Bacteria, Viruses, Membrane-Enclosed Microentities, and Fungi as the Environmental Evolutionary Entities Coexisting in Human Milk §

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Abstract

Traditionally, our understanding of the benefit of human milk and breastfeeding has focused on their nutrition and immunology: (i) breastmilk, as the natural food of newborns, offers various essential nutrients including carbohydrates, lipids, and proteins for the growth and development of suckling offspring; (ii) from an immunological perspective, breastmilk acts as a vector to transfer acquired and innate immune defense factors, including eukaryotic cellular entities and molecular entities, from the maternal body to suckling babies; and (iii) breastfeeding is a prerequisite for taking advantage of the above-mentioned nutritional and immunological values for offspring. Recently, in the fimpological UPOEE model, [1,2] a novel concept called "Evolutionary Background Entities" (EBEs) was proposed to refer to those entities of lower evolutionary levels which are the evolutionary "background organisms" of the entities at higher evolutionary levels. [1,2] In the most recent paper,[2] the following notion has been proposed: (i) animals are not only inhabitants of natural habitats, but they are also the "niches" or "habitats" of evolutionary micro-entities, including bacteria, viruses, and fungi; and (ii) the interaction between an evolutionary entity and its environment is actually the interaction between the entity and its environmental evolutionary entities at the same and/or different evolutionary levels. As the natural food, milk is one of primary environmental factors that mammalian newborns must contact for survival; and therefore, from the fimpological perspective, it is necessary to answer the question: what are the environmental evolutionary entities in human milk? In this paper, I try to review the physiological and pathological roles of prokaryotic bacterial cells, viruses, eukaryotic fungal cells, and membrane-enclosed microentities as the environmental evolutionary entities in human milk, and expect that a clear viral and fungal profile in normal human milk will be accomplished in the near future.

Keywords: Milk; Human milk; Breastmilk; Breastfeeding; Bacteria, Viruses; Membrane-enclosed microentities; Exosomes; Microvesicles; Fungi; Eukaryote; Prokaryote; Nutrition; Immune; Evolution; Pasteurization; Symbiotic bacteria; Bacterial diversity; Evolutionary Background Entities, EBE; Bio-Evolutionary Background Entities System, BEBES; Mammals

1. Background

1.1 Breastmilk and breast-feeding act as the postnatal extension of the maternal acquired and innate immune systems

Recently, the word “evolution” or evolutionary idea has been mentioned more frequently than ever before in articles of mammalian breastmilk research.[3-7] Milk produced by mammary glands from different mammalian species is diverse in its cellular and non-cellular components, which can only be attributed to their unique evolutionary signatures. [5,8-12] Traditionally, our understanding of human milk originated from its nutritional significance. As the natural food of newborns, breastfeeding is believed to be a distinctive behavior of mammals, including humans. Since the emergence of immunology around the beginning of the 20th century, [13] the study of human milk has entered its immunological era. Human milk was recognized as an immune system in the 1970s [6] and was theoretically hypothesized as an “anti-inflammation” system in the 1980s.[7,14]

The immunological role of human milk had been gradually revealed from an antimicrobial and anti-inflammatory to an immunoregulatory perspective over the past century.[6,15-18] Consequently, the following important recognitions have been formed: (i) In nature, breastfeeding is not only the extension of maternal nutrients to offspring, but it is also the maternal immune-feeding for newborns;[15-18] and (ii) milk is a naturally postnatal vehicle for transferring maternal acquired and innate immune defense factors, including both cellular and humoral components, to their suckling babies against pathogenic bacteria and viruses.[3,7,11,15,16,19-31] Therefore, it is not surprising that milk itself can be imagined to be the postnatal extension of the maternal acquired and innate immune systems.[7,10,11,15,16,20,30,32-34]

