The Role of the Gut-Breast Axis during Critical Windows of Development and its Effects on Mammary Gland Development Later in Life

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Cellular and Molecular Medicine

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Abstract

During critical developmental periods such as pregnancy, early life, and puberty, when the mammary gland is developing, any perturbations in the gut microbiome can lead to long-lasting disturbances in mammary gland homeostasis and may predispose individuals to breast cancer later in life. These perturbations, often triggered by exposure to immune stressors such as low-dose penicillin or lipopolysaccharides (LPS), can disrupt the gut microbiota equilibrium. Notably, prebiotics have emerged as a countermeasure to inflammation and microbiota imbalances caused by antibiotics or LPS exposure, though the mechanisms underlying their effectiveness have not been fully discovered. This thesis hypothesizes that antibiotic exposure during pregnancy and early life, or LPS exposure during puberty, could disrupt mammary gland homeostasis, thereby predisposing individuals to breast cancer in later life. Conversely, prebiotic intake may ameliorate disturbances associated with antibiotic- or LPS-induced inflammation by modulating the immune system epigenetically through microRNAs (miRNAs) and DNA methylation. This research aims to deepen our understanding of the mechanisms by which prebiotic intake, specifically Lentinula edodes cultured extract (AHCC), offers protective effects against the lasting immune deregulation associated with exposure to immune stressors during pivotal developmental phases. The objectives are:

1. To investigate the effectiveness of AHCC supplementation during puberty in mitigating LPS-induced inflammation and its corrective effects on mammary gland homeostasis and breast cancer risk. This will involve a comprehensive assessment of the structure and morphology of mammary glands, cytokines, miRNAs, and DNA methylation. Additionally, the study aims to measure tumor growth, the number of cancer stem cells, and the level of inflammatory cytokines in the tumor and adjacent mammary glands.

2. To explore whether AHCC intake can mitigate the potential impacts of prenatal and early-life antibiotic exposure on the development of mammary glands. This involves assessing the influence of early low-dose penicillin (LDP) on the maternal immune response, and gene expression of lactating mammary glands. The research will also analyze the immune response in offspring and gene expression of mammary glands at genetic and epigenetic levels.

This study utilizes Balb/c mice, exposing them to LPS at puberty or low-dose penicillin (LDP) during pregnancy to assess the effects on mammary glands and tumor development. LDP exposure involves feeding dams in the last week of gestation through weaning. The study examines the impact of AHCC intake on mitigating the long-term consequences of LPS or LDP by analyzing immune responses, gene and miRNA expression, and DNA methylation in mammary glands and tumors.

These studies highlight the effectiveness of AHCC, a prebiotic, in preventing gene dysregulation in critical genes such as Pdgfc, Ksr1, and Il2rb, especially in LPS-exposed groups. AHCC mitigates disruptions in cytokine levels (IL-1β, IL-10, IL-17a/f, IL-23) and miRNAs (let-7a/c, miR-34a, miR-130a) in mammary glands due to puberty LPS exposure. It also counters LPS-induced cytokine and miRNA alterations in tumor samples, particularly affecting IL-1β, IL-6, and IL-10 levels, along with the TGF-β family. AHCC regulates cytokine expression, reduces the IL-6 upsurge in mammary glands, and increases tumor-suppressing miRNAs (miR-125, miR-140, and miR-200c), contrasting the LPS-induced rise in oncogenic miRNAs (miR-21, miR-92, and miR-155). It also controls immune responses to LDP-induced dysbiosis by altering IL-10, IgG, and IL-6 levels, and affecting a broad range of genes (Fgf1, Fgfr2, Fzd7) and miRNAs (let-7a/c, miR-34b, and miR-146a), showcasing its regulatory impact on mammary gland homeostasis through gene and microRNA expression modulation.

This is the first time that the role of the gut-breast axis has been studied. The data will shed light on the importance of preventing gut dysbiosis during critical windows of development in the mammary gland during pregnancy and adolescence.

Acknowledgments

First and foremost, I extend my heartfelt thanks to Dr. Chantal for granting me the chance to embark on my Ph.D. journey within her laboratory. Her unwavering support and encouragement have been pivotal throughout my Ph.D. studies. I am deeply thankful for her guidance and inspiration, which have been crucial in advancing my research endeavors.

My sincere appreciation goes to my distinguished committee members, Dr. Dylan Burger, Dr. Lisheng Wang, Dr. Nafissa Ismail, and Dr. Benjamin Tsang, for their thorough review of my TAC reports and their valuable feedback and insights that have significantly enhanced my research work.

I am grateful to Dr. Zdenko Herceg, Dr. Felicia Fei-Lei Chung, Cyrille Cuenin, and Dr. Vincent Cahais at the International Agency for Research on Cancer, World Health Organization, for their assistance in epigenetic analysis. Their expertise has been instrumental in the progress of my research.

A special note of thanks to my colleague and friend, Roqia Shahbazi, for her invaluable support, recommendations, and collaboration throughout my research journey. Roqia has been more than just a lab colleague; she has become a cherished member of my family. I also extend my gratitude to all my friends in Dr. Matar's Lab, Nawal AlSadi, Mary Joe Hebbo, Darshan Babu Kambli, Dr. Maria Florencia Balacells, Dr. Taha Azad, Dr. Farzaneh Sharifzad, Dr. Jean-Francois Mallet, and Nour Ibrahim, for their support and camaraderie.

I am thankful for the cooperation of the staff at the animal care facility and the Louise Pelletier Histology Core Facility, with special thanks to Dr. Ardeshir Ariana and Dr. Marjan Khalili for their guidance and support. My gratitude also extends to Dr. Alex Green, Dr. George Cairns, and Abolfazl Nik-Akhtar for their training, mentorship, and valuable advice.

This thesis is a tribute to my parents, the pillars of my existence. My mother, Akram Ghasemkhani, whose sacrifices have paved the way for my pursuits, your faith in my aspirations and your constant support have been my guiding light. My father, Mehran Yasavli Sharahi, your sacrifices and

encouragement to pursue knowledge has ignited my passion for learning. Your faith in my dreams and your wisdom have been my guiding stars. To my precious siblings, Fatemeh and Mohammad, and to my dear sister Razieh, whose memory remains a beacon of love and hope in my heart.

To my dearest partner, Nasim B. Sahebi, your unwavering support has been the foundation of my strength. Your love, patience, and dedication have not only been a source of comfort but have also brought color and joy into my life. Your presence has been influential, guiding me towards becoming the best version of myself. I am deeply grateful for every moment of support and kindness you have shown me.

My heartfelt appreciation also extends to Mr. Ali B. Sahebi, Dr. Azita Bassiri, and Parsa B. Sahebi, for their gracious support and kindness. Your contributions have enriched this journey immeasurably.

I extend my gratitude to all those who have contributed to this research, named or unnamed. Your support has been invaluable.

Contents

List of Abbreviation

Short Chain Fatty Acids	SCFAS
Lipopolysaccharide	LPS
Aryl Hydrocarbon Receptor	AHR
Toll Like Receptors	TLR
Pattern Recognition Receptors	PRRS
Non-Alcoholic Fatty Liver Disease	NAFLD
Hypothalamic-Pituitary-Adrenal	HPA
Mucosal-Associated Lymphoid Tissue	MALT
Gut-Associated Lymphoid Tissue	GALT
Dendritic Cells	DCS
Messenger Rna	MRNA
Micrornas	MIRNAS
Primary Transcript	PRI-MIRNAS
Rna-Induced Silencing Complex	RISC
Argonaut	AGO
Intestinal Epithelial Cells	IECS
Fusobacterium Nucleatum	F NUCLEATUM
Escherichia Coli	E COLI
Inflammatory Bowel Disease	IBD
Extracellular Vehicles	EVS
Mirna-Induced Silencing Complex	MIRSC

Oncogenic Mirnas	ONCOMIR
S-Adenyl Methionine	SAM
Dna Methyltransferases	DNMTS
5-Methylcytosine	5MC
Base Excision Repair Pathway	BER
Thymine Dna Glycosylate	TDG
5-Carboxylcytosine	5CAC
5-Hydroxymethyluracil	5HMU
Pteridine Precursors	DHPP
P-Aminobenzoic Acid	PABA
Tricarboxylic Acid Cycle	TCA
Epigallocatechin-3-Gallate	EGCG
Terminal End Bud	TEBS
Matrix Metalloproteinases	MMPS
Extracellular Matrix	ECM
Epithelial To Mesenchymal Transition	EMT
Whey Acidic Protein	WAP
Estrogen	E2
Estrogen Receptor Alpha	ESR1
Amphiregulin	AREG
Fibroblast Growth Factor	FGF
Progesterone	P4
Progesterone Receptor	PR

Growth Hormone	GH
Insulin-Like Growth Factor 1	IGF-I
Epidermal Growth Factor Family	EGF
Epidermal Growth Factor Receptor	EGFR
Fibroblasts Growth Factor	FGF
Fibroblasts Growth Factor Receptors	FGFRS
Transforming Growth Factor Beta	TGFB
Vascular Endothelial Growth Factor	VEGF
N-3 Polyunsaturated Fatty Acids	PUFA
World Health Organization	WHO
Hydrogen Ion	H+
Non-Esterified Fatty Acids	FFAS
Estrogen Receptor	ER
Human Epidermal Growth Factor Receptor	HER2
Cancer Stem Cells	CSCS
Breast Cancer Stem Cells	BCSCS
Lentinula Edodes Cultured Extract	AHCC
Differentially Methylated Regions	DMRs
Differentially Methylated Positions	DMPs
Transcriptional Start Sites	TSS
Urinary Tract Infections	UTI
Sexually Transmitted Infections	STIs
Pelvic Inflammatory Disease	PID

Bacterial Vaginosis	BV

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Chapter 1: Introduction

1.1 Gut Microbiota

1.1.1 General introduction

The concept of microbiota consists of the entire range of microorganisms present in the human body, such as bacteria, fungi, and viruses. It is worth mentioning that the bacterial population in the body is roughly 30 trillion, a figure remarkably similar to the number of human cells (Sender, Fuchs et al. 2016). Moreover, the human gut alone has been identified to host over 9 million bacterial genes (Li, Jia et al. 2014). The majority of gut microbiota comprises commensal bacteria that coexist harmlessly with the body. Although the two terms "microbiota" and "microbiome" are frequently used synonymously, they have different meanings. Microbiota refers to the live microorganisms found in a specific environment like the gastrointestinal tract, while microbiome represents a broader concept, including the entire collection of genomes, microbial structure, and metabolites of these microorganisms (Berg, Rybakova et al. 2020). The composition of microbiota varies according to its anatomical location. The gastrointestinal tract, in particular, is recognized as the body's largest microbial community. The gastrointestinal microbial community is composed of six primary phyla, including Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia, with Firmicutes and Bacteroidetes being the dominant ones (Laterza, Rizzatti et al. 2016). As mentioned, bacteria are not the only microorganisms living in the gut; our gastrointestinal tract is the host of different types of fungi, including Candida, Saccharomyces, Malassezia, and Cladosporium (Auchtung, Fofanova et al. 2018), viruses, phages, and archaea (Lozupone, Stombaugh et al. 2012) (Figure 1.1).

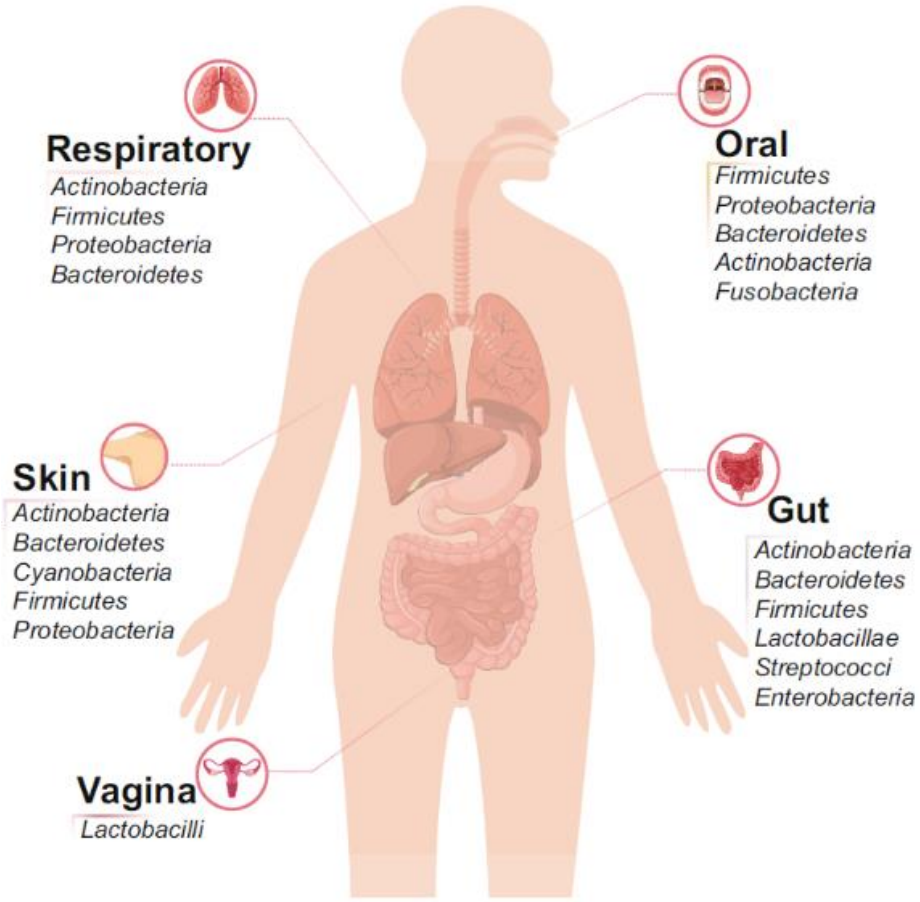

Figure 1.1. Illustration of human microbiota. The dominant microbiota of each anatomical site is highlighted (Hou, Wu et al. 2022).

1.1.2 Factors affecting gut microbiota:

Several factors affect the balance of the gut microbiota, including diet, birth mode and breastfeeding, lifestyle, medication, and genetics. **Diet** is one of the critical factors impacting gut microbiota composition. High consumption of saturated or monounsaturated fats adversely impacts the gut microbiota. On the other hand, diets rich in polyunsaturated fats appear to have a neutral effect. Diets high in polysaccharides are linked to an increased population of bacteria producing short-chain fatty acids (SCFAs), weight loss, and balanced cytokine and metabolome profiles (Cani, Bibiloni et al. 2008). **Birth mode and breastfeeding** significantly affect gut

microbiota composition, contributing to the infant's risk of developing allergies. A growing body of research reveals that cesarean birth can reduce the diversity of crucial bacteria like Bifidobacterium, Bacteroides, and Lactobacillus. This reduction is associated with a lower number of regulatory T cells and a higher chance of allergic reaction (Francino 2018). Breastfed infants tend to have less diverse but more beneficial gut microbiota, enriched with Bifidobacterium, Streptococcus, Staphylococcus, and Lactobacillus. In contrast, formula-fed infants have higher colonization rates of E. coli, Clostridium difficile, and Bacteroides (Kim, Sitarik et al. 2019). **Lifestyle** is another factor that significantly contributes to the gut microbiome and its composition. Obesity is linked to changes in gut microbiota, including an increase in the population of Firmicutes and a decrease in Bacteroidetes, which is associated with higher energy absorption and inflammation (Turnbaugh, Ley et al. 2006). In contrast, exercise modifies gut microbiota by enhancing the capacity to break down lactate and amino acids and increase short-chain fatty acid production (Allen, Mailing et al. 2018). The **genetic composition** of the host and medication usage, specifically antibiotics, are key influencers that affect the gut microbiota balance. People in developing countries often exhibit a reduction in gut microbiota diversity compared to traditional lifestyles, potentially due to the consumption of antibiotics. This reduction affects the population of beneficial gut microbiota including Bacteroides, Prevotella, Desulfovibrio, Lactobacillus, and Oxalobacter. Given the variety of factors that influence the formation of gut microbiota, there is no gold standard reference for the composition of gut microbiota.

1.1.3 The Impact of Gut Microbiota

Gut microbiota fulfills several essential functions such as maintaining the integrity of the gastrointestinal track, developing immune systems, and facilitating the digestion of food (Lynch and Pedersen 2016).

1.1.3.1 Maintaining gastrointestinal track's integrity: Substantial evidence suggests that gut microbiota are involved in maintaining the structure of the intestine by producing antimicrobial peptides, metabolites that regulate the immune system (Heintz-Buschart and Wilmes 2018). One-way commensal microbiota protect the gut barrier is by preventing pathogenic bacteria from colonizing the intestine. Previous studies have provided information concerning the effect of bacteria on tight junctions, crucial for barrier integrity. An imbalanced gut microbiota provides the chance for pathogenic bacteria to attach to cell walls and activate the MAPK signaling pathway, which is essential for regulating the expression of tight junctions. The activation of the MAPK signaling pathway, or its components JNK and ERK, inhibits the expression of occludin, claudin-1, and ZO-1, which are the main parts of tight junctions. Microbiome dysbiosis increases the level of lipopolysaccharide (LPS), leading to a leaky gut, which allows bacteria or their products (such as LPS) to cross through the epithelium and activate the immune response (Yu, Wang et al. 2012, Quigley 2017). Changes in gut permeability through diet-induced dysbiosis or immune stressors like LPS are accompanied by changes in gut function and systemic inflammation (Hiippala, Jouhten et al. 2018, Régnier, Van Hul et al. 2021). LPS is considered a major inducer of inflammatory responses, suggesting a possible association between LPS and susceptibility to cancer (Martinelli, Reginatto et al. 2020). Circulating LPS load in plasma adds the burden not only on the gut but also on distant organs, including the mammary gland (Kobayashi, Uejyo et al. 2013, Lai, Liu et al. 2017).

Another mechanism by which microbiota maintain gut barrier homeostasis is by producing a broad range of metabolites such as SCFAs and tryptophan metabolites. SCFAs boost antimicrobial peptide production and aid in the expansion and maturation of regulatory T cells (Schnupf, Gaboriau-Routhiau et al. 2018). Furthermore, SCFAs are vital for the modulation of the intestinal barrier by promoting the proliferation and maturation of intestinal cells (Lazar, Ditu et al. 2018). Tryptophan metabolites, products of dietary fermentation, act as ligands for the aryl hydrocarbon receptor (AhR) that is involved in the homeostasis of the intestine. Lack of this metabolite is associated with inflammatory bowel disease (Rannug 2020). Additionally, the metabolites produced by gut microbiota, such as SCFAs, are important in the proliferation of Tregs and innate lymphoid cells, which release IL22, important in the induction of antimicrobial molecules by epithelial cells (Yang, Yu et al. 2020).

1.1.3.2 Development of immune systems: Research involving germ-free mice has shown that these mice have less effective regulatory T cells and more naive T cells. Commensal microbiota not only provide immunity against pathogens but also expose T cells to antigens, aiding their maturation. Germ-free mice's Tregs show a lower level of essential proteins like Foxp3 and CTLA-4 for Treg development (Fontenot, Gavin et al. 2003). These mice also exhibit reduced IL-13 and IL-10 levels, indicating impaired Th2 responses. Underdeveloped Treg cells can lead to ineffective immune regulation and a higher risk of autoimmune diseases (Östman, Rask et al. 2006). Additionally, commensal bacteria prime the macrophages in the intestine, increasing the level of IL-1β and neutrophil cell numbers (Franchi, Kamada et al. 2012). Colonizing germ-free mice with Bacteroides fragilis has been associated with correcting $CD4^+$ T cell deficiencies and restoring the balance between Th1 and Th2 cells (Mazmanian, Liu et al. 2005). The absence of gut microbiota impacts immune cell development and Toll-like receptor (TLR) production

(Cario, Gerken et al. 2007). This suggests that gut microbiota regulate inflammation by affecting the expression of pattern recognition receptors (PRRs) like TLRs and NOD receptors (Sanderson and Walker 2007).

1.1.3.4 Food digestion: The gut microbiota plays a crucial role in digesting food, influencing various metabolic processes such as breaking down dietary fiber, fermenting proteins and peptides, and metabolizing bile acids. This activity supports essential functions like energy production, weight management, and appetite control. For instance, probiotic bacteria enhance bile acid conjugation, leading to lower cholesterol levels (St-Onge, Farnworth et al. 2000). The fermentation of carbohydrates by gut microbes produces short-chain fatty acids (SCFAs) like propionate and butyrate, which benefit gut and liver health and help combat obesity by promoting the release of hormones that suppress appetite (Bloemen, Venema et al. 2009). These SCFAs also reduce cholesterol production by downregulating the HMGCR gene in intestinal cells. Additionally, the gut microbiota synthesizes vital vitamins such as vitamin K, crucial for various bodily functions. Bacterial metabolism also produces phenols with anti-inflammatory and disease-preventing properties, contributing to the management of conditions like diabetes and cancer (Waldecker, Kautenburger et al. 2008, Ambriz-Pérez, Leyva-López et al. 2016).

1.1.4 The Role of Gut Microbiota in Health

As mentioned, gut microbiota can be easily affected by a wide array of factors including a sedentary lifestyle, unhealthy diet, and widespread antibiotic use either directly with clinical administration or indirectly through food (Shondelmyer, Knight et al. 2018). Antibiotic usage during pregnancy affects the bacterial environment of the mother and the fetus [6] and predisposes to childhood obesity and an increased risk of immune disorders that persist for several years after exposure, even after cessation of antibiotic intake (Kuperman and Koren

2016). In Western countries, antibiotic treatment during pregnancy accounts for 80% of prescribed medications. Projections of global antibiotic consumption by 2030 suggest increases of up to 200% (Klein, Van Boeckel et al. 2018). Moreover, it is estimated that by five years of age, up to 97% of children have already received at least one course of antibiotics, with a median of 8 antibiotic courses (Hobbs, Grant et al. 2017). Dysbiosis can occur even after a single course of antibiotics with long-lasting residual metabolic and immune consequences (Pushpanathan, Mathew et al. 2019). Gut dysbiosis is linked to different types of diseases, including obesity, type 2 diabetes, liver disease, and cancer.

1.1.4.1 Liver Disease: Non-alcoholic fatty liver disease (NAFLD), prevalent in 20–40% of the adult population in many countries (Younossi, Koenig et al. 2016), shows distinct gut microbiota patterns compared to healthy individuals, with increased levels of specific bacteria like Clostridium, Anaerobacter, Streptococcus, Escherichia, and Lactobacillus bacteria, and decreased populations of Oscillibacter, Flavonifaractor, Odoribacter, and Alistipes (Jiang, Wu et al. 2015). NAFLD patients often have more ethanol-producing bacteria such as E. coli, potentially damaging the gut barrier and increasing the risk of endotoxemia (Zhu, Baker et al. 2013).

1.1.4.2 Cancer: There is a growing body of evidence linking intra-abdominal infection and antibiotic use to an increased rate of colorectal cancer (Wang, Chang et al. 2014). Gut microbiota can directly affect intestinal cells, either promoting oncogenesis with hydrogen sulfate and Bacteroides fragilis toxin or inhibiting uncontrolled cell proliferation with SCFAs production (Louis, Hold et al. 2014). Dysbiosis, leaky gut, and resulting endotoxemia increase inflammatory factors and metabolic changes, leading to a higher incidence of hepatocellular carcinoma or breast cancer (Yoshimoto, Loo et al. 2013, Xuan, Shamonki et al. 2014). Certain bacteria such as

Helicobacter pylori and Escherichia coli are capable of inducing DNA breaks, contributing to genomic instability and tumor initiation (Frisan 2016). Some pathogenic bacteria can induce oxidative stress and inflammation or block immune cells, resulting in tumor growth. Bacteria like Clostridium leptum and Clostridium coccoides are able to increase the bioavailability of estrogen, affecting hormone metabolism and increasing the risk of hormone-responsive cancers such as breast cancer (Vivarelli, Salemi et al. 2019).

Cancer cells may develop drug resistance through interaction with the gut microbiome and its metabolites (Langdon, Crook et al. 2016). For instance, Tryptophan can be metabolized into various indole derivatives by Lactobacilli. These indoles act as ligands for the Aryl hydrocarbon Receptor. Research indicates that breast tumor cells often upregulate AhR expression and utilize Tryptophan metabolism to inhibit the apoptosis pathway (Bekki, Vogel et al. 2015). The efficacy of cancer drugs can be impacted by the gut microbiome. For instance, doxorubicin, a chemotherapy drug, can be inactivated through deglycosylation by Streptomyces WAC04685 and Parabacteroides distasonis. Studies have shown that mice lacking Parabacteroides distasonis exhibit a better response to doxorubicin treatment (Kverka, Zakostelska et al. 2011, Westman, Canova et al. 2012). These findings underscore the intricate relationship between the microbiome and cancer therapies, highlighting the potential for tailored treatment strategies that consider the gut microbiome's influence.

1.1.5. Prebiotics

The significant influence of prebiotics on immune response homeostasis within the gut is well-documented. They play a crucial role in various processes such as metabolism regulation, gene expression, inhibition of pathogens, and priming the immune system (Bengmark 2013, Williams, Stedtfeld et al. 2017). These elements are essential for maintaining and modulating the gut

microbial ecosystem and have been shown to reduce systemic inflammation (de LeBlanc, Matar et al. 2006, Mallet, Graham et al. 2016, Vuong, Mallet et al. 2016). Prebiotics are widely researched for their beneficial effects on microbiota and potential as chemoprotective agents. Research has highlighted the effectiveness of prebiotics in combating mammary carcinoma (de Moreno de LeBlanc, Matar et al. 2006, Mallet, Graham et al. 2016) and mitigating the adverse effects of antibiotics through dietary intervention. In the realm of cancer prevention and treatment, prebiotics have shown promising results, partly due to their ability to influence gene expression through epigenetic factors, affecting metastasis and carcinogenesis pathways (Riaz Rajoka, Jin et al. 2018). The restorative impacts of prebiotics on the gut microbiota include modulation of DNA methylation and microRNA expression (Kwa, Plottel et al. 2016) and preventing immune disorders (Cowan and Richardson 2019). These findings underscore the significant role of the gut microbiome in affecting health both within the gut and at distant sites (Kwa, Plottel et al. 2016).

Microbial metabolites, such as indoxyl sulfate, can reduce cancer stem cells' resistance to drugs by inhibiting the pregnane-X receptor (PXR) and the aryl hydrocarbon receptor (AXR) (Sári, Mikó et al. 2020).

Prebiotics like lithocholic acid can diminish breast cancer metastasis by priming the immune system and enhancing the anti-tumor properties of immune cells (Mikó, Vida et al. 2018). Urolithins, gut microbiome metabolites, suppress breast tumor development by targeting the Wnt signaling pathway (Lv, Shi et al. 2019) and modulate drug resistance by influencing ABCG2/BCRP transporters (González-Sarrías, Miguel et al. 2013). Short-chain fatty acids (SCFAs) produced by E. coli exhibit probiotic properties, inducing cytotoxicity in MCF-7 cells. N-(3-oxododecanoyl)-L-homoserine, produced by Pseudomonas aeruginosa, reduces the

viability of MDA-MB-231 breast cancer cells through apoptosis induction and proliferation inhibition (Balhouse, Patterson et al. 2017).

AHCC, recognized as a prebiotic with notable immunomodulatory properties, plays a significant role in modulating immune responses both at the gut level and throughout the body. Research indicates that AHCC supplementation enhances the population of IgA+ cells and boosts the production of secretory IgA within the intestinal tract, key components in the body's defense mechanism (Mallet, Graham et al. 2016). Additionally, AHCC exerts a regulatory influence on the production of inflammatory cytokines, managing to do so without triggering a strong pro-inflammatory reaction. Studies have demonstrated AHCC's capacity to mitigate the IL-6 responses following E. coli infection, an effect believed to be facilitated through its interaction with Toll-like receptors TLR2 and TLR4 on the intestinal epithelium, subsequently impeding the NF-kB and MAPK signaling pathways (Daddaoua, Martínez-Plata et al. 2013, Mallet, Graham et al. 2016). Further research has highlighted AHCC's potential to augment the levels of $CD8^+$ cells, a marker positively correlated with enhanced overall survival rates (Suknikhom, Lertkhachonsuk et al. 2017).

1.1.6 Gut Microbiota in Pregnancy and Infant

Research indicates that the composition of gut microbiota undergoes significant changes during pregnancy. In the first trimester, the gut microbiota resembles that of a healthy non-pregnant woman. However, as pregnancy progresses beyond the first trimester, the gut microbiota composition shifts, becoming dominated by Actinobacteria and Proteobacteria (Koren, Goodrich et al. 2012). These changes are important for a normal pregnancy, aiding in weight gain, boosting maternal metabolism, and supporting fetal growth (Koren, Goodrich et al. 2012, Gohir, Whelan et al. 2015). The imbalance of gut microbiota during pregnancy has significant

implications for maternal and fetal health and can cause health conditions like gestational diabetes mellitus (GDM), pre-eclampsia, and preterm birth (PTB). Studies have shown that fecal microbiota transplantation (FMT) from women who developed GDM later in pregnancy to germ-free mice demonstrated higher insulin resistance and higher levels of cytokines, two criteria leading to the development of GDM (Pinto, Frishman et al. 2023). This condition not only affects the women but also the infants born to these women, showing a different meconium metabolome compared to healthy ones (Chen, Qin et al. 2021). Preterm birth has been associated with the vaginal microbiome. Moreover, studies have shown that reduced alpha-diversity of the gut microbiota and higher excretion of omega-3 in stool are associated with PTB in women with a low-fiber and high-fat diet (Gershuni, Li et al. 2021). Studies on preterm infant meconium and germ-free mice showed growth restriction and abnormal hormonal levels. Moreover, the mice showed high levels of inflammation and metabolic disturbance (Hiltunen, Hanani et al. 2021, Koren, Konnikova et al. 2024).

The establishment of a healthy gut microbiota in infants is crucial for their survival and proper development. Human breast milk plays a significant role in this process, as it contains a unique microbiota that evolves throughout the breastfeeding period. The biological process whereby maternal gut microbiota is transferred to the mammary glands is known as the entero-mammary pathway.

Interestingly, the gut microbiota of infants is influenced not only by the mother’s skin bacteria but also by her mammary glands. Research has revealed that lactic acid bacteria in human breast milk differ from those on the skin (Martín, Heilig et al. 2007, Martín and Langella 2019). However, Lactobacillus and Bifidobacterium bacteria from breast milk originate from the mother's gut (Soto, Martín et al. 2014). Studies identifying identical genotypes of bacteria such

as *L. plantarum, L. fermentum, L. brevis, E. faecium, E. faecalis, and P. pentosaceus* in the mother's gut, breast milk, and infant's feces suggest a direct transfer from the mother's gut to the infant's gut (Albesharat, Ehrmann et al. 2011). Infants with a lower number of *Enterococci* and *Bifidobacteria* in their gut are more susceptible to gastrointestinal disease and allergies (Björkstén, Sepp et al. 2001). Furthermore, infants with colic have been found to have fewer Bifidobacteria and Lactobacilli in their feces (Sung, D'Amico et al. 2018).

1.2 Epigenetics

1.2.1 Overview of Epigenetic

The epigenetic field explores alterations in gene expression that are not a result of DNA sequence mutations. The underlying mechanisms of these changes include processes like DNA methylation, histone modification, and RNA-based processes including microRNA (Goldberg, Allis et al. 2007). Gene expression is crucial for cell survival, differentiation, and development, involving the conversion of genetic information into RNA, and ultimately protein (Bagot and Meaney 2010). This process begins with the transcription of DNA into messenger RNA (mRNA), which is then subjected to post-transcriptional modification. These modifications include the addition of a methylated cap and splicing of introns, transforming the mRNA into its mature form. Epigenetic modulate gene expression at both transcriptional and post-transcriptional stages (Cramer 2019).

1.2.2 MicroRNA (miRNA, miR):

MicroRNAs (miRNAs) are short, non-coding RNA sequences, typically consisting of almost 22 nucleotides. miRNAs play a pivotal role in post-transcriptional gene regulation. To date, over 3000 distinct miRNAs have been identified across species, including humans (Dexheimer and Cochella 2020). miRNAs are pivotal in maintaining critical biological functions, such as cell

differentiation, proliferation, and apoptosis (Huang, Shen et al. 2011). Dysfunctions in miRNA synthesis can result in developmental issues and the pathogenesis of various diseases, such as cancer (Garzon, Calin et al. 2009, Paul, Sadek et al. 2019). Advancements in technologies such as high-throughput sequencing have facilitated the identification of new miRNAs and the prediction of their target mRNAs (Osada and Takahashi 2007).

1.2.2.1 miRNA: From Biogenesis to Post-Transcriptional Gene Regulation

The synthesis of miRNAs begins with the transcription of the miRNA's gene by RNA polymerase II, resulting in the production of primary transcripts (pri-miRNAs) that range from a few hundred to several thousand nucleotides in length. The pre-miRNAs are subsequently processed by a protein complex composed of DROSHA and DGCR8 into pre-miRNAs of 60 to 100 nucleotides, which are then transported into the cytoplasm by exportin 5. In the final step, these pre-miRNAs are incorporated into the RNA-induced silencing complex (RISC) complex, which consists of Dicer, Argonaut (Ago) complex, and TRBP (Winter, Jung et al. 2009). Dicer's role is to remove the terminal loop (hairpin consisting of 55-70 nucleotides) from pre-miRNAs and transform them into double-stranded miRNAs. Subsequently, the passenger strand of miRNAs is removed, and the Ago complex is prepared for expression (Ha and Kim 2014) (Figure 1.2).

1.2.2.2 The Function of miRNA in Gene Regulation

A growing body of evidence has revealed the multifaceted role of miRNAs in gene expression regulation. Typically, mature miRNAs exert their regulatory effect by binding to the sequence of target mRNAs that contain complementary bases to their seed region, consisting of 2-8 nucleotides. This process is facilitated by the Ago protein complex, which carries the mature miRNA, thereby enabling effective base-pairing. Subsequently, the Ago complex, consisting of

Ago1 to Ago4, in conjunction with the TNRC6 protein, mediates repression of target mRNA, either by promoting degradation or inhibiting translation. Notably, within the Ago complex, Ago 2 is capable of directly cleaving the target mRNAs (Wahid, Shehzad et al. 2010). Interestingly, miRNAs have the capacity to bind to the 5'UTR of mRNA, potentially enhancing ribosome biogenesis, which in turn escalates protein biosynthesis at the cellular level (Ling, Fabbri et al. 2013).

1.2.2.3 Interaction Between miRNA and Host Microbiota in Gene Regulation

Emerging studies have indicated that the gut microbiota can influence miRNA expression in the gut. The interplay between gut microbiota and miRNAs occurs in the intestinal epithelial cells (IECs), triggering key signaling pathways and modulating gene expression dynamics (Dong, Tai et al. 2019).

Gut dysbiosis has been associated with disruption in the gut lining and inflammatory response. Notably, IECs have the capability to generate fecal miRNAs, which are essential in regulating cellular integrity on the intestinal lining and can target bacterial mRNA, thereby influencing bacterial survival and/or activity (Behrouzi, Ashrafian et al. 2020). For instance, miR-515-5p inhibits the growth of *Fusobacterium nucleatum* (*F. nucleatum*), while miR-1226-5p enhances the growth of *Escherichia coli* (E. coli) and *F. nucleatum.*

F. nucleatum is capable of upregulating oncogenic miR-21 expression (Yang, Weng et al. 2017) while E. coli has the ability to suppress miRNAs crucial for the maintenance of tight junction integrity, potentially enhancing intestine permeability or causing a "leaky gut" (Liu, Da Cunha et al. 2016). Variations in miRNA expression, such as miR-199a and miR-548a, can affect the population of *F. nucleatum*, E. coli, and segmented filamentous bacteria, potentially triggering inflammatory bowel disease (IBD) (Ji, Li et al. 2018). Furthermore, miRNAs modulate the

absorption of bacterial products. For instance, miR-193 has been reported to downregulate the expression of the PepT1 transporter, thereby reducing bacterial intake (Dalmasso, Nguyen et al. 2010). miRNAs exert a bidirectional effect on cellular processes within intestinal cells, playing a pivotal role in maintaining the integrity of the intestinal barrier. Studies have revealed that gut microbiota can exert a significant impact on miRNA expression, thus modulating host gene expression. Extracellular vesicles (EVs) produced by gut microbiota contain specific miRNAs that regulate gene expression (Severus, Florentina et al. 2021). Pathogenic bacteria such as Enteropathogenic Escherichia coli can inhibit the expression of miR-203, miR-483-3p, and miR-595, resulting in inflammation and compromised intestinal integrity (Veltman, Hummel et al. 2012). Similarly, bacteria from the Proteobacteria, Firmicutes, and Bacteroidetes phyla can enhance the expression of miR-182, miR-503, and miR-17-92, thereby elevating cancer risk (Yuan, Burns et al. 2018, Behrouzi, Ashrafian et al. 2020). *Helicobacter pylori* escalate the expression level of miR-143-3p, thus amplifying the risk of gastric cancer by inhibiting AKT2, a protein crucial for cell apoptosis (Wang, Liu et al. 2017). Additionally, E. coli has been identified to trigger extensive immune responses by suppressing Let-7b expression, leading to an increase in TLR4 expression (Guo, Cai et al. 2018).

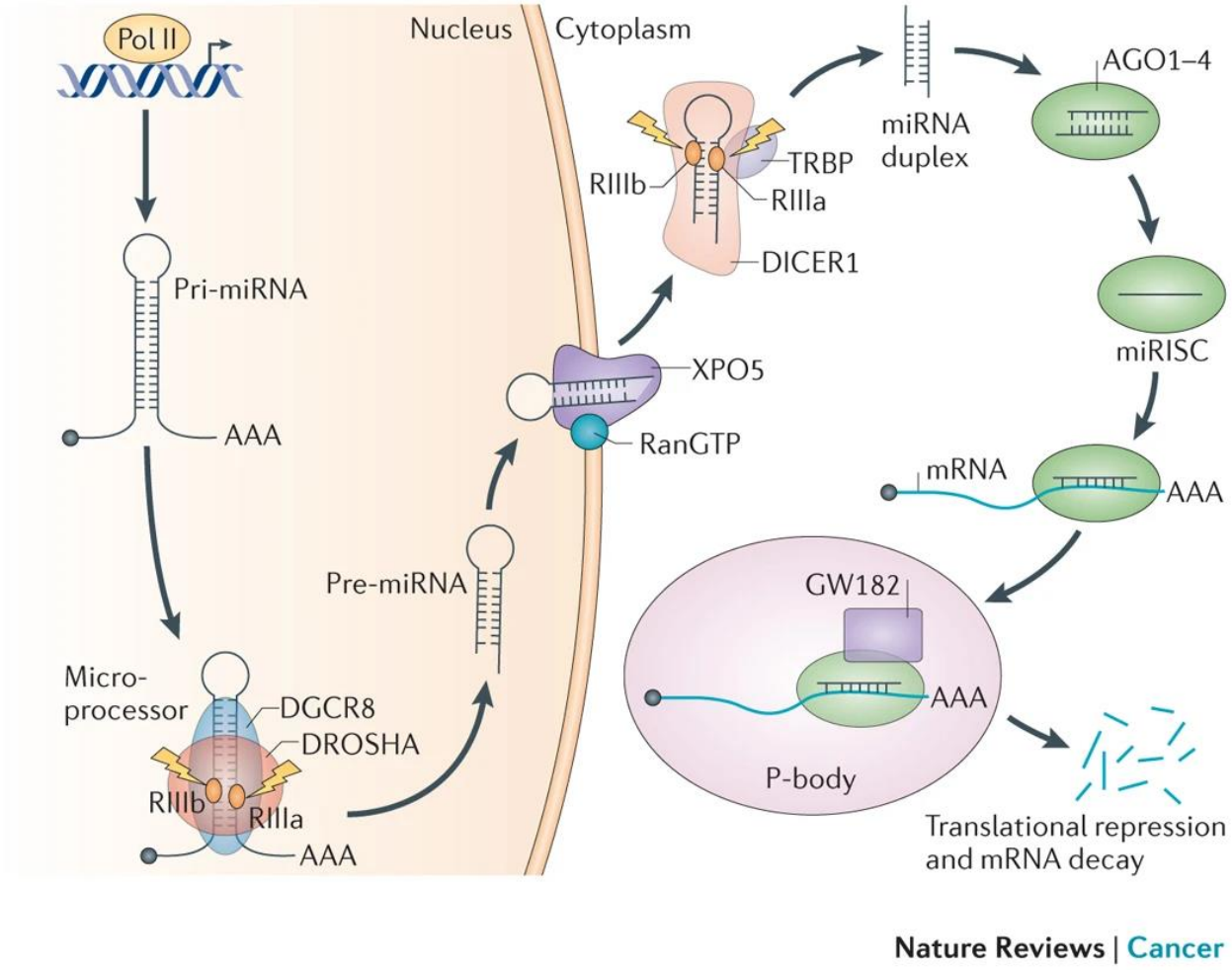

Figure 1.2. The biogenesis of microRNAs. miRNAs genes undergo transcription to form primary miRNAs (pri-miRNAs) via RNA polymerase II in the nucleus. There pri-miRNAs are then processed by the DROSHA and DGCR8 into pre-miRNAs. Subsequently, there pre-miRNAs are transported to the cytoplasm by exportin 5. In the cytoplasm, DICER1, further modifies the pre-miRNAs into mature miRNAs. The miRNAs incorporated one strand into the miRNA-induced silencing complex (miRSC), which includes both DICER1 and AGO proteins. This complex then directs itself to specific mRNAs based on sequence complementary, leading to gene suppression (Lin and Gregory 2015).

1.2.2.4 The Role of miRNA in Oncogenic Pathways

In the realm of cancer, miRNAs are classified into two categories: tumor suppressors or oncogenic miRNAs (oncomiRs). Dysregulation in miRNA expression results in changes in gene expression patterns, contributing to the development of various diseases such as cancer. For example, abnormal levels of miR-15 and miR-16, which are typically involved in suppressing

the anti-apoptotic protein BCL-2, have been associated with leukemia development (Cimmino, Calin et al. 2005). The Let-7 family, downregulated in lung and breast cancer, targets oncogenes like Ras, HMGA2, and c-MYC, thus inducing apoptosis and cell cycle arrest (Johnson, Grosshans et al. 2005, Lee and Dutta 2007). Another key miRNA, the miR-29 family, targets anti-apoptotic proteins such as MCL1 and plays a critical role in suppressing tumor growth (Mott, Kobayashi et al. 2007). miR-34a is noted for inhibiting the proliferation of mammary stem cells by targeting genes involved in the Wnt signaling pathway, thereby promoting differentiation (Bonetti, Climent et al. 2019). miRNA profiling of the pubertal mouse mammary gland identifies miR-184 as a candidate breast tumor suppressor gene (Phua, Nguyen et al. 2015). miR-184, identified as a potential breast tumor suppressor, inhibits metastasis in triple-negative breast cancer by regulating genes in the Akt/mTORC1 signaling pathway (Phua, Nguyen et al. 2015). Conversely, miR-21, upregulated in a wide array of cancers, can block apoptosis in cancers like glioblastoma, breast, and liver cancer through targeting PTEN (Pfeffer, Yang et al. 2015). The miR-17-92 cluster, consisting of six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1), is often found elevated in various tumors and promotes cell proliferation, angiogenesis, and apoptosis inhibition, mainly through targeting E2F and c-MYC genes (Olive, Jiang et al. 2010). miR-372 and miR-373 inhibit apoptosis by targeting LASTS2 and modulating the TP53 signaling pathway (Di Leva, Garofalo et al. 2014, Wang, Yu et al. 2018).

1.2.3 DNA Methylation

DNA methylation is a critical cellular process where a methyl group is transferred from S-adenyl methionine (SAM) to the fifth carbon of a cytosine, forming 5mC on CpG islands. This process is mediated by a group of enzymes called DNA methyltransferases (DNMTs), which include

DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L (Denis, Ndlovu et al. 2011, Lyko 2018). Within this family, DNMT1, DNMT3A, and DNMT3B are recognized as canonical cytosine 5 DNMTs, primarily responsible for the methylation of CpG islands. Conversely, DNMT2 and DNMT3L, lacking the catalytic sequence, represent evolutionary adaptations of the original DNMT enzymes (Lyko 2018).

DNA methylation plays a pivotal role in various cellular processes, including the regulation of gene expression, genomic imprinting, and X chromosome inactivation (Riggs 2002). This epigenetic mechanism is observed in diverse genomic regions, including intergenic regions, CpG islands, and gene bodies, and regulates gene expression (Smith and Meissner 2013). Notably, approximately 45% of the mammal genome consists of viral elements, predominantly silenced through methylation. The hypomethylation of these viral elements, particularly within intronic regions, poses a risk of genomic instability due to their potential to translocate within the genome (Schulz, Steinhoff et al. 2006).

CpG islands, categorized by lower nucleosome density, are part of the DNA sequence predominantly located near the promoter (Tazi and Bird 1990). Interestingly, about half of these CpG islands harbor specific transcriptional start sites (Carninci, Sandelin et al. 2006). These regions are usually rich in GC content, enhancing their role in facilitating transcription factor binding and accessibility to the DNA (Carninci, Sandelin et al. 2006). DNA methylation in the promoter regions is commonly associated with gene suppression. However, methylation patterns in other genomic regions, such as intragenic areas and gene bodies, often reflect tissue-specific methylation profiles (Maunakea, Nagarajan et al. 2010). CpG island shores, extending up to 2kb from the CpG islands, also exhibit patterns of tissue-specific methylation (Irizarry, Ladd-Acosta et al. 2009). Additionally, gene bodies, particularly the regions following the first exon, tend to

exhibit increased gene expression in actively dividing cells when methylated (Hellman and Chess 2007, Ball, Li et al. 2009, Brenet, Moh et al. 2011).

1.2.3.1 Role of Methylation in Gene Expression Changes

The underlying mechanism of DNA methylation involves three classes of enzymes: writers, erasers, and readers (Torres and Fujimori 2015). The **writers** category includes enzymes from the DNMTs family such as DNMT1, DNMT3A, and DNMT3B (Torres and Fujimori 2015). DNMT1 plays a critical role in maintaining the DNA methylation pattern. Over the course of cell division, it replicates the methylation pattern of the parental DNA strand onto the new strand (Hermann, Goyal et al. 2004). Additionally, DNMT1 has the capability to repair DNA methylation (Mortusewicz, Schermelleh et al. 2005). Disruption of DNMT1 in mouse embryos leads to significant apoptosis and embryonic fatality between stages E8 and E10.5 (Li, Bestor et al. 1992). DNMT3A and DNMT3B both have the capacity to methylate both naive and synthetic DNA strands (Okano, Bell et al. 1999, Xie, Wang et al. 1999). While DNMT3B's expression is primarily during embryonic development, DNMT3A is predominantly expressed in differentiated tissues (Xie, Wang et al. 1999). While DNMT3B's expression is primarily during embryonic development, DNMT3A is predominantly expressed in differentiated tissues (Ge, Pu et al. 2004). The specific targeting mechanism of DNMT3A and DNMT3B to DNA sequences is not fully understood. However, it is hypothesized that transcription factors may influence DNA methylation. These factors could potentially regulate methylation by binding to distinct DNA sequences, thus either directing DNMTs to methylate this sequence or preventing their methylation (Brenner, Deplus et al. 2005).

Erasers of DNA methylation consist of both passive and active demethylation processes. 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, on the other hand, occurs in both dividing and non-dividing cells and involves enzymatic processes to remove the methyl group from cytosine, converting 5-methylcytosine (5mC) back to cytosine (Oswald, Engemann et al. 2000).

The active process may involve deamination or oxidative reactions that produce intermediates recognizable by the base excision repair (BER) pathway. Various mechanisms have been proposed for active DNA demethylation, including the involvement of Tet enzymes, AID/APOBEC enzymes, Thymine DNA Glycosylase (TDG), and SMUG1 (Rai, Huggins et al. 2008, Cortellino, Xu et al. 2011). Typically, this process begins with DNA glycosylases identifying and removing modified bases (e.g., deaminated cytosine) from the DNA strand. Subsequently, DNA polymerase B fills the gap with new nucleotides, thus completing the demethylation process (Gehring, Reik et al. 2009) (Figure 1.3).

