ANTI-*CANDIDA* AND MICROBIOTA PROPERTIES OF BREASTMILK

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ANTI-*CANDIDA* AND MICROBIOTA PROPERTIES OF BREASTMILK

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LIST OF ABBREVIATIONS

VVC	Vulvovaginal candidiasis
IL	Interleukin
TGF	Transforming growth factor
EGF	Epidermal growth factor
HMO	Human milk oligosaccharides
CFU/mL	Colony forming units per mililiter
Ig	Immunoglobulin
YEPG	*Yeast* extract peptone glucose
PCR	Polymerase chain reaction
PERMANOVA	Permutational analysis of variance
OTU	Operational taxonomic unit
pIgR	Polymeric immunoglobulin receptor

CIRI-CIRI ANTI-KANDIDA DAN MIKROBIOTA SUSU IBU

ABSTRAK

Susu ibu dilaporkan memberi kesan yang baik dalam menentang pelbagai jangkitan yang telah terdedah kepada ibu semasa hayatnya, kerana antibodi dipindahkan ke susu ibu melalui kelenjar susu, struktur epitelium yang bercabang, yang terdiri daripada saluran laktiferus yang mengangkut susu. dan alveoli. Walaupun jangkitan faraj mungkin berlaku secara rawak pada wanita, Pusat Kawalan dan Pencegahan Penyakit (CDC) melaporkan bahawa wanita hamil mempunyai prevalens yang lebih tinggi, yang meningkatkan risiko jangkitan pramatang dan sistemik pada bayi baru lahir (CDC, 2022). Jangkitan faraj kekal menjadi kebimbangan utama dalam obstetrik dan ginekologi kerana telah ditunjukkan bahawa mikrobiota faraj ibu boleh terus kepada bayi selepas bersalin secara faraj. Kajian ini bertujuan untuk menyiasat perbezaan imunologi, sifat antimikrob dan profil mikrobiota susu ibu daripada wanita dengan (W) atau tanpa (WO) jangkitan yis faraj semasa mengandung dalam 85 wanita menyusu (W, n = 43; WO, n = 42). Ciri-ciri imunomodulasi susu ibu yang ditentukan oleh ujian immunoabsorbent berkaitan enzim komersial (ELISA) telah menunjukkan bahawa kepekatan IL-10, IgA, IgM, IgG, EGF, dan TGF-α adalah serupa dalam kedua-dua kumpulan. Walau bagaimanapun, susu ibu wanita berumur di bawah 31 tahun daripada kumpulan W menunjukkan kepekatan EGF yang lebih tinggi daripada kumpulan WO (p=0.031). Aktiviti perencatan susu ibu terhadap Kandida vagina dilakukan melalui pengagregatan dan perencatan pertumbuhan sel yis. Susu ibu daripada kumpulan WO menunjukkan sifat anti-Kandida yang lebih tinggi daripada kumpulan W (p<0.001). Analisis korelasi menunjukkan bahawa kepekatan susu

ibu TGF-α berkorelasi positif dengan kepekatan IL-10 (p=0.001) dan IgA (p=0.021) pada kumpulan W. DNA bakteria telah diekstrak daripada setiap sampel susu ibu untuk pemprofilan mikrobiota oleh amplikon penjujukan daya pengeluaran tinggi yang diperoleh daripada rantau V3-V4 gen 16S rRNA. Susu ibu daripada kumpulan W mempamerkan kepelbagaian alfa yang lebih tinggi daripada kumpulan WO pada tahap taksonomi class yang berbeza (p=0.015), order (p=0.011), family (p=0.020), dan genus (p=0.030). Perbezaan komposisi antara kumpulan seperti yang ditentukan melalui kepelbagaian beta menunjukkan perbezaan marginal pada tahap taksonomi phylum (p=0.087), family (p=0.064), dan genus (p=0.067). Kajian ini menunjukkan bahawa, walaupun susu ibu daripada wanita yang mengalami jangkitan faraj semasa mengandung mungkin tidak cukup menghalang pertumbuhan Kandida, bioaktif imunomodulasi lain boleh menggantikan kesan perlindungan tersebut. Di samping itu, walaupun jangkitan faraj semasa kehamilan mengubah komposisi mikrobiota susu ibu, ini mungkin tidak menimbulkan ancaman kepada pertumbuhan dan perkembangan bayi.

ANTI-*CANDIDA* AND MICROBIOTA PROPERTIES OF BREASTMILK

ABSTRACT

Breast milk has been reported to exert a beneficial effect in countering a variety of infections that has been exposed to the mother during her life, because antibodies are transferred to breast milk via mammary glands, a branching epithelial structure, consisting of milk-transporting lactiferous ducts and alveoli. While vaginal infections may occur randomly in women, the Centers for Disease Control and Prevention (CDC) reported that pregnant women had a higher prevalence, which increased the risk of preterm and systemic infections in the newborn (CDC, 2022). Vaginal infections remain a major concern in obstetrics and gynecology because it has been shown that the mother's vaginal microbiota can pass directly to the baby after vaginal delivery. This study aimed to investigate the different immunological, antimicrobial properties and microbiota profiles of breast milk from women with (W) or without (WO) vaginal yeast infections during pregnancy in 85 lactating women (W, n= 43; WO, n= 42). Immuno-modulatory properties of breast milk which was determined by commercial enzyme-linked immunoabsorbent assay (ELISA) had shown that concentrations of IL-10, IgA, IgM, IgG, EGF, and TGF-α were similar in both groups. However, breast milk of women aged below 31 years old from the W-group showed higher concentration of EGF than the WO-group (p=0.031). Inhibitory activity of breast milk against vaginal *Candida* was performed via aggregation and growth inhibition of yeast cells. Breast milk from WO-group exhibited higher anti-*Candida* properties than W-group (p<0.001). Correlation analysis showed that breast milk concentration of TGF-α positively correlated with concentrations of IL-10 (p=0.001) and