1.2 Breastmilk is not a sterile natural food and breast-feeding in nature is not a sterilized behavior

As early as in the 17th century, when examining scrapings of his tongue and teeth under lenses, Antoni van Leeuwenhoek became the first person to observe bacteria. Two centuries later, Louis Pasteur proposed his notion that fermentation, putrefaction, and infection were all caused by microbes or bacilli, and different bacilli seemingly caused different diseases. At the end of the 19th century, Koch further proposed his “one disease-one agent” model, or Koch’s postulates.[35] Therefore, since then, the pathogenic role of bacterial microorganisms in human diseases has become our mainstream recognition in modern orthodox medicine or modern Western medicine; [36,37] and because of it, bacteria have been portrayed as “the enemy of Man”. [38] As a result, human milk was traditionally considered “germ free”. [39, 40]

For a long time, it was believed that (i) maternal eukaryotic cells were the only cellular component existing in normal human breastmilk; (ii) if bacteria and/or viruses were found in human milk samples, they were habitually labeled “externally infectious agents”;[41-43] (iii) the vertical transmission of “infectious agents” from mother to infant via breastfeeding had the potential risk of transferring infections;[15,44,45] and (iv) under the powerful protection of maternal antimicrobial system in human milk, the number and role of milk-born bacteria could be ignored. In fact, three decades ago, West**,** Hewitt and Murphy had already pointed out that “breast milk is not sterile, even when collected aseptically.”[46,47] For instance, human colostrum normally contains cultivable bacteria at a range of 2.72 to 4.13 log10 colony-forming units per mL (cfu/ml).[48,49] Perez and colleagues reported that the number of cultivable bacteria in aseptically collected human breastmilk was less than 1,000 cfu/mL.[47] Lindemann and colleagues found that only 10.5% of breast milk samples had a total bacteria count of more than 10,000 cfu/ml.[48]

In generally, the total bacterial count in most breast milk is usually less than 100,000 cfu/mL. Martin and colleagues estimated that an infant consuming approximately 800 mL per day might ingest about 10^5 -10^7 bacteria.[51]

However, the actual number of bacteria that breastfed infants obtain from milk should be much higher than that figure when we consider that 60-80% of the observable bacteria under a light microscope cannot be cultivated.[52] Culture-dependent approches and culture-independent molecular methods have revealed diverse bacterial and fungal communities normally existing on the skin of healthy adults.[53-63] Most recently, diverse bacterial species were detected in normal and healthy breast tissue by Xuan and colleagues,[64] and Urbaniak and colleagues, [65] respectively. Moreover, the bacterial profile in healthy breast tissue showed its racial characteristic. For instance, *Staphylococcus*, *Corynebacterium* and *Sphingomonas yanoikuyae* normally existed in normal breast tissue of American women;[64] *Bacillus, Acinetobacter, Enterobacteriaceae, Pseudomonas, Staphylococcus, Propionibacterium, Comamonadaceae, Gammaproteobacteria,* and *Prevotella* were detected in healthy breast tissue from Canadian women;[65] and *Enterobacteriaceae, Staphylococcus, Listeria welshimeri, Propionibacterium,* and *Pseudomonas* were most abundant in Irish breast tissue.[65] Therefore, there is solid evidence to support the recognition that bacterial species exist not only in normal human breastmilk, but also in healthy human breast tissue; breastmilk is not actusally a sterile natural food for sucling infants; and breastfeeding in nature is not a sterilized behavioral operation of lactating mothers.

1.3 The pathological perspective: pathogenic bacteria

Up to now, our major attention and explanation around the phenomenon of “bacteriologically positive human milk” has mainly focused on its pathological significance. In order to eliminate the possible milk-born bacteria-induced pathogenic risk to neonates, donor human breast milk from milk banks in North America is usually pasteurized at 62.5 °C for 30 minutes to inactivate bacterial species.[66]

Indeed, pasteurization can effectively kill many bacteria (both pathogenic and non-pathogenic bacterial species) in milk samples, and therefore the potential milk-born bacteria-induced pathogenic risk is actually decreased and the milk samples could be stored for a longer time without quickly turning sour. However, recent studies showed that after pasteurization of human milk, some bacterial species in human milk, such as Bacillus cereus, are still alive.[48,49] On the other hand, to my knowledge, there has been little information about the effect of pasteurization on the viral component of human breast milk. Israel-Ballard and colleagues found that human immunodeficiency virus1 (HIV-1) in human breast milk was significantly inactivated by flash-heat treatment.[67] Therefore, in fact, microorganisms including bacteria and viruses in pasteurized milk (humans or non-humans) are a mixture of living and dead micro-entities.

