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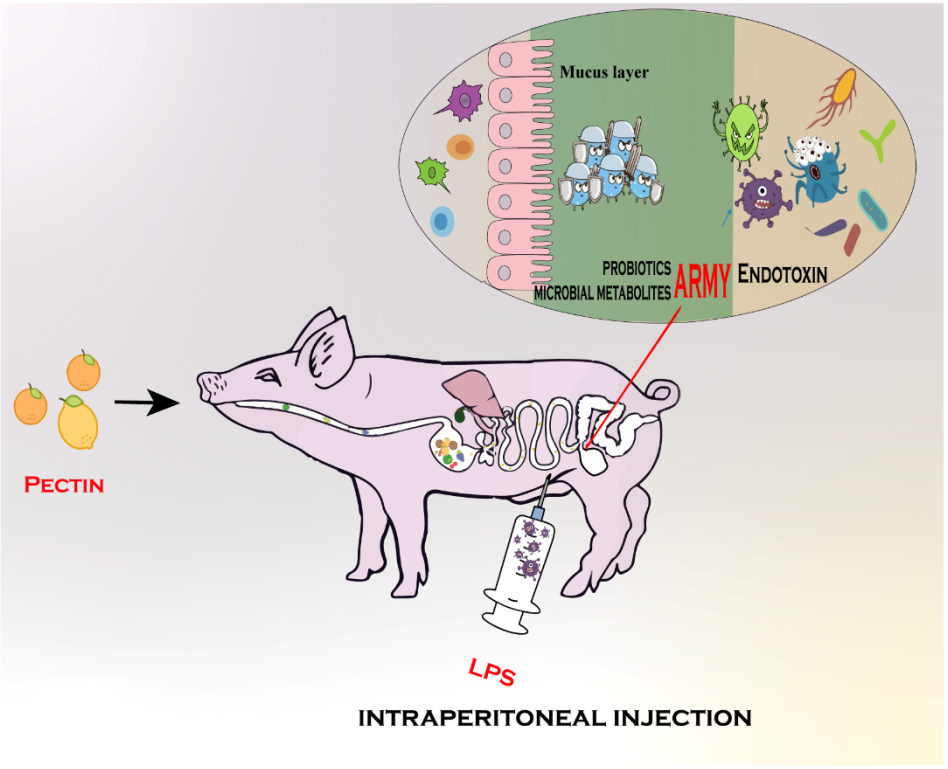
Unveiling Regulatory Mechanisms of Citrus Pectin on Intestinal Immunity in Piglets



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Unveiling regulatory mechanisms of citrus pectin on intestinal immunity in piglets

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Abstract

China is the largest pig-raising country globally. In 2022, global pork production reached approximately 125 million tons, with China accounting for 55.41 million tons, equivalent to around 44% of the total production. Despite being the largest consumer of pork, China lags behind as an advanced pig-raising country. Compared with developed countries in the livestock industry, the average number of live piglets contributed by sows of the same breed in China is only 18-19 piglets per year, which is significantly lower than the average of 24-25 piglets in developed countries such as Denmark, the Netherlands, and France. This lower number can be explained by the combination of lower proliferating sows and higher early life mortality. Especially this high mortality rate of weaned piglets is an important factor restricting the pig breeding industry, and there is room for improvement. Young animals are more susceptible to the external environment, which can disrupt the gut microbial composition and result in pathogen overgrowth, leading to intestinal inflammation and diseases. Farmers have traditionally used antibiotics added to feed to help piglets overcome this critical stage. However, since Sweden took the lead in completely banning the use of antibiotics in feed in 1986, followed by Denmark and other EU countries, there has been a global trend towards antibiotic restrictions. By 2020, China had also joined the wave of comprehensive antibiotic bans. Pectin, as a plant-derived polysaccharide, is one of the seventh major categories of nutrients known as dietary fiber. It has a direct and significant impact on both the intestinal mucosal layer and microbiota, exhibiting preventive effects against diarrhea, promoting growth, and regulating immunity in livestock. However, the mechanisms underlying the regulatory effects of dietary fiber on the intestinal function of livestock remain unknown. Therefore, the purpose of this study is: 1) What are the effects of pectin on jejunal development, gut microbiota, and indole-like metabolites in healthy piglets, and mucosal immunity in healthy piglets? 2) After piglets fed pectin are injected with LPS, what effect will pectin have on growth performance and immune tolerance of piglets? Here we thus address the effect of pectin in pigs undergoing a model of inflammation 3) What is the effect of pectin on the cecal morphology, microbiota structure, and microbial metabolites of LPS-challenged piglet models?

For the first objective, sixteen 21-day-old crossbred barrows (6.77 ± 0.92 kg; Duroc × Landrace × Yorkshire) were randomly assigned to two diets, with eight piglets per group. After a 3-day adaptation period, the piglets were fed a diet containing either 5% microcrystalline cellulose (control group) or 5% pectin (treatment group) for 3 weeks. The results demonstrated that pectin supplementation improved intestinal integrity, reduced inflammatory response, and down-regulated the expression of proinflammatory cytokines in the mucosa of the jejunum. Furthermore, pectin supplementation altered the jejunal microbiome and tryptophan-related metabolites in piglets, enhancing the *AhR-IL22-STAT3* signaling pathway.

Building on the first experiment, the second experiment aimed to investigate the resistance of piglets fed with pectin to LPS-induced stress. Twenty-four piglets (6.77 ± 0.92 kg BW; Duroc × Landrace × Large White; barrows; 21 days old) were randomly assigned to three groups: control, LPS-challenged, and pectin-LPS. The piglets received LPS or saline injections on days 14 and 21 of the experiment. The addition of pectin promotes the villus height in the ileum of piglets. At the same time, it restores the changes in tight junction proteins and inflammatory factors induced by LPS, thereby attenuating the inflammation caused by LPS stimulation in the small intestine. Additionally, it improved the mucin barrier function, increased *MUC2* mRNA expression, and modulated the gut microbiota composition, reducing harmful bacteria and increasing beneficial bacteria and short-chain fatty acids (SCFAs) production.

The third experiment focused on the cecum, as pectin is a dietary fiber known for its fermentation capability. Twenty-four piglets (Yorkshire × Landrace, 6.77 ± 0.92 kg) were divided into three groups: control, LPS-challenged, and pectin-LPS, with eight replicates per group. The piglets were fed corn-soybean meal diets containing 5% citrus pectin or 5% microcrystalline cellulose. Pectin supplementation mitigated the cecal morphological damage induced by LPS, improved the expression of tight junction proteins, mucin, and anti-inflammatory cytokines, while reducing proinflammatory cytokines. Pectin also modulated the gut microbiota composition, enriching beneficial bacteria and SCFAs, and activating relevant receptors.

In summary, this thesis focused on the intestinal development, microbiota, and immune response of piglets at different gut segments (jejunum, ileum, and cecum). It explores the effects of pectin on piglets in healthy and non-healthy conditions and provides valuable insights into the potential benefits of pectin as an alternative to antibiotics in piglet feed.

Key words: Pectin, weaned piglets, gut microbiota, metabolites, immunity

Résumé

La Chine est le plus grand pays d'élevage de porcs au monde. En 2022, la production mondiale de viande de porc a atteint environ 125 millions de tonnes, dont 55,41 millions de tonnes provenant de la Chine, ce qui représente environ 44% de la production totale. Malgré sa position de plus grand consommateur de porc, la Chine est en retard en tant que pays avancé dans l'élevage de porcs. Comparé aux pays développés de l'industrie de l'élevage, le nombre moyen de porcelets vivants produits par des truies de la même race en Chine est seulement de 18 à 19 porcelets par an, ce qui est significativement inférieur à la moyenne de 24 à 25 porcelets dans des pays développés tels que le Danemark, les Pays-Bas et la France. Ce nombre inférieur peut s'expliquer par la combinaison d'une prolifération plus faible des truies et d'une mortalité précoce plus élevée. En particulier, le taux de mortalité élevé des porcelets sevrés est un facteur important qui limite l'industrie de l'élevage de porcs, et il y a des possibilités pour améliorer. Les jeunes animaux sont plus sensibles à l'environnement externe, ce qui peut perturber la composition microbienne de l'intestin et entraîner une prolifération pathogène, conduisant à une inflammation intestinale et à des maladies. Les éleveurs ont traditionnellement utilisé des antibiotiques ajoutés à l'alimentation pour aider les porcelets à surmonter cette étape critique. Cependant, depuis que la Suède a été le premier pays à interdire totalement l'utilisation d'antibiotiques dans l'alimentation animale en 1986, suivi par le Danemark et d'autres pays de l'Union européenne, il existe une tendance mondiale à restreindre l'utilisation d'antibiotiques. En 2020, la Chine s'est également jointe à la vague d'interdiction complète des antibiotiques. La pectine, en tant que polysaccharide d'origine végétale, est l'un des sept principales catégories de nutriments connus sous le nom de fibres alimentaires. Elle a un impact direct et significatif à la fois sur la couche muqueuse intestinale et le microbiote, présentant des effets préventifs contre la diarrhée, favorisant la croissance et régulant l'immunité chez les animaux d'élevage. Cependant, les mécanismes sous-jacents des effets régulateurs des fibres alimentaires sur la fonction intestinale des animaux d'élevage restent inconnus. Par conséquent, l'objectif de cette étude est le suivant : 1) Quels sont les effets de la pectine sur le développement du jéjunum, le microbiote intestinal et les métabolites similaires à l'indole chez les porcelets en bonne santé, ainsi que sur l'immunité muqueuse chez les porcelets en bonne santé ? 2) Quel effet la pectine aura-t-elle sur les performances de croissance et la tolérance immunitaire des porcelets après les avoir nourris avec de la pectine et les avoir ensuite injectés avec du LPS ? Nous étudions donc ici l'effet de la pectine chez les porcs soumis à un modèle d'inflammation. 3) Quel est l'effet de la pectine sur la morphologie cœcale, la structure du microbiote et les métabolites microbiens des modèles de porcelets stimulés par le LPS ?

Pour le premier objectif, seize verrats croisés de 21 jours (6,77 ± 0,92 kg ; Duroc × Landrace × Yorkshire) ont été répartis au hasard dans deux régimes, avec huit porcelets par groupe. Après une période d'adaptation de 3 jours, les porcelets ont été nourris avec un régime contenant soit 5 % de cellulose microcristalline (groupe

témoin), soit 5 % de pectine (groupe traitement) pendant 3 semaines. Les résultats ont démontré que la supplémentation en pectine améliorait l'intégrité intestinale, réduisait la réponse inflammatoire et régulait à la baisse l'expression de cytokines pro-inflammatoires dans le jéjunum. De plus, la supplémentation en pectine modifiait le microbiote jéjunal et les métabolites liés au tryptophane chez les porcelets, renforçant la voie de signalisation AhR-IL22-STAT3.

Sur la base de la première expérience, la deuxième expérience visait à étudier la résistance des porcelets nourris avec de la pectine au stress induit par le LPS. Vingt-quatre porcelets (6,77 ± 0,92 kg de poids vif ; Duroc × Landrace × Large White ; verrats ; 21 jours) ont été répartis au hasard dans trois groupes : témoin, LPS-challenged et pectine-LPS. Les porcelets ont reçu des injections de LPS ou de solution saline les jours 14 et 21 de l'expérience. L'ajout de la pectine favorise la hauteur des villosités dans l'iléon des porcelets. Parallèlement, il rétablit les modifications des protéines de jonction serrée et des facteurs inflammatoires induits par le LPS, atténuant ainsi l'inflammation provoquée par la stimulation du LPS dans l'intestin grêle. De plus, elle améliorait la fonction barrière du mucus, augmentait l'expression de l'ARNm de MUC2 et modulait la composition du microbiote intestinale, réduisant les bactéries nuisibles et augmentant les bactéries bénéfiques et la production d'acides gras à chaîne courte (AGCC).

La troisième expérience portait sur le cæcum, car la pectine est une fibre alimentaire connue pour sa capacité de fermentation. Vingt-quatre porcelets (Yorkshire × Landrace, 6,77 ± 0,92 kg) ont été répartis en trois groupes : témoin, LPS-challenged et pectine-LPS, avec huit répétitions par groupe. Les porcelets ont été nourris avec des régimes à base de maïs et de tourteau de soja contenant 5 % de pectine d'agrumes ou 5 % de cellulose microcristalline. La supplémentation en pectine atténuait les dommages morphologiques du cæcum induits par le LPS, améliorait l'expression des protéines de jonction serrée, du mucus et des cytokines anti-inflammatoires, tout en réduisant les cytokines pro-inflammatoires. La pectine modulait également la composition du microbiote intestinale, enrichissant les bactéries bénéfiques et les AGCC, et activant les récepteurs correspondants.

En résumé, cette thèse s'est concentrée sur le développement intestinal, le microbiote et la réponse immunitaire des porcelets à différents segments intestinaux (jéjunum, iléon et cæcum). Elle explore les effets de la pectine sur les porcelets dans des conditions saines et non saines, et offre des aperçus précieux sur les avantages potentiels de la pectine en tant qu'alternative aux antibiotiques dans l'alimentation des porcelets.

Mots clés : Pectine, porcelets sevrés, microbiote intestinal, métabolites, immunité

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Guoqi Dang

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List of abbreviations

5-HT: Serotonin
ABA: Abscisic acid
ADFI: average daily feed intake
ADG: average daily gain
AhR: Aryl hydrocarbon receptor
B3GALT5: beta-1,3-galactosyltransferase 5
B3GNT3: beta-1,3-N-acetylglucosaminyltransferase 3
B3GNT6: beta-1,3-N-acetylglucosaminyltransferase 6
B4GALT1: beta-1,4-galactosyltransferase 1
BW: body weight
C1GALT1: core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1
C1GALT1C1: C1GALT1 specific chaperone 1
CD: crypt depth
ConA: concanavalin A
CYP1A1: Cytochrome P450 1A1
CYP1B1: Cytochrome P450 1B1
DAPI: 4_6-diamidino-2-phenylindole
F:G: feed-to-gain ratio
FITC: Fluorescein Isothiocyanate
FUT2: fucosyltransferases 2
GalA: D-galacturonic acid
GCNT1: glucosaminyl (N-acetyl) transferase 1
GCNT3: glucosaminyl (N-acetyl) transferase 3
GlcNAc: N-acetylglucosamine
HE: Ematoxylin-eosin
HIAA: 5-Hydroxyindole-3-acetic acid
IA: Indoleacrylic acid
IAA: 3-indoleacetic acid
IAALD: 3-indole acetaldehyde
IALD: 3-indole aldehyde
IE: 3-indole ethanol
IL: Interleukin
IPA: 3-indolepropionic acid
Kyn: Kynurenine
LPS: lipopolysaccharide
MCC: Microcrystalline cellulose
MCP1: monocyte chemotactic protein 1
MUC2: Mucin 2
NLS: Nuclear localization signal

PAS-AB: periodic acid-Schiff-Alcian Blue stain
PEC: pectin
PVDF: Polyvinylidene difluoride
SCFA: short-chain fatty acid
SRA: Sequence Read Archive
ST: Skatole
TFF3: Trefoil factor 3
TLR4: Toll-like receptor 4.
TNF- α: tumor necrosis factor- α
TPm: Tryptamine
Trp: Tryptophan
UEA-1: Ulex europaeus agglutinin 1
VH: Villus height
VH:CD: the ratio of villus height to crypt depth
WGA: wheat germ agglutinin
ZO-1: Zonula Occludens-1

Chapter I

General introduction

Chapter I. General introduction

1.1 Pig production and the critical post-weaning period.

In 2022, global pork production reached around 125 million tons, with China's production accounting for 55.41 million tons, or approximately 44% of the total production. The United States and the European Union follow closely in terms of pig farming progression. China's expanding economy is driving up the demand for pork, and while many pig farms in China remain small-scale, the emergence of factory farming as a large-scale production method has led to an increase in pork production (Shahbandeh, 2023). Additionally, China is the world's largest consumer of pork. In 2022, the average per capita consumption of pork for Chinese households was around 26.9 kg, representing a year-on-year increase of 6.75%. However, China is not an efficient pig breeding country. A study shows that in China, the average number of weaned piglets per breeding sow is 17 to 18 per year, and even for high level large-scale pig breeding enterprises, it only increases to 20 or 22, while in developed countries with livestock industry such as Europe and the United States, this number can reach 25 to 28, and some Danish farms have even reached 35 (Bjorkman et al., 2017;Kemp et al., 2018). There is an interesting saying in the livestock industry: "The greatest sorrow of a pig farm is not that the sows do not produce piglets; the greatest sorrow of a pig farm is that the sows produce piglets but cannot keep them alive". The high mortality rate of weaned piglets is one of the important reasons affecting the development of pig breeding in China.

1.1.1 Weaning stress in piglets

The weaning stage is the most difficult and critical period in a piglet's entire life, affecting growth and development. Currently, the exact weaning age in production primarily ranges from 3 to 6 weeks, and it may vary in different farms and management systems. Typically, farms aim to increase the frequency of sow farrowing by shortening the weaning age of piglets. Therefore, in commercial pig farms, the common weaning age for piglets is 3-4 weeks (Buchet et al., 2017;Vodolazska et al., 2023).

The impact of weaning on piglets varies considerably. Firstly, there are obvious changes in the farming environment before and after weaning, including the separation of mothers and their offspring, and the competition for the position after regrouping. Secondly, the temperature is a sensitive factor affecting the stress of weaned piglets. In addition, the digestive organs of early weaned piglets are not fully developed, and the diet of weaned piglets changes from the previous liquid breast milk to a solid starch-based feed. This leads to an incomplete digestion of nutrients in the intestine, with as result undigested proteins flowing into the hindgut and being fermented by microorganisms. This in turn produces potentially toxic metabolites and induces an inflammatory response in the intestine, which eventually can lead to diarrhea in weaned piglets (Pieper et al., 2012).

1.1.2 Pig intestinal anatomy

In general, the mammalian intestinal tract is physiologically structured from front to back as follows: the duodenum, jejunum, ileum, cecum, and colon. The duodenum, jejunum, and ileum collectively constitute the small intestine, while the cecum and colon make up the hindgut (Xu et al., 2021b).

The small intestine functions as the primary site for digestion, secreting various digestive enzymes to assist in the breakdown of proteins, carbohydrates, and fats (Zhao et al., 2022). Moreover, the small intestine is lined with microvilli, which increase the surface area for nutrient contact and facilitate nutrient absorption (Haussner et al., 2019). Additionally, the small intestine hosts an immune system that aids the body in defending against invading pathogens, thus maintaining intestinal health. The hindgut consists of the cecum and colon, renowned for their unique anaerobic environment and potent fermentation capabilities. This results in a rich population of microorganisms in the hindgut, along with microbe-associated metabolites, such as short-chain fatty acids, which play a significant role in modulating the body's immune responses (Zhang et al., 2019; Wahlstrom et al., 2016).

The composition of the intestinal tract from the inside out includes the mucosal layer, submucosal layer, muscular layer, and serosal layer (Bauer et al., 2020). The mucosal layer is the innermost layer of the intestinal wall, primarily composed of mucosal cells, villi, lymphocytes, immune cells, and more. The intestinal mucosa is lined with microvilli and villi, promoting the absorption of nutrients. Additionally, mucosal cells secrete mucus, serving as a lubricant to prevent friction between food and the intestinal wall. Moreover, the intestinal mucosa, as part of the immune system, acts as a barrier against invading pathogens and helps maintain intestinal balance. Moving to the submucosal layer, it maintains the blood supply and lymph drainage of the intestinal mucosa. The intestinal muscular layer and serosal layer play roles in intestinal motility, enzyme and food mixing, and other functions (Holman et al., 2017; Mosenthin., 1998).

1.1.3 Effects of weaning on the digestive system

After weaning, the piglet's stomach acid secretion capacity as well as the source of available lactose decrease, which leads to lower lactic acid production. This results in an increase of the pH of the weaned piglet's gastrointestinal tract and reduces the digestive capacity of the gastrointestinal tract. In addition, weaning reduces the piglet's gastrointestinal dynamics and prolongs the residence time of food in the stomach, which causes the growth of some harmful bacteria and leads to diarrhea. Lactating piglets acquire a large amount of IgA through breast milk, which enters the mucus-covered surface of the intestinal villi and effectively reduces the attachment of harmful bacteria to the intestinal villi. After weaning, the IgA provided by breast milk disappears. Bacteria and food can damage the intestinal villi causing them to atrophy, leading to a reduction in the absorption capacity of the intestine and resulting in diarrhea. In addition, the mammalian small intestine secretes carbohydrate catabolic, proteolytic, and lipase enzymes (Lebenthal et al., 1999;Han, 2000;Lim et al., 2022). It has been reported that the

activity of the carbohydrate enzymes maltase and sucrase in the small intestine of newborn piglets is low, while the activity of lactase is high (Corring, 1978). After weaning, the activity of pancreatic amylase in the small intestine decreases significantly, with the activity of lactase decreasing significantly one week after weaning and being lower than that of lactating piglets at the same age (Lindemann et al., 1986).

1.1.4 Effects of weaning on the gut microbiome

A multitude of studies agree that the intestine of a newly born animal is free from microbiota (Steegenga et al., 2014;Neu, 2016;Grech et al., 2021). After parturition, with changes in the environment, the intestinal microorganism begins to colonize due to a combination of genetic, dietary, and environmental factors. However, other studies have shown that a variety of bacteria are already present in the gut of the animal during the embryonic period (Neu, 2016). *Lactobacillus* and *Streptococcus* are the dominant microorganism in the stomach and small intestine of lactating piglets, which facilitates the digestion and absorption of nutrients from breast milk (Pluske, 2016). After weaning, the structure and function of the piglet's intestinal tract and the intestinal micro-organisms change accordingly with changes in the diet and environment (Guevarra et al., 2019). It has been observed that the gut microbiota of piglets shifts quickly after weaning and reaches a relatively stable level 10 days after weaning. *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Tenericutes*, *Actinobacteria* and *Fusobacteria* are the six dominant phyla that inhabit the piglet intestine (Arfken et al., 2020;Caffarelli et al., 2021). Age has a significant impact on alpha diversity, increasing species richness with time. After weaning, beta diversity is reduced, pointing to the convergent evolution of individuals. Higher relative abundance of *Bacteroides* and *Lactobacillus* in the gut of piglets is associated with a milk-oriented microbiome during lactation. As the piglets age and after weaning, increasing abundances of genera such as *Prevotella* and *Butyricimonas* have been observed (Chen et al., 2017b;Saladrigas-Garcia et al., 2022).

1.1.5 Common Pathogenic Microbial Communities in the Intestines of Piglets during Weaning

Pathogenic microorganisms are a group of organisms that can cause diseases or health problems, such as bacteria, viruses, fungi, or parasites, among others (Boeckman et al., 2022). They can lead to infections or diseases, typically because they have the ability to multiply and invade the host's tissues.

The impact of pathogenic microorganisms on the intestines of weaned piglets is generally negative. Research has shown that the intestinal tract of piglets is susceptible to the invasion of pathogenic microorganisms during the weaning period. These microorganisms can cause intestinal infections, resulting in health issues like diarrhea, delayed development, and stunted growth (Jiao et al., 2021). Moreover, some pathogenic microorganisms may compete for nutrients, leading to inadequate nutrient intake by piglets, which can affect their health and growth. Specifically, the pathogenic microorganisms that commonly induce illness in weaned piglets during the weaning stage include *Escherichia coli*, *Salmonella spp.*, *rotavirus* and *Enterococcus faecalis*. *Escherichia coli* is a conditionally pathogenic

bacterium that can cause gastrointestinal infections or infections in various local tissue and organs under specific conditions (Liu et al., 2022b). *Salmonella spp*. are Gram-negative bacteria with six subtypes, and the infection symptoms primarily include diarrhea and vomiting (Casanova-Higes et al., 2019). Rotavirus is a common pathogenic microorganism that can cause intestinal diseases in piglets (Vlasova et al., 2017). *Enterococcus faecalis* is another common pathogenic microorganism that can cause intestinal diseases in piglets (Kau et al., 2005).

1.2 The intestinal barrier

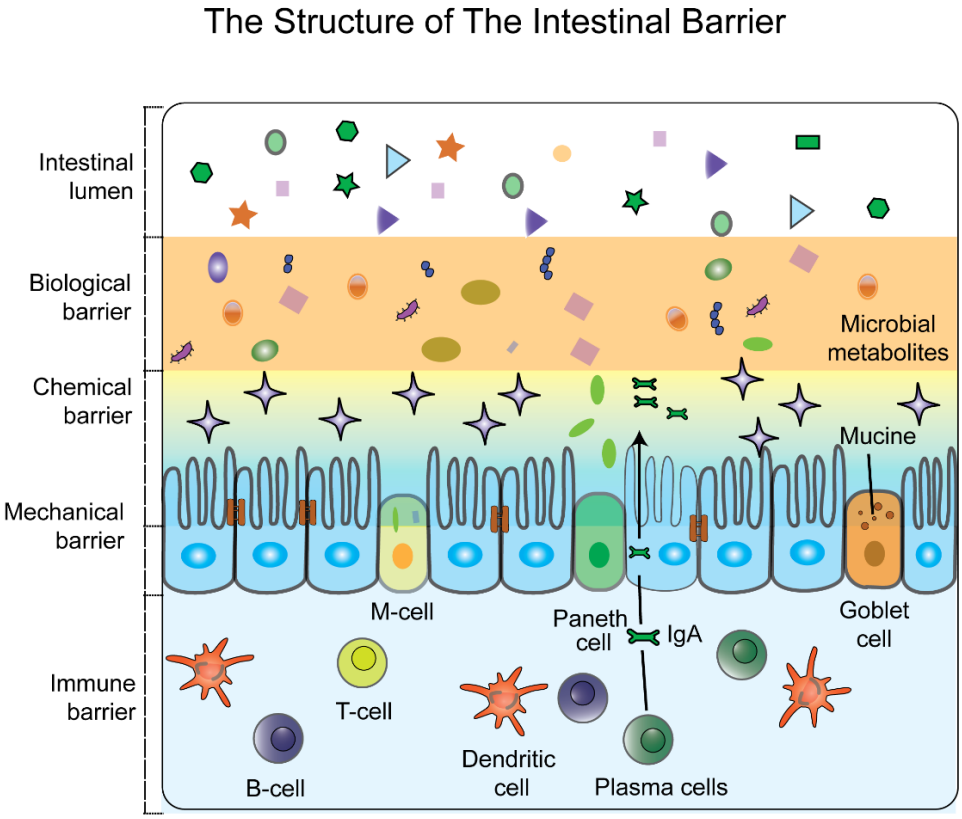


Figure 1-1. The illustration of the structure of the intestinal barrier. Starting from the intestinal lumen, there are four main components of the intestinal mucosal barrier. The first is the biological barrier, which is formed by the intestinal commensal microbiota. The second is the chemical barrier, which is mainly composed of mucus. The third is the mechanical barrier, which is composed of intestinal mucosal epithelial These three components work together to prevent bacteria from penetrating the mucosa and entering deep tissues, and form the structural basis of the intestinal mucosal barrier. The fourth and final component is the immune barrier, which is mainly composed of gut-associated lymphoid tissue (GALT) and secretory immunoglobulin (SIgA)."

The intestine is the longest and most functionally important section of the digestive tube, which runs from the pylorus of the stomach to the anus. In addition, the intestine is the largest immune organ in the mammalian body. Studies have shown that over 70% of immune cells are concentrated in the gut, including macrophages, T cells, NK cells, and B cells, and that 70% of immunoglobulin A,

which is beneficial to the gut, is made in the gut (Belkaid et al., 2014;Ma et al., 2021a).

The human intestine is also inhabited by a large number of microorganisms that have formed a symbiotic relationship with their hosts over a long period of evolution (Wu et al., 2021c). Under normal circumstances these microorganisms do not harm the host, but this is entirely dependent on the function of the body's intact intestinal mucosal barrier. The intestinal mucosal barrier consists of four main components: a mechanical, an immune, a chemical and a biological barrier, each of which has its own structural basis and is important to prevent harmful substances and pathogens from entering the internal environment of the body and to maintain the stability of the internal environment of the body (Figure 1-1).

1.2.1 Mechanical barrier

The mechanical barrier, the "security guard" of the intestine, consists of mucosal epithelial cells (IEC), tight intercellular junctions, and bacterial membranes. The IEC are composed of various cell types such as enteroabsorptive cells, goblet cells, and Paneth cells (Di Tommaso et al., 2021). Together they maintain effective defenses against various toxins and antigens in the intestinal lumen. Enterocytes are the main cell type (80-90%) of the intestinal epithelium, which is a simple columnar epithelial cell. They can not only absorb ions, water, sugars, lipids, and other nutrients but are also involved in the secretion of immunoglobulin (Kong et al., 2018). Goblet cells, another type of intestinal epithelial cells, secrete mucus. Paneth cells are distributed throughout the small intestine and secrete defensins in response to exposure to bacteria or antigens, which help maintain the intestinal barrier (Wilson et al., 1999; Ayabe et al., 2000). Microfolded cells (M cells) can ingest intestinal contents and transport antigens to immune cells, thus controlling the immune response (Beumer et al., 2021). Enteroendocrine cells regulate appetite and the release of insulin, and also regulate the activation of intestinal immune cells through peptide hormones such as glucagon-like peptide 1 (GLP-1) (Worthington et al., 2018). Tuft cells are referred to as sentinels of the gastrointestinal tract, which can secrete a wide range of effector molecules, including *interleukin 25* (*IL-25*), prostaglandin E2 and D2, acetylcholine, and β-endorphin, some of which have immune regulatory functions (Hendel et al., 2022).

The tight junctions, an important component of the epithelium, often receive threats from pro-inflammatory mediators, pathogenic viruses, and bacteria (Camilleri, 2019). Tight junction proteins can be divided into cell membrane proteins and cytoplasmic proteins, depending on their distribution. The intracellular structural domains of cell membrane proteins, including transmembrane proteins such as occludin, claudin, tight junction adhesion molecules (JAM) and tricellulin, interact with intracellular cytoskeletal proteins and are the structural proteins that form the selective barrier. Occludin proteins are involved in the signaling regulation of tight junction structures by binding in a zipper-like manner through the outer ring to produce a tight paracellular closure (Nusrat et al., 2000). Claudin is a member of a family of tight junction proteins that has only been identified in recent years. Changes in its expression level and distribution directly affect the structure and function of the tight junctions. The PDZ structural domain of cytoplasmic proteins, abbreviated from the postsynaptic dense protein 95 (PSD-95), discs-large

(Dlg), and Zonula occludens-1 (ZO-1), is one of the most commonly identified structural domains and interacts with the cytoskeleton to play an important role in maintaining the structural integrity (González-Mariscal et al., 2003).

Also, a biofilm can be seen as an interesting barrier (Yin et al., 2019). Commensal bacteria in the intestinal tract form a physical barrier through their adhesion and colonization (Coates et al., 2019). As we all know, the surface of bacteria is covered with a film of molecules such as polysaccharides and proteins, which can also prevent the destruction of the intestinal barrier by binding toxins and other harmful substances in the intestinal tract (Riu et al., 2022). In addition, bacteria can also act as antigens to stimulate the immune system to generate an immune response, thereby improving intestinal immunity (Belkaid et al., 2014).

The mechanical barrier ensures the absorption of nutrients, electrolytes and water. It also maintains effective defense against various toxins and antigens in the intestinal lumen (Groschwitz et al., 2009).

1.2.2 Chemical barrier

The chemical barrier, the "cleaner" of the intestine, is made up of chemicals such as mucus, digestive enzymes, mucopolysaccharides, glycoproteins and glycolipids secreted by the intestinal epithelium (Bertero et al., 2020).Stomach acid kills bacteria that enter the gastrointestinal tract and inhibits bacterial adhesion and colonization of the gastrointestinal epithelium. Lysozymes destroy the cell wall of bacteria and assure bacterial lysis (Xue et al., 2010). The mucous layer is primarily composed of mucins secreted by intestinal goblet cells, with MUC2 being the predominant form in the intestine, along with MUC3 (Hansson et al., 2020). The mucus produced by the goblet cells contains complement components that increase the antibacterial effect of lysozymes and immunoglobulins. In addition, the large amount of digestive fluid secreted by the intestine dilutes toxins, and flushes and cleanses the intestinal tract, making it difficult for potentially pathogenic bacteria to adhere to the intestinal epithelium and thus protecting the health of the intestinal tract (Camilleri, 2019).

1.2.3 Microorganisms barrier

The microorganisms as a barrier are the "manager" of the intestine. The intestine is known to be the largest bacterial reservoir in the body, hosting about $10^{\wedge 13}$ to $10^{\wedge 14}$ bacteria, 99% of which are exclusively anaerobic (Khan et al., 2021). The number and distribution of resident microbiota in the gut are relatively constant, forming an interdependent and interacting micro-ecosystem, the balance of which constitutes the biological barrier of the gut (Wei et al., 2013).

The intestinal microbiota consists mainly of the mucosal microbiota and the luminal microbiota. The mucosal microbiota is dominated by *Bifidobacteria* and lactic acid bacteria. The intestinal luminal microbiota are mostly *E. coli* and *Enterococcus*, which mainly adhere to the intestinal mucosa layer and form a multi-layered intestinal microbial barrier. These microorganisms can not only compete to inhibit pathogenic bacteria, such as certain intestinal parthenogenic anaerobes and foreign bacteria, from binding to the intestinal epithelium but also inhibit their colonization and growth (Axarlis et al., 2021). They can also secrete short-chain

fatty acids such as acetic acid, propionic acid, butyric acid and lactic acid, which lower the intestinal pH and redox potential. Moreover, the commensal bacteria also compete with pathogenic bacteria for the use of nutrients, thus inhibiting the growth of pathogenic bacteria (Flint et al., 2015).

1.2.4 Immune barrier

The immune barrier is the "soldier" of the intestinal tract. The immune components associated with the intestine are divided into the lymphoid tissues and lymphocytes scattered throughout the intestinal wall (Pan et al., 2019;Ren et al., 2019), including Peyer's patches, isolated lymph nodes in the intestinal mucosa, macrophages, T helper cells, B cells, plasma cells and intraepithelial lymphocytes that are diffused throughout the intestinal mucosa. The central role in the intestinal immune system is played by secretory IgA (sIgA) (Zhang et al., 2019). SIgA is mainly produced by plasma cells and is distributed on the surface of the intestinal mucosa. It is the most abundant immunoglobulin in intestinal secretions and is the main immune defense factor against pathogens (Ren et al., 2016). The main functions of sIgA in the intestine is to prevent the adhesion of pathogens to the intestinal mucosal surface, neutralize toxins produced by bacteria, neutralize viruses, enhance phagocytosis of cells and coordinate antibacterial activity with complement and lysozymes. A decrease in sIgA in the intestine decreases the immune barrier function of the intestinal mucosa against infection, increasing the opportunity for intestinal bacteria and endotoxins to interact with the mucosal epithelium and promoting bacterial translocation and endotoxin uptake (Brandtzaeg, 2013).