IgA (p=0.021) in the W-group. The DNA of bacteria was extracted from each breast milk sample for microbiota profiling by high-throughput sequencing amplicons derived from the V3-V4 region of the 16S rRNA gene. Breast milk from the W-group exhibited higher alpha diversity than that from the WO-group across different taxonomic levels of class (p=0.015), order (p=0.011), family (p=0.020), and genus (p=0.030). Compositional differences between groups as determined via beta diversity showed marginal differences at taxonomic levels of phylum (p=0.087), family (p=0.064), and genus (p=0.067). This study shows that, although breast milk from women with vaginal infections during pregnancy may not sufficiently hinder *Candida* growth, other immuno-modulatory bioactives may substitute for such a protective effect. In addition, even though vaginal infection during pregnancy alters the composition of breast milk microbiota, this may not pose a threat to infant growth and development.

CHAPTER 1

INTRODUCTION

1.1 Background

Vaginal infection is an inflammation that can cause discharge, itchiness, and pain in the vagina. The cause is typically an infection or a shift in the balance of vaginal microbiome (Machado *et al.*, 2017). The most prevalent vaginal infections are provoked by bacteria (such as bacterial vaginosis, BV and aerobic vaginitis, AV), and fungi (vulvovaginal candidiasis, VVC) (Palmeira-de-Oliveira *et al.*, 2015). After bacterial vaginosis, vulvovaginal candidiasis (VVC) is regarded as the second most common causes of vaginal infection (Gonçalves *et al.*, 2016).

The symptoms of VVC include white vaginal discharge, localized itching, burning, discomfort, and pain during urination and sexual activity. These symptoms are brought on by an excessive proliferation of yeast cells of the *Candida* species in the vaginal mucosa. Infection with *Candida albicans* occurs in 80% to 90% of confirmed VVC cases, while less commonly occurring infections with *Candida glabrata* or *Candida tropicalis* (Sobel, 2007). According to epidemiological research, 75% of women will have at least one episode of VVC over their lifetimes (Gonçalves *et al.*, 2016). VVC risk is roughly 20% for non-pregnant women, whereas it frequently rises to 30% during pregnancy. The risk can reach 50%, particularly during the final trimester (Gonçalves *et al.*, 2016; Sangaré *et al.*, 2018). The likelihood of a woman getting VVC will be increased during pregnancy due to higher levels of estrogen and higher glycogen content in vaginal secretions (Monif and Baker, 2003).

Related to vulvovaginal candidiasis, antibodies in breast milk may function as an immunological memory by shielding the mother from infections she may have encountered during her life, including VVC. The mammary glands, a branching epithelial structure containing lactiferous ducts that transport milk and alveoli that can transfer antibodies to breast milk (Atyeo and Alter, 2021). The adaptive and innate immune components of the immune system, as well as other nutrients, are delivered to the newborn and infant during breastfeeding by the mother's breast milk to help them develop their immune systems (McDade *et al.,* 2016).

Breast milk contains a broad range of protective components, including immunoglobulin (Ig) A, immunocompetent cells, fatty acids, oligosaccharides, lysozyme, or lactoferrin (Newburg, 2005), that prevent breast-fed infants from infectious diseases (Hanson and Korotkova, 2002; Morrow and Rangel, 2004). Lactobacilli, lactococci, enterococci, and *Leuconostoc* spp., which are commonly found in breast milk from healthy women, can also be considered as essential aspects of the defense mechanism that this biological fluid provides to the infant (Heikkilä and Saris 2003; Martin *et al.* 2003). Breastfeeding has been linked to the transmission of bacteria from mother to child that at least belong to the genera *Lactobacillus, Staphylococcus, Enterococcus,* and *Bifidobacterium*, according to several studies (Soto *et al,* 2014).

Although evidence have shown that dietary intake and lifestyles have a minor effect on composition of breast milk, most biochemical properties of human breast milk remain stable even among women with different health statuses (Samuel *et al.*, 2020; Pham *et al.*, 2020). Breast milk from women with or without either gestational diabetes mellitus or insulin-dependent diabetes mellitus also showed similar compositions of IgA,

total saccharides, total lipid, major vitamins, and minerals (Peila *et al.*, 2020). Nevertheless, how a mother's health influences certain aspects of breast milk that impact the health of infants remains unknown with limited information. An area where this is particularly unclear is the relationship between vaginal infections during pregnancy, the composition of breast milk, and the resulting potential health benefits for newborns.

One of the main concerns in obstetrics and gynaecology continues to be vaginal infections because it has been demonstrated that during vaginal delivery, the vaginal microbiota can be directly transferred from the mother to the newborn as well. Transfer of pathogenic vaginal microorganisms from mother to newborns during vaginal delivery is not new and has been reported involving *Trichomonas vaginalis* (Smith *et al.*, 2002) and Group B *Streptococcus* (Brokaw *et al.*, 2021) while vaginal *Candida* has been reported to transmit vertically to the mouth of newborns during labour (Rusan *et al.*, 2017). Thus, I postulated that i) women who have vaginal infections during pregnancy produce breastmilk with lower immune profiles than women who do not have vaginal infections during pregnancy; ii) women with vaginal infections during pregnancy will have a different breastmilk microbiota profile than women without vaginal infections during pregnancy. The purpose of this study is to investigate whether the composition of breast milk from mothers with or without vaginal infections would alter and whether it can defend newborns from the same microorganisms that cause vaginal infections.

