UNIVERSITY OF OTTAWA

FACULTY OF HEALTH SCIENCES

The Anti-Inflammatory and Immunomodulatory Role of Protein Hydrolysates: Involvement of Epigenetics and Gut Microbiome

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THESIS

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Abstract

Maintaining gut immune system and microbiota homeostasis is crucial to the individual's health. Any disruptions in gut homeostasis can lead to immune dysfunction not only at the gut level, but also at the distant organs from the gut. Bioactive peptides and hydrolysates from natural sources, including eggshell membrane hydrolysate (ESMH), have been tested in animal models of human diseases for potential anti-inflammatory roles. ESMH has been found to reduce inflammation in the gut-lung axis, prevent dysbiosis, and improve gut microbiota and immune response. Puberty is a critical developmental window of life during which substantial changes in immune system and gut microbiota happens. Previous studies have shown puberty as a sensitive period to immune stressor in which uncorrected inflammation and dysbiosis is accompanied by peripheral and central immune dysfunction and long-term changes is brain function. However, acute, and long-term effects of pubertal exposure to an immune stressor on gut-lung axis immunity has not been sufficiently explored. Therefore, in the current study, we used pubertal lipopolysaccharide (LPS)-induced inflammation in female Balb/c mice to simulate inflammation and dysbiosis during puberty. We hypothesized that LPS-induced inflammation during puberty induces dysbiosis and causes acute and/or enduring alterations in selected cytokines, miRNAs profile and DNA methylation of genes related to the Th17 mediated immunity at the gut and lungs level, while ESMH intake can mitigate effects of pubertal LPS on gut microbiota perturbation and epigenetic alterations. The objectives of this project are:

1. To investigate the role of ESMH in mitigating gut microbiota dysbiosis induced by the acute exposure to the LPS during puberty
2. To study the role of ESMH in mitigating the acute and lasting effects of pubertal exposure to the LPS on cytokines and epigenetics alterations, including miRNAs related to the Th17

mediated immunity at the gut/lung levels. Furthermore, to examine the DNA methylation of the genes in the lungs, related to the Th17, in the enduring phase

Our results revealed that ESMH consumption partially reduced LPS mediated changes in the gut microbiota, by reducing the levels of *Alistipes* and segmented filamentous bacterium *Candidatus arthromitus*. and increasing the abundance of *Lactobacillus* The results revealed an acute effect of LPS in increasing IL-6 and IL-10 levels in the gut, while ESMH intake was associated with an increase in IL-6 and IL-10 levels in the lungs. ESMH intake also mitigated lasting stimulatory effects of LPS on proinflammatory miRNAs, including miR-155, miR-222, and miR-223 at the lung levels. DNA methylation analysis revealed the role of ESMH in modulating LPS-induced alteration in methylation status of several genes related to Th17 mediated immunity in the lungs, including *Tnfaip3, Tnip1, Lcn2, Sox2ot, Ermap, Cxcl11, Ror*α, *Mcc, Prkcb, Socs7* and *Nlrc4.* These results in mice may suggest ESMH as a potential strategy for alleviating inflammatory response accompanying a challenge by an immune stressor at puberty by maintaining immune and epigenetic homeostasis in the gut-lung axis.

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List of Abbreviations:

5hmU: 5-hydroxymethyluracil

5mC: 5-methyl-carbon

AGO: Argonaute

AhR: Aryl hydrocarbon receptors

AID/APOBEC: Activation-induced cytidine deaminase/apolipoprotein B mRNA-editing enzyme complex

ALI: Acute lung injury

AMP: Antimicrobial proteins

APCs: Antigen presenting cells

BER: Base excision repair

CLRs: C-type lectin receptors

CpG: Cytosine-phosphate-guanosine

Cxcl11: C-X-C Motif Chemokine Ligand 1

DAMPS: Damage-associated molecular patterns

DGCR8: Drosha-DiGeorge syndrome critical region gene 8

DMP: Differentially methylated probes

DMRs: Differentially methylated regions

DNMTS: DNA methyltransferases

EGM: Eggshell membrane

ESMH: Eggshell membrane hydrolysate

Ermap: Erythroblast membrane associated protein

Foxp3: Forkhead box P3

GAGS: Glycosaminoglycans

GALT: Gut-associated lymphoid tissue

GF: Germ free

GPCRs: G-protein coupled receptors

HDACs: Histone deacetylases

HMO: Human mil oligosaccharide

IBD: Inflammatory bowel disease

IFN-γ: Interferons γ

IgA: Immunoglobulin A

IL-17: Interleukin-17

IP: Intraperitoneally

IRAK1: Interleukin 1 receptor associated kinase 1

JAK-STAT 3: Janus kinase-signal transducer and activator of transcription 3

Lcn2: Lipocalin 2

LPS: Lipopolysaccharide

MBDs: Methyl-binding proteins

Mcc: Mutated colorectal cancer regulator of WNT Signaling Pathway

miRISC: miRNA-induced silencing complex

miRNA: microRNA

MyD88: myeloid differentiation factor

NF-κB: Nuclear factor-kB

Nlrc4: NLR Family CARD Domain Containing 4

NLRs: NOD-like receptors

PAMPS: Pathogen-associated molecular patterns

PBS: Phosphate-buffered saline

PI3K-AKT-mTOR: Phosphoinositide 3-kianse protein kinase B-mammalian target of rapamycin

Pol II: RNA polymerase II

Prkcb: Protein kinase C Beta

PRR: Pattern recognition receptor

RISC: RNA-induced silencing complex

RORα: Retinoic acid receptor-related orphan receptor alfa

RORγt: Retinoic acid receptor-related orphan receptor-gamma-t

SAM: S-adenosyl methionine

SCFA: Saturated fatty acids

SMUG1: Single-strand-selective monofunctional uracil-DNA glycosylase 1

Soc7s: Suppressor of cytokine signaling 7

Sox2ot: SOX2 overlapping transcript

TDG: Thymine DNA glycosylase

TET: Ten-eleven translocation

TGF-β: Transforming growth factor-β

Th17: T-helper 17

TLR: Toll-like receptors

Tnfaip3: TNF-alfa-induced protein 3

TNF-α: Tumor necrosis factor-α

Tnip1: TNFAIP3 interacting protein 1

TRAF6: Tumor necrosis factor receptor-associated factor 6

TRBP: TAR RNA-binding protein

UHRF:Ubiquitin Like With PHD And Ring Finger

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Chapter 1: Introduction

Gut Microbiota

The human gut harbors a diverse community of microbes with an extensive metabolic repertoire essential for the host's digestive and immune systems (Jandhyala, 2015). It is composed of a complex and dynamic population of microorganisms, and is a key factor in shaping the biochemical profile of individuals, thus influencing the host's health and development of diseases (Thursby & Juge, 2017). The two terms are often used interchangeably: "microbiota" is defined as the living microorganisms that colonizes a particular environment or region (Jandhyala, 2015), whereas the "microbiome" encompasses the collective human genomes of all the microorganisms in the community spread around the human body (Hou et al., 2022; Thursby & Juge, 2017). The dominant gut microbial phyla are Actinobacteria, Proteobacteria, Fusobacteria, Verrucomicrobia along with Bacteroidetes and Firmicutes representing 90% of the gut microbiota (Jandhyala, 2015; Rinninella et al., 2019; Rowland et al., 2018; Thursby & Juge, 2017). The most common fungi, known as the gut mycobiota, are Candida, Saccharomyces, Malassezia and Cladosporium (Hou et al., 2022). The human gut microbiota also contains viruses, phages and archaea (Hou et al., 2022). Microbiota is also found in other organs of the body including oral cavities, lungs, skin and vagina (Hou et al., 2022) (Figure 1.1). The human gut microbiota varies from one individual to another. Moreover, any disturbance or imbalance of the gut microbiota is associated with several diseases and metabolic disorders such as inflammatory bowel disease (IBD), diabetes, obesity, cardiovascular and autoimmune diseases (Bull & Plummer, 2014; Hou et al., 2022).

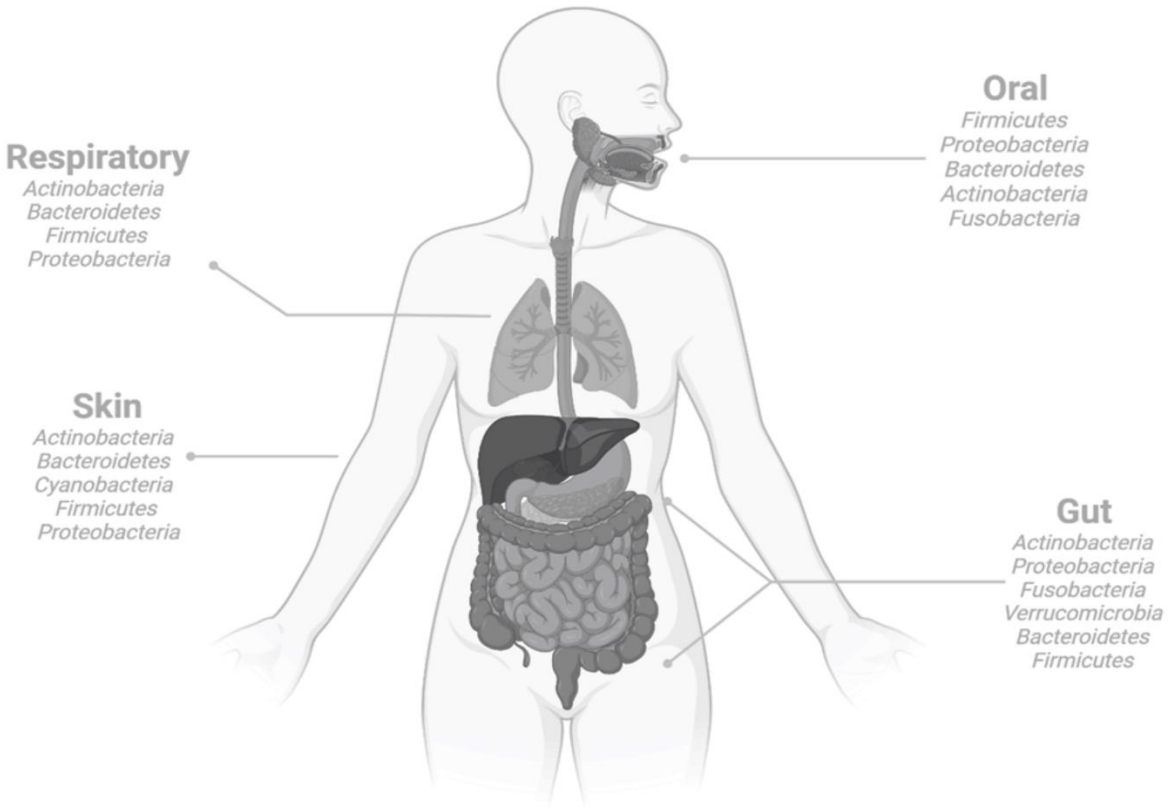

Figure 1.1. Schematic representation of the human microbiota composition in different regions of the body, including the oral cavity, gut, skin, and lungs. Image was created by BioRender.com (Hou et al., 2022).

1.1 Factors affecting the gut microbiota

Some variables play an important role in shaping the gut microbiota. Dietary habits are one of the crucial factors affecting the gut microbiota composition. Extensive studies have showed that Western diets, rich in processed meats and saturated/trans fat are associated with an alteration in the microbiota composition and its inflammatory capacities; it promotes anaerobic microorganisms such as Bacteroides and Bilophila. However, the consumption of Mediterranean/Asian diets rich in fibers, starch and mono/unsaturated fatty acids are associated with a higher abundance of Bifidobacterium (Al Bander et al., 2020). Second, lifestyle such as the frequency of exercise and daily habits influences the microbiota composition (Rinninella et al.,

2019). Third, genetics and the use of drugs such as antibiotics, can impact the diversity of the gut microbiota (Al Bander et al., 2020; Jandhyala, 2015; Rinninella et al., 2019). The frequent intake of antibiotics leads to an imbalance between the Firmicutes and Bacteroidetes (Pérez-Cobas et al., 2013). Moreover, the mode of delivery can affect the balance of the gut microbiota. When the newborn is delivered vaginally, they will be exposed to the vaginal bacteria including Lactobacillus and Prevotella genera (Dominguez-Bello et al., 2010; Jandhyala, 2015; Rinninella et al., 2019). On the other hand, neonates born through caesarean section are affected by the maternal skin flora, leading to a less diverse microbiota, and having a higher portion of skin dwelling microbes such as Staphylococcus, Corynebacterium, and Propionibacterium (Dominguez-Bello et al., 2010; Jandhyala, 2015; Matamoros et al., 2013; Rinninella et al., 2019). Furthermore, the type of feeding early in life is also considered as one of the characteristics impacting the gut microbiota. In formula-fed infants, Enterococcus, Enterobacteria, Bacteroides, Clostridia and other anaerobic Streptococcus dominate the gut niche where as in breast-fed infants, Bifidobacterium and *Lactobacillus* are more common, as breast milk contains different bioactive compounds and nutrients, such as the human milk oligosaccharides (HMO) (Dominguez-Bello et al., 2010; Jandhyala, 2015; Rinninella et al., 2019) (Figure 1.2). So, many variables can affect the characteristics of the gut microbiota. Therefore, there are no specific standard references for its composition.

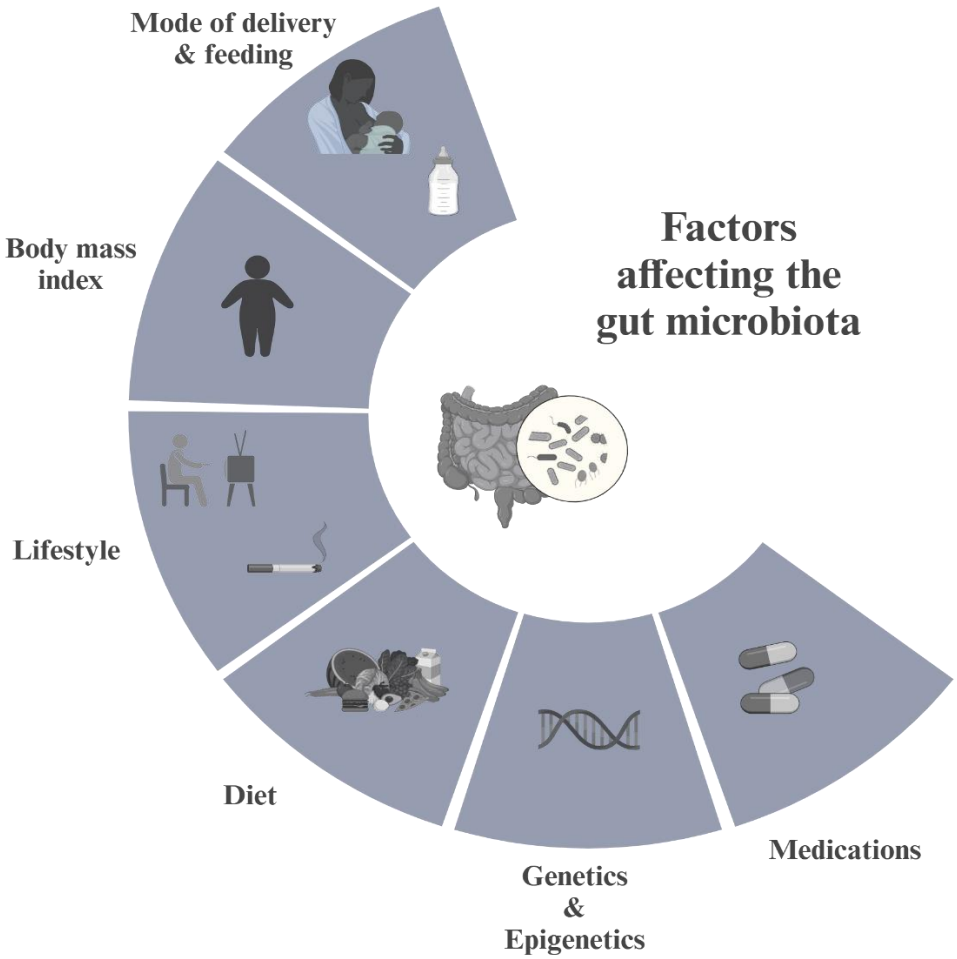

Figure 1.2. Schematic representation of the factors affecting the human gut microbiota composition including mode of delivery and feeding, body mass index, lifestyle, diet, genetics, and intake of medications. Image was created by BioRender.com (Al Bander et al., 2020; Rinninella et al., 2019).

1.2 Functions of the gut microbiota

The gut microbiota maintains a symbiotic relationship with the gut mucosa and provides significant metabolic, immune and protective functions in healthy individuals (Jandhyala, 2015). It utilizes nutrients from the host's diet and possesses a wide range of metabolic and adaptable functional plasticity. Some of the significant characteristics include, digestion of food, strengthening the structural integrity of the gut mucosal barrier, immunomodulation, immune response, and prevention of pathogen invasion (Jandhyala, 2015).

1.2.1 Nutrient metabolism

The gut microbiota promotes essential capacities for the digestion of food such as the breakdown of dietary fibers, carbohydrates, lipids and proteins (Valdes et al., 2018) and the production of metabolites such as short-chain fatty acids (SCFA) and bile acids (Al Bander et al., 2020). The fermentation of carbohydrates leads to the production of SCFA such as propionate, acetate, and butyrate (Heintz-Buschart & Wilmes, 2018; Rinninella et al., 2019). The gut microbiota metabolizes lipids by supressing the inhibition of lipoprotein lipase enzyme activity in adipocytes (Valdes et al., 2018), as well as breaking down proteins into peptides and amino acids. Moreover, the gut microbiota is responsible for the synthesis of vitamins and minerals such as vitamin K, as well as the fermentation of polyphenols, known as polyphenolic secondary metabolites found in plants, fruits and plant-derived products such as flavanols, anthocyanidins and others (Jandhyala, 2015).

1.2.2 Homeostasis of the gut immune system

The largest compartment of the immune system is in the intestine, which frequently encounters a variety of antigens and immunomodulatory components. Therefore, the intestinal epithelial cells and immunity play a major role in maintaining the immune homeostasis and protection against pathogens and infections (Sichetti et al., 2018).

An increasing body of evidence highlighted the role of the gut microbiota in the host's immunity (Takiishi et al., 2017). The initiation of the innate immune response in the intestine is triggered by pathogen-recognition receptors (PRRs) (Santaolalla & Abreu, 2012). These PRRs serve as sensors of pathogen-associated molecular patterns (PAMPs) from the intestinal lumen and damage-associated molecular patterns (DAMPs), known as intracellular molecules produced by the cells in response to any stimulus such as injury or stress (Jandhyala, 2015; Santaolalla & Abreu, 2012).

The most common PRRs are the Toll-like receptors (TLRs), defined as transmembrane proteins that are expressed by intestinal epithelial cells on the cell surface or endosomes (Broad et al., 2007; Santaolalla & Abreu, 2012). Furthermore, PRRs can activate inflammatory responses characterized by nuclear factor-kB (NF-κB), cytokine production and chemokine recruitment of acute inflammatory cells. TLR signaling in the intestine has been shown to promote epithelial cell proliferation (Fukata et al., 2006; L. Shang et al., 2008), immunoglobulin A (IgA) production (Fukata et al., 2009) , maintenance of tight junctions and antimicrobial peptide expression (Cario et al., 2004); critical characteristics for maintain a healthy gut barrier function.

Paneth cells, Goblet cells, and the gut-associated lymphoid tissue (GALT) collectively contribute to the function of the gut immune system (Mowat & Agace, 2014; Santaolalla & Abreu, 2012; Takiishi et al., 2017). Paneth cells are responsible for producing antimicrobial peptides (AMPs) like lysozyme as well as are key regulators of the intestinal microbiota and defective differentiation leads to the absence/decrease in number and decrease in antimicrobial production (Fukata et al., 2009), whereas Goblet cells generate mucus (Santaolalla & Abreu, 2012). Peyer's patches, found in the GALT, contain B cell and T cell zones, serving as the primary site to produce small intestine immunoglobulin A (IgA) plasma-blasts. The mucus layer, antimicrobial peptides, and IgA play pivotal roles in maintaining gut barrier integrity and supporting mucosal immunity.