The process of **reading** DNA methylation involves the repression of DNA transcription, facilitated by various proteins such as MBD proteins, UHRF proteins, and Zinc-finger proteins (Moore, Le et al. 2013). MBD proteins, characterized by their methyl-CpG-binding domain, exhibit a strong affinity for methyl groups on cytosine residues. Their binding to these sites generally leads to the suppression of transcription in the respective DNA regions (Nan, Meehan et al. 1993).

UHRF proteins, distinguished by their ubiquitin-like, PHD, and RING finger domains, are capable of identifying hemimethylated DNA sites. Unlike MBD proteins, the primary role of UHRF proteins extends beyond transcription suppression. They actively participate in the maintenance of DNA methylation, facilitating the recruitment of DNMT1 to hemimethylated DNA, thereby preserving the methylation status (Bostick, Kim et al. 2007).

The zinc-finger protein family, including Kaiso, ZBTB4, and ZBTB38, demonstrates a unique binding preference (Filion, Zhenilo et al. 2006). While the ZBTB family generally attaches to unmethylated cytosine, Kaiso specifically binds to two consecutive methylated CpG sites (Daniel, Spring et al. 2002). Similar to MBD proteins, the zinc-finger proteins also play a role in suppressing DNA transcription, contributing to the regulation of gene expression influenced by the methylation status (Filion, Zhenilo et al. 2006).

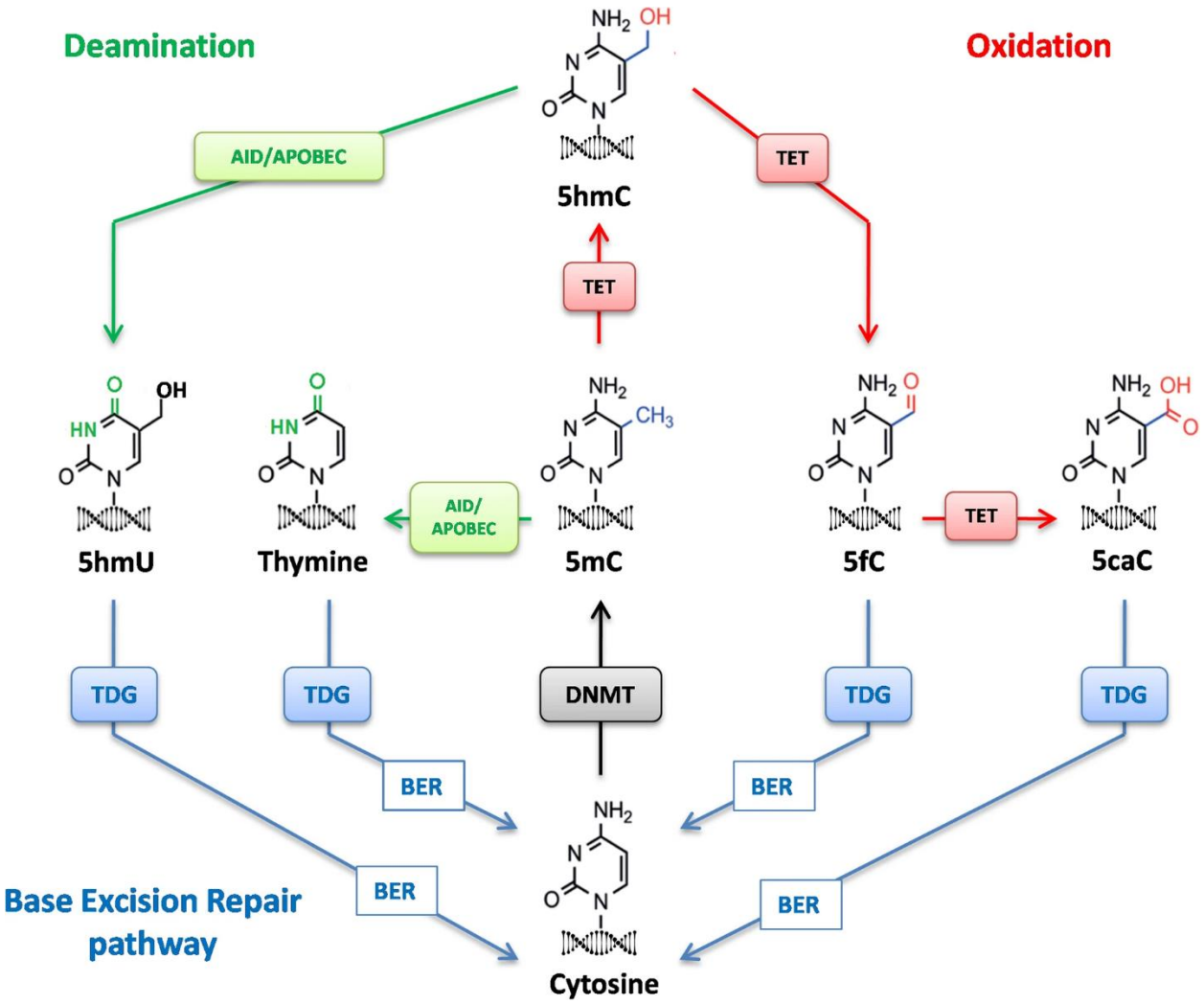

Figure 1.3. The metabolism of DNA methylation. This process involves a series of enzymatic processes that modify cytosine bases within the DNA. The primary step in this pathway is the addition of a methyl group to a naked cytosine, which is catalyzed by DNMTs (The black arrows). Once a cytosine is methylated from 5mC, it can undergo further modifications. The TET

enzymes oxidize 5mC to 5hmC. This oxidation can progress further, converting 5hmC to 5-formylcytosine and then to 5-carboxylcytosine (5caC), as indicated by the red arrow. Parallel to this, there is a deamination pathway, shown in green arrows, where enzyme AID or APOBEC can deaminate 5hmC to 5-hydroxymethyluracil (5hmU) or 5mC to thymine. This pathway represents another route by which modified cytosines can be created. Finally, these various modified bases are recognized and processed by the enzyme TDG. TDG plays a critical role in the BER pathway (Shown in blue arrows). Through BER, these modified bases are eventually converted back to naked cytosine, thus completing the cycle of DNA methylation and demethylation. This intricate process allows for dynamic regulation of gene expression through epigenetic modifications (Celarain and Tomas-Roig 2020).

1.2.3.2 DNA Methylation and microRNAs

DNA methylation and miRNAs are interconnected in a complex regulatory network that influences gene expression. This interplay can occur in various ways:

1.2.3.2.1 Regulation of miRNAs by DNA Methylation: DNA methylation, specifically involving enzymes like DNMT1 and DNMT3B, has been observed to impact the expression of miRNAs. Studies have shown that the loss of these DNMTs leads to a 10% increase in miRNA expression (Han, Witmer et al. 2007). This suggests that DNA methylation acts as a regulatory mechanism for controlling miRNA levels.

1.2.3.2.2 miRNAs Affecting DNA Methylation Patterns: miRNAs can also indirectly influence DNA methylation, particularly through histone modifications. This is exemplified in studies involving mice deficient in Drosha, an essential enzyme in the miRNA process. Despite unchanged DNMT1 levels in these mice, their cells displayed hypomethylation. This observation suggests that the Drosha complex, rather than Dicer, interacts with DNMT1 to enhance its activity (Benetti, Gonzalo et al. 2008).

1.2.3.2.3 Targeting of DNA Methylation Machinery by miRNAs: Some miRNAs can directly target and regulate proteins involved in DNA methylation. For instance, miR-125 targets the

3’UTR of Drosha and Dicer, leading to a reduction in their expression (Frixa, Sacconi et al. 2018). This mechanism provides a feedback loop whereby miRNAs can control their own production by influencing the enzymes that are crucial for their maturation. Another example is miR-29b, which targets proteins like DNMTs and TET2, directly involved in DNA methylation processes (Zhang, Cao et al. 2018).

1.2.3.3 DNA Methylation and Gut Microbiota

The interaction between gut microbiota and host epigenetics, particularly DNA methylation, is a critical aspect of the microbiota-nutrient metabolism-host epigenetic axis. This interaction is influenced by dietary factors and significantly impacts the host’s epigenetic landscape. Key points in this interaction include:

1.2.3.3.1 Association Between Gut Microbiota and DNA Methylation Patterns: Studies, including those on pregnant women, have demonstrated a strong correlation between the composition of gut microbiota and DNA methylation patterns in the host. For instance, an abundance of Firmicutes in mothers is linked to distinct methylation profiles, with specific genes being hypermethylated or hypomethylated, affecting pathways related to cardiovascular health, lipid metabolism, obesity, and inflammatory response (Kumar, Lund et al. 2014). Studies on germ-free mice indicate that in the absence of gut microbiota leads to increased DNA hypermethylation in colonic epithelial cells. This is attributed to reduced TET activity and lowered circulating folate levels (Takahashi, Sugi et al. 2011). Additionally, these mice exhibit altered methylation in the promoter of TLR4, which plays a role in immune regulation (Takahashi 2014). Infections with bacteria like Helicobacter pylori and Klebsiella spp have been linked to hypermethylation of specific genes such as LOX, THBD, FLNC, HRASLS, and HAND1 associated with gastric cancer risk (Maekita, Nakazawa et al. 2006). Individuals with

varying ratios of Firmicutes:Bacteroidetes show differences in DNA methylation genes related to glucose and energy homeostasis (Ramos-Molina, Sánchez-Alcoholado et al. 2019). This suggests the role of gut microbiota composition in influencing metabolic gene regulation.

1.2.3.3.2 Gut Microbiota Metabolites and DNA Methylation Patterns: Essential components like folic acid, vitamin B2, B6, and B12, produced by gut microbiota, are crucial for generating S-adenosylmethionine, the primary methyl donor for DNMTs and histone methyltransferases. Lactobacillus and Bifidobacterium, known for their role in regulating folate by producing pteridine precursors (DHPP) and p-aminobenzoic acid (pABA), are affected by antibiotics and the overall composition of the gut microbiota (Kok, Steegenga et al. 2018). This suggests that the biosynthesis of folate is highly dependent on the composition of gut microbiota. SCFAs, particularly Acetyl-CoA, influence the tricarboxylic acid cycle (TCA), influencing DNA methylation. The product of TCA, Alpha-Ketoglutarate, assists in DNA methylation, whereas fumarate and succinate can inhibit TET enzymes, leading to increased DNA methylation (Miro-Blanch and Yanes 2019). Studies on mice have shown that butyrate-producing bacteria like Anaerostipes can exert a tumor-suppressive effect on colon cancer. Butyrate decreases the expression of DNMT1 and hypermethylates the promoter of key proteins such as β-catenin and e-cadherin involved in the Wnt signaling pathway (Pan, Lam et al. 2017). In tumor cells, butyrate accumulation can trigger apoptosis and inhibit proliferation due to its function as a histone deacetylase inhibitor (Donohoe, Holley et al. 2014).

1.2.3.3.3 Effects of Dietary Compounds on DNA Methylation: Epigallocatechin-3-Gallate (EGCG), a compound found in certain plants, has been observed to influence DNMTs' activities, particularly in some cancers like breast cancer (Paluszczak, Krajka-Kuźniak et al. 2010). This effect of EGCG extends to modifying the gut microbiota composition, as seen in studies where

its supplementation is associated with a decreased ratio of Firmicutes:Bacteroidetes, leading to lower levels of the inflammatory cytokine IL-6 in the colon. Additionally, EGCG has been shown to impact DNA methylation patterns, specifically leading to the hypermethylation of the promoters of MLH1 and DNMT1 genes in the liver of mice (Remely, Ferk et al. 2017). These findings suggest that EGCG not only has a potential role in cancer therapy but also modulating gut microbiota composition and epigenetic modifications.

1.2.3.4 Distinct Methylation Alterations Associated with Cancer

Disruption in the DNA methylation process can lead to diseases, including cancer. Cancer cells often have distinct DNA methylation patterns compared to normal cells. In general, cancer cells exhibit a global reduction in DNA methylation, particularly in CpG islands (Lakshminarasimhan and Liang 2016). This hypomethylation contributes to genomic instability and the activation of oncogenes. Conversely, hypermethylation in the promoter regions of specific genes, especially tumor suppressor genes, can alter cell characteristics, leading to increased proliferation (Paz, Fraga et al. 2003).

1.2.3.4.1 DNA Hypomethylation in Cancer: In normal cells, genomic integrity is preserved by methylation of pericentromeric heterochromatin (Fioriniello, Marano et al. 2020) and repetitive intronic sequences like LINE, SINE, IAP, Alu elements (Weisenberger, Campan et al. 2005). In cancer cells, hypomethylation in these regions can lead to genomic instability through mitotic recombination or activation of transposable elements. Hypomethylation in specific regions of certain genes, such as SSX, DDX43, MAEL, and PIWIL1, has been linked to tumorigenesis (Van Tongelen, Loriot et al. 2017). For example, in colorectal and breast cancers, hypomethylation in the CDH3 promoter region causes an overexpression of p-cadherin, which is associated with increased cell invasion and migration. Additionally, the hypomethylation of viral

sequences integrated into the genome, such as in HPV infections, can lead to cervical cancer (Milicic, Harrison et al. 2008). Interestingly, while demethylating enzymes are not active in cancer cells, DNMT genes are often overexpressed. It is proposed that overexpression of inactive DNMT3b variants, which lack the catalytic domain, might negatively regulate DNA methylation by competing with the active DNMT3B forms (Ostler, Davis et al. 2007).

1.2.3.4.2 DNA Hypermethylation in Cancer: In cancer cells, hypermethylation of specific regions, leading to silencing of genes, is critical. For example, in retinoblastoma, there is a high level of hypermethylation in the promoter of the tumor suppressor gene RB1. This DNA methylation-associated silencing is a hallmark in many types of cancer. The hypermethylation of the promoter regions of CDKN2A and CDKN2B, crucial in cell cycle regulation, results in increased cell proliferation (Drexler 1998). Additionally, genes involved in DNA repair, such as MGMT, MLH1, and BRCA1, which are involved in DNA alkylation, DNA mismatch repair, and DNA repair of double-stranded break repair, are often hypermethylated in various cancers (Esteller, Hamilton et al. 1999, Fleisher, Esteller et al. 2001, Catteau and Morris 2002).

1.3 Overview of Mammary Glands

Mammary glands originated from surface ectoderm and are made from highly modified sweat glands of the skin (Khan and Sajjad 2019). Mammary gland development begins embryonically with the formation of bilateral milk lines, which evolve into mammary placodes determining the future location of the nipple (Hinck and Silberstein 2005). These placodes then develop into epithelial bulbs, which infiltrate the mesenchyme layer (Sakakura, Kusano et al. 1987). Following this, mammary buds advance into the fat pad, establishing a basic ductal tree that remains inactive until puberty (Figure 1.4).

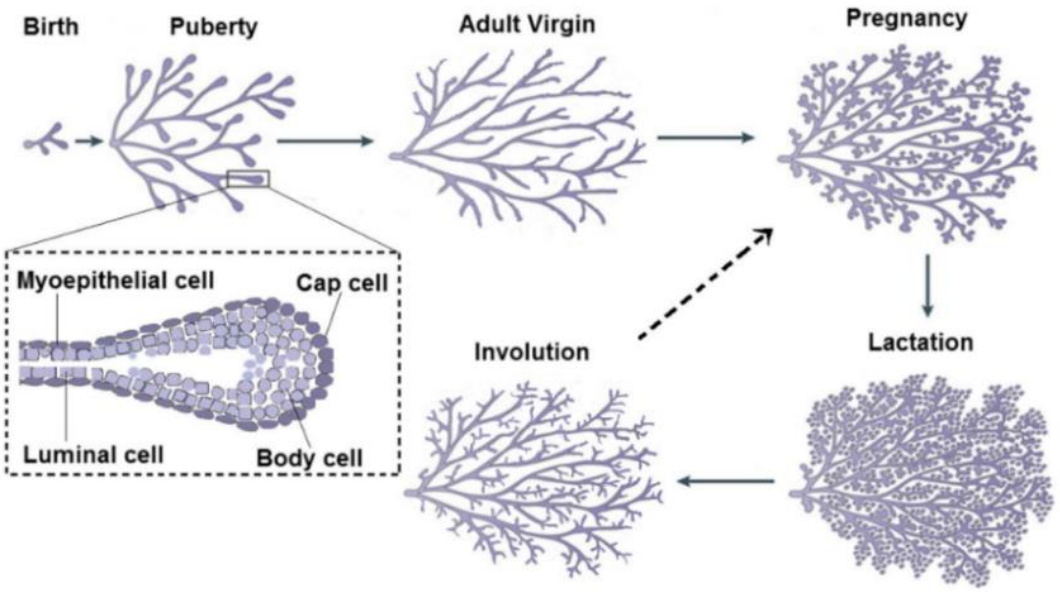

Figure 1.4. The anatomy of the mammary gland and its development. At the beginning of puberty, with the increased level of ovarian products, TEBs start to invade the fatty pad. Through pregnancy by increasing estrogen and progesterone, the TEBs start to proliferate and enhance the branches of the mammary glands. The presence of macrophages is essential to remodeling the extracellular cellular matrix and the production the important growth factors such as EGF (Paine and Lewis 2017).

At the onset of puberty, heightened levels of estrogen hormone stimulate the elongation of the ducts, the formation of terminal end buds (TEBs), and an increase in fat accumulation in the breast (Inman, Robertson et al. 2015). These TEBs create the main ductal network of the mammary glands and cease once reaching the edges of the fat pad (Williams and Daniel 1983).

The cellular composition of TEBs is diverse, including body cells, cap cells, myoepithelial, and luminal cells. TEBs are of special interest due to their unique characteristics, including high invasiveness, a high level of proliferation, and a low apoptosis rate, angiogenesis, and the ability to recruit immune and stromal cells (Daniel and Smith 1999, Paine and Lewis 2017). TEBs consist of two special compartments. The outer layer comprises cap cells at the TEBs' tip, which can differentiate into myoepithelial cells contributing to the elongation of the TEB structure (Williams and Daniel 1983, Hinck and Silberstein 2005). These cap cells also play a role in

modifying the composition of the extracellular matrix, facilitating penetration through the fat pad (Fata, Werb et al. 2003). The inner compartment is composed of multilayer body cells, which eventually differentiate into luminal cells (Fata, Werb et al. 2003) (Figure 1.5).

The stroma within mammary glands plays a pivotal role in their development across various life stages. In virgin mice, for instance, the stroma facilitates branching and duct elongation, while in pregnant mice, it aids in alveolar differentiation. Additionally, the stroma of the involuting glands promotes apoptosis of epithelial cells (Sakakura, Nishizuka et al. 1976). The stroma is a complex and dynamic structure consisting of a diverse array of cells such as adipocytes, fibroblasts, macrophages, eosinophils, neutrophils, and endothelial cells (Paine and Lewis 2017). Fibroblasts situated around TEBs are responsible for producing local growth factors such as FGF and regulating the extracellular matrix by synthesizing various components including laminin, collagen, fibronectin, proteoglycans, and matrix metalloproteinases (MMPs) (Unsworth, Anderson et al. 2014). **Macrophages**, another integral component, significantly influence mammary gland development. Their depletion leads to a reduction in the number of TEBs, diminished ductal growth, fewer branches, and altered TEB morphology (Ingman, Wyckoff et al. 2006). They also play a role in maintaining the luminal space by phagocytosing apoptotic cells (Gouon-Evans, Rothenberg et al. 2000). **Eosinophils**, found around the tip of TEBs, are recruited by exotoxins secreted by the TEBs. Although their absence does not affect the elongation of mammary ducts, it results in reduced branching and altered morphology of TEBs (Gouon-Evans, Rothenberg et al. 2000, Unsworth, Anderson et al. 2014) (Figure 2).

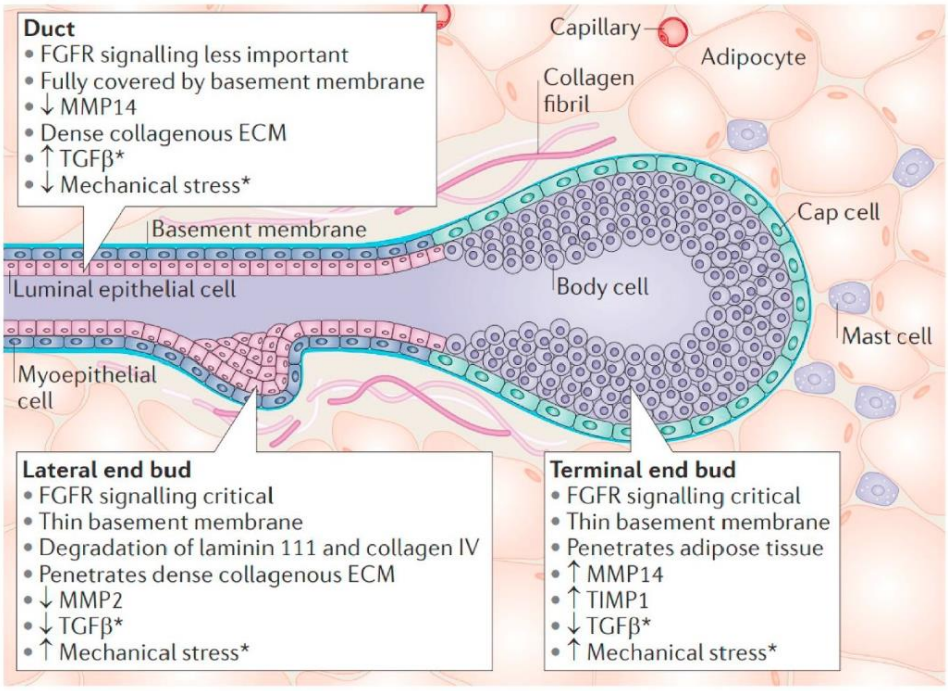

Figure 1.5. Graphical summary of terminal end buds. In the duct region, cells are surrounded by a substantial base membrane and compact extracellular matrix (ECM). In this region, the concentration of MMP14 is relatively low, and while FGFR signaling is inactive, the TGFβ signaling remains active. Conversely, in the TEBs, there is an escalated level of MMP14 and TIMP1, along with more active FGFR signaling. The base membrane in this region is thinner, allowing for the TEB to advance into adipose tissue. The lateral end bud shares similarities with TEB in its structure but penetrates a collagen-rich ECM. A notable difference in the lateral end bud is the diminished level of MMP2, which correlates with the degradation of laminin 111 and collagen IV (Gjorevski and Nelson 2011).

1.3.1 Factors contributing to mammary gland development:

The development of mammary glands is governed by intricate epigenetic factors and signaling pathways that regulate cellular behaviors such as differentiation, proliferation, and apoptosis. Mammary glands undergo complex developmental processes during each menstrual cycle and are significantly influenced by microRNAs, DNA methylation, and signaling pathways such as estrogen, progesterone, growth hormone, TGFβ, and Wnt signaling.

1.3.1.1 Epigenetics in mammary glands development:

1.3.1.1.1 microRNAs: The mammary glands go through various stages of development, including embryonic, puberty, pregnancy, and involution phases. miRNAs play a critical role in these stages, influencing differentiation, apoptosis, proliferation, and epithelial to mesenchymal transition (EMT).

During pregnancy, miR-137 is significantly upregulated in the mammary glands, almost three times higher than usual. Overexpression of miR-137 in mouse embryos leads to the thickening of mammary epithelial cells and inhibits epithelial cells from integrating into the fat pad (Lee, Cho et al. 2015). This overexpression in breast cancer has been linked to the inhibition of metastasis through the modulation of DUSP4, resulting in increased E-cadherin and decreased N-cadherin levels (Du, Yu et al. 2019).

In early embryonic development stages, miR-206 is highly expressed; however, its levels significantly drop around day 13.5 (Lee, Yoon et al. 2013). Elevated miR-206 levels have been associated with impaired mammary bud formation. This miRNA is also highly expressed during pregnancy, where it is believed to inhibit the differentiation of progenitor cells (Wang, Aydoğdu et al. 2019).

At puberty, miR-34a plays a vital role in the morphology of terminal end buds (TEBs). Depletion of miR-34a leads to increased TEB size due to a rise in progenitor cell population. In breast cancer, miR-34a targets genes associated with stemness, such as CD23, NOTCH1, NOTCH3, and HDAC1 (Bonetti, Climent et al. 2019). Alongside miR-34a, miR-184 regulates proteins involved in the PI3K/Akt and Wnt pathways. miR-184 levels are higher in mature ducts compared to TEBs and contribute to reduced activation of PI3K/Akt (Phua, Nguyen et al. 2015).

During pregnancy, the miR-17/92 cluster and miR-21 are increased compared to adulthood, suggesting a role in cellular invasion or ductal growth (Feuermann, Robinson et al. 2012). miR-142 is highly expressed during the involution of mammary glands, promoting apoptosis of mammary epithelial cells by regulating the JAK/STAT and MAPK signaling pathways, assisting the mammary glands in reverting to their normal state (Tian, Zhang et al. 2019, Wu, Thompson et al. 2022). The dysregulation of miRNAs can lead to breast cancer, highlighting their crucial role in maintaining the health and proper functioning of mammary glands.

1.3.1.1.2 DNA methylation: The DNA methylation pattern in mammary glands is distinct and varies based on both location and developmental stage. Extensive research has been conducted to discern the methylation profile of mammary stem cells and differentiated luminal and myoepithelial cells. Comparatively, mammary stem cells exhibit more hypomethylated genes, indicating the pivotal role of DNA methylation in cell lineage determination and differentiation(Platenburg, Vollebregt et al. 1996, Berdasco and Esteller 2011).

Mammary stem cells specifically show reduced methylation in genes crucial for maintaining stemness, such as HOXA1 and TCFL1 (Bloushtain-Qimron, Yao et al. 2008). DNA methylation profiling in mouse mammary epithelial subpopulations has revealed that luminal cells possess a higher degree of methylation in genes associated with stemness and basal cell traits, such as Angptl2 and Krt5. Conversely, genes involved in the differentiation of epithelial cells, like Elf5, Cldn4, and Krt8, tend to be hypomethylated (Lee, Hinshelwood et al. 2011, Huh, Clement et al. 2015).

The expression of DNMT1, essential for the maintenance of adult mammary stem cells, escalates during pregnancy (Pathania, Ramachandran et al. 2015) DNMT1 knockout mice show significant impacts on the development of TEBs and ductal elongation, leading to a reduction in

proliferative mammary epithelial cells (Pathania, Ramachandran et al. 2015). Additionally, inhibiting DNMTs has been linked to an increase in apoptosis and a decrease in the number of mammary cells (Huh, Clement et al. 2015).

Throughout lactation, essential protein genes like the αs1-casein gene exhibit a reduction in methylation. In contrast, during puberty or involution, this gene undergoes hypermethylation (Platenburg, Vollebregt et al. 1996). Similarly, the Whey Acidic Protein (WAP) gene, which plays a crucial role in the functionality of mammary glands during lactation, demonstrates a pattern of higher hypomethylation in its promoter region during lactation, signifying enhanced expression. Conversely, during puberty, this region is more hypermethylated, indicating a potential decrease in gene expression (Montazer-Torbati, Hue-Beauvais et al. 2008). This dynamic shift in DNA methylation patterns underscores the intricate regulatory mechanisms that govern the functional changes in mammary glands across different developmental stages.

1.3.1.2 Estrogen (E2): Primarily secreted by the ovaries or synthesized by adipose tissue, estrogen acts on mammary cells through estrogen receptor alpha (ESR1) (Yart, Lollivier et al. 2014). Estrogen is crucial for sustained cell proliferation and gland elongation (Bocchinfuso and Korach 1997). Upon binding to ESR1, estrogen translocate to the nucleus and enhances gene transcription, such as amphiregulin (Areg). Areg, upon paracrine secretion, binds to EGFR and stimulates the secretion of growth factors such as fibroblast growth factor (FGF) (Ciarloni, Mallepell et al. 2007). While estrogen independently has the capacity to form terminal end buds (TEBs), its combination with growth hormone synergistically enhances the formation of TEBs (Kleinberg, Feldman et al. 2000). Over the course of the menstrual cycle, estrogen levels peak around ovulation, facilitating the maturation of mammary glands in preparation for potential pregnancy. Following ovulation, in the absence of pregnancy, estrogen levels gradually decline

towards the end of the cycle (Figure 1.6) (Owen Jr 1975). The gut microbiota also plays a significant role in regulating estrogen levels within the body. Once estrogen metabolites are transported to the liver, they are conjugated with glucuronic acid and transferred to the gut via the bile duct. Studies have demonstrated that microbiota members, including those from the Bacteroidetes, Firmicutes, and Clostridium phyla, are capable of producing β-glucuronidase enzymes. This enzyme can convert inactive estrogen into its active form, which can then re-enter systemic circulation through the enterohepatic pathway (Sui, Wu et al. 2021).

1.3.1.3 Progesterone (P4): Progesterone, another key hormone from the ovaries, regulates Areg expression upon binding to the progesterone receptor (PR) and induces ductal elongation alongside estrogen (Haslam and Levely 1985, Ruan, Monaco et al. 2005, Aupperlee, Leipprandt et al. 2013). P4 is essential for the development of side branching and alveoli in mammary glands. Detailed studies reveal distinct functions of PR in epithelial and stromal cells. It has been shown that PR in epithelial cells is necessary for duct elongation, whereas PR in stromal cells is crucial for alveolar formation (Figure 1.6) (Humphreys, Lydon et al. 1997). Additionally, studies on gut microbiota have shown that elevated progesterone levels during pregnancy and lactation result in an overgrowth of Bifidobacterium in the intestine, which is associated with the health of both the mother and infant (Mallott, Borries et al. 2020).

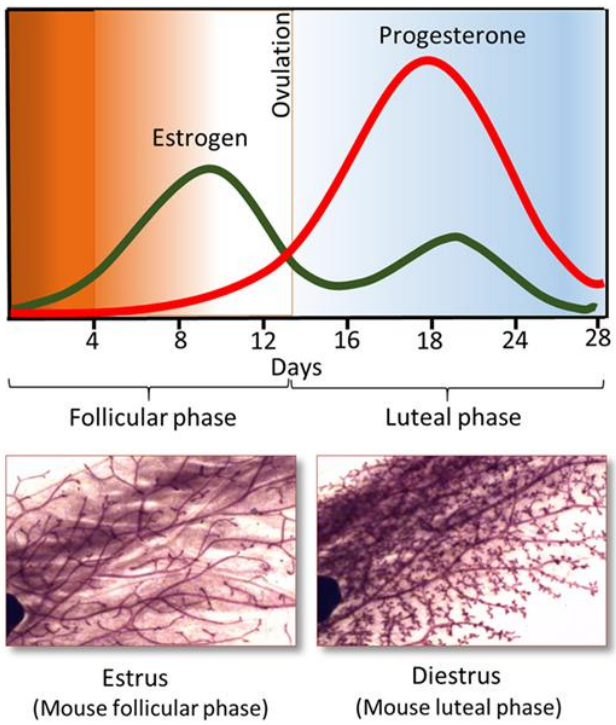

Figure 1.6. The effect of estrogen and progesterone on mammary glands. The level of estrogen (green) and progesterone (red) throughout the different phases of the menstrual cycle. The fluctuation of these hormones affects the morphologic of mammary glands (Atashgaran, Wrin et al. 2016).

1.3.1.4 Growth Hormone (GH): Growth hormone, secreted by the pituitary gland at puberty, primarily affects stromal cells and stimulates Insulin-like growth factor 1 (IGF-I) production (Macias and Hinck 2012, Hull and Harvey 2014). IGF-I is crucial for terminal end bud (TEB) formation and branching. Studies have shown that administering GH or IGF-I to mice results in the formation of a limited number of TEBs. However, the combined presence of IGF-I and estrogen significantly enhances TEB formation (Loladze, Stull et al. 2006). Gut microbiota influences GH/IGF-I production, with the absence of Lactobacillus resulting in less sensitivity to GH/IGF-I (Schwarzer, Makki et al. 2016). Short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate can directly inhibit GH production through the suppression of PRL and Pit-1 in bovine anterior pituitary cells (Wang, Fu et al. 2013, Jensen, Young et al. 2020).

1.3.1.5 Epidermal Growth factor family (EGF): Upon binding of EGF to its receptor epidermal growth factor receptor (EGFR), various signaling pathways are initiated, including Ras/MEK, PLC-γ1, and PI3K/AKT. These pathways are crucial for biological processes such as cell division, migration, and survival (Wang 2016). EGF and EGFR are mainly expressed during the developmental stages of mammary glands, especially during puberty and lactation (Schroeder and Lee 1998). Throughout puberty, EGFR is highly expressed on TEBs and stromal cells, playing a pivotal role in TEB formation and ductal elongation (Coleman, Silberstein et al. 1988). Studies on impaired EGFR mice have revealed reduced mammary gland outgrowth and fewer TEBs, although these mice were capable of undergoing alveologenesis and lactation during pregnancy (Xie, Paterson et al. 1997). Within the EGFR family, ErbB proteins, particularly ErbB2 and ErbB3, are critical for mammary gland development. Knockout studies of these proteins revealed incomplete mammary gland development (Tidcombe, Jackson-Fisher et al. 2003). ErbB3 knockout mice exhibited an increased number of smaller-sized TEBs compared to the wild type. ErbB2, known for its oncogenic role in breast cancer, is also important for mammary gland elongation (Jackson-Fisher, Bellinger et al. 2004).

1.3.1.6 Fibroblasts growth factor (FGF): FGF ligands and their receptors (FGFRs) play crucial roles in mammary gland development, particularly in ductal elongation (Hynes and Watson 2010). FGF ligands typically function in a paracrine manner and, upon binding to FGFRs, activate essential signaling pathways such as Ras-MAPK and PI3K-AKT, which regulate cell proliferation and survival (Fata, Mori et al. 2007, Pond, Bin et al. 2013). During puberty, FGF1, FGF2, FGF4, FGF7, and FGF10 are the predominant ligands in this family involved in ductal elongation (Hynes and Watson 2010). Overexpression of FGF4 can result in inhibition of ductal elongation and abnormal side branching in TEBs, with TEBs often presenting enlarged lumens

filled with cells, sometimes lacking a defined neck, and exhibiting low levels of apoptosis. Overexpression of FGF7 results in activation of TGFα signaling and inhibition of branching (Astigiano, Damonte et al. 2003, Cui and Li 2013). FGFR2 is notably vital among the receptors, as its deficiency results in an inability to form TEBs and an increased rate of apoptosis (Parsa, Ramasamy et al. 2008).

1.3.1.7 Transforming Growth Factor Beta (TGFβ): TGFβ ligands have a multifaceted role in mammary gland development. The binding of TGFβ ligands to their receptors triggers the activation of SMAD proteins, which regulate gene expression, motility, invasion, apoptosis, proliferation, and ECM remodeling (Moses and Barcellos-Hoff 2011, Vaidya and Kale 2015). During puberty and the phase of ductal elongation, TGFβ1 and TGFβ3 are particularly overexpressed, with TGFβ3 predominantly located in the cap cells. In contrast, TGFβ2 expression is relatively lower during puberty but increases during pregnancy (Silberstein, Strickland et al. 1990, Soriano, Pepper et al. 1998). Generally, high estrogen levels during puberty suppress TGFβ expression. A low concentration of TGFβ ligands stimulates mammary gland branching, whereas a higher concentration inhibits growth (Pierce, Johnson et al. 1993). Mutation in TGFβ1 can lead to enhanced ductal elongation, and increased proliferation of TEBs, but compromised DNA damage response (Factor 1997).

1.3.1.8 Wnt signaling pathway: Through β-catenin activation, the Wnt signaling pathway plays a pivotal role in various aspects of mammary gland development. Upon activation, β-catenin translocates to the nucleus and binds to the LEF transcription factor, crucial for stem cell maintenance, branching, and alveologenesis (Komiya and Habas 2008). Proteins like Lgr5 and Lgr6 are integral for TEB formation, and their absence leads to reduced side branching in mammary glands (Badders, Goel et al. 2009). Additionally, the Wnt signaling pathway is

involved in cytoskeletal rearrangement (Lindvall, Zylstra et al. 2009). Wnt5, in particular, regulates the branching and proliferation of TEBs. Components of the Wnt pathways are expressed in various locations within the mammary glands during developmental stages. For example, Lrp6 is predominantly found in epithelium and stroma, while Wnt4 and Wnt5a are present in the luminal layer, and Wnt6 is typically located in the basal layer (Roarty, Shore et al. 2015). The non-canonical receptor, Ror2, plays a significant role in controlling TEB proliferation and the differentiation of myoepithelial cells. Meanwhile, Wnt/β-catenin activity is essential for maintaining stemness in progenitor cap cells (Roarty and Serra 2007, Roarty, Shore et al. 2015).

1.3.2 Terminal End Buds as Models for Cancer

The developmental behavior of terminal end buds (TEBs) shares notable similarities with breast cancer, including rapid cell proliferation, substantial production of Vascular Endothelial Growth Factor (VEGF), and invasive characteristics (Visvader and Stingl 2014). TEBs demonstrate a high proliferation rate and a diverse cell population akin to breast cancer. They have the capability to attract macrophages, which in turn recruit endothelial cells, a crucial process for angiogenesis, a common feature in tumor growth (Rossiter, Barresi et al. 2007).

TEBs also exhibit invasive properties, altering the extracellular matrix and invading the surrounding fat pad. This invasion is facilitated by processes similar to epithelial-mesenchymal transition, marked by the expression of EMT-related proteins such as Slug, ZEB1, and TWIST (Nassour, Idoux-Gillet et al. 2012). Additionally, mammary cells produce Matrix Metalloproteinases like MMP-14 and MMP-2, which degrade the ECM, aiding the advancement of TEBs. These MMPs are essential for ductal growth during puberty (Wiseman, Sternlicht et al. 2003, Mori, Lo et al. 2013).

Breast tumors exhibit behavior similar to TEBs. Studies in mice have shown that a diet rich in n-3 polyunsaturated fatty acids (PUFA) leads to a reduction in the number of TEBs (MacLennan, Anderson et al. 2011, Anderson, MacLennan et al. 2014). Exposure of TEBs to carcinogens like 7,12-Dimethylbenz(a)anthracene during puberty has been linked to an increased risk of breast cancer later in life (Russo and Russo 1978).

The mammary gland fully develops during puberty in humans. Exposure to inflammatory agents or immune stressors during this critical development period, if unresolved, can lead to developmental disturbances and potentially increase the risk of cancer later in life.

1.4 Breast cancer

According to the World Health Organization (WHO), breast cancer is the most common cancer among women globally, with nearly two million new cases each year and approximately 685,000 deaths reported in 2020 ((WHO) 2020). In Canada, projections for 2023 indicate that at least 80 Canadians will be diagnosed with breast cancer daily, with over 15 deaths each day (Society 2020). The pathogenesis of breast cancer is multifactorial, predominantly influenced by genetic predisposition, family history, environmental factors, and lifestyle choices. These factors include prolonged exposure to estrogen, early onset of menstruation, late menopause, and factors related to childbirth such as age (Nik-Zainal, Davies et al. 2016).

1.4.1 Genetic predisposition and family history:

Approximately 10% of breast cancer cases are associated with genetic mutations and family history. The main genes involved in hereditary breast cancer include BRCA1, BRCA2, ATM, CHEK2, PALB2, PTEN, STK11, and TP53 (Taylor, Brady et al. 2018), with BRCA1 and BRCA2 being integral to genomic repair mechanisms. The probability of inheriting these mutations from parents carrying BRCA1 or BRCA2 is about 50%. Women with BRCA1

mutations have a 72% risk, and those with BRCA2 mutations have a 69% risk of developing breast cancer by the age of 80. Men with BRCA2 mutations have a 6% risk of developing breast cancer (Kuchenbaecker, Hopper et al. 2017). Ethnic background also affects these genetic risks, with the Asian population showing a lower tendency and higher risks observed in Ashkenazi Jewish families (Daly, Pilarski et al. 2016).

Moreover, miRNAs have been identified as key factors in cancer development, displaying varied expressions in breast cancer. Dysregulation of specific miRNAs, including miR-10b, miR-20, miR-21, miR-30a, miR-125, miR-126, miR-143, miR-145, miR-146, miR-155, miR-205, miR-223, miR-376, miR-451, miR-575, miR-371a, and let-7a, has been reported to critically impact the progression of breast cancer (Mattie, Benz et al. 2006, Iorio, Casalini et al. 2009, Li, Zhang et al. 2010, Zhang, Zhao et al. 2013, Singh, Pochampally et al. 2014). In breast cancer, miR-145 was reported to be significantly down-regulated, whereas miR-155 was upregulated (Iliopoulos, Polytarchou et al. 2009).

1.4.2 Lifestyle and environmental factors:

A growing body of evidence highlights that early pregnancy and elevated estrogen levels during pregnancy may lower breast cancer risk, potentially due to increased differentiation of stem or progenitor cells and altered gene expression leading to reduced cell proliferation (Siwko, Dong et al. 2008, Hilakivi-Clarke, De Assis et al. 2013). Additionally, obesity, physical inactivity, and alcohol consumption are significant risk factors, with 20% of breast cancer cases associated with these elements (Danaei, Vander Hoorn et al. 2005). Notably, every 10 grams of alcohol consumed daily is linked to a 7% to 10% increased risk of breast cancer (Chen, Rosner et al. 2011).

Alcohol boosts the expression of estrogen receptor alpha, thereby enhancing cellular proliferation, and is often found in ER-positive breast tumors (Shield, Soerjomataram et al. 2016). Alcohol also raises levels of IGF-1, which is known for promoting high cell proliferation and denser mammary glands (Vachon, Kushi et al. 2000, Lakhani, Ellis et al. 2012). When metabolized, ethanol forms acetaldehyde and then acetate, leading to the production of NADH and hydrogen ions (H^+). This process results in the generation of reactive oxygen species that potentially facilitate DNA damage (Francisco, Peddi et al. 2008, Fanelli, Maciel et al. 2011).

Dietary patterns play a crucial role in either promoting or preventing breast cancer. Studies indicate that a diet high in total meats, processed meats, and foods with a high glycemic index correlates with an increased risk of breast cancer. In contrast, the consumption of vegetables, carotenoids, citrus fruits, and mushrooms has been associated with a decreased incidence of breast cancer (Thomson 2012). The Mediterranean diet (MD), known for its high content of antioxidants, is particularly noted for its protective effects against breast cancer, especially in postmenopausal women. The efficacy of this diet is largely attributed to its potential to suppress cancer-promoting growth factors (Turati, Carioli et al. 2018). Conversely, the Western diet, marked by high consumption of refined sugars, saturated fats, and alcohol, is strongly associated with elevated breast cancer risk, primarily due to its role in enhancing pro-inflammatory cytokines (Stoll 1998).

Obesity, another critical environmental factor, contributes to increased local estrogen production. Studies indicate that obesity elevates levels of the IL-6 cytokine, which, along with increased body mass, raises the level of the aromatase enzyme responsible for converting androgens into estrogen (Park, Park et al. 2005, Bowers, Brenner et al. 2015). Obesity also causes adipose tissue to release non-esterified fatty acids (FFAs) into the bloodstream, prompting the liver and muscles

to store fat instead of glucose, thus reducing tissue efficiency in glucose metabolism (GORDON 1960, Kahn and Flier 2000). To counteract elevated glucose levels, the pancreas increases insulin production, leading to hyperinsulinemia. Hyperinsulinemia is associated with higher synthesis and activity of IGF-1 (Kulkarni, Holzenberger et al. 2002). Together, insulin and IGF-1 can activate and increase the expression of estrogen receptor alpha, which is linked to ER-positive breast cancer (Lorincz and Sukumar 2006, James, Wootton et al. 2015).

1.4.3. Breast cancer classifications:

Breast cancer is primarily classified into two categories: histological and molecular, with molecular classification being more integral in clinical settings (King, Marks et al. 2003, (WHO) 2020). From a molecular perspective, breast cancer is divided into five subtypes based on the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2):

1. **Luminal A** (60-70% of cases): Characterized by positive ER expression, negative PR, low Ki67 activity (indicative of cell proliferation), and mutations in PIK3CA, MAP3K1, GATA3, and FOXA1 genes.
2. **Luminal B-like HER2-positive** (13-15% of cases): Exhibits positivity for all receptors (ER+/PR+/HER2+), high Ki67 activity, and frequent mutations in TP53 and PIK3CA.
3. **Luminal B-like HER2-negative** (10-20% of cases): Defined by positive ER and PR expression, negative HER2, and high Ki67 activity.
4. **Triple-Negative** (10-15% of cases): Lacks expression of ER, PR, and HER2, has high Ki67 activity, and is associated with mutations in TP53, RB1, and BRCA1 genes. This subtype is noted for its high level of proliferation.

5. **HER2 Enriched** (Non-luminal) (13-15% of cases): Negative for ER and PR, but positive for HER2, with high Ki67 activity. Mutations in TP53, PIK3CA, and FGFR4 are common in this subtype (Vuong, Simpson et al. 2014, Harbeck, Penault-Llorca et al. 2019).

1.4.4 Cancer stem cells

Cancer stem cells (CSCs) are a small subpopulation of cells within the tumor that functionally are able to self-renew and differentiate from other types of cells. Moreover, these cells can give rise to the bulk of the tumor, initiate the metastasis process, and show resistance against chemotherapy. CSCs share the same pathways with normal stem cells to grow and generate a population of cells with various stages of differentiation (Reya, Morrison et al. 2001, Prager, Xie et al. 2019). Furthermore, CSCs can secrete specific cytokines to recruit immune cells, evade immune surveillance, or suppress the defense system (Alvarado, Thiagarajan et al. 2017). CSCs can be identified by expressing specific intracellular markers such as ALDH1 or surface markers such as CD133, CD24, CD44, and EpCAM. These markers vary depending on the type of cancer. For instance, breast cancer stem cells (BCSCs) express CD44, ALDH, and CD133, while prostate cancer stem cells express CD44, CD49f, CD133, and p63 (Klonisch, Wiechec et al. 2008). Previous studies have shown that the expression of CD24 and CD44 leads to differentiation of cells and metastasis of BCSCs, respectively. Therefore, one of the markers for BCSCs is $CD44^{+}/CD24^{-}$/low (Fillmore and Kuperwasser 2007). Another marker for BCSCs is ALDH or Aldehyde Dehydrogenase, which induces mammosphere formation, chemoresistance, and invasion and is suggested as a marker for poor survival (Suman, Das et al. 2013). It is also indicated that not all BCSCs express the same markers. Liu et al. showed that ALDH+ BCSCs are generated by hypoxia and are enriched with hypoxia factors, behaving like epithelial-like

cells. On the other hand, BCSCs with $CD44^{+}/CD24^{-}$/low are usually located at the tumor-invasive edge of the tumor bulk and behave like mesenchymal cells (Liu, Cong et al. 2014). EpCAM is another BCSCs marker that plays an important role in the proliferation and metastasis of BC and is associated with poor overall survival in BC. It is also shown that EpCAM contributes to E-cadherin degradation and subsequently leads to β-catenin accumulation in the cytoplasm, activating the Wnt signaling pathway (Osta, Chen et al. 2004).

1.4.5 Breast Cancer Microbiome

The tumor-associated microbiome, characterized by unique intracellular bacteria, plays a pivotal role in tumor development and heterogeneity (Nejman, Livyatan et al. 2020). These bacteria are actively involved in tumor initiation and progression through mechanisms such as inducing inflammation, suppressing the immune system, and fostering drug resistance.

In breast cancer specifically, bacteria from the gut may travel via the entero-mammary pathway, potentially causing DNA damage, stimulating cell proliferation, altering hormonal factors, and initiating tumor development (Alizadehmohajer, Shojaeifar et al. 2020, Eslami-S, Majidzadeh-A et al. 2020, Laborda-Illanes, Sanchez-Alcoholado et al. 2020). Breast tumors are noted for having a rich and diverse microbiome, which is linked to epigenetic changes associated with breast cancer and the perpetuation of inflammation (Alizadehmohajer, Shojaeifar et al. 2020).

The tumor microenvironment, often hypoxic, provides favorable conditions for the growth of certain bacteria. For example, Bacteroides fragilis, an oncogenic bacterium from the colon, has been observed to colonize mammary ducts, instigating carcinogenesis and metastasis through the activation of the Notch and Wnt signaling pathways (Parida, Wu et al. 2021). *Methylobacterium radiotolerans*, found in two-thirds of ER+ breast cancers, is associated with estrogen receptor regulation (Xuan, Shamonki et al. 2014).

