Original Article

Breast Milk as a Source of Prebiotic Human Milk Oligosaccharides and Bacteria from the *Lactobacillaceae* Family

(breastfeeding / *Lactobacillaceae* / human milk oligosaccharides / breast milk)

KATARYZNA ŁUBIECH[1], MAGDALENA TWARUŻEK[1], ELENA SINKIEWICZ-DAROL[1,2], DOROTA MARTYSIAK-ŻUROWSKA[3], BARBARA KUSZNIEREWICZ[3]

[1]Department of Physiology and Toxicology, Faculty of Biological Sciences, Kazimierz Wielki University, Bydgoszcz, Poland
[2]Human Milk Bank, Ludwik Rydygier Provincial Polyclinical Hospital in Toruń, Toruń, Poland
[3]Department of Food Chemistry, Technology and Biotechnology, Chemical Faculty, Gdansk University of Technology, Gdańsk, Poland

Abstract. Breast milk, as the optimal food for infants and young children, contains all the components necessary for proper growth and development. It is a rich source of both essential nutrients and biologically active factors, making breast milk a unique food with scientifically proven health-promoting properties. Among the entire range of biologically active factors, breast milk microorganisms and prebiotic factors, in the form of breast milk oligosaccharides, occupy an important place. The aim of our research was to determine the occurrence of bacteria with probiotic potential, belonging to the *Lactobacillaceae* family, in the environment of breast milk and breast milk oligosaccharides. The study included 63 human milk samples from breastfeeding women at various stages of lactation. Microorganism identification based on culture tests and MALDI TOF/MS, macronutrient analysis using the MIRIS human milk analyser, as well as analysis of human milk oligosaccharides using ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry were performed. The results have shown that breast milk from different breastfeeding women is characterized by great diversity in terms of the presence of *Lactobacillaceae* bacteria in its microbiological composition. These bacteria were present in 22.2 % of the tested breast milk samples. Analysis of the human milk oligosaccharide profile revealed a slightly higher content of prebiotic factors in breast milk samples containing *Lactobacillaceae*, including 2′-fucosyllactose, oligosaccharide occurring in the highest amount in breast milk.

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Corresponding author: Katarzyna Łubiech, Department of Physiology and Toxicology, Faculty of Biological Sciences, Kazimierz Wielki University, J.K. Chodkiewicza 30 Street, Bydgoszcz, Poland. E-mail: katarzyna.lubiech@ukw.edu.pl

Abbreviations: 2'FL – 2′-fucosyllactose, HMO – human milk oligosaccharides, HRMS – high-resolution mass spectrometry, MALDI TOF/MS – matrix-assisted laser desorption ionization time-of-flight mass spectrometry, MIRIS HMA – The Miris Human Milk Analyzer, UHPLC – ultra-high-performance liquid chromatography.

Introduction

Breast milk provides all the components necessary for the proper growth and development of a child in the early stages of life. This applies primarily to basic nutrients such as fat, protein and carbohydrates. Also, the energy value adjusts to the needs of the growing body, changing as lactation progresses. However, mother's milk is also a source of many biologically active ingredients that have health-promoting effects (Andreas et al., 2015). An important element of bioactive factors are microorganisms that inhabit the environment of breast milk and can be transported to the child's body. Scientific research to date has identified a diverse range of bacteria in breast milk (Zimmermann and Curtis, 2020). They are an important factor, among others, in shaping the immune system, preventing infections and ensuring the microbiological homeostasis of the child's intestinal microbiota (Notarbartolo et al., 2022). Among the microorganisms of breast milk, there may be bacteria with probiotic potential, characterized by multidirectional health-promoting effects. This group includes microorganisms belonging to the *Lactobacillaceae* family

(Dombrowska-Pali et al., 2024). The microbiological composition of breast milk is not constant and depends on many factors, both environmental and individual (Zimmermann and Curtis, 2020). Differences in the composition of the breast milk microbiota also influence the presence of bacteria from the *Lactobacillaceae* family. To date, not all factors determining the presence or absence of these microorganisms in human milk are known.

Another important element of the breast milk composition are oligosaccharides, factors with prebiotic effects (Menzel et al., 2021). Human milk oligosaccharides (HMO) constitute a group of complex glycans, compounds of various structures, consisting of three to 22 monosaccharides linked together in various combinations. The main monosaccharides included in HMO are D-glucose, D-galactose, L-fucose, N-acetylglucosamine and N-acetylneuraminic acid (sialic acid). HMO may also contain a lactose molecule and other mono- and disaccharide molecules. Due to the nature of the molecules that constitute them, we distinguish sialylated and fucosylated HMO, which are formed as a result of the attachment of sialic acid or fucose molecules to the structural skeleton of the oligosaccharide. This process occurs thanks to the action of catalytic enzymes. Therefore, based on the presence or absence of fucose and sialic acid in the HMO structure, these oligosaccharides are divided into acidic-sialylated (containing sialic acid, acidic fucosylated and acidic non-fucosylated) and neutral (core, containing GlcNAc and fucosylated, containing fucose) (Marcobal et al., 2011).