1.3 The source, extraction and properties of pectin

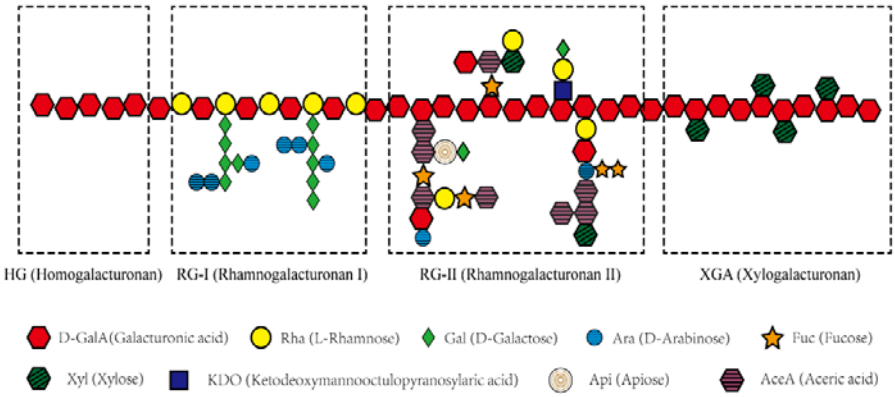


Figure 1-2. The structure of pectin. Pectin has four structural domains, including homogalacturonan (HG), a linear pectic polysaccharide of α-1,4-linked galacturonan; followed by rhamnogalacturonan I (RG-I), which consists of a repeating backbone of (α-1,2)-l-rhamnose-(α-1,4)-d-galacturonic acid, with various side chains; rhamnogalacturonan-II (RG-II) is the most complex type of fructose, consisting of a HG backbone of at least eight 1,4-linked α-D-GalA residues, decorated with 12 different types of side chains. The primary structure of XGA is composed of a linear polymer composed of α-1,4-D-GalAp residues, and the C-3 hydroxyl group of GalA on the polymer backbone is covered by monosaccharide D -Xyl to form the XGA domain.

Pectin is a type of important natural polymer compound that widely exists in all higher plants and is one of the main components of the plant cell wall (Mellinas et al., 2020). It deposits heavily in the primary cell wall and middle lamella, and cross-links with microfibrils of cellulose, hemicellulose, lignin with varying proportions, and certain extensins, to collectively maintain the structure and shape of various cell tissues (Sarkar et al., 2009). Pectins are also heterogeneous polysaccharides(Le Normand et al., 2021). It was for the first time discovered in 1824 by Bracennot, a famous pathologist in France who extracted it from carrot (Maxwell et al., 2012). Pectin consists of an alpha;-(1,4)-linked d-galacturonic acid (d-GalA) backbone, and contains some regions with alterating L-rhamnose and d-GalA (Willats et al., 2006). There are four structural types of pectin called pectin domains: Homogalacturonan (HG), Rhamnogalacturonan II (RG-II), Rhamnogalacturonan I (RG-I), xylogalacturonan (XGA). HG, a linear homopolymer of α-1,4-linked galacturonic acid that comprises 65% of the pectin, is the most abundant pectic polysaccharide (Lutz et al., 2009) (Figure 1-2). The second largest fraction of pectin is RG-I (20-35%), which comprises of a repeating backbone made up of (α-1,2)-l-rhamnose-(α-1,4)-d-galacturonic acid, along with various side chains (Wilmowicz

et al., 2022). RG-II is the pectin part with the most complex structure (10%), consisting of an HG backbone of at least 8 1,4-linked α-D-GalA residues decorated with side branches consisting of 12 different types of sugars in over 20 different linkages (Mohnen, 2008). The primary structure of XGA consists of a linear polymer composed of α-1,4-D-GalAp residues. However, the C-3 hydroxyl groups of GalA on the polymer backbone are substituted with monosaccharide D-Xyl to create the XGA domain (Martínez-Trujillo, 2009;Jin et al., 2021) (Figure 1-2).

1.3.1 The source of pectin

The sources for pectin compose of a wide variety of fruits and vegetables. Popular sources include citrus fruits (oranges, grapefruits, lemons, and limes - known as citrus pectin), apples, beets, carrots, apricots, plums, blackberries, and cherries.

1.3.1.1 Citrus pectin

Citrus pectin is a polysaccharide compound extracted from the peels and pulps of various fruits such as grapefruits, oranges, and lemons, and its molecules exist out of long-chain carbohydrates (Zhang et al., 2019). Recent studies have shown that citrus pectin extracted under ultrasound-assisted conditions has an enhanced emulsifying ability and stability when compared to untreated pectin. This is especially the case for lemon peel, which has a pectin content of up to 30% (Vogt et al., 2016;Wang et al., 2021a). This pectin has a good gel formation, thickening, and emulsifying properties, while both lemon and orange pectin show an ideal viscosity and protein stability, which can provide a good texture for food (Maxwell et al., 2012;Wongkaew et al., 2020).

1.3.1.2 Apple pectin

Apple pectin is a high molecular weight polysaccharide extracted from apple waste, which contains 15% to 18% pectin, making it another commercially produced fruit pectin besides citrus pectin (Lyu et al., 2020). As a natural food additive, it possesses excellent gel properties, stability, thickening, and emulsifying properties (Wang et al., 2016). The optimized apple pectin extracted through the process has gel and viscosity properties suitable for use as a gel agent (Wang et al., 2014a). Furthermore, acid-base modifications and thermal modifications of apple pectin result in a rich dehydrated GalA content, which enhances its antioxidant activity, possibly due to an increased level of GalA (Fraeye et al., 2007).

1.3.1.3 Other pectins

Beet pulp is a byproduct of beet sugar production and its raw materials are readily available (An et al., 2019). The pulp content ranges from 15-30% of the dry weight of the beet. The research of the effects of beet pulp pectin emerged later than that of the two previous mentioned types of pectins (Fissore et al., 2013). It however has good flower-like activity and gel properties. Studies have shown that beet pulp pectin has a small molecular weight, low viscosity, and strong thermal stability, making it effective in regulating gel properties (Mesbahi et al., 2005;Peighambardoust et al., 2021).

Sweet potato pectin is an acidic heteropolysaccharide extracted from sweet potato residue. Sweet potatoes are an important food crop worldwide, and their industrial

processing and production generate a significant amount of sweet potato residue, which contains 20-30% of pectin-like substances (Abang et al., 2017).

Furthermore, there are other pectins derived from different sources, such as sunflower head pectin obtained from sunflower heads (Muthusamy et al., 2019), watermelon pectin extracted from watermelon rind (Perez et al., 2022), and passion fruit pectin obtained from passion fruit rind (Kang et al., 2015;Perez et al., 2022). These pectins are mainly derived from byproducts, which not only avoids waste of resources but also exploit their potential value.

1.3.2 The extraction of pectin

There are many extraction methods for pectin, including the acid extraction method, the microbial extraction method, the microwave-assisted extraction method, the ion extraction method, the high voltage pulsed electric field method, the enzymatic extraction method and the ultrasonic method. In what follows we will give an overview of the working mechanism of all these extraction methods, their effectiveness, usability and potential drawbacks.

1.3.2.1 The acid extraction method

The acid extraction (AE) method is widely used in industry to convert protopectin in plant cells into water-soluble pectin (Joye, 2000). Inorganic acids like sulfuric, hydrochloric, and nitric acid are cost-effective but can potentially damage pectin due to their strong acidity (Rouse, 1976;Abang et al., 2020;Ostrozhenkova, 2020). Organic acids like citric acid, tartaric acid, and ammonium oxalate offer milder acidity but may result in pH instability (Zhao et al., 2015) (Kastner et al., 2014). Researchers have reported successful pectin extraction with organic acids, achieving good yields and desirable properties, although generating acidic wastewater remains an environmental concern (Yang et al., 2018). Balancing cost, yield, and environmental impact is essential when choosing the acid medium for pectin extraction.

1.3.2.2 The microbial fermentation extraction method

Microbial fermentation efficiently extracts pectin from plant tissues by breaking down complex polysaccharides. Sakai et al. (1980) used Trichosporon penicilatum to ferment peels, obtaining pectin powder by filtration, concentration, and drying (Sakai, 1980). Gomashe et al. (2019) employed Trichosporon penicillatum enzymes to ferment orange peel substrate, reducing production costs (Gomashe et al., 2019). Thibault et al. (1988) used a complex enzyme to extract pectin from citrus fruit. Microbial fermentation yields high-quality pectin with a stable quality, no need for crushing, heat or acid treatment, and low cost and pollution. This method holds promising prospects for further development (Thibault, 1988).

1.3.2.3 The microwave-assisted extraction method

Microwave-assisted extraction (MAE) is an efficient method that combines microwave energy with a solvent to extract organic compounds (Letellier et al., 1999). It is known for low energy use, short processing time, and high efficiency (Vinatoru, 2017). MAE heats polar molecules in plant cells, creating vapor pressure that ruptures cell walls, releasing substances into the extraction fluid (Hu et al., 2021a). This method preserves pectin's structure and accelerates extraction. For

instance, Maran et al. (2017) achieved a 25.8% pectin yield from watermelon rind in 2 minutes with 477W microwave power (Maran et al., 2017), while Hosseini et al. (2016) obtained a 29.1% yield from citrus peel in 3 minutes with 700W power (Hosseini et al., 2016).

1.3.2.4 The ion extraction method

Ion exchange process (IEP) uses resins to enhance pectin extraction by reducing intermolecular forces, yielding high-quality pectin with desired color. However, pectin can undergo depolymerization and denaturation during extraction. IEP is expensive and complex, limiting industrial use. Torkova et al. (2018) extracted pectin from pumpkin, citrus, and apple using cation exchange resin at 5% (wt) (Torkova et al., 2018). Double extractions at 85-88°C for an hour with a cationic resin produced higher yields and better gel strength compared to single extractions (Paresh, 2017)

1.3.2.5 High voltage pulsed electric field method

High Intensity Pulsed Electric Fields (HIPEF) is an innovative non-thermal technique gaining popularity for processing heat-sensitive materials. HIPEF disrupts plant cell membranes, enhancing intracellular substance solubilization (Rodrigo, 2003). Pectin, a large molecule, dissolves in water due to its polarity. Applying a high-frequency electromagnetic field induces molecular thermal motion, raising the material's temperature without thermal conduction or convection. Pectin in plant cells absorbs energy, facilitating separation from cellulose, boosting extraction efficiency. Optimal conditions for extracting pectin from apple pomace using HIPEF include an electric field strength of 15 kV/cm, pH 3, 10 electric pulses, 1:19 solid-liquid ratio, and 62°C (Yin, 2009). HIPEF offers a promising alternative to traditional thermal methods for heat-sensitive materials, enhancing pectin extraction efficiency (Lal et al., 2021).

1.3.2.6 Enzymatic assay

Enzyme-assisted extraction (EAE), also known as biological extraction, employs microorganisms or biological enzymes to break down plant cell walls, facilitating natural product release. Steps include adding enzyme-containing buffer to raw materials, constant temperature water bath shaking, ethanol precipitation, filtration, separation, drying, and pulverization for pectin production (Wikiera et al., 2015). Benefits include high extraction efficiency, minimal reverse reactions, precise enzyme control, better quality, and less solvent use. Drawbacks include longer reaction times, costly enzymes, and variable results due to strain differences. Vasco-Correa et al. (2017) extracted pectin from passion fruit peel using the fungal enzyme PPase-SE, yielding 26% GalA, 40% higher than chemical methods. Optimal conditions involve 30 U/mL raw pectinase-SE at pH 3.0 and 37°C (Canteri et al., 2010;Liew et al., 2016;Vasco-Corream etal., 2017)

1.3.2.7 The ultrasonic method

Ultrasonic-assisted extraction is a rapid, eco-friendly method that enhances substance dissolution in plant tissues using ultrasonic waves (Mehta et al., 2022). It offers advantages over traditional acid extraction, such as shorter cycles and reduced energy and solvent usage. However, excessive ultrasonic exposure can

over-hydrolyze raw materials, increasing pectin molecule fragmentation. Conversely, too short a time may lower pectin yield. Karbuz et al. (2021) found that ultrasonic waves boost solvent penetration into fruit peels and pectin release within 15-45 minutes, yielding higher pectin (Karbuz et al., 2021). Beyond 45 minutes, pectin structure modification and fragmentation reduce yield. Optimal conditions for maximum pectin yield are 45 minutes at 75°C (Karbuz et al., 2021). Kazemi et al. (2019) recommend 50W ultrasonic power and 30 minutes, resulting in a 33.64% yield (Kazemi et al., 2019).

1.3.3 The physical-chemical properties of pectin

The composition and physical-chemical properties of pectins vary greatly due to factors such as the type of raw source material and the extraction method. The physical-chemical properties include solubility, emulsifying, esterification degree, Gal-A content, and monosaccharide composition (Liu et al., 2022a). For instance, extraction temperature affects heat absorption performance (Leclere et al., 2013), and the emulsifying properties are greatly affected by structural factors such as the length of the straight chain region, the degree of esterification, and the content of hydrophobic groups such as acetyl groups. In order to enhance the emulsifying activity of pectin, measures such as enzymatic modification, acetylation reaction, and thermal treatment are commonly used to modify the structure, increase the number of hydrophobic groups, and reduce the molecular weight (Ren et al., 2016;Huang et al., 2018).

1.3.3.1 Solubility

Pectins can be classified into water-soluble and non-water-soluble pectins based on their solubility characteristics (Duan et al., 2008). There are many factors that influence the solubility of pectin, including the degree of esterification, and the distribution of the methoxy sites (Lofgren, 2000). Typically, pectins with a lower relative molecular mass and a higher degree of esterification have a better solubility (Kim et al., 1996). Additionally, pectin particles need to swell prior to dissolution, similar to hydrophilic colloids (Einhorn-Stoll et al., 2015).

1.3.3.2 Degree of esterification

The esterification degree of a pectin, also known as methoxylation, refers to the sum of the proportion of methyl esterification, acetylation, and amidation in the pectin. Its esterification degree affects the morphology and conformation of the pectin. Increasing the esterification degree can decrease the extension and curvature of the pectin molecules and increase the hydration volume (Karbuz et al., 2021). Decreasing the esterification degree can reduce the chain flexibility and increase the rigidity of the pectin molecules (Lima et al., 2010). According to the esterification degree, pectin can be divided into two categories: high ester pectin (DE > 50%) and low ester pectin (DE < 50%) (Ciriminna et al., 2017). This is usually due to differences in raw materials and processing technology. The esterification degree is of significant importance for the application of pectin. It also affects the ability of pectin to form a complex with other substances. Stone et al. (2018) found that high ester pectin has a stronger interaction with pea protein isolate under optimal mixing conditions (Warnakulasuriya et al., 2018). Pillai et al.

(2020) found that as the esterification degree decreases, the critical pH for pectin to form a complex with other substances significantly increases (Pillai et al., 2020).

1.3.3.3 Gal-A content and monosaccharide composition in general

Pectin is a heteropolysaccharide composed mainly of D-GalA, and in addition to D-GalA, it also contains neutral sugars such as L-rhamnose, D-galactose, and D-arabinose (Protzko et al., 2018). The composition of individual sugars can indirectly reflect the structure of pectin, and some scholars use the GalA content to indicate the purity of pectin (Parkar et al., 2021). The majority of neutral sugars in pectin are present in side-chains, so a high GalA content indicates a high content of side chains. A low content of neutral sugars in pectin means a lower content of side chains (Paniagua et al., 2014).

In this PhD thesis, the source of pectin was citrus peel, the concentration of galacturonic acid was 81.4%, and the degree of esterification was 13.5%.

1.4 Effects of pectin on gut immunity

Pectin has been demonstrated to have various beneficial effects on mammalian health, including immune modulation, anti-tumoral effects, and antioxidant activities.

1.4.1 Antioxidant effects of pectin

Antioxidants refer to the body's defense against the production of free radicals caused by continuous exposure to the external environment, such as through breathing (Wu et al., 2018). A vitro study by Tian et al. has shown that an excess of free radical production is often associated with cancer, aging, and other diseases (Tian et al., 2018). Many studies have shown that pectin exhibits strong antioxidant activity by reducing the production of free radicals and improving antioxidant capacity through free radical scavenging. In vitro research on the antioxidant effects of pectin has indicated that it significantly enhances the removal of DPPH (2,2-Diphenyl-1-picrylhydrazyl), which is a stable free radical widely considered as a tool for evaluating the free radical scavenging activity of antioxidants (Hu et al., 2004;Abdelmalek et al., 2015) and Hydroxy radical (OH), which has high reactivity and strong oxidizing ability and is an important parameter for evaluating antioxidant properties (Ma et al., 2021b;Wang et al., 2022).

DPPH scavenging activity of pectin increased in parallel with its concentration (Bayar et al., 2017). Studies revealed that pectins from medicinal plants have shown to have a great extent of DPPH scavenging activity in rat (Maria-Ferreira et al., 2014;Lefsih et al., 2016). Patova et al. (2019) has come to the similar conclusion, as they observed that the pectin extracted from horsetails scavenged DPPH radicals ranging from 49%-63% in vitro (Patova et al., 2019).

1.4.2 Regulation of the intestinal microbiota

Optimization of the gut microbiota is one of the main benefits of pectin consumption (Blanco-Perez et al., 2021) (Figure 1-3).

Pectin has been shown to promote the growth of certain beneficial microorganisms in human, such as those in the *Clostridium cluster XIV*, *Clostridium butyricum*, and *Clostridium beijerinckii* groups. At the same time, it

can inhibit the growth of harmful bacteria (Olano-Martin et al., 2002;Eliaz et al., 2006;Bang et al., 2018). It has also been reported that pectin increases the abundance of *Faecalibacterium prausnitzii* and *Roseburia intestinalis* in vitro. These two species have been linked to positive effects on colon cancer and ulcerative colitis (Pascale et al., 2022). Pectin could increase the relative abundance of *Bifidobacterium adolescentis*, *Prevotella spp*, *Bacteroides*. Acetate and butyrate, short-chain fatty acids (SCFAs) derived from the fermentation of pectin in the mice gut, have been shown to enhance digestibility (Tan et al., 2020). Probiotics may feed on pectin and produce secondary metabolites that inhibit the growth of non-probiotic microorganisms. A vitro study by Gómez et al. (2016) reported that pectic oligosaccharides isolated from sugar beet pulp and lemon peel waste increased the populations of *Bifidobacteria* and *Lactobacilli* by up to 34% and 29%, respectively

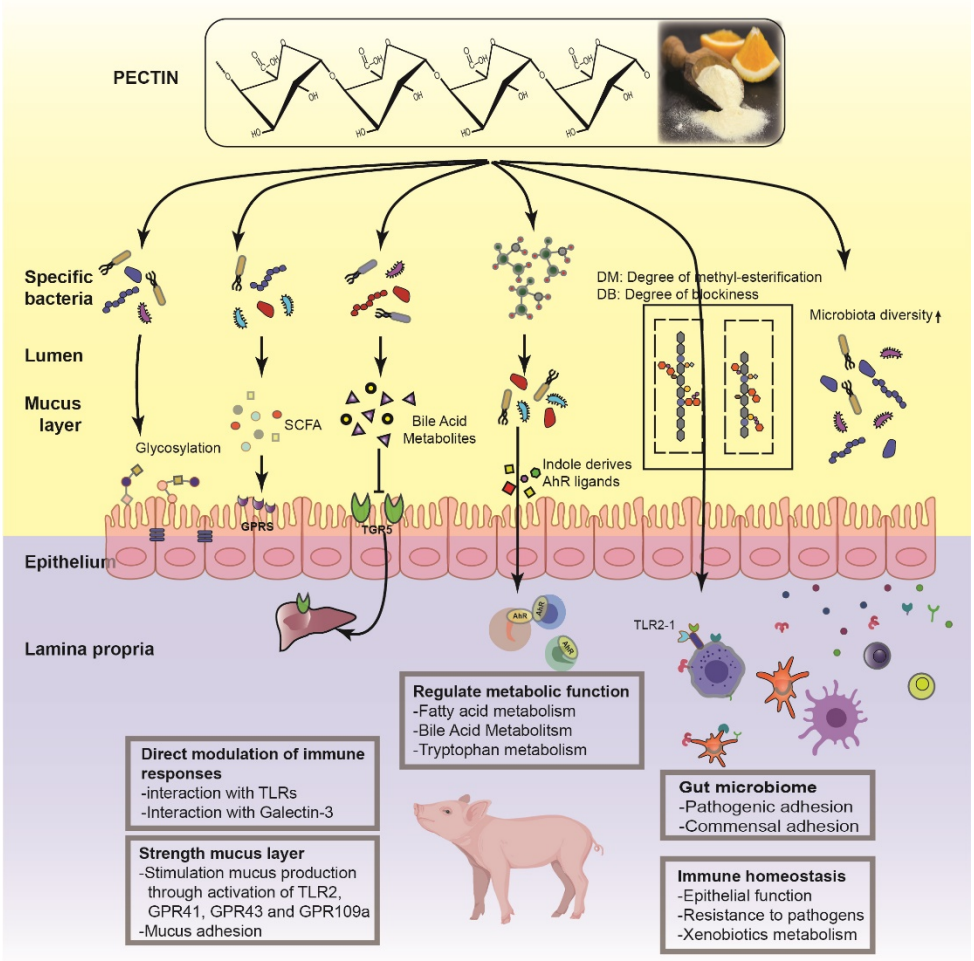


Figure 1-3. How pectin affects the host's immunity. The figure shows the possible ways in which the body's immunity can be affected when the body ingests pectin. The pathways in which pectin is directly involved in mediating immune responses include galectins and TLRs; indirect effects include stimulating microbial diversity (interfering with the adhesion of pathogens, etc.), stimulating glycosylation of mucins (strengthening the mucus layer, stimulating lymphocytes to produce IL -22) and short-chain fatty acids (stimulate epithelial integrity and mucus secretion by binding to GPR41, GPR43 or GPR109a, and participate in intestinal immune regulation), indoles (metabolites of tryptophan, which can act as AhR receptors Ligands, stimulate and activate type III lymphocytes, promote intestinal health), bile acid metabolites (bile acids can regulate blood sugar levels, cholesterol metabolism and immune signaling by activating receptors in the liver and gut).

(Gómez et al., 2016). An in vitro study using human feces as an inoculation substrate has also indicated that highly methylesterified pectin from oranges and lemons can increase the abundance of *Bifidobacteria*, *Lactobacilli*, and *Lactococci* (Gómez et al., 2016). In contrast, low DE pectin is more easily metabolized by the body than high DE pectin in children (Adam et al., 2016), with reports suggesting that high DE pectin stays in the gut for a longer duration of 24 hours than low DE pectin in human (Gómez et al., 2016). In addition to its impact on the microbial population, pectin also exerts its gel-forming properties, promoting gut health and peristalsis, as well as facilitating the formation of feces through its water-binding activity (Ohno et al., 2019).

1.4.3 Regulation of mucosal immunity through metabolites

The fermentation of dietary fiber into nutritious metabolites such as SCFAs is one of the important benefits that the gut microbiota provides to its host. Furthermore, pectin also effects the tryptophan metabolism as well as bile acid metabolism, important for many biochemical functions and overall health.

1.4.3.1 Effects of pectin on short-chain fatty acid metabolism

Pectin can increase the volume of chyme and accelerate gastrointestinal motility. Unlike insoluble fibers such as cellulose, pectin is almost 100% fermented in the colon (Pluske., 2000), leading to the production of SCFAs (Moore, 1998). SCFAs are the main energy source for colon cells and can lower colon pH, thus preventing colon cancer (Dang et al., 2021). Soluble dietary fiber apple pectin, after fermentation by intestinal microbiota, produces SCFAs, among which propionic acid may inhibit histone deacetylases (HDAC) expression, reduces the release of pro-inflammatory cytokine *IL-6*, alleviates tissue inflammation, and assists in the treatment of ulcerative colitis (Tao., 2022). Different genera can produce different SCFAs, for example, acetate can be produced by many genera, propionate is mainly produced by *Bacteroidetes* and *Firmicutes*, and butyrate is mainly produced by *Clostridium spp*. SCFAs bind to "metabolism-sensing" G protein-coupled receptors, such as *GPR41*, *GPR43*, and *GPR109A*, which promote the regulation of gut homeostasis and inflammatory responses (Dang et al., 2022) (Figure 1-3). GPRs and their metabolites affect Treg activation, epithelial integrity, gut homeostasis, dendritic cell activation, and IgA antibody response. By inhibiting HDAC expression or function, SCFAs also affect gene transcription in many cells and tissues.

1.4.3.2 Effects of pectin on tryptophan metabolism

The ingestion of pectin also affects tryptophan metabolism. Tryptophan is an essential amino acid for mammals and serves as a biochemical precursor for metabolites that significantly influence mammalian physiological functions, including gastrointestinal function, immune response, metabolism, and nervous system activity (Gao et al., 2020). Studies have shown that pectin administration significantly increases the abundance of *Bacteroides* both in human, mice and rat, which is capable of metabolizing tryptophan into indole derivatives (Roager et al., 2018;Kim et al., 2020;Wu et al., 2022a). Research conducted by Wrzosek et al. (2021) has demonstrated that supplementing pectin in the diets of mice lead to a

significant reduction in fecal tryptophan levels and promotes tryptophan metabolism (Ferrere et al., 2017;Wrzosek et al., 2021). The metabolism pathways of tryptophan in the intestine mainly include three types: the 5-hydroxytryptophan pathway, the quinolinic acid pathway, and the indole metabolism pathway. 5-hydroxytryptophan is an important neurotransmitter that can participate in the regulation of intestinal motility, appetite control, and emotions. The quinolinic acid pathway primarily regulates the immune system, affecting the activity of T lymphocytes and participating in immune balance. As for the indole metabolism, it not only activates lymphocytes, promoting the involvement of the immune factor IL-22 in immune regulation but also serves as a carbon source for some intestinal microorganisms to proliferate (Agus et al., 2018).

Moreover, pectin can reshape the gut microbiota of piglets, altering the direction of tryptophan metabolism and promoting the production of indole derivatives (Hendrikx, 2019;Dang et al., 2023). In the intestine, indole compounds are considered natural ligands and activators of aromatic hydrocarbon receptors (AHR). Clostridia convert tryptophan into serotonin, indolelactic acid (ILA), and indolepropionic acid (IPA). Another group of bacteria capable of metabolizing tryptophan is Lactobacillus. Lactobacillus species convert tryptophan into indolealdehyde (IAld) and ILA through aromatic amino acid transaminase (ArAT) and indolelactic acid dehydrogenase (ILDH).

1.4.3.3 Effects of pectin on bile acids metabolism

According to several studies, pectin can promote heart health by improving cholesterol metabolism (Dongowski, 2002;Dongowski et al., 2004;Fang et al., 2018;Hu et al., 2022) (Figure 1-3). In the liver, cholesterol is a precursor of bile acids. Pectin optimizes blood cholesterol levels by binding with bile acids in the pig small intestine (Gunness et al., 2010; Fang et al., 2018). As more bile acids are lost in the feces, more cholesterol is converted to bile acids, helping to optimize blood cholesterol levels. Research shows that pectin lowers low-density lipoprotein cholesterol without affecting high-density lipoprotein cholesterol. As a soluble dietary fiber, pectin can lower human cholesterol by 5% to 16% when the daily intake reaches 5g (Brown, 1999). However, there is also a different opinion as Singh et al. (2018) suggest that long-term addition of soluble dietary fiber such as inulin or pectin to the diet of mice with dysregulation can lead to an abnormal bile acid metabolism, bile stasis, and eventually liver cell cancer (Singh et al., 2018).

1.4.4 Pectin can interact directly with the mucosal barrier

Before dietary fiber is degraded by microbiota in the hindgut, it also interacts directly with the immune barrier cells in the small intestine of mice (Breton et al., 2015). The small intestine has a thin and loose layer of mucus that not only facilitates nutrient absorption but also allows dietary fiber molecules to directly interact with intestinal epithelial and immune cells, contributing to the regulation of gut immunity (Ermund et al., 2013) (Figure 1-3). Research suggests that pectin is directly involved in several pathways of intestinal immune regulation, including pathways where the TLR2-TLR1 receptor complex is implicated. Pectin can selectively block the binding of TLR2-TLR1, thereby inhibiting the release of pro-inflammatory cytokines (Beukema et al., 2019). Another direct mechanism

influenced by pectin involves glycosylation modifications. Pectin can undergo glycosylation modifications, attaching sugar molecules to specific positions on protein molecules (Gamage et al., 2020; Tardy et al., 1995). This modification alters the function and stability of proteins, providing protection to the intestine against harmful substances such as bacteria and viruses.

1.4.4.1 Pectin directly blocks the Toll-like receptor 2 (TLR2)-TLR1 pathway

Research has demonstrated that in addition to recognizing dietary fibers through pattern recognition receptors (PRRs), pectin can also directly interact with the intestinal tract by inhibiting the binding of bacterial lipopolysaccharide to TLR2. TLR2 forms heterodimers with TLR1, it is the prerequisite to recognize a wide spectrum of microbial pathogen-associated molecules. thereby blocking the TLR2-TLR1 heterodimer formation, pectin can effectively inhibit TLR2-1-induced IL-6 response (Beukema et al., 2021). This indicates that pectin not only affects TLR2-1 signaling, but also has an inhibitory effect on the initiation of its downstream IL-6 secretion (Atreya, 2005;Flynn et al., 2019). This inhibitory effect helps to prevent intestinal diseases that result from TLR2 activation, as TLR2 activation can exacerbate intestinal inflammation and produce certain cytokines that combat infection (Sahasrabudhe et al., 2018). Moreover, the efficacy of pectin is related to its degree of methylation (DM), with low-DM pectin being more effective than high-DM pectin. Similar studies have shown that low-DM pectin can directly interact with TLR2-1 receptors through electrostatic forces, reducing Toll-like receptor-2 mediated immune activation and decreasing peri-capsular fibrosis in mice (Beukema et al., 2021a;Hu et al., 2021b). In summary, dietary fibers can reduce inflammatory responses by directly interacting with TLR2-TLR1 receptors in the intestinal tract.

1.4.4.2 Effect of pectin on glycosylation modifications of intestinal mucin

Glycosylation refers to the process of attaching sugar molecules to protein molecules and is an important regulatory pathway for cellular signal transduction, protein stability, and function (Xia et al., 2022b). As a dietary fiber, pectin has emerged as a new research area with regulatory effects on the glycosylation modification of intestinal mucosal proteins. Studies in Caco2 cells have demonstrated that pectin can regulate the expression of glycosylation-related genes, such as galectin and glycocalyx genes, and inflammation genes, thus potentially preventing pathogen adhesion (Kong et al., 2021). Moreover, pectin can also affect the structure and composition of the gut microbiota, leading to the regulation of the glycosylation modification process in the intestine. Additionally, pectin can bind to specific proteins in the intestine and interfere with their glycosylation modification process, promoting the transformation of the O-glycosylation modification pattern of the jejunal mucosa to Core 3 type glycan chain structure and affecting the expression of *fucosyltransferase 2* (*FUT2*) and *O-GlcNAcase* (*OGA*). These research findings suggest that pectin has the potential to alleviate the damage caused by LPS stress to the piglet intestine by regulating the glycosylation modification pattern (Wen et al., 2022).

1.5 Concluding remarks and future perspectives

In pig production, the weaning period is a crucial and pivotal stage. Concerning the gastrointestinal tract, it involves the gradual adaptation to the digestion and absorption of solid feed, as well as the resistance against pathogens. Consequently, researchers have been actively exploring feed additives such as probiotics or prebiotics to enhance intestinal health and mitigate the occurrence of diarrhea through their judicious supplementation.

In this introduction, we summarized the preparation of pectin and its potential use as a feed additive for animal health. Pectin has the ability to reduce the abundance of potential pathogenic bacteria by promoting the proliferation of beneficial microorganisms in the gut. Moreover, pectin can regulate intestinal homeostasis by promoting the production of microbial-derived metabolites, such as short-chain fatty acid metabolism, bile acid metabolism, and tryptophan metabolism. It also has anti-inflammatory and immunomodulatory effects on inflammation and tumor development, and can directly act on pattern recognition receptors, such as TLR2-1 and TLR-4, to regulate the body's immune response.

Therefore, the purpose of this thesis was to study the effects of citrus pectin on the growth performance of weaned piglets. What effect does citrus pectin have on the immunity and disease resistance of different intestinal segments of weaned piglets? Are their underlying mechanisms consistent? Progress in these fields will be more conducive to the application value of pectin in animal production.

Chapter II

Thesis objectives, hypothesis, and structure

Chapter II. Objectives, hypothesis and outline of the thesis

2.1 Objectives

In our study, our overall objective was to find an alternative to antibiotics that can be added to piglet feed, aiding piglets in safely and smoothly transitioning through the weaning period. Specifically, we conducted research in the following three areas: (1) What are the effects of pectin on jejunal development, gut microbiota, and indole-like metabolites in healthy piglets, and mucosal immunity in healthy piglets? (2) After piglets fed pectin are injected with LPS, what effect will pectin have on growth performance and immune tolerance of piglets? Here we thus address the effect of pectin in pigs undergoing a model of inflammation. (3) What is the effect of pectin on the cecal morphology, microbiota structure, and microbial metabolites of LPS-challenged piglet models?

2.2 Hypothesis

We hypothesize that pectin may have a promoting effect on the overall intestinal health of piglets. Pectin may promote the intestinal morphology and health status of different segments of the intestine through non-uniform pathways. We hypothesize that intestinal microbiota play a major role in these positive effects caused by adding pectin to the feed of weaned piglets.

2.3 Outline of the thesis

Chapter 1: General introduction

Chapter 2: Thesis objectives, hypothesis and structure

Chapter 3 (Article 1): Pectin modulates intestinal immunity in a pig model via regulating the gut microbiota-derived tryptophan metabolite-*AhR-IL22* pathway. ***Dang G**, Wen X, Zhong R, Wu W, Tang S, Li C, Yi B, Chen L, Zhang H, Schroyen M. Journal of Animal Science and Biotechnology. 2023 Mar 8;14(1):38*

→ This chapter demonstrates that pectin promotes the development of a healthy piglet intestine and the diversity and richness of the jejunal mucosal microbiota, while also increasing the level of mucosal immunity in piglets.