Distinct breast cancer subtypes harbor different bacterial populations:

1. $ER^+/PR^+/HER2^-$ breast tumors are often colonized by bacteria such as *Arcanobacterium*, *Bifidobacterium*, *Cardiobacterium*, *Citrobacter*, *Escherichia*.
2. $ER^+/PR^+/HER2^+$ tumors are predominantly inhabited by *Bordetella*, *Campylobacter*, *Chlamydia*, *Chlamydophila*, *Legionella*, *Pasteurella*.
3. $ER^-/PR^-/HER2^+$ tumors commonly host *Streptococcus*.
4. $ER^-/PR^-/HER2^-$ tumors are associated with *Aerococcus*, *Arcobacter*, *Geobacillus*, *Orientia*, *Rothia* (Wang, Sun et al. 2021).

This diversity in bacterial populations across different breast cancer subtypes underscores their significant role in the development and progression of the disease.

1.5 Connection of Gut Microbiome, Mammary glands, Epigenetics, and Breast cancer

The intricate relationship between the gut microbiome, mammary glands, epigenetics, and breast cancer highlights a complex interplay crucial in maintaining the health and balance of the mammary gland ecosystem. The concept of the interconnected mucosal immune system suggests a communication pathway exists between the gut and distant organs, such as the mammary glands, underscoring the significance of immunological synergy across organs distant from the gut (McDermott, Clark et al. 1980).

The human lymphatic system, which drains lymph and supports immune system translocation, is implicated in this process (Rahman and Mohammed 2015). The mucosal-associated lymphoid tissue (MALT), including gut-associated lymphoid tissue (GALT), plays a crucial role in antigen sampling and plasma cell translocation to the mammary glands (Gutzeit, Magri et al. 2014, Kuper, Wijnands et al. 2017). Hormones such as estrogen and progesterone are believed to

influence the translocation of cells through MALT to the mammary glands [300]. The mother's immune system facilitates the transfer of gut microbiota to the mammary glands via an entero-mammary pathway (Milani, Mancabelli et al. 2015).

Dendritic cells (DCs) are instrumental in this process. Their primary function is to sample antigens in the intestinal lumen and present them to T cells in the lymph nodes, initiating their proliferation and differentiation (Aliberti 2016). Studies suggest that $CD11c^+$ DCs and $CX3Cr1^+$ macrophage cells can traverse intestinal tight junctions to capture bacteria or their antigens (Martín-Fontecha, Lanzavecchia et al. 2009, Jost, Lacroix et al. 2012). For instance, $CD103^+$ DCs have been shown to capture Salmonella bacteria in the intestinal lumen and transport them to lymph nodes (Farache, Koren et al. 2013, Bekiaris, Persson et al. 2014). This evidence supports the hypothesis that maternal gut microbiota is translocated to the mammary glands by DCs through this complex network (Jost, Lacroix et al. 2014, Rodríguez 2014).

Furthermore, the gut microbiota's modulation of the Th17 signaling pathway has a far-reaching impact on the immune system, affecting cells even in distant sites like the mammary glands (Luo, Leach et al. 2017). The differentiation of CD4+ cells into Th17 cells is triggered by the activation of T cell receptors through pathogen-associated molecular patterns in conjunction with specific sets of cytokines such as IL-1β, IL-6, and IL-23 (Geha, Tsokos et al. 2017, Cui 2019). This process is further facilitated by the activation of STAT3 signaling pathways (Lee, Smith et al. 2017, Woś and Tabarkiewicz 2021).

Investigations involving germ-free mice have revealed that the differentiation of Th17 cells is profoundly influenced by gut microbiota, specifically through interactions with segmented filamentous bacteria (SFB). This process involves the attachment of SFB to intestinal epithelial cells, setting off a chain reaction resulting in the generation of endocytic vesicles containing SFB

antigens. When encountered by immune cells, these vesicles significantly enhance the differentiation of Th17 cells (Sun, Yang et al. 2023).

IL-17, a pro-inflammatory cytokine often elevated in the breast tumor microenvironment and linked with more aggressive cancer subtypes, exemplifies the gut microbiome's influence on systemic immune readiness (Wang, Li et al. 2017, Mills 2023). This augmented Th17 response can disrupt critical signaling pathways like Transforming Growth Factor-β essential for controlling mammary gland development and implicated in the majority of human breast cancers (Hinck and Silberstein 2005, Novitskiy, Pickup et al. 2011).

The connection extends into the realm of epigenetics, where DNA methylation plays a crucial role in the differentiation and plasticity of Th17 cells (Renaude, Kroemer et al. 2020). *Fusobacterium* has been shown to elevate MYC expression levels, thereby increasing the expression of the miR-17-92 cluster (Yuan, Burns et al. 2018). Conversely, butyrate can reduce c-MYC overexpression and, consequently, the miR-17-92 cluster through histone deacetylase inhibition (Hu, Liu et al. 2015). *Fusobacterium* can activate the Wnt signaling pathway via its binding to E-cadherin and induce cell proliferation (Diosdado, Van De Wiel et al. 2009). *F. nucleatum* increases the expression of miR-4474 and miR-4717, targeting CREBBP, a key transcription factor in the Wnt signaling pathway, potentially inhibiting cell proliferation in colorectal cancer (Bordonaro and Lazarova 2015, Yang, Weng et al. 2017). The impact of *E. coli* has been associated with upregulated expression of miR-30c and miR-130a, which are involved in the regulation of autophagy, promoting the development of colorectal cancer (Rubinsztein, Codogno et al. 2012, Nguyen, Dalmasso et al. 2014). An imbalanced gut microbiome can significantly affect host metabolic and immune functions, leading to increased production of secondary bile acids and LPS. This imbalance may trigger the upregulation of COX2 and the

induction of PEG2, which, in turn, can diminish the recruitment of NK cells. Furthermore, such dysbiosis leads to the hyperactivation of CD8+ cells, fueling inflammation and resulting in T-cell exhaustion (Mohseni, Taghinezhad-S et al. 2023). This process underscores the intricate and impactful role of gut microbiota in influencing cancer development pathways and modulating immune responses.

Prebiotics play a vital role in nurturing and balancing the gut microbiota ecosystem. They have been proven to systemically reduce inflammation, including within the brain and mammary glands, demonstrating the broad reach of gut health on our overall well-being (de LeBlanc, Matar et al. 2006, Mallet, Graham et al. 2016, Vuong, Mallet et al. 2016). Similarly, dietary interventions incorporating probiotics have shown efficacy in counteracting the detrimental effects of antibiotics, showcasing the beneficial interplay between diet and health. The gut microbiota's contribution to immune regulation is particularly notable in its production of short-chain fatty acids (SCFAs). These compounds are instrumental in activating T cells by promoting the recruitment and infiltration of various immune cells, such as CD4+, ICOS+, and CD8+ cells, into the tumor microenvironment (Mohseni, Taghinezhad-S et al. 2023). This action enhances the expression of inflammatory mediators like INFγ and TNFα, thereby improving clinical outcomes. SCFAs like propionate are crucial in modulating the production of IL-17, keeping immune cells primed and ready for action (Dupraz, Magniez et al. 2021).

The restorative effects of probiotics on the gut microbiota also extend to the modulation of miRNA expression, particularly inducing changes in miR-192 and miR-215 [50]. This modulation plays a key role in preventing immune disorders, emphasizing the profound influence of gut health on the body's immune system and its capacity to fight diseases (Cowan and Richardson 2019). Such interventions underline the gut microbiome's significant effect on

both local and distant organs (Kwa, Plottel et al. 2016). In alignment with these findings, research has shown that the intake of probiotics can enhance immune protection by elevating levels of IgA and IL-10 in the mammary glands (de Moreno de LeBlanc, Matar et al. 2005, de Moreno de LeBlanc, Matar et al. 2006, de Moreno de Leblanc, Matar et al. 2007). Through dietary interventions and targeted 'biotic' therapies, the immune response in the gut-mammary glands axis early in life can be modulated to mitigate the risk of breast cancer later in life.

1.6 Developmental Origins of Health and Disease (DOHaD)

The Developmental Origins of Health and Disease (DOHaD) hypothesis explores the critical relationship between early life stages, including the periconceptual period, fetal development, and infancy, and the risk of developing long-term health problems. This theory suggests that cells can adapt to environmental conditions through changes in gene expression facilitated by epigenetic mechanisms (Gillman 2005). However, if these adaptations are inaccurate, they can disrupt cellular homeostasis in relation to the environment, potentially leading to disease. Moreover, these alterations may be transgenerationally inherited.

Research has demonstrated that children born to obese mothers possess a less optimal gut microbiome, thereby having a higher presence of bacteria that interfere with energy regulation, subsequently leading to metabolic disorders and obesity (Akagbosu, Nadler et al. 2022). Another study highlighted that a high intake of dietary linoleic acid during pregnancy can alter gut microbiota by increasing estrogen levels, which, in turn, may elevate the risk of breast cancer in offspring (Hilakivi-Clarke, Clarke et al. 1999).

Furthermore, undernutrition has been shown to impact the germline, affecting the health of future generations (Gillman 2005). Studies also have revealed that the dietary habits of fathers,

especially diets high in saturated fat, can influence the mammary glands of their offspring. This is attributed to epigenetic changes in sperm DNA, leading to structural changes in the mammary tree and an increased number of terminal end buds, thus heightening the risk of breast cancer (Fontelles, Guido et al. 2016).

The link between maternal diet and the disease susceptibility of offspring, governed by epigenetic mechanisms, furthermore sheds light on the significance of early life nutrition and the establishment of the microbiome in the context of DOHaD (Zheng, Zhang et al. 2021). A growing body of research points to the important role that the disruption of the gut microbiota in early life plays as a precursor to chronic diseases later in life (Stinson 2020).

In summary, related to gut microbiome disruption, the DOHaD hypothesis emphasizes the connection between early life stages and the establishment of the gut microbiome and long-term health implications. It highlights the need for precise and timely nutritional strategies to nurture a healthy microbiome, thus preventing the development of disease later in life.

1.7 Rationale for Study and Objectives

Exposure to antibiotics or LPS during early life or puberty stages has been linked to an elevated risk of developmental disruptions, potentially leading to increased susceptibility to cancer in later years. Considering the complex interaction of epigenetics and the gut microbiota, particularly in relation to the regulatory network governing the immune system, it is hypothesized that the administration of prebiotics can effectively modulate the epigenetic and immune signatures indirectly influenced by the gut microbiota. Prebiotics have the potential to impact systemic inflammation positively and rectify disturbances caused by antibiotics or LPS exposure during critical developmental windows, including early life and puberty.

Mammary gland development comprises two pivotal stages: embryonic and pubertal development (Macias and Hinck 2012, Smith 2012). Any disruption in epigenetic programming or exposure to inflammatory agents such as LPS during these critical periods can potentially alter the mammary gland's branching and ductal structure, increasing the risk of cancer development later in life (Tharp, Maffini et al. 2012). Consequently, these stages are the focus of our experimental approach in examining the gut-breast axis.

In mice, puberty typically occurs around 4 to 5 weeks of age, depending on the strain. An animal model developed by Dr. Nafisa Ismail demonstrated that disruption of the gut microbiota with LPS during puberty not only transiently affects the gut microbiota but also leads to endocrine changes and increases the risk of pre-carcinogenic lesions (Herrera-Covarrubias, Coria-Avila et al. 2017, Murray, Sharma et al. 2019). Furthermore, LPS exposure during puberty can program the hypothalamic-pituitary-adrenal (HPA) axis, correlating with long-term anxiety and depression-like behaviors (Smith, Murray et al. 2021). Interestingly, probiotic treatment has been observed to mitigate the stress impacts on mental health, indicating a potential role of the

gut microbiome in modulating the immune-neuroendocrine axis (Smith, Murray et al. 2021). The gut-organs axis during puberty is especially crucial in female adolescents, where significant brain reorganization and mammary gland development occur. Studies, including ours, have shown that prebiotic treatment positively influences the immune response and reduces inflammation linked to breast cancer (de LeBlanc, Matar et al. 2007, Aragón, Carino et al. 2015). The underlying common mechanism of these beneficial effects seems to be associated with the activation of transcription factors that counteract systemic inflammatory signals. The microbiota plays a significant role in modulating the immune response, and inflammatory factors, and altering host gene expression at the epigenetic level, potentially impacting tumor responses to therapy (Viaud, Daillere et al. 2015, Mani 2017, Fernández, Reina-Pérez et al. 2018). Prebiotics and probiotics are known to affect miRNA expression, thereby influencing pathways related to metastasis and carcinogenesis (Rajoka, Jin et al. 2018).

In this study, the selected prebiotic is *Lentinula edodes* Cultured Extract (AHCC). AHCC acts as an antagonist for TLR4, potentially preventing inflammatory cascades initiated by TLR4 from endogenous LPS, which can leak from imbalanced microbiota following antibiotic or LPS treatment (Mallet, Graham et al. 2016).

Disturbances to microbiota and epigenetic patterns caused by antibiotics can persist for periods ranging from two months to as long as four years before being restored (Masotti 2012, Thiemann, Smit et al. 2016). Even after microbiota recovery, the phenotypic changes remain, pointing to the long-lasting effects of these perturbations. A critical period exists during adolescence in females where epigenetic perturbations can affect mammary bud formation, subsequently influencing breast cancer risk (Phua, Nguyen et al. 2015). For instance, antibiotic use during pregnancy is associated with an increased risk of immune disorders in offspring.

Dietary interventions with prebiotics have been shown to mitigate the adverse effects of antibiotics (Dong, Li et al. 2018) and prebiotic intake has been effective in correcting LPS-induced dysbiosis during puberty (Murray, Sharma et al. 2019). Probiotic treatments have been observed to modulate miRNAs and alter the host transcriptome, including changes in miR-192 and miR-215 (Archambaud, Sismeiro et al. 2013). Additionally, prebiotics and probiotics are recognized for their roles in cancer prevention and treatment, partly due to their influence on miRNA expression and associated pathways in metastasis and carcinogenesis (Rajoka, Jin et al. 2018). Our research has demonstrated that prebiotics can increase key tumor-suppressor miR-145, significantly reducing the development of cancer stem cells and metastasis ex vivo, which are critical in cancer development and recurrence (Hernandez-Vargas, Ouzounova et al. 2011, Ouzounova, Vuong et al. 2013, Vuong, Mallet et al. 2016).

Therefore, our study aims to achieve the following objectives:

1.7.1 Objective 1: Examine if intake of prebiotics during prepuberty can correct LPS-induced dysbiosis and epigenetic alterations in the adult mammary gland and breast cancer.

1. Determine the influence of pubertal LPS exposure on immune and epigenetic changes, specifically DNA methylation, in adulthood.
2. Evaluate the impact on mammary gland structure and morphology due to pubertal LPS exposure.
3. Analyze the effects on tumor growth and cancer stem cell development from pubertal LPS exposure.
4. Measure inflammatory cytokine levels in the tumors and mammary glands.
5. Investigate the impact of pubertal LPS exposure on microRNA expression in breast tumors.

1.7.2 Objective 2: Investigate if prebiotic intake can counteract the potential role of postnatal and early-life antibiotic effects on mammary gland development.

1. Assess the influence of early-life antibiotics on the maternal immune system.
2. Examine the impact of early-life antibiotics on maternal mammary gland microRNAs.
3. Examine the impact of early-life antibiotics on maternal mammary gland gene expression.
4. Evaluate the effect of early-life antibiotics on the immune system of female pups.
5. Analyze the influence of early-life antibiotics on the mammary gland microRNAs of female pups.
6. Analyze the influence of early-life antibiotics on the mammary gland gene expression of female pups.

Chapter 2: *Lentinula edodes* Cultured Extract Intake at Puberty Mitigates Inflammatory Signals at the Mammary Glands by the Involvement of Epigenetic Mechanisms.

Submitted in Biomedicine & Pharmacotherapy Journal.

***Lentinula edodes* Cultured Extract Intake at Puberty Mitigates Inflammatory Signals at the Mammary Glands by the Involvement of Epigenetic Mechanisms.**

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2.1 Abstract

Exposure to lipopolysaccharide (LPS) during puberty can disrupt the gut microbiome and epigenetic regulation in mammary glands, potentially increasing breast cancer susceptibility. While the adverse effects of LPS on intestinal and brain function are documented, its impact on mammary glands is not well understood. An immunocompetent murine model (BALB/c mice) will be administered an acute dosage of LPS during the pubertal stage. The study aims to comprehensively evaluate the enduring effects of LPS exposure, as well as LPS exposure in combination with AHCC (*Lentinula Edodes* Cultured Extract), on various parameters, including DNA methylation patterns, cytokine profiles, and microRNA expression within the mammary gland, during adulthood, at the age of 9 weeks. Analysis was carried out by studying DNA methylation sequencing, multiplex immunoassays, qRT-PCR, and image processing. Results showed significant, persistent dysregulation in DNA methylation, cytokine levels, and microRNA expression. Specifically, critical genes such as Vav3, Pdgfa, Pdgfc, Jag2, Hras, Ksr1,

Il2rb, Il17b, and Il17rb exhibited hypermethylation at transcription start sites, promoters, and 5' UTRs in the LPS group. Altered cytokine levels (IL1b, IL-10, IL-17a/f, IL-23) and microRNAs (let-7a, miR-34a, miR-146a, miR-184, miR-200c, miR-211, and miR-219) further confirmed the dysregulation, indicating that LPS-induced inflammation may have lasting implications for mammary gland function through epigenetic alterations. Importantly, the inclusion of prebiotics like AHCC in the diet showed the potential to mitigate these effects, suggesting a strategy to protect the epigenetic integrity of mammary glands during puberty. This highlights the importance of dietary interventions in maintaining mammary gland health and preventing disease development.

Keywords: LPS, microbiome, gut immunity, prebiotics, Th17 cells, miRNAs, DNA methylation, proinflammatory cytokines, IL-17a, IL-17f, IL-10, IL-23.

2.2 Introduction

The commensal gut microbiota significantly impacts human health, particularly the development and function of the immune system (Shahbazi, Yasavoli-Sharahi et al. 2020). The intricate balance of the gut microbiome can be disrupted by various factors, including diet, medication, and obesity, leading to long-lasting metabolic and immune consequences, particularly in cases of childhood obesity and compromised immunity (Mensah, Opoku-Agyeman et al. 2017, Shondelmyer, Knight et al. 2018, Pushpanathan, Mathew et al. 2019).

AHCC's Potential in Modulating Cytokine Responses to LPS in Tumor and Mammary glands (LPS), which triggers an immune response (Lai, Liu et al. 2017). Changes in gut permeability and alterations in the gut microbiota, caused by factors like diet or immune

stressors, coincide with changes in gut function and contribute to dysfunctional immune responses characterized by systemic inflammation (Guo, Al-Sadi et al. 2013, Régnier, Van Hul et al. 2021). The inflammation-related modifications in the gut microbiome further compromise the integrity of the intestinal barrier. LPS, being a potent inducer of inflammatory responses, raises the possibility of an association between LPS and susceptibility to cancer. The circulating LPS or the increase in LPS load spreads to target tissues, imposing a burden not only on the gut but also on distant organs, including mammary glands (Martinelli, Reginatto et al. 2020).

The inflammatory response induced by LPS triggers events that lead to the modulation of the Th17 signaling pathway (Candelli, Franza et al. 2021). Within the gut-associated lymphoid tissue, LPS reduces the frequency of regulatory T cells (Tregs) while promoting the presence of Th17 cells. Th17 cells, essential components of the mucosal-associated lymphoid tissue, elicit an inflammatory reaction when encountering pathogens or LPS by releasing IL-17 and IL-23 (Ivanov, McKenzie et al. 2006, Singh, Schwartz et al. 2013). Activation of Th17 cells drives cellular responses, including the secretion of IL-1β, IL-6, IL-10, and epigenetic changes in gene expression (Ouyang, Kolls et al. 2008, Wilson 2008). These effects can impact various cell types, including cells in mammary glands. Importantly, activated Th17 cells possess the ability to migrate to and home in on lymph nodes situated in distant sites, such as breast tissue (Hsieh 2014).

Disruption in gene expression balance during critical developmental periods can potentially increase susceptibility to breast cancer later in life, especially considering that the mammary glands primarily develop after birth (Howlin, McBryan et al. 2006). Inflammation, acting through epigenetic mechanisms like DNA methylation and microRNAs, plays a significant role in altering gene expression patterns at both the gut level and distant sites, including mammary

glands (Gigli and Maizon 2013, Ivanova, Hue-Beauvais et al. 2023). Extensive research has highlighted the impact of inflammation on DNA methylation and microRNA expression (Hartnett and Egan 2012, Pekow and Kwon 2012). DNA methylation plays a critical role in the differentiation of mammary glands by influencing critical transcription factors, including HOXA1, TCF7L1, and GATA3 (Holliday, Baker et al. 2018). Additionally, microRNAs (miRNAs) also contribute significantly to the differentiation of terminal end buds. For instance, specific miRNAs, such as miR-184, exhibit pronounced upregulation during the differentiation of pubertal terminal end buds (Phua, Nguyen et al. 2015).

Prebiotics are essential for the maintenance and regulation of the gut microbial ecosystem (Sehrawat, Yadav et al. 2021). They not only contribute to reducing inflammation throughout the body but also counteract the detrimental effects of LPS when used as part of dietary manipulation with probiotics (de LeBlanc, Matar et al. 2006, Mallet, Graham et al. 2016, Vuong, Mallet et al. 2016). The beneficial effects of prebiotics primarily target the gut microbiota and have been demonstrated to exert influence on gene expression at the epigenetic level (Serban 2014). Remarkably, the gut microbiota possesses the capability to impact the Th17 signaling pathway, thereby affecting lymphocytes in distant sites (Luo, Leach et al. 2017). Furthermore, prebiotics interact with gut and immune cells, playing a regulatory role in modulating inflammatory pathways. This regulation is achieved through the modulation of pattern-recognition receptors (PRRs), specifically Toll-like receptors, as well as key signaling pathways such as NF-kB (Pujari and Banerjee 2021).

The mammary gland undergoes a unique developmental process, starting during the fetal stage, temporarily pausing after birth, and resuming in response to estrogen during puberty (Sternlicht 2005). The regulation of mammary gland morphogenesis, particularly the formation of terminal

end buds and secondary branch points, involves various mechanisms such as DNA methylation and miRNAs (Holliday, Baker et al. 2018, Wu, Thompson et al. 2022). At the onset of puberty, inflammation triggers the activation of crown-like structure macrophages, leading to the release of inflammatory factors that not only disturb the gene expression patterns of mammary stem cells but also impact adipokine expression within the fat pad surrounding mammary glands (Brown 2014). Additionally, pubertal exposure to LPS has been shown to elicit an immune response and influence brain function (Yahfoufi, Ah-Yen et al. 2021), yet its lasting effect on mammary glands remains understudied. The intake of prebiotics may potentially mitigate the inflammatory effect of LPS, although the impact of prebiotic intake in regulating the prolonged effects of inflammation on mammary glands has not been thoroughly investigated. Therefore, the objective of this study is to examine the effects of pubertal LPS exposure on mammary glands and evaluate whether dietary intervention during this critical period can reduce LPS-induced dysbiosis and rectify the enduring effects of LPS on mammary gland function.

In pursuit of our research goals, we employed a mouse model that induced inflammation during puberty through the administration of LPS. To explore potential interventions, we provided the mice with a prebiotic compound called *Lentinula Edodes* Cultured Extract (AHCC), known to possess potent immunomodulatory properties by inhibiting the activation of Toll-like receptor 4 (Mallet, Graham et al. 2016). Within the scope of this study, we aimed to delve into the intricate DNA methylation profiling of mammary glands, assess the level of cytokines, and investigate the expression pattern of microRNAs. We aimed to uncover valuable insights into the potential impact of inflammation and the efficacy of AHCC in modulating epigenetic factors, shedding light on new avenues for therapeutic intervention in mammary gland-related conditions.

2.3 Material and Method

Animals

Female BALB/c mice, aged three weeks and weighing between 13 and 17 grams, obtained from Charles River (Montreal, QC), were utilized in this study. The mice were housed in plastic cages with three mice per cage, providing a controlled environment with a 12-hour light/dark cycle and maintaining a temperature of 22 ± 2ºC and humidity at 55 ± 2%. Throughout the study, all groups of mice were provided with a conventional balanced diet without any restrictions. The treatment and maintenance of mice followed the guidelines outlined by the Canadian Council on Animal Care. The Animal Care Committee of the University of Ottawa approved the experimental protocol (HSe-3191).

Study Design

The experiment was conducted to investigate the long-term impact of pubertal exposure to LPS and prebiotic intake on the gut microbiota and immune system. A total of 36 mice were utilized for this experiment. Puberty onset was determined by observing the first occurrence of a pubertal event, specifically vaginal opening. The mice were divided into two groups consisting of 18 mice each: the prebiotic group received AHCC (2 gr/kg BW/day) in their drinking water, while the control group received regular drinking water for one week prior to puberty. Upon reaching puberty at five weeks of age, half of the mice in each group were intraperitoneally injected with LPS at a dose of 1.5 mg/kg body weight, known to induce inflammation and dysbiosis. The remaining half received an injection of sterile PBS. The LPS solution was prepared by dissolving LPS from Escherichia coli O26:B6 in sterile PBS at a concentration of 0.2 mg/ml. Thus, four experimental groups were formed after the LPS/PBS injection, with nine mice in each group: 1)

the control group receiving regular drinking water and PBS injection, 2) the LPS group receiving regular drinking water and LPS injection, 3) the prebiotic group receiving AHCC in drinking water and PBS injection, and 4) prebiotic+LPS group receiving AHCC in drinking water and LPS injection. The nutritional intervention continued for one week after injection, followed by a standard diet until early adulthood. At nine weeks of age, the mice were sacrificed, and relevant samples were collected to examine the enduring effects of the treatment on the immune system (Supplementary File 2, Fig. S2.1). The AHCC used in the experiment was provided by Amino Up Co., Ltd., based in Sapporo, Japan.

DNA Extraction and Mouse Methylation BeadChip Array

A segment of mammary glands from mice weighing 40-50 milligrams was subjected to homogenization using an electrical homogenizer (Bead Mill 24, Fisher Scientific, Canada). The homogenization was performed in tubes containing 500 µl of cell lysis buffer and 1.5 µl of proteinase K. Tissue DNA extraction was carried out using the Gentra Puregene Tissue Kit (33g) (Qiagen, Toronto, Canada) according to the manufacturer's instructions. The extracted DNA was subsequently diluted with DNA rehydration solution, provided with the kit, to a final concentration of 20 ng/µl and stored at -20ºC. The concentration of extracted DNA was determined using a Qubit 4 instrument (Thermo Fisher Scientific, Canada). DNA methylation analyses were conducted at the International Agency for Research on Cancer (IARC) in Lyon, France, as previously described (Bošković, Roje et al. 2022). Briefly, 500 ng of extracted DNA was subjected to bisulfite conversion using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) and was subsequently analyzed using the Infinium™ Mouse Methylation BeadChip (Illumina Inc., San Diego, CA, USA).

DNA Methylation Data Pre-Processing, Normalization, and Analysis

The bioinformatics analysis was performed following the pipeline developed in-house by the Epigenomics and Mechanisms Branch at the International Agency for Research on Cancer (available at https://github.com/IARCbioinfo/methylkey)), which utilizes R packages such as methylkey (v1), SeSAMe (v1.15.1), GenomicFeatures (v1.50.3), and minfi (v1.44.0). Briefly, IDAT files generated from the DNA methylation arrays were pre-processed and normalized using the SeSAMe R package. Differentially methylated probes were identified using robust linear regression, where significantly differential methylation was defined as probes in which the average inter-group difference in DNA methylation was more than |5%| and were assigned a false discovery rate (FDR)-adjusted p-value of less than 0.05. Differentially methylated regions were identified using the DMRcate (v2.11.0) package, defined by FDR-adjusted p-value < 0.05 and |max methylation difference| > 5%. KEGG pathways enrichment analyses were performed using the Enrichr (v3.0) package.

Multiplex Luminex Immunoassay

The concentration of interleukins IL-1β, IL-6, β0, IL-17a, IL-17f, and IL-23 were determined using a multiplex Luminex immunoassay. The extracted proteins from mammary gland samples were diluted with assay buffer solution at a 1:2 ratio. Mouse cytokine standards were prepared at concentrations of 3.2, 12, 80, 400, 2000, and 10000 pg/mL according to the protocol. Cytokine bead solutions were added to each well of the plate containing the standards, samples, and controls. The plate was then covered and incubated on a plate shaker at 2-8ºC overnight. Following incubation, two washes were performed, and detection antibodies were added to the wells. The plate was further incubated on a plate shaker at room temperature for one hour. Streptavidin-phycoerythrin was subsequently added to each well and incubated at room

temperature for 30 minutes. After another wash, the beads were resuspended in drive fluid and the cytokine concentrations were measured using a MAGPIX system.

Real-Time Quantitative Reverse Transcription PCR (RT-qPCR)

A small section of mammary gland tissue was preserved in RNAlater stabilization solution (Invitrogen, USA) at 4ºC for 24 hours and subsequently stored at -80ºC. Total RNA was extracted from samples using the miRNeasy Mini Kit (Qiagen, Toronto, Canada). The concentration and purity of the extracted RNA were determined using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). For assessing the expression of specific miRNAs, a reverse transcription reaction was performed to synthesize cDNA using the miRCURY LNA RT Kit (Qiagen, USA). The expression levels of microRNA Let-7a, Let-7c, miR-34, miR-130a, miR-146a, miR-184, miR-200c, miR-211, and miR-219 were measured by RT-qPCR using the specific miRCURY LNA miRNA PCR assay Primers (Qiagen, Toronto, Canada) and the miRCURY LNA SYBR Green PCR Kit (Qiagen, Toronto, Canada) in a CFX 384 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). The expression of miRNAs was normalized to the reference gene SNORD65 (mmu) using the miRCURY LNA miRNA PCR assay. The relative expression levels of miRNAs were calculated using the ΔΔCT method.

Whole Mount Staining of Mammary Glands

The fourth inguinal mammary gland was dissected and immediately placed on a glass slide. Subsequently, the slide was immersed in Carnoy's fixative, in the chemical hood for 24 hours. Following this, the mammary glands were hydrated in 70% ethanol for 15 minutes and then rinsed in distilled water. The slides were then transferred to a carmine alum solution for storage

for 24 hours. The dehydration process of the mammary glands involved sequentially transferring the slides into ethanol solutions of increasing concentration from 70% to 100%, followed by an overnight submersion in xylene (Thermo Scientific, Waltham, MA, USA) (Tolg, Cowman et al. 2018).

Image Processing

The image of whole mount staining of mammary glands was obtained using optical microscopy (Fig. 2.1A). The image processing analysis was conducted using the Python programming language (v 3.9), in conjunction with ImageJ software (v 1.54h, https://imagej.net/ij/). The preliminary phase of the analysis was executed using scikit-image (version 0.16.0), and NumPy (v 1.26.2) libraries. This phase involved the manipulation and detailed examination of whole-mount staining images of mammary glands. The conversion of colored images to grayscale was achieved using the `rgb2gray` function (Fig. 2.1B). Subsequently, the `meijering` function was employed to filter the images, effectively highlighting ridge-like structures (Fig. 2.1C). To refine the image quality, a morphological filter was applied to eliminate small objects, and Gaussian smoothing with a sigma value of 0.5 was utilized to diminish the noise of the images (Supplementary File 1).

For the transformation of the grayscale images into binary format, Otsu's method was performed. This technique calculates an optimal threshold value to separate the pixel classes into distinct groups. The process then progressed to skeletonization, where the binary image was converted into a skeletonized form, revealing the essential structure of mammary ducts (Fig. 2.1D).

In the next phase of analysis, the AnalyzeSkeleton (http://fiji.sc/AnalyzeSkeleton) (Arganda-Carreras, Fernández-González et al. 2010) plugin for ImageJ was used (Fig. 2.1E). This plugin performs a comprehensive quantification of various morphological aspects, including the number of branches, junctions, and endpoints, measurement of junction and slab voxels, assessment of average and maximum branch lengths, and determination of triple and quadruple points.

Statistical Analysis

Statistical analysis for all experiments, excluding DNA methylation analysis, was conducted using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). The distribution of data was assessed using the Shapiro-Wilk test. Two-way analysis of variance (ANOVA), followed by Tukey's post-hoc test, was performed to compare the means of the experimental groups. The results are presented as mean ± SEM, and a p-value < 0.05 indicates a statistically significant difference between groups.

2.4 Results

Long-term effects of LPS Exposure on DNA Methylation and Potential Impact of AHCC Treatment.

DNA methylation is a critical epigenetic mechanism that contributes to the repression of gene expression. The methylation status of specific regions of genes, including the 1-5 kb upstream of transcriptional start sites, promoters, and 5' untranslated regions (UTRs), can affect this repression. This study examines the long-term effects of LPS and AHCC exposure during puberty on the methylation profiles of mammary gland cells. To evaluate the data, the study used cut-off criteria of methylation status more than 3% and an adjusted p-value less than 0.05.

The study sought to investigate these regions in comparable genes in the control vs. AHCC, control vs. LPS, and LPS vs. AHCC+LPS groups (Supplementary File 2, Fig. S2.2). When comparing the control and AHCC groups, the analysis failed to identify any statistically significant differentially methylated regions (DMRs). However, when comparing the control and LPS groups, the results showed that 85.8% of genes were hypomethylated, while only 14.1% were hypermethylated (Supplementary Table 1). Fig. 2.1F indicates the global DNA methylation pattern across all chromosomes. Based on the genomic regulatory regions plot, there is a higher frequency of hypomethylated DMRs observed at the promoter, 1-5 kb upstream of TSS, and exonic regions (Fig. 1G). The proportion of hypermethylated regions is relatively small in CpG islands, shelves, and shores, as shown in the CpG density plots. In contrast, a larger proportion of hypomethylated regions are found in these CpG-rich regions (Fig. 2.1G). As DNA methylation on certain regions, such as TSS, the region 1-5 kb upstream of TSS, promoters, and 5'UTRs, can result in the repression of gene expression, subsequent analyses focused on DMRs within these regions as they were more likely to have a direct influence on gene expression.

The hypermethylated DMRs were involved in PI3K-Akt (4 DMRs), MAPK, Hippo, and Rap1 signaling pathways (3 DMRs each). In contrast, the hypomethylated DMRs were involved in cAMP (18 DMRs), PI3K-Akt (16 DMRs), Rap1 (14 DMRs), MAPK (14 DMRs), Ras signaling pathways (11 DMRs), Cytokine-cytokine receptor interaction (19 DMRs), microRNAs in cancer (13 DMRs), and Proteoglycan in cancer (12 DMRs) (Fig. 2.1H, Supplementary Table 2).

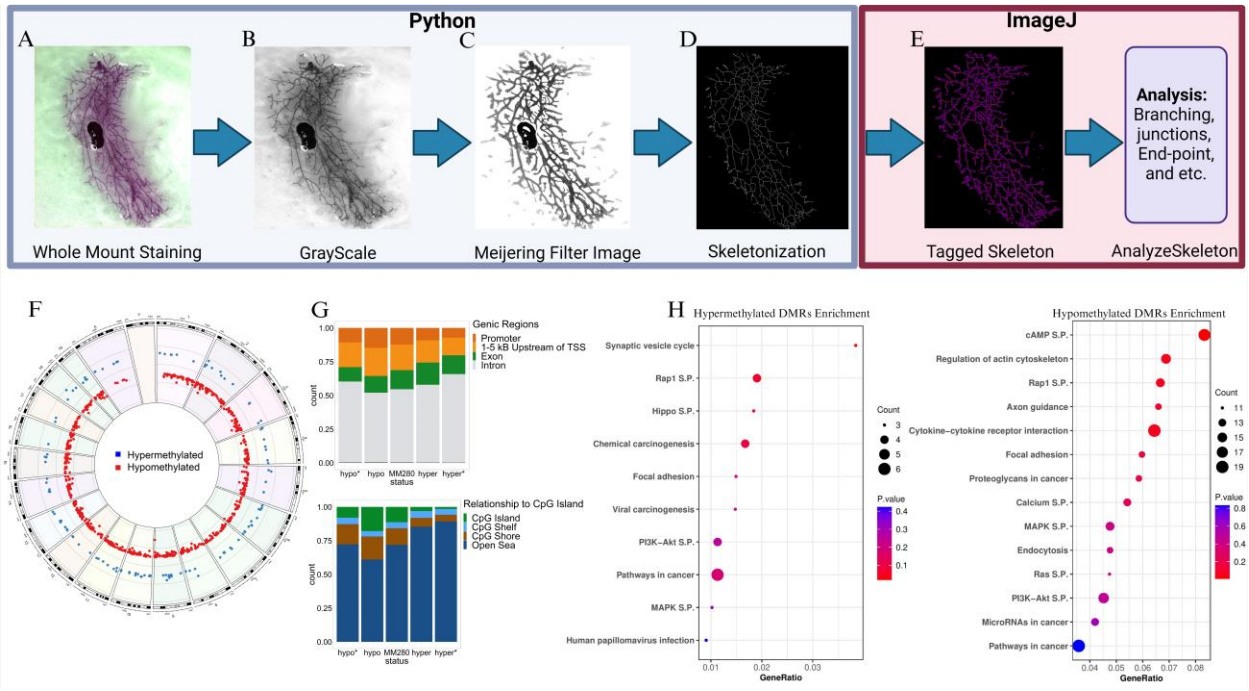

Fig. 2.1. Image Processing Procedure Flowchart and Genome-wide methylation analysis of the mammary glands, Comparing Control versus LPS Groups. (A) Whole mount staining of the mammary gland. **(B)** The grayscale image of the mammary gland using rgb2gray. **(C)** Meijering filter to enhance ridge-like structures. **(D)** The skeletonized image of mammary ducts**. (E)** Tagged skeleton, the processed image by AnalyzeSkeleton. Blue box, the processed performed in python, and the red box the processed performed in ImageJ software. **(E)** 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 the red dots represent hypomethylation. **(G)** illustrates the genomic regulatory region and CpG regulatory region of the DMRs, where the column with an asterisk represents significant differentially methylated regions (DMRs). The labels "hypo*" and "hyper*" indicate significant hypomethylation and hypermethylation, respectively. The absence of an asterisk signifies all hypo/hyper positions, regardless of their statistical

significance in differential methylation. Additionally, "MM280" represents the overall distribution of the array. **(H)** Depict the KEGG pathway enrichment analyses of hypermethylated and hypomethylated regions, respectively. S.P. refer to signaling pathway.

Additionally, when comparing LPS and AHCC+LPS, 29.9% of DMRs were hypomethylated, while 70.1% were hypermethylated (Supplementary Table 3). Fig. 2.2A illustrates the global DNA methylation pattern across all chromosomes. The genomic regulatory regions plot reveals a higher occurrence of hypermethylated DMRs in the promoter, 1-5 kb upstream of TSS, and exonic regions compared to hypomethylated DMRs (Fig. 2.2B). A small proportion of hypermethylated regions in CpG islands, shelves, and shores, as in the previous comparison of LPS vs. control. In contrast, a larger proportion of hypomethylated regions are found in these CpG-rich regions (Fig. 2.2B).

The hypermethylated DMRs were involved in T cell receptor (6 DMRs), Ras (8 DMRs), NOD-like receptor (6 DMRs), Rap1 (6 DMRs), cAMP (6 DMRs), MAPK (8 DMRs), PI3K-Akt signaling pathways (7 DMRs), Pathways in cancer (13 DMRs), microRNAs in cancer (8 DMRs), and Cytokine-cytokine receptor interaction (8 DMRs). On the other hand, the hypomethylated DMRs were involved in Tight junction (6 DMRs), Rap1 (6 DMRs), MAPK (6 DMRs), PI3K-Akt signaling pathway (5 DMRs), and Pathways in cancer (9 DMRs) (Fig. 2.2C, Supplementary Table 4).

In order to gain a deeper understanding of the potential for AHCC to mitigate the impact of LPS on DNA methylation, we conducted a thorough investigation into genes that were similarly differentially methylated in comparisons of control vs. LPS, as well as LPS vs. AHCC+LPS. Our analysis revealed 252 genes that overlapped in both comparisons. Of these genes, 14 were found to be involved in a range of important signaling pathways, such as Ras, Rap1, MAPK, PI3K-Akt,

Th1 and Th2 cell differentiation, cytokine-cytokine receptor interaction, and JAK-STAT, IL17 signaling pathways (Fig. 2.2D). These 14 genes included *Ntng1*, *Vav3*, *Ppp3ca*, *Pdgfa*, *Pdgfc*, *Jag2*, *Nr4a1*, *Hras*, *Ksr1*, *Camk2g*, *Mybl2*, *Il2rb*, *Il17b*, and *Il17rb* (Fig. 2.2E).

The analysis also identified 28 genes that were hypermethylated in the LPS group compared to the control, but hypomethylated in the AHCC+LPS group when compared to LPS. Among these genes, seven were directly involved in cancer development or tumor suppression, including *Rhobtb1*, *Dmtn*, *Zdhhc1*, and *Adarb1*, which play a role as tumor suppressors, and *Asph*, *Emp1*, and *Tmcc3*, which induce tumorigenesis and metastasis (Supplementary File 2, Fig. S2.3). These findings shed light on the potential lasting effects of LPS exposure on DNA methylation profiles, as well as the impact of LPS on cancer-related genes and immune system and microRNA signaling pathways. In addition, our results suggest that AHCC treatment may be effective in mitigating the effect of LPS on DNA methylation and gene expression.

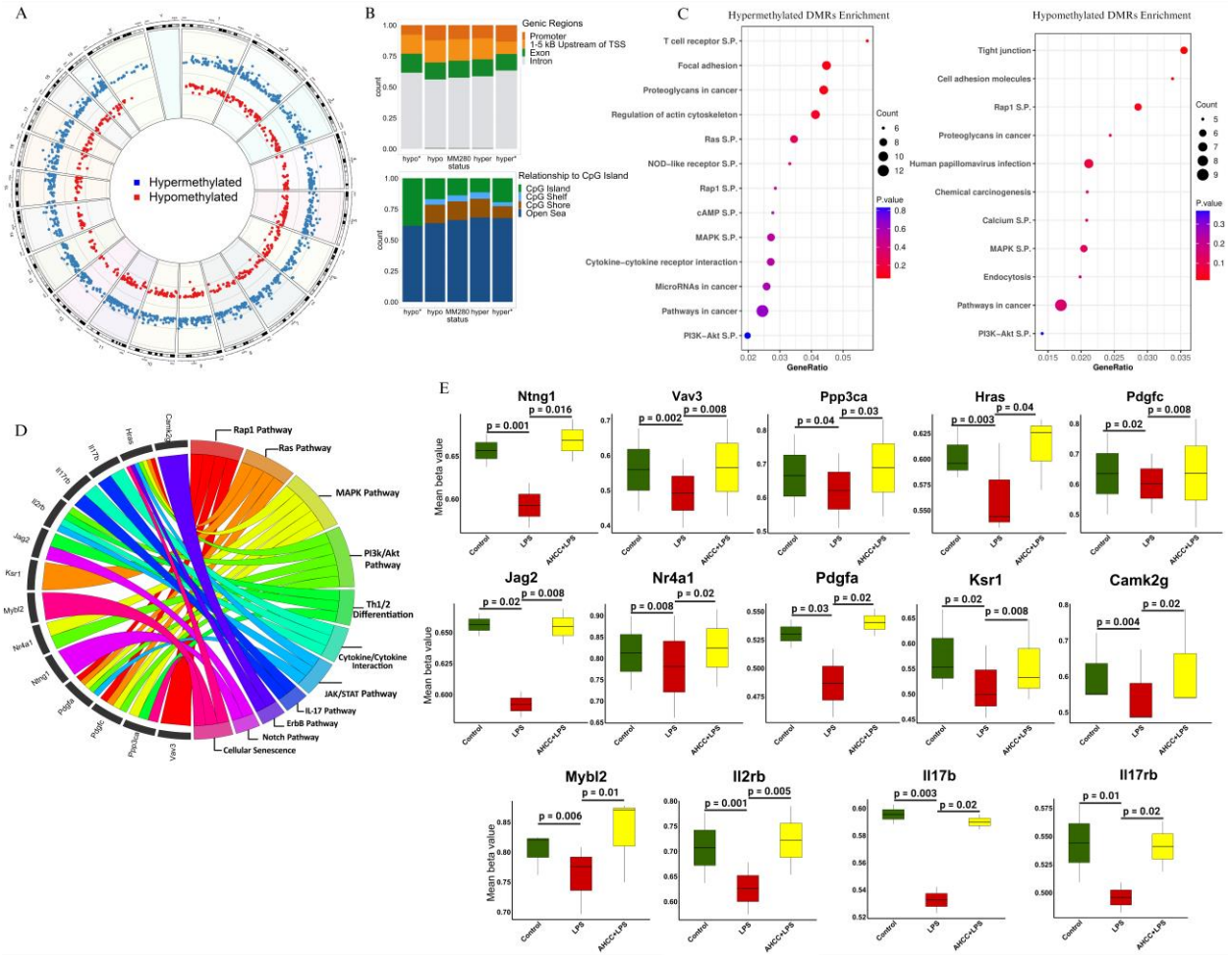

Fig. 2.2. Genome-Wide Methylation Analysis of the Mammary Glands, Comparing LPS versus AHCC+LPS Groups. **(A)** depict DNA methylation patterns across the genome. The outmost ring indicate the chromosome number 1 to 22, including X and Y chromosomes. Each dot on the plot marks a distinct genomic location with altered methylation. The blue dots represent hypermethylation and the red dots represent hypomethylation. **(B)** illustrate the genomic regulatory region and CpG regulatory region of the DMRs, where the column with an asterisk represents significant differentially methylated regions (DMRs). The labels "hypo*" and "hyper*" indicate significant hypomethylation and hypermethylation, respectively. The absence of an asterisk signifies all hypo/hyper positions, regardless of their statistical significance in differential methylation. Additionally, "MM280" represents the overall distribution of the array. **(C)** Depict the KEGG pathway enrichment analyses of hypermethylated and hypomethylated regions, respectively. S.P. refer to signaling pathway. (**D)** circular plot indicating the role of 14 genes in critical signaling pathways (S.P.). **(E)** The methylation level of 14 genes, *Ntng1*, *Vav3*, *Ppp3ca*, *Pdgfa*, *Pdgfc*, *Jag2*, *Nr4a1*, *Hras*, *Ksr1*, *Camk2g*, *Mybl2*, *Il2rb*, *Il17b*, and *Il17rb*, that were hypermethylated in AHCC+LPS and hypomethylated in LPS groups in mammary glands of adult mice four weeks after injection of a single dose of LPS at puberty.

Long-Term Effects of LPS Exposure on Pro-Inflammatory Cytokines and Immunomodulatory Effect of AHCC Treatment.

Our previous research established that LPS administration during critical developmental periods, such as puberty, leads to gut dysbiosis, notably an increase in gram-negative bacteria, which play a role in regulating the immune system. This phenomenon, combined with the observed shifts in DNA methylation of genes influencing immune functions and cytokine pathways, led us to investigate a wide array of cytokines in the mammary glands (Fig. 2.3A). Results showed that although IL1β levels were slightly decreased in the LPS group compared to the control group, the difference was not statistically significant. Interestingly, IL1β levels were significantly higher in the LPS group than in the AHCC+LPS group. Although the level of IL1β was lower in the AHCC and AHCC+LPS groups compared to the control group, this difference was not statistically significant.

The levels of IL6 were significantly decreased in the LPS, AHCC, and AHCC+LPS groups compared to the control group. However, no significant differences were found between the LPS and AHCC+LPS groups.

Regarding IL10, a tendency towards an increase in the LPS group compared to the control group was observed; however, this difference was not statistically significant. Interestingly, the level of IL10 was significantly lower in the AHCC group compared to the LPS and AHCC+LPS groups. No significant differences were observed between the LPS and AHCC+LPS groups.

Additionally, while IL17a levels were decreased in all groups, the only statistically significant difference was observed in the comparison of the control and AHCC+LPS groups. Moreover, the presence of IL17 was higher in the LPS group compared to the AHCC+LPS group.

The presence of IL17f was found to be significantly higher in the LPS group compared to the control group. Interestingly, IL17f levels were significantly lower in the AHCC group compared to the LPS group. Moreover, IL17f levels were lower in the AHCC+LPS group compared to the LPS group; however, this difference was not statistically significant.