HMO constitute the third largest group of solid components of breast milk after lactose and lipids. The amount of HMO changes during lactation, reaching 12–14 g/l in mature milk. Research to date has shown the presence of over 200 types of HMO in breast milk. The health-promoting effects of oligosaccharides are manifested primarily by supporting the intestinal microbiota of a child fed with human milk and supporting the development of the intestinal barrier (Kong et al., 2019). HMO are not digested by the child's digestive tract, providing the main support for the intestinal microbiota, which, thanks to specific bacterial enzymes, is able to use them as a source of energy (Chen, 2015).

In addition to supporting the intestinal microbiota, other health-promoting effects of breast milk oligosaccharides have also been demonstrated. In a child's digestive tract, HMO have the ability to block pathogens from adhering to the surface of the intestinal mucosa, thereby preventing intestinal infections. This is due to the specific structure of HMO, which are similar to the glycocalyx of the intestinal epithelium. The effect of this is to block the lectin receptors of pathogens and prevent them from adhering to the intestinal mucosa (Bode, 2015). They also have a beneficial effect on the condition of intestinal epithelial cells and influence the efficient functioning of the immune system, modulating its functions. HMO activate tolerogenic dendritic cells, characterized by high expression of inhibitory markers responsible for reduced production of pro-inflammatory cytokines. They also have the ability to induce regulatory T cells. This manifests itself in immunosuppressive effects, which are important from the point of view of preventing allergic and autoimmune diseases (Xiao et al., 2019). For premature infants, the supply of HMO contained in breast milk contributes to the beneficial effects of reducing the risk of necrotizing enterocolitis (Bode 2018; Wang et al., 2024). Studies have shown that a small amount of some of the HMO is absorbed in the child's digestive tract. It is assumed that this pathway is related to the action of oligosaccharides supporting the development of structures of the central nervous system (Ruhaak et al., 2014).

Material and Methods

Sampling and data collection

The study included 63 samples of human milk collected from Polish breastfeeding women in various stages of lactation (N = 63). All of them were microbiologically tested. Analysis of the composition of human milk in terms of macronutrients was performed with N = 56 samples, and studies on the content of human milk oligosaccharides were conducted with N = 44 samples of human milk. Differences in the number of samples analysed resulted from the abundance of the obtained material. Microbiological tests were treated as a priority.

The consent to conduct the study was granted by the Bioethics Committee of the Nicolaus Copernicus University in Toruń under number KB921/2018. The Committee also approved all required documents. The study participants were informed about the purpose and course of the study and asked to give written consent to participate in the study.

Breast milk samples were collected in the morning, after prior instruction and with the assistance of a person involved in the testing. A hospital-grade breast pump with disposable, sterile, replaceable kits (Medela Symphony, Medela AG, Baar, Switzerland) and sterile milk collection containers were used to obtain the material. Immediately after collection, the samples were transported to the laboratory under refrigerated conditions.

Sixty-three samples of breast milk were subjected to microbiological analysis, and as a result of the availability of research material, 44 milk samples were included in the oligosaccharide analysis. At the stage of obtaining research material, all study participants completed a survey regarding factors that may influence the breast milk composition, and sociodemographic characteristics. The questionnaire took into account eating habits, health condition, medications and dietary supplements taken, as well as perinatal factors. Samples and questionnaires were coded to maintain anonymity. Sensitive data obtained during the study were covered by a confidentiality clause. In Poland, GDPR regulations apply, which ensure the protection of sensitive data. The acquired data were stored in compliance with security rules.

Identification and isolation of Lactobacillaceae bacteria

In order to detect the presence of bacteria belonging to *Lactobacillaceae* and for their isolation, inoculation on solid De Man, Rogosa and Sharpe (MRS) medium and its modifications was used: MRS medium with the addition of vancomycin (10 mg/l) (Colombo et al., 2014), MRS medium with L-cysteine (0.5 g/l) (Soto et al., 2014), MRS medium with the addition of 3 % $CaCO_3$ (Jiang et al., 2016), and standard version of MRS medium without any additives. The tested material and its tenfold dilutions in the amount of 0.1 ml were spread on Petri dishes with MRS medium in duplicate. Incubation was carried out at 37 °C in anaerobic conditions for 72 ± 2 hours. After incubation, the grown bacterial colonies were subjected to further analysis using MALDI TOF/MS mass spectrometry (MALDI Biotyper IVD, Bruker Daltonik GmbH, Bremen, Germany). Material from the tested colonies was analysed in accordance with the recommendations of MALDI TOF/MS manufacturer. Each analysed colony was placed on the analyser plate, covered with the matrix and read using the device's detection. Bacteria identified as belonging to the *Lactobacillaceae* family were collected and stored in cryobanks.

Analysis of the macronutrients in breast milk

The breast milk macronutrient content was analysed using the MIRIS HMA human milk composition analyser (Miris AB, Uppsala, Sweden). The MIRIS HMA is based on semi-solid mid-infrared (MIR) transmission spectroscopy. Each raw human milk sample was analysed in triplicate, with the resulting value being the average of the three measurements. The procedures were performed in accordance with the device instructions and manufacturer's recommendations. The analysis took into account the content of individual macronutrients, such as total fat (g/100 ml), crude protein (g/100 ml), true protein (g/100 ml), carbohydrates (g/100 ml), dry matter (g/ 100 ml) and energy value (kcal/ml). Based on the lactation period, the study group was divided into subgroups as follows: milk samples from 1–6 months, 7–12 months, 13–18 months, 19–24 months of lactation, samples from women breastfeeding for more than 25 months.