1.2 Research Objectives

The main objective of the present study was to analyze and evaluate the different composition and antimicrobial activity of breast milk from women with and without vaginal infections against vaginal infection pathogen, especially *Candida*. The specific objectives of this study were as follows:

1. To evaluate immunological properties of breast milk from women with (W) or without (WO) vaginal yeast infections during pregnancy.
2. To determine and characterize inhibitory activity potentials of breast milk against prominent species of *Candida* involved in vaginal candidiasis via co-aggregation and growth inhibition.
3. To assess breast milk microbiota from women with (W) or without (WO) vaginal yeast infections during pregnancy.

CHAPTER 2

LITERATURE REVIEW

2.1 Breast Milk

Breast milk is a mammary gland secretion that contains a variety of nutrients and bioactive substances that support growth and immunological development in infancy. Breast milk is estimated to include between 0.9 and 1.2 g/dL of protein, 3.2 to 3.6 g/dL of fat, and 6.7 to 7.8 g/dL of lactose. Energy estimations have a strong association with breast milk fat levels, which range from 65 to 70 kcal/dL (Ballard and Morrow, 2013). Epidermal growth factor (EGF), nerve growth factor (NGF), insulin-like growth factors (IGFs), and interleukins (ILs) are among the growth modulators found in breast milk. Breast milk also contains granulocyte colony stimulating factor (G-CSF), transforming growth factor (TGF)-ß, and transforming growth factor (TGF)-α. These growth regulators are either produced by the mammary epithelial cells or by activated macrophages, lymphocytes (mostly T cells), or neutrophils in the milk (Tauber, 2021). Breast milk is not only important for infant, but also for probiotic microflora.

A variety of bacteria, including staphylococci, streptococci, corynebacteria, lactic acid bacteria, propionibacteria, and bifidobacteria can be found in breast milk. These populations include 10^4–10^5 colony forming units of lactobacilli per millilitre (Martin *et al.*, 2003). According to Soto *et al.* (2014), bifidobacteria were identified from 10.61% of breast milk samples while *Lactobacillus* bacteria were present in 40.91% of them. Probiotics, which contain *Lactobacillus*, have positive effects on human health generally. Probiotics are live bacteria that, when given in sufficient quantities, boost the host's health

(FAO/WHO, 2001). Despite the fact that bacteria from the genera *Lactobacillus* and *Bifidobacterium* are the most frequently used probiotics, other bacterial genera such as *Enterococcus, Streptococcus,* and *Escherichia* are also utilized. *Lactobacillus* are potentially hostile to spoilage and diseases because they produce compounds including bacteriocins, organic acids, and hydrogen peroxide (H_2O_2) with unique uses in nutrition and health because of their high antimicrobial action (Soto *et al.*, 2014).

2.1.1 Macronutrient and Micronutrient in Breast Milk

Mature breast milk contains 65–70 kcal per 100 mL of energy, with fat contributing for about 50% of those calories and carbohydrates for the remaining 40% (Table 2.1). However, the maternal diet, maternal health, the physiology of the mother's mammary glands, and other environmental factors could affect the nutritional composition of breast milk (Ballard and Morrow, 2013).

Human milk oligosaccharides (HMOs) are the second most common carbohydrate and third most common solid component in breast milk. The attachment of oligosaccharides and other carbohydrate-based bioactive components to lactose enhances the absorption of calcium and minerals (Thurl *et al.*, 2017). HMOs are known to play a substantial prebiotic role in the early postnatal phases of gut microbiota development. HMOs have been shown to shorten the duration of diarrhea, to encourage the growth of bifidobacteria, and to prevent the development of toxic gut microbiota by lowering intestinal pH while combined with short-chain fatty acids. (Harmsen *et al.*, 2000).

Whey, casein, and other peptides constitute the protein in breast milk. Depending on the stage of breast milk, the ratio of whey to casein varies (Liao *et al.*, 2017). The three

main components of whey proteins are alpha-lactalbumin, lactoferrin, and secretory IgA. For instance, alpha-lactalbumin is required for the synthesis of lactose as well as the binding of Ca and Zn ions. Calcium and phosphorus crystallize into masses with the aid of casein (Guo, 2014).

Casein clumps or curdles in the stomach, but whey stays liquid and is simpler to digest. Colostrum has a whey/casein ratio of roughly 90:10, but mature milk has a ratio of 60:40 (Liao *et al.*, 2017). Protein levels will decline over the first 4 to 6 weeks, regardless of when the baby is delivered. Due to the varied amino acid profiles of casein and whey proteins, breast milk has a variable total amino acid composition depending on the stage of lactation (Bauer *et al.*, 2011). Glutamine, the most common free amino acid, is present in mature milk at levels that are around 20 times higher than those in colostrum at their lowest concentrations. By supplying ketoglutaric acid, glutamine performs important functions in the citric acid cycle that possibly acting as a neurotransmitter in the brain, and a key source of energy for intestinal cells (Zhang *et al.*, 2013).