The gut microbiota is crucial for the development and homeostasis of the immune system (Thursby & Juge, 2017). Gut microbiota improves host immunity by various mechanisms, including competition with pathogens for nutrients and sites, and preventing pathogens' colonization. It also improves signal transduction by PPRs, and production of antimicrobial peptides, IgA, and mucin proteins (Binda et al., 2018; Kamada et al., 2013).

The gut microbiota is one of the major components responsible for maintaining the gastrointestinal barrier integrity (Alam & Neish, 2018). As previously mentioned, these processes are modulated by the PRRs, which include the TLR, NOD-like receptors (NLRs), C-type lectin receptors (CLRs) and some G-protein coupled receptors (GPCRs). Studies conducted on germ-free mice (GF) demonstrated the protective functions of commensal bacteria in the gut, such as the maturation of the immune systems, resistance to pathogenic infections, nutrient absorption and maintenance of intestinal barrier functions (Hooper et al., 2001). Commensal enteric bacteria can affect the epithelial permeability and integrity, particularly the tight junctions repair and maintenance such as Occludin, ZO-1, Claudin 3 and 4 and E-cadherin (Valdes et al., 2018). Moreover, one of the roles of the gut microbiota is the production of metabolites, such as SCFAs, which are capable to increase the number and function of the regulatory T-lymphocytes in the gut (Smith et al., 2013; Valdes et al., 2018) , as well as producing tryptophan derivatives, which bind to aryl hydrocarbon receptors (AhR) and trigger the IL-22 pathway, thus protecting the mucosa from various infections (Zelante et al., 2013). These metabolites are involved in promoting the gut barrier functions and influencing the intestinal immune system activity (Antonini et al., 2019). Gut microbial communities also enhances gut homeostasis by regulating gut microRNAs (miRNAs) profile therefore, improving gut innate and adaptive immune cells balance (Y. Li et al., 2020). Figure 1.3. illustrates role of gut microbiota in gut immunity.

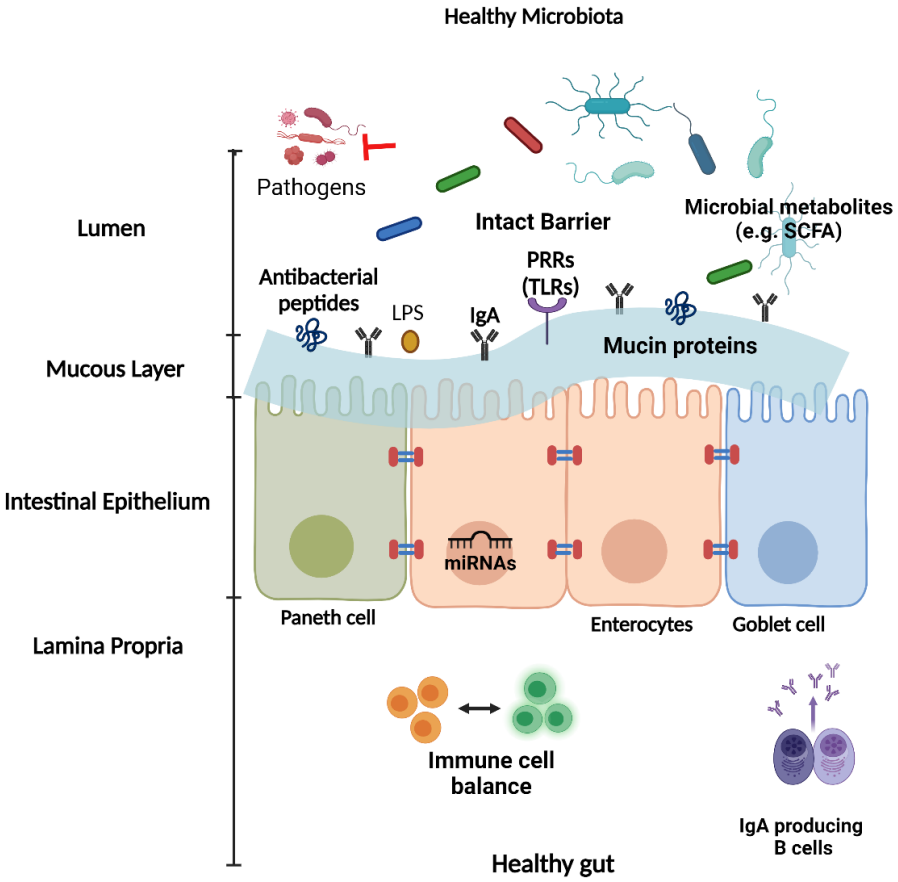

Figure 1.3. Role of gut microbiota in gut immunity. Gut microbiota improves host immunity by various mechanisms, including competition with pathogens for nutrients and sites, and preventing pathogens' colonization. It also improves signal transduction by pathogen-recognition receptors (PPRs), and production of antimicrobial peptides, immunoglobulin A (IgA), mucin proteins, and tight junction proteins, therefore, reinforcing gut barrier integrity. Gut microbial communities also enhance gut homeostasis by regulating gut microRNAs (miRNAs) profile and production of short-chain fatty acids (SCFAs), therefore, improving gut immune cells balance. Image was created by BioRender.com (Antonini et al., 2019; Y. Li et al., 2020; Smith et al., 2013).

1.2.3 Gut microbiota dysbiosis

Gut microbiota dysbiosis is defined as any alteration in the composition or function of the commensal microbial population living in the human's gut relative to the population found in normal gut microbiota in healthy hosts that negatively affects the symbiotic relationship among

microbial population and, therefore, host health (Carding et al., 2015; Hrncir, 2022; Petersen & Round, 2014; Shahbazi et al., 2020). Many factors can contribute to dysbiosis such as lifestyle modifications, diet, physical activity, medications such as to antibiotics, exposure to the toxins, and pathogens (Carding et al., 2015). Gut dysbiosis is associated with certain metabolic and inflammatory disorders such as inflammatory bowel disease (IBD), obesity, metabolic syndrome, diabetes, respiratory disorders and central nervous system disease related disorders (Carding et al., 2015; Petersen & Round, 2014) (Figure 1.4).

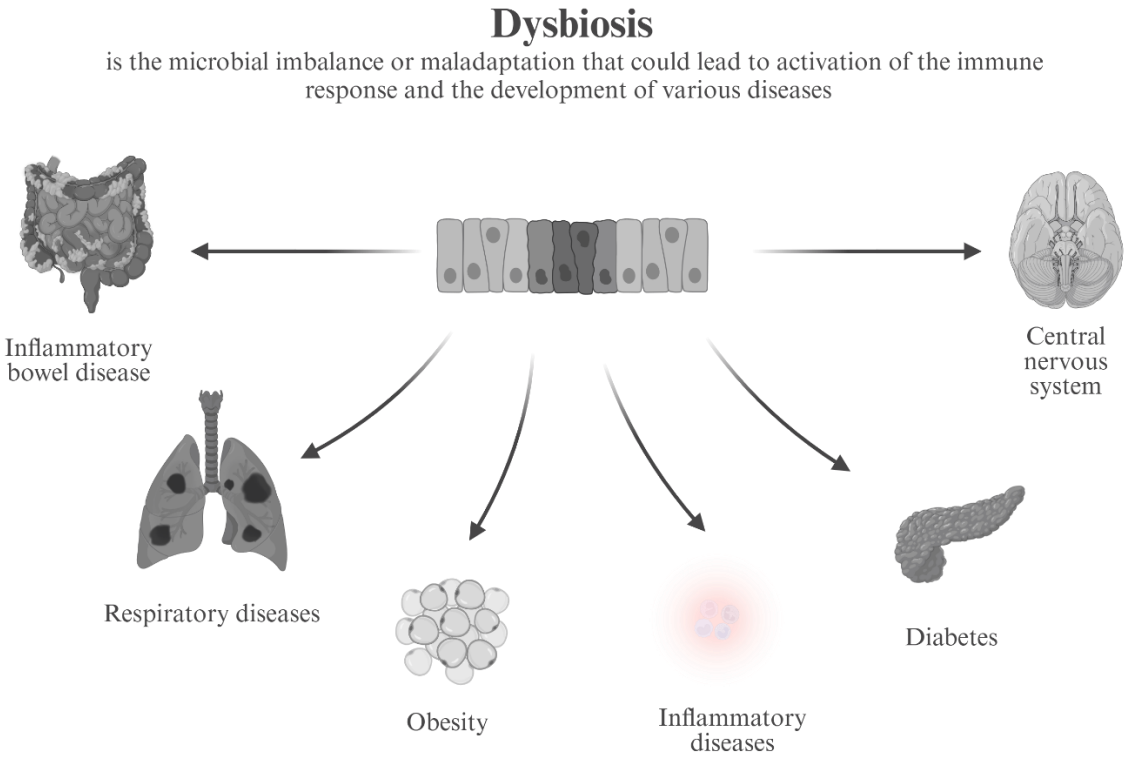

Figure 1.4. Schematic representation of some metabolic and inflammatory disorders associated with dysbiosis. Image was created by BioRender.com. (Thursby & Juge, 2017; Valdes et al., 2018).

1.4 Gut-Lung Axis

The gut microbiota has been characterized as a vital organ forming bidirectional communication or axis with other organs in the body such as the gut-lung axis (Afzaal et al., 2022). The effects of the gut microbiota on the mucosal immunity may affect immune responses at distal mucosal sites, including the lungs (Anand & Mande, 2018); highlighting the concept of the common interconnected mucosal immune system holds that there is an immunological cross talk between the gut and extra-intestinal sites. The gut-lung axis is a powerful mean of communication between the microbiome of the gut and inflammatory/immune environment of the lungs (Wenger et al., 2023). Any alterations in the gut microbiota composition and function, such as dysbiosis or inflammation, is associated with changes in the immune response and may trigger the development of pulmonary diseases or lung infections (Afzaal et al., 2022; Anand & Mande, 2018; Wenger et al., 2023).

The gut and the lungs can communicate through two different mechanisms, through the microbiota and its products or via immune cells. Based on the "gut-lymph" theory , some of the intestinal submucosa or mesenteric lymph nodes contain most translocating bacteria; therefore, surviving bacteria and other cell fragments, produced in the gut, travel through the systemic circulation (Samuelson et al., 2015). To elucidate more, translocated microorganisms are transferred to the mesenteric lymph nodes by antigen presenting cells (APCs) and used for priming naïve B and T cells. In other words, it reduces the host's ability to produce anti-inflammatory molecules leading to dendritic cell migration to local lymph nodes and priming the differentiation of T-cells (Bingula et al., 2017). The molecules can migrate out of the gut-associated lymphatic tissue (GALT) and reach mucosal and peripheral tissues, including bronchial epithelium, altering the immunological response, and improving the immunological response against pulmonary pathogens (Clarke et al.,

2010; Dumas et al., 2018) (Figure 1.5). Consequently, the pulmonary circulation may lead to dendritic cells and macrophages activation as well as priming of T-cells and their differentiation (Bingula et al., 2017).

Studies on murine models showed that dysbiosis in the lungs upon the administration of LPS is accompanied by disturbances in the gut microbiota due to the movement of the bacteria from the lungs into the bloodstream and vice versa (Sze et al., 2014) (Figure 1.6).

The gut-lung bidirectional axis is initiated at the gut level by bioactive components interacting with toll-like receptors (TLR), at the epithelial cell levels. TLRs are the key in maintaining immune homeostasis in the mucosal lymphoid associated tissues, which includes the lung and gut territories. Once TLR4 is stimulated by LPS, it can activate the nuclear factor NF-κB via two major pathways: myeloid differentiation factor (MyD) 88-dependent pathway which induces pro-inflammatory cytokines and MyD88-independent pathway which acts via type 1 interferons to increase the expression of interferon-inducible genes (Broad et al., 2007). TLR4 signaling has been implicated in innate immunity and inflammation following acute lung injury (ALI), intestinal permeability and dysbiosis.

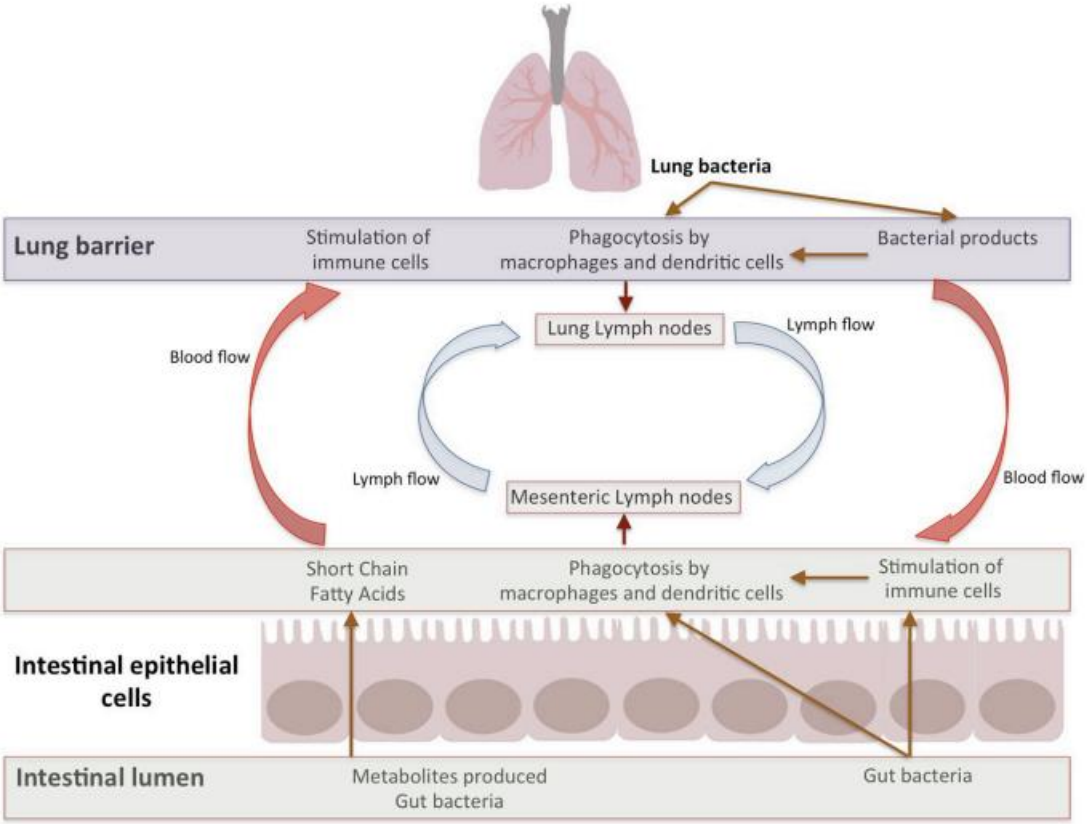

Figure 1.5. Bidirectional Gut-Lung Axis. Metabolites such as short-chain fatty acids (SCFAs), produced by the gut bacteria travel through the bloodstream to trigger immune responses in the lungs and vice versa (factors from the lungs influence the immune response in the gut). Immune cells prompted by numerous antigens move through the lymphatic ducts between organs, modulating the immune responses in both organs (Anand & Mande, 2018).

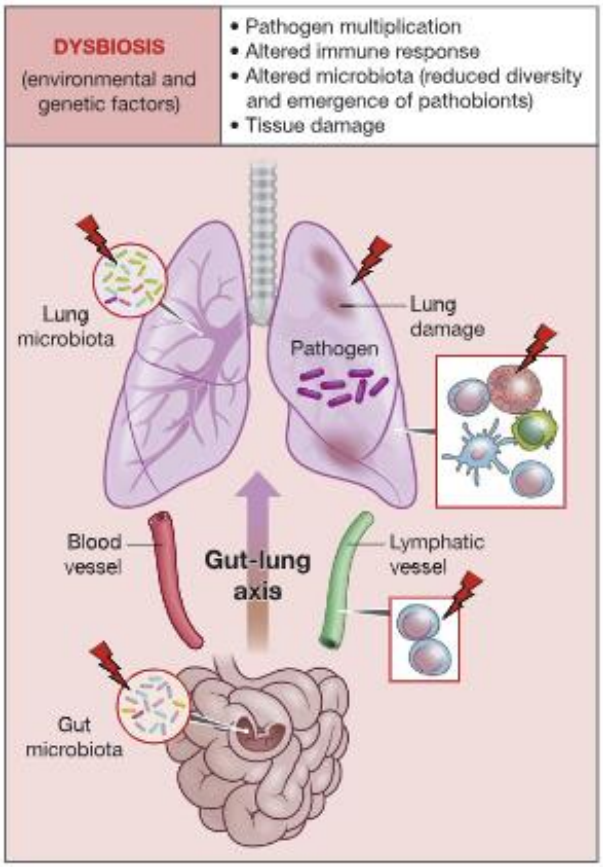

Figure 1.6. Gut-Lung Interaction during Dysbiosis. Dysbiosis can be caused by environmental or genetic factors. A respiratory pathogen causes dysbiosis where commensal bacterial diversity is affected. As a result of dysbiosis, disturbance at the gut-level is induced leading to lung damage (Dumas et al., 2018).

Moreover, immune cells residing in the intestine neutralize the translocating bacteria, which may travel from the lymphatic system through the systemic circulation. Therefore, some of the metabolites or bacterial fragments produced by the gut microbiota may modulate the lung immune response (Bingula et al., 2017; Gross et al., 2015).

In other words, the intestinal epithelium forms a tight barrier between the body's immune system and gut bacteria. Any impairment to the integrity of this epithelium may subsequently increase the risk of inflammation. LPS, which exhibits a pathogen-like behavior, causes destruction in the intestinal epithelial cells. Therefore, when the intestinal barrier is infected, this will affect the

pulmonary microbiota, due to the gut-lung axis as this system is interchangeable. So, dysbiosis of can result in lung disease via gut-lung mechanisms. Any alterations in the gut microbiota induced by respiratory influenza infection promotes Th17 cell production which consequently causes intestinal immune injury (Wang et al., 2014).

1.5 T-helper 17 (Th17)

T-helper (Th) cells or CD4+ cells engage in various immune system functions. They are critical mediators of immune response. Any dysregulation of Th-cell activation and differentiation is associated with inflammatory diseases (J. Huang et al., 2021a). The T-helper subsets are Th1, Th2 and Th17 (Luo et al., 2017; B. Wei & Pei, 2010). Th17, which is necessary for the adaptive immune response against infections, is characterized by the release of IL-17 (Atabaki et al., 2020a). There are six members of IL-17 family including IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F, where IL-17A and IL-17F are best understood (Jin & Dong, 2013; A. Zhang et al., 2017). IL-17A and IL-17F are pro-inflammatory cytokines, with differences depending on the site of inflammation (Bingula et al., 2017; Jin & Dong, 2013). They are crucial factors for the initiation of adaptive immunity by helping other cells and working effectively for an immune response. Th-17 cells are the main culprits behind the pathogenesis of inflammation and autoimmune diseases (Honardoost et al., 2015a; Jin & Dong, 2013). Th17 cells have a level of flexibility that allows them to express different effector cytokines simultaneously, like interferons γ (IFN-γ) or IL-10. This expands the pro-inflammatory impact of Th17 cells or transforms "pathogenic" Th17 cells into "protective" ones (J. Huang et al., 2021a).

Cytokines such as IL-1ß, IL-6, IL-23, and TGF-ß are major cytokines involved in the expression of the retinoic acid receptor-related orphan receptor-gamma-t (RORγt) transcriptional factor, which is the master transcription factor of Th17 cells, leading to the differentiation of naïve T

helper cells to Th17 cells, and production of IL-17A/F, which plays important role in the pathogenesis of inflammatory disease (Atabaki et al., 2020a; Honardoost et al., 2015b; Lee et al., 2020). IL-17 is known for its pro-inflammatory effects by the inducing expression of a variety of cytokines such as IL-6 (Atabaki et al., 2020b; Honardoost et al., 2015a). The exposure to immune stressors such as LPS is associated with production of different cytokines, including those inducing Th17 differentiation (Atabaki et al., 2020b; Honardoost et al., 2015a). Moreover, IL-10 is known to supress and inhibit Th17 cells development as well as immune responses (Huber et al., 2011).