Analysis of the HMO profiles

The analysis of HMO in breast milk samples was performed according to the method described by Remoroza et al. (2018) and Jarzynka et al. (2022). A total of 44 breast milk samples were tested. These were the samples for which the amount of material obtained allowed for additional analyses to be performed in addition to microbiological tests. As shown by the earlier stages of the study, 10 of them were positive for the presence of *Lactobacillaceae* bacteria. The HMO determination procedure began with the removal of fat and protein from the tested breast milk samples. The samples were degreased by centrifugation (1,100 *g,* 4 °C, 15 min). After the centrifugation process, the upper fractions, containing mainly fat, were discarded and the lower aqueous phases were transferred to separate vials. One ml of 99.6 % ethanol was added to the remaining fractions containing the precipitate. This mixture was centrifuged again in the same conditions. Precipitation was used to remove the protein by adding 1 ml of 99.6 % ethanol to the pooled supernatant and incubation at 4 °C overnight. The next stage was centrifugation performed at 1,100 *g* at 4 °C for 15 min. The remaining liquid phase was evaporated. As a result of this process, saccharides were obtained in powdered form, which were stored at –80 °C for further experiments. Oligosaccharide samples were analysed by ultra-high-performance liquid chromatography (UHPLC) coupled with high-resolution mass spectrometry (HRMS), using the Ultimate 3000 UHPLC System (Dionex,Thermo Scientific TM, San Jose, CA) coupled with a mass spectrometer Q Exactive TM Focus quadrupole-Orbitrap (LC-Q-OrbitrapMS/MS, Thermo Fisher Scientific, Bremen, Germany) with heated electrospray. An ionization source (HESI II) was used in the analysis, and chromatographic separation was performed using an ACQUITY Glycoprotein BEH Amide column (300 Å, 1.7 µm, 2.1 mm × 150 mm, Waters Corporation, Milford, MA) maintained at a temperature of 25 °C. The mobile phase consisted of: A: 5 mmol/l ammonium formate with 0.1 % (v/v) formic acid in water, B: 5 mmol/l ammonium formate with 0.1 % (v/v) formic acid in 90 % acetonitrile. The elution programme included: 2 min isocratic 95 % B, 5 min linear gradient to 80 % B, 30 min linear gradient from 80 % to 50 % B, 3 min washing the column with a linear gradient from 50 % to 2 % B and equilibration of the column from 2 % to 95 % B within 5 min. The acquisition time was 45 minutes and the mobile phase had a flow rate of approximately 200 µl/min. The isolated HMO were dissolved in an acetonitrile : water solvent (50 : 50, v : v) to a concentration of 20 mg/ml and the injection volume was 10 µl. In the analyses, commercially available HMO standards included non-fucosylated, fucosylated and sialylated oligosaccharides.

Statistics

In the statistical analysis, univariate analyses of the influence of multiple variables on the binary variable were performed using the logistic regression method. The results were presented as OR parameter values with 95% confidence intervals. The significance level of 0.05 was adopted in the analysis. Therefore, all P values below 0.05 were interpreted as indicating significant relationships. The analysis was performed in the R program, version 4.2.2. (R Core Team, 2022).

Results

Characteristics of the study group

The first element of the study was a survey dedicated to study participants, breastfeeding women who decided

to donate a sample of breast milk for scientific purposes. The main purpose of the questionnaire was to characterize the population included in the study and to indicate the possible relationship between specific factors and the results of the analysis. The survey results are presented in Table 1.

The table presents various maternal characteristics and related data. The analysed group consisted of women of reproductive age whose pregnancies ended at term. Most participants had one or two children and were not professionally active during the study period. The majority lived in urban areas. In most cases, the body weight of women both before and after pregnancy was within the norm. Vaginal delivery was the predominant mode of birth, and almost all new-borns had skin-to-skin contact immediately after birth. Most infants had a normal birth weight within the range of 3000–4000 g. Some mothers supplemented breastfeeding with formula milk (at the time of enrolment into the study), although the majority breastfed exclusively. The frequency of breastfeeding varied, and tandem breastfeeding was relatively rare.

Table 1. Characteristics of the study population based on the questionnaire

Maternal characteristic	**Mean ± SD**
Mother's age (years)	30.9 ± 4
Number of children	1.8
Gestational age at delivery (weeks)	39.4 ± 1.7
Breastfed child's age (months)	10.4 ± 9.2
Frequency of breastfeeding over 24 hours	8.3 ± 3.7
Child's sex	**N (%)**
Female	62.5 %
Male	37.5 %
Professional activity	**N (%)**
Yes	14.3 %
No	85.7 %
Place of residence	**N (%)**
City	77.8 %
Village	22.2 %
Tandem breastfeeding	**N (%)**
Yes	9.5 %
No	90.5 %
Type of delivery	**N (%)**
Natural childbirth	71.4 %
Caesarean section	28.6 %
Skin-to-skin contact	**N (%)**
Yes	95.2 %
No	4.8 %
Addition of formula	**N (%)**
Yes	39.7 %
No	60.3 %
Child's birth weight	**N (%)**
1000–2000 g	3.1 %
2000–3000 g	12.5 %
3000–4000 g	73.4 %
> 4000 g	10.9 %
Overweight/obesity before pregnancy	**N (%)**
Yes	27 %
No	73 %
Overweight/obesity after pregnancy	**N (%)**
Yes	34.9 %
No	65.1 %

