

Antibiotic Resistance of the Microflora of Women's Milk and the Suckling's Baby Intestine

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Breast Milk, Meconium, Microbiome, Antibiotics, Resistance.

ABSTRACT

Breast milk is a constant source of commensal and beneficial bacteria, including lactic acid bacteria and bifidobacteria. These microorganisms play an important role in the development and optimization of the intestinal microbiota of the newborn due to their pre- and probiotic components. In this experiment, we investigated the qualitative and quantitative composition of the microbiota of mothers' breast milk, and also assessed the influence of the mother's microbiota on the early bacterial colonization of the intestines of newborns. The most common breast milk microbial isolates were *Staphylococcus epidermidis*, *Staphylococcus hominis*, and *Streptococcus salivarius*. Among lactic acid bacteria, *Lactobacillus salivarius*, *Lactobacillus gasseri*, *Lactobacillus paracasei*, *Lactobacillus agilis*, and *Bifidobacterium longum* were the most common. We found that the bacterial profile of infant meconium is similar to that of mother's milk. The total number of microorganisms in 1 g of meconium varied from 3.6 log CFU/g to 5.8 log CFU/g. Staphylococci and fecal streptococcus were predominant (55.7% on average). The number of lactobacilli varied from 8% to 23%. Most of the microorganisms isolated from breast milk, meconium, and feces of monthly infants are multiresistant to antibiotics. The isolated microorganisms were most resistant to benzylpenicillin, doxycycline, ampicillin, oleandomycin, oxacillin, fosfomycin, and lincomycin.



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1. INTRODUCTION

Breast milk is considered the most optimal infant feeding product because it contains important nutrients and many biologically active compounds that support the growth and development of the infant immune system [1- 4]. Breast milk not only provides the growing baby with essential nutrients but is also a source of commensal bacteria that further improve the health of the baby by preventing pathogen adhesion and promoting colonization of the gut with beneficial microbes [4-6].

Breast milk was originally thought to be sterile. But studies in recent years have shown that breast milk is a constant source of bacteria during lactation [7- 9]. More recently, the presence of viable commensal, mutualistic, and potentially probiotic bacteria in healthy breast milk has been established [10- 12]. Among the microorganisms found in breast milk, strains belonging to the genera *Lactobacillus* and *Bifidobacterium*

subsp [3], [13]. In total, more than 200 different bacterial species, representing approximately 50 different genera, have been isolated from breast milk, including new bacterial species such as *Streptococcus lactarius* [14], [15].

Thus, it is now widely recognized that breast milk has an individual microbiota, which is mainly formed by the microbiome of the mother's skin during infant breastfeeding. Therefore, similarities between the microbiome of the mother's skin and the microbiome of her milk have been noted. Members of the genus *Staphylococcus* and *Corynebacterium* are particularly common in breast milk [16], [17]. Another source of breast milk bacteria is the retrograde flow of milk from the infant's mouth into the mother's mammary gland [18].

After birth, maternal and environmental commensal and probiotic bacteria gradually colonize the infant's gut. At the same time, almost one-third of the beneficial bacteria (from 8×10^4 to 8×10^6) enter the infant's gut from maternal milk [19], [20]. These microorganisms contribute to the development of the immune system and have trophic and metabolic effects [21]. They affect the integrity of the mucous membrane of the gastrointestinal tract of the baby and the production of antibodies, providing additional protection against colonization of the intestine by pathogens. The same bacteria also contribute to the prevention of mastitis [22], [23]. Strains derived from breast milk are potential probiotics. Therefore, many studies have focused on their isolation from breast milk for subsequent use in infant nutrition [24], [25].

The gut microbiota of newborns is characterized by low diversity, and its structure is generally unstable and very dynamic [26]. A few weeks after birth, the infant's gut forms its microbiome and begins the transition to a permanent bacterial population. This microbial population further significantly influences human biology throughout life [27], [28].