Table 2.1 Macronutrient Composition of Breast Milk

Component	Colostrum* (1-5 days)	Mature milk* (> 14 days)	References
Energy	50-60 kcal/ 100 mL	65-70 kcal/ 100 mL	Guo, 2014
Carbohydrate	50-62 g/L	60-70 g/L	Kim and Yi, 2020
Lactose	20-30 g/L	67-70 g/L	Kim and Yi, 2020
Oligosaccharides	20-24 g/L	12-14 g/L	Thurl *et al.*, 2017

Total protein	14-16 g/L	8-10 g/L	Guo, 2014
Total fat	15-20 g/L	35-40 g/L	Saarela *et al.*, 2005

*The range of each component is slightly different according to studies

In breast milk, fat ranks second in terms of macronutrient content. Triglycerides, which also contain the significant fatty acids linoleic and alpha-linolenic acids, make up the majority of the fatty acids in breast milk (95–98%). Alpha-linolenic acid and linoleic acid, which cannot be produced by the body, are the building blocks for eicosapentaenoic acid (EPA) and arachidonic acid, respectively, which is then transformed into docosahexaenoic acid (DHA). They are also essential for the development of in vivo signal transduction, retinal components, nervous system parts, inflammatory responses, growth, and immunological activity (Martin *et al.*, 2016). Due to the presence of bile salt-stimulated lipases and palmitic acid at breast milk triglycerides, fats in breast milk are effectively digested and absorbed in infants (Martin *et al.*, 2016). Joardar *et al.* (2006) reported that between the sixth week and sixth month of lactation, breast milk's lipid content and overall polyunsaturated fatty acid percentage both significantly increased.

The vitamins in breast milk, except for vitamins D and K, are sufficient to support the infant's normal growth. Infants who are exclusively breastfed have significantly lower vitamin D levels than recommended in their diets and below the minimal required amount. Sun exposure, a mother's nutrition, lifestyle, skin tone, and environment all have an impact on vitamin D levels. Breast milk typically contains vitamin D less than 1 mg or 40 IU/L, which is not enough to fulfill the needs of a newborn (Martin *et al.*, 2016). Vitamin D levels in breast milk can be increased by giving lactating moms 400–2000 IU

(International Unit) of vitamin D each day (Guo, 2014). Vitamin K is essential for protein due to its role in blood clotting. However, only a limited amount of vitamin K is delivered to the fetus through placenta. A newborn baby is commonly deficient in vitamin K that causes increasing their chance of developing hemorrhagic illness, therefore more vitamin K is recommended after delivery (Martin *et al.*, 2016; AAPC, 2003). Water-soluble vitamins are also significantly influenced by maternal status. Moms who do not consume balance diet may generally have low amounts of the vitamins folate, B_6, and B_{12} but still have appropriate levels of thiamin and riboflavin (Martin *et al.*, 2016).

Minerals are important for many physiological activities that occur in breast milk, such as the synthesis of various enzymes and the biological significance of molecules and structures. Compared to infant formula, breast milk has a lower concentration of minerals, but due to the high absorption of these elements, full breastfeeding does not necessitate any additional supplementation. In particular, iron is available in colostrum and mature milk at amounts of 0.5–1.0 mg/L and 0.3–0.7 mg/L, respectively, but its bioavailability is 20%–50%, thereby making it more efficient than iron in infant formula (4%–7%) (Domellöf *et al.,* 2004; Martin *et al.,* 2016).

2.1.2 Bioactive components and factors in breast milk

The term "bioactive components" refers to substances that have an effect on biological substrates or processes, and as a result, have an effect on how the body functions or is maintained, and ultimately how healthy the individual is (Ballard and Morrow, 2013). Breast milk contains a range of bioactive substances which some are created by the cells that are already present in the milk, while others are generated by the mammary

epithelium, carried from maternal serum, and transported through the mammary epithelium by receptor-mediated transport. Along with the milk fat globule (MFG), the mammary epithelium also secretes a range of proteins and lipids that are attached to membranes (Hendricks and Guo, 2014).

Proteins have the ability to bind important nutrients together, ensuring their solubility and promoting absorption by the intestinal mucosa. By restricting the activity of the enzymes that break down the proteins, protease inhibitors can enhance these binding proteins' physiological function and relative stability, thus facilitating the process of nutrient intake (Hendricks and Guo, 2014). Lactoferrin, α-lactalbumin, and secretory IgA compose most of the whey proteins. The main protein component is α -lactalbumin, which makes up to 25% of the total protein in breast milk. By producing peptides, it makes easier for the body to absorb essential minerals. It also aids in the production of lactose by binding both Ca and Zn ions (Lönnerdal and Lien, 2003).

A single polypeptide chain glycoprotein called lactoferrin has two α-helix connected lobes that individually bind iron. To restrict the spread of potentially hazardous bacteria by blocking microbes from reaching the iron, lactoferrin functions by firmly connecting with iron (up to two atoms of iron to each lactoferrin molecule). Lactoferrin concentration in colostrum ranges in 5-7 g/L and decreasing to 1-3 g/L in mature milk (Hendricks and Guo, 2014). S-IgA compose 80-90% of the immunoglobulins in milk, it may reach about 12 g/L in colostrum and then level off around 1 g/L (Lönnerdal and Lien, 2003). The secretory substance shields the mucosal surface from stomach acid and digestive enzymes, acting as a defensive mechanism for the antibody molecule, which attacks disease without generating inflammation (Lönnerdal and Lien, 2003).

Triglycerides is the majority of lipid component in milk, the other components are fatty acid, phospholipids, and sterols. Phospholipids compose an additional 0.5–1% of the total amount of lipids. Sterols such as cholesterol and other esters make up 0.2% of the milk fat in the membrane of the milk fat globule (Hendricks and Guo, 2014). Thormar and Hilmarsson (2007) reported that some unsaturated fatty acids can provide protective effects against microorganism and the disruption of viral envelopes. The major sterol in milk is cholesterol, which has concentration in range of 10 to 15 µg/ 100 mL (Guo and Hendricks, 2008).

Carbohydrate in breast milk comprises monosaccharide (e.g., glucose, galactose), disaccharides (e.g., lactose, lactulose), oligosaccharide, and more complex carbohydrates (e.g. glycoproteins) (Hendricks and Guo, 2014). Intestinal florae convert lactose to lactic acid, which aids in the absorption of minerals, especially calcium. As a result of the pH being lowered by the gut flora, the solubility of calcium salts is increasing. *Lactobacillus bifidus* and *Lactobacillus acidophilus* use lactulose, a galactose and fructose disaccharide, as a growth factor and energy source (Guo and Hendricks, 2008).