Chapter 4 (Article 2): Pectin supplementation ameliorates intestinal epithelial barrier function damage by modulating intestinal microbiota in lipopolysaccharide-challenged piglets. *Wen X, Zhong R, **Dang G**, Xia B, Wu W, Tang S, Tang L, Liu L, Liu Z, Chen L, Zhang H. The Journal of Nutritional Biochemistry. 2022*

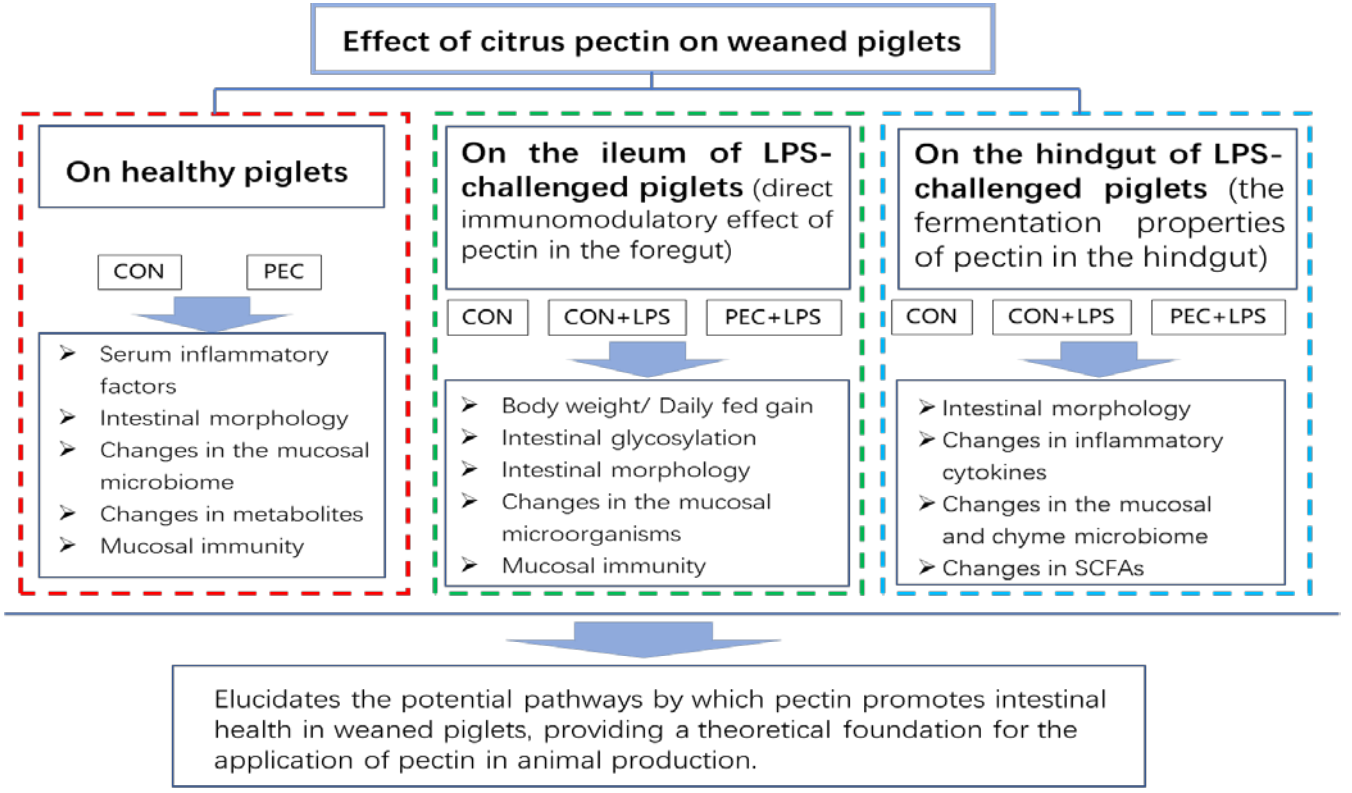
→ This chapter reveals that pectin can attenuate the degree of damage to ileal morphology in LPS-stressed piglets, while improving gut microbial homeostasis and intestinal immune defense.

Chapter 5 (Article 3): Pectin supplement alleviates gut injury potentially through improving gut microbiota community in piglets. ***Dang G**, Wang W, Zhong R, Wu W, Chen L, Zhang H. Frontiers in Microbiology. 2022 Dec 9;13:1069694.*

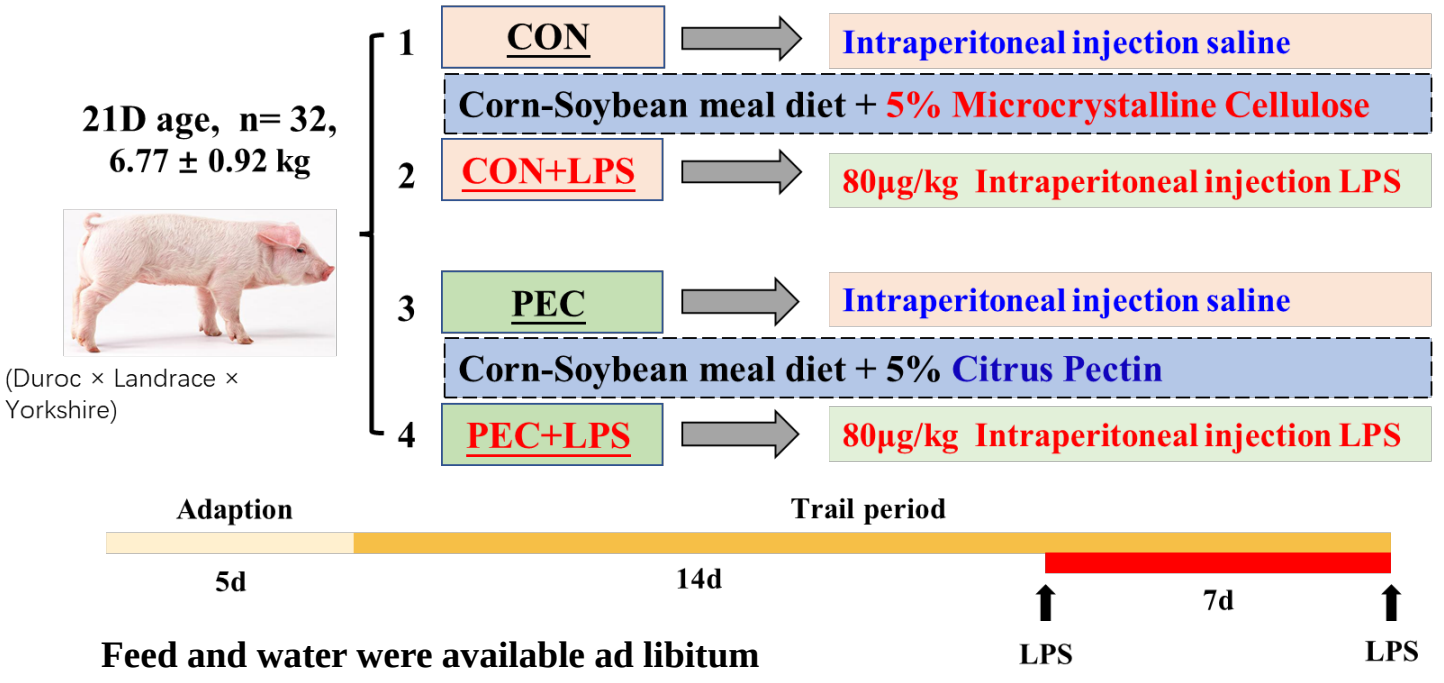
→ This chapter reveals that pectin can improve the immunity of piglets through intestinal microorganisms and short-chain fatty acids, thereby reducing damage to the cecum of piglets from LPS stress.

Chapter 6: General discussion and perspectives.

2.4 Technical route



Experimental Design


21D age, n= 32,
6.77 ± 0.92 kg
(Duroc × Landrace × Yorkshire)
1
CON
Intraperitoneal injection saline
Corn-Soybean meal diet + 5% Microcrystalline Cellulose
2
CON+LPS
80μg/kg Intraperitoneal injection LPS
3
PEC
Intraperitoneal injection saline
Corn-Soybean meal diet + 5% Citrus Pectin
4
PEC+LPS
80μg/kg Intraperitoneal injection LPS
Adaption
Trail period
5d
14d
7d
LPS
LPS
Feed and water were available ad libitum

Chapter III

Pectin modulates intestinal immunity in a pig model via regulating the gut microbiota-derived tryptophan metabolite-*AhR*-*IL22* pathway

Chapter III. Pectin modulates intestinal immunity in a pig model via regulating the gut microbiota-derived tryptophan metabolite-*AhR-IL22* pathway

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Key words: Dietary fiber, Gut microbiota, Immune pectin, Tryptophan metabolites

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Author Contributions: Guoqi Dang participated in animal husbandry experiments, data collection and analysis, as well as drafting and writing the paper.

3.1 Abstract

Background Pectin is a heteropolysaccharide that acts as an intestinal immunomodulator, promoting intestinal development and regulating intestinal microbiota in the gut. However, the relevant mechanisms remain obscure. In this study, pigs were fed a corn-soybean meal-based diet supplemented with either 5% microcrystalline cellulose (MCC) or 5% pectin for 3 weeks, to investigate the metabolites and anti-inflammatory properties of the jejunum. Result The results showed that dietary pectin supplementation improved intestinal integrity (Claudin-1, Occludin) and inflammatory response [interleukin (IL)-10], and the expression of proinflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α) was down-regulated in the jejunum. Moreover, pectin supplementation altered the jejunal microbiome and tryptophan-related metabolites in piglets. Pectin specifically increased the abundance of Lactococcus, Enterococcus, and the microbiota-derived metabolites (skatole (ST), 3-indoleacetic acid (IAA), 3-indolepropionic acid (IPA), 5-hydroxyindole- 3-acetic acid (HIAA), and tryptamine (Tpm)), which activated the aryl hydrocarbon receptor (AhR) pathway. AhR activation modulates IL-22 and its downstream pathways. Correlation analysis revealed the potential relationship between metabolites and intestinal morphology, intestinal gene expression, and cytokine levels. Conclusion In conclusion, these results indicated that pectin inhibits the inflammatory response by enhancing the *AhR-IL22*-signal transducer and activator of transcription 3 signaling pathway, which is activated through tryptophan metabolites.

3.2 Introduction

Weaning is one of the most critical periods in both animal production and infant growth and development. The gastrointestinal tract of animals is not fully developed at this stage (Wu et al., 2021a). It is susceptible to changes in feeding patterns and nutrition, leading to stress and diarrhea. Given the omnivorous and physiological similarities between weaned piglets and human infants, thepiglet is regarded as the most suitable animal model for studying gut health (Tang et al., 2022b).

Pectin, predominantly composed of α-1,4-linked *D*-galacturonic acid (GalA) monomers, is abundant in citrus, apple, lemon peels and pulp. As a typical fermentable dietary fiber, pectin can regulate the human and animal intestinal microbiota (Chung et al., 2016;Bang et al., 2018;Elshahed et al., 2021). It can also strengthen the mucus layer to restrict the entry of hazardous substances (Maria-Ferreira et al., 2018;Beukema et al., 2020). Furthermore, it can enhance the integrity of the epithelial cell layer (Wilms et al., 2019) and maintain intestinal integrity in piglets exposed to lipopolysaccharide or high-fat diet (Jiang et al., 2016).

The gastrointestinal tract is home to a diverse community of trillions of microorganisms collectively known as the gut microbiota (Sasaki et al., 2020), and this intricate community is central to gut health and disease (Zhang et al., 2020b). Moreover, the gut microbiota is associated with its ability to defend against enteropathogens, absorb nutrients, and maintain a healthy immune system (Ichikawa-Seki et al., 2019;Mills et al., 2019;Pilla et al., 2020). However, pectin

can also have direct effects in the small-intestinal sites (Beukema et al., 2020).It has been shown that the non-esterified GalA residues rich in pectin can bind to toll like receptor 2 (TLR2) via ionic bonds (Beukema et al., 2021b). The pectin suppresses the TLR2/1 signal (TLR2 can form heterodimers with TLR1), and thenIL-6 secretion is reduced, thereby reducing the inflammatory response and ameliorating the damage (Krishnan et al., 2017).

Recently, many studies have focused on the function of microbial tryptophan catabolites in the gut and their contributions to host physiology (Zhang et al., 2016). For instance, aryl hydrocarbon receptor (AhR) ligands; 3-indole ethanol(IE), 3-indole pyruvate (IPA), and 3-indole aldehyde (IA)reduce gut permeability (Scott et al., 2020). Serotonin (5-HT), another catabolite, plays an important role in gastrointestinal absorption, transit, and secretion. Besides, it also regulates mood, behavior, pain modulation, and cognitive function via the central nervous system (Mawe et al., 2013). According to a representative study, pectin supplementation may not only alter the intestinal microbiota of mice, but also increase the tryptophan metabolites of the microbiota by activating the AHR pathway (Wrzosek et al., 2021). Previous research from our laboratory has demonstrated the anti-inflammatory effects of pectin on gut immunity (Wu et al., 2020b). However, the precise mechanism by which pectin promotes gut health remains unknown.

Thus, the intriguing question was whether microbial tryptophan catabolite is the link between pectin and the gut health regulator. To bridge this knowledge, we examined the changes in serum, gut microbiota, and tryptophan metabolite following pectin supplementation in a pig model. This study gave a novel perspective for promoting a new understanding of how pectin promotes gut health.

3.3 Materials and methods

3.3.1 Ethics statement

All animal experiments were approved by the Animal Ethics Committees of Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (Ethics Code Permit IAS2019-37).

3.3.2 Standards and chemicals

Pectin extracted from citrus peel (Henan Yuzhong Biotechnology Co., Ltd., Zhengzhou, China) mainly consisted of galacturonic acid (white powder, with purity of > 81.4%, DM: 13.5%). Microcrystalline cellulose (MCC) is a β-1,4-multi-bonded linear carbohydrate consisting of glucose residues with 99.5% purity. (Beijing NCC Technology R&D Center, China). Reference standards for tryptophan (Trp), tryptamine (Tpm), 3-indoleacetic acid (IAA) and kynurenine (Kyn) were purchased from Sigma-Aldrich (St. Louis, MO, USA); 5-hydroxyindole- 3-acetic acid (HIAA) and skatole (ST) were obtained from Cato Research Chemicals Inc. (Eugene, OR, USA); 3-indolylpropionic acid (IPA) and serotonin (5-HT) were from Laboratory of the Government Chemist (Teddington, UK) and Beijing Wokai Biotechnology Co., Ltd. (China), respectively. Assay kits, including interleukin IL-17, IL-22 were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China).

3.3.3 Experimental design and animal care

A total of 16 crossbred barrows aged 21 d (6.77 ± 0.92 kg; Duroc × Landrace × Yorkshire) were randomly assigned to one of two diets with eight piglets per treatment. No antibiotics were administered to the piglets throughout the 4-week experiment. Piglets were fed ad libitum and had free access to water. A corn-soybean basal diet was formulated to meet nutritional requirements of National Research Council (NRC, 2012). After a 3-d of adaption, piglets were fed a diet containing either 5% microcrystalline cellulose (w/w) as the control (CON) group or 5% pectin (w/w) as the treatment (PEC) group for 3 weeks. All piglets were housed in separated pens with daily-cleaned plastic slatted floors.

3.3.4 Sample collection

Blood samples were acquired from the jugular vein via a sterilized syringe before the pigs were sacrificed at the end of the experiment. The serum was then separated by centrifugation for 10 min at 3000 × g at 4 °C and stored in aliquots at −80 °C for cytokines analysis. The middle section (2 cm) of the jejunum was obtained and fixed in 4% paraformaldehyde for histological examination. The intestinal segment was washed with ice-cold phosphate buffered saline (PBS), and the mucosa was scraped off using a glass microscope slide. Mucosa samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C to further investigate the bacterial community, genes, and protein expression.

3.3.5 Intestinal morphology

The hematoxylin-eosin (HE) staining of the jejunum was performed according to the methods as previously described (Xia et al., 2022a). Briefly, specimens of jejunum were embedded in paraffin, sectioned (5 μm thickness), and stained with HE for histological evaluation [villus height (VH)]. Microphotographs were taken with a Leica DM2000 light microscope (Leica, Wetzlar, Germany) at a magnification of 40. VH was performed using Image Pro software (Wen et al., 2022).

3.3.6 Serum inflammatory cytokines

The ELISA kit was employed to detect serum cytokines as previous describe (Shrestha et al., 2016). Quantitative analysis of proinflammatory cytokine (IL-17), and anti-inflammatory cytokine (IL-22) in serum were measured by ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the detection kit instructions.

3.3.7 Quantitative real-time (qRT) PCR analysis

Total RNA was extracted from the jejunum mucosa, using the RNeasy Mini Kit (GeneBetter, Beijing, China). The concentration of each RNA sample was quantified using the NanoDrop 2000 (Nanodrop Technologies, Wilmington, DE, USA). The cDNA was transcribed at 37 °C for 15 min and 85 °C for 5 s using the Prime- Script TM RT reagent kit with gDNA Eraser (Takara Biomedical Technology in Beijing, China). qRT-PCR with 40 amplification cycles was conducted with a commercial kit (PerfectStart Green qPCR SuperMix, TransGen Biotech, Beijing, China). In detail, a total of 10 μL reaction mixture contain 1 μL of cDNA, 0.4 μL forward primer, 0.4 μL reverse primer, 0.2 μL of ROX, and 3 μL of PCR grade water. The gene of β-actin was used as an internal control. Primers used were listed in Table 3-1. The relative gene expression level between the control

group and the treatment group was calculated by the 2- ΔΔCt method, and the value was normalized to the internal control.

3.3.8 Western blotting assay

Total protein was extracted from jejunum tissue using RIPA lysis buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA). It was quantified with the BCA protein assay kit (Cat# 23225, Thermo, Waltham, MA, USA). Total proteins in the amount of 30 μg were loaded for separation onto 10% SDS-PAGE. The proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane at 90 V for 1.5 h using the wet transfer method. The membranes were then incubated in 5% skimmed milk for 2 h at room temperature for blocking. After incubation with a primary antibody Occludin (Thermo Fisher Scientific Inc., #40-4700, 1:5000), Claudin- 1 (Thermo Fisher Scientific Inc., #51-9000, 1:5000), IL-22 (Abcam, #ab193813, 1:2000), STAT3 (Biowordtechnology; #AP0365, 1:1000), P-STAT3 (Biowordtechnology; #AP0248, 1:1000), and β-actin (CST, #4970 T, 1:4000) overnight at 4 °C, the membranes were incubated with HRP-labeled goat anti-mouse or goat anti-rabbit secondary antibodies (1:5000). Protein blots were visualized using a gel imaging system (Tanon 2500R; Tanon Science & Technology Co., Ltd., Shanghai, China). The band density was quantified using Image J 10.0 software and normalized to β-actin.

3.3.9 16S ribosomal RNA (rRNA) amplicon sequencing

Genomic DNA was extracted from the jejunum mucosa using the EZNATM Soil DNA Kit (D5625-02, Omega Bio-Tek Inc., Norcross, GA, USA), as directed by the manufacturer. The hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified by a two-step PCR using specific primers (338F, 5′-ACT CCT ACG GGA GGC AGC AG-3′ and 806R, 5′-GGA CTA CH VGGG TWT CTAAT-3′) with unique 8-bp barcodes to facilitate multiplexing. The amplicons were sequenced using the Illumina HiSeq sequencing platform, as previously described. The Majorbio Cloud Platform (www.majorbio.com) was used to analyze the raw data. The raw reads were deposited to the Sequence Read Archive (SRA) database (Accession Number: PRJNA876628) of NCBI. A more detailed methodology was described previously (Wu et al., 2020b).

3.3.10 Trp and its metabolites analysis by liquid chromatograph-mass spectrometer (LC-MS)

Methanol was used to extract Trp and its metabolites (ST, IAA, IPA, HIAA, Tpm, 5-HT, Kyn) from the jejunum mucosa. The methanol extraction solutions were pre-cooled for 30 min at −20 °C. After being vortexed for 1 min, the samples were grinded 3 times (30 s for each time and 10 s intervals) with high throughput Tissuelyser instrument (Scientz-48, Jingxin, Shanghai, China). The supernatant was collected after centrifugation at 10,000 × g for 5 min and filtered through 0.22 μm filter membranes (Jin Teng, Tianjin, China).

LC-MS analysis was performed on Agilent 1290 UHPLC electrospray ionization-time-of-flight mass spectrometer (ESI-TOF-MS) coupled with Agilent 1260 SFC-Ultivo equipped with an Agilent ZORBAX Eclipse XDB-C18 column (3.0 mm × 150 mm, 1.8 μm). A linear gradient was obtained by mixing eluent A (water + 0.1% formic acid) and eluent B (100% methanol). The elution gradient for 5-HT and ST

was as follows: 0–0.5 min (20% B), 0.5–1 min (20%–40% B), 1–3 min (65%–75% B), 3–4 min (75%–90% B), 4–7 min (90%–100% B) at the flow rate of 0.5 mL/min. For the remaining metabolites (Trp, IAA, IPA, HIAA, Tpm, Kyn), the elution gradient was set as follows: 0–0.5 min (20% B), 0.5–1 min (20%–40% B), 1–2 min (40%–50% B), 2–3 min (50%–80% B), 3–4 min (80% B), 4–7 min (80%–85% B), 7–9 min (85%–100% B), 9–11 min (100% B), and the flow rate was 0.3 mL/min, and the column temperature was 40 °C. The amount of each metabolite was calculated according to standard curves with known metabolite levels.

3.3.11 Statistical analysis

Data conforming to normal distribution were compared using Student t-test, while those with non-normally distributed were tested using Kruskal-Wallis test (CYP1A1, serum IL-17, TNF-α). These analyses were performed using the JMP software (JMP R version 10.0.0, SAS Institute, Cary, NC, USA) for Windows.

Raw data obtained from gut microbiota were processed using the free online platform of Majorbio I-Sanger Cloud Platform (www.i-sanger.com). For β-diversity, principal-coordinate analysis (PCoA) plots were produced using Bray-Curtis distances, and community significance was confirmed with a Wilcoxon Rank-Sum test. All data were presented as mean ± standard error of the mean (SEM). Acceptable significance levels were at $^{*}P < 0.05$ and $^{**}P \leq 0.01$.

Spearman's or Mantel's correlation was used to analyze the correlation between the mucosal tryptophan metabolites, gene expression (inflammatory cytokines, STAT3/ IL-22 pathway), and tryptophan and its derivatives in the jejunum.

3.4 Results

3.4.1 Dietary pectin supplementation improved the integrity of jejunum

To determine the effects of pectin supplementation on intestinal integrity, HE staining, qRT-PCR, and western blotting methods were used to examine the jejunum morphology and tight junctions. Histopathology staining results showed that the villus height was increased significantly ($P < 0.05$) in the PEC group than in control (Figure 3-1A–B). Additionally, the mRNA expression levels of tight junction proteins Claudin-1 ($P = 0.005$), Occludin ($P = 0.016$), and zonula occludens-1 (*ZO-1*, $P = 0.108$) were increased (Figure 3-1C–E). Western blotting results showed that the protein level of Claudin-1 increased greatly ($P < 0.05$), however, the level of Occludin did not change significantly (Figure 3-1F). It was indicated that pectin supplementation improved intestinal barrier and gut integrity.

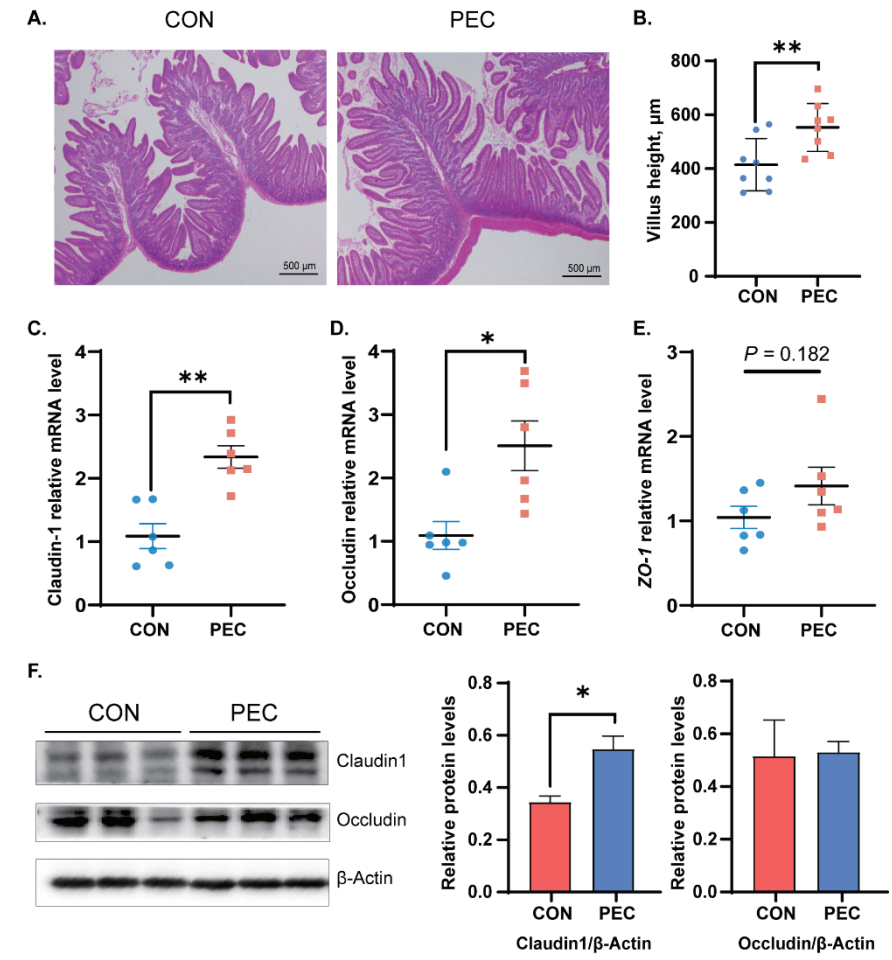

Figure 3-1 Effects of pectin on jejunal morphology in piglets. A representative images of hematoxylin-eosin staining in the jejunum; B Jejunal villus height; C Jejunal mRNA expression levels of Claudin-1; D Jejunal mRNA expression levels of Occludin; E Jejunal mRNA expression levels of *ZO-1* (n = 6). F Jejunal protein expression levels of tight junction proteins (Occludin, Claudin-1) (n = 4). Data were expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001

3.4.2 Pectin supplementation altered the expression levels of inflammatory cytokines in the jejunal mucosa and serum

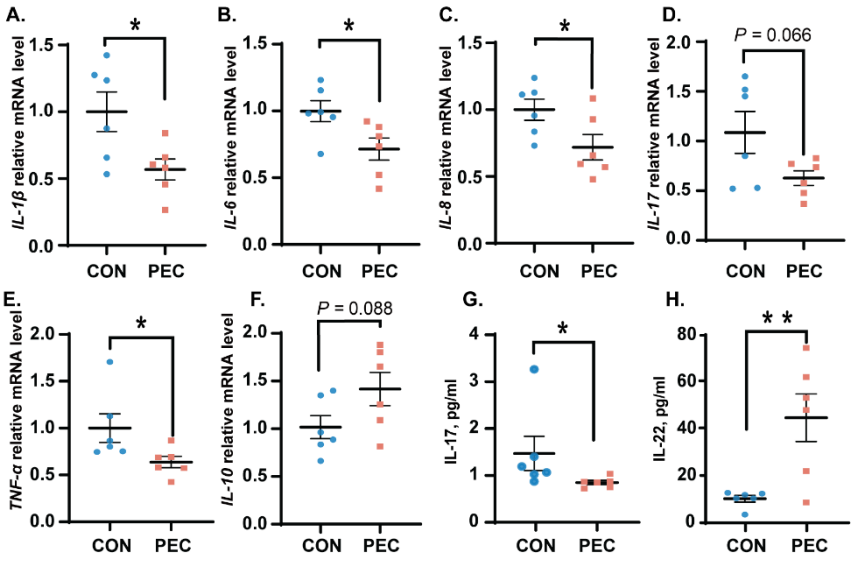

Figure 3-2 Pectin supplement altered the expression levels of inflammatory cytokines in the jejunum and serum. A *IL-1β*; B *IL-6*; C *IL-8*; D *IL-17*; E *TNF-α*; F *IL-10*; A–F were detected levels in Jejunum; G IL-17; and H IL-22 were detected in serum, $n = 6$. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; data are presented as the mean ± SEM ($n = 6$)

The inflammatory cytokines were also detected in the jejunal mucosa and serum. In the jejunal mucosa, pectin supplementation downregulated the expression of proinflammatory cytokines, *IL-1β* (Figure 3-2A; $P < 0.05$), *IL-6* (Figure 3-2B; $P < 0.05$), *IL-8* (Figure 3-2C; $P < 0.05$), *IL-17* (Figure 3-2D; $P = 0.066$), and *TNF-α* (Figure 3-2E; $P < 0.05$). On the other hand, the expression of the anti-inflammatory cytokine *IL-10* (Figure 3-2F; $P = 0.088$) was increased in the PEC group compared to the control group. Additionally, after pectin supplementation, a diminished expression level of IL-17 (Figure 3-2G; $P < 0.05$) and an enhanced expression level of IL-22 (Figure 3-2H; $P < 0.008$) was observed in the serum. Thus, pectin supplementation in the diet regulated the jejunum inflammatory responses in piglets.

3.4.3 Pectin supplementation altered the bacterial community in jejunum mucosa

Following size filtering, quality control, and chimera checking, 16S rRNA amplicon sequencing results revealed a total of 859,243 reads ranging from 35,227 to 74,138 reads per sample, to examine the effect of pectin on microbial population

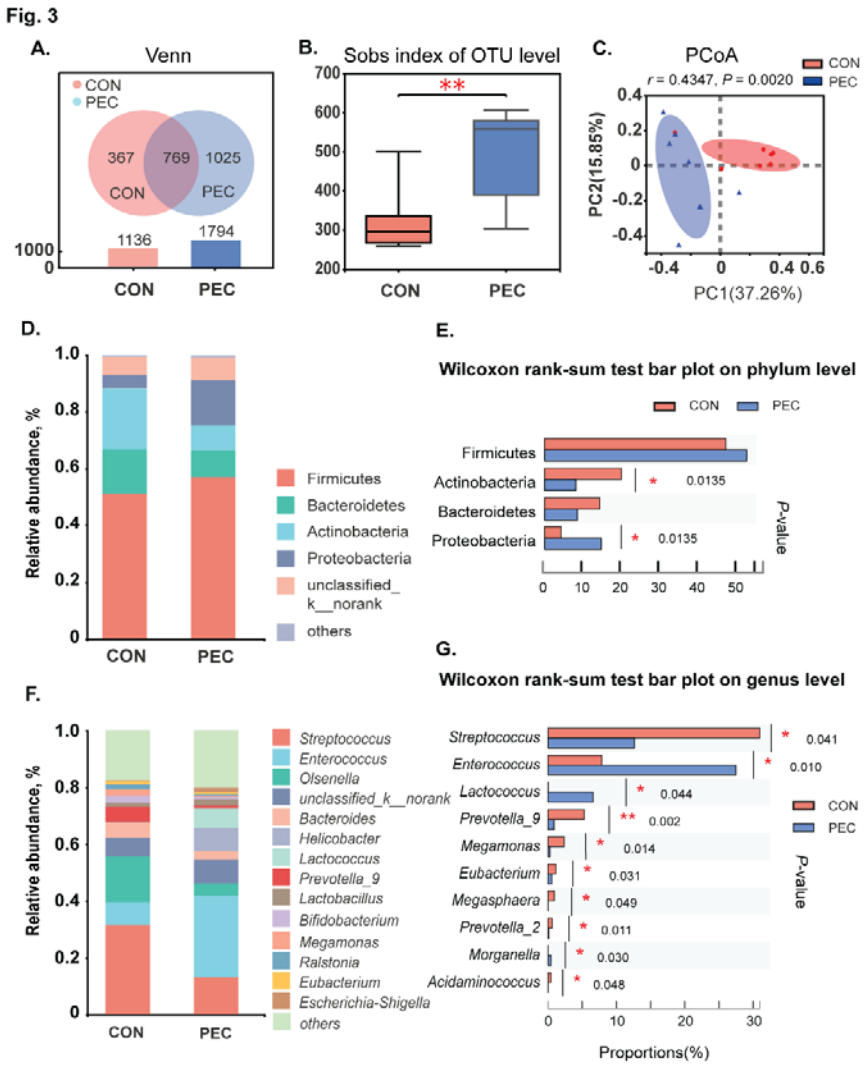

Figure 3.4.3-3 Effects of pectin on the jejunum microbial diversity. A Venn diagram; B The alpha diversity indices (Sobs); C The beta diversity presented by the PCoA plot based on the OTU level; D The abundance of the intestinal microbiota composition at the phylum level; E Differences in microbial community composition between two groups at phylum level; F The abundance of the intestinal microbiota composition at the genus level; G Differences in microbial community composition between two groups at phylum level. Data were expressed as mean ± SEM, $n = 8$. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

in the jejunum. Sequencing counts were normalized to acquire normalized reads for each sample into operational taxonomic units (OTUs) based on 97% identity.

As indicated in Figure 3-3, a Venn diagram was utilized to reveal the common and unique OTUs in the control and/ or pectin supplementation groups. Pigs in the CON and pectin groups had 367 and 1025 distinct OTUs, respectively, and 769 common OTUs (Figure 3-3A). Additionally, alpha diversity (Sobs indexes) revealed that the gut microbial microbiota diversity of pectin-treated piglets was significantly different from that of the control piglets, at the OTU level in the jejunal mucosa (Figure 3-3B). This was further supported by the beta diversity presented in PCoA (Figure 3-3C). The composition of the gut microbial community was then analyzed at the genus level. Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria constituted the majority of the microbiota at the phylum level (Figure 3-3D). Pectin boosted the abundance of Proteobacteria, whereas decreased the abundance of Actinobacteria (Figure 3-3E). Noticeable alterations in their microbial composition were detected at the genus level (Figure 3-3F). Pectin significantly reduced the relative abundance of *Streptococcus*, *Prevotella_9*, *Megamonas*, *Eubacterium*, *Megasphaera*, *Prevotella_2*, and *Actidaminococcus*, whereas it increased the relative abundance of *Enterococcus*, *Lactococcus*, and *Morganella* (Figure 3-3G, $P < 0.05$).

3.4.4 Pectin altered the levels of microbiota-derived tryptophan metabolites in jejunum

Trp is an important metabolite related to gut microbiota. Various diets and bacterial populations influenced the concentration of Trp and its derivatives. The Trp-derived metabolites in the jejunal mucosa were determined to evaluate whether a change in the intestinal microbiota affects the production of Trp and its related metabolites after pectin supplementation. The concentration of Trp was significantly lower in the pectin group compared to the CON group (Figure 3-4A). As for indole derivatives, the concentrations of ST (Figure 3-4B), IAA (Figure 3-4C), 3- IPA (Figure 3-4D), HIAA (Figure 3-4E), and Tpm (Figure 3-4F) were significantly higher in the pectin-fed piglets than in the CON group ($P < 0.05$). Particularly, the content of IPA reached extremely significant levels (Figure 3-4D; $P < 0.001$). Additionally, two other pathway metabolites, 5-HT and Kyn were not significantly different between these two groups (Figure 3-4G–H). Accordingly, adding pectin facilitated tryptophan metabolism towards the indoles as AhR ligands in the piglet intestine.