Finally, the level of IL23 was found to be lower in the AHCC group compared to the control, LPS, and AHCC+LPS groups. Although there was a trend towards an increase in IL23 levels in the LPS and AHCC+LPS groups, there were no statistically significant differences between these two groups (Fig. 2.3A).

Long-Term Effects of LPS Exposure on microRNAs and the Potential Impact of AHCC Treatment on Mammary glands.

DNA methylation study identified that Dicer1 and the intronic region of Ago2, both essential for microRNA maturation, were less methylated in the LPS group compared to the control group (Fig. 2.3B). The altered methylation patterns of these genes, which regulate microRNAs, along with the prominent association of the term "microRNAs in cancer" and the observed changes in key cytokine levels in mammary glands, led us to explore the expression of microRNAs linked to tumor progression and the development of mammary glands. In this study, the expression levels of several tumor suppressor microRNAs, including let-7a, let-7c, miR-34a, miR-130a, miR-146a, miR-184, miR-200c, miR-211, and miR-219 in response to exposure to LPS and AHCC were investigated (Fig. 2.3C).

The findings revealed a statistically significant downregulation of let-7a and let-7c in the LPS group, while no significant changes were observed in the AHCC group. Furthermore, the expression of let-7a and let-7c was slightly decreased in the AHCC+LPS group compared to the

control group. Notably, the expression of let-7a and let-7c was significantly higher in the AHCC+LPS group compared to the LPS group.

In this study, an upregulation of miR-34a was observed in all experimental groups compared to the control group, with a 1.4-fold increase observed in the AHCC group compared to the LPS group. Likewise, we observed a similar trend for miR-130a, another well-known tumor suppressor microRNA, which exhibited a significant upregulation in all experimental groups compared to the control group.

In contrast, no significant change was observed in the expression of miR-146a in the LPS and AHCC+LPS groups compared to the control group, indicating that LPS exposure did not have a significant effect on the expression of this microRNA in the mammary glands. However, it is noteworthy that the expression level of miR-146a was found to be significantly increased in the AHCC group compared to all other experimental groups.

The results demonstrate that miR-184 expression was significantly decreased in the LPS group compared to the control group. Additionally, there was a slight decrease in miR-184 expression in the AHCC+LPS group compared to the LPS group. It was observed that miR-184 expression was higher in the AHCC+LPS group compared to the LPS group. However, our findings indicate that AHCC treatment did not have a significant effect on miR-184 expression.

In the study, a slight downregulation of miR-200c was observed in both the LPS and AHCC+LPS groups compared to the control group. On the other hand, the expression of miR-200c was found to be significantly higher in the AHCC group compared to the control group.

The expression of miR-211 was found to be unaltered in the LPS group when compared to the control group. However, a significant increase was observed in the AHCC and AHCC+LPS

groups in comparison to the control group. Moreover, the expression level of miR-211 was observed to be higher in the AHCC+LPS group compared to the LPS group. These results suggest that AHCC treatment may positively regulate the expression of miR-211 in mammary glands and that LPS exposure alone may not affect its expression.

In addition, research findings revealed that miR-219 was significantly downregulated in response to LPS exposure, whereas it was significantly upregulated following AHCC treatment. Additionally, data showed that the expression level of miR-219 was decreased in the AHCC+LPS group compared to the control group; however, no significant difference was observed between the LPS and AHCC+LPS groups. These results suggest that LPS exposure can downregulate critical tumor suppressor microRNAs, such as let-7a, let-7c, miR-184, and miR-219, while AHCC treatment may mitigate this effect and restore their expression levels (Fig. 2.3C).

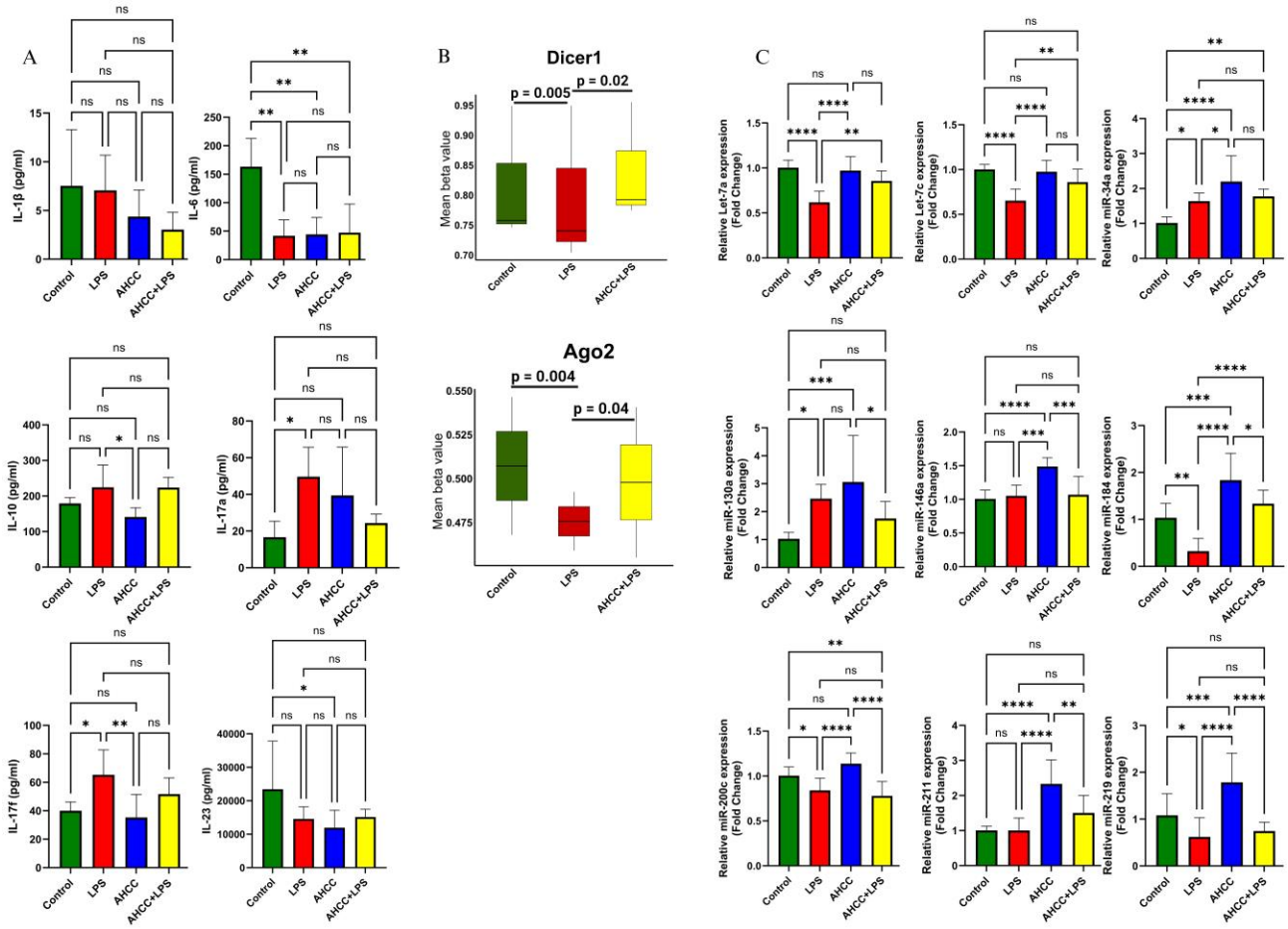

Fig. 2.3. Impact of Pubertal LPS Exposure and AHCC Treatment on Mammary Gland Cytokine Concentrations and microRNA Expression Levels. (A) Concentrations of IL1β, IL-6, IL-10, IL-17a, IL-17f, and IL-23 in the mammary glands of adult mice four weeks after the injection of a single dose of LPS at puberty. Two-way ANOVA and Tukey's post-hoc tests were used to compare groups. **(B)** DNA methylation patterns of Dicer1 and Ago2, two pivotal genes in microRNA maturation, in the LPS group compared to the control. **(C)** The relative expression of candidate tumor suppressor microRNAs: let-7a, let-7c, miR-34a, miR-130a, miR-146a, miR-184, miR-200c, miR-211, and miR-219, in the mammary glands of adult mice four weeks after a single LPS injection at puberty. Two-way ANOVA and Tukey's post-hoc tests were used to compare groups. All values are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Long-Term Effects of LPS Exposure and Potential Impact of AHCC Treatment on Mammary Glands Structure.

In the next step, following the observation of alterations in DNA methylation patterns, cytokine concentration changes, and the dysregulation of microRNAs within mammary glands, a detailed

structural analysis of mammary glands using image processing approaches was performed. This involved the evaluation of several parameters, including quantification of branching, junctions and endpoints, measurement of junction and slab voxels, assessment of average and maximum branch length, and determination of triple and quadruple points. Notably, the results revealed no statistically significant differences across these measurement criteria in relation to the LPS and AHCC treatment on mammary glands, with the exception of quadruple points. In this analysis, we observed that the number of quadruple points in the AHCC group is lower compared to the control and AHCC+LPS groups. This finding suggests that the morphological characteristics of the mammary glands remained largely unaffected by the treatments, except for a variation in quadruple points (Fig. 2.4).

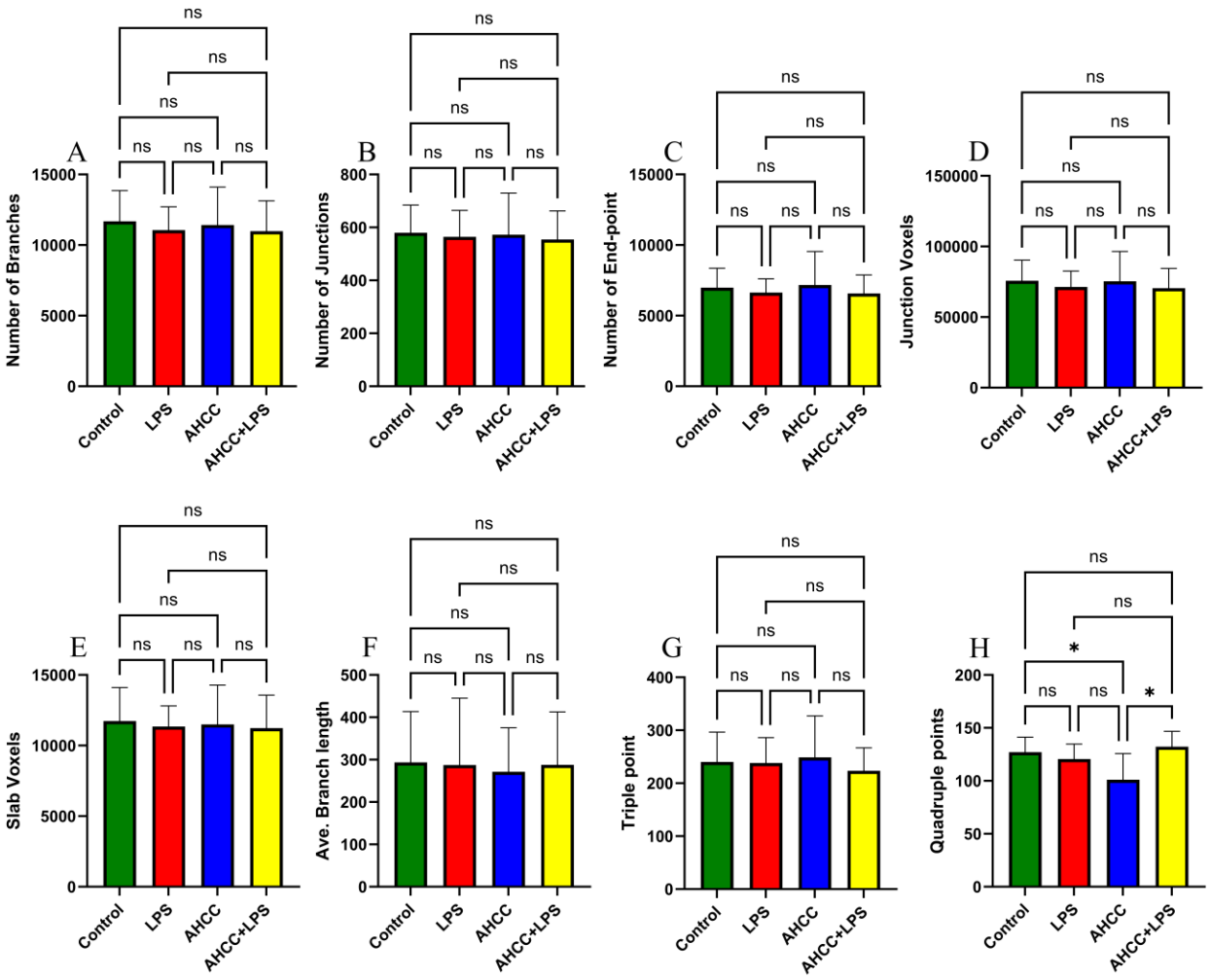

Fig. 2.4. The Measurement of Mammary Gland Structure. Different criteria, including **(A)** number of branches, **(B)** junctions, **(C)** endpoints, measurement of **(D)** junction and **(E)** slab voxels, assessment of **(F)** average branch length, and determination of **(G)** triple and **(H)** quadruple points of mammary glands were measured using the AnalyzeSkeleton plugin within ImageJ. Two-way ANOVA and Tukey's post-hoc tests were used to compare groups. All values are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

2.5 Discussion

Dietary intervention with prebiotics has been proven to counteract the negative effects of gut microbiota dysbiosis. Restorative effects of prebiotics on the gut microbiota have been reported to modulate miRNAs (Hasan and Yang 2019) and prevent immune disorders (Gourbeyre, Denery et al. 2011, Mallet, Graham et al. 2016). This study aimed to examine whether prebiotic

consumption could alleviate prolonged inflammatory immune responses in mice that were exposed to LPS during the developmental stage at puberty. The study focused on the effect of prebiotics on mammary glands, analyzing the regulatory signaling pathways and epigenetic mechanisms involved.

Microbiome dysbiosis leads to an increase in circulating LPS (Yu, Wang et al. 2012, Quigley 2017). While gut dysbiosis initially increases LPS levels, exposure to elevated LPS itself contribute to further gut microbiota dysbiosis. This cycle of dysbiosis and increased LPS levels triggers a cascade of systemic effects, including immune and epigenetic disruption beyond the gut, as it can also alter the DNA methylation profile of the cells, cause dysregulation in the expression of cytokines, and disrupt the levels of microRNAs (Cai, Zhang et al. 2012, Hartnett and Egan 2012, Liu, Yin et al. 2018). Disruption of the gut microbiota by an immune stressor (inflammatory LPS) during puberty has been shown not only to perturb gut microbiota but also to significantly increase the risk of pre-carcinogenic lesions (Herrera-Covarrubias, Coria-Avila et al. 2017). The impact of circulating LPS on gene expression in mammary glands is well established, including the induction of pro-inflammatory cytokines and abnormal growth of the virgin mammary gland through the activation of the NF-kB signaling pathway (Cao and Karin 2003, Li, Yin et al. 2017). Moreover, activation of the NF-kB signaling pathway and increased levels of IL-1β and IL-6 are potential precursors to mastitis (Shao, Shen et al. 2023). LPS-induced inflammation can disrupt the activity of DNA methyltransferases (DNMTs), leading to aberrant DNA methylation patterns throughout the genome. These altered methylation patterns resemble those observed in cancer cells (Maiuri and O'Hagan 2016). Additionally, LPS induces the differentiation of Th17 cells and the expression of their related cytokines, which are associated with an increase in chronic inflammation (McAleer, Liu et al. 2010). It is noteworthy

that miRNA expression is dependent on the gut microbiota. The interaction between gut microbiota and miRNAs at intestinal epithelial cells can activate signaling pathways and modulate gene expression (Dong, Tai et al. 2019). Several studies have shown the effect of LPS and inflammatory cytokines on miRNA dynamics and dysregulation in various tissues (Lindsay 2008, Sonkoly and Pivarcsi 2009).

In this study, the enduring and long-term effects of LPS exposure on DNA methylation and the potential impact of AHCC treatment on the DNA methylation profile of mammary glands were examined. This experiment was carried out by comparing the control vs. LPS group (85.8% hypomethylated and 14.1% hypermethylated) and LPS vs. AHCC+LPS groups (29.9% hypomethylated and 70.1% hypermethylated). Furthermore, the study revealed 252 genes that were both hypomethylated in the LPS group when compared to control and hypermethylated in the AHCC+LPS group when compared to LPS Of these genes, 14 genes including *Ntng1*, *Vav3*, *Ppp3ca*, *Pdgfa*, *Pdgfc*, *Jag2*, *Nr4a1*, *Hras*, *Ksr1*, *Camk2g*, *Mybl2*, *Il2rb*, *Il17b*, and *Il17rb* were involved in critical signaling pathways such as cytokine-cytokine receptor interaction, JAK-STAT, IL17 signaling pathways, and microRNA processes.

Several of these genes hold significant relevance in cancer biology and mammary gland development. For instance, Netrin G1 (*Ntng1*) and Jagged2 (*Jag2*) are pivotal players in cell survival signaling pathways and have been associated with colorectal and breast cancer development through the activation of the Notch signaling pathway(Mehlen and Mazelin 2003, Houde, Li et al. 2004, Zhang, Li et al. 2016, Mandula, Sierra-Mondragon et al. 2024). Vav3 plays a crucial role in hormone-dependent cancers, particularly breast cancer (Dong, Liu et al. 2006, Lyons and Burnstein 2006), where it influences estrogen receptor alpha (ERα) transcriptional activity, contributing to endocrine therapy resistance (Aguilar, Urruticoechea et

al. 2014, Chen, Chen et al. 2015). *Ppp3ca* is a key player in calcium-dependent signaling pathways, impacting cellular processes such as proliferation, differentiation, and apoptosis (Wójcik-Piotrowicz, Kaszuba-Zwoińska et al. 2016, Jones and Hazlehurst 2021). Moreover, it is involved in the regulation of the immune system and COX-2 expression, which leads to breast cancer invasion (Crabtree and Olson 2002, Yiu and Toker 2006, Guasch, Aranguren-Ibanez et al. 2015). The platelet-derived growth factor a and c, *Pdgfa* and *Pdgfc*, are two important genes involved in various cellular processes and have been associated with promoting epithelial-to-mesenchymal transition (EMT) in gastrointestinal tumors and breast cancer metastasis (Ren, Sun et al. 2019, Yoon, Tang et al. 2021, Shah and Sizemore 2022). Moreover, *Pdgfc* contributes to multiple functions including the VEGF signaling pathway, immune cell recruitment, and inflammation (Eitner, Bücher et al. 2008, Huang, Wang et al. 2017). *Nr4a1*, a member of the nuclear receptor subfamily, interacts with several key proteins including mTOR and HIF-1α, contributing to tumor progression and micro-metastasis induction (Zheng, Comaills et al. 2019, Deng, Chen et al. 2022). *Hras* is a critical regulator of Ras, PI3K/AKT, and Raf1 signaling pathways, influencing cell growth, differentiation, and survival (Espada, Galaz et al. 2009, Pązik, Michalska et al. 2021). *Hras* dysregulation is linked to gastric and breast cancer aggressiveness (Wu, Liu et al. 2016, Al-Eitan, Al-Ahmad et al. 2020, Kareff, Trabolsi et al. 2024). In contrast to *Hras*, *Ksr1* acts as a negative regulator of the Ras signaling pathway and has been associated with improved survival in breast cancer patients by stabilizing BRCA1 (Stebbing, Zhang et al. 2015, Zhang, Angelopoulos et al. 2015, Lavoie, Sahmi et al. 2018, Rao, Frodyma et al. 2021). *Camk2g* is involved in ovarian cancer and myelofibrosis, affecting cisplatin-induced reactive oxygen species (ROS) stress and sensitivity to Jak2 inhibitor, respectively (Li, Zheng et al. 2022, Miyauchi, Sasaki et al. 2022). *Mybl2* overexpression in breast cancer has been associated with

decreased overall survival (Kalinsky, Lim et al. 2014). It has been shown that *Mybl2* expression varies across BC subtypes, with the highest level observed in basal-like and the lowest in luminal-A tumors (Fuster, Llop et al. 2013, Li, Liu et al. 2019, Vera, Bok et al. 2021). *Il2rb* is significantly overexpressed in breast cancer tumors, influencing cytokine interaction and is associated with poor overall survival (Tan, Zuo et al. 2022). Lastly, *I17b* and its receptor *Il17rb* have critical functions in immune cell attraction and the inflammatory response, activating NF-kB and BCL2, and promoting cell growth, invasion, and survival through the ERK1/2 signaling pathway in breast cancer (Alinejad, Dolati et al. 2017).

In our study, we identified four tumor suppressor genes that were hypermethylated in the LPS group compared to the control, but hypomethylated in the AHCC+LPS group compared to LPS. These genes include Rhobtb1, Dmtn, Zdhhc1, and Adarb1. Rhobtb1 has been shown to inhibit tumor growth and invasion by interacting with Rhoch1 and Cul3, as well as preserving Golgi integrity by regulating Mettl7b activity and reducing breast cancer invasion (McKinnon and Mellor 2017, Haga, Garg et al. 2019). Overexpression of Zdhhc1 induces ER stress, resulting in enhanced apoptosis and pyroptotic cell death (Le, Mu et al. 2020). Adarb1 exhibits tumor-suppressive properties by regulating microRNAs, such as miR-589 and miR-200, to reduce glioblastoma cell growth and suppress metastasis in colorectal cancer (Chan, Qamra et al. 2016, Fu, Qin et al. 2017, Cesarini, Silvestris et al. 2018, Shelton, Duran et al. 2018). On the other hand, we identified three genes (Asph, Emp1, and Tmcc3) that exhibited the same DNA methylation pattern and play important roles in tumor progression. Asph is frequently overexpressed in solid tumors and regulates cbEGF-like domains, activating the Notch and Src signaling pathways to promote tumor growth (Gronke, VanDusen et al. 1989, Kanwal, Smahel et al. 2020). Emp1 overexpression is associated with increased tumor aggressiveness and poor

prognosis in various cancer types, including gastric and bladder cancer. Emp1 induces CD44 expression, maintains stem cell properties, and activates the PI3K-Akt and MAPK signaling pathways, contributing to tumor growth and metastasis (Sun, Zhao et al. 2014, Wang, Li et al. 2019, Lin, Zhang et al. 2020, Liu, Ding et al. 2020). Tmcc3 activates the Akt signaling pathway and promotes the progression and metastasis of breast cancer. Tmcc3 also preserves the stemness characteristics of breast cancer stem cells through its direct interaction with the Akt protein (Wang, Chan et al. 2021). These findings suggest that AHCC supplementation mitigates LPS-induced DNA methylation changes, potentially reversing hypermethylation of tumor suppressor genes affected by LPS. AHCC thus presents a promising strategy to counteract epigenetic modifications linked to cancer progression and mammary gland abnormalities.

Our research focuses on how DNA methylation affects genes involved in microRNA production and maturation. Notably, the promoter of *Dicer1*, as well as the intron of *Ago2*, demonstrate hypomethylation in the LPS group compared to the control. *Dicer1*, a key player in microRNA maturation, is heavily involved in cancer dynamics. While the loss of *Dicer1* is associated with tumorigenesis, studies have revealed that overexpression of *Dicer1* is linked to poor progression in colorectal cancer, indicating its dual role in cancer development. Argonaute 2 (*Ago2*) is a key component of the RISC protein complex, playing a crucial role in the regulation of gene expression, and any alteration in the expression of *Ago2* is associated with gene expression dysregulation (Pasquinelli, Hunter et al. 2005).

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 the genes affected, we identified several that are

involved in critical signaling pathways, including cytokine-cytokine receptor interaction, Jak-STAT, IL17 signaling pathways, and microRNA expression regulation. Inflammation plays a key role in DNA methylation modulation and is known to impact various cellular processes.

Our previous research revealed that pubertal LPS exposure alters the gut microbiome composition, significantly increasing the levels of Bacteroides and Parabacteroides genera in mice treated with LPS compared to the AHCC and AHCC+LPS groups (Shahbazi, Yasavoli-Sharahi et al. 2023). This dysbiosis is associated with colitis-induced damage to the mucosal lining. In addition, this dysbiosis is linked to an increase in proinflammatory cytokines including IL-17, IL-21, and IFN-γ (Shahbazi, Yasavoli-Sharahi et al. 2023).

In the context of the mammary glands, LPS exposure has been shown to induce abnormal growth and activate proinflammatory cytokines through the NF-κB signaling pathway (Page, Kell et al. 2022). Furthermore, LPS can also dysregulate microRNA expression (Taganov, Boldin et al. 2007). Previous studies have demonstrated the impact of LPS and inflammatory cytokines on microRNA dynamics and dysregulation in various tissues (Cai, Zhang et al. 2012). Therefore, based on the interplay between DNA methylation, microbiome composition, cytokines, and microRNAs, we decided to explore the effects of LPS and the potential mitigation by AHCC on cytokine expression and microRNAs in the mammary glands.

The study showed a consistent pattern of expression levels for IL-1β and IL-6 in the mammary glands. Notably, IL-1β levels were significantly higher in the LPS group compared to the AHCC and AHCC+LPS groups. The lower levels of IL-1β in the AHCC and AHCC+LPS groups suggest a potential modulatory effect of AHCC on IL-1β production in the context of LPS exposure. IL-1β plays a crucial role as a regulator of local tissue inflammation, capable of triggering the synthesis of various pro-inflammatory cytokines, chemokines, and adhesion

molecules (Edelman, Jiang et al. 2007, Dinarello 2018). Elevated IL-1β levels in mammary glands have been implicated in inflammation, tissue damage, and the development of breast cancer (Tulotta and Ottewell 2018).

IL-6 is a multifunctional cytokine involved in immune regulation, inflammation, and tissue homeostasis (Scheller, Chalaris et al. 2011). It plays a crucial role in the acute-phase response, immune cell activation, mammary gland development, and lactation (Palkowetz, Royer et al. 1994, Lund, Rømer et al. 1996, Tanaka, Narazaki et al. 2014). Moreover, elevated IL-6 levels have been associated with breast cancer progression and metastasis (Knüpfer and Preiß 2007). Interestingly, our study revealed unexpectedly low levels of IL-6 in the LPS, AHCC, and AHCC+LPS groups compared to the control group. This finding is intriguing, as LPS exposure generally induces the production of IL-6 (Liu, Zhang et al. 2017). One plausible explanation is the timing of mouse sacrifice, as IL-6 expression is highly dynamic, with a transient peak during the early phases of immune responses (Kishimoto 2010). Further investigation is needed to elucidate the dynamics and regulation of IL-6 expression in the context of LPS and AHCC treatment.

It is evident that LPS exposure can induce the production of proinflammatory cytokines such as IL-1β, IL-6, IL-17A, IL-17F, and IL-23, while simultaneously influencing the expression of anti-inflammatory cytokines such as IL-10. These findings suggest that LPS triggers an immune response characterized by both proinflammatory and anti-inflammatory components in the mammary glands.

In our study, we observed higher levels of IL-17A and IL-17F in the LPS-exposed group compared to the control group. Additionally, although not statistically significant, IL-10 and IL-23 showed a slight increase in the LPS group compared to the control group. Interestingly, the

AHCC+LPS group exhibited lower levels of IL-17A and IL-17F compared to the LPS group, suggesting that AHCC mitigates the prolonged effect of LPS on IL-17 levels. IL-17, produced by Th17, a distinct subgroup of CD4 cells, plays an important role in promoting immune cell recruitment, reducing IL-10 production, and increasing proinflammatory cytokines (Porcherie, Gilbert et al. 2016). Among IL-17 subtypes, namely IL-17A, IL-17B, IL-17D, and IL-17F, there is evidence of their ability to promote tumor development, proliferation, and metastasis in breast cancer through the NF-κB and MAPK signaling pathways (Fabre, Giustiniani et al. 2018, Mikkola, Almahmoudi et al. 2022). These findings suggest that exposure to LPS can affect IL-17 subtypes at both protein and DNA methylation levels, potentially predisposing mammary glands to inflammatory diseases or breast cancer later in life.

The LPS group showed a non-significant increase in IL-10 levels compared to the control, consistent with prior research on LPS effects (Pengal, Ganesan et al. 2006, Iyer, Ghaffari et al. 2010). In contrast, the AHCC group had significantly lower IL-10 levels than both the LPS and AHCC+LPS groups, indicating that AHCC has the potential to suppress IL-10 in LPS-exposed conditions. IL-10 has a complex role in inflammation, exhibiting both anti-inflammatory and proinflammatory characteristics depending on the context (Sanjabi, Zenewicz et al. 2009, Redford, Murray et al. 2011, Cui, Wang et al. 2024). The observed reduction in IL-10 levels in the AHCC group may indicate modulation of the immune response, potentially influencing the balance between pro and anti-inflammatory signals.

The LPS group exhibited a non-significant increase in IL-10 levels compared to the control, consistent with previous research on LPS effects. In contrast, the AHCC group demonstrated significantly lower IL-10 levels than both the LPS and AHCC+LPS groups, suggesting that AHCC has the potential to suppress IL-10 in LPS-exposed conditions.

Regarding IL-23, our findings indicate that its levels were reduced in the AHCC group compared to the control, LPS, and AHCC+LPS groups. This decrease in IL-23 levels associated with AHCC supplementation aligns with previous studies highlighting the immunomodulatory effects of AHCC on cytokine production (Daddaoua, Martinez-Plata et al. 2007, Terakawa, Matsui et al. 2008). While there was a tendency towards increased IL-23 levels in the LPS and AHCC+LPS groups, the differences were not statistically significant. These results suggest that LPS exposure alone may not have a significant impact on IL-23 production. IL-23 is recognized for its crucial role in promoting inflammation and its involvement in various inflammatory diseases (Geremia and Jewell 2012, Gaffen, Jain et al. 2014). The lower level of IL-23 in the AHCC group suggests a potential inhibitory effect of AHCC on IL-23 production, which could contribute to the modulation of inflammatory responses. Our finding indicates that AHCC shows the potential in mitigating adverse effects of LPS by modulating cytokine responses, particularly lowering IL-17 levels and potentially balancing pro-inflammatory and anti-inflammatory cytokines like IL-10 and IL-23. These results underscore AHCC's potential to mitigate LPS-induced effects by modulating cytokine responses, particularly by lowering IL-17 levels and potentially balancing pro-inflammatory and anti-inflammatory cytokines like IL-10 and IL-23. This supports AHCC supplementation as a therapeutic approach to manage inflammation and prevent related diseases in mammary glands, countering dysbiosis and elevated levels of proinflammatory cytokines induced by LPS exposure.

The dysregulation of microRNAs has been implicated in various aspects of mammary gland biology, including development, homeostasis, and tumorigenesis. In this study, we investigated the expression of several tumor suppressor microRNAs, including Let-7a, Let-7c, miR-130a, miR-146, miR-300c, miR-211, and miR-219, in response to LPS exposure and AHCC treatment.

The findings demonstrate a significant downregulation of Let-7a, Let-7c, miR-184, and miR-219 expression in the LPS group, indicating that LPS exposure suppresses these tumor suppressor microRNAs. This downregulation may have important implications, as Let-7 microRNAs play a crucial role in controlling cell proliferation, differentiation, and apoptosis in the mammary glands (Avril-Sassen, Goldstein et al. 2009, Piao and Ma 2012). The observed decrease in Let-7a and Let-7c expression could potentially disrupt these regulatory processes and promote cellular abnormalities in response to LPS exposure (Li, Wakao et al. 2024). Similarly, miR-184 expression was decreased in the LPS group compared to the control group. miR-184 regulates cell proliferation, apoptosis, and differentiation in the mammary glands (Phua 2013, Phua, Nguyen et al. 2015). Its downregulation in response to LPS suggests a disruption of these regulatory processes, contributing to mammary gland abnormalities. Furthermore, our findings revealed a significant downregulation of miR-219 in response to LPS exposure, while AHCC treatment significantly upregulated miR-219 compared to the control group. miR-219 is involved in regulating neuronal development, synaptic plasticity, and neuroprotection (Hudish, Blasky et al. 2013). Its downregulation due to LPS exposure suggests a potential disruption of neuroprotective mechanisms in the mammary glands (Ye, Wang et al. 2021). Interestingly, AHCC treatment did not significantly affect the expression of Let-7a, Let-7c, and miR-184, suggesting that AHCC may not directly modulate these microRNAs. However, AHCC supplementation appears to mitigate the effects of LPS and restore miR-219 expression levels. Further investigation is needed to understand the exact mechanisms by which AHCC regulates miR-219 expression.

In contrast to Let-7a, Let-7c, miR-184, and miR-219, both miR-34a and miR-130a demonstrated significant upregulation in all experimental groups compared to the control group. miR-34a is a

well-established tumor suppressor microRNA known for its role in regulating cell cycle arrest, senescence, and apoptosis (Yamakuchi, Ferlito et al. 2008, Li, Yuan et al. 2013). The observed upregulation of miR-34a in response to LPS exposure and AHCC treatment suggests a potential protective mechanism against LPS-induced cellular damage and inflammation. Similarly, miR-130a has been implicated in modulating cell proliferation, migration, and invasion in various cancer types, including breast cancer (Zhang, Jiang et al. 2017). Its upregulation in response to LPS exposure and AHCC treatment indicates a potential protective role against LPS-induced mammary gland abnormalities and inflammation.

It is worth noting that the AHCC group displayed a higher expression level of miR-34a compared to the LPS group, implying that AHCC supplementation may enhance the expression of this tumor suppressor microRNA, potentially contributing to its beneficial effect. However, further studies are required to elucidate the specific mechanisms by which miR-130a exerts its effects in the context of LPS and AHCC.

In this study, the expression of miR-146a did not show a significant change in the LPS and AHCC+LPS groups compared to the control group. miR-146a acts as a negative regulator of inflammation by targeting genes involved in pro-inflammatory signaling pathways (Rusca and Monticelli 2011, Labbaye and Testa 2012). The lack of significant change in miR-146a expression in response to LPS exposure suggests that LPS may not directly influence the levels of this microRNA in the mammary glands. However, it is noteworthy that the AHCC group exhibited a significantly higher expression level of miR-146a compared to all other experimental groups. This finding suggests that AHCC supplementation may have a potential modulatory effect on miR-146a expression, possibly contributing to its anti-inflammatory properties.

The expression of miR-200c showed a slight downregulation in both the LPS and AHCC+LPS groups compared to the control group. miR-200c plays a critical role in regulating epithelial-mesenchymal transition (EMT) and suppressing metastasis in breast cancer (Song, Liu et al. 2015, Mutlu, Raza et al. 2016). The downregulation of miR-200c in response to LPS exposure suggests a potential disruption of EMT regulation, which could contribute to cellular abnormalities and increased metastatic potential. On the other hand, the AHCC group exhibited a significantly higher expression level of miR-200c compared to the control group, suggesting a potential modulatory effect of AHCC on miR-200c expression.

Lastly, our findings reveal that miR-211 was significantly upregulated in the AHCC and AHCC+LPS groups compared to the control group. miR-211 is involved in regulating melanocyte development and pigmentation, with its dysregulation associated with melanoma (Boyle, Woods et al. 2011). The upregulation of miR-211 in response to AHCC treatment suggests a potential involvement in regulating mammary gland biology. Additionally, the higher expression level of miR-211 in the AHCC+LPS group compared to the LPS group suggests a potential modulatory effect of AHCC on miR-211 expression in the presence of LPS exposure.

Overall, these findings underscore the intricate interplay between LPS exposure, microRNA dynamics, and AHCC's potential therapeutic effects in managing mammary gland health. AHCC appears to selectively modulate key microRNAs, potentially offering protective and anti-inflammatory roles.

Following the evaluation of DNA methylation alterations, cytokine levels, and microRNA dysregulation, the morphological structure of mammary glands was assessed. This evaluation determined that neither LPS exposure during puberty nor AHCC treatment significantly altered

the structure of mammary glands. However, a notable observation was the decreased number of quadruple intersections in the AHCC group compared to the control and AHCC+LPS groups.

2.6 Conclusion

In summary, this study is the first to investigate the link between inflammatory exposure during puberty and the establishment of a pre-carcinogenic signature in mammary glands during adulthood. The results showed significant changes in DNA methylation patterns due to LPS exposure, with the majority of genes displaying hypomethylation. These altered patterns resemble those found in cancer cells, suggesting a potential link between LPS exposure and carcinogenesis. Additionally, LPS exposure disrupted the expression of proinflammatory cytokines and led to abnormal expression of inflammation-associated microRNAs and various signaling pathways.

The study also explored the impact of AHCC treatment on mitigating LPS-induced effects. AHCC treatment partially restored DNA methylation patterns, indicating its potential as a protective intervention against LPS-induced epigenetic changes. Furthermore, AHCC regulated key genes involved in critical pathways such as cytokine-cytokine receptor interaction, JAK-STAT, and IL-17, suggesting its ability to modulate the inflammatory response and restore immune-related processes in LPS-exposed mammary glands. While this study identifies an association between inflammation during puberty and an increased risk of breast cancer, it does not definitively establish causation due to the multifactorial nature of breast cancer. The findings underscore the need for further research to explore the roles of various factors in breast cancer pathogenesis. Further investigations are needed to elucidate the underlying mechanisms and validate the clinical relevance of AHCC in preventing LPS-induced dysfunction in mammary glands and associated pathologies. This insight is crucial for understanding the impact of gut

microbiome dysbiosis on mammary gland health and may pave the way for developing novel strategies for intervention and prevention of related disorders.

Declarations

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.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare they have no competing interests.

Funding

This research was funded by an NSERC Collaborative Research and Development Grant (532223-18), AHCC Research Association, and New Frontiers in Research Fund—Exploration NFRF-(2019-01497) grant to C.M and Z.H. Additionally, H.Y.S. was the recipient of an admission scholarship from the Faculty of Graduate and Postgraduate Studies at the University of Ottawa.

CRediT authorship contribution statement

All authors contributed to the data collection and analysis process. The manuscript was drafted by Hamed Yasavoli Sharahi, all authors reviewed the manuscript.

Acknowledgments

We would like to express our deepest gratitude to Dr. Farzaneh Sharifzad, Dr. Ardeshir Ariana, and Dr. Marjan Khalili for their invaluable support throughout this research.

2.7 Supplementary Data

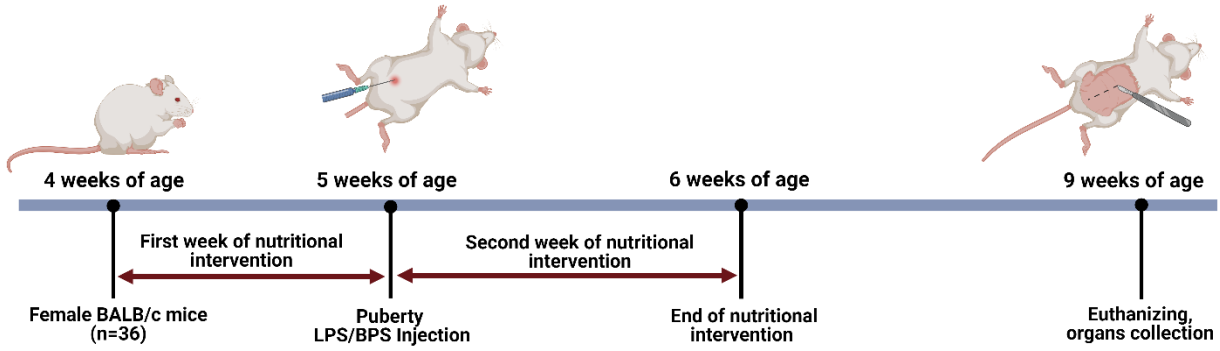

Fig. S2.1. Study design showing the schematic of the research methodology, including the timeline of experiment, administration of LPS/PBS, and dietary intervention. The study involves 36 mice, which are divided into two primary groups: control and AHCC. At the onset of puberty, 5 weeks of age, half of the mice within each group will receive LPS, while the remaining mice will receive PBS. This distribution results in the formation of four experimental groups with 9 mice in each group.

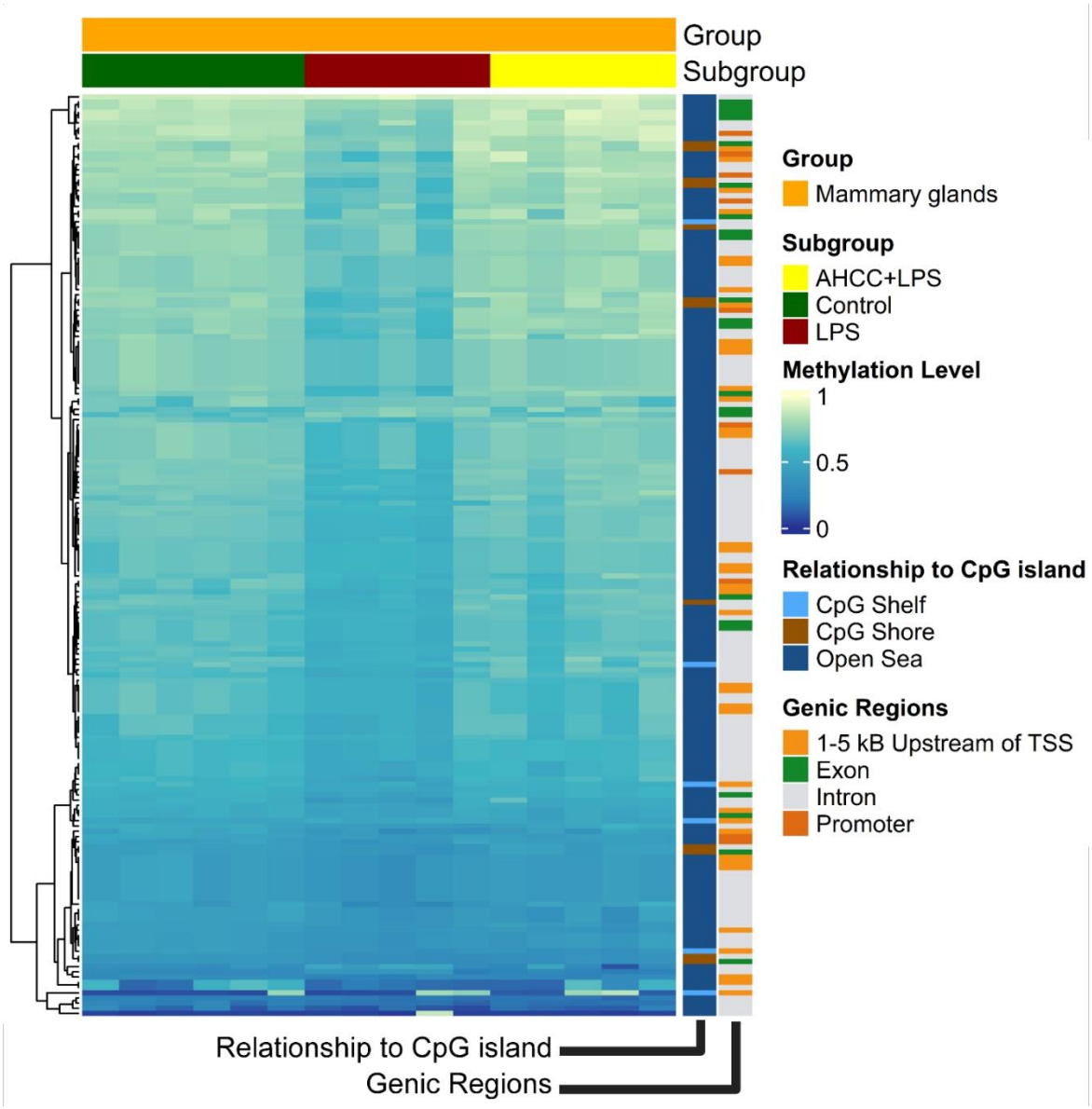

Fig. S2.2. Heatmap of DNA methylation level across all the treatment groups. The heatmap depicts the methylation levels of various genomic regions in mammary glands across three groups: Control, LPS, and AHCC+LPS. Annotations on the right denote the relationship of CpG sites to CpG islands (CpG Shelf in orange, CpG Shore in blue, Open Sea in grey), and the specific genic regions affected (1-5 KB upstream of TSS in green, Exon in orange, Intron in blue, Promoter in grey), providing insights into the genomic context of methylation changes.

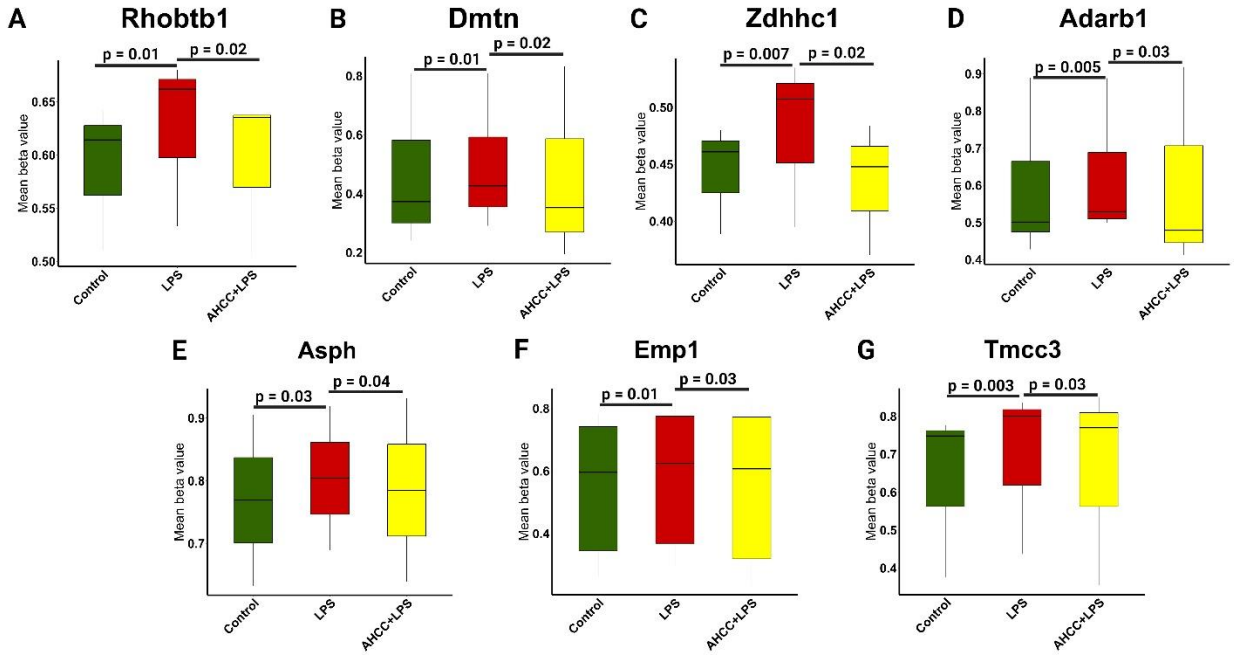

Fig. S2.3. Differential methylation response to LPS and AHCC treatment. Mean beta values for seven genes associated with cancer development and tumor suppression across three treatment groups. The genes analyzed include **(A)** Rhobtb1, **(B)** Dmtn, **(C)** Zdhhc1, **(D)** Adarb1, **(E)** Asph, **(F)** Emp1, and **(G)** Tmcc3.

Chapter 3. *Lentinula Edodes* Cultured Extract Prevents Immune Stress during Puberty and Modulates microRNAs Involved in Mammary Gland Development and Breast Cancer Suppression

Submitted in Breast Cancer Journal

***Edodes Cultured* Extract Prevents Immune Stress during Puberty and Modulates microRNAs Involved in Mammary Gland Development and Breast Cancer Suppression**

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3.1 Abstract

Immune stressors such as lipopolysaccharides (LPS) profoundly affect microbiota balance, leading to gut dysbiosis. This imbalance disrupts the metabolic phenotype and structural integrity of the gut, increasing intestinal permeability. During puberty, a critical surge in estrogen levels is crucial for mammary gland development. However, gut inflammation in this period may interfere with this development, potentially heightening breast cancer risk later. The long-term effects of pubertal inflammation on mammary development and breast cancer risk are underexplored. Such episodes can dysregulate cytokine levels and microRNA expression, altering mammary cell gene expression and predisposing them to tumorigenesis. This study hypothesizes that prebiotics, specifically *Lentinula Edodes* Cultured Extract (AHCC), can counteract LPS's adverse effects. Using BALB/c mice, an acute LPS dose was administered at puberty, and breast cancer predisposition was assessed at 13 weeks. Cytokine and tumor-related microRNA levels, tumor development, and cancer stem cells were explored through immunoassays and qRT-PCR. Results show that LPS induces lasting effects on cytokine and microRNA expression in mammary glands and tumors. AHCC modulates cytokine expression, including IL-1β, IL-17A/F IL-23, and mitigates LPS-induced IL-6 in mammary glands. It also regulates microRNA expression linked to tumor progression and suppression, particularly

counteracting the upregulation of oncogenic miR-21, miR-92, and miR-155. Although AHCC slightly alters some tumor-suppressive microRNAs, these changes are modest, highlighting a complex regulatory role that warrants further study. This underscores the potential of dietary interventions like AHCC to mitigate pubertal LPS-induced inflammation on mammary gland development and tumor formation, suggesting a preventive strategy against breast cancer.

