Manipulation of Gut Microbiota during Critical Windows of Development and its Effects on Immune System Function Later in Life

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Abstract

The potential long-term complications of gut microbiota perturbation during critical developmental periods of early life and puberty are a growing concern. Exposure to immune stressors such as antibiotics or lipopolysaccharides (LPS) in these periods disturbs the gut microbial balance and increases the risk of immunological disorders later in life. Probiotics and prebiotics can counteract inflammation and microbiota perturbation induced by antibiotic or LPS exposure. However, the underlying mechanisms remain inadequately explored. We postulated that exposure to LPS/antibiotic during puberty or early life negatively affects immune system homeostasis later in life while probiotic/prebiotic intake mitigates immune system disturbance related to LPS/antibiotic-induced inflammation and dysbiosis through modulating signaling pathways and epigenetic mechanisms, including miRNAs and DNA methylation. Therefore, this thesis aims to better understand the mechanisms underlying the protective effects of biotics intake against lasting immune deregulation associated with exposure to immune stressors during the developmental stages of life. The objectives of this project are:

1. To study the immunomodulatory properties of probiotic SV-53, viable and heat-inactivated forms, and prebiotic PCA, by assessing cytokines, miRNAs, and DNA methylation of genes related to the pro-inflammatory cytokine IL-17 signaling at the gut level.

2. To study if probiotic/prebiotic intake counteracts long-term immune system deregulation induced by inflammation and dysbiosis during puberty, through assessing cytokines, signaling pathways, miRNAs, and DNA methylation of genes related to the pro-inflammatory cytokine IL-17 pathway at the gut level.

3. To study if prebiotic intake counteracts early life antibiotic induced-dysbiosis and its long-term adverse effects on immune system homeostasis by assessing cytokines and miRNAs related to inflammatory pathways, including NF-κB and STAT3 at the gut level.

We used pre-clinical models involving Balb/c mice, exposing them to a single dose of LPS at puberty or to low-dose penicillin early in life by feeding dams with the antibiotic during the last week of gestation until weaning of pups. The role of probiotic/prebiotic intake in preventing and correcting long-term consequences of LPS or antibiotic was assessed by exploring the gut microbiota using fecal samples, as well as immune signaling, miRNA expressions, and DNA methylation in the ileum samples.

Results of these studies revealed the ability of probiotic SV-53 to improve gut immunity by increasing IgA levels and reducing inflammatory cytokines and miRNAs, such as IL-17A, IL-6, IL-23, miR-223, and miR-425, as well as epigenetic regulation of genes related to IL-17 signaling, including *Il6*, *Il17rc*, *Il9*, *Il11*, *Akt1*, *Ikbkg*, *Sgk1*, *Cblb*, and *Smad4*. In addition, probiotic and prebiotic intake alleviated immune system dysfunction induced by pubertal LPS by modulating IL-17A, IL-17F, IL-6, IL-1β, STAT3, FOXO1, miR-145, and DNA methylation of genes related to IL-17 signaling, including *Lpb*, *Rorc*, *Runx1*, *Il17ra*, *Rac1*, *Ccl5*, and *Il10*. Finally, maternal prebiotic intake could mitigate enduring immune dysfunction related to early-life antibiotic-induced dysbiosis in offspring by diminishing gut microbiota disturbances, reducing NF-κB levels, and inhibiting antibiotic-induced alterations in gut miRNAs, including miR-145.

These findings highlight the importance of dietary intervention as an effective approach to influence immune system programming during critical developmental stages and mitigate health issues stemming from early-life dysbiosis later in life.

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List of Abbreviations

AHCC	Active hexose correlated compound
A. muciniphila	Akkermansia muciniphila
AKT	Protein kinase B
APC	Antigen-presenting cell
B. fragilis	Bacteroides fragilis
CBLB	Cbl Proto-Oncogene B
CCL5	C–C chemokine ligand 5
CFU	Colony forming unit
DC	Dendritic cell
DMR	Differentially methylated region
DNMTs	DNA methyltransferases
E. coli	Escherichia coli
FOXOs	Forkhead box class O family member proteins
Foxp3	Transcription factor forkhead box protein 3
GALT	Gut-associated lymphoid tissue
HDACs	Histone deacetylases
IBD	Inflammatory bowel disease
IFN-γ	Interferon-gamma
IgA	Immunoglobulin A
IKBKG	Inhibitor of nuclear factor kappa B kinase regulatory subunit gamma
IL-17R	IL-17 receptor
ILC3	Type 3 innate lymphoid cell
ILF	Isolated lymphoid follicle
JAK	Janus kinase
LBP	LPS binding protein
LPS	Lipopolysaccharide
M cells	Microfold cells
MAPK	Mitogen-activated protein kinase
miRNA	microRNA
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor-kappa B
NGPs	Next generation probiotics
NK cell	Natural killer cell
NKT	Natural killer T cell
PAMPs	Pathogen-associated molecular patterns
PCA	Protocatechuic acid
PI3K	Phosphatidylinositol-3-kinase
PRRs	Pattern recognition receptors
Rac1	Rac family small GTPase 1

RORγt	Retinoic acid-related orphan receptor gamma t
RUNX1	Runt-related transcription factor 1
SAA	Serum amyloid A
SCFAs	Short-chain fatty acids
SFB	Segmented filamentous bacteria
SGK	Serum and glucocorticoid-regulated kinase
sIgA	Secretory IgA
SMAD4	SMAD Family Member 4
STAT3	Signal transducer and activator of transcription 3
SV-53	Rouxiella badensis subsp. acadiensis
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Th17 cell	T-helper 17 cell
TLR	Toll-Like receptor
TNF	Tumor necrosis factor
Treg	T-regulatory cell
TSS	Transcriptional start site
UTR	Untranslated region
ZO-1	Zonula occludens-1
γδ T cell	Gamma-delta T cell

Chapter 1: Introduction

1- Introduction

1-1- Gut Microbiota

The intestine harbors a complex and dynamic population of approximately 10^{13}-10^{14} microorganisms known as the gut microbiota. This microbial community has co-evolved with the host, maintaining a symbiotic relationship and providing mutual benefits. (Rinninella et al., 2019; Cheng et al., 2019). Gut microbiota performs essential functions in host metabolism and immunity. It also contributes to the metabolism of different nutrients such as vitamins, amino acids, and short-chain fatty acids (SCFAs). Therefore Gut microbiome equilibrium is necessary for optimal health (Rinninella et al., 2019).

Dysbiosis is defined as any alteration to the composition, function, metabolic activities, or local distribution of the resident commensal population relative to the healthy population that negatively affects the mutualistic relationships among microbial communities and thereby host health (Gagliardi et al., 2018; DeGruttola et al., 2016; Petersen and Round, 2014). Dysbiosis is linked to a variety of chronic inflammatory disorders, including Crohn's disease, ulcerative colitis, obesity, diabetes, and allergic diseases (DeGruttola et al., 2016).

The gut microbiota is primarily comprised of various bacterial phyla with unique roles in maintaining the homeostasis of the intestinal ecosystem, including Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria. Notably, Firmicutes and Bacteroidetes account for about 90% of the gut microbial population (Figure 1-1) (Rinninella et al., 2019).

The Clostridium genus dominates the Firmicutes phylum. Other important genera of this phylum are Lactobacillus, Bacillus, Enterococcus, and Ruminococcus (Rinninella et al., 2019). Firmicutes bacteria, including Clostridium species, and some species belonging to Ruminococcaceae and

Lachnospiraceae, are the primary sources of SCFAs production through the fermentation of prebiotics such as dietary fibers and complex carbohydrates in the gut (Houtman et al., 2022). SCFAs are one of the most important metabolites derived from gut commensals with a unique role in improving host immunity in the gut and other organs. The main SCFAs produced by gut microbiota are acetate, propionate, and butyrate (Liu et al., 2023). At the gut level, SCFAs modulate immune responses by regulating different pathways, including pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), phosphorylated mitogen-activated protein kinase (MAPK), and nuclear factor-kappa B (NF-κB) signaling pathways as well as regulation of immune cells, such as macrophages, natural killer cells, B cells, and T cells (Liu et al., 2023).

In addition, some Lactobacillus species are known for their health-promoting potential. Beneficial lactic acid bacteria belonging to this genus can metabolize carbohydrates to lactic acid. Many well-known probiotics, including *Lactobacillus acidophilus* (*L. acidophilus*), *L. brevis*, *L. casei*, *L. bulgaricus*, *L. gasseri*, *L. helveticus*, *L. reuteri*, and *L. rhamnosus* are members of this genus (Dempsey and Corr, 2022).

Bacteroidetes are known as the largest phylum of Gram-negative bacteria. Bacteroides, Alistipes, Parabacteroides, and Prevotella are some of the main representatives of this phylum (Gibiino et al., 2018). Host diet and mucus layer play an important role in forming and stability of gut bacteria composition, particularly Bacteroides species, by providing glycans. Mucin glycans are the major mediators of interaction between Bacteroides species and host, and their mutualistic function (Zafar and Saier, 2021). Bacteroides species possess polysaccharide utilization loci and are able to hydrolyze complex carbohydrates and, therefore, produce nutrients for the host and other gut commensals (Zafar and Saier, 2021). Dietary patterns high in fiber may increase the Bacteroides population (Zafar and Saier, 2021). Bacteroidetes species contribute to the

regulation of immune responses, metabolism, and the gut-brain axis (Gibiino et al., 2018). Bacteroidetes and host interaction play an important role in immune system development in early life (Gibiino et al., 2018).

The Firmicutes/Bacteroidetes ratio has been implicated in maintaining gut homeostasis and enhancing overall health. Changes in the Firmicutes/Bacteroidetes ratio are linked to inflammatory diseases. For instance, an elevated Firmicutes/Bacteroidetes ratio has been reported in obesity while a decreased ratio has been associated with IBD (Stojanov et al., 2020).

Proteobacteria are Gram-negative bacteria that are involved in nitrogen fixation. This phylum can be categorized into five subgroups, including Alpha, Beta, Gamma, Delta, and Epsilon Proteobacteria (Maheshwari and Sankar, 2023). Proteobacteria comprise a diverse array of pathogenic genera such as Escherichia, Shigella, Salmonella, and Yersinia (Rizzatti et al., 2017). Proteobacteria overgrowth may be associated with metabolic disorders such as obesity and nonalcoholic fatty liver disease, cardiovascular diseases, brain dysfunction, and gut inflammation (Rizzatti et al., 2017).

Actinobacteria are Gram-positive bacteria and constitute a small portion of the gut microbiota, but they play a significant role in maintaining gut homeostasis. The Bifidobacteria family is a well-known family in this phylum with distinct probiotic and health-promoting characteristics (Binda et al., 2018). These bacteria can produce SCFAs, mainly acetate that can protect the gut from invasion of pathogens, such as *Escherichia coli* (*E. coli*) and Shigella (Fukuda et al., 2012). Bifidobacteria may contribute to gut barrier defense by the production of SCFAs which, in turn, increase the mucin glycoproteins and tight junction protein expressions. Reduction of Bifidobacteria in the gut increases gut permeability, resulting in the translocation of bacterial

components to the circulation which leads to immune activation and sustained inflammation (Binda et al., 2018).

Fusobacteria, characterized as Gram-negative bacteria, constitute a minor percentage of gut microbiota. This phylum is divided into two microbial families, including the Leptotrichiaceae and Fusobacteriaceae (Brennan and Garrett, 2019). There is not an extensive amount of information available regarding the role of Fusobacteria in health and diseases; however, some evidence suggests a connection between Fusobacteria species, host inflammatory responses, and colon cancer (Kelly et al., 2018).

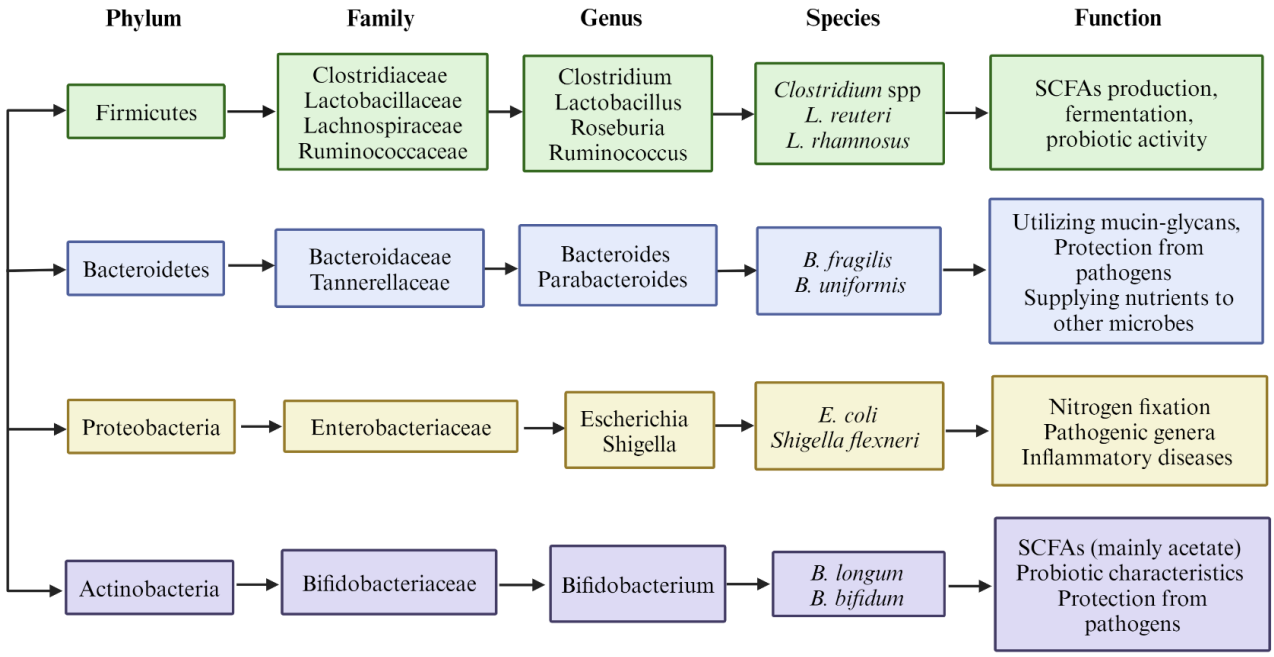

Figure 1-1. Main phyla in gut microbiota.

1-2- Gut Immune System

The intestine is recognized as the largest part of the immune system in the body, which remains in constant interaction with diverse antigens and immunomodulatory elements originating from both the diet and the resident commensals (Mowat and Agace, 2014).

The intestinal tract is composed of different regions with various anatomical and physiological properties. The surface epithelium of the intestine consists of absorptive enterocytes, Paneth cells, Goblet cells, and neuroendocrine cells. Mucosa comprises the epithelium, the lamina propria, and the muscularis mucosa, and is the major site of immunological processes. The majority of intestinal innate and adaptive immune cells are found in the lamina propria. The lamina propria includes B cells, T cells (Both $CD4^+$ T cells and $CD8^+$ T cells), and many innate immune cells, such as dendritic cells (DCs), and macrophages (Santaolalla and Abreu, 2012; Mowat and Agace, 2014).

Paneth cells, found in the small intestine with the largest number in the ileum, produce antimicrobial peptides such as lysozyme and β-defensins in response to cytokines and stimulation of PRRs (Marsal and Agace, 2012; Mowat and Agace, 2014; Santaolalla and Abreu, 2012). Goblet cells contribute to mucus secretion. Mucus has antibacterial activities by acting as a physical barrier, generating mucin glycoproteins that are toxic to numerous bacteria and providing a matrix for antibodies and antimicrobial peptide adhesion (Mowat and Agace, 2014).

The gut barrier protects against foreign antigens and pathogens that come into contact with the host's immune system. The commensal microbiota, the mucus layer housing secretory immunoglobulin A molecules (sIgA) and antimicrobial peptides, the epithelial cells, and the gut-associated lymphoid tissue (GALT) are the key constituents of the gut barrier (Dempsey and Corr, 2022). In the intestine, B cells produce IgA antibodies in response to commensals. IgA contributes to the shaping and balance of microbial communities and the maintenance of gut barrier function by inhibiting pathogens' attachment to epithelial cells and neutralizing toxins (Zheng et al., 2020). Gut barrier dysfunction correlates with the pathogenesis of various disorders such as IBD, diabetes, and obesity (Dempsey and Corr, 2022).

Lymphoid structures in the intestine, including GALT are the main sites for initiating adaptive immune system responses in the intestine. GALT includes Peyer's patches, intraepithelial lymphocytes, and isolated lymphoid follicles (ILFs) (Mowat and Agace, 2014; Marsal and Agace, 2012; Stahl and Belkind-Gerson, 2021). Peyer's patches contain many B and T cell zones and are the primary source of small intestine-IgA-secreting cells. The surfaces of intestinal lymphoid tissues are covered by a layer of follicle-associated epithelium. This layer contains conventional intestinal epithelial cells and a small number of specialized epithelial cells called microfold cells (M cells) (Mowat and Agace, 2014; Marsal and Agace, 2012).

The uptake of antigens from the lumen across the epithelium and presenting to the immune cells applies various transport mechanisms. In the intestine, pathogens are collected by M cells in the follicle-associated epithelium of Peyer's patches and ILFs to be transported to antigen-presenting cells (APCs) (Houston et al., 2016; Marsal and Agace, 2012).

1-3- Innate and Adaptive Immune Responses in the Gut

Innate immune response in the intestine is initiated by PRRs such as TLRs. PRRs recognize microbial products, known as pathogen-associated molecular patterns (PAMPs), which are essential for the microorganism's survival. Examples of these microbial components include lipopolysaccharide (LPS), lipoteichoic acid, peptidoglycan, and nucleic acids. TLRs are expressed on various immune cells, such APCs, including macrophages and DCs, as well as B cells, some types of T cells, and non-immune cells such as epithelial cells in response to pathogens, and some cytokines (Akira et al., 2006; Littman and Rudensky, 2010).

TLRs are the link between innate and adaptive immunity. Following exposure to TLR ligands, adaptor proteins such as myeloid differentiation factor 88 (MyD88) bind to the cytoplasmic portion

of the TLRs, resulting in initiating downstream signaling cascades like NF-κB and the production of pro-inflammatory cytokines and chemokines, and activation of APCs (Akira et al., 2006; Shi et al., 2016). APCs stimulate the adaptive immune response by presenting microbial antigens to naïve $CD4^+$ T cells leading to $CD4^+$ T cell differentiation such as T helper cells (Th cells) and regulatory T cells (Treg) (Akira et al., 2006). Activated APCs also induce the differentiation of naive $CD4^+$ T cells into Th1 cells or Th2 cells by expressing co-stimulatory molecules. Th1 cells produce interferon-gamma (IFN-γ) and mediate the elimination of bacterial and viral infection. Th2 cells, that produce IL-4, IL-5, and IL-13, eliminate helminth infection (Akira et al., 2006).

DCs in the lamina propria of ileum induce secretion of IL-6, IL-23, IL-1β, and transforming growth factor-β (TGF-β), which promotes Th17 differentiation. Th17 cells have recently been identified as a distinct $CD4^+$ T-helper subset characterized by IL-17 production that promotes inflammatory responses (Omenetti and Pizarro, 2015). Retinoic acid-related orphan receptor gamma t (RORγt) is the master transcriptional factor of Th17 differentiation (Bhaumik and Basu, 2017). IL-17A, IL-17F, IL-21, and IL-22 are the main cytokines produced by Th17 cells (Shen and Chen, 2018).

In addition to the Th17 cells, Treg cells are frequently found within the intestinal mucosa with opposing functions. Treg cells are a subpopulation of T cells that play a critical role in the suppression of immune response, thereby maintaining homeostasis and self-tolerance. It has been shown that Tregs can inhibit T helper proliferation and cytokine production and play a critical role in preventing autoimmunity (Kondělková et al., 2010). The intestinal microenvironment is one of the main places for Treg generation, because of the continuous exposure to pathogens which results in a high immune challenge, and therefore, the need for active immune suppression to sustain intestinal homeostasis (Harrison and Powrie, 2013). Transcription factor forkhead box protein 3

(Foxp3) is the most specific marker for Tregs (Kondělková et al., 2010) and is critical for the production and maintenance of a regulatory T cell phenotype (Harrison and Powrie, 2013).

The abnormal ratio of Th17 and Treg cells is associated with some metabolic or immunologic disorder-associated diseases such as colitis, allergic diseases, autoimmune diseases, and cancers (Littman and Rudensky, 2010).

1-4- Gut Microbiota and Gut Immune Homeostasis

Gut microbiota improves host innate and adaptive immunity by different mechanisms, including competition with invading and indigenous pathogens for nutrients and sites, and therefore, restricting pathogens' overgrowth and colonization. It also improves signal transduction by PPRs, antimicrobial substance and immunoglobulin productions, mucin secretion, and tight junction protein expressions, therefore, enhancing gut barrier defense (Kamada et al., 2013; Binda et al., 2018). Gut microbial communities also preserve gut homeostasis by regulating microRNAs (miRNAs) and, therefore, innate and adaptive immunity (Li et al., 2020). Gut microbiota metabolites also improve innate immune cell functions (Negi et al., 2019) as well as adaptive immunity by regulating immune cell differentiation, including Treg and T helper cells (Omenetti and Pizarro, 2015). Figure 1-2 illustrates the role of gut microbiota in gut immunity.

Disruption of the gut microbiota is associated with an imbalanced T cell population (Zhang and Chen, 2019). The role of gut microbiota in regulating the T cell balance was recognized when studies revealed an alteration in Th17 and Treg cells expression in the intestine of germ-free mice (Omenetti and Pizarro, 2015). Clostridium clusters IV and XIVa, segmented filamentous bacteria (SFB), and *Bacteroides fragilis* (*B. fragilis*) are the most important bacteria controlling T cell production in the gut (Cheng et al., 2019).

Clostridium clusters IV and XIVa are Gram-positive bacteria that participate in Treg production and intestinal inflammation prevention. The ability of Clostridium clusters to induce Treg expansion in the intestine is attributable to their SCFAs-producing characteristics. Specifically, SCFAs induce the secretion of TGF-β1 from epithelial cells and thus production of Treg cells (Cheng et al., 2019).

SFB are commensal bacteria that manifest as long, segmented filaments that adhere to the ileal epithelium. SFB represents the first commensal bacteria identified to influence the differentiation and development of Th17 cells in mice (Omenetti and Pizarro, 2015; Hedblom et al., 2018). Representation of SFB in germ-free mice induces differentiation of Th17 cells (Ravindran et al., 2016). In a study, colonization of the small intestine of Th17 cell-deficient mice with solely SFB induced the expression of Th17-related cytokines in $CD4^+$ T cells in the lamina propria, indicating the key role of SFB in Th17 generation (Ivanov et al., 2009). Furthermore, SFB colonization stimulates the production of serum amyloid A (SAA) in the ileum, which in turn, induces DCs in lamina propria, leading to Th17 cell differentiation and IL-17 expression (Ravindran et al., 2016; Sano et al., 2015). SAA-induced IL-23 secretion from DCs also contributes to Th17 survival and maintenance (Sano et al., 2015). Other APCs have also been found to secret IL-1β in response to SFB which acts together with IL-6, TGF-β, and SAA to enhance Th17 cell production (Cheng et al., 2019).

B. fragilis influences T cell generation and differentiation through TLRs signaling. This role of *B. fragilis* is namely related to polysaccharide A (PSA), a component of its capsule. PSA promotes $CD4^+$ T cells to acquire $Foxp3^+$ Treg phenotype and produce IL-10 in germ-free animals (Round and Mazmanian, 2010). In human fetal enterocytes, PSA also can suppress the production of Th17-inducing cytokines such as IL-1β through the TLR2 and TLR4 signaling, leading to suppression

of the differentiation of Th17 cells (Cheng et al., 2019). Other commensal bacteria such as Bifidobacterium also play an important role in T cell balance (Cheng et al., 2019). In addition, *Lactobacillus acidophilus*, *Faecalibacterium prausnitzii*, and *Roseburia intestinalis* may be linked to the differentiation of Th1 cells (Gehlhaar et al., 2022).

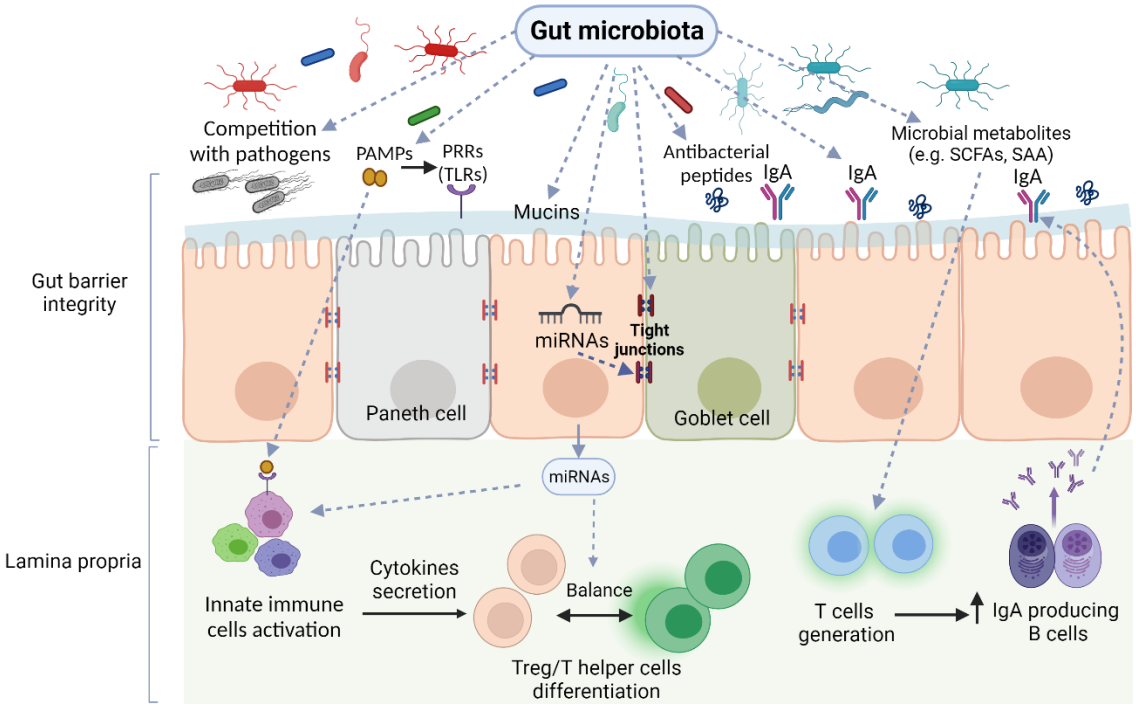

Figure 1-2. Role of gut microbiota in gut immunity. Role of gut microbiota in gut immunity homeostasis. Gut microbial communities inhibit the growth of pathogens, regulate signal transduction by PRRs, induce IgA, antimicrobial peptides, and mucin secretions, tight junction protein expressions, and modulate gut miRNAs, therefore, preserving gut barrier integrity. In addition, by regulating PRRs and antigen-presenting, modulating miRNAs, and secretion of different metabolites such as SCFAs and SAA, gut microbiota plays a crucial role in adaptive immunity balance. PAMPs: Pathogen-associated molecular patterns; PRRs: Pattern recognition receptors; TLRs: Toll-like receptors; IgA: Immunoglobulin A; SCFAs: Short chain fatty acids; SAA: Serum amyloid A; Treg: Regulatory T cells. The image was created by BioRender.com.

1-5- Signaling Pathways in Gut Immunity

1-5-1- IL-17 Signaling

The IL-17 family of cytokines comprises six members: IL-17A, B, C, D, E, and F. The most studied cytokines in this family are IL-17A and F which share structural and functional similarities. IL-

17A and F function through the IL-17 receptor (IL-17R), which is composed of two subunits, IL-17RA/IL-17RC, leading to various cellular responses involved in immune regulation and inflammation (Rex et al., 2023; Pappu et al., 2011). IL-17A and F are potent proinflammatory cytokines (Zenobia and Hajishengallis, 2015), and their over-expression has been reported in many autoimmune diseases, such as IBD and rheumatoid arthritis (Fujino et al., 2003; Pappu et al., 2011). In the gut, Th17 cells serve as the primary source of IL-17 production in adaptive immunity. (Jin and Dong, 2013). Differentiation of naïve $CD4^+$ T cells into Th17 cells happens following T cell receptor (TCR) stimulation by PAMPs in the presence of a set of upstream cytokines, including, IL-1β, IL-6, and IL-23 (Geha et al., 2017; Cui, 2019) and activation of signal transducer and activator of transcription 3 (STAT3) signaling which supports IL-17 expression, and inflammatory responses (Woś and Tabarkiewicz, 2021; Lee et al., 2017). Innate immune cells such as gamma-delta (γδ) T cells, type 3 innate lymphoid cells (ILC3s), and Natural killer T cells (NKT) are other main sources of IL-17A and F production in the intestine (Jin and Dong, 2013; Chung et al., 2021; Pappu et al., 2011). Additionally, gut commensals, their metabolites, and epigenetic processes control IL17A and F production at the gut level (Mukasa et al., 2010; Mikami et al., 2021; Dupraz et al., 2021; Ivanov et al., 2009).

1-5-2- Forkhead Box Class O-1 (FOXO1)

Forkhead box class O family member proteins (FOXOs) are transcription factors involved in various physiological processes, such as cell cycle, cell survival, and metabolism. FOXO1 is an important subclass of this family and is best studied for its role in metabolic pathways regulation, including adipogenesis and the inhibition of glucose production in response to insulin (Mallet et al., 2021; Tsuchiya and Ogawa, 2017). Besides, FOXO1 possesses a key role in hematopoietic stem cell maintenance and immune cell development (Cabrera-Ortega et al., 2017), including DCs,

B cells, and T cells (Cabrera-Ortega et al., 2017). FOXO1 increases the Foxp3 expression, thereby Treg differentiation, and function (Cabrera-Ortega et al., 2017), while it suppresses the Th17 cells differentiation and IL-17A expression (Cabrera-Ortega et al., 2017). Serum and glucocorticoid-regulated kinase (SGK) and AKT are the main inhibitors of FOXO1 (Wang et al., 2014). PI3K/AKT signaling leads to transcriptional inactivation of FOXO1 (Wang et al., 2014). Besides regulating T cell differentiation, a recent study found that FOXO is necessary for gut immunity homeostasis, and loss of FOXO in intestinal epithelial cells impairs mucus secretion, and tight junction integrity, and leads to dysbiosis (Chen et al., 2021).

1-5-3- STAT3

STAT proteins are a family of transcription factors with a critical role in signal transduction from the cell membrane to the nucleus, where they induce or repress gene expressions. STAT proteins control several cellular processes, such as cell growth, differentiation, and immune responses. Among STAT proteins, STAT3 is well known for its role in regulating immune responses and inflammation, most importantly Th17 cell differentiation (Seif et al., 2017). Janus kinase (JAK) proteins are activators of STAT proteins, including STAT3, which phosphorylate and activate STAT3 in response to cytokines (Seif et al., 2017; Fu, 2006). STAT3 exerts a crucial role in adaptive immunity (Egwuagu, 2009). Depending on the cytokine profiles, including IL-6, IL-1β, IL-23, TGF-β, and IL-10, STAT3 contributes to the production of either Treg or Th17 cells from naive $CD4^+$ T cells (59). For example, IL-6 stimulates STAT3 activation (Yang et al., 2007) and STAT3 induces IL-23 activity (Cho et al., 2006). STAT3/IL-23 stimulates Th-17/IL-17A inflammatory signaling and inflammatory responses (Cho et al., 2006).

1-5-4- NF-κB

The transcription factor NF-κB plays a crucial role in the regulation of differentiation, maintenance, and the function of innate cells such as M1 macrophages and inflammatory T cells such as Th1 and Th17, and acts as a mediator of inflammatory responses by increasing expression of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-12p40 (Liu et al., 2017).

NF-κB family includes p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB, and c-Rel. NF-κB activation involves canonical and non-canonical pathways. The canonical pathway is involved in the activation of canonical NF-κB family members, including p50, p65, and c-Rel, while the non-canonical pathway mediates the activation of p52 and RelB (Liu et al., 2017). Under physiological conditions, NF-κB is kept inactive in the cytoplasm, sequestered by inhibitors known as inhibitors of the κB (IκB) family. Signals from various stimuli, such as cytokines and LPS triggers activation of the canonical pathway, leading to IκB degradation and subsequent nuclear translocation of NF-κB subunits, where they induce expression of inflammatory genes (Wang and Shen, 2022; Liu et al., 2017). The canonical pathway is recognized for its role in directing inflammatory immune response against microbial pathogens (Trares et al., 2022). NF-κB non-canonical activation is mediated by NF-κB inducing kinase (NIK), primarily in response to signals from the tumor necrosis factor receptor (TNFR) superfamily (Wang and Shen, 2022; Liu et al., 2017).

Over-expression of NF-κB participates in intestinal inflammation (Pasparakis, 2008). In addition, TNF-α-induced activation of NF-κB is related to tight junction damage and gut permeability (Al-Sadi et al., 2016). IL-17 signaling also activates the NF-κB signaling cascade in intestinal epithelial cells (Awane et al., 1999). Probiotics and gut commensals, particularly members of Firmicutes, such as *L. casei*, *L. reuteri*, *L. rhamnosus*, and *L. salivarius*, may inhibit NF-κB signaling. This can be done by applying different mechanisms such as inhibiting pathogens-induced NF-κB

expression, suppressing TLR signaling, and inhibiting TNF-α, IL-1β, and LPS-induced NF-κB activation (Bhardwaj et al., 2020). Some strains of Clostridium may also suppress NF-κB signaling and alleviate intestinal inflammation (Giri et al., 2022).

1-6- Epigenetic Mechanisms and Gut Immunity

Epigenetic modifications are heritable, often reversible changes that regulate gene expression and function without altering the underlying DNA sequence. These mechanisms play a central role in determining how genes are expressed or silenced, directing various cellular processes. Unlike genetic changes, which involve alterations in the DNA sequence, epigenetic modifications involve chemical alterations to the DNA molecule and changes in DNA accessibility by DNA methylation, histone methylation, and histone acetylation (Liu et al., 2008a; Handy et al., 2011). Environmental factors, such as diet, stress, and drugs, influence epigenetic mechanisms (Liu et al., 2008a; Shepard and Nugent, 2020). Numerous studies have demonstrated that maternal diet during pregnancy can epigenetically impact the expression of critical genes that regulate metabolic and immune pathways in offspring, thereby increasing susceptibility to chronic diseases later in life (Nash et al., 2023; Masuyama and Hiramatsu, 2012; Masuyama et al., 2015). Notably, some of these epigenetic changes are not reversible through nutritional changes in offspring (Zhang et al., 2019b). This evidence highlights the crucial role of nutrition in fetal development by regulating epigenetic mechanisms and influencing long-term health consequences. Non-coding RNA molecules such as miRNAs, DNA methylation, and histone modifications are key epigenetic mechanisms affecting gene expression (Shepard and Nugent, 2020).

1-6-1- Gut miRNAs and Gut Immunity

miRNAs comprise a large family of about 22 nucleotide-long, highly conserved noncoding RNAs, which play significant roles in the post-transcriptional regulation of targets governing various biological processes (Park et al., 2017).

The biosynthesis of miRNAs is a multistep process and initiates with the transcription of miRNA genes by the action of RNA polymerase II or III. This process leads to the production of long, hairpin-shaped primary miRNA transcripts (pri-miRNAs). In the nucleus, these pri-miRNAs are cleaved by the Drosha-DGCR8 (Pasha) complex, resulting in precursor miRNAs (pre-miRNAs), which are subsequently exported to the cytoplasm by protein Exportin-5. In the cytoplasm, a second cleavage event occurs on pre-miRNAs through the action of the enzyme Dicer (RNase Dicer) along with the double-stranded RNA-binding protein (TRBP), leading to the generation of short double-stranded RNA molecules. The mature miRNA strand, together with argonaute (Ago2), is incorporated into the RNA-induced silencing complex (RISC). The miRNA directs the RISC complex to target mRNAs through base-pairing with target mRNAs at the 3′-untranslated region (3′UTR), resulting in mRNA degradation or translational repression. Dysregulation of miRNA expression or activity is involved in the pathogenesis of a variety of diseases (Winter et al., 2009; Macfarlane and Murphy, 2010).

miRNAs gene transcription in intestinal epithelial cells can be regulated through TLRs and associated downstream signaling pathways, including NF-κB and MAPK pathways, upon specific microbial recognition (Zhou et al., 2011). Gut miRNAs are key players in maintaining a healthy gastrointestinal environment by regulating the intestinal immune system (Bi et al., 2020). Specific miRNAs contribute to epithelial regeneration, differentiation of Paneth cells and their antimicrobial function, differentiation of Goblet cells, and mucus production (Park et al., 2017).

These molecules also affect gut barrier function by regulating tight junction proteins, including claudins, and occludin (Al-Sadi et al., 2020). Some miRNAs such as miR-155 and miR-223 influence innate immunity by regulating PRRs expression and signaling as well as APCs function (Bi et al., 2020). miRNAs participate in adaptive immunity by facilitating the differentiation of Th1, Th2, Th17, or Treg cells (Bi et al., 2020) via regulating different signaling pathways directing T cell generation and function such as STAT3 pathway (Mikami et al., 2021). For example, miR-155 has been shown to induce Th17 differentiation and inhibit Treg differentiation, while miR-125a induces Treg differentiation and inhibits Th17 differentiation (Bi et l., 2020).

Gut miRNAs play a crucial role in shaping the structure and distribution of microbial communities within the intestine (Bi et al., 2020). miRNAs can enter bacteria (such as *E. coli*), and regulate transcription of bacterial genes and bacterial growth. Mice deficient in miRNAs in intestinal epithelial cells exhibit uncontrolled gut microbiota and colitis (Viennois et al., 2019). On the other hand, gut microbiota regulates miRNA expressions in the host. miRNAs originating from intestinal epithelial cells are secreted in the lumen and accumulate in feces (Viennois et al., 2019). In a study, the effect of gut microbiota on specific miRNA expressions in feces was investigated using germ-free and antibiotic-treated animals. In germ-free animals, an altered miRNA profile was observed in the feces of germ-free mice compared to conventional mice. Also, following antibiotic-mediated depletion of gut microbiota, a reduction in fecal miRNAs was observed (Moloney et al., 2018) which indicates the role of gut microbiota in shaping gut miRNA signature.

1-6-2- DNA Methylation and Gut Immunity

DNA methylation involves the addition of a methyl group to the DNA molecule. Specifically, it takes place at the cytosine in 5′-C-phosphate-G-3′(CpG) dinucleotides and is catalyzed by enzymes known as DNA methyltransferases (DNMTs), including DNMT1, DNMT3A, and DNMT3B

(Handy et al., 2011; Li et al., 2022). CpG dinucleotides exhibit an uneven distribution across chromosomal DNA and can cluster in areas known as CpG islands, which are often associated with gene regulatory regions and may overlap gene promoters. Maintenance of DNA methylation state and epigenetic information is done by DNMT1 (Handy et al., 2011). The methylation status of the regions near the transcriptional start sites (TSS), including the 1-5 kb region before the promoter, promoter, and the 5′UTR sequence controls gene expression. DNA hypermethylation around TSS correlates with gene expression repression (Yuan et al., 2016; Hong and Rhee, 2022).

Dietary compounds regulate epigenetic mechanisms in the host. For instance, dietary fibers are utilized by gut microbiota leading to the production of various metabolites that serve as substrates for enzymes catalyzing epigenetic mechanisms (Li et al., 2022). Gut microbiota also affects the host by regulating epigenetic mechanisms. For example, in a mouse model of high-fat diet-induced obesity, gut microbiota disturbance by antibiotics was associated with alteration in the expression of genes related to obesity through modifying DNA methylation (Yao et al., 2020). Firmicutes and Bacteroides may also regulate host metabolism by DNA methylation (Li et al., 2022).

At the gut level, microbiota-mediated epigenetic programming is required for proper intestinal homeostasis and function, and controlling inflammation (Ansari et al., 2020). For example, the differentiation of Th17 and Treg cells from naïve $CD4^+$ T cells is controlled epigenetically by different mechanisms, including DNA methylation, miRNA expression, and histone modification (Luo et al., 2017). Commensal-derived metabolites (e.g. SCFAs) alleviate intestinal inflammation and enhance intestinal immunological homeostasis by promoting Treg differentiation epigenetically via inhibiting histone deacetylases (HDACs) and inducing histone H3 acetylation of the Foxp3 gene (Furusawa et al., 2013). Vitamins such as folic acid, B12, and B2 are other metabolites produced by gut microbiota that act as methyl group miRNAs gene transcription in

intestinal epithelial cells can be regulated through TLRs and associated downstream signaling pathways, including NF-κB and MAPK pathways, upon specific microbial recognition in epigenetic mechanisms. For example, Lactobacillus and Bifidobacterium produce folic acid which participates in S-adenosyl methionine generation, as the primary methyl group donor for DNA and histone methylation (Woo and Alenghat, 2022; Li et al., 2022). Furthermore, gut microbiota may control intestinal inflammation by inducing DNA methylation of the gene encoding TLR4 and suppressing its expression in the intestinal epithelial cells. This happens by gut microbiota-dependent adaptor molecules that recruit DNMT3 to the *TLR4* gene (Narabayashi et al., 2022).

