Human Milk Microbiome: A Perspective to Healthy and Infected Individuals

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Abstract

Human milk is a vital source of nutrient as well as a continuous source of bacteria to newborn. Microbes are present in milk aid to initiation and development of infant gut microflora. These bacteria play a vital role in lessening of incidences and severity of infection to the child. Breast milk protects the newborn against infectious diseases, as it consists of different antimicrobial compounds, immunoglobulin, immune component cells, and bacteriocins secreted by probiotic bacteria, which all together provoke the growth of the helpful bacteria in neonate gut. However, breastfeeding mothers may also experience a condition called mastitis. Mastitis, one of the most common conditions experienced by breastfeeding mother, is an inflammation of connective tissue within the mammary gland. It is caused by a mixture of pathogenic bacteria and often treated with antimicrobials. The recent advances in metagenomic sequencing and amplicon sequencing technologies, which try to capture all the DNA information from the biological sample, have been widely used for the characterization of microbial community present within a sample and identification of unknown etiological agents involved in diseased condition. In the present review, effort has been made to understand the development of milk microflora and also the microbial diversity in healthy and infected breast. The present article reveals that breast milk is a source of more life than we envision.

Keywords

Human milk • Microbiota • Metagenomics • Mastitis

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7.1 Introduction

The human microbiome is defined as collection of microbial species that colonize many body sites, including human milk. The human microbiome project was undertaken by the National Institutes of Health with a goal to conduct survey of microbes present within the body and those resting on human body and the potential impact these communities have on health. However, one of the key systems was ignored, human milk. Human milk was conventionally considered as sterile; however, recent examination discovered a constant foundation of commensal, mutualistic, and probiotic bacteria in human milk.

7.2 Human Milk

Human milk is an intricate biological fluid which fulfills the nutritional supplies of newborn baby, helps in the development of infant immune system, and provides defense against pathogens (Morrow and Rangel 2004). Bioactive molecules like polyamines, oligosaccharides, fatty acids, lactoferrin, lysozyme, immunoglobulin, immune-competent cells, and antimicrobial peptides present in colostrum and milk (Newburg 2005) are the main constituents involved in providing defense. Recent studies articulated the presence of not only environmental bacteria but also the symbiotic and probiotic bacteria in the milk which are transmitted through milk to the infant and hence contribute in initial colonization of gut microflora of the infant (Martín et al. 2009). Daily consumption of human milk by an infant is 800 ml/day; this in fact contributes to transport of 1×10^5 to 1×10^7 bacteria each day leading to their colonization in gut microflora (Heikkilä and Saris 2003). Human milk protects against gastrointestinal infections (Duijts et al. 2010), respiratory infections (Nishimura et al. 2009), and allergic diseases (Greer et al. 2008; Ip et al. 2008). According to the American Academy of Pediatrics (AAP), it also trims down the possibility of diseases like inflammatory bowel disease (IBD), obesity, or diabetes.

As neonates are born with immature immune system, they are more prone to get infected. At that time breastfeeding helps in building up the immune system by providing fatty acids, α-lactalbumin, sIgA, oligosaccharides, lactoferrin, lysozyme, antioxidants, and cytokine molecules bearing immune-protective role (Chirico et al. 2008; Goldman 2007). Human milk proteome consists of 976 proteins, out of which plentiful possess immunogenic property (Molinari et al. 2013; Gao et al. 2012). In addition to immune molecules, human milk also consists of blood-derived leukocytes which get transported to milk via the paracellular pathway. Bacteria present in human milk play numerous roles in the infant gut; they reduce the occurrence and severity of infections, produce antimicrobial compounds, or improve intestinal barrier function by enhancing mucin production and dropping intestinal permeability (Olivares et al. 2014). Studies have shown that accumulation of *Lactobacillus* strain, isolated from human milk, reduces the incidence of gastrointestinal infection, upper respiratory tract infections, and total number of infections to 46%, 27%, and 30% (Maldonado et al. 2012). These microorganisms also contribute in digestion by breaking down sugars and proteins and also participate in the right maturation of the infant immune system.

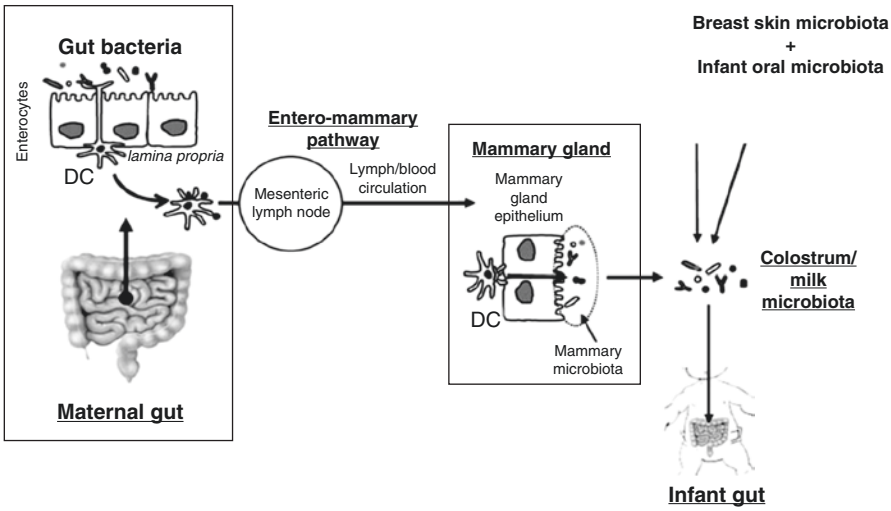

Fig. 7.1 Origin of microflora in human breast milk. Source: Fernández et al. (2013)