Of the 63 breastfeeding mothers, 62.5 % were mothers of a girl and 37.5 % of a breastfed boy. The proportion of 71.4 % mothers had vaginal deliveries and 28.6 % mothers had delivered by Caesarean section. The proportion of 95.2 % mothers had the opportunity to practice skin-to-skin contact after delivery; 77.8 % of the sampled women were residents of urban agglomerations and in 39.7 % of cases, a breastfed child was also fed with formula (at the time of enrolment into the study). The birth weight of 73.4 % of children was between 3000 and 4000 g. The proportion of 73 % of women included in the study had a normal body weight (BMI 18.5–24.9) and 27 % were overweight or obese (BMI > 25) before getting pregnant. After giving birth to a child, 34.9 % of women were overweight or obese. The proportion of 9.5 % of women included in the study breastfed their children in tandem – feeding both the younger and the older child at the same time; 85.7 % of women remained professionally inactive during the study. The mean gestational age at birth in the study population was 39.4 ± 1.7 weeks. Children were breastfed on average 8.3 ± 3.7 times per day.

The content of macronutrients in breast milk depending on the stage of lactation

The content of macronutrients in 56 breast milk samples was determined. Some of the women participating in the study encountered problems with collecting larger amounts [25–30 ml] of breast milk; therefore, in these cases, the priority was given to conducting microbiological tests. The average results (and standard deviation) for the tested parameters were as follows: total fat content: 4.4 ± 1.7 (g/100 ml), crude protein 1.3 ± 0.5 (g/100 ml), true protein 1.1 ± 04 (g/100 ml), carbohydrates 7.9 ± 0.6 (g/100 ml), dry matter 13.9 ± 2.0 (g/100 ml), energy value 78.7 ± 16.9 (kcal/100 ml).

The results were also analysed for the individual time intervals from which the breast milk samples examined were derived. Figs. 1–3 present selected average values for individual groups of breast milk.

Statistical analysis showed that the fat content, dry matter content and energy value were significantly higher after the 25th month of lactation than in the previous periods.

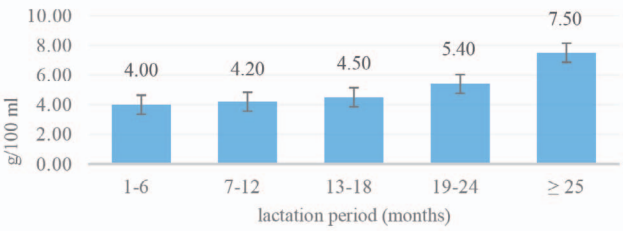

Fig. 1. Fat content in breast milk samples

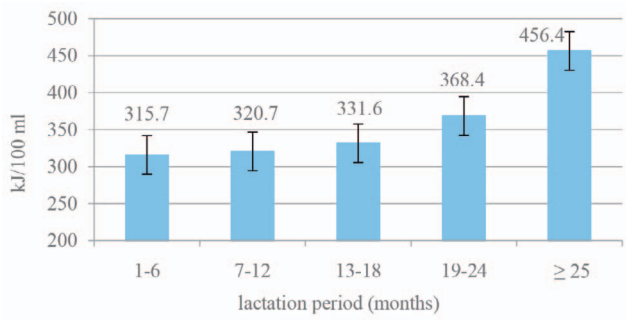

Fig. 2. Energy value of breast milk samples

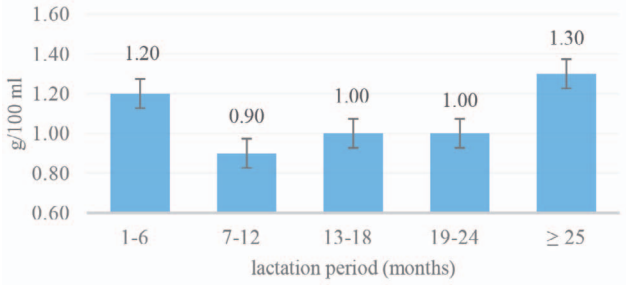

Fig. 3. True protein content in breast milk samples

Lactobacillaceae bacteria content in breast milk

The results indicated the presence of *Lactobacillaceae* bacteria in 22.2 % of the tested breast milk samples. In most cases, only one genus from the *Lactobacillaceae* family was identified in *Lactobacillaceae*-positive samples. Two types were identified in one breast milk sample. They were *Lactobacillus gasseri* and *Lactiplintibacillus plantarum.* The following types of bacteria belonging to *Lactobacillaceae* were identified in the breast milk samples: *Lacticaseibacillus paracasei*, *Lacticaseibacillus rhamnosus*, *Lactobacillus gasseri*, *Limosilactobacillus vaginalis* and *Limosilactobacillus fermentum*, which are listed in Table 2.