1-7- Antibiotics, Gut Microbiota, and Gut Immunity

Antibiotics are an indispensable medical treatment that has saved millions of lives since their discovery. In addition, the utilization of low levels of antibiotics in agriculture introduces these agents to the food and water supply, therefore exposing healthy individuals to antibiotics. Despite their beneficial effects, it is important to highlight the harmful lasting effects of antibiotic use on children and adults. Epidemiological studies have disclosed a correlation between exposure to various classes of antibiotics and the increased risk of developing inflammatory disorders, including allergies, asthma, obesity, and autoimmune conditions. This correlation is attributed to the disturbance of gut microbiota induced by antibiotics, rather than a direct side effect of the antibiotic itself (Knoop et al., 2016).

Besides targeting pathogenic bacteria, antibiotics can also affect commensal microbes. Antibiotics impact gut microbiota directly by reducing the diversity and abundance of microbiota communities and indirectly by impairing symbiotic relationships and codependency among the various subsets of the microbiota (Zhang and Chen, 2019). The metabolites produced by some species contribute to the expansion of other species. For instance, some species of Bifidobacterium generate lactate

and acetate by utilizing fructooligosaccharides which can be used by butyrate-producing bacteria as growth substrates (Zhang and Chen, 2019; Heinken and Thiele, 2015). On the other hand, some species, such as Lactobacilli, Bifidobacteria, and Clostridium, are able to metabolize toxic metabolites, such as conjugated bile acids, which act as growth inhibitors of other species (Rowland et al., 2018; Zhang and Chen, 2019). Therefore, the loss of some microbiota populations can change the metabolites and the microenvironment in the gut, which negatively impacts other populations (Zhang and Chen, 2019).

Gut microbiota communicates with the host through signal transduction by PPRs. Depletion of bacteria with antibiotics inhibits signal transduction from the gut microbiota to the host, disturbing innate and adaptive immune responses (Zhang and Chen, 2019; Ubeda and Pamer, 2012; Lazar et al., 2018).

Moreover, antibiotic intake may result in inflammation by releasing LPS derived from pathogenic Gram-negative bacteria (Ramirez et al., 2020; Breijyeh et al., 2020; Sun and Shang, 2015). LPS is a component of the outer membrane of the cell wall of Gram-negative bacteria. Among antibiotics, cell wall-active agents, such as β-lactams are often identified as the primary contributors to the release of LPS. These classes of antibiotics inhibit the activity of bacterial enzymes required for cell wall biosynthesis, known as penicillin-binding proteins, leading to bacterial lysis (Kirikae et al., 1998). In addition, antibiotic-induced translocation of commensal bacteria throughout the large intestine leads to inappropriate immune responses against translocated commensals and a predisposition for intestinal inflammation (Knoop et al., 2016).

Different strategies have been recommended to alleviate antibiotic-induced gut microbial perturbation and/or immune dysfunction, including administration of bacterial lysates or products

containing various PRR ligands to restore signal transduction by PPRs and immune homeostasis; oral administration of probiotics to prevent dysbiosis or restore the gut microbiota and immunity balance; and fecal microbiota transplantation which mitigates intestinal inflammation and restore intestinal homeostasis by different mechanisms, including inducing anti-inflammatory cytokines secretion, epithelial regeneration, and antimicrobial peptide production (Zhang and Chen, 2019).

1-8- LPS, Gut Microbiota, and Gut Immunity

LPS molecules consist of three distinct domains (Galgano et al., 2022). The lipid A moiety is a hydrophobic region that facilitates the interaction of LPS with TLR4 on immune cells via its phosphate groups and acyl chains, and is responsible for the endotoxic properties of LPS, activation of inflammatory signaling, and production of cytokines (Yamashita et al., 2021; Galgano et al., 2022). The central part, comprised of oligosaccharides, serves as the connection between the lipid A moiety with the O-antigen. The O-antigen consists of polysaccharides and is instrumental in the distinction between bacterial species (Galgano et al., 2022). Figure 1-3 illustrates the structure of the LPS molecule.

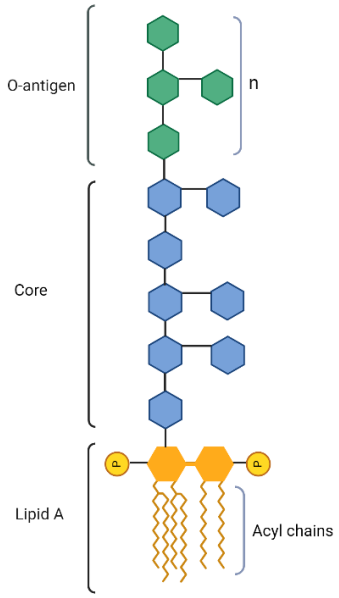

Figure 1-3. Structure of the LPS molecule. The image was created by BioRender.com

A notable aspect of the interaction between the host and microbiota in a healthy state is the host's ability to establish tolerance towards gut commensals along with retaining the capacity to initiate immune responses against pathogens (Zheng et al., 2020). Distribution of the balance between tolerance and immunity may result in the onset of various intestinal pathologies, such as IBD (Ahluwalia et al., 2018). In fact, within a healthy gut microbiota, the collective LPS originating from gut commensals exhibit immunoinhibitory activity. In a well-balanced gut microbiota, the primary contributors to LPS production are bacteria from the Bacteroidetes phylum that exerts immunoinhibitory properties by antagonizing TLR4, thereby suppressing downstream inflammatory pathways (d'Hennezel et al., 2017). Interaction between pathogenic LPS and TLR4 is mediated by different proteins, including LPS binding protein (LBP) and CD14 (Ciesielska et al., 2021). Inflammatory stimuli trigger the secretion of LBP by enterocytes (Richter et al., 2012). LBP forms a complex with LPS via binding to the lipid A moiety of LPS and subsequently transfers LPS to membrane-bound CD14, which delivers LPS to TLR4, leading to its activation (Ciesielska et al., 2021). However, in a steady state, the under-acylated structure of the lipid A in the LPS of non-pathogenic Gram-negative commensals is responsible for the silencing of TLR4 and immunoinhibitory properties of total LPS from the gut microbiota (d'Hennezel et al., 2017).

As stated earlier, healthy microbiota composition is crucial in maintaining the host gut barrier (Kamada et al., 2013; Binda et al., 2018). Inflammation-related alterations of gut microbiota can lead to a compromised intestinal barrier. In fact, gut epithelium barrier dysfunction and increased gut permeability known as leaky gut could be induced by different factors that change gut microbiota balance such as unhealthy diets (Western diet, high-fat diet), obesity, antibiotics intake, or exposure to exogenous LPS (Guo et al., 2013; Mohammad and Thiemermann, 2020; Feng et al., 2019). A leaky gut leads to the entrance of endogenous LPS originating from gut microbiota

to systemic circulation, causing metabolic endotoxemia, subsequent activation of inflammatory pathways such as NF-κB, and production of proinflammatory cytokines which results in low-level chronic inflammation with more severe outcomes than acute inflammation (Candelli et al., 2021; Mohammad and Thiemermann, 2020). In addition, an increase in the Gram-negative bacteria population in the gut contributes to the sustained production of LPS and results in sustained low-grade inflammation (Rizzatti et al., 2017). Persistent and uncorrected low-grade chronic inflammation ultimately leads to the development of chronic inflammatory disorders such as obesity, type 2 diabetes, fatty liver disease, and cardiovascular diseases (Mohammad and Thiemermann, 2020; Candelli et al., 2021).

Exposure to exogenous LPS is accompanied by gut dysbiosis (Murray et al., 2019), increased gut permeability (Guo et al., 2013), and inflammation-induced immune dysfunction (Yahfoufi et al., 2023). LPS exposure induces a shift in both gut microbiota and the immune system toward an inflammatory pattern. In GALT, LPS decreases Treg and increases Th17 cell frequencies (Candelli et al., 2021). Exogenous LPS can also damage the gut barrier through the production of inflammatory cytokines (Candelli et al., 2021). It has been shown that intraperitoneal injection of LPS increases intestinal permeability in mice by increasing TLR4 expression in the enterocyte membrane (Guo et al., 2013). Overexpression of TLR4 increases intestinal permeability through the down-regulation of tight junction proteins such as occludin in the intestinal epithelial barrier (Li et al., 2013). Figure 1-4 illustrates the effect of LPS/antibiotic exposure on gut immunity.

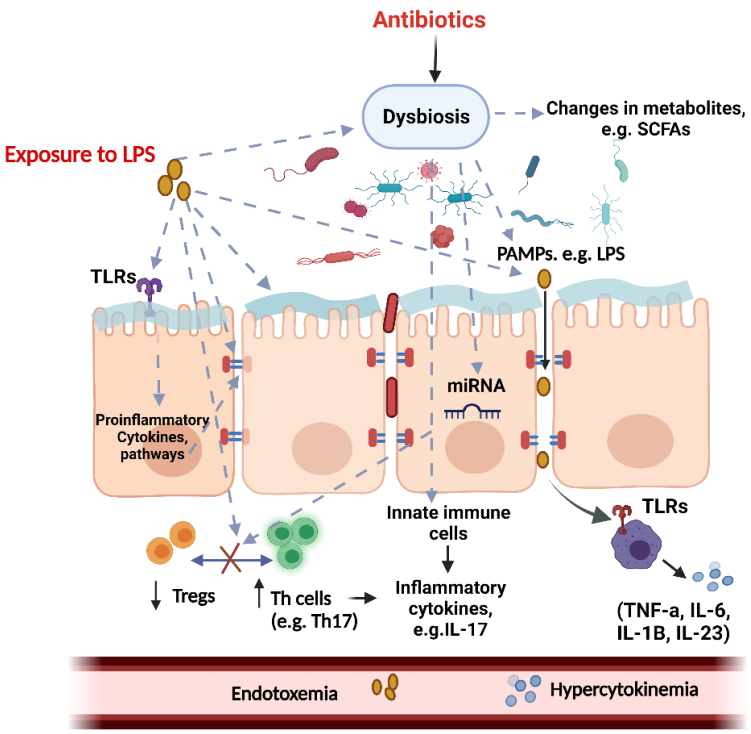

Figure 1-4. Effect of LPS and antibiotic exposure on gut immunity. LPS exposure may affect gut immunity by inducing gut dysbiosis, activating PRRs such as TLR4 and related inflammatory pathways, damaging mucus layer and tight junction proteins, and increasing gut permeability. LPS-induced TLR expression on immune cells leads to the secretion of inflammatory mediators and alters the adaptive immunity balance. Antibiotic exposure affects gut immunity by inducing gut microbiota dysbiosis, changing signal transduction by PRRs, changing gut microbiota metabolites and PAMPs, altering miRNA profile, and inducing the imbalance of innate and adaptive immune cells. The image was created by BioRender.com.

1-9- Probiotics, Prebiotics, and Gut Immunity

Probiotics are defined as live microorganisms that promote host health when consumed in adequate amounts (Hill et al., 2014). The most administrated probiotics belong to Gram-positive bacteria, including Lactobacillus, Bacillus, Bifidobacterium, and Enterococcus, and yeast such as *Saccharomyces boulardii* (Zhang and Chen, 2019). However, *E. coli* Nissle 1917 and *Akkermansia muciniphila* (*A. muciniphila*) are the most administrated Gram-negative probiotics (Behnsen et al., 2013; Xue et al., 2023). The main sources of probiotics in the human diet are dairy and non-dairy fermented foods (Syngai et al., 2016).

To be considered probiotics, microorganisms must have key characteristics, such as being non-pathogenic and non-toxic, being resilient in the gastrointestinal environment, and being able to effectively adhere to and colonize the intestinal epithelium, thereby exerting immunomodulatory activities (Syngai et al., 2016).

Scientific research has demonstrated the health benefits of probiotics by preventing or treating different health conditions, including antibiotic-associated diarrhea, irritable bowel syndrome, IBD, lactose intolerance, allergy, reduction in serum cholesterol, and anticancer effects due to their immunomodulatory activities (Syngai et al., 2016).

At the gut level, probiotics contribute to the homeostasis of the gut microenvironment. Probiotics have been shown to preserve gut barrier integrity by preventing overgrowth and colonization of pathogens, inducing the production of antimicrobial peptides such as β-defensin-2, and promoting mucus secretion by Goblet cells (Yahfoufi et al., 2018; Zhang and Chen, 2019; Bermúdez-Brito et al., 2012). Additionally, probiotics elevate the abundance of IgA-producing cells in the lamina propria and IgA secretion which improves mucosal immunity (Li et al., 2020). Probiotics induce DCs maturation and modulate adaptive immune responses. They down-regulate the differentiation of pathogenic Th17 cells and inflammatory IL-17A expression while upregulating Treg production (Yahfoufi et al., 2018).

Furthermore, probiotics modulate gut immunity by regulating epigenetic mechanisms. Probiotics direct the expression of gut miRNA associated with immunity (Davoodvandi et al., 2021). For instance, research has demonstrated that *E. coli* Nissle 1917 can mitigate gut inflammation in mice by restoring disrupted miRNA expression, including miR-143, miR-150, miR-155, miR-223, and miR-375, all involved in the inflammatory response during colitis (Rodríguez-Nogales et al.,

2018a). In addition, lactic acid bacteria have been identified to suppress NF-κB activity, as well as NF-κB-induced activation of IL-23/IL-17 signaling, as important factors involved in the pathogenesis of IBD, through the inhibition of histone acetylation and the stimulation of DNA methylation (Ghadimi et al., 2012).

Recently, there has been increasing interest in next-generation probiotics (NGPs). NGPs are defined as live microorganisms identified through comparative microbiota analyses, which, when consumed in adequate quantities, provide health advantages to the host. Advances in sequencing methods, tools, and computational techniques have facilitated the identification and isolation of NGPs (Kaźmierczak-Siedlecka et al., 2022; Martín and Langella, 2019). Many studies have demonstrated the health-promoting potential of this new category of probiotics. Similar to traditional probiotics, NGPs have been shown to promote gut barrier integrity and immunity, compete with pathogens, improve anti-cancer treatment efficacy, and regulate metabolic pathways. However, in contrast to traditional probiotics which their safety is proven, further studies, including clinical trials will help to prove NGPs safety for human consumption (Kaźmierczak-Siedlecka et al., 2022; Martín and Langella, 2019). *Faecalibacterium prausnitzii*, *Bacteroides fragilis*, *A. muciniphila*, *Prevotella copri* are some examples of NGPs. In addition, a novel NGP, named *Rouxiella badensis* subsp. *acadiensis* (Canan SV-53), was recently isolated from blueberry flora in our laboratory, exerting potential immunomodulatory activities. The health benefits of this novel NGP are discussed in subsequent sections.

Despite a substantial body of evidence regarding the health benefits of probiotics, there are some concerns about the safety of the application of live bacteria in neonates and vulnerable populations, such as systemic infections resulting from bacteria translocation from the gut to the circulation and the potential disruption of gut colonization in neonates. Therefore, there has been an increase in

interest surrounding the application of inactivated probiotics, their metabolites, and cell wall fractions in recent years (Piqué et al., 2019). Various strains of lactic acid bacteria have been shown to exert health-promoting effects in their heat-inactivated form (Chen et al., 2013).

Bacterial viability and cell wall integrity are not necessary for probiotics' interaction with the gut and their immunomodulatory activity. Many microbial functions, such as LPS and peptidoglycan, exert their immunomodulatory activities when are released from bacteria through interacting with mucosal sites (Ragland and Criss, 2017). While the mucous layer separates the bacteria and epithelial cells, microbial fractions pass through the mucus and directly act on epithelial cells (Piqué et al., 2019). The heat inactivation process facilitates the interaction between intracellular bioactive components and the host (Akter et al., 2020). Many studies have demonstrated the immunomodulatory and anti-inflammatory activities of heat-inactivated bacteria by modulating innate and adaptive immunity through regulating TLR signal transduction as well as by producing IgA, maintaining barrier function, and decreasing paracellular permeability (Piqué et al., 2019). A recent meta-analysis revealed the same effectiveness of live and heat-inactivated probiotics in improving IBD (colon length, disease activity index, and histological score) in animals (Poaty Ditengou et al., 2023).

Prebiotics are defined as selectively fermented compounds that lead to distinct alterations in the composition and/or function of the gastrointestinal microbial communities, providing health benefits to the host (Davani-Davari et al., 2019). To be considered as a prebiotic, a dietary compound must have the following characteristics: it must resist the stomach's acidic pH, resist degradation by digestive enzymes, evade absorption in the gastrointestinal tract, and undergo fermentation by the intestinal microbiota, ultimately contributing to the overall health of the host (Davani-Davari et al., 2019). Prebiotics are mainly subtypes of carbohydrates, including dietary

fibers and oligosaccharides such as inulin, fructo-oligosaccharide, and galacto-oligosaccharides (Davani-Davari et al., 2019; Plamada and Vodnar, 2021).

Prebiotics eliminate pathogens directly by acting as decoy receptors and indirectly by favoring probiotic commensal bacteria growth. Prebiotics also serve as the substrate for the bacteria to produce SCFAs, contributing to immune cell differentiation and functionality. Prebiotics also regulate the TLRs and associated signaling pathways such as NF-κB (Pujari and Banerjee, 2021).

Dietary phytochemicals, particularly polyphenols, may also serve as potential prebiotic compounds by increasing the growth and colonization of the probiotic commensals such as members of Bifidobacteriaceae and Lactobacillaceae families, diminishing the abundance of pathogenic bacteria such as *E. coli*, and *Helicobacter pylori*, and affecting SCFAs production (Plamada and Vodnar, 2021). In addition, polyphenols may regulate immune responses by modulating histone modification and DNA methylation. Polyphenols regulate the activities of DNMT1 and HDACs, and the expression of miRNAs. Some polyphenols regulate different signaling pathways such as FOXO and NF-κB via histone acetylation (Cuevas et al., 2013).

1-10- Gut Microbiota Development in Critical Developmental Periods of Life

Gut microbiota is formed in early life. Exposure to the maternal fecal and vaginal microbiota during birth is the first step in early-life microbial colonization (Nyangahu and Jaspan, 2019). Infants' microbiota is unstable with low diversity (Fouhy et al., 2012). Distinct transitions in gut microbiota happen during lactation characterized by the dominance of Bifidobacterium and during the weaning period characterized by the dominance of the phyla Bacteroidetes and Firmicutes. Over the first year of life, the diversity of the commensal bacteria increases and the infant's

microbiota composition starts to resemble that of the adult microbiota (Thursby and Juge, 2017; Bäckhed et al., 2015).

Gut microbiota composition during adolescence differs significantly from that observed in children and adults (Carson et al., 2023). Throughout adolescence, changes in the gut microbiota continue, marked by an augmented presence of Bifidobacteria and Clostridia, while maintaining lower diversity compared to adulthood microbiota (Novakovic et al., 2020; Agans et al., 2011). As puberty advances, there is a rise in Firmicutes abundance and a decline in Bacteroidetes abundance, resembling the composition observed in the adults (Calcaterra et al., 2022). In puberty, an increase in the abundance of class Betaproteobacteria has been reported (Yuan et al., 2020). However, there is no sex difference in gut microbiota composition in children, it may be influenced by sex starting at the onset of puberty which seems to be related to sex hormones (Calcaterra et al., 2022). For example, the prevalence of Adlercreutzia, Ruminococcus, Dorea, Clostridium, and Parabacteroides genera may be correlated with testosterone levels in the pubertal stage (Yuan et al., 2020).

The composition of the adult microbiome is marked by the prevalence of species from the Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria phyla (Calcaterra et al., 2022). Gut microbiota composition is relatively stable in adults, however, environmental factors may change this stability and lead to dysbiosis (Thursby and Juge, 2017).

The early-life microbiota's composition and functional potential are associated with both prenatal and post-natal factors. During gestation, different factors, including gestational weight gain, maternal BMI, antibiotic consumption, and maternal diet can lead to maternal microbial community disturbance and therefore, passing imbalanced microbiota to the infant (Mulligan and

Friedman, 2017). Post-natal factors, including mode of birth, type of feeding, diet, and antibiotic exposure are the main contributors to early-life microbiota development (Neuman et al., 2018).

Infants delivered vaginally inherit gut microbiota similar to their mothers' vaginal microbiota, characterized by the presence of Lactobacillus and Prevotella (Tanaka and Nakayama, 2017). In fact, these infants experience the initial colonization of their intestines primarily by facultative anaerobic bacteria. These bacteria play a pivotal role in altering the environment by influencing factors such as pH, redox potential, and nutrient production and therefore, creating favorable conditions for the subsequent colonization of beneficial bacteria (Jakobsson et al., 2014; Mulligan and Friedman, 2017; Palmer et al., 2007; Penders et al., 2006). At the phylum level, in early life, prior studies have reported an initial surge in Proteobacteria, followed by a decline within the first two years. Simultaneously, there is an expansion of Firmicutes starting at three months, and a peak in the Actinobacteria population is observed at the same time (Mulligan and Friedman, 2017).

Conversely, infants delivered via C-section have gut microbiotas resembling the mothers' skin microbiota (Tanaka and Nakayama, 2017; Bäckhed et al., 2015), primarily featuring Staphylococcus, Corynebacterium, and Propionibacterium. In addition, a reduction in bacterial diversity and Bacteroides has been observed in infants delivered through C-section. Due to gut microbiota disturbance, C-section delivery is associated with an increased susceptibility to immune-based disorders such as allergy, asthma, and celiac disease in children (Tanaka and Nakayama, 2017).

Infant feeding type, breastfeeding vs. formula feeding, has a significant impact on early-life microbiota maturation. Besides supplying immunoglobulins and nutrients, breast milk is a rich source of oligosaccharides. These oligosaccharides can undergo fermentation by Bifidobacteria,

leading to the production of SCFAs. The presence of these oligosaccharides favors the growth of a microbiota dominated by Bifidobacterium. There is a distinct difference between the gut microbiota of breast-fed infants and formula-fed infants. For example, lower α-diversity and higher abundance of Bifidobacterium and Lactobacillus have been reported in breast-fed infants when compared to formula-fed infants (Odiase et al., 2023).

Antibiotics are one of the most prescribed medications to children under 2 years of age in the Western world. About 45% of infants in Canada are exposed to antibiotics over the peripartum period (Stiemsma and Michels, 2018; Fouhse et al., 2019). In addition, about 25% of women intake antibiotics during pregnancy (Alhasan et al., 2023). Prenatal antibiotic exposure is associated with prolonged adverse effects on children's health (Leong et al., 2020; Loewen et al., 2018). Antibiotic usage in the first year of life has been reported to change the abundance of specific gut bacterial populations and decrease the maturation of the infant microbiota (Stiemsma and Michels, 2018; Neuman et al., 2018).

1-11- Gut Microbiota and Immune System Function in Critical Developmental Periods

1-11-1- Gut Microbiota and Immune System in Early Life

The development of the immune system such as mucosal immunity and T cell expansion, initiates during the embryonic stage (Rackaityte et al., 2020). Maternal gut microbiota plays an important role in fetal immune system development by producing different cell fragments, metabolites, antibodies, and bioactive molecules, which reach the fetus via the placenta (Koren et al., 2024; Macpherson et al., 2017). During early life, the immune responses of offspring against commensals are modulated by maternally acquired IgA and IgG (Koch et al., 2016). In addition, early-life IgG

responses to Gram-negative bacteria protect against Gram-negative pathogens such as *E. coli* (Zeng et al., 2016).

Studies utilizing germ-free models have signified the vital impact of gut microbiota on host immunity. Germ-free mice are characterized by a thin gut barrier, lack of SCFAs production, and immune system dysfunction (Behnsen et al., 2013). Notably, introducing microbiota to the intestine of these mice can restore gut functionality, immune responses, and immunotolerance, and protect the mice against intestinal inflammation (Behnsen et al., 2013; Zeissig and Blumberg, 2014; Olszak et al., 2012). However, this restoration is more effective when the mice's intestines are colonized with microbes during early life while microbiota exposure in adulthood may not adequately address missed opportunities during early life (Zeissig and Blumberg, 2014; Olszak et al., 2012). Significantly, the introduction of antibiotics to mice in their early postnatal stages leads to immune system changes that closely resemble the immune dysfunction observed in germ-free mice (Zeissig and Blumberg, 2014).

Growing evidence has demonstrated altered programming of the immune system due to microbiota changes which affect gene expression in the intestine (Schokker et al., 2015). In a study, early life exposure to antibiotics/stress led to changes in microbiota composition and differential gene expression in the ileum of adult animals. These genes were related to several immunological processes in ileal tissue, such as TNF/cytokine activity and processes involved in intestinal barrier function (tight junctions/cell adhesion) (Schokker et al., 2015). In addition, a single antibiotic course early in life was sufficient to cause alteration in intestinal microbial communities and ileal gene expression involved in immune processes, such as antigen-presenting pathways, B cells, and T cell developments (Ruiz et al., 2017). In another study, early life exposure of mice to low-dose

penicillin was associated with a general reduction in the expression of genes involved in intestinal immunity and a global reduction in intestinal immune responses (Cox et al., 2014).

Furthermore, animal studies have highlighted the significance of DNA methylation induced by commensal microbes in the early life development of the immune system (Woo and Alenghat, 2022). In the post-natal phase, shifts in transcript rates of DNA methylation enzymes, such as DNMT3A, have been observed in intestinal epithelial cells, resulting in microbiota-mediated differential DNA methylation and gene expression (Pan et al., 2018a). Additionally, the delayed establishment of microbiota resulting from antibiotic treatment in early life has been associated with alteration in DNA methylation and reduction in the expression of genes linked to innate immune responses in preterm animals (Pan et al., 2018b).

1-11-2- Gut Microbiota and Immune System in Puberty

Puberty, a crucial phase of development, is characterized by significant hormonal, metabolic, and immune alterations. Substantial changes during puberty may influence susceptibility to develop immune-related diseases in the later stages of life (Resztak et al., 2023). Sex difference in immune responses happens in puberty related to sex hormone functions with higher inflammatory responses in females compared to males post-puberty (Klein and Flanagan, 2016). Sex steroids affect the function of lymphocytes and antigen-presenting cells (Brenhouse and Schwarz, 2016). During puberty, a notable augmentation in both cellular and humoral immunity happens. The maturation of antigen-presenting cells and increased proficiency in their antigen-presenting capacity are associated with enhanced Th1 and Th17 immune responses (Ucciferri and Dunn, 2022). Following puberty, there is a notable shift in gene expression patterns, with increased expression of genes related to adaptive immunity in females and increased expression of genes related to innate immunity in males (Klein and Flanagan, 2016). However, despite substantial

changes in immunity during puberty, the molecular mechanisms governing these changes remain uncharacterized. There is some evidence suggesting the involvement of epigenetic mechanisms, particularly DNA methylation, in directing changes in immune cells and influencing immune and inflammatory responses during puberty (Resztak et al., 2023).

Puberty is a sensitive period to immune challenges with long-lasting health consequences (Sharma et al., 2019). For instance, LPS exposure during puberty may program both peripheral and central immunity, and cause a lasting effect on brain function later in life (Sharma et al., 2019; Yahfoufi et al., 2023). LPS exposure was associated with gut microbiota dysbiosis in pubertal mice indicating that long-term changes in the immune system and brain function induced by LPS may be related, in part, to gut microbiota dysbiosis (Sharma et al., 2019). However, adolescent probiotic intake could mitigate gut dysbiosis and pubertal LPS-mediated behavioral changes in adulthood (Sharma et al., 2019). These findings underscore the significance of gut microbiota during puberty in immune programming and long-term health outcomes. Figure 1-5 summarizes the gut microbiota and immune system development.

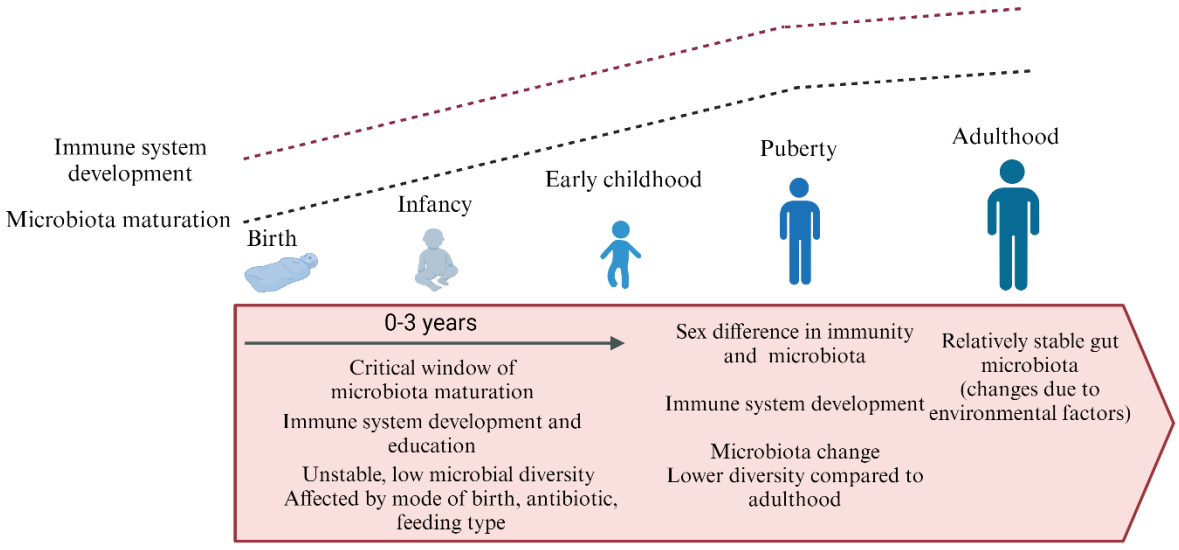

Figure 1-5. Gut microbiota maturation and immune system development from birth to adulthood. The image was created by BioRender.come.

1-12- Developmental Origins of Health and Disease

Developmental origins of health and disease (DOHaD) theory suggests that adulthood health and disease conditions are programmed in highly plastic developmental periods of life in response to environmental factors, including dietary factors (Stinson, 2020).

Recent evidence that indicates the causal effects of exposure to environmental stressors in early life and increased risk of chronic disease development later in life, mediated by epigenetic processes, highlights the role of epigenetic processes in underlying the DOHaD (Justulin et al., 2023). Notably, the connection between maternal diet and disease susceptibility of offspring later in life, governed by epigenetic mechanisms, clarifies the importance of early-life nutrition in DOHaD (Zheng et al., 2021). In addition, a substantial body of evidence emphasizing the correlation between gut microbiota disturbance in early life and the development of chronic diseases later in life also underscores the connection between the early-life gut microbiota and DOHaD (Stinson, 2020).

Moreover, recent studies emphasize puberty as a DOHaD programming window during which dietary factors contribute to the programming of chronic diseases later in life. For example, a recent study revealed that consumption of a high-fat diet during the pubertal period was associated with obese phenotype and related metabolic disorders, including hyperglycemia, hyperinsulinemia, hypertriglyceridemia, and fatty liver in adult animals (Armitage et al., 2023).

Overall, this evidence may elucidate the importance of proper timing in implementing directed interventional strategies, such as nutritional interventions, to enhance health and prevent disease later in life.

1-13- Rationale, Hypothesis, and Objectives of the Study

Most critical events in host immunity education take place during early life (Zheng et al., 2020). Growing evidence reflects the existence of a window of opportunity in early life that is important for microbiota-immune system interaction leading to immune system development and any alteration in this interaction results in an increased risk of immune-based disorders later in life probably through altered gene expression (Schokker et al., 2015) or durable epigenetic changes (Zeissig and Blumberg, 2014). Despite the substantial body of evidence exhibiting the enduring consequences of gut microbiota dysbiosis induced by immune stressors during early life and puberty (Murray et al., 2019; Leclercq et al., 2017), the precise underlying mechanisms remain inadequately understood.

In addition, some evidence highlights early life and puberty as the windows of opportunities during which nutritional interventions can alleviate long-term immunological changes related to gut microbiota dysbiosis (Murray et al., 2019; Leclercq et al., 2017). Whether gut microbiota-directed interventions, such as diets enriched with probiotics and prebiotics, could mitigate dysbiosis-related immune dysfunction by regulating epigenetic mechanisms and signaling pathways directing immune responses at the gut level, is an important question not explored yet. Therefore, we hypothesized that exposure to LPS/antibiotic during puberty or early life negatively affects immune system homeostasis later in life while probiotic/prebiotic intake mitigates immune system disturbance related to LPS/antibiotic-induced inflammation and dysbiosis through modulating signaling pathways and epigenetic mechanisms, including miRNAs and DNA methylation.

In this project, we first validated the immunomodulatory properties of the novel probiotic SV-53, both viable and heat-inactivated forms, and prebiotic protocatechuic acid (PCA) derived from the fermentation of blueberry juice by SV-53, via exploring their effects on selected cytokines and

epigenetic modifications related to Th17/IL-17A signaling in the steady-state in Balb/c mice. Next, to study the role of prebiotic/probiotic intake in mitigating lasting adverse effects of pubertal dysbiosis and inflammation on the immune system, we utilized a pubertal model of LPS-induce inflammation (Yahfoufi et al., 2023) using Balb/c mice treated with probiotic SV-53 and prebiotic AHCC (a standardized extract of cultured *Lentinula edodes* mycelia). For early life study, we used an antibiotic-induced dysbiosis model (Leclercq et al., 2017) where dams were exposed to low-dose penicillin and prebiotic AHCC one week before delivery and up to the weaning. The specific objectives of this project are as follows:

Objective 1: To study the immunomodulatory properties of probiotic SV-53, viable and heat-inactivated forms, and prebiotic PCA, by assessing cytokines, miRNAs, and DNA methylation of genes related to the pro-inflammatory cytokine IL-17 signaling at the gut level.

Objective 2: To study if probiotic/prebiotic intake counteracts long-term immune system deregulation induced by inflammation and dysbiosis during puberty, through assessing cytokines, signaling pathways, miRNAs, and DNA methylation of genes related to the pro-inflammatory cytokine IL-17 pathway at the gut level.

Objective 3: To study if prebiotic intake counteracts early life antibiotic induced-dysbiosis and its long-term adverse effects on immune system homeostasis by assessing cytokines and miRNAs related to inflammatory pathways, including NF-κB and STAT3, at the gut level.

Chapter 2: Novel Probiotic Bacterium *Rouxiella badensis* subsp. *acadiensis* (Canan SV-53) Modulates Gut Immunity through Epigenetic Mechanisms

2- Novel Probiotic Bacterium *Rouxiella badensis* subsp. *acadiensis* (Canan SV-53) Modulates Gut Immunity through Epigenetic Mechanisms

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Abstract

Gut immune system homeostasis is crucial for overall host health. Immune disturbance at the gut level may lead to systemic and distant sites' immune dysfunction. Probiotics and prebiotics consumption have been shown to improve gut microbiota composition and function and enhance gut immunity. In the current study, the immunomodulatory and anti-inflammatory effects of viable and heat-inactivated forms of the novel probiotic *bacterium Rouxiella badensis* subsp. *acadiensis* (Canan SV-53), as well as the prebiotic protocatechuic acid (PCA) derived from the fermentation of blueberry juice by SV-53, were examined. To this end, female Balb/c mice received probiotic (viable or heat-inactivated), prebiotic, or a mixture of viable probiotic and prebiotic in drinking water for three weeks. To better decipher the immunomodulatory effects of biotics intake, gut microbiota, gut mucosal immunity, T helper-17 (Th17) cell-related cytokines, and epigenetic modulation of Th17 cells were studied. In mice receiving viable SV-53 and PCA, a significant increase was noted in serum IgA levels and the number of IgA-producing B cells in the ileum. A significant reduction was observed in the concentrations of proinflammatory cytokines, including IL-17A, IL-6, and IL-23, and expression of two proinflammatory miRNAs, miR-223 and miR425 in treated groups. In addition, heat-inactivated SV-53 exerted immunomodulatory properties by elevating the IgA concentration in the serum and reducing IL-6 and IL-23 levels in the ileum. DNA methylation analysis revealed the role of heat-inactivated SV-53 in the epigenetic regulation of the genes related to Th17 and IL-17 production and function, including *Il6*, *Il17rc*, *Il9*, *Il11*, *Akt1*, *Ikbkg*, *Sgk1*, *Cblb*, and *Smad4*. Taken together, these findings may reflect the potential role of the novel probiotic bacterium SV-53 and prebiotic PCA in improving gut immunity and homeostasis. Further studies are required to ascertain the beneficial effects of this novel bacterium in the inflammatory state.

Keywords: Gut immunity; gut microbiome; probiotic SV-53; prebiotic; Th17 cell; miRNA; DNA methylation

2-1- Introduction

The largest immune system compartment in the body belongs to the gut (Shahbazi et al., 2020). Paneth cells, Goblet cells, and gut-associated lymphoid tissue (GALT) contribute to the gut immune system function (Santaolalla and Abreu, 2012; Mowat and Agace, 2014; Marsal and Agace, 2012). Paneth cells produce antimicrobial peptides such as lysozyme and β-defensins, while Goblet cells produce mucus (Marsal and Agace, 2012; Mowat and Agace, 2014; Santaolalla and Abreu, 2012). Peyer's patches, found in GALT, consist of B cell and T cell zones and are the main source of small intestine immunoglobulin A (IgA) plasmablasts (Mowat and Agace, 2014; Marsal and Agace, 2012). The mucus layer, antimicrobial peptides, and IgA play crucial roles in gut barrier integrity and mucosal immunity (Dupont et al., 2014).

Adaptive immune cells, including regulatory T cells (Treg) and T helper-17 (Th17), play a crucial role in maintaining gut immune system homeostasis. Signals from T cell receptors and cytokines direct T cells balance in the gut (Yan et al., 2020). Th17 cells are a distinct $CD4^+$ T-helper subtype characterized by interleukin (IL)-17 production that promotes inflammatory responses (Omenetti and Pizarro, 2015). Differentiation of naïve $CD4^+$ T cells into Th17 cells is regulated by the retinoic acid receptor-related orphan receptor-gamma-t (RORγt) transcriptional factor (Cabrera-Ortega et al., 2017). Transforming growth factor-β (TGF-β) and IL-6 are necessary for the early-stage differentiation of Th17 cells, while IL-23 plays a critical role in expanding Th17 cells and enhancing their pathogenic functions (Sharma et al., 2013; Kuwabara et al., 2017). IL-10 inhibits Th17 cells development and immune responses (Huber et al., 2011). Over-production of Th17

cells is associated with autoimmunity and inflammatory diseases such as inflammatory bowel disease (IBD) (Yan et al., 2020; Littman and Rudensky, 2010).

In addition to the role of cytokine milieu and transcription factors in the differentiation of naïve $CD4^+$ T cells (Luckheeram et al., 2012), the presence of Th17 in the small intestine is influenced by gut microbiota and microRNAs (miRNAs) profile (Hedblom et al., 2018; Luo et al., 2017; Park et al., 2017). Gut miRNAs play pivotal roles in both innate and adaptive immunity, directing various physiological and immunological processes in the intestine. Specific miRNAs contribute to the proliferation of intestinal epithelial stem cells, epithelial regeneration, differentiation of Paneth and Goblet cells, and the balance between Th17 and Treg cells (Park et al., 2017). Additionally, epigenetic mechanisms, including DNA methylation and posttranslational histone modifications, contribute to T cell generation (Luo et al., 2017).

Probiotics and prebiotics have been shown to enhance gut immunity by beneficially affecting gut microbial communities, maintaining the gut epithelial barrier, inhibiting pathogens' growth, and modulation innate and adaptive immune responses (Bermúdez-Brito et al., 2012; Pujari and Banerjee, 2021). Probiotics have also been found to modulate miRNA expression involved in gut immunity (Davoodvandi et al., 2021). In addition, heat-inactivated probiotics, their fractions, and purified components have been demonstrated to confer health benefits by protecting against pathogens and enhancing intestinal barrier function (Piqué et al., 2019). Probiotics and prebiotics may also enhance gut immunity by reducing proinflammatory cytokines such as IL-6 and IL-1β and increasing anti-inflammatory cytokines such as IL-10 (Wong et al., 2022). Besides, probiotics, prebiotics, and their metabolites have been shown to boost gut immunity by modulating epigenetic mechanisms, including DNA methylation and histone modification (Liu et al., 2021; Bhat et al., 2019; Kumar et al., 2013).

