Tanizaki et al., 1997).

Nagaoka et al. reported that ovomucin inhibited cholesterol uptake by Caco-2 cells and increased the fecal excretion of cholesterol and bile acids in rats (Nagaoka et al., 2002). Ovomucin could directly interact with cholesterol mixed micelles in the jejunal epithelia and inhibit reabsorption of bile acids in the ileum to suppress cholesterol absorption into serum and total lipids level in liver and attenuate hypercholesterolemia in rats. Ovomucin hydrolysates prepared by Alcalase, papain, and trypsin showed ACE inhibitory activity by test-tube assays (Abeyrathne et al., 2016), which needs to be verified by bioassays. These studies indicate the potential of ovomucin and its derived bioactive peptides in promoting metabolic syndrome, but further research is warranted. In summary, ovomucin is a crucial component of egg white proteins and possesses diverse bioactivities. It can be acknowledged as a promising source of bioactive peptides for developing novel functional foods.

In conclusion, numerous food-derived bioactive components have shown promise in mitigating bacterial infections and combating evolving AMR, providing a solid foundation for exploring the effects of ovomucin and ovomucin-derived bioactive peptides. Ovomucin and its hydrolysates exhibit significant potential in reducing pathogenic infections and preserving intestinal function and health. Therefore, this thesis aims to investigate the activities of ovomucin

and its hydrolysate products using *in vitro* and *in vivo* experimental models. The goal is to elucidate their mechanisms and assess their practical application in managing bacterial infections.

2.4 References

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Table 2.1 Anti-adhesive components against ETEC

Foods/components	Study model & design	Results & mechanisms	Ref
Milk oligosaccharides			
Fucosyloligosaccharides	Outbred Swiss mice 2-3 days old	Inhibited STa toxicity through binding to its receptor guanylate	(Newburg 1999; Newburg et al. 1990)
α1,2-linked Fucosyloligosaccharides, such as LNF I and 2′-FL	A cohort of 93 Mexican breastfeeding mother–infant pairs	Correlated with maternal genotype of Lewis blood group Le^{a-b-} and protected infants from ETEC-associated diarrhea	(Newburg et al. 2004)
2′-FL	Caco-2 cells; F18-susceptible pigs	Inhibited EPEC O119 adhesion to Caco-2 cells and ETEC F18 adhesion to the jejunum of pigs	(Cilieborg et al. 2017; Kong et al. 2021)
Sialyoligosaccharides from human milk and colostrum, such as LSTa, DSLNT, 3′S3-FL, and 6′-SL	Anti-hemagglutination assay	Inhibited CFA/I and CFA/II-positive ETEC human strains (O20 and O6:H16)-induced hemagglutination of calf and human erythrocytes	(Martín-Sosa, Martín, and Hueso 2002)
3-FL simulant di-fucosylated β-cyclodextrin	Caco-2 cells	Inhibited adhesion of ETEC ET8 and ETEC O78:H11	(Verkhnyatskaya et al. 2021)
Bovine milk and sialylated oligosaccharides	Anti-hemagglutination assay	Inhibited hemagglutination of horse erythrocytes induced by ETEC strains from diarrheic calves, α2,6-linked sialylated oligosaccharides showed preference,	(Martín, Martín-Sosa, and Hueso 2002)

		especially to several K99-, F41, and F17-fimbriated strains	
Milk glycoconjugates			
Human milk, colostrum, and non-immunoglobulin fractions	Anti-hemagglutination assay	Inhibited CFA/I and CFA/II-positive ETEC human strains (H10407 and E1392-75)-induced hemagglutination and suppressed LT binding to its receptor GM1 ganglioside	(Holmgren, Svennerholm, and Ahren 1981)
Human milk, immunoglobulin and non-immunoglobulin fractions, fSC	Caco-2 cells	Inhibited the adhesion of ETEC strains carrying diverse colonization factors including CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, and CS8	(Deoliveira et al. 2007)
Non-immunoglobulin fraction containing glycan structures from human milk and colostrum	Guinea pig intestinal epithelial cells and intestinal mucosa	Inhibited CFA/I and CFA/II-positive ETEC H10407 and E1392-75 adhesion	(Ashkenazi and Mirelman 1987)
hLf and bLf	*In vitro* culture	Bound to various diverse ETEC, EPEC, and EHEC independent on apo- and iron-saturated forms	(Naidu et al. 1991)
hLf and fSC	Anti-hemagglutination assay; HeLa cells	Inhibited CFA/I-positive ETEC induced hemagglutination of human group A erythrocytes, and suppressed EPEC O111ab:H2 adhesion	(De Araujo and Giugliano 2001; Giugliano et al. 1995)

fSC	*In vitro* culture	Interacted with bacterial CFA/II adhesin	(de Oliveira et al. 2001)
human milk ganglioside GM1, GM3	Caco-2 cells	Interacted with LT receptor to counteracted LT adhesion; reduced ETEC O6:K15:H16 and EPEC O111ab:K58:H21	(Idota and Kawakami 1995; Lægreid and Otnæss 1987)
Gangliosides supplement	Preterm infants born between 32 and 36 weeks of gestational age	Inhibited pathogenic *E. coli*-related infection in preterm infants and increased the abundance of bifidobacteria	(Rueda et al. 1998)
Bovine colostrum	ETEC F18-susceptible piglets	Inhibited ETEC colonization in piglet intestine and modulated mucosal immunity	(Sugiharto et al. 2015)
CMP	Anti-hemagglutination assay; IPEC-J2, Caco-2, and HT-29 cells	human CMP prevented human CFA/I and CFA/II positive ETEC strains-induced hemagglutination of human group A erythrocytes; bovine CMP inhibited ETEC K88ac adhesion to IPEC-J2 cells and porcine ileum through functioning as the receptor of K88 fimbriae by its terminal Galb(1-3)GalNAc and NeuAc(2-6)Gal; bovine CMP inhibited EPEC and EHEC strains to Caco-2 and HT-29 cells; camel	(Althnaibat et al. 2022; Brück et al. 2006; Feeney et al. 2017; Hermes et al. 2011; Hermes et al. 2013; Neeser et al. 1988; JR Rhoades et al. 2005)

		CMP inhibited ETEC K88-induced hemagglutination of porcine erythrocytes;	
Purified gangliosides GM3, GD3, and lactosylceramide	Anti-hemagglutination assay	Bound to K99-, F41-, and F17-fimbriated ETEC strains from calves and inhibited ETEC-induced hemagglutination of horse erythrocytes	(Martín et al. 2003; Sanchez-Juanes et al. 2009)
Fruits/Vegetables			
Tempe extracts rich in arabinose	Anti-hemagglutination assay; Caco-2 cells; jejunal brush border cells of weaned piglets	Prevented ETEC K88-induced hemagglutination of hamster erythrocytes, inhibited ETEC K88 adhesion, and alleviated piglet diarrhea	(Kiers et al. 2003; Kiers et al. 2002; Roubos-van den Hil et al. 2010; Roubos-van den Hil et al. 2009)
Tofu	Jejunal brush border cells of weaned piglets	Inhibited ETEC K88 adhesion	(Mo, Zhu, and Nout 2012)
Extracts and hydrolysates of faba bean hulls and pea	Small intestine segment perfusion model of weaned piglets	Bound to ETEC K88, inhibited LT adhesion to its receptor GM1 and attenuated ETEC	(Becker et al. 2012; Van der Meulen and Jansman 2010)

		O149:K91:F4:K88ac-induced hypersecretion in the small intestine of piglets	
Extracts from pumpkin, sesame seed, tomato, wheat bran, locust bean, and exopolysaccharides from olive fermentation	IPEC-J2 cells; porcine ileum mucus	Interacted with ETEC K88 and K99 and inhibited ETEC K88 adhesion	(Becker and Galletti 2008; Becker et al. 2007; González-Ortiz et al. 2013; González-Ortiz et al. 2014; Hermes et al. 2011)
Fibers of wheat starch, lentils, oat bran, and guar	Microplate assay; Caco-2 and HT-29 cells	Inhibited LT production and ETEC H10407 adhesion to type III mucin and cultured cells	(Sauvaitre et al. 2021)
Inulin	Hep-2, Caco-2, and HT-29 cells	Inhibited CFA/I-positive ETEC adhesion to HT-29 cells and neutralized the toxicity of ST and LT; inhibited EPEC O119 and ETEC ET8 adhesion to Caco-2 cells and Hep-2 cells	(Anand et al. 2018; Kong et al. 2021; Shoaf et al. 2006)
Pectin	Caco-2 cells	Inhibited EPEC O119 and ETEC ET8 adhesion	(Kong et al. 2021)
FOS	HT-29 cells	Inhibited CFA/I-positive ETEC adhesion and neutralized the toxicity of ST and LT	

Tea phenolics, epigallocatechin gallate	HEp-2 and IPEC-J2 cells	Inhibited ETEC K88 adhesion to IPEC-J2 cells, reduced adhesion and invasion of EPEC O127:H6, EHEC strain EDL933 to Hep-2 cells through dysfunctioning the T3SS	(Nakasone et al. 2017)
Phenolics from *Galla Chinensis* and terpenoids from *Chaenomeles*	*In vitro* culture	Neutralized the toxicity of LT by inhibiting its binding to the receptor GM1	(Chen et al. 2007; Chen et al. 2006)
Phenolics from the berry fruit jaboticaba	Microplate assay	Inhibited ETEC adhesion to mucin, disturbed biofilm formation, decreased cell surface hydrophobicity and bacterial motility, decreased the activities of efflux pump of ETEC and sensitized bacteria to antibiotics	(Lima et al. 2023)
Egg/Honey			
Ovomucoid-derived glycopeptides	Anti-hemagglutination assay	Inhibited CFA/II-positive ETEC induced hemagglutination of bovine erythrocytes	(Neeser et al. 1988)
Ovomucin hydrolysates	Anti-hemagglutination assay; IPEC-J2 cells	Inhibited hemagglutination of porcine erythrocytes induced by ETEC K88, and reduced ETEC K88 adhesion; neutralized the cytotoxicity of LPS from EPEC O111:B4	(Bao and Wu 2021; Sun, Gänzle, and Wu 2019; Sun, Gänzle, and Wu 2017)
Murine sources			

sulfated galactans from red algae *Eucheuma serra* and *Gracilaria verrucosa* of <20 kDa	ICR mice	Interacted with ETEC K88, destroyed bacterial cell membrane structure and attenuated ETEC K88-induced diarrhea and tissue damage in mice	(Liu et al. 2019; Ma et al. 2021)
Probiotics and exopolysaccharides			
L. fermentum and *L. paracasei*	HT-29 cells	Adhered to HT-29 cells and inhibited adhesion of ETEC isolates from stool specimens of children with acute diarrhea	(Pazhoohan et al., 2020)
L. rhamnosus/Streptococcus thermophilus with B-type lotus seedpod oligomeric procyanidin*	IPEC-J2 cells	Inhibited ETEC K88ac adhesion to cells, enhanced immune defense, and restored barrier function	(Tang et al., 2024)
L. rhamnosus NCDC 298 with FOS/inulin*	HT-29 cells	Autoaggregation and coaggregation with ETEC and inhibited ETEC adhesion to cell surfaces	(Anand et al. 2018)
d-glucan polysaccharides of *L. reuteri*	IPEC-1 cells	Inhibited ETEC adhesion to IPEC-1 cells and ETEC-induced expression of *IL-1β* and *IL-6*	(Kšonžeková et al., 2016)
Levan-type exopolysaccharide from *B. amyloliquefaciens*	HT-29 cells	With a main chain of β-(2,6)-linked Fru*f* residues and intensive branches of a single 2-linked Fru*f* at every six residues, inhibited ETE adhesion to cell surfaces	(Cai et al., 2019)

Reuteran and levan by *L. reuteri*	Anti-hemagglutination assay; small intestinal segment perfusion; 21-d-old weaned piglets	Adhered to both human and pig-associated ETEC strains; inhibited hemagglutination of porcine erythrocytes induced by ETEC K88 strains; reuteran decreased ETEC K88 levels in mucosal scrapings; reduced ETEC K88 colonization and STb levels in piglet gut	(Wang et al., 2010; Chen et al., 2014; Yang et al., 2015; Pramudito et al., 2024)

*Synergistic effects were observed.

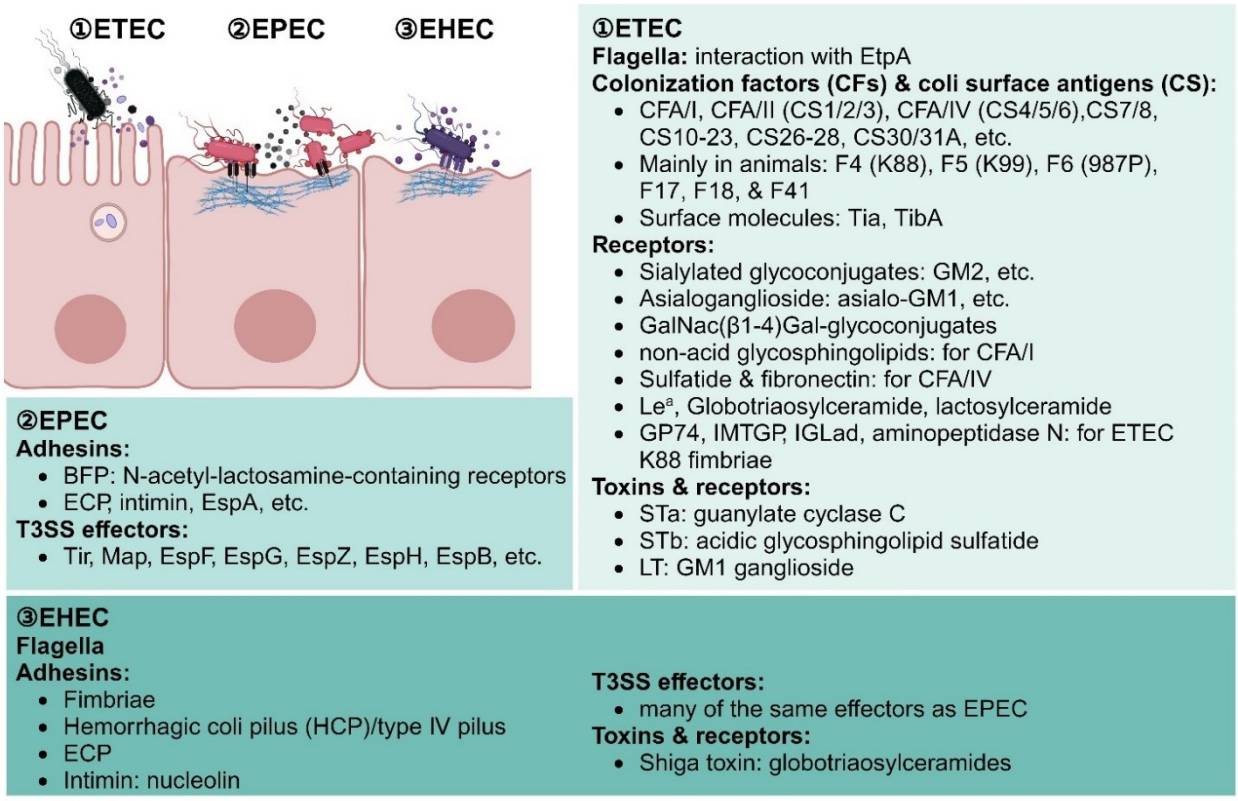

Fig. 2.1 ETEC, EPEC, and EHEC adhesion to intestinal epithelial cells. Adhesion and infection mechanisms are explained. ETEC: enterotoxigenic *Escherichia coli*; EPEC: enterotoxigenic *E. coli*; EHEC: enterohaemorrhagic *E. coli*; EtpA: a high-molecular-weight glycosylated exoprotein produced by ETEC two-partner secretion locus and a conserved adhesin of ETEC; Tia: a 25 kDa outer membrane glycoprotein encoded by the *tia* locus; TibA: a 104-kDa outer membrane glycoprotein encoded by the *tib* locus; GM1/GM2: ganglioside GM1/GM2 (M referring to monosialic); GP74: a 74 kDa transferrin glycoprotein; IMTGP: intestinal mucin-type sialoglycoproteins with a molecular mass of 210 and 240 kDa; IGLad: an intestinal neutral glycosphingolipid; Sta/STb: heat-stable toxin a/b; LT: heat-labile toxin; BFP: bundle-forming pilus; ECP: *E. coli* common pilus; EspA/B/F/G/H/Z: *E. coli* secreted protein A/B/F/G/H/Z; Tir: translocated intimin receptor; Map: mitochondrial-associated protein.

CHAPTER 3 – Ovomucin-Protex 26L Hydrolysate Inhibits Intestinal Integrity Damage in LPS-Treated Caco-2 Cells

3.1 Introduction

The intestinal barrier plays a crucial role in maintaining host health; compromised intestinal integrity contributes to intestinal inflammation, which is widely observed in inflammatory bowel disease (IBD) (Salim & Söderholm, 2011), irritable bowel syndrome (Piche et al., 2009), extraintestinal diseases such as diabetes (De Kort et al., 2011) and metabolic syndrome (Teixeira et al., 2012). Diverse factors like intraluminal antigens, toxins, pathogens, and enteric microorganisms can disturb the normal biological processes of enterocytes and impair the intestinal barrier function, triggering immune responses and causing inflammatory damage (Groschwitz & Hogan, 2009). Notably, inflammation can cause a leaky epithelial barrier, resulting in more antigens exposed to epithelia and submucosa and provoking more serious and persistent inflammatory responses. Various food components, such as proteins and derived bioactive peptides from milk (Tenore et al., 2019), soybean (Ren et al., 2014), proanthocyanidins (Gil-Cardoso et al., 2019), and vitamin A (He et al., 2019) have been studied about their protective effects on intestinal epithelial barrier function.

Ovomucin is a member of mucin family responsible for the viscous property and gel structure of fresh egg white (Kato et al., 1985). It is vital for the resistance of egg white to bacteria since breakdown of the physical structure of egg white and precipitation of ovomucin could promote egg white suitable to bacterial growth (Yadav & Vadehra, 1977). Ovomucin hydrolysates possess various biological activities, including anti-oxidative (Abeyrathne et al., 2016; Chang et al., 2013), anti-tumor (Oguro et al., 2000; Yokota et al., 2000), immunomodulatory (Otani & Maenishi, 1994; Tanizaki et al., 1997), and anti-inflammatory activity (Sun, Chakrabarti, et al., 2016). Our previous

studies showed that ovomucin-protex 26L hydrolysate (OP) could bind to enterotoxigenic *Escherichia coli* fimbriae (Sun et al., 2017) and inhibit the adhesion of the bacterial cells to porcine small intestinal epithelial cells (Sun et al., 2019), indicating the potential use against pathogenic infection. Therefore, the objectives of this study were to investigate the beneficial effects of OP on intestinal barrier integrity and its anti-inflammatory activity in differentiated Caco-2 cells challenged with LPS.

3.2 Materials & methods

3.2.1 Reagents and antibodies

Dulbecco modified Eagle's medium (DMEM), fetal bovine serum (FBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), nonessential amino acids (NEAA), penicillin − streptomycin (10,000 U/mL), and trypsin-EDTA solution (0.25%, phenol red) were obtained from Thermo Fisher Scientific (Burlington, ON, Canada). The cell counting kit-8, formalin solution (neutral buffered, 10%), Triton X 100, 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI), glass coverslips, and 12-well-transwell polyester permeable membrane support (0.4 μm pore size, 12 mm diameter, 1.12 cm^2 grown surface, Costar, Corning, NY) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rabbit monoclonal primary antibodies against claudin-2, claudin-3, occludin, ZO-1, phospho-NF-κB p65 (Ser536), NF-κB p65, phospho-p44/42 MAPK (ERK1/2) (Thr202/Try204), and p44/42 MAPK (ERK1/2) were obtained from Cell Signaling Technology (Whitby, ON, Canada). The goat antirabbit CF^{TM} 488A antibody and LPS from *Escherichia coli* O111:B4 was purchased from Sigma-Aldrich (St. Louis, MO, USA). The goat antirabbit IRDye 680RD secondary antibody and the donkey anti-mouse 800CW secondary antibody were obtained from LI-COR Biosciences (Lincoln, NE, USA).

3.2.2 Preparation of hydrolysate OP

Ovomucin and hydrolysate were prepared according to the previous research (Sun, Gänzle, et al., 2016; Wang & Wu, 2012). In brief, fresh eggs from White Leghorn within 24 h were collected from the Poultry Research Center of the University of Alberta (Edmonton, Canada). Fresh egg white was mixed with 3 times volume of Milli-Q water, and stirred constantly for 2 h before the pH was adjusted to 5.0. Then the slurry was placed at 4 ℃ for 24 h, followed by centrifugation at 15,344g for 10 min at 4 ℃ and lyophilization. The ovomucin hydrolysate was prepared by the enzyme Protex 26L (*Aspergillus niger* origin, Genencor Division of Danisco) for 4 h at 50 ℃ and pH 3.0 in a 1% (ovomucin/Milli-Q water, w/v) slurry. The enzyme was added at the ratio of 2% (enzyme/substate, w/w). The process was conducted using a Titrando (842, Metrohm, Switzerland). The suspension was heated in a water bath at 95 ℃ for 15 min, and was centrifuged at 15,344g for 20 min at 4 ℃ after cooled down to room temperature (RT) on ice. Finally, the supernatant was obtained, lyophilized, and stored at -20 ℃ for future use.

3.2.3 Cell culture

Caco-2 cells (20-35 passage) were cultured in DMEM with 10% FBS, 1% NEAA, and 1% penicillin-streptomycin solution in a humidified incubator under 5% CO_2 atmosphere at 37 °C. The culture medium was replaced every two days. The cells were incubated for 21 days to achieve differentiation for following studies.

3.2.4 Cell treatment

Ovomucin hydrolysate OP was diluted in medium to the final concentration of 0.1, 0.5, and 1.0 mg/mL and incubated with differentiated Caco-2 cells for 24 h. Then samples were collected for different analysis.

3.2.5 Cell viability analysis

Cells were seeded onto 96-well plate (1×10^4 cells/well) and cultured until reaching 80–90% confluence. The effect of OP on the Caco-2 cell viability was determined by the cell counting kit-8 according to the manufacturer's instructions. Briefly, 100 μL working reagent was added to the 96-well plate and incubated with cells for 1 h. The absorbance was measured at 450 nm on a microplate reader (SpectraMax M3, Molecular devices, CA, USA). Results were expressed as the percentage of cell survival (%) with respect to the control group.

3.2.6 Transepithelial electrical resistance (TEER) values and permeability tracer flux assay

Cells were seeded in 12-well transwell chambers (at a density of 5×10^4 cells/well) and cultured for 21 days until differentiation (with a TEER value $\geq 400\ \Omega \cdot cm^2$). OP or/and LPS was added to the upper chamber. TEER values were measured by an ohmmeter (World Precision Instruments, Sarasota, FL, USA). TEER values were shown in the percentage of the reduction of the initial values.

The tracers FITC-dextran of 4 and 40 kDa (Sigma-Aldrich) at the concentration of 100 μg/mL (in PBS) were added to the apical side of the permeable monolayer for 2 h. Samples were collected from the basolateral chamber. The fluorescent intensity was detected by a microplate reader (SpectraMax M3, Molecular devices, CA, USA). The excitation and emission wavelength are 490 and 520 nm, respectively.

3.2.7 Immunofluorescence analysis of tight junction proteins

Cells (2×10^5 cells/well) were grown on glass coverslips in 6-well plates for 21 days and treated with OP for 24 h before LPS (20 μg/mL) challenge for 2 h. After treatment, cells were washed with ice cold PBS for 3 times and fixed with formalin solution at 4 ℃ for 10 min. Then cells were permeabilized with 0.1% Triton X 100 (in PBS) for 10 min, followed by incubation with blocking

buffer (5% BSA in PBS) for 1 h at RT. Then the cells were incubated with anti-claudin2/3, anti-ZO-1, or anti-occludin antibody overnight at 4 ℃ on a slow shaker. Cells were washed with TPBS (0.1% tween 20 in PBS) 3 times, 5 min/time at RT, and incubated with CF^{TM} 488A antibody for 1 h at RT. DAPI was used to counterstain the nuclei. The coverslips were finally mounted in 50% glycerol and fluorescent images were captured using a CSU10 spinning disk confocal microscope (Quorum Technologies, Canada).

3.2.8 Western blot analysis

After treatment, cells grown in 6-well plates were collected using hot Laemmli's buffer with 2% DTT as previously described (Xu et al., 2017). Then the protein samples were applied to SDS-PAGE, transferred to nitrocellulose membranes, and blocked in 5% skim milk powder in PBS for 1 h at RT. The membranes were immunoblotted with different antibodies overnight at 4 ℃. After washing with TPBS 3 times, 5 min/time, membranes were incubated with secondary antibodies for 60 min at RT. The bands were detected with a LI-COR Odyssey Bioimager and quantified by densitometry using Image Studio software (LI-COR Biosciences, Lincoln, NB, USA). GAPDH was used as a reference. Data were presented by the percentage of untreated group.

3.2.9 Endotoxin-neutralizing activity (LAL assay)

Endotoxin neutralization was analyzed using a quantitative chromogenic Limulus amebocyte lysate (LAL) assay kit (Pierce™ LAL Chromogenic Endotoxin Quantitation Kit, Thermofisher, Canada). The assay was conducted according to the manufacture's instruction. Briefly, OP at different concentrations were incubated with LPS [0.5 endotoxin units (EU)/mL] in microtubes at 37 ℃ for 30 min to allow binding. An aliquot of 50 μL of the sample mixture and the standard (0.1-1.0 EU/mL) was dispensed into the prewarmed microplate. An equal volume of LAL reagent containing a chromogenic substrate [butyloxycarbonyl(Boc)-LeuGly-Arg-p-nitroanilide] prepared

from the circulating amebocytes of the horseshoe crab Limulus polyphemus was added into all plate wells followed by incubation at 37 ℃ for 10 min. Then 100 μL of prewarmed chromogenic substrate solution was added to each well for incubation for 6 min. Finally, 100 μL of stop reagent (25% acetic acid) was added and the absorbance at 405 nm was measured on a microplate reader (SpectraMax M3, Molecular devices, CA, USA). Endotoxin-free water was used as a control. The absorbance at 405 nm (A405) of the reaction mixture containing LPS (0.5 EU/mL) was expressed as 100%. The 50% effective (50% neutralizing) concentration (EC50) was calculated using plot od relative absorbances versus log hydrolysate concentrations (Taniguchi et al., 2017).

3.2.10 Statistical analysis

Data are expressed as mean ± standard error of mean (SEM) of 4–6 independent experiments. The data analysis was carried out by the PRISM 6 statistical software (GraphPad Software, San Diego, CA) using one-way analysis of variance with Dunnett's post hoc test. *P* less than 0.05 was considered significant.

3.3 Results

3.3.1 Effect of hydrolysate OP on Caco-2 cell viability

Cell viability was performed to determine the proper concentrations of OP used in the following tests. As shown in Fig. 3.1, OP at the tested concentrations showed no cytotoxicity to Caco-2 cells.

3.3.2 Effect of hydrolysate OP on TEER values

To determine the concentrations and timepoints that LPS could significantly alter the integrity of Caco-2 cell monolayer, LPS ranging from 10 μg/mL to 200 μg/mL was added to the upper chamber of the transwell plates for 2, 4, 6, and 24 h. The most profound reduction in TEER values were found at 6 h (Fig. 3.2A). LPS of 10 μg/mL did not decrease the TEER value when compared with the control group; LPS at concentrations of 20 μg/mL and higher levels significantly decreased

TEER values of Caco-2 cell monolayers. Therefore, LPS at a concentration of 20 μg/mL was applied to stress the cells. Cells pretreated with OP were exposed to LPS for 2, 4, and 6 h to study the protective effects of the hydrolysate against LPS. As shown in Fig. 3.2B-D, OP significantly restored LPS-induced TEER value decrease in differentiated Caco-2 cell monolayers.

3.3.3 Effect of hydrolysate OP on the permeability

The paracellular flux of fluorescent tracers across Caco-2 cell monolayers from the upper chambers to the bottom chambers was measured to evaluate the cell permeability. LPS exposure for 2 h significantly ($p < 0.001$) elevated the paracellular passage of FITC-Dextran (4 and 40 kDa) across the cell monolayers. Pretreatment with OP of different concentrations could significantly inhibit LPS-increased permeability of the monolayers (Fig. 3.3A-B). Together with the TEER value changes, OP showed a protective effect against LPS challenge on intestinal integrity.

3.3.4 Effect of hydrolysate OP on tight junction protein expressions

The expression levels of tight junction proteins were detected by Western blot to investigate the effect of OP against LPS-challenged integrity damage in Caco-2 cell monolayers. OP did not change the abundance of tight junction proteins (Fig. 3.4). The expressions of claudin-2 and claudin-3 were not significantly changed by LPS treatment (Fig. 3.4A-B). As shown in Fig. 3.4C-D, LPS significantly decreased the expression levels of occludin and ZO-1, while pretreatment with OP could prevent the decline ZO-1.

3.3.5 Effect of hydrolysate OP on the cellular distribution of tight junction proteins

The distribution of tight junction proteins was investigated by immunofluorescent staining since their localization besides expression levels was also crucial for the monolayer integrity and barrier function. In control and OP groups, they assembled at the cell membrane and formed a network between adjacent cells. As shown in Fig. 3.5, LPS treatment did not significantly disturb the

distribution and density of claudin-2 and claudin-3 on the cell surface when compared with the control group. However, the density and assembly of occludin and ZO-1 were affected as the network became discontinuous and vague, suggesting these components decreased from the cell-cell junctions. On the other hand, pretreatment with OP could maintain their structures and cell surface localization of occludin and ZO-1 compared with the LPS treatment group.

3.3.6 Hydrolysate OP inhibited LPS-induced activation of NF-κB and MAPK signaling pathways

As NF-κB and MAPK signaling pathways are the two main pathways associated with LPS-induced inflammation and intestinal dysfunction, effects of OP on LPS-activated NF-κB and MAPK signaling pathways were investigated. LPS treatment significantly induced the degradation of IκBα (Fig. 3.6A), provoked the phosphorylation of NF-κB (Fig. 3.6B) and promoted the translocation of phospho-NF-κB p65 to the nuclei (Fig. 3.6C). Pretreatment with OP could significantly prevent LPS-induced activation of NF-κB pathways. In addition, significant activation of ERK1/2 and p-38, members of MAPKs, were also detected in the LPS group, while pretreatment with OP inhibited the LPS-induced phosphorylation of ERK1/2 and p-38 (Fig. 3.6D-E). These results demonstrated the inhibitory effects of OP on LPS-induced activation of NF-κB and MAPK pathways.

3.3.7 LPS-neutralizing activity of hydrolysate OP

OP inhibited endotoxic activity of LPS in a concentration-dependent manner (Fig. 3.7). Moreover, the EC50 value was determined to be 0.74 mg/mL using plots of relative absorbances versus log peptide concentrations.

3.4 Discussion

Ovomucin is a mucin-like glycoprotein in egg white with extremely high molecular weight. It plays a key role in maintaining the structure of egg white and protects the embryo from microbial infection. Recent research progress consistently suggests that ovomucin and its hydrolysates possess various bioactive functions, including anti-viral, anti-bacterial, anti-tumor, anti-oxidative, and immunomodulatory functions (Tu et al., 2020). Besides, our previous research revealed that the hydrolysate OP could effectively inhibit the binding of microbial cells to porcine small intestinal epithelial cells (Sun et al., 2019). The latest review also indicated a possible role of ovomucin on intestinal health (Tu et al., 2020). Among the antigens that could impair the intestinal barrier architecture, LPS is the main component of Gram-negative bacteria's outer membrane and has been shown to increase intestinal permeability and trigger inflammatory responses (Im et al., 2012; Nighot et al., 2017). Here, the *in vitro* LPS-challenged Caco-2 cell model was used to study the protective effects of ovomucin-protex 26L hydrolysate on intestinal barrier function, trying to understand ovomucin's potential for intestinal health. The major finding of the present study is that OP restores LPS-induced TJ protein disruption and mitigates inflammatory response through neutralizing the endotoxic activity of LPS and inhibiting the activation of NF-κB and MAPK pathways in Caco-2 cells. These results suggest the potential application of OP as an anti-inflammatory substance to protect intestinal barrier function and prevent inflammation in gut diseases such as IBD.

Tight junction proteins are composed of the transmembrane strands (mainly the junctional protein occludin and the tetraspan proteins of the claudin family) and cytoplasmic scaffolding proteins (typical zonula occludens 1, ZO-1). These proteins are tightly associated and form the selective gates and diffusion barrier, sealing the paracellular space between epithelial cells and

playing a key role in maintaining the intestinal barrier function (Turner, 2009). Defects in the intestinal TJ barrier are observed in IBD patients and increased TJ permeability can accelerate disease development and severity (Kang et al., 2015). In the present study, LPS reduced the expression levels of occludin and ZO-1, meanwhile, vague and discontinuous protein strands were observed, which are consistent with previous studies (Park et al., 2010; Wu et al., 2018; Zhang et al., 2017). Decreased expression and destroyed distribution patterns of these two proteins were also commonly determined in IBD patients (Das et al., 2012; Zeissig et al., 2007). The expression of claudin-3 was reduced with redistributed pattern in IBD patients (Prasad et al., 2005). However, some results showed unchanged expression in active ulcerative colitis (Oshima et al., 2008). In our study, LPS treatment slightly decreased claudin-3 abundance and caused much less disturbance to its cellular localization in Caco-2 cells. The previous study (Ling et al., 2016) demonstrated significantly downregulated expression and disordered structure of claudin-3 after LPS treatment for 24 h, which is much longer than the time period used in the present study. Increased abundance and redistribution of claudin-2 were found in IBD and necrotizing enterocolitis (Ares et al., 2019; Oshima et al., 2008; Zeissig et al., 2007). Here we did not observe significant changes in claudin-2 expression and distribution after LPS stimulation, while claudin-2 expression was upregulated by 24 h treatment of LPS elsewhere (Feng et al., 2018). Similar to claudin-3, we considered this was due to a relatively shorter duration of the LPS challenge. TEER and paracellular tracer transport experiments are used to identify a compromised intestinal barrier function (Jimison et al., 2012; Sun et al., 2012). Our data showed that pretreatment with OP could restore LPS-induced intestinal permeability elevation and regulate TJ protein expression.