A newborn's first feces (meconium) are greenish-black in color and resinous in consistency. Meconium includes what the baby swallowed in the womb, including amniotic fluid, bile, and skin cells. In the newborn, the digestive tract functions too quickly, so the bile does not have time to digest, resulting in a greenish color of meconium. Over the next few days, the stools start to turn green, then become mustard yellow (if breastfed) or brownish-brown (if a formula is used). The composition of the gut microbiota stabilizes between the ages of 3 and 5 years [29].

The infant's developing gut microflora is often exposed to negative external influences, including antibiotics. The use of antibiotics is standard during a cesarean section or assisted vaginal delivery. In Western countries, approximately 50% of women take antibiotics during delivery, and between 2% and 5% of newborns receive antibiotics because of suspected infectious diseases. Perinatal antibiotics used during labor or after delivery rapidly alter gut colonization in both preterm and premature infants [30- 33,].

The increasing spread of antibiotic-resistant bacteria has become a serious problem not only for adult health but also in the perinatal period. Perinatal exposure to antibiotics causes microbiota dysbiosis in the vagina and intestines of newborns, as well as a significant decrease in lactobacilli [34]. Worldwide, about 214,000 neonatal deaths occur each year due to septic infections caused by antibiotic-resistant pathogens.

Acquired resistance to antibiotics can be realized through horizontal gene transfer between bacteria [35], as well as through vertical transmission from mother to infant at birth and during lactation [36- 38]. Despite many studies on the horizontal transmission of antibiotic resistance genes, not much research has been done on the mechanism of vertical transmission. Vertical transmission of antibiotic resistance genes from mother

to infant is important because early bacterial colonization affects infant health and immune programs throughout life.

Identifying risk factors for the acquisition of antibiotic-resistant bacteria in newborns has been the subject of many studies in recent years [39], [40]. However, most of these studies have looked at periods of a few weeks or months after birth, resulting in the omission of important aspects of early microbiome maturation, such as the acquisition of antibiotic resistance determinants immediately after birth [41]. Therefore, in the present study, we aimed to investigate antibiotic resistance in the maternal milk and meconium microflora on days 4-5 of the postpartum period

2. MATERIALS AND METHODS

Milk samples were collected from parturient women after informed consent on days 4-5 of the postpartum period. Four groups were formed, depending on the term of birth: at 38-40 weeks of pregnancy (10 samples, group 1); at 32-37 weeks of pregnancy (10 samples, group 2); at 28-32 weeks of pregnancy (10 samples, group 3); at one month postpartum (14 samples, group 4). Milk samples were collected according to the "Methodological Recommendations for Bacteriological Control of Breast Milk (USSR Ministry of Health, 1984)" and the "Instructions for Collection of Breast Milk for Testing. Microflora culture" (INVITRO, 2018). Meconium of infants and feces of one-month-old children were collected at the same time. The sampling site was the perinatal center of the regional clinical hospital of Kaluga region (groups 1-3) and volunteers (group 4).

Microbiological examination of breast milk, meconium, and feces of one-month-old infants was performed to determine the total number of microorganisms and to isolate and identify pure cultures of microorganisms. Identification of bacteria was performed in the following sequence: description of cultural features of isolated microorganism, obtaining pure daily culture by sowing on nutrient media, Gram staining, and microscopy of the preparation. The species composition of isolated microorganisms was studied by mass spectrometry on a MALDI-TOF MS autoflex speed analyzer (Bruker company).

The sensitivity of bacteria to antibiotics was determined by the diffusion method using discs impregnated with antibiotics (Table 1).

Table 1 List of antibiotics used

Name	Designation	Name	Designation
Ofloxacin	OF	Neomycin	NEO
Clarithromycin	KTM	Oleandomycin	OLE
Benzylpenicillin	PEN	Levomycetin	LEV
Doxycycline	DOK	Lincomycin	LIN
Fosfomycin	FOS	Cefoperazone	CPR
Tobramycin	TOB	Oxacillin	OKS
Tetracycline	TETR	Lomefloxacin	LOM
Ampicillin	AMP	Furazolidone	FRN

Statistical processing of the study results was performed using classical methods of mathematical statistics and the Microsoft Excel table processor.