We have previously shown the probiotic characteristics of a novel Gram-negative probiotic, *Rouxiella badensis* subsp. *acadiensis* (Canen SV-53), referred to as SV-53. This probiotic microorganism, which was isolated from the natural microflora of lowbush blueberry (Mallet et al., 2021), reinforces intestinal homeostasis by increasing the number of Paneth cells and production of the antimicrobial peptide α-defensin (Novotny-Nuñez et al., 2023). In the current study, the immunomodulatory properties of the live and heat-inactivated SV-53 and prebiotic protocatechuic acid (PCA)-derived from fermented blueberry juice by SV-53, were studied in female Balb/c mice by analyzing the effects of biotics intake on gut microbiota, gut mucosal immunity, selected cytokines, and miRNAs involved in Th17 cells differentiation and function. We also aimed to study whether SV-53 exerts potential immunomodulatory activities through modulating epigenetic mechanisms by analyzing its effect on the DNA methylation status of genes related to Th17 cell function.

2-2- Materials and Methods

2-2-1- Animals

Eight-week-old female Balb/c mice (Charles River, Montreal, QC) were used in the current study. Three mice were housed together in plastic cages in a controlled atmosphere (temperature 22 ± 2°C; humidity 55 ± 2%) with a 12-hour light/dark cycle. During the study, all groups received a conventional balanced diet ad libitum. Mice were maintained and treated following the guidelines of the Canadian Council on Animal Care. The protocol (HSe-3191) was approved by the Animal Care Committee of the University of Ottawa.

2-2-2- Probiotic and Prebiotic Solution Preparation

In the current study, we used the research bank cultures of the bacterium prepared by the National Research Council of Canada. The bacteria cultures were maintained in a tryptic soy broth (TSB)

culture medium (Difco Laboratories, Detroit, MI, United States) supplemented with 30% (v/v) glycerol at -80°C.

To prepare the probiotic solution, the bacteria were cultured in TSB for 17 hours at room temperature. Then, the bacteria culture was centrifuged at 5000 rpm for 10 min. The bacterial pellet was washed three times in sterile PBS (Sigma, Saint Louis, MO, United States) and resuspended in 5 mL of sterile 10% (wt/vol) non-fat milk. Bacterial suspensions were diluted 1:30 in water and administered ad-libitum to the mice at the final concentration of 5×10^7 CFU/mL. To prepare heat-inactivated bacteria, the same procedure was applied, and 5×10^7 CFU/mL bacterial preparation in the sterile water was heated at 70°C for 30 minutes. The heat-inactivated preparations were kept at −80°C to feed the mice. 2 mL probiotic solution was consumed daily by each mouse, therefore mice received a daily dose of 1×10^8 CFU of the live and heat-inactivated bacterium.

To prepare the prebiotic solution, 100 mg/kg BW of PCA (3,4-dihydroxybenzoic acid) was dissolved in sterile water. Then the pH of the prebiotic solution was adjusted to 7.4 using a pH meter. The fresh mixture was prepared twice a week and kept at 4°C.

2-2-3- Study Design

2-2-3-1- Probiotic-Prebiotic Experiment

Mice (n=24) were categorized into four groups: 1-control group; receiving sterile water, 2-probiotic group; receiving probiotic SV-53 (5×10^7 CFU/mL) in sterile drinking water, 3-prebiotic group; receiving 100 mg/kg prebiotic PCA in sterile drinking water, 4-probiotic+prebiotic group; receiving a mixture of SV-53 and PCA in sterile drinking water. The duration of the nutritional intervention was three weeks. Afterward, mice were sacrificed, and required samples, including

blood, feces, and ileum tissues, were collected and stored at -80°C to conduct corresponding experiments.

2-2-3-2- Heat-Inactivated Probiotic Experiment

Mice (n=18) were divided into three groups: 1-control; receiving sterile drinking water, 2-probiotic group; receiving live SV-53 (5×10^7 CFU/mL) in sterile drinking water, and 3-heat-inactivated probiotic group; receiving heat-inactivated SV-53 (5×10^7 CFU/mL) in sterile drinking water. At the end of three weeks of nutritional intervention, mice were sacrificed, and the required samples were collected and kept at -80°C until further experiments were performed.

2-2-4- Histological Sections Preparation

The ileum tissues of mice were removed, washed with ice-cold PBS, and small sections of the ileum were collected and fixed in a 4% paraformaldehyde solution for 48 hours. Subsequently, fixed tissues were dehydrated in increasing alcohol concentrations, cleared in xylene, and embedded in paraffin using conventional methods. Histological sections of 4µm were prepared from paraffin blocks using a rotational microtome (Leica RM2255 Automated Microtome). The processing, embedding, and preparation of histological sections were performed by the University of Ottawa Histology Core Facility.

2-2-5- Identification of IgA, IgG, IL-17A, IL-6, IL-23, and IL-10 Producing Cell Populations by Immunofluorescence

The numbers of IgA^+, IgG^+, and IL-$17A^+$ cells on histological sections from the ileum region were determined by direct immunofluorescence. The immunofluorescence tests were performed using FITC-conjugated goat (α-chain specific) polyclonal anti-mouse IgA (Sigma-Aldrich, St. Louis, MO, USA), FITC-conjugated goat (γ-chain specific) polyclonal anti-mouse IgG (Sigma-Aldrich,

St. Louis, MO, USA), and anti-Mo/Rat IL-17A (ebio17B7), (eBioscience, Thermo Fisher Scientific, Burlington, Canada). The histological sections were deparaffinized in xylene and rehydrated in a graded series of ethanol from 95% to 40%. The deparaffinized histological samples were stained with appropriate antibody dilution in 1X PBS (1:100 for IgA, 1:50 for IgG, and IL-17A) for 1 hour at 37°C. Indirect immunofluorescence was used to determine the number of IL-6 and IL-10-producing cells using polyclonal anti-murine IL-10 (PeproTech, NJ, USA) and polyclonal anti-murine IL-6 (PeproTech, NJ, USA) antibodies, respectively. The deparaffinized histological slides were incubated with appropriate primary antibody dilution in 1X PBS (1:50) overnight at 4°C and FITC-conjugated affinipure goat anti-rabbit IgG (H+L) secondary antibody (1:50 in 1X PBS) for 1 hour at 37°C in the dark. The slides were washed three times in PBS, mounted using Fluoromount (Sigma-Aldrich, St. Louis, MO, United States), and examined using a fluorescent light microscope (Evos FL Auto 2, Thermo Fisher Scientific, Bothell, USA). The results were expressed as the number of positive cells (fluorescent cells) per 10 fields at 40x magnification.

2-2-6- Determination of IgA, IgG, IL-17A, IL-6, IL-23, and IL-10 Concentrations by ELISA

Blood samples were collected and centrifuged at 10,000 g for 2 minutes to separate serum. Serum samples were kept at -80°C. Small pieces of ileum were snap-frozen and kept at -80°C until protein extraction. To examine the cytokine levels, a small part of the ileum tissues (20-25 mg) were collected in microtubes containing lysis buffer (Pierce IP Lysis, Thermo Fisher Scientific) and protease/phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor Cocktail, Thermo Fisher Scientific) and homogenized by an electrical homogenizer (Bead mill 24, Fisher Scientific, USA). IgA and IgG levels were determined in serum using mouse IgA and IgG uncoated ELISA kits (Invitrogen, Vienna, Austria). The levels of IL-6, IL-10, IL-17A, and IL-23 were measured in

ileum tissues using mouse-uncoated ELISA kits (Invitrogen, Vienna, Austria). All ELISA tests were performed according to the manufacturer's instructions. Absorbance was read at 450 nm with a wavelength correction of 570 nm using a microplate reader (Bio-TEK Instruments, Winooski, VT, USA).

2-2-7-Determination of miRNA Expression by Real-Time Quantitative Reverse Transcription PCR (RT-qPCR)

Small pieces of ileum were placed in tubes containing RNAlater Stabilization Solution (Invitrogen, USA) for 24 hours and then stored at -80°C until RNA extraction. Total RNA from the samples was extracted using miRNeasy mini kit (Qiagen, Toronto, ON, Canada). Samples purity was verified with a NanoDrop 2000 (Thermo Scientific, Waltham, MA, United States). A reverse transcription reaction was done to synthesize cDNA using the miRCURY LNA RT Kit (Qiagen, Toronto, ON, Canada). The expression of miR-223 and miR-425 was measured by RT-qPCR using hsa-miR-425-5p and hsa-miR-223-3p miRCURY LNA miRNA PCR assay primers (Qiagen, Toronto, ON, Canada) and miRCURY LNA SYBR Green PCR Kit (Qiagen, Toronto, ON, Canada) in a CFX 384 real-time PCR detection system (Bio-Rad, Laboratories, Hercules, CA, USA). miRNA expression was normalized to U6 as the reference gene using the U6 snRNA (hsa, mmu) miRCURY LNA miRNA PCR assay (Qiagen, Toronto, ON, Canada).

2-2-8- Gut Microbiome Analysis

For microbiome analysis, the cecum contents of mice were collected in sterile microtubes and snap-frozen in liquid nitrogen before storage at -80°C. Shallow shotgun sequencing was used for microbiome analysis. Microbiome analysis was done by Microbiome Insights Company, Vancouver, BC. DNA was extracted using the MagAttract PowerSoil DNA KF kit (Qiagen, Canada). The quality of extracted DNA was evaluated visually through gel electrophoresis and

quantified using a Qubit 3.0 fluorometer (Thermo-Fischer, Waltham, MA, USA). Libraries were prepared using an Illumina Nextera library preparation kit with an in-house protocol (Illumina, San Diego, CA, USA).

2-2-9- Methylome-Wide Profiling and Data Analysis

Approximately 15-20 mg of the mice ileum samples were homogenized using an electrical homogenizer in tubes containing 500 µL cell lysis buffer and 1.5 µL proteinase K. The Gentra Puregene Tissue Kit (33 g) (Qiagen, Toronto, Canada) was then employed to extract DNA from the tissues, following the manufacturer's instructions. The extracted DNA was quantified using Qubit 4 (Thermo Fisher Scientific, Waltham, MA, USA) and diluted with DNA rehydration solution to achieve a final concentration of 20 ng/µL, then stored at -20°C. Methylome-wide profiling was conducted as previously described (Bošković et al., 2022). Briefly, 500 ng of extracted DNA was subjected to bisulfite conversion using the EZ DNA Methylation kit (Zymo Research, Irvine, CA, USA). 250 ng bisulfite-modified DNA was analyzed using the Infinium Mouse Methylation BeadChip arrays which allow for the simultaneous assessment of DNA methylation at more than 285,000 CpG sites (Illumina Inc., San Diego, CA, USA). Methylome-wide data was analyzed using the methylkey pipeline developed by the Epigenomics and Mechanisms Branch at the International Agency for Research on Cancer (https://github.com/IARCbioinfo/methylkey). Briefly, raw data files were pre-processed, quality control was conducted, and normalization was performed by Noob normalization using the SeSAMe package (Zhou et al., 2018). Intergroup comparisons were conducted using linear regression analysis as implemented in the limma R package (Ritchie et al., 2015). Regional analysis to identify differentially methylated regions was conducted using the DMRcate package (Peters et al., 2015).

2-2-10- Statistical Analysis

All statistical analyses, except for the microbiome analysis, were conducted using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was employed to compare groups. Data were considered significantly different when the p-value < 0.05. All values are mean ± SEM, based on at least three independent tests. Microbiome analysis was performed using Qiime2, and for comparisons of differentially abundant taxa, negative binomial models (DESEq2 R package) were utilized. Alpha diversity was calculated from taxonomic profiles using Shannon's diversity index. Beta diversity analysis was conducted using Bray-Curtis dissimilarities, and the results were visualized using nonmetric multidimensional scaling (NMDS). A corrected/adjusted p-value < 0.05 was considered statistically significant. For Methylation analysis, differentially methylated genes were defined with false discovery rate (FDR)-adjusted p-value < 0.05 and an absolute inter-group beta value difference of > 0.05. Pathway visualization was performed using KEGG pathway enrichment analysis with Enrichr.

2-3- Results

2-3-1- Effect of Probiotic and Prebiotic Intake on Mucosal Immunity

The effect of the nutritional intervention on mucosal immunity was assessed by measuring IgA and IgG levels in the serum, as well as the population of IgA and IgG-producing cells in the lamina propria of the ileum tissues. Feeding mice with SV-53 and SV-53+PCA mixture significantly increased the serum IgA level ($p<0.05$) (Figures 2-1A). Furthermore, the population of IgA^+ cells exhibited a significant increase in the ileum tissues of treated groups compared to the untreated group ($p<0.05$, $p<0.01$, and $p<0.0001$, respectively) (Figures 2-1B). However, neither the serum IgG level nor the number of IgG+ cells in the ileum of mice changed following the feeding period

(Figures 2-1C, D). Figure 2-1E illustrates immunofluorescence images of histological sections stained with FITC-conjugated anti-IgA antibody.

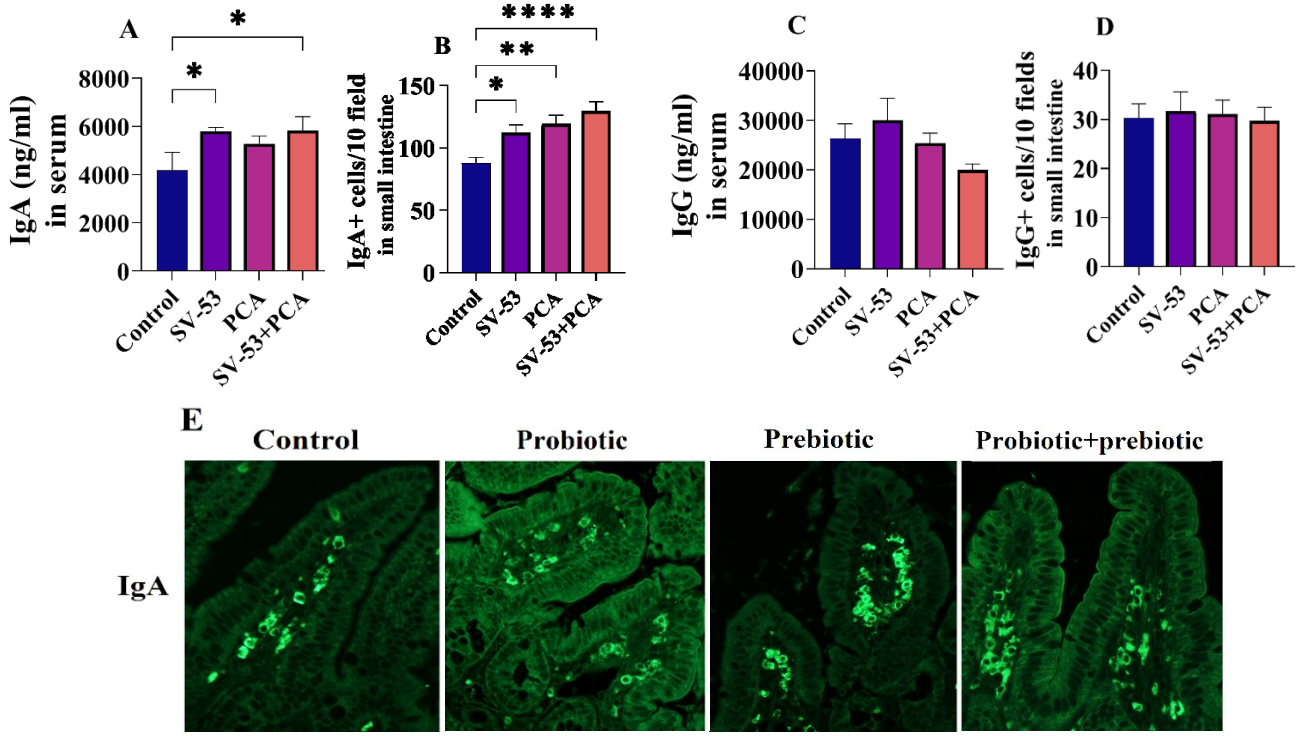

Figure 2-1. Effect of the probiotic and prebiotic intake on gut mucosal immunity. Female Balb/c mice were treated with the SV-53, PCA, or SV-53+PCA mixture in drinking water for three weeks. Serum levels of IgA and IgG and the number of IgA^+ and IgG^+ B cells in the ileum tissues of mice were measured by ELISA and direct immunofluorescence respectively; (A) the serum concentration of IgA, (B) the number of IgA^+ B cells, (C) the serum concentration of IgG, and (D) the number of IgG^+ B cells. One-way ANOVA followed by Dunnett's multiple comparisons were used to compare groups. All values are mean ± SEM. N=6, *p< 0.05, **p<0.01 and ****p<0.0001 vs. control. (E) Immunofluorescence images of histological sections of the ileum stained with the appropriate dilution of anti-IgA antibody (1:100) and imaged by a fluorescent light microscope at 40x magnification.

2-3-2- Effect of Probiotic and Prebiotic Intake on the Expression of Selected Cytokines and miRNAs

Oral administration of SV-53 and PCA for three weeks led to a significant reduction in IL-17A ($p<0.01$, $p<0.05$, and $p<0.01$, respectively) (Figure 2-2A), IL-6 ($p<0.01$, $p<0.05$, and $p<0.01$, respectively) (Figure 2-2B), and IL-23 ($p<0.01$, $p<0.05$, and $p<0.001$, respectively) (Figure2-2 C) concentrations in the ileum tissues of treated mice compared to the control counterparts. The level

of IL-10 increased in all treatment groups, however, statistical significance was reached only in the prebiotic group ($p<0.05$) (Figure 2-2D).

Additionally, the number of IL-17A, IL-6, and IL-10-producing cells in the lamina propria was measured through immunofluorescence. In accordance with ELISA results, the quantity of IL-17A-producing cells was significantly lower in all treatment groups than in the control ($p<0.01$, $p<0.05$, and $p<0.01$, respectively) (Figure 2-2E). IL-6 producing cells frequency was reduced in mice fed SV-53+PCA mixture compared to the control ($p<0.05$) (Figure 2-2F), while the abundance of IL-10-producing cells increased in SV-53 and PCA-fed mice ($p<0.01$ and $p<0.05$, respectively) (Figure 2-2G).

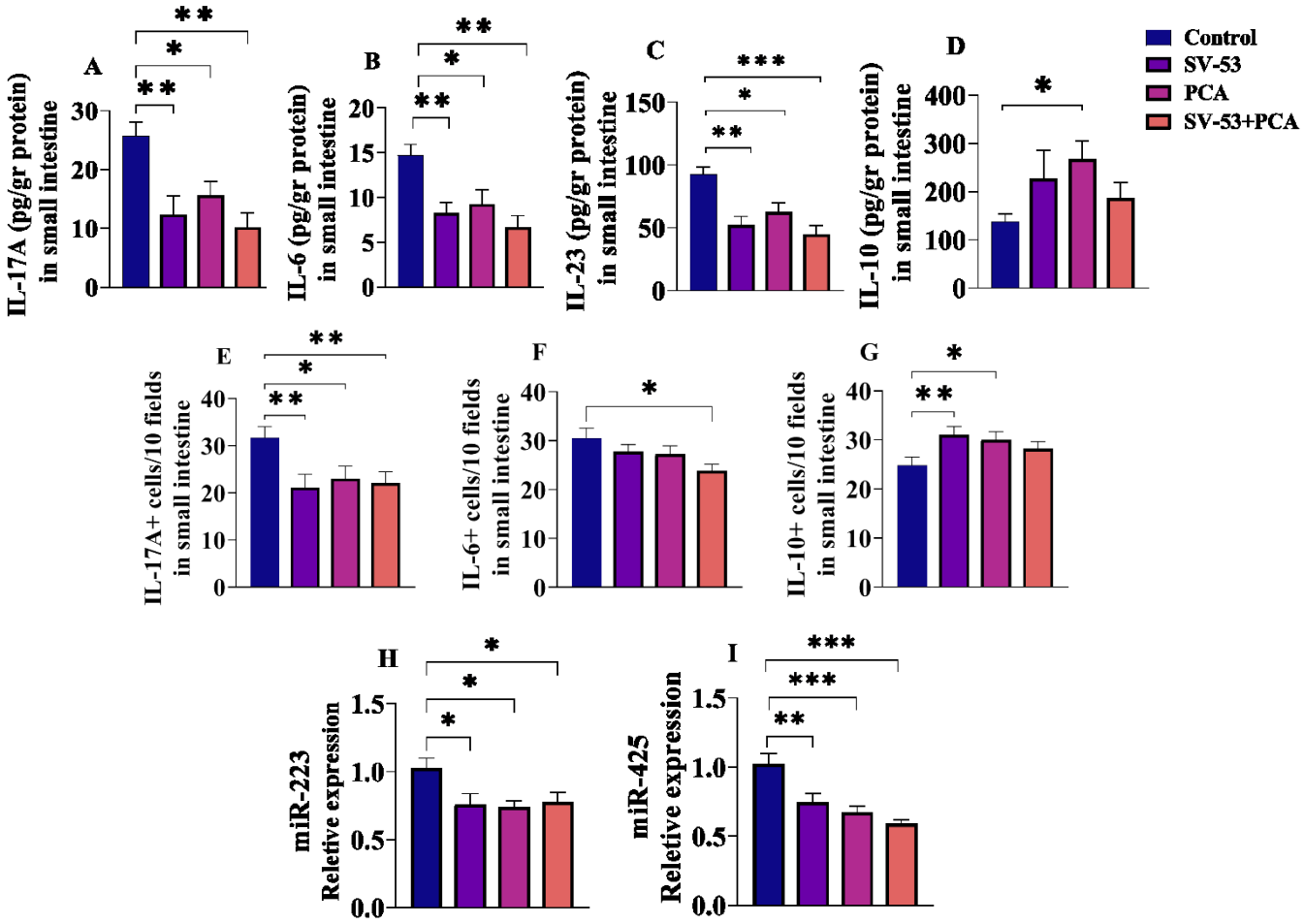

Figure 2-2. Effect of the probiotic and prebiotic intake on selected cytokines and miRNAs in the ileum tissues of mice. Female Balb/c mice were treated with the SV-53, PCA, or SV-53+PCA mixture in drinking water for three weeks. The concentrations of (A) IL-17A, (B) IL-6, (C) IL-23, and (D) IL-10 in the ileum tissues of mice were measured by ELISA. The frequencies of (E) IL-17A^{+}, (F) IL-6^{+}, and (G) IL-10^{+} cells were determined by immunofluorescence. The expression of (H) miR-223 and (I) miR-425 were measured by RT-qPCR. One-way ANOVA followed by Dunnett's multiple comparisons was used to compare groups. All values are mean ± SEM. N=6, *p< 0.05, **p<0.01, and ***p<0.001 vs. control.

Furthermore, the impact of SV-53 and PCA intake on the expression of two pro-inflammatory miRNAs, miR-223 and miR-425, was analyzed. Feeding mice with SV-53, PCA, and their mixture significantly decreased the expression of miR-223 ($p<0.05$) and miR-425 ($p<0.01$, $p<0.001$, and $p<0.001$, respectively) in ileum tissues of mice compared to the control group (Figure 2-2H, I).

2-3-3- Effect of the Probiotic and Prebiotic Intake on the Gut Microbiome

Alpha diversity, calculated from taxonomic profiles using Shannon's diversity index, did not show significant differences across groups (Figure 2-3A). Beta diversity was assayed using Bray-Curtis dissimilarities and samples were visualized using nonmetric multidimensional scaling (NMDS). Samples were very similar in general and no significant difference was seen (Figure 2-3B). Figure 2-3C displays the ten most abundant phyla in all groups. Microbiome analysis revealed the intestinal colonization of SV-53 in the mice that received SV-53 and SV-53+PCA ($p<0.0001$) (Figure 2-3D) and a significant decrease in the abundance of *Escherichia coli (E. coli)* in the SV-53 and SV-53+PCA groups ($p<0.0001$) (Figure 2-3E).

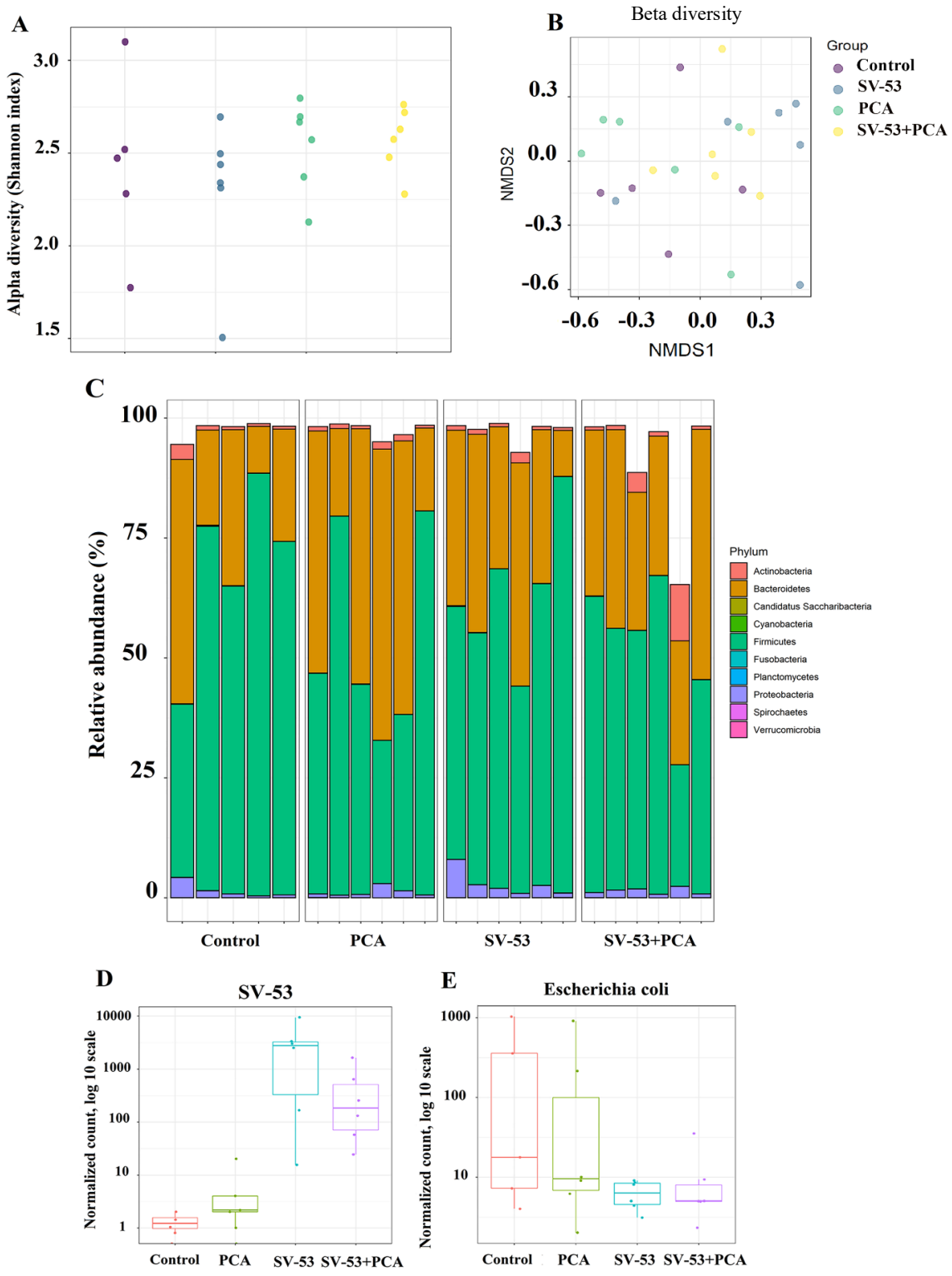

Figure 2-3. Effect of the probiotic and prebiotic intake on the gut microbiome. Female Balb/c mice were treated with the SV-53, PCA, or SV-53+PCA mixture in drinking water for three weeks. Then cecum contents of mice were used to analyze the gut microbiome by sallow shotgun sequencing; (A) alpha diversity, (B) beta diversity, (C) bacterial composition at the phylum level (ten most abundant phyla), and (D, E) differentially abundant species. N=5 in the control group and n=6 in the treatment groups.

2-3-4- Effect of Heat-Inactivated SV-53 Intake on Mucosal Immunity

The impact of heat-inactivated SV-53 on gut mucosal immunity was investigated in a separate experiment. Three weeks of treatment with heat-inactivated bacteria led to a significant increase in serum IgA levels ($p<0.05$) with no significant effect on the IgA^+ cells population in the lamina propria (Figures 2-4A, B respectively). Heat-inactivated bacteria also had no significant effect on IgG levels and the population of IgG^+ cells in lamina propria (Figures 2-4C, D respectively).

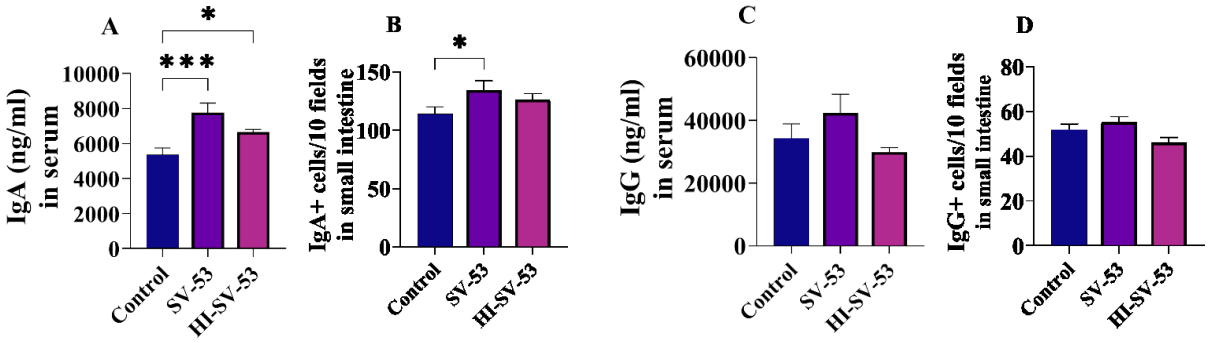

Figure 2-4. Effect of the heat-inactivated probiotic intake on gut mucosal immunity. Female Balb/c mice were treated with live SV-53 or heat-inactivated SV-53 (HI-SV-53) in drinking water for three weeks. Then, the serum levels of IgA and IgG, and the number of IgA^+ and IgG^+ B cells in the ileum tissues of mice were measured by ELISA and immunofluorescence, respectively; (A) the concentrations of IgA, (B) the number of IgA^+ B cells, (C) the concentrations of IgG, and (D) the number of IgG^+ B cells. One-way ANOVA followed by Dunnett's multiple comparisons were used to compare groups. All values are mean ± SEM. N=6, *p< 0.05 and ***p<0.001 vs. control.

2-3-5- Effect of Heat-Inactivated SV-53 Intake on the Expression of Selected Cytokines and miRNAs

IL-17A levels in the ileum tissues of mice in the heat-inactivated SV-53 group were insignificantly lower than those in the control group (Figure 2-5A). However, IL-6 and IL-23 concentrations significantly decreased in the ileum tissue of mice following treatment with heat-inactivated SV-53 ($p<0.05$ and $p<0.01$, respectively) (Figure 2-5B, C). The heat-inactivated SV-53 treatment had no significant effect on the IL-10 levels in the ileum of mice (Figure 2-5D). Administration of heat-inactivated SV-53 significantly reduced the numbers of $IL\text{-}17A^+$ and $IL\text{-}6^+$ cells compared to the control ($p<0.05$) (Figures 2-5E, F respectively), while it did not change

the IL-10-producing cells abundance (Figure 2-5G). Additionally, the administration of heat-inactivated bacteria did not alter the expression of miR-223 and miR-425 in the ileum tissues of mice compared to the untreated group (Figures 2-5H, I).

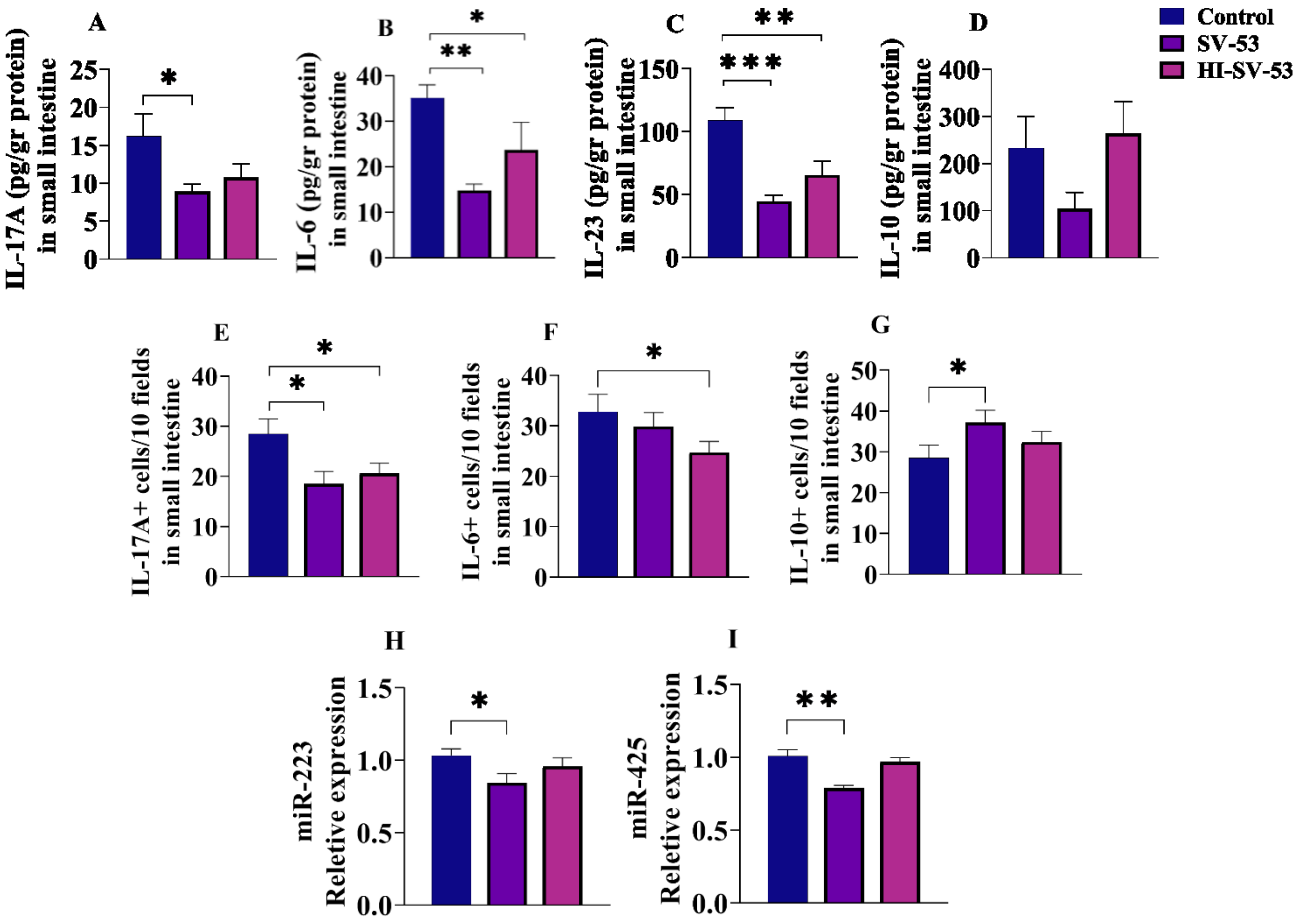

Figure 2-5. Effect of the heat-inactivated probiotic on selected cytokines and miRNAs expression in the ileum tissues of mice. Female Balb/c mice were treated with live SV-53 or heat-inactivated SV-53 (HI-SV-53) in drinking water for three weeks. The concentrations of (A) IL-17A, (B) IL-6, (C) IL-23, and (D) IL-10 in the ileum tissues of mice were measured by ELISA. The frequencies of (E) IL-17A^{+}, (F) IL-6^{+}, and (G) IL-10^{+} cells were determined by immunofluorescence. The expression of (H) miR-223 and (I) miR-425 were measured by RT-qPCR. One-way ANOVA followed by Dunnett's multiple comparisons was used to compare groups. All values are mean ± SEM. N=6, *p< 0.05, **p<0.01 and ***p<0.001 vs. control.

2-3-6- Effect of Heat-Inactivated SV-53 on DNA Methylation Status

Finally, the impact of heat-inactivated SV-53 on the methylation status of genes in the ileum samples of mice was analyzed. The methylation status of regions around the transcriptional start sites (TSS) plays a role in regulating gene expression (Yuan et al., 2016; Hong and Rhee, 2022),

where hypomethylation and hypermethylation correlate with transcriptional activation and suppression, respectively (Yakoob et al., 1998).

Absolute group mean difference in beta values of > 0.05 and FDR adjusted p-value < 0.05 were applied to identify differentially methylated probes (DMPs), which were used as a basis for regional analysis to find statistically significant differentially methylated regions (DMRs). We found significant DMRs related to different signaling pathways such as MAPK, PI3K-AKT, JAK-STAT, forkhead box O (FoxO), Th17 differentiation, and IL-17 signaling pathways. Figure 2-6A illustrates the top enriched pathways for genes associated with the identified DMRs. Notably, significant alterations in the methylation status of some genes regulating Th17 differentiation and function were observed in mice receiving heat-inactivated SV-53 compared to the control (Figure 2-6B). Significant hypermethylation of CpGs was identified within the promoters of *Il6* and IL-17 receptor c (*Il17rc*), and the 1 to 5 kb region of *Il11*. In addition, the 1 to 5 kb region of *Akt1* and the promoters of inhibitor of nuclear factor kappa B kinase regulatory subunit gamma (*Ikbkg*) and serum and glucocorticoid-regulated kinase 1 gene (*Sgk1*) were significantly hypermethylated. Furthermore, the promoter of *Il9*, the 5′ UTR region of casitas-B-lineage lymphoma protein-b (*Cblb*), and the 1 to 5 kb region of SMAD family member 4 (*Smad4*) were found to be hypomethylated in the heat-inactivated SV-53 group compared to the control group. No significant DMRs were found in mice receiving live SV-53 and PCA compared to untreated mice.

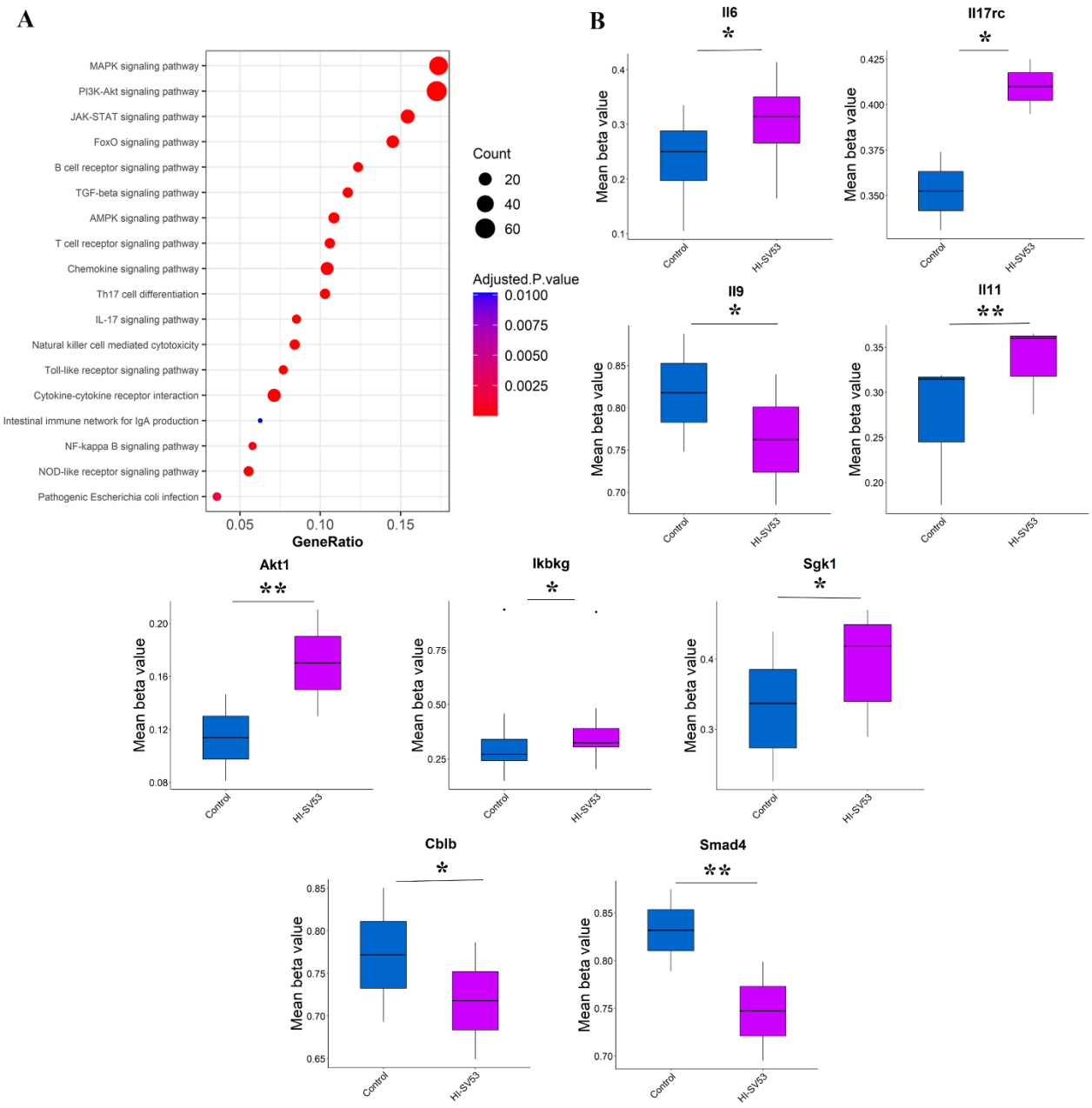

Figure 2-6. Effect of the heat-inactivated probiotic on DNA methylation. Female Balb/c mice were treated with live SV-53 or heat-inactivated SV-53 (HI-SV-53) in drinking water for three weeks. (A) Pathways enrichment analysis visualized by Enrichr, (B) boxplots of differentially methylated genes related to Th17 cells differentiation and function. *p< 0.05 and **p<0.01 vs. control.