Although to date there is no safe microbiological criteria to define the safety of human milk, traditionally, if the total bacterial count was more than 100,000 cfu/mL, or if it contained pathogenic bacteria such as *Escherichia coli*, *enterococci*, *Staphylococcus aureu*s, mold or yeast in a breastmilk sample, the breastmilk was usually considered to be contaminated and should be pasteurized or discarded.[68-70] Ng and colleagues found that 63% of expressed breastmilk samples from a group of Chinese women whose premature babies were in the neonatal intensive care unit satisfied the bacterial contamination standard.[68] Upon the collection of human breastmilk, which usually depends on hand-expression, how to control the external contamination usually becomes one of the major concerns.[68-70,72,73]

2. The fimpological perspective: the evolutionary background entities in human milk

For a long time, we have been unable to account for the fact that although breastmilk is not a sterile natural food and breastfeeding in nature is not a sterile process, in most cases, such food and behavior are safe to suckling neonates. We have often found ourselves in a dilemma when accounting for the presentation of microorganisms in breastmilk samples collected through sterile methods. If the lactating mother was suffering from a relevant disease, the positive result could be easily explained. However, if both the mother and the suckling baby were healthy, how can we explain the phenomenon of “microbiologically positive human milk”? Is it a pathological or a physiological consequence? In other words, during lactation, breastmilk-fed infants obtain not only huge amounts of cellular and noncellular anti-infection agents, but also many bacteria and viruses from breastmilk.

Indeed, despite the fact that our knowledge about the physiological role of human milk and breastfeeding in terms of anti-infection benefits and anti-inflammation theory has improved a lot over the past decades,,[7,14,15,74-77] we are still puzzled about the microorganisms in human milk and their co-existence with phagocytes, lymphocytes, and other non-cellular agents such as antibodies, lysozyme, and interleukins in breastmilk. In a recent study, researchers revealed further that the intrinsically ascribed antimicrobial properties of breastmilk didn’t inhibit the growth of some foodborn bacterial species in vitro, such as *Enterobacter sakazakii* [78] and that the bacterial composition of the infant fecal flora reflected the bacterial composition of their maternal breastmilk.[79,80]

Since the revival of the study on symbiotic bacteria in the human body in the 1990s, increasing evidence has reveal that the bacterial communities exist in normal human milk.[79,81-85] Moreover, although the discovery of virus-like particles in human milk since the early 1970s [86-90] has given us an opportunity to understand them from a microbiological perspective, the finding of microvesicles, a newly recognized normal component exsiting in human milk, clearly goes beyond the scope of microbiology. Therefore, the terms “bacteriologically positive human milk” and “symbiotic bacteria” cannot correctly explain the ecological and evolutionary significance of coexistence of various micro-entities in human milk. We need novel thoughts. Recently, in the UPOEE model,[1,2] viruses/phages and microvesicles are classified as structurally subcelllular evolutionary entities, and there may be an evolutionary link between them from a fimpological perspective. Morevoer, a novel concept called “Evolutionary Background Entities” (EBEs) was proposed to refer to those entities of lower evolutionary levels that are the evolutionary “background organisms” of the entities at higher evolutionary levels.[1,2] In this paper, I try to review prokaryotic bacterial cells, viruses, eukaryotic fungal cells, and microvesicles as the evolutionary background entities in human milk.