3. RESULTS AND DISCUSSION

Most breast milk samples (81.1%) contained microorganisms. The total number of viable bacteria in breast

milk ranged from 2.4 to 4.5 log CFU/ml, indicating the presence of a wide range of bacteria in maternal milk. At the same time, analysis of the data using standard deviation showed little difference in the total number of microorganisms in the breast milk of mothers of groups 1 to 3. But Group 4 mothers had a higher number of microorganisms in breast milk. This may be due to less hygienic conditions at home than in the hospital, or to closer bodily contact between mothers and their infants (Figure 1).

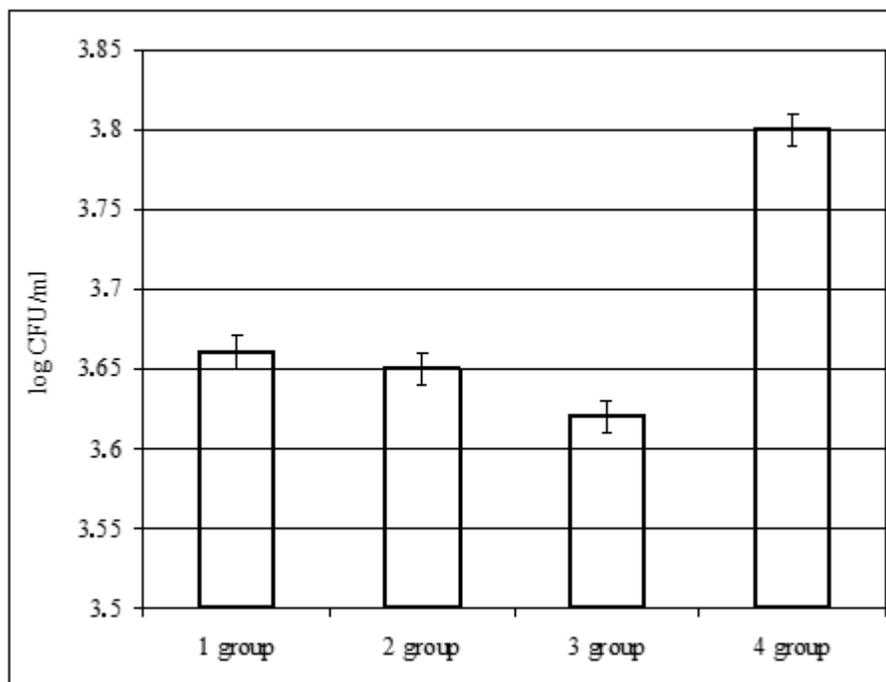


Figure 1 The total number of viable bacteria in breast milk

The most common microbial isolates in breast milk in all groups were *Staphylococcus epidermidis*, *Staphylococcus hominis*, and *Streptococcus salivarius*. *Lactobacilli* and bifidobacteria were a minority in the mother's milk (Table 2). Among lactic acid bacteria, *Lactobacillus salivarius*, *Lactobacillus gasseri*, *Lactobacillus paracasei*, *Lactobacillus agilis*, and *Bifidobacterium longum* were the most common and are the key subjects of the intestinal population.

Table 2 Bacterial associations of breast milk samples from mothers of different groups

Groups	Bacterial species	Frequency of detection, %
1–3	<i>Staphylococcus epidermidis</i>	95.1 ± 4.6
	<i>Staphylococcus hominis</i>	13.4 ± 1.3
	<i>Streptococcus salivarius</i>	2.1 ± 0.7
	<i>Lactobacillus salivarius</i>	1.7 ± 0.3
	<i>Lactobacillus gasseri</i>	2.4 ± 0.5
	<i>Lactobacillus paracasei</i>	0.8 ± 0.2
	<i>Lactobacillus agilis</i>	3.1 ± 0.9
	<i>Bifidobacterium longum</i>	1.4 ± 0.1
4	<i>Staphylococcus epidermidis</i>	86.2 ± 6.6
	<i>Staphylococcus hominis</i>	43.7 ± 4.5
	<i>Streptococcus salivarius</i>	12.1 ± 2.8
	<i>Lactobacillus salivarius</i>	2.3 ± 0.6