7.3 Origin of Microflora in the Human Milk

Physiological and hormonal alteration occurring during and after pregnancy increased gut permeability which in turn helps in the transfer of gut microflora to the mammary gland. Dendritic cells and macrophages also play an important role in the migration of microbes to the mammary gland (Fernández et al. 2013). These bacteria are transferred from maternal community to breast milk via the entero-mammary pathway (Fig. 7.1). Along with above apparent mechanisms, the retrograde flux between the mother's skin microbes and infant's oral microbes may also help in the development of the human milk microbiome (Makino et al. 2011; Albesharat et al. 2011).

7.4 Mechanism of Health-Promoting Probiotic Bacteria

The milk microbiota plays a significant role in decreasing the frequency of infection to the newborn babies due to their probiotic properties (Fig. 7.2). Probiotics have a potential to produce antimicrobial substance like bacteriocins which work as antagonists to the pathogenic bacteria and their efficient antagonistic activity is by alone or synergistically. These antimicrobial substances can be protein and bioactive peptides. Bacteriocins are important antimicrobial peptides which have therapeutic activity against intestinal pathogenic microbes (Thirabunyanon et al. 2009; Verdenelli et al. 2009; Gaudana et al. 2010). They also produce metabolites, i.e., acetic and lactic acids, which reduce the pH in the intestine and generate adverse environment for pathogen to survive (Ridwan et al. 2008). Probiotics can remove pathogens using competitive exclusion and/or blocking their attachment at the intestinal epithelium cells by competing for the glycoconjugate receptors (Vanderpool et al. 2008).

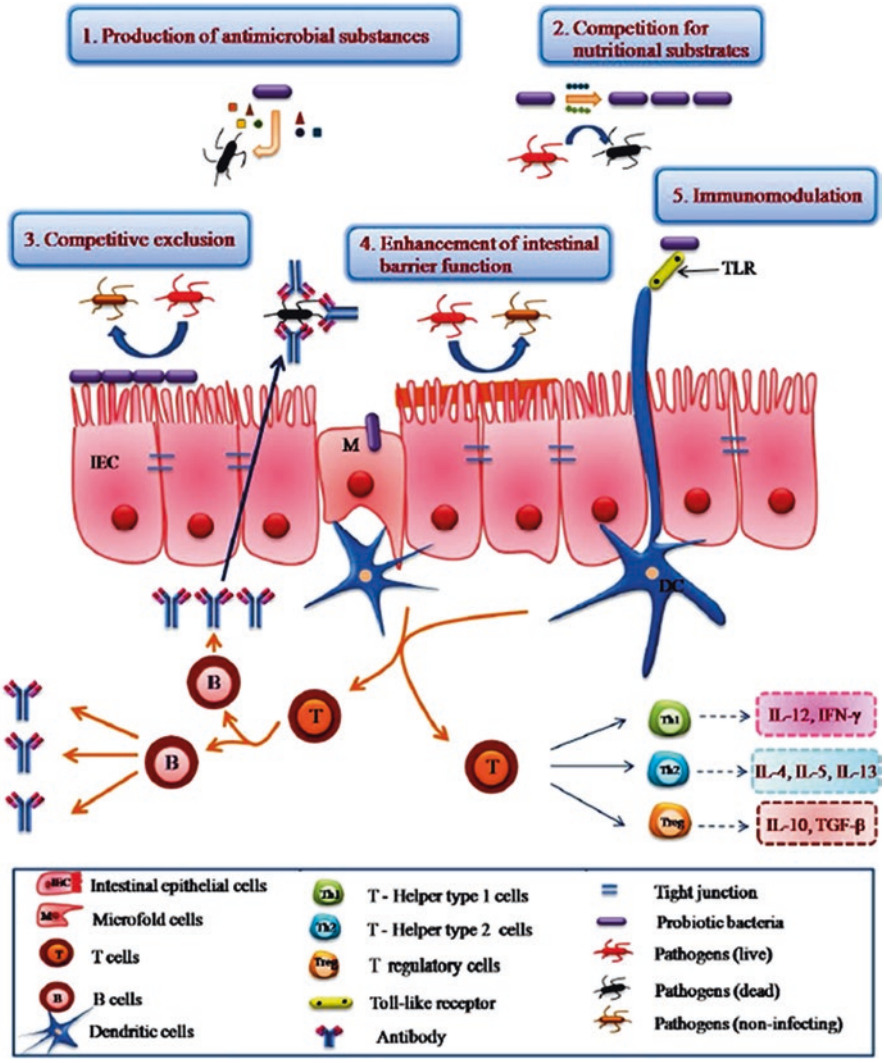

Fig. 7.2 Mechanisms of action of probiotic bacteria

7.5 Cell of Human Milk

Human milk alters in composition since colostrum to late lactation and varies within feeds and between mothers. Human milk consists of 75% leukocytes, i.e., neutrophils, erythrocytes, macrophages, and lymphocytes, and 25% epithelial cells (Paape and Weinland 1988). The epithelial cells of the glands are normally shed and get renewed, but at the time of infection, the number increases. The white blood cells work as a defense mechanism which fight against the infection and help in the repair of damaged tissue. During inflammation, it was observed that the level of neutrophils increases by 90% in human milk to fight against infection (Miller et al. 1985; Cooey