Keywords: LPS, microbiome and dysbiosis, mammary glands development, prebiotics, microRNAs, proinflammatory cytokines, Breast cancer, tumor development

3.2 Introduction

Gut microbiota homeostasis can be significantly affected by dietary choices and inflammatory stressors, such as lipopolysaccharides (LPS) (Dahl, Mendoza et al. 2020). Furthermore, an imbalanced population of gut microbiota not only triggers changes in gut phenotypes but also contributes to compromised gut barrier integrity, referred to as leaky gut (Camilleri 2019). As a result of this process, the gut's permeability is compromised, allowing bacteria or their byproducts such as LPS or LDP to breach the epithelial barrier and initiate a systemic immune response (Hiippala, Jouhten et al. 2018, Régnier, Van Hul et al. 2021). The adverse effects of dysbiosis can ultimately lead to dysregulation of gene expression in the mammary glands and increase the risk of breast cancer (Ruo, Alkayyali et al. 2021).

Lipopolysaccharide (LPS) plays a critical role as an inflammatory trigger, potentially linking it to cancer susceptibility, particularly in distant organs like the mammary glands (Kobayashi, Uejyo et al. 2013, Lai, Liu et al. 2017, Martinelli, Reginatto et al. 2020). This link is supported by the interconnected mucosal immune system theory, which proposes immunological communication between the gut and other mucosal sites (McDermott, Clark et al. 1980). Obesity-associated gut

microbiota alterations are linked to elevated IL-6 levels, which activate CD4+ T helper cells (Th17) and macrophages, increasing the production of inflammatory mediators like TNFα, IL-1β, and Cox-2 (Park, Park et al. 2005, Subbaramaiah, Howe et al. 2011, Paine and Lewis 2017). Th17 cells, crucial immune modulators, are known to migrate from the gut to other mucosal-associated lymphoid tissue (MALT) network organs, including the breast, where they can trigger inflammation in response to LPS or pathogens by releasing cytokines such as IL-17 and IL-23 (Ivanov, McKenzie et al. 2006, Singh, Schwartz et al. 2013, Hsieh 2014, Park, Jeong et al. 2015). This leads to further release of cytokines and chemokines like IL-8 and CXCL1 from various cells, promoting inflammatory responses and potentially enhancing carcinoma progression by disrupting Transforming Growth Factor-β (TGF-β) signaling (Hartupee, Liu et al. 2007, Ouyang, Kolls et al. 2008, Novitskiy, Pickup et al. 2011). Additionally, inflammation during key developmental stages, such as puberty, triggers not only Th17 cells but also macrophages to produce excessive inflammatory mediators. These mediators can alter the gene and microRNA expression in mammary stem cells, leading to the development of a cancer stem cell phenotype and potentially contributing to breast cancer onset (Figure 3.1A) (Korkaya, Liu et al. 2011, Brown 2014, Shi, Cao et al. 2014).

The mammary glands fully mature during puberty, undergoing a significant transformation from terminal end buds into a complex, branched ductal network through branching morphogenesis (Sternlicht 2005). Initiated in the fetal stage and pausing after birth, this development resumes under estrogen stimulation during puberty (Sternlicht 2006). Hence, the maturation of breast tissues consists of two phases, including embryonic and pubertal stages (Macias and Hinck 2012, Smith 2012). Disruptions from inflammatory agents or immune stressors during these phases can compromise ductal structure, raising the risk of developmental anomalies and subsequent cancer

susceptibility (Mantovani, Marchesi et al. 2007, Watson, Oliver et al. 2011, Tharp, Maffini et al. 2012). The research underscores the gut microbiome's vital influence on mammary gland development and its indirect role in cancer risk, with diet during puberty being a significant environmental factor (MacLennan, Anderson et al. 2011, Fernandez, Reina-Perez et al. 2018, Cryan, O'Riordan et al. 2019). For instance, a diet rich in n-3 polyunsaturated fatty acids has been shown to reduce terminal end bud numbers, reflecting healthier mammary gland development (Anderson, MacLennan et al. 2014).

In breast cancer, a small subpopulation of cells termed Cancer Stem Cells (CSCs) possesses a unique ability for self-renewal and differentiation into diverse cell types. These cells can generate the majority of a tumor’s mass, initiate metastasis, and exhibit resistance to chemotherapy, following similar developmental pathways as normal stem cells to proliferate and differentiate (Reya, Morrison et al. 2001, Prager, Xie et al. 2019). During puberty, inflammation can increase the likelihood of stem cells implicated in mammary gland formation acquiring a long-lasting epigenetic signature in adulthood that may increase susceptibility to breast cancer later in life.

MicroRNAs (miRNAs), short non-coding RNA sequences about 22 nucleotides long, play a crucial role in gene regulation by binding to the 3’UTR of mRNAs, affecting mRNA expression through processes like cleavage or suppression (Winter, Jung et al. 2009). Gut microbiota and miRNAs interact at intestinal epithelial cells, leading to the activation of key signaling pathways and modulation of gene expression (Dong, Tai et al. 2019). For example, miR-184 notably increases during pubertal mammary development (Phua, Nguyen et al. 2015). Disruptions in miRNA balance during key developmental stages could predispose to breast cancer later in life. Moreover, numerous miRNAs such as miR-10b, miR-20, and up to miR-575, have been

identified as influential in the progression of breast and gastric cancers, with specific miRNAs like miR-145 being downregulated and miR-155 upregulated in breast cancer (Mattie, Benz et al. 2006, Iliopoulos, Polytarchou et al. 2009, Iorio, Casalini et al. 2009, Li, Zhang et al. 2010, Zhang, Zhao et al. 2013, Singh, Pochampally et al. 2014). miR-184 notably curbs metastasis in triple-negative breast cancer by targeting the Akt/mTORC1 pathway (Phua 2013).

Prebiotics and probiotics play a crucial role in gut health by maintaining immune balance, influencing metabolism, and controlling pathogens (Bengmark 2013, Williams, Stedtfeld et al. 2017). Their consumption during puberty can counteract LPS-induced dysbiosis (Murray, Sharma et al. 2019). These compounds not only affect gut microbiota but also modulate miRNAs, altering transcriptome profiles including miR-192 and miR-215 expression (Archambaud, Sismeiro et al. 2013). They are beneficial in cancer prevention and treatment by impacting miRNA expression, which influences key pathways in metastasis and carcinogenesis (Rajoka, Jin et al. 2018, Trivieri, Panebianco et al. 2020). Specifically, *Lentinula Edodes* Cultured Extract (AHCC) acts as a TLR4 antagonist, reducing inflammation and the metastatic traits of breast cancer by suppressing ROS production and VE-cadherin phosphorylation(Haidari, Zhang et al. 2013).

In this study, mice exposed to the inflammatory agent LPS during puberty were subsequently treated with AHCC, a prebiotic derived from shiitake mushrooms known for its immunomodulatory effects, including inhibition of the TLR4 pathway (Mallet, Graham et al. 2016). This research aimed to explore how AHCC could mitigate the long-term impacts of LPS exposure on breast cancer development. We examined cytokines, microRNA expression patterns in mammary glands and tumors, tumor progression, and cancer stem cell spheres. Our findings

suggest that while LPS exposure during puberty may promote breast carcinoma, concurrent AHCC administration could offer protective benefits against breast cancer, highlighting potential preventive strategies for mammary-related diseases.

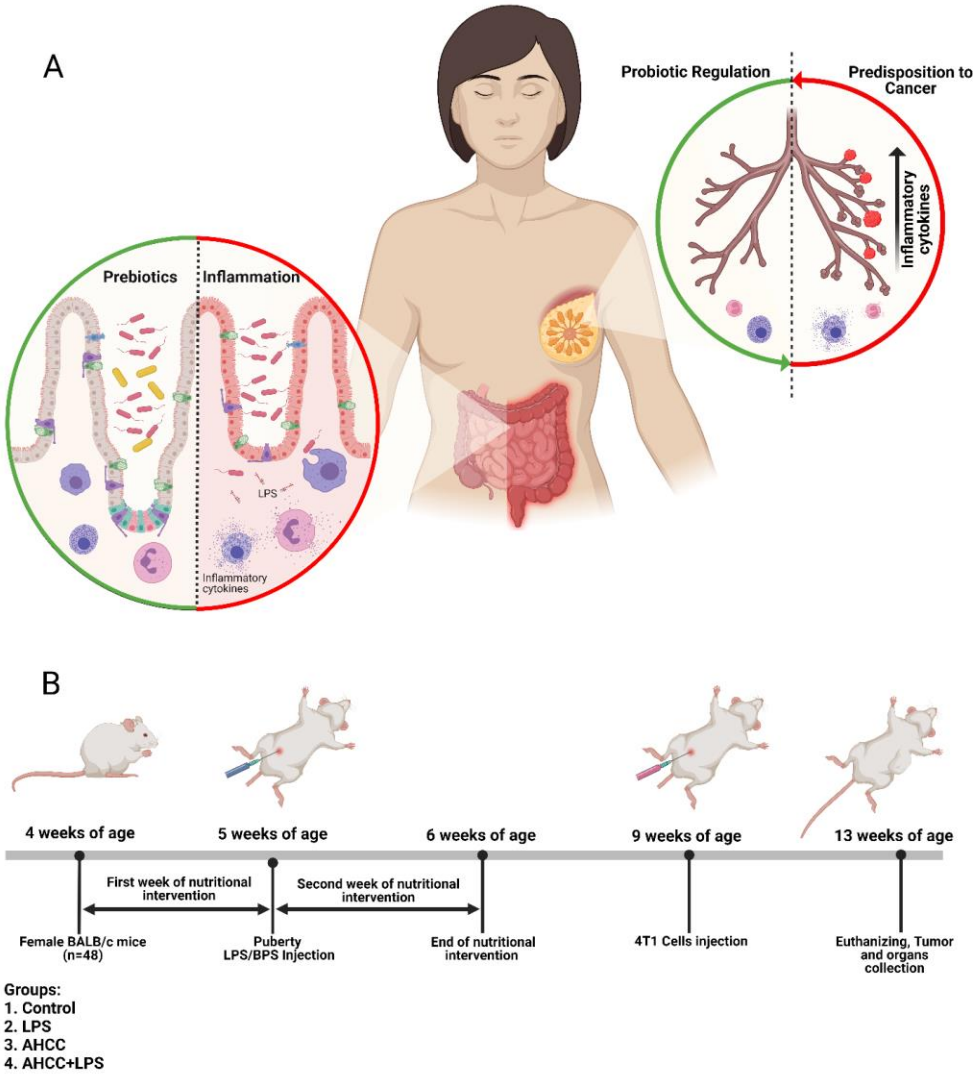

Figure 3.1: (A) The interplay between gut and microbiota and mammary gland development early in life and potential breast cancer later in life. An activated immune system produces pro-inflammatory factors such as TNFα, IL-1β, and Cox-2. Given that the gut immune system is a part of mucosal-associated lymphoid tissue (MALT), exposure to inflammatory agents or immune stressors during critical periods, such as puberty, has the potential to disrupt the normal development of mammary glands and alter their gene expression. This can ultimately contribute to the acquisition of a cancer stem cells (CSCs) phenotype and the development of breast cancer. **(B)** Study design showing the experimental timeline, groups, dietary intervention, LPS injection, and 4T1 cells injection.

3.3 Material and Method

Cell line

Murine breast cancer 4T1 cell line obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), were cultured in Roswell Park Memorial Institute (RPMI, Gibco, Gaithersburg, MD), supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD), and 1% penicillin/streptomycin mixture in a humidified 5% CO2 atmosphere at 37°C until reaching 80% confluence. Cells were collected using 0.25% trypsin-EDTA solution.

Animals

Female BALB/c mice, three weeks old and weighing 13 to 17 grams, were obtained from Charles River (Montreal, CA). They were maintained in plastic cages, four per cage, under a 12-hour light-dark cycle at 22±2ºC and 55±2% humidity. A standard balanced diet was provided. All procedures adhered to the Canadian Council on Animal Care's guidelines and received approval from the University of Ottawa's Animal Care Committee (Protocol HSe-3418).

Study Design

The study investigated the prolonged effects of pubertal LPS exposure and prebiotic (AHCC) intake on mammary gland development and tumor progression in 48 mice. Puberty onset was marked by the first vaginal opening. Mice were divided into two primary groups: one receiving AHCC-infused water aimed at a 2 g/kg dosage based on average consumption, and another receiving regular water. Daily water consumption was monitored to estimate and ensure consistent AHCC dosage. At five weeks of age, each group was subdivided: one subgroup received an intraperitoneal LPS injection (1.5 mg/kg body weight, Escherichia coli O26 LPS dissolved in sterile PBS at 0.2 mg/ml) and the other received a sterile PBS injection, creating

four groups: control, LPS, AHCC, and AHCC with LPS (AHCC+LPS). After one week of specific dietary interventions post-injection, a standard regimen was resumed. By the ninth week, 4T1 cells suspended in serum-free RPMI were subcutaneously injected into the abdominal mammary glands (2000 cells in 0.1 ml). Four weeks post-injection, tumors, mammary glands, and blood were collected for analysis post-euthanasia. Tumor size was measured using calipers, with volume calculated by V= 0.5×d^2×D (Bayat Mokhtari, Baluch et al. 2019). AHCC was provided by Amino Up Co., Ltd., Sapporo, Japan (Figure 3.1B).

Sphere Formation

Tumor samples were surgically removed, sectioned into small pieces, and digested using gentleMACS tumor dissociation (Miltenyi Biotec., Germany) following the manufacturer's protocol. The digested suspension was filtered through a 70-µm cell strainer and washed with serum-free RPMI. Dissociated cells were then seeded in ultra-low attachment 96-well plates (Millipore Sigma, Oakville, Canada) at a density of 1000 cells per 0.2 ml per well. The growth medium was DMEM-F12 (Invitrogen, USA) supplemented with 10 ng/ml EGF, 20 ng/ml BFGF, 5 µg/ml insulin, 1 mM sodium pyruvate, 0.5 µg/ml hydrocortisone, and 0.05 mg/ml penicillin/streptomycin. Spheres were counted after 24 hours.

Real-Time Quantitative Reverse Transcription PCR (RT-qPCR)

A tissue segment from the mammary glands and tumor was preserved in RNAlater stabilization solution (Invitrogen, USA) at 4°C for one day, then stored long-term at -80°C. Total RNA was isolated from these samples using the miRNeasy mini kit (Qiagen, Toronto, Canada). RNA concentration was measured using NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). For miRNA level measurement, cDNA was synthesized using the miRCURY LNA RT Kit

(Qiagen, Toronto, Canada). The expression levels of microRNAs Let-7a, Let-7c, miR-21, miR-34a, miR-92, miR-125, miR-140, miR-145, miR-155, miR-181, miR-184, miR-188, miR-200c, miR-205, miR-223, and miR-425 were quantified by RT-qPCR using the miRCURY LNA miRNA PCR (Qiagen, Toronto, Canada) on a CFX 384 real-time PCR detection system (Bio-Rad, Laboratories, Hercules, CA, USA). The expression of miRNAs was normalized to the reference gene SNORD65 (mmu). Relative expression levels of miRNAs were calculated using the ΔΔCT method.

Multiplex Luminex immunoassay

Levels of IL-1β, IL-6, IL-10, IL-17A, IL-17F, IL-23, TGF-β1, TGF-β2, and TGF-β3 in mammary glands and tumor tissues were analyzed from samples excised using surgical blades (Fisher Scientific, Toronto, Canada) and placed in microtubes containing lysis buffer (20 mM/L Tris-HCl at pH 7.5, 150 mM/L NaCl, 0.05% Tween-20) and a protease inhibitors cocktail (AEBSF, Hydrochloride, Millipore Sigma, Oakville, Canada). After vortexing and incubating at 4°C for 20 minutes, samples were homogenized and centrifuged at 14,000g for 10 minutes at 4°C to collect the supernatant. Total protein concentrations in the lysates were determined using the BCA method with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Toronto, Canada) and normalized to 2 mg/ml. The multiplex assay was conducted using the mouse Th17 Panel Magnetic, MTH17MAG-47K, and the TGF-BMAG-67K MAG bead kit (Millipore Sigma, Burlington, USA), following the manufacturer's instructions. Results were obtained using a MAGPIX System (Millipore Sigma, Burlington, USA).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). Data distribution was evaluated by the Shapiro-Wilk test. Differences in mean values among experimental groups were analyzed using a two-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. Results were presented as mean ± SEM, with a p-value below 0.05 considered statistically significant. Pathway analysis and miRNA roles were examined using the R programming language with the enrichR package (v 3.0) and miRPathDB (V 2.0)

3.4 Results

Measuring the Modulatory Effects of AHCC and LPS on Tumor Volume, Mass, and Cancer Stem Cell Spheres Abundance

In the initial phase, we examined the treatment's impact on tumor characteristics, focusing on tumor volume, mass, and the abundance of cancer stem cell spheres (CSCS) in each sample. Remarkably, mice treated with LPS exhibited a significant increase in tumor volume compared to both the control and AHCC+LPS groups. In contrast, AHCC treatment did not result in significant differences in tumor volume compared to the control group. Additionally, tumor weight was elevated in all groups relative to the control, although these differences were not statistically significant (Figure 3.2A).

Analysis of the CSCS count revealed higher numbers in both the LPS and AHCC+LPS groups compared to the control, although these differences were not statistically significant. The AHCC group showed a trend towards lower CSCS abundance compared to the control, LPS, and AHCC+LPS groups, but again, this difference was not statistically significant (Figure 3.2A). Figure 2B illustrates the CSCS number and size across the experimental groups.

Evaluating AHCC's Potential in Modulating Cytokine Responses to LPS in Tumor Context

Our main goal in this study was to investigate the levels of inflammatory cytokines, including IL-1β, IL-6, IL-10, IL-23, IL-17A, and IL-17F in the tumors (Figure 3.2C). Firstly, we were unable to detect any presence of IL-17A and IL-17F, the main cytokines produced by Th17 (Cui 2019), in the tumor samples. In terms of IL-1β expression, there was no significant difference between the LPS group and the control group. However, IL-1β expression was lower in the AHCC group compared to the control group. Furthermore, the AHCC+LPS group displayed a lower IL-1β level compared to the LPS groups. Notably, there was no significant difference between the AHCC and AHCC+LPS groups.

Our investigation into IL-6 revealed reduced levels in all experimental groups compared to the control group. Interestingly, no significant difference was observed between the LPS and AHCC+LPS groups.

Regarding IL-10, we observed decreased expression levels in all tumor groups compared to the control group. However, the difference was statistically significant only in the LPS group compared to the control group. Moreover, there were no statistically significant differences between the AHCC+LPS and LPS groups.

IL-23 showed a significant reduction in the LPS group, while its presence remained unchanged in the AHCC group compared to the control group. Furthermore, the expression level of IL-23 in the LPS group was significantly lower than that in the AHCC+LPS group.

Alongside our examination of inflammatory cytokines, we investigated the expression patterns of TGF-β1, TGF-β2, and TGF-β3. For TGF-β1, consistent expression was observed across all study groups, with no significant differences identified. As for TGF-β2, a decrease was noted in its

level in the LPS group. Meanwhile, although TGF-β3 levels declined in both the LPS and AHCC+LPS groups, the differences were not statistically significant (Figure 3.2C).

Evaluating AHCC's Potential in Modulating Cytokine Responses to LPS in Mammary Gland Context

We assessed the expression levels of inflammatory cytokines in normal mammary glands adjacent to the tumors, focusing on IL-1β, IL-10, IL-23, IL-17A, and IL-17F (Figure 3.2D). Interestingly, we observed a reduction trend in IL-1β expression in the AHCC group compared to the AHCC+LPS group; however, no statistically significant differences were found between the groups.

Regarding IL-6, we noted higher expression levels in the LPS group compared to the control group. Interestingly, AHCC attenuated the effect of LPS, as evidenced by lower IL-6 expression in the AHCC+LPS group compared to the LPS group.

IL-10 demonstrated reduced expression levels in all groups, with statistically significant differences observed only in the AHCC group compared to the control group.

IL-17A was significantly reduced in the LPS and AHCC+LPS experimental groups compared to the control group; however, no significant difference was observed among the other groups (Figure 5D). As for IL-17F, we found increased expression in the AHCC+LPS group compared to AHCC, but no other comparisons yielded statistically significant differences.

Turning to IL-23, an increase was observed in the LPS group compared to both the control and AHCC groups; however, the only statistically meaningful comparison was between AHCC and LPS. Moreover, IL-23 expression in the AHCC+LPS group did not significantly differ from that in the LPS group.

Regarding TGF-β1, higher expression levels were observed in the LPS group compared to the control group. No significant differences were found between the AHCC and AHCC+LPS groups compared to the control group. Furthermore, TGF-β1 expression in the LPS group was higher than that in the AHCC+LPS group; however, the differences were not statistically significant.

For TGF-β2, we found that its expression level was decreased in the AHCC group, but none of the comparisons reached statistical significance.

TGF-β3 exhibited no difference in the LPS group compared to the control group. In contrast, TGF-β3 expression demonstrated an increase in both the AHCC and AHCC+LPS groups compared to the control and LPS groups; however, none of the comparisons were statistically significant (Figure 3.2D).

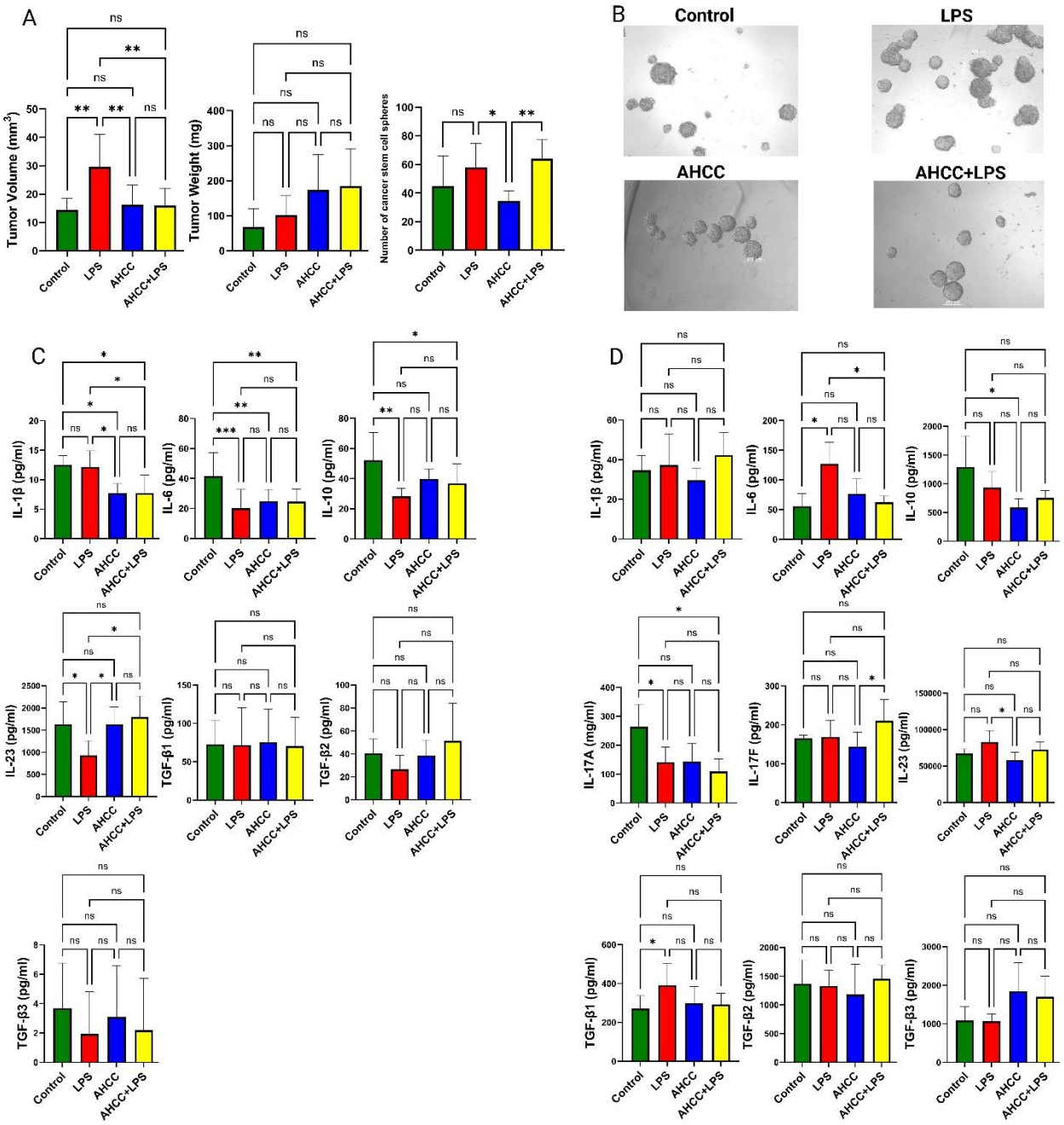

Figure 3.2. Effects of AHCC and LPS on tumor volume, weight, and the number of CSCS in 4T1 mice tumor. **(A)** represent the tumor volume, weight, and CSCs. **(B)** Indicate the number and size of CSCS in the treatment groups. **(C)** Concentrations of IL-1β, IL-6, IL-10, IL-23, TGF-β1, TGF-β2, TGF-β3 in the tumor samples. **(D)** Concentrations of IL-1β, IL-6, IL-10, IL-17A, IL-17F, IL-23, TGF-β1, TGF-β3 in the mammary gland samples. The mice were randomly assigned to receive either AHCC (2 gr/kg BW/d) in their drinking water or plain drinking water for two weeks, one week before and one week after the pubertal LPS injection. 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.

Investigating the miRNA Regulatory Effects of AHCC on LPS-Induced MicroRNA Patterns in Tumors

In the next step, we investigated the expression levels of microRNAs implicated in tumor progression (Figure 3.3A), including Let-7a, Let-7c, miR-21, miR-34a, miR-92, miR-125, miR-140, miR-155, miR-181c, miR-200c, and miR-425 (Figure 3.3B).

Let-7a exhibited no significant changes in the LPS group compared to the control group. In the AHCC and AHCC+LPS groups, an increase in Let-7a was observed compared to the control group; however, these comparisons were not statistically significant. Moreover, a significant upregulation was observed in the AHCC+LPS group compared to the LPS group.

Let-7c demonstrated a decrease in expression in the LPS group relative to the control group. In the AHCC group, the expression level slightly decreased, but the difference was not statistically significant. Moreover, Let-7c showed a significant increase in the AHCC+LPS group compared to both the control and LPS groups.

Notably, miR-21, miR-92, and miR-155 exhibited similar trends, with significant upregulation in the LPS group compared to the control, AHCC, and AHCC+LPS groups, indicating that AHCC may mitigate the effect of LPS. Although the expression levels of these three microRNAs were slightly higher in the AHCC and AHCC+LPS groups compared to the control, these differences were not statistically significant.

Regarding miR-34a and miR-181c, a reduction in expression was observed in the LPS group compared to the control group. Specifically, for miR-181c, when compared between the LPS and AHCC+LPS groups, a higher expression level was found in the AHCC+LPS group, suggesting that AHCC may mitigate the effects of LPS.

For miR-125 and miR-140, no significant changes were observed in the LPS group compared to the control group. However, their expression levels were increased in the AHCC group compared to the control group. miR-125 showed the highest expression level in the AHCC+LPS group compared to all other groups, while miR-140 tended to be lower in the AHCC+LPS group compared to all other groups, with a significant difference observed only in the comparison between AHCC and AHCC+LPS groups.

Surprisingly, miR-200c showed significantly increased expression in all groups compared to the control group. There were no significant differences between the LPS and AHCC+LPS groups, but the AHCC group displayed the highest expression level for miR-200c among all groups.

Concerning miR-425, it exhibited increased expression only in the AHCC+LPS group compared to all other groups. No differences were observed between the LPS and AHCC groups compared to the control group (Figure 3.3B).

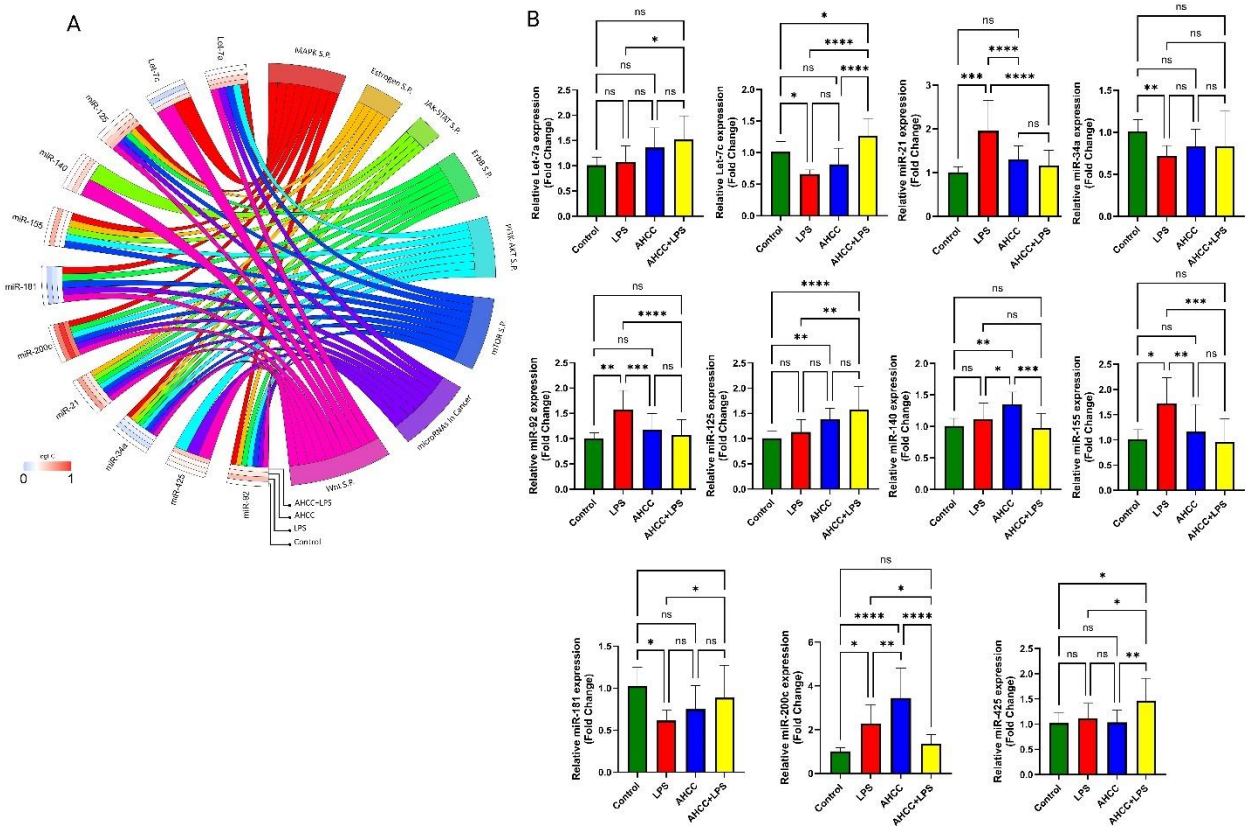

Figure 3.3. (A) The enrichment gene ontology plot indicating the role of microRNAs in the important pathways affecting tumor development. The left side of the plot is the heatmap, showing the log2 expression of the selected microRNAs. **(B)** The relative expression of candidate microRNAs, including let-7a, let-7c, miR-21, miR-34a, miR-92, miR-125, miR-140, miR-155, miR-181c, miR-200c, and miR-425 in the tumor of adult mice four weeks after 4T1 cells injection. The mice were randomly assigned to receive either AHCC (2 gr/kg BW/d) in their drinking water or plain drinking water for two weeks, one week before and one week after the pubertal LPS injection. 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<0.000.

Investigating the Regulatory Effects of AHCC on LPS-Induced MicroRNA Patterns in mammary glands

Next, we aimed to investigate the expression levels of several microRNAs that play crucial roles in mammary gland development and have effects on tumor growth (Figure 3.4A). The candidate microRNAs included miR-34a, miR-125, miR-145, miR-181c, miR-184, miR-188, miR-200c, miR-205, miR-223, and miR-425. Remarkably, we observed a consistent pattern of increased expression for microRNAs miR-34a, miR-145, miR-181c, miR-188, miR-223, and miR-425 in

all treatment groups (LPS, AHCC, and AHCC+LPS) compared to the control group (Figure 3.4B).

Specifically, miR-34a and miR-181c showed significant upregulation in all treatment groups relative to the control group, with the AHCC+LPS group exhibiting the highest expression levels among all treatment groups. For miR-188 and miR-223, although there were increases in expression in the LPS group compared to the control, these differences did not reach statistical significance. On the other hand, miR-145 and miR-425 showed statistically significant increases in expression in the LPS group compared to the control group.

Regarding miR-125, we observed increased expression in the AHCC group compared to the control group, while its expression remained unchanged in the LPS group compared to the control and AHCC+LPS groups.

Furthermore, miR-184 and miR-200c exhibited decreased expression in the LPS group compared to the control group. In contrast, their expression levels were higher in the AHCC group compared to the control group. Notably, miR-184 showed higher expression in the AHCC+LPS group compared to the LPS group, indicating that AHCC not only mitigated the effect of LPS but also promoted the expression of miR-184. Additionally, miR-200c exhibited slightly lower expression in the AHCC+LPS group compared to the control group, and higher expression compared to the LPS group, although these differences were not statistically significant.

Finally, miR-205 displayed higher expression in the LPS group compared to both the control and AHCC+LPS groups. Although miR-205 also showed higher expression in the AHCC group compared to the control and AHCC+LPS groups, these differences were not statistically significant (Figure 3.4B).

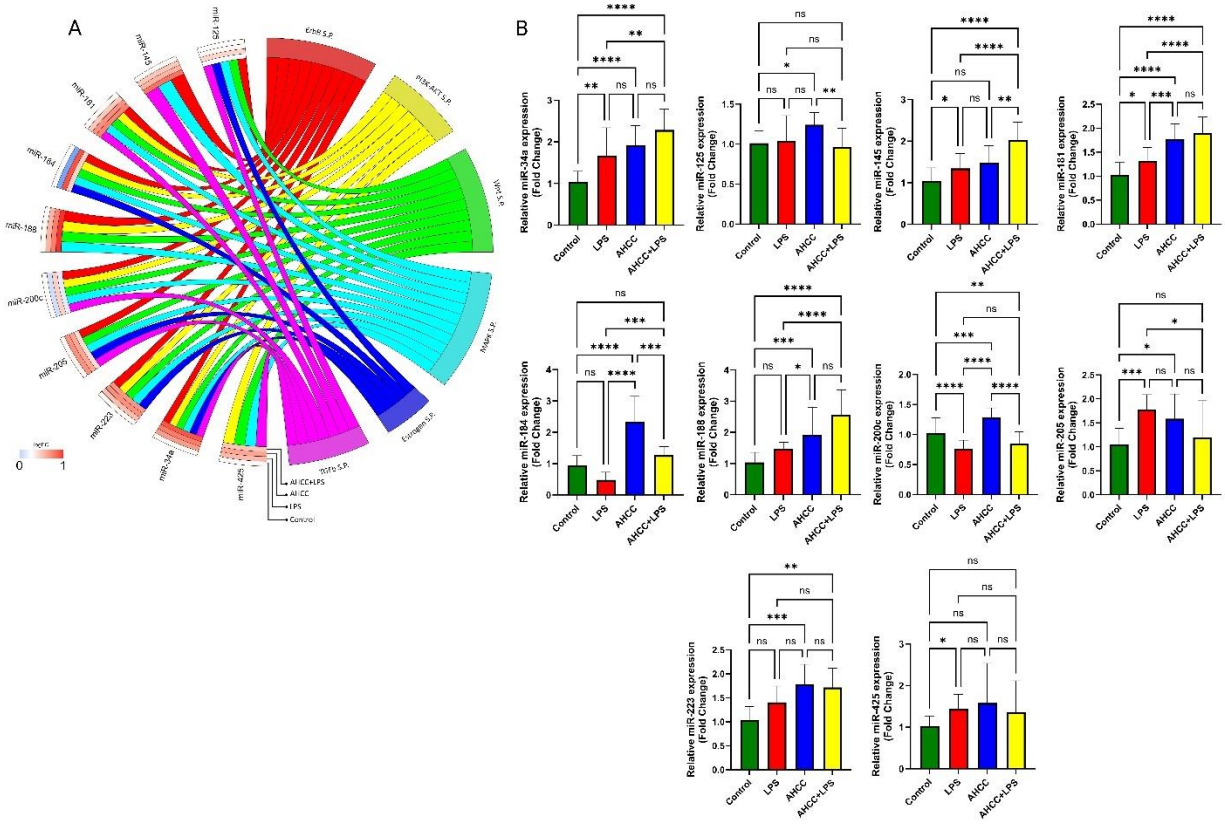

Figure 3.4. (A) The enrichment ontology plot indicating the role of microRNAs in the important pathways affecting tumor development. The left side of the plot is the heatmap, showing the log2 expression of the selected microRNAs. **(B)** The relative expression of candidate microRNAs, including miR-34a, miR-125, miR-145, miR-181, miR-184, miR-188, miR-200c, miR-205, miR-223, miR-425, in the mammary glands of adult mice four weeks after 4T1 cells injection. The mice were randomly assigned to receive either AHCC (2 gr/kg BW/d) in their drinking water or plain drinking water for two weeks, one week before and one week after the pubertal LPS injection. 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.

3.5 Discussion

Probiotics and prebiotics, known for belonging to the 'Biotics' family, play an important role in modulating gut microbial ecosystems by mitigating systemic inflammation (de LeBlanc, Matar et al. 2006, Mallet, Graham et al. 2016, Vuong, Mallet et al. 2016). They are also known for their chemopreventive agents against numerous types of cancers (Sunkata, Herring et al. 2014). We have previously provided evidence concerning the effect of a probiotic against mammary carcinoma (Vuong, Mallet et al. 2016, Graham, Mallet et al. 2017). These studies further

corroborate the potential role played by the gut microbiome at both local and long-distance sites from the gut (Kwa, Plottel et al. 2016).

The interaction between the gut microbiome and breast cancer is intricate and complex. The concept of the common interconnected mucosal immune system holds that there is an immunological cross-talk between the gut and extra-intestinal sites, including exocrine glands such as the mammary glands (McDermott, Clark et al. 1980). Furthermore, the MALT system enables the transportation of microorganisms from the maternal gastrointestinal tract to the mammary glands via immune cells (Rodríguez 2014). A disruption in the immune system, specifically in Th17 regulation, impacts lymphocyte activities at various sites, playing a pivotal role in deregulation of TGF-B signaling (Zhang 2018, Song, Wei et al. 2021). The intake of prebiotics has been identified as a modulating agent, influencing cytokine secretion, gene expression, and subsequently affecting tumor development, showing its potential therapeutic benefits (McDermott, Clark et al. 1980).

In this study, the effect of LPS and AHCC treatment on mammary glands and tumor characteristics, the concentration of inflammatory cytokines, and microRNA expression patterns in mice was systematically examined. Notably, LPS treatment led to a pronounced increase in tumor volume compared to the control and AHCC+LPS groups. This observation is consistent with literature suggesting that bacterial LPS can stimulate inflammatory pathways, which may contribute to the development of tumors (Chen and Nuñez 2010). Indeed, chronic inflammation independently linked to increased LPS circulation is a well-known factor that can facilitate tumor development and progression (Coussens and Werb 2002). Interestingly, the combination of AHCC and LPS mitigated the effects of LPS, implying that AHCC possesses anti-inflammatory properties (Gao, Zhang et al. 2006). The unchanged tumor volume in the AHCC-treated group

compared to the control suggests AHCC may have more immunomodulatory effects rather than direct tumor-suppressing action. The increase in tumor weight across all groups, compared to the control, may indicate that while LPS might predominantly influence tumor volume, the factors driving weight could be more intricate and not solely dependent on LPS or AHCC treatment. Tumor weight, unlike volume, is also affected by factors such as tumor density, necrosis, stromal content, or increased immune cell presence within the tumor. Therefore, additional investigation is required to identify factors associated with tumor weight precisely.

Regarding cancer stem cell spheres, these cells have gained significant attention due to their properties and role in resistance against therapy, metastasis, and tumor recurrence (Shibata and Hoque 2019). The elevated number of CSCs in the LPS and AHCC+LPS groups indicates a potential interaction of LPS treatment with pathways or critical proteins affecting stemness during critical stem cell activity in the pubescent mammary gland. Other studies have shown that LPS is able to induce stemness properties in certain cancer cells (Che, Yin et al. 2017). In Figure 3D, although the lower abundance of CSCS in the AHCC group was not statistically significant compared to the control, future studies are warranted. These studies should explore this potential effect more thoroughly and determine whether AHCC can modulate CSC dynamics in the context of LPS-induced stemness.

The complex connection of cytokines in tumorigenesis has become increasingly evident, with the inflammatory microenvironment playing a critical role in the development and progression of tumors (Yoshimura 2006). In this study, the intricate dynamics of certain cytokines within tumor samples and mammary glands, particularly their response to LPS and AHCC treatment, were assessed. The modulatory effects of AHCC on some cytokine levels are consistent with its immunomodulatory attributes (Daddaoua, Martínez-Plata et al. 2013). In particular, the reduced

IL-1β expression in tumors treated with AHCC, and in mammary glands, points towards its potential to mitigate inflammation (Tulotta and Ottewell 2018), which could be beneficial in the context of cancer. Interestingly, the concurrent administration of AHCC with LPS appeared to counteract the inflammatory effects induced by LPS in tumor samples, suggesting a potential anti-inflammatory role of AHCC in this context. IL-6 plays a multifaceted role in cancer pathogenesis. Elevated IL-6 has been associated with poor overall survival in breast cancer patients (Salgado, Junius et al. 2003). In this study, no significant differences were observed between the control and LPS in tumor samples. However, the observed reduction in IL-6 levels, particularly in the presence of AHCC, may suggest a potential therapeutic advantage. IL-10, commonly acknowledged as an anti-inflammatory cytokine, has been recognized for its complex roles in cancer, often exhibiting both tumor-promoting and tumor-suppressive behavior (Mocellin, Marincola et al. 2005). In this analysis, a reduced expression of IL-10 was evident across all groups relative to the control. The significant decrease in the LPS group specifically could be indicative of the inflammatory response triggered by LPS. Similarly, in the mammary glands, the AHCC and AHCC+LPS groups demonstrated a significantly lower IL-10 concentration. This suggests that AHCC may potentially influence the IL-10-mediated immune response within the mammary glands, which may be linked to the overall downregulation of genes related to cytokine expression (Moore, de Waal Malefyt et al. 2001). IL-23 and IL-17 are crucial to the cytokine network, modulating the balance between pro-inflammatory and anti-inflammatory effects (Rafa, Saoula et al. 2013). IL-23, unlike IL-10, is recognized as a pro-inflammatory cytokine pivotal in the differentiation of Th17 cells, thereby triggering inflammatory cascades in various diseases, including cancer (Langowski, Zhang et al. 2006, Croxford, Kulig et al. 2014). In the context of the mammary gland, our data demonstrated that

LPS administration resulted in elevated IL-23 expression. The consistent levels of IL-23, coupled with a slight increase in the AHCC+LPS group relative to AHCC alone, raise questions regarding the efficacy of AHCC in modulating IL-23 within the mammary gland environment.

In the tumor setting, a decline in IL-23 expression was observed post LPS administration. This observation appears to contrast with the typical inflammatory reaction induced by LPS, suggesting a different modulation of the IL-23 pathway specific to breast tumor cells when exposed to LPS. Furthermore, in examining IL-17 subsets, we observed an absence of both IL-17A and IL-17F in tumor samples. The pivotal role of these cytokines in an oncological context is significant, given their frequent association with the amplification of inflammation-driven tumorigenesis, thereby creating a favorable environment for tumor progression (Kolls and Lindén 2004). The decreased expression of IL-23 in the samples could potentially be a reason for the absence of IL-17A and IL-17F. However, it should be mentioned that the tumor-promoting effects of IL-23 could also be independent of IL-17 (Teng, Andrews et al. 2010). Tumor-associated macrophages are the predominant source of IL-23 in the tumor microenvironment. In a recent paper published in 2024, the unexpected immunosuppressive property of the pro-inflammatory cytokine IL-23 was reported (Wertheimer, Zwicky et al. 2024). The authors reported that a subset of tumor-infiltrating regulatory T (Treg) cells acts as a highly suppressive phenotype via their receptor Il23r. Therefore, a decrease in IL-23 contributes to decreasing its tumor-promoting effect by stabilizing an effector cell program in the tumor microenvironment.

The TGF-β family, including TGF-β1, TGF-β2, and TGF-β3, plays a crucial role in various biological functions such as cell growth, immune responses, and tissue repair. In the context of breast cancer, our findings showed consistent expression levels of TGF-β1 across all tested

groups. This suggests that TGF-β1 might play a stable role in the tumor microenvironment, which is consistent with prior research showing TGF-β1's dual role in both promoting and inhibiting growth (Neel, Humbert et al. 2012).

Our investigation indicated a decline in TGF-β2 across all groups, although it remained stable in the AHCC+LPS group. This highlights AHCC's potential to mitigate the effect of LPS, underscoring its potential therapeutic application. Previous studies have shown that TGF-β2 can modulate the immune system's ability to recognize and combat tumors (Tauriello, Sancho et al. 2022).

Regarding TGF-β3, despite a decrease in expression levels in the LPS and AHCC+LPS groups, these changes were not statistically significant. It is important to note that TGF-β3, like other family members, plays a critical role in various aspects of tumor development such as EMT and metastasis (Kudo-Saito, Shirako et al. 2009). The research highlights AHCC's capacity to influence cytokine activity, potentially altering the course of inflammation and tumor progression. This underscores the importance of further investigations into how these interactions can be effectively utilized in cancer treatment strategies.

In the context of mammary glands, the TGF-β family is considered the primary inhibitor of both ductal elongation and lateral branching (Hinck and Silberstein 2005). TGF-β1 exhibited a noticeable response to LPS by increasing its levels. However, when AHCC was introduced alongside LPS, TGF-β1 expression levels seemed to normalize. This suggests that AHCC may possess protective properties, potentially shielding mammary glands from inflammation or damage caused by LPS. This finding aligns with studies suggesting TGF-β1's role in tissue repair and inflammation (Roberts, Tian et al. 2006). The decrease in TGF-β2 levels in the AHCC group might indicate AHCC's direct influence on TGF-β2, despite the changes not reaching statistical

significance. As for TGF-β3, its increase in the AHCC and AHCC+LPS groups suggests AHCC's potential role in enhancing TGF-β3 levels. Since TGF-β3 is associated with tissue development and repair, higher levels may indicate a positive, protective response (Guo, Betts et al. 2017).

microRNAs (miRNAs) serve as post-transcriptional regulators involved in various cellular processes, including differentiation, proliferation, and apoptosis (Wahid, Shehzad et al. 2010). An imbalance in miRNA expression can significantly influence tumor progression and metastasis (Esquela-Kerscher and Slack 2006). In this study, we investigated crucial miRNAs associated with tumor progression, including let-7a/c, miR-21, miR-92, miR-140, and miR-155. We also explored miRNAs specifically linked to mammary gland development, such as miR-145, miR-184, miR-188, miR-205, and miR-223, in the presence of LPS and AHCC. Additionally, we examined the expression patterns of miRNAs, namely miR-34a, miR-125, miR-181c, miR-200c, and miR-425, which are involved in both mammary gland development and tumor progression.