2-4- Discussion

Probiotics, prebiotics, and symbiotic products have been shown to promote a balanced gut microbiota, reduce inflammation, and enhance immune system function, not only gut immunity

but also systemic and distant tissue immunity through the common mucosal immune system (Shahbazi et al., 2021; Zhou et al., 2021; Seifert and Watzl, 2007; Shahbazi et al., 2020; Yahfoufi et al., 2021a). In this study, the immunomodulatory and anti-inflammatory properties of the novel probiotic SV-53 and prebiotic PCA were explored. Certain probiotic bacteria and prebiotics, including small polyphenol oligomers, act as antagonist ligands for Toll-like receptors (TLRs), effectively inhibiting associated inflammatory pathways (Mallet et al., 2023; Najafi et al., 2023). SV-53 is a Gram-negative bacteria with probiotic characteristics (Novotny-Nuñez et al., 2023). The subtle distinction between bacterial lipopolysaccharides (LPS) derived from pathogenic Gram-negative bacteria and Gram-negative commensal bacteria in gut microbiota is responsible for the immunoinhibitory activity of Gram-negative commensals (d'Hennezel et al., 2017). On the other hand, PCA is a metabolite product resulting from the fermentation of blueberry juice by SV-53 and deriving from complex polyphenols and quercetin (Mallet et al., 2023), which is a TLR4 antagonist (Nam and Lee, 2018).

The results of the current study revealed that SV-53 and PCA administration could increase the population of IgA-producing B cells in the ileum, and SV-53 intake increased serum IgA concentration without increasing IgG levels. Within the intestinal environment, IgA-positive (IgA+) cells and secretory IgA (sIgA) play a vital role in maintaining intestinal mucosal immunity and homeostasis (Yahfoufi et al., 2021b). sIgA is the most abundant antibody class in the mucosal immune system and gut lumen (Li et al., 2020). It enhances gut mucosal immunity and homeostasis through various mechanisms, including quenching microbial components, neutralizing bacterial toxins, influencing gut microbial communities, enhancing antigens transport to immune cells in GALT, and downregulating proinflammatory responses (Mantis et al., 2011). On the other hand, IgG antibodies are potent effector molecules that activate innate immune cells and induce

inflammation (Aschermann et al., 2010; Castro-Dopico and Clatworthy, 2019). Increased IgG level is associated with intestinal inflammation and persistent immunopathology in the intestinal mucosa (Vinderola et al., 2006). In addition, an increase in local commensal-specific IgG during intestinal inflammatory diseases, such as colitis, has been reported by Castro-Dopico et al. (2019), and this increase was associated with the magnitude of intestinal inflammation (Castro-Dopico et al., 2019). They also found that IgG immune complexes-stimulated colonic macrophages demonstrated a Th17-polarizing phenotype (Castro-Dopico et al., 2019). No changes in IgG levels were observed in response to probiotic intake, reflecting the effectiveness of the treatment in improving gut mucosal immunity by producing sIgA without increasing IgG levels and inducing inflammation.

In addition, probiotic and prebiotic intake significantly decreased the level of IL-17A, the main proinflammatory cytokine secreted by Th17 cells (Omenetti and Pizarro, 2015), and levels of IL-6 and IL-23, major cytokines inducing pathogenic Th17 cells production and function, in the ileum tissues of mice (Sharma et al., 2013; Kuwabara et al., 2017). IL-17A is involved in the pathogenicity of inflammatory disorders (Kuwabara et al., 2017). For instance, the involvement of IL-17A/IL-23 in the pathogenesis of IBD has been shown in both human and animal studies (Cătană et al., 2015). We have previously shown that the fermentation of blueberry juice with SV-53 significantly increases the quantity of polyphenols naturally present in the juice (Martin and Matar, 2005). This novel product was found to inhibit IL-6/STAT3 signaling (Vuong et al., 2016). IL-6/STAT3/RORγt pathway induces Th17 cells differentiation (Chang et al., 2019), and IL-23/IL-23 receptor signaling phosphorylates the STAT3 and increases the expression of inflammatory cytokines such as IL-17A leading to intestinal inflammation (Schmitt et al., 2021). Moreover, increased IL-6 negatively affects gut mucosal immunity and leads to gut barrier

dysfunction by damaging tight junctions (Li et al., 2021; Al-Sadi et al., 2014). Our results may provide some evidence regarding the potential role of SV-53 and PCA in modulating the IL6/STAT3 pathway. However, further studies are required to explore the role of SV-53 and PCA in regulating STAT3 signaling.

Furthermore, we found an increase in the IL-10 concentration in the PCA group and an increase in the number of IL-10-producing cells in the SV-53 and PCA groups. IL-10 is an immunosuppressive cytokine secreted by various immune cells, including dendritic cells, macrophages, and Tregs. IL-10-producing $CD4^+$ $CD25^+$ Treg cells play a key role in controlling intestinal inflammation and self-tolerance (Wei et al., 2020). IL-10 contributes to B cells differentiation and the production of IgA by B cells, thereby enhancing mucosal humoral immunity (de Moreno de Leblanc et al., 2011). In addition, in a study using a chronic colitis mice model, IL-10 suppressed the inflammasome/IL-1β pathway, reduced the pathogenicity of Th17 cells, and suppressed IL-17 production, leading to the mitigation of intestinal inflammation (Zhang et al., 2014). Lactic acid bacteria have been shown to prevent intestinal inflammation and autoimmunity by increasing IL-10 and $CD4^+$ $CD25^+$ Treg cells (Di Giacinto et al., 2005; Lavasani et al., 2010). Moreover, studies have shown the ability of prebiotics to modulate IgA, IgG, proinflammatory, and anti-inflammatory cytokines (Yahfoufi et al., 2018). For example, prebiotic intake increased sIgA and IL-10 levels in the intestines of rats (Looijer-van Langen and Dieleman, 2009) and inhibited LPS-induced IL-17 release in mouse splenocytes (Capitán-Cañadas et al., 2014). Additionally, polyphenols inhibit inflammation by blocking TLR4 and suppressing the production of inflammatory mediators such as IL-1β, IL-6, and TNFα (Yahfoufi et al., 2018).

Gut miRNAs link the gut immune system and the microbial community by regulating signaling pathways critical for maintaining gut microenvironment homeostasis (Bi et al., 2020). In the

current study, the effect of nutritional intervention with probiotic SV-53 and prebiotic PCA intake on miR-223 and miR-425 expression was explored, and a significant decrease in the relative expression of both miRNAs was observed in the treatment groups. A marked increase in the expression of miR-223 and miR-425 has been reported in intestinal inflammatory conditions such as IBD (Yang et al., 2018; Rodríguez-Nogales et al., 2018a). miR-223 may promote the secretion of cytokines by dendritic cells and therefore regulate Th17 and Tregs differentiation from naïve T cells (Bi et al., 2020). miR-233 is downstream of the IL-23 cascade (Wang et al., 2016) and has been shown to promote pathogenic Th17 cells differentiation during autoimmunity (Jiao et al., 2021). miR-223 also increases intestinal barrier permeability by targeting tight junction proteins through IL23/Th17 pathway (Wang et al., 2016). Impaired barrier function is a major factor contributing to intestinal inflammation (Wang et al., 2016). miR-425 is involved in chronic-degenerative inflammations such as autoimmune diseases, age-related disorders, and cancer progression (Balzano et al., 2017). This miRNA promotes Th17 cells differentiation and pathogenicity. In a study conducted on colitis mice, overexpression of miR-425 was found to mediate Th17 cell production and IL-17A secretion, while inhibiting miR-425 alleviated intestinal mucosal inflammation and markedly decreased IL-17A levels (Yang et al., 2018). Therefore, these results suggest that SV-53 and PCA may enhance gut immunity and prevent inflammation by regulating gut miRNAs as well.

Similar to these results, several studies have reported the beneficial effects of probiotics in mitigating gut inflammation through the modulation of miRNA expression. For instance, the oral administration of two probiotics, *Lactobacillus fermentum* and *Saccharomyces boulardii,* significantly regulated the expressions of miR-223 and improved gut barrier function, restored dysbiosis, and ameliorated disease severity in a mouse model of dextran sodium sulfate (DSS)-

induced colitis (Rodríguez-Nogales et al., 2017; Rodríguez-Nogales et al., 2018b). In another study, feeding mice with probiotic *E. coli* Nissle 1917, a Gram-negative probiotic bacterium, prevented the DSS-induced colonic inflammation by enhancing the expression of various cytokines and proteins responsible for maintaining epithelial integrity and decreasing several miRNAs involved in the inflammatory response, including miR-223 (Rodríguez-Nogales et al., 2018a).

Full genome analysis of SV-53 has revealed a cluster of genes coding for bacteriocins against pathogenic Gram-negative bacteria (Salvetti et al., 2023). We have previously shown the antibacterial effect of SV-53 against *Staphylococcus aureus* and *Salmonella enterica* serovar Typhimurium (Novotny-Nuñez et al., 2023). In this study, the microbiome analysis signified a decrease in *E. coli* population in mice fed SV-53 and the mixture of SV-53 and PCA. An increase in pathogenic *E. coli* has been observed in IBD, and following antibiotic-induced dysbiosis (Martinez-Medina and Garcia-Gil, 2014; Looft and Allen, 2012). Furthermore, studies have observed that the Western diet can alter gut microbiota composition, increase gut permeability, induce overgrowth of *E. coli,* promote invasive *E. coli* colonization in the gut mucosa, and subsequently lead to inflammation and immune dysfunction (Martinez-Medina et al., 2014; Agus et al., 2016). Moreover, enterotoxigenic *E. coli* exposure has been shown to increase RORγt expression and IL-17 secretion in the intestines of mice (Ren et al., 2017). Several probiotics, such as *Lactobacillus plantarum* and *E. coli* Nissle 1917, have been reported to alleviate pathogenic *E. coli*-induced intestinal barrier dysfunction by modulating the expression of tight junction proteins (Raheem et al., 2021).

Furthermore, we demonstrated the immunomodulatory and anti-inflammatory properties of heat-inactivated SV-53 by increasing IgA concentration in the serum, reducing IL-6 and IL-23

concentrations, and the number of IL-17A and IL-6-producing cells in the ileum tissues of mice. However, our results indicated that viable SV-53 exhibited more robust immunomodulatory properties compared to the non-viable bacterium. Many of the ascribed benefits of live probiotics are facilitated by interactions of these organisms with the host's gut epithelium and immune system. They can interact with the host directly through cell-to-cell interaction or indirectly through the production of metabolites and the release of various components (Castro-Herrera et al., 2020). Heat-inactivated probiotics are also metabolically active and can interact with the host, resulting in health benefits (Castro-Herrera et al., 2020). In fact, heat-killed probiotics retain functionality due to the presence of cell wall components involved in interactions with the host, such as lipoteichoic acids and peptidoglycan (Castro-Herrera et al., 2020).

In an experimental colitis model, the administration of a mixture of heat-inactivated probiotics downregulated IL-6, IL-23, STAT3, and p-STAT3 expression in colonic tissues of rats (Sang et al., 2015). In another study, heat-inactivated *E. coli* Nissle 1917 induced the antimicrobial peptide β-defensin in intestinal epithelial cells (Wehkamp et al., 2004). We have previously shown the immunomodulatory and anti-inflammatory activity of heat-inactivated lactic acid bacteria using primary cultures of intestinal epithelial cells in mice fed *Lactobacillus casei CRL* 431 or *Lactobacillus helveticus* R389 (Vinderola et al., 2005). Similar to this study, Pyclik et al. demonstrated the significance of the viability status of *Bifidobacterium* strains for its immunomodulatory properties, with heat inactivation being shown to alter these properties (Pyclik et al., 2021). In addition, Sturm et al. reported that in contrast to heat-killed *E. coli* Nissle 1917, the viable bacterium might exert a more potent inhibitory effect against intestinal inflammation by inhibiting T cells proliferation and the expression of proinflammatory cytokines (Sturm et al., 2005).

To investigate whether the immunomodulatory properties of SV-53 are mediated, in part, through epigenetic modification, DNA methylation analysis was conducted. In mice exposed to heat-inactivated SV-53, an increase in CpGs methylation around TSS of several genes controlling Th17 differentiation and function, including *Il6*, *Il17rc*, *Il11*, *Akt1*, *Ikbkg*, and *Sgk1*, was found, which may indicate transcriptional repression of these genes.

As discussed earlier, IL-6 plays a central role in initiating the differentiation of naïve T cells into the Th17 lineage. IL-17RC is critical for signal transduction by IL-17A and the pathogenesis of autoimmune diseases. *In vitro* studies have shown that IL-17RC deficiency protects against autoimmunity (Hu et al., 2010). Moreover, it has been shown that Th17 cells can secret IL-9. IL-9 neutralization and IL-9 receptor deficiency led to a reduction in Th17 cells and IL–6-producing macrophages in the central nervous system, ultimately ameliorating the development of experimental autoimmune encephalomyelitis in mice (Nowak et al., 2009). IL-11 may induce Th17 cells generation and expansion *in vitro* and Th17 cells responses *in vivo*, and contribute to autoimmunity. IL-11 exerts its effect on Th17 cells by activating the IL-6/STAT3 pathway (Zhang et al., 2019d).

Furthermore, AKT plays an important role in Th17 production. Upon TCR stimulation, the PI3k/AKT pathway contributes to the proliferation and survival of Th17 cells by facilitating nuclear transportation of RORγ and downregulating negative regulators of Th17 differentiation (Kurebayashi et al., 2012). *Ikbkg*, also known as nuclear factor-kappa B (NF-κB) essential modulator (NEMO), encodes the regulatory subunit of the inhibitor of kappa B (IκB) kinase (IKK) complex, which is essential for NF-κB activation (Johnston et al., 2016). NF-κB contributes to the production of inflammatory cytokines such as IL-1, IL-6, IL-23, RORγ expression, and the generation of inflammatory T cells, including Th17 cells (Liu et al., 2017). *Sgk1* encodes SGK1,

a serine/threonine kinase, which is downstream of IL-23 signaling. *Sgk1* expression is specifically induced and maintained by exposure to IL-23. The kinase activity of SGK1 is higher in Th17 cells compared to other subsets of T cells. On the other hand, SGK1 is crucial for regulating IL-23R expression, thereby maintaining the Th17 cell phenotype by suppressing FOXO1, which negatively regulates IL-23R expression (Wu et al., 2013).

In addition, a significant hypomethylation was detected within CpGs around TSS sites of *Cblb,* and *Smad4* in heat-inactivated SV-53 mice compared to their untreated counterparts, which may suggest the transcriptional activation of these genes. Cblb is a Cbl family E3 ubiquitin ligase that restrains pathogenic Th17 cells generation and Th17-related autoimmunity by suppressing IL-6 secretion by macrophages (Zeng et al., 2022). IL-21 promotes Th17 cells differentiation and SMAD4 negatively regulates IL-21-induced Th17 by repression of *Rorc* gene expression (Zhang et al., 2019c). However, we could not find any changes in the methylation status of genes in mice receiving live bacteria, which indicates that in addition to cell wall components, other components released during the heat-inactivation process may contribute to epigenetic modification by heat-inactivated SV-53.

While results from cytokine and DNA methylation analyses may offer some insights into the potential role of SV-53 in regulating Th17 cells, flow cytometry analysis needs to be done to elucidate the effect of treatment on Th17 cells production.

In conclusion, the results of this study suggest the potential role of both viable and heat-inactivated probiotic SV-53, along with the prebiotic PCA, in improving gut immunity and homeostasis by modulating IgA, cytokines, and/or gut miRNAs expression. Additionally, these findings highlight the significant role of heat-inactivated SV-53 in regulating gut immunity through DNA

methylation. SV-53 also enhances gut immunity and prevents inflammation by decreasing the population of pathogenic bacteria. These findings may indicate the probiotic characteristics of SV-53 as a novel next-generation probiotic bacterium.

Author Contributions: R.S. performed experiments, wrote the manuscript, and performed data analysis; H.Y.-S. contributed to the sample collection, DNA methylation analysis, and RT-qPCR; J.-F.M. contributed to the bacterial culture and prebiotic mixture preparation, animal feeding, and sample collection; F.S. contributed to the sample collection and DNA extraction; N.A. contributed to the sample collection; C.C. conducted DNA methylation and 16S rRNA sequencing experiments; V.C. and F.F.-L.C. contributed to the DNA methylation analysis; Z.H. contributed to the review and editing; C.M. designed and supervised the work. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol (HSe-3191) was approved by the Animal Care Committee of the University of Ottawa on 13 July 2022.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Chapter 3: *Lentinula Edodes* Cultured Extract and *Rouxiella badensis* subsp. *acadiensis* (Canan SV-53) Intake Alleviates Immune Deregulation and Inflammation by Modulating Signaling Pathways and Epigenetic Mechanisms

3- *Lentinula Edodes* Cultured Extract and *Rouxiella badensis* subsp. *acadiensis* (Canan SV-53) Intake Alleviates Immune Deregulation and Inflammation by Modulating Signaling Pathways and Epigenetic Mechanisms

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Abstract

Puberty is a critical developmental period of life characterized by marked physiological changes, including changes in the immune system and gut microbiota development. Exposure to inflammation induced by immune stressors during puberty has been found to stimulate central inflammation and lead to immune disturbance at distant sites from the gut; however, its enduring effects on gut immunity are not well explored. Therefore, in this study, we used pubertal lipopolysaccharide (LPS)-induced inflammation mouse model to mimic pubertal exposure to inflammation and dysbiosis. We hypothesized that pubertal LPS-induced inflammation may cause long-term dysfunction in gut immunity by enduring dysregulation of inflammatory signaling and epigenetic changes, while prebiotic/probiotic intake may mitigate the gut immune system deregulation later in life. To this end, four-week-old female Balb/c mice were fed prebiotics/probiotics and exposed to LPS in the pubertal window. To better decipher the acute and enduring immunoprotective effects of biotics intake, we addressed the effect of treatment on interleukin (IL)-17 signaling-related cytokines and pathways. In addition, the effect of treatment on gut microbiota and epigenetic alterations, including changes in microRNA (miRNA) expression and DNA methylation, were studied. Our results revealed a significant dysregulation in selected cytokines, proteins, and miRNAs involved in key signaling pathways related to IL-17 production and function, including IL-17A and F, IL-6, IL-1β, transforming growth factor-β (TGF-β), signal transducer and activator of transcription-3 (STAT3), p-STAT3, forkhead box O1 (FOXO1), and miR-145 in the small intestine of adult mice challenged with LPS during puberty. In contrast, dietary interventions mitigated the lasting adverse effects of LPS on gut immune function, partly through epigenetic mechanisms. A DNA methylation analysis demonstrated that enduring changes in gut immunity in adult mice might be linked to differentially methylated genes, including *Lpb*,

Rorc, *Runx1*, *Il17ra*, *Rac1*, *Ccl5*, and *Il10*, involved in Th17 cell differentiation and IL-17 production and signaling. In addition, prebiotic administration prevented LPS-induced changes in the gut microbiota in pubertal mice. Together, these results indicate that following a healthy diet rich in prebiotics and probiotics is an optimal strategy for programming immune system function in the critical developmental windows of life and controlling inflammation later in life.

Keywords: Gut microbiota; *Lentinula edodes* mycelia; probiotic *Rouxiella badensis*; interleukin-17; FOXO1; STAT3; microRNA; DNA methylation

3-1- Introduction

Dynamic interactions between commensal bacteria living in the intestine and the host are crucial for the development and function of the immune system, and intestinal homeostasis (Shahbazi et al., 2020). Any alteration to the composition of the commensal population in the gut that negatively affects mutualistic relationships among microbial communities is considered dysbiosis. Uncorrected dysbiosis may lead to various chronic inflammatory disorders, including inflammatory bowel disease (IBD), obesity, and diabetes (Wu et al., 2016; Gagliardi et al., 2018).

Exposure to immune stressors such as exogenous lipopolysaccharide (LPS) is accompanied by gut dysbiosis and permeability (Murray et al., 2019; Yahfoufi et al., 2023; Guo et al., 2013) and mediates the production of inflammatory cytokines such as interleukin (IL)-6 and IL-23 by immune cells (Chang et al., 2014; Sutton et al., 2009). These cytokines along with IL-1β enhance IL-17 production in the presence of T cell receptor (TCR) stimulation by inducting T helper (Th)-17 cells differentiation (Chang et al., 2014; Sutton et al., 2009). Th17 cells are the major source of IL-17 production in adaptive immunity (Chung et al., 2021; Jin and Dong, 2013). Besides, innate immune cells such as γδ T cells and type 3 innate lymphoid cells (ILC3s) secret IL-17, in response

to IL-1 and IL-23, without TCR engagement (Chung et al., 2021; Jin and Dong, 2013). IL-6 and IL-23 favor IL-17 production through signal transducer and activator of transcription 3 (STAT3) signaling (Woś and Tabarkiewicz, 2021; Lee et al., 2017). IL-1β initiates the IL-6/STAT3 pathway (Mori et al., 2011). Evidence shows that IL-6/STAT3 inhibits forkhead box O (FOXO)-1 expression (Ichiyama et al., 2016). FOXO1 suppresses Th17 differentiation and pathogenicity, and IL-17A and IL-17F expression (Ichiyama et al., 2016). IL-17 is known for its ability to initiate inflammation and autoimmunity by induction of a variety of cytokines such as IL-6 and IL-1β (Zenobia and Hajishengallis, 2015).

Besides, gut commensal bacteria regulate IL-17 production (Dupraz et al., 2021). Gut microbiota-derived short-chain fatty acids control IL-17A production by repressing IL-17-producing γδ T cells (Dupraz et al., 2021). The segmented filamentous bacterium is a gut commensal that was found to favor the proliferation of Th17 cells (Littman and Rudensky, 2010) and stimulate the expression of IL-17A in $CD4^+$ T cells in the lamina propria (Ivanov et al., 2009). In addition, epigenetic modification such as DNA methylation and histone modification may regulate IL-17-producing cell differentiation, including Th17 and γδ T cells (Mukasa et al., 2010; Schmolka et al., 2013). Different cytokines such as IL-6, IL-1β, IL-23, and TGF-β may contribute to epigenetic regulation of IL-17 expression (Ghoreschi et al., 2010). Also, gut microRNAs (miRNAs) regulate the intestinal immune system (Bi et al., 2020). Dysregulation of gut miRNA profile correlates with inflammatory responses (Raisch et al., 2013). It has been shown that gut miRNAs regulate IL-17 production by modulating intestinal Th17 cell production (Mikami et al., 2021).

A growing body of evidence has shown the health-promoting benefits of prebiotics and probiotics related to their anti-inflammatory and immunomodulatory activities at the gut level and beyond (Robichaud et al., 2021; Shahbazi et al., 2021; Shahbazi et al., 2020). Manipulating the gut

microbiota by probiotics could be used as a strategy for maintaining gut microbial composition, gut barrier function, and gut immune system homeostasis and preventing diseases related to chronic inflammation (Mohammad and Thiemermann, 2020; Pujari and Banerjee, 2021). Prebiotics favor probiotic commensal bacteria growth and eliminate pathogens. Prebiotics also directly interact with the gut epithelial cells and innate immunity by regulating the Toll-like receptors (TLRs) and inflammatory signaling pathways (Pujari and Banerjee, 2021).

Puberty is a critical developmental window of life characterized by marked physiological changes (Yuan et al., 2020). Gut microbiota development continues throughout adolescence (Yahfoufi et al., 2020). The immune system as a potential mediator of developmental programming undergoes profound alteration throughout puberty (Brenhouse and Schwarz, 2016). It has been shown that pubertal LPS exposure can induce dysbiosis, program the peripheral and central immune responses, and affect brain function (Sharma et al., 2019; Yahfoufi et al., 2021a); however, its lasting effects on gut immunity are not well explored. Probiotic intake might mitigate pubertal LPS-mediated behavioral changes (Yahfoufi et al., 2021a), but the effect of prebiotic and probiotic intake on mitigating enduring LPS-induced inflammatory response at the gut level is not well studied.

Therefore, the current study aimed to investigate whether exposure to LPS in the pubertal window results in immune dysfunctioning in mice during early adulthood and whether dietary intervention during puberty can block LPS-induced immune deregulation later in life by exploring selected cytokines, signaling pathways, and epigenetic mechanisms related to IL-17 production and function. To this end, pubertal female Balb/c mice were exposed to LPS and fed a prebiotic compound, a standardized extract of cultured *Lentinula edodes* mycelia (ECLM) referred to as

AHCC, or probiotic bacterium *Rouxiella badensis* subsp. *acadiensis* (Canan SV-53) referred to as SV-53, to study the effects of treatment on the immune system at the gut level.

3-2- Results

3-2-1- Effect of the Treatment on the Cytokine Concentrations in the Small Intestine

First, we examined the immediate inflammatory effects of LPS exposure and prebiotic/probiotic intake on immune system function by measuring IL-17A and IL-17F levels in the small intestine of pubertal mice. The IL-17 cytokine family consists of six members, including IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. Among all the members, IL-17A and IL-17F share the highest sequence homology and are best studied (Jin and Dong, 2013). Dysregulated IL-17A and IL-17F contribute to chronic inflammation and autoimmunity (Jin and Dong, 2013). The concentration of IL-17A and IL-17F was higher in mice challenged with a single dose of LPS in puberty than in mice receiving AHCC and AHCC+LPS ($p<0.05$) (Figure 3-1A). Then, we measured levels of a variety of cytokines related to IL-17A production, such as TGF-β, IL-6, IL-1β, IL-23, and IL-10 (Figure 3-1A). TGF-β level was higher in the AHCC group compared to the control ($p<0.01$), LPS ($p<0.001$), and AHCC+LPS ($p<0.01$). LPS significantly induced IL-6 secretion ($p<0.05$) and AHCC intake decreased the effect of LPS on IL-6 concentration but did not reach significance. IL-1β concentration significantly was lower in the AHCC group compared to the other groups ($p<0.05$ vs. control and AHCC+LPS and $p<0.01$ vs. LPS). IL-23 level was also lower in the AHCC group compared to other groups ($p<0.05$ vs. control and AHCC+LPS and $p<0.001$ vs. LPS). The level of IL-10 was elevated in groups challenged with LPS compared to unchallenged mice receiving AHCC ($p<0.01$).

Next, the enduring effect of pubertal exposure to LPS and prebiotic intake on the same cytokine profile was examined in adult mice (Figure 3-1B). LPS exposure during puberty caused an enduring increase in IL-17A levels in comparison with control mice ($p<0.01$), while prebiotic intake mitigated the impact of LPS on IL-17A ($p<0.05$). In AHCC+LPS mice, the IL-17F level was lower than in the control and LPS groups ($p<0.05$). TGF-β level was higher in AHCC+LPS mice compared to control and LPS mice ($p<0.05$). AHCC intake in mice challenged with LPS was correlated with a lasting reduction in IL-6 concentration compared to mice that received pubertal LPS without receiving AHCC ($p<0.05$). Feeding mice with AHCC mitigated the stimulatory impact of LPS on IL-1β ($p<0.05$). No significant difference was seen in IL-23 concentration among groups. IL-10 level was lower in LPS mice compared to control and prebiotic groups ($p<0.05$).

Figure 3-1C illustrates probiotic SV-53's impact on the enduring consequences of LPS on immune responses. Probiotic administration to the LPS-treated mice mitigated the enduring effect of LPS on IL-17A ($p<0.05$). IL-17F concentration was significantly lower in the probiotic+LPS group compared to the control and LPS groups ($p<0.05$). A significant increase in the TGF-β levels was seen in mice receiving probiotic compared to untreated mice ($p<0.01$) and mice exposed to LPS ($p<0.0001$). Although probiotic intake decreased the inhibitory effect of LPS on TGF-β, the level of this cytokine remained lower in the probiotic+LPS group than in the probiotic group ($p<0.01$). Probiotic intake also inhibited the stimulatory effect of LPS on IL-6 ($p<0.05$). No significant difference was observed in IL-23 levels among groups. IL-10 concentration was lower in adult mice challenged with pubertal LPS compared to unchallenged and SV-53 mice ($p<0.05$).

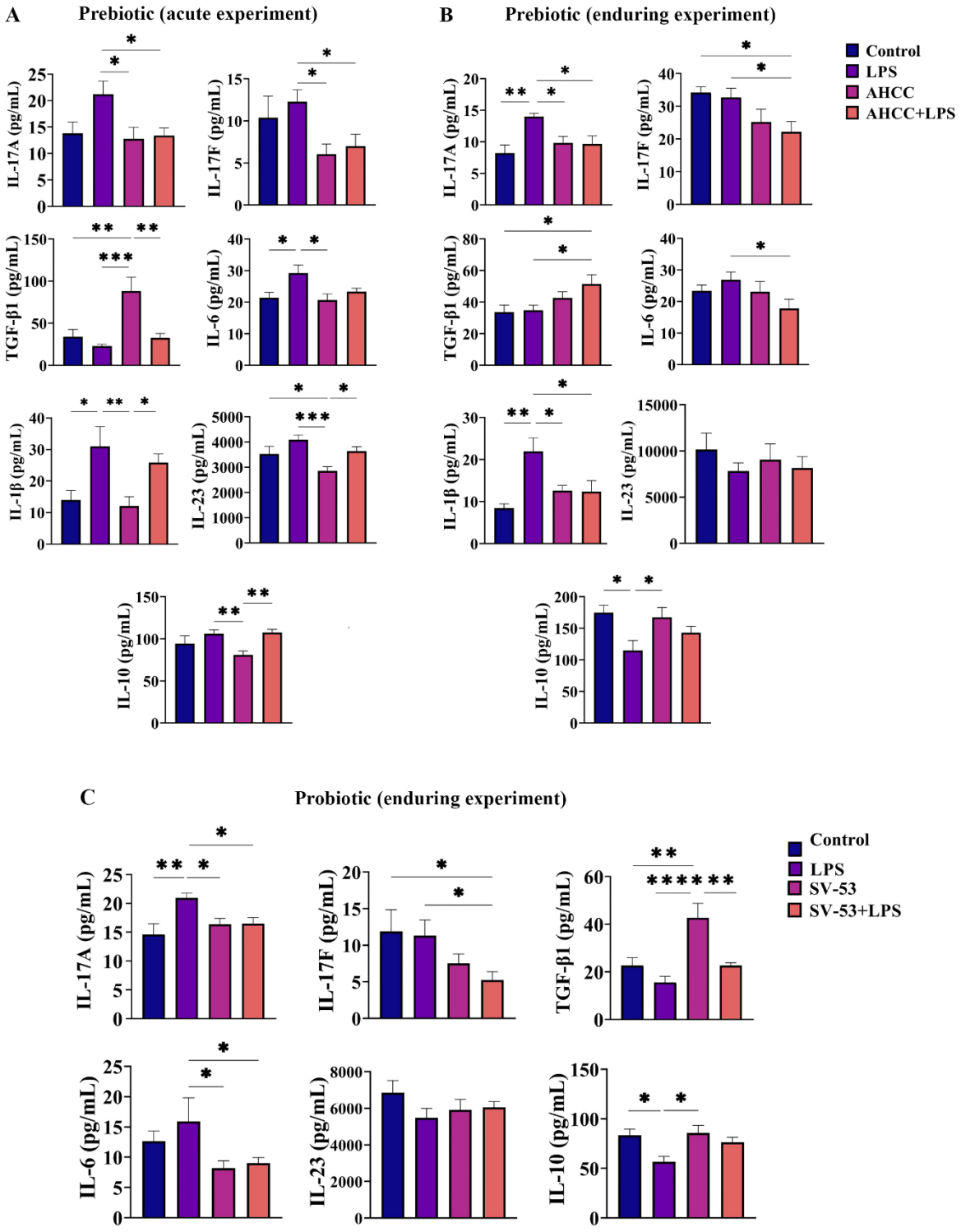

Figure 3-1. (A) Concentrations of IL-17A, IL-17F, TGF-β, IL-6, IL-1β, IL-23, and IL-10 in the small intestine of mice 8 h after injection of a single dose of LPS at puberty. Mice received AHCC (2 g/kg BW/d) in drinking water or drinking water without AHCC for one week before injection. (**B**) Concentrations of IL-17A, IL-17F, TGF-β, IL-6, IL-1β, IL-23, and IL-10 in the small intestine of adult mice four weeks after injection of a single dose of LPS at puberty. Mice received AHCC (2 g/kg BW/d) in drinking water or

drinking water without AHCC for two weeks, one week before, and one week after LPS injection. (**C**) Concentrations of IL-17A, IL-17F, TGF-β, IL-6, IL-23, and IL-10 in the small intestine of adult mice four weeks after injection of a single dose of LPS at puberty. Mice received SV-53 (10^9 CFU/mL) in drinking water or drinking water without SV-53 for two weeks, one week before, and one week after the LPS injection. One-way ANOVA and Tukey's post hoc tests were used to compare groups.. All values are mean ± SEM. N=9, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$

3-2-2-Effect of the Treatment on p-STAT3, STAT3, and FOXO1 Levels in the Small Intestine

To understand the underlying mechanisms governing the immunoprotective effects of the treatments, both acute and enduring effects of treatments on p-STAT3, STAT3, and FOXO1 levels were studied. IL-17 production by the lamina propria $CD4^+$ T cells is dependent on STAT3 activation (Yu et al., 2009) while FOXO1 is a negative regulator of Th17 differentiation and IL-17 production (Lainé et al., 2015). In the puberty window, LPS challenge increased the level of p-STAT3 as compared to the control and AHCC groups ($p<0.05$). AHCC intake could inhibit the stimulatory effect of LPS on p-STAT3 (Figure 3-2A). LPS challenge had no significant acute effect on STAT3 (Figure 3-2A). Also, in pubertal mice, a significant increase in FOXO1 level was found in mice receiving AHCC for one week compared to the control ($p<0.01$) (Figure 3-2A). In adult mice, p-STAT3 levels were significantly lower in AHCC and AHCC+LPS mice than in LPS mice ($p<0.01$ and $p<0.05$, respectively) (Figure 3-2B). In addition, a lasting increase in STAT3 production was observed in LPS mice compared to the control mice ($p<0.05$), while AHCC intake for two weeks significantly inhibited the effect of LPS on STAT3 ($p<0.01$) (Figure 3-2B). Regardless of LPS exposure, prebiotic consumption during puberty stimulated FOXO1 production in adult mice ($p<0.05$) (Figure 3-2B).

Furthermore, in mice challenged with LPS at puberty, probiotic SV-53 administration was able to prevent the stimulatory effect of LPS on p-STAT3 and STAT3 in adult mice ($p<0.05$ and $p<0.01$, respectively) (Figure 3-2C). An increase in the FOXO1 protein was found in the probiotic group

compared to the control ($p<0.05$) and LPS ($p<0.01$) groups and in the SV-53+LPS mice compared to the LPS counterparts ($p<0.05$) (Figure 3-2C).

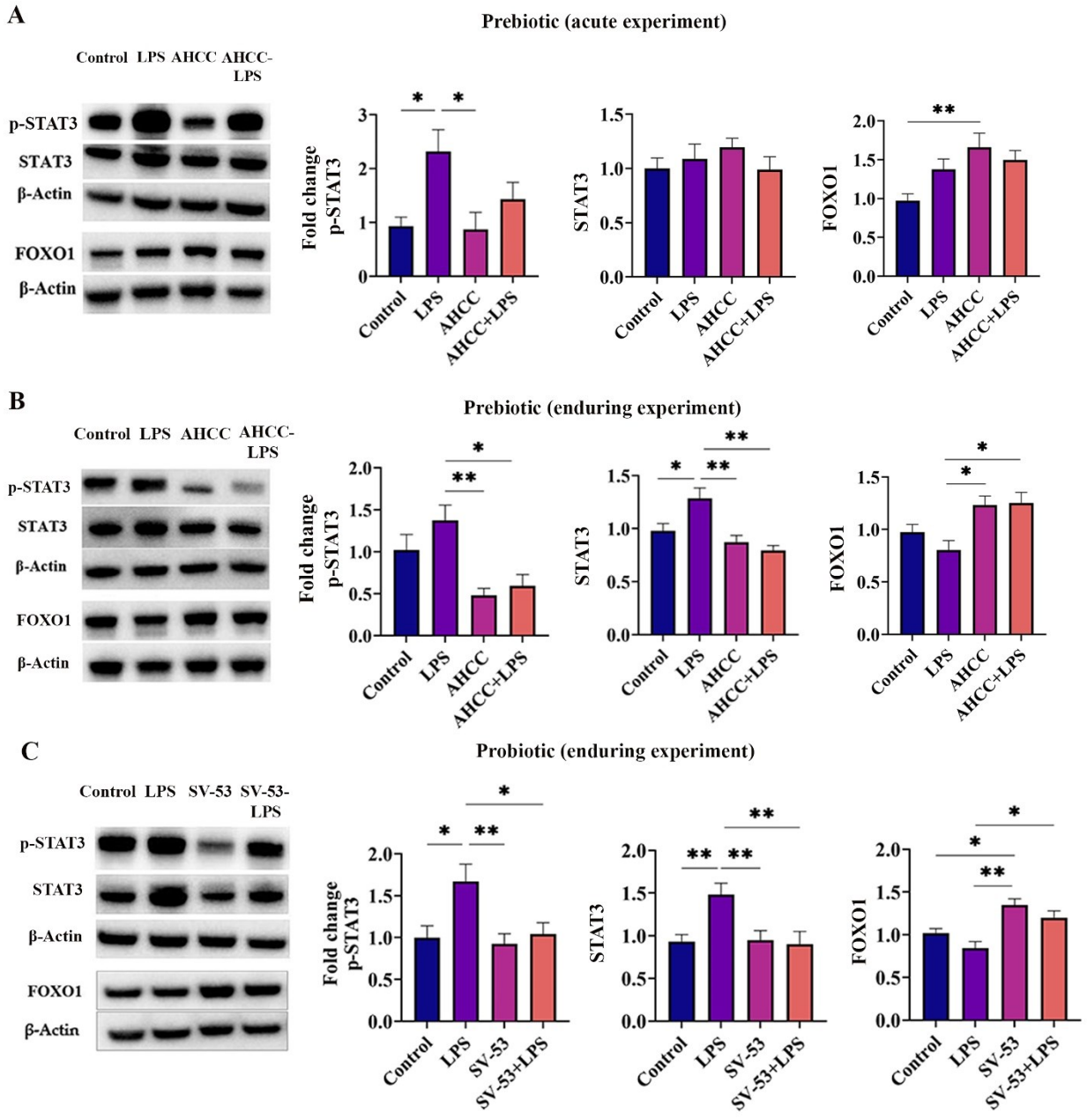

Figure 3- 2. (A) Levels of p-STAT3, STAT3, and FOXO1 in the small intestine of mice 8 h after injection of a single dose of LPS at puberty. Mice received AHCC (2 g/kg BW/d) in drinking water or drinking water without AHCC for one week before injection. (**B**) Levels of p-STAT3, STAT3, and FOXO1 in the small intestine of mice four weeks after LPS injection at puberty. Mice received AHCC (2 g/kg BW/d) in drinking water or drinking water without AHCC for two weeks, one week before, and one week after LPS injection. (**C**) Levels of p-STAT3, STAT3, and FOXO1 in the small intestine of mice four weeks after LPS injection at puberty. Mice received SV-53 (10^9 CFU/mL) in drinking water or drinking water without SV-53 for two weeks, one week before, and one week after LPS injection. One-way ANOVA and Tukey's post hoc tests were used to compare groups. Values are mean ± SEM. N=9, * $p < 0.05$, and ** $p < 0.01$.