3. Bacterial Entities in Human Milk

Since the use of culture-independent molecular approaches in the study of human milk bacteria in recent years, the dynamic, diversity, and complexicity of the bacterial community in normal human milk have gradually been uncovered.[47,80,82-84,91-97] Perez and colleagues reported that the the concentration of cultivable bacteria in breast milk was less than 1000 cfu/ml.[47] *Staphylococci*, *streptococci* and *bifidobacteria* were among the cultivable bacteria isolated in human milk.[98] Using culture-dependent and culture-independent molecular approaches, it has been shown that there are over 360 bacterial genera in human milk,[80] with the predominant bacterial species belonging to *Proteobacteria*, *Firmicutes*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus*.[80,93,99] Collado and colleagues reported that bacterial species in the genera *Staphylococcus*, *Streptococcus*, *Bifidobacterium*, and *Lactobacillus* were detected in all human milk samples using culture-independent techniques.[84] Cabrera-Rubio and colleagues revealed that in human colostrum samples, the predominant bacterial species belonged to the genera *Weisella*, *Leuconostoc*, *Staphylococcus*, *Streptococcus*, and *Lactococcus*, and in 1- and 6-mo milk samples, oral bacterial species belonging to *Veillonella*, *Leptotrichia*, and *Prevotella* increased significantly.[82] Clearly, the coexisting of many bacterial species in human milk is an undisputable fact, confirmed by conventional bacterial culture analysis and culture-independent molecular approaches.

3.1 Staphylococcus

Staphylococci, including *Staphylococcus epidermidis* and *Staphylococcus lugdunensis*, are the predominant cultivable bacterial species in human milk.[42,79,82,83,96] Moreover, *Staphylococcus aureus*, *Klebsialla* species, *Enterobacter* species, Serratia species, and *Escherichia coli* have been found to co-exist in human milk.[50] Heikkila and Saris showed that some *Staphylococcus epidermidis* isolates can inhibit the growth of *Staphylococcus aureus*.[79] Budagovskaia and colleagues found that the presence of 500 bacterial cells of *Staphylococcus aureus* in human milk was not harmful to the health of breastfeeding infants.[100] Although Huber and colleagues reported that methicillin-resistant coagulase-negative *staphylococci* (MR-CNS), such as *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*, were detected in almost half of human milk samples,[101] Jimenez and colleagues showed that the isolates of *Staphylococcus epidermidis* were sentitive to most of the antibiotics tested, including vancomycin.[102] Hunt and colleagues found *Staphylococcus* in human milk showed a remarkable inter-individual variation.[83]

3.2 Streptococcus

Streptococcus salivarius and *Streptococcus Mitis* are the predominant bacterial species in human milk.[79,82,83,96] Heikkila and Saris showed that some *Streptococcus salivarius* isolates can inhibit the growth of *Staphylococcus aureus*.[83] Group B *streptococcus* (GBS) is usually associated with Group B streptococcal disease, but most breastfed infants remain healthy despite GBS in human breast milk [103] and the question of transmission of *Streptococcus agalactiae* (Group B streptococcal, GBS) via breast milk and breastfeeding from mother to infant was discussed well in a recent review article by Le Doare and Kampmann.[103]

3.3 Lactobacillus

Some species of *Lactobacillus*, such as *Lactobacillus gasseri*, *Lactobacillus rhamnosus*, *Lactobacillus crispatus*, and *Lactobacillus fermentum*, have been isolated from human milk.[51,104] Martin and colleagues found that lactic acid bacteria in human milk were not obtained exoenously from maternal breast skin, and may be endogenously from the maternal gut microbiota.[51]

3.3.1 Antibacterial properties

Heikkila and Saris showed that some strains of *Lactobacillus rhamnosus*, *Lactobacillus crispatus*, *Lactobacillus lactis*, and *Lactobacillus mesenteroides* from human milk samples were effective against *Staphylococcus aureus*.[83] Olivares and colleagues indicated that the antibacterial activity of *Lactobacillus salivarius* CECT5713, *Lactobacillus gasseri* CECT5714, *Lactobacillus gasseri* CECT5715, and *Lactobacillus fermentum* CECT5716 against *Salmonella choleraesuis* was displayed both in vitro and in vivo.[105]