and Harmon 1994). Moreover, composition of somatic cells in human milk changes with respect to lactation cycle and type of secretion (Table 7.1). Generally, the number of SCC in human milk from healthy mammary gland is approximately 1×10^5 cells/ml, while challenge with bacterial infection causes it to increase above 1×10^6 cells/ml (Bytyqi et al. 2010). Of the somatic cells, leukocyte is the most studied cell type in human milk, and depending on stage of lactation and health status of breastfeeding dyad, it may account for considerable portion of human milk (Boutinaud and Jammes 2002; Hassiotou et al. 2012; Cregan 2002; Ho et al. 1979). Many of these leukocytes are activated, motile, and interactive (Smith and Goldman 1970). This suggests that they confer active immunity to the infant (Wirt et al. 1991).

Table 7.1 Microbial diversity of human milk studied by culture-dependent and culture-independent methods

Sr. no	Author's name	Country and sample size	Experimental techniques	Identified microbial profiles
1	Martín et al. (2003)	*Spain* No. of samples 8 4 days postpartum	Culturing and identification of lactic acid bacteria using RAPD analysis	Lactic acid bacteria, specifically *Lactobacillus gasseri* and *Enterococcus faecium*, were present in all the milk samples
2	Grönlund et al. (2007)	*Finland* No. of samples 61 mothers and infant pairs	Real-time PCR	Bifidobacteria were noticed in all milk samples with the *Bifidobacterium longum* being most abundant
3	Collado et al. (2009)	*Spain* No. of samples 50	qPCR	*Lactobacillus*, *Bifidobacterium*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Clostridium* clusters XIVa–XIVb were the mainly abundant
4	Solís et al. (2010)	*Spain* No. of samples 20 mothers and infants. At day 1, 10 days, 1 month, and 3 months postpartum	Culturing and identification of lactic acid bacteria and bifidobacteria using 16S rRNA sequencing and RAPD	*Streptococcus*, i.e., *Streptococcus salivarius*, was predominant followed by *Lactobacillus* and *Bifidobacterium*
5	Albesharat et al. (2011)	*Syria* No. of samples 30 mothers and infant pain (1 month to 2 years postpartum). Human milk, maternal/infant feces, and fermented foods were collected	Culturing and identification of lactic acid bacteria using RAPD, 16S rRNA sequencing, and matrix-assisted laser desorption/ionization (MALDI)	Lactic acid bacteria like *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Streptococcus*, *Staphylococcus*, and *Weissell*a were isolated

(continued)

Table 7.1 (continued)

Sr. no	Author's name	Country and sample size	Experimental techniques	Identified microbial profiles
6	Hunt et al. (2011)	*United States* No. of samples 16 22–26 weeks postpartum three samples collected from each subjects	Pyrosequencing approach	Most abundant genera were *Streptococcus*, *Staphylococcus*, *Serratia*, and *Corynebacterium*. "Core" microbiome includes *Staphylococcus*, *Streptococcus*, *Serratia*, *Pseudomonas*, *Corynebacterium*, *Ralstonia*, *Propionibacterium*, *Sphingomonas*, *Bradyrhizobium*
7	Collado et al. (2012)	*Finland* No. of samples 56 mothers (22 overweight and 34 normal weight) and their infants. 1–2 days (colostrum), 1 month, and 6 months postpartum	qPCR	Most abundant genera were *Lactobacillus*, *Bifidobacterium*, and *Staphylococcus* *Staphylococcus* occurred in higher abundance, and *Bifidobacterium* and *Lactobacillus* were observed in lower abundance in overweight mother
8	Cabrera-Rubio et al. (2012)	*Finland* No. of samples 18 0–2 days, 1 month, and 6 months postpartum	Pyrosequencing, qPCR	*Weissella*, *Leuconostoc*, *Staphylococcus*, *Streptococcus*, and *Lactococcus* dominant in colostrum, whereas *Leuconostoc*, *Weissella*, *Lactococcus*, and *Staphylococcus* in mature milk
9	Bhatt et al. (2012)	*India* No. of samples 7 Randomly milk samples collected	Cultured probiotic bacteria	*Lactobacillus fermentum*, *Enterococcus mundtii*, *Enterococcus faecium*, *Lactobacillus reuteri*, *and Bacillus subtilis* were identified by 16S approach
10	Gonzalez et al. (2013)	*Mozambique* No. of samples 55 (29 of whom tested positive for HIV) 14 days, 15–90 days, 91–180 days, and 181–360 days postpartum	Culturing of nonfastidious bacteria, yeasts, molds, qPCR	44 genera and 124 species were identified; commonly cultured isolates belonged to *Staphylococci*, *Streptococci*, and *Lactobacilli*

Table 7.1 (continued)