3-2-3- Effect of the Treatment on the Gut Microbiota of Mice in Pubertal Window

To study the effect of treatment on microbial richness and evenness, different metrics, including observed features, Chao1, Shannon, and Simpson indices were calculated to assess alpha diversity. At the level of 97% sequence similarity, varied alpha metric results showed no significant difference in alpha diversity. Figure 3-3A illustrates the alpha diversity inferred by the Shannon index plot. For beta diversity, principal coordinates analysis (PCoA) based on the weighted UniFrac Matrix was used to visualize group differences (Figure 3-3B). Then, the beta diversity quantitative distance metric was performed to assess the significance of differences between the groups using the PERMANOVA pairwise test. Our results indicated a significant difference between LPS and prebiotic (Pseudo-F: 5.859, adjusted p-value or q-value: 0.03) and between LPS and prebiotic+LPS (Pseudo-F: 3.959, q-value: 0.03) groups (Figure 3-3C).

Tannerellaceae, Bacteroidaceae, and Flavobacteriaceae were the most abundant families seen in all groups (Figure 3-3D). Differential abundant analysis revealed no significant difference between control and treated groups at the various taxonomic levels. We found differential abundant taxa between LPS and prebiotic, and between LPS and prebiotic+LPS groups at the various levels (Figure 3-3E). Most importantly, the abundance of Bacteroides and Parabacteroides genera, and the abundance of *Bacteroides intestinalis* (*B. intestinalis*) species was markedly higher in mice that received only LPS compared to mice that received only AHCC. The same results at the genus level were obtained when comparing LPS and AHCC+LPS groups, indicating the potential of prebiotic intake in alleviating LPS's impact on gut bacterial communities (Figure 3-3E). AHCC intake one week before the LPS challenge decreased the impact of LPS on *B. intestinalis,* without reaching a significant level (Figure 3-3E).

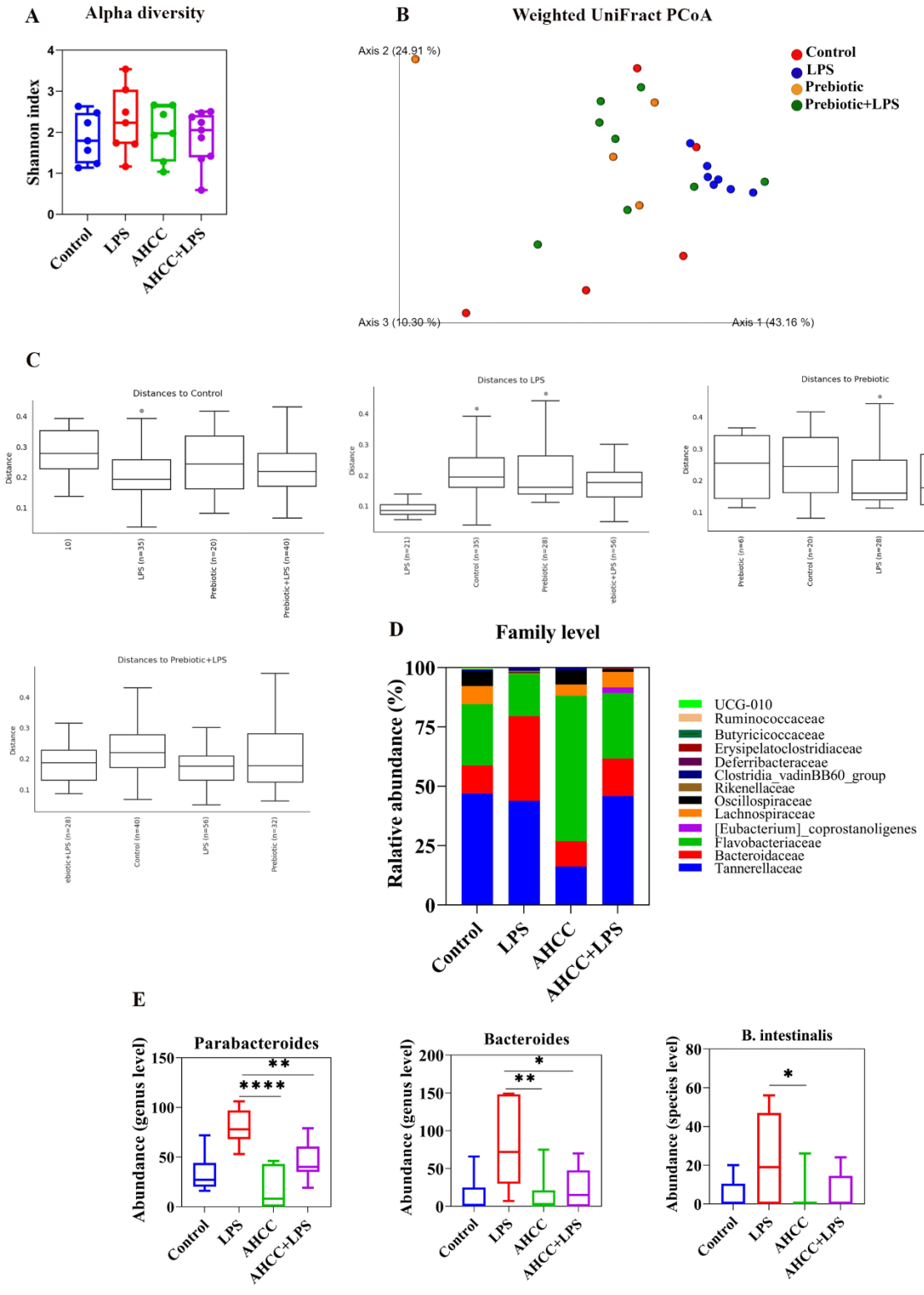

Figure 3-3. Gut Microbiome analysis of pubertal mice. Four-week-old female Balb/c mice received AHCC (2 gr/kg BW/d) in drinking water or drinking water without AHCC for one week before LPS injection. At 5 weeks of age, mice were injected with LPS, and 8 h after injection, mice were sacrificed, and feces

samples were collected. (**A**) alpha diversity, (**B**) weighted UniFrac PCoA, (**C**) weighted UniFrac distance, (**D**) relative abundance of most abundant taxa at the family level, and (**E**) differentially abundant taxa at the genus and species levels. N=7 in the LPS group and 9 in other groups, * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$.

3-2-4- Effect of the Treatment on the miR-145 and miR-425 Expressions in the Small Intestine

miR-145 has been shown to improve chronic inflammatory diseases by targeting various proteins (He et al., 2020). FOXO1 enhances the miR-145 expressions (Gan et al., 2010; Mallet et al., 2021). In addition, miR-145 may suppress STAT3 activation in cancer (Jiang et al., 2017). Inhibition of FOXO1 by miR-425 has been reported (Yang et al., 2018). miR-425 also increases pathogenic Th17 differentiation and IL-17 production (Yang et al., 2018). We have previously shown the role of a polyphenolic mixture fermented by SV-53 in the chemoprevention of mammary carcinoma and controlling breast cancer stem cells by increasing miR-145 and FOXO1 (Mallet et al., 2021; Mallet et al., 2023). We also observed the ability of SV-53 to inhibit miR-425 (unpublished research). Therefore, in the current research, we studied the effect of the treatment on miR-145 and miR-425 expression. LPS and prebiotic intake did not affect miR-145 expression in mice at puberty (Figure 3-4A), however, a significant decrease in miR-425 expression was observed in the AHCC+LPS group compared to LPS ($p<0.01$) (Figure 3-4A). In adult mice, receiving AHCC during puberty correlated with an enduring elevation in miR-145 expression when compared to control ($p<0.0001$ for AHCC, and $p<0.001$ for AHCC+LPS groups) and LPS ($p<0.001$ for AHCC, and $p<0.05$ for AHCC+LPS groups) (Figure 3-4B). In mice challenged with pubertal LPS, AHCC intake led to an enduring reduction in miR-425 compared to the LPS group ($p<0.01$) (Figure 3-4B).

Moreover, feeding mice with probiotic SV-53 upregulated miR-145 expression compared to control ($p<0.05$) and LPS-injected mice ($p<0.0001$) (Figure 3-4C). In mice challenged with LPS,

probiotic intake reduced the inhibitory effect of LPS on miR-145 expression ($p<0.05$) (Figure 3-4C). We found a significant downregulation in miR-425 expression in the SV-53+LPS group compared to the control and LPS groups ($p<0.05$) (Figure 3-4C).

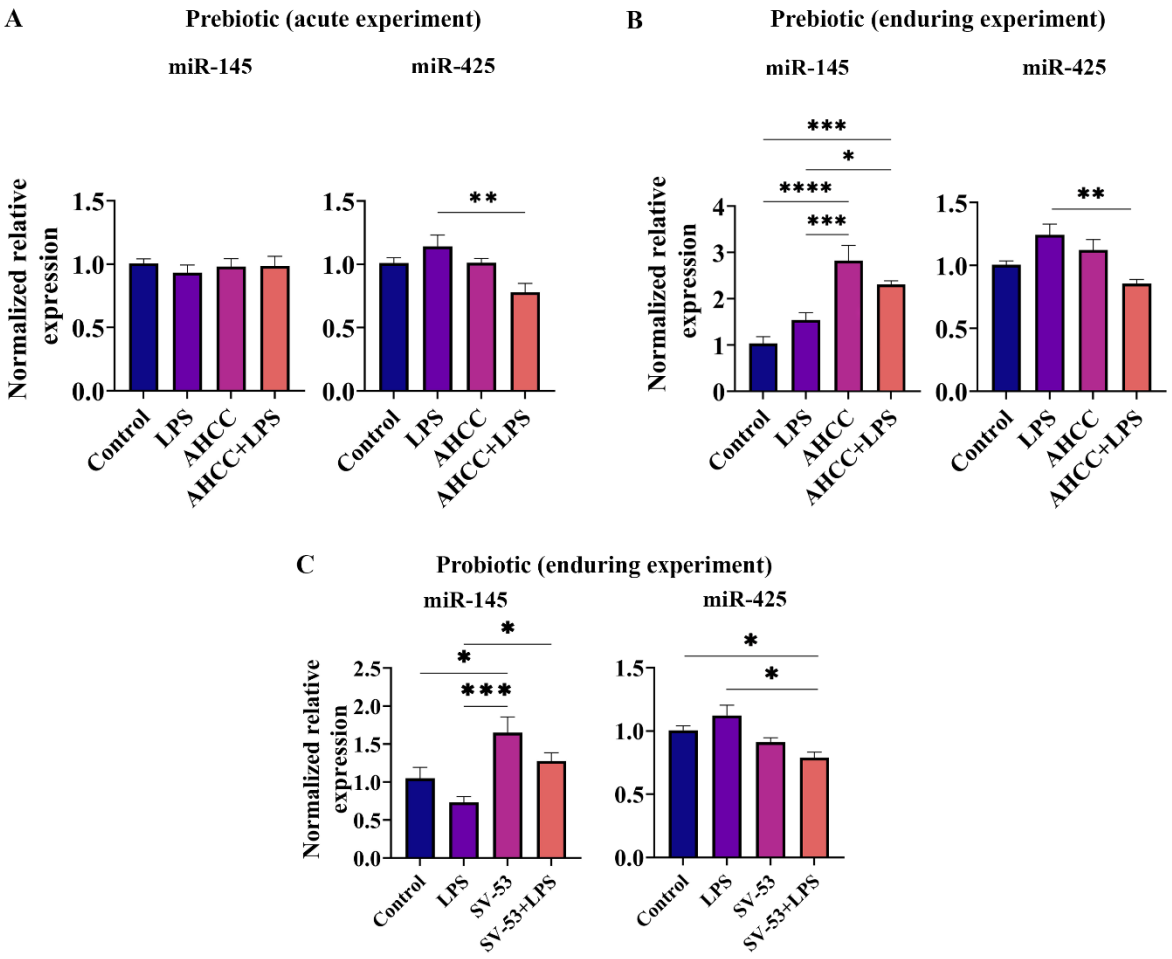

Figure 3-4. (A) Relative expressions of miR-145 and miR-425 in the small intestine of mice 8 h after injection of a single dose of LPS at puberty. Mice received AHCC (2 g/kg BW/d) in drinking water or drinking water without AHCC for one week before injection. (**B**) Relative expressions of miR-145 and miR-425 in the small intestine of adult mice four weeks after LPS injection at puberty. Mice received AHCC (2 g/kg BW/d) in drinking water or drinking water without AHCC for two weeks, one week before, and one week after LPS injection. (**C**) Relative expressions of miR-145 and miR-425 in the small intestine of adult mice four weeks after LPS injection at puberty. Mice received SV-53 (10^9 CFU/mL) in drinking water or drinking water without SV-53 for two weeks, one week before, and one week after the LPS injection. One-way ANOVA and Tukey's post hoc tests were used to compare groups. All values are mean ± SEM. N=9, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

3-2-5-Effect of the Treatment on DNA Methylation Status of Genes in Small Intestine of Mice

The methylation status of the regions around the transcriptional start sites (TSS) is important for gene expression. DNA hypermethylation within TSS is associated with gene expression repression (Yuan et al., 2016; Hong and Rhee, 2022).

To determine if the lasting effects of pubertal challenge with LPS and prebiotic intake on immune system function are mediated partly through epigenetic modifications, the methylation status of genes in the ileum samples of adult mice in AHCC+LPS vs. LPS (Figure 3-5) and LPS vs. control (Supplementary figure S3-1) were examined. By applying the cut-off of absolute group mean difference in beta values of > 0.05 and FDR adjusted p-value < 0.05, differentially methylated probes (DMPs) were identified, and were used as a basis for regional analysis where we found statistically significant differentially methylated regions (DMRs) related to various immune and metabolic pathways such as MAPK, PI3K-AKT, JAK-STAT, and T cell receptor signaling pathways. Figure 3-5A and supplementary figure S3-1A represent the distribution of DMRs within different genomic regions and figure 3-5B and supplementary figure S3-1B illustrate the top enriched pathways for genes associated with the identified DMRs. Notably, when focusing on genes related to the objectives of our study, significant hypermethylation of CpGs was identified within the promoter of the LPS-binding protein gene (*Lbp*) in LPS-challenged and AHCC-fed mice in comparison to LPS-challenged mice (Figure 3-5C). We also found significant hypermethylation of promoter regions of genes directly controlling IL-17A production or function, including RAR-related orphan receptor C (*Rorc)*, runt-related transcription factor 1 (*Runx1*), and IL-17 receptor A gene (*Il17ra*) in AHCC+LPS mice compare to LPS counterparts (Figure 3-5C). 1 to 5 kb regions within chemokine (C-C motif) ligand 5 (*Ccl5*), and Rac family small GTPase 1 (*Rac1*) were also significantly methylated in AHCC+LPS (Figure 3-5C). In addition, the promoter of *Il10* and 1 to

5 kb region of nuclear factor of activated T cells c1 (*Nfatc1*) were hypomethylated in the AHCC+LPS group compared to the LPS group (Figure 3-5C).

In LPS vs. control mice, hypomethylation of *Runx1*, *Il17ra*, *Rac1*, and *Ccl5*, and hypermethylation of *Il10* and *Nfatc1* genes around TSS were observed; however, by applying the cut-off, only *Rac1*, *Ccl5*, and *Il10* were significantly different between groups (Supplementary figure S3-1C). None of these genes were found among detected differentially methylated genes when the AHCC+LPS group was compared to the control.

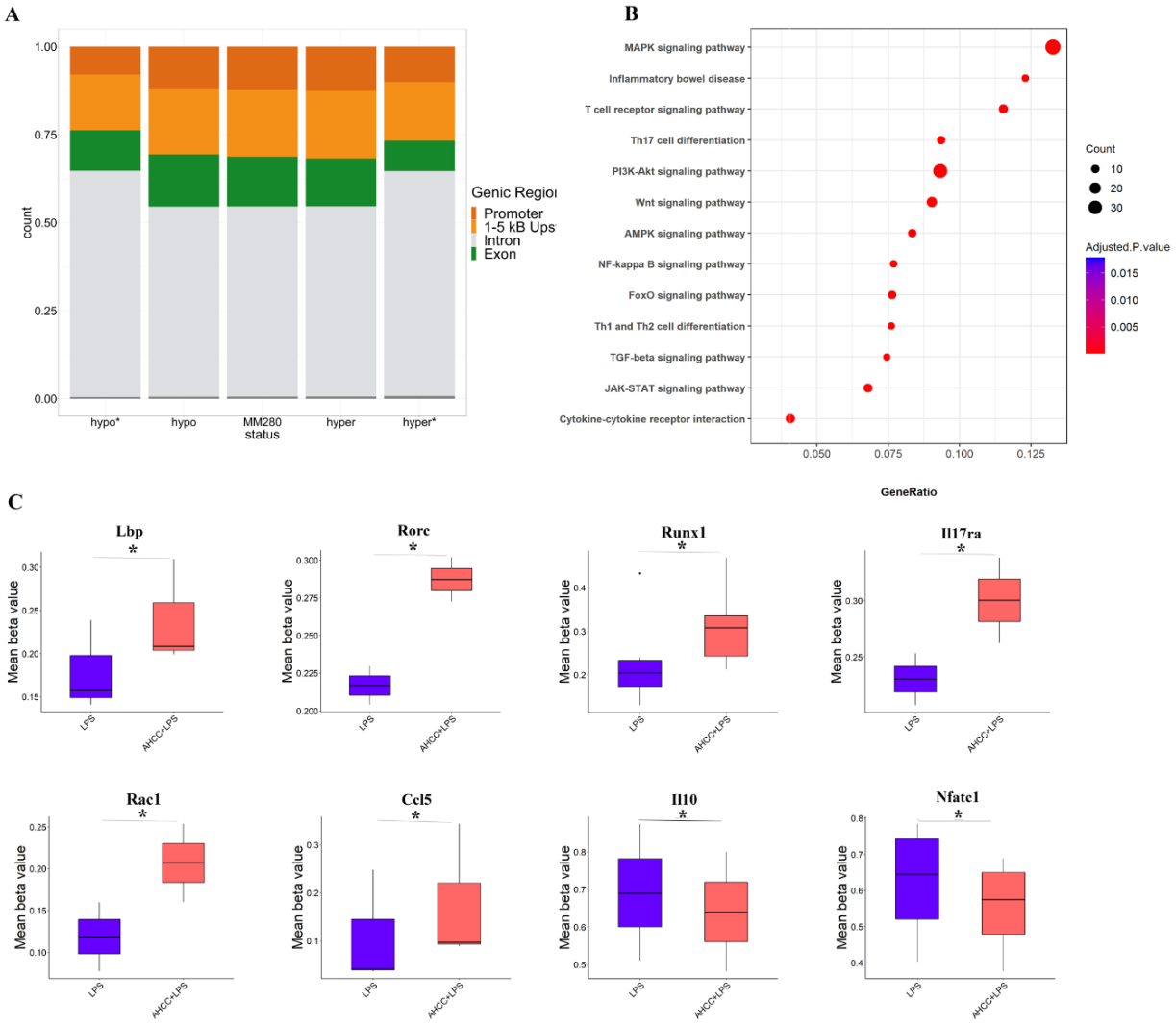

Figure 3-5. DNA methylation analysis in adult mice four weeks after LPS injection at puberty. Mice received AHCC (2gr/kg BW/d) in drinking water or drinking water without AHCC for two weeks, one

week before and one week after LPS injection. (**A**) Genomic regulatory regions, where the hypo and hyper columns indicate all hypo- and hypermethylated regions, respectively and hypo* and hyper* columns represent significant hypomethylated and hypermethylated DMRs, respectively. MM280 column represents the overall distribution of the array. (**B**) Pathways enrichment analysis visualized by Enrichr, (**C**) Boxplots of differentially methylated genes.*$p < 0.05$ vs. LPS

3-3- Discussion

Disruption of gut microbiota by an immune stressor (inflammatory LPS) during puberty has been shown not only to transiently perturb gut microbiota but also to induce immune alterations that could significantly affect developmental programming at distant sites from the gut (Herrera-Covarrubias et al., 2017). Even after microbiota recovery, phenotypic changes such as immune and metabolic changes remain with alteration in long-term developmental programming (Cho et al., 2012; Leclercq et al., 2017). However, dietary interventions can mitigate these changes and prevent immune perturbation (Yahfoufi et al., 2023). Probiotics and prebiotics are an integral part of a balanced diet such as the Mediterranean diet which is well-known as an effective dietary pattern against dysbiosis (Garcia-Montero et al., 2021).

In the present study, we investigated the potential role of a prebiotic compound, AHCC, and a probiotic bacterium, SV-53 in mitigating lasting inflammatory immune responses in mice exposed to inflammation and dysbiosis during the developmental period of puberty through regulation signaling pathways and epigenetic mechanisms. Both AHCC and SV-53 have been shown to protect against inflammatory challenges by potentially mimicking immunobiotic effects (Yahfoufi et al., 2021a; Yahfoufi et al., 2023; Mallet et al., 2016). Prebiotic AHCC is a cultured mushroom mycelium extract shown to favorably modulate the immune system and alleviate cancer burden. AHCC plays an immunomodulatory role by priming the TLR-2 and TLR-4 at the intestinal epithelium (Mallet et al., 2016). Evidence also suggests that AHCC exerts inhibitory activity on cancer stem cells by modulating miRNAs involved in immune evasion (Graham et al., 2017).

Probiotic SV-53 is a novel Gram-negative probiotic bacterium isolated from the microbiota of wild blueberry fruit which exerts significant immunomodulatory effects at the gut level by improving gut mucosal immunity and gut barrier integrity, regulating IgA, IL-10, and IL-6 secretion, modulating gut miRNA expression, and reducing the population of pathogenic bacteria such as *Salmonella Typhimurium* and *Staphylococcus aureus* in intestinal fluid (Matar; Novotny-Nuñez et al., 2023; Yahfoufi et al., 2021b; Salvetti et al., 2023).

In the current paper, we first studied the immediate effect of LPS on cytokine profiles, eight hours post-exposure in pubertal mice, and the role of AHCC intake in inhibiting LPS effects. The effect of LPS treatment on increasing inflammatory cytokine levels, shortly after exposure, in the serum and tissues has been reported (Sylvia and Demas, 2018; Yahfoufi et al., 2023). A difference in the acute immune responses to LPS exposure has been found between puberty and adulthood, indicating that immune responses to immune stressors are highly affected by age (Esposito and Ismail, 2022). We found that prebiotic administration diminished the immediate effects of pubertal LPS exposure on IL-17A and F, TGF-β, IL-6, IL-1β, and IL-23.

Along with the acute effect, the long-term impact of pubertal LPS and prebiotic/probiotic exposure on immune responses in adult Balb/c mice was also studied. Interestingly, in adult mice, prebiotic intake during puberty diminished the lasting stimulatory effect of pubertal LPS challenge on IL-17A, IL-1β, and IL-6 production. Also, in adulthood, the level of TGF-β was higher in mice concomitantly exposed to LPS and AHCC in the pubertal window. Although LPS exposure immediately increased the IL-10 production, in adult mice the levels of IL-10 dropped compared to untreated and prebiotic-treated mice. LPS and dietary intervention had no long-term impact on IL-23 levels. We also observed that probiotic SV-53 intake contracted LPS impact on IL-17A, TGF-β, and IL-6 production. LPS exposure exerted an enduring inhibitory impact on IL-10

production, while probiotic intake elevated IL-10 levels in mice exposed to LPS but did not reach significance.

Based on the results from the cytokines, the lasting impact of the treatment on STAT3, p-STAT3, and FOXO1 levels was studied in the small intestines of adult mice. Studies have shown the effect of natural products on the inhibition of IL-17 secretion by targeting the STAT3 pathway (Chen et al., 2015). In a study, feeding mice with a *Lactobacillus acidophilus* led to the alleviation of colitis-associated inflammatory responses related to the IL-23/Th17 pathway through inhibition of STAT3 and secretion of IL-17 (Chen et al., 2015). In the acute phase, AHCC intake could decrease the stimulatory effect of LPS on p-STAT3 in pubertal mice. In the long term, our result showed the significant role of prebiotic/probiotic intake in mitigating LPS-derived increase in STAT3 and p-STAT3 in the small intestine of adult mice. Moreover, prebiotic/probiotic administration caused a lasting increase in the intestinal level of FOXO1.

Secreted cytokines following antigen recognition by innate immune cells activate different transcription factors such as STAT3 and instruct naïve T cells to acquire effector or regulatory phenotypes (Egwuagu, 2009). The master transcription factor of Th17 cells, retinoic acid receptor-related orphan receptor-gamma-t (RORγt), regulates Th17 cell differentiation and is necessary to induce IL-17A expression (Lee et al., 2020). In the gut, IL-6 promotes STAT3-dependent expression of RORγt and subsequent Th17 cell proliferation and IL-17 expression (Chen et al., 2022), while IL-1 and IL-23 induce delayed differentiation and expansion of Th17 cells (Sutton et al., 2009; Ghoreschi et al., 2010). Studies have shown that the deletion of STAT3 in $CD4^+$ T-cells protects mice against the development of experimental autoimmune diseases and suppresses the production of IL-17-expressing T cells (Liu et al., 2008b). In addition, p-STAT3 activates the transcription of the STAT3 gene, resulting in an accumulation of unphosphorylated STAT3.

Unphosphorylated STAT3, in turn, triggers a second wave of cytokines production, specifically IL-6, causing prolonged cytokine-dependent signaling at later stages (Yang et al., 2007). On the other hand, IL-10 signaling in T cells blocks the emergence of IL-17A-producing cells (Huber et al., 2011). IL-10 along with TGF-β stimulates regulatory T cell (Treg) development and limits Th17 cell-induced inflammation in the gut (Hsu et al., 2015).

Some evidence indicates that IL-6 and IL-23 in the presence of IL-1β efficiently promote IL-17 secretion from Naïve $CD4^+$ T cell, possibly by histone modification of the *Il17a*/*Il17f* and *Rorc* promoters directed in the absence of TGF-β1 while TGF-β1 suppresses this polarization (Ghoreschi et al., 2010). IL-1 signaling in T cells not only promotes early Th17 cell differentiation but it is also critical for the maintenance of Th17 cells in the lack of TCR stimuli (Chung et al., 2009). IL-1β facilitates IL-17 A and F expression by promoting STAT3 phosphorylation and its binding to key cis-elements that control *Il*17*a*/*f* transcription (Whitley et al., 2018). Our results may indicate that LPS may affect IL-17 production, in part, through IL-1β signaling while prebiotic intake may diminish this effect by inhibiting IL-1β. In a study, systemic LPS administration to mice did not increase Th17 differentiation but drove pre-committed IL-17A-producing Th17 cell expansion independent of IL-23 and possibly through IL-1 and IL-6-dependent manner (McAleer et al., 2010).

On the other hand, overexpression of IL-17A, in turn, drives the production of IL-6, IL-23, and IL-1β resulting in inflammation amplification (Chen and Zhou, 2015; Sutton et al., 2009). IL-17-stimulated release of IL-6 activates the STAT3 pathway and promotes the differentiation and maturation of Th17 cells and further IL-17 production (Chen and Zhou, 2015).

In the present research, at the acute phase, a rapid elevation in IL-17 levels in LPS-challenged mice may also indicate the role of innate immune cells such as ILC3s in IL-17 induction which directly happens in response to IL-1β and IL-23 without further differentiation of $CD4^+$ T cells (Chung et al., 2021).

FOXO1 transcriptional factor is highly expressed in Treg which mediates the expression of IL-10, and TGF-β (Graves and Milovanova, 2019; Cabrera-Ortega et al., 2017) and suppresses Th17 cells differentiation by binding to RORγt via its DNA binding domain and inhibiting its transcriptional activity, as well as decreasing IL-17A production and IL-23 receptor expression (Lainé et al., 2015; Cabrera-Ortega et al., 2017). LPS activates AKT signaling leading to FOXO1 phosphorylation and inactivation (Graves and Milovanova, 2019). Moreover, in a study, IL-6/STAT3 signaling highly stimulated miR-183 cluster (miR-183C) expression. This miRNA cluster is connected to the pathogenicity of Th17 due to pathogenic cytokine production, including IL-17A and IL-17F, and mediates autoimmunity by repression of FOXO1 and induction of IL-1R1 expression (Ichiyama et al., 2016). Of interest, TGF-B inhibited the IL-6/STAT function in inducing miR-183C in Th17 cells and reduced their pathogenicity (Ichiyama et al., 2016). Hence, our results may indicate that prebiotic and probiotic intake exert immunomodulatory activity partly by modulating STAT3 and FOXO1 signaling and regulating the expression of cytokines that control these pathways, including IL-17A, IL-1β, IL-6, and TGF-β.

Furthermore, we studied the effect of AHCC on the gut microbiota of pubertal mice eight hours after LPS injection. We have previously shown that the administration of LPS and SV-53 to pubertal mice changed gut microbiota in a sex-dependent manner (Yahfoufi et al., 2023). In the current research, the abundance of Bacteroides and Parabacteroides genera was markedly higher in mice that received only LPS compared to mice in AHCC and AHCC+LPS counterparts. An

increase in Bacteroides and Parabacteroides was positively correlated with colitis-induced mucosal injury (Shi et al., 2021). Also, a positive correlation between the augmentation of Parabacteroides and Bacteroides genera and cytokines involved in the pathogenesis of immune-related disorders, including IL-17, IL-21, and IFN-γ has been reported in patients (Guo et al., 2020).

We found a higher abundance of *B. intestinalis* in the gut microbiota of mice challenged with LPS compared to prebiotic-fed mice, and an insignificant decline in *B. intestinalis* in AHCC+LPS compared to LPS groups. A recent study revealed the critical role of *B. intestinalis* and IL-1β signaling in immune checkpoint blockade (ICB) therapy-induced ileitis (Andrews et al., 2021). Andrews et al. (2021), found that ICB-induced intestinal inflammation is strongly related to overexpression of ileal *Il1b*. Gut microbiota analysis revealed a marked elevation of *B. intestinalis* in patients with ICB toxicity along with overexpression of mucosal IL-1β in patient samples of colitis and mice. Furthermore, colonizing mice with *B. intestinalis* upon microbiota ablation by antibiotics accompanied by induction of ileal *Il1b* transcription and increased ileal damage (Andrews et al., 2021).

To investigate whether the persisting effects of LPS exposure and prebiotic intake on gut immunity are mediated by durable epigenetic modifications, we studied miRNA expression and DNA methylation status in the small intestine of adult mice. Epigenetic mechanism regulates microbiota-host interactions (Pan et al., 2018b). miRNAs derived from intestinal epithelial cells modulate the composition of the intestinal microbial communities (Bi et al., 2020). Commensal microbes can regulate DNA methylation by providing primary substrates (Woo and Alenghat, 2022).

In this study, it was shown that prebiotic and probiotic intake stimulated miR-145 expression. Also, feeding LPS-exposed mice with the prebiotic/probiotic significantly diminished the relative

expression level of miR-425. Similarly, in a study using a murine model of colorectal cancer, the administration of probiotic *Bifidobacterium longum* correlated with an increase in miR-145 and a decrease in IL-6 concentration (Fahmy et al., 2019). Down-regulation of miR-145 is associated with an increase in pathogenic Th17 cell responses (Wang et al., 2013). In a study, over-expression of miR-145 improved metabolic inflammation in mice and prevented LPS-induced inflammation *in vitro* (He et al., 2019). Also, miR-145 knockdown was associated with increased secretion of TNF-α, IL-6, and IL-1β *in vitro (Li et al., 2018)*. In bladder cancer cells miR-145 expression inhibited STAT3 activation, stimulated FOXO1 expression, and suppressed cell growth (Jiang et al., 2017). Upregulation of miR-425 has been reported in inflammatory conditions such as some types of cancers which is correlated with cancer progression (Xiao et al., 2019; Wu et al., 2021). Overexpression of miR-425 promotes Th17 cell production and IL-17A secretion by suppressing FOXO1 in colitis mice (Yang et al., 2018). IL-1β was found to induce miR-425 expression in gastric cancer cells by activating NF-κB signaling (Ma et al., 2014).

Additionally, DNA methylation analysis revealed an increase in CpGs methylation around TSS of *Lbp*, *Rorc*, *Runx1*, *Il17ra*, *Rac1*, and *Ccl5* in adult mice received AHCC+LPS during puberty compared to the mice only challenged with LPS which may indicate the transcriptional repression of these genes by AHCC.

TLR4 recognizes LPS with the help of diverse proteins such as LBP. LBP binds to LPS molecules and delivers them to CD14. CD14 subsequently transfers LPS to the TLR4/MD-2 complex which leads to inflammatory signal initiations and proinflammatory cytokines production (Park and Lee, 2013). *Rorc* encodes RORγt (Ma et al., 2022). Conditional deletion of *Rorc* in IL-17A-producing Th17 cells has revealed the critical role of RORγt in Th17 cell stability (Hall et al., 2022). Moreover, genetic ablation of *Rorc* in mature Th17 has been found to suppress their pathogenic

activity (Chi et al., 2022). Overexpression of *Runx1* is associated with the pathogenesis of autoimmunity through Th17 cell induction (Zhong et al., 2016). RUNX1 promotes Th17 generation by enhancing RORγt expression and forming a complex and interacting with RORγt which upregulates *Il17* transcription (Zhang et al., 2008). IL-17RA is required for IL-17 cytokines signaling. IL-17/IL-17RA signaling regulates different inflammatory pathways resulting in the release of pro-inflammatory cytokines such as IL-1β and IL-6 (Rex et al., 2023). IL-17/IL-17RA signaling exerts a pathogenic role in multiple inflammatory and autoimmune diseases and targeting this signaling is a remarkable strategy in the treatment of autoimmunity (Zhang et al., 2023; Zhang et al., 2019a).

Evidence demonstrates that LPS may induce NF-κB and proinflammatory cytokine responses through a Rac1-dependent pathway (Sanlioglu et al., 2001). Rac1 is necessary for IL-17A expression and induction of autoimmunity in mice. A reduction in IL-17A, IL-17F, and IL-22 levels, and IL-23 receptor expression in Rac1 deficient Th17 cells has been reported (Kurdi et al., 2016). Moreover, LPS induces CCL5 expression in macrophages through a TLR4-dependent pathway (Bandow et al., 2012) and higher expression of proinflammatory chemokines, including CCL5 by pathogenic Th17 cells has been reported (Lee et al., 2012).

In addition, the *Il10* promoter was hypomethylated in the AHCC+LPS group, which may indicate transcription activation of the *Il10*. Evidence shows the protective function of IL-10 against autoimmunity by controlling pathogenic Th17 differentiation (Zhang et al., 2013). In colitis mice, IL-10 potently suppresses the pro-IL-1β production transcriptionally in macrophages and its maturation to IL-1β, and alters the Th17 cytokine dependency required for colitis pathogenesis (Li et al., 2015). IL-10 preserves FOXO1 function by inhibiting the AKT pathway (Hsu et al., 2015).

NFATc1 is a member of the NFAT family of transcription factors which is activated following TCR stimulation and subsequent dephosphorylation and nuclear import. NFATc1 contributes to Th17 cell differentiation and its deficiency is correlated with reduced expression of RORγt and Th17-related cytokines such as IL-17A, IL-17F, and IL-21 (Reppert et al., 2015). However, we observed a decrease in methylation of *Nfatc1,* the gene coding NFATc1 protein, in AHCC+LPS mice than in LPS mice.

Our study has some limitations. Although our results, particularly p-STAT3, FOXO1, and DNA methylation results, may suggest the role of AHCC in regulating immune responses in inflammatory conditions, in part, by modulating Th17 cells, fluorescence-activated cell sorting or FACS analysis could be instrumental in determining the different immune cells' involvement in the anti-inflammatory activity of AHCC. Moreover, although sex differences in immune responses in mice have been previously demonstrated (Yahfoufi et al., 2023), we only studied female mice in this paper, because this research is part of a larger study on the gut-mammary gland axis utilizing female Balb/c mice. We also did not perform DNA methylation for the probiotic SV-53 experiment.

In conclusion, we found that exposure to an immune stressor in critical windows of development that alters gut microbiota may cause enduring dysregulation in cytokines and central proteins involved in gut immunity, in part, by epigenetic modification, and therefore, lead to lasting immune system dysfunction at the gut level; while prebiotic or probiotic intake may mitigate these negative consequences. Together, these results may indicate that following a healthy dietary pattern such as a diet rich in prebiotics and probiotics could be a useful strategy in priming the immune system in early life and preventing health problems later in life.

3-4- Materials and Methods

3-4-1- Animals

Four-week-old female Balb/c mice weighing 13–17 g (Charles River, Montreal, QC) were used in the current study. Three mice were housed together in plastic cages in a controlled atmosphere (temperature 22 ± 2°C; humidity 55 ± 2 %) with a 12 h light/dark cycle. During the study, all groups received a conventional balanced diet ad libitum. Mice were maintained and treated following the guidelines of the Canadian Council on Animal Care. The protocol (HSe-3191) was approved by the Animal Care Committee of the University of Ottawa.

3-4-2- Study Design

3-4-2-1 Pubertal LPS-Prebiotic Model

We first examined the acute and enduring effect of pubertal LPS exposure and prebiotic intake on the gut microbiota and immune system. In total, 72 mice were used in this experiment. The timing of puberty was determined in mice by examining the first pubertal event or vaginal opening (Nelson et al., 1990). Mice were categorized into two groups (n=36): 1- prebiotic group; receiving AHCC (2 gr/kg BW/day) in drinking water and 2- control group; receiving regular drinking water for one week before puberty. At puberty (5 weeks of age), half of the mice in each group were injected intraperitoneally (IP) with LPS at a dose of 1.5 mg/kg, which has been shown to provoke inflammation and dysbiosis (Yahfoufi et al., 2023), and the other half were injected with 1X sterile phosphate buffer saline (PBS) (NaCl 2.7 mM KCl 10 mM Na2HPO4, 1.8mM mM KH2PO4, pH7 (Sigma Aldrich, Oakville, Canada). LPS solution was prepared by dissolving 0.2 mg/ml lyophilized LPS from *Escherichia coli* O26:B6 (Sigma Aldrich, Oakville, Canada) into sterile PBS. Therefore, we had four experimental groups after LPS/PBS injection (n=18 in each group): 1-control; receiving regular drinking water and injected with PBS, 2-LPS; receiving regular

drinking water and injected with LPS, 3-prebiotic; receiving AHCC in drinking water and injected with PBS, and 4-prebiotic+LPS; receiving AHCC in drinking water and injected with LPS. Eight hours after the LPS/PBS injection half of the mice in each group were sacrificed to study the acute effect of treatment on gut microbiota and immune system. For the remaining mice, nutritional intervention continued for one week after injection, after which the mice received a standard diet until early adulthood. Mice were sacrificed at nine weeks of age, and the required samples were collected to study the lasting effects of treatment on the immune system. AHCC® is a standardized extract of cultured *Lentinula edodes* mycelia, produced by Amino Up Co., Ltd. (Sapporo, Japan).

3-4-2-2- Pubertal LPS-Probiotic Model

Based on the results from the first experiment, we conducted another experiment to study the durable effect of pubertal LPS exposure and probiotic intake on the gut immune system. Thirty-six mice were categorized into two groups (n=18): 1-probiotic group; receiving SV-53 (10^9 CFU/mL) in drinking water, and 2-control group; receiving regular drinking water for one week before puberty. At puberty, half of the mice in each group were injected with LPS or PBS as stated above. Thus, we had four experimental groups after LPS/PBS injection (n=9 in each group): 1-control; receiving regular drinking water and injected with PBS, 2-LPS; receiving regular drinking water and injected with LPS, 3-probiotic; receiving SV-53 in drinking water and injected with PBS, and 4-probiotic+LPS; receiving SV-53 in drinking water and injected with LPS. One week after injection, the nutritional intervention was terminated and mice were sacrificed at nine weeks of age. The bacterial culture and preparation have been explained elsewhere (Yahfoufi et al., 2021b). Figure 3-6 illustrates the study design of the pubertal LPS-prebiotic model (acute and enduring experiments) and pubertal LPS-probiotic model (enduring experiment).