Based on microbiological data and data from completed questionnaire responses, the occurrence of potential relationships between the presence of *Lactobacillaceae* bacteria in the composition of human milk and various factors was analysed. However, none of the considered variants brought statistically significant results, which was likely due to the limited number of human milk samples positive for the presence of the discussed bacteria. Nevertheless, certain trends were noticeable and are worth considering in future studies. A higher probability of identifying *Lactobacillaceae* in human milk was noted in the following cases: breastfeeding women from rural areas, high BMI during lactation, absence of gestational diabetes during pregnancy, no supplementary feeding with formula, avoidance of highly processed food in the mother's diet, no use of antibiotic therapy during pregnancy. A lower probability of the occurrence of *Lactobacillaceae* in human milk was observed in cases with advanced lactation (long period of breastfeeding) and with the advanced age of the breastfeeding woman. No influence of factors such as the type of delivery or occurrence of thyroid diseases in breastfeeding women was observed in the microbiological test results. Statistical significance was not obtained for any of the above relationships. Concerning human milk composition, it was observed that bacteria belonging to *Lactobacillaceae* were more frequently identified in human milk samples with a higher share of fats, carbohydrates and higher energy value. Also in this case, the results were not statistically significant.

Table 2. Bacterial strains identified in the tested breast milk samples

Bacterial strain	Number of breast milk samples in which the bacterial strain was identified
Lacticaseibacillus paracasei	3
Lactobacillus gasseri	7
Lactiplintibacillus plantarum	1
Lacticasibacillus rhamnosus	2
Limosilactobacillus vaginalis	1
Limosilactobacillus fermentum	1

HMO in breast milk

Twenty types of oligosaccharides were identified in the tested material. The content of individual oligosaccharides was estimated based on surface area recorded peaks, taking into account the molecular weight of individual HMO. The presented data concern breast milk from a population perspective. Table 3 shows the identified HMO divided into neutral fucosylated oligosaccharides, neutral non-fucosylated oligosaccharides and acidic sialylated oligosaccharides, along with their average content in the milk [µg/1 ml].

The results for the individual groups of HMOs are presented in Figs. 4–6.

Based on the study results, the dominant group of HMO was represented by neutral, fucosylated oligosaccharides. Among them, 2'FL had the highest content. High concentrations of DFLNT and total LNFP were also recorded. In the group of neutral, non-fucosylated oligosaccharides, LNnT and LNT had the highest content. In the group of sialylated HMO, the oligosaccharide 3'-SL dominated quantitatively.

Table 3. Identified breast milk oligosaccharides and their average content

Type of HMO	µg/1 ml
HMO neutral, fucosylated	6536.3 ± 1845.90
HMO neutral, non-fucosylated	1518.3 ± 423.40
HMO acidic, sialylated	1710.5 ± 544.61

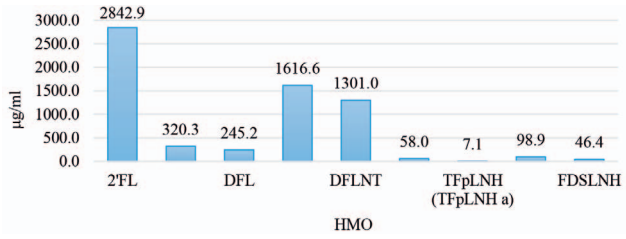

Fig. 4. Estimated average content of neutral, fucosylated oligosaccharides in breast milk samples (µg/1 ml)

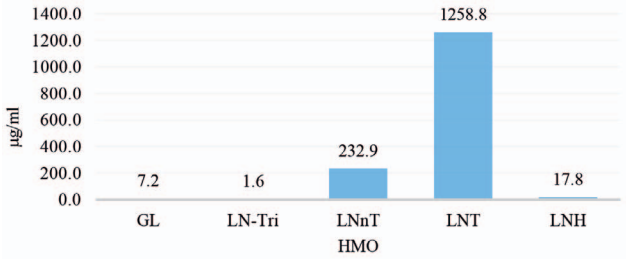

Fig.5. Estimated average content of neutral, non-fucosylated oligosaccharides in breast milk samples (µg/1 ml)

Fig. 6. Estimated average content of sialylated oligosaccharides in breast milk samples (µg/1 ml)

Fig. 7 shows the content of oligosaccharides in breast milk in two sample pools: those that showed the presence of *Lactobacillaceae* in the microbiota composition and those that did not contain these microorganisms.

In breast milk samples positive for bacteria belonging to *Lactoibacillaceae,* a higher content was observed for 2'-FL oligosaccharide compared to samples not containing these microorganisms. A slightly higher content was also observed in the case of HMO such as LNFP I, II and III, as well as LNT and DFLNT. Due to the disproportion in the number of both groups of breast milk samples (positive vs negative for the presence of bacteria belonging to *Lactobacillaceae*), no statistical significance test was performed regarding the results.

The statistical analysis performed did not show statistical significance in any of the analysed relationships between the occurrence of *Lactobacilleceae* bacteria and other factors taken into account. For this reason, future studies conducted using a larger research group seem justified.