1.6 Lipopolysaccharides

Inflammation occurs in response to a various stimulus, such as bacterial/viral/fungal infections, tissue injury, toxins and cell death, and it is defined as a biological response to the immune system activation (L. Chen et al., 2018; Sichetti et al., 2018). LPS, a cell wall gram-negative constituent, is one of the most potent stimulators for triggering the innate immune response and generates a network of inflammatory responses involving inflammatory cytokines (Matsuura, 2013). It can disrupt the intestinal epithelial barriers by binding to the Toll-like receptor-4 (TLR4) and upregulating all inflammatory parameters at the intestinal levels (Håkansson et al., 2012).

Exposure to immune stressors such as exogeneous lipopolysaccharide (LPS) is accompanied by gut dysbiosis and mediates the production of inflammatory cytokines such as IL-6 and tumor necrosis factor-α (TNF-α) by immune cells (Murray et al., 2019; Yahfoufi et al., 2023). In mice, intraperitoneal injection of LPS has been reported to induce gut permeability through stimulating TLR4 overexpression in the intestinal epithelial cells (Guo et al., 2013). Overexpression of TLR4 is associated with increased gut permeability by down-regulating expression of tight junction proteins (X. Li et al., 2013). In addition, studies have shown that acute exposure to a single dose

of LPS disrupts the airways, leading to the translocation of bacteria into the bloodstream (Sze et al., 2014). Furthermore, LPS-induced inflammation is associated with an increase in proinflammatory cytokine IL-17A, the main cytokine produced by Th17 cells (Xu et al., 2023).

On the other hand, it has been shown that ESMH may promote the proliferation of intestinal cells and inhibited the LPS-induced expression of inflammatory cytokines as well as preventing changes in the gut microbiota (Benson et al., 2012; Jia et al., 2017a). ESMH was capable of reducing the inflammatory responses and the magnitude of IL-6 in epithelial cells by acting as antagonists to TLR4 and TLR2 during the stimulation of LPS; increasing IgA+ cells while orchestrating Th1/Th2 response (Nelson et al., 2007a). Figure 1.7 illustrates effect of ESMH and LPS on gut immunity.

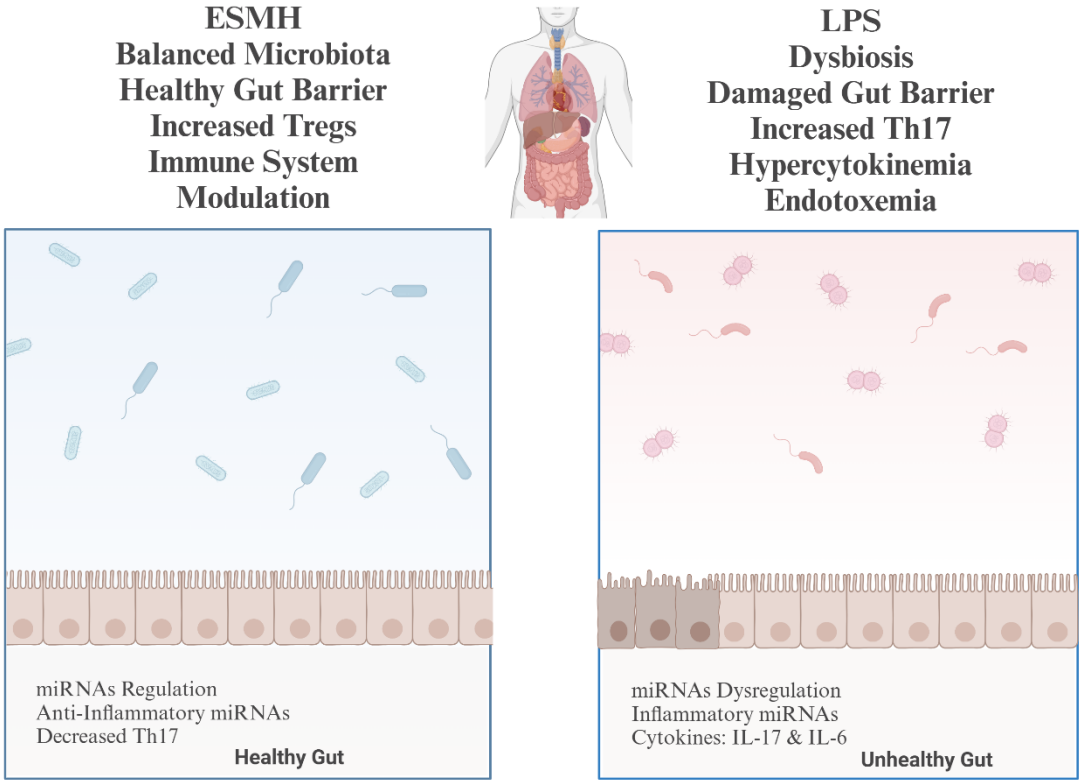

Figure 1.7. Schematic representation comparing the effect of eggshell membrane hydrolysate (ESMH) and lipopolysaccharide (LPS) on the intestinal immunity. Image was created by BioRender.com. (Nelson et al., 2007b; Yahfoufi et al., 2023).

Epigenetic mechanisms and immunity

Nowadays, the role of epigenetic determinants has been extensively studied and recognized. Epigenetics is the study of heritable changes in the gene activity or function without altering the underlying DNA sequence. Epigenetic modifications involves chemical modifications of the DNA sequence or its associated proteins, which can be influenced by external cues such as inflammation and can be transmitted from one generation to another (Gonzalez-Jaramillo et al., 2019).

Epigenetic modifications can be directed by environmental factors, such as diet and stress (L. Liu, 2008; Shepard & Nugent, 2020). For example, it has been shown that early life nutrition can epigenetically modulated the expression of genes regulating metabolic and immune pathways, therefore, affecting the susceptibility to chronic diseases later in life (Masuyama et al., 2015; Masuyama & Hiramatsu, 2012; Nash et al., 2023).The molecular basis of epigenetic is complex and constitutes DNA methylation, histone modification, and gene regulation by noncoding RNAs such as miRNAs (Feinberg, 2008) .

1.7.1 MicroRNAs

MicroRNAs are endogenously encoded single stranded RNAs of about 22 nucleotides. miRNAs play important roles in the regulation of gene expression for cellular differentiation, homeostasis and immune regulation ; they are vital anti-viral immunity in orchestrating immune responses (B. Wei & Pei, 2010). miRNAs shape immunity by modulating the repertoire of genes expressed in immune cells , the magnitude and the duration of responses to a particular pathogen (Baltimore et al., 2008; Sato et al., 2016). Most of the miRNAs originate within the genes, intragenic, mostly from introns and the others from exons of protein-coding genes; the rest are transcribed independently and regulated by their own promoters (R. Shang et al., 2023). miRNA genes are

transcribed by RNA polymerase II (Pol II) to produce primary transcripts (pri-miRNAs). These pri-miRNAs are initially processed by the Drosha-DiGeorge syndrome critical region gene 8 (DGCR8) complex, also known as the Microprocessor complex, generating pre-miRNAs of approximately 70 nucleotides in length (Iorio & Croce, 2012). Pre-miRNAs have a short stem, which is recognized by the nuclear export factor exportin 5. After being exported from the nucleus, the cytoplasmic RNase III Dicer catalyzes the production of miRNA duplexes. Dicer, along with TAR RNA-binding protein (TRBP; also known as TARBP2) and Argonaute (AGO) 1–4, mediates the processing of pre-miRNA and the assembly of the RNA-induced silencing complex (RISC) (Carthew & Sontheimer, 2009). Within the RISC complex, one strand of the miRNA duplex is removed, resulting in a single-stranded miRNA that remains in the complex and is partially complementary to the target mRNA (Cappelletti et al., 2015). Interaction of the miRNA complex with mRNA induces posttranscriptional silencing through both mRNA destabilization and translational repression (S.-Y. Wei et al., 2021) (Figure 1.8).

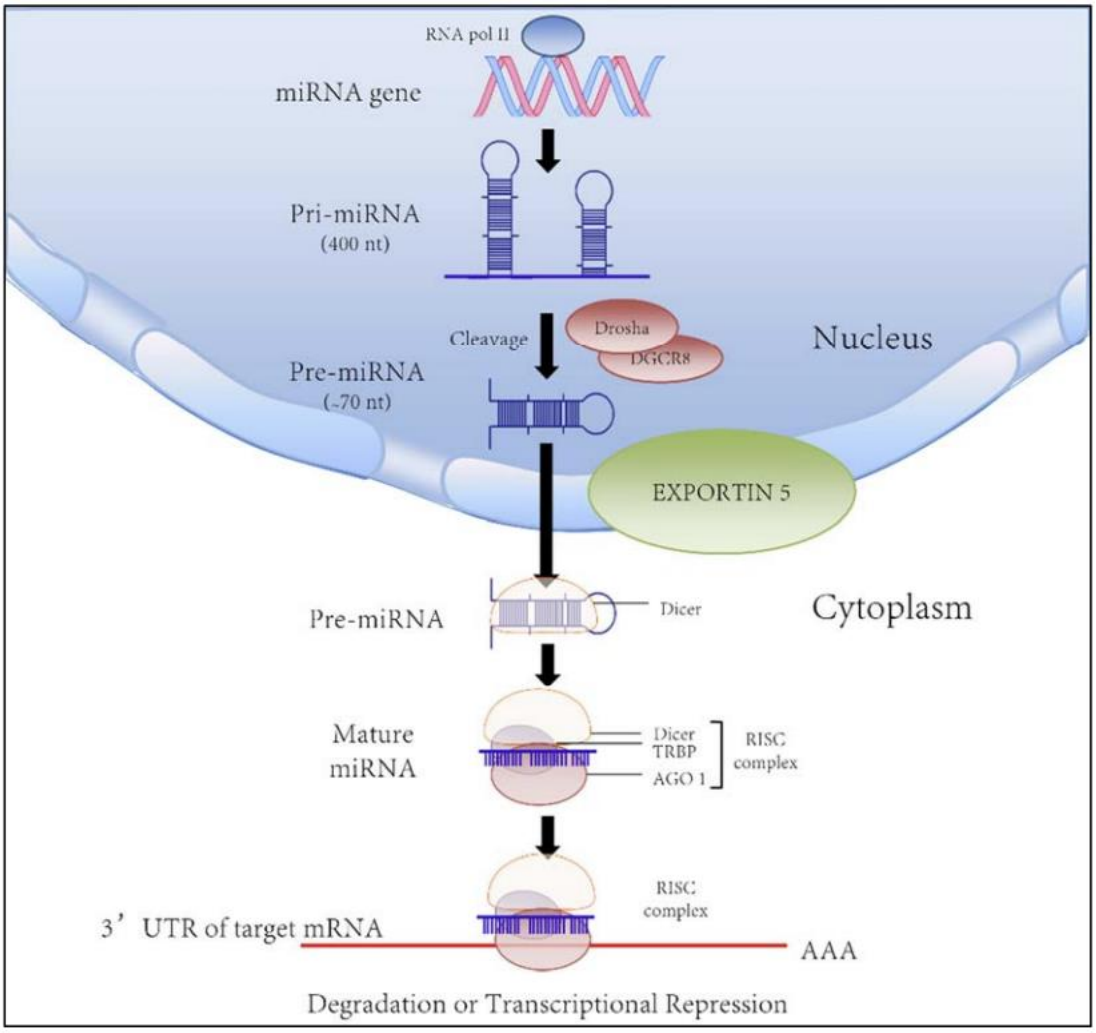

Figure 1.8. microRNA biogenesis. miRNA genes are transcribed by RNA polymerase II in the nucleus, forming primary miRNAs (pri-miRNAs). These pri-miRNAs are then processed by DROSHA and Drsoha-DiGeorge syndrome critical region gene 8 (DGCR8) into pre-miRNAs. Subsequently, the pre-miRNAs are transported to the cytoplasm by exportin 5. In the cytoplasm, DICER1 further modifies the pre-miRNAs into mature miRNAs. The miRNAs are incorporated into the miRNA-induced silencing complex (miRISC), which includes DICER1 and argonaute (AGO) proteins. This complex then targets specific mRNAs based on sequence complementarity, leading to gene suppression (Pisarello et al., 2015).

1.7.2 The Role of miRNA in Th17

miRNAs can positively or negatively regulate the Th17 differentiation. The four main pathways that affect the Th17 differentiation are: Transforming growth factor-β (TGF-β), which is critical in

the differentiation of Th17 cells, Janus kinase-signal transducer and activator of transcription 3 (JAK-STAT3), Nuclear factor kappa-B (NF-κB), and Phosphoinositide 3-kianse protein kinase B-mammalian target of rapamycin (PI3K-AKT-mTOR). Cytokines IL-6, IL-21 and IL-23 positively regulate the Th17 differentiation through JAK-STAT3 signaling pathway. NF-κB activation is important for Th17 cell activation/differentiation by regulating RORγt which is an important transcriptional regulator of Th17 cell differentiation (J. Huang et al., 2021b; Jin & Dong, 2013).

Therefore, the cluster of miRNAs studied in current research are directly or indirectly related to Th17. miRNAs used for both intestine and lungs are the following: miR-155 which is required for cell differentiation, germinal center B cell responses, and response to bacterial/viral infections; it plays a vital role in the stability of Th17 phenotype (B. Wei & Pei, 2010; A. Zhang et al., 2017) as well as inhibits some signaling pathways for the promotion of Th17 differentiation (J. Huang et al., 2021b). miR-146a is a negative regulator of NF-κB signaling in response to a bacterial infection (Casciaro et al., n.d.; B. Wei & Pei, 2010) as well as targets the tumor necrosis factor receptor-associated factor 6 (TRAF6) and interleukin 1 receptor associated kinase 1 (IRAK1) which regulate the NF-κB signaling in T-helper cells, blocks the NF-κB-mediated secretion of IL-6/IL-21, and inhibits the development of pathogenic Th17cells (J. Huang et al., 2021b). miR-223 has a pro-inflammatory role involved in modulating the immune responses by activating and promoting the pathological Th17 cells differentiation during autoimmunity (Casciaro et al., n.d.; S. Yuan et al., 2021). miR-222 forms an integral part of the intestinal Th17 response (Mikami et al., 2021a).

1.7.3 DNA methylation

DNA methylation is an epigenetic mechanism which includes the transfer of a methyl group (CH_3) onto the carbon 5 position of the nucleotide cytosine to produce 5-methylcystosine. (Feinberg,

2008). This process regulated gene expression by recruiting proteins involved either in the gene repression or inhibiting the binding transcription factors to DNA (Moore et al., 2013). DNA methylation is catalyzed by a family of DNA methyltransferases (DNMTS) that transfer a methyl group from S-adenosyl methionine (SAM) the cytosine residue on cytosine-phosphate-guanosine (CpG) islands. The majority of the gene promoters reside within the CpG islands, particularly the promoters for housekeeping genes (Illingworth et al., 2010). During replication, DNMT1 and DNMT2 are accountable for DNA re-methylation (Kaiser et al., 2017; Rountree et al., 2000), where as DNMT3A and DNMT3B initiate new methyl groups addition to unmethylated DNA (Bachman et al., 2001). Moreover, DNA demethylation can be modified by Ten-eleven translocation methyl cytosine dioxygenase (TET) proteins which convert methyl to hydroxymethylcytosines (Y. Huang et al., 2014). DNA methylation is crucial for silencing retroviral elements, regulating tissue-specific gene expression, genomic imprinting and X-chromosome inactivation (Moore et al., 2013).

The underlying mechanisms of DNA methylation involves three classes of enzymes: writers, erasers, and readers (Illingworth et al., 2010; Moore et al., 2013).

The writer's category involves the methyltransferase enzymes (DNMTs), as previously discussed. DNMT1 plays a pivotal role in maintaining the DNA methylation sequence; it replicates the parental DNA strand onto the newly synthesized daughter strand (Jeltsch & Jurkowska, 2014; Moore et al., 2013). On the other hand, DNMT3A and DNMT3B can establish new methylation sequences to unmodified DNA and are known as *de novo* DNMT (Jeltsch & Jurkowska, 2014); the specific targeting characteristics of DNMT3A and DNMT3B has not been fully understood.

The eraser's classification includes the DNA demethylation, characterized by passive or active. In dividing cells, passive DNA demethylation occurs when DNMT1 is inhibited, leading to the

maintenance of unmethylated cytosine in newly synthesized DNA strands after cell division. Active demethylation occurs in both, dividing and nondividing cells; however, the process requires enzymatic reactions to convert 5-methylcytosine (5mC) back to cytosine (Mahadevan et al., 1998). Various mechanisms have been processed for active DNA methylation, may involve deamination or oxidative reactions that produces intermediates known by the base excision repair (BER) pathway. It may include activation-induced cytidine deaminase/apolipoprotein B mRNA-editing enzyme complex (AID/APOBEC) enzymes, Ten-eleven translocation (Tet) enzymes, thymine DNA glycosylase (TDG) and single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1) (Figure 1.9) (Ito et al., 2010; Rai et al., 2008; Tahiliani et al., 2009).

The reader DNA methylation process includes the repression of the DNA transcription facilitated by various protein such as the Methyl-binding proteins (MBD), and Ubiquitin Like With PHD And Ring Finger (UHRF) and zinc-finger proteins (Moore et al., 2013). The MBD proteins include a conserved methyl-CpG-binding domain that confers a higher affinity for single methylated CpG sites, where the sites generally lead to the suppression of transcription in the respective DNA regions (Nan et al., 1993). The UHFR1 and UHFR2 are multidomain proteins that bind to cytosines through a SET and RING-associated DNA-binding domains. The role of UHFR extends beyond the transcription suppression. They participate in the maintenance of DNA methylation, facilitating the recruitment of DNAMT1 to hemi-methylated DNA, preserving the methylation status (Bostick et al., 2007; Moore et al., 2013). The last family of methyl-binding proteins binds to methylated DNA by a zinc-finger domain and is composed of Kaiso, ZBTB4 and ZBTB38 (Filion et al., 2006). ZBTB4 and ZBTB38 can bind to a single methylated CpG, while Kaiso specifically binds to two consecutives methylated CpG sites. Similarly to MBD proteins, the zinc-finger proteins also play

a role in supressing DNA transcription, contributing to the regulation of gene expression (Filion et al., 2006; Moore et al., 2013).