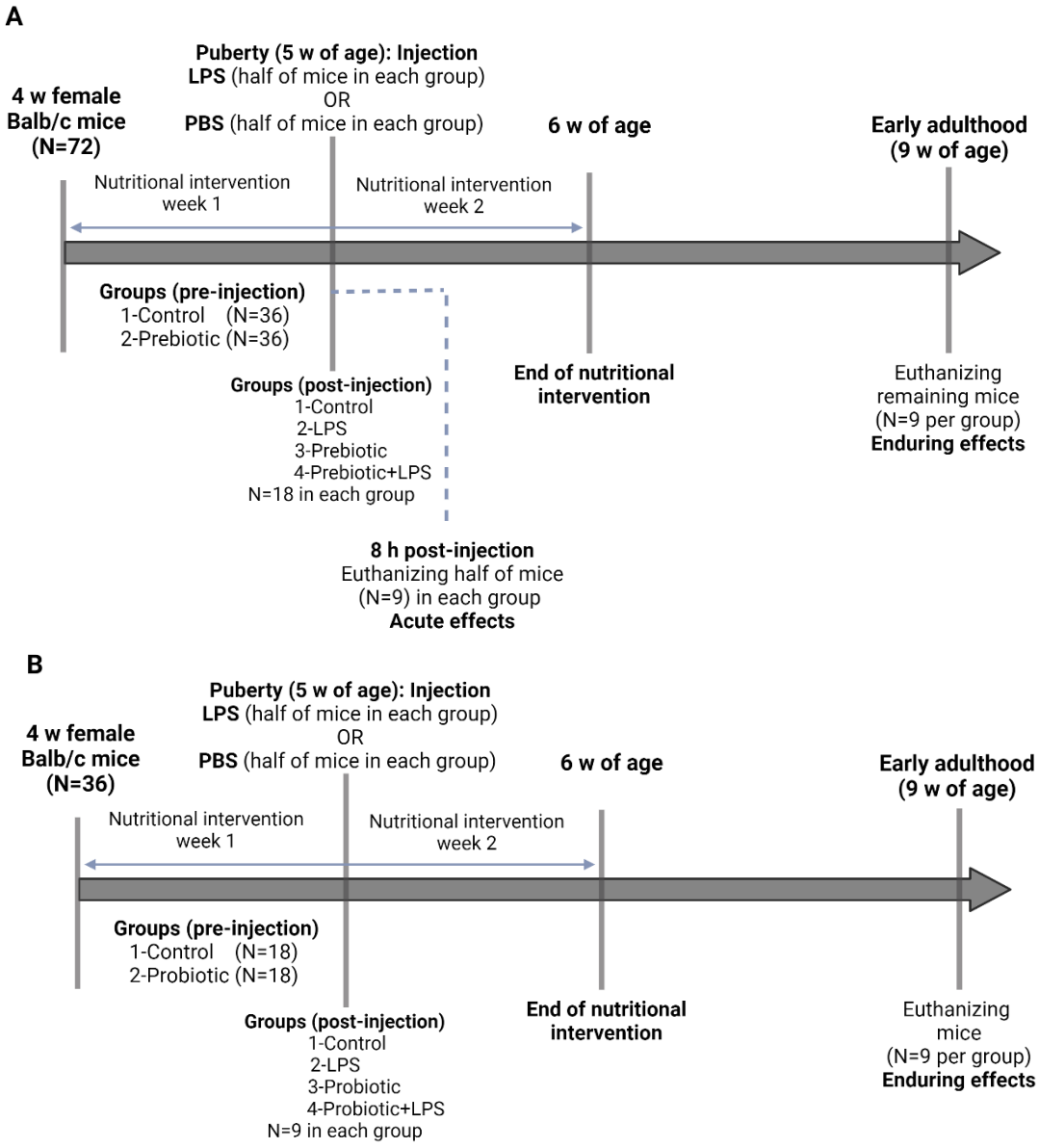

Figure 3-6. Study design showing the experimental timeline, groups, LPS injection, and dietary intervention for (**A**) the pubertal LPS-prebiotic model (acute and enduring experiments) and (**B**) the pubertal LPS-probiotic model (enduring experiment).

3-4-3- Determination of Cytokine Concentrations in the Small Intestine of the Mice by ELISA and Luminex Multiplex Assay

To examine the IL-17A, IL-17F, TGF-β, IL-1β, IL-6, IL-23, and IL-10 levels in the intestinal tissue, after washing the small intestine with 1X PBS, small parts of the ileum (20-25 mg) were cut into very small pieces using surgical blades (Fisher Scientific, Toronto, Canada) and collected

in microtubes containing lysis buffer (20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.05% Tween-20) and a cocktail of protease inhibitors (AEBSF, Hydrochloride, Millipore Sigma, Oakville, Canada). After vortexing and incubation on a rocker at 4°C for 20 minutes, homogenized samples were centrifuged at 14000g for 10 minutes at 4°C, and supernatants were collected. The concentrations of total proteins in tissue lysates were measured by the BCA method using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Toronto, Canada) and adjusted to 2 mg/mL using the same lysis buffer. Then, the multiplex assay was done using Mouse Th17 Panel Magnetic, MTH17MAG-47K (Millipore Sigma, Burlington, USA) and TGFBMAG-64K MAG Bead Kit (Millipore Sigma, Burlington, USA) based on the manufacturer's instruction, and plates were read using a MAGPIX® System (Millipore Sigma, Burlington, USA). The IL-17A levels were assayed using an ELISA kit (Invitrogen, Vienna, Austria) and the results were calculated by dividing each sample's observed concentrations of cytokines by the total protein concentration of that sample.

3-4-4-Determination of p-STAT, STAT3, and FOXO1 Levels in the Small Intestine of the Mice by Western Blotting

Small segments of ileum were cut into very small pieces and lysed in the appropriate amount of RIPA buffer (Thermo Fisher Scientific, Toronto, Canada) and the Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Toronto, Canada) for 1 hour on a rocker at 4°C. The protein lysates were collected by centrifuging homogenized samples at 14000g, for 20 min, at 4°C, and total protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Toronto, Canada) following the manufacturer's protocol. The equal amount and concentration of protein samples were run on the 12% Bis-Tris Mini Protein Gels (Invitrogen, Toronto, Canada), using MES SDS Running buffer (Life Technologies, Toronto, Canada) at 200

V for 22 min and then transferred to PVDF membrane in a Trans-Blot Cell (Bio-Rad, Hercules, CA, USA), at 100 V for 1 hour. Membranes were incubated with anti-FOXO1 (1:1000), anti-STAT3 (1:1000), anti-phospho-STAT3 (phospho Y705) (1:1000), and anti-β-actin (1:1000) primary antibodies (Abcam, Toronto, ON, Canada) at 4ºC overnight. Then, blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10000) (Jackson Immuno Research Laboratories, West Grove, PA, USA) at room temperature for 1 hour. Bands were visualized by chemiluminescence assay using ECL substrate (Bio-Rad, Mississauga, ON, Canada) and quantified by the Bio-Rad Image Lab Software using β-actin as loading control.

3-4-5- Determination of miRNAs Expression in the Small Intestine of the Mice by Real-Time Quantitative Reverse Transcription PCR (RT-qPCR)

Small pieces of ileum were stored in RNAlater Stabilization Solution (Invitrogen, USA) for 24 h at 4 °C and then stored at -80°C until RNA extraction. The total RNA of samples was extracted using miRNeasy mini kit (Qiagen, Toronto, ON, Canada), and their concentrations and purity were determined by a NanoDrop 2000 (Thermo Scientific, Waltham, MA, United States). To assay the expression of selected miRNAs, first reverse transcription reaction was done to synthesize cDNA using miRCURY LNA RT Kit (Qiagen, USA), and then the expression levels of miR-145 and miR-425 were measured by RT-qPCR using hsa-miR-145-5p and has-miR-425-5p miRCURY LNA miRNA PCR assay primers (Qiagen, Toronto, Canada) and miRCURY LNA SYBR Green PCR Kit (Qiagen, Toronto, Canada) in a CFX 96 real-time PCR detection system (Bio-Rad, Laboratories, Hercules, CA, USA). Expression of miRNAs was normalized to SNORD65 (mmu) miRCURY LNA miRNA PCR Assay (Qiagen, Toronto, Canada) as the reference gene. The relative expression level of miRNAs was calculated using the ΔΔCT method.

3-4-6- Methylome-Wide Profiling and Data Analysis

In total, 15–20 mg of mice ileum samples were homogenized by an electrical homogenizer (Bead Mill 24, Fisher Scientific, Canada) in tubes containing 500 µL cell lysis buffer and 1.5 µL proteinase K. The Gentra Puregene Tissue Kit (33 g) kit (Qiagen, Toronto, ON, Canada) was used to extract tissues' DNA according to the manufacturer's instructions. The extracted DNA was then diluted with DNA rehydration solution, provided with the kit, to the final concentration of 20 ng/µL and stored at −20 ◦C. The concentration of extracted DNA was measured using a Qubit 4 instrument (Thermo Fisher Scientific, Canada). For methylome-wide profiling, 500 ng of extracted DNA was subjected to bisulfite conversion using the EZ DNA Methylation kit (Zymo Research, Irvine, CA, USA). Then, 250 ng bisulfite-modified DNA was analyzed using the Infinium Mouse Methylation BeadChip arrays, which allow for the simultaneous assessment of DNA methylation at more than 285,000 CpG sites (Illumina Inc., San Diego, CA, USA). Methylome-wide data were analyzed using the methylkey pipeline developed by the Epigenomics and Mechanisms Branch at the International Agency for Research on Cancer (https://github.com/IARCbioinfo/methylkey (accessed on 21 January 2023)). Briefly, raw data files were pre-processed, quality control was conducted, and normalization was performed by Noob normalization using the SeSAMe package (Zhou et al., 2018). Intergroup comparisons were conducted using linear regression analysis as implemented in the limma R package (Ritchie et al., 2015). Regional analysis to identify differentially methylated regions was conducted using the DMRcate package (Peters et al., 2015).

3-4-7- Gut Microbiome Analysis

For microbiome analysis, the feces samples of mice were collected in sterile microtubes and stored at -80°C following snap-freezing in liquid nitrogen. Microbiome analysis was done by IARC, Lyon, France using amplicon-based sequencing of V3 and V4 variable regions of the 16S rRNA

gene according to the 16S metagenomics sequencing library preparation protocol (Amplicon et al., 2013).

3-4-8- Statistical Analysis

Statistical analysis for all experiments except for DNA methylation and microbiome analysis was conducted by GraphPad Prism 5.0 Software (GraphPad Software Inc., San Diego, CA, USA). The distribution of data was assayed by the Shapiro–Wilk test. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was performed to compare the means of experimental groups. Data are reported as mean ± SEM of three independent experiments and p-value < 0.05 indicates a statistically significant difference between groups. For microbiome analysis, the 16S analysis was conducted by Qiime2, while for differentially abundant taxa comparison, the DESeq2 package for analysis and Wald test were used. Differences in alpha diversities (α-diversity) were assessed by the Chao1, observed species, Shannon and Simpson indexes, and Kruskal–Wallis pairwise test. Sample beta diversity (β-diversity) clustering was assessed using weighted UniFrac PCoA and PERMANOVA pairwise test. Graphs related to α-diversity and taxa abundance were created by GraphPad Prism. Corrected/adjusted p-value < 0.05 was considered statistically significant. Differentially methylated genes were defined with false discovery rate (FDR)-adjusted p-value < 0.05 and absolute inter-group beta value difference of $>$ 0.05. For pathway visualization, KEGG pathway enrichment analysis was performed using Enrichr. Figures 3-6 A and B were generated by BioRender.com (accessed on 19 September 2023).

Supplementary Materials: The supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms241914610/s1.

Author Contributions: R.S. contributed to animal feeding and sample collection, performed all experiments, and wrote the manuscript; H.Y.S. contributed to animal feeding, LPS injection, sample collection, and DNA methylation analysis. N.A. contributed to sample collection and running western blot; F.S. contributed to sample collection and DNA extraction; S.F contributed to protein extraction; C.C. conducted DNA methylation and 16S rRNA sequencing experiments; V.C., and F.F.-L.C. contributed to DNA methylation analysis; Z.H. contributed to the review and editing; C.M. designed and supervised the work. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol (HSe-3191) was approved by the Animal Care Committee of the University of Ottawa on 13 July 2022.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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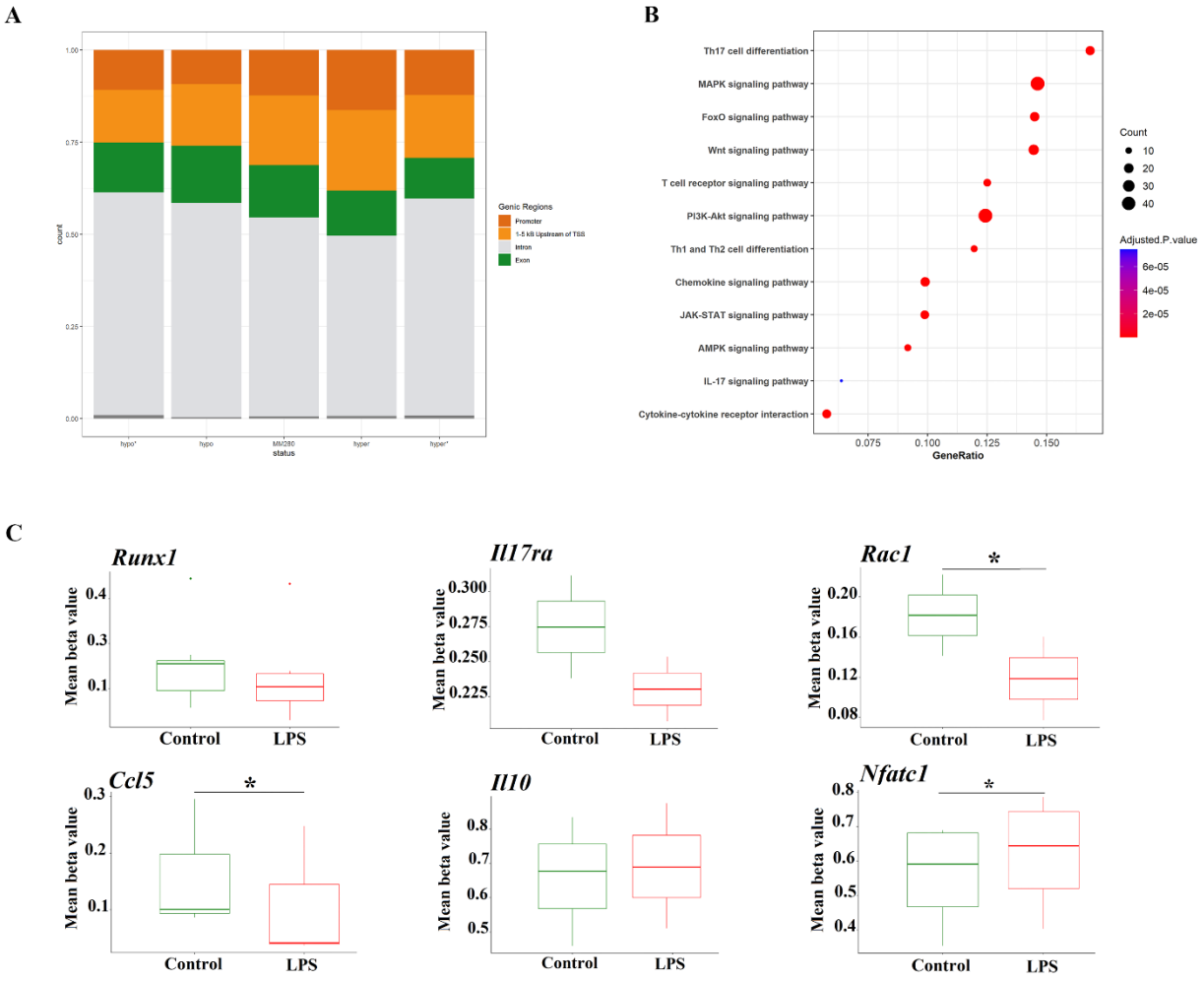
Conflicts of Interest: The authors declare no conflict of interest. Where authors are identified as personnel of the International Agency for Research on Cancer/World Health Organization, the authors alone are responsible for the views expressed in this article and they do not necessarily represent the decisions, policies, or views of the International Agency for Research on Cancer/World Health Organization.

Supplementary Figure S3-1. Effect of the Treatment on DNA Methylation Status in Small Intestine of Mice



Supplementary Figure S3-1. DNA methylation analysis in adult mice four weeks after LPS injection at puberty. Mice received AHCC (2gr/kg BW/d) in drinking water or drinking water without AHCC for two weeks, one week before and one week after LPS injection. (A) Genomic regulatory regions, where the hypo and hyper columns indicate all hypo- and hypermethylated DMRs, respectively and hypo* and hyper* columns represent significant hypomethylated and hypermethylated DMRs, respectively. MM280 column represents the overall distribution of the array. (B) Pathways enrichment analysis visualized by Enrichr, (C) Boxplots of differentially methylated genes.* $p < 0.05$ vs. control.

Chapter 4: *Lentinula Edodes* Cultured Extract Intake Alleviates Long-Term Immune Deregulation Induced by Early-life Gut Microbiota Dysbiosis

4- *Lentinula Edodes* Cultured Extract Intake Alleviates Long-Term Immune Deregulation Induced by Early-life Gut Microbiota Dysbiosis

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Submitted to the "mSystems"

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Abstract

The establishment of gut microbiota during early life is crucial for immune system development and its disturbance within this critical period exerts enduring adverse effects on health. Perinatal antibiotic exposure, a common environmental stressor, disrupts early-life microbiota and leads to long-term immune dysregulation. However, the underlying mechanisms remain inadequately explored. We investigated the persistent consequences of perinatal exposure to low-dose penicillin on gut immunity and the potential protective role of a prebiotic compound, AHCC, against antibiotic-induced dysbiosis and immune dysregulation. Pregnant mice were subjected to penicillin and AHCC treatment from the third week of gestation until weaning of pups. Subsequently, the offspring were evaluated for gut microbiota at weaning as well as immune function, and epigenetic changes at eight weeks of age. Microbiome analysis revealed substantial alterations in gut microbiota composition, characterized by an increase in Proteobacteria and a decrease in Firmicutes following antibiotic exposure. Lactobacillus, and short-chain fatty acid (SCFA)-producing bacteria, crucial for gut homeostasis, were diminished by the antibiotic, emphasizing a potential link to inflammatory responses. AHCC intake prevented antibiotic effects on Proteobacteria in dams and offspring and some SCFA-producing bacteria in male offspring. In adult offspring, AHCC exhibited immunomodulatory activity by decreasing pro-inflammatory cytokines, including IL-2, IL-6, IL-15, and IL-21. In addition, lasting antibiotic-induced increase in NF-κB was mitigated by AHCC. Early-life antibiotic exposure altered gut miRNA expression, increasing pro-inflammatory miR-221 and decreasing anti-inflammatory miR-145 in males while AHCC intake prevented antibiotic-mediated dysregulation of miRNA-145. These results highlight the potential of prebiotic intake as a promising strategy to prevent and mitigate persistent health issues arising from early-life dysbiosis.

Keywords: Antibiotic, Prebiotic, Early-life gut microbiota, Dysbiosis, Immune regulation, Signaling pathways, microRNAs

4-1- Introduction

Gut microbiota establishment and development take place within a critical window in early life. In addition, the most critical events in host immunity education occur during early life, where early-life microbiota plays a pivotal role in the healthy development, education, and long-term functionality of the immune system. The interplay between these fundamental processes highlights the importance of this critical developmental period on long-term health outcomes (Gagliardi et al., 2018; Tanaka and Nakayama, 2017). Growing evidence indicates that microbiota disturbance in early life is associated with altered programming of the immune system due to enduring changes in the expression of genes involved in immunological processes (Schokker et al., 2015).

Postnatal antibiotic exposure is one of the primary early-life environmental stressors affecting microbiota development and has been shown to alter the diversity and maturation of neonatal microbiota (Stiemsma and Michels, 2018). Several studies in murine models have demonstrated that perinatal exposure to a clinically relevant antibiotic treatment (one week before birth until weaning) which is sufficient to induce dramatic perturbations in gut microbiota, causes long-term adverse effects on various aspects of health, including metabolic, immunologic, and neurocognitive disturbances (Cox et al., 2014; Leclercq et al., 2017; Champagne-Jorgensen et al., 2020). Notably, despite the recovery of gut microbiota shortly after the cessation of antibiotic treatment, physiological damage related to early-life dysbiosis persists into adulthood (Cox et al., 2014) which emphasizes the significance of implementing interventional strategies, such as nutritional intervention, to prevent or correct dysbiosis in this critical period of life in order to

prevent permanent detrimental health consequences. However, regardless of the substantial evidence indicating the long-term adverse effects of perinatal dysbiosis, the underlying mechanisms remain insufficiently explored.

Gut miRNAs are key players in maintaining a healthy gastrointestinal environment by regulating both innate and adaptive immunity (Bi et al., 2020). miRNAs derived from the intestinal epithelial cells modulate the composition and distribution of the gut microbial populations. The complex crosstalk among gut microbiota and gut immunity mediated by miRNAs is vital for maintaining gut homeostasis (Bi et al., 2020). Besides, gut miRNAs regulate the gut immune system by modulating key signaling pathways involved in immune system homeostasis such as phosphoinositide 3-kinase (PI3K)/AKT, Toll-like receptor 4/nuclear factor-kappa B (TLR4/NF-κB), and Janus kinase/signal transducer and activator of transcription (JAK/STAT3) signaling pathways (Zhang et al., 2015; Anzola et al., 2018; Chu et al., 2018).

Gut microbiota perturbation, on the other hand, leads to dysregulation in the gut miRNA profile (Moloney et al., 2018) which correlates with inflammatory responses (Raisch et al., 2013). Furthermore, changes in gut microbiota and their products correlate with the production of proinflammatory cytokines, and an imbalance in immune cells such as an increase in inflammatory T helper (Th)-17, activation of inflammatory signaling such as NF-κB, and development of inflammatory disorders (Zhao et al., 2023; Peng et al., 2020).

Recent research has demonstrated the protective role of prebiotics and probiotics in preventing gut microbiota disturbance, as well as mitigating inflammation and immune system dysfunction at the gut level and beyond during critical windows of development (Shahbazi et al., 2023a; Yahfoufi et al., 2023). Prebiotic intake has been shown to alleviate gut immune system deregulation by

modulating central signaling pathways and epigenetic mechanisms, including STAT3, miRNAs expression, and DNA methylation (Shahbazi et al., 2023a).

In experiments using germ-free models, the transfer of microbiota from antibiotic-exposed mice to germ-free mice has indicated that the adverse effects of early-life antibiotic exposure are primarily related to disruptions in the gut microbiota, rather than a direct effect of antibiotics (Cox et al., 2014). Interestingly, nutritional intervention with probiotics has been shown to alleviate the negative health consequences of early-life antibiotic-induced dysbiosis (Leclercq et al., 2017). Whether gut microbiota-directed interventions, such as diet enrichment with prebiotics will prevent lasting immune deregulation linked to early-life dysbiosis by modulating miRNAs and inflammatory signaling pathways is an important question that remains unexplored.

Hence, in this research, we investigated the enduring consequences of perinatal exposure to a low dose of penicillin, alongside concurrent administration of a prebiotic compound, a standardized extract of cultured *Lentinula edodes* mycelia referred to as AHCC, to examine the protective role of AHCC intake in mitigating immune system disturbance associated with the early-life antibiotic-induced dysbiosis by addressing selected miRNAs and signaling pathways that play a crucial role in modulating immune responses at the gut level. To this end, pregnant mice were exposed to the antibiotic and AHCC from the last week of gestation until the waning of pups, and the long-term effects of treatment were assessed in offspring.

4-2- Materials and Methods

4-2-1- Animals

Pregnant Balb/c mice (gestation day 13) (Charles River, Montreal, QC, Canada) were used in the current study. Mice were housed in plastic cages in a controlled atmosphere (temperature 22 ±

2°C; humidity 55 ± 2 %) with a 12 h light/dark cycle. During the study, all groups received a conventional balanced diet ad libitum, and treatment was delivered via drinking water. Mice were maintained and treated following the guidelines of the Canadian Council on Animal Care. The protocol (HSe-3866) was approved by the Animal Care Committee of the University of Ottawa on March 14, 2023.

4-2-2- Study Design

Sixteen pregnant Balb/c mice were used in the current study. One week before delivery, at gestation day 14, the mice were housed individually, and the treatment was initiated. The pregnant mice were divided into four groups (n = 4 in each group) as follows: 1- receiving regular drinking water as the control group, 2- receiving low doses of penicillin V (Sigma Aldrich, Toronto, ON, Canada) at a dose of 31 mg/Kg BW/day in drinking water, 3- receiving prebiotic AHCC (4 g/kg BW/day) in drinking water, and 4- receiving penicillin V (31 mg/Kg BW/day) plus AHCC (4 g/kg BW/day) in drinking water. The treatment process continued to the weaning of pups (postnatal day (PND) 21). Pups, therefore, were exposed to the treatment early in life (in utero and early postnatal life). At weaning, the intervention was discontinued, and dams were sacrificed. Ileum tissues and fecal samples were then collected for corresponding experiments. Additionally, at weaning, male and female pups were separated and randomly assigned to non-littermate, same-sex, and same-treatment cages. The pups received a standard diet until they reached eight weeks of age (PND 56). In early adulthood (PND 56), pups were sacrificed and required samples were collected to examine the long-term effects of the treatment on the immune system at the gut level. Figure 4-1 illustrates the study design. AHCC® is a standardized extract of cultured *Lentinula edodes* mycelia, provided by Amino Up Co., Ltd. (Sapporo, Japan).

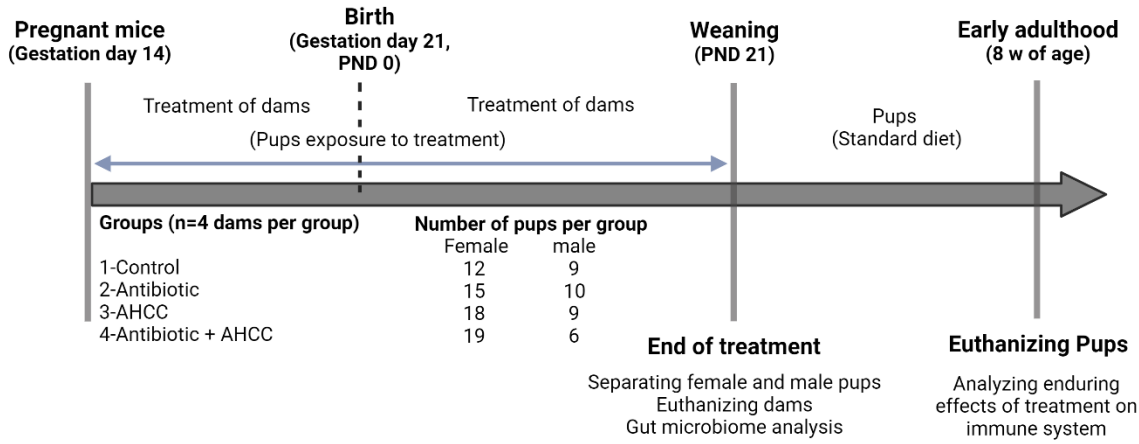

Figure 4-1. Study design. Pregnant mice were treated with the antibiotic (penicillin V, 31 mg/Kg BW/day), AHCC (4 g/kg BW/day), or a combination of antibiotic and AHCC, administrated in their drinking water, from the last week of gestation (gestation day 14) until the weaning of the pups (postnatal day 21). The control group received regular drinking water. At weaning, the treatment was discontinued, the male and female pups were separated, and dams were sacrificed. Pups were then provided with a standard diet until reaching eight weeks of age. At this age, the offspring were sacrificed to study the long-term effects of treatment on the immune system at the gut level. The image was created by BioRender.com.

4-2-3- Gut Microbiome Analysis

For microbiome analysis, the feces samples of dams and pups were collected at weaning using sterile microtubes and stored at -80°C following snap-freezing in liquid nitrogen. The total DNA of samples was extracted using the QIAamp PowerFecal Pro DNA kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's instructions. The concentration of extracted DNA was measured using a Qubit 4 instrument (Thermofisher Scientific, Toronto, ON, Canada). 16s rRNA V4 amplicon sequencing was conducted for microbiome analysis.

4-2-4- Determination of Cytokine Concentrations by Luminex Multiplex Assay

The small intestines of the mice were washed with 1X PBS, and small segments of ileum tissues were finely cut into small pieces using surgical blades (Fisher Scientific, Toronto, ON, Canada). Chopped tissues were collected in microtubes containing lysis buffer (20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.05% Tween-20), and a cocktail of protease inhibitors (AEBSF,

Hydrochloride, Millipore Sigma, Oakville, ON, Canada). After vortexing and incubation on a rocker for 20 minutes at 4°C, homogenized samples were centrifuged at 14000g for 15 minutes at 4°C, and the resulting supernatants were collected. The concentrations of total proteins in tissue lysates were determined by the BCA method using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Toronto, Canada) and adjusted to a concentration of 2 mg/mL using the same lysis buffer. Next, the multiplex assay was conducted using Mouse Th17 Panel Magnetic, MTH17MAG-47K (Millipore Sigma, Burlington, USA), following the manufacturer's instructions. Plates were then read using Bio-Plex® 200 System (Bio-Rad, Hercules, CA, USA).

4-2-5- Determination of p-STAT3, STAT3, and NF-κB Levels by Western Blotting

Small sections of ileum were homogenized using an electrical homogenizer in tubes containing the appropriate amount of RIPA buffer (Thermo Fisher Scientific, Toronto, ON, Canada), along with a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Toronto, ON, Canada). Samples were incubated on a rocker for 20 minutes at 4°C. Subsequently, protein lysates were collected by centrifuging homogenized samples at 14000g, for 15 min, at 4°C, and total protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Toronto, ON, Canada) according to the manufacturer's instruction. The equal amount and concentration of protein lysates were loaded onto the 12% Bis-Tris Mini Protein Gels (Invitrogen, Toronto, ON, Canada), using MES SDS Running buffer (Life Technologies, Toronto, ON, Canada) at 200 V for 22 min and subsequently, transferred to a PVDF membrane in a Trans-Blot Cell (Bio-Rad, Hercules, CA, USA), at 100 V for 75 minutes. The membranes were then incubated with anti-STAT3 (1:1000), anti-phospho-STAT3 (phospho Y705) (1:1000), anti-NF-κB p65 (1:1000), and anti-β-actin (1:1000) primary antibodies (Abcam, Toronto, ON, Canada) overnight at 4°C. Afterward, the blots were incubated with horseradish peroxidase-conjugated

secondary antibodies (1:10000) (Jackson Immuno Research Laboratories, West Grove, PA, USA) at room temperature for 1 hour. Bands were visualized by chemiluminescence assay using ECL substrate (Bio-Rad, Mississauga, ON, Canada) and quantified by the Bio-Rad Image Lab 6.1 Software using β-actin as loading control.

4-2-6-Determination of miRNAs Expression by Real-Time Quantitative Reverse Transcription PCR (RT-qPCR)

Small sections of ileum tissues were preserved in RNAlater Stabilization Solution (Invitrogen, USA) for 24 h at 4°C and then stored at -80°C until RNA extraction. The extraction of total RNA from the samples was carried out using miRNeasy mini kit (Qiagen, Toronto, ON, Canada). The concentrations and purity of extracted RNA were determined by a NanoDrop 2000 (Thermo Scientific, Waltham, MA, United States). To assay the expression of selected miRNAs, a reverse transcription reaction was conducted to synthesize cDNA using miRCURY LNA RT Kit (Qiagen, Toronto, ON, Canada), and subsequently, the expression levels of miR-15, miR-let-7C, miR-21, miR-221, and miR-145 were measured by RT-qPCR using hsa-miR-15b-5p, hsa-let-7c-5p, hsa-miR-21-5p, hsa-miR-221-3p, and hsa-miR-145-5p miRCURY LNA miRNA PCR assay primers (Qiagen, Toronto, ON, Canada) and miRCURY LNA SYBR Green PCR Kit (Qiagen, Toronto, ON, Canada) in a CFX 384 real-time PCR detection system (Bio-Rad, Laboratories, Hercules, CA, USA). Expression of miRNAs was normalized to SNORD65 (mmu) miRCURY LNA miRNA PCR Assay (Qiagen, Toronto, ON, Canada) as the reference gene. The relative expression level of miRNAs was calculated using the ΔΔCT method.

4-2-7- Statistical Analysis

Statistical analyses for all experiments, excluding microbiome analysis, were conducted using GraphPad Prism Software (GraphPad Software Inc., San Diego, CA, USA). Data distribution was

assessed with the Shapiro-Wilk test. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was performed to compare the means of the experimental groups. The results are presented as mean ± SEM and a p-value < 0.05 indicates a statistically significant difference between the groups. For microbiome analysis, the 16S analysis was conducted using Qiime2, and for the comparison of differentially abundant taxa, negative binomial models (DESeq2 R package) and Wald test or Kruskal-Wallis were employed. Differences in alpha diversities (α-diversity) were assessed using the Chao1 and Shannon indices, along with Kruskal-Wallis pairwise tests. Beta diversity (β-diversity) was evaluated through principal coordinates analysis (PCoA) based on the weighted UniFrac matrix to measure distinctions between groups. Then, the β-diversity quantitative distance metric was applied to assess the significance of differences between the groups using the PERMANOVA pairwise test. An adjusted p-value < 0.05 was considered statistically significant. All graphs related to microbiome results, except for the PCoA graph, were generated using GraphPad Prism.

4-3- Results

4-3-1- Effect of the Treatment on the Gut Microbiome

Analysis of the gut microbiota was done in dams and offspring at the weaning of the pups. To assess the effect of treatment on microbial richness and evenness, α-diversity was measured using different metrics. In dams, Chao1 results showed lower diversity in groups receiving antibiotic compared to the control, and AHCC groups ($p < 0.05$), however, Shannon metric results showed no significant difference between groups (Figure 4-2A). β-diversity results showed a distinction in the clustering of groups receiving antibiotic from control and AHCC groups, but it did not reach statistical significance ($p = 0.054$ vs. control and $p = 0.057$ vs. AHCC) (Figure 4-2A).

In male offspring, results from Chao1 and Shannon indices signified a decrease in microbial diversity in both groups exposed to antibiotic compared to the control and AHCC counterparts ($p < 0.01$) (Figure 4-2B), and β-diversity results demonstrated significant changes in the antibiotic and antibiotic + AHCC treated groups compared to control and AHCC ($p < 0.01$) (Figure 4-2B).

In female offspring, a significant decrease in microbial diversity was observed in groups treated with antibiotic compared to the control mice ($p < 0.001$ for both Chao1 and Shannon) and AHCC mice ($p < 0.001$ for Chao1 and $p < 0.01$ for Shannon) (Figure 4-2C). β-diversity analysis revealed a significant dissimilarity in antibiotic and antibiotic + AHCC mice compared to the control ($p < 0.01$) and AHCC ($p < 0.05$) (Figure 4-2C).

Figure 4-3 represents the most abundant taxa at the phylum and class levels and differentially abundant taxa among different groups in dams and offspring. As is evident in figures 4-3A, C, and E, antibiotic exposure was associated with a marked rise in the Proteobacteria phylum due to an increase in the Gammaproteobacteria class and a drop in the Firmicutes phylum due to a decrease in mainly Bacilli class in dams and Clostridia and Bacilli classes in offspring. In fact, the decrease in Firmicutes was compensated for by the expansion of Proteobacteria in the antibiotic group, and predominantly by Bacteroidota, with a lesser extent of Proteobacteria, in the antibiotic + AHCC group.

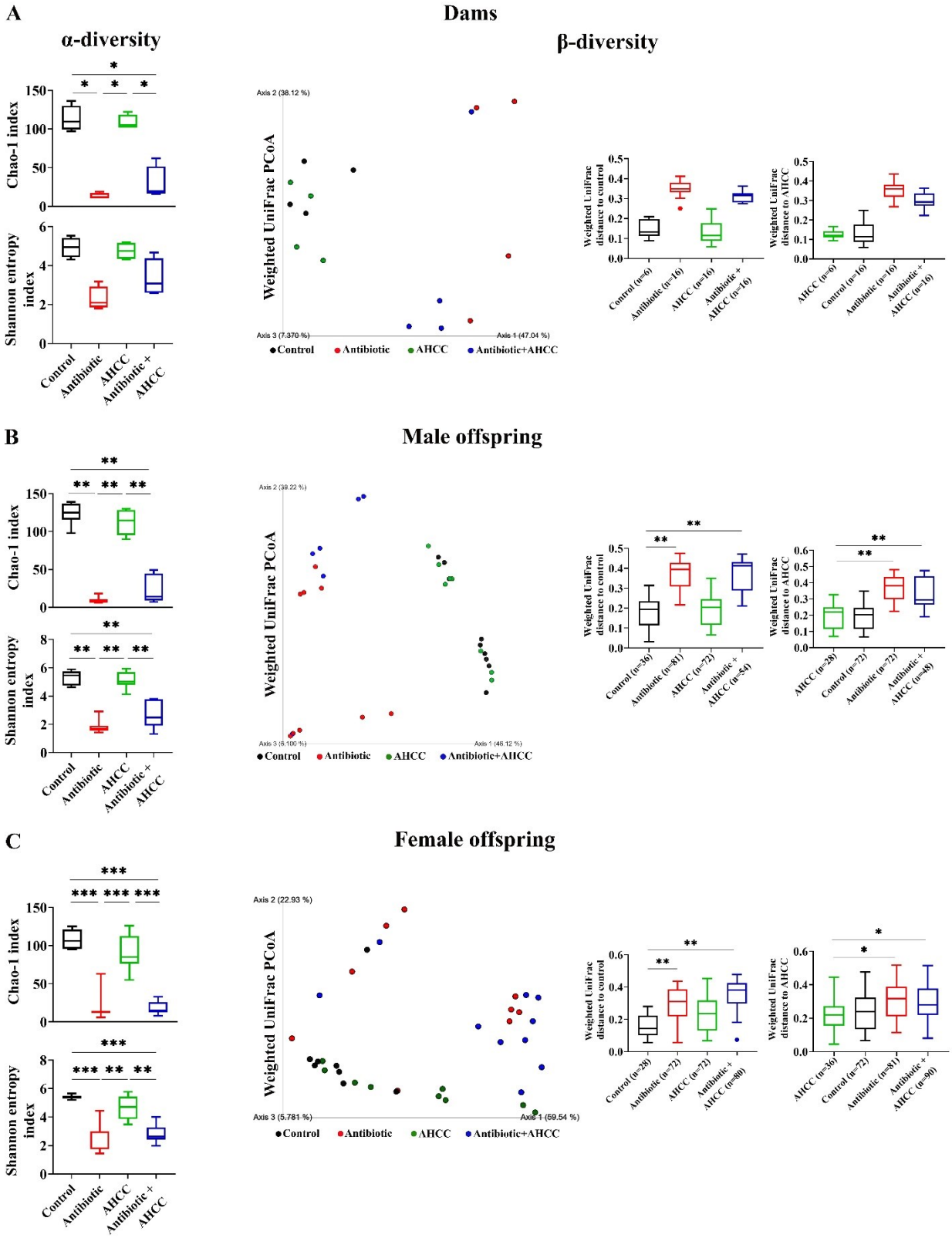
A
Dams
α-diversity
β-diversity
Chao-1 index
Shannon entropy index
Control
Antibiotic
AHCC
Antibiotic + AHCC
Weighted UniFrac PCoA
Axis 2 (38.12 %)
Axis 3 (7.370 %)
Axis 1 (47.04 %)
Control
Antibiotic
AHCC
Antibiotic+AHCC
Weighted UniFrac distance to control
Weighted UniFrac distance to AHCC
Control (n=6)
Antibiotic (n=16)
AHCC (n=16)
Antibiotic + AHCC (n=16)
AHCC (n=6)
Control (n=16)
Antibiotic (n=16)
Antibiotic + AHCC (n=16)
B
Male offspring
Axis 2 (39.22 %)
Axis 3 (8.100 %)
Axis 1 (48.12 %)
Control (n=36)
Antibiotic (n=81)
AHCC (n=72)
Antibiotic + AHCC (n=54)
AHCC (n=28)
Control (n=72)
Antibiotic (n=72)
Antibiotic + AHCC (n=48)
C
Female offspring
Axis 2 (22.93 %)
Axis 3 (5.781 %)
Axis 1 (59.54 %)
Control (n=28)
Antibiotic (n=72)
AHCC (n=72)
Antibiotic + AHCC (n=80)
AHCC (n=36)
Control (n=72)
Antibiotic (n=81)
Antibiotic + AHCC (n=90)

Figure 4-2. Effect of the treatment on gut microbiota diversity. Pregnant mice were treated with the antibiotic (penicillin V, 31 mg/Kg BW/day), AHCC (4 g/kg BW/day), or a combination of antibiotic and AHCC, administrated in their drinking water, from the last week of gestation (gestation day 14) until the weaning of the pups (postnatal day 21). The control group received regular drinking water. Fecal samples from dams and pups were collected at weaning for microbiome analysis using 16S rRNA V4 amplicon sequencing. (**A**) α-diversity and β-diversity in dams, (**B**) α-diversity and β-diversity in male offspring, and (**C**) α-diversity and β-diversity in female offspring. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Differential abundance analysis in dams revealed a significant increase in the Proteobacteria population in the antibiotic group compared to the control and AHCC groups ($p < 0.05$) while AHCC intake reduced the impact of the antibiotic on this phylum (Figure 4-3B). In male offspring, there was a significant increase in Proteobacteria in the antibiotic group compared to the control and AHCC groups ($p < 0.0001$), however, AHCC intake diminished the effect of the antibiotic on this phylum ($p < 0.05$) (Figure 4-3D). Additionally, the abundance of Firmicutes significantly decreased in the antibiotic and antibiotic + AHCC groups compared to the control ($p < 0.01$) and AHCC groups ($p < 0.01$ for the antibiotic group and $p < 0.05$ for the antibiotic + AHCC group) (Figure 4-3D). In female offspring, the Proteobacteria abundance was significantly higher in antibiotic mice than those in the control and AHCC groups ($p < 0.001$), and AHCC intake mitigated antibiotic effect ($p < 0.01$ and $p < 0.05$ vs. control and AHCC, respectively) (Figure 4-3F). Furthermore, Firmicutes abundance was lower in antibiotic + AHCC mice than in the control mice ($p < 0.001$) and Deferribacterota abundance was lower in groups receiving antibiotic than in the control ($p < 0.01$) (Figure 4-3F). In both male and female offspring, the Bacteroidetes population was higher in the antibiotic + AHCC group than in the control ($p < 0.05$ for males and $p < 0.01$ for females) (Figures 4-3D and F).

