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Analysis of the Major Probiotics in Healthy Women's Breast Milk by Realtime PCR. Factors Affecting the Presence of Those Bacteria

Georgia Nikolopoulou [1], Theofania Tsironi [2], Panagiotis Halvatsiotis [3], Ekaterini Petropoulou [4], Nikolaos Genaris [4], Despina Vougiouklaki [4], Dionyssios Antonopoulos [4], Apollon Thomas [4], Anastasia Tsilia [5], Anthimia Batrinou [4], Efstathia Tsakali [4,6,*], Jan F. M. Van Impe [6] and Dimitra Houhoula [4,*]

1 National Public Health Organization, 15123 Maroussi, Greece; georgianik@hotmail.com
2 Department of Food Science and Human Nutrition, Agricultural University of Athens, 11855 Athens, Greece; ftsironi@aua.gr
3 2nd Propaedeutic Department of Internal Medicine, "ATTIKON" University Hospital, Medical School, National and Kapodistrian University of Athens, 12462 Chaidari, Greece; pahalv@gmail.com
4 Department of Food Science and Technology, University of West Attica, 12243 Egaleo, Greece; ft14066@uniwa.gr (E.P.); nikos_genaris@hotmail.com (N.G.); dvougiouklaki@uniwa.gr (D.V.); antondion@uniwa.gr (D.A.); ft16029@uniwa.gr (A.T.); batrinou@uniwa.gr (A.B.)
5 General Maternity Hospital Helena Venizelou, 11521 Athens, Greece; anatsilia@yahoo.gr
6 Department of Chemical Engineering, BioTeC + − Chemical and Biochemical Process Technology and Control, KU Leuven, 9000 Gent, Belgium; jan.vanimpe@kuleuven.be
* Correspondence: etsakali@uniwa.gr (E.T.); dhouhoula@uniwa.gr (D.H.)

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Abstract: Breast milk has been reported as a bacteria source that affects infant gut microbiota development. The present study utilizes a realtime PCR method to identify *Lactobacillus* and *Bifidobacterium* spp. in the breast milk of healthy women and attempts to identify factors affecting those human milk bacteria. Breast milk samples—both colostrum and mature milk—of 100 healthy women, were collected in Greece along with data about the demographic factors and nutritional habits of the volunteers. The colostrum samples were found to have higher percentages of either *Bifidobacterium* or *Lactobacillus* (76.9% and 48.6%, respectively) compared to the mature milk samples. For younger women, aged from 18 to 29 years, and women from rural areas, bacteria were detected in higher incidence than for older groups and women in urban areas, respectively. Moreover, for high-BMI women, bacteria were detected in lower incidence than for those with normal BMI. Probiotic supplements did not affect the composition of the breast milk-identified bacteria. Various factors such as lactation stage, maternal age, maternal weight, and residential location may contribute to the presence of those species in human milk. RT PCR has significant potential for the microbiological analysis of human milk.

Keywords: probiotic bacteria; realtime PCR; breast milk; *Bifidobacterium* spp.; *Lactobacillus* spp.; breast milk

1. Introduction

Human breast milk is considered a high-quality source of nutrients for babies as it contains a large number of bioactive compounds, especially antioxidants, but also growth factors, hormones, and cytokines [1] and protective factors [2,3]. Breastfeeding has many established benefits for maternal and child health as the human breast milk is a known source of probiotic bacteria such as *Bifidobacterium* and lactic acid bacteria (LAB), which are reported to have a beneficiary effect in the infant gut [4,5].

These bacteria may also play a significant role in the incidence and severity of infections of the suckling infant. Specific strains of LAB isolated from human milk have shown an ability to inhibit growth of a wide range of pathogenic bacteria by competitive

exclusion or through the production of antimicrobial components, as for example, bacteriocins and organic acids [4,6]. It is estimated that breastfed infants receive 10^4–10^6 bacteria per day (based on an average daily consumption of 800 mL of milk) with most isolated species belonging to the genera *Staphylococcus*, *Streptococcus*, *Lactobacillus*, and *Bifidobacterium* spp. [7]. Formula-fed infants lack exposure to these potentially health-promoting bacteria, thus delivery of probiotic *Bifidobacterium* and *Lactobacillus* spp. in infant formula or milk substitutes remains a priority [8]. The diversity of LAB isolated from human milk and their characteristics have been investigated in the literature [4,9]. Kansandee (2010) [5] identified LAB from human colostrum and the results were supportive of the protective role of probiotics. Meanwhile, it has been reported that many factors can influence the composition of human milk. such as lactation stage, maternal age, maternal body mass index (BMI), residence location, smoking, dietary patterns, e.g., dairy consumption [10], but also number of pregnancies, other environmental factors [1], and maternal health [11].