<i>Lactobacillus gasser</i> ,	3.1 ± 0.8
<i>Lactobacillus paracasei</i>	0.6 ± 0.2
<i>Lactobacillus agilis</i>	3.0 ± 1.1
<i>Bifidobacterium longum</i>	7.7 ± 3.3

The number of *Staphylococcus epidermidis* decreased in the breast milk of Group 4 women, but the number of *Staphylococcus hominis* and *Streptococcus salivarius* increased 3 and 6-fold, respectively. The number of *Lactobacillus salivarius* (1.4-fold) and *Bifidobacterium longum* (5.5-fold) also increased. In a single case, *Aeromonas caviae* was isolated from breast milk. *Staphylococcus aureus* was not detected in any sample.

In contrast to streptococci and staphylococci, which are widely present in mother's milk, the presence of lactobacilli and bifidobacteria is more variable. Overall, the quantitative and qualitative composition of the microorganisms found in the breast milk samples showed that none of the women had mastitis at the time of sample collection.

Further studies showed that the meconium of newborns was also not sterile. It contains a variety of bacteria characteristic of mother's milk. This supports the hypothesis that the formation of the meconium microflora and subsequent infant intestinal tract microflora is due to the microflora of breast milk, which is formed through intestinal translocation and blood transport, describing the internal entero-milk pathway [6], [42].

The total number of microorganisms in 1 g of meconium in group 1-3 infants ranged from 3.6 log CFU/g to 5.8 log CFU/g. Staphylococci and fecal streptococci were predominant (mean 55.7%), characteristic of the maternal microbiota. The number of lactobacilli ranged from 8% to 23% (Figure 2). In group 4 infants (gestational age 1 month), the number of staphylococci and bifidobacteria slightly increased. This supports evidence that breast milk oligosaccharides have a prebiotic effect on bacteria that are frequently found in breast milk, including bifidobacteria, *Staphylococcus epidermidis* и *Enterococcus faecalis* [43].