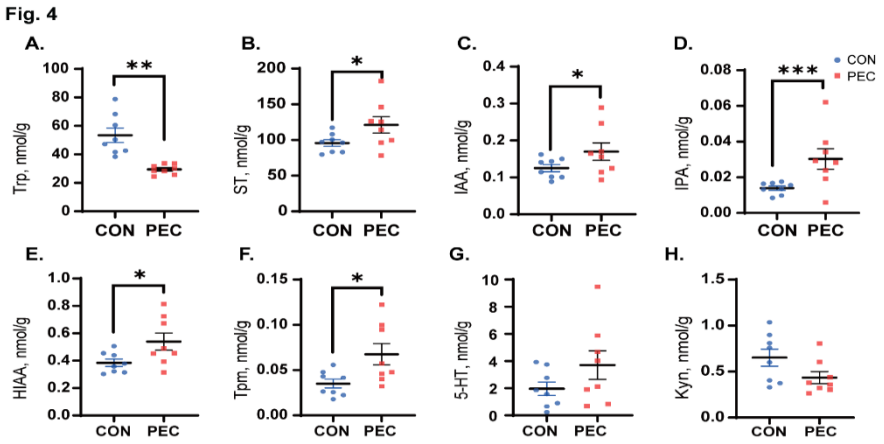

Figure 3.4.4-4 Effect of pectin on the jejunum microbial tryptophan metabolism concentration. A Trp (tryptophan); B ST (Skatole); C IAA (3-Indole acetic acid); D IPA (3-indolepropionic). E 5-Hydroxyindole-3-acetic acid; F Tpm (Tryptamine); G 5-HT (5-hydroxytryptamine); H Kyn (Kynurenine). Data are presented as the mean ± SE, (n = 8). *P < 0.05; **P < 0.01; ***P < 0.001

3.4.5 The changed Trp metabolism by pectin supplementation activated the AhR/IL-22/STAT3 signaling pathway in jejunal mucosa of piglets

Microbial derived tryptophan catabolites (indole compounds) are ligands for the AhR and act on the AhR in lymphoid cells. Therefore, we investigated the effect of pectin on the AhR signaling pathway. We analyzed the expression of *AhR* activation in the jejunum. All changes in expression [*AhR* (Figure 3-5A), *IL-22* (Figure 3-5B), cytochrome P450 1A1 (*CYP1A1*, Figure 3-5C), cytochrome P450 1B1 (*CYP1B1*,

Figure 3-5D), recombinant regenerating islet derived protein 3 gamma (*RegIIIγ*, Figure 3-5E)] were significantly increased ($P < 0.05$). Similar results were obtained using WB analysis of the IL-22-STAT3 pathway (Figure 3-5F). The protein levels of IL-22 and P-STAT/STAT3 were increased, however, not to a significant level (Figure 3-5G–H, $P > 0.05$). These results mentioned above suggested that pectin

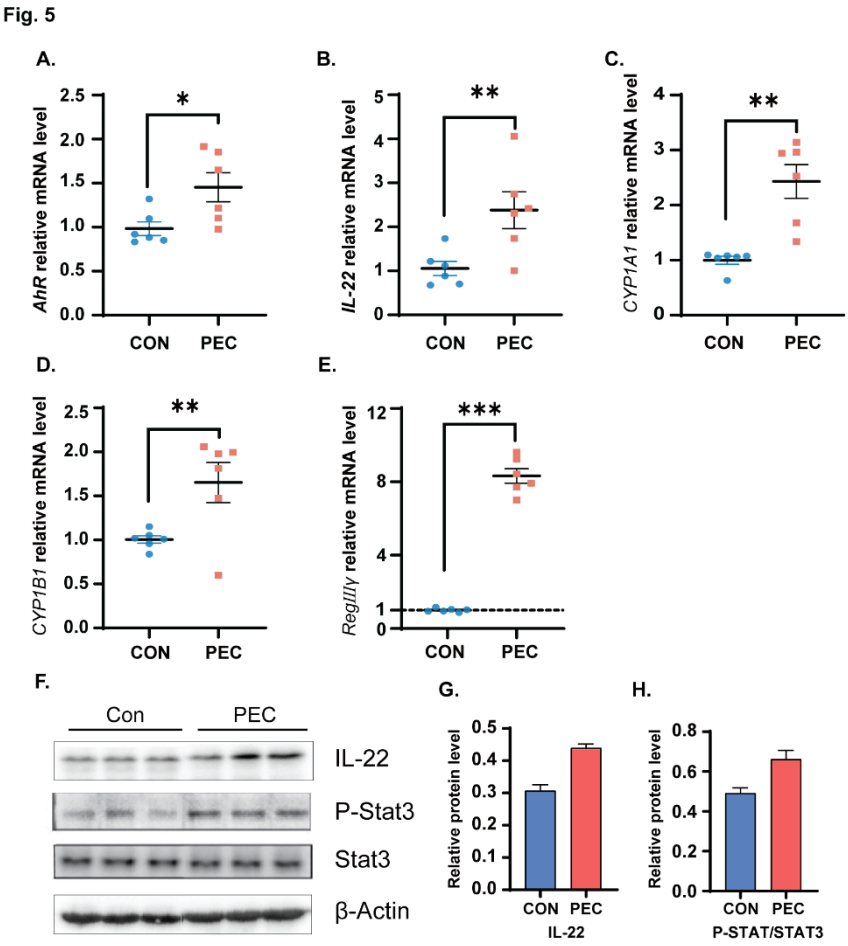

Figure 3-5 Dietary pectin supplementation influenced AhR activation and relative downstream genes expression in the jejunum of weaned piglets. A *AhR*; B *IL-22*; C *CYP1A1*. D *CYP1B1*. E *RegIIIγ*. F Protein abundances of IL-22, STAT3 and p-STAT3; G Protein abundances of IL-22; H The protein rate of p-STAT3/STAT3. Data are presented as the mean ± SE, ($n = 6$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

can activate the AhR-IL-22-STAT3 signaling pathways.

3.4.6 AhR activation in mucosa is potentially correlated with mucosal tryptophan metabolites

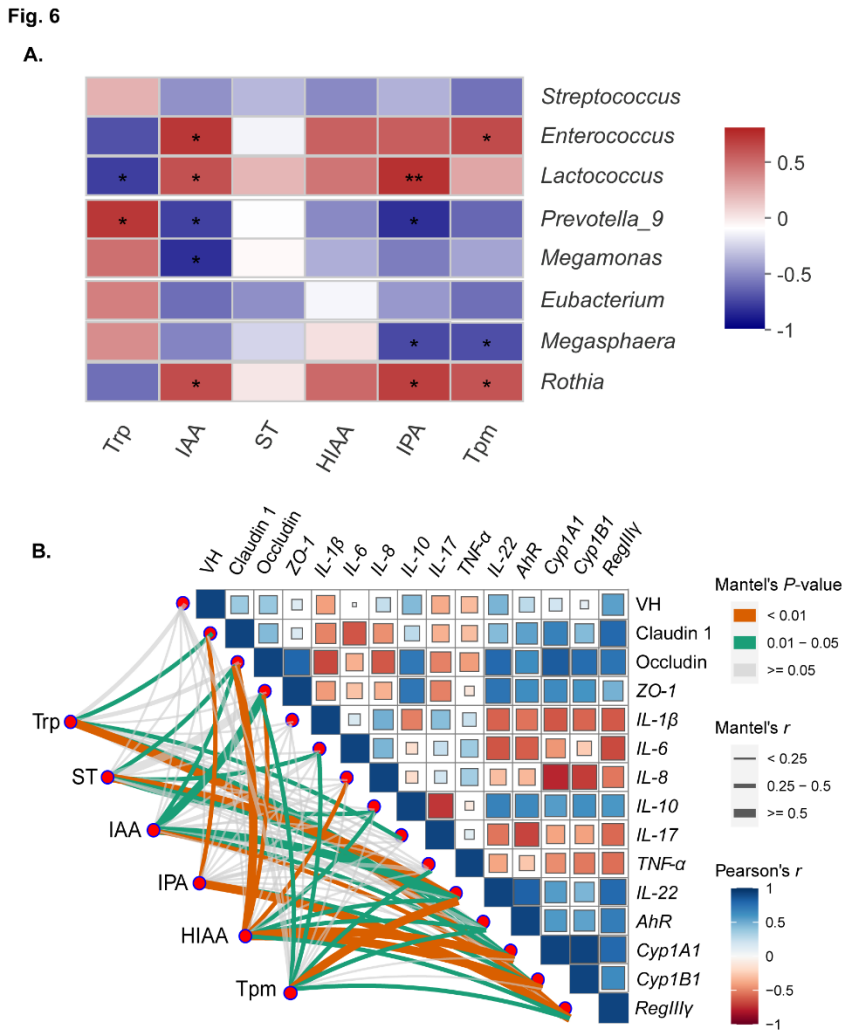

Figure 3-6 Heat maps of the Spearman rank correlation coefficient and significant tests between the differential bacteria and tryptophan metabolites (A). Pairwise comparisons if metabolites are demonstrated with a color gradient denoting Spearman's correlation coefficient. Trp, ST, IAA, IPA, HIAA, and Tpm are related to inflammatory cytokines, jejunum morphology indices by partial spearman tests. Edge width corresponds to the Partial Spearman's r statistic for the corresponding distance correlations and edge color denotes the statistical significance (B). Data are presented as the mean ± SE, (n = 6). *P < 0.05; **P < 0.01; ***P < 0.001

Spearman rank correlations coefficient and significance tests revealed a correlation between the various bacteria and the tryptophan metabolites (Figure 3-6A). The concentration of Trp was significantly and negatively correlated with the abundance of *Lactococcus*, whereas it was significantly and positively linked to the abundance of *Prevotella_9*. The concentration of IAA was positively correlated with the abundance of *Enterococcus*, *Lactococcus*, and *Rothia*, but inversely correlated with *Prevotella_ 9* and *Megamonas*. IPA was positively correlated with the abundance of *Lactococcus* and *Rothia*, while it was negatively correlated with *Prevotella_9* and *Megasphaera*. Tpm had a significantly positive relationship with *Enterococcus* and *Rothia*, whereas it had a significantly negative relationship with *Megasphaera*. Moreover, the Mantel test demonstrated a significant correlation between Trp and the gene expression levels of Claudin-1, *IL-17*, *CYP1A1*, and *RegIIIγ* (Mantel's $r > 0.25$, $P < 0.05$, Figure 3-6B). Beyond that, five genes (Occludin, *IL-6*, *IL-10*, *IL-22*, *AhR*) showed a significant correlation with ST (Mantel's $r > 0.25$, $P < 0.05$). IAA significantly correlated with Occludin, *ZO-1*, *IL-22*, *CYP1A1*, and *CYP1B1*. Additionally, we found that Claudin-1, *CYP1B1*, and *RegIIIγ* showed a significant association with IPA. HIAA was also associated with eight genes (Occludin, *ZO-1*, *IL-10*, *IL-22*, *AhR*, *CYP1A1*, *CYP1B1*, and *RegIIIγ*). There was a significant relationship between Tpm and *ZO-1*, *IL-6*, *TNF-α*, *IL-22*, *AhR*, and *RegIIIγ*.

3.5 Discussion

During the weaning transition period, piglets are susceptible to infection by various pathogens and non-pathogens, associated with a disrupted state of microbiota and an immature immune system (Wang et al., 2020b). Emerging evidence demonstrated that pectin could enhance anti-inflammatory properties, regulate the host microbiome (Yu et al., 2021), boost markers of mucus barrier function, modulate immunological activity (Wu et al., 2020b;Xie et al., 2020), and promote gut integrity (Schanz et al., 2020). In this study, we demonstrated that supplementing piglets with pectin can boost their anti-inflammatory activity, which may be associated with changes in microbial tryptophan metabolites induced by pectin supplementation. In this study, pectin was found to increase the villus height, suggesting that it enhances intestinal health status and promotes nutrient absorption. Previous research has also shown that dietary fiber treatment can improve the morphological structure of the jejunum of piglets, as evidenced by an increase in VH and a decrease in crypts depth (CD), resulting in decreased intestinal mucosal permeability (Abdollahi et al., 2021;Wen et al., 2022) and increased the intestinal barrier protection (Wang et al., 2014b;Tejeda et al., 2021).

As an essential protein in the intestine, tight junction proteins play a crucial role in gut barrier function, particularly in maintaining the integrity of the intestinal barrier and preventing the spread of harmful substances (Stan et al., 2020). Recent research showed that feeding piglets inulin or pectin increases the gene expression of Claudin-1, Occludin, and *ZO-1* (Wu et al., 2020b;He et al., 2021). As expected, the results of our study were consistent with the previous work. Thus, pectin supplementation during the weaning transition period improved the intestinal barrier function of piglets. Subsequently, we also investigated whether pectin may have an additional beneficial effect on the intestinal tract. As is well-known, *TNF-*

α, *IL-1β*, and *IL-6* are essential proinflammatory response indicators. Additionally, macrophages produce *IL-8*, a small inflammatory cytokine. This indicates that variations in these proinflammatory cytokine levels may reflect the inflammatory response status. A previous study revealed that low-methoxyl pectin might downregulate the mRNA levels of *TNF-α*, *IL-1β*, and *IL-6* in ileum colonic tissues (Sun et al., 2017b). Furthermore, pectin extract from apples might decrease the gene expression of *IL-6* in mouse ileum tissue (Jiang et al., 2016). Similarly, pectin derived from oranges and lemons can improve intestinal inflammation by inhibiting the initiation of *IL-6* secretion (Beukema et al., 2021b). In this study, we observed that administration of pectin reduced the mRNA expression of *IL-1β*, *IL-6*, *IL-8*, and *TNF-α* in jejunal mucosa of piglets. Moreover, there are several trials suggested that a low degree of methyl-esterification-(DM) pectin could suppress TLR2- TLR1 by directly blocking of TLR2 receptor (Sahasrabudhe et al., 2018), then slow down capsule implantation induced increasing in pro-inflammatory cytokine (*TNF-α*, *IL-6*), and promoted the release of *IL-10* (Jermendi et al., 2022). *IL-17* is also a proinflammatory cytokine mainly produced by Th17 cells and is associated with the pathogenesis of many autoimmune inflammatory diseases (Patel et al., 2020). *IL-10*, which is primarily secreted by Tregs, reduces Th17 development and function, as well as inhibits the secretion of proinflammatory cytokines and chemokines (Stankovic et al., 2019). Research showed that the decreased expression of *IL-10* and the increased expression of *IL-17* might aggravate intestinal inflammation (Shi et al., 2020). Interestingly, the pectin treatment decreased the mRNA expression level of *IL-17* while increasing the mRNA expression level of *IL-22*, which was consistent with the data mentioned above from the previous studies. Moreover, the levels of serum cytokines *IL-17* and *IL-22* were consistent with those of the jejunal mucosa. Thus, we proposed that pectin may modulate mucosal immunity by increasing anti-inflammatory cytokines and decreasing pro-inflammatory cytokines. Due to its fermentation properties on microorganisms, most previous studies on dietary fiber focused on the hindgut (Fischer et al., 2020;Chen et al., 2021). In contrast, pectin was found to modulate the microbial composition of the foregut in this study, although the foregut is not the primary site of microbial fermentation. Wu et al. (Wu et al., 2020b) reported that the supplementation of pectin in the piglet diet decreased the diversity and abundance of microorganisms in the small intestine. Similar results were obtained in other studies (Wu et al., 2020a). In contrast, other studies observed an increased abundance and diversity of ileal microbial in piglets (Pu et al., 2020;Tang et al., 2021b). We also hypothesized that pectin supplementation could affect gut health by altering the microbial composition or in other ways. As predicted by the preceding analysis, our data revealed that pectin significantly changed the composition and structure of the gut microbiota and increased the OTU number and alpha diversity. A healthy gut microbiota typically consists of four main phyla: Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (Jin et al., 2019). In the present study, we observed that pectin reduced the abundance of Actinobacteria in the jejunal mucosa. It has been reported that the abundance of Actinobacteria was highly expressed in the gut of goats with diarrhea (Li et al., 2021). Proteobacteria, an intestinal commensal bacteria, was also significantly increased

by pectin in the present study. However, pectin significantly decreased the abundance of Streptococcus, a known facultative-anaerobe bacterium and an opportunistic pathogen. Specifically, Streptococcus infection may cause mucosal damage (Flemer et al., 2018) and is associated with an increased risk of colorectal cancer (Kasai et al., 2016). Our study also found that the indole-derivatives-producing bacteria (*Lactobacillus* and *Enterococcus*) in the gut showed a notable increase in pectin group. Additionally, pectin significantly reduced the abundance of *Prevotella_9*, *Megamonas*, *Eubacterium*, *Megasphaera*, *Prevotella_2*, and *Acidaminococcus*. *Prevotella_9* and *Megasphaera* are generally regarded as opportunistic pathogens (Li et al., 2017;Chen et al., 2018a). Moreover, *Megamonas*, *Eubacterium*, and *Acidaminococcus* are commonly believed to be associated with fatty acid metabolism (Dai et al., 2011;Dai et al., 2012;Polansky et al., 2015;Stinson et al., 2020), which significantly decreased in pectin group. *Morganella*, the Gram-negative bacillus, belongs to the Enterobacteriaceae family. This study observed that pectin supplementation significantly increased the abundance of *Morganella*. Therefore, our study showed that adding pectin significantly reduced the abundance of harmful bacteria (*Streptococcus*) and increased the abundance of beneficial bacteria (*Enterococcus* and *Lactobacillus*) in the intestinal mucosa, to promote intestinal health.

Lactobacillus and *Enterococcus* are related to tryptophan metabolism. Specifically, tryptophan is an important amino acid that mammals must obtain from their diet, and it can be transformed into indole and indole derivatives by the gut microbiota (Zelante et al., 2014;Zhang et al., 2020a). Then, it can alter the immune-related signaling pathways that regulate inflammation in the gut (Keszthelyi et al., 2009). Only a few commensal species, including *Enterococcus* (Wlodarska et al., 2017;Zhu et al., 2021) and *Lactobacillus* (Lamas et al., 2018), are known to produce indole derivatives, and many more are likely to be discovered. In this study, pectin supplementation decreased the concentration of Trp while increasing the amount of indole derivatives (ST, IAA, IPA, HIAA, Tpm). Previous studies suggested that dietary fiber supplementation increased the levels of indole derivates, including IPA (Kundi et al., 2021). Other studies also showed that pectin can alleviate alcoholic hepatitis by promoting the elevation of microbial metabolites, IAA, and Indole- 3-lactic acid (Wrzosek et al., 2021). This accorded with our research that pectin promotes the metabolism of tryptophan, shifting the metabolic direction toward the metabolism of indoles. As a whole, pectin increased the content of indole derivatives in the gut, which had a beneficial effect on intestinal immunity.

Group 3 innate lymphoid cells (ILC3) are greatly enriched in the lamina propria of the jejunum, the highly expressed AhR in ILC3 can be activated by tryptophan metabolites (indole derivates) as ligands, thereby promoting the production of *IL-22* by ILC3 cells (Zelante et al., 2013;Alexeev et al., 2018;Dong et al., 2020;Qian et al., 2022), and activates downstream pathways by inducing phosphorylation of Stat3, further promoting the production of antimicrobial peptides and mucins (Wang et al., 2018b;Elshaer et al., 2021). Representative research showed that pectin supplementation altered the intestinal microbiota of mice, increased the tryptophan metabolites of the microbiota, and reduced alcohol-induced liver damage by activating the AhR pathway (Schanz et al., 2020;Wrzosek et al., 2021). In this study, we found that pectin treatment enhanced the levels of *AhR*, *IL-22* and

p-Stat3 downstream of *AhR*, which plays as an essential role in promoting the production of monooxygenase enzymes (*CYP1A1*, *CYP1B1*) and antimicrobial molecule (*RegIIIγ*). Consequently, these antimicrobial molecules exerted the protective function of the intestinal barrier. This indicated that the altered in the microbiota structure and metabolite concentration in jejunal mucosa observed following pectin treatment was the basis of immunomodulation, with the activated AhR/IL-22/Stat3 signaling pathway providing a plausible mechanism.

3.6 Conclusions

In conclusion, our study found that adding pectin to the model could improve the intestinal integrity and gut immunity by promoting tryptophan metabolism. This indicated that dietary pectin supplementation altered jejunal microbial composition, thus promoting microbial tryptophan metabolism. Increased metabolites can act as ligands or signaling molecules to modulate the intestinal immune response through the AhR/IL-22/State3 pathway, ultimately reducing proinflammatory factors and enhancing intestinal barrier function. These results provided a reliable theoretical foundation and guidance for using pectin in mammals as a prospective intestinal health defender.

Table 3-1. Primer sequences used for real-time PCR.

Gene	Primer	Nucleotide sequences (5’ to 3’)
β-actin	F	GCGTAGCATTTGCTGCATGA
	R	GCGTGTGTGTAACTAGGGGT
ZO-1	F	CTCCAGGCCCTTACCTTTCG
	R	GGGGTAGGGGTCCTTCCTAT
Occludin	F	CAGGTGCACCCTCCAGATTG
	R	TATGTCGTTGCTGGGTGCAT
Claudin-1	F	TCGACTCCTTGCTGAATCTG
	R	TTACCATACCTTGCTGTGGC
IL-1β	F	GCCAGTCTTCATTGTTCAGGTTT
	R	CCAAGGTCCAGGTTTTGGGT
IL-6	F	TCCAATCTGGGTTCAATCA
	R	TCTTTCCCTTTTGCCTCA
IL-8	F	TACGCATTCCACACCTTTC
	R	GGCAGACCTCTTTTCCATT
IL-10	F	TCGGCCCAGTGAAGAGTTTC
	R	GGAGTTCACGTGCTCCTTGA
IL-17	F	CTCTCGTGAAGGCGGGAATC
	R	GTAATCTGAGGGCCGTCTGG
TNF-α	F	CGTCGCCCACGTTGTAGCCAAT
	R	GCCCATCTGTCGGCACCACC
AhR	F	CATGCTTTGGTCTTTTATGC
	R	TTCCCTTTCTTTTTCTGTCC
CYP1A1	F	CCTTCACCATCCCTCACAGT
	R	ATCACCTTTTCACCCAGTGC
CYP1B1	F	AATAACGGGGGAAATTCCTG
	R	CACCGAAACACAATGCAATC
RegIIIγ	F	AACCTGGATGGGTGCAGACGTG
	R	TTGGTTCCAAGCCCTCGGTG
IL-22	F	CTACATCACCAACCGCACCT
	R	TCAGAGTTGGGGAACAGCAC

Chapter IV

Pectin supplementation ameliorates intestinal epithelial barrier function damage by modulating intestinal microbiota in lipopolysaccharide-challenged piglets

Chapter IV. Pectin supplementation ameliorates intestinal epithelial barrier function damage by modulating intestinal microbiota in lipopolysaccharide-challenged piglets

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Key words: Pectin; Piglet; Lipopolysaccharide; Intestinal barrier function; Glycosylation; Microbiota.

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Author Contributions: Guoqi Dang conducted animal husbandry experiments, collected data, performed some sample testing (qRT-PCR, WB), conducted data analysis (16S rRNA microbiome), and contributed to paper editing and refinement.

4.1 Abstract

During weaning, infants and young animals are susceptible to severe enteric infections, thus inducing intestinal microbiota dysbiosis, intestinal inflammation, and impaired intestinal barrier function. Pectin (PEC), a prebiotic polysaccharide, enhances intestinal health with the potential for a therapeutic effect on intestinal diseases. One 21-d study was conducted to investigate the protective effect of pectin against intestinal injury induced by intraperitoneal injection of *Escherichia coli* lipopolysaccharide (LPS) in a piglet model. A total of 24 piglets (6.77 ±0.92 kg BW; Duroc × Landrace × Large White; barrows; 21 d of age) were randomly assigned into three groups: control group, LPS-challenged group, and PEC + LPS group. Piglets were administrated with LPS or saline on d14 and d21 of the experiment. All piglets were slaughtered and intestinal samples were collected after 3 h administration on d21. Pectin supplementation ameliorated the LPS-induced inflammation response and damage to the ileal morphology. Meanwhile, pectin also improved intestinal mucin barrier function, increased the mRNA expression of *MUC2*, and improved intestinal mucus glycosylation. LPS challenge reduced the diversity of intestinal microbiota and enriched the relative abundance of *Helicobacter*. Pectin restored alpha diversity and improved the structure of the gut microbiota by enriching anti-inflammatory bacteria and short-chain fatty acids (SCFAs)-producing bacteria, and increased the concentrations of acetate. In addition, Spearman rank correlation analysis also revealed the potential relationship between intestinal microbiota and intestinal morphology, intestinal inflammation, and intestinal glycosylation in piglets. Taken together, these results indicate that pectin enhances intestinal integrity and barrier function by altering intestinal microbiota composition and their metabolites, which subsequently alleviates intestinal injury and finally improves the growth performance of piglets.

4.2 Introduction

During weaning, piglets are prone to occur immune stress and intestinal injury due to low immune function, hypoplasia of the digestive tract, and environmental stresses, which lead to postweaning diarrhea and affect the normal growth of piglets (Campbell et al., 2013;Chen et al., 2017a;Gresse et al., 2017). Previous studies indicated that the intestinal microbiota played a very important role in host health and disease throughout life, particularly in infancy (Xie et al., 2021). The colonization of intestinal microbiota in infancy is a critical period for the formation of intestinal microbiota. During this phase, the intestinal microbiota can not only promote intestinal health, maintain normal intestinal barrier functions, and escape from pathogenic bacteria, but also improve the intestinal and systemic immune system development and maturation (Morelli, 2008;Buffie et al., 2013;Pickard et al., 2017;Shanahan et al., 2018). It has been confirmed that intestinal dysbiosis has been implicated in the pathogenesis of certain inflammatory diseases and infections (Thursby et al., 2017). Furthermore, the intestinal microbiota influences the host nutritional metabolism and immune response as well as intestinal homeostasis, and the microbiota makeup is, in turn, regulated by the host, the environment, and inter-microbial interactions (David et al., 2014;Larabi et al., 2020;Sun et al., 2021b). From this, intestinal microbiota modulation has become a new strategy to enhance

intestinal health, which has been verified in some studies (Wang et al., 2019b;Hu et al., 2020;Sun et al., 2021c).

Pectin (PEC) is a complex polysaccharide, the main ingredient is an α-(1,4)-linked *d*-galacturonic acid (d-galUA) that is account for over 70% (Protzko et al., 2018). Pectin can modulate the intestinal microbiota and their metabolic products, thereby improving intestinal homeostasis and intestinal health (Ishisono et al., 2019;Singh et al., 2019). Meanwhile, pectin can ameliorate intestinal inflammation by reducing pro-inflammatory cytokines and enhancing the mRNA expression of *MUC2* (Chen et al., 2006;Hino et al., 2013;Jiang et al., 2016). Our previous study also indicated that pectin exerts beneficial biological functions by regulating the intestinal microbial community (Wu et al., 2020c). At present, there are few studies about adding pectin to the diet of weaned piglets. Therefore, pectin supplementation may be an effective strategy to regulate the development of early intestinal microbiota and thus maintain the long-term health of the host.

Given the interaction between intestinal health and intestinal microbiota balance, we hypothesized that pectin would restore intestinal injury by altering intestinal microbiota in weaning piglets. Therefore, we used a lipopolysaccharide (LPS) challenge to trigger intestinal inflammation and damage that post-weaning piglets generally suffered (Xu et al., 2021a), to evaluate whether pectin could ameliorate LPS-induced gut microbial dysbiosis, inflammatory response, and intestinal barrier dysfunction.

4.3 Materials and methods

4.3.1 Experimental design, animals, and diets

All animal experimental procedures were performed by the Guidelines for Care and Use of Laboratory Animals of Chinese Academy of Agriculture Sciences and experiments were approved by the Animal Ethics Committee of Experimental Animal Welfare and Ethicals of Institute of Animal Science, Chinese Academy of Agriculture Sciences (IAS2019-37). Twenty-four piglets (6.77 ±0.92 kg BW; Duroc × Landrace × Large White; barrows; 21 d of age) with good health and similar physical condition were randomly divided into three groups: control group (CON) and LPSchallenged group (LPS) were fed with the control diet, and PEC + LPS group (PECL) was fed with the pectin diet. The adaption period was 3 d, and the experimental period was 21 d. On d14 and d21 of the entire experiment, piglets in the LPS group and PECL group were injected intraperitoneally with LPS (80 μg per kg BW, E. coli 055: B5, Sigma), and the piglets in the CON group were injected with the same amount of normal saline intraperitoneally. During the whole trial period, all pigs were housed in separated pens with a plastic slatted floor and the pens were cleaned daily to avoid disease occurrence. Meanwhile, all piglets were given ad libitum access to clean drinking water and corresponding diets (Table 4-1). The control diet and pectin diet are corn-soybean meal diets containing 5% cellulose or pectin, respectively. The diets had been formulated to meet the nutritional requirements suggested by NRC (2012) for pigs, among which cellulose was used to formulate equal fiber diets as previously described (Singh et al., 2019). The amount of supplemental pectin used in this experiment was based on our

reported results (Fang et al., 2018;Wu et al., 2020c). Cellulose was purchased from the Beijing Engineering Research Center of Cellulose and Its Derivatives (Beijing, China). Pectin was purchased from the Yuzhong Biotech Corporation (Zhengzhou, China). The injection method and dosage of the LPS treated model were chosen based on the results of our preliminary trials. The experimental scheme is shown in Figure 4-1 A.

4.3.2 Sample collection

After 3 h injection of LPS or normal saline on d21, all piglets were anesthetized using an intravenous injection of pentobarbital sodium (25 mg/kg of BW) and killed by exsanguination. The sections of the ileum were in situ ligated before the whole gut was removed from the abdominal cavity. An ileal segment of approximately 3 cm was preserved in 4% paraformaldehyde for routine morphological measurement. Ileal mucosa or digest was collected in 2-mL sterile tubes, immediately frozen in liquid nitrogen, and stored at -80 °C for sequencing of microbial 16S genes and specific gene expression or analysis for short-chain fatty acids (SCFAs) quantification, respectively. Previous studies proved that LPS causes serious damage to the intestinal structure and barrier function within 2–6 h after injection, so the time of sample collection was carried out at 3 h after LPS or normal saline injection (Liu et al., 2012;Xu et al., 2020).

4.3.3 Growth performance

During the whole experiment, the daily feed intake of each replicate was accurately recorded, and the health status of piglets was observed. The piglets were weighted on the morning of the d1, d14, and d21 of the experiment, respectively. The average daily feed intake (ADFI), average daily gain (ADG), and feed-to-gain ratio (F:G) for each stage and over the whole period of the experiment was calculated.

4.3.4 Intestinal morphology analysis

After 24 h fixation, the ileum segment was dehydrated with gradient alcohol, made transparent with xylene, and embedded in paraffin. To determine the effects of pectin supplementation on the LPS challenge-induced damage to intestinal integrity, we evaluated the ileal morphology using hematoxylin and eosin (H&E staining kit, Solarbio, Beijing, China) staining. Five cross-sections (5 μm thick) of each ileum specimen were taken to prepare histological sections and stained, then observed on a Leica DM20 0 0 light microscope (Leica Microsystems, Wetzlar, Germany). The images were analyzed with ImageJ v1.8.0 software. Fifteen well-oriented and intact villi and their associated crypts were taken from each segment. Villus height (VH), crypt depth (CD), and the ratio of villus height to crypt depth (VH:CD) were measured. Goblet cells were examined by periodic acid-Schiff-Alcian Blue stain (PAS-AB staining kits, Solarbio, Beijing, China). Briefly, deparaffinized slides were exposed to 3% acetic acid, followed by staining with Alcian Blue, 1% periodic acid-Shiff reagent, and sodium metabisulfite. Finally observed and analyzed on a Leica DM20 0 0 light microscope (Leica Microsystems, Wetzlar, Germany).

4.3.5 Intestinal glycosylation analysis

The expression of intestinal glycosylation oligosaccharides was examined using

FITC-conjugated lectins: Ulex europaeus agglutinin 1 (UEA-1; fucose), concanavalin A (ConA; mannose), and wheat germ agglutinin [WGA; N-acteylglucosamine (Glc- NAc); Vector Laboratories, Burlingame, CA] were used as previously described (Engevik et al., 2015). Briefly, sections were deparaffinized, blocked with Carbohydrate blocking solution (Vector Laboratories, Burlingame, CA, USA), and stained with FITC-labeled lectin (5 μg/mL, added 0.05% Tween 20) for 1 h at room temperature. Sections were then washed three times in PBS (added 0.05% Tween 20), counterstained with DAPI Mounting medium (Vector Laboratories, Burlingame, CA), and analyzed by confocal laser scanning microscopy (Leica LSM Confocal TCS SP8; Leica, Germany). Eight animal samples from each treatment group were analyzed. Positively stained cells were counted. A minimum of 1,0 0 0 cells were counted for each sample of each experiment. The data were then normalized to the control.

4.3.6 Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from the ileal mucosa using the RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Took 1 μg of total RNA and quantified it with an Ultramicro Protein Nucleic acid analyzer (BioDrop-μLite, UK) to ensure that the detection value of the RNA sample was A260/280 = 1.8 ~2.0. cDNA was transcribed using a PrimeScript RT reagent kit with gDNA Eraser (Takara, Kusatsu, Shiga, Japan). qRT-PCR was performed according to the TB Premix Ex Taq instructions (Takara, Kusatsu, Shiga, Japan), and conducted on ABI Q7 Flex Real-time PCR System (ABI, Singapore). The amplification procedure was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. β-actin and GAPDH were used as the internal reference for relative expression of each gene in ileal mucosa, and gene expression was calculated by $2^{-\Delta\Delta}$CT method (Schmittgen, 2008). All the above operations are completed on the clean bench. All primer sequences were provided in Table 3-1.

4.3.7 Western blotting analysis

The total protein of ileum tissue was extracted using RIPA (Thermo Fisher Scientific Inc., MA, USA) and preserved at -80 °C for subsequent analysis. Then the concentration of total protein was quantified using BCA assays (Thermo Fisher Scientific Inc., MA, USA), separated them using SDS-PAGE, and then transferred to membranes for western blotting. To determine the effects of pectin supplementation on the LPS challenge-induced damage to intestinal barrier integrity, we evaluated the protein expression of tight junction protein using western blotting. Occludin (Thermo Fisher Scientific Inc., MA, USA, #40-470 0, 1:50 0), Claudin-1 (Thermo Fisher Scientific Inc., MA, USA, #51-90 0 0, 1:50 0), and β-actin antibody (Proteintech, Chicago, USA, #20536-1-AP, 1:1,0 0 0) were used as primary antibodies and incubated with membranes overnight at 4 °C. The membranes were then incubated with the HRP-labeled goat anti-rabbit secondary antibodies (Abcam, Cambridge, UK, #ab6721, 1:50 0 0). The experiments were repeated three times and the band density of the target protein was quantified after normalization to β-actin using Image lab software.

4.3.8 The 16S rRNA sequencing and analysis of ileal microbiota

DNA extraction, PCR amplification, DNA quantification, and Illumina MiSeq sequencing by the standardized protocol of Shanghai Majorbio Bio-pharm Technology (Shanghai, China). Briefly, DNA was extracted from the mucosa using the Qiagen DNA isolation kit (Qiagen, Hilden, Germany) and followed by the provided protocol. The V3-V5 hypervariable region of the 16S bacterial rRNA was amplified using primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'- GGACTACHVGGGTWTCTAAT-3'). The amplicons were sequenced on the Illumina HiSeq sequencing platform, as described before (Wu et al., 2020c). Raw data obtained from gut microbiota were analyzed on the free online platform of Majorbio Cloud Platform (www.majorbio.com) to understand the gut microbial diversity and composition. To gain insights into how the function alteration responded to the structural shift of intestinal microbiota, we investigated the intestinal microbial function using the PICRUST2 method.

4.3.9 Short-chain fatty acids (SCFAs) analysis

Quantification of SCFAs was analyzed using gas chromatography (GC) based on our previous studies (Wu et al., 2016). Briefly, the digesta were dissolved in and extracted with distilled water. The extracted samples were obtained by centrifuge at 9,000 g. Metaphosphoric acid (25%, w/v) was added to the extracts at a ratio of 1:9. After centrifugation at 10,0 0 0 g, the supernatant was subjected to SCFAs analysis with Agilent 6890N GC (Palo Alto, CA, USA).