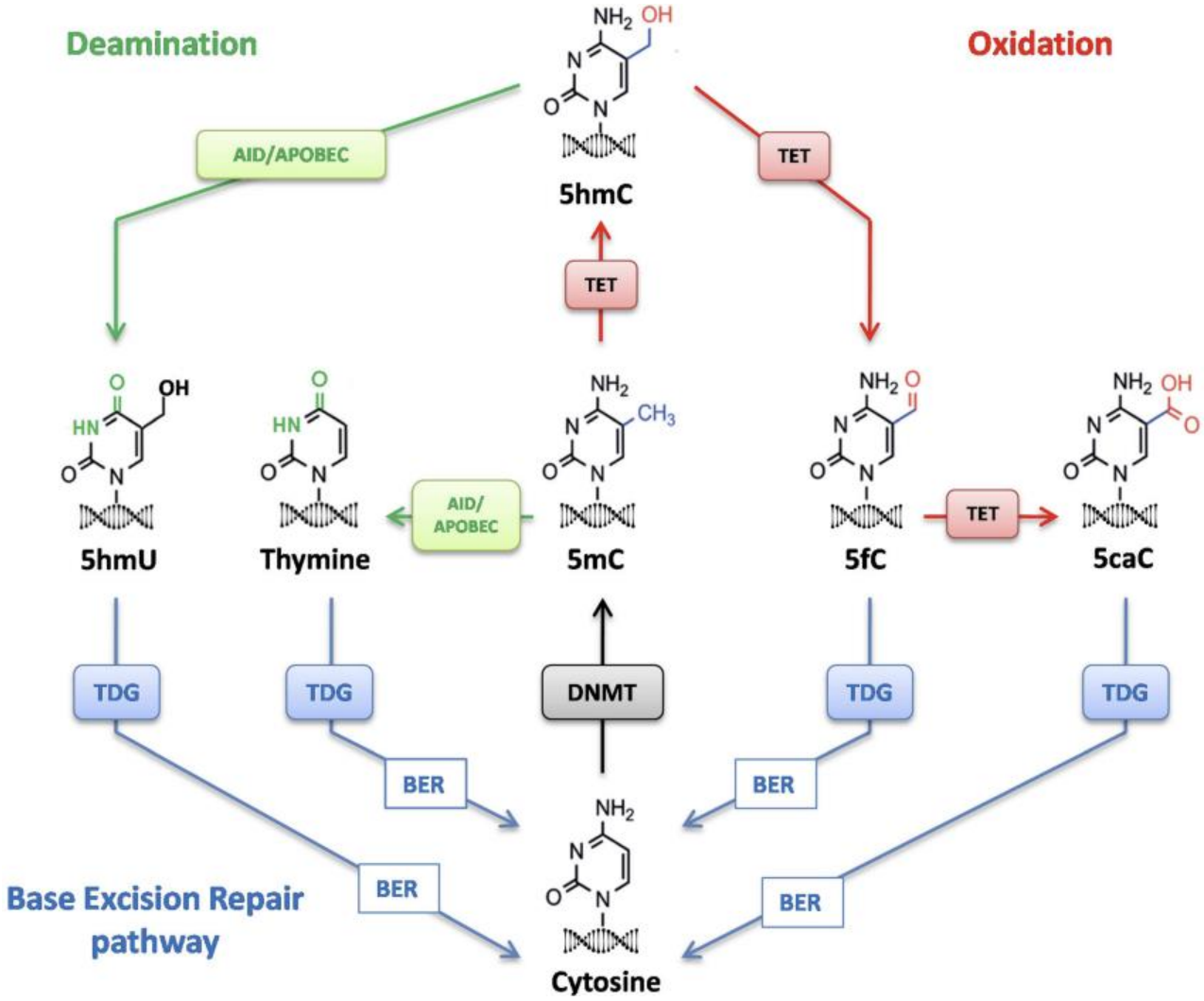

Figure 1.9. The metabolism of DNA methylation. This process involves a series of enzymatic steps that alter the cytosine bases within the DNA. The initial step is the addition of a methyl group to an unmodified cytosine, catalyzed by DNA methyltransferase (DNMTs) (shown by the black arrows). Once cytosine is methylated to 5mC, it can undergo further modifications/alterations. Ten-eleven translocation methyl cytosine dioxygenase (TET) enzymes oxidize 5-methyl cytosine (5mC) to 5-hyroxymethyl cytosine (5hmC). This oxidation process can proceed, converting 5hmC to 5-formylcytosine and then to 5-carboxylcytosine (5caC) (as indicated by the red arrow). The deamination pathway (shown by the green arrows) includes the activation-induced cytidine deaminase/apolipoprotein B mRNA-editing enzyme complex (AID or APOBEC), which

deaminate 5hmC to 5-hydroxymethyluracil (5hmU) or 5mC to thymine, creating additional cytosines. These modified bases are recognized and processed by the enzyme thymine DNA glycosylase (TDG). It is crucial in the base excision repair (BER) pathway (depicted by blue arrows). Via BER, the modified bases are eventually converted back to unmodified cytosine, completing the cycle of DNA methylation and demethylation. This complex process allows for the dynamic regulation of gene expression through epigenetic modifications (Celarain & Tomas-Roig, 2020).

1.7.4 DNA methylation in immunity

Changes in methylation levels at specific gene locations such as *Tbet*, *Il4*, *interferon γ* (*Ifn γ*), and *Forkhead box P3* (*Foxp3*) are crucial for directing CD4+ T cells towards different phenotypes like Th1, Th2, Th17, or Tregs (Floess et al., 2007; Lee et al., 2020). Similar modifications in certain promoter regions regulate the differentiation and functions of CD8+ T cells, as well as genes involved in B-cell proliferation, migration, and NF-κB signaling, where DNA methylation inversely correlates with gene expression (Raghuraman et al., 2016).

Dietary factors contribute to epigenetic modifications in the host. For example, dietary fibers fermentation by gut microbiota leads to the generation of various metabolites that function as substrates or cofactor for enzymes regulating epigenetic processes (D. Li et al., 2022). Furthermore, gut microbiota controls epigenetic mechanisms by producing SCFAs, which contributes to histone modification, and vitamins such as folic acid, which serves as methyl group donors for DNA methylation and histone methylation (Furusawa et al., 2013; D. Li et al., 2022).

1.7.5 Epigenetic crosstalk

DNA methylation works along with histone modification and microRNA (miRNA) to regulate transcription (Han et al., 2007). Histones undergo chemical modifications, such as methylation and acetylation, which influences gene expression. DNMTS interact with enzymes that regulate histone modifications, to impose a repressive state on the gene regions (Y. Zhang et al., 2010). Conversely, histone modifications can influence DNA methylation patterns. Methyl-binding

proteins (MBDs) such as UHRF proteins link DNA methylation with histone modification, enhancing gene repression (Moore et al., 2013). miRNAs, which regulate gene expression by binding to target mRNAs, are also influenced by DNA methylation (Berezikov, 2011). The interaction between miRNAs and DNA methylation is bidirectional, as miRNAs can regulate DNA methylation and histone modifications. Overall, DNA methylation, histone modifications, and miRNAs cooperate to influence the gene expression (Figure 1.10).

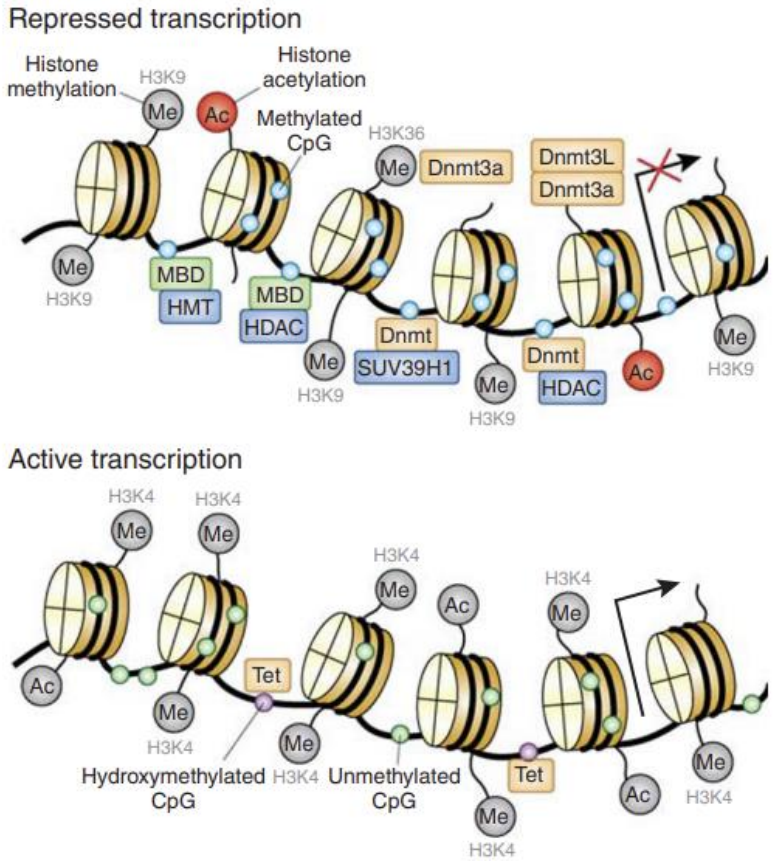

Figure 1.10. Epigenetic crosstalk. Transcription is regulated by the interaction of several epigenetic mechanisms that activate or silence the gene expression. Methylation, regulated by proteins like DNA methyltransferases (DNMT) and ten-eleven translocation (Tet) (purple), involves adding or modifying DNA methylation (hydroxy methylation in red). DNA methyltransferases (DNMTS) methylate DNA at the cytosine-phosphate-guanosine (CpG) sites to suppress gene expression, sometimes aided by histone tails and DNMT3L. Methyl-binding proteins such as MBDs (yellow), along with DNMTS, recognize DNA methylation and recruit enzymes like histone deacetylases (HDACs), which removes the acetylation (red) and HMTs

which methylates histone (green), which further repress gene expression by modifying histone tails (orange). In actively transcribed DNA regions, ten-eleven translocation (Tet) removes DNA methylation, and histone modifications like H3K4me3, prevent DNMT binding, allowing transcription (Moore et al., 2013).

1.8 Gut microbiota, immune system, puberty

Puberty, is a crucial phase of development with significant changes in hormonal, metabolic, and immune system status (Resztak et al., 2023). In addition, significant alteration in gut microbiota during puberty happens, characterized by an increase in some microbial families, including Bifidobacteria and Clostridia, with lower microbial diversity than adults' microbiota (Agans et al., 2011; Novakovic et al., 2020). As puberty advances, an increase in Firmicutes population and a decrease in Bacteroidetes population happens resembling the gut microbiota composition during adulthood (Calcaterra et al., 2022).

Puberty is a critical stage of development during which presence of immune stressors can lead to enduring health problems (Sharma et al., 2019). For example, in pubertal mice, exposure to an immune stressor such as LPS during puberty altered peripheral and central immunity programming, and was associated with a lasting effect on brain function during adulthood (Sharma et al., 2019; Yahfoufi et al., 2023). LPS exposure was associated with gut microbiota dysbiosis in pubertal mice which may suggest that lasting changes in the immune system and brain function induced by pubertal immune stressor may be mediated by gut microbiota disturbance, highlighting the importance of gut-brain axis (Sharma et al., 2019). However, dietary intervention during

puberty alleviated gut microbial perturbation and enduring LPS-induced behavioral changes in adult mice (Sharma et al., 2019).

1.9 Eggshell membrane hydrolysate (ESMH)

A growing body of evidence has shown the health promoting effects of eggshell membrane hydrolysate (ESMH). Protein hydrolysates and peptides derived from eggshell membrane will likely exhibit an immunomodulatory action affecting the function of immune cells that migrate or release effectors beyond the gut and into the lung (Cesta, 2006; Nelson et al., 2007a). Eggs are an important part of the human diet, as they provide many essential nutrients, including essential amino acids. Eggshell membrane (EGM) is a natural, low-cost, food-source ingredient obtained from the membrane of the chicken eggshell. It is a bioactive material composed of different networks of complex carbohydrates including glycosaminoglycans (GAGS) which act as either pro or anti-inflammatory, collagen (type I, V and X) along with other glycoproteins such as fibronectin, which activates/binds proteins, egg white proteins such as ovotransferrin and lysozyme, and eggshell matrix proteins such as Ovocalyxin-36, osteopontin which contains various binding sites for cells, calcium and serine/threonine (Shi, Zhou, et al., 2021) and hyaluronic acid (specifically in the matrix) (Benson et al., 2012; Vuong et al., 2017).

Several studies have shown that peptides derived from ovocalyxin-36 play a role in reducing LPS-induced inflammatory symptoms and inhibiting the production of pro-inflammatory mediators in the small intestine (Kovacs-Nolan et al., 2014). Moreover, eggshell membrane can be separated from the eggshell through mechanical, chemical and enzymatic treatment (Shi et al., 2021). Enzymatic hydrolysis is a common procedure used to reduce macromolecules in byproducts, therefore making it easier for extraction and separation; proteins are tightly bound to the eggshell, therefore proteases and other enzymes such as pepsin, trypsin and bromelain have been used for

extraction and separation (Tsopmo et al., 2019). The product would be protein hydrolysates and peptides. One study indicated that pepsin has been successfully used to solubilize collagen from eggshell membrane, due to proteolysis (Mohammadi et al., 2016). The bioactive components of eggshell enhance the overall well-being, nutrient absorption/bioavailability and exert emulsifying, coagulating, antimicrobial, antioxidant, antihypertensive and immunomodulatory properties (Bhat et al., 2015; Pasarin et al., 2023; Tsopmo et al., 2019). One study showed that fermented eggshell scavenged free radicals and reduced ferric ions; this might be caused by the electron-donating amino acid residues, such as tyrosine and histidine, composition of ESMH and their small molecular size which increases their accessibility to the radicals (Jain & Anal, 2017). This highlights the mineral binding and antioxidant properties of eggshell hydrolysate. Lysozymes are antimicrobial proteins found in eggshell membrane. They can damage the bacterial cell wall by attacking the peptidoglycan in the cell wall; mainly gram-positive bacteria such as *Clostridium tyrobutyricum* (Tsopmo et al., 2019). Ovotransferrin can deprive microbes of iron essential for their multiplication (Makkar et al., 2015). The presence of tryptophan and arginine can increase the antimicrobial benefits of peptides, since tryptophan initiates the folding of peptides in aqueous solutions, whereas arginine exerts positive charges on peptides that can form hydrogen bonds with anionic components of bacterial cell wall (Chan et al., 2006). Additionally, egg phosvitins can disrupt bacterial cell wall and cause DNA leakage in gram-positive bacteria, *Escherichia coli,* due to its metal-chelating and high surface activity (Tsopmo et al., 2019). These hydrolysates can be used to enhance functional properties such as solubility, foaming and emulsifying properties (Jain & Anal, 2016).

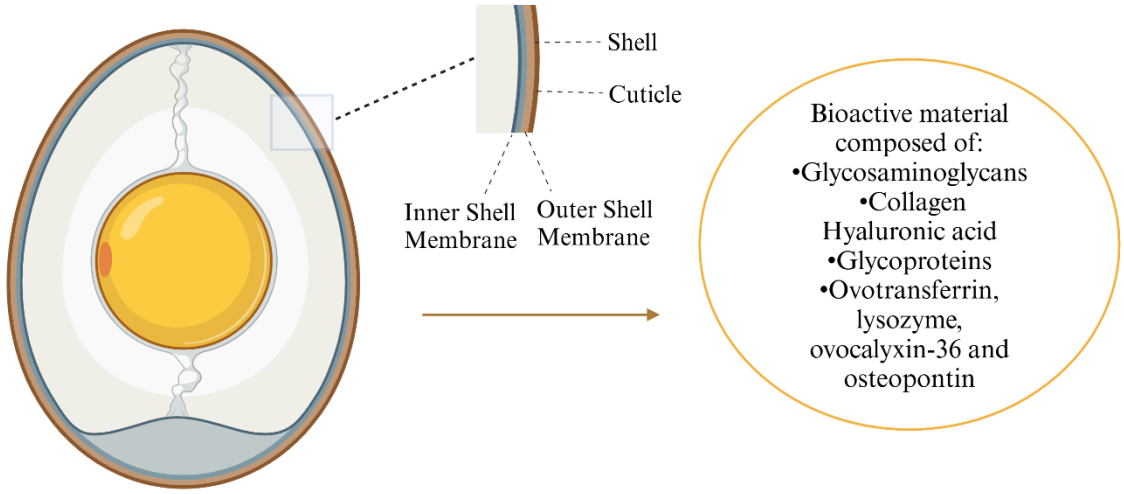

Figure 1.11. Schematic representation of the components of eggshell membrane. Image created by BioRender.com (Benson et al., 2012; Mohammadi et al., 2016; Shi et al., 2021).

Eggshell powder is useful in many applications such as the preparation of bioactive materials containing calcium and phosphate; specifically it can be used for cosmetics, wrinkles, skin burns, and wound/bone healing (Pasarin et al., 2023; Shi et al., 2014; Tsopmo et al., 2019).

Following digestion, bioactive peptides can be either absorbed through the intestine to enter the blood circulation intact and exert systemic benefits or produce promising roles/effects in the gastrointestinal tract (Moreno-Fernández et al., 2020). ESMH is a source of non-digestible protein which may have some physiological functions and characteristics like that of the dietary fiber including the stimulation of cecal fermentation and alteration of the intestinal bacterial composition (Jia et al., 2017a). For example, continuous studies are showing that milk-derived bioactive peptides are signaling via TLR4, and casein hydrolysates inhibited LPS-induced cytokine synthesis (Marcone et al., 2017).

Furthermore, ESMH has been shown to enhance gut immunity by beneficially affecting the gut microbial communities, preventing dysbiosis, maintaining the gut epithelial barriers, reducing inflammation, inhibiting the growth of pathogens, and modulating innate/adaptive immune responses (Jia et al., 2014, 2017b). It may regulate Th17 cells expansion by inhibiting the overgrowth of some filamentous bacteria (Shi, Zhou, et al., 2021).

In a study, ESMH inhibited inflammatory cytokine production induced by lipopolysaccharide (LPS); these effects were related to the significant improvements in gene expressions of inflammatory mediators, intestinal epithelial cell proliferation and antimicrobial peptides (Jung et al., 2017).

Other studies highlighted the protective role of ESMH in reducing TNF- α induced IL-8 secretion; IL-8 was significantly decreased with the treatment of ESMH indicating the beneficial effect of ESMH in alleviating inflammation (Shi et al., 2014). Additionally, the reduction of TNF- α-associated expression of IL-8 cytokine has been associated with the inhibition of NF-kB activation, which has been found to be correlated with anti-inflammatory activity in mice (Ruff & DeVore, 2014). TNF- α and IL-6 act as critical pro-inflammatory cytokine and are recognized as inflammatory mediators in IBD. TNF- α is involved in the improvement of IL-1β and IL-6; treatment with ESMH inhibited the concentration of IL-6, which induces STAT-3 and NF-kB activation (Mudter & Neurath, 2007).So, ESMH might reduce inflammation in colitis-mice models via IL-6 mediated pathway and promotes T-cells apoptosis to restore immune homeostasis in the gut (Shi et al., 2014).

1.10 Rationale, hypothesis, and objectives of the study

In early life, there are critical windows of development directly after birth or at puberty where immune and non-immune cells can acquire characteristics of inflammatory phenotype if a stressor is present and not corrected. As stated earlier, scientific evidence emphasizes puberty as an immune programming window during which immune stressors and dietary factors are among major determinants of health and diseases condition of individuals later in life (Sharma et al., 2019). For example, evidence shows that following a high-fat dietary pattern during puberty correlates with developing an obese phenotype and metabolic disorders in adulthood (Ferreira-Junior et al., 2023). Overall, this evidence highlights the importance of proper timing of interventional strategies to improve health conditions later in life.