The carbohydrate component of glycoconjugates, such as glycolipids and glycoprotein, form the oligosaccharides in breast milk. Breast milk contains glycoprotein, which prevents enteropathogenic *E. coli, Campylobacter jejuni*, and *Streptococcus pneumoniae* from adhering to the intestinal wall cells (Guo and Hendricks, 2008). Mucin, a long macromolecule also present in human milk, combines with oligosaccharides. The breast milk mucin complex, or mucin-associated glycoprotein known as lactadherrin, binds to rotavirus and renders the viral particles harmless by surrounding and coating

them, preventing them from adhering to target tissues (Lönnerdal and Lien, 2003). Breast milk also has a lot of other bioactive factors (Table 2.2).

Table 2.2 Bioactive Factors in Breast Milk

Bioactive Factor	Effect or Function in Newborn Health
Nucleotides: Five bases—adenine, guanine, cytosine, uracil, and thymine	Precursors of RNA and DNA, also provide high energy source ATP, as regulatory signals (cyclic AMP and cyclic GMP), as components of coenzymes, and as important methyl donors
Casomorphins	Opioid-like substances that may affect infant behavior and mood in addition to a range of other functions
Galanin, neuropeptide Y, substance P	Neuropeptide galanin is widely distributed in the nervous and endocrine systems. It appears to facilitate the growth and repair of sensory neurons in the peripheral nervous system and gut. In addition, substance P induces interleukin (IL)-12 production by macrophages.
Thyrotropin-releasing hormone, gonadotropnin-releasing hormone, growth hormone-releasing hormone	Act directly on the developing gut tissue, lengthening of the villi, enhancing the activities of lactase, maltase and sucrase. There is evidence that certain agents may interact not only with the GI tract, but may also be absorbed into the systemic circulation on act on other target tissues
Cytokines, chemokines	Anti-inflammatory and immune-enhancement properties as well as growth-promoting action

Antimicrobial peptides (AMPs)	AMPs are short chains of amino acids that offer defense against microbial threats. Broadly, they are diverse. set of molecules in terms of form and exact function.
microRNAs	Immunomodulatory component hypothesized to be a key mechanism through which the infant immune system develops

*According to the studies from Guo and Hendricks (2008) and Christian *et al.* (2021)

2.1.3 Breast Milk Microbiota and Their Properties

Breast milk consists of a low-biomass, low-diversity microbiome, primarily composed of bacteria. Microbiota in breast milk have ability to reduce the frequency and severity of infection in breastfed infants by various mechanisms, including a competitive exclusion, production of antimicrobial compounds, or enhancement of mucine synthesis and intestinal permeability in the function of the intestinal barrier (Olivares *et al.,* 2006). The overall bacterial content is approximately 10^3-10^4 CFU/mL in healthy breast milk by enumeration bacteria on non-selective media. *Staphylococcus* and *Streptococcus* are among the most commonly mentioned taxa that have been shown to be present in all breast milk samples (Fitzstevens *et al.,* 2016). In addition, numerous additional taxa, such as *Corynebacterium, Bifidobacterium, Lactobacillus, Propionibacterium, Bacteroides, Enterococcus, Faecalibacterium, Veillonella, Serratia, Ralstonia, Acinetobacter, Rothia,* and several members of the Lachnospiraceae and Ruminococcaceae families, have been frequently mentioned, indicating the existence of a core of breast milk microbiota (Boudry *et al.,* 2021).

In fact, some *Staphylococcus epidermidis* strains that prevent *Staphylococcus aureus* from colonizing have been proposed as a potential method for eliminating the pathogen from mucosal surfaces (Iwase *et al.,* 2010). Viridans streptococci have also been found to prevent oral colonization by methicillin-resistant *S. aureus* in high-risk babies that exposed to a hospital environment (Fernández *et al.,* 2013). *Streptococcus infantis* is a non-inflammatory and commensal species that has frequently been found in breast milk samples, as well as in pairs of samples of breast milk and mouth swabs from breastfed infants (Biagi *et al.,* 2017). Even though *S. infantis* is a typical oral pathogen, this species frequently functions as a mediator in processes to eliminate the gut pathogens like *E. coli* (He *et al.,* 2014).

According to Johnstone *et al.* (2017), the presence of corynebacteria in deep breast tissue and within vacuoles that are encircled by granulomatous inflammation confirms the harmful role of the *Corynebacterium.* Inflammatory granulomatous mastitis is caused by *Corynebacterium kroppenstedtii* (Paviour *et al.,* 2002). *Bifidobacterium* have a significant impact on how the gut microbiota of breastfed newborns is structured, due to their capacity to consume the oligosaccharides present in human milk (LoCascio *et al.,* 2010). Some bifidobacteria do breakdown glycans externally, such as *Bifidobacterium bifidum,* which produces external glycosyl hydrolases that have been proven to encourage cross-feeding (Egan *et al.,* 2014). *Bifidobacterium longum* subsp. *infantis* colonized premature infants better than *Bifidobacterium animalis* subsp. *lactis* when given concurrently with human milk, likely due to the capacity of *B. longum* subsp. *infantis* has the ability to fully consume a wider variety of complex Human milk glycans (HMGs) (LoCascio *et al.,* 2010).