Sr. no	Author's name	Country and sample size	Experimental techniques	Identified microbial profiles
11	Jost et al. (2013)	*Switzerland* No. of samples 7 3–6 days, 9–14 days, and 25–30 days postpartum	Pyrosequencing, RAPD, Sanger sequencing	Firmicutes and Proteobacteria dominated. *Staphylococcus*, *Streptococcus*, *Pseudomonas*, and *Ralstonia* were the most abundant genera
12	Ward et al. (2013) and Khodayar-Pardo et al. (2014)	*Canada* No. of samples 1 (ten milk samples pooled) 9–30 days postpartum	Metagenomic sequencing on Illumina	360 genera were identified. Proteobacteria (65%) and Firmicutes (34%) dominated; *Pseudomonas* and *Staphylococcus* were the most abundant genera
13	Khodayar-Pardo et al. (2014)	*Spain* No. of samples 32 1–5 days, 6–15 days, and 17–18 days postpartum	qPCR	*Lactobacillus*, *Streptococcus*, and *Enterococcus* spp. were most prevalent
14	Olivares et al. (2014)	*Spain* No. of samples 24 (half with celiac disease) 1 month postpartum	qPCR	*Bifidobacterium* spp. were observed in all milk samples. *Bifidobacterium bifidum* and *Bifidobacterium breve* were the most abundant
15	Urbaniak et al. (2014)	*Canada* No. of samples 9 (one undergoing chemotherapy related to Hodgkin's lymphoma)	Ion Torrent sequencing	Chemotherapy was associated with lower microbial diversity and altered bacterial profiles: decreased percentage abundances of *Acinetobacter* and Xanthomonadaceae with chemotherapy
16	Soto et al. (2014)	*Germany and Austria* No. of samples 160 Mainly 1–4 weeks postpartum	Culturing of *Lactobacilli* and bifidobacteria Its identification by 16S sequencing	*Lactobacilli* and bifidobacteria were isolated and identified

(continued)

Table 7.1 (continued)

Sr. no	Author's name	Country and sample size	Experimental techniques	Identified microbial profiles
17	Vaidya et al. (2015)	*India* No. of samples 32	Culture-dependent method Sanger sequencing	At species level, *Enterococcus faecalis*, *Lactococcus lactis*, *Bacillus litoralis*, *Bacillus licheniformis*, *Bacillus safensis*, *Lactobacillus oris*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermis*, *Lysinibacillus* spp. were identified
18	Cabrera-Rubio et al. (2015)	*Spain* No. of samples 10 (six vaginally and four cesarean delivered mothers)	Pyrosequencing qPCR	Alteration in microbiome of human milk based on mode of delivery. *Streptococcus*, *Staphylococcus*, *Enterobacter*, and *Pseudomonas* were the most abundant genera
19	Urbaniak et al. (2016)	*Canada* No. of samples 39	Illumina sequencing	No statistical difference was observed in human milk microbiome based on birthing method, gestation time, and infant gender. *Staphylococcus*, *Pseudomonas*, *Streptococcus*, and *Lactobacillus* were the most abundant genera
20	Sakwinska et al. (2016)	*China* No. of samples 90 (30 samples without aseptic cleansing and 60 samples collected aseptically)	Illumina 16S sequencing qPCR	*Streptococci* and *Staphylococci* dominated in both collection procedures. *Acinetobacter* was predominant in milk collected without aseptic cleansing

This was further supported by in vivo studies in animal models showing active transfer of milk leukocyte through the intestinal epithelium into the blood circulation, and movement to and engraftment in different organs, including the mesenteric nodes, liver, and spleen (Weiler et al. 1983; Zhou et al. 2000; Michie et al. 1998; Schnorr and Pearson 1984).

7.6 Microbial Profiling of Human Milk

During the last decades, microbiological studies that focused on human milk were restricted to the identification of potential pathogenic bacteria in stored milk or milk retrieved from maternal infected human milk, but microbes present in healthy

human milk are unexplored (El-Mohandes et al. 1993; Wright et al. 1998). Standard microbiological based culturing methods can only detect small proportion of bacteria because the great majority of bacteria on earth are not culturable in laboratory condition. To identify these unculturables and estimate real bacterial diversity, culture-independent method is required. Sequence-based identification of microbial species through sequencing has overcome the limitation. The nine hypervariable regions of 16S rRNA can be used for identification of bacterial species. Amplification of 16S rRNA region using universal primer is useful for estimation of bacterial diversity.

7.7 Culture-Dependent Assessment of Human Milk Microbial Diversity

Initial report of culture-dependent methods for studying human milk microbial diversity came in 2003 by Dr. Juan Rodriguez with his associate researcher R. Martin. They isolated a total of 178 isolates from each mother and infant pair (human milk, nipple areola, infant's mouth and feces) and subjected it to randomly amplified polymorphic DNA (RAPD) analysis and identified by 16S rDNA sequencing. Bacteria having identical profiles in mother and child pair were identified as *Lactobacillus gasseri* and *Enterococcus faecium*. Surprisingly, none of the lactic acid bacteria isolated from breast skin shared RAPD profiles into other sources (Martín et al. 2003).

After that Grönlund et al. (2007) studied the association of maternal fecal and breast milk bifidobacteria and infant fecal bifidobacteria using real-time PCR from 61 mother-infant pairs. They found that *Bifidobacterium longum* was the most abundant species isolated from breast milk. Moreover, they concluded that *Bifidobacterium adolescentis* and *Bifidobacterium bifidum* colonization frequency and count correlated significantly among mother and infant pairs (Grönlund et al. 2007).