4.3.10Statistical analysis

Each animal is the experimental unit. One-way ANOVA of the data on growth performance (BW, ADG, ADFI, and F:G), intestinal morphology and glycosylation, SCFAs, qRT-PCR (cytokines, *MUC2*, *TFF3*, and glycosyltransferase), WB (Claudin-1 and Occludin) and bacterial alpha diversity indices (Sobs, Shannon, Simpson, ACE, Chao, and Coverage) was performed using the JMP software (JMP 10.0.0, SAS Institute, Cary, NC, USA) for Windows. Statistical differences among the treatments were separated by the "least-squares means Student t" method and significant differences among various groups are represented as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. $P < 0.05$ was regarded as statistically significant, while $0.05 < P < 0.1$ was set as a significant trend. In addition, the R package "ggalluvial" was used to profile microbial communities and Spearman's correlation analysis was applied using the R package "pheatmap".

4.4 Results

4.4.1 Growth performance

Pectin supplementation significantly decreased the feed-to-gain ratio (F:G) of piglets from d1–14 ($P < 0.05$; Table 4-2). From d15–21, the LPS challenge, compared with that in the CON group, significantly decreased the average daily feed intake (ADFI) and average daily gain (ADG) of piglets and increased the F:G by 5.49% ($P < 0.05$). Pectin supplementation significantly increased ADFI and ADG of piglets compared with the LPS group ($P < 0.05$), and F:G tended to be increased ($P = 0.05$).

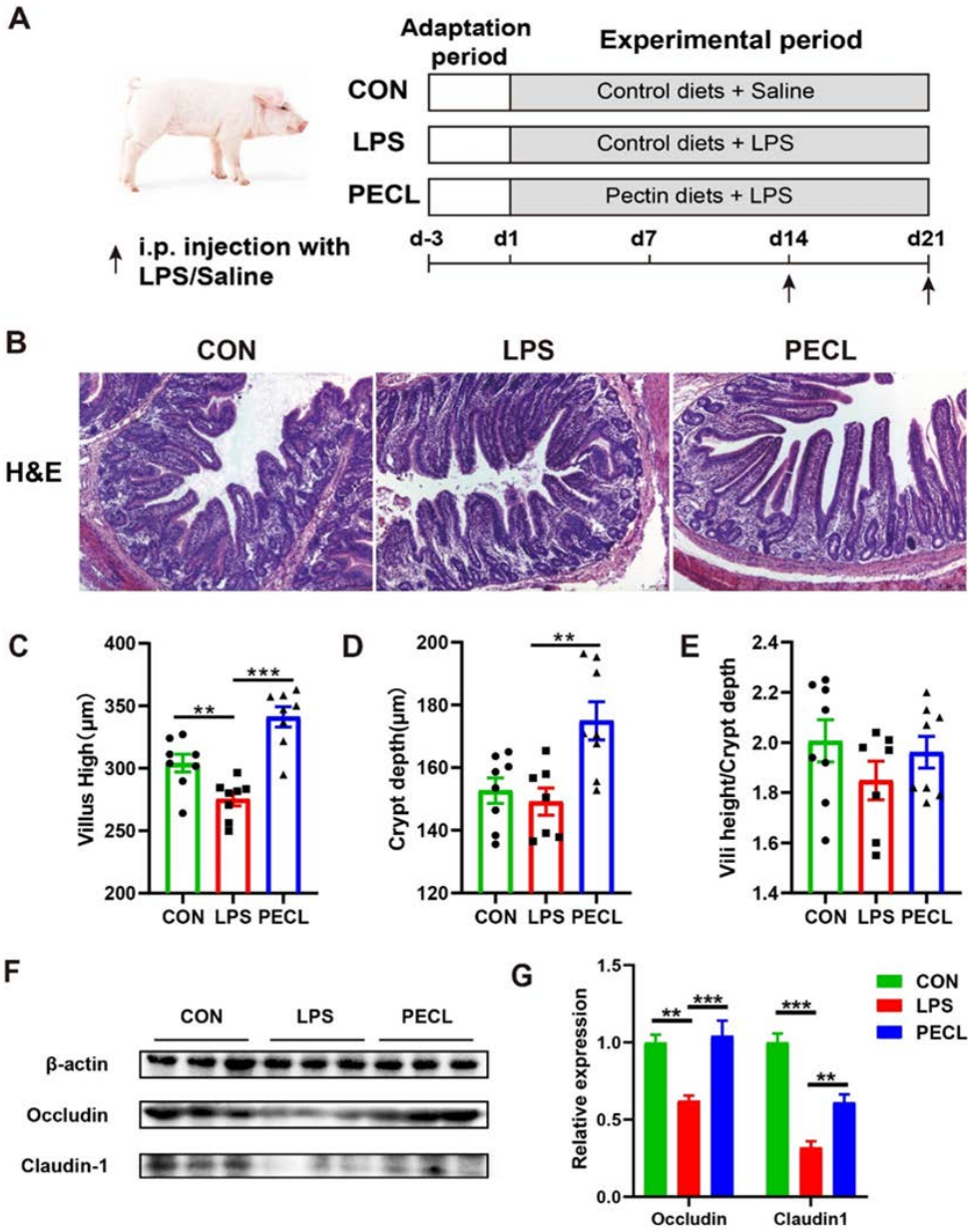

Figure 4-1 Changes in intestinal barrier function of piglets in different groups. (A) The schematic of the experimental timeline. (B) Representative H&E -stained ileum sections (C) Villus height. (D) Crypt depth. (E) Villus height/crypt depth. Scale bar 100μm; (F and G) Western blot analysis of tight junction proteins occluding and claudin-1. Signification is presented as * P<0.05, ** P<0.01, and *** P<0.001; data are presented as the mean ± SE (n=8).

4.4.2 Intestinal barrier function

LPS challenge caused fever, diarrhea, anorexia, shivering, and inactivity within 1 h in all piglets. LPS challenge damaged the ileal morphology (Figure 4-1 B) and decreased the VH (Figure 4-1 C), compared with that in the CON group ($P < 0.05$). Pectin supplementation significantly increased the VH (Figure 4-1 C), compared with the LPS group ($P < 0.05$). Pectin supplementation significantly increased the CD ($P < 0.05$), but the VH: CD showed no significant difference (Figure 4-1 C–E). Moreover, our results showed that the LPS challenge reduced the protein expression

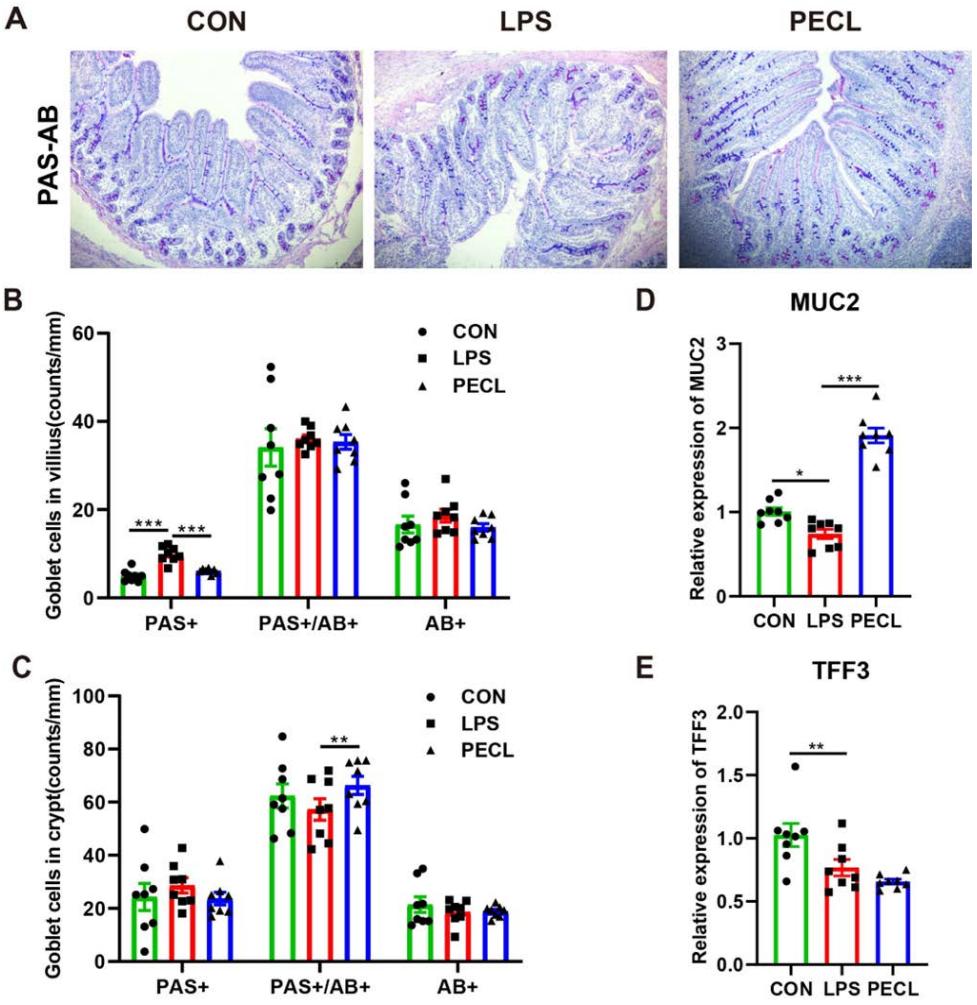

Figure 4-2 Changes in intestinal mucus barrier function of piglets in different groups. (A) Representative PAS-AB -stained ileum sections. The positive goblet cell in (B) ileum villus and (C) crypt (counts/mm). Scale bar 100μm; The mRNA expression of their marker *MUC2* (D) and *TFF3* (E) were measured in ileum mucosa by Real-time PCR. Signification is presented as * P<0.05, ** P<0.01, and *** P<0.001; data are presented as the mean ± SE (n=8).

of Claudin-1 and Occludin ($P < 0.05$). The protein expression of Occludin and

Claudin-1 in the PECL group was significantly elevated compared with that in the LPS group (Figure 4-1 F and G).

4.4.3 Intestinal glycosylation

LPS challenge increased the number of PAS-positive goblet cells in the ileal villus, compared with the CON group ($P < 0.05$). Pectin supplementation significantly restored the number of PAS-positive goblet cells in the ileal villus ($P < 0.05$; Figure 4-2 B). Additionally, Pectin supplementation significantly increased the number of PAS/ABpositive goblet cells in the ileal crypt ($P < 0.05$; Figure 4-2 C). The mRNA

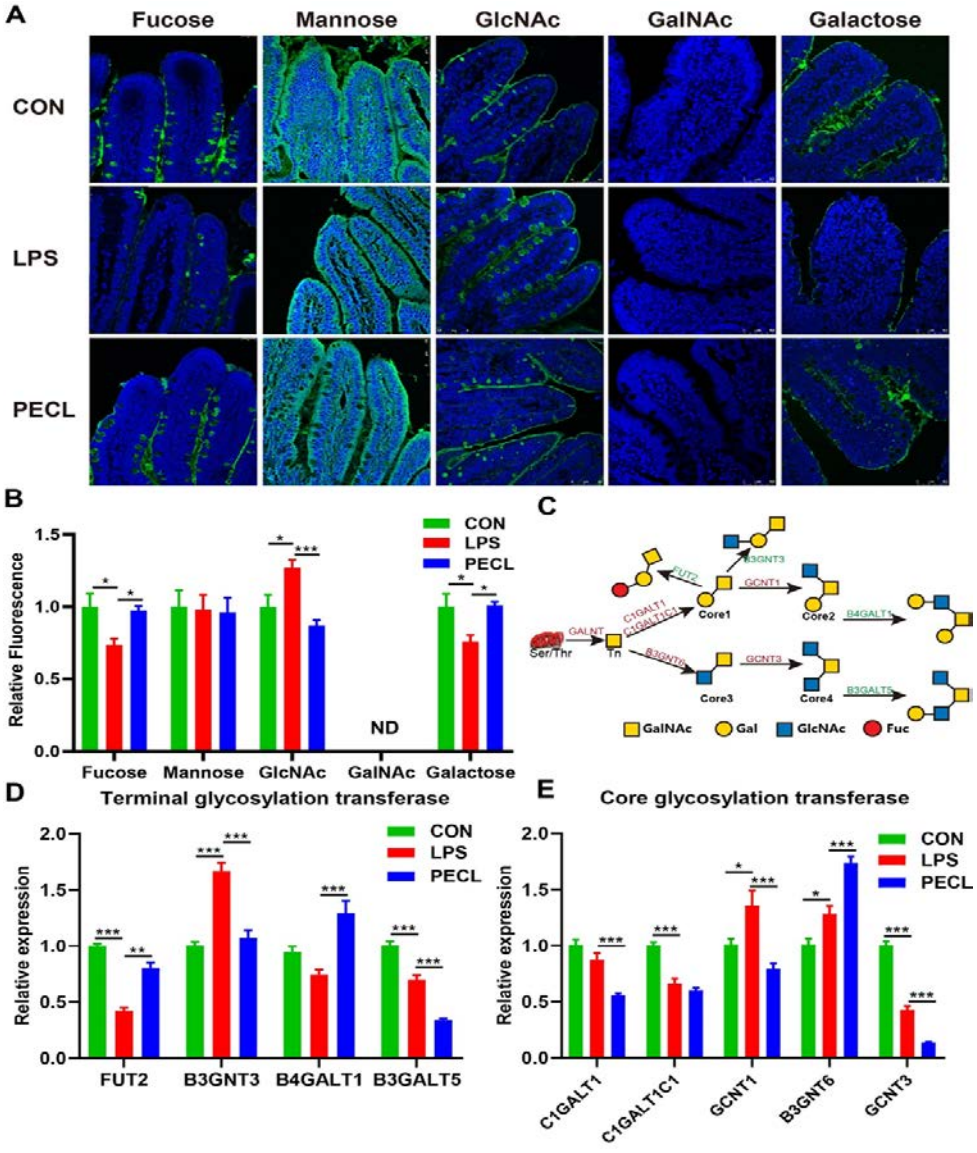

Figure 4-3 The main mucin-type O-glycosylation pathways in the piglet intestine. (A) Confocal images of the ileum section stained with a panel of lectins to identify mucus oligosaccharides (green) fucose, mannose, N-acetylglucosamine, N-acetylgalactosamine, and galactose counterstained with DAPI (blue). Scale bar = 50 μM. (B) Relative fluorescence of lectin stain where fluorescence of oligosaccharide was calculated relative to CON. (C) The mucin-type O-glycosylation biosynthesis process. (D) The mRNA expression levels of intestinal (D) terminal and (E) core glycosyltransferase of piglets in different groups. The fold change in mRNA expression relative to GAPDH and β-actin for glycosyltransferase is shown. Only differentially abundant taxa at the genus or higher taxonomic ranks were indicated. Signification is presented as * P<0.05, ** P<0.01, and *** P<0.001; data are presented as the mean ± SE (n=8).

expression of *MUC2* and *TFF3* was decreased after the administration of LPS compared with the CON group, while pectin supplementation significantly restored the level of *MUC2* (P < 0.05; Figure 4-2 D and E). Gut mucus oligosaccharides N-acetyl-galactosamine (Glc- NAc), galactose (Gal), GalNAc, fucose (Fuc), N-acetylneuraminic acid (NeuNAc), and mannose (Man) (Johansson et al., 2011a;Johansson et al., 2011b) are attached to mucin glycoproteins and can be used by bacteria to maintain their relative niches. The result showed that there are some differences in the expression of these oligosaccharides in each group (Figure 4-3 A). The LPS group exhibited increased levels of GlcNAc and decreased levels of Fuc and Gal, compared with the CON group (P < 0.05). Pectin supplementation significantly restored the level of GlcNAc, Fuc, and Gal (P < 0.05). No changes in intensity were observed in mannose levels between the groups and we do not detect GalNAc expression in the ileum (Figure 4-3 B). Moreover, LPS challenge significantly decreased the mRNA expression of terminal glycosylation transferase including fucosyltransferases2 (*FUT2*) and galactosyltransferase (*B4GALT1* and *B3GALT5*) but increased the mRNA expression of GlcNAc transferase (*B3GNT3*) in the ileum of piglets compared with that in the CON group (P < 0.05), and pectin supplementation restored those shifts. Furthermore, LPS challenge significantly decreased the mRNA expression of the *C1GALT1C1* and *GCNT3* and increased the mRNA expression of *GCNT1* and *B3GNT6* compared with the CON group (P < 0.05). Pectin supplementation decreased the mRNA expression of *C1GALT1* and *GCNT1*, but further decreased the mRNA expression of GCNT3 and increased the mRNA expression of *B3GNT6* (P < 0.05; Figure 4-3 D and E). In all, the LPS challenge changed the proportion of core1 and core2 glycan, and pectin supplementation restored these changes to some extent and promoted the shifts of core1, 2, and 4 glycans to core3 glycans in the mass.

4.4.4 Intestinal inflammation

LPS challenge significantly increased the mRNA expression of *TLR4*, *MCP1*, and pro-inflammatory cytokines *IL-1β* and *TNF-α*, but decreased the mRNA expression of anti-inflammatory cytokine *IL-10* in the ileum of piglets compared with that in the CON group ($P < 0.05$; Figure 4-4). Pectin supplementation significantly restored the level of *TLR4*, *MCP1*, and *IL-1β*, decreased the mRNA expression of *TNF- α* ($P < 0.05$), and the mRNA expression of *IL-10* tended to increase ($P =$

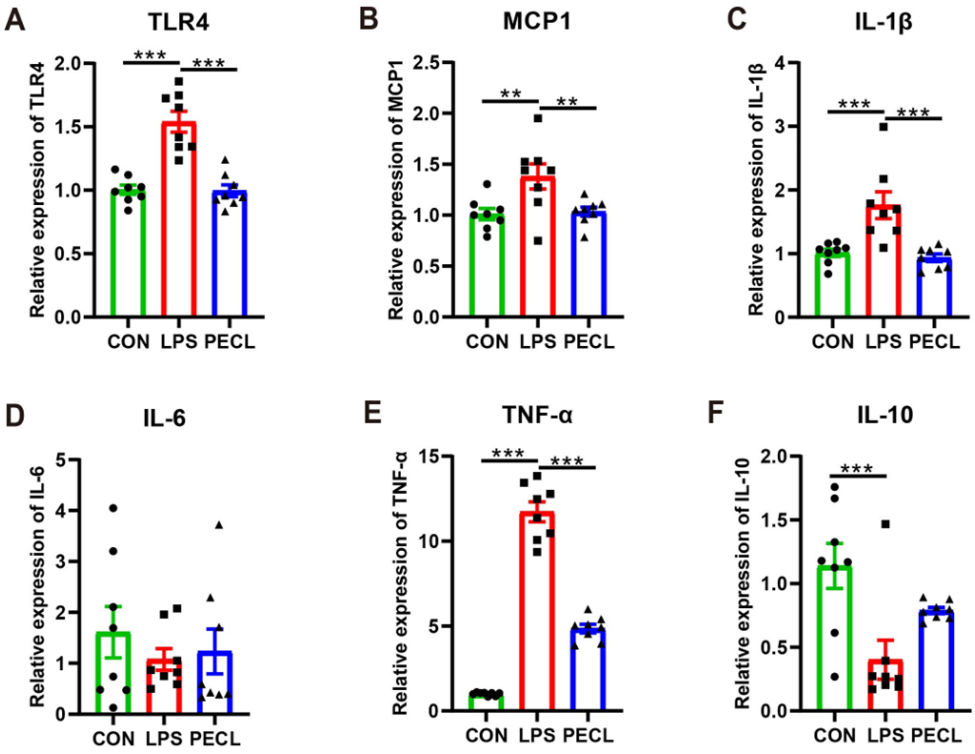

Figure 4-4 The mRNA expression levels of intestinal inflammatory cytokines of piglets in different groups. The fold change in mRNA expression relative to *GAPDH* and *β-actin* for (A) *TLR4*, (B) *MCP1*, (C) *IL-1β*, (D) *IL-6*, (E) *TNF-α*, (F) *IL-10* is shown. Signification is presented as * $P<0.05$, ** $P<0.01$, and *** $P<0.001$; data are presented as the mean ± SE ($n=8$).

0.06).

4.4.5 Structure and diversity of intestinal microbiota

Among the groups, the PECL group shared more OTU with the CON group than the LPS group (Figure 4-5 A). This indicates that the structure of intestinal microbiota in the PECL group was more similar to that of the CON group than that of the LPS group (Figure 4-5 A). This was further supported by the beta diversity presented by PCoA which illustrated that the LPS group formed a distinct cluster markedly away from that of the CON group and the PECL group (Figure 4-5 B). LPS challenge reduced the Simpson, ACE, and Chao, but increased the Coverage

compared with the CON group ($P < 0.05$). Pectin supplementation restored those shifts but did not reach statistical significance (Figure 4-5 C–H).

4.4.6 Composition and difference of intestinal microbiota

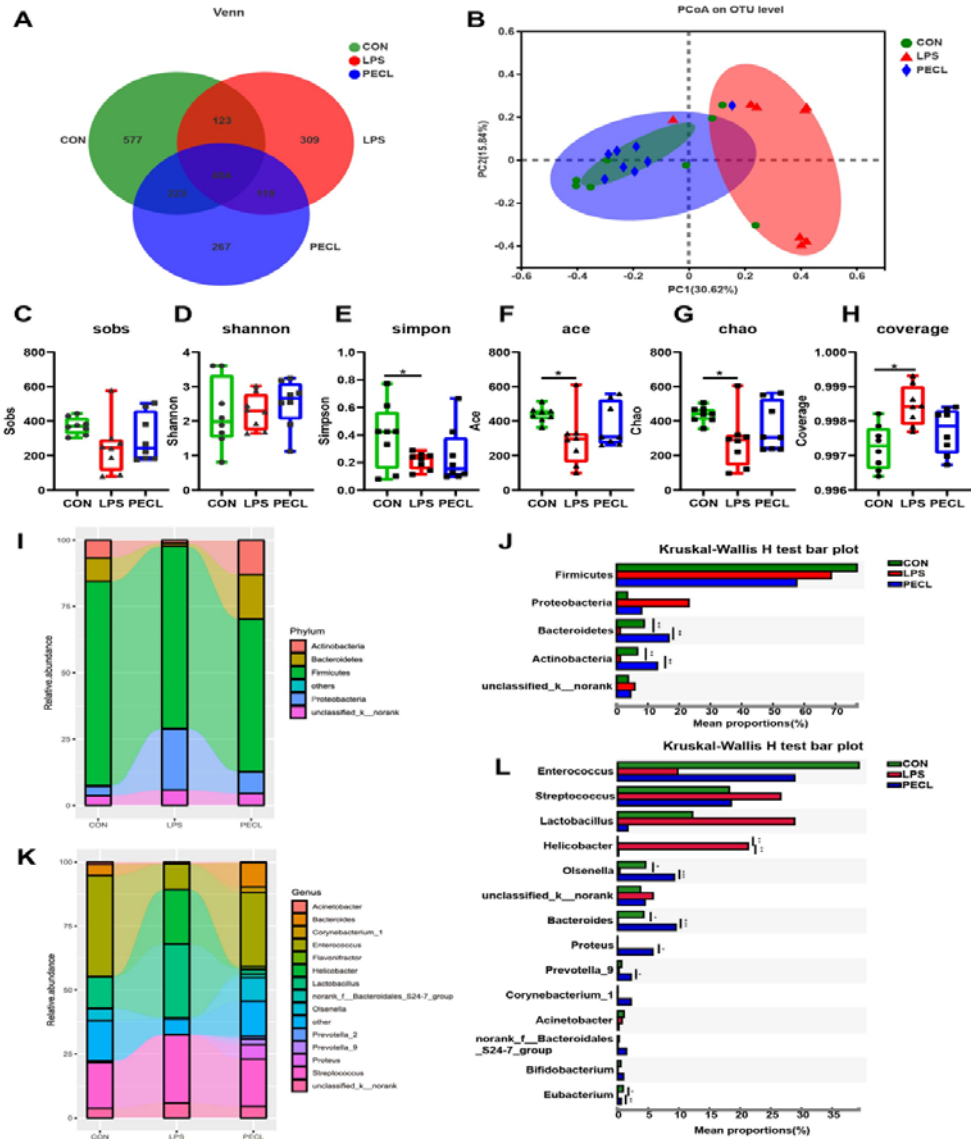

Figure 4-5 Structure and diversity of intestinal microflora of piglets in different groups. (A) The common/unique OTUs number among the groups. (B) The structure shifts (beta diversity) presented by the PCoA plot based on the OTU level. (C-H) The alpha diversity indices observed species, Chao1, ACE, Shannon, Simpson, and Good-coverage. Relative abundance of the intestinal microbiota composition in the ileum in weaned pigs at the (I) phylum level and (K) genus level. Changes in the intestinal microbiota of piglets in different groups at the (J) phylum level and (L) genus level. Signification is presented as * P<0.05, ** P<0.01, and *** P<0.001; data are presented as the mean ± SE (n=8).

The relative abundance of microbiota was analyzed at the phylum level and genus level (Figure 4-5 I and K). The linked bar plots illustrated that LPS challenge markedly shifted the relative abundance of bacteria at different taxon levels, which were restored by pectin supplementation. In detail, at the phylum level, the ileum microbiota mainly consisted of Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. After the LPS challenge, compared with the CON group, the relative abundance of Bacteroidetes and Actinobacteria was significantly decreased ($P < 0.05$). Pectin supplementation signif icantly increased the relative abundance of Bacteroidetes and Actinobacteria ($P < 0.05$; Figure 4-5 I and J). At the genus level, the relative abundance of *Olsenella*, *Bacteroides*, *Proteus*, *Prevotella_9*, and *Eubacterium* were lower in ileum mucosa due to LPS challenge, whereas *Helicobacter* was more abundant, compared with the CON group ($P < 0.05$). The relative abundance of *Olsenella*, *Bacteroides*, *Proteu*s, *Prevotella_9*, and *Eubacterium* was higher in the PECL group. Besides, pectin supplementation significantly decreased the relative abundance of *Helicobacter* compared with the LPS group ($P < 0.05$; Figure 4-5 K and L).

4.4.7 Function and metabolism of intestinal microbiota

The predicted results can be enriched at three different levels of the KEGG pathways (Figure 4-6 A). LPS challenge significantly decreased the function of metabolic pathways, microbial metabolism in diverse environments, carbon metabolism, starch and sucrose metabolism, glycolysis/gluconeogenesis, phosphotransferase system (PTS), fructose and mannose metabolism, and galactose metabolism ($P < 0.05$). Pectin supplementation restored the function shifts of intestinal microbiota induced by the LPS challenge. Moreover, we also found that most feature predictions of the altered predicted bio function are related to sugar metabolisms, such as carbon metabolism, starch, and sucrose metabolism, glycolysis/ gluconeogenesis, fructose, and mannose metabolism, and galactose metabolism. Among these shifted functions of the gut microbiota, the carbohydrate metabolism function is responsible for the gut microbial fermentation of carbohydrates under a strictly anaerobic environment to produce SCFAs which benefit the host (Besten et al., 2013). Accordingly, pectin supplementation significantly increased the concentration of acetate compared with that in the LPS group ($P < 0.05$; Figure 4-6 B–H).

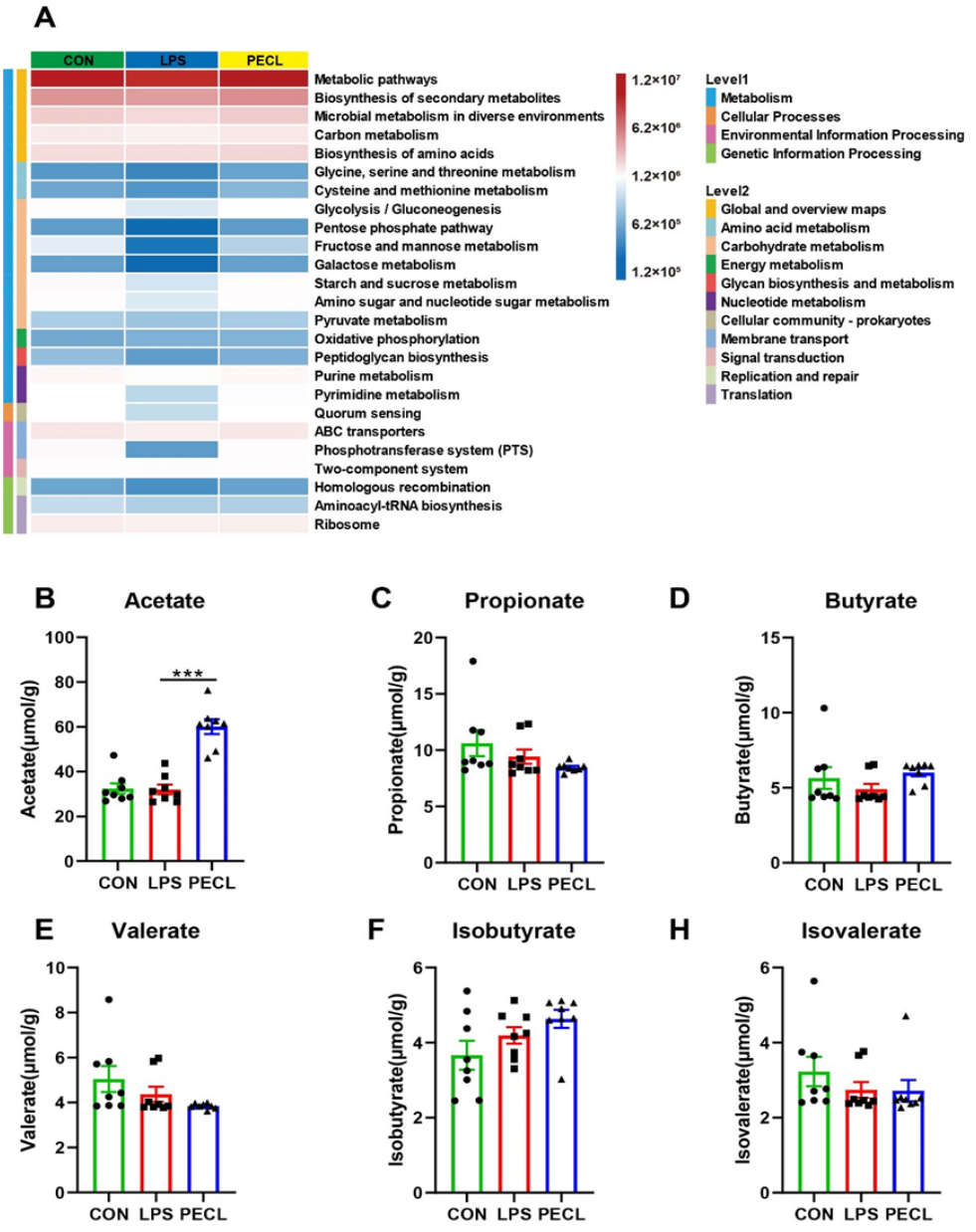

Figure 4-6 Biofunction prediction of the intestinal microbial community using PICRUSt2 at the KEGG pathway(A). Concentrations of SCFAs in the intestinal contents of piglets in different groups. The intestinal contents SCFAs including (B) acetate, (C) propionate, D) butyrate, (E) valerate, (F) isobutyrate, (H) isovalerate. Signification is presented as * P<0.05, ** P<0.01, and *** P<0.001; data are presented as the mean ± SE (n=8).

4.4.8 The correlation between the differential bacteria and the examined indices

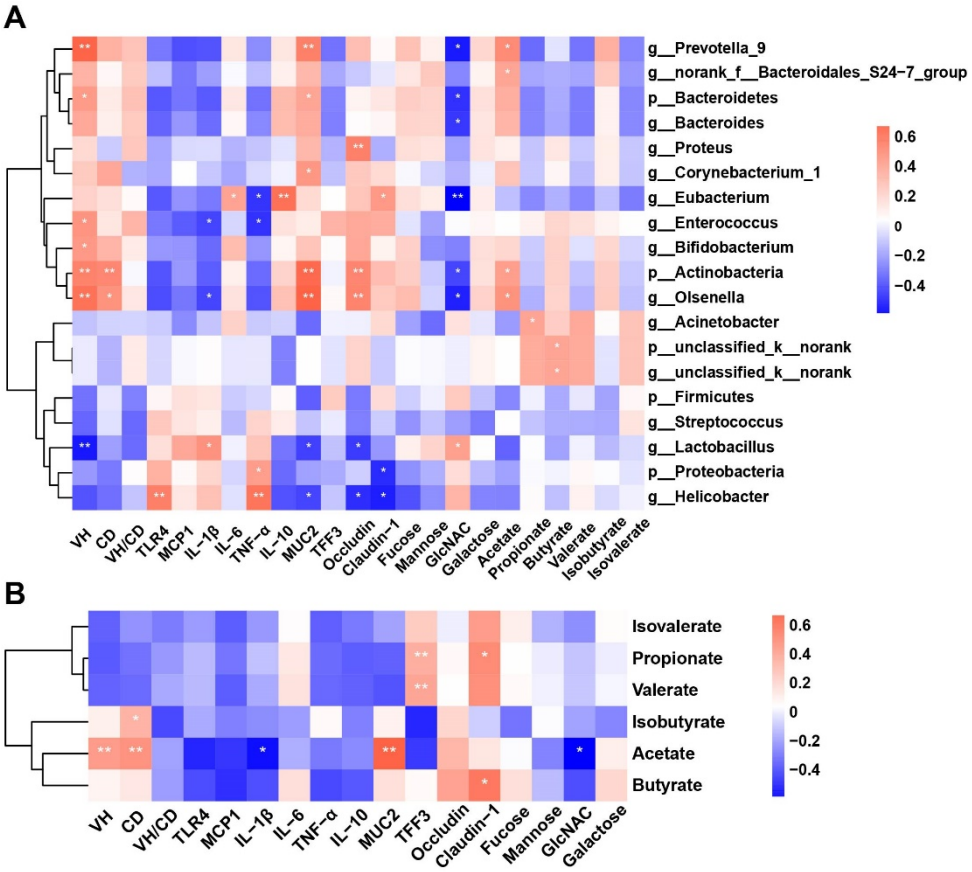

Figure 4 -7 (A) Heatmaps of the Spearman rank correlation coefficient and significant tests between the intestinal microbiota and ileum morphology, inflammatory cytokines, mucus makers, tight junction proteins, glycosylation, microbial metabolite SCFAs. (B) Heatmaps of the Spearman rank correlation coefficient and significant tests between the differential microbial metabolite SCFAs and ileum morphology, inflammatory cytokines, mucus makers, tight junction proteins, glycosylation. The red color represents a positive correlation, while the blue color represents a negative correlation. Signification is presented as * P<0.05, ** P<0.01, and *** P<0.001; data are presented as the mean ± SE (n=8).

Spearman correlations were used to further reveal the potential relationships between intestinal microbiota and ileal parameters including morphology, inflammatory cytokines, mucus makers, tight junction proteins, glycosylation, and microbial metabolite SCFAs (Figure 4-7 A). For ileal morphology, *Bacteroidetes*, *Actinobacteria*, *Olsenella*, *Prevotella_9*, *Acinetobacter*, and *Bifidobacterium* were positively but *Lactobacillus* was negatively correlated with VH (P < 0.05); *Actinobacteria*, *Olsenella* showed a significant negative correlation with CD (P < 0.05). For inflammatory cytokines, *Helicobacter* was positively correlated with

TLR4 ($P < 0.05$); *Lactobacillus* was positively but *Olsenella* and *Enterococcus* were negatively correlated with *IL-1β* ($P < 0.05$); *Eubacterium* was positively correlated with *IL-6* and *IL-10* ($P < 0.05$); *Proteobacteria* and *Helicobacter* were positively but *Enterococcus* and *Eubacterium* were negatively correlated with *TNF-α* ($P < 0.05$); For intestinal mucus, *Actinobacteria*, *Bacteroidetes*, *Olsenella*, *Prevotella_9*, and *Corynebacterium_1* were positively but *Lactobacillus* and *Helicobacter* were negatively correlated with *MUC2* ($P < 0.05$). For tight junction proteins, *Actinobacteria*, *Proteus*, and *Olsenella* were positively but *Lactobacillus* and *Helicobacter* were negatively correlated with Occludin ($P < 0.05$); *Eubacterium* was positively but *Proteobacteria* and *Helicobacter* were negatively correlated with Claudin-1 ($P < 0.05$).