Discussion

Breast milk is characterized by variations in the presence or content of individual components. These variations are influenced by a number of factors, such as maternal genetic, perinatal and environmental factors, as well as the stage of lactation (Czosnykowska-Łukacka et al., 2018). These changes can be observed in breast milk macronutrient and energy content, as well as its bioactive components, which include oligosaccharides and breast milk microbiota.

Occurrence of Lactobacillaceae in breast milk

Research on the presence of bacteria of a potential probiotic nature, including bacteria belonging to the *Lactobacillaceae*, is conducted by various research groups

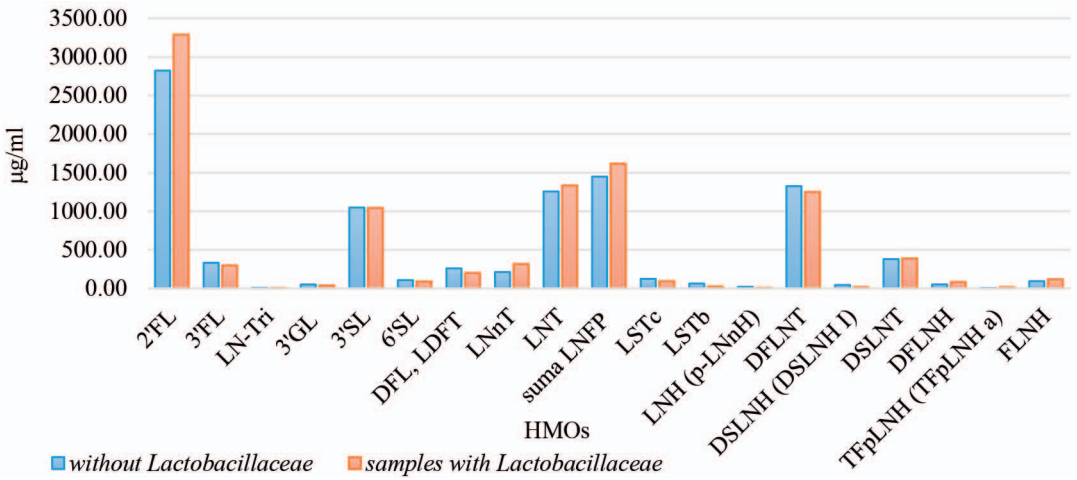

Fig. 7. Average content of HMO (mg/1 ml) in *Lactobacillaceae*-positive breast milk samples and in the group of samples in which no bacteria belonging to *Lactobacillaceae* were identified. The values for samples containing bacteria belonging to *Lactobacillaceae* are marked in orange, while the values for samples that did not contain the discussed microorganisms are marked in blue.

in various centres, often using differing techniques or introduced modifications. These differing approaches and numerous factors may influence the differences in the results. Nevertheless, the results of these studies indicate how much the composition of breast milk may differ in relation to bioactive factors, which include the microbiota of breast milk.

Soto et al. (2014) studied the microbiota of human milk and obtained high values for the presence of *Lactobacillus* in the composition of human milk. As a result of microbiological culture tests, bacteria belonging to *Lactobacillaceae* were isolated in 40.91 % of the 66 samples tested. In a PCR study of 160 breast milk samples, *Lactobacillus*-specific sequences were detected in 67.50 % of the analysed samples. The most common isolates were *L. salivarius* (35 %), *L. fermentum* (25 %) and *L. gasseri* (21.88 %). The authors also indicated that the number of samples positive for lactobacilli was lower in women who underwent antibiotic therapy during pregnancy or lactation, which was also confirmed in our study.

In studies by Jost et al. (2013), the authors investigated breast milk samples collected from seven women at three sampling points (3–6, 9–14, and 25–30 days after delivery). Inoculation on microbiological media resulted in isolation of several *Lactobacillus* strains. These bacteria were detected at an average relative abundance of only less than 0.1 %. Interestingly, the isolation of strains belonging to *Lactobacillaceae* was possible on media that were not dedicated to this purpose. No growth of the expected bacteria was observed on selective media designed to support the growth of *Lactobacillus.*

Since the frequency of identification of *Lactobacillaceae* bacteria in human milk is variable, research is underway to identify compounds and factors that could influence the microbiological composition of human milk. Previous studies conducted in various research centres have shown a relationship between a lower incidence of bacteria from the *Lactobacillcaeae* family and antibiotic therapy, delivery of a child by Caesarean section or use of anaesthesia during labour (Martín et al., 2007; Soto et al., 2014). A study by Martín et al. (2007) indicated the presence of bacteria of the genus *Lactobacillus* in 40.91 % of breast milk samples, including: *L. casei*, *L. fermentum*, *L. gasseri*, *L. gastricus*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*, *L. salivarius* and *L. vaginalis*, with the most common strains being *L. salivarius*, *L. fermentum* and *L. gasseri*. The authors noticed that *Lactobacillaceae* bacteria occurred most often singly in milk samples, which is consistent with our observations. There were also breast milk samples in which two strains of bacteria from the genus *Lactobacillus* could be identified at the same time. Our research also indicates a limited diversity of bacteria belonging to *Lactobacillaceae* identified in a single breast milk sample. Only one milk sample included in the study contained two types of bacteria*, Lactobacillus gasseri* and *Lactiplantibacillus plantarum*. Other breast milk samples in which *Lactobacillaceae* bacteria were identified contained only one genus.