Through binding to toll-like receptors (TLRs), LPS can activate downstream nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathways, promoting the

expression of proinflammatory cytokines (Akira et al., 2006). In brief, cytoplasmic IκB is phosphorylated and then degraded by the proteasome, leading to the liberation of NF-κB, which translocates into the nucleus and initiates the transcription of proinflammatory cytokine genes. MAPK signal transduction pathways are highly conserved stress-activated mechanisms in eukaryotic cells in response to diverse stimuli such as environmental stresses and mediate various cellular biological processes, including immunity and inflammation. MAPK core signaling modules contains different individual elements that participate promiscuously in several pathways, among which the extracellular signal-regulated kinases 1/2 (ERK1/2) and p38 are well-demonstrated to be recruited and activated along with the NF-κB pathway by stresses and inflammatory stimuli (Akira et al., 2006; Kyriakis & Avruch, 2012). Data from our lab demonstrated that different ovomucin hydrolysates by enzymes of diverse origins could serve as the receptor of pathogenic fimbriae and block the attachment of pathogen cells to porcine small intestinal epithelial cells (Sun et al., 2019; Sun et al., 2017). In these studies, enterotoxigenic *Escherichia coli* K88 was used, which is the primary cause of piglet diarrhea and can trigger intestinal inflammation and dysfunction (Wan et al., 2019; Wu et al., 2016). These findings indicate the potential immunoregulatory effects of ovomucin hydrolysates. Here, the NF-κB and MAPK signaling pathways were detected to investigate the anti-inflammatory effect of OP and illuminate the mechanisms in modulating the intestinal barrier function. The results showed that LPS-induced IκB degradation was alleviated by OP. The activation and translocation of NF-κB were also reversed by OP treatment. Moreover, OP repressed the activation of MAPK ERK 1/2 and p38 molecules. To better understand the molecular mechanisms of OP alleviating LPS toxicity, its endotoxin-neutralizing activity was determined by LAL assay. The EC50 of OP was calculated to be 0.74 mg/mL, indicating that OP components could bind to LPS and thereby promote the LPS

detoxification. Peptides derived from rice and soybean have been reported with LPS-neutralizing activity (Taniguchi et al., 2017; Taniguchi et al., 2019). This study is the first to describe that the hydrolysate OP shows LPS-neutralizing activity. As described in the previous research, OP contains the amounts of essential amino acids but shows high hydrophobicity. Additionally, OP comprises protein fragments with a molecular weight of less than 12.4 kDa (Sun, Gänzle, et al., 2016) and glycopeptides contained in OP are crucial for its anti-adhesive activity (Sun et al., 2019). Thus, enzymatic treatment of ovomucin is likely to induce the release of bioactive peptides. Further study is needed to identify the responsible peptides in the hydrolysate and validate their potential.

In conclusion, our current study revealed that OP showed LPS-neutralizing activity and reduced the TJ permeability, and that it mitigated inflammatory response induced by LPS likely via inhibiting the activation of the NF-κB and MAPK signaling pathways in Caco-2 cells. These data indicate the possible application of the hydrolysate OP as an intervention strategy to prevent inflammation in pathogenic infection and IBD development. Further experiments in animal models of pathogenic infection and IBD are necessary to advance our understanding of the activities of ovomucin hydrolysates and support the potential application.

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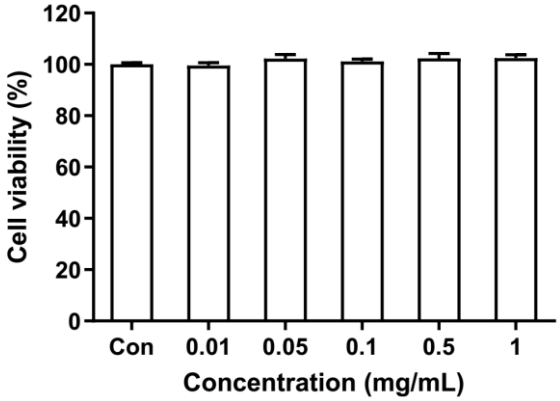

Fig. 3.1 Cell viability at different concentrations of OP. Caco-2 cells were treated with ovomucin-protex 26L hydrolysate for 24 h. Cell viability was detected by the Enhanced Cell Counting-8 kit following the manufacturer's instruction. The data are expressed as mean ± SEM of six independent experiments. Con means control group.

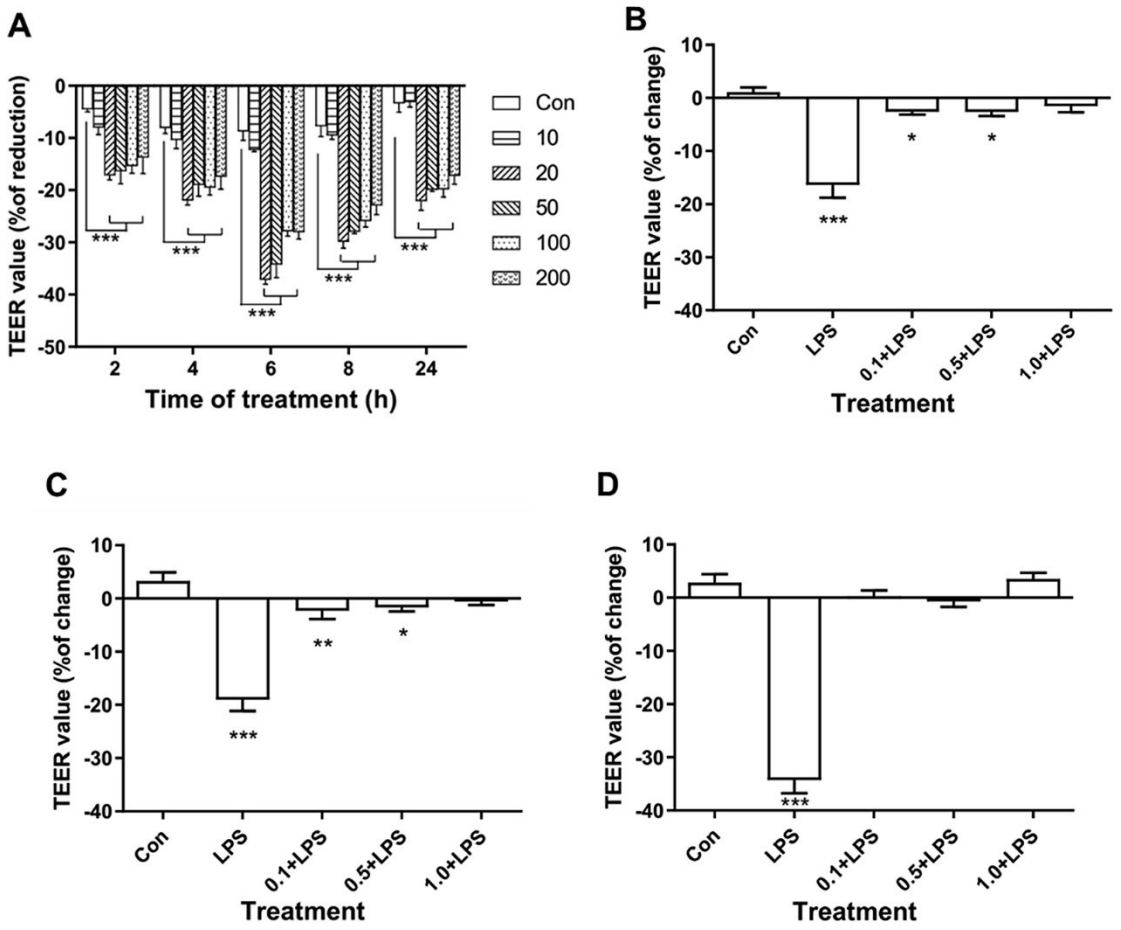

Fig. 3.2 Effect of LPS and OP on the trans-epithelial electrical resistance (TEER) values of differentiated Caco-2 cell monolayers. Different shapes of bars represent different concentrations of LPS from 10 μg/mL to 200 μg/mL as indicated in the Panel A. Cells on transwell plates were pretreated with ovomucin-protex 26L hydrolysate of 0.1, 0.5, and 1 mg/mL for 24 h and then exposed to LPS (20 μg/mL) for 2 (B), 4 (C), and 6 (D) h. The TEER values were measured by a voltmeter (Millicell-ERS; Millipore, MA, USA). Data are expressed in the percentage of the reduction of the initial values from four independent experiments. Con means the control group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, significantly different from the control group.

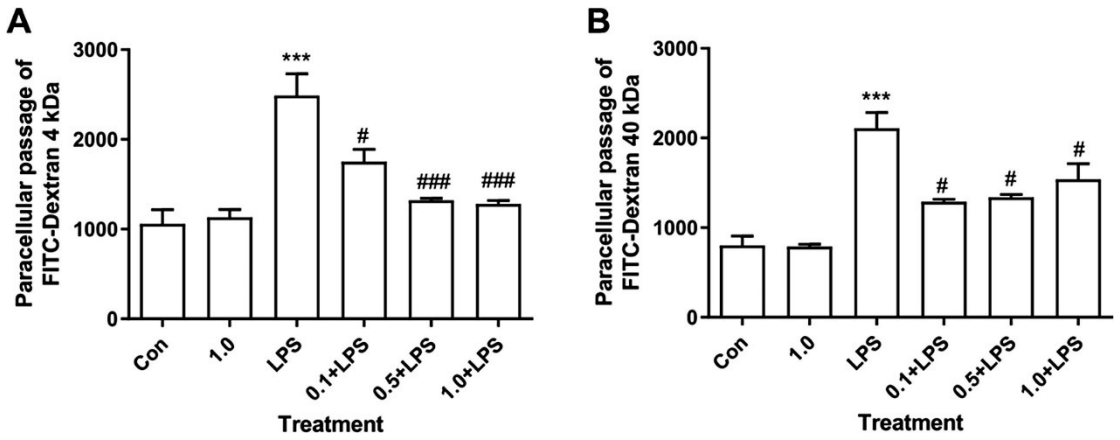

Fig. 3.3 Paracellular passage changes of FITC-Dextran 4 (A) and 40 kDa (B) after differentiated Caco-2 cell monolayers were treated with OP (0.1, 0.5 and 1.0 mg/mL) for 24 h and then LPS (20 μg/mL) for 2 h. The results are representative of four independent experiments expressed as mean ± SEM. Con means control group. ***$P < .001$, significantly different from the control group. #$P < 0.05$, ###$P < 0.001$, significantly different from the LPS group.

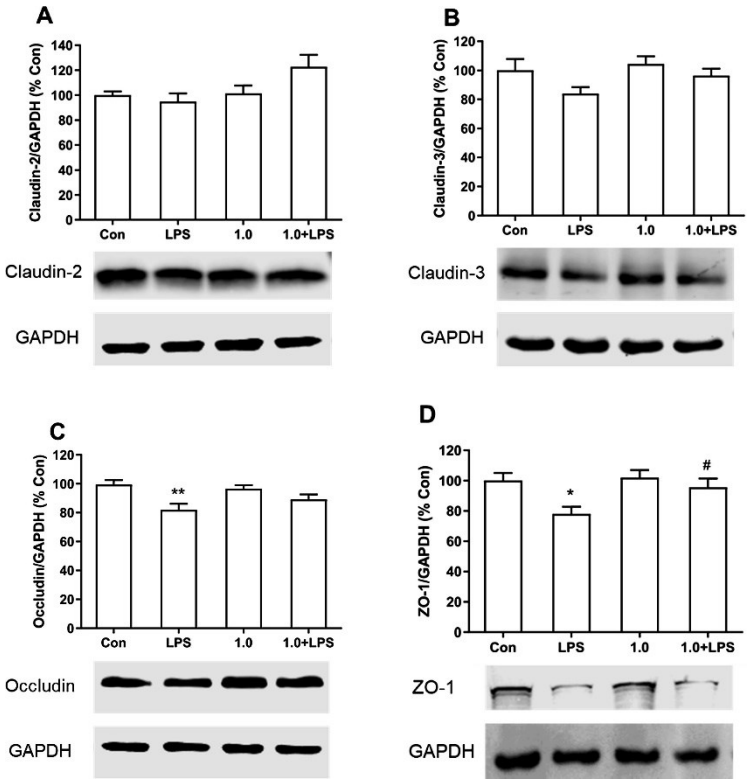

Fig. 3.4 Abundance changes of tight junction proteins in differentiated Caco-2 cells treated with OP (1.0 mg/mL) for 24 h and LPS (20 μg/mL) for 2 h. Whole cell lysates were used for Western blot analysis of claudin-2 (A), claudin-3 (B), occludin (C), and ZO-1 (D), respectively. Band densities were quantified using Image Studio software (LI-COR Biosciences). The data are expressed as mean ± SEM of 4 independent experiments. Con means control group. $*P < 0.05$, $**P < 0.01$, significantly different from the control group. $\#P < 0.05$, significantly different from the LPS group.

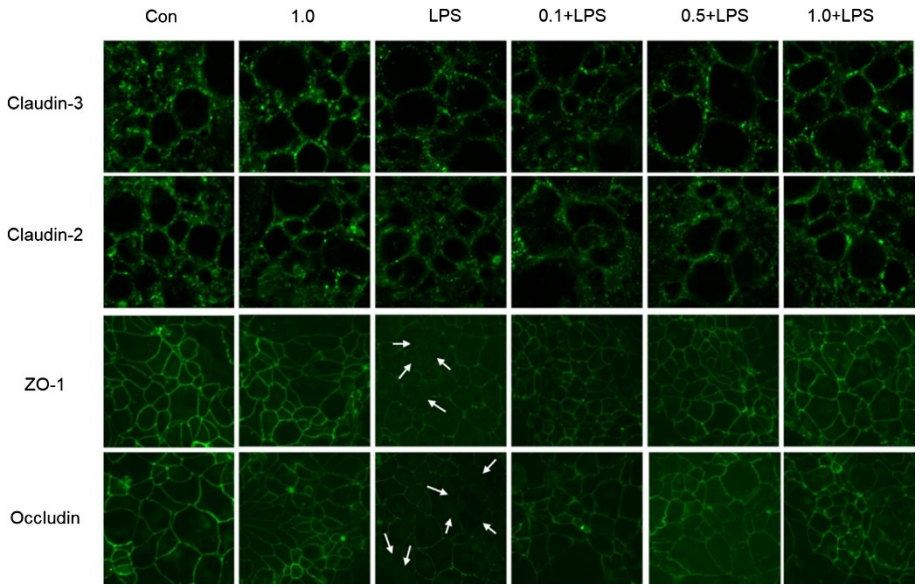

Fig. 3.5 Cellular distribution of tight junction proteins. Caco-2 cells were cultured on the coverslips for 21 d to undergo differentiation and treated with ovomucin-protex 26L hydrolysate for 24 h followed by LPS (20 μg/mL) for 2 h. After treatment, cells were fixed and stained with primary anti-claudin-2/3, anti-occludin, and anti-ZO-1 antibodies at 4 ℃ overnight. Anti-rabbit CF^{TM} 488A antibody (Sigma) was used to visualize the localization of tight junction proteins by a CSU10 spinning disk confocal microscope (Quorum Technologies, Canada). Con means the control group.

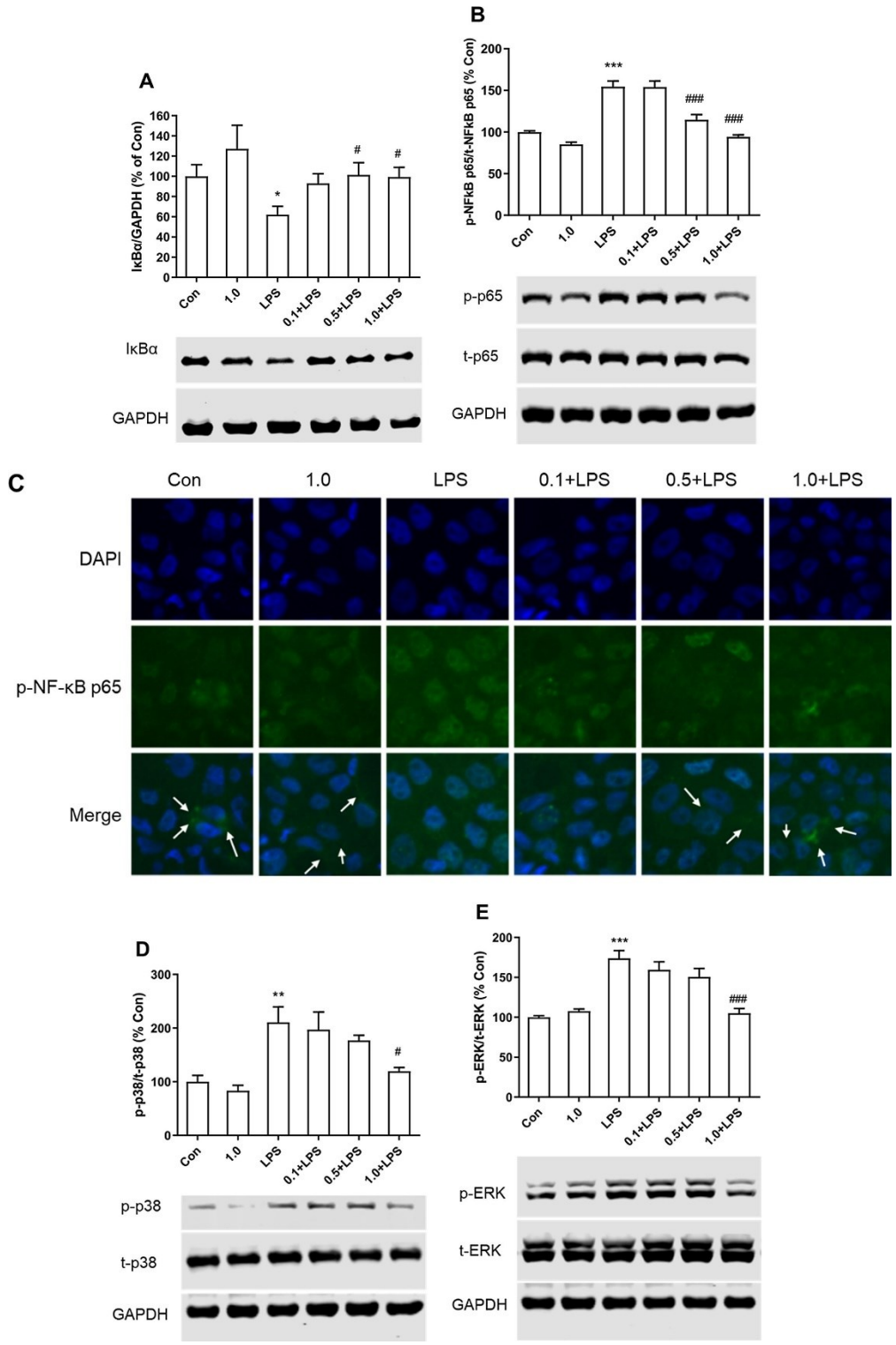

Fig. 3.6 Effect of OP on LPS-activated NF-κB and MAPK signaling pathways. Differentiated Caco-2 cells were pretreated with the hydrolysate (0.1, 0.5, and 1.0 mg/mL) for 24 h followed by

LPS treatment (20 μg/mL) for 2 h. Whole cell lysate samples were collected for Western blot analysis of IκBα (A), p-NF-κB p65 (Ser536)/NF-κB p65 (B), p-p38 (Thr180/Tyr182)/p38 (D), p-ERK1/2 (Thr 202/Tyr 204)/ERK1/2 (E). The cellular distribution of p-NF-κB p65 was studied by a CSU10 spinning disk confocal microscope (Quorum Technologies, Canada) (C). The data are expressed as mean ± SEM of 4–6 independent experiments. Con means the control group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significantly different from the control group. #$P < 0.05$, ###$P < 0.0001$, significantly different from the LPS group.

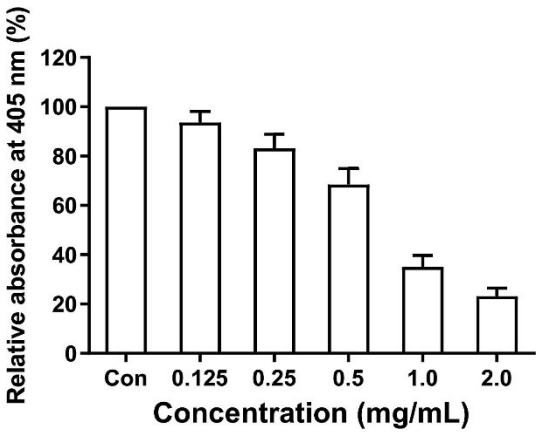

Fig. 3.7 Evaluation of LPS-neutralizing ability of OP by a quantitative chromogenic LAL assay. The hydrolysate at different concentrations was incubated with LPS (0.5 EU/mL) at 37 ℃ for 30 min to allow the binding to LPS. Absorbance was measured at 405 nm. The A_{405} in the reaction mixture without hydrolysate was expressed as 100% and the relative absorbance was indicated as the percentage by the ratio of A_{405} of the reaction mixture containing hydrolysate to that (100%) of the reaction mixture without hydrolysate. The data are expressed as mean ± SEM of three independent experiments.

CHAPTER 4 – Ovomucin Hydrolysates Reduce Bacterial Adhesion and Inflammation in Enterotoxigenic *Escherichia coli* (ETEC) K88-Challenged Intestinal Epithelial Cells

4.1 Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is the leading cause of diarrhea in children and travelers (Gupta et al., 2008; Qadri et al., 2005). It is also the most common cause of diarrhea in neonatal and postweaning pigs, which leads to economic losses in the swine industry (Dubreuil et al., 2016; Sjölund et al., 2014). ETEC expresses diverse virulence factors mediating its adhesion to the microvilli of small intestinal epithelial cells and initiating infections, with the K88 fimbriae (also termed F4) being the most prevalent one (Luppi et al., 2016). K88 receptors, such as the 74-kDa transferrin glycoprotein, the 210-kDa and 240-kDa intestinal mucin-type glycoprotein, aminopeptidase N, and the intestinal neutral glycosphingolipid, have been identified in intestinal brush border and mucus preparations (Xia et al., 2015). After binding to the receptors, ETEC produces two main enterotoxins, heat-stable toxins (STa, STb) and heat-labile toxin, which change the absorption of intestinal epithelium and increase the secretion of fluid and electrolytes, resulting in watery diarrhea (Dubreuil et al., 2016). ETEC K88 could also impair the intestinal integrity (Roselli et al., 2007; Yu et al., 2015), modulate immune and inflammation responses via toll-like receptors (TLRs)/nuclear factor κB (NF-κB)/mitogen-activated protein kinase (MAPK) pathways (Wan et al., 2019; Yu et al., 2015), and induce the disruption of intestinal histology and dysbiosis of gut microbiota (Wang et al., 2018). Antimicrobials are the most commonly prescribed drugs in the food-producing animal industry; in the swine industry, over 80% of antimicrobial substances are administered between birth and 10 weeks of age for disease management, including severe diarrhea in neonatal and weaned piglets that is mainly caused by ETEC (Lekagul et al., 2019). With

the increasing concern about antimicrobial resistance, reducing antimicrobial use in the agricultural sector and developing effective antimicrobial alternatives are crucial for combating the antimicrobial resistance crisis.

Given that bacterial adhesion to the host cell surface is the initial step of pathogenesis, anti-adhesive therapy provides a promising strategy to interfere with the interactions of microbes and toxins with host cells, inhibiting pathogenic colonization and infection (Shoaf-Sweeney & Hutkins, 2008). Various dietary components act as decoy receptors for pathogens and are considered safe to inhibit the development of multi-resistant bacteria (Lane et al., 2010; Sun & Wu, 2017). For example, peptide fractions from wheat bran (Gonzalez-Ortiz et al., 2014) and fermented soy bean extracts (Roubos-van den Hil et al., 2009) inhibited ETEC K88 binding to intestinal epithelial cells. Feed ingredients, including microbial exopolysaccharides (Chen et al., 2014; Wang et al., 2010; Yang et al., 2015) and casein macropeptide released during cheese making, were reported to reduce ETEC binding to the intestinal mucosa of piglets (González-Ortiz et al., 2014; Hermes et al., 2013). These studies indicated the potential of food-derived glycoconjugates as anti-adhesive substances and antimicrobial alternatives to prevent pathogenic colonization and infection.

Ovomucin, a glycoprotein in chicken egg white, contains ~33% carbohydrate and 2.6-8% sialic acid (Mine, 1995). It is vital for the resistance of egg white to bacteria since the breakdown of the physical structure of egg white and precipitation of ovomucin renders egg white suitable for bacterial growth (Yadav & Vadehra, 1977). Ovomucin and its hydrolysates possess various biological activities, including anti-oxidative and immunomodulatory activities (Omana et al., 2010). In addition to interacting with various viruses, ovomucin hydrolysates generated by Pronase digestion was able to bind to *E. coli* O157:H7, with sialic acids in the hydrolysate playing a crucial role in mediating the interaction (Kobayashi et al., 2004). Ovomucin hydrolysates inhibited ETEC

K88-induced hemagglutination (Sun et al., 2017), among which two hydrolysates (OP, prepared by enzyme protex 26L, and OPP, prepared by pepsin and pancreatin) were subsequently shown to decrease ETEC K88 adhesion to porcine small intestinal epithelial cells by serving as decoy receptors for ETEC fimbriae K88 (Sun et al., 2019). It remains unknown, however, whether ovomucin hydrolysates additionally exert protective effects that are unrelated to binding to the lectin domain of K88 fimbriae. Thus, the aim of this study was to further investigate the effects of these two ovomucin hydrolysates on ETEC K88-induced epithelial cell integrity damage and inflammation. Ovomucin hydrolysates treated with sialidase were used to determine mechanisms in addition to inhibiting pathogenic adhesion, in porcine intestinal epithelial (IPEC-J2) and human Caco-2 cells.

4.2 Materials & methods

4.2.1 Materials and reagents

Dulbecco modifed Eagle's medium (DMEM)/ Ham's F-12 (1:1), fetal bovine serum (FBS, Sigma F1051), penicillin–streptomycin, insulin–transferrin–selenium (ITS), epidermal growth factor (EGF), 0.25% trypsin-EDTA, CO_2-independent medium, and Luria–Bertani medium were purchased from Thermo Fisher Scientific Inc. (Ottawa, ON, Canada). The neuraminidase (NANase, *Clostridium perfringens* origin), N-acetylneuraminic acid (sialic acid, Neu5Ac), formalin solution (10%), Triton X-100, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), glycerol, glass coverslips, 24-well transwell plates (6.5 mm diameter with 0.4 μm pore polyester membrane inserts), dithiothreitol (DTT), bovine serum albumin (BSA), and the goat antirabbit CF 594 antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). Minca agar (modified) and Minca supplements were bought from Sifin Diagnostics GmbH (Berlin, Germany). Rabbit monoclonal primary antibodies against occludin, claudin-3, and ZO-1 were obtained from Abcam

(Toronto, ON, Canada). Rabbit monoclonal primary antibodies against phospho-NF-κB p65 (Ser 536), phospho-ERK1/2 (Thr 202/Tyr 204), p38 MAPK, and GAPDH, and mouse monoclonal primary antibodies against NF-κB p65, ERK1/2, and phospho-p38 MAPK (Thr180/Tyr182) were purchased from Cell Signaling Technology (Whitby, ON, Canada). The donkey antimouse 800CW secondary antibody and the goat antirabbit IRDye 680RD secondary antibody were purchased from LI-COR Biosciences (Lincoln, NE, USA). Fluo-4 AM and Pluronic F-127 were obtained from Invitrogen (Burlington, ON, Canada). Porcine calcium/calmodulin-dependent protein kinase 2 (CaMK II) ELISA kit (ABIN778503) was purchased from Antibodies-online Inc. (Limerick, PA, USA). Dihydroethidium (DHE) was purchased from Molecular Probes (Eugene, OR).

4.2.2 Bacterial strains and culture

The porcine K88ac ETEC strain ECL13795 (O149; virotype STb:LT:EAST1:F4) was kindly provided by the *Escherichia coli* Laboratory at the University of Montreal (Montreal, Quebec, Canada). It was revived on LB agar plate and single colony was selected for culture in 10 mL LB broth for 16 h. Then 200 μL of cultures was spread on Minca agar and cultivated aerobically at 37 °C overnight as previously described (Chen et al., 2014; Sun et al., 2019). Cell suspensions collected in phosphate-buffered saline (PBS, pH 7.2) were quantified based on the optical density of 1.2 at 600 nm corresponding to a cell count of 10^9 CFU/mL and subsequently diluted to corresponding concentrations used in the following tests.

4.2.3 Preparation of ovomucin and ovomucin hydrolysates

Ovomucin (OVM), OP, and OPP were prepared according to the established protocols (Sun et al., 2016; Wang & Wu, 2012). Briefly, egg white was mixed thoroughly with three times volume of Milli-Q water for 2 h and adjusted to pH 5.0 by HCl. After keeping at 4 °C for 24 h, the slurry was centrifuged at 15,344*g* for 10 min at 4 °C (Beckman Coulter, Rotor JA10, USA) to obtain the

precipitate, which was lyophilized and stored at -20 °C. Ovomucin slurry (1%, W/V, OVM/Milli-Q water) was adjusted to their respective conditions: enzyme protex 26L (pH 3.0 at 50 °C for 4 h; *Aspergillus niger* origin, Genencor Division of Danisc) and pepsin/pancreatin (pH 2.0 for 3h followed by pH 6.2 for 3 h at 37 °C; porcine pancreas and gastric origin, Sigma-Aldrich). Enzymes were added at the level of 2% (enzyme/substrate, w/w). The hydrolysis procedures were conducted on a Titrando (842, Metrohm, Herisan, Switzerland) connected with a circulating water bath to ensure a constant pH and temperature. The suspension was heated at 95 °C for 15 min, followed by centrifugation at 15,344*g* at 4 °C for 20 min. Finally, the supernatant was lyophilized and stored at -20 °C until further use.

4.2.4 Adhesion test

Procedures were adapted from the methods described by González-Ortiz et al. (González-Ortiz et al., 2014). OVM, OP, OPP, and casein macropeptide (CMP, Arla Foods Ingredients, Inc., Aarhus, Denmark) were suspended in PBS (1%, w/v). Then 300 μL of the solutions was added to 96-well microplates. After overnight incubation at 4 °C, the plates were gently washed with sterile PBS to remove nonbinding material and blocked with 1% BSA at 4 °C for 1 h. Then 300 μL of ETEC K88 suspensions (1×10^9 CFU/mL) was added for incubation at room temperature for 30 min. Nonadherent bacterial cells were removed by sterile PBS wash. Finally, 200 μL sterile PBS was added to resuspend the adherent bacteria and counted on LB agar. In the desialydation process, materials coated onto the bottom of microplates were incubated with NANase (1 mU/μg glycoprotein) in 50 mM sodium acetate buffer (pH 5.0) containing 150 mM NaCl at 37 °C for 24 h before adding ETEC K88 suspensions (Parker et al., 2010).

4.2.5 Cell culture

IPEC-J2 cells (North Carolina State University, Raleigh, North Carolina, USA) were cultured in DMEM/Ham's F-12 (1:1) medium supplemented with 5% of FBS, 1% of penicillin–streptomycin, 1% of ITS, and 5 ng/mL of EGF and maintained in an atmosphere of 5% of CO_2 at 37 °C as previously described (Sun et al., 2019). The culture medium was replaced every other day. Cells were grown for 14 days to allow differentiation (Vergauwen, 2015).

Caco-2 cells were grown in DMEM supplemented with 10% of FBS, 1% of NEAA, and 1% of penicillin–streptomycin in the humidified incubator at 37 °C under 5% of CO_2 atmosphere. Cells were cultured for 21 days to achieve differentiation (Bao & Wu, 2021).

4.2.6 ETEC binding to cell surfaces

The test was conducted according to the previous description with some modifications (Hermes et al., 2011). After differentiation on 48-well plates, cells were incubated with 400 μL of preincubated material–bacteria mixture for 30 min at RT in the CO_2-independent medium. For sialic acid (Neu5Ac) incubation with bacteria, 25 mM Neu5Ac in 10 mM Tris-HCl buffer (pH 6.9) was mixed with bacteria at 37 °C for 30 min (Sakarya et al., 2010; Salcedo et al., 2013). After coincubation at 37 °C for 30 min, cells were washed by sterile PBS three times to exclude nonadherent bacteria and lysed by 400 μL of 0.1% Triton X-100 at RT for 30 min. Serial dilutions of the adherent bacteria were counted on LB agar. To determine the role of epithelial surface Neu5Ac in ETEC K88 adhesion, cells were pretreated with 0.1 U/mL of NANase in 50 mM sodium acetate buffer (pH 6.9) containing 150 mM NaCl for 4 h at 37 °C or media alone before incubation with bacterial suspension or the material–bacteria mixtures. NANase was heated at 100 °C for 1 h for inactivation (Redondo et al., 2004; Sakarya et al., 2010).

4.2.7 Detection of transepithelial electrical resistance (TEER) values

Cells were grown on 24-well transwell plates to allow differentiation. OP and OPP (0.1%, 0.5%, and 1.0%) were preincubated with ETEC K88 at RT for 30 min in the CO_2-independent medium. Then 100 μL of mixtures was added to the upper chambers. TEER values were determined by an ohmmeter (World Precision Instruments, Sarasota, FL, USA) at different time points.

4.2.8 Western blot analysis

Differentiated cells were stressed with hydrolysate–bacteria mixture in the CO_2-independent medium. Ovomucin hydrolysates (0.1%, 0.5%, and 1.0%) were incubated with ETEC K88 at RT for 30 min. After treatment, cell lysates were collected in heated Laemmli's buffer containing 2% DTT as described (Bao & Wu, 2021). Cell lysate samples were run in SDS–PAGE, transferred to nitrocellulose membranes, blocked in 5% skim milk solution, and immunoblotted with different antibodies of interest overnight at 4 °C. After incubation with secondary antibodies for 60 min at RT, the bands were visualized with a LI-COR Odyssey Bioimager and quantified by the Image Studio software (LI-COR Biosciences, Lincoln, NB, USA). GAPDH was used as the reference. Data were presented as the percentage of untreated samples.

4.2.9 Immunofluorescence analysis of tight junction proteins

Differentiated Caco-2 cells grown on glass coverslips in 6-well plates were treated with bacteria, 1.0% ovomucin hydrolysate, or the preincubation of 1.0% ovomucin hydrolysate and ETEC K88 (1×10^8 CFU/mL) in the CO_2-independent medium. After treatment at 37 °C for 2 h, cells were fixed with formalin at 4 °C for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Subsequently, cells were blocked in 5% BSA at RT for 1 h and incubated with primary anti-claudin3/occludin/ZO-1 antibody overnight at 4 °C. The goat antirabbit CF 594 antibody in TPBS (0.1% Tween 20 in PBS) was added for incubation at RT for 1 h. Finally, the coverslips were

mounted on 50% glycerol in H_2O. The images were captured by a confocal laser scanning microscope (Olympus FV3000) under the 10× objective lens.

4.2.10 Lipopolysaccharide (LPS) treatment

IPEC-J2 cell were treated with 10 μg/mL of LPS (*E. coli* O111:B4 origin, Sigma-Aldrich) for 15 min to 4 h. Then whole cell lysate samples were collected to determine the activation of MAPK p38 and NF-κB p65 molecules. To test the effects of ovomucin hydrolysate samples against LPS stress, IPEC-J2 cells were preincubated with OP or OPP (1%) for 24 h before being exposed to LPS.

4.2.11 Calcium staining

After treatment, intracellular calcium signals were visualized by the Fluo-4 AM dye with adaptations (Spinelli & Gillespie, 2012). In brief, Fluo-4 AM dye (10 mM in DMSO) was thoroughly mixed with the same volume of Pluronic F-127 (20% solution in DMSO) by rigorous vortex and bath sonication. Then the dye was diluted by HEPES (final 10 μM) and added to cells for incubation at 37 °C for 60 min in dark. After three times of HEPES wash, images were captured using an Olympus IX81 fluorescent microscope (Olympus, Tokyo, Japan) under the FITC channel. ImageJ software (https://imagej.net/Welcome) was used to determine the fluorescence density. Data were expressed as the fold change in the mean fluorescence density per cell over the control group.