S-layer proteins are produced by *L. brevis* strains discovered in the human milk microbiota. S-proteins from *Lactobacillus* species, namely *L. brevis* strains, play a regulatory role in modifying the effector activities of dendritic cells (DCs), but S-proteins from other *Lactobacillus* species exhibit immunomodulatory activity in the gut. A balance in the cytokine response is achieved when the macrophage-inducible C-type lectin (Mincle) recognizes the S-layer of *L. brevis*. This interaction causes the generation of both pro- (IL-6 and TNF) and anti-inflammatory (IL-10 and TGF- β) cytokines. Furthermore, the intestinal immune barrier is reinforced, microbial translocation is restricted, and systemic inflammation and its metabolic effects are prevented by promoting the synthesis of IL-6 (Prado *et al.*, 2021). Another properties from *Lactobacillus* is in the absence of an inflammatory stimulus, *Lactobacillus fermentum* CECT 5716 and *Lactobacillus salivarius* CECT 5713 increased macrophage production of Th1 cytokines, such as IL-2 and IL-12 and the inflammatory mediator TNF- α (Fernández *et al.*, 2013). They also have a wide range of effects on the immune system, acting as moderately strong activators of CD4+ and CD8+ T cells and regulatory T cells and potent activators of NK cell (Pérez-Cano *et al.*, 2010). In terms of allergic diseases, the human breast milk strain *Lactobacillus gasseri* CECT 5714 decreased the incidence and intensity of the allergic response in an animal model of cow's milk protein allergy (Fernández *et al.*, 2013).

Enterococcus spp., particularly *E. faecium* and *E. faecalis*, have been identified as the common species in the breastfed infant's gut microbiota (Rahmani *et al.*, 2020). *Enterococcus faecalis* can cause beta-haemolysis, meanwhile *E. faecium* C2 strain isolated from human breast milk might be a potential novel probiotic (Khalkhali and Mojgani, 2018). *E. faecium* might carry antibiotic resistance genes on plasmids and it has

also been demonstrated that antibiotic resistance could be transmitted through breast milk (Pärnänen *et al.,* 2018). Breast milk can take on a pinkish hue because of *Serratia marcescens* colonization. This rod-shaped gram-negative bacterium is responsible for producing the reddish-orange tripyrrole pigment called prodigiosin, which has been linked to a number of illnesses and even newborn deaths (del Valle and Salinas, 2014). *Acinetobacter* species are gram-negative, non-fermenting, aerobic, non-motile, catalase-positive, indole-negative, and oxidase-negative bacteria (Kurcik-Trajkovska, 2009). Most of *Acinetobacter* species are opportunistic pathogens with low levels of virulence and clinical significance, such as *Acinetobacter baumannii* that associated with nosocomial infections. Additionally, the presence of *Acinetobacter* has also been linked to a decrease in allergies in 6-month-old newborns, indicating that these bacteria may have a significant immunomodulatory effect (Ruokolainen *et al.,* 2020).

Besides bacteria, viruses can be transmitted via breast milk from mother to infant in some maternal viral infections. Exposure to modest amounts of virus in human milk numerous times per day during breastfeeding likely incorporates transmission from breastfeeding. The most frequent viral infections for which human milk consumption may provide a risk of transmission include HIV, cytomegalovirus (CMV), and human T-lymphotropic virus (HTLV) (Madore and Fisher, 2021). HIV has been found in both cell-free and cell-associated human milk, and the rate of transmission is linked to maternal viral load. Although antiretroviral therapy is anticipated to reduce the amounts of free HIV in human milk, the presence of cell-associated virus (intracellular HIV DNA) may persist and constitute a transmission risk (Gaillard *et al.,* 2004).

Due to a highly developed immune system and the acquisition of transplacental pathogens, postnatally acquired CMV seldom causes clinically severe illness in full-term newborns. Preterm neonates with CMV IgG-positive moms have transmission rates ranging from 6% to 58%, with the median rates of symptomatic disease being 3.7%, and the most vulnerable infants being the more immature ones. (Kurath *et al.,* 2010).

In 90% of infants who are infected before or at birth, chronic hepatitis B virus (HBV) infection develops. If infected after birth, approximately 30% acquire chronic HBV, which can present as chronic active infection, chronic persistent hepatitis, cirrhosis, and an increased risk of hepatocellular cancer later in life (AAPC, 2018). In this case, the benefits of human milk outweigh the theoretical danger of transmission. Infants born to hepatitis B surface antigen positive mothers should get HBV immunoglobulin and the first dose of monovalent HBV vaccine within 12 hours of birth, according to standard recommendations (Shi *et al.,* 2011).

Women who are infected with human T-lymphotropic virus (HTLV) type I or II should not breastfeed or supply expressed milk. HTLV type I is linked to malignant neoplasms and neurological problems, and early life transmission is linked to an increased risk of leukemia. Breastfeeding is the primary method of infant transmission. HTLV type II is a virus that causes chronic ataxia and multisystem diseases and has been found in human milk. Breastfeeding or providing expressed milk is not suggested for HTLV-II seropositive women, as it is for HTLV-I (Carneiro-Proietti *et al.,* 2014).

2.2 Vaginal Infections

The most common identified vaginal infection were bacterial vaginosis (BV) as the leading cause of vaginal infection in symptomatic women (22–50%), followed by vulvovaginal candidiasis (17–19%) and finally aerobic vaginitis (approximately 11%). No significant difference was observed for each etiology compared to socio-demographic data, but variety of different risk factors, such as ethnicity and geographic location, have been found to influence the prevalence of BV. Several authors reported different bacterial vaginosis prevalence in Asia, Europe, Africa, and Latin America (Salinas *et al.*, 2020).