In our study, the prevalence of bacteria from the *Lactobacillaceae* family was 22.2 %. The analysis did not show statistically significant relationships between specific factors and the presence of the tested bacteria due to the small group of samples positive for *Lactobacillaceae*. However, statistically insignificant differences in some factors can be highlighted, which requires further research in a larger study group. A statistically insignificant relationship was observed between the age of the mother and the occurrence of bacteria from the *Lactobacillaceae* family in human milk. The probability of the occurrence of these microorganisms decreased with age. Another statistically insignificant relationship was the lower probability of the occurrence of the tested bacteria in the milk of women who supplementary fed children with formula milk or who frequently consumed highly processed food. A statistically insignificant relationship was also observed between the increase in the fat content and energy value of human milk and the higher probability of the occurrence of *Lactobacillaceae* in the microbiota of human milk.

In studies conducted by Sinkiewicz and Ljunggren (2008), only 15 % of samples out of 220 included in the analyses turned out to be positive for the presence of *L. reuteri*, a bacterium belonging to the *Lactobacillaceae*. In these studies involving samples from women living in different geographical areas, differences in the frequency of identification of the tested bacteria were noticed. *L. reuteri* was most often identified in the milk of breastfeeding women living in Japan and South Korea. This diversity may have a multifactorial basis, ranging from genetic conditions to dietary habits and environmental influences. In the same study, no differences in the level of *L. reuteri* occurrence were observed between the milk of women living in rural areas and urban agglomerations. An important factor influencing the content of breast milk composition is the stage of lactation. In a study by Lopez Leyva et al. (2021), bacteria belonging to *Lactobacillaceae* were present in greater amounts in the environment of breast milk at the stage of early lactation than in later periods. In this study, breast milk samples were collected from women in early lactation (5–46 days after delivery) as well as from a group of women in stable lactation (4–6 months after delivery). The authors compared the results of microbiological analyses of breast milk taking into account the following criteria: mother's age, BMI, number of deliveries, stage of lactation and method of breastfeeding (exclusive, dominant or mixed feeding). The results indicated that bacteria from the genus *Lactobacillus* were more numerous in the early stages of lactation. In our presented test results, there was also a statistically insignificant difference between samples from different stages of lactation. A decreasing trend was seen in the probability of occurrence of *Lactobacillaceae* in relation to breastfeeding period (period of lactation). As time

passed, the probability of occurrence of *Lactobacillaceae* bacteria decreased.

A lower incidence of *Lactobacilaceae* was observed in the milk of women who took antibiotics during pregnancy compared to the group of women who were not subjected to antibiotic therapy. The results of other authors also indicate the importance of taking antibiotics during the indicated period on the composition of breast milk microflora. The already mentioned study by Soto et al. (2014) with 66 breast milk samples showed a lower frequency of bacteria belonging to *Lactobacilaceae* in samples from women using antibiotic therapy during pregnancy or lactation. The authors did not demonstrate a relationship between the length of antibiotic administration and the incidence of *Lactobacillus* bacteria. However, it was possible to indicate a statistically insignificant relationship between the less frequent detection of *Lactobacillaceae* in the breast milk of women who have undergone a Caesarean section and/or anaesthesia during childbirth.

Breast milk oligosaccharides

As with the composition of breast milk microbiota, the presence of oligosaccharides in breast milk varies and depends on many factors. The composition of HMO is largely genetically determined. The key genes involved are the Secretor and Lewis blood group genes, which regulate the expression of transferases, enzymes that shape the structure of the resulting HMO. The Secretor gene encodes the FUT2 enzyme, while the Lewis blood group gene encodes the FUT3 enzyme (Salli et al., 2021).

An important element shaping the HMO content is the stage of lactation. Studies have shown that the content of HMO in breast milk decreases as lactation progresses. The highest concentration of oligosaccharides is observed in colostrum. According to Thurl et al. (2010), colostrum contains 20–23 g/l of HMO. Over time, the content of oligosaccharides decreases, reaching the amount of 12–15 g/l (Thurl et al. 2010) or 5–20 g/l (Mao et al., 2024) in mature milk. This was also confirmed by a review of studies on HMO content in breast milk from 31 countries by Soyyılmaz et al. (2021), reporting the values for individual groups depending on the duration of lactation as follows: the average total HMO content in colostrum was 17.7 ± 3.3 g/l, in transitional milk 13.3 ± 6.5 g/l, and the lowest level of oligosaccharides in mature milk was –11.3 ± 2.2 g/l. The above-mentioned review lists the most abundant oligosaccharides from various groups. The most common compounds were the same that were also determined in our study. These include 2-FL, DFLNT, LNFP-I, LNFP-II, LNT and 3-FL. Numerous studies have shown that despite identification of over 200 different structures of breast milk oligosaccharides, approximately 15 of them constitute the majority of the total HMO fraction. Other factors, apart from genetics, that may have a significant impact on the composition of breast milk oligosaccharides are the mother's age, diet, health status, BMI and health problems (Soyyılmaz et al., 2021).