4.2.12 CaMK II activity assay

CaMK II activity in IPEC-J2 cells was determined according to the manufacture's instruction. Briefly, after treatment IPEC-J2 cells grown in 6-well plates were detached by trypsin and collected by centrifugation. Then cells in each well were washed with PBS three times and finally resuspended in 100 μL of sterile PBS, followed by three cycles of freezing at −20 °C and thawing

by gentle vortex. Cell debris was removed by centrifugation at 1000 *g* for 15 min at 4 °C. Then 100 μL of cell lysate samples was dispensed onto the coated wells and mixed with 10 μL of the balance solution included in the kit. After adding 50 μL of conjugate and mixing by pipetting, the plate was incubated at 37 °C for 1 h followed by five times of manual wash using the 1× wash solution. Finally, 50 μL of substrates A and B were added subsequently to allow 20 min incubation at 37 °C in dark. After adding 50 μL of the stop solution and mixing by pipetting, the optical density at 450 nm was determined immediately by a microplate reader (SpectraMax M3, Molecular devices, CA, USA).

4.2.13 DHE staining

Superoxide generation in IPEC-J2 cells was determined by the DHE staining method (Fan et al., 2022). Briefly, after being exposed to ETEC K88 or the preincubation of bacteria–hydrolysate for 30 min, IPEC-J2 cells were gently washed with sterile PBS for 3 times and incubated with DHE (20 μM in CO_2-independent medium, protected from light) at 37 °C for 30 min. Cells were then washed with nonphenol-red DMEM (Thermo Fisher Scientific, Burlington, Canada) for three times, followed by imaging using an Olympus IX81 fluorescent microscope (Olympus, Tokyo, Japan) under a 10× objective lens and under the TRITC channel. For each group, images were randomly captured from more than nine fields. The fluorescence density was quantified by ImageJ (https://imagej.net/Welcome) and expressed by the mean fluorescence density per field of view (integrated density/area). Superoxide generation was measured as the fold change over the untreated control group.

4.2.14 Statistical analysis

All data are expressed as mean ± standard error of mean (SEM) of 4–6 independent experiments. The PRISM 6 statistical software (GraphPad Software, San Diego, CA) was used for the analyses

using one-way analysis of variance with Bonferroni *post hoc* test. $P < 0.05$ was considered statistically significant.

4.3 Results

4.3.1 Ovomucin hydrolysates inhibited ETEC K88 adhesion to intestinal epithelial cells

Intact ovomucin (OVM) coated onto the bottom of 96-well plate exhibited the highest adhesion to ETEC K88 (load of 3×10^8 CFUs/well), and removing sialic acids significantly decreased its interaction with the bacterial cells (Table 4.1). OP and OPP both interacted with ETEC K88. Removal of the terminal sialic acid residues by NANase significantly reduced ETEC K88 interaction with OVM and OP, but did not significantly interfere with bacterial adhesion to OPP (Table 4.1). At higher concentrations, OP (2.5% and 5.0%) and OPP (5%) significantly inhibited bacterial adhesion to IPEC-J2 cell surfaces (Table 4.2). Sialic acids on the glycan structures attached to ovomucin peptide backbones contributed partially to their anti-adhesive properties because desialydation by NANase impaired their anti-adhesive capacities ($P = 0.0306$ and 0.0466 by *t* test, respectively, Table 4.2). However, incubation of free sialic acids with ETEC K88 did not impact bacterial adhesion to IPEC-J2 cells.

In Caco-2 cells (Table S4.1), removing sialic acid residues on cell surface glycoconjugates reduced the number of adherent bacteria, in line with that in IPEC-J2 cells (Table 4.2). Free sialic acids decreased ETEC K88 adhesion to Caco-2 cell surface. OP and OPP at higher concentrations also significantly suppressed ETEC K88 adhesion to the surface of differentiated Caco-2 cells. However, removing sialic acid residues in OPP did not influence its anti-adhesive effect, indicating ETEC K88 adhesion to Caco-2 cell surface through sialic acid-independent mechanisms. This discrepancy suggested the species-specificity and differences of receptor/glycan-recognition patterns of ETEC K88 in IPEC-J2 and Caco-2 cells.

4.3.2 Ovomucin hydrolysates restored tight junction integrity in Caco-2 Cells

ETEC K88 (1×10^7 CFU/mL) treatment significantly decreased the expression levels of tight junction protein occludin and claudin-3 at different time points (Fig. S1). However, preincubation of OP or OPP (1% of concentration) with bacteria did not restore the abundance of claudin-3 and occludin in IPEC-J2 cells after 1 h (Fig. 1A & B) or 2 h challenge (Fig. 1C & D). ETEC K88 challenge for 1 h decreased ($P < 0.05$) TEER values, while OP preincubation could significantly protect the epithelial cell integrity against ETEC K88 (Fig. 1E). However, OPP preincubation did not show significant protection at different time points.

In Caco-2 cells, ETEC K88 challenge for 2 h significantly downregulated the expression of claudin-3, occludin, and ZO-1 in Caco-2 cells (Fig. S2). Preincubation of bacteria with 1.0% of OP alleviated ($p < 0.05$) bacteria-induced downregulation of these tight junction proteins; 0.5% of OP also showed protective effects on ZO-1 expression (Fig. S2A–C). Preincubation with 1.0% of OPP prevented ($p < 0.05$) bacterial effects on the abundances of claudin-3 and occludin (Fig. S2D & E). OPP slightly but not significantly increased ZO-1 expression (Fig. S2F). ETEC K88 challenge for 1 h slightly decreased the TEER values of Caco-2 cell monolayers, with significant reduction observed after bacterial stress for 2 h and longer periods (Fig. S3). Preincubating bacteria with different concentrations of OP (0.1%, 0.5%, and 1.0%) at RT for 30 min could significantly mitigate ETEC K88-induced reduction of TEER values, while OPP only exerted protective effect within 2 h infection (Fig. S3).

Under normal conditions, tight junction proteins were localized on cell membrane surface, forming closed networks and sealing paracellular pathways of adjacent intestinal epithelial cells, as shown in the control group in Fig. S4. ETEC K88 (1×10^8 CFU/mL) treatment for 2 h dramatically destroyed the structures of tight junction proteins as the signals became vague and

discontinuous. Co-incubation of bacteria with OP or OPP (1.0%) restored the fluorescent density and assembly of tight junction proteins. Specifically, occludin and ZO-1 in bacteria–hydrolysate groups exhibited stronger signals and better organized structures compared with ETEC K88 groups, in which the networks shown in the image were rarely captured. ETEC K88 weakened claudin-3 signals, while preincubation with OP and OPP increased claudin-3 assembly on cell surface. Meanwhile, the intracellular fraction of claudin-3 was also enhanced by these two ovomucin hydrolysates (Fig. S4). Whether ovomucin hydrolysates functioned through mediating the migration and diffusion of claudin-3 to maintain functional tight junction permeability needs further research. Taken together, these data suggested that ovomucin hydrolysates could preserve epithelial cell integrity in Caco-2 cells, supported by the ability of ovomucin hydrolysates in maintaining the abundance and distribution of tight junction proteins against ETEC K88 challenge.

4.3.3 Ovomucin hydrolysates suppressed NF-κB p65 and MAPK p38 signaling pathways

NF-κB p65 and MAPK p38 signaling pathways in IPEC-J2 cells were significantly activated after ETEC K88 challenge for 1 and 2 h, respectively (Fig. S5A-B). OP at 1% could significantly inhibit the phosphorylation of NF-κB p65 (Fig. 2A), while OPP downregulated the phosphorylation of NF-κB p65 and MAPK p38 (Fig. 2A-B), consistent with what were detected in Caco-2 cells (Fig. S6). In Caco-2 cells, apart from NF-κB p65 and MAPK p38 molecules, ETEC K88 stress for 1 h significantly increased phosphorylation of MAPK ERK1/2 (Fig. S6). Preincubation of OP (1.0%) and OPP (0.1%, 0.5%, and 1.0%) with ETEC K88 at RT for 30 min could significantly suppress the activation of MAPK ERK1/2 (Fig. S6C-F).

Our previous study showed that OP could effectively neutralize LPS, a component in the cell wall of Gram-negative bacteria, and mitigate LPS-induced inflammatory responses in differentiated Caco-2 cells (Bao & Wu, 2021). Hence, effects of OP and OPP were also tested in

LPS-stimulated IPEC-J2 cells. LPS stress induced significant phosphorylation of NF-κB p65 and MAPK p38 after 30 and 15 min of exposure, respectively (Fig. S7A-B). Preincubation of IPEC-J2 cells with OPP inhibited LPS-induced activation of NF-κB p65 and MAPK p38 (Fig. 2C-D), while OP only significantly inhibited phosphorylation of MAPK p38 (Fig. 2D). OP showed different modulatory effects on NF-κB p65 and MAPK p38 in ETEC K88- and LPS-stressed IPEC-J2 cells, which was assumed to result from the different mechanisms by which LPS and ETEC cause inflammatory responses in intestinal epithelial cells. Collectively, our results demonstrated that both hydrolysates inhibited both proinflammatory signaling pathways in ETEC K88-challenged cells.

4.3.4 Ovomucin hydrolysates inhibited ETEC K88-induced intracellular calcium influx

Given that intracellular calcium Ca^{2+} is a crucial mediator of intestinal ion and fluid movement and that ETEC-associated watery diarrhea involves CaMK II activation and Ca^{2+} influx (Dubreuil et al., 2016), Ca^{2+} levels and CaMK II activity were detected. ETEC K88 challenge for 30 min elevated intracellular Ca^{2+} concentrations, and preincubation of bacterial cultures with OP and OPP at RT for 30 min could significantly inhibit Ca^{2+} accumulation in IPEC-J2 cells (Fig. 3A-B). CaMK II activity assay further showed that both ovomucin hydrolysates decreased intracellular Ca^{2+} influx through suppressing ETEC K88-induced activation of CaMK II (Fig. 3C). Similar effects were also observed in Caco-2 cells (Fig. S8). These data demonstrated the potential of ovomucin hydrolysates to restore intracellular signaling molecules against ETEC K88 challenge. Notably, CaMK II and Ca^{2+} are also stimulated by the toxin STb (Dubreuil et al., 2016). Further studies are worthy of investigating the effects of OP and OPP against STb, such as whether OP and OPP can decrease enterotoxin production/secretion, detoxify STb molecules, or inhibit STb

interacting with the cell surface receptor sulfatide, thus eliminating STb toxicity in opening a GTP-binding regulatory protein-linked receptor-operated Ca^{2+} channel in the cell membrane.

4.3.5 Ovomucin hydrolysates mitigated ETEC K88-induced superoxide generation

Since ETEC K88 infection can induce oxidative stress (Jiménez et al., 2020) and oxidative stress has been well elaborated to influence calcium channels and induce intracellular Ca^{2+} accumulation (Ermak & Davies, 2002), superoxide generation in IPEC-J2 cells was subsequently determined. DHE staining showed that ETEC K88 could significantly elevate the superoxide level in IPEC-J2 cells when compared with the control group, while preincubation with ovomucin hydrolysates could effectively decrease superoxide generation (Fig. 4). These data suggested that both OP and OPP exhibited anti-oxidative activity against ETEC K88 challenge by mitigating superoxide-induced oxidative stress in IPEC-J2 cells.

4.4 Discussion

Ovomucin from chicken egg white and its hydrolysates have been demonstrated with multiple biological activities, including the abilities to interact with diverse microorganisms, which indicate their potential to interfere with pathogenic adhesion, colonization, and resulting infectious diseases (Omana et al., 2010). Recent research about ovomucin modulating intestinal health also suggested its promising role in mediating gut function and combating antimicrobial resistance (Sun et al., 2019; Tu et al., 2021; Tu et al., 2020). Here, ETEC K88-stressed IPEC-J2 and Caco-2 cell models were used to investigate the anti-adhesive and anti-inflammatory properties of two ovomucin hydrolysates that were determined from our previous studies (Sun et al., 2019; Sun et al., 2017), aiming to gain fundamental knowledge about their effects on intestinal epithelial barrier function, inhibiting pathogenic infection, and potentiating its application as an antimicrobial alternative. The major findings of the current study are that OP and OPP effectively inhibited

ETEC K88 adhesion to intestinal epithelial cells. In addition to inhibiting ETEC adhesion that was mediated by the lectin domain of K88 fimbriae, ovomucin hydrolysates decreased ETEC toxicity by interfering with bacterial virulence factors, suppressing inflammatory responses, and alleviating oxidative stress.

While free sialic acids are generally utilized as a nutrient by bacteria, sialic acids prevalently located at the termination of diverse glycoconjugates are acknowledged as the ligands for bacterial recognition and adhesion (Bell et al., 2023; Jennings et al., 2022). Ovomucin contains 2.6-8% sialic acids with α2,3- and α2,6-linkages (Mine, 1995; Xu et al., 2018); ovomucin and its hydrolysates have been reported to interact with different microorganisms, where terminal sialic acid residues on the glycan structures play an important role (Kobayashi et al., 2004; Sun et al., 2019). Removing sialic acids from ovomucin and OP decreased their interactions with ETEC K88 (Table 4.1); similarly, removing sialic acids from cell surfaces by NANase reduced ETEC K88 adhesion to IPEC-J2 and Caco-2 cells (P = 0.0474 and 0.0048 by t test, respectively, Tables 4.2 and S4.1). These findings were consistent with previous studies that sialic acids mediated the binding of enteric bacteria (Parker et al., 2010; Sakarya et al., 2010; Salcedo et al., 2013). Free sialic acids decreased ETEC adhesion to Caco-2 cells (Table S4.1), aligning with previous research that sialic acids interfered with ETEC adhesion to Caco-2 cells (Salcedo et al., 2013). Desialydation of OP and OPP impaired their anti-adhesive activities in IPEC-J2 cells (P = 0.0306 and 0.0466 by t test, respectively, Table 4.2), but not to Caco-2 cells, suggesting other sialic acid-independent factors involved in mediating ETEC K88 binding. OPP contains higher contents of carbohydrate moieties and peptides of larger molecular weights than OP (Sun et al., 2016). These differences could account for their distinct effects in preventing ETEC K88 adhesion as both glycans and peptides were involved in interacting with bacterial fimbriae (Sun et al.,

2019). OP and OPP at 0.5% and 1% concentrations showed much higher anti-adhesive activities against ETEC K88 strain ECL13795 in undifferentiated IPEC-J2 cells (Sun et al., 2019), similar to what we observed in differentiated Caco-2 cells in the current study. Differences can be explained by the features of IPEC-J2 cells and Caco-2 per se (Lea, 2015; Vergauwen, 2015), as well as the physiological and characteristic changes after cell differentiation (Pi et al., 2022). Overall, the IPEC-J2 cell line that has been well characterized and remodeled to mimic porcine jejunal physiology forms a suitable model for investigating porcine-specific bacterial infections (Koh et al., 2008; Schierack et al., 2006). Caco-2 cells do not express the glycan receptor for K88 fimbriae but share similar morphological and functional properties with IPEC-J2 cells and thus can be utilized to investigate pathogenic mechanisms and preliminarily identify potential compounds for managing pathogenic infections (Ding et al., 2021; Geens & Niewold, 2011).

The paracellular space between adjacent intestinal epithelial cells is sealed by the epithelial junctional complex, including tight junctions. With increased understanding of the composition and functionalities of tight junction proteins beyond controlling paracellular permeability and maintaining intestinal epithelial integrity, they have been well acknowledged as bidirectional signaling platforms that can transduce signals to cell interior to regulate cell biological processes in response to different stimuli, and that can adapt to signals from the cell interior for their trafficking and assembly (Zihni et al., 2016). A dysfunctional intestinal barrier is prevalently associated with diverse disorders, including enteric infections and chronic diseases (Stamatovic et al., 2017). ETEC K88 infection was reported to increase intestinal permeability, decrease expression of tight junction proteins, and promote inflammatory response through activating MAPK and NF-κB signaling pathways in IPEC-J2 (Johnson et al., 2010; Yu et al., 2018) and Caco-2 cells (Roselli et al., 2016; Yu et al., 2015), as well as in piglets (Garas et al., 2018; Xu et

al., 2014). In the present study, ETEC K88 reduced TEER values and increased tight junctional permeability in both cell lines, indicating glycan-independent mechanisms of ETEC K88-induced epithelial integrity defects as these two cells have distinctive glycosylation patterns. OP and OPP showed protective effects on tight junction proteins only in Caco-2 cells (Fig. 1E, S2 and S3), although they showed similar anti-adhesive capacities at 1% dosage in both cell lines (Tables 4.2 and S4.1). Also, the distribution patterns of occludin, claudin-3, and ZO-1 proteins were dramatically disturbed by ETEC K88 challenge in Caco-2 cells (Fig. S4.4), while OP and OPP could partially maintain their assembly on cell membrane surfaces. This suggested the sialydation-independent mechanisms by which OP and OPP alleviate ETEC K88-induced tight junction damage since sialic acid contents in OP and OPP were different (0.1% and 1.7%, respectively) (Sun et al., 2016). The intracellular fusions of claudin-3 were obviously enhanced in OP and OPP groups compared with those in the ETEC K88 group (Fig. S4). This suggested the possibility that OP and OPP pretreatment could preserve the expression of tight junction proteins and that they were likely to mediate the migration/fusion of tight junctions from/to cell membrane under conditions of ETEC K88 infection, as recycling by endocytosis is the dynamic nature of tight junction and plays a crucial role in mediating or responding to physiological variations including bacterial infection (Chalmers & Whitley, 2012; Stamatovic et al., 2017).

NF-κB and MAPK signaling pathways are activated to initiate inflammatory response by various stressors including microbial components, such as LPS, also termed pathogen-associated molecular patterns (Akira et al., 2006). Further, activation of NF-κB and MAPK pathways can impair intestinal integrity via myosin-mediated interaction of tight junction proteins with the cytoskeleton (Al-Sadi et al., 2010; Costantini et al., 2009). In the present study, ETEC K88 triggered activation of NF-κB p65 and MAPK pathways in both porcine and human cells (Fig. 2A-

B and S5-6), indicating ETEC K88-induced pro-inflammatory response through glycosylation-independent or receptor recognition-independent pathways. OP significantly inhibited ETEC K88-induced activation of NF-κB p65 and MAPK ERK1/2, while OPP also suppressed MAPK p38 in intestinal epithelial cells. In addition, LPS activated NF-κB and MAPK pathways in IPEC-J2 cells, while both OP and OPP mitigated the effects of LPS (Fig. 2C-D). This was consistent with our previous study showing that OP could inhibit LPS-induced activation of NF-κB and MAPK pathways and epithelial barrier defects in Caco-2 cells and neutralize LPS toxicity (Bao & Wu, 2021). These data indicated the potential of OP and OPP to attenuate inflammation and suggested their application in managing pathogenic infection. Further study is needed to advance our understanding of underlying molecular mechanisms and to prove their efficacies in preventing ETEC infection in piglets.

In addition to LPS, heat-stable toxin and heat-labile toxins (STa and STb) are two crucial virulence factors that determine ETEC K88-induced watery diarrhea and intestinal dysfunction through different receptors and cellular molecules (Dubreuil et al., 2016). After binding to its receptor, STb acts primarily through elevating intracellular Ca^{2+} levels, upregulating CaMK II activity, and disturbing tight junction proteins (Dreyfus et al., 1993; Fujii et al., 1997; Ngendahayo Mukiza & Dubreuil, 2013). Ca^{2+} is a vital second messenger in cellular signal transduction and it can interact reciprocally and form a feedforward loop with reactive oxygen species (Ermak & Davies, 2002; Peng & Jou, 2010). Accordingly, intracellular Ca^{2+} concentrations, CaMK II activity, and superoxide generation were investigated in this study. Results showed that OP and OPP could effectively alleviate the detrimental actions of ETEC K88 in IPEC-J2 and Caco-2 cells (Fig. 3, 4, and S8), indicating they alleviated inflammation and oxidative stress possibly through diminishing the toxicity of ETEC virulence factors in cells. Future studies using purified STb

(Bossé et al., 1993) are necessary to confirm the mechanisms by which OP and OPP interfere with STb and mitigate STb toxicity. Furthermore, the specific bioactive peptides in OP and OPP remain to be identified to support their future application in managing ETEC K88 infection in practice. However, a reasonable concern arises when expecting the outcome of *in vivo* study because continuous fluid shear stress induces nonadherent pathogen shedding from the gut and complexity of intestinal microenvironment will influence the interaction of ovomucin hydrolysates with ETEC K88.

In conclusion, our present study demonstrated that ovomucin hydrolysates OP and OPP inhibited bacterial adhesion, wherein sialic acids were partly involved. OP partially restored epithelial cell integrity against ETEC K88 challenge in both cells, but the abundance and networks of tight junction proteins were maintained only in Caco-2 cells, but not in IPEC-J2 cells. Both hydrolysates could effectively alleviate ETEC-induced inflammatory response and oxidative stress, whereas the interactions between ovomucin hydrolysates and the virulence factors especially toxins are to be established. Overall, our results indicate possible applications of ovomucin hydrolysates in preventing ETEC K88 infection, thus alleviating antimicrobial resistance issues arising from agricultural sectors. Further *in vivo* studies using ETEC K88-challenged piglet models and on-farm studies are needed to validate their efficacies in practice.

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Table 4.1 Number of bacteria (log CFU per well) attached to wells coated with different materials in the adhesion test

Treatment	**Adherent bacteria**	***P* value**
PBS	5.69±0.09	
1.0%OVM	6.95±0.05***	< 0.0001
1.0%OVM-NANase	6.62±0.06$^{\#}$	0.0151
1.0%OP	6.37±0.09***	< 0.0001
1.0%OP-NANase	6.00±0.03$^{\#\#}$	0.0051
1.0%OPP	6.30±0.07***	< 0.0001
1.0%OPP-NANase	6.32±0.06	> 0.9999
1.0%CMP	7.00±0.08***	< 0.0001
1.0%CMP-NANase	6.89±0.04	> 0.9999

Data are represented as mean ± SEM of four independent experiments.

$^{****}P < 0.0001$, compared with PBS group.

Italic, $\#P < 0.05$, $\#\#P < 0.01$, compared with corresponding OVM/hydrolysate without NANase removing sialic acid.

Table 4.2 Number of bacteria (log CFU per well) attached to differentiated IPEC-J2 cell surface

Treatment	**Adherent bacteria**	**Inhibitory percentage (%)**	***P* value**
PBS	5.60±0.03		
NANase	5.33±0.12	45.00±15.00	0.2327
Nanase buffer	5.62±0.03	No activity[1]	> 0.9999
Free Neu5Ac	5.46±0.16	23.19±26.45	0.9716
0.5%OP	5.52±0.06	14.70±12.38	0.9988
1.0%OP	5.39±0.03	37.47±5.79	0.3413
2.5%OP	4.19±0.10****	95.63±1.20	< 0.0001
5.0%OP	4.43±0.04****	93.13±0.62	< 0.0001
1.0%OP-NANase	*5.53±0.02*	*15.00±5.44*	*0.8767*
0.5%OPP	5.56±0.06	6.90±13.56	> 0.9999
1.0%OPP	5.39±0.05	36.69±8.78	0.3283
2.5%OPP	5.35±0.03	43.13±5.44	0.1278
5.0%OPP	5.01±0.08****	72.50±6.21	< 0.0001
1.0%OPP-NANase	*5.58±0.04*	*3.08±10.40*	*0.4439*
1.0%CMP	5.22±0.05**	57.47±6.01	0.0021

[1], no activity means inhibitory percentage ≤ 0.

Data are represented as mean ± SEM of four independent experiments.

***P* < 0.01, *****P* < 0.0001, compared with the PBS group.

Italic, compared with corresponding hydrolysate without NANase treatment.

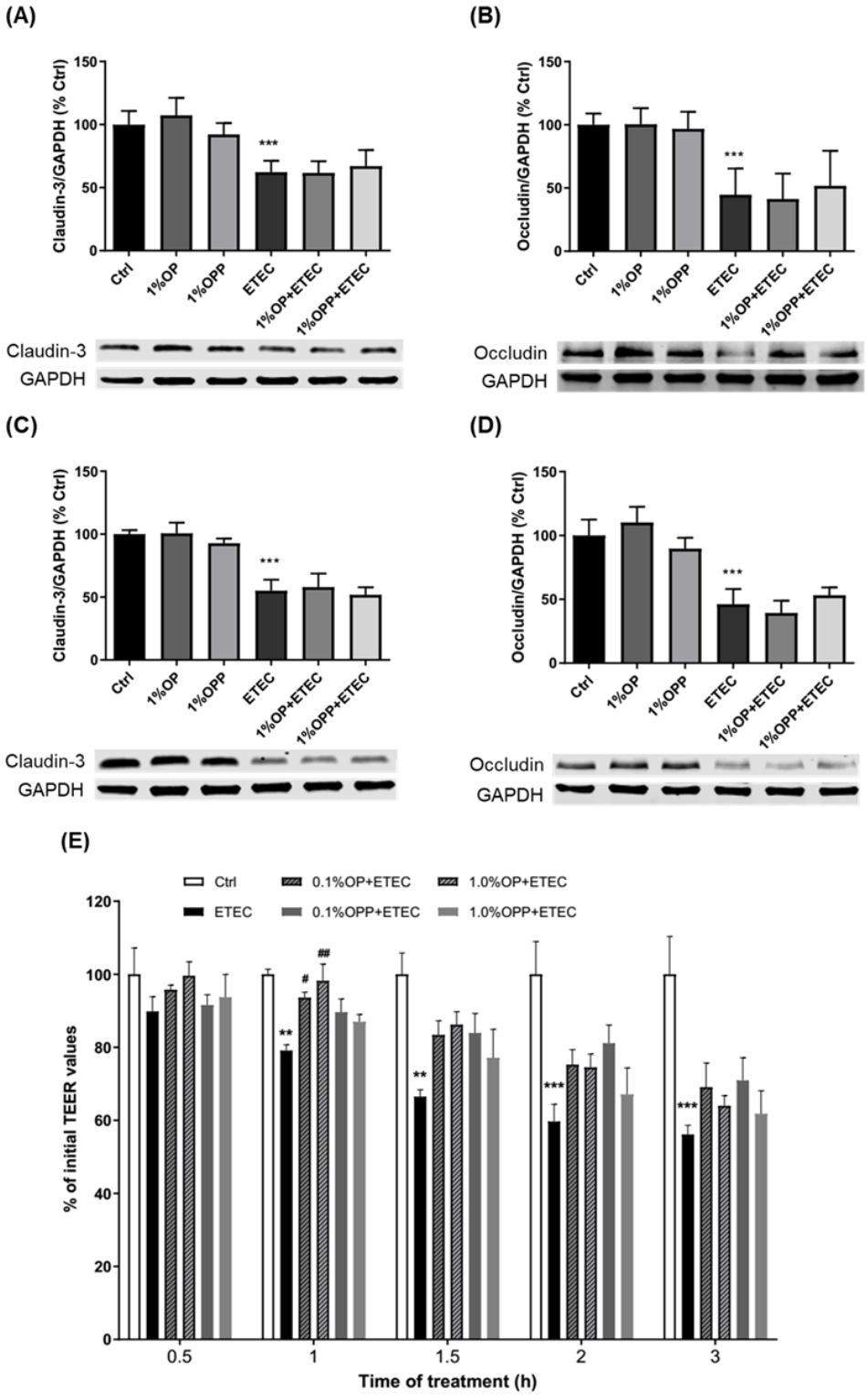

Fig. 4.1 Effects of OP and OPP on ETEC-induced tight junction protein degradation (Panels A and D) and TEER reduction (Panel E). IPEC-J2 cells cultured for 14 days to undergo differentiation.

Ovomucin hydrolysates OP and OPP were mixed with ETEC (final concentration of 1×10^7 CFU/mL) at RT for 30 min before incubated with IPEC-J2 cells for 1 h (Panels A-B) or 2 h (Panels C-D). Panel E, TEER values of IPEC-J2 cells grown on transwell filters were measured by a voltmeter after treatment for different time points and expressed as the percentages of initial values. Data are represented as mean ± SEM of four independent experiments. Ctrl means control group. OP means ovomucin-protex 26L hydrolysate. OPP means ovomucin-pepsin/pancreatin hydrolysate. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significantly different from the control group. #$P < 0.05$, ##$P < 0.01$, significantly different from the ETEC K88 group.

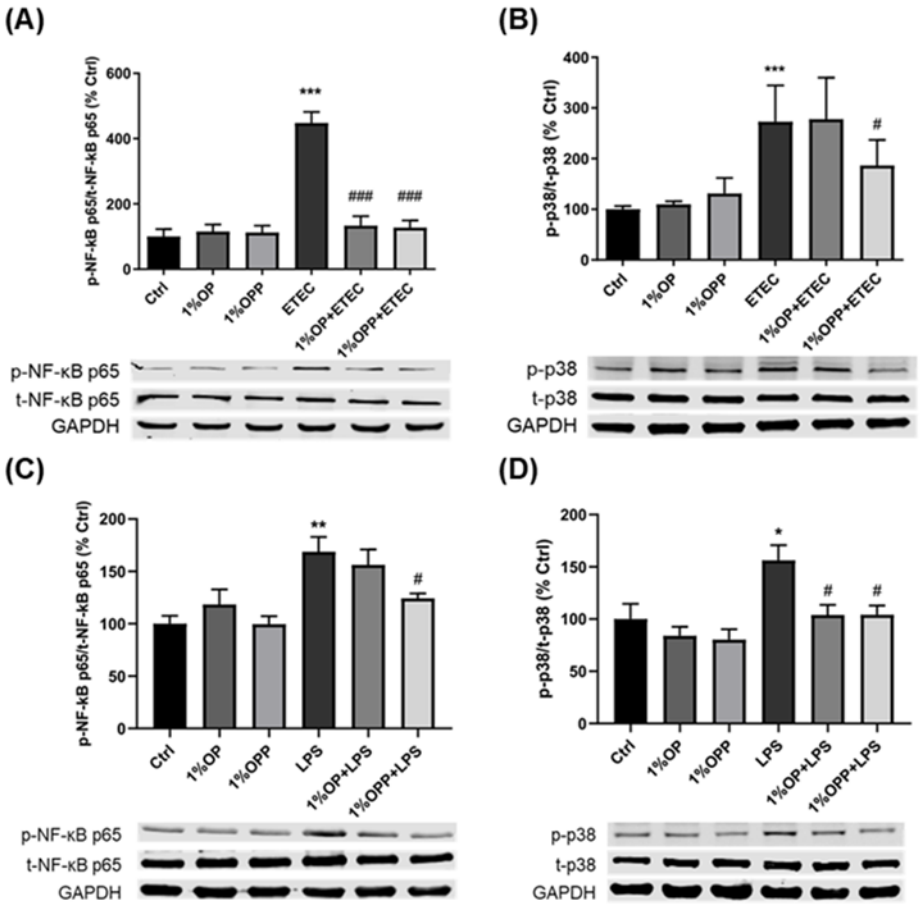

Fig. 4.2 Effects of OP and OPP on activation of NF-κB p65 and MAPK p38 molecules induced by ETEC K88 (Panel A and B) and LPS (Panels C and D). OP and OPP were mixed with ETEC K88 (final concentration of 1×10^7 CFU/mL) at RT for 30 min before incubated with IPEC-J2 cells. Preincubation of OP and OPP for 24 h downregulated LPS-induced phosphorylation of NF-κB p65 (Panel C, 10 μg/mL LPS stress for 0.25 h) and MAPK p38 (Panel D, 10 μg/mL LPS stress for 1 h). Data are represented as mean ± SEM of four independent experiments. Ctrl means control group. OP means ovomucin-protex 26L hydrolysate. OPP means ovomucin-pepsin/pancreatin hydrolysate. CMP means casein macropeptide. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significantly different from the control group. #$P < 0.05$, ###$P < 0.001$, significantly different from the ETEC K88 group.

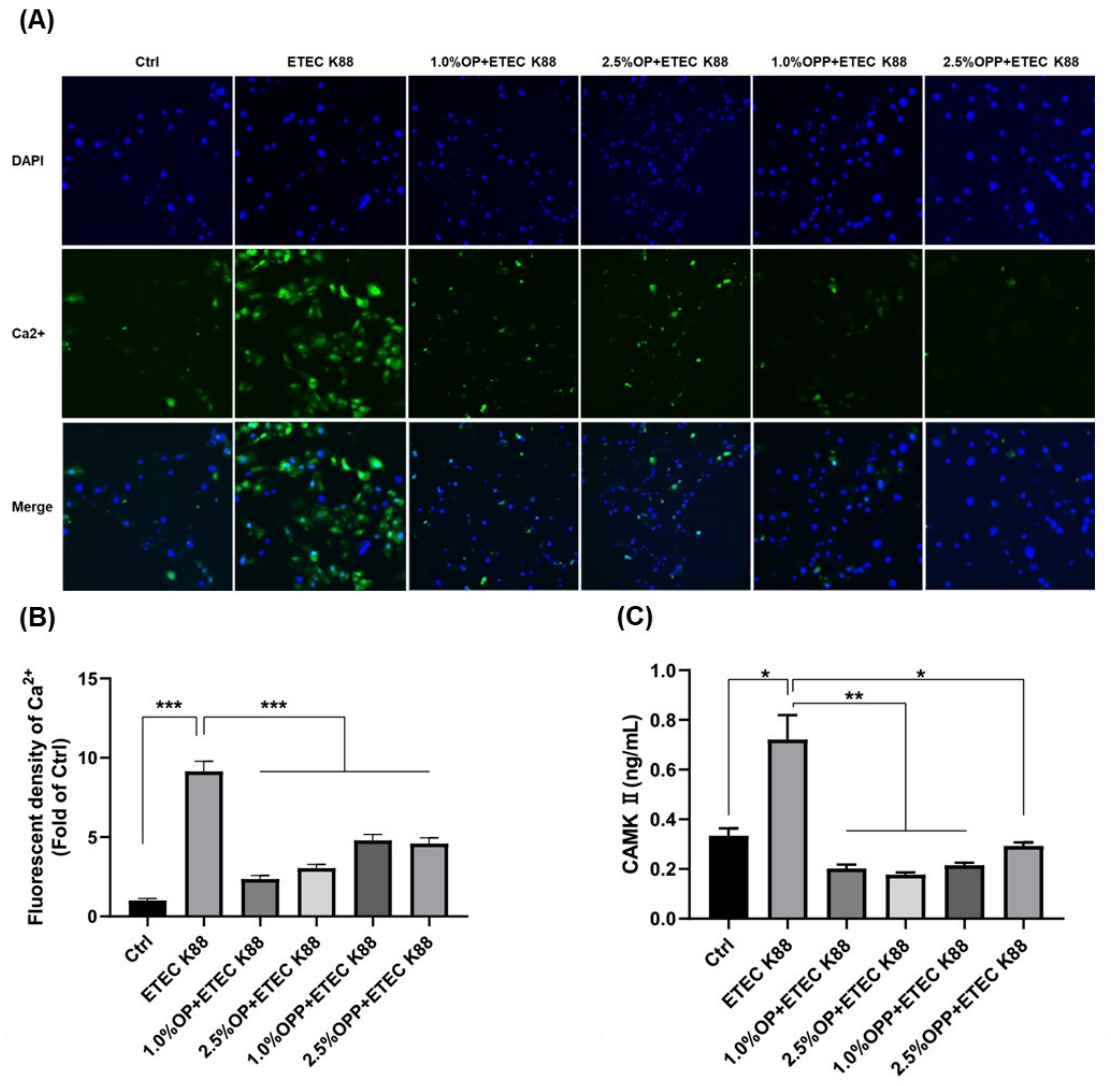

Fig. 4.3 Ca^{2+} influx and CaMK II activity in IPEC-J2 cells. Cells were treated with ETEC K88 (1×10^7 CFU/mL) or bacteria-hydrolysate preincubation (at RT for 30 min) for 30 min. Intracellular Ca^{2+} was stained with the fluorescent dye Fluo-4 (Panels A and B). Cell nuclei were stained with DAPI. Images were captured by a fluorescent microscope under the 10× objective lens. Fluorescence density of Ca^{2+} was quantified by ImageJ software (https://imagej.net/Welcome). Data were expressed as the fold change in the mean fluorescence density per cell over the control group. CaMK II activity in whole cell lysates were measured (Panel C). Data were indicated as mean ± SEM of six independent determinations. Ctrl indicates the untreated control group. OP

means ovomucin-protex 26L hydrolysate. OPP means ovomucin-pepsin/pancreatin hydrolysate. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with ETEC K88-treated group.