3.3.2 Antiviral properties

Intranasal administration of *Lactobacillus rhamnosus* GG was found to protect mice from H1N1 influenza virus.[106] *Lactobacillus rhamnosus* GG and heat-killed *Lactobacillus plantarum* L-137 (HK-LP) were shown to protect mice from influenza virus infection.[107,108] Heat-killed *Lactobacillus* and *Pediococcus* significantly inhibited infection with HIV-1.[109]

3.3.3 Impact on host eukaryotic cells

In an experiment on mice, the commensal bacterial strain *Lactobacillus reuteri* 100-23 was found to induce transiently reactive changes in intestinal epithelial cells.[110] Probiotic *Lactobacillus reuteri* strain ATCC PTA 6475 was shown to suppress TNF production by lipopolysaccharide-activated monocytes and primary monocyte-derived macrophages from children with Crohn's disease.[111] A bacteria-free solution derived from *Lactobacillus plantarum* was shown to inhibit NF-kappaB binding activity, degradation of IkappaBalpha, and the chymotrypsin-like activity of the proteasome in intestinal epithelial cells, macrophage, and primary culture murine dendritic cells.[112] *Lactobacillus fermentum* CECT5716 and *Lactobacillus salivarius* CECT5713, two probiotic strains isolated from human milk were shown in vitro to active NK and T cell subsets, increase Treg cells, and induce a broad array of cytokines including TNFα, IL-1β, IL-8, MIP-1α, MIP-1β, and GM-CSF.[113] *Lactobacillus fermentum* CECT5716 was shown to induce pro-inflammatory cytokines and enhance the production of Th1 cytokines by spleen cells, and increase the IgA concentration in faeces in vivo assays in mice, while *Lactobacillus salivarius* CECT5713 was shown to induce IL-10 production.[114]

3.3.4 Application at the individual level

Lactobacillus fermentum CECT5716, *Lactobacillus salivarius* CECT5713, and *Lactobacillus gasseri* CECT5714 from breast milk were shown in vivo to be effective in treating infectious mastitis during lactation.[115,116] *Lactobacillus fermentum* CECT5716 was shown to be nonpathogenic to mice even in doses 10,000 times higher (expressed per kg of body weight) than those normally consumed by humans.[117] *Lactobacillus salivarius* CECT5713, isolated from breast milk was showed in vivo to increase plasmatic levels of immunoglobulins M, A and G, and the regulatory cytokine IL-10, the percentage of NK cells and monocytes, fecal lactobacilli counts, and the frequency of defecation.[118] *Lactobacillus gasseri* BNR17 from human breast milk has a suppressing effect on the blood glucose level of mice.[119] *Lactobacillus reuteri* can significantly reduce intestinal mucosal levels of KC/GRO (~IL-8) and IFN-γ in vivo when newborn rat pups are induced with lipopolysaccharide (LPS).[120]

3.4 Bifidobacterium

Among the bifidobacterial species in human milk, Bifidobacterium longum was the most widely found species, followed by *Bifidobacterium animalis*, *Bifidobacterium bifidum*, and *Bifidobacterium catenulatum*.[121] Additionally, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium pseudocatenulatum*, and *Bifidobacterium longum* from human milk and infant feces were shown to produce exopolysaccharide.[122] Oral administration of *Bifidobacterium longum* strain BB536 significantly decreased gut-derived sepsis caused by *Pseudomonas aeruginosa* in immunocompromised mice.[123] Oral administration of *Bifidobacterium longum subsp. infantis* 157F-4-1 and *Bifidobacterium longum subsp. longum* NCC2705 was shown to protect against the lethal infection of *Escherichia coli* O157:H7 in gnotobiotic mice by preventing Shiga toxin production.[124]

3.5 Enterococcus

There is a high concentration of *enterococci* in milk from healthy mothers.[46,51,83,125,126] *Enterococci* including *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus hirae*, *Enterococcus casseliflavus*, and *Enterococcus durans*, were identified in the milk samples of healthy mothers.[92] Some Enterococci isolates, such as *Enterococcus faecalis*, were also effective against *Staphylococcus aureus*.[83] Reviriego and colleagues inferred that the milk of healthy mothers may be a source of avirulent Enterococcus faecium isolates for newborns.[96,126]