Collado et al. (2009) in their study examined 50 breast milk samples for the presence of differential bacterial genera by using qPCR technique. They found that *Staphylococcus*, *Streptococcus*, *Bifidobacterium*, and *Lactobacillus* were the most abundant genera in all the samples. In addition, Collado et al. (2012) studied the effect of maternal weight and weight gain during pregnancy on milk microbiota (56 mothers, 22 overweight and 34 normal weight) using qPCR. *Staphylococcus* group bacteria were observed in higher number, whereas *Bifidobacterium* group was in lower level, in overweight mother compared to normal-weight mother. Moreover, they found higher prevalence of *Akkermansia muciniphila* in higher number in breast milk of overweight mothers (Collado et al. 2009).

Solís et al. (2010) studied the development of lactic acid bacteria and bifidobacteria during the first 3 months of life in 20 vaginally delivered breastfed infants and mothers. *Streptococcus*, *Lactobacillus*, and *Bifidobacterium* were the most dominant genera in breast milk contributing to the initial establishment of microbiota in newborn (Solís et al. 2010).

Albesharat et al. (2011) isolated a total 700 isolates of LAB from fecal sample of breastfeeding mother, feces of their infant, from breast milk, and from fermented

food that is normally consumed in Syria, and characterized it by RAPD and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Their study demonstrates occurrence of 36 different species of *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Weissella*, and *Pediococcus*. Interestingly, they found identical RAPD genotype of *L. plantarum*, *L. fermentum*, *L. brevis*, *Enterococcus faecium*, *Enterococcus faecalis*, and *P. pentosaceus* in feces of mother, in breast milk of mother, and in feces of her babies (Albesharat et al. 2011).

In 2014, Khodayar-Pardo et al. studied the bacterial population present in 32 Spanish breastfeeding women using quantitative PCR and determine the influence of lactational stage, gestational age, and delivery mode on milk microbiota. They identified *Enterococcus*, *Lactobacillus*, and *Streptococcus* spp. as the dominant bacterial group. They also concluded that *Bifidobacterium* is found more commonly in vaginal than cesarean deliveries (Khodayar-Pardo et al. 2014).

Afterward, Soto et al. (2014) isolated *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, and *Staphylococcus* species from breast milk of 47 Slovenian lactating mother. Moreover, Gonzalez et al. (2013) also found *Staphylococcus*, *Streptococcus*, and *Lactobacillus* genera in breast milk collected from 121 Mozambique women (Albesharat et al. 2011).

7.8 Culture-Independent Assessment of Human Milk Microbial Diversity

In 2011, Hunt et al. used a new approach (454 pyrosequencing), which utilized specific primer targeting the V1–V2 hypervariable region of 16S rRNA gene of bacteria. They characterized microbial diversity and temporal stability of bacterial profiles in healthy human milk collected from 16 US women over a 4-week period (Hunt et al. 2011). Half of the bacterial sequences were contributed by nine "core" OTUs which include *Pseudomonas*, *Staphylococcus*, *Serratia*, *Corynebacterium*, *Ralstonia*, *Streptococcus*, *Sphingomonas*, *Bradyrhizobium*, and *Propionibacterium*. Moreover, the proportion of these core OTUs varied greatly between subjects.

Similarly, Cabrera-Rubio et al. (2012) studied bacterial diversity in human milk over three different time points (colostrum 1 and 6 months postpartum) in 18 Finnish women (Cabrera-Rubio et al. 2012). They found that human milk microbiome changes over lactation period. Bacteria belonging to *Weissella*, *Leuconostoc*, *Staphylococcus*, *Streptococcus*, and *Lactococcus* were more abundant in colostrum. While in 1- and 6-month milk samples, *Veillonella*, *Leptotrichia*, and *Prevotella*, typical inhabitants of oral cavity, increased significantly. Moreover, they concluded that milk from obese mother tends to be altered and less diverse than normal-weight mothers.

Jost et al. (2013) examined bacterial diversity in breast milk of seven mothers at three different sampling points (days 3–6, 9–14, and 25–30 postpartum) using culture-dependent and culture-independent techniques. They found that Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria were the most abundant phyla, including representatives from the genera *Pseudomonas*, *Staphylococcus*, *Ralstonia*, *Streptococcus*, *Bacteroides*, *Blautia*, and *Bifidobacterium*. Moreover, they also

found, for the first time, bacteria belonging to *Faecalibacterium* and *Roseburia*, which are butyrate producers and important for colonic health (Jost et al. 2013).

After that, Ward et al. (2013) performed metagenomic functional analysis of a pooled milk samples from ten donor mothers using Illumina sequencing. Over 360 bacterial genera were identified with predominance of sequences belonging to Proteobacteria and Firmicutes. In addition, they also concluded that human milk is less diverse than the feces of infant and mother at the phylum level. Human milk contained prominent amounts of genetic component which link with nitrogen membrane transport, stress response, metabolism, and immunomodulatory functions (Ward et al. 2013).

In addition, Urbaniak et al. (2014) studied bacterial diversity in human milk collected every 2 weeks over a 4-month period from lactating mothers undergoing chemotherapy of Hodgkin's lymphoma. They found that chemotherapy causes substantial alteration in microbiome from healthy controls, with reduction in genera such as *Bifidobacterium*, *Eubacterium*, *Staphylococcus*, and *Cloacibacterium* (Urbaniak et al. 2014).