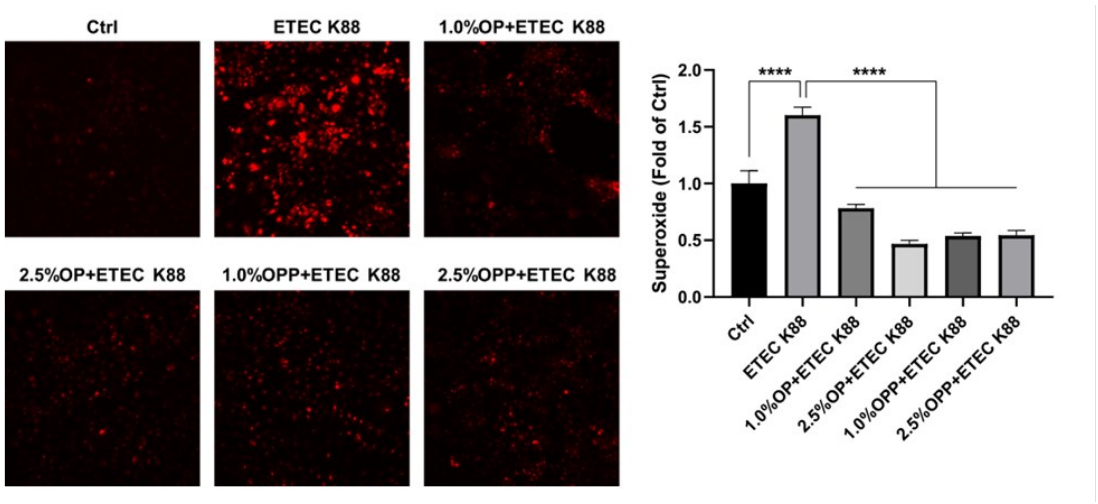

Fig. 4.4 Effects of OP and OPP on ETEC-induced superoxide generation in IPEC-J2 cells. Cells were treated with ETEC K88 or the preincubation of bacteria and ovomucin hydrolysates (at RT for 30 min) at 37 °C for 30 min before DHE staining. A set of representative images from three independent experiments were shown and fluorescence density per field of view was quantified by the software ImageJ. Data were presented as the fold change over the untreated control group (Ctrl). OP means ovomucin-protex 26L hydrolysate. OPP means ovomucin-pepsin/pancreatin hydrolysate. ****$P < 0.0001$, compared with ETEC K88-treated group.

Supplementary data

Table S4.1 Number of bacteria (log CFU per well) attached to Caco-2 cell surface

Treatment	Adherent bacteria	Inhibitory percentage (%)	*P* value
PBS	6.28±0.08		
NANase	5.82±0.06*	63.50±5.47	0.0397
Heated NANase	6.08±0.10	31.00±18.88	0.9329
Free Neu5Ac	5.68±0.12**	71.85±7.80	0.0015
0.5%OP	5.94±0.08	51.43±8.86	0.2997
1.0%OP	5.82±0.19*	53.66±19.38	0.0366
2.5%OP	5.81±0.04**	65.52±3.18	0.0070
5.0%OP	5.26±0.05****	90.18±1.23	< 0.0001
1.0%OP-NANase	*5.05±0.08*$^{\#\#\#\#}$	*93.47±1.00*	< 0.0001
0.5%OPP	5.92±0.16	40.10±12.63	0.0503
1.0%OPP	5.85±0.08	61.07±6.65	0.0584
2.5%OPP	5.56±0.08****	79.24±3.37	< 0.0001
5.0%OPP	4.82±0.07****	96.34±0.51	< 0.0001
1.0%OPP-NANase	*5.74±0.08*	*67.35±6.81*	*0.9999*
1.0%CMP	5.90±0.07	57.14±6.40	0.2016

Data are represented as mean ± SEM of six independent experiments.

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, compared with PBS group.

Italic, $^{\#\#\#\#}P < 0.0001$, compared with corresponding hydrolysate without NANase treatment.

Table S4.2 Information of antibodies used in this study

Antibody	**Supplier**	**Catalogue No.**	**Species**
Occludin	Abcam	ab216327	Rabbit
Claudin-3	Abcam	ab15102	Rabbit
ZO-1	Abcam	ab96587	Rabbit
phospho-NF-κB p65 (Ser 536)	Cell signaling technology	3033T	Rabbit
NF-κB p65	Cell signaling technology	6856S	Mouse
phospho-ERK1/2 (Thr 202/Tyr 204)	Cell signaling technology	9101S	Rabbit
ERK1/2	Cell signaling technology	4696S	Mouse
phospho-p38 MAPK (Thr180/Tyr182)	Cell signaling technology	9216S	Mouse
p38 MAPK	Cell signaling technology	8690S	Rabbit
GAPDH	Cell signaling technology	2118S	Rabbit
goat anti-rabbit CF™ 594	Sigma-Aldrich	SAB4600322	Goat
donkey anti-mouse 800CW secondary antibody	LI-COR Biosciences	P/N 926-32212	Donkey

goat anti-rabbit IRDye 680RD secondary antibody	LI-COR Biosciences	P/N 926-68071	Goat

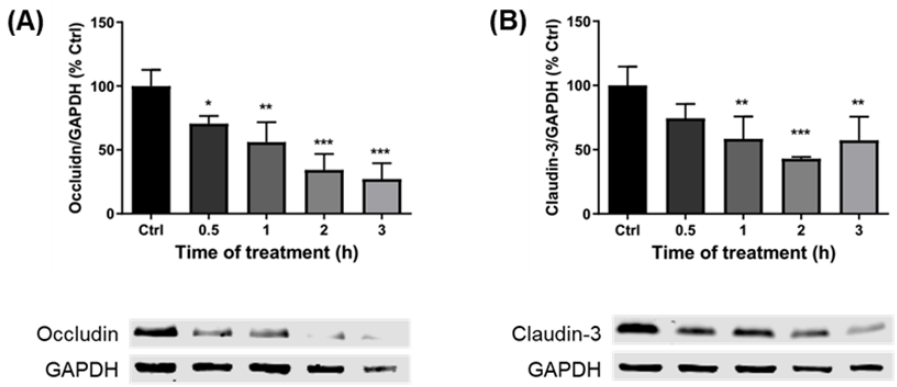

Fig. S4.1 Expression changes of tight junction proteins in ETEC K88-challenged IPEC-J2 cells. IPEC-J2 cells cultured for 14 days were treated with ETEC K88 (1×10^7 CFU/mL) for 0.5, 1, 2, and 3 h and whole cell lysates were applied to Western blot analysis for tight junction protein occludin (Panel A) and claudin-3 (Panel B). Data are represented as mean ± SEM of four independent experiments. Ctrl means control group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significantly different from the control group.

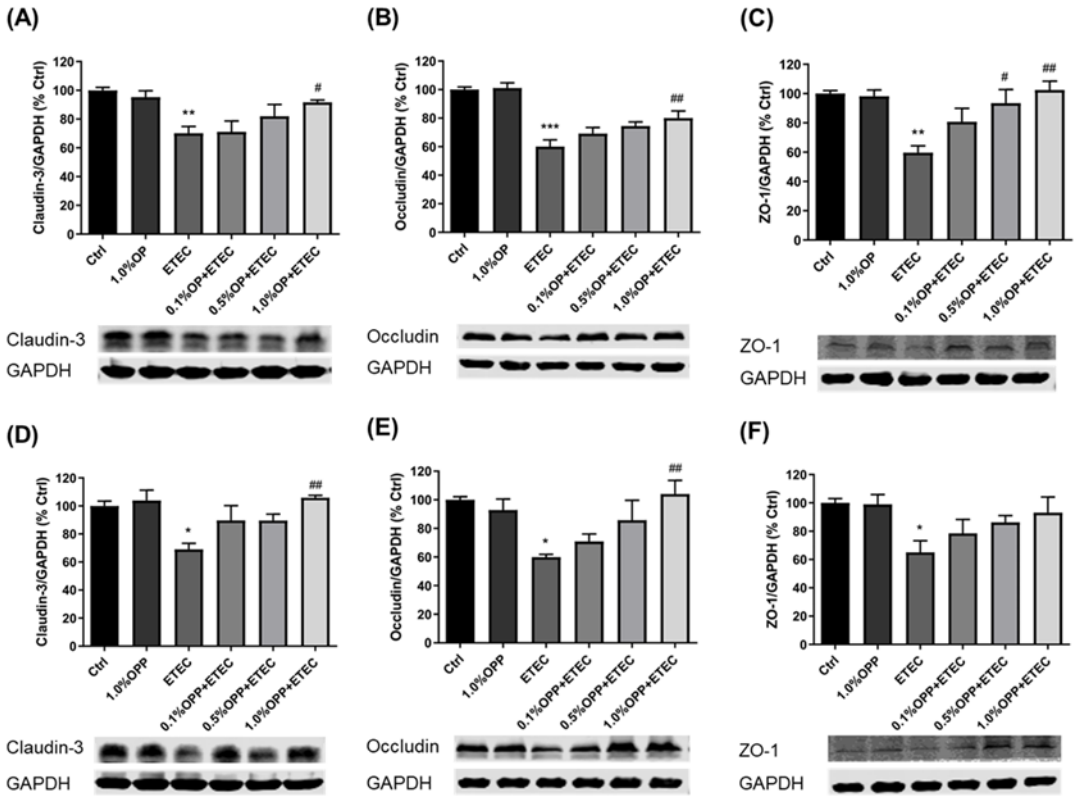

Fig. S4.2 Effects of OP and OPP on the expression levels of tight junction proteins in differentiated Caco-2 cells. OP or OPP were preincubated with ETEC K88 (final concentration of 1×10^8 CFU/mL) at RT for 30 min. Panels A-C, Caco-2 cells were treated with OP (1.0%), ETEC K88, or the mixture of OP at different concentrations with bacteria at 37°C for 2 h. Panels D-F, Caco-2 cells were treated with OPP (1.0%), ETEC K88, or the mixture of OPP with bacteria at 37°C for 2 h. Whole cell lysates were applied to Western blot analysis of claudin-3, occludin, and ZO-1. Data are represented as mean ± SEM of four independent experiments. Ctrl means control group. OP means ovomucin-protex 26L hydrolysate. OPP means ovomucin-pepsin/pancreatin hydrolysate. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significantly different from the control group. #$P < 0.05$, ##$P < 0.01$, significantly different from the ETEC K88 group.

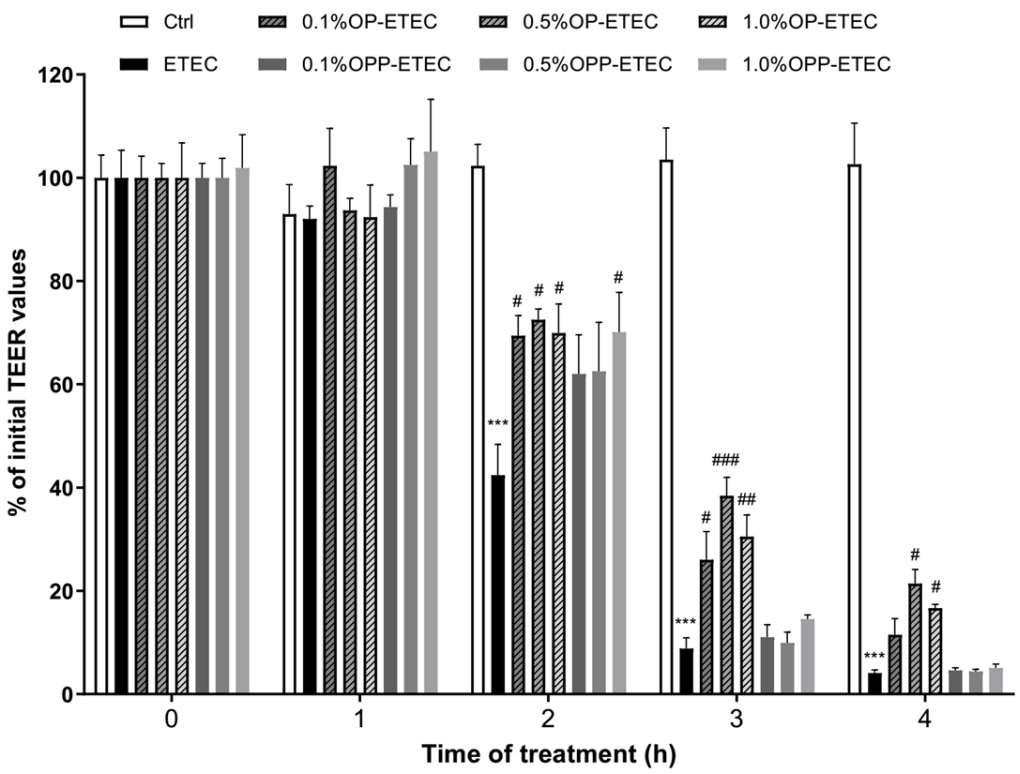

Fig. S4.3 Effects of OP and OPP on TEER values of Caco-2 cell monolayers. Mixture of ETEC K88 (1×10^8 CFU/mL) and ovomucin hydrolysates (0.1%, 0.5%, and 1.0%) at RT for 30 min were applied to cells for 1-4 h. TEER values were measured by a voltmeter (Millicell-ERS; Millipore, MA, USA). Ctrl means control group. OP means ovomucin-protex 26L hydrolysate. OPP means ovomucin-pepsin/pancreatin hydrolysate. Data are expressed in the percentage of the initial values from four independent experiments. ***$P < 0.001$, significantly different from the control group. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, significantly different from ETEC K88 group.

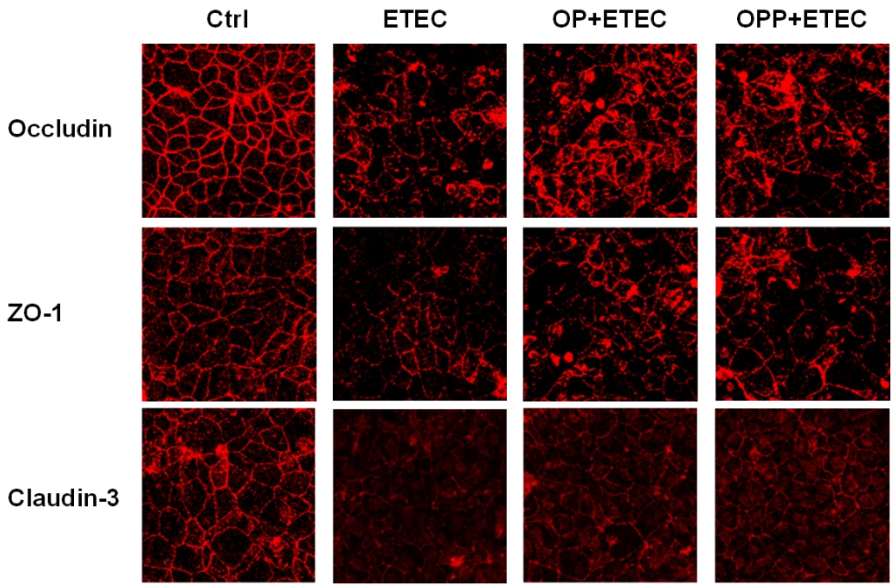

Fig. S4.4 Localization of tight junction protein occludin, ZO-1, and claudin-3 in Caco-2 cells. Cells grown on glass coverslips were treated with ETEC K88 (1×10^8 CFU/mL) or the preincubation of bacteria and ovomucin hydrolysates (1.0%) at 37°C for 2 h. After treatment, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X 100, followed by overnight incubation with primary anti-occludin/ZO-1/claudin-3 antibody at 4°C. Finally, cells were stained with the anti-rabbit CF^{TM} 594 antibody. Distribution of tight junction proteins were visualized by a confocal laser scanning microscope (Olympus FV3000) with the 10× objective lens. Ctrl indicates the control group. OP means ovomucin-protex 26L hydrolysate. OPP means ovomucin-pepsin/pancreatin hydrolysate.

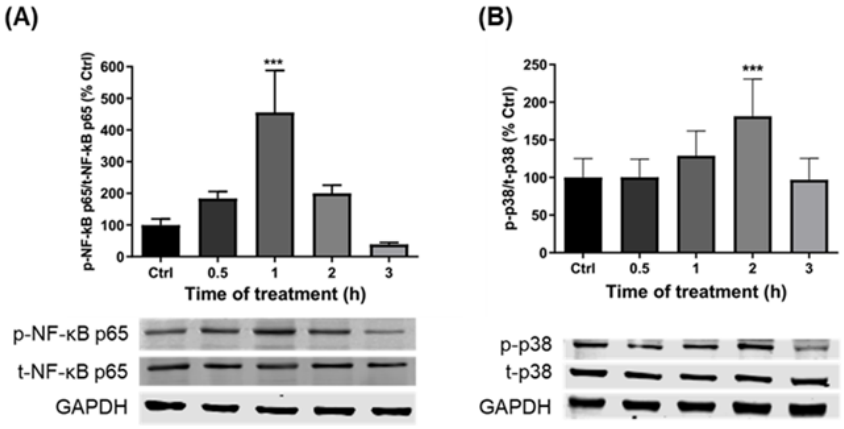

Fig. S4.5 ETEC K88-induced activation of NF-κB p65 (Panels A) and MAPK p38 (Panel B) in IPEC-J2 cells. ETEC K88 (final concentration of 1×10^7 CFU/mL) was incubated with IPEC-J2 cells for 0.5-3 h. Whole cell lysates were applied to Western blot analysis. Data are represented as mean ± SEM of four independent experiments. Ctrl means control group. ***$P < 0.001$, significantly different from the control group.

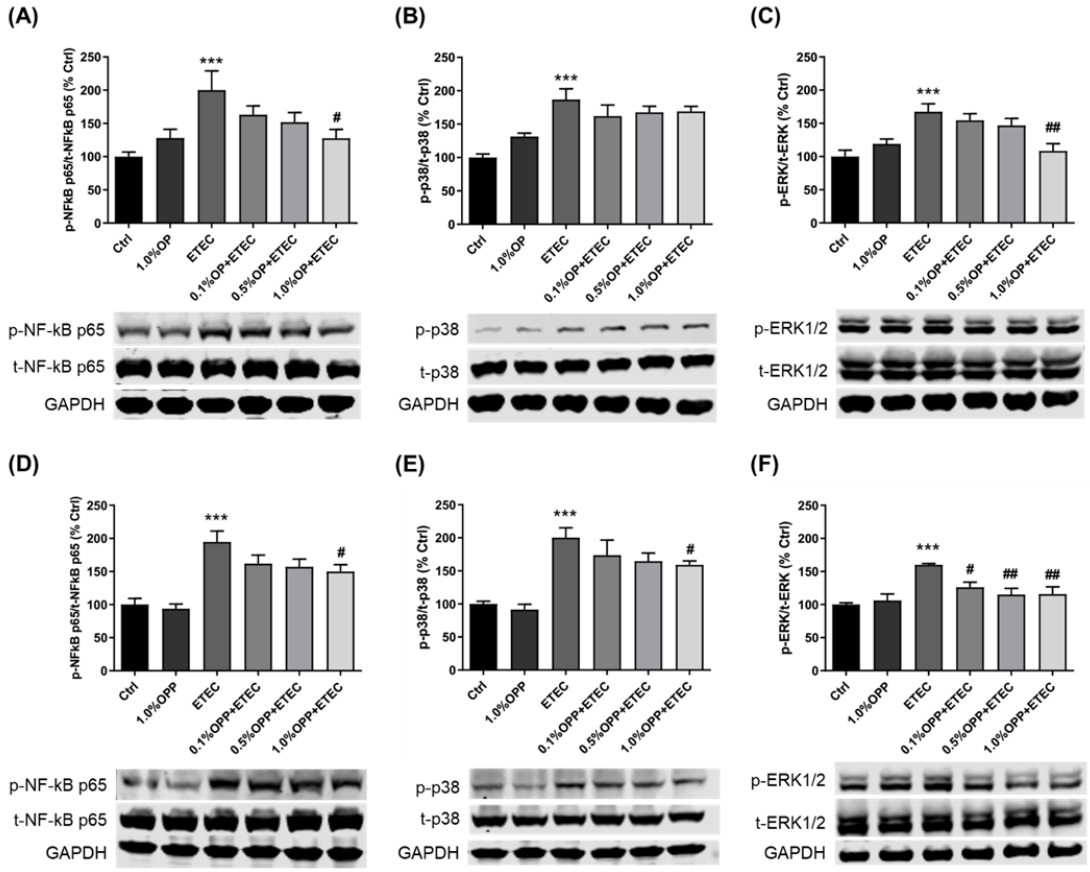

Fig. S4.6 Activation of NF-κB p65 and MAPK (p38 and ERK1/2) pathways in Caco-2 cells. Panels A-C, Caco-2 cells were treated with OP (1.0%), ETEC K88, or the mixture of OP at different concentrations with bacteria at 37°C for 1 h. Panels D-F, Caco-2 cells were treated with OPP (1.0%), ETEC K88, or the mixture of OPP with bacteria at 37°C for 1 h. After treatment, whole cell lysates were collected for Western blot analysis of NF-κB p65, p38, and ERK1/2. The results were expressed as mean ± SEM of four independent experiments. Ctrl means control group. OP means ovomucin-protex 26L hydrolysate. OPP means ovomucin-pepsin/pancreatin hydrolysate. ***$P < 0.001$, significantly different from the control group. #$P < 0.05$, ##$P < 0.01$, significantly different from the ETEC K88 group.

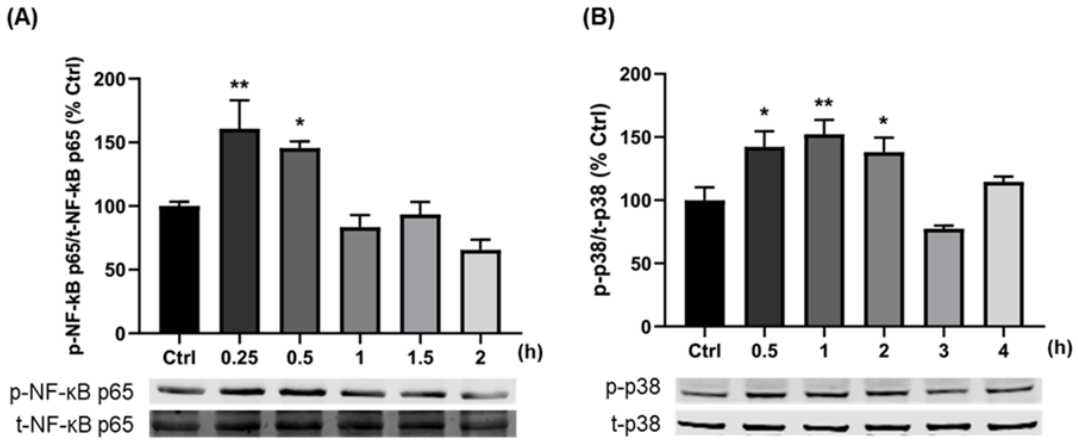

Fig. S4.7 LPS-induced activation of NF-κB p65 (Panel A) and MAPK p38 (Panel B) in IPEC-J2 cells. Cells were treated with LPS (10 μg/mL) for different periods as indicated. Data were expressed as mean ± SEM (n=4-6) and presented as percentage of the untreated control group. Ctrl means the control group. **P* < 0.05, ***P* < 0.01, significantly different from the control group.

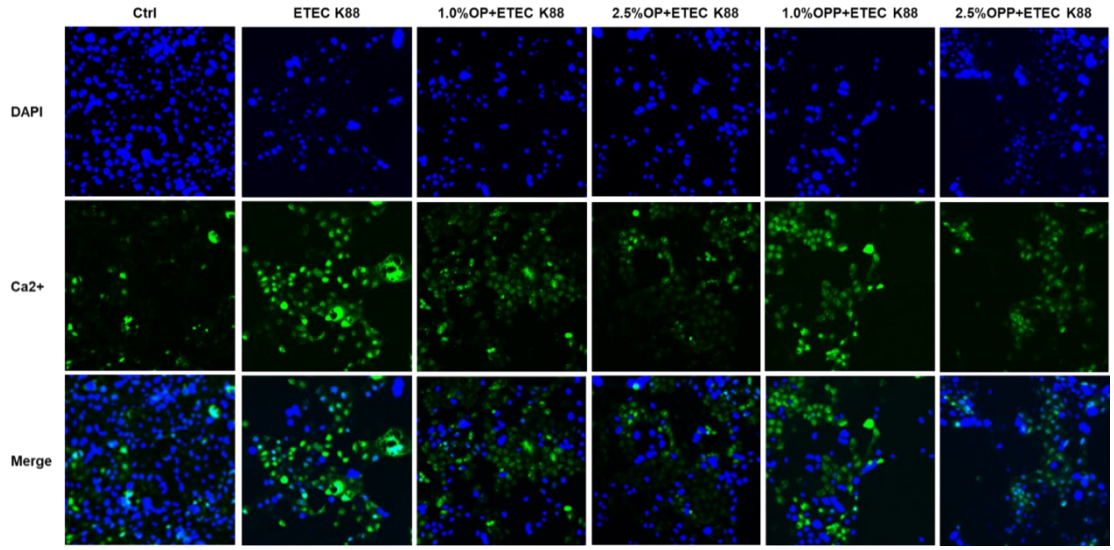

Fig. S4.8 Ca^{2+} staining in Caco-2 cells. Cells were treated with ETEC K88 (1×10^8 CFU/mL) or bacteria-hydrolysate preincubation for 30 min. Intracellular Ca^{2+} was stained with the dye Fluo-4. Cell nuclei were stained with DAPI. Images were captured by a fluorescent microscope under the 10× objective lens. Ctrl indicates the control group. OP means ovomucin-protex 26L hydrolysate. OPP means ovomucin-pepsin/pancreatin hydrolysate.

CHAPTER 5 – Ovomucin and its Hydrolysates Differentially Influenced Colitis Severity in *Citrobacter rodentium*-Infected Mice

5.1 Introduction

Citrobacter rodentium, a natural murine pathogen, is closely related to attaching and effacing pathogens, enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) that cause human diarrheal diseases worldwide. During infection, *C. rodentium* employs a type III secretion system to inject effector proteins including the translocated intimin receptor (Tir), mitochondrial-associated protein, and the type III-secreted proteins (e.g. *E. coli* secreted protein, EspA) (Collins et al., 2014). The pathogen-associated molecular patterns of *C. rodentium* are recognized by Toll-like receptor 2 (TLR2) and TLR4 on the surface of intestinal epithelial and myeloid cells. Subsequently, the signaling is transduced to the adaptor protein myeloid differentiation factor 88, the nuclear factor-κB (NF-κB), and the mitogen-activated protein kinase (MAPK) pathways which leads to downstream signaling cascades, including pro-inflammatory cytokine/chemokine production, macrophages and neutrophils recruitment, and colonic crypt hyperplasia (Gibson et al., 2008a; Gibson et al., 2008b; Khan et al., 2006). *C. rodentium*-induced mouse colitis has long been used as a robust model to elaborate the molecular mechanisms of EPEC and EHEC infection *in vivo*, as well as to study host-microbe interaction, mucosal immunology, and health benefits of bioactive substances (Collins et al., 2014).

Various food-derived bioactive substances have been revealed to modulate *C. rodentium* colonization and infection. For example, egg protein-derived peptides (Ma et al., 2019) and phytochemicals, including quercetin from citrus fruits (Lin et al., 2019), eugenol primarily from clove (Marta Wlodarska et al., 2015), and organosulfur compounds in garlic extracts (Zhu et al., 2022), reduced *C. rodentium* adhesion to the intestinal epithelium, inhibited *C. rodentium*

colonization in colon, elevated host anti-oxidative activity, decreased the production of pro-inflammatory cytokines, modulated the abundance of gut microbes, and alleviated colitis severity. Furthermore, probiotics like *Lactobacillus* strains (Chen et al., 2005; Rodrigues et al., 2012) and *Saccharomyces boulardii* (Wu et al., 2008) attenuated *C. rodentium* infection through maintaining colonic barrier function, interfering with virulence factors, facilitating pathogen clearance, and ameliorating inflammation. These studies elucidated multiple mechanisms of bioactive compounds reducing *C. rodentium* pathogenesis and highlighted their potential in protecting the host from enteric infections. Notably, natural bioactive agents that enhance colonization resistance against pathogens may represent a viable approach to decrease antimicrobial use in managing infectious diseases and thus combating antimicrobial resistance (Álvarez-Martínez et al., 2020).

Ovomucin is a mucin-type glycosylated protein in chicken egg white, composed of a carbohydrate-rich β-subunit and a carbohydrate-poor α-subunit that are connected via disulfide bonds to form a linear structure (Donovan et al., 1970; Omana et al., 2010). As a viscous molecule, its primary biological role is to protect the embryo against microbial assaults by restraining the migration of pathogens towards egg yolk (Guyot et al., 2016). Ovomucin, along with its digests, have demonstrated numerous biological functions, including anti-inflammatory and anti-adhesive activities and promoting intestinal barrier integrity (Omana et al., 2010; Tu et al., 2020). For example, ovomucin restored intestinal integrity defects, preserved gut microbial communities, and attenuated mucosal inflammation in dextran sulfate sodium-induced mouse colitis (Tu et al., 2021). Ovomucin-Pronase hydrolysate could bind to *E. coli* O157:H7 through its carbohydrate moieties (Kobayashi et al., 2004). Ovomucin hydrolysate prepared by protex 26L (*Aspergillus niger* origin) mitigated lipopolysaccharide (LPS)-induced inflammation in human colorectal adenocarcinoma cells, an *in vitro* model for intestinal epithelia (Chapter 3) (Bao & Wu, 2021). Ovomucin-protex

26L and -pepsin/pancreatin hydrolysates inhibited ETEC adhesion to porcine intestinal epithelial cells through serving as decoy receptors for the fimbriae K88 (Sun et al., 2019). These findings indicated a potential of ovomucin and its hydrolysates in interfering with bacteria colonization and modulating mucosal immune defense against enteric infections (Tu et al., 2020). This study aimed to investigate the effects of ovomucin and its hydrolysates on pathogenic colonization as well as underlying mechanisms in *C. rodentium*-induced murine colitis.

5.2 Materials and Methods

5.2.1 Ovomucin extraction and hydrolysis

Ovomucin extraction from liquid egg white was adapted from procedures reported previously (Ji et al., 2020; Omana & Wu, 2009). Briefly, 20 L of liquid egg white (Egg Solutions, Lethbridge, Canada) was diluted with 60 L of ddH_2O. Lysozyme was removed using Amberlite FPC 3500 ion-exchange resin (1 g/10 mL of egg white) (Abeyrathne et al., 2014). The supernatant containing ovomucin was subsequently collected and mixed with NaCl (0.1 M). After adjusting pH to 6.0 and standing overnight at 4℃, ovomucin precipitation was collected by centrifugation at 10,000 rpm at 4℃ for 10 min and then resuspended in 0.5 M NaCl solution, followed by centrifugation, ultrafiltration, and lyophilization (Omana & Wu, 2009).

Ovomucin hydrolysis by enzyme protex 26L or pepsin-pancreatin was performed based on methods published previously (Sun et al., 2016). Ovomucin was dispersed in Milli-Q water (1% w/v) to make a slurry. pH and temperature were adjusted as follows: pH 3.0 at 50℃ for protex 26L hydrolysis for 4 h; pH 2.0 at 37℃ for pepsin hydrolysis for 3 h prior to 3 h of pancreatin hydrolysis at pH of 6.5 at 37℃. After hydrolysis, samples were heated at 95℃ for 15 min before cooling to room temperature (RT). The supernatant was subsequently collected by centrifugation

and lyophilized. Ovomucin and hydrolysates were added to the mouse chow diet (ENVIGO T.7913M.15) at a final concentration of 2.5% (w/w).

5.2.2 Mice

The animal study was approved by the Animal Care and Use Committee of the University of Alberta and performed in accordance with the guidelines of the Canadian Council on Animal Care. Forty 5-week-old female C57Bl/6J mice (Jackson Laboratory, Bar Harbor, ME) were housed in an animal facility under specific pathogen-free conditions at the University of Alberta. All mice were housed in sterilized filter-topped cages and the room was environmentally controlled for temperature and light cycle (12-h light and 12-h darkness). Upon arrival, mice were randomly grouped into ten cages with 4 mice per cage by a blinded laboratory animal technician. After an acclimation period of one week, mice were allocated to 5 treatments: a control group (Ctrl), *C. rodentium* infection (CR), ovomucin supplementation (OVM), ovomucin-protex 26L hydrolysate supplementation (OP), and ovomucin-pepsin/pancreatin hydrolysate supplementation (OPP). Mice were given water and food *ad libitum* throughout the experiment.

To perform *C. rodentium* challenge, mice were screened by plating fecal samples on MacConkey agar (BD Difco) to confirm that mice were free of coliforms. CR, OVM, OP, and OPP mice were challenged with *C. rodentium* culture with approximately 1×10^8 colony forming units (CFUs) by oral gavage whereas Ctrl mice received the vehicle control. Fresh fecal pellets were collected at 1-, 3-, 5-, and 7 days post-infection (dpi) for *C. rodentium* enumeration using MacConkey agar. *C. rodentium* abundance was normalized to fecal sample weight. Body weights were recorded, and fresh fecal pellets were collected before and after *C. rodentium* challenge (Fig. 5.1A).

5.2.3 Bacterial strain

For C. *rodentium* infection, *C. rodentium* (DBS100) was cultured in Luria-Bertani broth at 37℃ for 16 h with shaking at 250 rpm. *C. rodentium* cells were enumerated using MacConkey agar.