4.5 Discussion

It is well known that the intestine is not only an important organ for nutrient digestion, absorption, and metabolism but also the first intestinal barrier against food-derived pathogens. Thus, maintaining the integrity and functions of intestinal barrier is essential for human and animal health (Hu et al., 2020). The weaning stress of piglets can cause immune system destruction, intestinal microbiome dysbiosis, and intestinal barrier dysfunction, then leading to intestinal injury and poor health, thus reducing the growth performance, diarrhea occurrence, and even death (Campbell et al., 2013;Gresse et al., 2017;Tao et al., 2017;Cui et al., 2020;Sun et al., 2020;Tang et al., 2020). LPS is a component of the outer cell wall of Gram-negative bacteria. Previous studies showed that LPS stimulation could cause immune cells to release a large number of inflammatory cytokines, and the increase of inflammatory cytokines could activate the hypothalamus-pituitaryadrenal axis and inhibit the growth axis, resulting in growth inhibition (Liu et al., 2003;Liu et al., 2008;Zhu et al., 2016;Liu et al., 2021). Therefore, the intraperitoneal injection of LPS is widely used as a model of intestinal injury and a common tool for exploring the effects of dietary regimes (Liu et al., 2008;Liu et al., 2021). Whether LPS challenge can completely simulate the immune stress suffered in a feeding environment is a subject of much debate. However, LPS challenge could cause immune stress and intestinal damage, which allowed a better understanding of the physiology of infection and inflammation during weaning. In the present study, LPS challenge could decrease growth performance, and induce an inflammatory response, as well as lead to intestinal barrier dysfunction, which was consistent with previous studies (Luo et al., 2020;Sun et al., 2020;Xu et al., 2020;Sun et al., 2021c;b). Thus, it is proposed that the LPS challenge model was successfully established. Dietary fiber is an important nutrient that can improve intestinal health (Kohn, 2016;Fischer et al., 2020). In the present study, pectin supplementation significantly decreased the F: G of piglets from d 1–14 compared with the CON and LPS group, which was consistent with previous studies (Chen et al., 2017a). Previous studies found that pectin supplementation could increase the total tract apparent digestion of dry matter, total energy, total carbohydrate, and crude protein, because it can increase the activities of digestive enzymes in the intestine (Chen et al., 2017a;Zhang, 2018). Moreover, pectin supplementation had beneficial impacts on the intestinal health status of piglets (Chen et al., 2017a;Tian et al., 2017;Wu et al., 2020c). Therefore, the improvement of growth performance is associated with

a better intestinal environment and increasing intestinal digestion and absorption. Additionally, pectin supplementation can ameliorate the adverse effects of LPS challenge on growth performance in piglets. Chen et al. reported that pectin supplementation could improve the growth performance of the weaned pigs infected with rotavirus, which causes intestinal inflammation of piglets (Chen et al., 2017a). Moreover, the same results were got in other soluble dietary fibers, such as betaglucan (Luo et al., 2020), inulin (Rodríguez-Sorrento et al., 2020), and xylooligosaccharides (Chen et al., 2021). In short, this result suggested the importance of pectin supplementation during inflammation.

Intestinal morphology is an important indicator of intestinal health, including VH, CD, and VH:CD. It can be used to measure the integrity of the intestinal epithelial barrier, and the ability to digest and absorb nutrients (Zhang et al., 2018b). Numerous researches have shown that LPS challenge can cause various intestinal morphological changes, such as the decrease of VH and the increase of CD (Wang et al., 2019b;Sun et al., 2021b;Xu et al., 2021a), villus shedding, submucosal edema, epithelial vacuolation, and necrosis (Liu et al., 2008;Fan et al., 2019), and we also found intestinal hyperemia. As common indicators for estimating intestinal integrity, the VH, CD, and the VH:CD can reveal some information on gut health and the functional capacity of the intestine in piglets. The present study showed that LPS challenge decreased VH and VH:CD, indicating that LPS challenge negatively affects intestinal integrity and function in piglets. Whereas these adverse effects of LPS challenge were attenuated by pectin supplementation in piglets, which was similar to an earlier finding (Buraczewska et al., 2007b). Tight junctions maintain gut homeostasis by physiologically functioning as the “great wall”against the penetration of luminal bacteria and harmful substances into the mucosa (Lee et al., 2010), and intestinal injury is closely associated with changes in tight junctions(Weight et al., 2015). In this study, Claudin-1 and Occludin were decreased by the LPS challenge and restored by pectin supplementation. These data demonstrated that pectin supplementation improved intestinal epithelial barrier function and alleviated tissue injury.

The mucus layer, the first defensive line of the intestinal barrier, is composed primarily of mucins secreted by goblet cells. The mucus layer not only provides a physical barrier to protect the intestinal epithelium from the intestinal pathogenic bacteria but also provides a habitat and energy source for intestinal symbiotic bacteria (Johansson et al., 2011a;Johansson et al., 2011b). Only by destroying the mucus layer can pathogenic bacteria invade intestinal epithelial cells and trigger an intestinal immune response. Among them, *MUC2* and *TFF3* are important markers for detecting the secretion function of goblet cells. Chen et al. reported that pectin supplementation increased the mRNA expression of *MUC2*, which is beneficial to maintain the intestinal chemical barrier of the piglet and protecting the intestinal tract from the intrusion of harmful pathogens (Chen et al., 2017a). Our previous studies found pectin supplementation could increase the number of PAS/AB-positive goblet cells in the ileum of growing pigs (Zhang, 2018). Consistent with these reports, we found that LPS challenge decreased the mRNA expression of *MUC2* and *TFF3*, while pectin supplementation could restore their expression.

Furthermore, we found that LPS challenge could increase the number of neutral goblet cells in the villus, while pectin supplementation has no significant effect on this compared with the CON group. Based on previous studies and our study, we speculated that pectin supplementation could have a direct interaction with epithelial tissue, fostering the release of mucin from individual goblet cells accompanied by *MUC2* up-regulation (Hino et al., 2013). Besides, several bacterial taxa were positively associated with the mRNA expression of *MUC2* (e.g., *Prevotella_9*, *Bacteroidetes*, *Actinobacteria*, and *Olsenella*). The potential mechanisms of this association may be that some microorganisms can degrade mucus and use it as an energy source. Conversely, they have the capability to alter host glycan expression after invasion (Ottman et al., 2017;White et al., 2021). The results of this study indicated that pectin supplementation could promote intestinal mucus secretion and the capability to defend against harmful bacteria, thus exerting a protective effect on the intestinal epithelial structure.

In addition to serving as a barrier, the mucus serves as an interface between the bacteria and the host: the mucus provides binding sites for not only commensal but also pathogenic bacteria and bacterial energy/food sources. In addition, mucin glycosylation impacts the function of the mucus layer, as well as its ability to control microbe adherence and microbial nutrition at this key microbe-host interface (Allaire et al., 2018). The mucin type O-glycosylation biosynthesis process was shown in Figure 1-3 C. Gut mucus oligosaccharides GlcNAc, Gal, GalNAc, Fuc, and Man are attached to mucin glycoproteins and can be used by bacteria to maintain their relative niches (Engevik et al., 2015). Moreover, unique glycosylated structures in the mucus layer and intestinal epithelial cells have crucial roles in physiological protection and functional and immunological processes, including cell attachment and signal transduction (Wagner et al., 2018). Previous studies reported that the change of glycosylation patterns of intestinal mucins is related to intestinal diseases such as irritable bowel syndrome, idiopathic chronic diarrhea, colorectal cancer (CRC), and inflammatory bowel diseases (IBD), which are accompanied by disturbances in the intestinal microbiota (Reily et al., 2019;Westreich et al., 2019;Crouch et al., 2020;Kudelka et al., 2020). Microbial products (such as LPSs) have been reported to influence the glycosylation of intestinal mucins, because these products may create glycan-altering environments (including the energy level, pH, and cytokine expression). Meanwhile, microbial products could be recognized by pattern recognition receptors, such as TLRs (Qu et al., 2021). As expected, LPS challenge changed intestinal glycosylation, such as increasing GlcNAc and decreasing fucose and galactose, while pectin supplementation restored the shifts, which is consistent with the gene expression of relative transferase. Such a change in orientation might correspond to mucin maturation, which greatly affects the role of mucin in the mucus barrier. For instance, mucin in intestinal epithelial cells is generally fucosylation, which is closely related to colonization of intestinal microorganisms and microbial-host interaction. Blocking the synthesis of fucose in intestinal epithelial cells caused intestinal microbiota imbalance and aggravated the susceptibility of pathogenic bacteria in FUT2 −/ −mice (Chessa et al., 2008). And the increased fucosylation level can inhibit the growth of harmful bacteria and promote the growth of commensal bacteria in mice during disease state (Goto et al., 2014;Pickard et al.,

2014). The abundance of Glc-NAc was negatively correlated with most beneficial bacteria and acetate, which was exactly the opposite of the result for *MUC2*. Previous studies also found that the GlcNAc was associated with *NF-κB* activation and intestinal inflammation (Yang et al., 2008). However, increased GlcNAc levels reverse *NF-κB* activation in macrophages and inhibit inflammatory responses have also been reported (Zou et al., 2009). The discrepancies among them may attribute to a major difference in modes of GlcNAc transferase activation, duration, and site of GlcNAc modulation, type of inflammatory stimulator, cell and animal models adopted, and even the oxygen levels. Moreover, the dynamics of GlcNAc transferase mediated GlcNAc modification are complex and remain incompletely elucidated (Wells et al., 2001;Hart et al., 2010;Liu et al., 2014). Furthermore, we found that pectin supplementation increased core3 while decreased core1,2 and 4 glycans, indicating the shifts of core1,2 and 4 glycans to core3 glycans. Previous studies have shown that the core3 structure promoted mucin stability in the presence of bacterial-derived proteases. This critical function preserves mucus barrier integrity, and prevents unrestricted microbial invasion of the mucosa that would otherwise lead to spontaneous chronic inflammation (Bergstrom et al., 2017). Loss of core3 structure led to decreasing mucin secretion and increasing intestinal permeability (An et al., 2007). These data demonstrated the importance of core3 glycans in intestinal function. Therefore, to a certain extent, the results of this study indicated that pectin could regulate the mucus barrier by changing the structural types of core glycan, thus affecting intestinal health.

Intestinal epithelial cells can provide an immune barrier, which can maintain the delicate balance between tolerance to intestinal commensal bacteria and invasion immunity to intestinal pathogenic bacteria. *TLR4*, which is best known for recognizing LPS, and its downstream signaling molecules are key members of inflammatory signaling pathways, which eventually lead to destructive and systemic inflammatory response syndrome such as fever, shock, and even death (Liu et al., 2012). Research has suggested that pectin supplementation attenuated endotoxin shock via suppression of Toll-like receptor signaling (Ishisono et al., 2017). Similarly, some studies revealed that the upregulated the mRNA expression of *TLR4* can cause the release of related inflammatory factors, including *IL-1β*, *IL-6*, and *TNF-α*, and participate in the immune response against bacterial pathogens (Sabroe et al., 2008;Fukata et al., 2009). Consistent with this, our study found that LPS challenge increased the mRNA expression of *TLR4*, whereas pectin supplementation decreased the mRNA expression levels of *TLR4*. Furthermore, we hypothesized that pectin exerts a beneficial effect on the intestine by reducing the inflammatory response. And we did find that higher pro-inflammatory cytokines *MCP1*, *IL-1β*, and *TNF-α* in the ileum of LPS-challenged piglets, which might be associated with the breakdown of intestinal integrity and epithelial function (Wang et al., 2019b). Adding pectin to the diet alleviated these impacts of LPS and increased the mRNA expression of anti-inflammatory cytokines *IL-10*. Comparing well with our results, Sun et al. reported that pectin supplementation reduced the mRNA expression of *IL-1β* and *IL-6* (Sun et al., 2017a). Our previous studies found pectin supplementation reduced the mRNA expression of *IL-1β* in growing pigs and

increased the mRNA expression of *IL-10* in IPEC-J2 cells (Zhang, 2018). Pectin probably exerts a direct regulatory effect on intestinal inflammation. Therefore, pectin supplementation was effective in alleviating intestinal inflammation in weaned piglets.

The gut microbiota of mammals has numerous roles benefiting the host, such as digestion and fermentation of carbohydrates, maintenance of intestinal barrier functions, increasing the expression of tight junctions, regulating mucin biosynthesis and catabolism, regulating the immune responses, and protection from pathogenic bacteria (Ashida et al., 2011;Gresse et al., 2017). Several epidemiological and experimental studies have shown that the health benefits of dietary fibers were related to the composition and function of the intestinal microbiota (Riva et al., 2019;Fischer et al., 2020). Thus, we speculated that pectin supplementation might affect the microbiota composition in the ileum of LPS-challenged piglets. As expected, our data showed that LPS challenge profoundly shifted the structure of gut microbiota, and reduced the OUT number and alpha diversity. Pectin supplementation restored these LPS-induced shifts of intestinal microbiota, including the structure of the intestinal microbiota and alpha diversity. In detail, we found that fewer *Bacteroidetes* and *Actinobacteria* abundance in the ileum of LPS-challenged piglets, whereas pectin supplementation had the reverse effects. Although they represent only a small percentage, however, they are pivotal in the maintenance of gut homeostasis. As a main contributor of SCFAs, *Bacteroidetes* promote the balance of intestinal microbiota and play an important role in many major metabolic activities involving fermentation of carbohydrates, utilization of nitrogenous substances, and prevention of pathogen colonization (Xu et al., 2012;Zhang et al., 2016). A higher abundance of *Bacteroidetes* in the ileum suggested that the pectin supplementation might change bacterial fermentation. *Actinobacteria* have been supposed to the modulation of gut permeability, immune system, metabolism, and the gut-brain axis. An unbalanced abundance has been evidenced in several pathological conditions as a recent review indicated (Binda et al., 2018). Moreover, within the *Proteobacteria* phylum, there exist numerous potential opportunistic pathogens such as *Escherichia*, *Salmonella*, *Campylobacter*, and *Helicobacter*, and the elevation in their abundance could be regarded as a possible indicator of intestinal disorders (Shin et al., 2015a;Hughes et al., 2017b), and this is also evidenced by a correlation between *TNF-α* and Claudin-1 and *Proteobacteria* in this study. Our study also found that *Proteobacteria* abundance was observed to increase in LPS-challenged piglets, while pectin supplementation decreased Proteobacteria abundance, consistent with an earlier study by Wu et al. (Wu et al., 2020c). Therefore, we speculated that this might be a mechanism of pectin regulating intestinal health by regulating the abundance of *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* to improve intestinal barrier dysfunction and inhibit intestinal endotoxin-mediated inflammation.

LPS or pectin also alters microbiota composition at the genus level. We found the boom of Helicobacter after LPS challenge, while pectin supplementation decreased it. *Helicobacter* belongs to the phylum *Proteobacteria*, and harmful effects on the intestine were mentioned. Specifically, previous studies showed that *Helicobacte*r could contribute to inflammatory bowel disease (IBD) by inducing alterations in intestinal permeability or by causing immunological derangements resulting in

absorption of antigenic material and autoimmunity via various immunological pathways (Papamichael et al., 2014;Castaño-Rodríguez et al., 2017;Wu et al., 2020c). Our study also found that a decrease in the abundance of Helicobacter organisms in intestinal mucosa was accompanied by the exacerbation of intestinal inflammation and damage to the intestinal barrier. In addition, *Olsenella*, *Bacteroides*, *Proteus*, *Prevotella_9*, and *Eubacterium* abundance were observed as the major up-regulated microbes by pectin. *Olsenella* and *Prevotella_9* are generally considered beneficial to intestinal function (Kong et al., 2019), and *Prevotella* is one of the predominant fiber fermenters and SCFAs producers in the intestinal tracts of pigs (Zhou et al., 2016). In this study, pectin supplementation significantly increased the abundance of *Olsenella* and *Prevotella_9*, which may help to provide energy for intestinal cells and protect the intestinal barrier. Another study showed that the long-term diet was strongly associated with the gut microbial composition, that is, those who ate plenty of carbohydrates, particularly dietary fiber, had *Prevotella* as the dominant species (Wu et al., 2011;Zhou et al., 2016), which is similar to our findings. Moreover, *Bacteroides* can break down complex plant polysaccharides (Xu et al., 2003) and the proportion of *Bacteroides* in the intestinal microbiota is dependent on the intestinal inflammatory status (Steimle et al., 2019). In ulcerative colitis patients, the proportion of *Bacteroides* is markedly decreased (Kumari et al., 2013), indicating *Bacteroides* were important beneficial players in the intestinal microbiota. Similar alterations were also found in other studies (Chung et al., 2017). *Proteus* is the most common opportunistic pathogen (O'Hara et al., 2000), which has been significantly increased in the PECL group. In addition, correlation analysis showed that *Proteus* had a positive correlation with the expression of Occludin, indicating that pectin supplementation is potentially beneficial to the intestinal barrier. Previous studies have already suggested that *Eubacterium* was a specialist pectin degrading *Firmicutes* species that has the potential function to deliver anti-inflammatory activity by promoting the production of *IL-10* by epithelial cells (Chung et al., 2017). Our results were consistent with this founding. In the present study, the abundance of *Eubacterium* in the ileum of piglets was increased after the addition of pectin, and *Eubacterium* had a positive correlation with the production of *IL-10*. Thus, pectin supplementation may play an important role in reducing intestinal inflammation and injury to maintain intestinal health by modulating intestinal microbiota composition at different taxonomic levels, such as increasing the concentration of anti-inflammatory bacteria and SCFAs-producing bacteria.

SCFAs, a group of saturated aliphatic organic acids that are comprised of no more than six hydrocarbons, closely related to the composition of intestinal microbiota, are produced through fermentation of polysaccharides by anaerobic intestinal microbiota (Xu et al., 2013;Zhang et al., 2019;Wu et al., 2021b). Growing evidence has demonstrated that SCFAs regulate immune response and could potentially function as therapeutic agents to prevent various inflammatory diseases (Kobayashi et al., 2017). SCFAs also play a crucial role as energy sources for intestinal epithelial cells, maintaining intestinal cell function and serving as an important link connecting intestinal cell metabolism, microbiota, and gene expression regulation

(Trompette et al., 2014;Kasubuchi et al., 2015;Koh et al., 2016). Zacharias et al. reported that pectin supplementation increased the digestibility of pectin and the concentration of total SCFAs and acetate, and speculated that pectin might be beneficial to the development of fermentative processes (Zacharias et al., 2004). Similarly, fibers containing uronic acids such as pectin induced the production of acetate by inoculating pig fecal in vitro (Melliana et al., 2012). These are consistent with the present study, where pectin supplementation increased the concentrations of acetate in the ileum, presumably due to the increase in the abundance of certain acetate-producing bacteria, including *Bacteroidetes* and *Prevotella_9* (Stevenson et al., 2007;Besten et al., 2013). In particular, they produce a high concentration of acetate that can protect the host from enteropathogenic infections. This may help improve intestinal integrity and reduce pro-inflammatory cytokines expression. Correlation analysis and significance test further demonstrated that acetate contributed significantly to improving intestinal injury caused by LPS. Consistently, earlier research reported that acetate can reduce LPS-induced *TNF-α* release from human blood-derived neutrophils, and also inhibit *TNF-α* mediated activation of the downstream inflammatory signaling pathways in the human colon adenocarcinoma cell line (Tedelind et al., 2007). It could be seen that the effect of pectin on the intestinal microbiota of piglets was closely related to the changes in SCFAs composition, which was beneficial to the improvement of intestinal function and growth performance of the host by improving the composition of intestinal microbiota and its metabolites.

To better comprehend the functional roles of the intestinal microbiota, we used PICRUST2 to investigate the functional profiles. Carbohydrate metabolism was the primary function of piglets in each group, which might be because the feed components and substrate types fermented by dominant microorganisms were carbohydrates (Sun et al., 2021b). LPS challenge significantly decreased the function of metabolic pathways, microbial metabolism in diverse environments, carbon metabolism, starch, and sucrose metabolism, glycolysis/gluconeogenesis, phosphotransferase system (PTS), fructose and mannose metabolism, and galactose metabolism, while pectin supplementation restored the function shifts. Therefore, pectin supplementation may help to regulate the abnormal function of intestinal microbiota caused by stress and thus maintain homeostasis of the intestinal tract during weaning. Moreover, we also found that most changes in predicted bio function caused by LPS or pectin are related to sugars metabolisms, such as carbon metabolism, starch, and sucrose metabolism, glycolysis/gluconeogenesis, fructose and mannose metabolism, and galactose metabolism. The altered sugars metabolism-related functional pathway provides the possibility for pectin to regulate intestinal glycosylation through microbiota, because intestinal microbiota is closely related to intestinal glycosylation (Bergstrom et al., 2020;Kudelka et al., 2020;Qu et al., 2021), and this is consistent with the intestinal glycosylation results.

From correlation analysis, we could conclude that LPS challenge caused intestinal microbiota disorder, altered mucus glycosylation, and permeability, and upregulation of pro-inflammatory cytokine expression, which triggers the impaired intestinal barriers, intestinal inflammation, and damaged intestinal morphology, ultimately reducing the growth performance of piglets. This conclusion was in line with the previous study (Sun et al., 2021b), and the process could be reversed by

the addition of pectin. Specifically, pectin supplementation could regulate the intestinal microbiota structure, increase the relative abundance of acetate-producing bacteria and the content of acetate, improve mucus glycosylation and permeability, then reduce the expression of intestinal pro-inflammatory cytokines, thus improving intestinal morphology, maintaining intestinal barrier function, alleviating the inflammatory reaction, and eventually improving intestinal health and growth performance of piglets.

In summary, our results revealed that pectin supplementation had beneficial impacts on the improvement of intestinal integrity, intestinal injury, and ultimately growth performance of piglets. We speculated that the improvement of growth performance of piglets challenged by LPS may be attributed to the beneficial effects of pectin supplementation on intestinal microbiota and SCFAs, thus improving intestinal barrier function, reducing the inflammatory response, and enhancing body health. These findings provide a new perspective for the development of pectin as a functional food ingredient and strong support for regulating the intestinal health and growth performance of piglets via dietary pectin. Our data also provide insights for studying the role of pectin in regulating human infant intestinal health.

Table 4-1 The composition and nutrient content of basal diets (air-dry basis) %

Ingredients	**CON / LPS**	**PECL**
Expanded corn	46.73	46.73
Expanded soybean	10.00	10.00
Soybean meal	13.00	13.00
Fish meal	4.50	4.50
Dried whey	10.00	10.00
Soybean oil	2.00	2.00
$CaHPO_4$	0.50	0.50
NaCl	0.30	0.30
Limestone	0.51	0.51
Choline chloride	0.09	0.09
Lysine	0.80	0.80
Met	0.17	0.17
Thr	0.32	0.32
Trp	0.08	0.08
Sugar	2.50	2.50
Glucose	2.50	2.50
Premix[1)]	1.00	1.00
Cellulose	5.00	
Pectin		5.00
Nutrient levels[2)]		
GE Mcal/kg	3.94	4.01
Crude protein	18.01	17.90
Ca	0.67	0.67
TP	0.54	0.54
AP	0.39	0.39
Lys	1.51	1.51
Met	0.44	0.44
Met+Cys	0.68	0.68
Thr	0.91	0.91
Trp	0.26	0.26

1) The premix provides per kg of diet: VA: 18 000 IU; VD3: 4 500 IU; VE: 22.5 mg; VK3: 4.5 mg; VB1: 4.32 mg; VB2: 12 mg; VB6: 4.86 mg; VB12: 30 μg; Biotin: 480 μg; Folic acid: 1.764 mg; Calcium Pantothenate: 33.12 mg; Nicotinamide: 41.58 mg; Cu: 20 mg; Fe: 140 mg; Zn: 140 mg; Mn: 40 mg; Se: 0.3 mg; I: 0.5 mg.
2) GE and Crude protein are measured values, while the other nutrient levels are calculated values

Table 4-2 Growth performance of piglets in different groups

Parameter and period (days)	**Piglet group**			***P-Value***
	CON	**LPS**	**PECL**	
Body Weight（BW）				
Initial BW (kg)	6.73±0.38	6.93±0.96	6.60±0.69	0.76
d14 BW (kg)	10.06±0.41	10.42±0.32	10.38±0.41	0.77
d21 BW (kg)	11.57±0.45	11.24±0.33	12.07±0.35	0.32
Day 1 to14				
ADFI (g)	378.88±11.98	402.75±15.51	390.13±42.76	0.83
ADG (g)	238.12±6.66	248.93±8.25	269.64±26.36	0.40
F:G	1.60±0.06^{a}	1.62±0.04^{a}	1.43±0.03^{b}	<0.05
Day 15 to 21				
ADFI (g)	350.11±17.86^{B}	199.02±16.06^{C}	482.79±43.40^{A}	<0.01
ADG (g)	215.71±16.47^{A}	116.43±9.26^{B}	241.07±17.05^{A}	<0.01
F:G	1.64±0.07^{b}	1.73±0.09^{ab}	2.01±0.13^{a}	<0.05

Values are means ± SE. In the same row, values with the same or no letter superscripts mean no significant difference (P>0.05), while with different small letter superscripts mean significant difference (P<0.05), and with different capital letters superscripts mean significant difference (P<0.01). ADFI (average daily feed intake), ADG (average daily gain), F:G = Average Daily Feed Intake (F) / Average Daily Gain (G)

Chapter V

Pectin supplement alleviates gut injury potentially through improving gut microbiota community in piglets

Chapter V. Pectin supplement alleviates gut injury potentially through improving gut microbiota community in piglets

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5.1 Abstract

As pectin is widely used as a food and feed additive due to its tremendous prebiotic potentials for gut health. Yet, the underlying mechanisms associated with its protective effect remain unclear. Twenty-four piglets (Yorkshire × Landrace, 6.77 ± 0.92 kg, 21d of age) were randomly divided into three groups with eight replicates per treatment: (1) Control group (CON), (2) Lipopolysaccharidechallenged group (LPS), (3) Pectin-LPS group (PECL). Piglets were administrated with LPS or saline on d14 and 21 of the experiment. Piglets in each group were fed with corn-soybean meal diets containing 5% citrus pectin or 5% microcrystalline cellulose. Our result showed that pectin alleviated the morphological damage features by restoring the goblet numbers which the pig induced by LPS in the cecum. Besides, compared with the LPS group, pectin supplementation elevated the mRNA expression of tight junction protein [*Claudin-1*, *Claudin-4*, and *zonula occludens-1* (*ZO-1*)], mucin (*Muc-2*), and anti-inflammatory cytokines [interleukin 10 (*IL-10*), and *IL-22*]. Whereas pectin downregulated the expression of proinflammatory cytokines (*IL-1β*, *IL-6*, *IL-18*), tumor necrosis factor-&alpha (*TNF-α*), and *NF-κB*. What is more, pectin supplementation also significantly increased the abundance of beneficial bacteria (*Lactobacillus*, *Clostridium_sensu_stricto_1*, *Blautia*, and *Subdoligranulum*), and significantly reduced the abundance of harmful bacteria, such as *Streptococcus*. Additionally, pectin restored the amount of short-chain fatty acids (SCFAs) after being decreased by LPS (mainly Acetic acid, Propionic acid, and Butyric acid) to alleviate gut injury and improve gut immunity via activating relative receptors (*GPR43*, *GPR109*, *AhR*). Mantel test and correlation analysis also revealed associations between intestinal microbiota and intestinal morphology, and intestinal inflammation in piglets. Taken together, dietary pectin supplementation enhances the gut barrier and improves immunity to ameliorate LPS-induced injury by optimizing gut microbiota and their metabolites.

5.2 Introduction

The weaning transition is a critical period for mammalian growth (Holman et al., 2021;Gonzalez-Sole et al., 2022). Due to the inadequate development of immunological and digestive systems (Hughes et al., 2017a), they are more susceptible to viruses and harmful bacteria, which can damage intestinal function and cause debilitating diarrhea. However, gut microbial dysbiosis is a major cause of neonatal and post-weaning diarrhea in pigs (Hughes et al., 2017a). Trillions of microorganisms are colonized in the mammalian gut (Lynch et al., 2016). The intestinal microbiota is a mutually beneficial relationship between the host and the intestinal microbiota that regulates the body's food intake and metabolism, serves as a defense against toxins and external antigens, and fosters the growth of the intestinal immune system (Reyman et al., 2019). Very recently, the effect of intestinal microbiota on the host might be mediated by influences on the microbial metabolites (Wan et al., 2021). Acetic acid, propionic acid, and butyric acid are the three of the most representative short-chain fatty acids (SCFAs). The acetic acid in the intestine is mainly produced by the fermentation of *Bifidobacterium spp*. and *Lactobacillus spp*. (Zuniga et al., 2018). It constitutes the highest proportion of short-chain fatty acids produced by intestinal bacterias. Acetic acid regulates the

pH level, maintains the homeostasis of the intestinal environment, nourishes beneficial microorganisms, and prevents the invasion of harmful bacteria and opportunistic pathogens (Konda et al., 2020). *Escherichia coli*, *Mycobacterium fragilis* and *Microcystis aeruginosa* are the main propionic acid-producing bacterias. In addition to their functional similarities with acetic acid, propionic acid can also regulate appetite through *PYY* and *GLP-1* (Canfora et al., 2019). Butyric acid is absorbed directly into the colonic epithelium, where it is oxidized to produce butyryl coenzyme and used in the synthesis of ATP. It also has the vital function of maintaining the integrity of the intestinal wall (Dang et al., 2021).

Dietary fiber (DF) is a carbohydrate polymer with more than 10 monomeric units, making it difficult to be hydrolyzed and absorbed by endogenous enzymes in the small intestine (Nevara et al., 2021). According to its solubility, DF is usually divided into two categories: soluble dietary fiber (SDF, e.g., pectin) and insoluble dietary fiber (IDF, e.g., cellulose;) (Xia et al., 2021). There are many ways for pectin to regulate the host, such as regulating the composition of intestinal microbes, regulating intestinal permeability, and reducing intestinal inflammation. Beyond that, the effect of microbial metabolites (short-chain fatty acids) is a non-negligible regulatory pathway. Pectin is mainly a group of acids heteropolysaccharides consisting of *D*-galacturonic Acids (D-Gal-A) linked by α-1,4-glycosidic bonds (Wu et al., 2020b). Besides, it also contains neutral sugars, such as *L*-rhamnose, *D*-galactose, and *D*-arabinose. And it is abundant in the peels of citrus, lemon, and grapefruit.

In recent years, the application of microbiology in the analysis of dietary fibers on the intestinal immune factors has extensively increased, but biological meaningful pathways, for instance, the regulatory and metabolic pathways, are still poorly understood. Thus, in this study, lipopolysaccharide (LPS) intraperitoneal injection was used to establish the intestinal injury model of piglets, and the aim of this research was to explore whether pectin supplementation in the diet could alleviate intestinal injury via gut microbiota community in piglets.

5.3 Materials and methods

5.3.1 Animal management, experimental design, and sample collection

All procedures in this study received ethical approval from the Experimental Animal Welfare and Ethical Committee of Institute of Animal Science of Chinese Academy of Agricultural Sciences (IAS2019-37). A total of 24 21-day-old pigs (Yorkshire × Landrace, 6.77 ± 0.92 kg), half male and female piglets in each group, were randomly distributed into three groups with eight replicates per treatment: (1) Control group (CON), (2) LPS-challenged group (LPS), (3) Pectin-LPS group (PECL). The initial body weight and health status of the piglets were no significant difference among the groups in this study. Each group of piglets was fed with corn-soybean meal diets containing 5% citrus pectin (PEC, [with a purity of >81.4%] purchased from Yuzhong Biotech Corporation, Henan, China) or microcrystalline cellulose (MCC [99.5% purity], purchased from Engineering research center of cellulose and its derivatives, Beijing, China) respectively.

The experiment lasted for 28 days which consisted of a pre-starter period (7 days) and a starter period (21 days). During the whole test period, the diets had been formulated to meet the nutritional requirements suggested by NRC (2012) for pigs within the corresponding weight range (Table 4-1). On day 14 and day 21, piglets from LPS group and PECL group received a simulated bacterial challenge by an intraperitoneal injection of LPS solution (80 μg per kg BW, *E. coli* 0111: B4, Sigma). And meanwhile, piglets in the CON group received an intraperitoneal injection of 200 μl of saline. Previous studies proved that LPS causes serious damage to the intestinal structure and barrier function within 2 to 6 h after injection (Xu et al., 2020). Hence, 3 h after the 2nd injection of LPS or Saline, blood samples were collected from the anterior vena cava before being anaesthetized. After slaughtering, samples of cecal contents and mucosa were obtained and immediately frozen in liquid nitrogen and then transferred to −80°C for further analysis.

5.3.2 Counting the number of goblet cells

The cecum segment was fixed in paraformaldehyde, dehydrated, and embedded in paraffin to prepare paraffin sections (section thickness of 5um), and then stained with PAS solution. The number of goblet cells was assessed by random measurement of 10 crypts per section using DS-U3 (Nikon, Japan). For more specific details about this process, we refer to (Tang et al., 2022a).

5.3.3 DNA extraction, real-time PCR analysis

We extract the total RNA from cecal mucosa using the RNeasy Mini Kit (GeneBetter, Beijing, China). The concentration of each RNA sample was quantified using the NanoDrop 2000 (Nanodrop Technologies, Wilmington, DE, USA). The cDNA was transcribed by using the High-Capacity cDNA Archive kit (Takara, Takara Biomedical Technology in Beijing, China). qRT-PCR was conducted with a commercial kit (PerfectStart Green Qpcr SuperMix, Transgen in Beijing, China). The mRNA level of *β-actin* was used as an internal control. The relative genes expression of mRNA of tight junction protein (Claudin-1, *Claudin-4*, *ZO-1*), mucin (*Muc-2*) inflammatory cytokine genes (*IL-1β*, *IL-6*, *IL-8*, *IL-18*, *TNF-α*, *NF-κB*, *IL-10*, and *IL-22*), and immune-related receptors (*GPR41*, *GPR43*, *GPR109*, *AHR*) was detected by qRT-PCR. A single peak was checked to confirm the specificity of each primer (Table 5-1) set in the melting curves after 40 PCR amplification cycles. (Primer efficiency was checked by using different cDNA concentrations and only primer with mathematical efficiency between 90 and 110% were used). Relative expression of each primer between the control group and treatment group was calculated by $2^{-\triangle\triangle Ct}$ method, and the values were normalized to the reference house-keeping genes *β-actin*.