Two recent independent studies by Cabrera-Rubio et al. (2015) and Urbaniak et al. (2016) studied the milk microbiota composition of healthy women and correlated it to birthing method. In addition to birthing method, Urbaniak et al. (2016) also studied alteration of milk microbiota with gestation time and infant gender. Urbaniak et al. (2016) in their study collected human milk from 39 Canadian mothers and analyzed microbial profiles by 16S rRNA sequencing using Illumina platform. They found Proteobacteria and Firmicutes as most dominant phyla and *Staphylococcus*, *Pseudomonas*, *Streptococcus*, and *Lactobacillus* as most abundant genera. However, comparison of bacterial profile between term and preterm infants, vaginal and C-section deliveries, and male and female showed no statistical significant difference (Urbaniak et al. 2016). In contrast, Cabrera-Rubio et al. (2015), in their study, compared milk microbiome of six vaginally delivered mothers and four cesarean delivered mothers and found significant separation of milk microbiome based on mode of delivery (Cabrera-Rubio et al. 2015).

The microbiota of breast milk from 90 Chinese lactating women was analyzed with two different collection procedures (without aseptic cleansing and after aseptic cleaning) by Olga Sakwinska et al. (2016). They found that *Streptococci* and *Staphylococci* were the most abundant in both the group and results were consistent with that of previous study. However, they revealed that breast milk collected without aseptic cleansing and rejection of foremilk had higher abundance of *Acinetobacter* sp. Moreover, bifidobacteria and *Lactobacilli* were present in few samples but with low abundance (Sakwinska et al. 2016).

7.9 Overview of Mastitis

Mastitis is an inflammation of connective tissue within the mammary gland (Gianneechini et al. 2002; Zhao and Lacasse 2008). The term comes from the Greek word masto-referring to the mammary gland and its meaning inflammation. It is characterized by physical, chemical, and bacteriological changes in the breast milk.

It is the most common condition experienced by lactating mothers. Incidence of occurrence of mastitis varies extensively because of difference in breastfeeding method. As per the data of WHO (World Health Organization), overall 2–33% of breastfeeding mothers are thought to be infected with mastitis (WHO 2000). Studies conducted in the USA, New Zealand, Finland, and Australia suggest that 20–25% of breastfeeding women have chances of developing mastitis (Kinlay et al. 1998; Fetherston 1997; Foxman et al. 2002). Although mastitis is a very common condition, very few studies are conducted on it till date (Foxman et al. 2002). Mastitis usually affects lactating women; hence, it is known as lactational mastitis.

Mastitis is a deliberately painful condition experienced by breastfeeding mothers. It is mainly found to be prevalent during second and third week postpartum. Mastitis can be caused by an infection or excess of milk remaining in the milk tissue (milk stasis). Mastitis is usually the result of a blocked milk duct that hasn't cleared. Milk banked up behind the blocked duct can be forced into nearby breast tissue, causing the tissue to become inflamed. Sometimes it may occur due to sudden stop of breastfeeding. Infectious mastitis develops when bacteria commonly found on skin enter the mammary gland through cracked nipples and multiply in the fatty tissue of mammary gland resulting in infection.

7.9.1 Mastitis: A Dysbiosis of Breast Milk Bacteria

Breast milk has got vibrant bacterial diversity mainly that related with skin and non-skin. These bacteria are transported from maternal community to breast milk via the entero-mammary pathway. Pathogenesis of mastitis could have resulted from enrichment of pathogenic bacteria in milk and mutual healthy milk microflora killed due to toxins released by pathogenic bacteria.

7.9.1.1 Types of Mastitis

Scandinavian researchers suggested in the 1980s that mastitis should be classified into two classes: clinical mastitis and subclinical mastitis.

Clinical mastitis: It is characterized by the presence of gross inflammatory signs and symptoms.

Clinical mastitis can be divided into three types:

1. *Preacute mastitis*: Inflammation and changes in milk composition. Systemic signs like fever, depression, shivering, loss of appetite, and loss of weight.
2. *Acute mastitis*: Similar to preacute mastitis, but with mild signs like fever and mild depression.
3. *Subacute mastitis*: In this type of mastitis, signs of inflammation are minimal and no visible systemic signs.

Subclinical mastitis: This form of mastitis is characterized by change in milk composition with no signs of gross inflammation or milk abnormalities.

Mastitis is associated with increased somatic cells, free fatty acids, and interleukin-8 concentrations (Hunt et al. 2013). However, fresh milk produced by a mastitis gland has free fatty acids (FFAs) and when stored at 4 °C exhibits greater rates if lipolysis occurs (Randolph and Erwin 1974; Murphy et al. 1989).

7.9.2 Mastitis and Somatic Cell Count

Somatic cells are white blood cells; their number increases during bacterial infection in order to fight against pathogenic bacteria (Sharma et al. 2011). Thus somatic cells can be a better indicator of infectious condition in mammary gland. Somatic cell count in women with mastitis usually has an elevated count compared to healthy women (Hunt et al. 2013; Hassiotou et al. 2013). Intramammary infection results in a significant increase in the somatic cell count level in the breast milk. In response to invasion of mammary gland by bacteria, leukocytes are released into the milk to kill the bacteria, which results in increases in somatic cell numbers and ultimately leads to inflammation and blocked milk ducts. Moreover somatic cells contain lipolytic and proteolytic enzymes, which degrade fats and proteins, respectively. Upon challenge by bacterial infection, the amount of destructive enzymes carried out by increased somatic cells results into deterioration of milk fat and protein. Somatic cell count is often used for diagnosis of mastitis in case of bovine animals.