According to the literature, Let-7a and Let-7c have emerged as interesting targets. Let-7a is known to suppress tumor growth and metastasis in various cancers (Mi, Liu et al. 2019). The observed upregulation of Let-7a in the AHCC and AHCC+LPS groups might suggest a potential protective role against tumor progression. Similarly, Let-7c, which has previously been associated with tumor-suppressive effects in breast cancer (Fu, Mao et al. 2017), showed a marked increase in the AHCC+LPS group, emphasizing the potential therapeutic role of AHCC in modulating miRNA expression.

Another important observation was the upregulation of miR-21, miR-92, and miR-155 in the LPS group. These miRNAs are often effective in promoting tumor growth and inhibiting

apoptosis in various cancers (Si, Zhu et al. 2007, Al-Nakhle, Burns et al. 2010, Mattiske, Suetani et al. 2012). However, their expression seemed to be mitigated by AHCC, highlighting the potential role of AHCC in reducing the tumor-promoting effects induced by LPS.

miR-140, a crucial microRNA known for its role in inhibiting cancer stem cell proliferation and migration, exhibited increased expression in the AHCC group compared to all other groups. Interestingly, a decrease in miR-140 expression was observed in the AHCC+LPS group. Additionally, miR-140 can suppress the Wnt signaling pathway and enhance the efficacy of doxorubicin treatment (Li, Yao et al. 2014, Wu, Zhang et al. 2019).

Within the context of mammary glands, miR-145, miR-188, and miR-223 showed significant upregulation across all groups, with the highest expression seen in the AHCC and AHCC+LPS groups. MiR-145 and miR-188 play a pivotal role in modulating lipogenesis and fatty acid metabolism by suppressing key proteins such as FOXO1 and PTEN (Mobuchon, Marthey et al. 2015, Wang, Shi et al. 2017, Lyu, Li et al. 2023). Meanwhile, miR-223 is recognized for its regulatory effect on the EGFR signaling pathway. Studies have demonstrated its capacity to regulate the organization, growth, and expansion of mammary epithelial cells and ducts, which are essential for lactation (Citron, Segatto et al. 2020).

Focusing on miR-184, a tumor suppressor, it crucially influences mammary gland development by regulating the PI3K/AKT/mTOR pathway. Evidence suggests that this microRNA regulates the differentiation of terminal end bud cells into ductal epithelial cells (Jena 2017). Interestingly, we found that LPS reduced the expression of miR-184, whereas AHCC and AHCC+LPS increased its level.

miR-205 is part of a complex regulatory network that modulates the YAP and Wnt signaling pathways and is predominantly expressed in the basal cells of mature mammary glands. Any

disruption in its expression can have adverse effects on stem cell self-renewal and the normalcy of mammary epithelial cells (Lu, Cao et al. 2018).

We also investigated miRNAs that impact both mammary gland development and tumor progression, including miR-34a, miR-125, miR-181c, miR-200c, and miR-425. Regarding miR-34a and miR-181c, which are often associated with tumor suppression roles through the inhibition of proliferation by downregulating Bcl-2 and MAP4K4 in various cancers (Li, Yuan et al. 2013, Huang, Ye et al. 2015, Xie, Li et al. 2022). we observed significant downregulation in the LPS group. However, administration of AHCC alongside LPS showed relatively higher levels of miR-34a compared to LPS alone, emphasizing AHCC's protective potential against the LPS-induced suppression of this tumor-suppressive miRNA (Li, Yuan et al. 2013). In the context of mammary glands, we found that both miR-34a and miR-181c increased in all treatment groups, with their levels being highest in the AHCC+LPS group. This phenomenon indicates the complex effects of LPS and AHCC on the regulation of these microRNAs involved in mammary gland development. MiR-34a is able to maintain the homeostasis of mammary epithelial cells by suppressing the Wnt signaling pathway through the inhibition of β-catenin (Ivanova, Le Guillou et al. 2021). According to the literature, miR-181c is involved in the regulation of important pathways including the PI3K-Akt pathway, which is crucial for the proliferation and cell survival of mammary glands (Paine and Lewis 2017, Strotbek, Schmid et al. 2017).

miR-125, known for its crucial roles in cell differentiation and the suppression of tumor progression and metastasis through targeting ENPEP, CK2-α, CCNJ, and MEGF9, showed elevated levels in the AHCC group, suggesting the regulatory influence of AHCC on miR-125 (Feliciano, Castellvi et al. 2013).

An increase in miR-200c expression was noted across all treatment groups compared to the control. This miRNA has been linked to the suppression of tumor proliferation and epithelial-mesenchymal transition (EMT) in various cancers by targeting key transcription factors such as ZEB1 and ZEB2 (Zhou, Men et al. 2018). The upsurge in miR-200c expression in AHCC also strengthens its anti-inflammatory properties. Interestingly, although LPS typically induces immune stress, an increase in miR-200c was observed, suggesting a complex interplay in the tumor microenvironment (Zhao, Liu et al. 2022). In mammary glands, the decreased expression level of miR-200c is associated with duct elongation through the inhibition of EMT.

miR-425 has been implicated in different types of cancers, playing a dual role as both an oncogene and a tumor suppressor (Liu, Hu et al. 2015, Xiao, Zhu et al. 2019). In this study, we found that exposure to either LPS or AHCC alone did not modulate miR-425 expression significantly. However, the combination of both treatments led to a noticeable increase in miR-425 expression. It has been shown that miR-425 is linked to pathways that modulate the immune response (Nakagawa, Muroyama et al. 2017).

microRNAs play a crucial role in regulating signaling pathways necessary for both mammary gland development and cancer progression, as well as modulating inflammatory pathways. For instance, the Let-7 microRNA family directly suppresses IL-6, further suppressing the activation of NF-κB and STAT3 (Iliopoulos, Hirsch et al. 2009). Studies have indicated that miR-21 and miR-155 modulate the production of IL-10 expression (Pena-Philippides, Caballero-Garrido et al. 2016, Castro-Leyva, Arenas-Huertero et al. 2022), whereas miR-92 is essential for IL-10 production (de Kouchkovsky, Esensten et al. 2013). We observed a decrease in IL-10 expression in the LPS group in our investigation.

miR-140, in addition to modulating cell proliferation, represses the expression of IL-6 and IL-8 by regulating TLR4 (Li, Guan et al. 2017). Literature reveals that miR-145 inhibits mTOR and AKT3, thereby regulating IL-1β and IL-6 (Shi, Jiang et al. 2018). In addition, miR-188 inhibits TLR4, influencing NF-κB activity and subsequently decreasing the expression of inflammatory cytokines (Liu, Zhang et al. 2022). Though miR-223 is primarily recognized as an anti-inflammatory miRNA, mitigating LPS-induced inflammation by targeting TLR4 and NLRP3 (Yan, Lu et al. 2019, Qu, Liu et al. 2020), There's evidence suggesting the proinflammatory role of miR-223, particularly in inhibiting CLDN8 and increasing IL-23 in IBD (Wang, Chao et al. 2016). In our study, we observed an elevation in miR-223 across all groups, a decline in IL-23 in the LPS group, and an increase in the AHCC and AHCC+LPS groups. Significantly, LPS exposure increases miR-205 levels, while AHCC seems to counteract this effect. Moreover, miR-205 can elevate inflammatory cytokines like IL-1β through targeting COMMD1 (Yeh, Chen et al. 2016). Studies indicate that both miR-34a and miR-181c modulate the NF-κB signaling pathway, thereby decreasing LPS-induced cytokines such as IL-6 and IL-1β (Jiang, Liu et al. 2012, Hutchison, Kawamoto et al. 2013). Interestingly, LPS did not exhibit any detrimental effect on miR-125. This miRNA inhibits IL-6, JAK1, and STAT3, effectively regulating inflammation by inhibiting the JAK1/STAT3 and NF-κB pathways (Yao, Zhou et al. 2021). miR-200c inhibits LPS-induced macrophage polarization and reduces IL-6, IL-8, and CCL-5 expression (Zhao, Liu et al. 2022). Within mammary glands, our study detected a downregulation of miR-200c and a concomitant upregulation of IL-6 in the LPS group. Literature suggests that miR-425 is capable of regulating IL-1β and TNFα levels, thereby mitigating inflammation (Nakagawa, Muroyama et al. 2017). Our findings highlight the potential

therapeutic effects of AHCC in mitigating the adverse effects of LPS on specific miRNAs, including miR-34a, miR-125, miR-181c, miR-200c, and miR-425.

Pathological examination of tumors revealed no significant differences across a spectrum of parameters, including necrosis, vessel formation, gland formation, nucleoli, pleomorphism, and neutrophil infiltration (data not shown).

In conclusion, this research sheds light on the intricate relationship between prebiotics and their influence on inflammation, cytokine levels, and miRNA expression in the context of mammary gland development and breast cancer. The data suggests that inflammation prompted by LPS during puberty can increase tumor size, certain inflammatory cytokines, and some oncogenic miRNAs, possibly via activation of inflammatory pathways.

Key observations include the increased presence of tumor-inhibiting microRNAs such as Let-7a/c, and decreased levels of cancer-promoting microRNAs like miR-21, miR-92, and miR-155 when AHCC is introduced. Furthermore, AHCC increased levels of microRNAs associated with mammary gland development, such as miR-145, miR-188, and miR-223. The alterations in microRNAs affecting both mammary glands and tumor growth, including miR-34a, miR-125, miR-181c, miR-200c, and miR-425, further emphasize AHCC's potential therapeutic advantages. The patterns of microRNA expression in both mammary glands and tumor samples underscore the importance of dietary interventions during the critical period of pubescent mammary gland development and its potential impact on reducing the risk of breast cancer later in adulthood. Although this study identifies a correlation between pubertal inflammation and its effects on mammary glands and tumor development, it is important to note that such inflammation does not necessarily lead to breast cancer, given the multifactorial nature of the disease.

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.

Funding

This research was funded by an NSERC Collaborative Research and Development Grant (532223-18), AHCC Research Association. Additionally, H.Y.S. was the recipient of an admission scholarship from the Faculty of Graduate and Postgraduate Studies at the University of Ottawa.

Author contributions

All authors contributed to the data collection and analysis process. The manuscript was drafted by Hamed Yasavoli Sharahi, all authors reviewed the manuscript.

Chapter 4. Prebiotic Intake during Pregnancy Mitigates Long-Term Antibiotic Disturbance in the Gut-Breast Axis and Regulates Mammary Gland Development

Submitted in Nature Communications Journal

Prebiotic Intake during Pregnancy Mitigates Long-Term Antibiotic Disturbances in the Gut-Breast Axis and Regulates Mammary Gland Development

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4.1 Abstract

There are three main developmental periods for the mammary gland: pregnancy, puberty, and lactation. During the last phase of pregnancy, the mammary gland undergoes massive remodeling. Any immune, endocrine, and epigenetic perturbations during this critical period may lead to acquired dysfunctions in the long term. The equilibrium of the gut microbiome during pregnancy and early stages of infant development is known to be a health determinant in adulthood. Exposure to antibiotics during the prenatal period, a prevalent environmental factor, may lead to enduring effects on distant organs from the gut. This is particularly true regarding the development of the mammary gland during pregnancy. Such exposure can entail potential

disturbances in the epigenetic signature that may persist into adulthood. However, despite the importance of this developmental period in the gut-breast axis, the mechanisms underlying these effects are still not fully understood. This study delves into the long-term implications of prenatal/postnatal exposure to low-dose penicillin on immune response in mothers and mammary gland development in offspring, alongside examining the potential protective effect of the prebiotic compound AHCC against antibiotic-induced immune and epigenetic changes. Pregnant mice received low-dose penicillin (LDP) and AHCC from the third week of gestation until the weaning of their pups. The dams' immune responses were analyzed through the measurement of immunoglobulin and cytokine levels, and the structural integrity of the blood-milk barrier was examined. At eight weeks old, offspring were assessed for immune response and mammary gland development, and inter-group differences in gene expression at the genetic and epigenetic levels were evaluated. The findings indicated an inflammatory response in the dams linked to antibiotic exposure during pregnancy. The integrity of the blood-milk barrier was not compromised following LDP exposure. However, the intake of AHCC led to an improvement in the blood-milk barrier in the mammary glands of dams. In adult offspring, AHCC showed immunomodulatory properties by maintaining balanced levels of immunoglobulins and cytokines, including INFγ, IL-1β, IL-2, IL-15, IL-17A/F, and IL-22. The homeostasis of offspring's mammary glands was notably compromised, with significant dysregulation in key genes such as *Fgf1*, *Fgfr2*, *Igf1r*, *Egfr*, *Igf-1r*, *Fzd7*, *Dvl3*, *c-Jun*, *Ptk2*, as well as microRNAs including Let-7a/c, and miR-146a, involved in the regulation of the ERK/MAPK signaling pathway. Additionally, DNA methylation analysis of mammary glands from offspring that received LDP revealed dysregulation in DNA methylation levels in several genes, including *Sox10*, *Igfbp7*, *Pax5*, *Mia2*. These findings underscore the detrimental impact of early-life

antibiotic exposure, potentially predisposing offspring to breast cancer, and suggest the intake of prebiotics like AHCC as a strategy to prevent and alleviate long-term disturbances in mammary gland homeostasis following exposure of dams to antibiotics during pregnancy and postnatally.

Keywords: **Antibiotic, Prebiotic, Early-life gut microbiome, Dysbiosis, Immune regulation, Mammary glands development, ERK/MAPK Signaling pathways, breast cancer, microRNAs**

4.2 Introduction

The gut microbiome, a complex and diverse community comprising bacteria, fungi, and viruses, is pivotal in maintaining immune homeostasis and consequently influences the development of various organs distant from the gut (Li, Wang et al. 2014, Malmuthuge, Griebel et al. 2015, Lu and Claud 2019). Growth in the body of evidence involving germ-free mice has revealed that an absence of the gut microbiome correlates with an underdeveloped immune system, increased vulnerability to pathogens, and abnormal growth of distant organs such as the brain (Tlaskalová-Hogenová, Štěpánková et al. 2011, Cryan and Dinan 2012). Notably, an imbalance in the gut microbiome has been associated with the onset of numerous diseases, including liver disorders and cancer (Gopalakrishnan, Helmink et al. 2018, Wang, Tang et al. 2021).

The intake of some antibiotics, either directly or indirectly through antibiotic-laden food, might lead to gut dysbiosis (Shondelmyer, Knight et al. 2018). Worldwide, the prescription of antibiotics to pregnant women can range from 35% to up to 50%, with North America being on the higher end (Yu, Tran et al. 2020). Looking forward, projections indicate a substantial surge in global antibiotic intake, with expectations of an increase by up to 200% by the year 2030 (Klein, Van Boeckel et al. 2018). Studies have shown that by the age of five, about 97% of children will have received at least one course of antibiotics, with an average of eight treatments (Hobbs, Grant et al. 2017). Even a single course of antibiotics can disrupt the balance of the gut microbiome, potentially leading to long-term metabolic and immune dysregulation (Pushpanathan, Mathew et al. 2019). Additionally, exposure to antibiotics during childbirth, including those deemed safe during pregnancy, has been linked to an increased risk of childhood obesity and compromised immunity (Mensah, Opoku-Agyeman et al. 2017). Furthermore, enduring phenotypic changes such as microbiome-induced obesity have been observed to persist

even after the restoration of the microbiome, highlighting the long-term impact of microbiome alterations on metabolic and developmental programming (Cox, Yamanishi et al. 2014).

Pregnancy frequently coincides with the onset of certain conditions such as urinary tract infections (UTIs), sexually transmitted infections (STIs)(Mullick, Watson-Jones et al. 2005), pelvic inflammatory disease (PID) due to an elevated population of bacterial vaginosis (BV) (Taylor, Darville et al. 2013), Streptococcus B infection (Gonçalves, Procter et al. 2022), prolonged rupture of membranes (Walker, Picklesimer et al. 2014), and postpartum conditions such as mastitis of the mammary glands (Li, Xu et al. 2023). While antibiotic administration is crucial for treating infections, it is not without drawbacks, including potential risks like preterm birth and the development of allergies (Tran, Zureik et al. 2023). Importantly, antibiotic use during pregnancy not only alters the bacterial environment of the mother and fetus but also predisposes children to obesity and a heightened risk of immune disorders. These effects can persist for years following the discontinuation of antibiotics (Kuperman and Koren 2016).

The disruption of gut bacteria balance does more than just alter phenotypic characteristics; it also leads to a compromised gut barrier, commonly referred to as "leaky gut" (Camilleri 2019). This condition arises from the activation of MAPK signaling pathways, which subsequently disrupt the gene expression of critical tight junction proteins, including occludin, claudin-1, and ZO-1. This disruption results in the leakage of bacteria or their components, such as LPS, into the circulation, triggering an immune response (Yu, Wang et al. 2012, Quigley 2017). Dysbiosis caused by antibiotic use can lead to endotoxemia and impairment of signaling transduction from the gut microbiome to the host through the toll-like receptors pathway, adversely affecting both innate and adaptive immune responses. Maternal antibiotic treatment was reported to impact

viral immunity with less secreted interferon γ from $CD8^+$ cells (Gonzalez-Perez, Hicks et al. 2016).

The development of a healthy gut microbiome in infants is essential for their survival and optimal development. Human breast milk significantly contributes to this process by providing a unique microbiome that evolves over the course of breastfeeding. The infant gut microbiome influences several processes, including the differentiation of intestinal epithelium, communication with the gut-associated lymphoid tissue (GALT), the morphology of the gastrointestinal tract, as well as nutrition and metabolism (Groer, Luciano et al. 2014). The infant's gut bacteria are shaped not only by the mode of birth, and the mother's skin bacteria but also by the bacteria present in the mammary glands. The entero-mammary pathway (Fig. 4.1) enables the transfer of the maternal gut microbiome to the mammary glands, mediated by mucosal-associated lymphoid tissue and antigen-sampling cells such as dendritic cells (Farache, Koren et al. 2013, Bekiaris, Persson et al. 2014, Gutzeit, Magri et al. 2014, Kuper, Wijnands et al. 2017).

The gut microbiome exerts a regulatory effect over epigenetic factors, thereby influencing gene expression. Epigenetic modifications regulate gene expression at the transcriptional stage through DNA methylation and at the post-transcriptional stage through microRNAs (miRNAs) (Cramer 2019). The interaction between the gut microbiome and host epigenetics, particularly concerning DNA methylation, forms a vital part of the microbiome-nutrient metabolism-host epigenetic axis. This interaction is modulated by dietary factors and has significant implications for the host’s epigenetic landscape. Studies on germ-free mice have demonstrated that the absence of the gut microbiome leads to increased DNA hypermethylation in colonic epithelial cells due to reduced TET activity and lower circulating folate levels (Takahashi, Sugi et al.

2011). Short-chain fatty acids (SCFAs), especially Acetyl-CoA, affect the tricarboxylic acid cycle (TCA), influencing DNA methylation. TCA products like Alpha-Ketoglutarate aid in DNA methylation, while fumarate and succinate can inhibit TET enzymes, leading to increased DNA methylation (Miro-Blanch and Yanes 2019). miRNAs play a crucial role in various cellular processes within intestinal cells, particularly in maintaining the integrity of the intestinal barrier. Extracellular vesicles (EVs) originating from the gut microbiome carry specific miRNAs that are instrumental in regulating host gene expression (Severus, Florentina et al. 2021).

Research has shown that dysbiosis of the gut microbiome can significantly alter miRNA expression, thus modulating host gene expression and impacting key signaling pathways like the phosphoinositide 3-kinase (PI3K)/AKT, Toll-like receptor 4/nuclear factor-kappa B (TLR4/NF-κB), and Janus kinase/signal transducer and activator of transcription (JAK/STAT3) signaling pathways (Zhang, Shen et al. 2015, Anzola, González et al. 2018, Chu, Wang et al. 2018). Furthermore, gut microbiome dysbiosis is involved in immune responses, promoting the production of proinflammatory cytokines and activating related pathways such as NF-kB (Peng, Ouyang et al. 2020, Zhao, Chu et al. 2023).

The mammary gland is one of the unique organs, undergoing stages of development stemming from the last phase of pregnancy and peaking during puberty. Exposure to immune stressors during this critical window of age, if unresolved, may elevate the risk of developmental anomalies, potentially increasing susceptibility to cancer later in life (McNally and Martin 2011). The gut-breast axis plays a pivotal role, both directly and indirectly, in governing the development of the mammary glands and predisposing it to neoplastic lesions (Laborda-Illanes, Sanchez-Alcoholado et al. 2020). Notably, studies on dietary interventions during puberty indicate that diet can serve as a key modulator of long-term breast cancer risk. Specifically, mice

consuming a diet rich in n-3 polyunsaturated fatty acids (PUFA) exhibited a reduced number of terminal end buds, indicating altered mammary gland development (MacLennan, Anderson et al. 2011, Anderson, MacLennan et al. 2014, Shahbazi, Yasavoli-Sharahi et al. 2023).

Research on prebiotics and probiotics has underscored their crucial involvement in numerous biological functions, including the regulation of metabolism, influencing gene expression, and priming the immune system (Bengmark 2013). These components play a key role in preserving and regulating the gut microbial ecosystem and are known to reduce systemic inflammation (Ewaschuk and Dieleman 2006, McLoughlin, Berthon et al. 2017). Dietary interventions using prebiotics are effective in mitigating the adverse effects of antibiotics, leading to the restoration of the gut microbiome, modification of epigenetic markers, and prevention of immune system disorders (Cowan and Richardson 2019). Experiments with germ-free mice have demonstrated that the absence of a gut microbiome leads to improper development of various organs, particularly the immune system (Östman, Rask et al. 2006, Luczynski, McVey Neufeld et al. 2016). Remarkably, dietary interventions involving prebiotics have been successful in enhancing immune system functionality.

Hence, this study investigates the long-term impact of prenatal exposure to low doses of penicillin, in conjunction with the administration of a prebiotic known as AHCC (a standardized extract of cultured *Lentinula edodes* mycelia (ECLM)). AHCC acts as an antagonist for TLR4, possessing the capability to inhibit inflammatory pathways associated with TLR4 (Vinderola, Matar et al. 2005, Mallet, Graham et al. 2016). The primary aim is to investigate the potential regulatory effects of AHCC in counteracting the adverse effects of low-dose penicillin on the development and function of mammary glands.

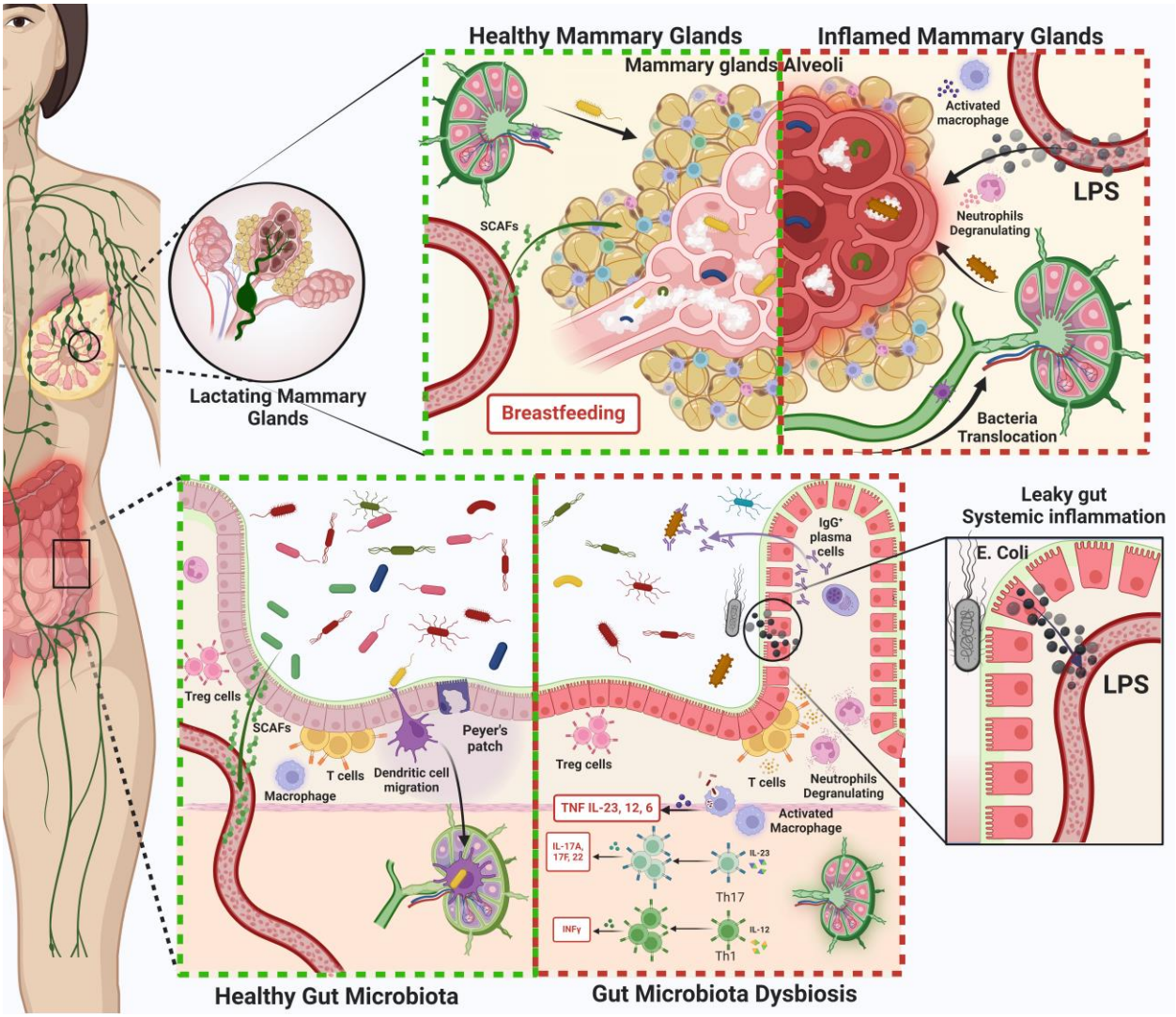

Fig. 4.1: Illustration depicting the entero-mammary pathway linking the gut and mammary glands. Illustration depicting the entero-mammary pathway linking the gut and mammary glands. Under normal circumstances, the mammary glands, being part of the mucosa-associated lymphoid tissue (MALT), allow dendritic cells to capture certain bacteria and translocate them to the mammary glands (Rodríguez 2014, Kuper, Wijnands et al. 2017). However, gut dysbiosis can result in a leaky gut, allowing bacterial products such as LPS to enter the bloodstream and trigger systemic inflammation (Camilleri 2019). This inflammatory response can disrupt mammary gland homeostasis, particularly during lactation. The infant's gut microbiome is shaped not only by skin flora but also by certain bacteria from the maternal gastrointestinal tract (Walker and Iyengar 2015). Maternal gut microbiome dysbiosis increases the risk of gut dysbiosis in infants (Wang, Ryan et al. 2020). Intake of prebiotics during pregnancy can aid in balancing the gut microbiome, regulating the immune system, improving the mammary gland microbiome, and maintaining mammary gland homeostasis (Jarde, Lewis-Mikhael et al. 2018).

4.3 Materials and Methods

Animals

In this investigation, pregnant Balb/c mice (gestation day 13), sourced from Charles River, Montreal, QC, Canada, were utilized. The mice were housed in plastic cages under controlled conditions with a temperature of 22 ± 2°C, humidity levels maintained at 55 ± 2%, and a 12-hour light/dark cycle. Throughout the study, all groups were provided with a standard balanced diet ad libitum, and treatments were administered via their drinking water. The care and treatment of the mice strictly adhered to guidelines set by the Canadian Council on Animal Care. The protocol (HSe-3866) was approved by the Animal Care Committee of the University of Ottawa on March 14, 2023.

Study Design

Sixteen pregnant Balb/c mice were used in the study. Starting one week before expected delivery, at gestation day 14, the mice were individually housed and began receiving their assigned treatments. The pregnant mice were divided into four groups, each comprising four mice: 1. The control group received regular drinking water. 2. The second group was given low doses of penicillin V (Sigma Aldrich, Toronto, ON, Canada) at 31 mg/kg body weight (BW)/day administered through drinking water. 3. The third group received a prebiotic, AHCC (2 g/kg BW/day), in their drinking water. 4. The fourth group was treated with a combination of penicillin V (31 mg/kg BW/day) and AHCC (2 g/kg BW/day) in their drinking water. Water consumption was monitored daily to ensure consistent dosage, with AHCC administration adjusted accordingly.

The treatments continued until the weaning of the offspring at postnatal day (PND) 21, thereby subjecting the offspring to prenatal and early postnatal exposure to the treatments. After weaning, all dietary interventions ceased, and the dams were euthanized for collection of mammary glands and blood plasma.

Simultaneously, at the end of weaning, the offspring were separated by sex and randomly reassigned to non-littermate, same-sex, and same-treatment groups. The pups then received a standard diet until they reached eight weeks of age (PND 56). At early adulthood (PND 56), the pups were euthanized, and mammary glands and blood plasma were collected to examine the enduring impacts of maternal treatment on their immune system and mammary gland development (Supplementary Fig. 1a). AHCC® is a standardized extract of cultured Lentinula edodes mycelia, provided by Amino Up Co., Ltd. (Sapporo, Japan).

Determination of Cytokine Concentrations by Luminex Multiplex Assay and Elisa

The mammary glands of the mice were washed with 1X PBS, and small segments of mammary gland tissues were finely cut into small pieces using surgical blades (Fisher Scientific, Toronto, ON, Canada). Chopped tissues were collected in microtubes containing lysis buffer (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.05% Tween-20) supplemented with a cocktail of protease inhibitors (AEBSF, Hydrochloride, Millipore Sigma, Oakville, ON, Canada). After vortexing and incubation on a rocker at 4°C for 20 minutes, homogenized samples were centrifuged at 14,000g for 15 minutes at 4°C, and the resulting supernatants were collected. Total protein concentrations in tissue lysates were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Toronto, Canada), and samples were adjusted to a concentration of 2 mg/mL with the same lysis buffer. The multiplex assay was performed using the Mouse Th17 Panel Magnetic, MTH17MAG-47K (Millipore Sigma, Burlington, USA), following the

manufacturer's instructions. Plates were read using the Bio-Plex® 200 System (Bio-Rad, Hercules, CA, USA). Additionally, the levels of immunoglobulins IgA and IgG were measured using ELISA kits from Invitrogen (Vienna, Austria), while the concentrations of cytokines IL-6 and IL-10 were determined using kits from BD Biosciences (Mississauga, ON, Canada). Analytically, the results for each sample were normalized by dividing the observed concentrations by the total protein concentration of that sample.

Determination of Protein Levels by Western Blotting

Small sections of mammary glands were homogenized using an electrical homogenizer in tubes containing RIPA buffer (Thermo Fisher Scientific, Toronto, ON, Canada) supplemented with a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Toronto, ON, Canada). Samples were incubated on a rocker for 20 minutes at 4°C. Protein lysates were collected by centrifuging homogenized samples at 14,000g for 15 minutes at 4°C, and total protein concentrations were measured using the Pierce BCA Protein Assay Kit according to the manufacturer's instructions. Equal amounts and concentrations of protein lysates were loaded onto 12% Bis-Tris Mini Protein Gels (Invitrogen, Toronto, ON, Canada) using MES SDS Running Buffer (Life Technologies, Toronto, ON, Canada) at 200 V for 22 minutes. Subsequently, proteins were transferred to a Nitrocellulose membrane using the Trans-Blot Turbo RTA Mini Transfer Kit (Bio-Rad, Hercules, CA, USA) at 25 V for 7 minutes. The membranes were then incubated overnight at 4°C with primary antibodies: anti-Occludin (1:1000), anti-Claudin 1 (1:1000), anti-β-actin (1:1000), PTEN (1:1000) (Abcam, Toronto, ON, Canada), anti-ERK1/2 (1:1000), and anti-phospho-ERK1/2 (Cell Signaling, CA, USA). Following primary antibody incubation, blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000) (Jackson ImmunoResearch Laboratories, West

Grove, PA, USA) at room temperature for 1 hour. Protein bands were visualized using a chemiluminescence assay with ECL substrate (Bio-Rad, Mississauga, ON, Canada) and quantified using Bio-Rad Image Lab 6.1 Software, with β-actin used as the loading control.

Determination of Gene, and miRNAs Expression Level by Real-Time Quantitative Reverse Transcription PCR (RT-qPCR)

Small sections of mammary gland tissues were preserved in RNAlater Stabilization Solution (Invitrogen, USA) for 24 hours at 4°C and subsequently stored at -80°C until RNA extraction. Total RNA and total microRNAs were extracted using the RNeasy Plus Mini Kit and miRNeasy Mini Kit (Qiagen, Toronto, ON, Canada). The concentrations and purity of the extracted RNA were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). For mRNA and miRNA expression analysis, reverse transcription reactions were performed to synthesize cDNA using the QuantiTect

Reverse Transcription Kit and miRCURY LNA RT Kit (Qiagen, Toronto, ON, Canada). The expression levels of selected mRNAs (*Fgf1, fgfr2, Fzd7, Itgb4, Ptk2, Magi1, Crebbp, Lama2, Akt1, Mapk1, Tp53, c-Jun, Apc, Pten, Egfr, Igf1r, Esr2, Ocln, Cldn1, Cldn3*) and miRNAs were assessed by RT-qPCR using QuantiNova LNA PCR Assays and miRNA PCR assay primers (Table 1). The reactions were conducted with QuantiNova SYBR Green PCR Kit and miRCURY LNA SYBR Green PCR Kit (Qiagen, Toronto, ON, Canada) on a CFX 384 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Normalization of mRNA expression levels was performed using average expression of the reference genes Actb and B2m, while Cldn4 was used for tight junction proteins. miRNA expression levels were normalized using SNORD65 (mmu)

miRCURY LNA miRNA PCR Assay (Qiagen, Toronto, ON, Canada) as the reference gene. Relative expression levels of miRNAs were calculated using the ΔΔCT method.

Table 4.1. List of primer

MM_PTEN_2476269	MM_JUN_2289604
MM_FGFR2_2057749	MM_MAPK1_2044721
MM_FGF1_2318822	MM_APC_2026135
MM_AKT1_2264597	MM_TRP53_2085532
MM_ESR2_2213470	MM_ACTB_2476263
MM_EGFR_2277473	MM_B2M_1919401
MM_IGF1R_2013373	MM_CLDN3_2246430
MM_FZD7_2300051	MM_CLDN1_2003889
MM_CREBBP_2170702	MM_OCLN_2327602
MM_ITGB4_2053175	hsa-let-7c-5p
MM_LAMA2_1917339	hsa-let-7a-5p
MM_PTK2_2232290	mmu-miR-34b-5p
MM_MAGI1_2158566	hsa-miR-146a-5p

DNA Extraction and Mouse Methylation BeadChip Array

A segment of mammary gland tissue, weighing 40-50 milligrams, from offspring mice was processed using an electrical homogenizer (Bead Mill 24, Fisher Scientific, Canada).

Homogenization was performed in tubes filled with 500 µl of cell lysis buffer and 1.5 µl of proteinase K. DNA extraction from mammary tissue was carried out using the Gentra Puregene Tissue Kit (Qiagen, Toronto, Canada), following the manufacturer's instructions. After extraction, DNA was diluted to a final concentration of 20 ng/µl using DNA rehydration solution and stored at -20ºC. The DNA concentration was measured using the Qubit 4 instrument (ThermoFisher Scientific, Canada). DNA methylation analysis was performed at GenomeQuébec, Quebec, Canada. Briefly, 500 ng of extracted DNA underwent bisulfite conversion using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) and was subsequently analyzed using the Infinium™ Mouse Methylation BeadChip (Illumina Inc., San Diego, CA, USA).

DNA Methylation Data Pre-Processing, Normalization, and Analysis

The bioinformatics analysis was performed following the pipeline developed in-house by the Epigenomics and Mechanisms Branch at the International Agency for Research on Cancer (available at https://github.com/IARCbioinfo/methylkey) which utilizes R packages such as methylkey (v 1), SeSAMe (v 1.15.1), GenomicFeatures (v 1.50.3), and minfi (v 1.44.0). Briefly, IDAT files generated from the DNA methylation arrays were pre-processed and normalized using the SeSAMe R package, which executed dye bias correction, detection *p*-value masking, background subtraction, and quality control. Probes in which the detection *p*-value exceeded 0.02 or were missing in 20% of the analyzed samples were excluded from the analyses. Differentially methylated probes were identified using robust linear regression, where significantly differential methylation was defined as probes in which the average inter-group difference in DNA methylation was more than |3%| and were assigned a false discovery rate (FDR)-adjusted *p*-value of less than 0.05. Differentially methylated regions were identified using

DMRcate (v 2.11.0) package, defined by FDR-adjusted p-value < 0.05 and |max methylation difference| > 3%. KEGG pathways enrichment analyses were performed using Enrichr (v 3.0) package.

Statistical Analysis

Statistical analyses for all experiments, excluding DNA methylation analysis, were performed using GraphPad Prism Software (GraphPad Software Inc., San Diego, CA, USA). Data distribution was assessed with the Shapiro-Wilk test. Due to the limited sample size, dam-related data were analyzed using a two-way analysis of variance (ANOVA) followed by Fisher's post-hoc test. For offspring-related data, Tukey's post-hoc test was applied. Results are presented as mean ± SEM, and a p-value < 0.05 indicates a statistically significant difference between groups.

4.4 Results

Assessing the Impact of Low Dose Penicillin and AHCC Exposure During Pregnancy and Early-Life on Weight Patterns in Dams and Offspring

In this study, we closely monitored weight changes in dams from before delivery through postpartum week three, as well as the weight of their offspring at weaning and eight weeks of age. We observed that dams experienced a weight increase following delivery, peaking in the second postpartum week, followed by a decline in the third week (Fig. 4.2a). Using three-way ANOVA, no significant differences were found between treatment groups in terms of dams' weight during this period.

Given that the primary focus of this study was on mammary gland development, only female samples were selected for further analysis. We noted that female pups in the LDP and AHCC+LDP groups had decreased weights compared to those in the control and AHCC groups at the end of weaning (week 3) (Fig. 4.2a). This weight pattern persisted into adulthood, although the difference at nine weeks of age did not reach statistical significance (Fig. 4.2a). AHCC supplementation alone did not significantly impact mouse weight. These findings suggest a potential association between LDP and offspring weight, as evidenced by consistent weight patterns observed up to 8 weeks of age.

Evaluating the Impact of Low Dose Penicillin and AHCC Exposure on Immune Response in Plasma and Mammary glands of Postpartum Dams

In our previous study, we conducted a metagenomics analysis using 16S rRNA gene sequencing to examine the gut microbiome in both maternal and female offspring cohorts. This analysis revealed significant shifts in microbial populations due to the administered treatments. Specifically, dams exposed to LDP showed an increased prevalence of *Proteobacteria* and

Robonsoneilla, alongside a diminished presence of *Lactobacillus*, *Clostridia UCG-014*, and *Eubacterium xylanophilum* (Data not shown).

Building on these observations, the current study examined the potential inflammatory response of microbiome dysbiosis on maternal health. The investigation focused on quantifying the levels of immunoglobulins IgA and IgG, and the inflammatory cytokine IL-6 in plasma. The results indicated a significant elevation in plasma IgA levels in dams treated with LDP compared to the control ($P<0.01$). Interestingly, the IgA level remained the same across the AHCC and AHCC+LDP groups (Fig. 4.2b). IgG levels exhibited increased levels across all groups, with the highest in the LDP and AHCC+LDP groups, with a p-value < 0.05. The AHCC group showed a slight increase in IgG levels compared to the control; however, this comparison was not statistically significant (Fig. 4.2b).

The ELISA assay did not detect IL-6 in the plasma, likely due to the timing of sample collection. IL-6 expression is known to be highly dynamic and tends to peak in the early stages of the immune response (Kishimoto 2010).

Additionally, differences in immunoglobulin levels within mammary tissue were observed. The AHCC groups displayed a higher concentration of IgA compared to the control, while the LDP group exhibited slightly lower levels, though these differences were not statistically significant. Similarly, a slight elevation in IgG was detected across all treatment groups compared to the control, yet these differences were not statistically significant (Supplementary Fig. 4.1b).

The Influence of Low Does Penicillin and AHCC on Inflammatory Cytokines in Lactating Mammary Glands of Dams

The previous findings on IgA and IgG prompted further investigation into the concentration levels of various inflammatory cytokines, including IFNγ, TNFα, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-15, IL-17A, IL-17F, IL-22, and IL-23 in the lactating mammary glands of dams. Among all the measured cytokines, only IL-1β, IL-2, IL-4, IL-6, IL-10, and IL-17A exhibited statistically significant changes across the different study groups.

IL-1β levels were elevated in the LDP group and remained consistent across all groups. However, statistical significance was observed only when comparing the LDP group to the AHCC+LDP group (Supplementary Fig. 4.1c). An increase in the levels of IL-2 and IL-17A was observed in lactating dams treated with LDP compared to the control group, while these cytokine levels remained the same in the AHCC and AHCC+LDP groups as in the control group (Fig. 4.2c).

The concentration levels of IL-4 remained consistent in the LDP and control groups. However, IL-4 was notably reduced in the AHCC and AHCC+LDP groups, with a significant decrease observed only in the AHCC group compared to the control group (Fig. 4.2c). For IL-6, a reduction was observed across all treatment groups compared to the control group. The decline in IL-6 levels within the AHCC group was significant when compared with the control group (Fig. 4.2c). In the case of IL-10, a slight decrease was observed in the AHCC group; however, no statistically significant difference was found across all groups (Supplementary Fig. 1c).

Impact of Low-Dose Penicillin and AHCC Exposure on the Integrity of Blood-Milk Barrier

To assess whether alterations in inflammatory factors in blood plasma and mammary glands could affect the blood-milk barrier (BMB), the levels of specific messenger RNAs for Occludin (Ocln), Claudin 1 (Cldn1), and Claudin 3 (Cldn3) were measured.

Ocln expression was markedly higher in the AHCC and AHCC+LDP groups compared to the control and LDP groups, with fold changes of 4.5 and 5.2, respectively, and a p-value of < 0.01 (Fig. 4.2d). Cldn1 exhibited an overall increase across all groups compared to the control group. However, statistical analysis revealed no significant difference between the groups (Fig. 4.2d). Cldn3 expression was significantly elevated in the AHCC+LDP group compared to both the control and LDP groups, with a fold change of 2 and a p-value of < 0.05 (Supplementary Fig. 4.1d).

Western blot analysis further supported these findings, confirming the overexpression of Ocln in the AHCC group compared to the control group with a p-value < 0.01. While Ocln levels were also elevated in the AHCC+LDP group compared to the LDP group, this difference was not statistically significant. Regarding Cldn1, the LDP group exhibited consistent protein levels. Although there was an observed increase in Cldn1 levels in the AHCC group, this difference did not reach statistical significance (Fig. 4.2d).

Assessing the Impact of Low Dose Penicillin and AHCC Exposure on Key Genes Associated with Mammary Glands Homeostasis in Dams

In the subsequent step of the study, the expression of critical genes associated with cellular homeostasis of mammary glands and breast cancer, including *Mapk1*, *Akt1*, *Pten*, *Esr2*, *Egfr*, and *Igf-1r*, was examined. *Akt1* expression remained relatively consistent across all groups, with no statistically significant differences observed (Supplementary Fig. 4.1e). A general downregulation trend in *Mapk1* expression across all groups was observed, although the differences did not reach statistical significance (Fig. 4.2e). *Pten* expression remained stable in the LDP group but was significantly higher in the AHCC and AHCC+LDP groups compared to the control and LDP groups, with fold changes of 2 and 1.7, and p-values < 0.01 and 0.05,

respectively (Fig. 4.2e). *Egfr* expression showed a declining trend in the LDP and AHCC groups and an increase in the AHCC+LDP group, yet these differences did not achieve statistical significance (Supplementary Fig. 4.1e). *Igf-1r* expression demonstrated a general decrease across all groups relative to the control, with the comparison between the AHCC group and the control being the only one to reach statistical significance, showing a fold change of 0.4 and a p-value of < 0.05 (Supplementary Fig. 4.1e). *Esr2* expression was downregulated in the LDP group compared to the control and increased in the AHCC+LDP group compared to the LDP group, although these changes were statistically significant only for the comparison between LDP vs AHCC+LDP (Supplementary Fig. 4.1e).

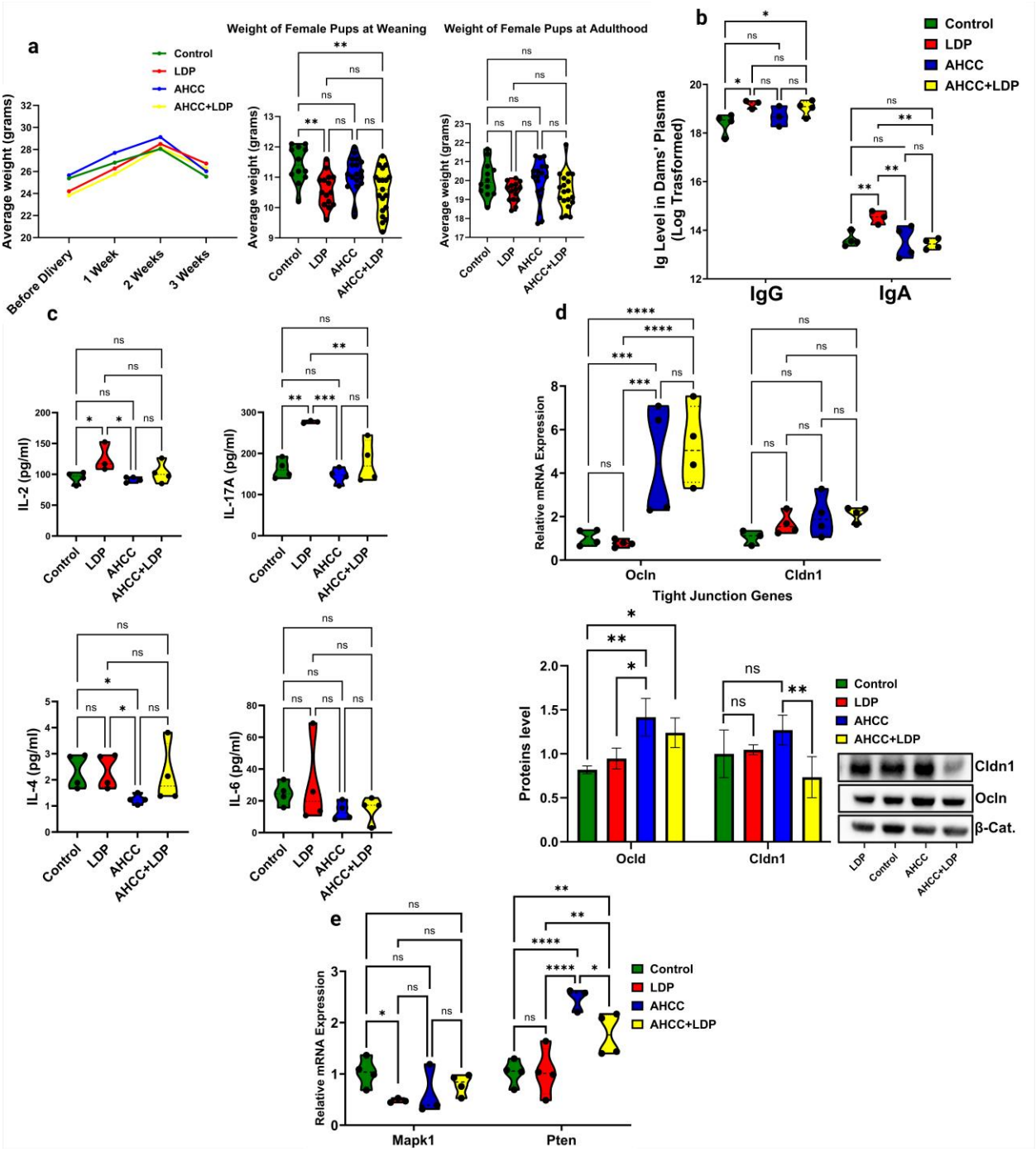

Fig. 4.2. Assessment of Treatment Effects on Maternal and Offspring Weights, Maternal Immune Responses, and Tight Junction in Dams. Pregnant mice received treatments via drinking water from the last week of gestation (gestation day 14) to the weaning of their pups (postnatal day 21). Treatments included penicillin V (31 mg/kg BW/day), AHCC (2 g/kg BW/day), or a combination of both. The control group was given regular drinking water. Data are presented as mean ± SEM. **(a)** Dam weight monitoring pre- and post-delivery (n = 4 per group). Differences between groups were analyzed using three-way ANOVA followed by Fisher's LSD post-hoc tests. **(b)** Offspring weight trends at weaning (n > 12 per group, postnatal day (PND) 21) and at 8 weeks of age (PND 63). **(c)** Changes in blood plasma concentrations of immunoglobulins IgA and IgG in dams at the end of weaning (n > 3 per group). **(d)** Concentration of inflammatory cytokines (IL-2, IL-4, IL-6, and IL-17A) in the dams (n > 3 per group). **(e)** Changes in the expression of occludin (Ocln) and claudin-1 (Cldn1) in the lactating mammary

glands. Expression was measured at the mRNA level by RT-qPCR and at the protein level by Western blot (n > 3 per group). **(f)** Alterations in the expression of *Mapk1* and *Pten* genes in lactating mammary glands, quantified by RT-qPCR (n > 3 per group). Statistical analysis of normality was conducted using the Shapiro-Wilk test. Group comparisons were made with two-way ANOVA and Tukey's post-hoc tests. Significance levels are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Evaluating the Impact of Early-Life Exposure of Low Dose Penicillin and AHCC on Cytokines IL-6, IL-10, and Immunoglobulin IgG in the Plasma of Offspring

In our recent publication, it was revealed that exposure to LDP results in the depletion of certain gut bacteria populations in the female offspring, including *Firmicutes Anaerotruncus*, *Firmicutes Lactobacilli*, and *Firmicutes Roseburia* (Data not shown). These bacteria are crucial for the development of the gut and immune system. The disruption of gut microbiome balance is linked to inflammation, which led to the investigation of inflammatory markers such as IL-6, IL-10, and IgG in the blood plasma of offspring

IL-6 concentration was notably higher in the AHCC+LDP group compared to the LDP group alone, with a p-value of <0.01. In the LDP and AHCC groups, IL-6 concentration was significantly lower than that in the control group, with p-values of <0.05 and 0.01, respectively (Fig. 3a). IL-10 levels were elevated in all groups, with the comparison between the AHCC and control group showing statistical significance (p-value < 0.01). While IL-10 concentration was higher in the LDP group, this difference did not reach statistical significance (Fig. 4.3a). IgG levels exhibited a downward trend across all groups. The differences between the AHCC and the control group, as well as between the AHCC+LDP and control/LDP groups, were statistically significant, indicating a meaningful reduction in IgG levels associated with the AHCC treatment (Fig. 4.3a).