5.3.4 16S rRNA gene sequencing

Total bacterial DNA was extracted from the intestinal chyme and mucosa using the EZNATM Soil DNA kit (D5625-02, Omega Bio- Tek Inc., Norcross, GA, USA) according to the instructions of the manufacturer. The V3-V4 hypervariable regions of the bacterial 16S rDNA were amplified by a two-step PCR method using primers 338F (5′-ACTCCTRCGGGAGGCAGCAG-3′) and 806R (5′-GGACTACCVGGGTATCTAAT-3′) with unique 8-bp barcodes to facilitate multiplexing, and sequencing was carried out with an Illumina sequencing platform

using Miseq PE300 (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by fastp version 0.20.0 (Chen et al., 2018b), and merged by FLASH version 1.2.7 (Magoc and Salzberg, 2011). Operational taxonomic units (OTUs) with 97% similarity cutoff (Edgar, 2013) were clustered using UPARSE version 7.1, and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier version 2.2 (Wang et al., 2007) against the 16S rRNA database using a confidence threshold of 70% (PRJNA889391).

5.3.5 Western blot

Tissue total protein was extracted in a RIPA lysis buffer on ice. Protein content was quantified using the BCA protein assay kit (Cat# 23225, Thermo, Waltham, MA, USA). A total of 30 μg protein was loaded per lane and boiled for 15 min before separation by 10% SDS-PAGE gel. The SDS-PAGE gel result was transferred onto a polyvinylidene difluoride membrane under 90 V for 1.5 h using the wet transfer method. Then the membranes were incubated in 5% skimmed milk for 2 h at room temperature for blotting. After incubation with a primary antibody of Occlaudin (Thermo Fisher Scientific Inc., MA, USA, #40–4,700, 1:500)), Claudin-1 (Thermo Fisher Scientific Inc., MA, USA, #51–9,000, 1:500), and β-actin (Proteintech, Chicago, USA, #20536-1-AP, 1:1,000) overnight at 4°C, the membrane was washed with TBST buffer and incubated with the HRP-labeled goat anti-rabbit/mouse second antibody (Abcam, Cambridge, UK, 10#ab6721, 1:5,000) for 40 min at room temperature. Protein blots were visualized using SuperSignal® West Femto Maximum Sensitivity Substrate (Cat # 34094, Thermo) and a gel imaging system (Tanon Science & Technology Co., Ltd., China). Band density was quantified by the Image J 10.0 software and normalized to β-Actin.

5.3.6 Metabolite determination of gut microbiota

Quantification of Cecum Short-Chain Fatty Acids (SCFAs) in pig cecal contents were measured as described in our previous report (Tang et al., 2021a). Briefly, cecal contents (200.0 mg) were thoroughly mixed with ultrapure water at a ratio of 1:9, then shocked for 30 min to mix evenly, and then incubated at 4°C overnight and then centrifuged at 10000 g for 10 min. After this, 0.9 milliliters of the supernatant were mixed with 0.1 ml of metaphosphoric acid (25% (v/v)) and kept for 3 h. The sample was then cleared by centrifugation at 10,000 g for 10 min and passed through a 0.45-μm Milled-LG filter (Jinteng, Tianjin, China) and subjected to SCFA analysis.

5.3.7 Data analysis and statistical test

Data on cytokines, bacterial α-diversity indices (Chao1, and Shannon), microbial metabolites (SCFAs), and gene expression were analyzed by the Tukey–Kramer test and the Duncan multiple comparison method (JMP version 10.0, SAS Institute, Inc., Cary, NC, USA). Significance is presented as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. In addition, the correlation analysis was performed using the Mental test and Spearman's correlation (R package "pheatmap").

5.4 Results

5.4.1 Pectin supplementation affected cecal permeability and histomorphology

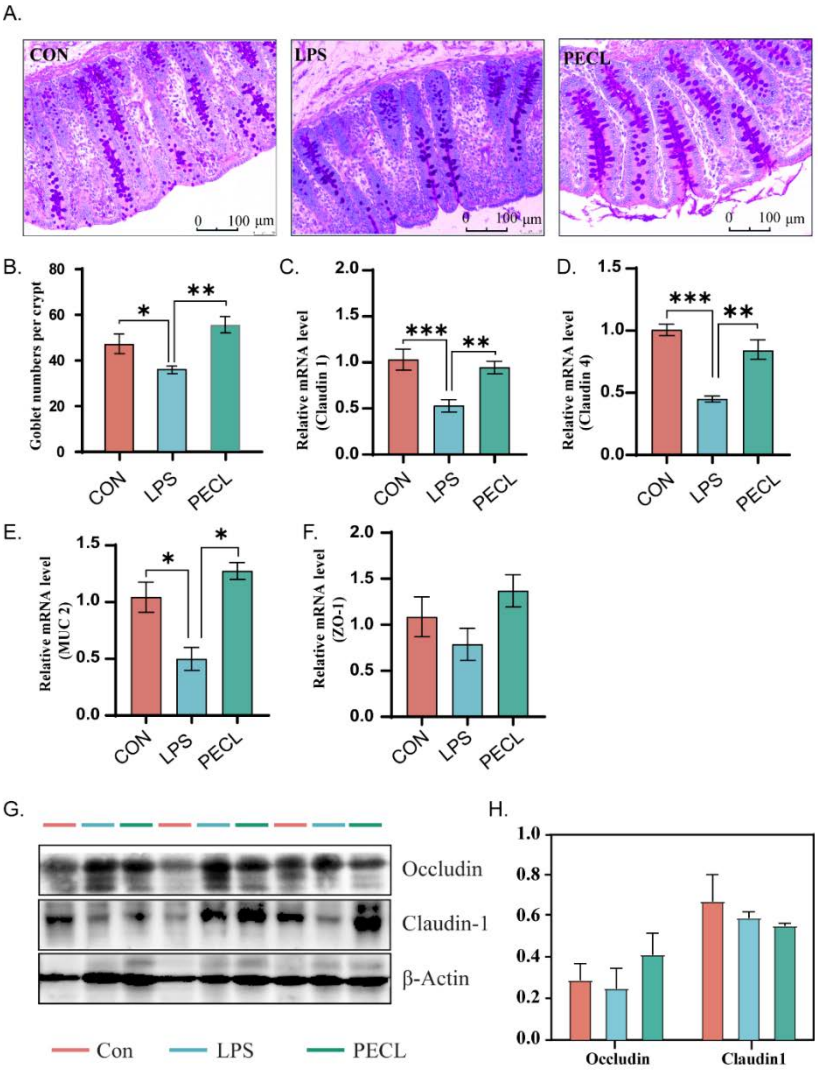

Figure 5-1 Effects of dietary supplemented with pectin on gut morphology of LPS challenged piglets. (A) Representative PAS-stained cecum sections. (B) Goblet number. The fold change in mRNA expression relative to β-actin for (C) *Claudin-1*, (D) *Claudin-4*, (E) *ZO-1*, (F) *Muc-2* is shown. And (G,H) Western blot analysis of *Occludin* and *Claudin1*. Signification is presented as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$; data are presented as the mean ± SE (mRNA, $n = 8$; WB, $n = 3$).

The piglets challenged with LPS showed signs of diarrhea, fever, and cough. To observe morphological and histological changes in the cecum, we performed PAS staining. It was apparent from Figure 5-1 that LPS decreased the number of goblet cells compared with that in the CON group ($P < 0.05$). Addition of pectin significantly increased the number of goblet numbers when compared with the LPS group (Figures 1A, B; $P < 0.01$). Furthermore, LPS significantly decreased the mRNA expression levels of tight junction protein [*Claudin 1*, Figure 5-1C, ($P < 0.001$), *Claudin 4*, Figure 5-1D, ($P < 0.001$)], pectin supplementation notably increased the mRNA levels of *Claudin 1* ($P < 0.01$), *Claudin 4* ($P < 0.01$), compared with LPS group (Figure 5-1C, D). Similarly, piglets challenged with LPS significantly reduced the mRNA expression of *Muc2*, and it was restored after the addition of pectin in PEC group ($P < 0.05$). Although WB data did not reach the significant levels (Figures 1G, H). Accordingly, pectin supplementation markedly restored the intestine damage in piglets due to LPS stress.

5.4.2 Pectin modified inflammatory cytokine gene expression in the cecum

As shown in Figure 5-2, piglets in the LPS group had higher expression levels of *IL-1β* ($P < 0.01$), *IL-6* ($P < 0.01$), *IL-18* ($P < 0.01$), *TNF-α* ($P < 0.001$), and *NF-κB* (Figures 2A, B, D, E), and lower expression levels of *IL-10* ($P < 0.001$), *IL-22* ($P < 0.001$; Figures 2G, H) than piglets in CON group. After adding pectin, the mRNA expression levels of pro-inflammatory was significantly reduced, including *IL-1β* (Figure 5-2A; $P < 0.01$), *IL-6* (Figure 5-2B; $P < 0.01$), *IL-18* (Figure 5-2D; $P < 0.001$), *TNF-α* (Figure 5-2E; $P < 0.001$), and *NF-κB* (Figure 5-2F; $P < 0.001$), and restored the levels of the anti-inflammatory *IL-10* (Figure 5-2G; $P < 0.001$) and *IL-22* (Figure 5-2H; $P < 0.01$).

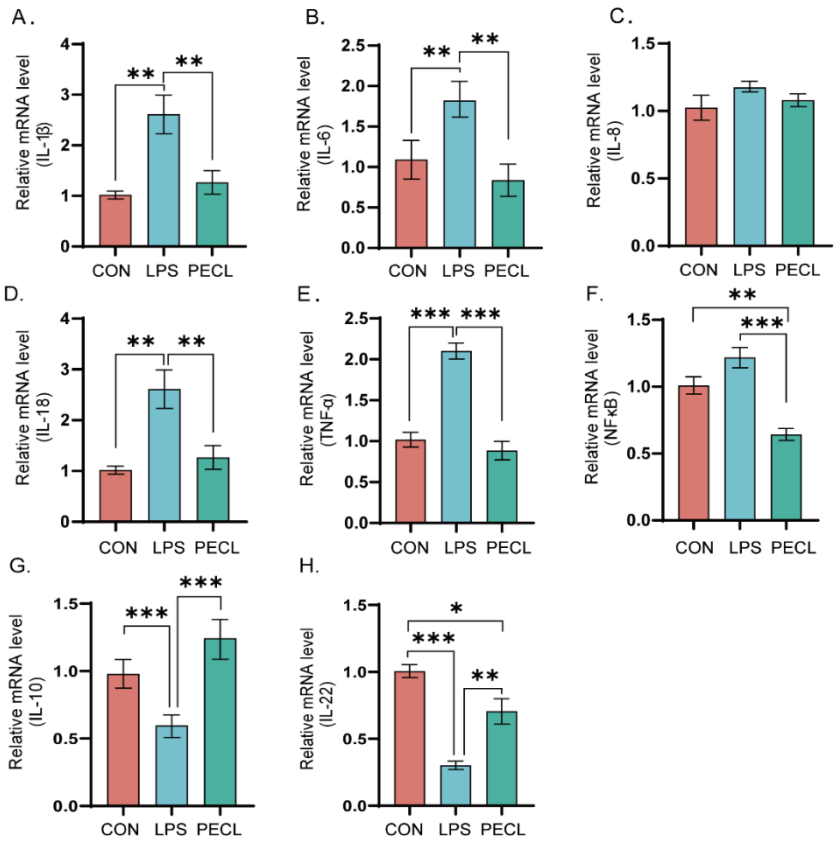

Figure 5-2 Effects of dietary pectin on inflammatory cytokines in cecum. The fold change in mRNA expression relative to β-actin for (A) *IL-1β*, (B) *IL-6*, (C) *IL-8*, (D), *IL-18*, (E) *TNF-α,* (F), *NF-κb*, (G) *IL-10*, (H) *IL-22* is shown. Signification is presented as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$; data are presented as the mean ± SE ($n = 8$).

5.4.3 Pectin supplementation affected cecal intestinal microbiota

We profiled the composition and structure of the microbial communities in both

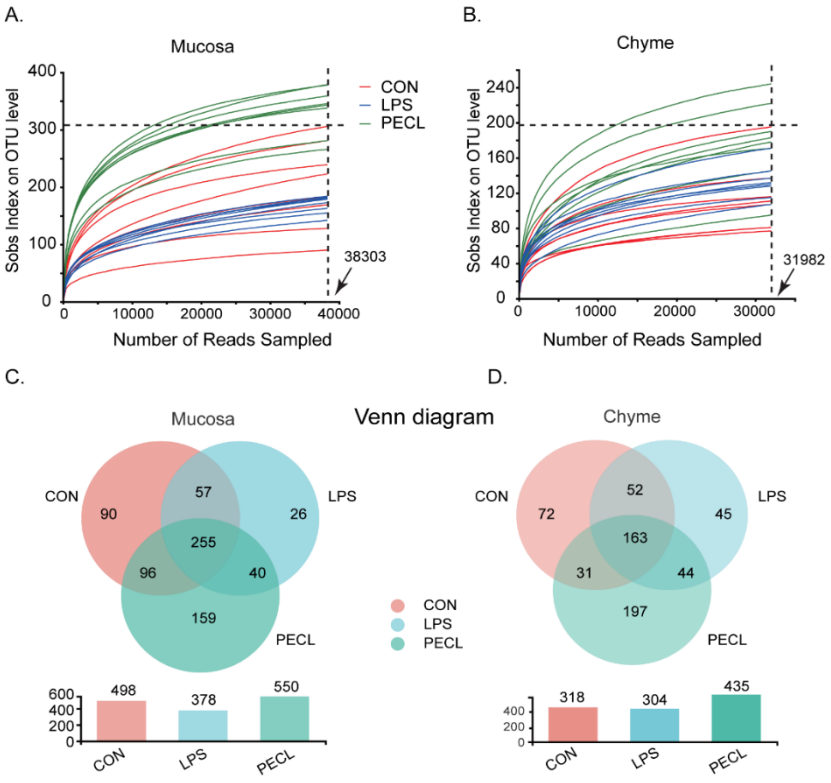

Figure 5-3 Effects of pectin on Gut Microbiota diversity. (A, B) The rarefaction curves of sobs index on OUT levels of mucosa and chyme in piglets. (C, D) Venn diagrams showing the overlap of the OTUs identified in intestinal chyme microbiota and mucosa. Signification is presented as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$; data are presented as the mean ± SE ($n = 8$).

mucosa and chyme of cecum using 16S rRNA amplicon sequencing. Rarefaction curves approached asymptotes across all samples, implying that the sequencing depth was sufficient to cover almost all microbes in the samples (Figures 3A, B). The Venn diagram in the cecal mucosa showed that piglets in the CON, LPS, and PECL groups contained 255 same OTUs and 90, 26, and 159 unique OTUs, respectively (Figure 5-3C). When it comes to cecal chyme, there were 163 common OTUs between these three groups. Meantime, the CON, LPS, and PECL groups contained individual 72, 45, and 197 OTUs, respectively (Figure 5-3D).

And after quality-filtered and merged, 723 OTUs in mucosa and 704 OTUs in chyme were obtained, respectively. In mucosa, the α-diversity was significantly higher in PECL than in CON and LPS group, while without any difference between

CON and LPS group (Figures 4A–D). Similarly, the α-diversity of chyme in PECL was higher than that in other two groups (Figures 4B–D). As for beta diversity result clearly showed dissimilarity among the communities in mucosal and chyme bacteria (Figures 4E, F).

As for the microbial composition in mucosa between these three groups. At the

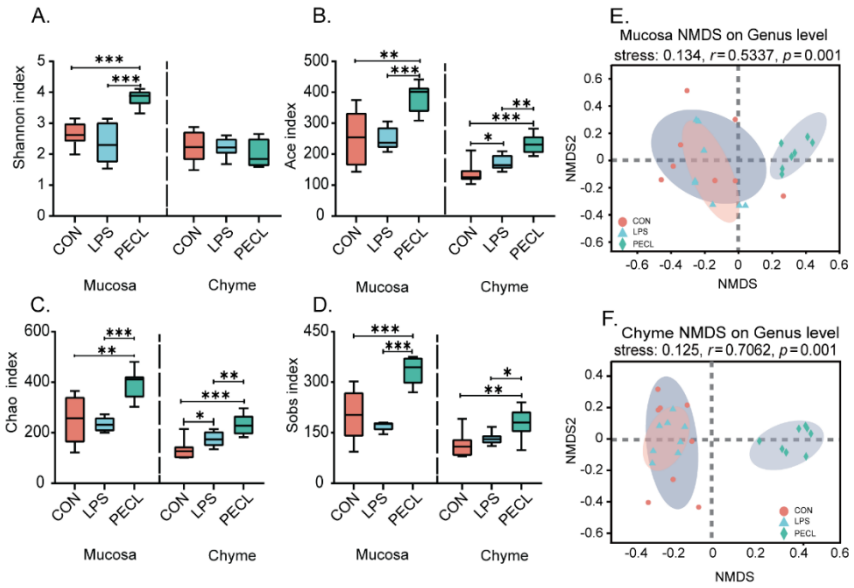

Figure 5-4 Structure and diversity of intestinal microbiota of piglets in different groups. The alpha diversity indices observed species, (A) Shannon, (B) Ace, (C) Chao, and (D) Sobs among the three groups. NMDS plot of the chyme microbiota (E) and mucosa microbiota (F) based on weighted UniFrac distance.

phylum, the results showed that *Firmicutes*, *Bacteroides*, *Actinobacteria*, as well as *Proteobactere* were the four main bacterial genera phylum levels in cecum chyme (Figure 5-5A). Meanwhile, the abundance of *Firmicutes* was higher than that in other groups and decreased the abundance of *Actinobacteria*. As for bacteria genera in genus level of the mucosa, the composition was more complex. The main bacteria genera in the CON group and LPS group were *Streptococcus*, *Olsenella* and *Bacteroides*. These three dominant bacteria accounted for more than 50 and 70% of the intestinal microbiota, respectively, in the CON group and LPS group, whereas it decreased in the PECL group (Figure 5-5C).

In cecal chyme, the predominant bacterial communities in CON groups and LPS were *Firmicutes*, *Bacteroides*, and *Actinobacteria* in cecal chyme (Figure 5-5B). Piglets treated with LPS which fed pectin led to a significant increase in *Firmicutes* as well as a clear decrease in that of *Bacteroidetes* and *Actinobacteria*. Moreover, the ratio of *Firmicutes* / *Bacteroidetes* was significantly higher in the PECL group compared with the CON and LPS group ($P < 0.01$). As for the community abundance on genus level, *Streptococcus*, *Enterococcus*, and *Prevotella_9* were

important bacterial genera in the CON group, while the LPS group showed enhanced relative abundance of *Streptococcus* and decreased relative abundance of *Enterococcus* and *Prevotella*. And in the PECL group, the abundance of *Streptococcus* was dramatically decreased.

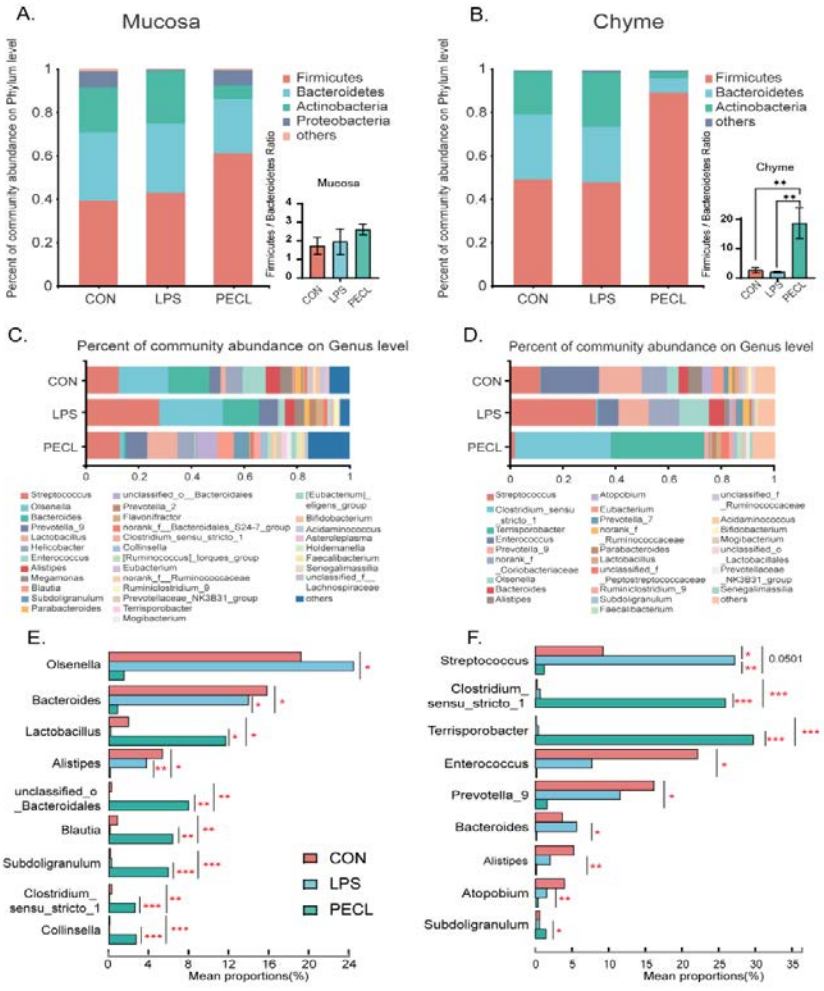

Figure 5-5 Pectin altered the cecum microbiota in LPS-challenged piglets. Structure comparison of intestinal microbiota in (A) mucosa and (B) chyme between CON, LPS and PECL groups at Phylum level and Genus level (C, D). Kruskal–Wallis H test bar plot shows the changes in the intestinal microbiota in (E) mucosa and (F) chyme of piglets in different groups at the genus level. Signification is presented as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$; data are presented as the mean ± SE ($n = 8$).

Oppositely, the abundance of *Clostridium* and *Terrisporobacter* was increased (Figure 5-5D). The differences in abundance between CON, LPS, and PECL groups at the genus level were calculated using the Kruskal-Wallis with FDR correlation. Figures 5E, F listed the top 9 different species (Figures 5E, F). In mucosa, LPS challenge, compared with the CON group, appeared to have some effect on the relative abundance of *Olsenella*, *Bacteroides*, *Lactobacillus*, *Alistipes*, *unclassified_o_Bacteroidales*, *Blautia*, *Clostridium_sensu_stricto_1*, but the difference did not reach statistical significance. However, in PECL group, the relative abundance of *Olsenella*, *Bacteroides*, and *Alistipes* was decreased, *Lactobacillus*, *unclassified_o_Bacteroidales*, *Blautia*, *Subdoligranulum*, *Clostridium_sensu_stricto_1*, and *Collinsella* were increased compared with LPS group (Figure 5-5E; $P < 0.05$). And in chyme, the relative abundance of *Streptococcus* was higher due to LPS challenged ($p < 0.05$), whereas *Enterococcus*, *Prevotella_9*, *Alistipes*, and *Atopobium* were decreased, compared with the CON group. After being treated with pectin, the relative abundance of *Clostridium_sensu_stricto_1*, *Terrisporobacter* and *Subdoligranulum* were increased ($P < 0.05$), *Streptococcus*, *Enterococcus*, *Prevotella_9*, *Bacteroides*, *Alistipes*, and *Atopobium* were decreased (Figure 5-5F; $P < 0.05$).

5.4.4 Pectin promoted mRNA expression of metabolite-associated receptors in the cecum

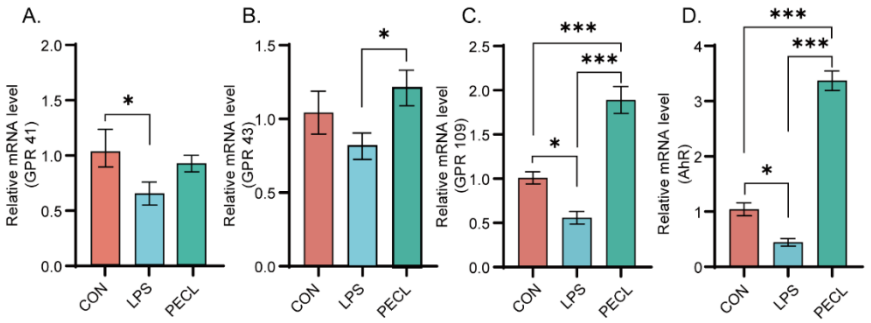

Figure 5-6 Relative mRNA expression of receptors in cecal mucosa. (A) *GPR41*, (B) *GPR43*, (C) *GPR109*, (D) *AHR*. Signification is presented as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$; data are presented as the mean ± SE.

For the expression of metabolite-associated receptors, the data showed in Figure 5-6. Piglets challenged with LPS decreased the mRNA expression of *GPR41* ($P < 0.05$; Figure 5-6A), *GPR43* (Figure 5-6B), *GPR109* ($P < 0.05$; Figure 5-6C) and *AhR* ($P < 0.05$; Figure6D). In PECL group, the decreased mRNA expression levels of *GPR43* ($P < 0.05$; Figure 5-6B), *GPR109* ($P < 0.001$; Figure 5-6C) and *AhR* ($P < 0.001$; Figure 5-6D) were restored by pectin supplementation. Even more, the expression of *GPR109* and *AhR* were notably higher than that in the CON group.

5.4.5 Addition of pectin increased the concentration of short-chain fatty acids in cecal contents

Among the groups, LPS challenge reduced the concentration of Acetic acid, Propionic acid, Butyric acid, and total SCFA compared with the CON group (Figures 7A; $P < 0.05$). Pectin supplementation markedly restored the levels of Acetic acid, Propionic acid, Isobutyric acid, Butyric acid, and total SCFA ($P < 0.01$), and the concentration of Isovaleric acid tended to increase.

Beyond this, the SCFA composition also varied greatly (Figure 5-7B). LPS decreased the percentage of propionic acid and butyric acid, whereas increased the percentage of isobutyric acid, isovaleric acid, and Valeric acid. Pectin supplementation restored those shifts, especially in butyric acid. These results demonstrated that supplementation of pectin changed not only the levels of SCFA after being challenged with LPS but also changed the composition ratio of SCFA.

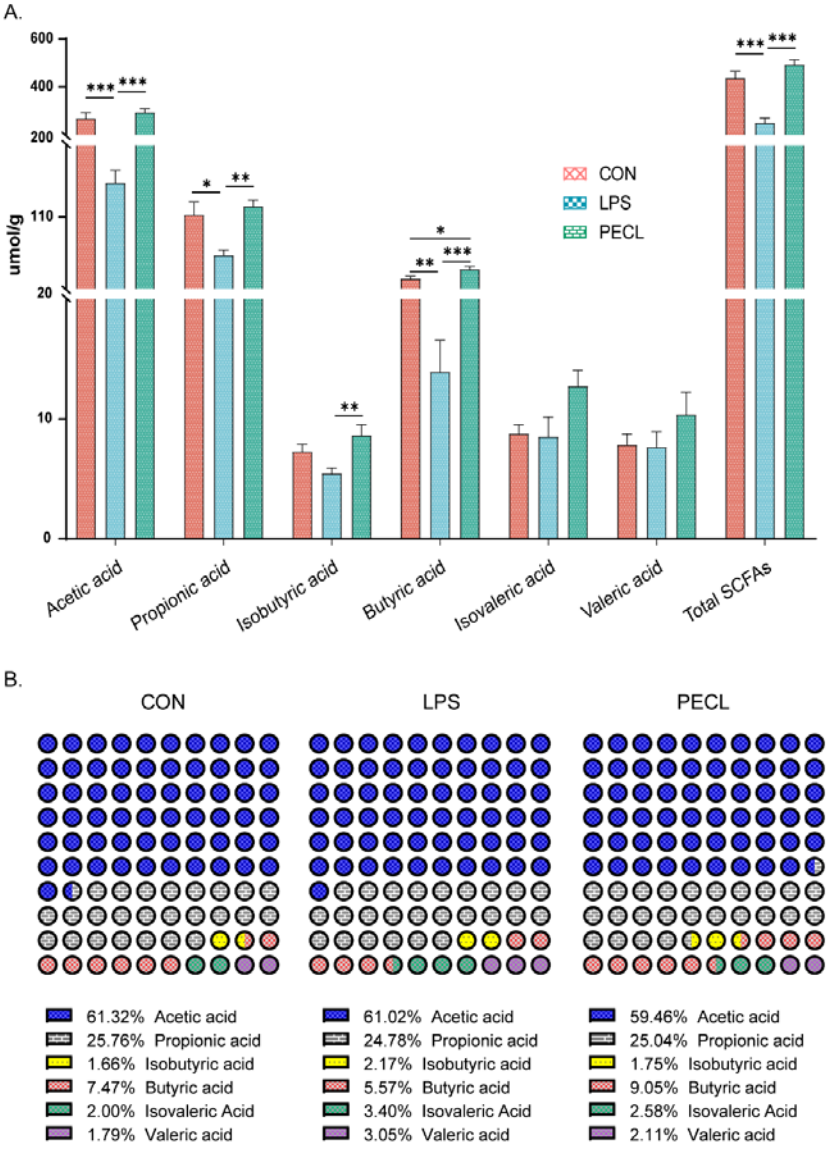

Figure 5-7 Effects of Pectin on SCFA concentrations (A) and compositional proportion (B) in the cecum of pigs. Signification is presented as **P* < 0.05, ***P* < 0.01, and ****P* < 0.001; data are presented as the mean ± SE.

5.4.6 Effects of dietary pectin supplementation on microbiota-metabolites correlation

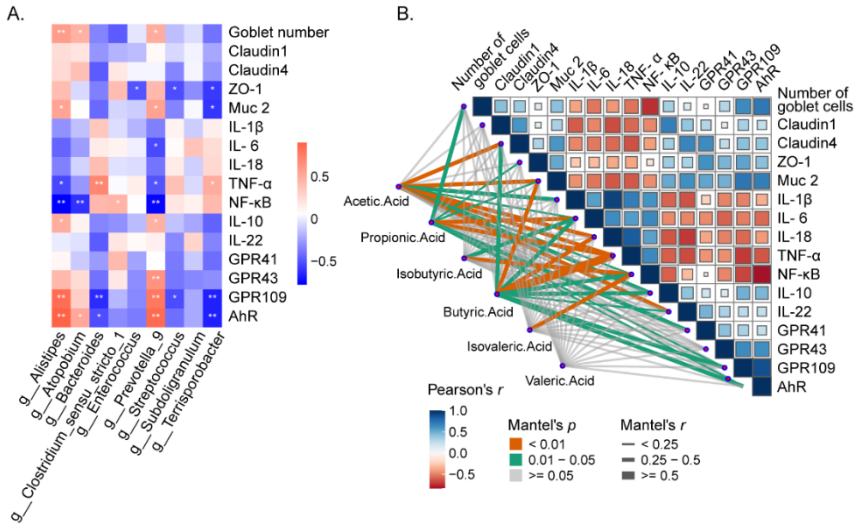

Figure 5-8 Effects of dietary Pectin supplementation on Microbiota-Metabolites Correlation. (A) Spearman's correlation matrix of Short-chain fatty acids (SCFA), and relative microorganisms in piglets. The direction of ellipses represents positive or negative correlations, and the width of ellipses represents the strength of correlation (narrow ellipse = stronger correlation). Signification is presented as $*P < 0.05$, $**P < 0.01$, data are presented as the mean ± SE (B) Pairwise comparisons of cecal genera are shown with a color gradient denoting Spearman's correlation coefficient. Short-Chain Fatty acids were related to other relevant indicators by partial Spearman tests. Edge width corresponds to the Partial Spearman's r statistic for the corresponding distance correlations and edge color denotes the statistical significance.

To find the correlation between the chyme intestinal microbiota and cecal parameters in piglets, spearman's correlation analysis was carried out based on experimental parameters. As shown in Figure 5-8A, *g_Terrisporobacter* was positively correlated with Goblet numbers, *Muc-2*, *GPR109*, and *AhR*, but negatively correlated with *TNF-α*, and *NF-κB* ($P < 0.05$). For *g_Subdoligranulum*, Goblet numbers and *AhR* were positively but *NF-κB* was negatively related with it ($P < 0.05$). *g_Streptococcus* was positively correlated with *TNF-α* and negatively correlated with *GPR109*, and AhR ($P < 0.05$). *g_Prevotella_9* was notably positively with *NF-κB* ($P < 0.05$). *g_Enterococcus* and *g_Bacteroides* had a negative correlation with *ZO-1* ($P < 0.05$). *g_Clostridium_sensu_stricto_1* was positively correlated with the number of Goblet cells, *Muc-2*, *IL-10*, *GPR43*, *GPR109*, and *AhR* ($P < 0.05$), but negatively correlated with *IL-6*, *TNF-α*, *NF-κB* ($P < 0.05$). *g_Alistipes* was positively correlated with *TNF-α* and negatively related with *ZO-1*, *Muc-2*, *GPR109*, and *AhR* ($P < 0.05$).

Mantel tests were performed to detect the correlation between SCFAs and cytokines in cecal mucosa (Figure 5-8B). The mantel correlation analysis demonstrated that a significant correlation was observed between Claudin-4, *Muc-2*, *IL-1β*, *IL-6*, *IL-18*, *TNF-α*, *NF-κB*, *IL-10*, *IL-22*, and Acetic acid (Mental's $r >$ 0.25, $P < 0.05$). We also found Propionic acid had a powerful relationship with Goblet number, *ZO-1*, *Muc-2*, *IL-6*, *TNF-α*, *GPR41*, *GPR109*, and *AhR*; Isoburic acid had a significant relationship with *IL-6*, and *NF-κB*; Butyric acid had dramatically correlations with *Claudin-4*, *Muc-2*, *TNF-α*, *NF-κB*, *IL-10*, *IL-22*, *GPR109*, and *AhR*; Isovaleric acid was associated with *NF-κB*. Thus, SCFAs were closely associated with the goblet cells, tight junction protein, inflammatory cytokines, mucus, and metabolite-related receptors.

5.5 Discussion

Weaning is considered as the most critical period during pig production because of its enormous negative impact on health status and performance (Wang et al., 2018a). LPS intraperitoneal injection is widely used in animal studies to establish intestinal injury models to simulate diarrheal pigs in weaning period. Lipopolysaccharide (LPS) intraperitoneal injection is widely used in animal studies to establish intestinal injury models (Wen et al., 2022). Extensive studies showed that LPS could produce a plethora of inflammatory cytokines and damage the intestinal epithelial structure of piglets, resulting in reduced feed intake and diarrhea (He et al., 2019). Besides, recent studies also showed that intestinal injury increases intestinal permeability and bacterial translation (Borisova et al., 2020;Sharma et al., 2020).

Over the years, numerous studies have shown the beneficial effects of pectin and its potential to regulate the inflammatory response (Li et al., 2020), cholesterol (Zofou et al., 2019), and blood glucose (Carvalho et al., 2019). Previous study in our laboratory showed that dietary supplementation of pectin could enhance intestinal barrier function (Wen et al., 2022), increase the expression of *Claudin-4* and *Muc-2* in the cecum of piglets (Wu et al., 2020b), and abolish the abnormal expression of *ZO-1* and *Occludin* caused in the acute pancreatitis model (Xiong et al., 2021). In this study, pectin supplementation could improve gut barrier function to alleviate LPS-induced intestinal injury in the cecum by increasing the number of goblet cells, and improving the expression of tight junction proteins and *Muc-2*. Thus, pectin supplementation improves the function of the intestinal barrier and reduces gut inflammation in the pig model.