7.9.3 Etiology of Mastitis

Etiological agents of mastitis can be infectious or noninfectious. Organisms which may cause mastitis are bacteria, viruses, mycoplasma, yeasts, and algae. Gram-positive, catalase-positive bacteria are mostly isolated from mastitis-infected milk. It can be caused by microbes, such as *Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Streptococcus agalactiae*, *Staphylococcus epidermidis*, *Corynebacterium bovis*, *Corynebacterium pyogenes*, *Klebsiella* sp., and *Candida albicans*. Among all of these microorganisms, the most important are *Staphylococcus aureus* and *Staphylococcus epidermidis*, which is a common cause of mastitis, and it is commonly isolated from mastitis-infected milk. In Brazil, studies reported the predominance of *Staphylococcus aureus* over other disease-causing agents in all regions of the country (Rodrigues et al. 2015). Other than this, coagulase-negative *Staphylococci* are considered as minor mastitis-causing pathogens. Nineteen distinct species of coagulase-negative *Staphylococci* have been revealed to date. Members of the *Staphylococcus epidermidis* subgroup include *S. hominis*, *S. warneri*, *S. capitis*, and *S. haemolyticus*. Variety of pathogenic organism causing mastitis can be divided into two groups: contagious mastitis pathogens and environmental mastitis pathogens.

Contagious mastitis pathogens: These are commonly found on the skin and enter into the mammary gland through cracked or sore nipples. The major contagious pathogens are *Staphylococcus aureus* and *Streptococcus agalactiae*.

Environmental mastitis pathogens: Environmental mastitis pathogens are also known as opportunistic mastitis pathogens because they will take the opportunity to cause mastitis and cause intramammary infections sporadically. The most common environmental mastitis pathogens are *Staphylococcus chromogenes*, *Staphylococcus simulans*, *Staphylococcus xylosus*, *Staphylococcus epidermidis*, *Staphylococcus hyicus*, and *Staphylococcus haemolyticus*.

7.9.3.1 *Staphylococcus aureus* and Its Virulence Gene

Staphylococcus aureus is a gram-positive bacteria associated with many serious diseases in humans as well as animals, and it is found to be the most predominant bacteria causing human mastitis with relevant losses in the dairy industry (Bjork et al. 2014; Li et al. 2009). *S. aureus* is the most common species of *Staphylococci* to cause *Staphylococcus* infections. It is frequently found in the human respiratory tract and on the skin. Although *S. aureus* is not always pathogenic, it is a common cause of skin infections (e.g., boils), respiratory disease (e.g., sinusitis), and food poisoning. Other than this *S. aureus* can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils (furuncles), cellulitis folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening disease such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia, and sepsis. Virulence factors, such as surface proteins that promote colonization of host tissues, surface factors that inhibit phagocytic engulfment (protein A), biochemical properties that enhance their survival in phagocytes (catalase production), immunological disguises (protein A, coagulase clotting factor), and acquired resistance to microbial agents, are often expressed by *S. aureus*. Clumping factor is the surface agent that acts as adhesions. Coagulase is tightly bound to the surface of *S. aureus* and coats its surface with fibrin upon contact with blood. This fibrin-coated *S. aureus* resists phagocytosis. Protein A binds to IgG in wrong orientation in serum, thus preventing opsonization and phagocytosis.

The role of bacteria in lactational mastitis is still questionable. Although it is most common among lactating women, there is lack of scientific analysis on it. Culture-dependent and culture-independent assessment of mastitis-infected breast milk can provide in-depth analysis of microflora involved in diseased condition. Culture-dependent studies involve classical microbiological techniques and it has several drawbacks, while culture-independent studies involve assessment of microflora by metagenomic approach with the recent next-generation sequencing technology.

7.9.4 Culture-Dependent Assessment of Mastitis

There are many conventional techniques used for isolation and identification of pathogenic bacteria. Isolation of pathogenic bacteria on the sheep blood agar is

widely used in many laboratories because pathogenic bacteria grow via engulfing the red blood cell and appear as greenish colony. Otherwise if pathogens are not present in breast milk, they cannot grow on blood agar. Molecular typing (molecular markers) techniques such as polymerase chain reaction (PCR) technology provided additional approaches that have been reported and is considered as the most powerful technique for the control and investigation of pathogens. But culture-based approach to isolate microorganism from any environment does not provide comprehensive information on composition of bacterial communities. This technique also failed to determine microorganism which cannot grow in laboratory condition. Most of the studies performed till date on mastitis involve classical microbiological techniques to identify etiology of mastitis.

Kvist et al. (2008) compare bacterial composition in milk samples collected from 192 women with a clinical mastitis and 466 healthy donors. They found that *S. aureus* was present in 45% of women with mastitis and 31% of healthy donors. In both the group, mean colony counts were identical and no correlation was observed between colony counts and symptom severity. Finding hints that the presence of *S. aureus* in breast milk does not always result in clinical mastitis and it is always present in healthy human milk.