The published research on the composition of the human milk microbiota and the factors that affect it, is currently limited and their introduction and utilization will open novel perspectives in the field. Among the currently available molecular methods, realtime PCR (RT-PCR) can provide accurate and sensitive detection of individual species or specific bacterial groups. The aim of the present study is to identify the probiotic *Biffidobacterium* spp. and *Lactobacillus* spp. in human milk samples by using an RT-PCR technique. *Bifidobacterium longum* and *Bifidobacterium bifidum* were selected as two of the four human-derived *Bifidobacterium* species known to be abundant in the intestine of healthy human infants but relatively diminished in adults [12]. Moreover, a correlation of possible factors that influence the human breast bacteria is evaluated.

2. Materials and Methods

2.1. Human Milk Samples

One hundred independent human breast milk samples were obtained from healthy women at a time frame between 09:00 and 10:00 a.m., until one month from the postnatal day, approximately 25 mL in one aliquot, and analyzed for the presence of some probiotic microorganisms (76 milk samples were provided by the Human Milk Bank of the General Maternity Hospital Helena Venizelou, Athens, Greece, and 24 samples from volunteer mothers). Before sample collection, mothers were given written instructions for standardization purposes. After washing their hands with soap and cleaning their nipples in order to minimize milk contamination, they were asked to use a BM pump with an automatic regulator to suction milk from the breast opposite to that from which their babies had previously suckled. Bottles and suction funnels were autoclaved before their use.

All samples were collected in bottles, immediately aliquoted under sterile conditions and transported in sterile tubes and stored at −20 °C immediately after sampling. The research was approved by the Scientific Advisory Board of the Helena Venizelou Hospital and complied with all rules of bioethics (No 452/2021).

2.2. Genomic DNA Extraction

The milk samples were centrifuged at 14,000× *g* for 15 min. DNA was directly extracted from the cell pellet using an automatic extractor with the Nucleic Acid Extraction Kit, (ZYBIO Company, Chongqing, China) following the protocol recommended by the supplier. The purity and quantity of extracted DNA was evaluated spectrophotometrically by calculating OD_{260}/OD_{280} (spectrophotometer Epoch, Biotek).

2.3. Realtime PCR

The DNA was used in subsequent realtime PCR using the MeltDoctorTM HRM MASTER Mix (Applied Biosystems) that had the fluorescent dye MeltDoctorTM HRM Dye (initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, hybridization at 59 °C for 30 s, and elongation at 72 °C for 1 min). Primers were used as

previously described [13–16] for the *Bifidobacterium* spp. and *Lactobacillus* spp. and for the species *Bifidobacterium bifidum* and *Bifidobacterium longum* as shown in Table 1.

Table 1. Primers used for the detection of genera *Bifidobacterium* spp., *Lactobacillus* spp., and *Bifidobacterium bifidum* and *Bifidobacterium longum*.

Target Microorganism	Primer	Sequence (5′ to 3′)	Reference
Bifidobacterium	Bifid-F Bifid-R	CTC CTG GAA ACG GGT GG GGT GTT CTT CCC GAT ATC TAC A	Requena et al., 2002
Lactobacillus	Lab 159 Lab 677	GGA AAC AGT TGC TAA TAC CG CACCGC TAC ACA TGG AG	Heiling et al., 2014
Bifidobacterium bifidum	B.biFIdum FWD B.biFIdum REV	CCA CAT GAT CGC ATG TGA TTG CCG AAG GCT TGC TCC CAA A	Haarman et al., 2005
Bifidobacterium longum	B. longum FWD B. longum REV	TTC CAG TTG ATC GCA TGG TC GGG AAG CCG TAT CTC TAC GA	Haarman et al., 2005

PCR products were analyzed with 1.2% (w/vol) agarose (Sigma, Kanagawa, Japan) gel with ethidium bromide staining. A 100-bp ladder (Invitrogen, Paisley, UK) was used as a molecular weight standard. Gels were run for approximately 1 h at 100 V, and the DNA was visualized and analyzed in a gel documentation system (Gel Doc 2000, Bio-Rad, Hercules, CA, USA).

Five samples were chosen randomly in order to assess the repeatability of the method for the detection of *Lactobacillus* and *Bifidobacteria*. The proposed assay was repeated 5 times for each DNA extract.

2.4. Questionnaire

The study evaluated maternal demographics, anthropometric characteristics (age, BMI, and residence in urban or rural areas), and nutritional habits (consumption of dairy products, probiotic supplements, and dairy products enriched with probiotics).

Biomedical Ethics issues: The collection of clinical and epidemiological data from patients was correlated with the laboratory research results, and was conducted in such a way as to fully guarantee the patients' anonymity and personal data confidentiality. All the questionnaires were collected with the consent of the patients.

2.5. Statistical Analysis

Analysis of variance (one way ANOVA) with a probability value of significant difference equal to 5% was used for the evaluation of the differences between the prevalence of probiotic bacteria in breast milk in the tested groups, in terms of age, education level, BMI, place of residence, and dietary behavior (STATISTICA® 7.0; StatSoft Inc., Tulsa, OK, USA).