Cytokines can dynamically regulate the intestinal barrier. The pro-inflammatory factors *IL-1β*, *IL-6* and *TNF-α* can increase intestinal epithelial permeability, induce the pathological opening of the intestinal tight junction barrier, and mediate the inflammatory response. Meanwhile, the anti-inflammatory factors *IL-10* and *IL-22* can maintain homeostasis in the intestine (Xia et al., 2021;Yu et al., 2021). Research showed that mice fed dietary fiber could reduce the concentration of pro-inflammatory cytokines, including *TNF-α* and *IL-6*, while increasing the concentrations of *IL-10* in sera (Zhang et al., 2018a). Besides, Chen et al. found that insoluble fiber supplementation decrease the gene expression levels of *IL-1β* and *TNF-α* (Chen et al., 2020). Again, remarkably similar results were obtained by Sun et al. (2021) (Sun et al., 2021a). In consistence with previous studies, our

results showed that pectin supplementation decreased the mRNA expression levels of *IL-1β*, *IL-6* and *TNF-α* after being increased by LPS. Therefore, pectin may exert an anti-inflammatory effect by regulating the expression levels of cytokines.

Pigs have a developed cecum, which gives piglets a larger relative population of gut microbes and stronger capacity for carbohydrate fermentation. Moreover, when the diversity, constitution, and functions of the gut microbiota are disturbed, the imbalance of the microbiota affects the intestinal immune system via metabolite signals or microbial composition (Huang et al., 2020;Lee et al., 2021). Recent research showed that the action of dietary fiber in gut disease caused by intestinal microbiome disorders is priceless (Guan et al., 2021;Hua et al., 2021;Usuda et al., 2021). Thus, we assessed the gut microbiota diversity in the cecum mucosa and chyme by 16 s rRNA sequencing of microorganisms. The results revealed that LPS treatment did not exert many differences on the α-diversity (Shannon, Ace, Chao, Sobs). After fed with pectin, the Ace, Chao, and Sobs indexes were boosted. What is more, we found that dietary supplementation with pectin optimized the composition of the intestinal chyme and mucosa microbiota of the LPS challenged including increasing the ratio of *Firmicutes* / *Bacteroides* and the abundance of *Firmicutes*. These observations suggest that the protective effect of pectin is apparently realized by alerting the microbiota. Specifically, at the genus level, we found that the addition of pectin increased the relative abundances of *Clostridium sensu_strict_1*, *Terrisporobacter*, *unclassified_f_Peptostreptococcaceae*, *Subdoligranulum*, and *Faecalibacterium*. *Clostridium_sensu_stricto_1* and *Terrisporobacter* may directly ferment polysaccharides to SCFAs (Niu et al., 2015;Lu et al., 2021). Besides, *Faecalibacterium* and *Subdoligranulum* are also the major butyrate and Lactic producers in the hindgut (Holmstrom et al., 2004;Bjerrum, 2006;Louis et al., 2009) which echoes the above that SCFAs were rich in cecal chyme.

At the genus level in the mucosa, *Olsenella* was notably decreased in the pectin supplementation group, which is a propionate-producing bacteria (Adamberg et al., 2018). It has also been confirmed that the percentage of propionate in the pectin group is lower than that in other groups. *Lactobacillus* is an important probiotic which is capable of metabolizing and producing bioactive substances, strengthening the intestinal mucosal barrier, reducing endotoxins, and regulating the body's immunity (Sarkar et al., 2016). Besides, *Alistipes*, *Blautia*, *Subdoligranulum* as well as *Collinsella* are all microorganisms associated with SCFA producers (Zhou et al., 2017;Gavin et al., 2018;Qin et al., 2019;Das et al., 2021;Kim et al., 2021). Among them, Alistipes is a well-known butyric acid producer. *Blautia* is another most abundant member of gut microbiota responsible for the production of butyric acid and acetic acid, which are associated with the improvement in glucose metabolism (Kiros et al., 2019) and the decrease in obesity via G-protein coupled receptors 41 and 43 (Ozato et al., 2019). Consistent with the findings of the former study, SCFAs production-related bacteria were significantly higher in the fermented dietary fiber treatment group. This suggests that at least part of the pathway of pectin mitigation of LPS stress is via the SCFAs pathway.

Metabolites derived from microbiota have been proven to alter the host's metabolism and intestinal health (Wu et al., 2021b). SCFAs, also known as volatile fatty acids, play an essential role in the storage of energy and the regulation of osmolality in the body. In addition, they are involved in maintaining the function of the intestinal cells, regulating the intestinal immune response, and reducing various inflammatory diseases (den Besten et al., 2013b;Koh et al., 2016;Dang et al., 2021). In this study, the supplementation of dietary fiber significantly enhanced the concentration of short-chain fatty acids other than isovaleric acid in the contents of the cecum.

SCFAs have been shown to repair intestinal mucosa and reduce intestinal inflammation by activating GPRs and inhibiting histone deacetylases (HDAC) and downregulating the expression of pro-inflammatory cytokines (Tang et al., 2022a). *GPR41*, expressed in gut and adipose tissue, is activated equally by propionate and butyrate (Horiuchi et al., 2020), whereas *GPR43* is more responsive to acetate and propionate than to butyrate. Besides, both *GPR109* and *AhR* can only be activated by butyrate (Dang et al., 2021). In this study, LPS decreased the expression of *GPR41*, *GPR43*, *GPR109*, and *AhR*. And pectin increased the expression of *GPR43*, *GPR109*, and *AhR*, which might be because of the restores of acetate, and propionate in the gut that was reduced by LPS challenge. SCFA further activates *GPR43*, *GPR109*, and *AhR* receptors on the surface of lymphocytes in the cecal mucosa, thereby promoting the host's immunity and protecting intestinal health.

To better understand the correlations, spearman's correlation analysis was conducted between the 16sRNA sequencing analysis identified several genera associated with pectin intake, and some of these genera were correlated with levels of Histomorphology, cytokines, and receptors. In a previous study, Alistipes was positively correlated *with GPR109, AhR* (He et al., 2022). An increased proportion of *Alistipes* may associated with higher levels of, which is identical with our results. Besides, *Prevotella_9* is also a well-known beneficial bacteria (Wang et al., 2019b), it showed a positive correlation with *AhR*, *GPR109*, *GPR43*, which the receptors may activated by SCFAs. To some extent, these results are consistent with previous studies. In our research, we further found butyric acid was positively correlated with the mRNA expression levels of *Claudin 1*, *ZO-1*, *IL-10*, and *IL-22* in the cecum mucosa, suggesting that butyric acid may play a role in boosting anti-inflammatory capacity. These results obtained are consistent with the previous work carried out by others. These results suggested that: the active state of gene expression and tight junction protein in cecum, being found in pectin treatment, may be only the basis of other biological processes. At the same time, the enhanced beneficial bacteria and increased SCFAs can provide a viable mechanism to support the accurate progress of repairing intestinal damage.

5.6 Conclusion

In conclusion, the present study showed that dietary pectin supplementation, during the weaning period, may improve the ability of piglets to resist intestinal injury induced by LPS challenges. The addition of pectin to the diet improved the mucosal and chymous microbial disruption, and further augmented the SCFAs. Noteworthy, SCFAs improved the intestinal barrier, elevated anti-inflammatory cytokines, and reduced pro-inflammatory cytokines by activating *GPR43*, *GPR109*,

and *AhR* receptors. Last, this study makes an essential contribution to the evidence base on the use of pectin in fed additives.

Table 5-1. Primer sequences used for real-time PCR.

Gene	Primer	Nucleotide sequences (5' to 3')
β-actin	F	GCGTAGCATTTGCTGCATGA
	R	GCGTGTGTGTAACTAGGGGT
Claudin-1	F	TCGACTCCTTGCTGAATCTG
	R	TTACCATACCTTGCTGTGGC
Claudin-4	F	CAACTGCGTGGATGATGAGA
	R	CCAGGGGATTGTAGAAGTCG
MUC2	F	CGCATGGATGGCTGTTTCTG
	R	ATTGCTCGCAGTTGTTGGTG
ZO-1	F	CTCCAGGCCCTTACCTTTCG
	R	GGGGTAGGGGTCCTTCCTAT
IL-1β	F	GCCAGTCTTCATTGTTCAGGTTT
	R	CCAAGGTCCAGGTTTTGGGT
IL-6	F	TCCAATCTGGGTTCAATCA
	R	TCTTTCCCTTTTGCCTCA
IL-8	F	TACGCATTCCACACCTTTC
	R	GGCAGACCTCTTTTCCATT
IL-10	F	TCGGCCCAGTGAAGAGTTTC
	R	GGAGTTCACGTGCTCCTTGA
IL-18	F	TCCGGATCACTTCCTCTCGT
	R	CCGATTCCAGGTCTTCATCGT
IL-22	F	AGCAAGCGTGAAGGTGCGGTT
	R	GCGGACATCTGGGAGCCCTTT
TNF-α	F	CGTCGCCCACGTTGTAGCCAAT
	R	GCCCATCTGTCGGCACCACC
NF-κB	F	AGTACCCTGAGGCTATAACTCGC
	R	TCCGCAATGGAGGAGAAGTC
GPR41	F	GTCTGTGCCCTCATGGGTTT
	R	GACGTTCATACCTTCGGCCT
GPR43	F	CCTGACGCTGGCAGACCT
	R	GCTGCTGTAGAAGCCGAAACC
GPR109	F	AGCCATCATCTCCTGCCTCCTG
	R	ATCATGCCAGCGGAAGGTATTGC
AhR	F	CATGCTTTGGTCTTTTATGC
	R	TTCCCTTTCTTTTTCTGTCC

Chapter VI

General discussion, conclusion and perspectives

6.1 General discussion

As previously mentioned in the introduction, pectin can be categorized into various types based on its source and extraction process, and there are distinctions between different pectin varieties (Chandel et al., 2022). Specifically, pectin can be classified into low-methoxyl pectin and high-methoxyl pectin based on their degree of esterification (Molino et al., 2022). Low-methoxyl pectin, characterized by high solubility and low viscosity, not only influences blood glucose levels and provides dietary fiber but also promotes the production of fermentation byproducts in the hindgut. In contrast, high-methoxyl pectin, owing to its excellent gelling properties, is commonly used in the food industry.

For low-methoxyl pectin from various sources, although their monosaccharide composition may differ, they share common physiological functions, which include promoting intestinal health, maintaining blood sugar stability, reducing lipid levels, increasing satiety, and mitigating systemic inflammation (Beukema et al., 2022).

6.1.1 Intestinal injury model

In scientific research, several common methods are employed to establish intestinal injury models. These methods primarily include chemical induction, bacterial infection, and genetic engineering models (Caradonna L, 2000; Baydi et al., 2021).

Chemical Induction: Chemical induction in animal experiments involves administering specific chemicals (Oxazolone, DSS, TNBS.) to induce intestinal damage, mainly in mice and rats, through oral gavage or injection (Perse et al., 2012; Chassaing et al., 2014; Cheon et al., 2012). These chemicals can harm the epithelial cells of the intestines, leading to mucosal inflammation and ulcer formation. This method is straightforward, allows for control of dosage and exposure time, but the model's complexity is limited. Additionally, these drugs may have adverse effects on overall health (Jiminez et al., 2015).

Bacterial Induction: Bacterial induction of intestinal injury entails administering specific bacteria (such as *Escherichia coli*, *Salmonella*, etc.) or their products to induce intestinal inflammation and damage (Wu et al., 2010;Lawhon et al., 2011). This method allows researchers to study intestinal lesions caused by bacterial infections and gain insights into the mechanisms of intestinal infections, making it a more complex approach (Jiminez et al., 2015).

Genetic Engineering Models: Genetic engineering models involve altering specific genes to create specific intestinal disease or injury models. The IL-10-/- and IL-2-/- gene knockout models you mentioned are used to study the association between the immune system and intestinal diseases (Anderson et al., 2011; Mombaerts et al., 1993). This method offers high specificity, enabling precise control of disease-related gene alterations and aiding in the exploration of the relationship between genes and diseases. However, it often requires complex genetic engineering techniques, may produce abnormal compensatory mechanisms affecting model integrity, and its biological applicability may be limited (Baydi et al., 2021).

In our study, we utilized lipopolysaccharide (LPS), a common endotoxin found

in bacterial cell walls, which, upon entry into the body, interacts with TLR4, activating immune cell signaling pathways, particularly in the context of intraperitoneal injection-induced inflammation models (Tang et al., 2010; Liu et al., 2009). It can activate monocytes, macrophages, endothelial cells, and epithelial cells via the cell signaling transduction system, inducing the synthesis and release of various cytokines and inflammatory mediators, thereby triggering a series of host responses (Li et al., 2021). In pigs, LPS induces symptoms resembling bacterial infection, including reduced appetite, lethargy, and fever, which are employed to induce injury models in the piglet's gastrointestinal tract.

In fact, there are primarily two ways to induce animal injury models using LPS: intravenous injection and intraperitoneal injection (Mizobuchi et al., 2020). Intravenous injection of LPS elicits a systemic immune response, as LPS rapidly circulates throughout the body via the bloodstream (Galanos et al., 1979). This approach is suitable for studying systemic inflammatory responses and immune system activation (Shi et al., 2021; Engelhardt et al.,1991). On the other hand, intraperitoneal injection of LPS leads to more localized effects, particularly within the peritoneum and abdominal tissues. This method is useful for investigating peritonitis or inflammation related to intra-abdominal organs.

Furthermore, an interesting observation is that, according to existing literature, oral administration of LPS does not appear to have any adverse effects on the body. Instead, it shows potential for improving pathology and disease prevention (Márquez et al., 2007; Iguchi et al., 1992).

6.1.2 Pectin used in this study

As mentioned earlier, there are various types of pectin, and commercially available pectin is mainly derived from three main residues: sugar beet pulp, citrus peel and apple pomace. In this PhD thesis we used pectin originated from citrus peel. Moreover, we used citrus pectin with a concentration of galacturonic acid at 81.4% and a degree of esterification of 13.5%. However, with regard to the esterification, there can be important differences when comparing low-ester pectin and high-ester pectin. Low-ester pectin has high solubility, low viscosity, which not only can impact blood glucose levels and provide dietary fiber but also promote the production of fermentation products in the hindgut. On the other hand, high-ester pectin, due to its excellent gelling properties, is typically used in the food industry. For low-ester pectin, despite variations in monosaccharide composition, they share common physiological functions, such as promoting gut health and controlling blood sugar, and this is independent of the source of pectin.

6.1.3 Effect of pectin on the growth performance of livestock

In order to enhance pig farming practices, early piglet weaning measures bring a range of benefits to the industry. These include improved reproductive performance of sows, shorter slaughter cycles, enhanced utilization of farrowing (Campbell, 2013;Koketsu et al., 2017). However, the weaning process is associated with the weaning stress syndrome, which has a significant negative impact on piglet growth and performance and increases the risk of gastrointestinal diseases (Kim et al., 2012). For a long time, farmers have administered antibiotics (such as gentamycin, oxytetracycline, guillamycin, etc.) to prevent diseases and promote growth in weaned piglets (Strom et al., 2018;Adekanye et al., 2020). Nevertheless, the

dangers associated with the excessive use of antibiotics have gradually emerged. These risks include the development of drug-resistant strains of bacteria, the presence of drug residues in the animal's body, the occurrence of secondary infections, and the pollution of the ecological environment (Kraemer et al., 2019;Hu et al., 2020).

Conventional nutrition suggests that the viscosity of pectin affects its intestinal sticking properties, reducing feed intake and affecting animal performance (Fleming et al., 1983; Hedemann et al., 2006). Recent studies have shown that the addition of pectin oligosaccharides to the diet significantly increased average daily feed intake (ADFI) and average daily gain (ADG), improved the feed to meat ratio (F/G) and reduced the diarrhoea index in weaned piglets (Chen et al., 2017a). Mao et al. (2016) reported an improved growth performance (increased ADG and ADFI and reduced FCR) in weaned rats by supplementing 800 mg/kg pectic oligosaccharides (POS) in the diet (Mao., 2016). In our research, pectin supplementation significantly decreased the F/G of piglets from d 1–14 compared with the CON and LPS group, which was consistent with previous studies (Chen et al., 2017a).

The ratio of villus height to crypt depth serves as an indicator of the degree of nutrient absorption and utilization in the foregut. Higher ratios indicate a larger surface area for effective nutrient absorption and a greater absorption capacity (Czernichow., 1996). A study conducted by Wang et al. (2019) demonstrated that supplementing feed with POS can increase villus height in the duodenum of broilers (Wang et al., 2019c). Similar findings have been observed in studies involving other animal species. In the aforementioned research of Mao et al. (2016), adding POS to the diet of mice, significantly increased the ratio of villus height to crypt depth in the jejunum (Mao., 2016). Additionally, Mao et al. (2017) found that supplementing the diet of weaned piglets with 200 mg/kg POS resulted in an increased height of jejunal villus and the villus/crypt ratio (Mao., 2017).

The addition of pectin was also seen to reduce the standardized ileal amino acid (AA) digestion rate, with an average decrease of up to 5% digestive units (Buraczewska et al., 2007a). Buraczewska et al. (2007) reported that pectin supplementation did not alter the weight or length of the small intestine, but it did induce changes in intestinal morphology. The width of the muscular layer in the duodenum, mid-jejunum, and ileum significantly increased, while the length of the villi in the duodenum and ileum also increased (Buraczewska et al., 2007a). Furthermore, the inclusion of 4% pectin in the diet lead to changes in intestinal morphology, characterized by a significant increase in villus height and muscular width in the duodenum and ileum (Buraczewska et al., 2007a). In chapters 3 and 4 of this PhD study, we describe that the addition of our citrus pectin significantly enhanced villus height and tight junction protein expression in the jejunal mucosa of weaned piglets compared to the control group receiving microcrystalline cellulose. Additionally, pectin supplementation reduced villus loss, lowered the occurrence of atrophy, and diminished the decrease in tight junction proteins caused by lipopolysaccharide (LPS) stress. Therefore, while there may be some disagreements regarding the effects of pectin on growth performance and nutrient utilization in mammals, likely due to variations in species, sources, and doses of

pectin used, there is a consensus that pectin promotes small intestinal development and protects intestinal integrity.

6.1.4 Effect of pectin on tight junction proteins and goblet cells

Tight junction proteins play a crucial role in maintaining the integrity of the intestinal mucosal barrier and its barrier function (Suzuki, 2013). These proteins form multiple complexes that include transmembrane proteins such as Occludin and *Claudin-2*, as well as the cytosolic protein *ZO-1* (Chiba et al., 2008). Claudin proteins, being transmembrane proteins, are responsible for the formation of tight junctions between cells and primarily regulate the permeability of the barrier structure (Gunzel et al., 2013). The stability of these cell-cell tight junctions relies on complex interactions between claudin proteins among themselves and with other tight junction proteins (Venugopal et al., 2019). Extensive evidence consistently supports the notion that downregulation of *ZO-1* leads to an increased permeability of the gut, contributing to gut dysfunction and initiating host inflammation (Miao et al., 2015).

Research conducted by Wu et al. (2020) and Xiong et al. (2021) has demonstrated that the addition of pectin to piglet feed significantly enhances the expression of tight junction proteins in the piglet intestine, thereby improving immunity and promoting intestinal homeostasis (Wu et al., 2020b;Xiong et al., 2021). Consistent with these studies, our own investigation found that the addition of pectin to piglet feed significantly increased the gene expression of *Claudin-1* and *Occludin* in the jejunal mucosa (Chapter 3). Similarly, in the ileum and cecum (Chapters 4 and 5), a notable decrease in tight junction protein expression was observed in the LPS group. However, in the pectin supplemented group where animals received LPS (PECL), there was a significant upregulation of *Claudin-1*, *Occludin*, and *ZO-1* gene expression compared to the control with LPS group, thereby mitigating the damage caused by LPS challenge.

Goblet cells are a type of mucus-secreting cells found in the intestine, predominantly distributed adjacent to the mucosal epithelial cells (Hooper., 2015). Due to their cytoplasm rich in mucins, they are an integral part of the innate immune barrier in the intestines. They contribute to the protective mucus layer covering the intestinal surface by secreting mucins (MUC) and trefoil factors (TFF) along with water (Yang et al., 2021). Previous studies have demonstrated that *MUC2*, a core component of mucin, possesses significant host defense functions (Kufe, 2009;Birchenough, 2016). Tadesse et al. (2017) demonstrated through their study on *MUC2* knockout mice that the absence of *MUC2* can increase the incidence of tumors in the intestinal tract (Tadesse, 2017). There is limited research on the addition of pectin to piglet feed and its effect on goblet cells or their secretion, but several mouse studies have shown that pectin can regulate goblet cell development and mucin production in the intestine. Adding 5% pectin, as well as soluble fiber glucomannan and soluble corn fiber, to the diet increased the depth of crypts, goblet cell numbers, and acidic mucins in the cecum and colon of rats (Knapp et al., 2013). Adding 50g/kg pectin to the diet increased the expression of *MUC2* and luminal mucin content in the rat intestine but did not affect goblet cell numbers in the ileum (Hino et al., 2013). In vitro studies have demonstrated that pectin can stimulate HT-29MTX cell line to secrete mucins (Hino et al., 2013). In our study (chapter 4),

PAS+/AB+ staining showed that the addition of citrus pectin to the diet significantly increased the number of goblet cells in the ileal crypts of weaned piglets. The qPCR results also showed that the addition of pectin to the diet significantly increased the expression of *MUC2* in the mucosa, which is thus consistent with our hypothesis. Moreover, this is also in line with the findings of Turner et al. (2009) (Turner, 2009).

6.1.5 Effects of pectin on spatial heterogeneity of mammalian gut microbiota

The mammalian intestinal tract is physiologically structured from front to back as the duodenum, jejunum, ileum, cecum, and colon (Xu et al., 2021b). The duodenum and jejunum are broadly categorized as the foregut, as they are connected to the stomach, and therefore, the foregut contains both aerobic and anaerobic microorganisms. The cecum and colon, on the other hand, are defined as the hindgut, where the microbial composition consists solely of anaerobic microorganisms (Wang et al., 2019a). There are a large number of microorganisms inhabiting the gastrointestinal tract of mammals. These microorganisms are involved in important physiological processes such as intestinal development, nutrient digestion, vitamin synthesis, defense against pathogenic bacteria, and immune regulation.(Szabo et al., 2023).

The niches in which the microbiota reside are considered the deterministic factors responsible for shaping the composition of the gut microbial community. These niches are determined by a combination of factors, including the availability of substrates, oxygen concentrations, pH levels, and interactions among microbial species, among others (Hollister et al., 2014; Pereira et al., 2017). These factors collectively give rise to distinct niches in various segments of the gastrointestinal tract, thereby supporting the existence of discrete microbial communities (Stearns et al., 2011; Zhao et al., 2015). Notably, along the length of the intestinal tract, a natural pH gradient emerges, ranging from 6 in the duodenum to 7.4 in the terminal ileum of humans (Fallingborg, 1999). The hindgut, as an anaerobic environment rich in diverse microbial populations, serves as an excellent site for investigating spatial microbial heterogeneity (Martinez et al., 2019; Donaldson et al., 2016). Research conducted by Gu (2013) and others, conducted in mice, has shown that the stomach and small intestine are enriched with facultative bacteria, including Bacilli (class), Lactobacillaceae (family), and Lactobacillus (genus). In contrast, strictly anaerobic bacteria such as Clostridia (class) and Lachnospiraceae (family) are enriched in the large intestine (Gu et al., 2013).

Therefore, to assess the impact of pectin on the spatial microbial heterogeneity along the longitudinal axis of the piglet's gastrointestinal tract, we chose to conduct a comprehensive analysis of both the foregut (small intestine and ileum) and the hindgut (cecum).

Research shows that the composition and structure of the intestinal microbiota fluctuates dramatically after weaning of piglets (Cremonesi et al., 2022). There is growing evidence that dysbiosis of the intestinal microbiota is responsible for many inflammatory diseases that cause antibiotic-associated diarrhea (Heinsen et al., 2015;Degruttola et al., 2016). Therefore, the composition and function of the

microbial community is critical for maintaining homeostasis in the gastrointestinal tract and host health. The entire mammalian gut has proximal to distal segments that form a distinct microenvironment that well supports the growth of a very different microbial community in the gut (Tropini et al., 2017). In the digestive system, simple and easily metabolised carbohydrates are mainly digested and absorbed in the small intestine, while structurally complex polysaccharides and fiber accumulate mainly in the large intestine (Roy et al., 1994;Clemens et al., 2016). The differences in fermentation substrates and pH between the foregut and hindgut contribute to the different microbiota.

In this PhD study, we demonstrated an effect of citrus pectin addition to the diet of healthy piglets on piglet intestinal mucosa microorganisms (Chapter 3), showing that pectin increased the diversity of microorganisms in the jejunal mucosa, indicating an increase in community diversity and an increase in the variety of microorganisms. Among the microorganisms comprising the piglet jejunum mucosa, the phylum level was dominated by *Firmicutes*, *Actinobacteria*, *Bacteroides* and *Proteobacteria*, which is consistent with results previously reported for pig intestinal microorganisms (Shin et al., 2015b;Jin et al., 2019). The pectin group showed a significant decrease in *Actinobacteria* and a significant increase in *Proteobacteria* compared to the control group. At the genus level, *Lactobacillus* and *Enterococcus* were significantly higher in the pectin group, two bacteria known to be associated with tryptophan metabolism, which is consistent with the previous description of the jejunum as the main site of digestion and absorption of protein-like substances (Lamas et al., 2018).

In chapters 4 and 5, weaned piglets were fed different diets (maize and soybean meal-based diet + 5% microcrystalline cellulose in the control group (CON) and control with LPS group (LPS), and basal diet + 5% pectin in the pectin with LPS group (PECL)), where the LPS was intraperitoneally injected inducing intestinal damage. This was used to study the immune tolerance of weaned piglets fed pectin when exposed to LPS stress. The results showed that in the ileal PECL group, the abundance and diversity of microorganisms was greatly moderated compared to the LPS group. Interestingly, in the cecum, the abundance and diversity of intestinal microorganisms were significantly higher in the PECL group than in the LPS group, and even in the CON group. This may be due to the fact that pectin, as a dietary fibre, underwent fermentation in the hindgut and significantly increased the abundance and diversity of microorganisms in the cecum (Wu et al., 2022b;Xu, 2023). *Firmicutes*, *Actinobacteria*, *Bacteroides* and *Proteobacteria* were also predominant in the ileum and cecum at the phylum level, similar to the microbial composition of the jejunum and previous studies (Shin et al., 2015b;Wlodarska et al., 2017). At the genus level, the addition of pectin moderated the decrease in *Enterococcus* (associated with protein digestion and absorption) and the increase in *Helicobacter*. Notably, *Helicobacter* is a potential opportunistic pathogen associated with LPS stress (Shin et al., 2015b;Hughes et al., 2017a). It can be concluded that although there is structural continuity between intestinal segments, there are highly significant differences in microbial abundance, alpha diversity and beta diversity between jejunum, ileum and cecum. The combination of microbial abundance and structure contributes to the diversity of the microbial communities in the different intestinal segments. In this study, pectin altered the richness and

structure of the microbiota in all parts of the gastrointestinal tract measured, namely the jejunum, ileum, and cecum. However, there is currently few research that investigates the changes in dietary fiber on the microbiota of the entire intestinal tract as a whole. Furthermore, the relationship between dietary fiber and microbial migration between intestinal segments remains understudied. Therefore, we speculate that there might be a potential connection between the effects of dietary fiber addition on microorganisms in different intestinal segments when weaned piglets are used as pathological models. Further research is required for elucidation.

In addition to the differences in the composition of gut microbiota (abundance, diversity, and composition) due to spatial heterogeneity in the gut, our analysis of the foregut and hindgut suggests that pectin, being resistant to enzymatic hydrolysis and fermentation in the small intestine, primarily exerts its effects in the foregut by directly interacting with pattern recognition receptors (Vogt et al., 2016). In a similar vein, Christiane (2016) posited a similar viewpoint by stimulating dendritic cells derived from murine bone marrow in vitro, suggesting that the cytokine response is induced by dietary fiber rather than short-chain fatty acids and the microbiota Correspondingly, in related research, Bermudez-Brito (2015) co-cultured DCs with dietary fiber, revealing that dietary fiber induced Treg cell production of IL-10 while reducing IL-6 levels through direct interaction with the mucosa (Bermudez-Brito., 2015).

In the hindgut, the pathway through which dietary fiber modulates gut immune competence is indirect. This process involves altering gut microbiota diversity and composition, promoting the proliferation of beneficial bacteria while inhibiting harmful ones. It also facilitates the generation of microbial metabolites, such as short-chain fatty acids, thereby contributing to the regulation of gut health (den Besten et al., 2013).

6.1.6 Effect of pectin on intestinal microbiota-related metabolites

Another way in which gut microbes influence gut health is through the secretion of metabolites (Parker et al., 2020). As the piglet gut extends, differences in the microbial composition of the various intestinal segments give rise to variations in the composition of the main metabolites (Gresse et al., 2019). The small intestine is dominated by aerobic or partly anaerobic bacteria, which play a crucial role in nutrient digestion and absorption. Nearly all proteins, lipids, and carbohydrates are absorbed in the small intestine, including amino acids such as tryptophan (Kanauchi., 2003).

Tryptophan is an essential amino acid in mammals and is involved in regulating inflammation, immunity, and intestinal homeostasis (Comai et al., 2020). The findings of this PhD study demonstrate that pectin can increase the production of metabolites such as, indole-3-acetic acid (IAA), indole-3-propionic acid (IPA), and 5-hydroxyindole-3-acetic acid (HIAA) by promoting the metabolism of tryptophan towards indole derivatives (Chapter 3). In the host, factors that can participate in the activation of the AHR receptor include indole derivatives (Dong et al., 2020), butyric acid (Modoux et al., 2020), and polyphenolic compounds (Brinkmann et al., 2022). In this experiment, pectin, as a polysaccharide devoid of polyphenols, was studied for its immunomodulatory effects on the intestinal mucosa concerning

tryptophan metabolites - indoles (as the cecum does not produce butyric acid).

Research on the promotion of tryptophan metabolites originating from dietary fiber in the gut microbiota, further activation of the Ahr receptor in type 3 lymphocytes in the gut, improvement of gut barrier function, and enhancement of gut immunity has been reported in mice (Zou et al., 2018;Wrzosek et al., 2021). Similar studies in humans have also confirmed this (Wang et al., 2017). However, research on pigs, a species with a high degree of physiological similarity to humans and mice, is relatively scarce. Huang's study in 2023 confirmed that fiber-based feed can increase the concentration of indole metabolites in the intestinal tract of growing pigs, while activating Ahr (Huang et al., 2023). In other studies, it was further confirmed that in the immune regulation process of piglets, the secretion of IL-22 in the gut can participate in the regulation of host immunity (Beaumont et al., 2018;Swimm, 2018; Yang et al., 2017). In this study, pectin can promote the production of indole metabolites by altering the tryptophan metabolic pathway, thereby activating the AHR-IL-22 metabolic pathway and promoting gut immunity

The hindgut is less exposed to oxygen and therefore also has a higher abundance of anaerobic bacteria. The main metabolites include short-chain fatty acids (SCFAs) and secondary bile acids (Zhang et al., 2023). SCFAs are a widely studied group of small molecule metabolites that are produced by the fermentation of dietary fibers by gut microbiota (Al et al., 2020). In chapter 4 of this PhD study, there were no significant differences in the levels of SCFAs between the ileum groups, except in the pectin group, where acetic acid levels were significantly higher than in the other groups. The low levels of SCFAs found there are consistent with the theory that the foregut is less fermentable and produces lower levels of microbial metabolites. In chapter 5, the LPS group significantly reduced the levels of acetic acid, propionic acid and butyric acid in the piglets' cecum compared to the CON group. In the PECL group, pectin not only reversed the reduction in SCFAs brought about by LPS stress, but even reached significantly higher levels of butyric acid than in the CON group. Numerous studies have demonstrated that butyric acid produced by microbial fermentation is a good source of energy for epithelial cells and can effectively promote the proliferation of epithelial cells (Peng et al., 2009;Becattini et al., 2016;Kaisar et al., 2017;Dang et al., 2021). In addition, the proportion of short-chain fatty acids in the intestine of mammals can, to some extent, reflect the potential health status of the host (Arora et al., 2011;Carroll., 2011;den Besten et al., 2013a). Previous studies have shown that the addition of pectin to the diet can significantly increase the proportion of acetic acid in the short-chain fatty acids (Rao., 1998). In the present study (Chapter 5), the proportion of acetic acid decreased and butyric acid increased in the PECL group. This may be due to the fact that there is also some interconversion between fatty acids. For instance, some *Clostridium* in the intestine can synthesize butyric acid from lactic and acetic acid (Detman et al., 2019). Interestingly, the *Clostridium* in the cecum were also significantly higher in the PECL group than in the LPS group. This could also explain the phenomenon in our experiments.

6.2 Conclusion and perspectives

In this PhD thesis, we investigated the effects of pectin on the development, microbiota, and immunity of the foregut and hindgut of piglets in both healthy and

challenged model states.

In healthy weaned piglets, the addition of 5% citrus pectin to the diet was studied for its impact on the development and immunity of the jejunum. We demonstrated that pectin promotes the metabolic direction of tryptophan in the jejunum, leading to an increase in indole derivatives. Indole derivatives can act as ligands to specifically bind to the AHR receptor present in the cytoplasm of widely distributed intestinal lymphocytes, activating the AHR-IL22 immune pathway. This promotes the proliferation of goblet cells in the intestinal mucosa, increases mucin-2 expression, and enhances the expression of downstream inflammatory factors, promoting the abundance of beneficial microbiota such as Enterococcus and Lactococcus in the mucosa.

In weaned piglets subjected to intestinal injury, the addition of pectin to the diet was found to effectively alleviate the decrease in productivity induced by LPS stress. Dietary pectin significantly increased villus height and crypt depth, enhanced intestinal nutrient absorption capacity, reduced intestinal permeability, preserved intestinal integrity, inhibited the upregulation of inflammatory cytokines MCP-1 and IL-1β induced by LPS, lowered intestinal inflammation, improved piglet growth performance, promoted mucin secretion by goblet cells, and enhanced the intestinal mucous barrier. Dietary pectin also altered the glycosylation structure of mucins in the ileum of LPS-stressed piglets, promoting the development and maturation of goblet cell mucins. This greatly impacted the barrier function of mucins, strengthening the mucous barrier function, protecting intestinal epithelial cells, reducing intestinal inflammation, and enhancing intestinal nutrient absorption capacity. In the cecum, pectin promoted the proliferation of beneficial bacteria such as Lactobacillus and Blautia while inhibiting the growth of harmful bacteria like Streptococcus. Additionally, it stimulated the abundance of short-chain fatty acids, activating the GRPs regulatory pathway, and improving gut health.

However, several bottlenecks still exist in the preparation and application of pectin. Currently, there are differences in the composition of pectin from different batches and sources, including homogalacturonan (HG), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII). Additionally, pectin, as a typical acidic and easily fermentable dietary fiber, can serve as a nutrient source for microorganisms in the hindgut, promoting the proliferation of beneficial bacteria and also promoting the development of goblet cells and pattern recognition receptors in the mucosal barrier in the foregut, thereby promoting gut health. More research is needed to elucidate its specific molecular regulation mechanism in the foregut. Progress in these fields will be more conducive to the application value of pectin in the prevention and treatment in animal production.

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Appendix

Scientific publications

Scientific publications

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