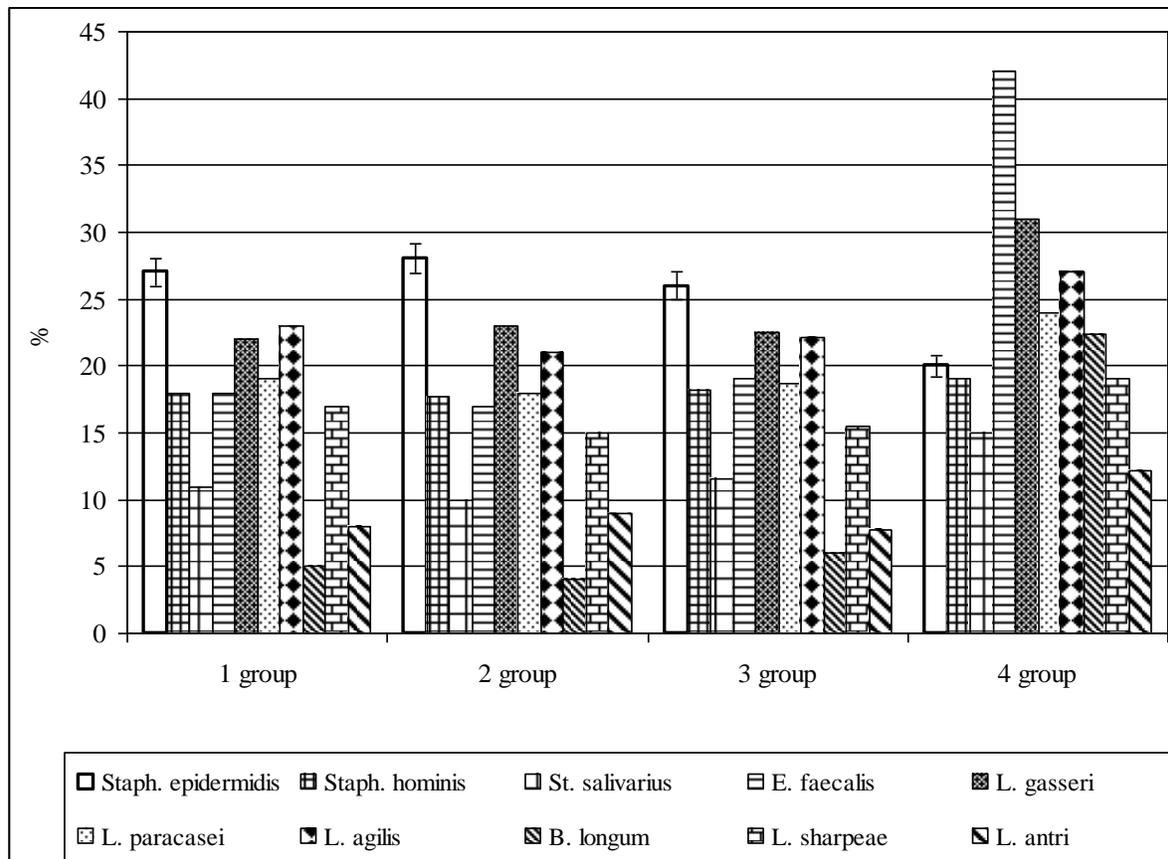


Figure 2 Meconium microbiome of newborns (group 1-3) and feces of monthly children (Group 4)

Microorganisms isolated from women's breast milk and infants' intestines were tested for antibiotic resistance. Most microorganisms isolated from breast milk, meconium, and feces of monthly infants were found to be multiresistant to antibiotics and may function as reservoirs of antibiotic resistance. Breast milk microorganisms showed the greatest resistance to benzylpenicillin (PEN), doxycycline (DOK), ampicillin (AMP), oleandomycin (OLE), oxacillin (OKS), phosphomycin (FOS), and lincomycin (LIN) (Figure 3). Similar results were obtained in antibiotic sensitivity testing of microorganisms isolated from the meconium and feces of monthly infants. However, the percentage of resistant microorganisms was slightly lower (Figure 4).

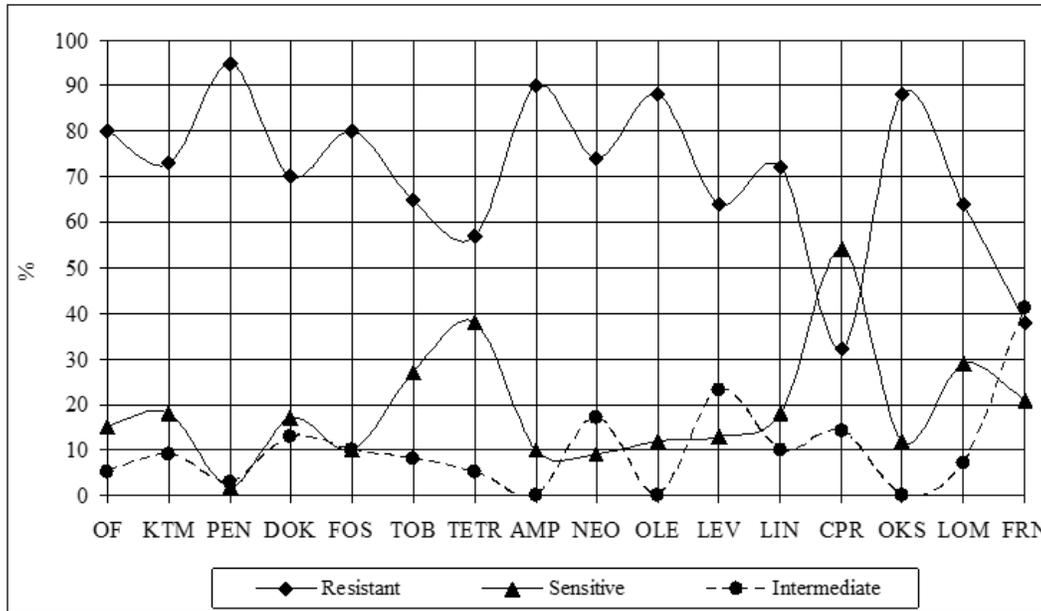


Figure 3 Cumulative antibiotic resistance of microorganisms isolated from breast milk