Whether gut microbiota-directed interventions will mitigate immune dysfunction induced by pubertal inflammation by regulating epigenetic mechanisms directing immune responses at the gut-lung axis, is an important question not explored sufficiently. Therefore, we hypothesized that LPS-induced inflammation during puberty induces dysbiosis and causes acute and enduring alterations in cytokines, miRNAs profile and DNA methylation of genes related to the Th17 cell mediated immunity at the gut and lung levels, while ESMH intake can mitigate effects of pubertal LPS on gut microbiota perturbation and acute/enduring epigenetic alterations. The overarching aim of this study is to test the effectiveness of ESMH intervention in preventing inflammation and dysbiosis in the gut-lung axis. Specific objectives of current projects are:

1. To investigate the role of ESMH in mitigating gut microbiota dysbiosis induced by the acute exposure to the LPS during puberty
2. To study the role of ESMH in mitigating the acute and lasting effects of pubertal exposure to the LPS on cytokines and epigenetics alterations, including miRNAs related to Th17

mediated immunity at the gut/lung levels. Furthermore, to examine the DNA methylation of the genes in the lungs, related to the Th17, in the enduring phase

Chapter 2: Immunomodulatory Role of Protein Hydrolysates in the Gut-Lung Axis: Involvement of Epigenetic Mechanisms

Immunomodulatory Role of Protein Hydrolysates in the Gut-Lung Axis: Involvement of Epigenetic Mechanisms

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2.1 Abstract

Maintaining gut immune homeostasis is crucial to the overall health. Any disturbances in gut immunity can lead to immune dysfunction in distant organs. Puberty represents a crucial period of development where disturbances in immune homeostasis can lead to long-term immune dysregulation in organs distant from the gut. The gut-lung axis is part of a common immune network where immune cells can migrate from one site to another. Induced dysbiosis and inflammation during puberty is often accompanied by immune deregulation. However, their effect on gut-lung axis have not been sufficiently explored. Natural products, such as protein hydrolysates, derived from eggshell membrane (ESMH) has been shown to prevent dysbiosis and reduce inflammation. In the current study, we studied the effect of pubertal exposure to LPS on gut microbiota, cytokines, as well as acute and enduring epigenetic alterations at the gut and lung levels. The role of ESMH intake in mitigating LPS-induced dysbiosis as well as acute and/or lasting changes in cytokines, miRNAs and DNA methylation of genes related to the Th17 mediated immunity at the gut-lung axis was also explored. Our results revealed that the administration of ESMH partially prevented LPS-induced changes in the gut microbiota by reducing the levels of *Alistipes* and *Candidatus arthromitus and* increasing the abundance of *Lactobacillus*. Moreover,

an acute effect of LPS increased IL-6 and IL-10 levels in the gut, while ESMH intake was associated with an increase in IL-6 and IL-10 levels in the lungs. We also observed a lasting deregulation in miR-222, miR-223, and miR-155 at the lung levels in mice exposed to pubertal inflammation. However, dietary interventions mitigated the effect of LPS on these miRNAs. A DNA methylation analysis, in the lung tissues, demonstrated an enduring change in the methylation status of several genes related to Th17 mediated immunity, including *Tnfaip3, Tnip1, Lcn2, Sox2ot, Ermap, Cxcl11, Ror*α, *Mcc, Prkcb, Socs7* and *Nlrc4.* These findings may demonstrate the effectiveness of ESMH, in improving the gut microbiota composition and gut/lung immunity in mice by regulating epigenetic mechanisms. ESMH may be considered a potential nutraceutical alternative due to its immune-protective effects in the gut and the lungs, through mechanisms related to the gut microbiome modulation the gut microbiome and epigenetic regulations in the lungs.

Key words: Eggshell Membrane (ESMH); miRNAs; DNA methylation; Gut-lung axis; Gut immunity; Gut microbiota; LPS.

2.2 Introduction

The largest compartment of the immune system is in the intestine, which frequently encounters a variety of antigens and immunomodulatory components (Shahbazi et al., 2020). Therefore, the intestinal epithelial cells and immunity play a major role in maintaining the gut homeostasis and protection against pathogens and infections (Mowat & Agace, 2014; Santaolalla & Abreu, 2012). Any alterations to the composition of the commensal population in the gut, that negatively affects the symbiotic relationship among microbial communities, is known as dysbiosis (Petersen & Round, 2014).

Furthermore, disturbances affecting the gut microbiota are associated with respiratory disorders in the lungs, hence, creating a bidirectional cross-talk between the gut and the lungs (Anand & Mande, 2018) . The gut microbiota is central on the mucosal immunity may affect immune responses at distal mucosal sites, including the lungs (McDermott & Bienenstock, 1979); highlighting the concept of the common interconnected mucosal immune system holds that there is an immunological cross talk between the gut and extra-intestinal sites. The immune cells residing in the intestine neutralize most of the translocating bacteria, which may travel from the lymphatic system through the systemic circulation. Therefore, some of the metabolites or bacterial fragments produced by the gut microbiota may modulate the lung immune response (Bingula et al., 2017; Gross et al., 2015). The gut microbiota communicates with the lungs via soluble microbial components and bacterial metabolites, which reach the systemic circulation, and through the migration of immune cells from the intestine to the respiratory tract (Eladham et al., 2024). This bidirectional communication emphasizes that the lung bacterial population/metabolites can also enter the gut through the lung lymphatic system or via the bloodstream into the intestinal lymph nodes reaching the gut (Eladham et al., 2024; Enaud et al., 2020).

Gut miRNAs regulate the intestinal immune system by playing a vital role in innate and adaptive immunity, hence affecting various immunological and physiological processes in the intestine (Bi et al., 2020a). Any dysregulations in the gut miRNAs are correlated with inflammatory responses (Mikami et al., 2021b). Evidence showed that gut miRNAs regulate IL-17 production by modulating intestinal Th17 cell response (Raisch, 2013). Moreover, epigenetic mechanisms, including DNA methylation and posttranslational histone modifications can contribute to T-cell generation (Luo et al., 2017). Changes in the genomic DNA methylation have important regulatory functions in normal and pathological cellular processes and contribute to the normal development and differentiation of cells and tissues (Horsburgh et al., 2015). Studies have shown that infections may lead to the hypomethylation of inflammation-associated genes (Horsburgh et al., 2015). A study suggests an inverse correlation between DNA methylation of TLR4 and inflammatory response to LPS stimulation in intestinal epithelial cells (Takahashi et al., 2009).

Exposure to immune stressors such as exogeneous lipopolysaccharide (LPS) is accompanied by gut dysbiosis and mediates the production of inflammatory cytokines such as IL-6 and tumor necrosis factor-α (TNF-α) by immune cells (Murray et al., 2019; Yahfoufi et al., 2023). LPS can disrupt the intestinal epithelial barriers by binding to the Toll-like receptor (TLR4) and upregulating various inflammatory parameters at the intestinal levels (Håkansson et al., 2012). However, the gut-lung bidirectional axis is initiated at the gut level by bioactive components interacting with TLRs, at the epithelial cell levels. TLR is the key in maintaining immune homeostasis in the mucosal lymphoid associated tissues, which includes the lung and gut territories. Once TLR4 is stimulated by LPS, it can activate the nuclear factor NF-κB which induces pro-inflammatory cytokines production (Broad et al., 2007). TLR4 signaling has been implicated in innate immunity and inflammation following acute lung injury (ALI), intestinal

permeability and dysbiosis. Furthermore, LPS-induced inflammation is associated with an increase in IL-17A (Xu et al., 2023). LPS also can induce Th17 cells differentiation (Park et al., 2015), a subset of CD4+ t cells characterized by proinflammatory cytokine IL-17A production (Atabaki et al., 2020a). IL-6 plays a key role in Th17 differentiation (Atabaki et al., 2020a), while IL-10 inhibits Th17 cells differentiations and inflammatory responses (Huber et al., 2011).

Puberty is a critical developmental phase in life, characterized by physiological changes. The immune system undergoes profound alterations throughout puberty (X. Yuan et al., 2020). It has been shown that pubertal LPS-exposure can initiate dysbiosis, inflammation and affect the neurological functions (Brenhouse & Schwarz, 2016; Sharma et al., 2019; Yahfoufi et al., 2023). However, the long-lasting effects of pubertal LPS-induced inflammation on gut-lung axis immunity has not been sufficiently explored.

A growing body of evidence has shown the health promoting effects of eggshell membrane hydrolysate (ESMH). Protein hydrolysates will likely exhibit an immunomodulatory action affecting the function of immune cells that migrate or release effectors beyond the gut and into the lung (Cesta, 2006; Nelson et al., 2007b). ESMH has been shown to enhance gut immunity by beneficially affecting the gut microbial communities, maintaining the gut epithelial barriers, reducing inflammation and dysbiosis, counteracting obesity, inhibiting the growth of pathogens and modulating innate/adaptive immune responses (Jia et al., 2014, 2017b). ESMH has been shown to reduce intestinal inflammation by facilitating the restoration of the integrity of the epithelium and mitigating the effects of microbial dysbiosis (Jia et al., 2017b). In one of the studies, ESMH inhibited inflammatory cytokine production induced by LPS; these effects were related to the significant improvements in gene expressions of inflammatory mediators, intestinal epithelial cell proliferation and antimicrobial peptides (J. Y. Jung et al., 2017). Moreover, ESMH might

reduce inflammation in colitis-mice models via IL-6 mediated pathway and promotes T-cells apoptosis to restore immune homeostasis in the gut (Shi et al., 2014).

Therefore, the current study aimed to investigate whether supplementation by ESMH during puberty can mitigate the pubertal LPS-induced gut dysbiosis and epigenetic alteration at the gut-lung axis by exploring gut microbiota, Th-17 related cytokines, miRNAs, and DNA methylation of genes related to the Th17 mediated immunity. To this end, pubertal Balb/c mice were exposed to LPS and fed eggshell membrane hydrolysate (ESMH), to study the effects of the treatment on gut microbiota and epigenetic modifications at the gut-lung axis.

2.3 Materials and Methods

Animals

Three-weeks-old female Balb/c mice weighing 10-11 g (Charles River, Montreal, QC, Canada) 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. All groups received conventional balanced diet ad libitum. Mice were treated and properly maintained according to the guidelines of the Canadian Council on Animal Care. The protocol (#Hse-3419-R3) was approved by the Animal Care Committee of the University of Ottawa.

Eggshell Membrane Hydrolysate (ESMH) Solution Preparation

In this study, eggshell membrane hydrolysate was purchased, in a powdered form. Five grams of powdered ESMH were measured, using a scale, and dissolved in one liter of sterilized water by placing the container, with a magnet, on the magnetic stirrer. Then, the fresh mixture was prepared twice a week and kept at 4°C.

Study Design

Pubertal LPS-Mice Models

We first examined the enduring and acute effects of pubertal LPS exposure and eggshell membrane hydrolysate intake on the gut microbiota, gut-lung axis, and immune system. In total, 84 mice were used in this experiment. Mice were categorized into two groups: 1- control group; receiving regular drinking water, 2- treatment group receiving ESMH (0.10 g/kg/BW) in drinking water. At puberty, half of the mice were injected intraperitoneally (IP) with LPS at a dosage of 1.5 mg/kg, which has been shown to induce inflammation and dysbiosis (Håkansson et al., 2012; Yahfoufi et al., 2023) and the other half were injected with 1X sterile phosphate-buffered saline (PBS) (NaCl 2.7 mM KCl 10 mM Na2HPO4, 1.8 mM mM KH2PO4, pH7 (Sigma Aldrich, Oakville, ON, Canada). LPS solution was prepared by dissolving 0.2 mg/mL lyophilized LPS from Escherichia coli O26:B6 (Sigma Aldrich, Oakville, ON, Canada) into sterile PBS. Therefore, we had four experimental groups after LPS/PBs injection (n=12 in each group in the enduring and n=9 in each group in acute) in enduring and acute experiments: 1- control; receiving regular drinking water and injected with PBS, 2-LPS; receiving regular drinking water and injected with LPS; 3-eggshell membrane hydrolysate; receiving ESMH in drinking water and injected with PBS and 4-eggshell membrane hydrolysate; receiving ESMH in drinking water and injected with LPS. In the acute and enduring experiments, mice were fed for 2 weeks; in the acute, we sacrificed the mice 24 hours post-injection, to examine the short-term effect of the treatment on the immune system, gut microbiota, and the gut-lung axis, whereas in the enduring experiment, we continued feeding the mice, 4 weeks post-injection. Mice were sacrificed at nine weeks of age and the required samples were collected to study the long-lasting effect of the treatment on immunity. Figure (2.1) illustrates the study design of the LPS-induced mice models (enduring and acute experiments).

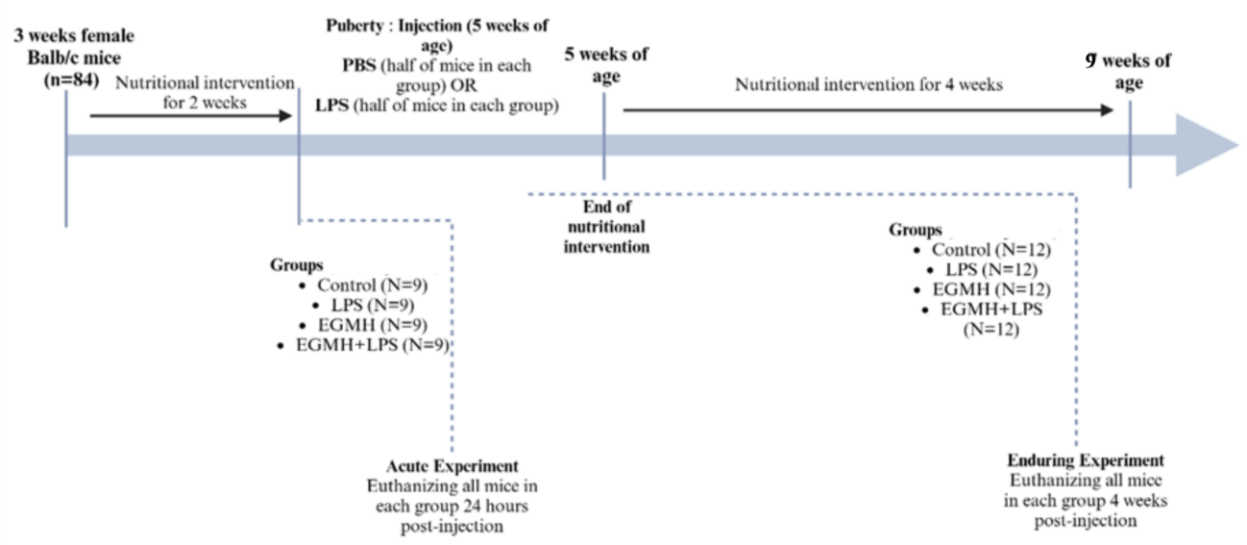

Figure 2.1. (A) Study design showing the experimental timeline, groups, lipopolysaccharide (LPS) injection, and dietary intervention for the pubertal LPS-induced mice models (acute and enduring experiments).

Gut Microbiota

Feces samples were collected in sterile microtubes and stored in -80°C following snap-freezing in liquid nitrogen, for microbiome analysis. 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 used for microbiome analysis. Microbiome was analyzed by Microbiome Insights Company (Vancouver, BC, Canada).

Determination of Cytokine Concentrations of IL-17A, IL-6, and IL-10 in the Small Intestine and Lungs of the Mice by ELISA

To determine the levels of IL-6 and IL-17A in intestinal and lung tissues, after washing both tissues with 1X PBS, small parts of the ileum and lungs (20-25 mg) were cut into small pieces using surgical blades (Fisher Scientific, Toronto, ON, Canada) and collected in microtubes containing lysis buffer (Pierce IP Lysis, Thermo Fisher Scientific) and a mixture of protease/phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor Cocktail, Thermo Fisher Scientific). Then, homogenized samples were centrifuged at 4°C for 10 minutes at 14,000x g, where the supernatant was collected. The concentration of the total proteins in the tissue lysates was measured by the BCA method using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Toronto, ON, Canada). IL-17A, IL-6 and IL-10 levels were assayed using mouse-uncoated ELISA kit (Invitrogen, Vienna, Austria) and the results were calculated by dividing each sample's observed concentrations of cytokines by the total protein of the same sample. Absorbance was read at 450 nm with a wavelength correction of 570 nm using a microplate reader (TECAN, NC, USA).

miRNA Expressions by Real-Time Quantitative Reverse Transcription PCR (RT-qPCR):

Small pieces of the ileum and lung tissues were stored in RNA later Stabilization Solution (Invitrogen, Carls-bad, CA, USA) for 24 hours at 4°C and then stored at -80°C till RNA extraction takes place. RNA was extracted from the samples using the miRNAeasy Mini Kit (Qiagen, Toronto, ON, Canada). The sample's purity and concentration were verified using the NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). A reverse transcription reaction was performed to synthesize the cDNA, using the miRCURY LNA RT Kit (Qiagen, Toronto, ON, Canada). Then, expression levels of miR-155, miR-146a, miR-222 and miR-223 were measured by RT-qPCR using hsa-155-5p , miR-146a-5p , hsa-miR-222-3p, 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 96 real-time PCR and CFX 384 real-time PCR detection system (Bio-Rad, Laboratories, Hercules, CA, USA). SNORD65 (mmu) miRCURY LNA miRNA PCR Assay (Qiagen, Toronto, ON, Canada) was used as a reference gene. The expression levels of the miRNAs were calculated using the ΔΔCT method.

DNA methylation

15-20 mg of mice lung samples were homogenized by an electrical homogenizer (Bead Mill 24, Fisher Scientific, Canada) in tubes containing 500 µL of cell lysis buffer and 1.5 µL proteinase K. The Gentra Puregene Tissue Kit (33g) (Qiagen, Toronto, ON, Canada) was used for the extraction of tissue DNA, based on the manufacturer's instructions. The extracted DNA was diluted with DNA rehydration solution, to a final concentration of 20 ng/ µL, based on the kit, and stored at -20°C. The final concentration of the extracted DNA was measured using the Qubit 4 instrument (Thermo Fisher Scientific, Canada). Then, 500 ng of extracted DNA was exposed to bisulfite conversion using the EZ DNA Methylation kit (Zymo Research, Irvine, CA, USA). After, bisulfite-modified DNA was analyzed using the Infinium Mouse Methylation BeadChip arrays (Illumina Inc., San Diego, CA, USA). Bioinformatic analysis of methylome-wide data was conducted using the methylkey pipeline developed by Epigenomics and Mechanisms Branch at the International Agency for Research on Cancer (https://github.com/IARCbioinfo/methylkey). Raw data files were processed, and normalization was conducted by Noob normalization using SeSAMe package. Comparisons between groups was performed using linear regression analysis and differentially methylated regions were analyzed using the DMRcate package. 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).

Statistical analysis

Statistical analysis for miRNA expression was conducted by GraphPad Prism Software (GraphPad Software Inc., San Diego, CA, USA). Two-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 Shannon index and Kruskal–Wallis pairwise test. Sample beta diversity (β-diversity) clustering was assessed using PCoA and PERMANOVA pairwise test. Corrected/adjusted p-value < 0.05 was considered statistically significant.

2.4 Results

Effect of the treatment on the Gut Microbiota of Mice

To analyze how the treatment impacts the diversity and even distribution of gut microbiota, alpha and beta-diversities were computed. All groups have higher alpha diversity as compared to the control. Moreover, results showed that all groups have a similar significant difference as compared to the control group: in LPS+ ESMH-Control (adjusted *p*-value < 0.0001), in LPS-Control (adjusted *p*-value <0.001) and ESMH-Control (adjusted *p*-value < 0.001). Figure 2.2.A depicts the alpha diversity as shown by the Shannon index plot. For beta diversity, pairwise contrasts results showed a significant difference between all groups as compared to the control group: Control vs.

LPS (adjusted *p*-value < 0.004), Control vs. ESMH (adjusted *p*-value < 0.005), Control vs. LPS+ESMH (adjusted *p*-value < 0.004), LPS vs. ESMH (adjusted *p*-value < 0.004) and ESMH vs. LPS+ESMH (adjusted *p*-value < 0.006) (Figure 2.2.B).

Firmicutes and Bacteroidota were the most abundant phyla seen among all groups (Figure 2.2.C). Figure 2D shows the most abundant taxa at the family levels. Results show that Lactobacillacaeae was the most abundant in the control and ESMH group; Bacteroidaceae population increased in groups exposed to LPS and Lachnospiraceae was the most abundant taxa in the LPS+ESMH group. Furthermore, differential abundance analysis was done to detect taxa which were significantly different among groups at the genus levels. The results showed that the abundance of *Lactobacillus* was lower and the abundance of *Alistipes* was higher in LPS mice compared to control (adjusted *p*-value <0.05) (Figure 2.2.E). In LPS + ESMH group *Alistipes* abundance was higher than control, while *Ligilactobacillus, Lactobacillus, Limosilactobacillus* and *Candidatus arthromitus* abundances were lower than control (adjusted *p*-value < 0.05) (Figure 2.2.F). In addition, in LPS+ESMH group, *Alistipes* was higher and *Candidatus arthromitus, Acetatifactor* and *Limosilactobacillus* were lower when compared to ESMH group (adjusted *p*-value <0.05) (Figure 1G). In ESMH mice, *Alistipes* and *Butyricimonas* were lower compared to LPS group (adjusted p-value <0.05) (Figure 2.2.H). Results showed that there was no difference at the genus levels between ESMH vs. Control group and between LPS+ESMH vs. LPS.