2.2.1 Type of Vaginal Infections

Vaginal infection is the frequent gynecological condition affecting millions of women of reproductive age annually and the primary reason for seeking gynecological medical attention (Machado *et al.,* 2017). The most prevalent vaginal infections are brought on by bacteria (such as bacterial vaginosis, BV and aerobic vaginitis, AV), fungi (vulvovaginal candidiasis, VVC), and protozoa (trichomoniasis) (Palmeira-de-Oliveira *et al.*, 2015). Vaginal infections are typically characterized by a shift in the microbial communities, including the gradual replacement of some *Lactobacillus* species by pathogenic or opportunistic microorganisms (Machado *et al.,* 2017). BV usually associated with several anaerobic or facultative bacteria, the most prevalent being: *Gardnerella vaginalis; Atopobium sp.; Prevotella sp.; Bacteroides sp.; Peptostreptococcus sp.; Mobiluncus sp.; Sneathia sp.; Leptotrichia sp*.; and genital *Mycoplasma,* such as *Mycoplasma hominis* and *Ureaplasma urealyticum* (Salinas *et al.*, 2020). A feature of BV is an elevated vaginal pH (diagnostic threshold pH>4.5), which is blatantly at odds with a typical healthy vaginal milieu. This is followed by a considerable

decrease in the number of protective lactobacilli (Palmeira-de-Oliveira *et al.*, 2015). The diagnostic criteria and Nugent rating are based on semiquantitative microscopic assessment of Gram-stained vaginal smears. If a sample has a Nugent score of 7 or more, it is considered to have BV according to this scale because anaerobic bacteria have replaced the lactobacilli in the sample. Lactobacilli are prevalent in healthy vaginal samples, in contrast (Nugent score of 3 or lower). Vaginal smears that match to the 'intermediate flora' are found between these groups (Nugent *et al.*, 1991).

Aerobic vaginitis (AV) is associated with inflammation, as shown by microscopic examination of leukocytes and parabasal cells in vaginal samples and a considerable rise in the quantity of vaginal proinflammatory cytokines in vaginal washings (Donders *et al.*, 2002). AV can be frequently caused by group B streptococci, *E. coli*, *E. faecalis*, enterococci, and among other aerobic bacteria (Salinas *et al.*, 2020). AV and BV are frequently linked with an increased risk of acquiring human immunodeficiency virus (HIV), Herpes simplex type 2, and other sexually transmitted infections with *Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis*, among others (Datcu, 2014).

Vulvovaginal candidiasis (VVC) is the second most common vaginal infection, after BV, that caused by *Candida* spp. According to estimates, 70–75% of women will experience VVC at some point in their lives, and by the age of 25, almost half of women will have experienced at least one episode (Sobel, 2007). Typical symptoms of VVC include vaginal discomfort and discharge, vulvar burning, pruritus, and swelling. VVC is mostly due to *Candida albicans, Candida glabrata*, and *Candida tropicalis* (Salinas *et al.*, 2020). Trichomoniasis is the most prevalent sexually transmitted disease in the world, that brought on by the protozoan *Trichomonas vaginalis*. Up to 80% of trichomoniasis

infections are asymptomatic, and they might last for several months. When symptoms do occur, they are distinguished by diffuse, foul-smelling, yellow-green, thin, watery vaginal discharge with a pH of >4.5 and vulvar discomfort (Palmeira-de-Oliveira *et al.*, 2015).

2.2.2 Factors Affecting Vaginal Infection during Pregnancy

Pregnancy causes an increase in estrogen levels, which can create a more favorable environment for yeast growth. Some studies found that estrogen reduces the resistance of epithelial cells in vaginal against pathogen, meanwhile progesterone enhances the pathogens to adhere in vaginal epithelium (Fidel *et al.*, 2000). Estrogen and progesterone may lead to colonization and virulence of pathogen strains, leading to severe diseases (Li *et al.*, 2022). In particular, increased level of estrogen can increase glycogen production in vaginal epithelium. This increase in glycogen becomes favorable for glucose-fermenting microorganism (Li *et al.*, 2022). The immune system is naturally suppressed during pregnancy, making it harder for the body to fight off infection that enhance susceptibility to vaginal infection, especially vulvovaginal candidiasis (Disha and Haque, 2022).

Vaginal epithelial mucosa has a role as physiological barrier against invasion of the intrauterine compartment by microorganisms during pregnancy. The vaginal microbiota undergoes significant changes during pregnancy by increased stability, a decrease in overall diversity, and predominance of *Lactobacillus* species (Bagga and Arora, 2020). Abundance of *Lactobacilli* in pregnancy results in reduction of vaginal pH and an increased vaginal gland secretion which acts as a barrier against pathogen (Prince *et al.*, 2014). The big changes in the vaginal microbiome happens during early pregnancy,

while during later stages of pregnancy and the puerperium, the vaginal microbiome gets back to baseline with an increase in diversity, decrease in *Lactobacilli* and enrichment of bacterial associates (Bagga and Arora, 2020). A hostile environment is created for the colonization of pathogenic bacteria and fungi when lactic acid produced by lactobacilli acidifies the pH of the vagina (O'Hanlon *et al.,* 2013). High intake of sugar can promote the growth of yeast. Vaginal colonization with *Candida* is more frequent in diabetic women than in non-diabetics. Women with type 2 diabetes are more prone to colonisation with *Candida glabrata* (de Leon *et al.,* 2002).

2.2.3 Prominent *Candida* that Causes Vaginal Candidiasis

An infection of the vulva and/or vagina brought on by a *Candida* species is known as vulvovaginal candidiasis (VVC). After bacterial vaginosis, it is the second most frequent cause for vaginal infections (Nagashima *et al.,* 2016). About 90% of the time, it is caused by an overgrowth of *Candida albicans,* which is a common vaginal flora member and an opportunistic pathogenic yeast. This dimorphic commensal yeast typically contributes to the colonisation of the skin, gastrointestinal tract, and reproductive systems (Disha and Haque, 2022). When the equilibrium between the colonising yeast and the host becomes momentarily off, *Candida* spp. can result in an infection like VVC. The most common non-albicans *Candida* spp. that causes VVC is *Candida glabrata* (Erdem *et al.,* 2003). *Candida parapsilosis, Candida tropicalis,* and *Candida krusei* are rarely the causes of vaginitis, despite the fact that the majority of *Candida* species have been linked to the disease. Clinically, vaginitis produced by non-*Candida albicans* species is identical to vaginitis caused by *C. albicans*, and these species are frequently more treatment-resistant.