3.6 The dynamic of bacterial community in human milk

Recently, studies on human milk bacteria have shown that the composition of the bacterial community in human milk is dynamic during lactation.[82,127] Cabrera-Rubio and colleagues found that the predominant bacteria in colostrum were *Weisella*, *Leuconostoc*, *Staphylococcus*, *Streptococcus*, and *Lactococcus*, while the predominant bacteria in 1- and 6-mo milk samples were those bacterial inhabitants of the oral cavity, such as *Veillonella*, *Leptotrichia*, and *Prevotella*.[82] Bacteria in human milk were found to be affected by antibiotic therapy and mode of delivery.[82,127] Soto and colleagues revealed that the number of *lactobacilli*- or *bifidobacteria*-positive breastmilk samples was significantly lower in mothers who received antibiotic therapy during pregnancy or lactation.[127] Cabrera-Rubio and colleagues showed that the bacterial community in breastmilk samples of mothers who underwent elective cesarean delivery was different from that of mothers who experienced vaginal delivery,[82] which further accounted for the early finding that fecal bacterial composition of cesarean-delivered infants was different from that of vaginally-born infants.[128-133]

4. Subcellular Entities: Viruses and Membrane-Enclosed Microentities in Human Milk

4.1 Viruese in human milk

Virus-like subcellular entities were first identified in human milk in the 1970s.[86-90] Although various viral species, such as herpes viruses, parvovirus, hepatitis A, B and C, and rubella are often detected in breastmilk, viral transmission is very rare.[134-136] However, because of a lack of a universal marker gene for detecting viruses,[137] we have not achieved a similar level of understanding of symbiotic viruses as we have of bacterial communities over the past decades. The recognition in viral origins, diversity, and continuity is still a challenge, [138-140] and we do not yet have a clear viral profile in normal human milk. This is expected to change in the near future. When viral entities are considered to be a kind of subcellular evolutionary entities that normally coexist in breastmilk, the term of ‘bacteriologically positive human milk’ alone cannot accurately reflect the coexistence of different evolutionary microentities in normal human milk.

4.1.1 The pathological perspective: pathogenic vieuses

Viral entities in human milk have been associated with the risk of vertical transmission from mother to infant of certain viral pathogens, which has especially been highlighted since the 1980s when HIV and AIDS were recognized and the safety of breastfeeding has been questioned.[45,141-143]

Another reason for the attention to viruses in human milk is the possible pathogenic relation between virus-like particles and human breast cancer.[80,144-148] *Human papillomaviruses* (HPV), *mouse mammary tumor virus* (MMTV), and *Epstein-Barr virus* (EBV) were believed to be involved in breast cancer.[149] Laboratory studies showed that the human mammary carcinoma cell line T47D can release retrovirus-like particles (95 nm in diameter) via a steroid-dependent manner.[150] Wu and colleagues showed that neonatal mice acquired *murine cytomegalovirus* (MCMV) from mothers with acute or latent MCMV infection through breast milk.[151] “What are the roles of milk-born viruses?” and “do they associate with canceration in offspring?” are two of the recently concerned questions.[152]

4.1.2 Human Papillomavirus

Sarkola and colleagues reported that *human papillomavirus* (HPV) DNA was detected in breastmilk.[153] However, Mammas and colleagues, using polymerase chain reaction techniques, reported that no HPV type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 or 58 DNA were detectable in human milk.[154] Human papillomavirus type 16, 18, 39, 59, and 73 are oncogenic HPV types in cervical cancer, and some HPV types are non-oncogenic.[155,156]

4.1.3 Human Herpesvirus

Human herpes simplex virus 1, 2

Human herpes simplex virus (HSV) types 1 and 2 (HSV-1 and HSV-2) are enveloped DNA viruses belonging to the viral family Herpesviridae [157] and have been detected in human milk.[158,159]