The Influence of Early-Life Exposure of Low Dose Penicillin and AHCC on Inflammatory Cytokines in Mammary Glands of Offspring

The concentration of INFγ, TNFα, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-15, IL-17A, IL-17F, IL-22, and IL-23 cytokines was evaluated in the mammary glands of pups at 8 weeks old. Among the mentioned cytokines, only INFγ, IL-1β, IL-2, IL-4, IL-10, IL-15, IL-17A, IL-17F, IL-22 showed statistically significant variation across all groups. It was found that pups receiving LDP treatment exhibited a decrease in INFγ, IL-1β, IL-2, IL-4, IL-10, IL-15, IL-17A, IL-17F, IL-22 cytokines (Fig. 3b). In contrast, the group treated with AHCC displayed cytokine levels that were similar or slightly changed compared to the control group. Notably, the group treated with AHCC+LDP showed higher levels of cytokines than those observed in the LDP group, indicating that AHCC might have the potential to neutralize the effects of LDP.

IL-4, and IL-10 levels remained unchanged in the LDP group relative to the control group, whereas a minor increase was observed in the AHCC and AHCC+LDP groups. (Supplementary Fig. 4.2a).

The Impact of Early-Life Exposure of Low Dose Penicillin and AHCC on the Expression of Key Genes Related to Breast Cancer Susceptibility

In the next step, the expression of critical genes including *Apc*, *Magi1*, *Tp53*, *Pten*, *Akt1*, *Mapk1*, *c-Jun*, *Egfr*, *Igf-1r*, *Ptk2*, *Fgf1*, *Fgfr2*, *Fzd7*, *Dvl3*, *Crebbp*, and *Esr2* was examined.

It was noted that the expression levels of tumor suppressor genes Apc and Magi1 were elevated across all groups (Fig. 4.3c). *Tp53* was elevated in both the AHCC and AHCC+LDP groups compared to the control group, while its expression remained unchanged in the LDP group relative to the control (Fig. 4.3c). Notably, *Pten* showed upregulation in the AHCC group, while its expression in the LDP and AHCC+LDP groups was slightly lower compared to the control, although this difference was not statistically significant (Fig. 4.3c). *Egfr*, *Igf-1r*, and *Ptk2*

exhibited similar expression trends across all groups, showing upregulation overall. However, the only statistically significant difference was observed in the LDP group compared to the control group (Fig. 4.3c). The expression levels of *Fgf1*, *Fgfr2*, *Fzd7*, *Dvl3*, and *Crebbp* were elevated in the LDP group compared to the control group. Conversely, in the AHCC and AHCC+LDP groups, the expression levels of these genes either remained consistent or exhibited a slight increase, although these alterations did not reach statistical significance (Fig. 4.3c).

Esr2 showed a broad range of variation within the LDP group, yet the comparison between the LDP and control groups was not statistically significant. Nonetheless, *Esr2* was upregulated in both the AHCC and AHCC+LDP groups with a p-value of < 0.05, and its expression was higher in the AHCC+LDP group compared to the LDP group (Fig. 4.3c).

In addition, *c-Jun* exhibited significant upregulation in the LDP group compared to the control. *c-Jun* expression remained stable in AHCC and AHCC+LDP compared to the control and LDP groups, suggesting a modulatory effect of AHCC on c-Jun expression (Fig. 4.3c).

Akt1 showed elevated expression levels in the AHCC and AHCC+LDP groups compared to the control group. Conversely, its expression level remained unchanged in the LDP group (Supplementary Fig. 4.2b). For *Mapk1*, an increase in expression was observed in the AHCC group alone, while its expression levels remained consistent in both the LDP and AHCC+LDP groups (Supplementary Fig. 4.2b).

KEGG enrichment analysis confirmed the involvement of these genes in signaling pathways associated with breast cancer such as PI3K/Akt, estrogen, MAPK, Ras, ErbB, mTOR, Wnt, focal adhesion, cancer pathways, and stem cell regulation (Fig. 4.3d).

Potential Dysregulation of MAPK signaling pathway upon Early-Life Exposure of Low Dose Penicillin in Offspring

The study aimed to elucidate the potential pathways through which specific gene dysregulation induced by various treatments might influence cell development and proliferation, thereby increasing susceptibility to breast cancer. To this end, an enrichment analysis was conducted using EnrichR on genes found to be dysregulated by LDP treatment and modulated by AHCC. The results highlighted their involvement in pivotal signaling pathways such as MAPK, PI3K-AKT, pathways regulating pluripotency of stem cells, and Wnt (Fig. 4.3d).

Further validation of these findings involved assessing the protein levels of key markers within the PI3K-Akt and MAPK pathways through western blot analysis. This included FOXO1, pFOXO1, AKT1, pAKT1, PTEN, ERK1/2, pERK1/2, MEK1/2, and pMEK1/2. Despite not detecting FOXO1 and pFOXO1, AKT1, pAKT1, MEK1/2, and pMEK1/2, an overall increase in PTEN protein levels was observed in the AHCC and AHCC+LDP groups compared to the control, although these differences did not reach statistical significance (Fig. 4.3e).

The analysis also showed consistent levels of total ERK1/2 across all groups. However, pERK1/2 levels were elevated in the LDP group compared to both the control and AHCC+LDP groups. The protein levels of AHCC and AHCC+LDP groups in relation to pERK1/2 remained consistent with the control (Fig. 4.3e).

The Impact of Early-Life Exposure of Low-Dose Penicillin and AHCC on Key miRNAs Related to Mammary Glands Development in Offspring

Alongside measuring the expression of critical genes in the mammary glands, the study evaluated the expression levels of microRNAs involved in mammary gland development and breast cancer, specifically Let-7a, Let-7c, miR-34b, and miR-146a.

The findings indicated significant influences of LDP and AHCC on the expression of these microRNAs. Let-7a and Let-7c showed a consistent pattern of downregulation in the LDP group, while they were overexpressed in both the AHCC and AHCC+LDP groups. Additionally, miR-146a was downregulated in the LDP group but remained stable or slightly decreased in the AHCC and AHCC+LDP groups, with an increase observed specifically in the AHCC+LDP group compared to all other groups (Fig. 4.3f). In contrast, miR-34b did not show significant changes in the LDP group but was significantly upregulated in both the AHCC and AHCC+LDP groups (Supplementary Fig. 4.2d). These observations underscore the potential dysregulation of the ERK/MAPK pathway and highlight the therapeutic implications of AHCC in modulating both gene and microRNA expression. These effects are particularly relevant in understanding mammary gland development and the pathogenesis of breast cancer.

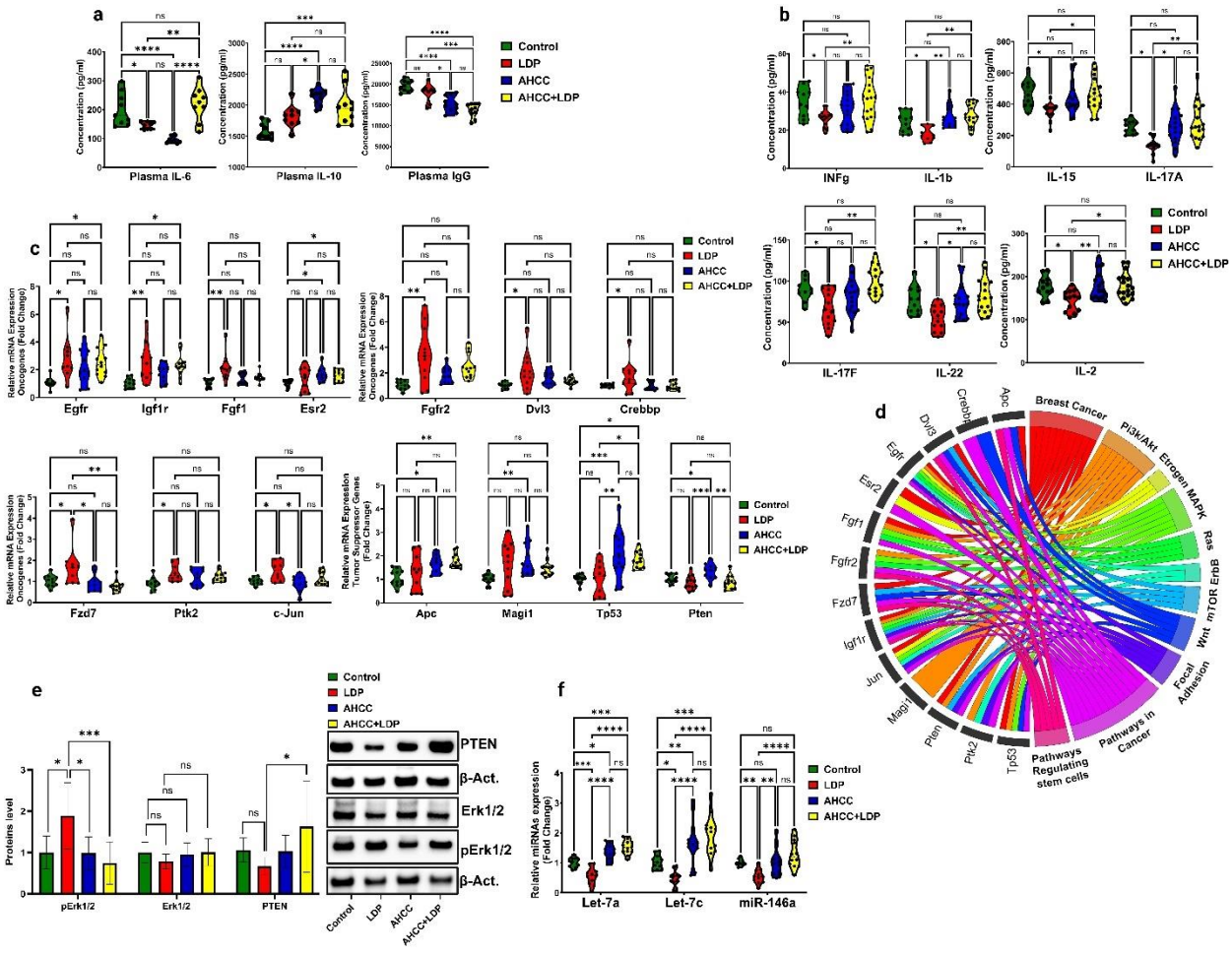

Fig. 4.3: The Impact of Treatment on Immunological Markers and Gene Expression in Offspring (n > 9/group). (a) Concentration changes in immunoglobulins (IgG) and cytokines (IL-6 and IL-10) in the blood plasma of offspring post-treatment, quantified using ELISA and Luminex Multiplex Assays. **(b)** Levels of inflammatory cytokines (IFNγ, IL-1β, IL-2, IL-15, IL-17A/F, IL-22) in mammary glands, measured by Luminex Multiplex Assay. **(c)** Post-treatment alterations in the expression levels of genes (Apc, Magi1, Tp53, Pten, Egfr, Igf-1r, Fgf1, Esr2, Fgfr2, Dvl3, Crebbp, Fzd7, Ptk2, and c-Jun) in mammary glands of offspring, assessed using RT-qPCR. **(d)** KEGG signaling pathway enrichment plot for the 14 analyzed genes. **(3)** Protein levels of phosphorylated Erk1/2, total Erk, and Pten were quantified via Western blot assay (n > 8/group). **(f)** Changes in the expression levels of microRNAs Let-7a, Let-7c, and miR-146a (n > 9/group). Data normality was assessed using the Shapiro-Wilk test. Statistical comparisons between groups were performed using two-way ANOVA and Tukey's post-hoc tests. All values are presented as mean ± SEM. Significance levels are denoted as *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

Long-Term Effects of Early-Life Exposure of Low Dose Penicillin and AHCC on DNA Methylation and Potential Impact of AHCC Treatment

DNA methylation is a crucial epigenetic mechanism involved in numerous cellular processes, notably the regulation of gene expression (Riggs 2002). Methylation patterns in specific gene regions, such as 1-5 kb upstream of transcription start sites, promoters, and the 5' untranslated region (UTR), are frequently linked with the suppression of gene expression (Smith and Meissner 2013). Conversely, methylation within the gene body, especially in regions beyond the exon, is associated with enhanced gene expression (Hellman and Chess 2007, Ball, Li et al. 2009, Brenet, Moh et al. 2011). Epigenetic profiling was carried out using the Illumina Infinium Mouse Methylation BeadChip arrays, which allow for the simultaneous interrogation of approximately 285,000 probes. All interrogated samples passed all quality control metrics (Supplementary Fig. 4.2e).

Unsupervised clustering of the 500 probes with the highest variability among the samples, determined by the coefficient of variation, showed that control and AHCC-treated samples grouped together. Conversely, samples from mice treated with AHCC+LDP tended to cluster with those treated with LDP alone, as visualized in the heatmap (Fig. 4.4a). In line with this, a comprehensive analysis of differential methylation highlighted that the LDP-treated group exhibited the most pronounced methylation changes compared to the control group, with 1955 CpG sites showing statistically significant variation after multiple tests. The majority of these changes in the LDP group mice were hypermethylated (Supplementary Table 1). On the other hand, AHCC treatment compared to the control, and AHCC+LDP compared to the LDP group, resulted in altered methylation at 382 and 639 sites, respectively (Supplementary Table 1).

Regional analysis was conducted with differentially methylated probes (DMPs) identified from the above three comparisons to account for variations across regions resulting from treatments (Supplementary Table 2). When comparing LDP treatment to the control, 348 unique

differentially methylated regions (DMRs) were discovered, including genes involved in critical cellular pathways such as cGMP-PKG, cAMP, PI3K-Akt, TGF-beta signaling, estrogen signaling, focal adhesion, and Rap1 signaling pathways (Supplementary Fig. 4.2f). However, enrichment analysis showed no pathways significantly enriched in genes associated with the DMRs after correcting for multiple tests. The investigation highlighted certain genes like *Nfatc1*, *Itgb4*, *Hyal1*, *Sox10*, *Igfbp7*, *Pax5*, *Mia2*, *Tiam1*, *Smad6*, *Bmp7*, *Ksr2*, *Itga9* that were differentially methylated. Specifically, the 1-5 kb and intronic regions of *Nfatc1*, as well as the exon and intron regions of Itgb4, were found to be hypomethylated in the LDP group compared to the control, but not statistically significant in the comparison of LDP and AHCC+LDP. Promoter regions of genes *Hyal1*, *Sox10*, *Igfbp7*, *Pax5*, and *Mia2* were found to be hypermethylated in the LDP group relative to the control, while comparisons between control vs AHCC and LDP vs AHCC+LDP groups did not show significant differences. Additionally, the intronic regions of *Tiam1*, *Smad6*, *Bmp7*, *Ksr2*, and *Itga9* genes were hypermethylated in the LDP group relative to the control (Fig. 4.4b). Upon comparing LDP and AHCC+LDP, 116 DMRs were identified. Subsequent enrichment analysis of signaling pathways did not yield statistically significant results. Additionally, some of these DMRs were found outside of gene coding regions or have limited information available in the literature. Notably, among these DMRs, the intronic regions of *Smad3* and *Foxp1* were found to be hypomethylated, while *Lrig1* and *Bcl6* were hypermethylated (Fig. 4.4b).

To further elucidate the LDP-induced DNA methylation changes that can be attenuated or exacerbated by the addition of AHCC, we focused on the DMRs shared between the control vs LDP and LDP vs AHCC+LDP comparisons. Thus, 28 DMRs were identified as common between these comparisons. Notably, within these genes, the promoter regions of *Tiam1* and

Pax5, as well as the intronic regions of *Lrig1* and *Itga9*, displayed higher methylation levels in the AHCC+LDP group relative to the LDP group alone.

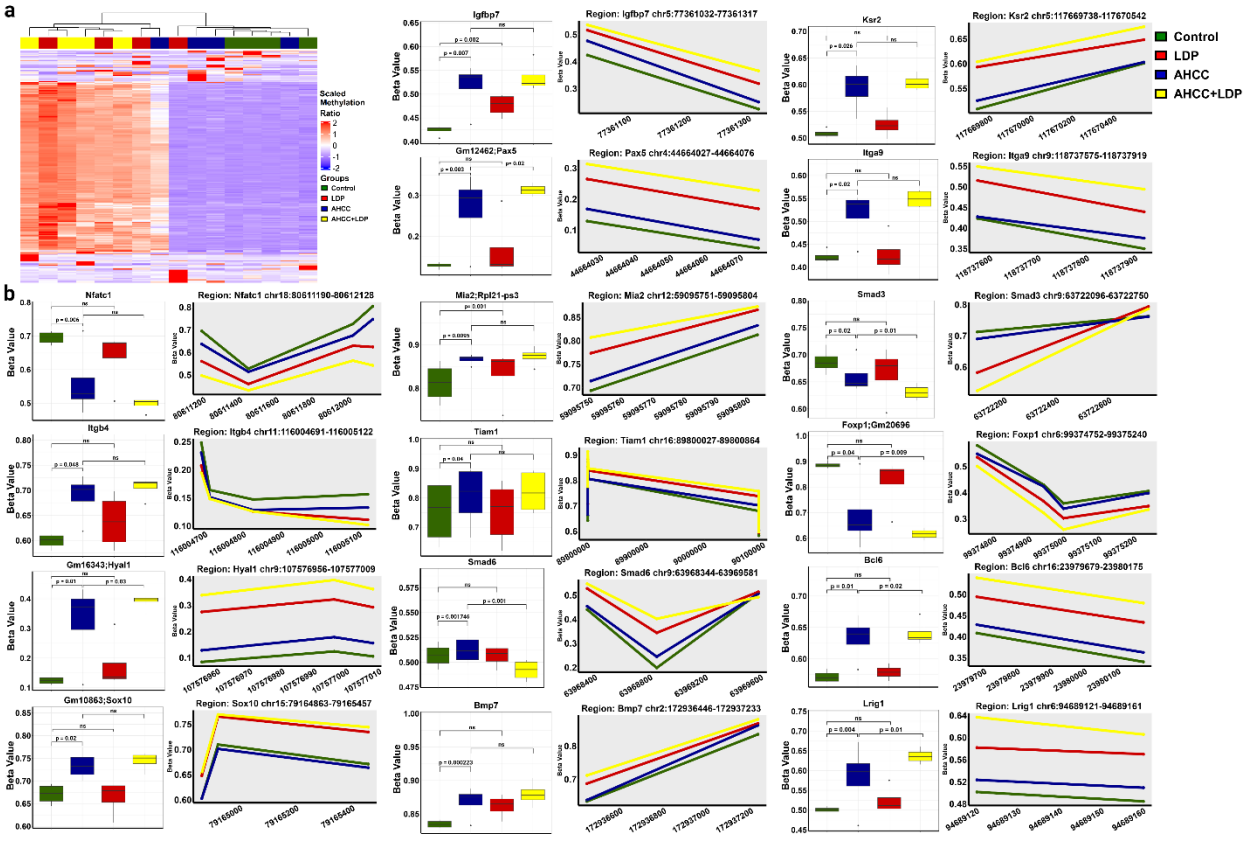

Fig. 4.4: The Impact of Treatment on Protein Levels in the MAPK Signaling Pathway, microRNA Expression, and DNA Methylation in Offspring. (a) Heatmap representing unsupervised clustering of the 500 probes with the highest variability, based on samples and determined by the coefficient of variation (n > 4/group). **(b)** Average differences in DNA methylation across specific gene regions: Nfatc1, Itgb4, Hyal1, Sox10, Igfbp7, Pax5, Mia2, Tiam1, Smad6, Bmp7, Ksr2, Itga9, Smad3, Foxp1, Bcl6, Lrig1.

4.5 Discussion

Antibiotics are one of the most prescribed medications during pregnancy (Yu, Tran et al. 2020). A growing body of evidence has established a link between early-life antibiotic consumption and a heightened risk for allergies, autoimmune conditions, and obesity (Cox, Yamanishi et al. 2014, Schokker, Zhang et al. 2015, Neuman, Forsythe et al. 2018). These studies indicate that the use of antibiotics leads to gut microbiome dysbiosis and lasting changes to the immune system,

thereby facilitating the development of various diseases (Vijay and Valdes 2022). Recent findings have shed light on the mechanism known as the entero-mammary pathway, which is implicated in the translocation of bacteria to the mammary glands, suggesting that an imbalanced gut microbiome could influence both lactating mammary glands and the neonatal gut microbiome (Kwa, Plottel et al. 2016, Patangia, Ryan et al. 2022, Zhao, Bao et al. 2022). Concurrently, natural compounds are increasingly recognized for their potential to mitigate disruptions in the gut microbiome and regulate the immune system (Shahbazi, Yasavoli-Sharahi et al. 2020, Shahbazi, Sharifzad et al. 2021, Shahbazi, Yasavoli-Sharahi et al. 2023).

In our recent study, we demonstrated that exposure to low-dose penicillin induces dysbiosis in the gut microbiome of offspring. Prior studies have elucidated how dysbiosis in the gut microbiome and the subsequent activation of the MAPK signaling pathway suppresses the expression of fundamental proteins of tight junctions. This process compromises the integrity of tight junctions, paving the way for systemic inflammation by allowing bacterial products such as lipopolysaccharide (LPS) to enter the bloodstream (Yu, Wang et al. 2012, Quigley 2017).

Furthermore, we aimed to shed light on the link between antibiotic-induced dysbiosis during pregnancy and the early-life immune system in lactating mammary glands. Our research also delved into the potential of the prebiotic compound AHCC, derived from cultured mushroom mycelium extract, to counteract the enduring effects of antibiotic consumption in offspring. AHCC has been shown to beneficially alter the gut microbiome and enhance the immune system by influencing Toll-like receptor 4 (Mallet, Graham et al. 2016). Furthermore, it possesses the ability to inhibit the development of cancer stem cells by regulating gene expression, offering promising avenues for further research into its therapeutic potential (Mallet, Graham et al. 2016, Graham, Mallet et al. 2017).

Systemic inflammation is commonly associated with raised levels of IL-6. However, in the current study reported here, IL-6 was not detected in the blood plasma of lactating dams, aligning with the fact that IL-6 typically exhibits brief surges at the onset of immune responses (Dijstelbloem, Kallenberg et al. 2001, Kishimoto 2010). IgA, the most abundant immunoglobulin in mucus, is crucial for bolstering anti-inflammatory responses and immunity, primarily by neutralizing toxins and bacteria (Mkaddem, Christou et al. 2014, Hansen, Hoepel et al. 2017).

The levels of IgG and IgA were notably higher in the group treated with LDP compared to other groups. This elevation suggests ongoing inflammation possibly stemming from a disrupted gut microbiome. Subsequently, the impact of gut microbiome dysbiosis and systemic inflammation was investigated in the lactating mammary glands. Interestingly, the levels of IgA and IgG were found consistent across all experimental groups. However, when assessing the cytokine profiles, a significant increase in the concentration of IL-2 and IL-17A within the LDP group was observed. IL-17A, known for its role in initiating inflammatory responses and recruiting immune cells, is crucial for priming the immune system against bacterial invasions (Mills 2023). On the other hand, IL-2, primarily produced by $CD4^+$ T cells, plays a critical role in guiding the differentiation of these cells into several subsets, such as regulatory T cells, while also potentially inhibiting the differentiation of Th17 cells (Abbas, Trotta et al. 2018, Spolski, Li et al. 2018). Furthermore, the activation of Th-17 cells is facilitated by a broad cellular response, including secretion of TNFα, IL-1β, and IL-6 (Ouyang, Kolls et al. 2008). An increase in the levels of IL-2 and IL-17 signals an intensified activity of the immune system. Moreover, IL-1β and IL-6, both key proinflammatory cytokines, are fundamental in enhancing defense against pathogens. IL-1β is essential in activating inflammation, mainly by promoting Th-17 cells to generate IL-1, whilst also suppressing the production of IL-10 (Dinarello 2019, Mills 2023). The

activation of IL-6 often correlates with the involvement of the NF-κB and PI3K signaling pathways (Luo and Zheng 2016). A trend toward an increase in IL-1β levels in the LDP group was noted, which was then followed by a reduction in the AHCC+LDP group compared to the control. IL-6 concentration was decreased across all groups, with a significant decrease in the AHCC group.

IL-4 and IL-10 are pivotal cytokines in modulating inflammation, yet their levels remained unchanged or decreased across all experimental groups. IL-4 and its receptor are known to initiate signaling cascades that include STAT 6 activation, which in turn promotes the transcription of GATA3. This process fosters the development of regulatory T cells and inhibits Th1 cells by suppressing the production of INFγ. On the other hand, IL-10 serves an anti-inflammatory role by reducing the production of key cytokines such as IL-1, IL-6, IL-12, and TNF-α, and therefore inhibiting the presentation of MHC class II molecules, thereby diminishing the activation of T cells (Chatterjee, Chiasson et al. 2014).

The integrity of the blood-milk barrier (BMB) is crucial for preventing the transfer of specific components from blood to milk. A compromised BMB leads to alterations in milk composition and an increase in the presence of blood proteins like plasma albumin, lactate dehydrogenase (LDH), or IgG within the milk (Wellnitz and Bruckmaier 2021). There is a growing body of evidence suggesting that inflammation in lactating mammary glands triggers signaling pathways that affect the expression of tight junction proteins such as Occludin, Claudin-1, and Claudin-3, thereby impairing the BMB (Guo, Liu et al. 2019).

Our findings have shown a significant increase in the expression and protein level of Occludin in the AHCC and AHCC+LDP groups, with no change observed in the LDP group. Although Claudin-1 levels were higher across all groups, these differences were not statistically

significant. Western blot analysis indicated a slight increase in Claudin-1 levels in the AHCC-treated group, but a decrease in the AHCC+LDP-treated group. Conversely, Claudin-3 expression levels were elevated in the AHCC+LDP group. This suggests that inflammation stemming from an imbalanced gut microbiome due to LDP does not compromise the integrity of the BMB, and that AHCC supplementation may enhance the BMB's strength. In the next phase of the study, the impacts of antibiotic exposure during pregnancy and inflammation from gut dysbiosis on gene expression within lactating mammary glands were measured. *Pten* expression was significantly elevated in the AHCC and AHCC+LDP groups, suggesting the potential of AHCC in enhancing cellular immunity. *Pten* is essential for regulating cell proliferation, differentiation, and apoptosis through the PI3K/AKT signaling pathway (Carnero, Blanco-Aparicio et al. 2008). *Pten* regulates key components within the PI3K/AKT pathway, and its deficiency is associated with an aggressive form of breast cancer (Ortega-Molina and Serrano 2013).

Mapk1, another critical gene for cell proliferation and differentiation, showed a decrease in expression following antibiotic exposure in pregnant dams across all groups, though these findings were not statistically significant (Yager and Davidson 2006, Ahn, Schatzkin et al. 2007, Sun, Casbas-Hernandez et al. 2012, Che, Liu et al. 2014, Bowers, Brenner et al. 2015). Dysbiosis in the gut microbiome is associated with systemic inflammation, leading to an elevated level of estrogen within mammary tissue. The increase in estrogen potentially activates the *Igf1r* signaling pathway predominantly through ERα, increasing the risk of breast cancer (Kahlert, Nuedling et al. 2000). *Esr2* and *Egfr* maintained consistent expression patterns across all groups. *Esr2* regulates the activity of estrogen, which, by binding to its receptor, activates the proliferation of mammary cells. *Esr2* modulates the action of estrogen to control cell

proliferation (Colitti and Pulina 2010). *Egfr* plays a crucial role in mammary gland development by recruiting immune cells necessary for gland formation, with no significant observed changes in the LDP and AHCC groups compared to the control (Aupperlee, Zhao et al. 2014).

In our recent publication, the effects of early life antibiotic exposure on the gut microbiome of offspring have shown that LDP leads to a reduction in specific gut microbiome populations, notably within the *Firmicutes Anaerotruncus*, *Firmicutes Lactobacillie*, and *Firmicutes Roseburia*. These bacterial species play a pivotal role in both gut and immune system development (Domingues, Cabral et al. 2023).

Subsequent to these findings, the levels of IL-6, IL-10, and IgG in the blood plasma of offspring were analyzed. Increased levels of IL-10 in both AHCC and AHCC+LDP groups were noted, whereas IgG and IL-6 levels were lower in the AHCC group, with IL-6 levels remaining consistent in the LDP and AHCC+LDP groups. Notably, IL-10 is recognized for its capacity to mitigate inflammation and suppress IL-6 production.

Further investigation into inflammatory cytokines, including INFγ, IL-1β, IL-2, IL-15, IL-17A/F, and IL-22, present in the mammary glands of offspring revealed that most cytokines were significantly downregulated in the LDP group compared to the control. Conversely, the AHCC and AHCC+LDP groups exhibited similar levels of cytokine expression. Prior research indicated that antibiotic-treated mice typically show a general decline in T cells, particularly T helper and T memory cells, with a reduction in cytotoxic T cells following antibiotic administration (Ubeda and Pamer 2012). Additionally, a decrease in pro-inflammatory cytokines is commonly reported, with microbiome depletion linked to reduced production of the IL-1 family, IL-17, INFγ, and TNFα (Hill, Hoffmann et al. 2010). This study observed a similar trend in cytokine reduction, suggesting that the gut microbiome is instrumental in educating and modulating the immune

system in offspring. Another contributing factor to this trend is the gut microbiome's role in maintaining the balance between Th1 and Th2 cells, with evidence indicating a shift towards Th2 dominance in mice treated with antibiotics (Kennedy, King et al. 2018).

In the next step, genes known for their role in breast cancer pathogenesis, including *Apc*, *Magi1*, *Tp53*, *Pten*, *Egfr*, *Igf-1r*, *Fgf1*, *Esr2*, *Fgfr2*, *Dvl3*, *Crebbp*, *Fzd7*, *Ptk2*, and *c-Jun* were evaluated. *Apc*, and *Magi1* showed an increase in AHCC group only. APC is involved in the degradation of β-catenin in the Wnt signaling pathway destruction complex (Anastas and Moon 2013). MAGI1 enhances epithelial-like morphology and stabilizes key adherence junction proteins. It also acts as an inhibitor of the Wnt signaling pathway, suppressing migration and invasion of cancer cells (Zaric, Joseph et al. 2012). *Tp53* and *Pten* showed consistent expression levels in the LDP group but an increase in the AHCC and AHCC+LDP groups compared to the control. TP53, a well-known tumor suppressor, halts the cell cycle to prevent uncontrolled tumor growth and is mutated in approximately 50% of all cancers (Kastenhuber and Lowe 2017). PTEN, through its lipid phosphatase activity, suppresses the PI3K/Akt signaling pathway, influencing the ECM. Loss of PTEN is linked with changes in the ECM, transforming fibroblast cells into a myofibroblast-like state (CAFs), which produces tumor-associated collagen, facilitating breast cancer metastasis (Jones, Hammer et al. 2019).

In comparison to the control group, *Egfr*, *Igf-1r*, and *c-Jun* showed overexpression in the LDP group, while no significant difference was noted in the AHCC and AHCC+LDP groups. EGFR, part of the ErbB family, initiates a cascade leading to activation pathways like ERK/MAPK, stimulating proliferation, cell growth, and differentiation (Olayioye 2001). HER2, a crucial protein involved in breast cancer, activates through dimerization with other family members, especially EGFR (Hsu and Hung 2016). IGF-1R activation also triggers the ERK/MAPK

pathway, enhancing cell survival and proliferation, and is essential for terminal end bud (TEB) formation and duct elongation (Farabaugh, Boone et al. 2015). Overexpression of IGF-1R is linked to estrogen receptor-positive breast cancer and is also present in triple-negative breast cancer, where high levels correlate with poor prognosis (Rigiracciolo, Nohata et al. 2020). c-JUN is downstream of the MAPK signaling pathway and is involved in many cellular processes, including proliferation, apoptosis, and cell survival (Meng and Xia 2011). Moreover, studies have shown that c-Jun induces breast cancer invasion and metastasis (Jiao, Katiyar et al. 2010). *Fgf1, Fgfr2, Fzd7, Dvl3, Crebbp,* and *Ptk2* exhibited significant upregulation in the LDP group, while their expression levels remained consistent across the AHCC and AHCC+LDP groups.

FGF1 and FGFR2, serving as crucial ligand and receptor pairs, are involved in cellular processes such as proliferation, differentiation, and development by activating the ERK/MAPK and PI3K/AKT signaling pathways (Xie, Su et al. 2020). FZD7 and DVL3, components of the Wnt signaling pathway located at the cell membrane and cytoplasm respectively, play roles in cell proliferation, differentiation, and migration (Lee, Gao et al. 2008, Peghaire, Bats et al. 2016). The binding of Wnt ligands to the FZD7 receptor allows DVL3 and other proteins to prevent the degradation of β-catenin, thus activating the canonical Wnt signaling pathway (Holzem, Boutros et al. 2024). CREBBP, a transcriptional co-activator with histone acetyltransferase activity, modifies chromatin structure to enhance gene expression and is implicated in activating the TGFβ signaling pathway by overexpressing Smad4 (Das, Roy et al. 2014). ESR1 and ESR2 play complementary roles in mammary gland development; ESR1 promotes cell proliferation, while ESR2 inhibits it, likely by suppressing Cyclin A and D1 expression (Paruthiyil, Parmar et al. 2004). PTK2, or focal adhesion kinase (FAK), is critical for cell migration, adhesion, proliferation, and apoptosis (Wu, Wang et al. 2022). Its overexpression has been linked to higher

metastasis levels in breast cancer and poorer overall survival (Zhang, Li et al. 2022). PTK2 binds to P53 in the nucleus, initiating its degradation (Frame, Patel et al. 2010). Additionally, PTK2's ability to bind to EGFR and IGF-1R promotes cell migration and metastasis in breast cancer (Long, Yi et al. 2010, Taliaferro-Smith, Oberlick et al. 2015). Interestingly, PTK2 acts upstream of ERK1/2, and its activation results in overexpression of ERK1/2 (Wang, Chen et al. 2012).

The observed increase in gene expression of *Fgf1*, *Fgfr2*, *Igf1r*, *Egfr*, *c-Jun*, and *Ptk2* suggests the activation of the ERK/MAPK and PI3K/Akt signaling pathways, essential for processes such as cell proliferation and survival (He, Sun et al. 2021, Bahar, Kim et al. 2023). Despite the inability of the western blot assay to detect Akt1, pAkt1, Foxo, and pFoxo, and while total ERK1/2 levels remained unchanged, an increase in pERK1/2 was noted. Thus, these data suggest that activation of the ERK/MAPK signaling pathway by early-life antibiotic exposure, linked to a higher proliferation rate via cyclin D1 activation, facilitates the G1 to S phase transition. Moreover, ERK1/2 activation leads to an increase in *Egr*, *Fos*, *Jun*, and *Myc* expression, enhancing the proliferation rate and contributing to breast cancer development (Lavoie, Gagnon et al. 2020) (Fig. 4.5).

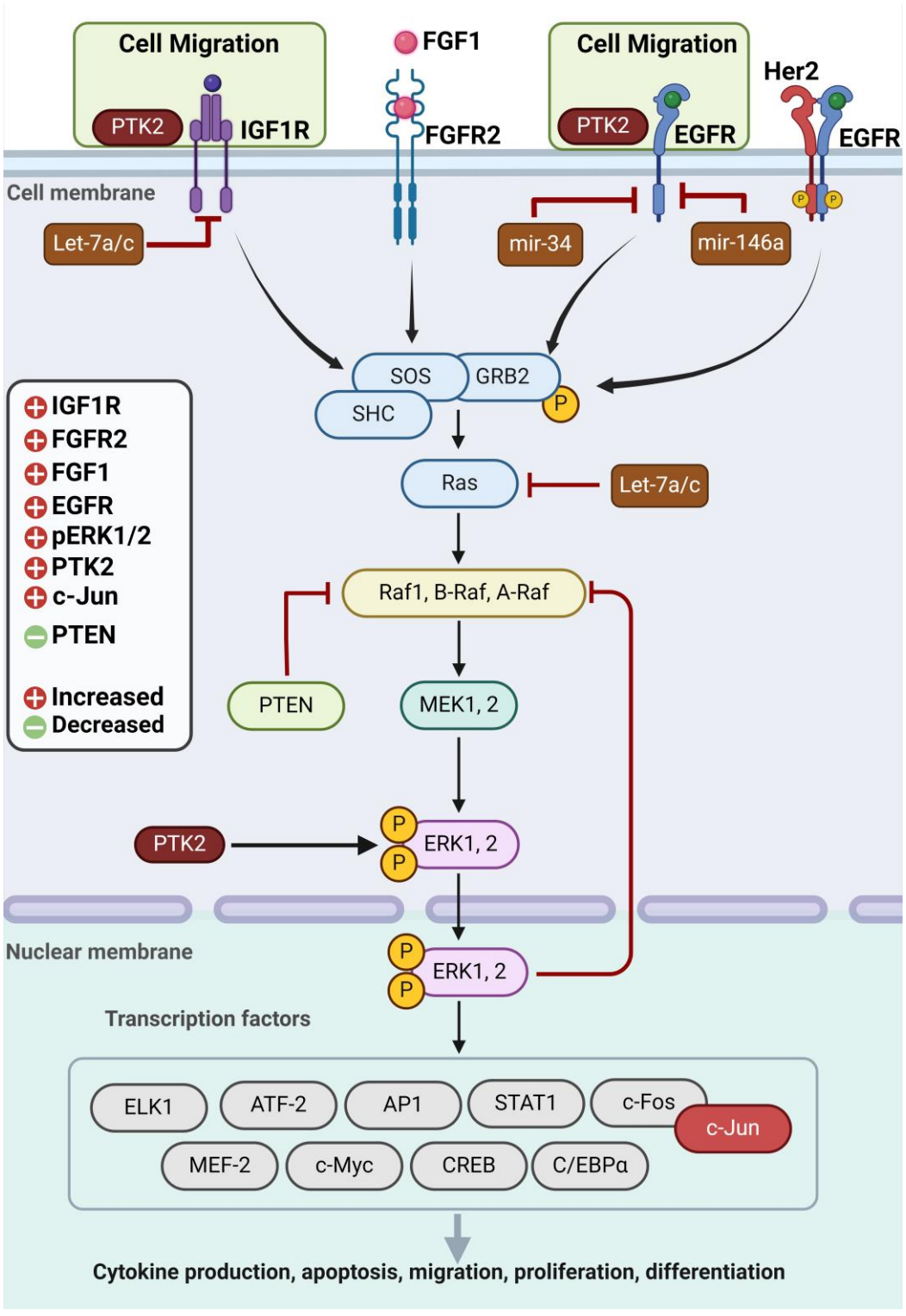

Figure 4.5. The Potential Impact of Treatment on ERK/MAPK signaling pathway. Treatment with LDP dysregulated the expression levels of Egfr, Igf1r, Fgf1, Fgfr2, and Ptk2. Dysregulation of these genes leads to the activation of the Ras protein, which subsequently activates the ERK1/2 protein. The activation of ERK1/2 propagates to the nucleus, enhancing the expression of genes involved in cytokine production, cell

proliferation, and migration. Moreover, dysregulation of microRNAs, such as Let-7a/c, which target Egfr, Igf1r, and Ras, further amplifies the activation of the ERK/MAPK signaling pathway.

To further elucidate the role of microRNAs, in this study, we examined the expression of microRNAs already identified as regulators of the ERK/MAPK signaling pathway and involved in mammary gland development. It was discovered that Let-7a/c, miR-34b, and miR-146a were downregulated in the LDP group compared to others. Let-7a/c and miR-34b are recognized for their tumor-suppressing role and are often found to be downregulated in various diseases, including cancer (Stahlhut and Slack 2015). Specifically, Let-7a/c can directly target Ras protein, thereby regulating the ERK/MAPK signaling pathway. Additionally, Let-7a/c has the capability to regulate IGF-1R and suppress the ERK/MAPK signaling pathway (Johnson, Grosshans et al. 2005, Li, Pan et al. 2018). The Let-7 family has also been demonstrated to play a vital role in the development of mammary glands at various stages, including puberty, early gestation, and involution. Consequently, dysregulation of Let-7 can lead to abnormalities in mammary gland development (Avril-Sassen, Goldstein et al. 2009). miR-146a, another tumor suppressor, can regulate TLR4 and mitigate inflammation, as well as control the ERK/MAPK signaling pathway by inhibiting EGFR (Xu, Wang et al. 2012). Moreover, this microRNA is involved in the specification and maintenance of the alveolar lineage in mammary epithelial cells (Elsarraj, Stecklein et al. 2012).

In the next step, the enduring and long-term effects of early life exposure to LDP on DNA methylation and the potential counteractive effects of AHCC treatment on DNA methylation profiles in the mammary glands of offspring were assessed. The analysis revealed that AHCC-treated samples predominantly clustered with control samples, while the LDP-treated groups showed the most significant changes in DNA methylation. Specifically, the LDP group displayed differences in 1955 probes, whereas comparisons between AHCC vs control and AHCC+LDP vs

LDP did not show substantial differences. A regional analysis indicated that the LDP groups had 349 differentially methylated regions (DMRs) involved in various signaling pathways such as cAMP, PI3K-Akt, and TGFβ signaling. Nonetheless, due to the diverse array of biological processes represented by these genes, the enriched pathways were not statistically meaningful. Generally, DNA methylation occurring at the promoter, 1 to 5 kb upstream of transcription start sites (TSS), or within the 5' UTR is typically linked to transcriptional inhibition and consequently gene suppression (Jones 2012, Wang, Xiong et al. 2022). Additionally, hypermethylation observed in gene bodies, including exonic and intronic regions, is typically linked to enhanced transcriptional elongation or gene overexpression. Analysis of DMRs obtained from the comparison of control vs LDP showed hypomethylation within 1 to 5 kb of *Nfatc1* and in the exonic regions of *Itgb4*. *Nfatc1* plays a critical role in the colony formation of a subset of mammary epithelial cells. Furthermore, this gene is implicated in the lineage of stem cells and contributes to the migration and invasion of breast cancer cells (McNeil, Han et al. 2021). *Itgb4*, a subunit of the integrin protein, is essential for cell adhesion and signal transduction that regulates cell growth. Increased expression of *Itgb4* has been noted in various cancers, especially in breast cancer, where it is linked with enhanced proliferation and invasion (Sung, Kang et al. 2020). Promoter regions of genes such as *Hyal1*, *Sox10*, *Igfbp7*, *Pax5*, and *Mia2* showed hypermethylation levels, suggesting potential suppression of these genes. HYAL1, a hyaluronidase enzyme, plays a pivotal role in degrading hyaluronan, a key component of the ECM. This activity is essential for the development and remodeling of ECM, particularly for mammary glands development (Witschen, Elfstrum et al. 2023). In the realm of cancer, *Hyal1* is often overexpressed in a multitude of cancers and can function either as an oncogene or a tumor suppressor depending on chromosomal conditions and mutations (Lokeshwar and Selzer 2009).

PAX5 serves as a regulator of E-cadherin, stabilizing mammary epithelial cells, and capable of reversing the epithelial-mesenchymal transition (EMT). While PAX5 can both promote and inhibit cancer, it is particularly noted for its potential to inhibit the proliferation and invasion of breast cancer cells (Benzina, Beauregard et al. 2017, Chen, Chen et al. 2023). SOX10 is integral in maintaining the lineage of ectoderm-originating cells, including neural crest cells, melanocytes, and potentially mammary gland cells (Mollaaghababa and Pavan 2003, Macias and Hinck 2012). The loss of *Sox10* has been observed to reduce cell proliferation; however, it can lead to activation of the TGFβ signaling pathway and the overexpression of *Egfr* and *Pdgfrβ* (Zhang and Herlyn 2014). This contributes to increased drug tolerance and induces resistance to targeted therapies such as BRAF or MEK inhibitors (Capparelli, Purwin et al. 2022). The dual role of SOX10 highlights its complex influence on cellular processes and cancer therapy resistance. Overexpression of *Igfbp7* is associated with apoptosis and tumor suppression. Research indicates that IGFBP7 inhibits activation of the IGF-1R receptor, thereby suppressing cell survival and proliferation in breast cancer (Evdokimova, Tognon et al. 2012). MIA2 is recognized as a tumor suppressor gene in hepatocellular carcinoma. It has been proposed that activation of the TGFβ signaling may be associated with inhibition of *Mia2* expression (Hellerbrand, Amann et al. 2008, Kurihara, Kirita et al. 2013).

Additionally, hypermethylation in gene bodies of *Tiam1*, *Smad6*, *Bmp7*, *Ksr2*, and *Itga9* has been observed, which could be associated with the overexpression of these genes (Yang, Han et al. 2014). TIAM1 is linked to carcinogenesis and promotes higher invasive traits in breast cancer (Li, Liu et al. 2016). SMAD6 plays a critical role in inhibiting the activation of the BMP signaling pathway and is involved in regulating the cytoskeleton of cells, enhancing the invasive properties of breast cancer (De Boeck, Cui et al. 2016). In contrast, BMP7 facilitates the

activation of BMP signaling through its interaction with specific receptors, leading to the activation of SMAD1/5/8 (Patel and Dressler 2005). BMP7's function in cancer is context-dependent; it can act either as an oncogene to promote invasion and metastasis or as a tumor suppressor that inhibits the proliferation of breast cancer cells (Buijs, Van Der Horst et al. 2012, Sakai, Furihata et al. 2012). KSR2 serves as a scaffold that supports and enhances the ERK signaling pathway. The expression of KSR2 also boosts the signal transduction from Ras, thereby increasing the proliferation rate of cells by modulating the RAS/ERK signaling pathway (Fernandez, Henry et al. 2012). ITGA9, a component of the ECM, is critical for cell-cell and cell-matrix adhesion, promoting both proliferation and the migration of cancer cells (Mostovich, Prudnikova et al. 2011).