Delgado et al. (2008) recognized the role of coagulase-negative *Staphylococcus* spp. in human mastitis. Employing pulsed-field gel electrophoresis, they found that *S. epidermidis* was present in 85% (17/20) of samples collected, while *S. aureus* in 40% (8/20) of samples. After that they compared strains of *S. epidermidis* present in women with mastitis and women with healthy human milk. They found that women with clinical signs of infection were more likely to harbor strains of *S. epidermidis* with the *icaD* (33 vs. 11%, p ¼ 0.03), which was correlated with biofilm production. Thus, virulence factors of *S. epidermidis* strains found in breast milk may play a vital role in pathogenesis.

Using 16S-specific PCR primers, Shriram et al. (2015) identified bacteria belonging to *Staphylococcus* and *Pseudomonas* genera from human milk of 32 mastitis women. Moreover, the authors found 17 genera and 30 different species from mastitis milk suggesting diverse community in diseased condition (Patel et al. 2016).

7.9.5 Culture-Independent Assessment of Mastitis

Traditionally microbial genome sequencing has been restricted to only a small number of organisms which can be grown in pure culture in laboratory. Progressive development of culture-independent methods has allowed researchers to sequence microbial communities directly from environmental samples. Culture-independent techniques deal with the isolation of total DNA from the environmental sample. Culture-independent approach is commonly referred to as "metagenomic" or "community genomics." Metagenomics is applied literally to describe any culture-independent analysis of microbial communities. With the recent development in more advances sequencing techniques, which try to capture all the DNA information from the biological sample have been widely used

for the characterization of microbial community present within a sample and identification of unknown etiological agents involved in diseased condition. Moreover, this type of technology provides identification of thousands of sequences per sample, which increases the possibility to observe less frequent phylotypes that may have significant importance in disease condition. Metagenomics can also be applied to solve practical challenges in the field of medicine, agriculture, sustainability, and ecology. Numerous microbiome studies have been carried out to assess the composition of the bacterial communities inhabiting a variety of human body locations, including the gut (Zhao and Lacasse 2008), oral cavity (Nasidze et al. 2009; Belda-Ferre et al. 2012), vagina (Ravel et al. 2011), skin (Costello et al. 2009), and human milk (Jost et al. 2013; Belda-Ferre et al. 2012; Ward et al. 2013). All of these studies were focused on the bacterial component of the microbiome.

So far only one study has been reported discussing metagenome of breast milk from mastitis-infected women. Jimenez et al. (2015) performed shotgun sequencing of ten healthy and ten mastitis-infected breast milk samples. They found that *Staphylococcus aureus* clearly dominated the microbiome in the samples from the women with acute mastitis, whereas high abundance of *Staphylococcus epidermidis*-related reads was observed in the milk of those suffering from subacute mastitis (Jimenez et al. 2015).

7.9.6 Prevention and Control

Antibiotics are regularly used to treat mastitis. But nowadays development of multiple resistances by different bacteria has led to failure of treatment. It is due to indiscriminate use of antimicrobials without checking its in vitro sensitivity to the causing bacteria (Oliver and Murinda 2012). In addition to antibiotic resistance, formation of biofilm is also an important virulence factor implicated by mastitis-causing pathogens, which allow survival of bacteria at high antimicrobial concentration (Hoiby et al. 2010). Alternative treatment for the antibiotics can be probiotic therapy and herbal therapy.

7.9.7 Probiotic Therapy

Development of new strategies based on probiotics is an alternative or complement to antibiotic therapy for the management of mastitis and is particularly appealing. Use of lactic acid bacteria as oral administration of lactobacilli isolated from breast milk for the treatment of mastitis has been used by researchers (Jimenez et al. 2008). Human milk consists of bacterial species like *Lactobacillus gasseri*, *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactobacillus fermentum*, or *Bifidobacterium breve* with probiotic properties. These bacteria have shown promising results as probiotic agents that might be useful in treating mastitis.

7.9.8 Herbal Therapy

There has been not a single study published till date indicating use of herbal therapy on human mastitis. But in veterinary field, there are some studies focused on the use of natural herbal plant as a remedy for mastitis. It has been reported that garlic tincture or aloe gel can be used as a fast remedy from mastitis (Pol and Ruegg 2007). In literature antimicrobial properties of garlic extracts and *Aloe vera* gels have already been reported (Ross et al. 2001; Agarry et al. 2005). But the use of these compounds to successfully treat mastitis has not been described. In one clinical trial, they have specifically evaluated the clinical efficacy of a botanical treatment to treat subclinical mastitis (Abaineh and Sintayehu 2001). Two different doses of a dried leaf powder of an African perennial herb (*Persicaria senegalense*) were fed for 3–5 days to cows infected with subclinical mastitis. Results of this trial indicated positive effect of herbal medicines in eradication of mastitis but conceded that more research is necessary.

7.10 Conclusion and Future Aspects

In conclusion, there are now convincing proofs that human milk consists of diverse and feasible microbial population, which initially colonize the infant gut. Somehow, variations in microbial profiling in different studies were due to behavioral, environmental, and genetic differences or a consequence of methodological variation. As such, the era has moved away from the past belief that breast milk is sterile and acknowledged the rich microbial community present in human milk.

However, dysbiosis of breast milk microbial community results in a development of mastitis. Monitoring changes in mastitis-causing microflora with metagenomic platforms might be helpful in building a strategy to overcome this problem.

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