Since prebiotic and probiotic factors strongly support the health of the babies fed with breast milk, it is worth understanding the relationships between these components in the breast milk composition. The diversity of HMO affects the composition of the microbiota and the development of the immune barrier. Breast milk oligosaccharides are particularly important for the growth of bacteria such as *Bifidobacterium* strains, including *B. longum*, *B.bifidum*, *B. breve* and *Bacteroides*, with particular emphasis on *B. fragilis*. The enzymes of these bacteria have the ability to degrade fucosylated α1-2 oligosaccharides. A study by Sánchez et al. (2021) showed that *Lactobacillus* spp. in infant faeces are correlated with the total galactose concentration in sulphonated milk oligosaccharides, despite the fact that for most bacteria belonging to the *Lactobacillus* spp. HMO are not a good source of carbohydrates. Schwab and Gänzle (2011) showed the inability of lactic acid bacteria to use complex HMO while metabolizing HMO components, which confirms the indirect support of lactic acid bacteria by breast milk oligosaccharides.

Bacteria from the *Lactobacillaceae* family are, therefore, not direct beneficiaries of the supportive function of HMO, but benefit from it indirectly. Most bacteria from the *Lactobacillaceae* family are not able to directly use complex HMO, although individual strains differ in their ability to ferment oligosaccharides. Examples are strains such as *Lactobacillus delbrueckii* ssp. lactis characterized by moderate potential for using fucosylated and sialylated HMO, *L. casei* BL23 using type I HMO, or *L. casei*, *L. acidophilus* and *L. plantarum*, which produce enzymes capable of hydrolysing HMO; however, this does not always stimulate their growth (Thongaram et al., 2017). A study by Mollova et al. (2023) analysed the probiotic potential of strains isolated from human milk and also examined the enzymatic hydrolysis of human milk oligosaccharides by lactic acid bacteria. According to the authors, the ability of bacteria belonging to the *Lactobacillaceae* genus to use HMO seems to be somewhat limited. It was found that the strongest inducer of the synthesis of beta-galactosidase, one of the main enzymes involved in the absorption of HMO, was observed for the *L. fermentum* St5 strain (2.4 U/mg). These studies focused not only on the concentration of individual oligosaccharides in breast milk but also on the potential relationship between the breast milk microbiota and the content of HMO. In our study, the content of breast milk oligosaccharides was determined for the entire studied population, taking into account two subgroups: a group of breast milk samples positive for *Lactobacillaceae* and a group of samples in which the discussed bacteria were not identified. The results allowed us to notice statistically insignificant differences in both groups of samples. In the pool of breast milk samples containing *Lactobacillaceae* bacteria, a higher total HMO content was found, as well as a higher con-

tent of the dominant 2'FL oligosaccharide. In this group, the LNnT content was also higher. Similar observations were made by Cabrera-Rubio et al. (2019), who showed a higher concentration of total and neutral HMO in the tested samples and a higher content of 2'FL in colostrum and mature milk in the presence of *Lactobacillaceae* bacteria. Additionally, higher contents of LNFP I and LDFT and lower contents of LNFP II and LNDFH II were found in samples containing larger amounts of *Lactobacillaceae.*

Breast milk is the optimal food for babies in the early stages of life. Its properties and composition adapt to the needs of the developing young organism, but may also depend on factors such as maternal, environmental or perinatal factors. It is important to learn about emerging relationships in order to make the most of the potential of breast milk, but also to gain a deeper understanding of human physiology. Bacteria with potential probiotic properties may be part of the microbiological composition of breast milk and support the homeostasis of the child's body. Additionally, the presence of components with probiotic effects, including breast milk oligosaccharides, is of significant importance for health promotion. Our study demonstrated the presence of bacteria from the *Lactobacillaceae* family in 22.2 % of the tested breast milk samples. The relationships between these bacteria and the various identified factors were statistically insignificant due to the limited number of samples testing positive for *Lactobacillaceae*. A statistically insignificant association of the presence of *Lactobacillaceae* in breast milk with factors such as the stage of lactation, age of the breastfeeding woman, geographic origin of the sample or use of antibiotic therapy during pregnancy was shown. Additionally, a negligible relationship was also demonstrated between the presence of bacteria from the *Lactobacillaceae* family and a higher total concentration of breast milk oligosaccharides, including 2'FL, in samples testing positive for *Lactobacillaceae*. There is a need to conduct research using a larger study group.

Bacteria from the *Lactobacillaceae* family exhibit various health-promoting properties. However, under natural conditions, such as infant feeding with human milk, the number of these microorganisms reaching the child's digestive tract is relatively low. At this point, it is essential to consider variations in the presence of probiotic potential, which require thorough research and evaluation. Proper nutrition is a fundamental pillar of maintaining the bodily homeostasis, contributing to optimal growth and development, particularly in the early period of life. Breast milk, as the optimal source of nutrition during this critical period, remains the subject of ongoing research regarding its composition and importance of individual components. Although breast milk contains a set of constant elements, compositional differences among individual women are also observed. Understanding these variations and potential factors influencing them will provide deeper insights into human physiology. The findings from this study highlight the variability of breast milk, which is rich in bioactive components essential for the infant while also emphasizing its individual variability.

Conflict of interest

All authors declare that they have no conflict of interests.

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