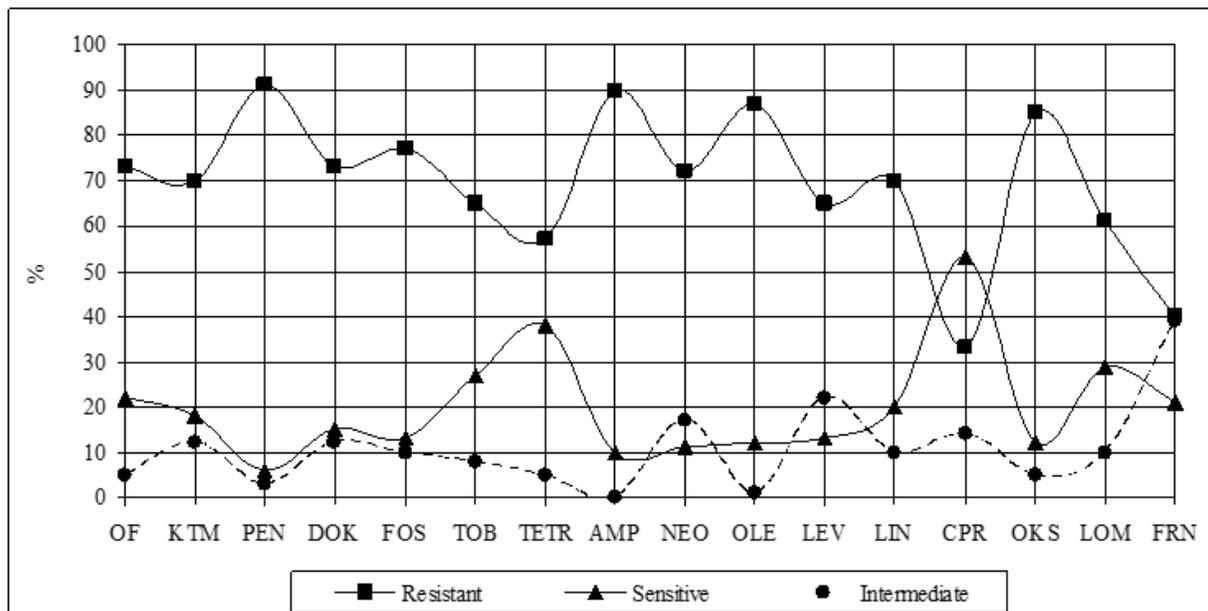


Figure 4 Cumulative antibiotic resistance of microorganisms isolated from meconium and feces of one-month-old children

Antibiotic sensitivity was also analyzed in bacterial isolates isolated from maternal milk, meconium, and fecal samples of one-month-old infants (Tables 3 and 4). Most of the isolated strains in the milk samples are multidrug-resistant and may play a role in the risk of antibiotic resistance.

Table 3 Antibiotic sensitivity testing for microorganisms in breast milk and feces of infants

Bacterial species	AMP		PEN		DOK		NEO		OLE		FOS		LEV		LIN	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
<i>Staphylococcus epidermidis</i>	-	-	-	±	+	+	-	-	-	±	+	+	±	±	±	±
<i>Staphylococcus hominis</i>	-	-	-	-	+	±	-	±	-	±	+	+	±	±	-	±

<i>Streptococcus salivarius</i>	-	-	-	±	±	±	-	+	-	±	-	±	±	±	+	+
<i>Lactobacillus salivarius</i>	+		-	+	+	+	+	+	-	±	+	+	-	-	-	±
<i>Lactobacillus gasseri</i>	-	-	-	-	+	+	-	±	-	±	-	-	±	±	-	-
<i>Lactobacillus paracasei</i>	-	-	-	-	-	±	+	+	-	-	±	+	±	+	-	-
<i>Lactobacillus agilis</i>	-	-	-	-	-	-	-	-	+	+	±	+	-	-	±	±
<i>Bifidobacterium longum</i>	+	+	-	-	+	+	+	+	±	+	±	±	±	±	+	+