For the *in vitro* culture study, M9 medium (12.8 g $Na_2HPO_4 \cdot 7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, and 1 g NH_4Cl dissolved in 1 L of ddH_2O containing 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.4% glucose) was used to give a minimal growth condition for *C. rodentium*. Approximately 5×10^6 CFUs of *C. rodentium* was inoculated to 10 mL of M9 medium or M9 medium supplemented with 2.5% of OP or OPP, followed by incubation at 37℃ for up to 12 h with shaking at 250 rpm. At each time point, bacterial cultures were taken for measuring optical density (OD_{600nm}) and enumeration on MacConkey plates.

5.2.4 *In vitro C. rodentium* adhesion

Mouse rectal epithelial cells (CMT-93) were seeded in 24-well plates at a density of 5×10^5 cells/well. Cells were grown until reaching confluence in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Ottawa, ON, Canada) supplemented with 10% of fetal bovine serum (FBS) in an atmosphere of 5% of CO_2 at 37°C as previously described (Bording-Jorgensen et al., 2021; Qiaochu Liang et al., 2023). Prior to infection, the medium was changed to serum and antibiotic-free DMEM. *C. rodentium* was added at the multiplicity of infection (MOI) of 50 for 5 h. After PBS wash for three times, cells were treated with 200 μL of 0.1% Triton X-100 at RT for 5 min. Adherent bacteria were counted by plating the serial dilutions of lysate samples onto MacConkey agar.

5.2.5 Sample collection

At 7 dpi, mice were euthanized by carbon dioxide asphyxiation. Ileal and colonic contents were collected, snap frozen in liquid nitrogen, and stored at -80℃ for downstream analyses. Terminal

5-mm segments of distal colon were collected and fixed in 10% formalin for histological analysis; the remaining colonic tissues were snap frozen in liquid nitrogen and stored at -80℃ for cytokine and tight junction protein analyses.

5.2.6 Histological analysis

Colonic tissues fixed in 10% neutral buffered formalin overnight were transferred into 70% ethanol and subsequently embedded in paraffin and cut into 5-µm sections. Fixed tissue sections were stained with hematoxylin and eosin(Ju et al., 2017; Wlodarska et al., 2011) and imaged by an EVOS FL Auto Imaging System (Thermo Scientific, Nepean, Ontario, Canada). Well-oriented cross sections were assessed for pathological scores of lumen, surface epithelium, mucosa, and submucosa by a pathologist using a scoring system as described previously (Forgie et al., 2023).

5.2.7 Colonic cytokine/chemokine analyses

Colonic tissues were ground with mortars and pestles in liquid nitrogen. Fifty to one hundred milligrams of tissue powder were homogenized in 1 mL of Tris lysis buffer (Meso Scale Discovery, Gaithersburg, MD) containing 150 mM NaCl, 20 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and protease inhibitors (Sigma Aldrich) to extract protein. The homogenates were centrifuged at 14,000 rpm at 4℃ for 10 min and the supernatant was collected. Protein concentrations were determined by a bicinchoninic acid protein assay kit (Thermo Scientific, Nepean, Ontario, Canada). A Pro-inflammatory Panel 1 (mouse) kit (Meso Scale Discovery, Gaithersburg, MD) was used to measure cytokine/chemokine levels according to the manufacturers' instructions. Cytokine/chemokine concentrations were normalized to the protein input (100 µg).

5.2.8 Western blot for tight junction protein expression

Protein extraction from colonic tissue was performed as mentioned above and concentrations were quantified using a bicinchoninic acid assay. Samples were mixed with Laemmli's buffer containing 2% 1,4-dithiothreitol and heated at 98°C for 5 min. Subsequently, samples with equal protein input (30 μg) were electrophoresed in 12% polyacrylamide gels and transferred to nitrocellulose membranes, which were blocked in 5% bovine serum albumin (dissolved in PBS containing 0.05% Tween 20) at RT for 1 h. The membranes were subsequently immunoblotted with primary anti-occludin (1:1000 dilution, Abcam) and anti-ZO-1 (1:1000 dilution, Abcam) antibodies overnight at 4°C. After 3 times of PBST wash (5 min/time), the membranes were incubated with a goat anti-rabbit secondary antibody at RT for 1 h and blots were developed with a LI-COR Odyssey Bioimager and quantified by Image Studio software (LI-COR Biosciences, Lincoln, NB, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping protein. Data were presented by the percentage of the Ctrl group.

5.2.9 DNA extraction and *C. rodentium* quantification

Total DNA from colonic contents was extracted using the QIA Fast DNA Stool Mini Kit (Qiagen, Valencia, CA) with an additional bead-beating step at 6.0 m/s for 60 s (FastPrep-24 5G instrument, MP Biomedicals). DNA concentrations were measured by a NanoDrop 2000c spectrophotometer (Thermo Scientific). *C. rodentium* genes and total bacterial load were quantified using real-time PCR on an ABI StepOne real-time system (Applied Biosystems, Foster City, CA) with SYBR Green PCR Master Mix (Applied Biosystems) following the program: 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C/58°C for 1 min. Primers for *espB* (Table S5.1) were applied for determining *C. rodentium* load and normalized by total bacteria abundance.

5.2.10 Microbial composition analysis

Total DNA was extracted from colonic and fecal contents as described above. Amplicon libraries were constructed targeting the V3-V4 region of the 16S rRNA gene according to a protocol from Illumina (16S Metagenomic Sequencing Library Preparation). A paired-end sequencing run with 2 x 300 cycles was performed on an Illumina MiSeq platform (Illumina Inc., San Diego, CA). Sequences were analyzed using the Quantitative Insights Into Microbial Ecology 2 (QIIME2) pipeline (Bolyen et al., 2019). The Divisive Amplicon Denoising Algorithm 2 plugin incorporated in QIIME2 was used to perform merging and denoising of de-multiplexed paired end reads. Forward and reverse reads were truncated at 270 bp and 220 bp, respectively. An amplicon sequence variant (ASV) feature table was constructed, and a pre-trained Naïve Bayes classifier (SILVA database (Quast et al., 2012), release 138, 99% identity) was used for taxonomical assignment. A tree was generated using the align-to-tree-mafft-fasttree pipeline in QIIME2. Downstream analyses were performed in R using multiple packages including phyloseq (McMurdie & Holmes, 2013) (v1.34.0), vegan (Oksanen et al., 2019) (v2.5-7), and DESeq2 (Love et al., 2014) (v1.30.1). The ASV table was filtered to exclude ASVs that showed up less than 1 time across all samples and that were shown in less than 3 times in at least 20% of analyzed samples. Taxa were agglomerated at each taxonomic rank using the tax_glom function.

5.2.11 Statistical analysis

Results were analyzed using the Prism 6 software (GraphPad Software, San Diego, CA) or R (v4.1.0) and data were expressed as the mean ± standard error of the mean (SEM). Body weight data were analyzed by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. *C. rodentium* load, cytokine/chemokine concentrations, gene and tight junction protein expression levels, and alpha diversity indices were tested for normality of distribution by

Shaporo-Wilk test and analyzed by one-way ANOVA, followed by Tukey's HSD *post-hoc* test for parametric data or Kruskal-Wallis test for nonparametric data with Dunn's *post-hoc* test, respectively. For microbial composition analyses, the Permutational Multivariate Analysis of Variance (PERMANOVA) test was performed to assess the difference in community structure (999 permutations, adonis2 function, vegan package, R v4.1.0). Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarities was plotted using the phyloseq package. Comparisons of individual ASVs between treatment groups were conducted using DESeq2 at a controlled false discovery rate (FDR) level of 5%. $P < 0.05$ was considered statistically significant.

5.2.12 Data availability

Raw sequence reads of the 16S rRNA gene amplicon data were deposited in the Sequence Read Archive under accession number PRJNA1035782.

5.3 Results

5.3.1 OP reduced *C. rodentium* colonization in the mouse gut

During the three weeks of feeding prior to *C. rodentium* infection, no significant difference in the body weight was observed among treatment groups. However, mice in the OPP group showed a higher body weight increase than the OP group at day 14 ($P = 0.030$, Fig. 5.1B). At the end of the third week, mice were orally inoculated with *C. rodentium* or vehicle control. After infection, body weights of mice in CR, OP, and OPP groups were not significantly influenced when compared with the Ctrl group throughout the infection period, while the OVM group showed decreased body weights compared with CR and OP groups at 7 dpi ($P = 0.027$ and 0.030, respectively). OVM group showed a lower body weight increase than the CR group at 1 dpi ($P = 0.037$, Fig. 5.1B). Throughout the infection period, OVM and OPP treatments had numerically higher *C. rodentium* counts than the CR group, while OP supplementation had the lowest *C. rodentium* level throughout

the challenge, reaching significance at 5 dpi compared with OVM and OPP groups ($P = 0.030$ and 0.019, respectively, Fig. 5.1C). At 7 dpi, OP treatment led to lower *C. rodentium* load compared with OVM, OPP and CR groups ($P = 0.004$, 0.004, and 0.003, respectively, Fig. 5.1C & D). Quantitative measure of *C. rodentium* virulence factor *EspB* relative to total bacteria consistently showed that OP mice had lowest *C. rodentium* load at 7 dpi (Fig. 5.1D).

5.3.2 OP attenuated *C. rodentium*-induced colitis

Histological analyses of colon sections showed that mice developed colonic hyperplasia after *C. rodentium* infection, a typical feature of the colitis model (Fig. 5.2A-C). OVM and OP groups showed less pathological damage to the colonic epithelium and mucosa when compared with the CR group (Fig. 5.2D-E & G, respectively). Specifically, mice fed the OVM diet had less surface regeneration and ulceration, and the OP group exhibited less goblet cell depletion when compared with the CR group (Fig. 5.2H & I). Mice in the OPP group had more severe damage to the surface epithelium than that in the CR group as indicated by increased surface regeneration and desquamation (Fig. 5.2F-H). Tight junction proteins (occludin and ZO-1) that play a crucial role in regulating intestinal barrier integrity in colon tissues were determined (Fig. S5.1A & B). *C. rodentium* colonization did not alter the expression of occludin and ZO-1 in the mouse colon, while mice in the OPP group showed significantly decreased expression of occludin when compared with the CR group ($P = 0.012$). The highest expression level of occludin and ZO-1 were observed in the OP group (Fig. S5.1A & B). Together with the results from histological analysis, OP showed protective effects by decreasing *C. rodentium* colonization and mitigating pathological changes in colonic mucosa.

5.3.3 OVM and OP treatments decreased colonic inflammatory tone after *C. rodentium* infection

Concentrations of inflammatory cytokines and chemokines in the colon at 7 dpi were measured to investigate the impact of OVM, OP, and OPP on *C. rodentium*-induced inflammation. Compared with the Ctrl group, *C. rodentium* infection significantly increased the production of colonic chemokines and cytokines including MCP-1, IFN-γ, IL-10, IL-1β, IL-2, IL-6, TNF-α, and KC/GRO (Table 5.1). OVM and OP mice showed a trend of decreased proinflammatory responses after *C. rodentium* colonization, in line with lower abundance of *C. rodentium* in the mouse colon at 7 dpi (Fig. 5.1C) and improved pathological scores (Fig. 5.2). On the contrary, OPP supplementation promoted the production of pro-inflammatory chemokines and cytokines after *C. rodentium* infection (Table 5.1), which was consistent with higher *C. rodentium* burden in the mouse colon at 7 dpi (Fig. 5.1C) and more severe pathological damage to the surface epithelium compared with the CR group (Fig. 5.2H).

5.3.4 OVM, OP, and OPP differentially modulated gut microbial community

Gut microbial composition was characterized by sequencing 16S rRNA gene amplicons from mouse fecal samples before and 7 days after *C. rodentium* infection. Three weeks of feeding on OVM, OP, or OPP did not significantly alter gut microbial community structures (adonis $P > 0.05$, Fig. 5.3A). However, the relative abundance of certain bacterial taxa was influenced as indicated by results from DESeq2 analyses (Fig. S5.2). Differences were observed in the relative abundance of bacterial taxa including *Lachnospiraceae*, *Ruminococcaceae*, *Oscillospiraceae*, *Clostridia vadinBB60 group*, *Monoglobaceae*, and *Staphylococcaceae* among treatment groups. Specifically, *Anaerotruncus* (ASV78) and *Oscillibacter* (ASV27) tended to be lower in the OP group when compared with the Ctrl group (both $P = 0.079$), while the abundance of *Romboutsia* (ASV79) was

significantly lower in the OPP group compared with Ctrl, OVM, and OP groups (all $P < 0.001$, Fig. S5.2).

After *C. rodentium* challenge, OVM and OP supplementation both maintained the richness of microbial communities, while OPP significantly decreased α-diversity indices of gut microbial communities as indicated by Chao1 and PD indices (Fig. 5.3B), suggesting that the enhanced damage to the colonic epithelium of mice in the OPP group in response to *C. rodentium* challenge was accompanied with alterations in the gut microbial structure. *C. rodentium* colonization induced major shifts in microbial composition at 7 dpi compared with that in the Ctrl group (Fig. 5.4) with higher *Clostridia vadinBB60 group* (ASV23) and a trend for lower abundance of *Ruminococcaseae* (ASV98) in CR mice ($P = 0.020$ and 0.085, respectively, Fig. S5.3A). With the presence of *C. rodentium*, OVM supplementation tended to result in higher relative abundance of families *Oscillospiraceae* (ASV11, ASV16, and ASV15, $P = 0.097$, 0.093, and 0.093, respectively), *Lachnospiraceae* (ASV47 and ASV25, $P = 0.088$ and 0.093, respectively), and *Ruminococcaceae* (ASV76, $P = 0.093$), as well as lower abundance of *Muribaculaceae* (ASV1, $P = 0.088$) and *Lachnospiraceae NK4A136 group* (ASV45, $P = 0.088$) compared with the CR group (Fig. S5.3B). OP supplementation significantly increased the population of the genus *Ruminococcaceae* (ASV98) when compared with the CR group ($P = 0.047$, Fig. S5.3C), while the OPP treatment dramatically reduced the population of *Romboutsia* (ASV79) when compared with CR, OVM, and OP groups (all $P < 0.001$, Fig. S5.3D, F & G). Additionally, OPP showed lower abundance of *Lachnospiraceae* (ASV59, $P = 0.006$) than the OVM group and higher abundance of Turicibacter (ASV7, $P = 0.047$), *Akkermansia* (ASV10, $P = 0.047$) and *Anaerotruncus* (ASV78, $P = 0.021$) than the OP group.

Spearman correlation analysis was applied to assess the relationship between significantly changed microbes and colonic cytokine/chemokine profiles. *C. rodentium* (ASV4) and *Akkermansia muciniphila* (ASV10) were positively associated with levels of MCP-1, KC/GRO, TNF-α, IL-6, IL-1β, IL-10, and IFN-γ (Fig. 5.5). The genus *Clostridia vadinBB60* group (ASV23) was positively associated with IL-2 level. In contrast, *[Eubacterium] coprostanoligenes* group (ASV86) was negatively associated with TNF-α, IL-6, IL-2, IL-1β, and IFN-γ. A *Lachnospiraceae* member (ASV24) had a negative association with TNF-α, IL-6, and IFN-γ, whereas *Lachnospiraceae NK4A136* group (ASV45) and *Romboutsia* (ASV79) were negatively associated with IFN-γ. These results suggested that the different effect of OVM, OP, and OPP on *C. rodentium*-induced inflammatory cytokines/chemokines in the colon were related to their impact on intestinal microbial communities.

5.3.5 OPP supported *in vitro* growth of *C. rodentium*

In vitro culture of *C. rodentium* in M9 minimal media supplemented with 2.5% of OP or OPP was conducted, aiming to explain the different impact of OP and OPP on *C. rodentium* colonization. The growth rate of *C. rodentium* was measured 4, 6, 8, and 10 h after incubation. Increased proliferation of *C. rodentium* was observed at 6 h of incubation in OPP-supplementation group when compared with that in the OP group (P = 0.011, Fig. 5.6 & S5.4A), while no obvious difference was determined at the other timepoints (Fig. S5.4B). In DMEM, OP and OPP supplementation supported *C. rodentium* proliferation and adhesion to CMT-93 cells after incubation for 5 h, compared with the control group. The levels of adherent bacteria to CMT-93 cells were comparable in OP and OPP groups, although the OPP group showed numerically higher bacterial adhesion (Fig. S5.5).

5.4 Discussion

The present study demonstrated that ovomucin and its hydrolysates can differentially influence the severity of *C. rodentium*-induced colitis. While OP supplementation resulted in lower *C. rodentium* loads and pathology, OPP was consistently associated with the highest pathogen loads and enhanced pathological changes in the gut. Mice fed OVM and OP both showed decreased proinflammatory tone in response to *C. rodentium* infection, in accordance with less severe damage to the surface epithelium and mucosa in colon.

It is well established that mice infected with *C. rodentium* show colonic hyperplasia, disruption of normal colonic architecture, and goblet cell depletion (Collins et al., 2014). In the current study, mice in the CR group showed thickening of colon and enhanced damage to colonic lumen, epithelium, and mucosa. Specifically, *C. rodentium* induced significant ulceration, desquamation, and epithelial regeneration in colonic epithelial surface with profound depletion of goblet cells. Mice in the OP group exhibited the lowest pathological scores in the colon with maintained population of goblet cells, which was in line with lower levels of *C. rodentium* colonization during the infection period. Goblet cells are the source of mucin that lubricates the intestinal epithelium, shapes the gut microbial community, and protects against luminal antigens and pathogens (Pelaseyed et al., 2014), suggesting the positive effect of OP on restoring *C. rodentium*-induced goblet cell depletion. On the other hand, OVM did not influence pathogenic load in the mouse colon while also protected mice from *C. rodentium*-induced damage to colonic epithelial surface. These results indicated different mechanisms by which OVM and OP alleviated the impairment of the gut barrier function in *C. rodentium*-induced colitis. The OPP group showed no ameliorative effect on colonic pathology, consistent with its numerically higher load of *C. rodentium* than the CR group at 7 dpi. Tight junction proteins, including claudins, ZO-1, and

occludin, have been well acknowledged in regulating intestinal integrity in response to *C. rodentium* infection (Kim et al., 2017; Liu et al., 2023; Zhang et al., 2021). Generally, *C. rodentium* induces disorganization of tight junction proteins in epithelial cell surface and increases intestinal permeability that is in parallel with inflammation, hyperplasia, and bacterial dissemination to spleen and liver (Ahmed et al., 2010; Guttman et al., 2006). However, contradictory results have been reported regarding changes in tight junction proteins in the context of *C. rodentium* infection. *C. rodentium* administered at 2.5×10^8 CFUs did not significantly impact ZO-1 expression in C57Bl/6J mice at 6 dpi (Gibson et al., 2008b). In the present study, *C. rodentium* infection (at 1×10^8 CFUs) did not significantly influence the expression of ZO-1. OP treatment showed the highest levels of occludin and ZO-1 in the mouse colon at 7 dpi, which was consistent with findings from our LPS-induced Caco-2 cell study (Chapter 3) (Bao & Wu, 2021). Collectively, our data indicated the possible role of OP in reducing *C. rodentium* colonization, modulating tight junction proteins and protecting intestinal integrity to alleviate *C. rodentium*-induced murine colitis.

Chemokine MCP-1 and KC/GRO in mouse colon tissues were significantly upregulated by *C. rodentium* infection in the CR group when compared to that in the Ctrl group, consistent with previous studies (Gibson et al., 2008b). Mice in the OVM and OP group demonstrated consistently lower levels of MCP-1 and KC/GRO, in accordance with reduced colonic pathological scores. However, OPP treatment induced higher production of MCP-1 in infected mice compared to the CR group ($P > 0.05$), which suggested a distinct action of OPP on regulating colonic mucosal immunity in response to *C. rodentium* infection. Upregulation of IL-6, TNF-α, IFN-γ, and IL-1β has been considered as a protective effect against *C. rodentium* infection reflected by increased susceptibility to *C. rodentium*, exacerbated colonic mucosal injury, and delayed bacterial eradication in mice with a deficiency of these cytokines (Gibson et al., 2008b; Gonçalves et al.,

2001; Kitamura et al., 2004; Simmons et al., 2002; Spahn et al., 2004). However, excess secretion of cytokines can exert an opposite effect, highlighting the importance of immune homeostasis in the context of pathogenic infection (Alipour et al., 2013; Kayagaki et al., 2011). IL-10 and IL-2 are also induced during *C. rodentium* infection, which are demonstrated to worsen colitis severity because a deficiency of IL-10 can mitigate infection-associated colitis and facilitate pathogen clearance and IL-2 deficiency in *C. rodentium*-infected mice can generate effective host defense (Dann et al., 2014; Krause et al., 2015; Laurence et al., 2007; Wang et al., 2014). In the current study, OP treatment resulted in lower production of these chemokines and cytokines, which was consistent with lower *C. rodentium* load in the mouse gut at 7 dpi. OVM treatment resulted in lower secretion of chemokines and cytokines except for IL-6 when compared with the CR group, suggesting that OVM might confer protection through distinct mechanisms from OP. OPP treatment triggered a higher but not significant secretion of MCP-1, IL-1β, and IL-6 with a lower production of IL-2 and TNF-α when compared with the CR group, in line with modest reduction of pathology in mucosa but no obvious alteration in the overall colonic pathologic score. Collectively, OPP group showed enhanced inflammation in response to *C. rodentium* infection, indicating the importance of considering different hydrolysis methods for generating bioactive peptides from proteins.

The gut microbiota shapes immune responses and maintains intestinal homeostasis, and *C. rodentium* relies on gut commensals for colonization in the distal colon (Mullineaux-Sanders et al., 2017). Therefore, we investigated the impact of dietary treatment on gut microbial structure before and after infection. OVM, OP, and OPP administration for 3 weeks did not shift the overall structure of the gut microbiota. However, there were alterations in certain bacterial families including *Lachnospiraceae* and *Ruminococcaceae* that are known for short-chain fatty acid (SCFA)

production (Vital et al., 2014). Specifically, the SCFA-producing *Clostridial vadinBB60* was enriched in OVM and OP groups compared to the Ctrl group. In murine colitis, a decreased abundance of *Lachnospiraceae, Ruminococcaceae*, and *Clostridiales vadinBB60* has been characterized (Harrison et al., 2018). OP group also exhibited a moderate increase in *Lachnospiraceae NK4A136 group* than the OVM group, while OVM group showed increased *Anaerotruncus* and *Oscillibacter*-related taxa. *Lachnospiraceae NK4A136 group* has been negatively correlated with the enteric pathogens including *C. rodentium* due to colonization resistance (Forgie et al., 2023; Mullineaux-Sanders et al., 2017; Perez-Lopez et al., 2019). *Anaerotruncus* and *Oscillibacter* can produce SCFAs from non-plant-derived glycans and backbones of mucin protein (Gophna et al., 2017; Raimondi et al., 2021), consistent with the fact that ovomucin is a highly glycosylated protein belonging to the mucin family (Donovan et al., 1970; Omana et al., 2010). OVM modulating SCFA-producers and OP reinforcing colonization of resistance-related microbial members, both before and after *C. rodentium* infection, might indicate their distinct protective mechanisms. However, compared to the other groups, the OPP group showed a consistently lower level of *Romboutsia* regardless of *C. rodentium* infection, which is a SCFA-producing bacterium involved in chronic inflammation in the intestinal mucosa (Cao et al., 2021). *Romboutsia* also exhibits a positive correlation with inflammatory cytokines including IL-6, IL-1β, TNF-α, and IL-23, suggesting its role in mediating the development of chronic inflammatory diseases (Cao et al., 2021; Li et al., 2021). Additionally, at 7 dpi, the OPP group had higher abundance of *Akkermansia*, *Anaerotruncus*, *Turicibacter*, and *Clostridium sensu stricto 1* than OP and/or OVM groups. *Akkermansia muciniphila* is a mucin-degrading bacterium belonging to the phylum Verrucomicrobiota (Luo et al., 2022). A higher abundance of *Turicibacter* is observed in murine colitis and patients with inflammatory bowel disease (IBD) (Bosshard et al.,

2002), with *Clostridium sensu stricto 1* overgrowth involved in porcine colonic inflammation (Hu et al., 2021) and poultry necrotic enteritis (Yang et al., 2019). Thus, increased mucus degradation in the OPP group, along with a depletion of goblet cells and presence of opportunistic pathogens, might explain its different efficacy in response to *C. rodentium* infection from OP and OVM.

Latest research has demonstrated that *C. rodentium* could utilize sialic acid to fuel their growth and production of virulence factors, facilitating their transition and adhesion to the mucosal niche (Liang et al., 2023). Compared with OP, OPP contains higher contents of carbohydrate moieties, sialic acid (1.7% versus 0.1%), and peptides of larger molecular weights and lower amounts of essential amino acids (Sun et al., 2016). Given that glycoprotein-derived O-glycan sugars, including sialic acid, N-acetylglucosamine, mannose, and galactose can be catabolized by A/E bacterial pathogens facilitating proliferation and mucosal adhesion, and exacerbating colitis (Liang et al., 2023; Liang et al., 2002; Liang et al., 2019; Mslati et al., 2022), it was speculated that different contents of glycans/sialic acid in OP and OPP, to some extent, serve as a potential contributor to their discrepant efficacies in regulating *C. rodentium* colonization. This is supported by research demonstrating that increased sialidase activity and upregulated sialic acid catabolism in the intestine indeed triggered microbial dysbiosis and intestinal inflammation (Hasler et al., 2022; Huang et al., 2015). In this study, *in vitro* culture of *C. rodentium* in the minimal media with a supplementation of OP or OPP showed that OPP exhibited a better promotive effect on *C. rodentium* growth than OP. OP and OPP consistently promoted *C. rodentium* growth in DMEM, wherein OPP supplementation showed numerically higher *C. rodentium* adhesion to CMT-93 cells than OP. Collectively, these data suggested that the supplementation of OPP might facilitate the growth rate of *C. rodentium* to support their early colonization in the gut, while the higher content of sialic acids contained in OPP might contribute to favoring *C. rodentium* pathology and

exacerbating colitis severity. On the other hand, OVM includes higher sialic acid (~4.0% with the β-subunit containing 13.8%) (Donovan et al., 1970; Omana et al., 2010), but did not trigger colitis severity as that in the OPP group, which could be due to increased accessibility and utilization of sialic acids to microbes after liberation from the termination of glycans by enzymatic hydrolysis in the OPP group (Ng et al., 2013). However, further studies are warranted to investigate other components and factors in OP and OPP that account for their different effects on *C. rodentium* infection. OP supported *C. rodentium* growth *in vitro* but did not affect bacterial adhesion to cultured CMT-93 cells as the ratios of adherent bacteria to total bacteria were comparable between the OP and DMEM control groups (0.12 versus 0.14, respectively, without significant difference). Although the adherent *C. rodentium* to mouse colonic mucosa was not directly quantified in this study, however, *C. rodentium* load in mouse colon (Fig. 5.1C) could indirectly reflect potential levels of *C. rodentium* adhesion to intestinal epithelial cells since microbial colonization in the outer layer of mucus contributes to subsequent mucus penetration and epithelial adhesion. Combining with the gut microbial analyses, OP was therefore concluded to reduce *C. rodentium* colonization in mice through modulating gut microbiota, rather than through directly inhibiting *C. rodentium* growth.

In conclusion, this study demonstrated that OP could attenuate *C. rodentium*-induced colitis through inhibiting *C. rodentium* colonization, mitigating proinflammatory response, and modulating the abundance of beneficial gut microbes that produce SCFAs and improve colonization resistance to *C. rodentium*. OVM alleviated colonic inflammation and exerted protective effect to a lesser extent which was likely through regulating SCFA producers but not directly suppressing *C. rodentium* colonization. However, OPP supplementation showed reduced gut microbial richness and enhanced pathological severity. Our study indicated the potential of

OVM and OP in preventing enteric infection and managing intestinal disease like IBD and suggested different efficacy of OVM's hydrolases such as OP in impacting host resistance to *C. rodentium* infection. This study also provided insights into the practical application of ovomucin protein hydrolysates in the treatment of intestinal disease.

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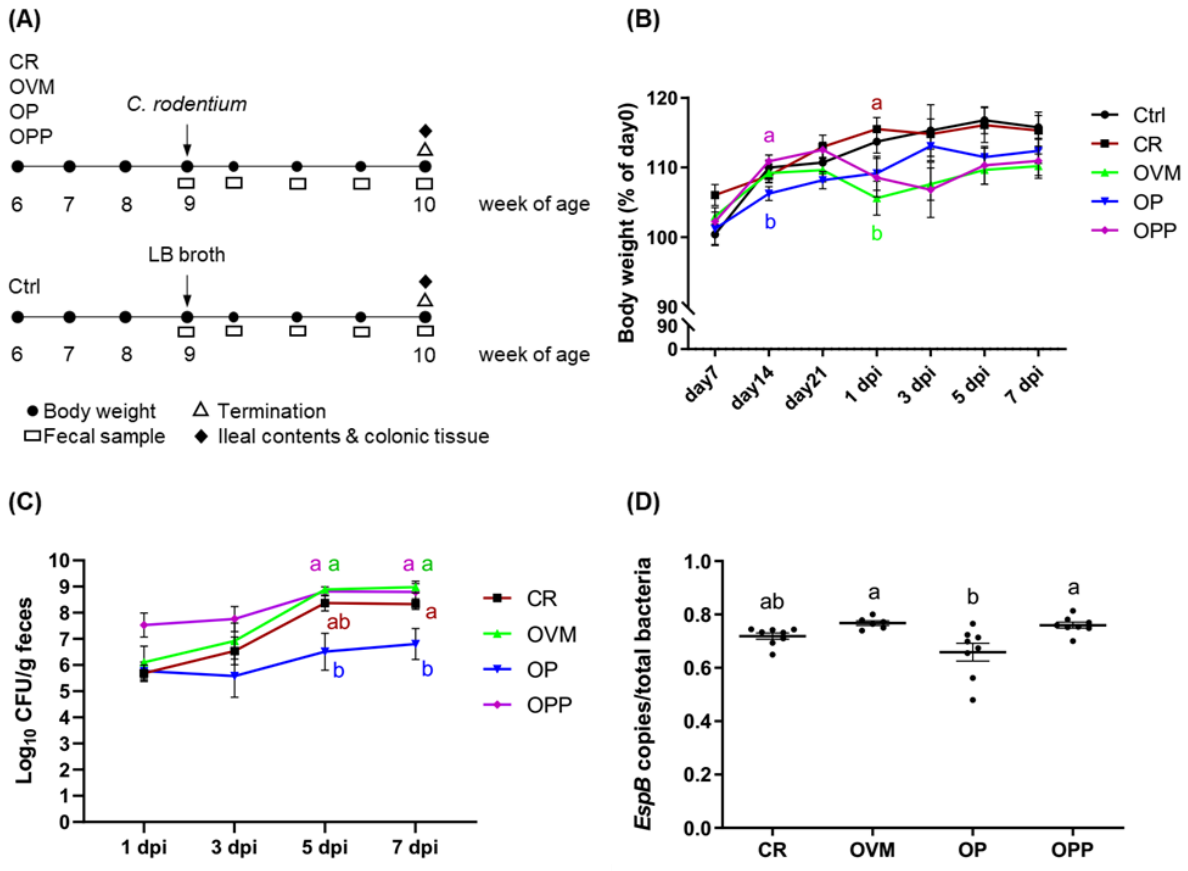

Fig. 5.1 (A) Experimental timeline. The body weight of mice before infection was recorded weekly. *C. rodentium* at a concentration of 1.0×10^9 CFUs/mL was given to mice (0.1 mL/mouse). After infection, body weight (B) and fecal samples were collected at 1, 3, 5, and 7 dpi. (C) *C. rodentium* load in mouse gut. Comparison was conducted at the same day. (D) Relative abundance of *C. rodentium* in the mouse gut at 7 dpi measured by qPCR analysis. Each dot represents an individual mouse. All data are expressed as mean ± SEM. Ctrl, untreated control group; CR, *C. rodentium* group; OVM, ovomucin group; OP, ovomucin-protex 26L hydrolysate group; OPP, ovomucin-pepsin/pancreatin hydrolysate group; CFUs, colony-forming units. Groups that do not share a letter (a or b) are significantly different.

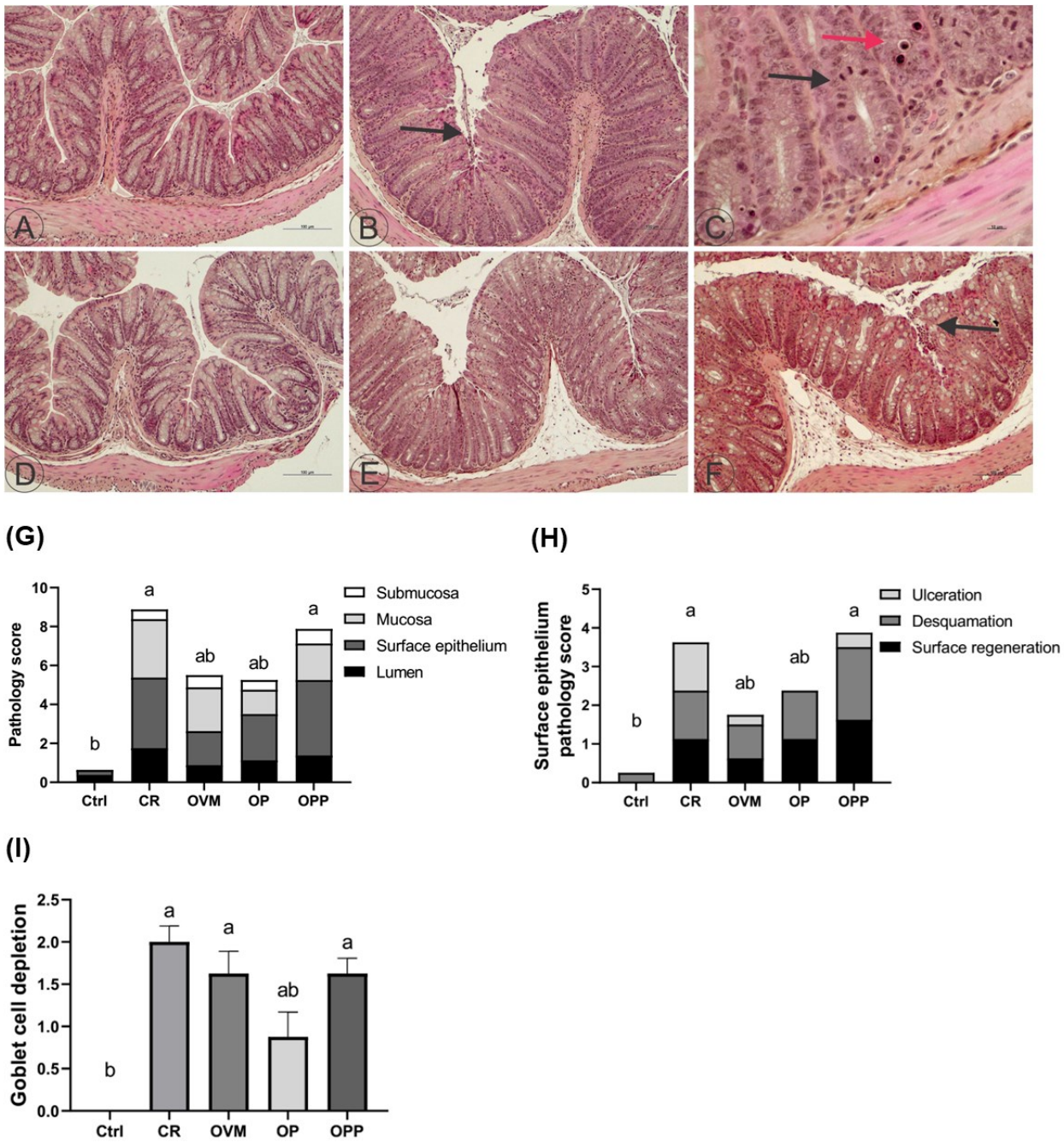

Fig. 5.2 Histological staining and pathological scoring of distal colon sections. (A) Mice in the Ctrl group showing normal architecture and no neutrophils infiltration, ulceration, or desquamation. (B-C) CR mice at low and high magnification, respectively, showing some epithelial shedding with mononuclear cells (arrow in panel B) and increased surface regeneration with mitotic figures (black arrow in panel C), along with nearby goblet cell depletion, neutrophils, and apoptotic figures

in crypts (red arrow in panel C). Mice in the OVM (D), OP (E), and OPP groups (F) exhibited increased cellularity in the lamina propria, with goblet cell depletion and prominent ulceration (arrow in panel F). Magnification and bars are shown in the lower right corner: ×100 original magnification for panels A-B and D-F, and ×400 original magnification for the panel C. (G-I) Pathological scores of colon tissue at 7 dpi. The assessment included the inflammation and damage to lumen, surface epithelium, mucosa, submucosa, and the number of goblet cells. Ctrl, untreated control group; CR, *C. rodentium* group; OVM, ovomucin group; OP, ovomucin-protex 26L hydrolysate group; OPP, ovomucin-pepsin/pancreatin hydrolysate group. All data are expressed as mean ± SEM (n = 8). Groups that do not share a letter are significantly different according to the Kruskal-Wallis test and Dunn's test ($\alpha = 0.05$).