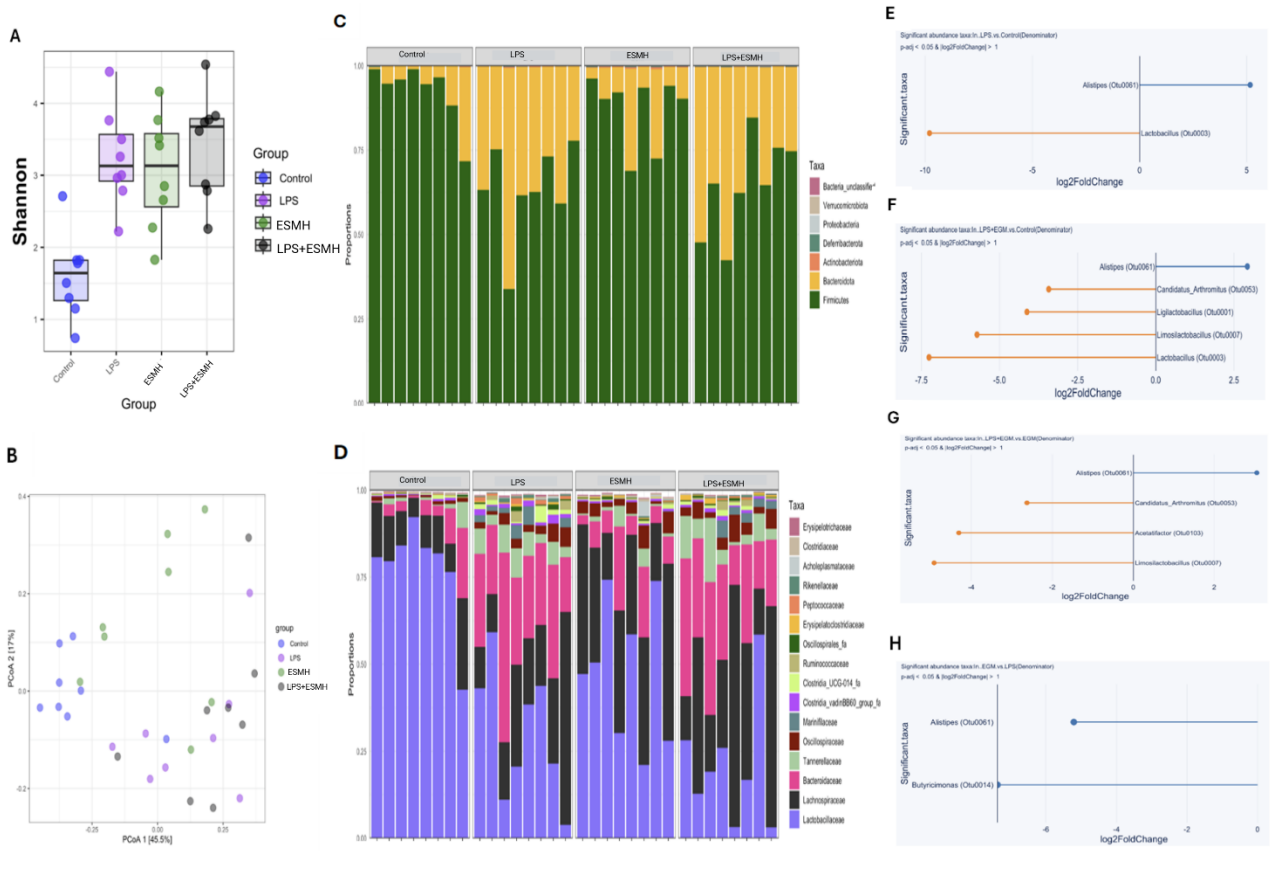

Figure 2.2. Effect of treatment on gut microbiome 24 hours after lipopolysaccharide (LPS) injection at puberty. Mice received eggshell membrane hydrolysate (ESMH) (0.10 g/kg BW/day) in drinking water or drinking water without eggshell membrane hydrolysate (ESMH) for two weeks before LPS injection. At puberty mice were injected with either lipopolysaccharide (LPS) or phosphate buffered saline (PBS) and 24 hours after injection, feces samples were collected for microbiome analysis. (**A**) Alpha diversity, (**B**) beta-diversity, (**C**, **D**) the most abundant taxa at the phylum and family levels, (**E**-**H**) significant differentially abundant taxa among groups.

Effect of the Treatment on the IL-17A, IL-6, and IL-10 levels in the Small Intestine and Lungs of Pubertal Mice

In pubertal mice at the gut level, LPS exposure was related to an increase in IL-17A levels in both LPS and LPS + ESMH groups compared to the control, however it did not reach significant. ESMH decreased IL-17A level compared to control and other groups, but it was not significant (Figure 2.3.A). IL-6 levels were significantly higher in both groups exposed to LPS. IL-10 levels were higher in all treated groups compared to the control and exposing mice to LPS + ESMH was associated with higher increase in the IL-10 levels compared to the LPS and ESMH alone (Figure 2.3.A).

At the lung level, IL-17A level was not significantly higher in mice exposed to LPS alone compared to all other groups. IL-6 levels were significantly higher in ESMH than in the control and LPS. IL-10 levels were higher in both groups fed ESMH than in the control and LPS groups (Figure 2.3.B).

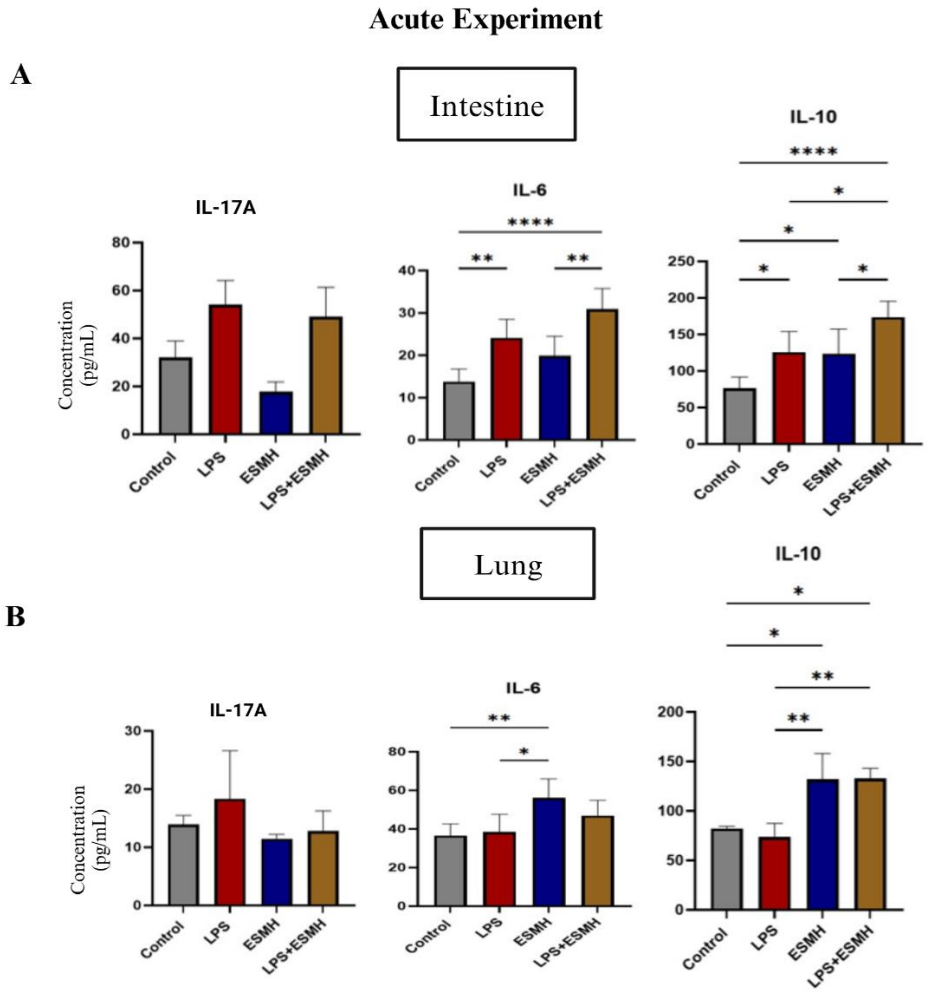

Figure 2.3. Effect of treatment on the selected cytokines related to TH17 cells 24 hours after lipopolysaccharide (LPS) injection at puberty. Mice were fed eggshell membrane hydrolysate (ESMH) (0.10 g/kg BW/day) in drinking water or drinking water without eggshell membrane hydrolysate (ESMH) for two weeks before lipopolysaccharide (LPS) injection at puberty (5 weeks of age). At puberty mice were injected with either lipopolysaccharide (LPS) or phosphate buffered saline (PBS) and 24 hours after injection, small intestine and lung samples were collected. (**A**) Levels of IL-17A, IL-6, and IL-10 in the intestine, (**B**) levels of IL-17A, IL-6, and IL-10 in the lungs. Statistical analysis was done using Two-Way ANOVA and Tukey's post hoc tests were used to compare groups. All values are mean ± SEM. *$p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$.

Effect of the Treatment on the miRNA Expressions in the Small Intestine and Lungs of Mice

In the acute experiment at the gut level, relative expression of miR-146a was lower in LPS and ESMH groups compared to the control and LPS + ESMH groups. miR-222 and miR-223 expression was lower in all treated groups compared to the control (Figure 2.4.A). ESMH significantly decreased miR-222 when compared to LPS (Figure 2.4.A). At the lung level, miR-146a, miR-222, and miR-223 expressions was higher in both groups treated with ESMH compared to control and LPS (Figure 2.4.B).

In enduring experiment, at the gut level, a lasting increase in relative expressions of miR-155 and miR-222 was observed in mice exposed to pubertal LPS compared to the mice exposed to ESMH and LPS + ESMH. miR-223 expression was lower in LPS + ESMH compared to the control (Figure 2.4.C). At the lung level, expressions of miR-155, miR-222, and miR-223 were increased in adult mice exposed to the LPS during puberty compared to all other groups, while ESMH intake during puberty decreased effect of LPS on these miRNAs (Figure 2.4.D).

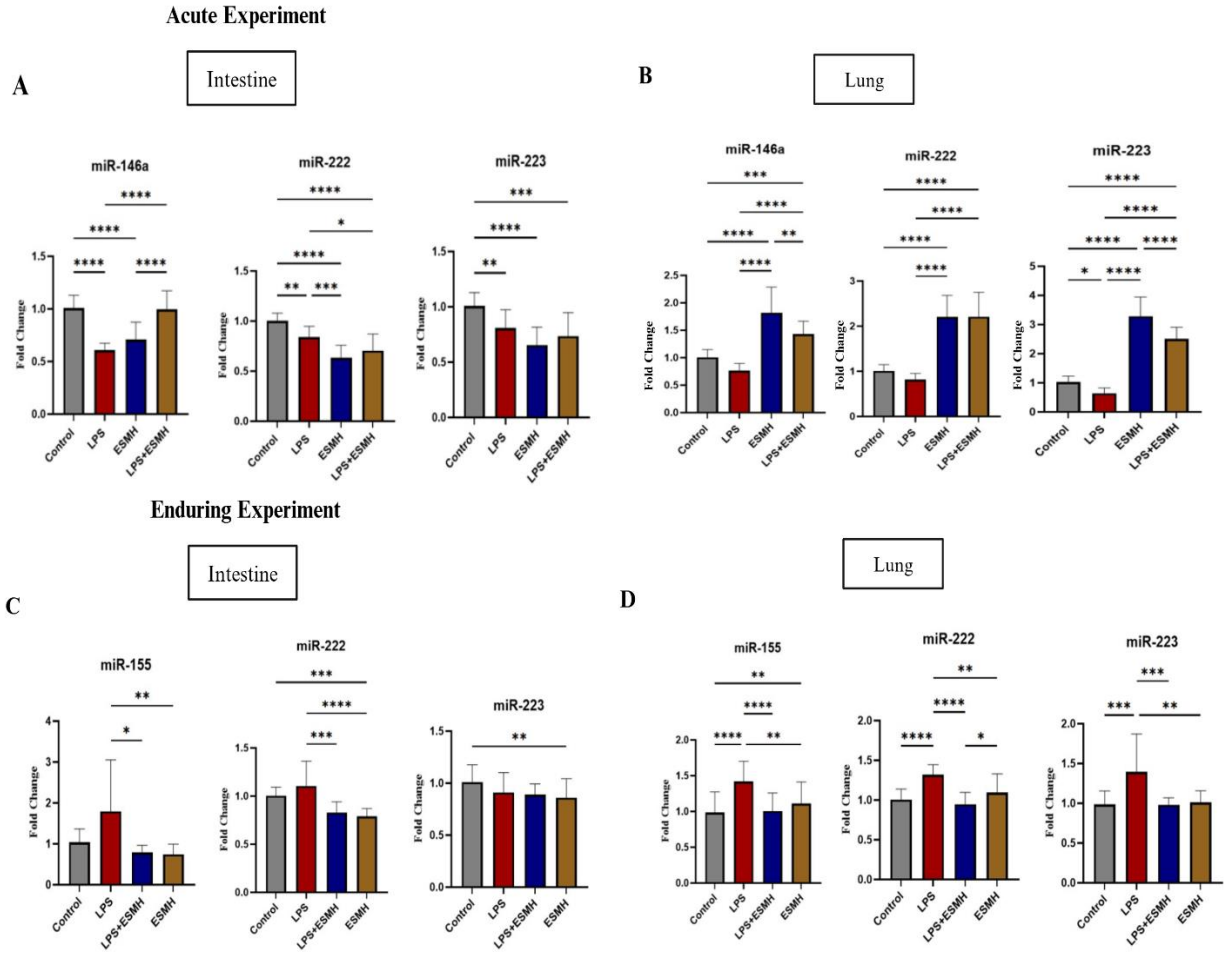

Figure 2.4. (A, B) Relative expressions of miR-146a, miR-222 and miR-223 in the small intestine and lungs of mice, respectively, 24 h after injection of a single dose of lipopolysaccharide (LPS) at puberty (Acute experiment). Mice received eggshell membrane hydrolysate (ESMH) (0.10 g/kg BW/day) in drinking water or drinking water without eggshell membrane hydrolysate (ESMH) for two weeks before injection. **(C, D)** Relative expressions of miR-155, miR-222 and miR-223 in the small intestine and lungs of mice, respectively, 4 weeks after injection of a single dose of lipopolysaccharide (LPS) at puberty (enduring experiment). Mice received eggshell membrane hydrolysate (ESMH) (0.10 g/kg BW/day) in drinking water or drinking water without eggshell membrane hydrolysate (ESMH) for two weeks before injection, and for four weeks after. Statistical analysis was done using Two-Way ANOVA and Tukey's post hoc tests were used to compare groups. All values are mean ± SEM. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Effect of the Treatment on the DNA Methylation Status of Genes in the Lungs

To determine the long-lasting effects of pubertal challenge with LPS and ESMH on the gut-lung axis, the methylation status of genes in the lung samples of adult were examined. Differentially methylated probes (DMPs) were identified, with 45.5 % showing hypermethylation and 54.5 % hypomethylation in the control vs. LPS group. In LPS versus ESMH+LPS comparison, 26.3 % of DMPs were hypermethylated, while and 73.6 % were hypomethylated. Additionally, the analysis of differentially methylated regions (DMRs) identified 72 DMRs with statistically significant p-values. Enrichment analysis of these DMRs revealed their involvement in various immune and metabolism pathways such as IL-17 signaling pathway, NF-kappa B, JAK-STAT and PI3K-AKT signaling pathways. Unsupervised clustering of the 500 probes with the highest variability based on the samples, determined by the coefficient of variation. The four groups are shown: control, ESMH, ESMH+LPS and LPS (figure 2.5 B). In line with this, a comprehensive analysis of differentially methylation highlighted that LPS treated groups exhibited the most pronounced methylation changes compared to the control group. When focusing on the genes related to the objectives and aim of this study, in LPS-challenged mice, a significant hypomethylation of CpGs was identified within the promoter of TNF-α-induced protein 3 *(Tnfaip3)*, TNFAIP3 interacting protein 1 (*Tnip1),* Lipocalin 2 *(Lcn2),* SOX2 overlapping transcript *(Sox2ot),* Erythroblast membrane associated protein *(Ermap),* and C-X-C Motif Chemokine Ligand 1 *(Cxcl11)* as compared to the control *($p < 0.05$)* (Figure 2.5 C). Additionally, hypomethylation within the intronic regions was observed in RAR Related Orphan Receptor A *(Rorα),* Mutated colorectal cancer regulator of WNT Signaling Pathway (*Mcc*) and Protein kinase C Beta *(Prkcb*). In contrast, Suppressor of Cytokine Signaling 7 (*Socs7*) was hypermethylated within the exon region in LPS-induced mice in comparison to the control *($p < 0.05$)* (Figure 2.5 C). In ESMH+LPS group,

hypermethylation of promoter regions of *Tnfaip3, Tnip1* and *Nlrc4* genes was observed compared to LPS *($p < 0.05$)* (Figure 2.5 C). *Socs7* was hypomethylated within the exon region *($p < 0.05$)* (Figure 2.5 C). Furthermore, no significance was observed in *Tnfaip3* and *Socs7* genes in both groups, control vs. LPS and LPS vs. ESMH+LPS, whereas a significant difference was shown in *Tnip1*. However, we saw a difference in *Lcn2, Sox2ot, Ermap, Cxcl11, Rorα, Mcc* and *Prkcb* between both groups, but we couldn't find them in LPS vs. LPS+ESMH group probably due to the lack of *p-value* or the mean difference was not more or less than 2.5.

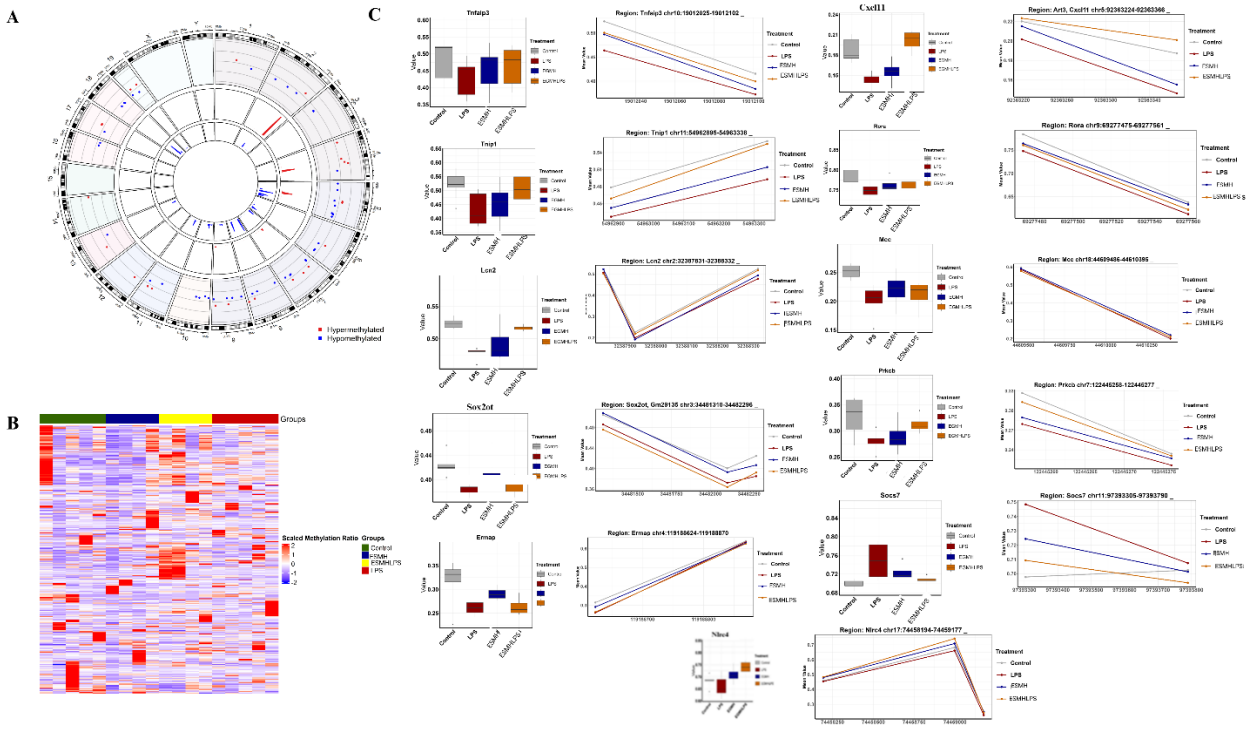

Figure 2.5. DNA methylation analysis in adult mice, four weeks after lipopolysaccharide (LPS) injection at puberty. Mice received eggshell membrane hydrolysate (ESMH) (0.10 g/kg BW/day) in drinking water or drinking water without eggshell membrane hydrolysate (ESMH) for two weeks before injection and 4 weeks post injection. (A) depict DNA methylation across the genome. The outmost ring indicates the chromosome number 1 to 22, including X and Y chromosomes. Each dot on the plot marks a distinct genomic location with altered methylation. The blue dots represent hypermethylation and red dots represent hypomethylation. (B) represents a heatmap pf unsupervised clustering of 5oo probes with the highest variability based on the samples, determined by the coefficient of variation. (C) The methylation levels of 11 genes, *Tnfaip3, Tnip1,*

Lcn2, Sox2ot, Ermap, Cxcl11, Rorα, *Mcc, Prkcb, Socs7* and Nlrc4 and their average differences across the regions.