M-milk; F-meconium and feces; - Resistant; + Sensitive; ± Intermediate

Table 4 Antibiotic sensitivity testing for microorganisms in breast milk and feces of infants

Bacterial species	CPR		KTM		OKS		LOM		TETR		OF		TOB		FRN	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
<i>Staphylococcus epidermidis</i>	+	+	-	±	-	-	-	-	±	+	-	-	-	-	+	+
<i>Staphylococcus hominis</i>	+	+	+	+	-	-	+	+	-	±	+	+	-	-	±	+
<i>Streptococcus salivarius</i>	+	+	-	±	-	-	-	-	-	±	-	-	-	-	+	+
<i>Lactobacillus salivarius</i>	+	+	+	+	+	+	-	-	+	+	-	±	-	±	+	+
<i>Lactobacillus gasseri</i>	+	+	-	-	±	+	+	+	-	-	+	+	±	+	+	+
<i>Lactobacillus paracasei</i>	+	+	±	±	-	-	+	+	+	-	+	+	+	+	-	±
<i>Lactobacillus agilis</i>	+	+	-	-	-	-	-	-	-	-	-	±	-	-	-	-
<i>Bifidobacterium longum</i>	+	+	+	+	-	-	+	+	-	-	±	-	+	+	+	+

M, milk; F, meconium and faeces; - Resistant; + Sensitive; ± Intermediate

Evidence suggests that bacteria resistant to clinically relevant antibiotics from the mother's breast milk can be transmitted to the infant's gut and affect the resistance of the emerging microbiota. This can harm the baby's treatment outcome for infectious diseases. Although in some cases, infant gut microorganisms are more sensitive to antibiotics than the mother's original milk microorganisms. This phenomenon requires further research.

4. Conclusion

Breast milk has a microbial association consisting of cutaneous and gut-associated bacteria. The total number of viable bacteria in breast milk ranged from 2.4 to 4.5 log CFU/mL.

The findings suggest that bacteria from breast milk can be transferred to the infant's gut and affect the developing gut microflora. The most common microbial isolates in breast milk in all groups were *Staphylococcus epidermidis*, *Staphylococcus hominis*, and *Streptococcus salivarius*. Lactobacilli and bifidobacteria were a minority in maternal milk.

Among the lactic acid bacteria, *Lactobacillus salivarius*, *Lactobacillus gasseri*, *Lactobacillus paracasei*, *Lactobacillus agilis*, and *Bifidobacterium longum* were the most common, which are the key subjects of intestinal probiotic microflora.

After one month of lactation, the number of *Staphylococcus epidermidis* decreased in the woman's breast milk, and the number of *Staphylococcus hominis* and *Streptococcus salivarius* increased 3 and 6-fold, respectively. The number of *Lactobacillus salivarius* (1.4-fold) and *Bifidobacterium longum* (5.5-fold) also increased.

The total number of microorganisms in 1 g of meconium ranged from 3.6 log CFU/g to 5.8 log CFU/g. Staphylococci and fecal streptococci were predominant (mean 55.7%), characteristic of the maternal

microbiota. The number of lactobacilli ranged from 8% to 23%. In group 4 infants (gestational age 1 month), the number of staphylococci and bifidobacteria increased slightly.

Most microorganisms isolated from the breast milk, meconium, and feces of one-month-old infants were multiresistant to antibiotics. The microorganisms isolated were most resistant to benzylpenicillin, doxycycline, ampicillin, oleandomycin, oxacillin, fosfomicin, and lincomycin.

Bacteria from maternal breast milk that are resistant to clinically relevant antibiotics determine the resistance of the baby's emerging gut microbiota, which may adversely affect the outcome of his treatment for infectious diseases.

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