***Human herpesvirus* (HHV) *type 8* (HHV-8)**

Human herpesvirus (HHV) type 8, also known as Kaposi's sarcoma associated herpesvirus (KSHV) was detectable in maternal saliva samples and breastmilk samples.[160]

***Human herpesvirus 5, Human Cytomegalovirus* (HCMV)**

HCMV-DNA was detectable in human breastmilk and was also detected in infant saliva.[161,162] HCMV mother-to-child transmission is considered to be a route of primary infection.[161,162] Most healthy individuals seem to readily control latent CMV infection, while a subpopulation (1/3) of individuals uses a large proportion of their CD8+ T cell repertoire to control the infection.[163,164] Zhang and colleagues reported that CMV positive rate was 39.58% in milk samples from women in Qinba Mountain area of Shanxi Province in China.[165] *Human cytomegalovirus* (HCMV) was shown to be inhibited in vitro by lactoferrin.[166] Wu and colleagues showed that *murine cytomegalovirus* (MCMV) was detected in breastmilk leukocytes collected from lactating mice and demonstrated that breastmilk is a source of *murine cytomegalovirus*.[151]

4.1.4 Epstein–Barr virus (EBV)

Epstein-Barr virus (EBV) is ubiquitous in healthy populations throughout the world, and 100% of individuals shed EBV in saliva.[164] Primary infections of infants with EBV are usually asymptomatic.[167-169] EB viruese were detected in human milk samples.[170]

4.1.5 Human Circovirus

Torque Teno virus (TTV), a single-stranded and circular DNA virus, was the first known human circovirus.[171] TTV was first found in a Japanese patient (named Torque Teno, initials T.T.) in 1997.[172,173] TTV viremia is widespread, with a very high incidence in general populations worldwide, and it may be a nonpathogenic DNA virus in humans.[174-178] TTV was detected in breastmilk.[179-182] Although the majority of breastmilk samples from viremic mothers are positive by TTV PCR, there is no need to discourage women from breastfeeding, because most children are TTV viremic even before breastfeeding begins.[182]

4.1.6 Enterovirus

Neonatal enteroviral infections are common.[183] Jenista, Powell and Menegus reported that 13% of infants were infected with an enterovirus in their first month of life.[184] Although enteroviruses have been reported to cause severe disease and even death in preterm and full-term infants within the first 10 to 14 days of life,[185-191] and the virus has also been associated with epidemic diarrhea in infants, neonatal sepsis, exanthema, and leukoencephalitis,[192-194] the majority of enteroviral infections are actually asymptomatic.[195] Most enterovirus-infected neonates are presumed to have acquired infection either perinatally, by exposure to maternal blood or urogenital secretions, or postnatally, via fecal-oral or respiratory transmission. Some in utero infections may occur following transplacental spread with the onset of disease within the first few hours of birth.[195] Previous studies have found that breastfeeding is protective against symptomatic enteroviral infections due to the presence of neutralizing antibodies.[28,184] Chang and colleagues reported that *Coxsackievirus* B3 was detcted in the mothers' milk of two severely ill neonates.[196] Sane and colleagues found that human milk can neutralize *Coxsackievirus* B4 in vitro.[31] Maus and colleagues detected enteroviral RNA and cultured infectious virus from breastmilk samples from the mother of an ill infant.[195]

4.1.7 Human Immunodeficiency Virus (HIV)

As early as in the 1980s, breastfeeding was thought to be an important route of *human immunodeficiency virus type 1* (HIV-1) vertical transmission.[197] Consistent HIV viral shedding and high breastmilk HIV viral load were further confirmed in more recent epidemiological and laboratory studies[198-203] and the risk of HIV transmission via breastfeeding comes from both cell-free HIV-1 virus and HIV-1-infected breastmilk cells.[204-206] Dring the past decades, an interesting phenomenon has been revealed in studies of mother-to-child HIV-1 transmission: HIV-infected women who exclusively breastfeed their infants are less likely to transmit HIV postnatally.[207-216] In other words, the majority of breastfed infants born to HIV-positive mothers remain uninfected despite prolonged HIV viral exposure via breastfeeding. This puzzling phenomenon raised many hypotheses.[215,217,218]