At the genus level in dams, there was a substantial decrease in Lactobacillus abundance following antibiotic treatment compared to the control and AHCC groups ($p < 0.05$). Additionally, antibiotic intake led to a significant increase in the Robinsoniella population when compared to the control

and AHCC-treated mice ($p < 0.05$) and a significant decrease in the population of Clostridia UCG-014 compared to the AHCC ($p < 0.05$). Antibiotic intake also abolished the Eubacterium xylanophilum group ($p < 0.05$) and AHCC mitigated the antibiotic effect (Figure 4-3B).

In male offspring, antibiotic exposure correlated with a significant decrease in Lactobacillus ($p < 0.05$), Roseburia ($p < 0.01$), Intestinimonas ($p < 0.0001$), and Lachnospiraceae NK4A136 group ($p < 0.0001$) populations compared to the control, while AHCC exposure could not inhibit the effect of antibiotic on these genera. Antibiotic exposure also significantly reduced the abundance of Incertae Sedis ($p < 0.05$), Colidextribacter ($p < 0.001$), Oscillibacter ($p < 0.0001$), Eubacterium xylanophilum group ($p < 0.01$), and Clostridia vadin BB60 group ($p < 0.001$), when compared to the control. However, AHCC intake diminished the antibiotic-induced changes in these genera. Lachnospiraceae FCS020 group population was higher in the AHCC group than in the antibiotic ($p < 0.01$) and the antibiotic + AHCC groups ($p < 0.05$) (Figure 4-3D).

In female offspring, a significant reduction in the Lactobacillus genus was observed in both groups treated with the antibiotic compared to the control and AHCC-treated groups ($p < 0.05$). Additionally, the abundance of Roseburia, Mucispirillum, Anaerotruncus, Family XIII UCG-001, and Incertae Sedis significantly decreased in both groups exposed to antibiotic compared to the control ($p < 0.05$). Intestinimonas abundance was lower in the antibiotic and antibiotic + AHCC mice compared to the control ($p < 0.001$), and AHCC ($p < 0.05$) (Figure 4-3F).

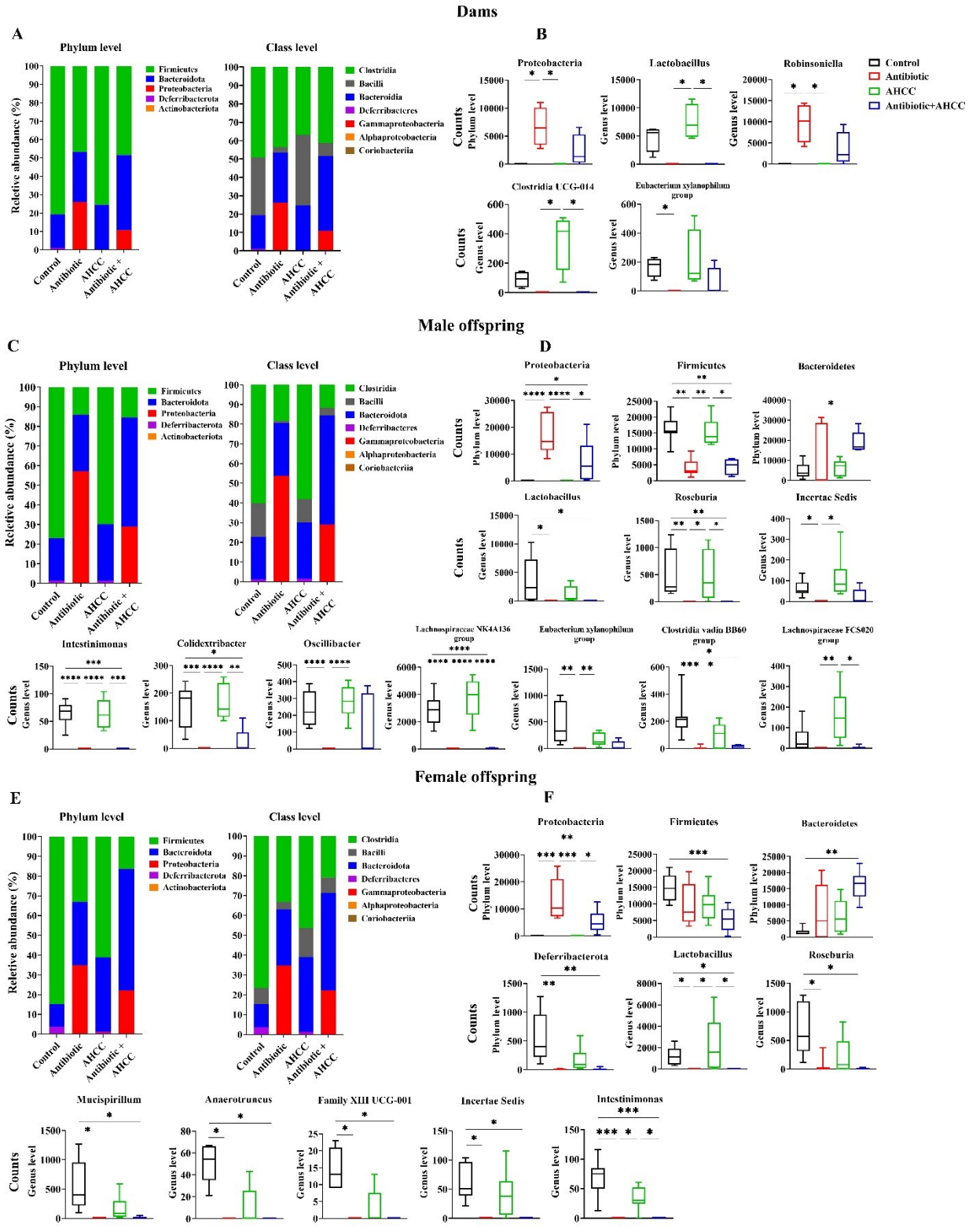

Figure 4-3. Effect of the treatment on gut microbiota composition. Pregnant mice were treated with the antibiotic (penicillin V, 31 mg/Kg BW/day), AHCC (4 g/kg BW/day), or a combination of antibiotic and AHCC, administrated in their drinking water, from the last week of gestation (gestation day 14) until the

weaning of the pups (postnatal day 21). The control group received regular drinking water. Fecal samples from dams and pups were collected at weaning for microbiome analysis using 16S rRNA sequencing. (A, B) the most abundant taxa and differentially abundant taxa in dams (C, D) the most abundant taxa and differentially abundant taxa in male offspring, and (E, F) the most abundant taxa and differentially abundant taxa in female offspring. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

4-3-2- Effect of the Treatment on Cytokine Concentrations

To investigate the impact of early-life dysbiosis on long-term immune system function, the levels of a variety of cytokines, including IFN-γ, IL-2, IL-6, IL-10, IL-15, IL-21, IL-22, and IL-23 were assayed in the ileum tissues of offspring during early adulthood. The same cytokine panel was also assessed in dams following the weaning of pups. In dams, no significant differences in the measured cytokines were noted across the groups, except for IFN-γ, which was significantly lower in the antibiotic + AHCC group than in the control and AHCC groups ($p < 0.05$) (Figure 4-4A).

In male offspring, a reduction in IFN-γ levels was evident in the antibiotic + AHCC-treated mice compared to the control ($p < 0.05$). The level of IL-2 was significantly higher in mice exposed to early-life antibiotic than in the AHCC and antibiotic + AHCC groups ($p < 0.05$ and $p < 0.01$, respectively). AHCC intake could mitigate the effect of the antibiotic on IL-6 and IL-15 ($p < 0.05$ vs. antibiotic). IL-22 concentration decreased in mice exposed to a combination of antibiotic and AHCC compared to the untreated group ($p < 0.05$) (Figure 4-4B). No significant changes were observed in the levels of IL-21, IL-23, and IL-10 among different groups (Figure 4-4B).

In female offspring, IL-6 concentration was lower in adult mice exposed to a mixture of antibiotic and AHCC in early life compared to those exposed solely to antibiotic ($p < 0.05$). The level of IL-15 was significantly lower in both groups receiving AHCC compared to the mice receiving only antibiotic ($p < 0.05$). IL-21 level was higher in the antibiotic group compared to all other groups ($p < 0.05$). A significant reduction in IL-22 concentration was also found in the antibiotic + AHCC

group compared to the untreated mice ($p < 0.05$) (Figure 4-4C). No significant changes were detected in the IFN-γ, IL-2, IL-10, and IL-23 levels across the various groups (Figure 4-4C).

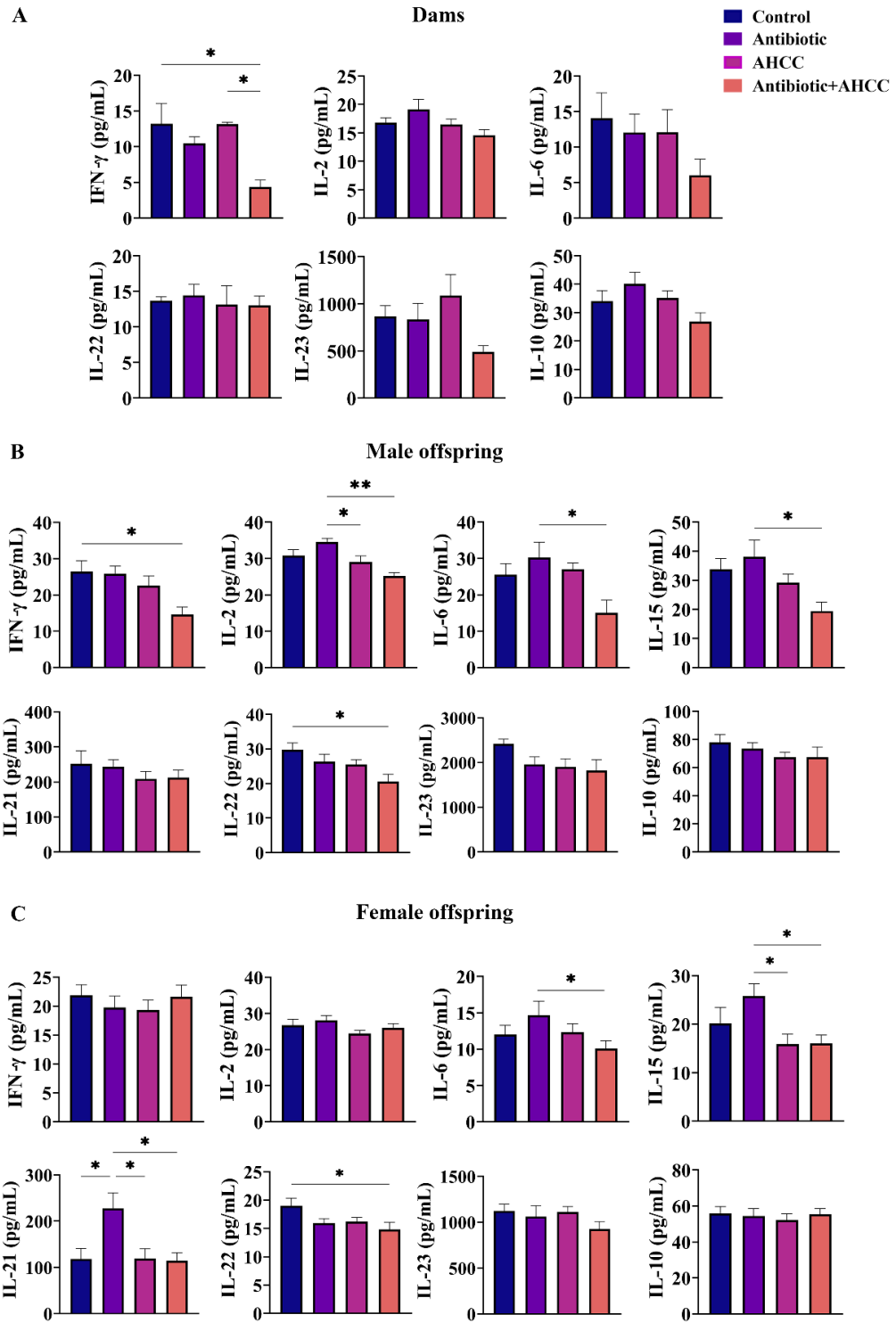

Figure 4-4. Effect of treatment on cytokine levels. Pregnant mice were treated with the antibiotic (penicillin V, 31 mg/Kg BW/day), AHCC (4 g/kg BW/day), or a combination of antibiotic and AHCC, administrated

in their drinking water, from the last week of gestation (gestation day 14) until the weaning of the pups (postnatal day 21). The control group received regular drinking water. Cytokine concentrations were measured in the ileum tissues of dams and offspring. (**A**) Concentration of IFN-γ, IL-2, IL-6, IL-10, IL-22, and IL-23 in dams at weaning, (**B**) concentration of IFN-γ, IL-2, IL-6, IL-10, IL-15, IL-21, IL-22, and IL-23 in male offspring at eight weeks of age, and (**C**) concentration of IFN-γ, IL-2, IL-6, IL-10, IL-15, IL-21, IL-22, and IL-23 in female offspring at eight weeks of age. All values are mean ± SEM. * $p < 0.05$ and ** $p < 0.01$.

4-3-3- Effect of the Treatment on the p-STAT3, STAT3, and NF-κB

Based on cytokine results, the impact of the antibiotic and AHCC on p-STAT3, STAT3, and NF-κB levels was evaluated in dams at weaning and in adult offspring. Antibiotic intake resulted in an increase in p-STAT levels in dams when compared to all the other groups; however, this increase did not reach statistical significance (Figure 4-5A). No difference was observed in STAT3 and NF-κB levels among the different groups (Figure 4-5A).

In male offspring, a significant decrease was observed in p-STAT3 levels in mice treated with AHCC and antibiotic + AHCC compared to the untreated mice ($p < 0.05$). STAT3 levels did not change in response to treatment with antibiotic and AHCC (Figure 4-5B). Early-life antibiotic exposure led to a lasting increase in NF-κB levels in adult males when compared to the control ($p < 0.05$). However, AHCC intake counteracted the lasting effect of antibiotic intake on NF-κB levels ($p < 0.05$ vs. antibiotic) (Figure 4-5B).

In female offspring, no significant difference was observed in p-STAT3 and STAT3 levels in the treated groups compared to the untreated mice (Figure 4-5C). However, early-life antibiotic exposure was associated with a sustained increase in NF-κB levels as compared to the control and AHCC-treated mice ($p < 0.05$ and $p < 0.001$, respectively). Notably, AHCC exposure mitigated the lasting effect of antibiotic on NF-κB levels (Figure 4-5C).

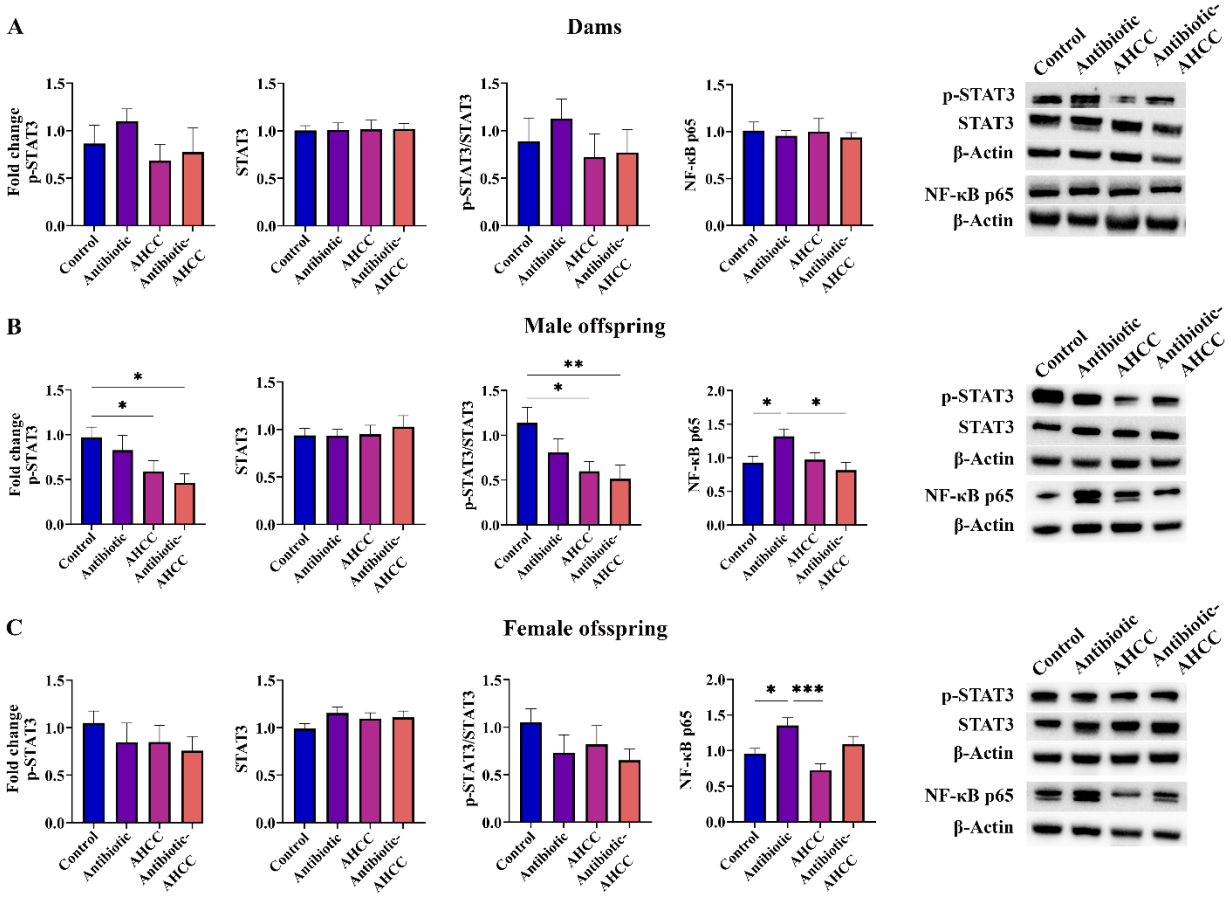

Figure 4-5. Effect of the treatment on the p-STAT3, STAT3, and NF-κB levels. Pregnant mice were treated with the antibiotic (penicillin V, 31 mg/Kg BW/day), AHCC (4 g/kg BW/day), or a combination of antibiotic and AHCC, administrated in their drinking water, from the last week of gestation (gestation day 14) until the weaning of the pups (postnatal day 21). The control group received regular drinking water. (**A**) Levels of p-STAT3, STAT3, and NF-κB in the ileum tissues of dams at weaning, (**B**) levels of p-STAT3, STAT3, and NF-κB in the ileum tissues of male offspring at eight weeks of age, and (**C**) levels of p-STAT3, STAT3, and NF-κB in the ileum tissues of female offspring at eight weeks of age. All values are mean ± SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

4-3-4- Effect of the Treatment on Expressions of miRNAs

Next, the effect of antibiotic and AHCC exposure on the expression of selected miRNAs related to STAT3 and NF-κB pathways, including miR-15b, miR-let-7c, miR-21, miR-221, and miR-145, was assessed in the ileum tissues of dams and adult offspring. Among the dams, none of the tested miRNAs were differently expressed across groups (Figure 4-6A).

In male offspring, miR-15b expression did not change among various groups. miR-let-7c expression was lower in the antibiotic + AHCC group compared to the AHCC group ($p < 0.05$) (Figure 4-6B). Expression of miR-21 was significantly reduced in antibiotic and antibiotic + AHCC groups compared to the control ($p < 0.01$ and $p < 0.001$, respectively) and AHCC-exposed mice ($p < 0.01$ and $p < 0.001$, respectively) (Figure 4-6B). Furthermore, antibiotic consumption increased the expression of miR-221 compared to the control ($p < 0.05$), and in the antibiotic + AHCC group, miR-221 expression decreased compared to the antibiotic alone ($p < 0.01$) (Figure 4-6B). Antibiotic treatment led to a reduction in miR-145 levels compared to the control and AHCC groups ($p < 0.01$ and $p < 0.0001$, respectively), however, AHCC treatment mitigated the inhibitory impact of the antibiotic on this miRNA ($p < 0.05$ vs. antibiotic) (Figure 4-6B).

In female offspring, the expression of miR-21 was lower in all treated groups compared to the untreated mice ($p < 0.001$ for antibiotic and antibiotic + AHCC, and $p < 0.01$ for AHCC). miR-145 levels markedly increased in the AHCC and antibiotic + AHCC groups when compared to the control ($p < 0.0001$ and $p < 0.01$, respectively) and antibiotic counterparts ($p < 0.0001$ and $p < 0.01$, respectively) (Figure 4-6C). No significant changes were observed in the levels of other miRNAs among the various groups (Figure 4-6C).

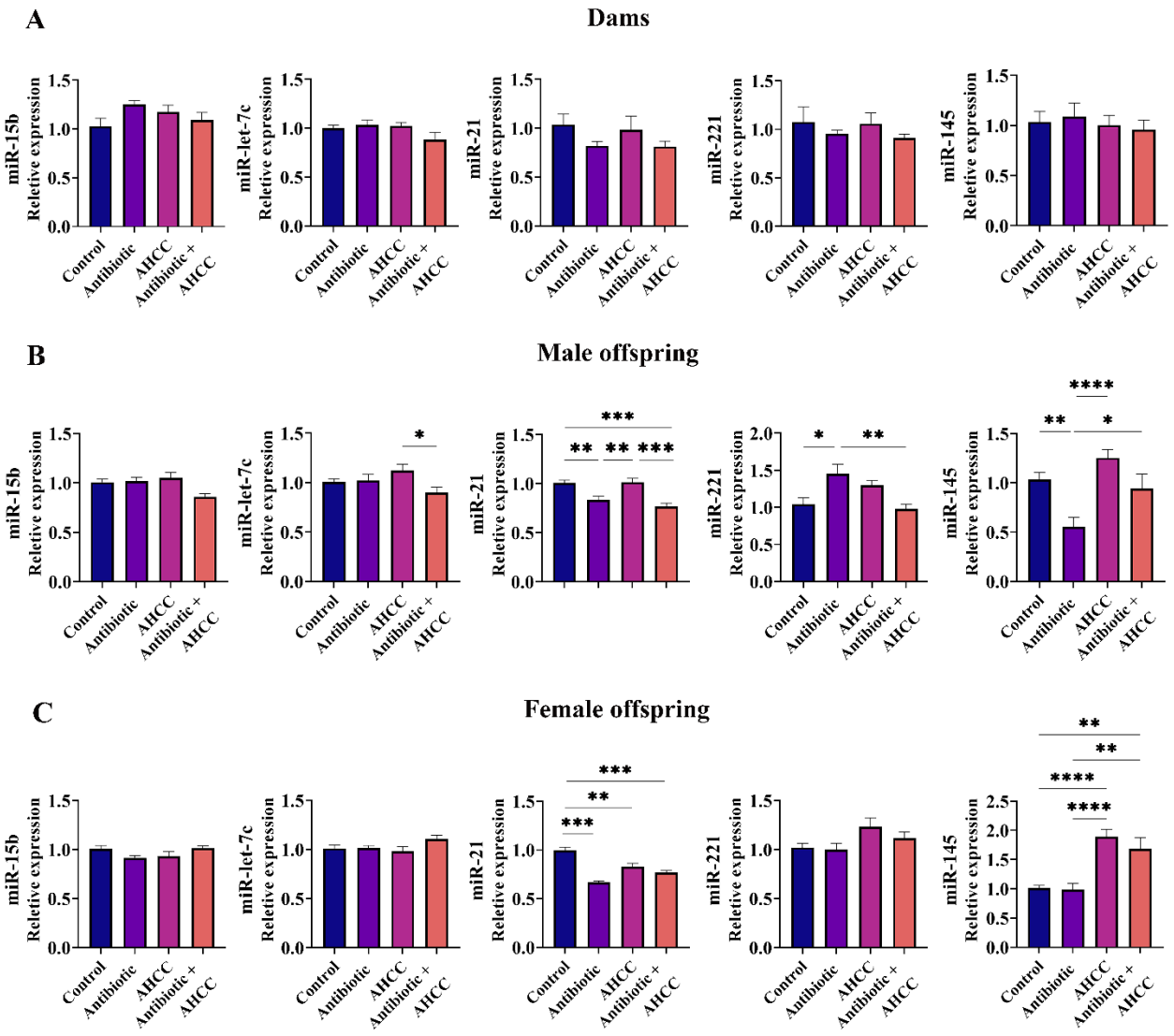

Figure 4-6. Effect of the treatment on expression of miRNAs. Pregnant mice were treated with the antibiotic (penicillin V, 31 mg/Kg BW/day), AHCC (4 g/kg BW/day), or a combination of antibiotic and AHCC, administrated in their drinking water, from the last week of gestation (gestation day 14) until the weaning of the pups (postnatal day 21). The control group received regular drinking water. Relative expressions of miRNAs were measured in the ileum tissues of dams and offspring. (**A**) Relative expression of miR-15b, miR-let-7c, miR-21, miR-221, and miR-145 in dams at weaning, (**B**) relative expression of miR-15b, miR-let-7c, miR-21, miR-221, and miR-145 in male offspring at eight weeks of age, and (**C**) relative expression of miR-15b, miR-let-7c, miR-21, miR-221, and miR-145 in female offspring at eight weeks of age. All values are mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

4-4- Discussion

There is increasing concern regarding potential long-term complications of antibiotic use during early life due to perturbation in the infant's microbiome establishment and maturation (Neuman et al., 2018). Since gut microbiota is a key player in immune and metabolic programming, many clinical and animal studies have found a strong association between early-life antibiotic exposure and developing allergies, autoimmunity, and obesity later in life (Neuman et al., 2018; Schokker et al., 2015; Cox et al., 2014). Early-life antibiotic exposure is linked to overall alteration in genes involved in intestinal immune responses such as genes regulating differentiation of immune cells and those governing the functions of T cells, B cells, and antigen-presenting in the ileum (Cox et al., 2014). In recent years, probiotics and prebiotics have attained considerable attention due to their ability to prevent gut microbiota disturbance and prime the immune system (Shahbazi et al., 2021; Shahbazi et al., 2020; Shahbazi et al., 2023b).

In the current study, we studied the potential role of a prebiotic compound, AHCC, in mitigating lasting inflammatory immune responses in adult mice exposed to dysbiosis during the critical developmental stage of early life via regulating signaling pathways and epigenetic mechanisms. The prebiotic AHCC has been demonstrated to improve gut immunity by modulating pattern recognition receptors and to protect against breast cancer development through modulating miRNAs expression (Graham et al., 2017; Mallet et al., 2016).

We initially assessed the impact of early-life antibiotic exposure on gut microbiota and the potential protective effects of AHCC intake against antibiotic-induced dysbiosis in dams and their offspring following weaning. Earlier research has observed the efficacy of the probiotic *Lactobacillus rhamnosus* JB-1 in partially alleviating gut microbiota alterations caused by early-life antibiotic exposure (Leclercq et al., 2017). Similarly, some studies have shown the role of

maternal prebiotic intake in affecting early-life diet-induced dysbiosis in offspring (Paul et al., 2016). We found a significant increase in the Proteobacteria population, namely Gammaproteobacteria, following antibiotic administration while AHCC intake could reduce the antibiotic effect. Gammaproteobacteria is a class of Gram-negative bacteria that consists of a diverse array of pathogenic genera, including *Escherichia coli* (*E. coli*) and Shigella (Rizzatti et al., 2017). An increase in Proteobacteria has been reported in many inflammatory conditions such as inflammatory bowel diseases (IBD) and lung diseases (Rizzatti et al., 2017). Our results are consistent with other studies that found an increase in Proteobacteria in mice exposed to low-dose antibiotic early in life (Leclercq et al., 2017; Candon et al., 2015).

In addition, a decrease in Firmicutes was observed in groups receiving antibiotic (statistically significant in male offspring) and antibiotic + AHCC (statistically significant in male and female offspring), and an increase in Bacteroidetes was found in antibiotic + AHCC groups (statistically significant in male and females offspring). Similarly, in a study, early-life antibiotic exposure was associated with a decrease in Firmicutes and an increase in Bacteroidetes and Proteobacteria (Jin et al., 2017). Furthermore, antibiotic exposure during late pregnancy and throughout lactation was associated with a reduction in the relative abundance of Firmicutes in offspring during adulthood (Cho et al., 2020). However, in another study, low-dose antibiotic exposure was associated with a decrease in Firmicutes and Bacteroidetes in dams after one week of treatment and an increase and decrease in Firmicutes and Bacteroidetes, respectively in pups following treatment until weaning (Leclercq et al., 2017). Human and animal studies have shown the role of prebiotic consumption in decreasing Firmicutes and increasing Bacteroidetes (Dewulf et al., 2013; Parnell and Reimer, 2012). In the current study, low-dose penicillin was used to induce dysbiosis, a narrow-spectrum β-lactam antibiotic, which functions best against Gram-positive bacteria (Sturød et al., 2020).

Firmicutes predominantly consist of Gram-positive bacteria, whereas Bacteroidetes consist of Gram-negative bacteria (Panda et al., 2014). Therefore, the alteration observed at the phylum level may be attributed to both the type of antibiotic used in this study and the impact of the prebiotic AHCC.

Noteworthy, under healthy conditions, Bacteroidetes species are significant contributors to lipopolysaccharide (LPS) biosynthesis in the gut microbiota. Notably, a structural difference in the lipid A domain, responsible for the endotoxic and immunostimulatory activity of LPS, has been identified in Bacteroidetes species which leads to the immunosuppressive activity of these bacteria, potentially through the inhibition of the TLR4/NF-κB pathway and the suppression of anti-inflammatory cytokine production, such as IL-6 and IL-1. On the contrary, Proteobacteria species contribute to the production of pathogenic LPS in the gut (d'Hennezel et al., 2017). Therefore, the administration of AHCC may offer protection against antibiotic-related adverse effects, to some extent, by promoting a reduction in Proteobacteria and an increase in the Bacteroidetes population.

At the genus level, the most alteration was observed in the population of the short-chain fatty acid (SCFA)-producing bacteria belonging to Firmicutes, class Clostridia, including Clostridia UCG-014, and Eubacterium xylanophilum group in dams, Roseburia, Ruminococcaceae Incertae Sedis, Intestinimonas, Colidextribacter, Oscillibacter, Lachnospiraceae NK4A136 group, Eubacterium xylanophilum, Clostridia vadin BB60 group, and Lachnospiraceae FCS020 in male offspring, as well as Roseburia, Anaerotruncus, Family XIII UCG-001, Incertae Sedis, and Intestinimonas, in female offspring, where antibiotic intake substantially declined these genera in dams and offspring. However, AHCC intake diminished antibiotic impacts on Eubacterium xylanophilum in dams. It also alleviated alteration in Incertae Sedis, Colidextribacter, Oscillibacter, Eubacterium

xylanophilum, and Clostridia vadin BB60 in male offspring. In female offspring, AHCC intake could not mitigate the antibiotic impact on these genera.

SCFA-producing bacteria primarily belong to the phylum Firmicutes. A decline in SCFA-producing bacteria, particularly within the Ruminococcaceae and Lachnospiraceae families, along with reduced SCFA levels, has been reported in IBD which might be associated with inflammatory responses potentially stemming from inhibition of anti-inflammatory signaling and activation of inflammatory signaling (Parada Venegas et al., 2019). Studies have demonstrated that diminished SCFA levels correlate with the activation of NF-κB and NF-κB-induced pro-inflammatory mediator productions such as TNF-α, IL-6, and IL-12 (Parada Venegas et al., 2019). Diets rich in fiber, such as the Mediterranean diet, have been demonstrated to enhance the population of SCFA-producing bacteria in the gut microbiota (Garcia-Mantrana et al., 2018).

Furthermore, Lactobacillus (belonging to Firmicutes, class Bacilli) was the other genus highly affected by treatment in both dams and offspring. Lactobacillus species also contribute to SCFA production (Markowiak-Kopeć and Śliżewska, 2020). There is evidence indicating that maternal antibiotic intake causes reduced colonization of infants' gut by Lactobacillus due to reduced vertical transmission (Keski-Nisula et al., 2013). A low abundance of Lactobacillus in breast milk due to maternal antibiotic exposure during pregnancy or lactation has been reported (Soto et al., 2014; Cortes-Macías et al., 2021). Lactobacillus bacteria inhibit NF-κB by preventing its main subunit (p65 (RelA)) nuclear translocation (Iyer et al., 2008). In this study, antibiotic exposure abolished this genus in dams and offspring and AHCC intake could not prevent antibiotic effects.

Next, we investigated the impact of early-life gut microbiota disruption and nutritional intervention on gut immunity in dams, as well as on enduring immune system function in offspring at 8 weeks

of age. Among dams, there were no discernible alterations in cytokine levels, except for IFN-γ, which could be attributed to the relatively small number of dams in each group. Our results demonstrated the ability of AHCC to reduce the long-term effect of early-life antibiotic exposure on proinflammatory cytokines, including IL-2, IL-6, and IL-15 in male offspring, and IL-6, IL-15, and IL-21 in female offspring.

IL-6 exerts a pivotal role in the pathogenesis of intestinal inflammation (Yang et al., 2014). NF-κB activation induces the expression of IL-6, which, in turn, results in the phosphorylation and activation of STAT3 (Yang et al., 2014). This cascade leads to the expression of transcriptional factors and inflammatory cytokines, including the master transcription factor of Th17 cells and IL-17, contributing to the inflammatory responses (Shahbazi et al., 2023a; Yang et al., 2014). IL-6 itself may activate the NF-κB pathway via the PI3K pathway (Luo and Zheng, 2016). NF-κB/IL-6/STAT3 pathway may link intestinal chronic inflammation to colitis-associated colorectal cancer (Yang et al., 2014). Biotics intake has demonstrated efficacy in preventing colorectal cancer by modulating gut microbiota and NF-kB/IL6-STAT3 signal transduction pathway (Jiang et al., 2020).

IL-2, IL-15, and IL-21 receptors have a shared component known as the common gamma chain (γc) within their subunits. IL2 and IL15 receptors also share IL2/IL15Rβ subunit (Waldmann, 2015). Both IL-2 and IL-15 activate the JAK/STAT signaling pathway and play crucial roles in T cell proliferation, and natural killer (NK) cell maintenance (Waldmann, 2015). IL-2 exhibits a dual role in inflammation (Hoyer et al., 2008) and has been found to activate various cascades, including NF-κB in different immune cells (Fung et al., 2003; Chan et al., 2010). IL-15, on the other hand, is a proinflammatory cytokine, and its chronic dysregulation has been observed in autoimmunity possibly by affecting different immune cells such as NK cells and $CD8^+$ T cells (Waldmann et al.,

2020). IL-15 exerts a pathogenic role in colitis by activating STAT3 signaling (Sugimoto, 2008). Besides, IL-15 activates other signaling, including NF-κB (Giron-Michel et al., 2003). IL-15 stimulates inflammatory infiltration of macrophages by up-regulating NF-kB expression (Yan et al., 2016). Furthermore, IL-21, a pro-inflammatory cytokine primarily produced by activated $CD4^+$ T cells and NKT cells, has been found to initiate and conserve inflammatory signals related to intestinal inflammation and tumorigenesis (De Simone et al., 2015). Notably, IL-21 deficiency is linked to the suppression of STAT3 and NF-κB, resulting in decreased production of inflammatory cytokines such as IL-17A and IL-6 (De Simone et al., 2015).

Based on cytokines results, NF-κB and STAT3, as the primary targets of these cytokines, were studied to elucidate the molecular mechanisms underlying the impact of early-life antibiotic exposure and prebiotic intake on immune system function later in life. We have previously shown the ability of AHCC to inhibit the enduring effects of pubertal LPS-induced inflammation on the immune system by regulating inflammatory cytokines and signaling pathways, including IL-17, IL-6, IL-1β, and STAT3 (Shahbazi et al., 2023a). Although we anticipated an increase in p-STAT levels in groups receiving antibiotics, no significant differences were detected in dams and female offspring, while in male offspring a reduction in p-STAT3 levels was found in all treated groups compared to the control, but the impact of antibiotic did not achieve statistical significance. Some studies have shown that antibiotics inhibit STAT3 activation by different mechanisms, including suppressing the JAK family kinases (Nelson et al., 2008), directly binding to STAT3 (He et al., 2021), and inhibiting transcription factor aryl hydrocarbon receptor (Wang et al., 2018). Moreover, early-life dysbiosis was associated with a lasting increase in NF-κB levels in offspring while maternal prebiotic intake diminished this effect. NF-κB cascade regulates multiple functions of the immune system and is tightly controlled by gut bacteria especially Firmicutes bacteria (Zhang et

al., 2022). A decrease in these bacteria may lead to improper NF-κB signaling. Under normal conditions, NF-κB signaling is crucial for enhancing tolerance against gut commensals and maintaining gut homeostasis, however, in the presence of dysbiosis, it triggers inflammatory responses, contributing to the development of various chronic diseases (Zhang et al., 2022). Therefore, these results indicate the potential immunomodulatory activity of AHCC by modulating NF-κB signaling and related cytokines.

Next, to investigate whether early-life antibiotic-induced dysbiosis can affect the immune system by enduring epigenetic changes, the expression of selected miRNAs related to NF-κB and STAT3 pathways was examined in offspring. Gut miRNA profile dysregulation participates in the pathogenesis of intestinal inflammation (Xiao et al., 2022). AHCC has been shown to exhibit immunomodulatory activity at the gut level through modulation of the DNA methylation of genes related to the Th17 and IL-17 pathways as well as the expression of miRNAs (Shahbazi et al., 2023a). Here, miR-221 expression was lower in antibiotic + AHCC group compared to the antibiotic group. AHCC could diminish the inhibitory impact of the antibiotic on the anti-inflammatory miR-145 in males. In females, an increase in miR-145 expression was observed in both groups receiving AHCC. miR-221 overexpression has been reported in colorectal cancer and is associated with constitutive activation of NF-κB and STAT3, which in return stimulates the expression of miR-221 (Liu et al., 2014). On the other hand, miR-145 may inhibit inflammation and tumor progression by suppressing NF-κB activation (Sui et al., 2019; Mei et al., 2017; He et al., 2020). It also exerts anti-tumor activity, partly by inhibiting p-STAT3 expression (Gao and Ding, 2021).

Besides, over-expression of miR-21 has been demonstrated in inflammatory conditions, including IBD, which is related to tissue injury (Shi et al., 2013). miR-21 exhibits inflammatory activity in

IBD, partly by affecting STAT3 and NF-κB expression (Lu et al., 2020). In this study, reduced expression of miR-21 was observed in male offspring within antibiotic-exposed groups and in female offspring within all treated groups. Some evidence has demonstrated the ability of a specific class of antibiotics to attenuate miR-21 expression by inhibiting the maturation of pre-miR-21 (Garner et al., 2019), whereas other studies have indicated the overexpression of miR-21 due to the antibiotic-induced aberration of gut microbiota in the gut and other organs (Yang et al., 2021).