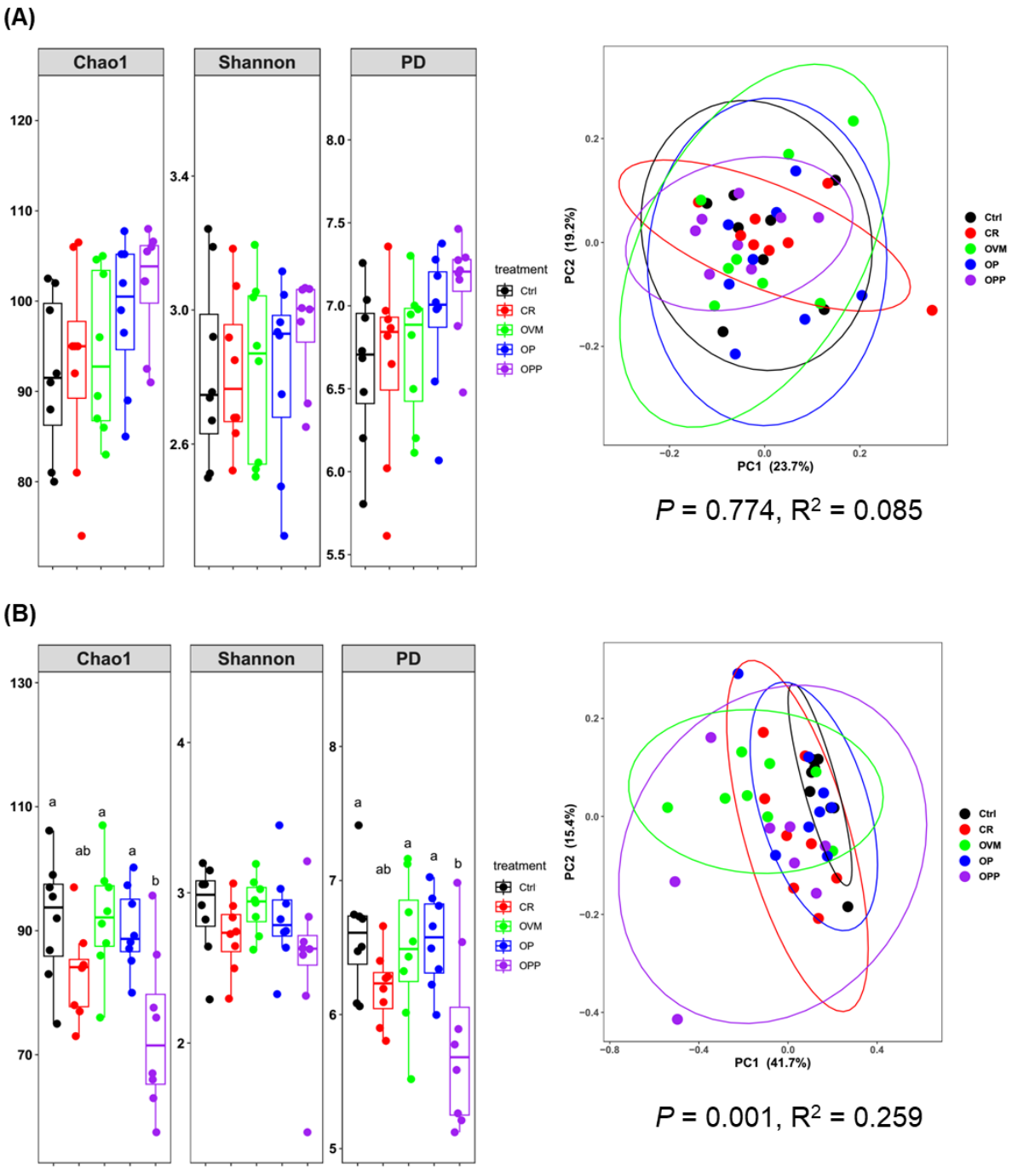

Fig. 5.3 Principal coordinate analysis (PCoA) plots based on Bray-Curtis dissimilarities and α-diversity indices of microbial community structures before (A) and after (B) *C. rodentium* infection. Each point represents an individual mouse. Ctrl, untreated control group; CR, *C. rodentium* group; OVM, ovomucin group; OP, ovomucin-protex 26L hydrolysate group; OPP,

ovomucin-pepsin/pancreatin hydrolysate group. Data are expressed as box plots in which the boxes represent the 25^{th}-75^{th} percentiles and the lines in the boxes indicate the medians. Significant difference was indicated by different letters ($P < 0.05$; n = 8).

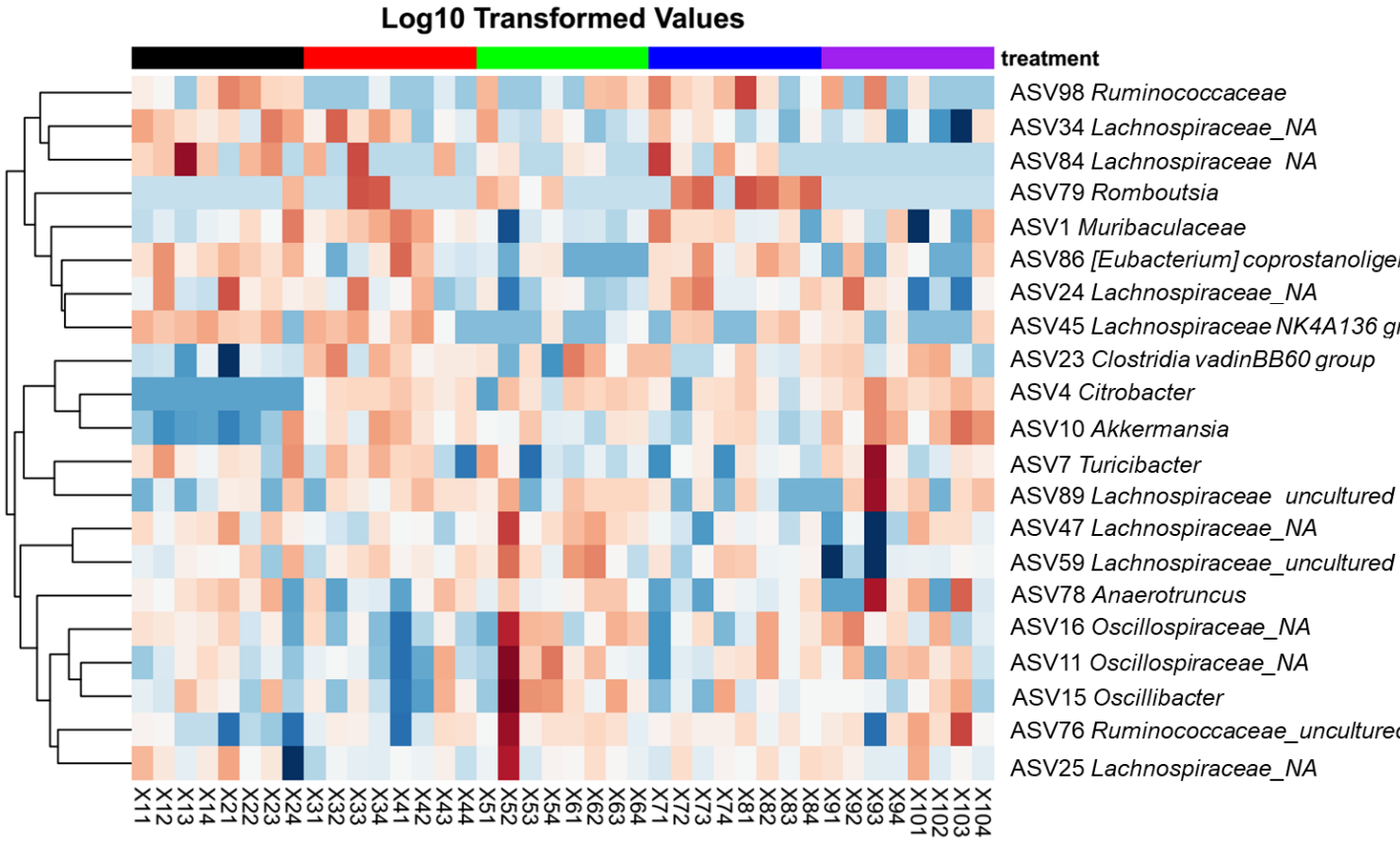

Fig. 5.4 Heatmap showing changes in the relative abundance of identified microbes at the genus level among different groups at 7 dpi of *C. rodentium* colonization. Ctrl, untreated control group; CR, *C. rodentium* group; OVM, ovomucin group; OP, ovomucin-protex 26L hydrolysate group; OPP, ovomucin-pepsin/pancreatin hydrolysate group.

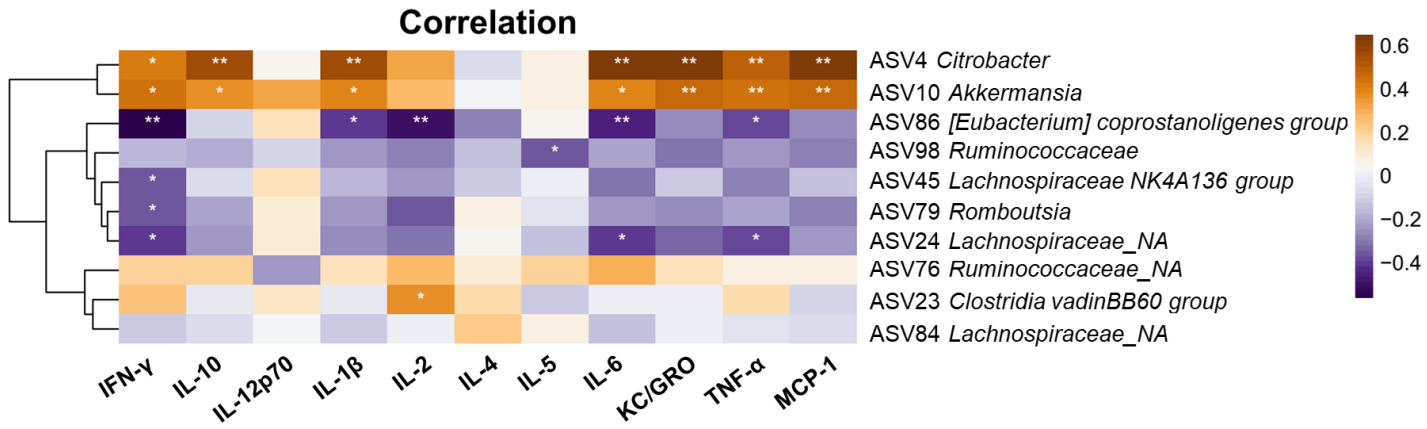

Fig. 5.5 The relationship between microbial composition at the genus level and cytokines/chemokines in mouse colon. The color depth corresponded to the extent of relevance between gut microbiota and cytokines/chemokines. The colors represent r values. *$P < 0.05$, and **$P < 0.01$.

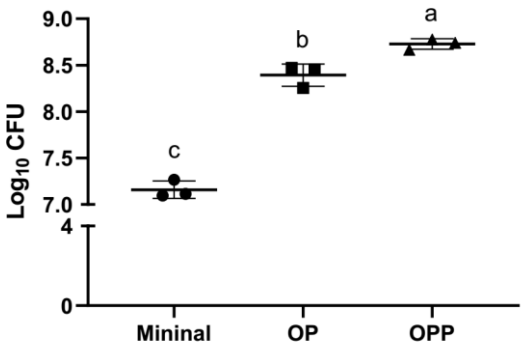

Fig. 5.6 Growth of *C. rodentium* in M9 minimal media supplemented with 2.5% of OP or OPP. Data are expressed as mean ± SEM. OP, ovomucin-protex 26L hydrolysate group; OPP, ovomucin-pepsin/pancreatin hydrolysate group. Groups that do not share a letter are significantly different (one-way ANOVA and Tukey's test) ($\alpha = 0.05$).

Table 5.1 Colonic cytokine/chemokine levels at 7 dpi

Cytokines/	Ctrl (pg/mg protein)		CR (pg/mg protein)		OVM (pg/mg protein)		OP (pg/mg protein)		OPP (pg/mg protein)		
Chemokines	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	*P*
MCP-1	6.52^{b}	0.30	53.89^{a}	12.61	35.56^{ab}	11.83	27.45^{ab}	8.84	103.77^{a}	35.99	0.0013
IFN-γ	0.08^{b}	0.02	3.44^{a}	1.36	2.46^{ab}	1.23	0.82^{ab}	0.41	3.82^{a}	0.89	0.0007
IL-10	1.54^{b}	0.11	2.36^{a}	0.16	2.16^{ab}	0.18	1.97^{ab}	0.26	2.71^{a}	0.24	0.0029
IL-12p70	12.69	0.66	14.27	0.57	12.32	0.74	13.03	0.36	12.94	0.50	0.1976
IL-1β	2.56^{c}	0.19	25.49^{ab}	4.16	12.92^{bc}	3.69	12.13^{bc}	4.37	35.99^{a}	8.51	0.0004
IL-2	0.28^{b}	0.02	4.67^{a}	1.86	3.13^{a}	0.76	1.07^{ab}	0.29	3.36^{a}	0.63	0.0013
IL-4	0.03	0.01	0.07	0.02	0.03	0.01	0.03	0.01	0.03	0.01	0.1753
IL-5	0.16	0.03	0.15	0.02	0.13	0.02	0.12	0.01	0.14	0.01	0.2069
IL-6	1.30^{c}	0.07	42.07^{ab}	10.17	60.59^{abc}	24.73	7.34^{bc}	2.77	169.11^{a}	67.97	0.0003
KC/GRO	2.68^{b}	0.11	33.95^{a}	5.98	22.68^{ab}	5.54	15.96^{ab}	4.46	39.05^{a}	7.26	0.0003
TNF-α	1.01^{b}	0.10	9.04^{a}	1.83	6.45^{ab}	2.27	3.62^{ab}	1.20	8.69^{a}	1.75	0.0008

OVM, ovomucin group; CR, *C. rodentium* group; OP, ovomucin-protex 26L hydrolysate group; OPP, ovomucin-pepsin/pancreatin hydrolysate group; MCP-1/CCL2, monocyte chemoattractant protein-1/(C-C motif) ligand 2; Keratinocyte chemoattractant/human growth-regulated oncogene (KC/GRO), C-X-C-motif chemokine ligand 1 (CXCL1).

[a, b, c] Mean values within a row with unlike superscript letters are significantly different according to one-way ANOVA or the Kruskal–Wallis test combined with the Tukey or Dunn's test ($\alpha = 0.05$).

The lower detection limit of the listed cytokines is 0.015 pg/mg protein.

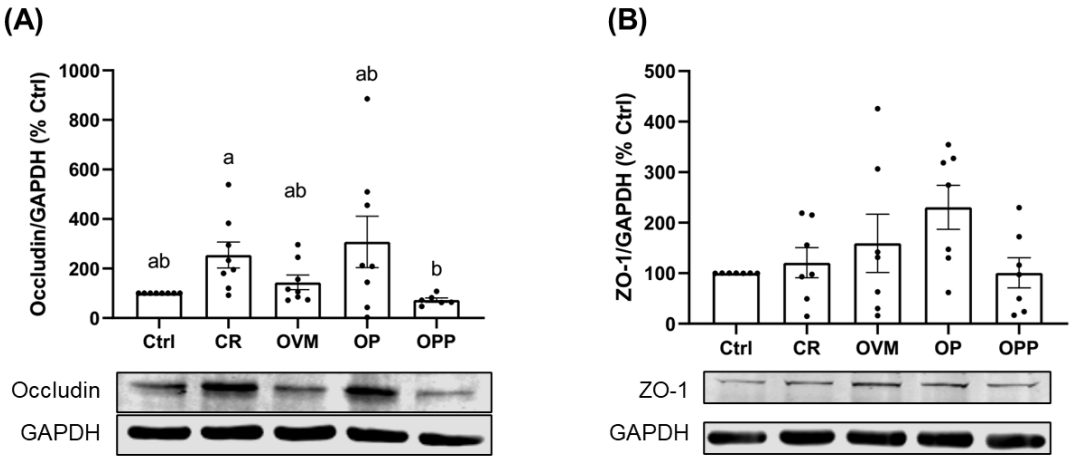

Fig. S5.1. Expression levels of tight junction protein occludin and ZO-1 in mouse colon tissues at 7 dpi. Data are expressed as mean ± SEM (n=8). Ctrl, untreated control group; OVM, ovomucin group; OP, ovomucin-protex 26L hydrolysate group; OPP, ovomucin-pepsin/pancreatin hydrolysate group.

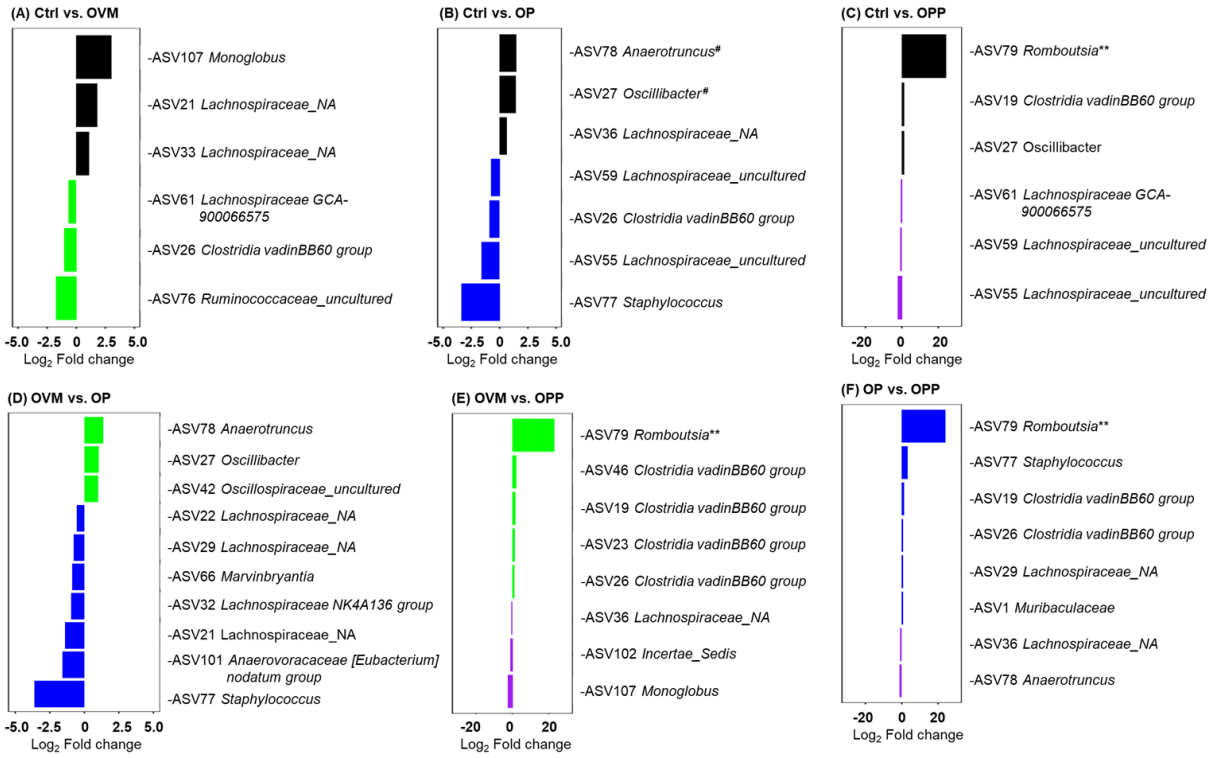

Fig. S5.2 Comparison of relative abundance of bacterial taxonomies before *C. rodentium* infection. *$P < 0.05$, **$P < 0.01$, #$P < 0.1$, according to the Kruskal-Wallis test combined with Dunn's test.

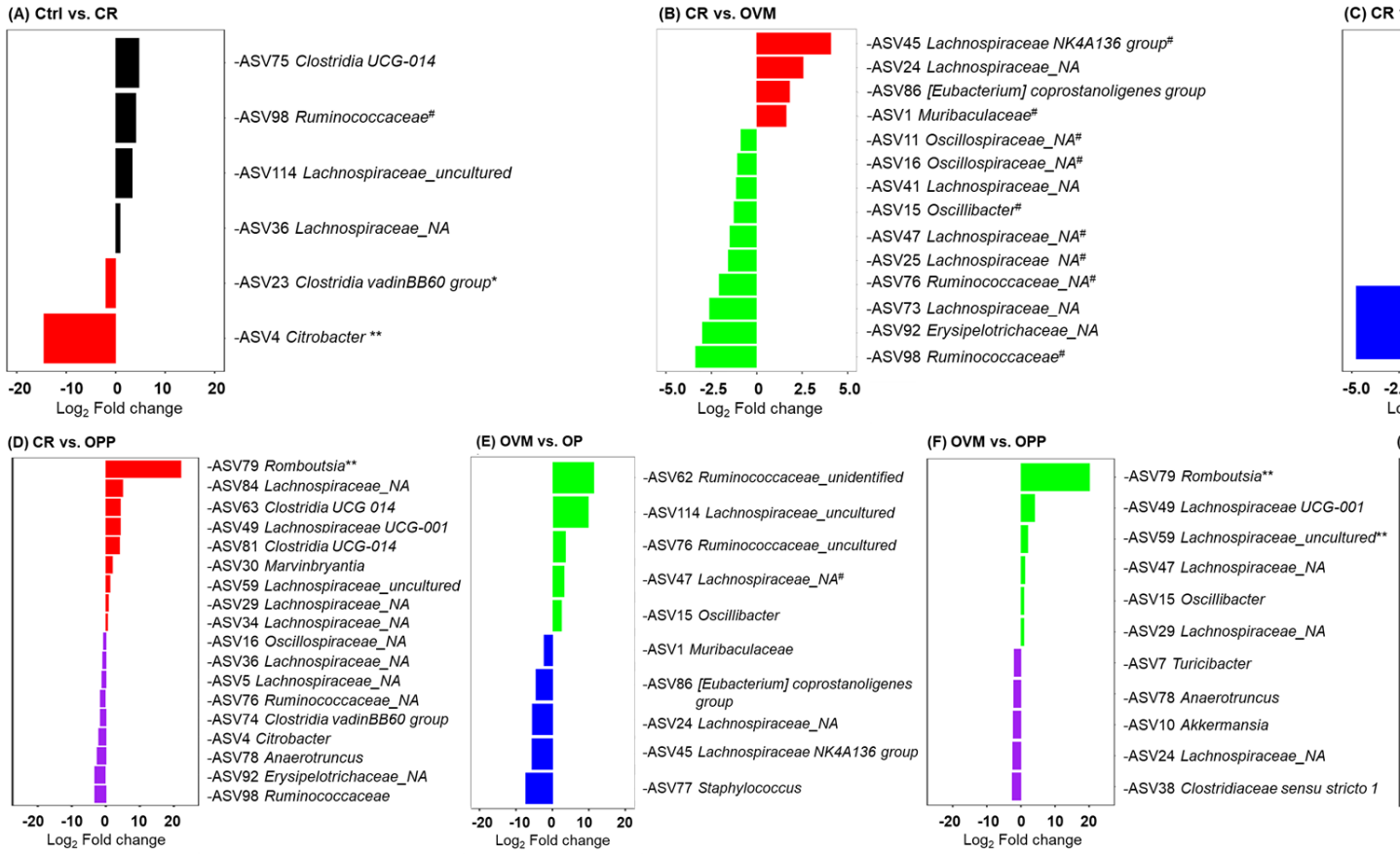

Fig. S5.3 Comparison of relative abundance of bacterial taxonomies at 7dpi. **P* < 0.05, ***P* < 0.01, #*P* < 0.1, according to the Kruskal-Wallis test combined with Dunn's test.

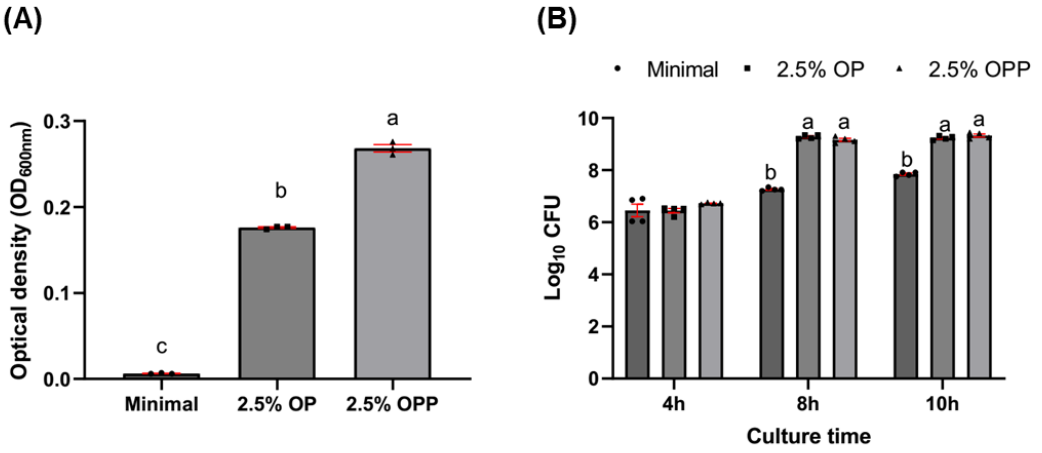

Fig. S5.4 Growth of *C. rodentium* in M9 minimal media supplemented with 2.5% of OP and OPP. (A) Optical density at 600nm of *C. rodentium* after 6 h culture in M9 media. (B) *C. rodentium* quantification after cultivation in M9 media for different time periods. Data are expressed as mean ± SEM (n=3-4). Groups that do not share a letter are significantly different (one-way ANOVA and Tukey's test) ($\alpha = 0.05$).

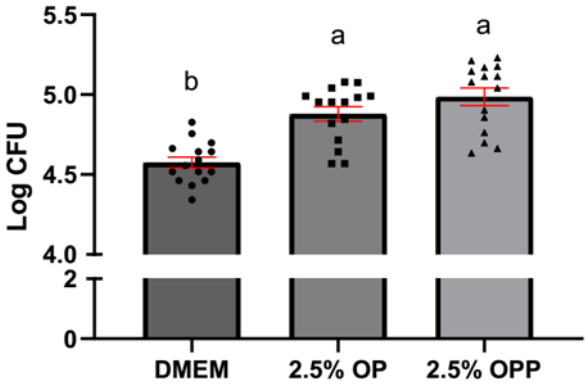

Fig S5.5 *C. rodentium* adhesion to CMT-93 cells after 5 h incubation in DMEM supplemented with 2.5% of OP and OPP. Data are expressed as mean ± SEM. Groups that do not share a letter are significantly different (Kruskal-Wallis test with Dunn's test) ($\alpha = 0.05$).

Table S5.1 Primers for real-time PCR analysis

Targeted gene	**Oligonucleotide sequence (5'–3')**	**Reference**
EspB	Forward: ATGCCGCAGATGAGACAGTTG Reverse: CGTCAGCAGCCTTTTCAGCTA	(Sagaidak et al., 2016)
Total bacteria	Forward: AAACTCAAAKGAATTGACGG Reverse: CTCACRRCACGAGCTGAC	(De Gregoris et al., 2011)

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CHAPTER 6 – Ovomucin and Ovalbumin Hydrolysates Protect Early-Weaned Piglets against Enterotoxigenic *Escherichia coli* K88

6.1 Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is the most common cause of severe diarrhea in neonatal and young farm animals, particularly piglets (Nagy & Fekete, 2005). ETEC attaches to small intestinal epithelial cells through the recognition of glycoprotein receptors by fimbriae(Kulkarni et al., 2010). Fimbriae that bind to glycoproteins in piglets include K88 (F4), K99 (F5), F6 (987P), F17, F18, and F41, with K88 being the most extensively studied due to their critical roles in ETEC infections in neonatal and weaned piglets (Luppi et al., 2016; Nagy & Fekete, 2005). K88 fimbrial protein AB (FaeG) is the major subunit of K88 fimbriae that mediate the binding of K88. Receptors for K88 fimbria in the intestinal brush border include the 74-kDa transferrin glycoprotein, the 210-kDa and 240-kDa intestinal mucin-type glycoprotein, aminopeptidase N, and intestinal glycosphingolipids (Xia et al., 2015). After adhesion, ETEC produces one or more of three main types of enterotoxins: heat-stable toxins (STa, STb) and heat-labile toxin (LT). These toxins disrupt the balance of absorption and secretion in the intestinal epithelium, leading to watery diarrhea (Dubreuil, 2012). ETEC infections cause significant economic losses due to piglet mortality, morbidity, medication costs, and decreased production performance (Fairbrother et al., 2005; Gresse et al., 2017). First-line treatment for severe diarrhea in neonatal and weaned piglets involves penicillin and tetracycline, which are also medically important to humans (Cutler et al., 2020; Nagy & Fekete, 2005; Zeineldin et al., 2019). However, resistance to these antimicrobial classes is common and poses a threat to human and animal health (Bassi et al., 2023; Landers et al., 2012). The World Health Organization has called for restrictions on antimicrobial use in food animal production (WHO, 2017), and the European Union and other countries have banned the

use of antimicrobials as growth promotors in food animal production (Administration, 2011; Bond, 2018; Canada, 2008; EU, 2003). This underscores the importance of the development of effective alternatives in antibiotic-free production.

Because the engagement of bacterial adhesins with their receptors on host tissue is a prerequisite for colonization and pathogenesis of pathogens, molecules with the capacity to impede bacterial adhesion, particularly those derived from natural food sources, are considered ecologically safe for controlling infections without accelerating the development of resistant strains (Pecoraro et al., 2023; Sun & Wu, 2017). In the context of ETEC K88 infection, various food-derived substances, primarily glycoconjugates, have been demonstrated to inhibit bacterial adhesion and attenuate disease severity. For example, casein macropeptide, released during cheese making, has been shown to bind to K88 fimbriae and inhibit ETEC K88 adhesion to porcine intestinal epithelial cells as well as ileal mucosal scrapings of weaned piglets (González-Ortiz et al., 2014; Hermes et al., 2011). In ETEC K88-infected weaned piglets, casein macropeptide preserved the functional morphology of ileum, reduced ileal bacterial adhesion, decreased the overgrowth of enterobacteria, and increased the lactobacilli population in the piglet gut after ETEC K88 challenge (Hermes et al., 2013; Rong et al., 2015). Similarly, pea hull extracts strongly attached to ETEC K88 bacterial cells and improved net fluid absorption in the small intestinal segments of ETEC K88-challenged weaned piglets through preventing the adhesion of ETEC K88 to intestinal mucosa as decoy receptors for the pathogen (Becker et al., 2012; Van der Meulen & Jansman, 2010). These studies suggested the potential of dietary oligosaccharides or glycoconjugates in managing ETEC K88 infection.

Ovomucin is a high molecular weight glycoprotein found in egg white, comprising approximately 33% of carbohydrate content (Mine, 1995; Omana et al., 2010). The carbohydrate

structures mainly consist of 3-5 units of N-acetylgalactosamine, galactose, N-acetylglucosamine, and sialic acid (Strecker et al., 1987, 1989). Ovomucin hydrolysates prepared by various enzymes exhibited anti-adhesive activity against K88 fimbria and ETEC K88 bacteria to intestinal epithelial cells, along with anti-inflammatory activity against ETEC K88 stress (Bao et al., 2024; Sun et al., 2019; Sun et al., 2017). Given these properties, it is hypothesized that these ovomucin hydrolysates can confer protective effects against ETEC K88 challenge *in vivo*. Therefore, the primary aim of this study is to test the *in vivo* efficacy of ovomucin hydrolysate prepared by enzyme protex 26L (OP) in mitigating ETEC K88-infection in weaned piglets. Additionally, ovalbumin, as the most abundant protein in chicken egg white, has demonstrated binding capacity to *Salmonella* strains and Shiga toxin-producing *E. coli* O84 (Leusch et al., 1991a; Leusch et al., 1991b). It has been reported that with high-mannose-type carbohydrate side chains, ovalbumin-generated short Man5GlcNAc2-contaning glycopeptides act as decoy receptors for type I pilus-mediated adhesion of enteropathogenic *E. coli* strains (Neeser et al., 1986; Tai et al., 1975). Therefore, ovalbumin-protex 26L hydrolysate (OVAP) was also used in the present study to explore its activity in ETEC K88-challenged piglets.

6.2 Materials and methods

The animal use protocol (F23-009, AC1181) was approved by the Animal Care Committee of the University of Manitoba. Piglets were housed and managed in T.K. Cheung Center of the University of Manitoba according to the Canadian Council on Animal Care guidelines (CCAC, 2009).

6.2.1 Preparation of protein hydrolysates

Ovomucin was extracted from liquid egg white according to the two-step precipitation procedure (Kharbanda, 2022). Briefly, 100 L of liquid egg white (Egg Solutions, Lethbridge, Canada) was diluted with 300 L of ddH_2O. After lysozyme removal using Amberlite FPC 3500 ion-exchange

resin (2 g/100 mL egg white) at room temperature (RT) for 3 h, the supernatant was collected by siphoning and the pH was adjusted to 6.0 by 1 M of HCl. The precipitate (~10 L) was collected after overnight stand at 4°C by centrifugation at 10,000 rpm for 10 min and resuspended in 10 × volume of ddH_2O. The pH was subsequently adjusted to 6.0 to allow reprecipitation overnight at 4°C. Finally, the precipitate was lyophilized and stored at 4°C for further use.