In comparison between LDP and AHCC+LDP, 116 DMRs were identified. Among these, only a few were found in gene coding loci. Notably, the intronic region of four DMRs, Smad3 and Foxp1 were hypomethylated, while Lrig1 and Bcl6 were hypermethylated. SMAD3 plays a role in the TGF-β signaling pathway, influencing angiogenesis and bone metastasis in breast cancer (Petersen, Pardali et al. 2010). FOXP1 is regulated by the PI3K/Akt signaling pathway, and its suppression is linked to worse overall survival in breast cancer. FOXP1 also modulates immune factors such as cytokines and transcription factors, creating a suppressive tumor microenvironment. Conversely, FOXP1 expression has also been associated with worse overall survival in hormone receptor-positive breast cancer (De Silva, Garaud et al. 2019).

LRIG1 acts as a tumor suppressor by regulating the ErbB and EGFR signaling pathways. Loss of Lrig1 leads to increased activity in the ErbB1-3 and ERK1/2 pathways (Wang, Poulin et al. 2013).

Bcl6 is overexpressed by a factor of 2 in nearly all breast cancer cell lines. It influences the cell cycle, promoting cell proliferation. Additionally, BCL6 directly regulates STAT3, amplifying tumorigenesis (Walker, Liu et al. 2015).

In this study, 28 DMRs were found to be common between the control vs LDP and LDP vs AHCC+LDP comparisons. Notably, the promoter region of *Tiam1* and *Pax5*, along with intronic regions of *Itga9*, exhibited hypermethylation levels in the AHCC+LPD group compared to the LDP group. In these regions, the addition of AHCC to LDP did not significantly alter the DNA methylation profile, suggesting that AHCC prebiotic exposure does not attenuate or amplify the effects of LDP on DNA methylation. This is particularly interesting, as the administration of AHCC alone did not trigger significant hyper- or hypomethylation in treated animals. One possibility is that LDP treatment triggers instability in the epigenome, and AHCC may not have a discernible impact in the presence of LDP.

AHCC may not exert its influence at the DNA methylation level, which could explain the lack of observed effects on DNA methylation from AHCC treatment. However, AHCC's mechanism of action may relate to other epigenetic pathways, such as the modulation of microRNAs. This study has shown that AHCC can impact microRNAs Let-7a/c, miR-146a, and miR-34b.

This paper represents a pioneering effort to investigate the lasting impact of early-life antibiotic-induced disturbance on immune system dysfunction, delving into underlying mechanisms that include signaling pathways and epigenetic modifications involved in mammary gland development. However, the study is not without its limitations. It focuses on the effects of early-life antibiotic exposure on mammary glands at 8 weeks of age. To fully understand the treatment's impact on cancer development, subsequent research phases should examine pregnancy and post-weaning in the first generation, or directly initiate cancer induction. Another

crucial step involves conducting experiments focused on the offspring's immune system. In summary, the findings of this study suggest that persistent immune dysregulation due to early-life gut microbiome dysbiosis may be associated with long-lasting alterations in immune function and mammary gland development. Interestingly, maternal intake of prebiotics could offer partial protection against alterations in the gut microbiome and subsequent changes in gene expression within mammary glands. These insights add to the accumulating evidence on the long-term effects of early-life gut microbiome disturbances and underscore the potential for preventive measures to address health complications later in life.

Author Contributions: H.Y.S. contributed to the methodology, animal feeding, and sample collection, performed all experiments, and wrote the manuscript; R.S. contributed to the methodology, animal feeding, sample collection, and performing experiments; D.B.K. contributed to performing experiments, the M.J.H. contributed to the sample collection, DNA, and protein extraction and normalization; M.F.B. contributed to performing experiments, N.A. contributed to the animal feeding, and sample collection; Z.H. contributed to design and reading the manuscript; F.C.F.L. contributed to bioinformatics and epigenetic analysis, C.M. designed and supervised the work. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded - Exploration NFRFE-(2019-01497) grant to Z.H and C.M and an admission scholarship by the Faculty of Graduate and Postgraduate Studies of the University of Ottawa to H.Y.S.

Institutional Review Board Statement: The protocol (HSe-3866) was approved by the Animal Care Committee of the University of Ottawa on 3-14-2023.

Informed Consent Statement: Not applicable.

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

Acknowledgments: We would like to acknowledge the support provided by the University of Ottawa Library.

Conflicts of Interest: The authors declare no conflict of interest.

4.6 Supplementary Data

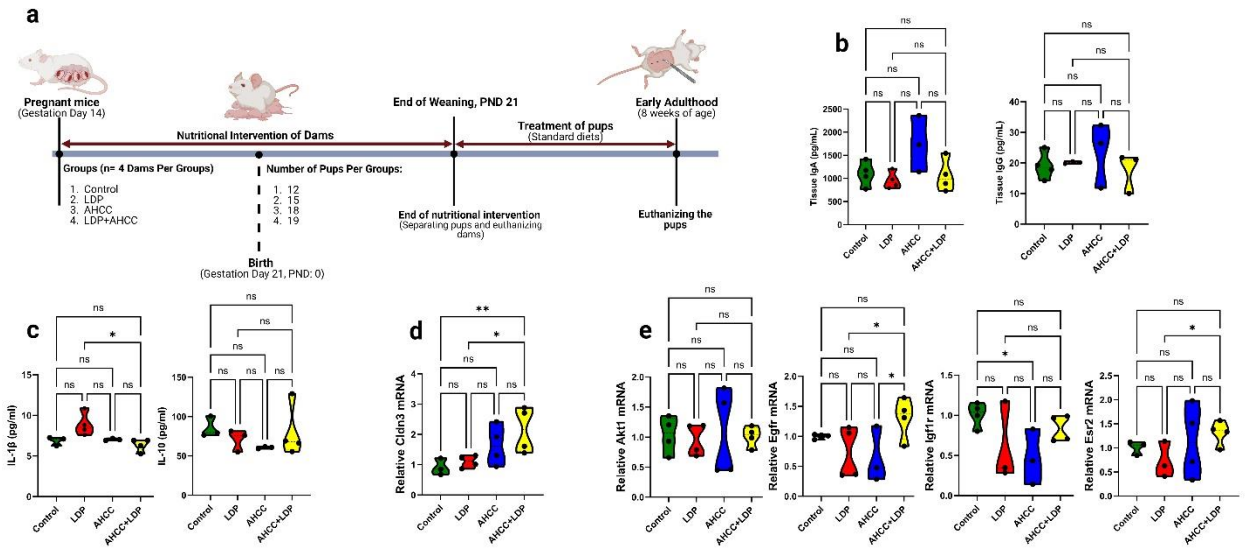

Fig. 4.1: (a) Study Design: Pregnant mice were administered treatments via their drinking water from the last week of gestation (gestational day 14) until the weaning of the pups (postnatal day 21). Treatments included penicillin V (31 mg/kg BW/day), AHCC (2 g/kg BW/day), and a combination of both. The control group received regular drinking water. Upon weaning, treatments were discontinued, male and female pups were separated, and dams were sacrificed. Pups received a standard diet until eight weeks of age, at which point they were sacrificed to assess the long-term effects of the treatments on gut-level immune system responses. This illustration was created using BioRender.com. **(b)** IgA and IgG Concentrations: Measured in the lactating mammary tissue of dams at weaning, with a sample size exceeding three per group (n > 3/group). **(c)** Inflammatory Cytokines: Concentrations of IL-1β and IL-10 were quantified. **(d)** Gene Expression in Mammary Glands (Cldn3), assessed by RT-qPCR post-treatment. **(e)** Gene Expression in Mammary Glands (Akt1, Egfr, Igf1R, Esr2), evaluated by RT-qPCR following treatment. Data normality was verified using the Shapiro-Wilk test. Group comparisons were made using two-way ANOVA and Fisher's post-hoc tests. All values are presented as mean ± SEM. Significance levels are denoted as *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

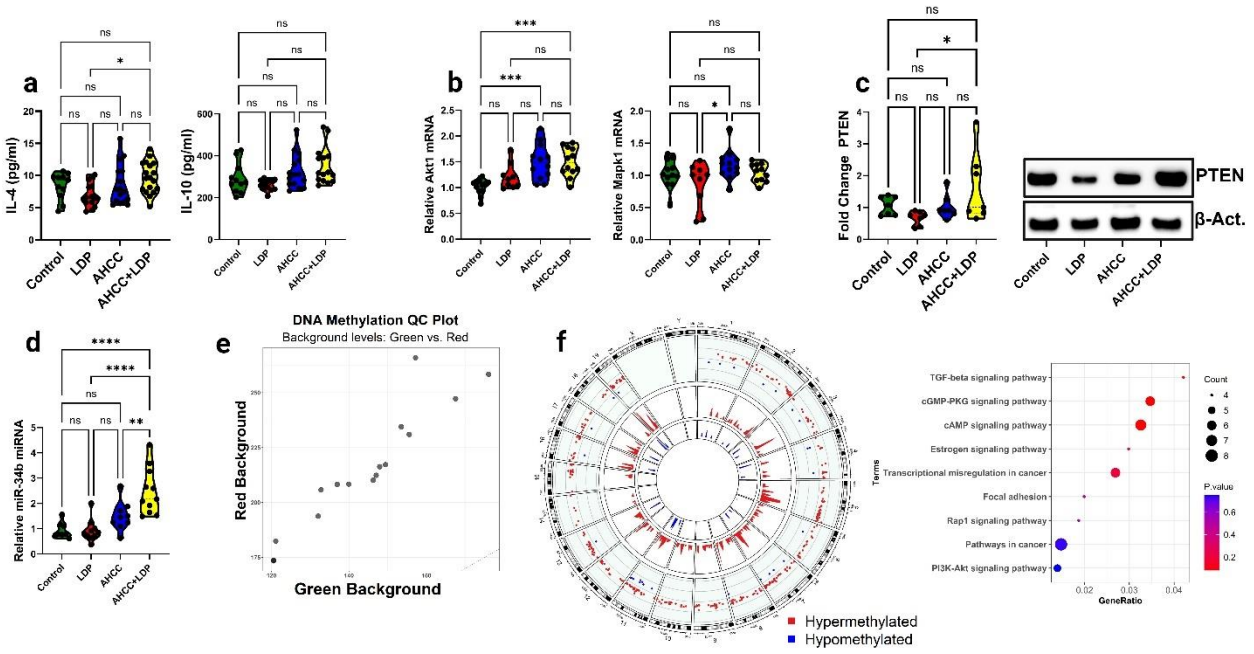

Fig. 4.2: (a) Concentrations of inflammatory cytokines, including IL-4 and IL-10, in the mammary glands of offspring. **(b)** Changes in the expression levels of Akt1 and Mapk1 in the mammary glands of offspring, as measured by RT-qPCR. **(c)** Protein levels of PTEN were assessed using Western blot assays ($n \geq 8$ per group). **(d)** Alterations in the expression of microRNA miR-34b ($n > 9$ per group). **(e)** The QC plot demonstrates the normal distribution of the samples. **(f)** Visualization of DNA methylation patterns across the genome. The outer ring represents chromosomes 1 through 22, along with the X and Y chromosomes. Each dot on the plot denotes a unique genomic location exhibiting altered methylation levels; and KEGG enrichment analysis for differentially methylated regions (DMRs), derived from comparisons between control and treatment groups (LDP).

Chapter 5. Discussion

5.1 Discussion

The gut-organ axis is the subject of intense ongoing research. The gut and the mammary glands are part of mucosal-associated lymphoid tissue where there is circulation of immune cells, cytokines, and chemokines (Selvamani, Dailin et al. 2021). Inflammation occurring in the intestine can lead to disruption in immune signaling and homeostasis at distant sites. Disruption of the gut microbiome during critical periods of development in females, prenatally or at puberty, can lead to a pre-malignancy signature with long-lasting effects into adulthood, predisposing to breast cancer later in life (Herrera-Covarrubias, Coria-Avila et al. 2017).

The interplay between gut microbiota and overall health, particularly in relation to immune responses and disease development, is attracting significant interest, highlighted by recent research into the effects of dietary interventions through prebiotics and the impact of gut dysbiosis. Prebiotics have been shown to play a crucial role in modulating the gut microbiota, thereby exerting systemic anti-inflammatory effects and acting as chemopreventive agents against various types of cancers, including mammary carcinoma (de Moreno de LeBlanc, Matar et al. 2006, Sunkata, Herring et al. 2014). These dietary components help restore the balance of the gut microbiota, which in turn can alleviate prolonged inflammatory immune responses and potentially reduce the risk of precancerous lesions and other immune disorders (Fernández, Reina-Pérez et al. 2018). This study aimed to investigate the role of induced dysbiosis by LPS at puberty or by antibiotics during pregnancy and its effects on mammary gland development. The intricate connections between gut microbiota dysbiosis and its far-reaching impacts on health,

from modulating epigenetic dynamics and cytokine expression to affecting gene expression and signaling pathways involved in inflammation and tumor development, were also studied.

The widespread use of antibiotics, especially during critical periods such as pregnancy, has been linked to adverse long-term health outcomes, including an increased risk for allergies and autoimmune conditions (Tlaskalová-Hogenová, Štěpánková et al. 2011). This connection is attributed to antibiotics' role in causing gut microbiota dysbiosis and subsequently lasting alterations to the immune system. Interestingly, the entero-mammary pathway highlights how an imbalanced gut microbiota can influence not only lactating mammary glands but also the neonatal gut microbiome, underscoring the significant impact of maternal gut health on offspring well-being (Rodríguez 2014). The emerging evidence supporting the use of natural compounds to mitigate these disruptions further emphasizes the potential of dietary and microbiota interventions in maintaining health and preventing disease (Cowan and Richardson 2019). Collectively, this study demonstrates the mechanisms underlying inflammatory signals acquired during puberty or pregnancy in disrupting immune and epigenetic homeostasis in the mammary gland and the increased risk of breast cancer later in life.

Chapter 2. Long-term Effects of LPS Exposure on DNA Methylation and Potential Impact of AHCC Treatment.

Inflammation triggered by LPS impairs the function of DNA methyltransferase, resulting in atypical DNA methylation across the genome resembling patterns observed in tumors (Maiuri and O'Hagan 2016). Furthermore, this inflammation promotes Th17 cell differentiation and cytokine production, leading to increased chronic inflammation (McAleer, Liu et al. 2010).

In the first step of the research, the lasting influence of LPS exposure on DNA methylation and the prospective corrective effect of AHCC on methylation patterns in mammary glands have been investigated. Through comparative analysis, DNA methylation between control and LPS-exposed groups (noting 85.8% hypomethylation and 14.1% hypermethylation) and between LPS-exposed and LPS+AHCC treated groups (revealing 29.9% hypomethylation and 70.1% hypermethylation) was assessed. The study identified 252 genes that changed from hypomethylation in the LPS group to hypermethylation in the AHCC+LPS group compared to the LPS group alone, indicating the potential of AHCC to counteract the detrimental effect of LPS. Among these, 14 genes including *Pdgfa*, *Jag2*, *Hras*, *Ksr1*, *Il2rb*, *Il17b*, and *Il17rb*, among others, were highlighted for their roles in crucial signaling pathways such as cytokine receptor interactions, JAK-STAT, and IL-17 signaling pathways, all linked to cancer biology and mammary gland development. For instance, *Pdgfa*, a key factor in cell growth and development, has been shown to promote metastasis in breast cancer (Ren, Sun et al. 2019). JAG2 is key in pathways critical for cell survival and is linked to colorectal and breast cancer via the Notch signaling pathway (Houde, Li et al. 2004). HRAS plays a pivotal role in regulating various signaling pathways including Ras and PI3K/Akt, influencing cell growth, differentiation, and survival (Wu, Liu et al. 2016). KRAS, which serves as a negative regulator of the Ras signaling pathway, has been linked to improved survival in breast cancer by stabilizing BRCA1 (Lavoie, Sahmi et al. 2018). IL2RB is notably overexpressed in breast cancer, affecting cytokine interactions and linked with poor overall survival, underscoring its significance in immune response and cancer progression (Tan, Zuo et al. 2022). IL17b and its receptor IL17RB are essential in recruiting immune cells and promoting cell growth, invasion, and survival through the ERK1/2 signaling pathway in breast cancer (Alinejad, Dolati et al. 2017).

This investigation sheds light on the intricate interplay between DNA methylation and predisposition to cancer development. This effect can be mitigated by a dietary approach in the form of a prebiotic intake, *Lentinula edodes* cultured extract (AHCC), also pointing out the therapeutic potential of AHCC in adjusting DNA methylation landscapes.

Chapter 2. Long-term Effects of LPS Exposure on Pro-inflammatory Cytokines and microRNAs on Mammary Glands and Immunomodulatory Effect of AHCC Treatment.

Following LPS exposure, changes were noted in DNA methylation patterns, exhibiting both hyper- and hypo-methylation levels within genes critical to key signaling pathways, including cytokine-cytokine receptor interactions, Jak/STAT signaling, IL-17, and microRNA regulation. This suggests that inflammation, a consequence of LPS exposure at puberty, plays a significant role in modifying gene expression and affecting cellular functions.

Previous research indicated that LPS exposure during puberty alters the gut microbiome, elevating levels of Bacteroides and Parabacteroides, which correlates with colitis-induced mucosal damage and a surge in proinflammatory cytokines like IL-17, IL-21, and INFγ. LPS's influence extends to mammary glands, promoting abnormal growth and activating proinflammatory cytokines via the NF-κB pathway, and affecting microRNA expression (Taganov, Boldin et al. 2007, Alinejad, Dolati et al. 2017). The study explored LPS's effects on mammary gland cytokines and microRNAs, observing a distinct expression pattern for IL-1β and IL-6, with IL-1β significantly elevated in the LPS group but reduced in the AHCC and AHCC+LPS groups, suggesting AHCC's regulatory impact (Edelman, Jiang et al. 2007, Dinarello 2018). Elevated IL-1β is linked to inflammation and breast cancer development, while IL-6 plays a role in immune regulation and breast cancer progression (Palkowetz, Royer et al. 1994). Interestingly, IL-6 levels were unexpectedly low across groups, hinting at a potentially

dynamic IL-6 expression influenced by the timing of sample collection (Kishimoto 2010). The study also noted LPS-induced proinflammatory cytokine production, with higher IL-17A and IL-17F levels in LPS-exposed mice, whereas AHCC intake seemed to mitigate these effects. IL-17's involvement in immune cell recruitment and promotion of proinflammatory cytokines highlights its potential to predispose mammary glands to inflammatory conditions that may favor cancer development (Fabre, Giustiniani et al. 2018).

microRNA dysregulation was also investigated, focusing on tumor suppressor miRNAs such as Let-7a/c and miR-219, which were downregulated following LPS exposure but showed differential responses to AHCC treatment. The study found miR-34a and miR-130a upregulated across all groups, suggesting a protective mechanism against LPS-induced damage (Li, Yuan et al. 2013, Zhang, Jiang et al. 2017). AHCC appeared to specifically upregulate miR-146a, indicating its anti-inflammatory properties, and influence miR-200c expression, involved in EMT regulation and metastasis suppression (Rusca and Monticelli 2011, Mutlu, Raza et al. 2016). Additionally, miR-211 was notably upregulated in AHCC-treated groups, indicating AHCC's potential upregulatory effect on mammary gland biology in the presence of LPS (Boyle, Woods et al. 2011).

This step of research elucidates the complex interplay between LPS exposure, DNA methylation, cytokine expression, and microRNA regulation in mammary glands, highlighting AHCC's potential as a protective agent.

Chapter 2. Long-Term Effects of LPS Exposure and Potential Impact of AHCC Treatment on Mammary Glands Structure

After examining changes in DNA methylation, cytokine levels, and microRNA dysregulation, this study also investigated the impact of LPS exposure at puberty on the morphological structure of mammary glands later in life. This step utilized image processing packages such as scikit-image and NumPy in Python, as well as ImageJ software. It was found that puberty exposure to LPS or treatment with AHCC did not significantly alter the structure of mammary glands. A noteworthy finding was a reduction in quadruple intersections observed in the AHCC group compared to the control and AHCC+LPS groups, suggesting AHCC's capability to lower mammary gland density and potentially lessen the risk of developing breast cancer later in life (Boyd, Guo et al. 2007).

Our research collectively indicates that consumption of AHCC supplements during puberty may prevent enduring immune dysregulation in mammary glands caused by LPS through modulation of epigenetic mechanisms like microRNAs and DNA methylation within this critical developmental window. This suggests a protective role of AHCC against immune challenges affecting mammary gland health during puberty.

In obesity, a disrupted gut barrier can lead to endotoxemia or increased circulation of LPS from pathogenic gut bacteria, affecting distant organs including the mammary gland (Camilleri 2019). Therefore, the use of this preclinical experimental model may be somewhat translatable to clinical models and real-life situations.

Chapter 3. Measuring the Modulatory Effects of AHCC and LPS on Tumor Volume, Mass, and Cancer Stem Cell Abundance

In the next step of the project, we delve into the complex relationship between the inflammatory signature acquired during mammary gland development and the occurrence of breast cancer in

adulthood following a challenge with a cancerous cell line. To this end, the effect of pubertal LPS injection on breast cancer development was examined. LPS treatment notably increased tumor volume, aligning with existing studies that associate bacterial LPS with the activation of inflammatory pathways conducive to tumor development (Chen and Nuñez 2010). The observation that AHCC treatment did not significantly change tumor volume compared to the control group suggests that AHCC's impact may result from its immunomodulation effect rather than direct tumor suppression. The across-the-board increase in tumor weight, regardless of treatment group, points to potentially complex factors influencing tumor weight, including tumor density, necrosis, stromal content, and the infiltration of immune cells. Thus, further research is needed to fully decipher the relationships and dynamics among these factors.

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Cancer stem cells (CSCs), due to their role in therapy resistance, metastasis, and tumor recurrence, are a focal point of the study. An increase in CSC counts in the LPS and AHCC+LPS groups suggests LPS's potential to interact with pathways or proteins that enhance stemness, especially during pivotal stages of mammary gland development (Shibata and Hoque 2019). Other research supports the notion that LPS can induce stemness properties in cancer cells (Che, Yin et al. 2017). While the decreased CSC abundance in the AHCC group was not statistically

significant compared to the control, it nevertheless points to an area of interest regarding AHCC's influence on CSC populations, warranting further investigation.

Chapter 3. Evaluating AHCC's Potential in Modulating Cytokine Responses to LPS in Tumor and Mammary glands

The research focused on cytokine expression in tumor samples and mammary gland development, particularly in response to treatment with LPS and AHCC. AHCC's modulation of cytokine levels aligns with its known immunomodulatory properties, notably demonstrated by the reduced expression of IL-1β in AHCC-treated tumors and mammary glands, hinting at its anti-inflammatory potential (Daddaoua, Martínez-Plata et al. 2013, Tulotta and Ottewell 2018). The combination of AHCC with LPS counteracted LPS-induced inflammation in tumors, further corroborating its anti-inflammatory capabilities (Gao, Zhang et al. 2006). IL-6, associated with poor prognosis in breast cancer, did not show significant variation between control and LPS samples, though AHCC's presence indicated a potential therapeutic benefit by reducing IL-6 levels (Salgado, Junius et al. 2003).

IL-10's complex role in cancer, possessing both pro-tumorigenic and antitumorigenic properties, was examined, revealing reduced expression across all study groups, hinting at the inflammatory response triggered by LPS (Mocellin, Marincola et al. 2005). The study also delved into the pro-inflammatory cytokines IL-23 and IL-17, critical in the cytokine network and inflammatory responses. Interestingly, LPS treatment elevated IL-23 expression in mammary glands, yet this was not effectively modulated by AHCC (Rafa, Saoula et al. 2013). The study highlighted the role of IL-23 in the tumor context and its potential immunosuppressive effects, as other studies suggested a nuanced role of IL-23 in the tumor microenvironment (Wertheimer, Zwicky et al. 2024).

The research further explored the TGF-β family's involvement in breast cancer, showing stable TGF-β1 levels across groups and highlighting AHCC's capacity to stabilize the effects of LPS on TGF-β2 expression (Neel, Humbert et al. 2012, Tauriello, Sancho et al. 2022). TGF-β3's role in tumor development and the observed changes in its expression in response to LPS and AHCC treatments underscore the complexity of these interactions (Kudo-Saito, Shirako et al. 2009).

In summary, this part of the research investigated AHCC's potential as a modulatory agent against LPS-induced inflammatory responses, with implications for understanding cytokine behavior in cancer progression and treatment strategies. The findings contribute valuable insights into the cytokine-mediated mechanisms underlying breast cancer development and the therapeutic potential of AHCC in modulating these processes.

Chapter 3. Investigating miRNAs Regulatory Effects of AHCC on LPS-Induced microRNA Patterns in Tumors and Mammary glands:

microRNAs are involved in regulating cellular processes like differentiation, proliferation, and apoptosis, highlighting how an imbalance in miRNA expression can significantly affect tumor progression and metastasis (Wahid, Shehzad et al. 2010). Specific miRNAs associated with tumor progression or suppression (let-7a/c, miR-21, miR-92, miR-140, miR-155) and those linked to mammary gland development (miR-145, miR-184, miR-188, miR-205, miR-223) were examined to decipher the effects of pubertal LPS exposure on miRNAs and the regulatory effect of AHCC. Additionally, miRNAs (miR-34a, miR-125, miR-181c, miR-200c, miR-425) relevant to both mammary gland development and tumor progression were also analyzed.

Let-7a/c, with known tumor-suppressive effects in various cancers, are able to directly target IL-6 and have shown increased expression in groups treated with AHCC, suggesting AHCC's

potential role in protecting against tumors (Fu, Mao et al. 2017, Mi, Liu et al. 2019). Conversely, miR-21, miR-92, and miR-155, known for promoting tumor growth and inhibiting apoptosis, were upregulated by LPS exposure but mitigated by AHCC treatment, highlighting AHCC's potential anti-tumor effects (Si, Zhu et al. 2007, Mattiske, Suetani et al. 2012).

miR-140, which inhibits cancer stem cell proliferation and migration and suppresses the expression of IL-6 and IL-8 by targeting TLR-4, showed an interesting expression pattern with an increase in the AHCC group and a decrease in the AHCC+LPS group, pointing to AHCC's inability to control miR-140 expression in the presence of LPS (Li, Yao et al. 2014, Wu, Zhang et al. 2019). In the context of mammary glands, miR-145, miR-188, and miR-223 were notably upregulated, especially in the AHCC-treated groups, implicating their roles in lipid metabolism and EGFR signaling regulation. miR-145 and miR-188 both regulate TLR4 and consequently modulate mTOR and NF-κB activity by decreasing inflammatory cytokines. miR-223 is an anti-inflammatory miRNA shown to target TLR4 and reduce LPS-induced inflammation (Wang, Shi et al. 2017, Citron, Segatto et al. 2020, Liu, Zhang et al. 2022). Additionally, we observed that LPS exposure raised miR-205 levels, which are associated with an increase in inflammatory cytokines like IL-1β, whereas AHCC treatment appeared to counteract the effect of LPS (Lu, Cao et al. 2018).

miR-184, acting as a tumor suppressor by regulating the PI3K/AKT/mTOR pathway, and miR-205, involved in modulating YAP and Wnt signaling pathways, were also regulated by LPS and AHCC treatments, underscoring the complexity of their roles in mammary gland development and tumor suppression (Huang, Ye et al. 2015, Lu, Cao et al. 2018). In this study, we further investigated miRNAs like miR-34a and miR-181c, recognized for their tumor-suppressive activities through modulating the NF-κB pathway, and observed their downregulation in the LPS

group, which was counteracted by AHCC treatment (Li, Yuan et al. 2013, Huang, Ye et al. 2015). miR-125, involved in the regulation of inflammation by inhibiting IL-6, JAK1, and STAT3, showed an increase in the AHCC group (Feliciano, Castellvi et al. 2013), while miR-200c's increased expression across treatments highlights its role in regulating tumor suppression and inhibiting EMT (Zhou, Men et al. 2018). miR-425, with its dual role in various cancers, was examined for its ability to regulate IL-1β and TNFα levels, potentially serving as a modulator of inflammation (Liu, Hu et al. 2015). This investigation into miRNA expression patterns revealed their significant roles in both mammary gland development and tumor progression, with AHCC treatment showing potential in modulating these miRNAs to exert protective effects against LPS-induced inflammation. This part of the thesis aimed to investigate miRNA involvement in cancer and mammary gland biology, offering insight into AHCC's potential therapeutic benefits in modulating inflammatory pathways and miRNA expression related to cancer progression.

In the next phase of the study, the effect of antibiotics during pregnancy and early life on lactating mammary glands in dams and the immune response and development of mammary glands in offspring will be investigated.

Chapter 4. Evaluating the Impact of LDP and AHCC on Immunoglobulin IgA and IgG of in Dams

In the next step, the effect of antibiotic-induced disruption of maternal gut microbiota on the immune system and lactating mammary glands was investigated. Despite the association of systemic inflammation with elevated IL-6 levels, this study did not detect IL-6 in lactating dams' blood plasma, consistent with the reported pattern that IL-6 shows a brief surge during the initial immune response (Kishimoto 2010). In contrast, levels of IgG and IgA, crucial for their role in immunity by neutralizing pathogens and toxins, were significantly higher in the LDP group,

indicating ongoing inflammation likely due to gut microbiome disruption (Leusen and Nimmerjahn 2013, Mkaddem, Christou et al. 2014).

Chapter 4. The influence of Dietary Interventions on Immunoglobulins IgG and IgA, and Inflammatory Cytokines and Integrity of Tight Junction in Lactating Mammary Glands of the Dams.

We further examined the effect of inflammation originating from gut microbiota and systemic inflammation on lactating mammary glands, revealing interesting findings regarding immunoglobulin levels and cytokine profiles. The levels of IgA and IgG remained consistent across all experimental groups, while IL-2 and IL-17A showed significant increases in the LPD group. IL-17A is pivotal for initiating inflammatory responses and immune cell recruitment, playing a vital role in preparing the immune system for bacterial infections (Mills 2023). IL-2, mainly produced by $CD4^+$ T cells, can have inhibitory effects on Th17 cell differentiation (Abbas, Trotta et al. 2018, Spolski, Li et al. 2018). The activation of Th17 cells is further reinforced by increased secretion of TNFα, IL-1β, and IL-6 signaling (Ouyang, Kolls et al. 2008).

Moreover, pro-inflammatory cytokines IL-1β and IL-6 play essential roles in pathogen defense. IL-1β primarily activates inflammation and promotes Th17 cell activity while inhibiting IL-10 production (Dinarello 2019, Mills 2023). IL-6 activation is closely associated with the NF-κB and PI3K signaling pathways (Luo and Zheng 2016). We also observed an upward trend in IL-1β levels in the LDP group, with a subsequent decrease in the AHCC+LDP group compared to controls. Additionally, a decrease in IL-6 levels was noted across all groups, particularly in the AHCC group, reinforcing the anti-inflammatory role of AHCC.

The analysis of IL-4 and IL-10 revealed that their levels either remained unchanged or decreased across all experimental groups. IL-4, upon binding to its receptor, initiates a signaling cascade leading to the activation of STAT6, which increases the activation of GATA3, a process related to the development of Treg cells. IL-10 plays a critical anti-inflammatory role by reducing the production of major cytokines including IL-1, IL-6, IL-12, and TNFα (Chatterjee, Chiasson et al. 2014).

The integrity of the blood-milk barrier (BMB) is essential to prevent the transfer of specific blood components into milk. When the BMB is compromised, it can result in altered milk composition and increased inflammation in lactating mammary glands, leading to the activation of signaling pathways related to the expression of tight junction proteins such as Occludin, Claudin-1, and Claudin-3 (Guo, Liu et al. 2019, Wellnitz and Bruckmaier 2021).

Our study found a significant increase in Occludin expression in the AHCC and AHCC+LPD groups, with no changes observed in the LDP-only group. Western blot analysis showed a minor increase in Claudin-1 levels in the AHCC group and a decrease in the AHCC+LPD group. In contrast, Claudin-3 expression was higher in the AHCC+LDP group, suggesting that LDP-induced inflammation does not affect the integrity of the BMB.

Chapter 4. The Influence of Dietary Interventions on Lactating Mammary Glands' Gene Expression in Dams.

Next, we examined the effect of LDP exposure on gene expression in lactating mammary glands. *Akt1* expression remained stable across all groups, whereas *Pten* expression increased significantly in the AHCC and AHCC+LDP groups, indicating AHCC's potential to enhance cellular immunity. Disruption in *Akt1* and *Pten* deficiency is associated with the activation of the

PI3K/Akt signaling pathway, which can lead to cancer development and aggressive breast cancer (Carnero, Blanco-Aparicio et al. 2008, Wang, Chen et al. 2017).

Mapk1, vital for cell proliferation and differentiation, showed a decrease in expression following LDP exposure, although this decrease was not statistically significant (Sun, Casbas-Hernandez et al. 2012). Dysbiosis is linked to an increase in estrogen levels, potentially raising breast cancer risk by activating the Igf1r signaling pathway (Che, Liu et al. 2014). *Esr2* and *Egfr* expressions, which regulate estrogen activity and are key in mammary gland development, remained unchanged, indicating no significant effect from LDP and AHCC treatment (Colitti and Pulina 2010, Aupperlee, Zhao et al. 2014).

Chapter 4. Evaluating the impact of LDP and AHCC on immunoglobulin IgG, cytokines IL-6 and IL-10 in the plasma of offspring

Parallel to this study, one of our lab members demonstrated that LDP exposure affects the gut microbiota composition of offspring, noting a decrease in specific gut microbiota populations, particularly *Anaerotruncus*, *Lactobacilli*, and *Roseburia* within the Firmicutes phylum. These species are crucial for gut and immune system development (Domingues, Cabral et al. 2023).

Further analysis of inflammatory cytokines in the mammary glands of offspring showed a significant downregulation of most cytokines in the LDP group compared to the control. In contrast, cytokine levels in the AHCC and AHCC+LPD groups were similar to those in the control group. Previous studies have indicated a decrease in T cells, including T helper and T memory cells, cytotoxic T cells, and pro-inflammatory cytokines following antibiotic treatment, due to microbiota depletion. This depletion leads to reduced production of the IL-1 family, IL-17A, INFγ, and TNFα (Hill, Hoffmann et al. 2010, Ubeda and Pamer 2012). Our findings align

with these observations, underscoring the gut microbiota's critical role in immune system education and modulation. Studies have also highlighted the microbiota's importance in maintaining Th1/Th2 cell balance, with a shift towards Th2 dominance observed in antibiotic-treated mice (Kennedy, King et al. 2018). Overall, this illustrates the complex interactions between gut microbiota, antibiotics, and immune response in early life.

Chapter 4. Differentially Methylated Genes in the Mammary Gland of Offspring Exposed to LDP

In this study, we investigated the prolonged effect of early life exposure to LDP on DNA methylation in the offspring's mammary glands, exploring the potential mitigating influence of AHCC treatment. Our findings revealed that samples treated with AHCC closely clustered with the control group, showing minimal variation in DNA methylation. In contrast, exposure to LDP resulted in significant alterations, affecting 1955 probes and 349 differentially methylated regions associated with signaling pathways such as cAMP, PI3K-Akt, and TGFβ.

Specifically, the LDP-exposed group exhibited methylation changes in genes including *Nfatc1* and *Itgb4*. NFATC1 plays a crucial role in mammary epithelial cell colony formation and is implicated in the lineage development and migration of breast cancer cells (McNeil, Han et al. 2021). Meanwhile, ITGB4 is essential for cell adhesion and signaling transmission and has been linked to increased proliferation and invasion across various cancer types, particularly breast cancer (Sung, Kang et al. 2020).

Additionally, genes such as *Hyal1*, *Sox10*, *Igfbp7*, *Ltbp3*, *Pax5*, and *Mia2* displayed hypermethylation at their promoters, indicative of a potential gene suppression. HYAL1 is critical for hyaluronan breakdown, essential for extracellular matrix remodeling, and can act

variably as an oncogene or tumor suppressor in different cancers (Witschen, Elfstrum et al. 2023). *Sox10* maintains cell lineages derived from the ectoderm, impacting TGFβ signaling and resistance to cancer treatments (Mollaaghababa and Pavan 2003). *Igfbp7* suppresses tumors by blocking the *Igf-1r* receptor, influencing cell survival and proliferation. *Ltbp3* is associated with the TGFβ pathway and correlates with less favorable survival outcomes in cancer (Evdokimova, Tognon et al. 2012). *Pax5* stabilizes mammary epithelial cells and plays dual roles in promoting and inhibiting cancer progression (Benzina, Beauregard et al. 2017). *Mia2,* known as a tumor suppressor in liver cancer, exhibits variable expression in breast cancer, associated with TGFβ signaling (Hellerbrand, Amann et al. 2008).

Moreover, the study observed hypermethylation within the genes bodies of *Tiam1*, *Deptor*, *Smad6, Bmp7, Ksr2,* and *Itga9,* which could potentially lead to their overexpression (Yang, Han et al. 2014). These genes are involved in enhancing invasive characteristics, modulating mTOR kinase activity, and increasing cell migration and proliferation in cancer contexts (Catena and Fanciulli 2017). The study also identified genes with consistent methylation patterns between the control vs. LDP and LDP vs. AHCC+LDP comparisons, highlighting significant methylation changes affecting *Tiam1*, *Pax5*, and *Itga9* regions.

Overall, while AHCC did not significantly alter DNA methylation, it may influence other epigenetic mechanisms such as microRNA modulation (e.g., Let-7a/c, miR-146a, miR-34). This suggests a potential pathway through which AHCC may exert its effects.

Chapter 4. The Impact of LDP on Key Genes Expression Level Involved in MAPK Signaling Pathway and Breast Cancer in Mammary Glands of Offspring

In addition, we investigated the expression levels of genes associated with breast cancer, such as *Apc, Magi1, Tp53, Pten, Akt1, Mapk1, c-Jun, Egfr, Igf-1r, Ptk2, Fgfr2, Fzd7, Dvl3, Crebbp, Esr2,* and *Lama2*. APC and MAGI1 are involved in β-catenin degradation and regulation of the Wnt signaling pathway (Zaric, Joseph et al. 2012, Anastas and Moon 2013). TP53, a tumor suppressor, plays a crucial role in suppressing cell cycle progression and preventing tumor growth (Kastenhuber and Lowe 2017). PTEN, a suppressor of the PI3K/Akt pathway, is involved in ECM remodeling, which suppresses tumor-associated collagen and inhibits breast cancer metastasis (Jones, Hammer et al. 2019). *Apc*, *Magi1*, *Tp53*, and *Pten* showed increased expression in AHCC and AHCC+LDP.

Akt1 and *Mapk1* expression remained unchanged in the LDP group but increased in the AHCC groups. AKT1 is essential for mammary gland development, regulated by estrogen and progesterone, and involved in duct elongation. It is also regulated by IGF1R and promotes cell proliferation (LaRocca, Pietruska et al. 2011). MAPK1 is crucial for mammary gland organization; its overactivation disrupts cell organization, potentially contributing to breast cancer (Whyte, Bergin et al. 2009). We observed that EGFR, IGF-1R, and c-Jun were overexpressed in the LDP group compared to the control but not in the AHCC and AHCC+LDP groups. EGFR activates the ERK/MAPK pathways, promoting proliferation and differentiation. HER2, when dimerized with EGFR, promotes breast cancer development (Olayioye 2001). IGF1R activation enhances cell survival and proliferation, crucial for terminal end bud (TEB) formation and duct elongation. Overexpression of *Igf-1r* is associated with poor prognosis in breast cancer (Farabaugh, Boone et al. 2015). FGF1 and FGFR2 are crucial for cellular processes such as proliferation, differentiation, and development through the ERK/MAPK signaling pathways (Xie, Su et al. 2020). FZD7 and DVL3 are key components of the Wnt signaling

pathway, influencing cell proliferation, differentiation, and migration (Holzem, Boutros et al. 2024). CREBBP acts as a transcriptional co-activator influencing gene expression and is involved in TGFβ signaling (Das, Roy et al. 2014). PTK2 (FAK) is essential for cell migration, adhesion, and apoptosis, with its overexpression associated with increased metastasis and poorer survival in breast cancer (Wu, Wang et al. 2022). ESR2 inhibits proliferation by suppressing Cyclin A and Cyclin D1 expression, both of which play critical roles in mammary gland development (Paruthiyil, Parmar et al. 2004).

The observed overexpression of *Fgf1*, *Fgfr2*, *Igf1r*, *Egfr*, *c-Jun* and *Ptk2* suggests activation of the ERK/MAPK and PI3K/AKT signaling pathways. Subsequently, western blot analysis was performed on AKT1, pAKT1, FOXO, pFOXO, ERK1/2, and pERK1/2. Despite failing to detect AKT1, pAKT1, FOXO, and pFOXO by western blot assay, an increase in pERK1/2 was noted, while ERK1/2 levels remained unchanged, indicating activation of the ERK/MAPK signaling pathway. This activation is linked to higher proliferation rates via Cyclin D1, facilitating the transition from G1 to S phase and contributing to breast cancer development. Additionally, ERK1/2 activation leads to increased expression of *Egr*, *Fos*, *Jun*, and *Myc*, further enhancing proliferation rates (Lavoie, Gagnon et al. 2020).

Further investigation into microRNAs involved in the MAPK signaling pathway revealed downregulation of Let-7a/c, miR-34b, and miR-146a in the LDP group. Let-7a/c and miR-34b, known for their tumor-suppressing roles, are downregulated in various diseases, including cancer. Let-7a/c directly targets the RAS protein, regulating the ERK/MAP pathway and suppressing *Igf-1r* (Johnson, Grosshans et al. 2005). miR-34b suppresses *Egfr*, influencing cell proliferation and inhibiting tumor growth (Stahlhut and Slack 2015). miR-146a, a tumor suppressor microRNA, regulates TLR4 by mitigating inflammation and controls the

ERK/MAPK pathway by inhibiting EGFR, but it was downregulated by LDP (Xu, Wang et al. 2012).

This study expands our understanding of how gut dysbiosis influences immune response and mammary gland development. It highlights the beneficial effects of prebiotics, particularly AHCC supplements, in mitigating gut dysbiosis during critical developmental stages such as pregnancy, early life, and puberty. Moreover, it underscores the mechanisms through which disruptions in gut microbiota trigger immune responses and alter gene expression patterns at both genetic and epigenetic levels. Additionally, it emphasizes the importance of elucidating how gut microbiota dysbiosis may predispose individuals to severe diseases, including cancer. This investigation provides essential scientific evidence regarding the influence of the gut-breast axis and how gut dysbiosis may impact breast cancer development. Furthermore, the study focuses on the role of prebiotics in preventing and potentially treating breast cancer, enhancing our understanding of regulatory processes within the gut-breast axis.

5.2 Limitation of the study

The intricate hormonal changes orchestrated primarily by estrogen and progesterone during puberty and pregnancy are pivotal for mammary gland development and have implications for breast cancer risk. Imbalances in estrogen and progesterone levels can lead to mammary cell proliferation, inhibition of apoptosis, thereby elevating the risk of breast cancer development. In this study, one of our primary objectives was to understand the detrimental effects of inflammation during puberty on the numbers and structure of terminal end buds using whole-mount staining and computational analysis (Haslam and Levely 1985, Ruan, Monaco et al. 2005, Aupperlee, Leipprandt et al. 2013). However, to better decipher the impact of inflammation on mammary gland structure, it is essential to complementarily determine the levels of estrogen and progesterone in these cases to distinguish changes due to inflammatory triggers by LPS from those due to naturally occurring hormonal cycles (Stingl 2011).

Next, we examined the effects of pubertal inflammation on breast cancer by administering 4T1 cells to the mice. These cells, known for their invasiveness and high metastatic potential, are suitable for studying mammary gland development in an immunocompetent murine model. However, as these cells lack hormone receptor expression and represent triple-negative breast cancer characteristics, using cell lines that express estrogen receptors or are less aggressive may yield more insightful results, considering the crucial role of the gut microbiome in estrogen regulation.

The DNA methylation analysis uncovered various genes with altered methylation levels, suggesting potential epigenetic changes in genes crucial for mammary gland development and breast cancer. Beyond DNA methylation, the research also identified significant dysregulation in

microRNAs, which play key roles in essential signaling pathways. However, further analysis of mRNA levels for additional candidate genes from DNA methylation analysis or microRNA targets would further consolidate the role of AHCC in decreasing inflammation due to LDP-induced dysbiosis.

In studying early-life antibiotics, mating the dams on-site is recommended to minimize stress on pregnant dams. However, accurately determining the timing of menstruation and ovulation poses challenges, potentially leading to variable birth rates. Additionally, we evaluated the effects of treatments on mammary glands during early adulthood at 8 weeks of age, focusing on puberty when terminal end buds are forming to gain insights into mammary gland formation under treatment influences. Yet, pinpointing the exact onset of puberty is challenging due to environmental factors such as stress and male mouse odors, which can result in early puberty.

Gut microbiota dysbiosis leads to dysregulation in short-chain fatty acid (SCFA) production. However, we did not measure SCFA levels, which could elucidate the mechanistic connection between immune dysregulation and mammary glands affected by early-life dysbiosis.

Finally, this study exposed offspring to treatment from one week before birth until weaning. Future research using separate prenatal and postnatal models will enhance our understanding of the adverse effects stemming from dysbiosis in maternal and/or offspring gut microbiota during critical developmental stages before and shortly after birth.

5.3 Future directions

Our findings suggest that AHCC supplementation may mitigate the effects of LPS-induced inflammation during puberty by modulating DNA methylation and microRNA expression. Previous studies have elucidated the immunoregulatory mechanisms of AHCC. Further analysis,

combining gene expression profiling through high-throughput sequencing with DNA methylation studies, could provide insights into how AHCC regulates gene expression. Given the critical roles of estrogen and progesterone in mammary gland development and function, a thorough examination of these hormones is essential to understand the impact of LPS-induced inflammation and AHCC's potential counteractive effects. LPS can trigger systemic inflammation, activating immune cells including macrophages. M2 macrophages, typically resident in mammary glands, promote cell proliferation and are vital for mammary gland development; under LPS influence, these may shift to M1 pro-inflammatory macrophages (Zheng, Hong et al. 2013). Cytokines secreted by macrophages alter gene expression in adipose tissue and increase aromatase production, crucial for estrogen synthesis. Elevated aromatase levels can raise estrogen levels, heightening the risk of ER+ breast cancer. Therefore, investigating LPS's effects on mammary gland-resident macrophages and aromatase levels, alongside AHCC's regulatory potential, could provide valuable insights into mammary gland health and disease pathways.

In this study, we evaluate the effects of LPS and AHCC on tumor growth. Further avenues for investigation into inflammation levels and immune cell infiltration via flow cytometry should be considered. Recent studies in breast tumors have identified various bacteria within tumor cells, suggesting that elevated inflammatory cytokine levels might compromise intestinal barrier integrity, leading to leaky gut and facilitating bacterial translocation to distant tumor sites (Harbeck, Penault-Llorca et al. 2019). AHCC's potential to inhibit TLR4 and possibly prevent gut permeability suggests that metagenomic analysis within tumor cells could shed light on the impact of LPS and AHCC on intratumoral microbial loads.

Furthermore, considering that early-life antibiotic exposure may result in persistent immune dysfunction, future research could employ an early-life gut microbiota dysbiosis model extending into adulthood, focusing on non-virgin or post-lactation mice. This approach might lead to a better understanding of the underlying mechanisms linking immune stressors and inflammation to increased cancer susceptibility in adults with a history of early-life gut microbiota alterations.

Research has also highlighted the lasting impact of maternal diet-induced dysbiosis on both immediate and subsequent offspring generations, with evidence suggesting that epigenetic changes can be inherited by the second generation. Investigating the second-generation offspring of mice subjected to early-life gut microbiota dysbiosis could reveal vulnerabilities to immune-related disorders under immune stress conditions.

Our findings hint at the role of specific genes in mammary gland development. Using mouse models with targeted gene knockouts could clarify how genes modulate long-term immune responses to early-life or pubertal immune challenges and inflammation. This strategy would enhance our understanding of the molecular mechanisms underlying immune dysfunction and disease susceptibility, potentially paving the way for targeted therapeutic interventions.

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Author: Ingrid S. Paine et al

Publication: Journal of Mammary Gland Biology and Neoplasia

Publisher: Springer Nature

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The Terminal End Bud: the Little Engine that Could

Author: Ingrid S. Paine et al

Publication: Journal of Mammary Gland Biology and Neoplasia

Publisher: Springer Nature

Date: Feb 6, 2017

Figure .15:

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