Our study has several limitations. Firstly, we assessed the impact of treatment on gut microbiota only after weaning, and the adult offspring microbiome was not analyzed. Nevertheless, prior research has indicated a robust effect of antibiotics on gut microbiota post-weaning, with this impact diminishing after antibiotic cessation. However, the adverse health effects of uncorrected early-life dysbiosis persist into adulthood (Cox et al., 2014). Therefore, in this study, we focused on examining the weaning microbiome. Secondly, despite our microbiome results revealing a significant impact of antibiotic exposure on SCFA-producing genera, we did not measure SCFA levels, which could represent a potential mechanism of immune dysregulation induced by early-life gut microbiota dysbiosis. Lastly, in this study, mice were exposed to antibiotic and nutritional intervention from the third week of gestation to weaning. However, further investigations are required to explore prenatal and postnatal exposures separately to provide more insights into the adverse consequences of gut microbiota dysbiosis during various critical stages of development.

In conclusion, this study demonstrates that persistent immune dysregulation stemming from early-life gut microbiota dysbiosis could be linked, to some extent, to enduring changes in gut miRNA profiles and inflammatory signaling pathways. Notably, maternal prebiotic intake could partially prevent gut microbiota alteration as well as changes in miRNAs and NF-κB. Overall, these results may contribute to the growing body of evidence regarding the long-term consequences of early-

life gut microbiota dysbiosis and highlight potential strategies for preventive interventions to mitigate health issues later in life.

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Chapter 5: Discussion

5- Discussion

Most effects of immune stressors on microbiota composition in critical developmental periods persist for weeks to several months, although some studies have found significant differences in microbial composition even 2 to 4 years after exposure (Cox et al., 2014). Even after microbiota recovery, phenotypic changes such as microbiota-induced obesity remain with long-term metabolic and developmental programming (Cox et al., 2014). Because of the interaction between diet, gut microbiota, and immunity, numerous research studies have been recently conducted to determine the functional consequences of manipulating the microbial community to promote health (Zhang and Chen, 2019; Yoon and Yoon, 2018). In this regard, natural components, especially probiotics, and prebiotics, have attracted considerable attention due to their potential roles in restoring gut microbiota and alleviating dysbiosis adverse effects (Neuman et al., 2018). However, the molecular mechanisms underpinning these effects have not been sufficiently investigated.

In this research, the potential immunomodulatory and anti-inflammatory activities of prebiotic compounds, PCA and AHCC, and probiotic SV-53, through regulation of epigenetic mechanisms and/or signaling pathways were investigated. These compounds may exert their anti-inflammatory roles by modulating TLRs (Nam and Lee, 2018; Mallet et al., 2016; Grabig et al., 2006). SV-53 is a Gram-negative bacterium with probiotic-like features that was isolated from the microbiota of wild blueberry fruit and has been shown to improve gut immunity by increasing sIgA, IL-10, Goblet cells, and preventing pathogenic bacteria growth (Matar; Novotny-Nuñez et al., 2023; Yahfoufi et al., 2021b; Salvetti et al., 2023). Gram-negative probiotics may ameliorate inflammation via TLR-2 and TLR-4-dependent pathways (Grabig et al., 2006). PCA is a polyphenolic compound with antioxidant and anti-inflammatory effects. PCA has been found to

suppress the production of IL-6, IL-1β, and IL-8 and inhibit LPS-induced inflammatory mediator productions by suppressing TLR4 and TLR4-dependent pathways, including AKT, mTOR, and NF-κB pathways (Nam and Lee, 2018). AHCC is rich in bioactive oligosaccharides such as α-glucans. Previous studies have reported the immunomodulatory activity of AHCC in disease conditions such as viral infection, colitis, and cancer, by modulating innate immune responses, NKT, CD4+ and CD8+ T cells production and activity, and cytokines production (Shin et al., 2019). Our previous research has also revealed the ability of AHCC to enhance immune responses through modulating TLRs in the intestinal epithelial cells (Mallet et al., 2016).

5-1- Validation of Immunomodulatory Activity of SV-53 and PCA in the Steady-State

5-1-1-SV-53 and PCA Improve Mucosal Immunity and Modulate IL-6, IL-10, and IL-23 Cytokines

Previous research in our lab has utilized a blueberry juice fermented by SV-53, referred to as polyphenol-enriched blueberry preparation or PEBP, containing a mixture of polyphenols and SV-53, and found its effectiveness against breast cancer (Mallet et al., 2021). Further analyses revealed many small oligomers with anti-inflammatory activity derived from the degradation of blueberry juice polyphenols by SV-53, including a phenolic mixture containing PCA (Mallet et al., 2023).

Therefore, in this project, we first designed a study to assay the immunomodulatory and anti-inflammatory activity of SV-53 and PCA (alone and not in the form of PEPB or polyphenolic mixture) in adult Balb/c mice in the homeostatic condition. Since, it is known that exposure to LPS is associated with an increase in IL-6, IL-23, and subsequent differentiation of Th17 cells, and IL-17 expression through TLR4-dependent pathways (Park et al., 2015) and given our preliminary data revealed the role of SV-53 in modulating cytokines related to Th17 differentiation, including

IL-10, IL-6, IL-17, therefore, we explored selected cytokines and epigenetic mechanisms related to Th17 differentiation and IL-17A signaling.

In addition, inactivated non-viable probiotic bacteria exhibit comparable immunomodulatory and health-promoting properties to their viable counterparts. Besides, non-viable microorganisms offer additional advantages such as extended shelf life, easier transportation, simplified storage methods, and fewer safety concerns (Akter et al., 2020). These inactivated microbial cells have recently been introduced as "paraprobiotics" which refers to "non-viable microbial cells (intact or broken) or cell extracts when administered in adequate amounts, confer a health benefit to the consumer" (Taverniti and Guglielmetti, 2011). Therefore, we also investigated the potential immunomodulatory activity of the heat-inactivated form of SV-53.

Probiotics and prebiotics can modulate immune responses by regulating cytokines and immunoglobulin secretions (Yahfoufi et al., 2018). The results of this study indicated the role of SV-53 and PCA in improving mucosal and humoral immunity. Both SV-53 and PCA administration elevated IgA-producing B cell frequency in the ileum and SV-53 intake increased the concentration of serum IgA. On mucosal surfaces, IgA combats invading pathogens by forming an immune complex that can be recognized by specific receptors on the immune cells which leads to the activation of different mechanisms in immune cells, finally eliminating the pathogens. It also may be involved in the downregulation of inflammatory responses (Mantis et al., 2011). Conversely, IgG triggers inflammatory responses and contributes to the exacerbation of inflammation and Th-17 polarization by stimulating macrophages during colitis (Aschermann et al., 2010; Castro-Dopico and Clatworthy, 2019; Castro-Dopico et al., 2019). Our treatment did not change IgG levels. Stimulating the production of sIgA without concurrent elevation of IgG levels emphasizes the efficacy of the SV-53 and PCA in improving gut mucosal immunity.

In addition, an increase in the frequency of the IL-10-producing cells in both SV-53 and PCA-fed mice further indicates the role of these compounds in improving mucosal immunity due to the role of IL-10 in the production of IgA by B cells (de Moreno de Leblanc et al., 2011). IL-10 can inhibit antigen presentation and subsequent pro-inflammatory signaling activation and cytokine release, therefore is effective in inhibiting intestinal inflammation (Li and He, 2004). These results are in line with other studies exploring the immunomodulatory activity of probiotics or prebiotics (Di Giacinto et al., 2005; Lavasani et al., 2010; Yahfoufi et al., 2021b; Roller et al., 2004; Hoentjen et al., 2005). SV-53 and PCA intake were associated with a decrease in the levels of IL-17A, IL-6, and IL-23. IL-17A/IL-23 signaling contributes to the pathogenesis of IBD (Cătană et al., 2015). Additionally, increased IL-6 expression damages gut mucosal immunity by targeting tight junctions (Li et al., 2021; Al-Sadi et al., 2014).

Heat-inactive probiotics are also metabolically and functionally active due to the release of various components during the inactivation process, including cell wall components (Castro-Herrera et al., 2020). Heat-inactivated probiotics have been shown to downregulate IL-6, and IL-23 expressions, and STAT3 activation in colitis animals (Sang et al., 2015). In this study, feeding mice with heat-inactivated SV-53 led to an increase in serum IgA concentration, a reduction in IL-6 and IL-23 concentrations, and a decrease in the number of IL-17A and IL-6-producing cells in the ileum tissues of mice. Our results revealed more potent immunostimulatory activity of live bacteria compared to non-viable bacteria. These results are in line with other studies reporting more effect for viable bacteria, where heat inactivation of some probiotic bacteria such as *E. coli* Nissle 1917 (Sturm et al., 2005) and Bifidobacterium led to the alteration of their immunomodulatory and anti-inflammatory activities (Pyclik et al., 2021). Noteworthy, in this study, heat-inactivated SV-53 was applied under normal conditions. Analyzing the activity of heat-inactivated bacteria in the

presence of inflammation may offer more insights into the potential immunomodulatory properties of these bacteria. Previous research has demonstrated that heat-inactivated *A. muciniphila* is more effective in alleviating inflammatory responses in colitis mice compared to the live bacteria (Xue et al., 2023).

5-1-2- SV-53 Colonizes the Intestine and Decreases *E-coli* Population

Results from gut microbiome analysis revealed SV-53 colonization in the groups treated with this compound. Comprehensive genome analysis of SV-53 has identified no potentially pathogenic genes, while a cluster of genes responsible for coding bacteriocins that target pathogenic Gram-negative bacteria was found (Salvetti et al., 2023). Interestingly, in the current study, we observed the ability of SV-53 to reduce the population of *E. coli*, a Gram-negative pathogenic bacterium in the gut microbiota, which has been shown to increase IL-17 secretion (Ren et al., 2017) and disturb gut barrier integrity, leading to inflammation (Raheem et al., 2021). Similarly, some probiotics, such as *L. plantarum*, *L. rhamnosus, L. fermentum*, and *E. coli* Nissle 1917, have been reported to prevent the detrimental effects of pathogenic *E. coli* on gut barrier defense (Raheem et al., 2021).

5-1-3- SV-53 and PCA Modulate Gut Immunity through Epigenetic Mechanisms

To investigate whether the immunomodulatory activities of SV-53 and PCA are governed by epigenetic processes, we examined miRNA expression and DNA methylation status of genes related to the IL-17 pathway. A significant decrease in the relative expressions of miR-425 and miR-223 was observed in the mice treated with live SV-53 and PCA, while heat-inactivated bacteria did not affect these miRNAs. Over-expression of both miRNAs contributes to intestinal inflammation (Yang et al., 2018; Rodríguez-Nogales et al., 2018a). miR-233 is downstream of the IL-23/IL-23R signaling which promotes autoreactive Th17 cells during autoimmunity through the IL-23/STAT3 pathway (Wei et al., 2019) and its expression correlates with IL-17A expression (Xu

et al., 2020). miR-223 also increases gut permeability by damaging tight junction proteins such as claudin-8 through the IL-23/Th17 pathway. miR-425 participates in inflammatory diseases such as autoimmunity (Balzano et al., 2017), and its overexpression is related to intestinal mucosal inflammation, Th17 cell pathogenicity, and IL-17A secretion (Yang et al., 2018).

Next, a DNA methylation analysis was conducted. While we could not find any differentially methylated regions (DMRs) in mice receiving live bacteria and PCA, heat-inactivated probiotic intake was associated with increased methylation levels of probes within CpG islands surrounding the transcription start sites (TSS) of genes related to IL-17A signaling, such as *Il6*, *Il17rc*, and *Il11*. As stated earlier, IL-6 is indispensable for the initiation of Th17 differentiation from naïve $CD4^+$ T cells (Tanaka et al., 2014), while IL-17RC, in the form of the IL-17RA/IL-17RC receptor complex, is essential for signal transduction mediated by IL-17A and IL-17F (Rex et al., 2023; Pappu et al., 2011). IL-11 appears to play roles in the generation, expansion, or response of Th17 cells, consequently contributing to the development of autoimmunity (Zhang et al., 2019d).

PI3K/AKT/NF-κB pathway plays an important role in Th17 differentiation and IL-17A gene expression (Park et al., 2014). We noted hypermethylation in the regulatory regions of two genes associated with this pathway: *Akt1* and *Ikbkg*. AKT participates in the activation of RORγ and facilitates Th17 differentiation (Kurebayashi et al., 2012), while *Ikbkg* encodes the regulatory subunit of the inhibitor of the IκB kinase complex, which participates in NF-κB activation (Johnston et al., 2016). NF-κB binds to the RORγt and RORγ promoter regions and induces their expressions (Park et al., 2014). It also induces the expression of IL-1, IL-6, IL-23, IL-17, and IL-23R (Liu et al., 2017; Park et al., 2014). In addition, the promoter of *Sgk1* underwent hypermethylation following treatment with heat-inactivated bacteria. This gene encodes SGK1,

which participates in the maintenance of the Th17 cell phenotype by regulating the expression of IL-23R (Wu et al., 2013).

CpGs around the TSS of *Cblb* and *Smad4* were hypomethylated, which may be related to the transcriptional activation of these genes. CBLB and SMAD restrain Th17 cell differentiation by restricting IL-6 secretion from immune cells (Zeng et al., 2022) and suppressing Th17 transcriptional factor expression (Zhang et al., 2019c), respectively. These findings suggest that, in addition to cell wall components, other elements released during the heat-inactivation process may be involved in epigenetic modifications induced by heat-inactivated SV-53. Together, this result revealed the anti-inflammatory and immunomodulatory effects of probiotic SV-53 by regulation epigenetic mechanisms.

5-2- AHCC Alleviates Acute Impact of Pubertal LPS Challenge on Gut Microbiota

We have previously shown the effect of LPS and SV-53 on gut microbiota in pubertal mice exposed to LPS (Yahfoufi et al., 2023). Therefore, in the current study, we only studied the AHCC effect on gut microbiota at puberty. β-diversity results showed significant differences between mice challenged with only LPS and both groups receiving AHCC. At the family level, the Bacteroidaceae population was observed to be higher in the LPS group compared to the other groups, while the Lachnospiraceae population decreased in the LPS group. Lachnospiraceae species are known as key producers of SCFAs in the gut, consequently leading to lower levels of inflammatory mediators (Vacca et al., 2020). In the AHCC group, Flavobacteriaceae were the most abundant bacterial population. Genome analysis of Flavobacteriaceae has found a significant abundance of gene clusters associated with antimicrobial compound synthesis (Gavriilidou et al., 2020). In addition, LPS exposure was associated with the increased abundance of Bacteroides and Parabacteroides genera and *B. intestinalis* species while AHCC intake prevented the effect of LPS

on this bacteria. Overgrowth of Bacteroides and Parabacteroides correlates with colitis-induced mucosal injury (Shi et al., 2021), and inflammatory cytokine production, such as IL-17, IL-21, and IFN-γ (Guo et al., 2020). Furthermore, studies have identified a positive correlation between colonizing mice with *B. intestinalis* and the overexpression of IL-1β, leading to ileal damage (Andrews et al., 2021).

5-3- AHCC and SV-53 Mitigate Pubertal LPS-Induced Inflammatory Responses by Modulation of Cytokines and Signaling Pathways Related to IL-17 Signaling

The protective effects of AHCC or SV-53 administration during the pubertal window against acute and/or enduring inflammatory responses induced by pubertal LPS challenge were explored at the gut level. AHCC administration was found to inhibit the immediate effects of pubertal LPS exposure on IL-17A, Il-L-17F, TGF-β, IL-6, IL-1β, and IL-23.

Notably, AHCC intake during puberty also reduced the lasting effects of pubertal LPS exposure on IL-17A, IL-1β, and IL-6 production in adult mice. Furthermore, SV-53 intake was observed to mitigate the LPS impact on IL-17A, TGF-β, and IL-6 production. Both compounds prevented the lasting inhibitory effect of pubertal LPS on IL-10.

Then, based on cytokine results, the impact of treatment was investigated on STAT3 and FOXO1 levels. In the acute phase, AHCC intake reduced the stimulatory impact of LPS on p-STAT3 in pubertal mice. In the long term, both pubertal AHCC and SV-53 intake suppressed the LPS-induced increase in STAT3 and p-STAT3 levels. Additionally, AHCC and SV-53 administration in the pubertal window resulted in a lasting increase in FOXO1 levels during adulthood. Similarly, a study by Chen et al. demonstrated that *Lactobacillus acidophilus* administration could mitigate inflammatory responses associated with the IL-23/Th17 signaling by suppressing the STAT3

pathway, thereby reducing IL-17 expression in colitis mice (Chen et al., 2015). In another study, prebiotic inulin inhibited gut injury and promoted mucosal immunity in Salmonella-infected animals by inhibiting IL-6, IL-1β, IFN-γ, and JAK/STAT3 pathway (Song et al., 2020).

In the intestine, IL-6 contributes to the early differentiation of Th17 by inducing STAT3-dependent expression of RORγt (Chen et al., 2022), while IL-1 and IL-23 participate in delayed proliferation, and maintenance of $CD4^+$ Th17 phenotype (Sutton et al., 2009; Ghoreschi et al., 2010). In fact, early differentiation of Th17 cells is IL-23 independent because naïve T cells do not express IL-23R (Ivanov et al., 2006). IL-1R signaling is also critical for sustaining Th17 cells, in the absence of TCR stimuli. Notably, the results of a study uncovered that in the absence of TCR stimuli, the addition of IL-23 or IL-6 to the Th17 culture did not improve cytokine production by these cells, while the addition of IL-1 significantly enhanced the cytokine secretion (Chung et al., 2009).

IL-10 is involved in limiting Th17 cell-induced inflammation in the gut (Hsu et al., 2015). Also, signals from TGF-β inhibit pathogenic Th17 production, while may enhance non-pathogenic th17 cell generation (Ghoreschi et al., 2010). Some evidence has also been suggested that TGF-β isoforms might be related to the pathogenicity of Th17 cells and TGF-β3-induced Th17 cells may exert more pathogenicity compared to IL-6 and TGF-β1-induced cells (Lee et al., 2012).

On the other hand, IL-17A overexpression, in turn, directs the production of IL-6, IL-23, and IL-1β, and IL-6-derived phosphorylation and activation of STAT3, leading to further IL-17 secretion and sustained inflammation (Chen and Zhou, 2015; Sutton et al., 2009). Our results may suggest that the lasting inflammatory responses induced by LPS may be mediated, partially, by IL-1β. In addition, it may indicate the involvement of innate immune cells in IL-17 production as previous

studies have shown the ability of ILC3s to produce IL-17A and IL-17F in response to IL-1β and IL-23, independent of TCR involvement (Chung et al., 2021).

In contrast to STAT3, FOXO1 induces IL-10, and TGF-β expression (Graves and Milovanova, 2019; Cabrera-Ortega et al., 2017), suppresses IL-23R expression, and RORγt transcriptional activity, resulting in inhibition of IL-17A production by Th17 (Lainé et al., 2015; Cabrera-Ortega et al., 2017). LPS has been shown to inhibit FOXO1 activity through activation of TLR4/PI3K/AKT signaling (Graves and Milovanova, 2019). In addition, the IL-6/STAT3 pathway may inhibit FOXO1 expression epigenetically, leading to subsequent induction of IL-1R1/IL-1 signaling. Conversely, this process is inhibited by TGF-β (Ichiyama et al., 2016). Our results suggest that prebiotic and probiotic consumption may exert immunomodulatory properties partly by modulating STAT3, FOXO1, and cytokines related to these pathways.

5-4- AHCC and SV-53 Intake Alleviate Enduring Immune Dysfunction Associated with Pubertal LPS-Induced Inflammation through Modulating Epigenetic Mechanisms

Finally, to evaluate if the enduring impacts of LPS exposure as well as AHCC/SV-53 intake on gut immunity involve lasting epigenetic alterations, an analysis of miRNA expression and/or DNA methylation was conducted. Our treatment was associated with an enduring increase in miR-145 expression in adult mice exposed to the pubertal prebiotic and probiotic. Also, prebiotic/probiotic administration around puberty significantly diminished the enduring effect of pubertal LPS exposure on miR-425 expression in adult mice. Loss of miR-145 has been shown to induce pro-inflammatory signals of innate immunity (Pekow et al., 2012), increase secretion of TNF-α, IL-6, and IL-1β (Li et al., 2018), and augment pathogenic Th17 cells responses (Wang et al., 2013). miR-145 expression inhibits LPS-mediated inflammatory responses by suppressing NF-κB (He et al., 2020). It also exerts an anti-inflammatory role by suppressing STAT3 and inducing FOXO1

(Jiang et al., 2017). The inflammatory properties of miR-425 were discussed earlier. IL-1β has been identified to derive miR-425 expression through the NF-κB pathway (Ma et al., 2014). miR-425 represses FOXO1 expression in the experimental colitis causing over-expression of IL-17A (Yang et al., 2018). In line with these results, in a study, administration of the *Bifidobacterium longum* was associated with an elevation in miR-145 expression and a concurrent reduction in IL-6 concentration in an animal model of colorectal cancer (Fahmy et al., 2019).

Then, DNA methylation analysis was done for the enduring LPS-AHCC experiment. We found hypermethylation of CpGs around the TSS of some genes directly or indirectly related to IL-17 signaling, including *Lbp*, *Rorc*, *Runx1*, *Il17ra*, *Rac1*, and *Ccl5*, in adult mice exposed to pubertal LPS+AHCC compared to mice solely exposed to LPS. This observation may suggest the transcriptional repression of these genes by AHCC. *Lbp* encodes for LBP, which as discussed in the introduction section, is necessary for LPS-mediated TLR4 signaling initiation by delivering LPS to CD14. *Rorc* encodes the Th17 master transcription factor RORγt (Ma et al., 2022). RUNX1 enhances RORγt expression, Th17 generation, and IL-17 transcription (Zhang et al., 2008). *Il17ra* encodes IL17RA which is essentil for IL-17 signaling initiation (Rex et al., 2023). Rac1 contributes to LPS-induced proinflammatory cytokine expressions (Sanlioglu et al., 2001) and is required for IL-17A expression in autoimmunity (Kurdi et al., 2016). Lack of Rac1 in Th17 cells leads to suppression of IL-17A, IL-17F, and IL-23R expression (Kurdi et al., 2016). Moreover, LPS induces CCL5 expression through a TLR4-dependent pathway (Bandow et al., 2012). Pathogenic Th17 cells express higher levels of CCL5 (Lee et al., 2012) and IL-17A/F signaling blockade may reduce CCL5 expression (Solá et al., 2023). Furthermore, the *Il10* promoter was hypomethylated in the AHCC+LPS mice, which may indicate transcription activation of the *Il10*. IL-10 preserves FOXO1 function (Hsu et al., 2015) and suppresses IL-17A production (Gu et al., 2008).

Together, our finding suggests that prebiotic/probiotic intake in the pubertal window inhibits lasting immune deregulation mediated by immune stressors in this period through the preservation of gut microbiota balance, and modulation of signaling pathways and epigenetic mechanisms.

5-5- Early-Life Antibiotic Exposure Induces Gut Microbiota Dysbiosis

To investigate the correlation between early-life gut microbiota dysbiosis and immune dysfunction in adulthood, mice were exposed to low-dose penicillin perinatally, and the potential protective effects of AHCC intake against antibiotic-induced dysbiosis were studied at the weaning.

At the phylum level, notable changes were identified: a significant rise in the Proteobacteria population in mice exposed to antibiotic in both dams and offspring; a decline in the Firmicutes population in offspring exposed to the maternal antibiotic, and an increase in Bacteroidetes in both male and female offspring exposed to antibiotic + AHCC. These results are in line with previous research studying the effect of early-life antibiotic intake on gut microbiota (Leclercq et al., 2017; Candon et al., 2015; Jin et al., 2017; Cho et al., 2020). At the genus level, a reduction in the main genera involved in the SCFAs production (belonging to Firmicutes phylum and class Clostridia) and in the population of Lactobacillus (belonging to Firmicutes phylum, class Bacilli) was observed in mice exposed to the antibiotic in dams and offspring. AHCC intake could mitigate the overgrowth of Proteobacteria induced by the antibiotic in dams and offspring, and partially alleviate the inhibitory effect of maternal antibiotic on SCFAs-producing bacteria in dams and male offspring. Consistent with our results, several studies have highlighted the impact of diets abundant in prebiotics on reducing Firmicutes and increasing Bacteroidetes (Dewulf et al., 2013; Parnell and Reimer, 2012), as well as promoting short-chain fatty acid (SCFA)-producing bacteria in the gut microbiota (Garcia-Mantrana et al., 2018).

As stated in the introduction section, under homeostatic conditions, the majority of gut-derived LPS originate from Bacteroidetes, producing non-pathogenic LPS with immunoinhibitory properties, potentially by inhibiting the production of inflammatory mediators through the NF-κB-dependent pathway (d'Hennezel et al., 2017). Conversely, Proteobacteria are a primary source of pathogenic LPS, raising the risk of inflammatory responses (d'Hennezel et al., 2017). Overgrowth of Proteobacteria correlates with intestinal inflammation (Rizzatti et al., 2017). Furthermore, SCFA-producing bacteria may suppress intestinal inflammation by inhibiting NF-κB and cytokine production (TNF-α, IL-6, IL-12, and IL-17A) (Morgan et al., 2012; Zhu et al., 2018). Maternal gut microbiota and a prebiotic-rich diet during pregnancy and lactation play a crucial role in early-life metabolic, neurologic, and immunologic development, facilitated by SCFA production (Kimura et al., 2020; Nakajima et al., 2017). Hence, our results suggest that AHCC may enhance immune function and mitigate antibiotic-induced inflammation, potentially by reducing Proteobacteria, elevating Bacteroidetes, and preserving SCFA-producing bacteria.

5-6- AHCC Intake Alleviates Long-Term Immune Deregulation Induced by Early-Life Gut Microbiota Dysbiosis

The impact of AHCC on mitigating the lasting effects of early-life gut microbiota disturbance on immune function was assessed in offspring at 8 weeks. AHCC could mitigate the enduring effect of early-life antibiotic exposure on proinflammatory cytokines, including IL-2, IL-6, IL-15, and IL-21 in offspring. These cytokines play an important role in regulating two inflammatory pathways: NF-κB and STAT3. IL-6 induces the NF-κB pathway via the activation PI3K pathway (Luo and Zheng, 2016). On the other hand, NF-κB itself induces the expression of IL-6, leading to phosphorylation and activation of STAT3 (Yang et al., 2014), and inflammatory responses by producing inflammatory mediators such as IL-17 (Yang et al., 2014). IL-2, IL-15, and IL-21 induce

STAT3 and NF-κB cascades. IL-2 plays a dual role in inflammation, while IL-15 and IL-21 are proinflammatory cytokines contributing to intestinal inflammation and colitis (Waldmann, 2015; Fung et al., 2003; Chan et al., 2010; Sugimoto, 2008; Giron-Michel et al., 2003; De Simone et al., 2015).

Next, based on cytokines results, we hypothesized that antibiotic exposure in early life is associated with a lasting increase in STAT3 phosphorylation and activation as well as NF-κB levels. However, p-STAT3 levels were not affected by antibiotic and prebiotic treatment in dams and female offspring. In male offspring, its level decreased insignificantly in antibiotic-exposed mice and significantly in AHCC and antibiotic + AHCC-exposed mice. Similarly, bioactive compounds have been reported to protect against colitis-related gut inflammation by suppressing JAK1, p-STAT3, inflammatory cytokines such as IL-1β, IL-6, IFN-γ, and TNF-α, improving Th17/Treg balance, preventing dysbiosis, and increasing SCFA-producing bacteria (Li et al., 2023). In addition, inhibition of phosphorylation and transcriptional activity of STAT3 by specific antibiotics has been reported (Heppler et al., 2022; Ye et al., 2018) which is mediated by different mechanisms, such as suppressing the JAK family kinases (Nelson et al., 2008) or binding to STAT3 (He et al., 2021). Furthermore, early-life dysbiosis was associated with an enduring elevation in NF-κB levels in offspring while prebiotic intake prevented this effect. Similar to our results, other studies have found the efficacy of bioactive compounds in decreasing the content of NF-κB p65 and IL-6 in inflammatory conditions (Jin et al., 2022). NF-κB signaling is controlled by gut bacteria, especially Firmicutes (Zhang et al., 2022), however, in dysbiotic conditions, NF-κB over-activation initiates inflammatory cascades, leading to the development of chronic inflammation (Zhang et al., 2022). These results suggest the potential immunomodulatory activity of AHCC by modulating NF-κB signaling and related cytokines.

5-7- AHCC Intake Alleviates Long-Term miRNAs Deregulation Induced by Early-Life Gut Microbiota Dysbiosis

To further explore the potential impact of early-life antibiotic-induced dysbiosis and AHCC intake on the immune system through persistent epigenetic alterations, we investigated the expression of specific miRNAs associated with the NF-κB and STAT3 pathways in the offspring. Antibiotic intake increased pro-inflammatory miR-221 and antibiotic + AHCC exposure reduced miR-221. In addition, antibiotic intake decreased anti-inflammatory miR-145 expression in males which was prevented by AHCC. AHCC intake also increased miR-145 expression in females. Dysregulation of the gut miRNA profile participates in the pathogenesis of intestinal inflammation (Xiao et al., 2022). Overexpression of miR-221 is correlated with the constitutive activation of NF-κB and STAT3 which further upregulates miR-221 expression (Liu et al., 2014). On the contrary, miR-145 exerts anti-inflammatory and antitumor activities by inhibiting NF-κB (Sui et al., 2019; Mei et al., 2017) and p-STAT3 (Gao and Ding, 2021). It also attenuates inflammatory responses related to metabolic disorders by inhibiting NF-κB activation (He et al., 2020).

Together, these findings suggest that dietary interventions incorporating prebiotics have the potential to prevent the enduring consequences of early-life gut microbiota dysbiosis through the regulation of signaling pathways and miRNAs, thereby promoting health in later stages of life.

5-8- Critical Findings and Summary

In summary, our study contributes to generating new insights into the immunomodulatory properties of prebiotics and probiotics in critical developmental windows of life. The efficacy of these compounds to preserve gut microbial balance, regulate cytokine responses, and modulate epigenetic processes, suggests a promising approach for dietary strategies aimed at promoting long-term immune health. Figure 5-1 summarizes the potential mechanisms of action of AHCC/SV-53.

Our findings emphasize the significant impact of AHCC and SV-53 in preventing persistent inflammatory responses and immune dysregulation triggered by pubertal immune challenges. Biotics intake effectively regulated cytokine profiles and signaling pathways related to IL-17, including IL-6, IL-1β, TGF-β, STAT3, and FOXO1, showing a comprehensive immunomodulatory effect. In addition, we showed that immune deregulation induced by pubertal immune challenge might be mediated, in part, by enduring changes in DNA methylation and that nutritional intervention can prevent these changes, shedding light on a connection between pubertal immune challenges, epigenetic modifications, and the efficacy of dietary intervention in immune regulation. Furthermore, our result revealed that early-life antibiotic-induced gut microbiota dysbiosis leads to lasting deregulation in NF-κB and miRNAs such as miR-221 and miR-145, while prebiotic intake could mitigate gut microbiota disturbances and related adverse effects, suggesting the role of nutritional intervention in the window of opportunity in early life to prevent immune-related disorders later in life.

This research expands our current knowledge and highlights the potential translational applications of biotics in preventing immunological alternations associated with exposure to immune stressors and dysbiosis in the developmental phases of life.

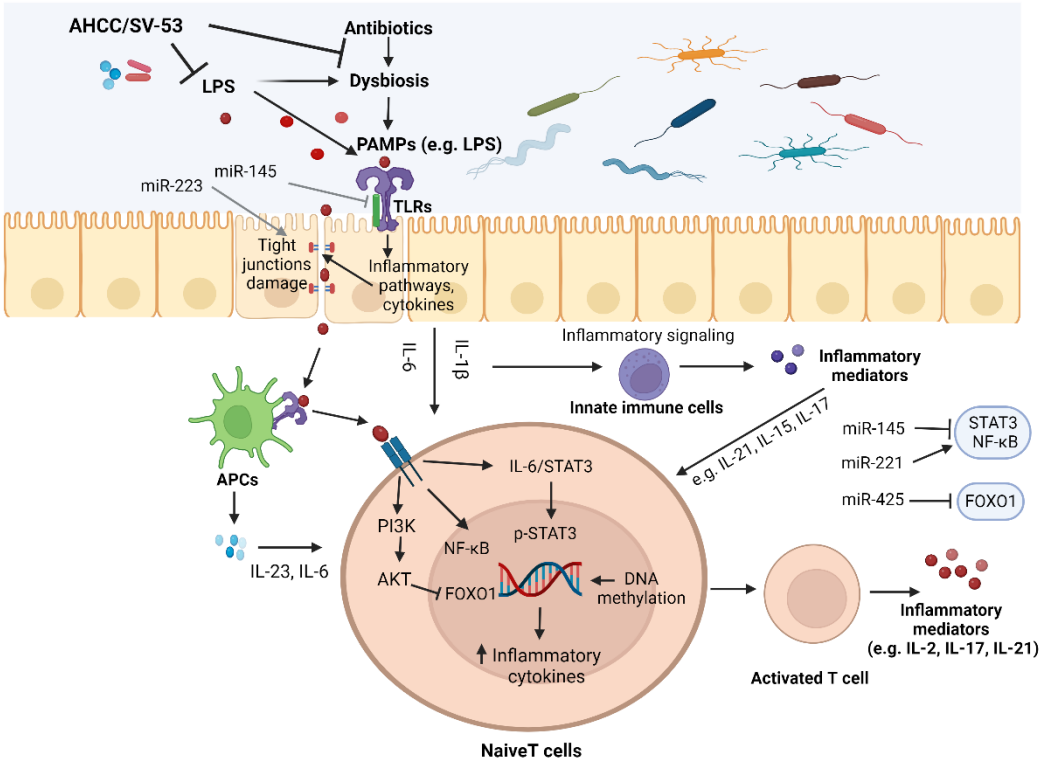

Figure 5-1. The potential mechanisms of action of AHCC/SV-53 in modulating gut immunity. LPS exposure activates TLRs by either directly interacting with receptors or inducing dysbiosis. Antibiotic exposure is associated with dysbiosis, increased PAMPs production, and subsequent activation of TLRs. TLRs and downstream inflammatory signaling activation lead to overexpression of pro-inflammatory cytokines and, therefore, activation of innate immunity and secretion of more pro-inflammatory cytokines. LPS and other PAMPS can also be detected by APCs and be transferred to T cell receptors on the surface of naïve T cells, in the presence of cytokines such as IL-6 and IL-23. This process may induce STAT3 and NF-κB and inhibit FOXO1, leading to the differentiation of naïve T cells to mature T cells such as Th17. Cytokines produced by innate immune cells as well as activated T cells can further activate inflammatory pathways such as STAT3 and NF-κB. These changes may also lead to the alteration of the gut miRNA profile and DNA methylation status of genes controlling gut immunity. AHCC/SV-53 may prevent gut microbiota dysbiosis, as well as signaling pathways and epigenetic deregulation induced by LPS/antibiotic exposure. PAMPs: Pathogen-associated molecular patterns; APCs: Antigen-presenting cells. The image was created by BioRender.com.

5-9- Limitations of the Study

This project has some limitations:

In the first and second studies only female mice were utilized to study the effect of treatment on the immune system, however, there is a sex difference in immune system function (Kane and Ismail, 2017; Klein and Flanagan, 2016). Males and females exhibit differences in their immune responses in embryonic stages and early life, such as differences in the production of cytokines by immune cells, indicating that some sex-specific variations may be encoded in the germline (Klein and Flanagan, 2016). Sex differences in immune responses are significantly influenced by the differential expression of genes located on the X and Y chromosomes. These genetic differences contribute to variations in both innate and adaptive immune responses between males and females observed in normal conditions and disease states. Gene expression analysis in immune cells has revealed that males and females exhibit differences in the expression of transcripts for both proteins and non-coding RNAs. Notably, approximately 7% of these differentially expressed transcripts are encoded on the sex chromosomes (Dunn et al., 2024). In addition, sex hormones direct sex differences in immune responses between males and females. In both animals and humans, immune system function changes throughout adolescence, especially in the puberty period (Brenhouse and Schwarz, 2016). Testosterone has an anti-inflammatory and immunosuppressive effect due to its ability to induce the production of IL-10 by T cells which leads to less susceptibility to immune-related disorders following the onset of puberty in males compared to females (Brenhouse and Schwarz, 2016). Whereas estrogen seems to have a dual effect on various immune functions, like cell-mediated immune function, humoral-mediated immune function, and cytokine levels (Kane and Ismail, 2017). In addition, sex differences in immune responses to the pubertal immune stressor have previously been reported (Yahfoufi et al.,

2023). So, it is recommended to take into account the factor of sex when investigating the consequences of immune challenges in puberty and adulthood (Kane and Ismail, 2017).

While our findings, especially those related to p-STAT3, FOXO1, and DNA methylation, may provide some indication regarding the potential role of SV-53 and AHCC in regulating immune responses during inflammatory conditions, by influencing Th17 cells, performing a flow cytometry test could offer an additional tool to determine various immune cell affected by treatment with LPS and AHCC.

The DNA methylation results revealed several differentially methylated genes impacted by the treatment, offering insights into the potential epigenetic modulation of genes associated with Th17/IL-17 signaling. However, measuring mRNA levels of these genes could provide more conclusive evidence regarding the treatment's effectiveness in modulating gene expressions.

In the early-life antibiotic-induced dysbiosis experiment, the treatment's impact on gut microbiota was analyzed after weaning, and the analysis of the microbiome during adulthood was not performed. However, studies have consistently highlighted a significant impact of antibiotics on gut microbiota post-weaning, with this effect declining after the discontinuation of antibiotic treatment. Nevertheless, the adverse health consequences of uncorrected early-life dysbiosis, despite antibiotic cessation, persist into adulthood (Cox et al., 2014). Therefore, in this study, we focused on examining the weaning microbiome.

Furthermore, although our microbiome findings indicated a significant change in the population of bacteria responsible for SCFA production following treatment with antibiotic, we did not assess SCFA levels, while changes in SCFA levels could potentially serve as a mechanistic link to the immune dysregulation induced by early-life gut microbiota dysbiosis.

Lastly, in this study, offspring were exposed to treatment in utero one week before birth and continued until weaning. However, conducting further studies using prenatal and postnatal models separately will help gain a better understanding of the detrimental outcomes associated with maternal and/or offspring gut microbiota dysbiosis across critical developmental stages before and shortly after birth.

5-10- Future Directions

Our results indicate that heat-inactivated SV-53 plays a role in the epigenetic modulation of the immune system by influencing the DNA methylation of certain genes involved in immune responses. This suggests the involvement of other components, apart from LPS, which are released during the heating process and contribute to the epigenetic modulatory properties of the heat-inactivated bacterium. Notably, studies have demonstrated that surface lipids and proteins extracted from the outer membrane of the Gram-negative probiotic *A. muciniphila* are potent immunomodulatory molecules. These molecules exert their effects through interactions with TLRs (Bae et al., 2022; Wang et al., 2021). Therefore, characterizing cell wall components of SV-53, including LPS, as well as outer membrane proteins and phospholipids can be the next step in our research to identify bioactive molecules mediating the anti-inflammatory and immunomodulatory properties of this novel probiotic bacterium.

In addition, in this study, we assayed the potential immunomodulatory activity of heat-inactivated SV-53 in a homeostatic condition, while future studies can explore the effects of the heat-inactivated SV-53 in the presence of immune challenges, gut dysbiosis, or intestinal inflammation using different concentrations of bacteria to identify the effectiveness of bacteria in inflammatory conditions and to determine the optimal dose of heat-inactivate bacteria preparation.

Further studies addressing epigenetic modifications, such as DNA methylation, followed by gene expression analysis, will help to gain deeper insights regarding underlying mechanisms governing the long-term effect of early-life gut microbiota dysbiosis on the immune system.

Since antibiotic exposure in early life leads to lasting immune dysfunction, further studies can be designed utilizing the early-life gut microbiota dysbiosis model, with a longer period during adulthood to explore the effect of exposure to immune stressors and inflammation on susceptibility of developing immune-based disorders in adult mice experiencing early-life gut microbiota dysbiosis.

Studies have indicated that maternal diet-induced dysbiosis is not only linked to enduring outcomes in the offspring but also contributes to long-term effects in the second generation of offspring (Di Gesù et al., 2022). Additionally, it is well-established that epigenetic alterations can be transmitted intergenerationally (Nilsson et al., 2022). Therefore, further studies can be conducted to investigate whether the offspring of mice exposed to gut microbiota dysbiosis in their early life (second-generation offspring) are susceptible to developing immune-based disorders in the presence of immune stressors.

Our results provide some evidence about the involvement of miRNAs in regulating immune responses; however, utilizing targeted miRNA knockout mice can help elucidate the role of specific miRNAs in preventing/inducing long-term immune dysfunction in response to early-life/pubertal immune stressors and inflammation.

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