Recurrent vulvovaginal candidiasis is commonly brought by non-albicans *Candida* spp., particularly *Candida glabrata* (Sobel, 2007).

Compared to non-albicans species, *C. albicans* attaches to vaginal epithelial cells in much greater quantities. All *C. albicans* strains appear to attach to exfoliated buccal and vaginal epithelial cells equally effective. *Candida* organisms mainly enter the vaginal lumen and secretions from the surrounding perianal region (Barousse *et al.*, 2004). Contrarily, in adhesion experiments, there is a significant interindividual difference in the in vitro vaginal epithelial cell receptivity to *Candida* organisms. However, women with recurrent infection have not been associated with an increase in receptivity (Sobel, 2007).

2.3 Immunological and Antimicrobial Properties of Breast Milk

2.3.1 Immunological Properties of Breast Milk

Breast milk contains antibodies that may act as an immunological memory by protecting the mother against various infections she may have been exposed to during her life. The mammary glands, a branching epithelial structure with milk-transporting lactiferous ducts and alveoli able to deliver or transfer antibodies to breast milk (Atyeo and Alter, 2021).

2.3.1(a) Immunoglobulin (Ig)

As the immunoglobulin that makes up 80 to 90% of the total immunoglobulins in breast milk, immunoglobulin A (IgA) is thought to be the immunoglobulin that is most abundant in breast milk. Breastfed newborns receive about 0.3 g/kg/day of this protein. IgA contains anti-inflammatory properties that able to prevent microbial invasion (Lepage

and Van De Perre, 2012). Royle *et al.* (2003) stated that milk IgA possesses an "additional binding site" for bacterial lectin receptors, i.e., sialylated and/or fucosylated glycans, which depict the connection between innate and acquired immunity. Early colostrum (days 2–5) has the highest reported concentration of IgA (around 2.5 g/L), and the level remains largely stable through transitional (days 8–12, around 1 g/L) to mature milk (days 26–30, around 0.7 g/L) milk. (Trend *et al.*, 2016). IgA is high at the first month of lactation and then gradually decreasing until 6 months (Goonatilleke *et al.*, 2019).

Immunoglobulin M (IgM) antibodies can be found in quantities of up to 2.5 mg/mL. These antibodies are crucial for protecting their mucosal surfaces of infants due to their high avidity with viruses and bacteria. IgM against pathogen via opsonization of gram-negative bacteria (Lepage and Van De Perre, 2012). Strong affinities of dimeric IgA and pentameric IgM antibodies are carried via the pIgR and may even bind to dormant viruses (such as rotavirus and influenza) inside of epithelial cells, and bring these pathogens and their byproducts back into the lumen, so preventing cytolytic damage to the epithelium (Johansen and Brandtzaeg, 2004). Goonatilleke *et al.* (2019) showed the gradual decrease in IgM concentration from the beginning of lactation until 6 months.

In both the term and preterm groups, the concentration of milk IgG is highest in the first three days of lactation (27.9 ± 23.2 and 41.7 ± 17.3 mg/L, respectively). However, in the following days of lactation, from 4 to 55 days, it decreased and stayed at a similar level, 16.6 ± 10.6 and 16.7 ± 8.8 mg/L, respectively, regardless of the week of delivery (Czosnykowska-łukacka *et al.*, 2020). IgG can activate phagocytes, display anti-inflammatory activity, and reduce excessive inflammatory reactions in response to allergens in term of their antimicrobial function (Czosnykowska-łukacka *et al.*, 2020).

2.3.1(b) Interleukin

Leukocytes and tissue cells go through dimerization and oligomerization in order to produce cytokines. Disulfide bridges between cysteine residues give cytokines their three-dimensional structure. Interleukins are cytokine components that are involved in the immune system's signaling pathways (Kielbasa *et al.*, 2021). The body's immunological response is triggered and maintained by cytokines, which also have anti-inflammatory effects. The production of irregular cytokines can have a negative impact on health and play a role in the emergence of food allergies, immunological illnesses, and jaundice in the future. Pro-inflammatory cytokines are consisting of TNF-α, IL-6, IL-8, IL-12, IL-2, and INF-γ. Meanwhile, anti-inflammatory cytokines including TGF-ß, IL-7, IL-10, IL-18, and G-CSF (Dinarello, 2007).

Certain cytokines, such IL-1 and IFN-γ, have an impact on the mammary gland's ability to produce defensive agents, SIgA, or other cytokines. Other cytokines like IL-6 and TNF-α are linked with controlling the growth and functioning of the mammary gland (Palmeira and Carneiro-Sampaio, 2016). Both the whey fraction and fat of breast milk include IL-10, an important anti-inflammatory cytokine. Since IL-10's molecular weight in breast milk (>80 kD) is larger than its molecular weight in serum, it is likely that IL-10 has undergone post-transcriptional alteration or is otherwise interacting with other molecules. IL-10, which likewise reduces Th1 responses, encourages B cell proliferation and survival, downregulates the expression of the major histocompatibility complex-II on these cells, and also limits the ability of monocytes to function as antigen-presenting cells. IL-10 has been closely associated in reducing gastrointestinal inflammation and regulating responses to the microbiota (Dawod *et al.*, 2019).