Ovalbumin extraction was conducted using the ovomucin-devoid supernatant (~390 L) (Abeyrathne et al., 2014). The ovomucin-devoid supernatant was heated at 60°C for 30 min and cooled down to RT before overnight incubation at 4°C to allow the precipitation of ovotransferrin. The ovotransferrin-devoid supernatant (~320 L) was collected and ultrafiltered to final ~30 L, which was lyophilized for preparing the hydrolysate OVAP.

Protein samples were dispersed in Milli-Q water (1% w/v) and the slurries were adjusted to pH 3.0 and 50°C before adding 2% of the enzyme protex 26L (w/w, enzyme/substrate) (Sun et al., 2016). After hydrolysis for 4 h, the samples were heated at 95°C for 15 min and subsequently cooled down to RT. Finally, the samples were lyophilized and added to the piglet diet at a final concentration of 1% (w/w).

6.2.2 Genetic susceptibility and piglet selection

ETEC F4ab/ac susceptible piglets were selected as described (Jensen et al., 2006). Genomic DNA was extracted from tails obtained on day 3 after farrowing (Truett et al., 2000). The *MUC4* gene was amplified using polymerase chain reaction that comprised 2.5 uL of 10× buffer, 1 U DreamTaq DNA polymerase (ThermoFisher Scientific, Ottawa, ON, Canada) with 2 mM $MgCl_2$, 5 mM dNTP mix, and 10 mM of each primer (Table 6.1) in a total volume of 25 µL. Thermocycling was performed as follows: 95°C for 3 min, 35 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 1 min, followed by 72°C for 10 min. PCR products (5 µL) were treated with FastDigest XbaI

(ThermoFisher Scientific, Ottawa, ON, Canada) at 37°C for 5 min according to the manufacturer's instructions. The digested DNA products were subsequently applied to electrophoresis on a 2% agarose gel in Tris-borate-EDTA buffer. With the SYBR Safe stain (Invitrogen, Burlington, ON, Canada), DNA samples with fragments of 151 bp and 216 bp were identified as the susceptible allele, while those remained whole and undigested were identified as the resistant allele. Piglets with the susceptible allele and similar body weight were selected for the ETEC K88 challenge study.

6.2.3 Experimental design

Thirty-eight susceptible piglets based on *MUC4* polymorphism from the Glenlea Swine Research Unit were weaned at 21-day-old (TN Tempo × TN70, 20 female and 18 castrated male piglets) with average body weight of 6.43 ± 0.16 kg (mean ± SEM) and housed individually in a temperature-controlled room (30 ± 1°C from day 1 to day 7, and 29± 1°C from day 8 to day 12). Piglets were given a corn-soybean meal basal diet according to the NRC (2012) recommendations (Table 6.2) (Choi et al., 2020). The piglets were randomly assigned to five groups based on their body weights with pig as the experimental unit: (1) the control group fed the basal diet (Ctrl, n=8); (2) the infection control group fed the basal diet (ETEC, n=7); (3) the positive control group fed the basal diet with 80 mg/kg avilamycin (AVI, n=7); (4) ovomucin-protex 26L hydrolysate group (OP, n=8) fed the basal diet supplemented with 1% of OP; (5) ovalbumin-protex 26L hydrolysate group (OVAP, n=8) fed the basal diet supplemented with 1% of OVAP. Piglets had *ad libitum* access to water and diet throughout the trial. Body weight (BW) and feed intake were recorded before ETEC K88 challenge and at 4 days post-infection (dpi). On day 11 (4 dpi), piglets were anesthetized by an intramuscular injection of ketamine:xylazine (20:2 mg/kg BW) and sacrificed by a captive bolt gun.

6.2.4 ETEC K88 challenge

The ETEC K88 strain P4 positive for Sta, Stb, and LT virulence factors was streaked on a tryptic soy agar and grown anaerobically at 37°C overnight. A single colony was inoculated in 10 mL of tryptic soy broth and cultivated anaerobically overnight at 37°C with shaking at 150 rpm (MaxQ SHKE4000; ThermoFisher Scientific, Ottawa, ON, Canada). The culture (300 µL) was subsequently inoculated to 300 mL of tryptic soy broth. After cultivation for 2.5 h at 37°C, sterile phosphate buffered saline (pH 7.4) was used to dilute the bacterial culture to the targeted concentration of 5×10^6 colony forming units (CFUs)/mL.

At day 8 postweaning, piglets in the ETEC, AVI, OP, and OVAP groups were inoculated with the overnight ETEC K88 culture (5 mL of 5×10^6 CFUs/mL), whereas piglets in the Ctrl group received the vehicle control. Rectal temperature was measured before inoculation and at 3, 24, and 48 hpi (hour post-inoculation) through a digital thermometer. Fecal scores ranging from 0-3 (0 = normal solid faeces, 1 = soft faeces; 2 = mild diarrhea with yellowish fluid faeces; 3 = severe diarrhea with watery and projectile faeces) were evaluated before infection and every 4 h post-infection (hpi) (Choi et al., 2020; Marquardt et al., 1999).

6.2.5 Sample collection

At 4 days post-infection, piglets were sacrificed for sample collection. A 10-cm mid-jejunum segment (400 cm from the stomach-duodenum junction) was collected in the ice-cold Krebs ringer buffer (154 mM Na^+, 6.3 mM K^+, 137 mM Cl^-, 0.3 mM H_2PO_4, 1.2 mM Ca^{2+}, 0.7 mM Mg^{2+}, 24 mM HCO_3^-, 1 µM indomethacin; pH 7.4) with 10 mM glucose for subsequent Ussing chamber analysis. Mid-duodenum, jejunum, and ileum (~2 cm) were collected in the 10% formaldehyde solution for histological analysis. Additional jejunal, ileal, and colonic segments and colonic contents were snap frozen in liquid nitrogen and stored at -80°C for further analysis.

6.2.6 Ussing chamber analysis

The electrophysiological properties of mid-jejunum segments were determined using modified Ussing chambers (VCC-MC8; Physiologic Instruments Inc., San Diego, CA) as previously described (Choi et al., 2020). Briefly, tissues without serosal and muscle layers were mounted in Ussing chambers to allow equilibration for 10 min before the short-circuit current and transepithelial electrical resistance (TEER) values were recorded. Additionally, to determine intestinal permeability, 0.1 mg/mL of FITC-D40 (molecular weight of 40 kDa) was added to the mucosal side and 1 mL of sample in the serosal side was collected after 1 h treatment. The fluorescence was detected at the excitation and emission wavelength of 485 nm and 528 nm, respectively, using a Bio-Tek PowerWave HT Microplate Scanning Spectrophotometer (BIO-TEK Instruments, Inc., Santa Clara, CA).

6.2.7 RNA extraction and quantification of mRNA

Total RNA was extracted from jejunum, ileum, and colon tissue samples by the Trizol method (TRIzol® Reagent, ThermoFisher Scientific, Nepean, Ontario, Canada). RNA concentrations were measured by a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Nepean, Ontario, Canada) and 1 μg of total RNA was used for reverse transcription using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Nepean, Ontario, Canada). Expressions of *IL-1β*, *IL-6*, *TNF-α*, *CLDN3*, *ZO-1*, and *OCLN* were analyzed by reverse-transcription quantitative PCR on an ABI StepOne real-time system (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with primers listed in Table 6.1. The housekeeping gene *β-actin* was referenced for normalizing the fold change of gene expression relative to the Ctrl group using the $2^{-\Delta\Delta Ct}$ method.[44]

6.2.8 Histology analysis

Jejunal, ileal, and colonic tissues were fixed in 10% formaldehyde solution overnight and transferred to 70% ethanol. The tissues were subsequently embedded in paraffin and cut into 5-μm sections. Fixed tissue sections were stained with hematoxylin and eosin and imaged by a light microscope (EVOS™ M7000 Imaging System, Invitrogen, Germany). For each sample, villus height (VH) and crypt depth (CD) were measured from 50 to 150 villi and crypts using the Infinity Analyze software.

6.2.9 DNA extraction and quantitative PCR analysis of intestinal bacteria

Bacterial DNA was extracted from colonic contents using the QIA Fast DNA Stool Mini Kit (QIAGEN, Valencia, CA) according to the manufactures' instructions. DNA concentrations were measured by a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, Nepean, Ontario, Canada) and subsequently diluted to the final concentration of 50 ng/μL.

DNA samples randomly taken from ETEC K88-infected piglets were pooled for fimbria K88 amplification with primers targeting *faeG* (Table 6.1). The amplicon was purified by a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) and its concentration was measured by a NanoDrop 2000c spectrophotometer. Ten-fold serial dilutions with concentrations ranging from 102 to 1011 gene copies/μL were applied to obtain the standard curve. The level of ETEC K88 was quantified using real-time PCR on an ABI StepOne real-time system (Applied Biosystems, Foster City, CA) with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) following the program: 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Data are expressed as means of the $\log_{10}$ number of K88 copies/g feces ± standard error of the mean (SEM).

An individual high-resolution melting curve qPCR using a Rotor-Gene Q (QIAGEN, Valencia, CA) HRM-thermocycler and the Type-it HRM kit (QIAGEN) was further applied to determine the ETEC K88 abundance with primers targeting *faeG*. The reaction system contained 12.5 μL of 2×HRM PCR Master Mix, 2 μL of template DNA and 0.7 μM primers to a final volume of 25 μL. The PCR was programmed according to the previous study (Wang et al., 2017): denaturation at 95°C for 5 min, 45 cycles of denaturation at 95°C for 10 s, annealing at 62°C for 30 s, and extension at 72°C for 25 s with the temperature increased from 65°C to 95°C at the speed of 0.1°C/step and held for 2 s at each step.

6.2.10 Statistical analysis

Results were analyzed using the Prism 6 software (GraphPad Software, San Diego, CA) and data were expressed as means ± SEM. Survival curves were analyzed by Log-rank (Mantel-Cox) test. Diarrhea scores, rectal temperature, and BW data were analyzed by mixed-effects model (REML) followed by Dunnett's and Tukey's multiple comparison test, respectively. Growth performance, gut permeability, histomorphology, gene expression levels, and ETEC K88 abundance were tested for normality of distribution by Shapiro-Wilk test. The effects of ETEC K88 challenge (ETEC versus Ctrl) were analyzed by unpaired t test for parametric data or Mann-Whitney test for nonparametric data. Comparisons of ETEC K88-challenged groups (ETEC, AVI, OP, and OPP) were analyzed by one-way ANOVA followed by Dunnett's *post-hoc* test for parametric data or Kruskal-Wallis test for nonparametric data with Dunn's *post-hoc* test, respectively. $P < 0.05$ was considered statistically significant.

6.3 Results

6.3.1 Growth performance and diarrhea scores

Due to extensive diarrhea and poor clinical condition, 1 out of 7 ETEC K88-piglets, 1 out of 7 AVI-piglets, 2 out of 8 OP-piglets and 2 out of 8 OVAP-piglets were euthanized before the completion of the experiment (Fig. 6.1). There was no difference among the survival curves (Log-rank P=0.660). Additionally, there was no difference in the BW, average daily feed intake (ADFI), and feed conversion ratio (indicated by gain:feed ratio) among all groups regardless of ETEC K88 infection (Table 6.3). During the 7-d feeding period before ETEC K88 inoculation, piglets in the ETEC, AVI, OP, and OVAP groups had similar BW, average daily gain (ADG), ADFI, and gain:feed ratio. Piglets assigned to the ETEC group before challenge showed higher ADG compared to those in the Ctrl group (P=0.027), while tended to have lower ADG than the Ctrl group during the post-challenge period (P=0.076; Table 6.3).

ETEC K88 inoculation induced diarrhea in piglets, nevertheless there was no significant difference among groups (Fig. 6.2). Notably, the diarrheic severity in piglets within the ETEC group peaked at 32, 36, and 40 hpi, showing differences compared to the diarrhea scores observed at 4 hpi (P=0.004, 0.001, and 0.001, respectively). Piglets in the AVI and OVAP groups showed intermittent episodes of diarrhea. Diarrhea in piglets from the OP group was postponed and peaked from 56 hpi, while no difference was observed within the OP group when compared to the diarrhea scores at 4 hpi. Furthermore, there was no difference in the rectal temperature among piglets in all groups during the 48-hpi period (Fig. S6.1).

6.3.2 Jejunal permeability and histomorphology

There was no difference in the TEER values and FITC-D40 kDa fluxes of mid-jejunum among groups (Table 6.4). In addition, ETEC K88 inoculation exerted no influence on VH, CD, and

VH:CD values of mid-jejunum in the ETEC group versus the Ctrl group (P=0.151, 0.729, and 0.297, respectively). Compared to the ETEC group, OP supplementation increased mid-jejunal VH and CD (P=0.005 and 0.023, respectively), while the VH:CD value was not altered. The OP group also had higher VH and CD than the AVI group (P=0.003 and 0.072, respectively). OVAP treatment showed no alterations of VH and CD compared to the ETEC and AVI groups.

6.3.3 Expression of tight junction proteins and cytokines in the intestinal mucosa

The expression levels of *OCLN* and *ZO-1* in jejunum were increased in the ETEC group than the Ctrl group, while the *CLDN3* expression tended to be higher in the ETEC group (Fig. 6.3). No changes were observed in ileal mucosa of piglets in the context of ETEC K88 infection (Fig. 6.4). In colonic mucosa, ETEC K88 challenge decreased the expression of *ZO-1* compared to the Ctrl group (Fig. 6.5). No differences were observed in the expression levels of *OCLN*, *ZO-1*, and *CLDN3* in jejunal and ileal mucosa among all ETEC K88-challenged groups (ETEC, AVI, OP, and OVAP; Fig. 6.3-6.4). However, in colonic mucosa the OP group showed higher expression of *OCLN*, *ZO-1*, and *CLDN3* than the OVAP group, along with a higher level of *OCLN* compared to the AVI group (Fig. 6.5).

ETEC K88 infection increased *IL-6* expression in the jejunum (Fig. 6.3), while elevated expression of *IL-1β* and *TNF-α* was observed in colon tissues (Fig. 6.5). No differences were detected in ileal mucosa by ETEC K88 challenge when compared to the Ctrl group (Fig. 6.4). Moreover, the expression of *IL-6*, *IL-1β*, and *TNF-α* was not altered in jejunum and ileum among ETEC K88-chalenged groups (Fig. 6.3-6.4), while the level of *TNF-α* in colonic mucosa tended to be lower in OP and OVAP groups compared to the ETEC group (Fig. 6.5).

6.3.4 ETEC K88 abundance in colonic contents

ETEC K88 load in the ETEC group, as indicated by *faeG* gene copy numbers, was higher compared to the Ctrl group where all samples were below detection by qPCR (Fig. 6.6). OP supplementation did not result in any statistically significant changes in the ETEC K88 abundance compared to the ETEC and AVI groups, while the OVAP group had lower ETEC K88 load than the ETEC and AVI groups (Fig. 6.6). A higher abundance of ETEC K88 in the ETEC group than the Ctrl group was confirmed by the HRM-qPCR test (Fig. S6.2). The ETEC K88 load of OP and OVAP groups were not different when compared to the ETEC group (Fig. S6.2).

6.4 Discussion

ETEC K88 exploits diverse glycan structures on piglet enterocytes to establish adhesion and initiate infection. Consequently, glycans that disrupt the adhesion of K88 fimbria with their receptors represent attractive anti-adhesive strategies for managing infections (Asadi et al., 2019; Sun & Wu, 2017). The present study demonstrated that supplementation with OP and OVAP altered the diarrhea of weaned piglets upon ETEC challenge, influenced the morphology and permeability of piglet jejunum, and modulated the mucosal immune responses in the context of ETEC K88 infection. Notably, OVAP demonstrated a reduced level of ETEC K88 than the AVI group.

The ETEC K88 challenge model in weaned piglets has been extensively applied in the investigation of feeding interventions and vaccination strategies to counteract postweaning diarrhea. Since the *MUC4* gene polymorphism on porcine chromosome 13 has been associated with ETEC K88 susceptibility with higher and faster diarrhea incidence (Jørgensen et al., 2003; Jensen et al., 2006; Roubos-van den Hil et al., 2017), the *MUC4* marker was employed as a criterion for selecting piglets, aiming to minimize individual animal variations in response to

ETEC K88 infection. In this study, piglets in the ETEC group developed severe diarrhea during 32 to 40 hpi and showed intermittent patterns, while 2 of the 7 piglets maintained normal solid feces throughout the experimental period with low or undetectable levels of ETEC K88 in the colonic contents. This is consistent with previous research indicating that the worst fecal scores were typically observed from 2 to 4 d post-inoculation in ETEC K88-challenged weaned piglets and that not all *MUC4* susceptible piglets appeared diarrhea even at the peak of infection (Jensen et al., 2006; Luise et al., 2019). This also implies the importance of including multiple timepoints in the challenge protocols, in addition to the *MUC4* biomarker, to successfully establish ETEC K88 infection and effectively evaluate the efficacies of feeding strategies. Compared to the ETEC group, OP supplementation delayed the peak of diarrhea to 56 hpi while the OVAP group showed an intermittent diarrhea pattern similar to that of the Ctrl group. These findings suggested the promising potential of OP and OVAP in modulating ETEC K88 infection in early-weaned piglets.

ETEC K88 pathogenicity in weaned piglets following the successful adhesion and colonization is commonly characterized by several clinical parameters (e.g., diarrhea occurrence, rectal temperature changes, fecal shedding of pathogens) and biomarkers (intestinal proinflammatory cytokines and ETEC K88-specific immunoglobulins and receptors) (Dahmer et al., 2023; Luise et al., 2019). However, variations in some of these response parameters are documented after oral inoculation of ETEC K88 due to complex factors, including but not limited to the preconditions of piglets, virulence traits of the ETEC K88 strains, doses and timing of the bacterial inoculation, and sampling timepoint (Dahmer et al., 2023; Luise et al., 2019). For example, the virulence traits of ETEC is highly affected by environmental factors like oxygen availability (Crofts et al., 2018). ETEC senses environmental oxygen to globally influence virulence factor expression, and therefore changes in oxygen levels along the intestinal region and

proximity to the oxygenated zone bordering intestinal epithelial cells influence ETEC virulence *in vivo* (Crofts et al., 2018). Notably, discrepancies regarding intestinal mucosal immunity and barrier function in response to ETEC K88 were previously noted (Fairbrother et al., 2017; Lallès et al., 2004; McLamb et al., 2013; Van den Broeck et al., 1999; Verdonck et al., 2002; Zhang et al., 2023). ETEC K88 colonization is typically accompanied by the presence of K88-specific IgM in mucosal tissues and serum from 4 dpi onward, while maximal levels of K88-specific IgG and IgA are observed at 7 dpi thereafter due to fast activation and recruitment of mast cells and neutrophils in intestinal mucosa (Fairbrother et al., 2017; Van den Broeck et al., 1999; Verdonck et al., 2002). Similarly, the kinetics of cytokine production in different intestinal regions/structures of piglets weaned at different ages with ETEC infection have been reported (Lallès et al., 2004; McLamb et al., 2013; Zhang et al., 2023). The variations in cytokine production can be attributed to the anatomical differences of immune apparatus along the intestinal tract (Bailey, 2009) and the less developed mucosal immune systems in younger piglets (Bailey et al., 2005; Stokes et al., 2004) ETEC K88 also induces the production of cytokines, among which IL-6 and TNF-α are the most studied pro-inflammatory cytokines and show great variability across studies (Dahmer et al., 2023). In the current study, piglets weaned at 21 days of age were used and ETEC K88 inoculation was performed at 7 days postweaning when the passive immunity from sow milk immunoglobulins decreased yet the mucosal immune function remained immature. ETEC K88 infection did not trigger robust gene expression of *IL-1β*, *TNF-α*, or *IL-6* in the ileum of piglets, while elevated mRNA levels of jejunal *IL-6* as well as colonic *IL-1β* and *TNF-α* compared to the uninfected control piglets were observed. This indicated the activation of immune systems in the intestinal mucosa, while some early signs might be overlooked in the small intestine at 4 dpi (Heim et al., 2014). Dietary supplementation of OP and OVAP showed no changes in cytokine levels in the

small intestine when compared to the ETEC group, however a tendency to decrease the expression of *TNF-α* in colonic tissues was observed. OP group showed decreased colonic *IL-1β* and *IL-6* compared to the ETEC-piglets. This was consistent with lower colonic ETEC K88 levels in OP and OVAP groups, although the significant reduction was only observed in the OVAP group.

The intestinal morphology and permeability are disturbed by ETEC K88 infection during postweaning period, as evidenced by changes in parameters such as VH, CD, VH:CD, fluxes of fluorescence tracers with different molecular weights, and tight junction proteins (Dubreuil, 2017; Kim et al., 2022). However, these responses are variable across studies in ETEC K88-infected weaned piglets compared to uninfected control piglets (Dahmer et al., 2023). In this study, ETEC K88 did not deteriorate the jejunal morphology, while OP treatment improved morphological markers such as VH and CD in the presence of ETEC K88, indicating its protective effects in maintaining functional nutrient absorption for piglet growth performance and preserving the intestinal barrier integrity to restrict antigen diffusion. The dynamics and plasticity of tight junctions in response to pathogen infections have been widely demonstrated since disruption of tight junctions generally leads to increased permeability which facilitates the entry and spread of pathogens. However, concurrently some pathogens exploit junctional molecules for their advantage, indicating the complex role of tight junctional structures during infectious diseases (Eichner et al., 2017; Guttman & Finlay, 2009). There are still controversies, at least regarding the expression levels of tight junction proteins, that need to be rectified during pathogen infections, as well as in the context of ETEC K88 challenge in weaned piglets. In the current study, ETEC K88 downregulated *ZO-1* expression in colonic tissues, likely due to a higher pathogenic load and immune responses in the colonic mucosa. An interesting observation is that the expression of genes encoding tight junction proteins (i.e. *OCLN*, *ZO-1*, and *CLDN3*) in the jejunum of weaned piglets

were upregulated by ETEC K88 infection, contrary to most of the current findings indicating downregulation of tight junctions following ETEC K88 challenge (Dubreuil, 2017). Detailed mechanisms are elusive to understand the distinct outcomes, such as whether the pathogen differentially mediates tight junctional molecules in the jejunum and colon, or if the colonization and infection dynamics differ in different intestinal regions following ETCE K88 infection. In any case, compared to uninfected healthy piglets, ETEC K88 challenge induced perturbation in tight junction proteins, while OP and OVAP supplementation did not effectively attenuate these disturbances.

As demonstrated with different glycoconjugate receptors, K88 exhibits lectin activities with glycans containing a Galβ1-3GlcNAc, such as α1-3-galactose and asialo-GM1 (Dubreuil et al., 2016). Ovomucin carries glycan structures that can function as decoy receptors for ETEC K88, and OP has exhibited anti-adhesive activity against pathogens including ETEC K88 (Bao et al., 2024; Sun et al., 2019; Sun et al., 2017). However, the current piglet trial did not strongly support the *in vivo* efficacy of OP to reduce ETEC K88 colonization in the piglet colon. Future *in vivo* studies are warranted to test the impact of higher doses of this product on ETEC K88 adhesion and colonization. Ovalbumin is the major protein in egg white and consists of approximately 3% carbohydrate content being high mannose type, hybrid-type, and complex-type oligosaccharides (Hwang et al., 2014). OVAP, generated from ovalbumin and possessing high-mannose-type carbohydrate side chains (e.g. Man5GlcNAc2-structures), effectively reduced ETEC K88 abundance in the colonic contents, which is therefore considered to function through interacting with other adhesins, such as the type 1 pilus that is highly conserved in ETEC strains (Sheikh et al., 2017), to inhibit ETEC K88 adhesion and growth in the piglet intestine. Indeed, whole egg white and specific protein components such as lysozyme and ovotransferrin have also

demonstrated diverse bioactivities, including antimicrobial and anti-inflammatory activities against bacterial infections (Bao et al., 2023). These findings inspire the future studies on non-food functional uses of egg protein(s) and egg products, particularly in controlling infectious diseases in the era of antimicrobial resistance.

In summary, ETEC K88 challenge induced diarrhea in weaned piglets, decreased their growth performance, triggered mucosal immune responses, and compromised intestinal barrier integrity. Supplementation of OP improved the morphology of jejunum and potentially modulated the tight junction integrity and immune response in colon. However, this trial did not confirm the *in vitro* findings that suggested the anti-adhesive activity of OP against ETEC K88 colonization in piglet gut. Additionally, OVAP showed the potential in maintaining the growth performance of piglets and exhibited more significant activity in reducing ETEC K88 load in piglet colon. These data underscored the potential of OP and OVAP in preserving intestinal health and controlling ETEC K88 infection through distinct modes of action. Future studies are needed to evaluate different dosages and/or the combination of these hydrolysate products and optimize their practical applications.

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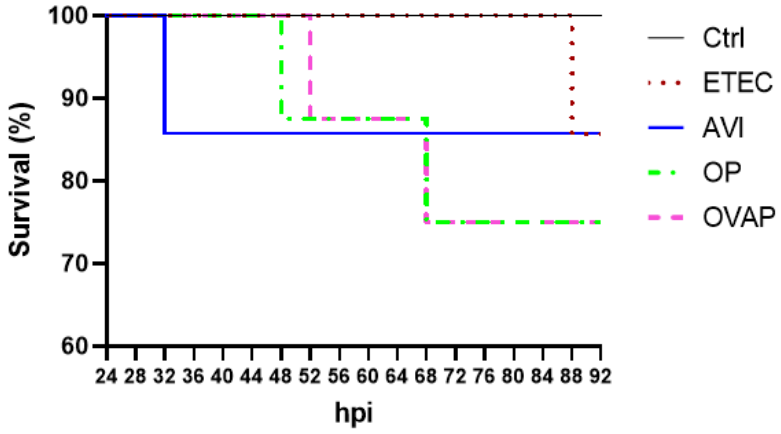

Fig. 6.1. Survival curves of piglets during the 92 h post-inoculation study. Ctrl: the control group (n=8); ETEC: the ETEC K88 infection control group (n=7); AVI: avilamycin (n=7); OP: ovomucin-protex 26L hydrolysate (n=8); OVAP: ovalbumin-protex 26 L hydrolysate (n=8).

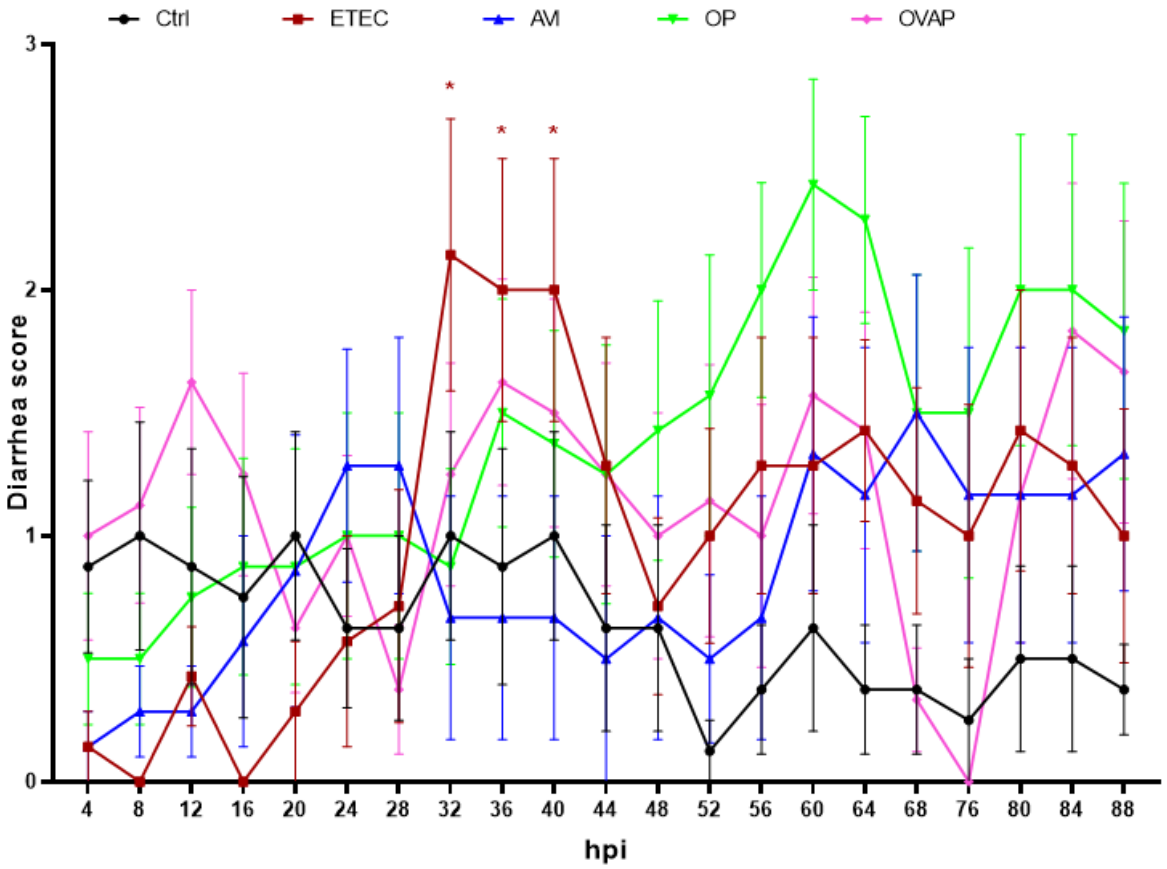

Fig. 6.2. Diarrhea scores of piglets assessed according to the criterion: 0 = normal solid faeces, 1 = soft faeces; 2 = mild diarrhea with yellowish fluid faeces; 3 = severe diarrhea with watery and projectile faeces. Data are expressed as mean ± SEM. Ctrl: the control group; ETEC: the ETEC K88 infection control group; AVI: avilamycin; OP: ovomucin-protex 26L hydrolysate; OVAP: ovalbumin-protex 26 L hydrolysate. *P< 0.05 by one-way ANOVA with Dunnett's test compared to the ETEC-piglets at 4 h post-inoculation.

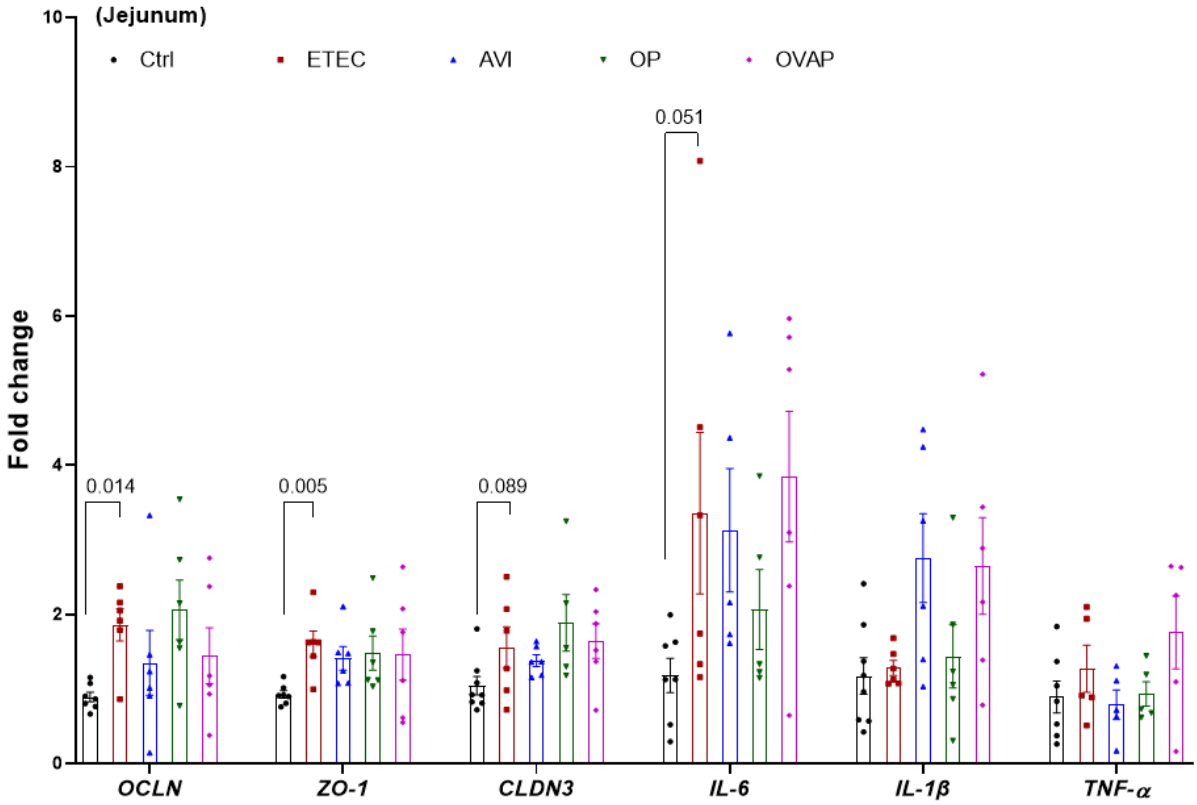

Fig. 6.3. Relative expression of genes encoding tight junction proteins and cytokines in the jejunum of piglets. Ctrl: the control group; ETEC: the ETEC K88 infection control group; AVI: avilamycin; OP: ovomucin-protex 26L hydrolysate; OVAP: ovalbumin-protex 26 L hydrolysate. The ETEC group was compared with the Ctrl group by unpaired t test or Mann-Whitney test. ETEC-challenged groups were analyzed according to the one-way ANOVA and Dunnett's test ($\alpha = 0.05$).