2.5. Discussion

While many studies highlighted the effects of immune stressors on microbiota composition during critical developmental periods are usually temporary (Cox et al., 2014) , certain phenotypic changes, such as dysbiosis-induced obesity can persist even after the gut microbiota has been normalized (Cox et al., 2014). This underscores the long-term metabolic and developmental impacts of dysbiosis. Since dietary factors play a pivotal role in maintaining a healthy gut microbiota and immune homeostasis, research has been conducted to examine the effectiveness of nutritional interventions in preventing gut microbial perturbations to enhance overall health and prevent immune related disorders (Yoon & Yoon, 2018; S. Zhang & Chen, 2019). Our understanding of the molecular aspects of several bioactive compounds and natural components in mitigating the adverse effect of dysbiosis and impaired gut microbiota are not yet fully understood (Neuman et al., 2018). Therefore,

The gut-lung axis aspect has emerged; it refers to the reciprocal crosstalk between the gut and the lungs (Trivedi & Barve, 2020). It is associated with the transfer of metabolites and immunomodulatory signals between both organs, through the lymphatic system or via the bloodstream (Haldar et al., 2023). For example, intake of probiotics, during COVID-19 infection may play an immune-protective role in the gut-lung axis (Robichaud et al., 2021; Shahbazi et al., 2020). Therefore, any disruptions of the gut microbiota by an external immune stressor such as inflammatory LPS during puberty has been shown to induce perturbations to the gut microbiota, as well as immune alterations that can significantly affect the developmental programming of other distal organs, such as the lungs.

Many dietary interventions have been shown to enhance the gut microbiota and modulate innate and adaptive immune responses. Probiotics and prebiotics have been shown to enhance the gut immunity by positively affecting gut microbial communities, maintain gut epithelial barriers and altering immune responses (Bermudez-Brito et al., 2012; Pujari & Banerjee, 2021). Dietary supplements such as fermentable fibers can change the composition of the gut microbiota by modulating the Firmicutes/Bacteroidetes ratio (Conte & Toraldo, 2020). Moreover, natural products, such as eggshell membrane, is used as an essential ingredient in nutraceuticals and functional food industries. Egg protein hydrolysates has been shown to exert anti-inflammatory and immunomodulatory effects (Jia et al., 2017b; Shi et al., 2014), improve gut immunity by beneficially affecting the gut microbial communities, prevent dysbiosis, alleviate inflammation and immune response and inhibit the growth of pathogens (Jia et al., 2017b).

Dysbiosis and increased LPS levels induce a cascade of systemic effects, including immune and epigenetic disruptions; as it can also alter the DNA methylation profile of the cells, causing dysregulation in the expression of cytokines and disrupting the microRNA levels (Cai et al., 2012; Hartnett & Egan, 2012)

In this study, we explored the role of ESMH in reducing inflammation in the gut-lung axis using a mice model of LPS-induced inflammation and dysbiosis during puberty. We examined the effects of ESMH on gut microbiota composition and enduring epigenetic modifications.

We investigated the impact of ESMH on the gut microbiota in pubertal mice 24 hours after LPS injection. LPS exposure led to an increase in Bacteroidota, known as Bacteroidetes, in both groups exposed to LPS compared to the control and ESMH groups, reflecting that LPS increases Bacteroidota population, commonly associated with chronic intestinal inflammation, and alters the Firmicutes/Bacteroidota ratio. This ratio is crucial for gut homeostasis. An increase in

Bacteroidetes and a decrease in Firmicutes/Bacteroidota ratio are associated with inflammatory bowel disease (IBD) which correlates with the progression of IBD (Stojanov et al., 2020). At the family level, the Lachnospiraceae population increased in all treated groups, with a higher increase in the LPS+ESMH group. Therefore, ESMH increased the Lachnospiraceae species, favoring the production of SCFAs in the gut, which subsequently reduces inflammation (Rønning et al., 2024; Vacca et al., 2020).

We observed a higher abundance of *Alistipes*, which plays a critical role in inflammation and disease, in the LPS group compared to the control, however, ESMH reduced the effect of LPS on this genus in the LPS+ESMH group when compared to the control. *Alistipes* is relevant in dysbiosis and inflammation-related diseases, including anxiety and chronic fatigue syndrome (Parker et al., 2020). Moreover, changes in *Alistipes* are positively correlated with serum IL-6 (Shi, Zhong, et al., 2021). Additionally, a reduction in *Lactobacillus* population was found in LPS-exposed group compared to the control, however, ESMH could decrease the effect of LPS on this genus in LPS+ESMH group when compared to the control. *Lactobacillus* species have been shown to decrease inflammatory responses in animals with necrotizing enterocolitis by inhibiting IL-6, TLR4, and NF-κB signaling (Liu et al., 2012). Furthermore, the abundance of *Candidatus arthromitus* decreased in the LPS+ESMH group compared to the ESMH and control groups. *Candidatus arthromitus* is a segmented filamentous bacterium (SFB), which are the main commensals involved in Th17 cell differentiation in the gut. Colonization of the gut by SFB induces antigen-presenting cells in the lamina propria by the secretion of serum amyloid secretion, which stimulates Th17 cell differentiation and IL-17 expression (Ravindran et al., 2016; Sano et al., 2015). Thus, ESMH reduced the levels of *Alistipes* and segmented filamentous bacterium, *Candidatus arthormitus*, and increased the abundance of *Lactobacillus*. This highlights the

potential role of ESMH in reducing intestinal inflammation by facilitating the restoration of the integrity of the epithelium and mitigating the effects of microbial dysbiosis (Jia et al., 2017b).

Next, we examined the effect of treatment on selected cytokines related to the Th17 cells differentiation or function, including IL-17A, IL-6 and IL-10. In this study, we observed a trend in IL-17A at the gut and lung levels, but it was not significant. Th17 cells are proinflammatory CD4+ T cells, known to secret proinflammatory cytokine IL-17A, that contribute in the pathogenesis of intestinal inflammation and airway diseases (Fogli et al., 2013; Zhao et al., 2021). In the lung, IL-17 overexpression has been shown to induce neutrophils infiltration and lung epithelium alterations, leading to an inflammatory state followed by declined lung function (Fogli et al., 2013). Further investigation is needed to elucidate the expression of IL-17A in the context of LPS and ESMH treatment.

At the gut level, IL-6 concentrations increased in pubertal mice exposed to LPS when compared to the control and ESMH, however, ESMH intake in mice exposed to LPS could not inhibit the stimulatory effects of LPS on these cytokines. In addition, IL-6 levels increased, in the lungs, in ESMH (significant) but not significantly in LPS + ESMH. IL-6 is a multifunctional cytokine involved in various biological processes; it is known as a proinflammatory cytokine, however, it is also known to possess anti-inflammatory characteristics such as its ability to downregulate LPS-induced monocyte IL-1 and TNF-α mRNA expression (Vinderola et al., 2005). Moreover, IL-6 plays a central role in the Th17 differentiation and IL-17A production by inducing phosphorylation and activation of STAT3 pathway, which is key signaling pathway in regulating differentiation of naïve T cells into Th17 cells by stimulating expression of RORγt, the master transcription of Th17 cells differentiation (Shahbazi et al., 2023). One plausible explanation for the increase in IL-6 in ESMH is the timing of the mouse sacrifice and sample collection, as IL-6 expression is highly

dynamic, with a temporary peak during the early stages of immune responses (Kishimoto, 2010). Therefore, further investigation is needed to elucidate the dynamics and regulation of IL-6 expression in the context of LPS and ESMH treatment.

Is it evident that LPS exposure can initiate the production of pro-inflammatory cytokines such as IL-6, IL-17A, IL-23 while simultaneously influencing the expression of anti-inflammatory cytokines such as IL-10.

Although results have shown that IL-10 levels, in the gut, increased in all treated groups, in the ESMH-fed groups the increase was higher compared to LPS group. These results reveal strong immunomodulatory effects of acute exposure to LPS leading to an increase in both anti- and pro-inflammatory cytokines production at the gut level. They are consistent with previous studies showing stimulatory effects of LPS on different anti- and pro-inflammatory cytokines expression, such as IL-6, Il-10, Il-1β in bloods and/or other organs such as brain and intestine (Shahbazi et al., 2023; Yahfoufi et al., 2023). In the lungs, IL-10 levels were higher in mice fed ESMH regardless of LPS exposure. These results may indicate immunomodulatory effect of feeding mice for two weeks with ESMH which resulted in an increase in both IL-10 and IL-6 levels. Noteworthy, the low dose of intraperitoneal LPS used in this study (1.5 mg/Kg BW) compared to other studies applying higher dosage of intraperitoneal LPS to induce acute lung injury (Abdelmageed et al., 2016) or using other routes of LPS injection, such as intratracheal instillation (Tsikis et al., 2022) should also be taken into consideration when interpreting the results related to IL-10. IL-10 inhibits Th17 differentiation and its inflammatory responses in mice challenged with LPS but receiving prebiotics as well (Shahbazi et al., 2023). The observed increase in IL-10 levels may indicate modulation of the immune response, potentially influencing the balance between pro and anti-inflammatory signals.

This supports ESMH supplementation as a therapeutic approach to manage inflammation and prevent related diseases in the gut, countering dysbiosis and elevated levels of proinflammatory cytokines induced by LPS exposure.

To investigate whether the persisting effect of LPS exposure and ESMH intake on the gut-lung axis and immunity are mediated by durable epigenetic modifications, we studied the miRNA expression in the intestine and lungs and DNA methylation status of immune-related genes.

In the acute experiment, in both, intestine and lungs, miR-146a, and pro-inflammatory miR-222 and miR-223 were downregulated by LPS. One could argue that short term effect of LPS after 24 hours of injection did alter the miRNAs expression due to the acute inflammation while pro-inflammatory miRNAs were shown to increase in the LPS group in the enduring experiment. In the enduring experiment, in the intestine and lungs, the pro-inflammatory miRNAs, miR-155, miR-222 and miR-223 were upregulated by LPS, where ESMH treatment could have mitigated the effect of LPS on these miRNAs. miR-146a is a negative regulator of NF-κB signaling in response to a bacterial infection (Casciaro et al., n.d.; B. Wei & Pei, 2010) as well as targets the tumor necrosis factor receptor-associated factor 6 (TRAF6) and interleukin 1 receptor associated kinase 1 (IRAK1) which regulate the NF-kB signaling in T-helper cells, blocks the NF-kB-mediated secretion of IL-6/IL-21, and inhibits the development of pathogenic Th17cells (J. Huang et al., 2021b).The upregulation of miR-222 is associated with increased proliferation and initiating oxidative stress in the lungs (Tepebaşı & Öztürk, 2023), as well as contributing to the pathogenesis of gut mucosal atrophy, damaged mucosa, and epithelial barrier dysfunction (Wang et al., 2017). 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., 2020a) and has been shown to initiate pathological Th17 cells differentiation during autoimmunity (Atabaki et al., 2020a; Jiao et al.,

2021). However, some studies indicate that miR-223 might have a dual role; regulating the inflammatory response during acute lung injuries (Haneklaus et al., 2013). A study demonstrated that miR-223 reduced inflammation, supressed NLRP3 inflammasome and negatively regulated the TLR4/NF-kB signaling pathways, which served as a potent positive regulator of inflammation in lung injury (Yan et al., 2019). miR-155 is associated with various disorders such as cardiovascular diseases, inflammation and cancer (Faraoni et al., 2009) and plays a vital role in the stability of Th17 phenotype. Increased levels of miR-155 has been seen in patients with inflammatory bowel disease (IBD) and colorectal cancer (Xiao et al., 2009). It has been shown to promote the secretion of cytokine by dendritic cells, therefore to further regulate Th17 cell differentiation, which was associated with the development of chronic intestinal inflammation and other autoimmune diseases (Bi et al., 2020b). This miRNA can activate the release of Th17 cells (Atabaki et al., 2020b). Therefore, these results suggest that ESMH may enhance gut immunity and prevent inflammation in the gut-lung axis by regulating gut and lung miRNAs.

Overall, these findings underscore the intricate play between LPS exposure, miRNA dynamics and ESMH potential therapeutic effect in reducing inflammation and maintaining immune homeostasis in the gut and the lungs. ESMH appears to selectively modulate key miRNAs, potentially offering protective, anti-inflammatory and immunomodulatory roles.

In this study, the enduring long-term effect of LPS exposure on DNA methylation and potential impact of ESMH treatment on DNA methylation of lungs was examined. This experiment was carried out by comparing the control vs. LPS group (45.5 % were hypermethylated and 54.5 % were hypomethylated) and LPS vs. ESMH+LPS (26.3 % were hypermethylated and 73.6 % were hypomethylated). Of these genes, 10 genes including *Tnfaip3, Tnip1, Lcn2, Sox2ot, Ermap,*

Cxcl11, Rorα, *Mcc, Prkcb* and *Socs7* which were involved in critical signaling pathways such as IL-17 signaling pathway, NF-kappa B, JAK-STAT and PI3K-AKT signaling pathways.

Several of these genes are involved in Th17/inflammatory signaling pathways. TNFAIP3, also known as A20*,* has been proven to act as a negative regulator of NF-kB (Momtazi et al., 2019) , functions as a crucial regulator of inflammation and immunity and acts as a tumor suppressor in lung cancer (H. Chen et al., 2020). Therefore, studies have shown that *Tnfaip3* overexpression-induced cell apoptosis and inhibition of tumor growth in the lungs via downregulation of key proinflammatory signaling pathways, including NF-kB (H. Chen et al., 2020). However, TNFAIP3 downregulation was also associated with Th1 and Th17 cell differentiation as well as the activation of inflammatory p38 activation seen in patients with dysfunction immune response (Jiang et al., 2020).

It was found that the two genes, *Cxcl11* and *Rorα,* both involved in Th17 polarization, were hypomethylated in their promoter and intronic regions. The methylation status of specific genomic regions, including the 1–5 kb upstream of transcriptional start sites, promoters, and 5' untranslated regions (UTRs), can significantly influence gene expression. Additionally, the methylation status of intronic regions is often associated with alternative splicing or suppression of gene activity.

CXCL11 has been shown to decrease the transcription of RORγ, leading to the polarization of type 1 regulatory T cells (Tr1) or type 2 T helper cells (Th2) from naïve T4 helper cells by stimulating the p70 kinase/mTOR pathways (Cole et al., 1998). Other studies have demonstrated that CXCL11 can supress the macrophage polarization, thereby inhibiting the breast cancer tumor growth (Oghumu et al., 2014).

Furthermore, RORα is upregulated during Th17 cell differentiation, directing the expression of IL-17 and contributing to the effector functions of Th17 cells (Huh et al., 2011). Overexpression of RORα has been shown to promote Th17 cell differentiation, which in turn upregulates IL-17 expression (Yang et al., 2008). RORα has been suggested to play a role in the regulation of Th17 gene expression (Dzhagalov et al., 2004). Studies have found that *Nlcr4*-driven necroptosis and IL-18 supress IL-17A signaling from γδ T cells leading to neutrophil recruitment, which is important to eradicate *Staphylococcus aures* from the lungs (Jeyaseelan et al., 2006).

TNIP1 is a key anti-inflammatory protein that plays a critical role in supressing inflammatory signaling pathways downstream of TLRs. Loss of TNIP1 expression in immune cells has been linked to the development of autoimmune diseases (Shamilov & Aneskievich, 2018). Nuclear and cytoplasmic *Tnip1* was found in the malignant keratinocytes of squamous cell carcinomas and esophageal cancer whereas it was to be decreased in prostate cancer (Gurevich et al., 2011).

LCN2 functions by limiting bacterial growth through the sequestration of iron-containing siderophores (B.-K. Jung & Ryu, 2023). Studies in mice have shown that the absence of the Lcn2 gene increases susceptibility to bacterial infections (Santiago-Sánchez et al., 2020). Beyond this antibacterial role, LCN2 also regulates immune and inflammatory responses, induces apoptosis, and helps maintain skin homeostasis and insulin sensitivity (Asaf et al., 2023, p. 2).

ERMAP inhibits T-cell proliferation and activation, reduces cytokine production, and plays a protective role in preventing autoimmune diseases (Su et al., 2021). Additionally, the Ermap-Ig fusion protein has been shown to supress T-cell activity and promote the generation of anti-inflammatory macrophages (H. Liu et al., 2021; Song et al., 2021). In this study, hypomethylation in the promoter regions of *Tnip1, Lcn2* and *Ermap* was observed, which may suggest increased expression of these genes.

Hypermethylation in the exonic region of *Socs7*, associated with gene suppression, and hypomethylation in the intronic regions of *Mcc* and *Prkcb*, linked to alternative splicing or gene overexpression, were observed.

SOCS7 is a regulator of various signaling pathways including JAK/STAT pathway that controls cell proliferation (P. Huang et al., 2022). It is negatively correlated with signal transducer and activator of transcription 3 (STAT3) hyperactivation, regulating tumorigenesis. A reduction in *Socs7* levels promoted expression of immunosuppressive and tumor-inducing factors such as TGF-β (Cornebois et al., 2024; P. Huang et al., 2022) .

Overexpression of *Mcc* , a known tumor suppressor gene, has been shown to inhibit entry into S phase in cell cycle (Benthani et al., 2018). MCC also inhibits Wnt/β-catenin signaling and has been found to act as an oncogene in B cells, with its knockdown inducing apoptosis in human multiple myeloma (Song et al., 2021). PRKCB plays diverse roles in cellular regulation and survival, particularly in apoptosis, and studies have demonstrated hypermethylation in the promoter regions of Prkcb in various adenocarcinomas (Wang et al., 2022). Furthermore, *Prkcb* expression has been associated with immune cell infiltrations, underscoring its significance in the tumor microenvironment of lung adenocarcinoma through its role in regulating tumor immune cell interaction (Muppa et al., 2019). Altogether, the analysis of the differentially methylated genes of *Tnfaip3, Tnip1, Lcn2, Sox2ot, Ermap, Cxcl11, Rorα, Mcc, Prkcb* and *Socs7* by LPS were mitigated by ESMH.

The findings related to DNA methylation have provided valuable insights into the connection between DNA methylation, cytokine expression and microRNAs. We observed that DNA methylation profiles were altered in response to LPS exposure, resulting in both hypomethylation and hypermethylation of specific genes. Among those genes affected, we identified several that

are involved in critical signaling pathways including Il-17 signaling pathways and JAK-STAT and signaling pathways as well as microRNA expression regulation. Therefore, inflammation plays a key role in DNA methylation modulation and is known to affect various cellular processes.