4.2 Membrane-Enclosed Microentities in Human Milk

Membrane-enclosed microentities (MEMs),[138] also called extracellular membrane vesicles (EMVs)[219] or extracellular vesicles (EVs),[220-222] refer to various types of nanometer-sized membrane-enclosed subcellular entities, such as ectosomes, exosomes, microvesicles, and apoptotic bodies.[138,219,223-225] MEMs, EMVs, or EVs have been described as a novel form of mechanism for intercellular interaction without direct cell-to-cell contact for their capability in transferring evolutionary molecular entities including DNAs, mRNAs, microRNAs, small RNAs, proteins, and lipids.[219,223,224,226,227]

Despite their existence in different human body fluids such as cerebrospinal fluid (CSF),[228-230] amniotic fluid,[230-232] nasal lavage fluid,[233] urine,[229,234] saliva,[233,235] plasma,[222,236-238] tears,[229] and semen,[239-241] exosomes were also identified morphologically in human breastmilk;[229,242-244] and they bear MHC classes I and II, CD63, CD81, and CD86 molecular features.[244] Some exosomes in human breastmilk contain RNA or milk mRNA transcripts and possess reverse transcriptase activity;[245,246] and moreover, microRNA (miRNA) in human breastmilk is stable even in very acidic conditions,[243] which together suggests the possibility of milk exosomes transfering genetic signals from mother to neonate during the lactation period.[245,246] Several molecular entities, such as microRNAs, Hsc70, and CD81 contained in exosomes of breastmilk have immune modulatory features;[244,245,247] and for more information about regulating functions of milk exosomal microRNA on the immune system, please see the recent review written by Melnik, John and Schmitz.[247]

5. Fungal Entites in Human Milk

Due to limitations of available isolation techniques, fungal entities in normal human milk have only been revealed in a few studies in the past. *Candida albicans* is a common fungal microorgaism detected in normal human nipple and milk.[248] Some strains of *Candida albicans* were found to be pathogenic and their growth is usually inhibited by iron-free human lactoferrin.[249,250] Novak and colleagues isolated several fungal strains including *Aspergillus Niger group, Aspergillus sp., Paecilomyces sp., Penicillium sp., Rhizopus sp.*, and *Syncephalastrum sp.* from expressed human milk samples, but the authors' doubt that the possibility of contamination cannot be ruled out.[251]

Recently, in their recent paper, Huffnagle and Noverr pointed out that "nobody is fungus-free." [252] Indeed, as unicellular eukaryotes, fungi play an indispensable role in the evolution of multicellular eukaryotes; therefore, we can anticipate that research on fungal cells in normal breastmilk will attract much more attention in the near future.

6. Concluding Remarks

The coexistence of various bacterial, viral, and fungal entities in human milk has been associated with the risk of vertical transmission from mother to infant of certain pathogens. During the past decades, accumulating evidence has indicated that human milk contains various cellular and sucellular micro-entities such as eukaryotic and prokaryotic cells, viruses, and microvesicles. As the naturally primary food of neonates, human milk's ecological and evolutiological significance is beyond our traditional understanding of its nutritional and immunological roles. However, exploring human milk's new roles may require novel theories, especially, when new findings cannot be accounted for by our existing understanding or are beyond our exsiting theoretical system. In this paper, according to the fimpological UPOEE model,[1,2] the coexistence of fungal eukaryotic entities, bacterial prokaryotic entities, viral subcellular entities, and microvesicle subcellular entities reflects different evolutionary signatures at the cellular and subcellular evolutionary levels, respectively; they can be recognized as components of environmental evolutionary entities in human milk; and their complex interactions at the molecular, sucellular, and cellular levels determine the biological, ecological, and evolutiological consequences of human milk and breastfeeding.

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