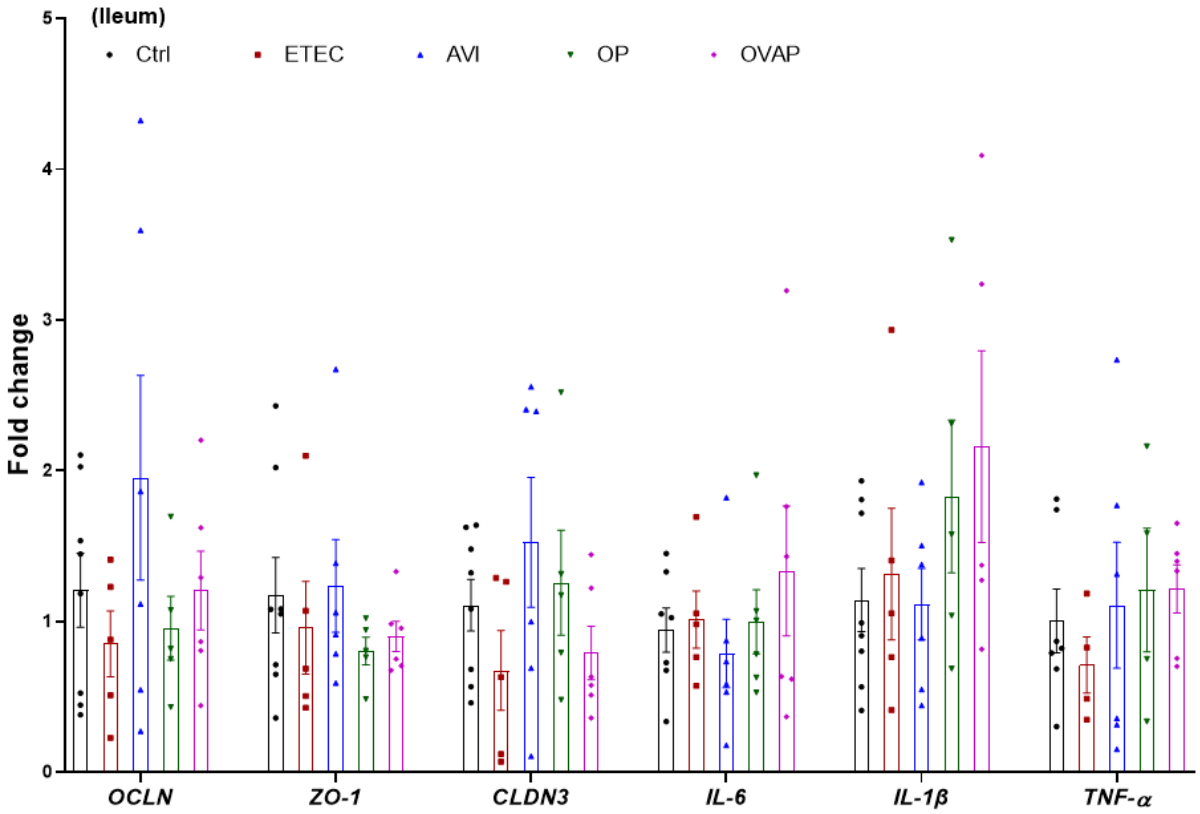

Fig. 6.4. Relative expression of genes encoding tight junction proteins and cytokines in the ileum of piglets. Ctrl: the control group; ETEC: the ETEC K88 infection control group; AVI: avilamycin; OP: ovomucin-protex 26L hydrolysate; OVAP: ovalbumin-protex 26 L hydrolysate. The ETEC group was compared with the Ctrl group by unpaired t test or Mann-Whitney test. ETEC-challenged groups were analyzed according to the one-way ANOVA and Dunnett's test ($\alpha = 0.05$).

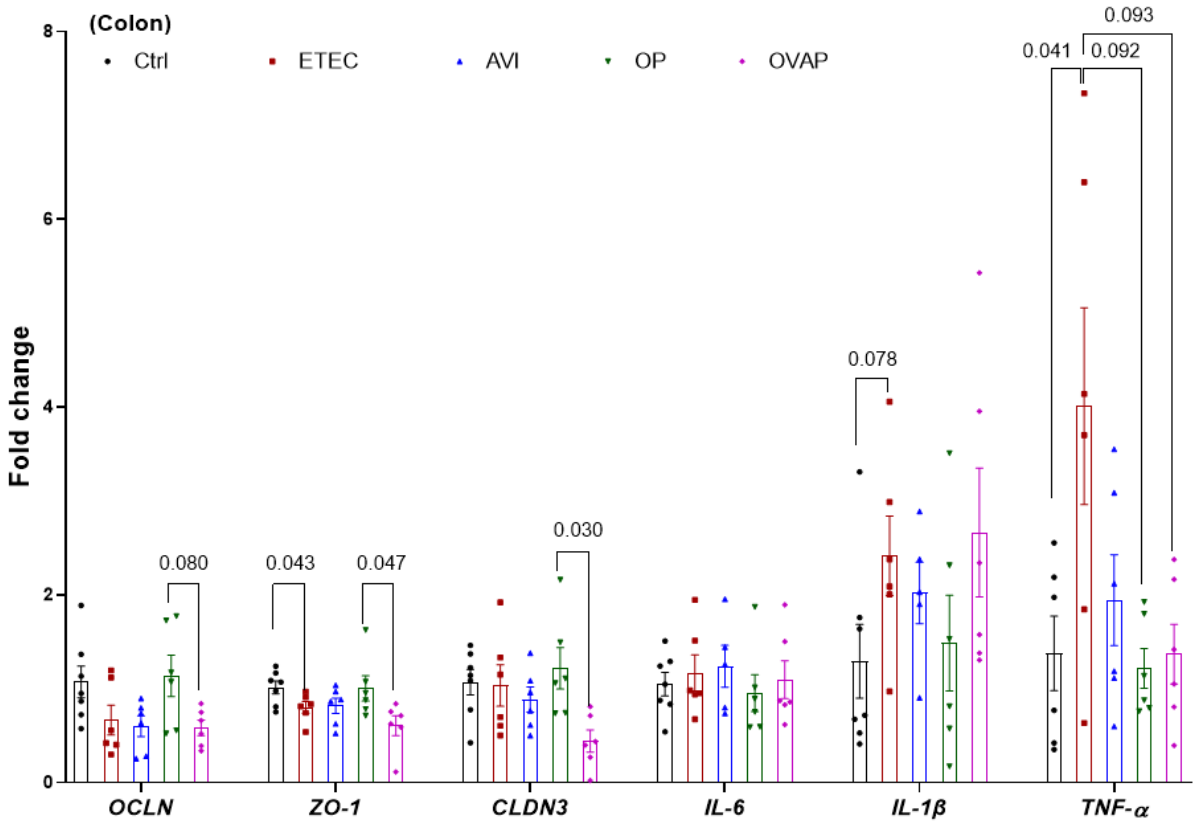

Fig. 6.5. Relative expression of genes encoding tight junction proteins and cytokines in the colon of piglets. Ctrl: the control group; ETEC: the ETEC K88 infection control group; AVI: avilamycin; OP: ovomucin-protex 26L hydrolysate; OVAP: ovalbumin-protex 26 L hydrolysate. The ETEC group was compared with the Ctrl group by unpaired t test or Mann-Whitney test. ETEC-challenged groups were analyzed according to the one-way ANOVA and Dunnett's test ($\alpha = 0.05$).

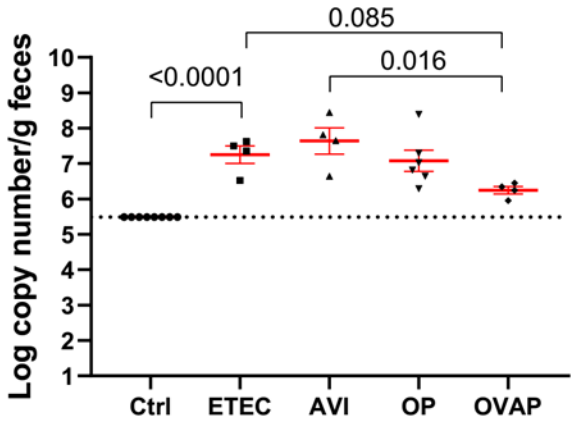

Fig. 6.6. Abundance of ETEC K88 as indicated by $\log_{10}$ number of *faeG* gene copies in colonic contents. Data are expressed as mean ± SEM. The dotted line represents the detection limit. The ETEC group was compared with the Ctrl group by Mann Whitney test. Difference among all ETEC K88-challenged groups were analyzed by one-way ANOVA with Dunnett's test. Ctrl: the control group; ETEC: the ETEC K88 infection control group; AVI: avilamycin; OP: ovomucin-protex 26L hydrolysate; OVAP: ovalbumin-protex 26 L hydrolysate.

Table 6.1. Primers used in this study

Target gene	Primer sequence (5′ - 3′)	Size (bp)	Reference
OCLN	CTGTGGATGTCCTGCGTGT	93	(Choi et al., 2020)
	GGTTGCTTGCAAAGTGGTGTT		
ZO-1	GATCCTGACCCGGTGTCTGA	200	(Choi et al., 2020)
	TTGGTGGGTTTGGTGGGTT		
CLDN3	CTACGACCGCAAGGACTACG	123	(Choi et al., 2020)
	TAGCATCTGGGTGGACTGGT		
IL-6	AAGGTGATGCCACCTCAGAC	151	(Choi et al., 2020)
	TCTGCCAGTACCTCCTTGCT		
IL-1β	TGGCTAACTACGGTGACAACA	91	(Choi et al., 2020)
	CCAAGGTCCAGGTTTTGGGT		
TNF-α	ATGGATGGGTGGATGAGAAA	151	(Choi et al., 2020)
	TGGAAACTGTTGGGGAGAAG		
β-actin	GGCGCCCAGCACGAT	66	(Che et al., 2017)
	CCGATCCACACGGAGTACTTG		
faeG	GCACATGCCTGGATGACTGGTG	439	(Wang et al., 2017)
	CGTCCGCAGAAGTAACCCCACCT		
MUC4	GTGCCTTGGGTGAGAGGTTA	367	(Dahmer et al., 2023)
	CACTCTGCCGTTCTCTTTCC		

Table 6.2. Ingredient composition of the basal diet

Ingredients	**Weight (kg)**
Corn	483.840
Soybean meal (480 g crude protein/kg)	160.000
Whey permeate	124.220
[1]Soybean protein concentrate (480 g crude protein/kg)	110.000
Fish meal herring	65.727
Soybean oil	15.000
Limestone	14.323
[2]Biofos (mono-calcium phosphate)	5.733
Salt-bulk fine	4.998
[3]Starter vitamin-mineral premix	10.000
L-lysine 78%	2.828
DL-methionine 99%	1.521
L-threonine	1.322
L-tryptophan	0.514
Calculated energy and nutrient levels	
Metabolizable energy (MJ/kg)	14.19
Net energy (MJ/kg)	10.36
Crude protein (%)	22.35
[4]SID lysine (%)	1.34
[4]SID methionine (%)	0.50
[4]SID threonine (%)	0.87

[4]SID tryptophan (%)	0.27

[1]Soybean protein concentrate (X-SOY600; CJ Selecta, Goiania, State of Goiás, Brazil).

[2]Biofos (mono-calcium phosphate with 21% Ca and 17% P; Mosaic Co., Plymouth, MN, USA)

[3]Starter vitamin-mineral premix contains the following ingredients per kilogram of feed: vitamin A, 2,200 IU; vitamin B1, 1.5 mg; vitamin B2, 4 mg; niacin, 30 mg; calcium pantothenate, 12 mg; pyridoxine, 7 mg; biotin, 0.2 mg; folic acid, 0.3 mg; vitamin B12, 0.02 mg; vitamin D3, 220 IU; vitamin E, 16 IU; vitamin K, 0.5 mg; choline chloride, 600 mg; calcium iodate, 0.14 mg; Cu (copper sulfate), 6 mg; Fe (ferrous sulfate), 100 mg; manganese oxide, 4 mg; sodium selenite, 0.3 mg; zinc oxide, 100 mg.

[4]SID, standardized ileal digestible amino acids.

Table 6.3. Growth performance of piglets during the pre-challenge and post-challenge period

	Ctrl		ETEC		AVI		OP		OVAP		*P*
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Initial BW	6.47	0.34	6.37	0.41	6.46	0.41	6.41	0.31	6.43	0.31	>0.999
Pre-challenge											
BW (kg)	6.88	0.36	7.41	0.34	7.40	0.24	7.21	0.25	7.27	0.32	0.773
ADG (g/d)	57.5	11.4	109*	15.8	134	29.5	113	23.0	120	22.7	0.917
ADFI (g/d)	96.3	13.8	121	10.5	158	15.5	133	18.6	152	12.4	0.404
Gain:Feed	0.68	0.21	0.81	0.07	0.83	0.03	0.88	0.08	0.90	0.07	0.670
Post-challenge											
BW (kg)	7.25	0.42	7.60	0.33	7.86	0.33	7.52	0.38	7.76	0.43	0.339
ADG (g/d)	141	20.1	53.1#	33.4	99.6	68.3	70.0	47.9	168	22.4	0.533
ADFI (g/d)	188	14.8	167	15.0	213	25.4	195	10.0	208	17.1	0.383
Gain:Feed	0.66	0.19	0.50	0.84	0.72	0.11	0.43	0.69	0.67	0.15	0.744

Ctrl: the control group; ETEC: the ETEC K88 infection control group; AVI: avilamycin; OP: ovomucin-protex 26L hydrolysate; OVAP: ovalbumin-protex 26 L hydrolysate. #0.05<*P*<0.10, **P*<0.05, ETEC versus Ctrl by unpaired t test.

Table 6.4. Mid-jejunal permeability and morphology

	Ctrl		ETEC		AVI		OP		OVA		*P*
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
TEER (Ω·cm2)	54.79	12.00	41.20	4.79	34.86	2.54	60.69	16.37	42.14	9.03	0.300
FITC-D40 kDa flux, μg·cm-2·hr-1·mL-1	1.12	0.09	1.02	0.07	1.32	0.19	1.14	0.15	0.92	0.05	0.223
VH (μm)	403.55	25.64	354.94^{b}	12.20	350.79^{b}	20.92	444.30^{a}	18.93	403.83^{ab}	11.12	0.002
CD (μm)	224.00	9.08	219.30^{b}	9.31	227.29^{ab}	8.51	261.97^{a}	14.48	253.98^{ab}	7.99	0.024
VH:CD	1.81	0.12	1.63	0.09	1.55	0.08	1.71	0.07	1.60	0.06	0.506

Ctrl: the control group; ETEC: the ETEC K88 infection control group; AVI: avilamycin; OP: ovomucin-protex 26L hydrolysate; OVAP: ovalbumin-protex 26 L hydrolysate. ETEC-challenged groups that do not share a letter are significantly different according to the one-way ANOVA and Dunnett's test ($\alpha = 0.05$).

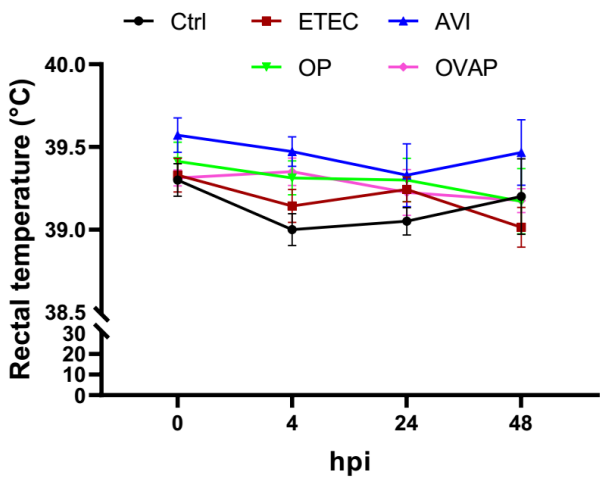

Fig. S6.1. Rectal temperature of piglets. Data are expressed as mean ± SEM. Ctrl: the control group; ETEC: the ETEC K88 infection control group; AVI: avilamycin; OP: ovomucin-protex 26L hydrolysate; OVAP: ovalbumin-protex 26 L hydrolysate.

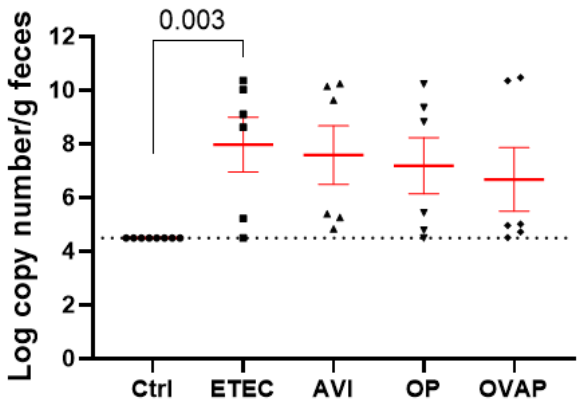

Fig. S6.2. Abundance of ETEC K88 as indicated by $\log_{10}$ number of *faeG* gene copies in colonic contents by HRM-qPCR analysis. Data are expressed as mean ± SEM. The dotted line represents the detection limit. The ETEC group was compared with the Ctrl group by Mann Whitney test. Difference among all ETEC K88-challenged groups were analyzed by one-way ANOVA with Dunnett's test. Ctrl: the control group; ETEC: the ETEC K88 infection control group; AVI: avilamycin; OP: ovomucin-protex 26L hydrolysate; OVAP: ovalbumin-protex 26 L hydrolysate.

CHAPTER 7 – Concluding Remarks and Future Directions

7.1 Key findings of this research

The major objectives of this research are to investigate the modulatory effect of ovomucin hydrolysates on preserving intestinal barrier integrity and mediating mucosal inflammation as an anti-adhesive substance, and to explore its potential application as an antimicrobial alternative against enterotoxigenic *Escherichia coli* (ETEC) K88 infection in post-weaning diarrhea. The key findings of each study are listed below:

1) The first objective was to investigate the anti-inflammatory and intestinal barrier integrity-benefiting activities of ovomucin-protex 26L hydrolysate (Chapter 3)

Ovomucin, the fourth major protein in egg white, is highly glycosylated and responsible for the antimicrobial property of the viscous egg white to protect the embryo development from pathogen infection (Guyot et al., 2016; Omana et al., 2010). Enzymatic hydrolysis of proteins is an efficient approach to generating various bioactive peptides, particularly for those with limited water solubility such as ovomucin. Studies have demonstrated that ovomucin and its hydrolysates possess various biological activities, including anti-oxidative, anti-tumor, and anti-inflammatory activities (Omana et al., 2010; Tu et al., 2020). Previous studies reported the anti-hemagglutinating activity of ovomucin hydrolysates against enterotoxigenic *Escherichia coli* (ETEC) K88 strains, indicating their potential in impeding the adhesion of ETEC K88 to receptors on host tissues (Sun et al., 2017). In a subsequent study, ovomucin-protex 26L hydrolysate (OP) was shown to inhibit the K88 fimbriae-mediated attachment of ETEC K88 to porcine intestinal epithelial cells through function as a decoy receptor for K88 (Sun et al., 2019). Notably, the anti-hemagglutination assay and dot-blot assay used in these two studies are extensively applied models in investigating bacterial adhesion and intestinal microbes-mucus interactions (Etienne-Mesmin et al., 2019; Wang

et al., 2010), while the intestinal epithelial cells are always acknowledged and exploited as the a robust tool in studying bacterial adhesion to host cells (Etienne-Mesmin et al., 2019; Koh et al., 2008; Verhoeckx et al., 2015). Therefore, in this study, the intestinal epithelial monolayer model with differentiated Caco-2 cells were induced by the lipopolysaccharide (LPS), the most potent immunomodulatory component of the bacterial cell wall, to investigate the anti-inflammatory and barrier benefiting activity of the hydrolysate OP.

LPS stress resulted in defects of the cell monolayer integrity, as evidence by decreased transepithelial electrical resistance, increased fluxes of fluorescence tracers, and disruption in the expression and distribution of tight junction proteins. LPS also caused the activation of IκBα/NF-κB and MAPK signaling pathways. OP incubation restored the barrier integrity and inhibited the activation of pro-inflammatory signalling pathways induced by LPS in Caco-2 cells, indicating its potential in protecting intestinal barrier function from pathogen-associated molecule assaults.

2) The second objective was to study the anti-adhesive and anti-inflammatory activities of ovomucin hydrolysates against enterotoxigenic Escherichia coli (ETEC) K88

ETEC K88 is the leading cause of diarrhea in neonatal and post-weaning pigs (Luppi et al., 2016). Several ovomucin hydrolysates, including OP and ovomucin-pepsin/pancreatin hydrolysate (OPP), exhibited binding capacities to the bacterial fimbriae K88 and inhibited the adhesion of ETEC K88 strains to porcine intestinal epithelial cells (IPEC-J2) (Sun et al., 2019). However, it remains unknown whether the hydrolysate products exert protective effects on cell monolayer integrity and if sialic acid residues attached to the glycans on peptide backbones play a crucial role in mediating bacteria-intestinal epithelia interactions. Therefore, OP and OPP were treated with sialidase to determine the role of sialic acid in terms of inhibiting ETEC K88 adhesion to porcine IPEC-J2 and

human Caco-2 cells. Additionally, the effects of OP and OPP on ETEC K88-induced intestinal integrity damage and inflammation were explored.

OP and OPP inhibited ETEC K88 adhesion to intestinal epithelial cells, while removing sialic acids in the hydrolysates compromised their anti-adhesive activity. OP and OPP both protected the intestinal epithelial monolayer integrity as indicated by restored transepithelial electrical resistance. However, the expression levels and network structures of tight junction proteins were only preserved in differentiated Caco-2 cells, not in IPEC-J2 cells. ETEC K88 challenge also induced superoxide generation and intracellular calcium accumulation and activated NF-κB and MAPK p38 signaling molecules. OP and OPP effectively attenuated ETEC K88-induced inflammation and oxidative stress in intestinal epithelial cells. These data supported the anti-adhesive property of OP and OPP and demonstrated their protective effects on intestinal barrier function from ETEC K88 challenge, implying their possible application in preventing ETEC K88 infection.

3) The third objective was to study the in vivo efficacies of ovomucin and hydrolysates in reducing pathogenic colonization and alleviating intestinal mucosal inflammation

Citrobacter rodentium is a natural murine pathogen and related to attaching and effacing pathogens, such as enteropathogenic *E. coli* and enterohemorrhagic *E. coli*, that causes diarrheal diseases in human beings (Collins et al., 2014). *C. rodentium*-induced murine colitis model has been prevalently used to understand the molecular mechanisms of mucosal immunity and pathogen-host interactions and evaluate the effectiveness of dietary interventions (Collins et al., 2014; Lin et al., 2019; Rodrigues et al., 2012; Wlodarska et al., 2015). Given the promising activities demonstrated by OP and OPP in mitigating inflammation induced by bacteria and bacterial components, as well as their properties in preventing bacterial adhesion to intestinal epithelial cells (Chapter 3 & 4), the *C. rodentium*-infected mouse colitis model was used to assess the *in vivo*

efficacies of intact ovomucin (OVM), OP, and OPP, prior to their inclusion in the study involving ETEC K88-infected weaned piglets. *C. rodentium* developed stable colonization in the mouse gut and induced severe colitis in the colonic mucosa as evidenced by colonic hyperplasia, disruption of normal colonic architecture, and goblet cell depletion. OP supplementation resulted in a significant reduction of *C. rodentium* load at 7 d post-infection (dpi) and ameliorated colitis severity as characterized by lower levels of colonic pathology and inflammatory cytokines/chemokines. OVM also mitigated inflammation in the colonic mucosa compared to the *C. rodentium*-infected control group, but potentially not through directly reducing pathogenic burden at 7 dpi. Gut microbial analysis showed that *C. rodentium* infection decreased the bacterial diversity, while OP and OVM maintained the microbial diversity in the gut microbiota in the context of *C. rodentium* infection. Specifically, OP modulated the relative abundance of beneficial gut microbes that produce SCFAs and improved colonization resistance to *C. rodentium*, while OVM mainly regulated the abundance of SCFA producers. Unexpectedly, OPP supplementation reduced gut microbial richness and enhanced pathological severity. These results demonstrated the potential of OVM and OP in preventing pathogenic infections and suggested the different outcomes of ovomucin hydrolysates such as OP in reinforcing host resistance to bacterial infections. This study also indicated the potential practical application of OP in controlling intestinal diseases.

4) The fourth objective was to study the effectiveness of OP in managing post-weaning diarrhea in ETEC K88-infected early weaned piglets

The *MUC4* gene polymorphism on porcine chromosome 13 is associated with ETEC K88 susceptibility (Jørgensen et al., 2003; Jensen et al., 2006). The *MUC4* marker was thus employed to screen piglets in order to control the individual animal variation. Avilamycin (AVI), 0.008% in

the complete feed, is a prescribed medication for reducing incidence and severity of post-weaning diarrhea in swine industry (Government of Canada, 2020). AVI was included in this study as a positive control to compare the efficacy of OP. Additionally, the protex 26L-prepared hydrolysate of ovalbumin, the most abundant protein in egg white, termed OVAP, was used in the present study considering that ovalbumin-derived peptides displayed anti-adhesive activity against enteropathogenic *E. coli* strains (Neeser et al., 1986; Tai et al., 1975).

In this study, piglets in the ETEC control group developed severe diarrhea during 32 hr post-inoculation (hpi) to 40 hpi and showed intermittent patterns, while 2 of the 7 piglets maintained normal solid feces throughout the experimental period with low or undetectable levels of ETEC K88 in the colonic contents. ETEC K88 infection decreased the growth performance of weaned piglets, disturbed intestinal barrier integrity, elevated the mRNA levels of jejunal *IL-6* and colonic *IL-1β* and *TNF-α* compared to the uninfected control piglets. OP and OVAP supplementation showed no changes in the cytokines in small intestine when compared to the ETEC group, while decreased *TNF-α* in colonic tissues. OP-piglets improved jejunal morphology and decreased the colonic *IL-1β* and *IL-6*, while it did not confirm its ability to prevent the ETEC K88 colonization in the pig intestine as indicated by the *in vitro* anti-adhesive property. OVAP showed potential in maintaining the growth performance of piglets and resulted in lower colonic ETEC K88 burden compared to the ETEC control group and the AVI group, potentially through interacting with adhesins such as the type 1 pilus, other than the K88 fimbriae. This study revealed the potential of OP and OVAP in controlling ETEC K88 infection and preserving intestinal health through distinct modes of action. However, future studies are needed to evaluate different dosages of these hydrolysate products and support their practical application.

7.2 Significance of this research

In addition to the nutrient value, egg proteins and derived peptides have been revealed to exhibit various biological properties, including but not limited to anti-oxidative, anti-inflammatory, and anti-hypertensive activities (Bao et al., 2023). Ovomucin, as one of the major proteins in egg white, has also been extensively studied for its bioactivities along with derived hydrolysates (Omana et al., 2010; Tu et al., 2020). Previous research from our lab has characterized the physiochemical properties of ovomucin hydrolysates prepared by different enzymes, including the protex 26L of *Aspergillus niger* origin (termed OP in this thesis) and pepsin-pancreatin of porcine source (termed OPP in this thesis), and preliminarily studied their bifidogenic and anti-adhesive activities (Sun et al., 2016; Sun et al., 2019; Sun et al., 2017). These data lay a foundation for the research in this thesis. This thesis, to the best of our understanding, is the first to report the *in vitro* (in both human and porcine intestinal epithelial cells) and *in vivo* (in both mice and piglets) intestinal health-benefiting effect of ovomucin hydrolysates in the context of enteric infections. This research supports future insights into various value-added investigations and application of ovomucin and hydrolysates.

1) Significance to the egg industry

In 2022, Canada counted 1,218 registered egg producers and 22 federally registered processing egg establishments across the country, generating $1.6 billion in farm cash receipts (AAFC, 2022). Egg production in Canada is increasing from 657 million of dozens in 2021 to 867 million of dozens in 2022 (Statistics Canada, 2024). Table eggs and processed eggs are two categories of the egg market in Canada, where the former accounts for approximately 70% of the Canadian egg production supplying the retail and food service market and the latter accounts for approximately 30% supplying the industrial, food services and retail sectors (AAFC, 2022). Processed eggs are

generally produced into whole eggs, albumen, and yolks in liquid, frozen, or dried form, transformed into other products such as scramble egg mix, hard cooked eggs and egg patties, and developed as many food items including bakery products, mayonnaise, and noodles (AAFC, 2022; American Egg Board, 2023b). Other non-food functional uses for eggs include pet foods, a protein reference used for feeding laboratory animals, culture media for laboratory microorganisms, the ground dried shells for laying hens, the egg white as ingredient for facial masks, and the egg yolk for shampoos and conditioners (Board, 2023a). Specifically, lysozyme from avian egg white is prevalently applied in food industry as an antimicrobial agent (Nawaz et al., 2022). And egg white avidin-based biotechnology is used as a versatile tool with broad applications in medical diagnosis, drug delivery, and targeted therapies (Jain & Cheng, 2017; Lesch et al., 2010). Therefore, the potential of ovomucin and hydrolysates in preventing enteric infections and improving intestinal health will add significant value to eggs and thus diversify egg uses.

2) Significance to the swine industry regarding the antimicrobial resistance crisis

Antimicrobials used in food animals is predicted to rise to 200,235 tons by 2030 (Van Boeckel et al., 2017). In livestock sector, pig production has been highlighted as one of the highest-users of antimicrobials (Matheson et al., 2022; Van Boeckel et al., 2015). And over 90% of antimicrobials used in pig production are administered to weaners and suckling piglets (Lekagul et al., 2019; Sarrazin et al., 2019). Given the global concerns about antimicrobial resistance (AMR) and the impact of the food animal industry, there is increasing interest in exploring promising alternatives in antibiotic-free production for reducing antimicrobial use and preserving animal welfare. The major functional use of egg products in relative sectors is the development and applications of egg yolk antibodies (IgY) in human and veterinary health (Pereira et al., 2019). IgY has also been used in pig production for the prevention and treatment of diarrhea diseases in neonatal and weaned

piglets caused by a number of bacterial and viral pathogens, including ETEC strains carrying different fimbriae adhesins and/or producing different enterotoxins (Jin et al., 1998; Marquardt et al., 1999; Li et al., 2015). Among the mechanisms by which IgY counteracts pathogen activity, inhibition of adhesion is regarded as the primary mode. IgY technology represent a successful pattern of its production, collection, and extraction from eggs and is an important therapeutic resource in times of increasing resistance and antibiotic-reducing production. However, IgY extraction and purification can be time-consuming and expensive that may be not suitable for industrial-scale manufacturing processes or large-scale application (Yakhkeshi et al., 2022). Additionally, poultry welfare is a critical consideration in immunization practices for IgY production, for example, tissue damage and lesions due to injections and adjuvant systems (Olbrich et al., 2002; Marcq et al., 2015). Ovomucin and its hydrolysates demonstrate similar bacteria-agglutinating and adhesion-inhibiting activities against K88-expressing ETEC strains. The findings of this study advance our understanding of anti-adhesive interventions for managing pathogenic infections and provide knowledge for future development of new therapeutic approaches to preventing infectious diarrhea in swine production. Meanwhile, all the potential interventions can contribute to reducing or replacing antimicrobial use and alleviating antimicrobial resistance, offering significant economic and social benefits.

7.3 Limitations and future research prospects

Studies included in this thesis are firstly based on *in vitro* intestinal epithelial cell lines, followed by a mouse model and a post-weaning diarrheic piglet model. This serial research follows a common workflow in the context of investigating natural substances for controlling bacterial infections. The research demonstrated the anti-adhesive, anti-inflammatory, and intestinal health benefiting activities of ovomucin and its hydrolysate. Nevertheless, there are some limitations that

should be acknowledged. The human Caco-2 and porcine IPEC-J2 cell lines are used in this research, however, they lack the complexity of *in vivo* microenvironment, especially for the anti-adhesive study. Pathogenic load in animal gut were quantified; bacterial adhesion to intestinal epithelia can be measured to directly reflect the effectiveness of ovomucin and its hydrolysates. Ovomucin-pepsin/pancreatin hydrolysate, termed OPP in this thesis, unexpectedly showed adverse effect in the context of *Citrobacter rodentium* infection, which remains unknown whether specified dietary care should be taken under the conditions of preexisting enteric infections. Additionally, the digestion, absorption, and metabolism of ovomucin and its hydrolysates in feeding supplementation are not fully understood; what the key components are, particularly in the ovomucin-protex 26L hydrolysates (OP) that significantly reduced pathogenic load in the mouse gut; and how the responsible components and the metabolites take actions in intestinal mucosa as well as in circulation. Finally, small amounts of early weaned piglets were used in this research and each piglet was single housed in a pen, which may not well represent the group conditions in practical production. Based on the key findings and limitations of this thesis, the future research prospects are outlined:

1) In *in vitro* studies involving cell lines, multiple strategies have been developed and applied to enrich the complexity and mimic the *in vivo* microenvironment, including introducing immune cells and enteric bacterial communities and combining engineering technology to establish tissue architecture in the culture system (Etienne-Mesmin et al., 2019; Fasciano et al., 2019). These models and approaches can be used to investigate the role of ovomucin and ovomucin hydrolysates in mediating bacteria-host interactions.

2) The K88 fimbriae of ETEC was of the major research target in this research. However, bacteria exploit various adhesins and virulence factors for adhesion and pathogenesis. Enterotoxins

of ETEC K88, their adhesion to receptors such as sulfatide for the heat-stable toxin (STb) and ganglioside GM1 for the heat-labile toxin (LT), as well as the related molecular signaling pathways and ion channels can be included in future studies to better understand the underlying mechanisms.

3) Ovomucin, OPP, and OP were found to distinctly modulate the gut microbiota of both healthy and infected mice, among which several bacterial genera, such as the *Lachnospiraceae NK4A136 group* with potential to enhance colonization resistance to enteric pathogens (Forgie et al., 2023; Mullineaux-Sanders et al., 2017) and the *Romboutsia* that is reported to widely associate with chronic inflammatory diseases (Cao et al., 2021; Li et al., 2021). Future studies involving model microorganisms and a mouse model system in the context of a controlled or complex microbial community will contribute to better understanding of the diet (ovomucin/hydrolysate)-bacteria-host interactions in health and disease.

4) *Citrobacter rodentium* is a natural murine pathogen and extensively used for understanding the pathogenic mechanisms of enterohemorrhagic and enteropathogenic *Escherichia coli*. Since Ovomucin and its hydrolysate OP showed great potential in mitigating *C. rodentium*-induced colitis, future research is worthy of investigating their activities against human pathogens.

5) The current study used a single dose of the ovomucin hydrolysate OP in the ETEC K88-infected piglet trial and showed potential protective effect from diarrhea and mucosal immune responses. Future research is needed to test higher doses of OP and inspect its long-term effect on piglet intestinal health and growth performance in larger sample size and in field research.

6) In the piglet study, ovalbumin-protex 26L hydrolysate (termed OVAP in this thesis) demonstrated significant reduction in ETEC K88 load in the piglet gut, which was considered through impeding type 1 pilus-mediated adhesion of ETEC K88. Therefore, it is worthy of further studying the effectiveness and mechanisms of OVAP.

Overall, while this thesis demonstrated the potential of ovomucin and its hydrolysate OP in mitigating intestinal infection, several critical areas remain underexplored, warranting further investigation. Future research should elucidate the underlying mechanisms of action of ovomucin and its hydrolysate(s), particularly in complex *in vivo* environments. Additionally, understanding the digestion, absorption, and metabolism of these bioactive compounds in the gastrointestinal tract will be crucial for translating these findings into practical applications, such as dietary supplements or therapeutic interventions. Notably, the promising findings related to OVAP's impact on reducing ETEC K88 load in piglets underscore the need for further studies to confirm these effects and uncover the underlying mechanisms. Expanding research on ovalbumin could reveal additional bioactivities and potential synergies with ovomucin in promoting intestinal health in the context of enteric infections. Furthermore, methods of extraction/production of ovalbumin, ovomucin, and their hydrolysates should be further optimized to reduce the cost of production and make these bioactive compounds feasible for practical use in animal production. Moreover, incorporating more realistic models, such as larger sample size, longer-term inspections, and field studies in piglet production, will enhance the applicability of these findings in practice. By addressing these gaps, future studies can advance the understanding and application of egg-derived bioactives and potentially offer cost-effective solutions in promoting intestinal health and preventing enteric infections.

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