The study has some limitations. Although our epigenetic results may suggest the role of ESMH in regulating immune responses under inflammatory conditions, by modulating Th17 cells, further analysis of miRNA as well as mRNA levels for more genes from DNA methylation or miRNA targets would further consolidate the role of ESMH in reducing inflammation in gut-lung axis, in LPS-induced mice.

In conclusion, we found that the exposure to an immune stressor in critical developmental window of puberty alters the gut microbiota and leads to immune deregulation in the gut and lungs and may cause enduring dysregulation in the epigenetic modifications which in turn can lead to lasting immune dysfunction at the gut and gut-lung axis levels. ESMH intake might mitigate these negative drawbacks. Together, these results may indicate that consuming eggshell protein hydrolysates could be a useful strategy in priming the immune system and maintaining immune homeostasis later in life. Research highlighted the mechanisms and characteristics of the exposure to any external stressor, such as inflammation that may result in persistent immune dysfunction, therefore affecting other organs such as the gut-lung axis.

Our findings may demonstrate the use of this product, ESMH, in improving gut homeostasis, immunity and microbiota composition, as well as reflecting an optimal strategy for immune function by controlling inflammation and dysbiosis. This project shed a light on the importance of the preventative and protective effects of ESMH in reducing inflammation and creating an integrated immune-based approach to mediate a systemic anti-inflammatory immunity in the gut and lungs, through epigenetic mechanisms. This process could enhance our understanding of the

molecular mechanisms underlying immune dysfunction and disease susceptibility, leading to a targeted therapeutic intervention.

Ethics approval and consent to participate

The experimental protocol for all animal experiments conducted in this study was approved by the Animal Care Committee of the University of Ottawa on 13th July 2022, under the approval code HSe-3191. The care, treatment, and maintenance of mice throughout the course of this research adhered strictly to the guidelines set forth by the Canadian Council on Animal Care, ensuring the highest standards of animal welfare. This study did not involve any human subjects.

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Chapter 3: Discussion

3.1 Discussion

The gut-organs axis is emerging in scientific research. The gut lung axis emphasizes the reciprocal exchange of metabolites and immunomodulatory signals between both organs. While numerous studies have shown that the impact of immune stressors on microbiota composition and function during the crucial developmental stages is usually temporary (Cox et al., 2014), certain alterations, such as dysbiosis-induced obesity, can persist even after the gut microbiota has returned to a normal state (Cox et al., 2014). This highlights the enduring metabolic and developmental impacts of dysbiosis. Given that diet plays a significant role in maintaining a healthy gut microbiota and immune homeostasis, recent research has focused on the effectiveness of nutritional interventions in preventing gut microbial disruptions to improve overall health and prevent immune-related disorders (Yoon & Yoon, 2018; S. Zhang & Chen, 2019). While natural components have garnered significant interest for their potential in rebalancing gut microbiota and mitigating the adverse effects of dysbiosis, our understanding of the molecular mechanisms driving these effects remains incomplete (Neuman et al., 2018).

External immune stressor, such as LPS during puberty can disrupt the gut microbiota and cause immune alterations that significantly impact the developmental programming of other organs, such as the lungs. Some dietary interventions can mitigate these changes and prevent immune dysregulation. ESMH demonstrated anti-inflammatory and immune-modulatory effects (Jia et al., 2017b; Shi et al., 2014).

In this study, we investigated the potential role of ESMH in mitigating inflammation in the gut-lung axis, using an LPS-induced inflammation and dysbiosis mice model at puberty. The effect of ESMH on the gut microbiota composition as well enduring epigenetic modifications was studied.

Effect of pubertal exposure to LPS and ESMH on gut microbiome

We studied the effect of ESMH on the gut microbiota in pubertal mice, twenty-four hours after LPS injection. LPS exposure led to an increase in the abundance of Bacteroidota in both groups exposed to LPS when compared to the control and ESMH groups, which suggests that LPS increases the Bacteroidota population and leads to an imbalance in the Firmicutes/Bacteroidota ratio. An increase in this ratio is associated with IBD and its progression (Stojanov et al., 2020). At the family level, Lachnospiraceae population increased in all treated groups, however this increase was higher in LPS+ESMH group. Lachnospiraceae species produce SCFAs in the gut, therefore lowering inflammation (Vacca et al., 2020). This highlights the protective role of ESMH in reducing Bacteroidetes population and initiating gut homeostasis and production of metabolites.

We found a higher abundance of *Alistipes* in the LPS group compared to the control, however, ESMH decreased the effect of LPS on this genus in LPS+ESMH group when compared to the control. In addition, a reduction in *Lactobacillus* population was found in both groups exposed to LPS mice compared to the control, however, ESMH intake could decrease the effect of LPS on this genus in LPS+ESMH group when compared to the control. *Lactobacillus* species have been shown to decrease inflammatory responses via the inhibition of IL-6, TLR4, and NF-κB signaling (Y. Liu et al., 2012). Furthermore, *Candidatus arthromitus* abundance decreased in LPS+ESMH compared to ESMH and control. *Candidatus arthromitus* is a segmented filamentous bacteria (SFB) involved in Th17 differentiation in the gut (Hedblom et al., 2018). This investigation

highlights the protective and anti-inflammatory role of ESMH on the microbial diversity in the gut, therefore reducing inflammation.

Effect of pubertal exposure to LPS and ESMH on selected cytokines related to Th17 cells in pubertal mice at the gut and lung levels

In this study, at the gut level we found an increase in the IL-17, which was not significant, and IL-6 levels in pubertal mice exposed to LPS when compared to the control and ESMH, however, ESMH intake in mice exposed to LPS could not inhibit the stimulatory effects of LPS on these cytokines. Moreover, IL-10 levels also were upregulated in all treated groups. These results reveal robust effects of acute exposure to LPS, causing an increase in anti- and pro-inflammatory cytokines production at the gut level. These results are constant with previous studies reflecting immunomodulatory effects of LPS on different anti- and pro-inflammatory cytokines expression, such as IL-10, IL-6, and IL-1β in bloods and/or other organs such as the intestine (Shahbazi et al., 2023; Yahfoufi et al., 2023).

At the lung level, IL-17A levels was not significantly higher in mice exposed to LPS alone, while IL-6 levels increased in ESMH, significantly, and the increase in LPS + ESMH was not significant. Additionally, IL-10 levels were higher in mice fed ESMH alone, regardless of LPS exposure. These results may show the effect of ESMH in mice, which upregulated IL-10 and IL-6 levels. However, acute LPS exposure did not exert any significant immunostimulatory effects at the gut level after ; this might be associated with the dosage or route of LPS injected in this study (1.5 mg/Kg BW) compared to other studies (Abdelmageed et al., 2016). However, an explanation for the increase in IL-6 expression could be that this cytokine is highly dynamic, often exhibiting a temporary peak during the early stages of immune responses (Kishimoto, 2010).

IL-6 plays central role in the Th17 differentiation and IL-17A production by inducing phosphorylation and activation of STAT3 pathway, which is a key signaling pathway in regulating differentiation of Naïve T cells into Th17 cells by stimulating expression of RORγt. Anti-inflammatory IL-10 inhibits Th17 differentiation and its inflammatory responses (Shahbazi et al., 2023) . Th17 cells are proinflammatory CD4+ T cells, known to secret proinflammatory cytokine IL-17A, that contribute to the pathogenesis of intestinal inflammation and airway diseases (Fogli et al., 2013; Zhao et al., 2021). Th17 cells contribute to the IBD progression by secreting proinflammatory cytokines such as IL-17A, IL-17F, IL-21, and IL-22 (L. Chen et al., 2023). However, in the lungs, overexpression of IL-17 has been shown to initiate lung epithelium alterations, leading to inflammation, and impaired lung function (Fogli et al., 2013).

In summary, this part of the research plan investigated ESMH's potential as a modulatory agent against LPS-induced inflammatory responses, with implications in understanding cytokine behaviors in inflammation and treatment strategies. These findings contribute valuable insights into the cytokine-mediated mechanism underlying dysbiosis and therapeutic potential of ESMH in modulating these processes in the gut-lung axis.

Effect of pubertal exposure to LPS and ESMH on lasting alteration in miRNAs expression related to Th17 cells in adult mice at the gut and lung levels

We studied the miRNA expression in the intestine and lungs and DNA methylation status of genes related to the immunity in the lungs of mice, to investigate the constant effect of LPS exposure and ESMH intake on the gut-lung axis and immunity.

In the acute experiment, in both intestine and lungs, miR-146a and pro-inflammatory miR-222 and miR-223 were reduced by LPS. In the enduring experiment, in the intestine and the lungs, the pro-

inflammatory miRNAs, miR-155, miR-222 and miR-223 were increased by LPS, where ESMH treatment could alleviate the effect of LPS on these miRNAs. This disparity might be because LPS might not have exerted its effect; due to the short-term exposure of LPS (24 hours post-injection).

miR-146a is a negative regulator of NF-κB signaling in response to a bacterial infection (Casciaro et al., n.d.; B. Wei & Pei, 2010) as well as targets the tumor necrosis factor receptor-associated factor 6 (TRAF6) and interleukin 1 receptor associated kinase 1 (IRAK1) which regulate the NF-kB signaling in T-helper cells, blocks the NF-kB-mediated secretion of IL-6/IL-21, and inhibits the development of pathogenic Th17cells (J. Huang et al., 2021b). It is a key regulator in innate immune response. LPS/TLR4 signaling pathway triggers an inflammatory cascade via the recruitment of IRAK1 and TRAF6 to promote NF-κB activation, thus initiating the production of pro-inflammatory cytokines (Kawai & Akira, 2007). Therefore, over expression of miR-146a inhibited LPS-induced pro-inflammatory cytokines secretion through the down regulation of the expressions of TLR4, IRAK1 and NF-kB (Y. Chen et al., 2016).

The upregulation of miR-222 is associated with increased proliferation and initiating oxidative stress in the lungs (Tepebaşı & Öztürk, 2023), as well as contributing to the pathogenesis of gut mucosal atrophy, damaged mucosa, and epithelial barrier dysfunction (Wang et al., 2017).

On the other hand, some studies indicate that miR-223 might have a dual role; regulating the inflammatory response during acute lung injuries (Haneklaus et al., 2013). Moreover, miR-223 has been shown to regulate the macrophage differentiation by targeting the NLRP3 inflammasome using mouse models, where lung inflammation is mediated by macrophages (D. Zhang et al., 2019). Also, recent evidence pinpoints the role of miR-223 in controlling innate immunity for protection against excess inflammation and tissue damage; modulating the immune response to inflammatory stimuli in myeloid cells (X. Yuan et al., 2018). Moreover, miR-223 can be down-

regulated in dendritic cells (Bros et al., 2018). Another study demonstrated that miR-223 reduced inflammation, supressed NLRP3 inflammasome and negatively regulated the TLR4/NF-kB signaling pathways, which served as a potent positive regulator of inflammation in lung injury (Yan et al., 2019).

miR-223 may promote the secretion of cytokines by dendritic cells, regulating Th17 and Tregs differentiation from naïve T cells, as well as promotes pathological Th17 cells differentiation during autoimmunity (Atabaki et al., 2020b; Jiao et al., 2021). miR-223 plays a potential role in maintaining the Th17/Treg balance (Jiao et al., 2021). miR-223 regulates immune cell proliferation, differentiation, and polarization of inflammatory macrophages, as its down-regulation in macrophages reduces the inhibition of STAT gene, promoting the release of LPS-induced IL-6 and IL-1β, ultimately promoting muscle tissue inflammation, exacerbation and injury (Chen et al., 2012).

Additionally, studies have shown that miR-155 is associated with various disorders such as cardiovascular diseases, inflammation and cancer (Faraoni et al., 2009) and plays a vital role in the stability of Th17 phenotype. Increased levels of miR-155 has been seen in patients with inflammatory bowel disease (IBD) and colorectal cancer (Xiao et al., 2009). miR-155, as a promoter of inflammatory response, plays a crucial role by regulating the activation of immunocytes and release of inflammatory cytokines (Wan et al., 2016). It has been shown to promote the secretion of cytokine by dendritic cells, therefore to further regulate Th17 cell differentiation, which was associated with the development of chronic intestinal inflammation and other autoimmune diseases (Bi et al., 2020b). This miRNA can activate the release of Th17 cells (Atabaki et al., 2020b).

This investigation into miRNA expression patterns revealed their significant role in inflammation in the gut and the lungs, with ESMH treatment showing potential in modulating these miRNAs to exert protective effects against LPS in the enduring and acute stages. This part of the thesis aimed to investigate miRNA involvement in inflammation/dysbiosis and gut-lung axis biology, offering insights into ESMH's potential therapeutic role in modulating inflammatory pathways and miRNA expressions related to inflammation.

Effect of pubertal exposure to LPS and ESMH on lasting alteration in DNA methylation of genes related to the Th17 cells mediated immunity in adult mice at the lung level

DNA methylation analysis revealed, in LPS-challenged mice, a significant hypomethylation of CpGs was identified within the promoter of *Tnfaip3*, *Tnip1, Lcn2, Sox2ot, Ermap,* and *Cxcl11* as compared to the control. Moreover, *Rorα, Mcc* and *Prkcb* were hypomethylated within the intronic region, however, *Socs7* was hypermethylated within the exon region in LPS-induced mice in comparison to the control. In ESMH+LPS group, hypermethylation of promoter regions of *Tnfaip3, Tnip1* and *Nlrc4* genes was observed compared to LPS. *Socs7* was hypomethylated within the exon region. Some of these genes were involved in critical signaling pathways such as IL-17 signaling pathway, NF-kappa B, JAK-STAT and PI3K-AKT signaling pathways. The analysis of the differentially methylated genes of *Tnfaip3, Tnip1, Lcn2, Sox2ot, Ermap, Cxcl11, Rorα, Mcc, Prkcb* and *Socs7* by LPS were mitigated by ESMH.

This investigation sheds light on the intricate interplay between DNA methylation and inflammation in the lungs. This effect can be mitigated by dietary approaches such as peptides and hydrolysates, specifically ESMH, also highlighting the therapeutic potential of ESMH in adjusting DNA methylation landscapes.

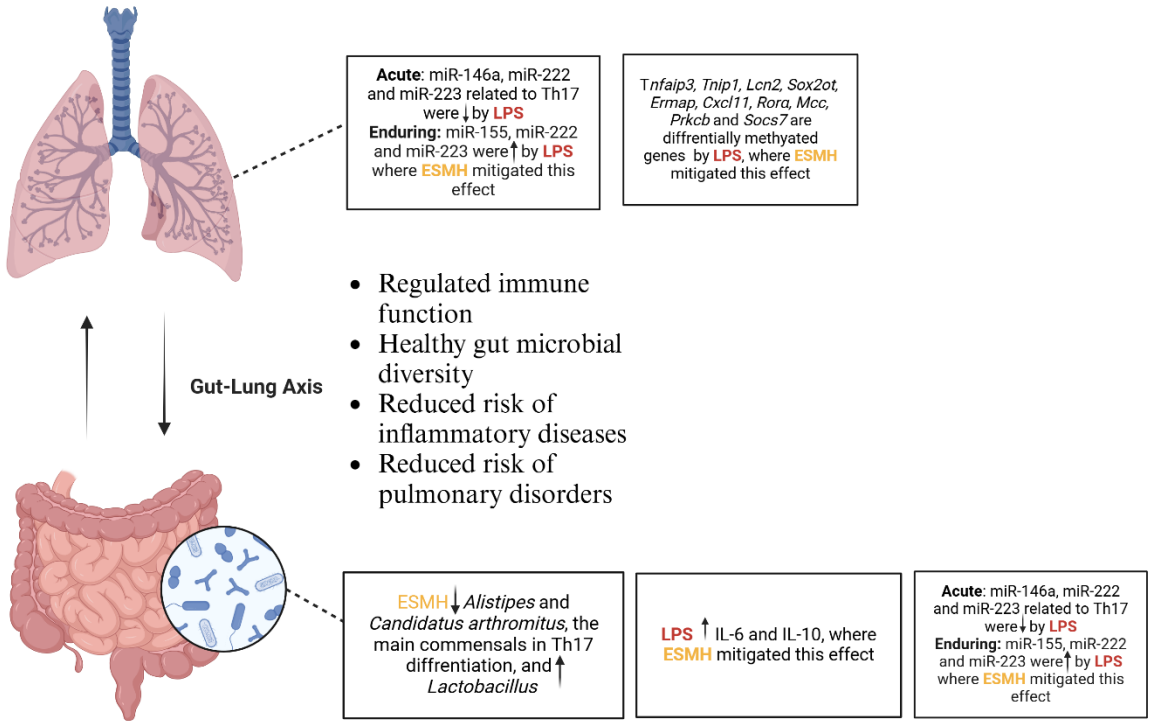

Figure 3. Summary of the outcomes of the study in the gut-lung axis. Image was created by BioRender.com (Eladham et al., 2024; Enaud et al., 2020; Robichaud et al., 2021).

3.2 Limitations of Study

Our study has some limitations as follows:

In this research only female mice were applied to study the effect of treatment on gut microbiome and epigenetic changes related to the gut-lung immunity, however, there is a sex difference in immune system function because of differences in sex hormones (Kane & Ismail, 2017). Also, there is a sex difference in gut microbiota composition, starting at the onset of puberty which is associated with sex hormones (Calcaterra et al., 2022).

To better decipher the impact of inflammation on the gut-lung axis, immune cells such as T cells population could have been measured by flow cytometry to rule out the changes triggered by LPS.

As the study is targeting the gut-lung axis, as well as Th17, mechanisms related to both, should be further discussed, and studied, to target the specific mechanisms related to this axis. Moreover, cytokines, associated with Th17, can also be elucidated to determine the levels/extent of inflammation. Moreover, flow cytometry studies would be helpful to discriminate if IL-17 is related to Th17 or other immune cells.

The gut dysbiosis leads to the dysregulation in the production of certain metabolites, such as SCFA production. We did not measure the SCFA levels, which could highlight the connection between immune dysregulation and inflammation between the gut and its distal organs, particularly the lungs.

The DNA methylation analysis showed various functions of the genes in the lungs with altered methylation levels, suggesting a potential epigenetic change in the genes crucial for the gut-lung axis development. However, some significant dysregulations in miRNAs, were shown, which play a significant role in essential signaling pathways. Therefore, further analysis of miRNA as well as mRNA levels for more genes from DNA methylation or miRNA targets would further consolidate the role of ESMH in reducing inflammation in gut-lung axis, in LPS-induced mice.

3.3 Future Directions

Research highlighted the mechanisms and characteristics of the exposure to any external stressor, such as inflammation that may result in persistent immune dysfunction and having a leaky gut, therefore affecting other organs such as the gut-lung axis; evidence suggesting that epigenetic changes can be inherited by the second generation.

Our findings may demonstrate the use of this novel product, ESMH, in improving gut homeostasis, immunity and microbiota composition, as well as reflecting an optimal strategy for immune

function in the critical developmental periods of life and controlling inflammation and dysbiosis later in life. ESMH can be studied in the prevention of gut-lung injury related to external stressors/challenges with respiratory microbes, as well as studying the interplay between the lung microbiota and the gut.

3.4 Conclusion

Our research demonstrated that the exposure to an immune stressor during the critical developmental stages of puberty disrupts the gut microbiota and leads to immune deregulation in both the gut and lungs. This may also cause lasting epigenetic modifications that contribute to persistent immune dysfunction at the gut-lung axis. Overall, our findings suggest that consuming eggshell membrane hydrolysate may serve as an effective strategy to prime the immune system and support its homeostasis throughout life. Furthermore, this study sheds light on the mechanisms by which external stressors, such as inflammation, can cause enduring/acute immune dysfunction, perturbation and dysbiosis, affecting the gut-lung axis; proving that epigenetic changes may be inherited by the upcoming generations. This study broadens our understanding of how gut dysbiosis influences immune response and the mechanisms underlying the crosstalk between the gut and the lungs. It highlights the beneficial effect of ESMH in mitigating gut dysbiosis during the critical developmental stages in life, specifically puberty. This research particularly illuminates the mechanisms by which perturbations in the gut microbiota affects the whole immune system/response as well as distal organ and alters the gene expression.

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