3. Results

Breast milk was collected from 100 healthy women in Greece, aged from 20 to 47 years. Twenty-six (26) samples were colostrum and seventy-four (74) mature breast milk. The probiotic bacteria *Bifidobacterium* were detected in 61.5% samples of colostrum, and 37.8% of mature breast milk. The presence of the genus *Lactobacillus* was identified in the 46.2% of colostrum and 24.3% of mature breast milk. The statistical analysis indicated significance differences in the two types of breast milk ($p < 0.05$). In 18% of the samples the presence of both genera was detected. The colostrum milk samples were found to have higher percentages of either *Bifidobacterium* or *Lactobacillus* when compared to the mature milk samples (76.9% and 48.6%, respectively) ($p < 0.05$) (Table 2). The samples positive for *Bifidobacterium* were further tested in order to determine the species. From the 44 positive samples for *Bifidobacterium* spp. tested, 20 (45.5%) were found to be *Bifidobacterium longum*, 14 samples (31.8%) were *Bifidobacterium bifidum*, and 10 (22.7%) were identified as other *Bifidobacterium* species.

Table 2. Realtime PCR detection of probiotic bacteria *Bifidobacterium* and *Lactobacillus* in human breast milk.

Type of Breast Milk	Number of Samples Analyzed (*n*)	Positive for *Bifidobacterium*	Positive for *Lactobacillus*	Positive Samples for either *Bifidobacterium* or *Lactobacillus*
colostrum	26	16 (61.5%)	12 (46.2%)	20 (76.9%)
mature	74	28 (37.8%)	18 (24.3%)	36 (48.6%)
total samples	100	44 (44.0%)	30 (30.0%)	56 (56.0%)

The highest percentages of samples positive in either *Bifidobacterium longum* or *bifidum* (37.5%) were found for colostrum, when compared to mature milk (14.3% and 28.6%, respectively) ($p < 0.05$) (Table 3). In the breast milk of all women aged under 29 years, both *Lactobacillus* spp. and *Bifidobacterium* spp. were detected.

Table 3. Realtime PCR detection of the probiotic species *Bifidobacterium longum* and *Bifidobacterium bifidum* in human breast milk.

Type of Breast Milk	Number of Samples Analyzed (*n*)	Positive for *B. longum*	Positive for *B. bifidum*
colostrum	16	6 (37.5%)	6 (37.5%)
mature	28	4 (14.3%)	8 (28.6%)
total samples	44	10 (22.7%)	14 (31.8%)

As age progresses, this incidence declines. In almost all women (97.1%) from smaller communities (villages), probiotic bacteria *Bifidobacterium* and *Lactobacillus* were detected (and this was statistically significant) more than in the milk of women from urban areas/major cities (33.8%) ($p < 0.05$). In the majority of women with a BMI less than 25, milk bacteria were detected, while in the other BMI categories the incidence decreased substantially. The consumption of probiotics did not affect the composition of the breast milk microbiota ($p > 0.05$). Women consuming yogurt regularly in their diet, had in their majority (64.3%) milk bacteria detected, and this was statistically significant more than that in the non-yogurt eaters ($p < 0.05$) (Table 4).

Table 4. Correlation of maternal demographics, anthropometric characteristics, and nutritional habits with positive samples in *Bifidobacterium* and/or *Lactobacillus*.

Data	Number of Women	Total Positive Samples
Age		
18–24	7	7 (100%)
25–29	15	15 (100%)
30–34	35	23 (65.7%)
35–39	23	6 (26.1%)
40–44	15	5 (33.3%)
≥45	5	0
BMI		
<20	15	13 (86.7%)
20–25	40	32 (80%)
25–30	35	8 (22.9%)
>30	10	3 (30%)

Table 4. *Cont.*

Data	Number of Women	Total Positive Samples
Place of residence		
Urban areas	65	22 (33.8%)
Rural areas	35	34 (97.1%)
Probiotics Supplements		
Yes	40	24 (60%)
No	60	32 (53.3%)
Dairy Products		
Yogurt	70	45 (64.3%)
Dairy products enriched with probiotics	30	11 (36.7%)

4. Discussion

Breast milk is an extremely important source of gut microbiota for newborns, since this is the single natural source of nutrients that they receive [17]. Up to 20 years ago, it was believed that breast milk was sterile and any bacteria present were associated with the infant's mouth or mother's skin [18]. However, Hunt et al. (2011) [19] identified the diversity and temporal stability of bacterial communities in three different milk samples collected from sixteen women in the USA within a period of one month. According to this analysis, nine genera were identified in every sample, including *Staphylococcus*, *Streptococcus*, *Serratia*, *Pseudomonas*, *Corynebacterium*, *Ralstonia*, *Propionibacterium*, *Sphingomonas*, and *Bradyrhizobium*. Jost et al. (2014) [20] characterized the human milk microbiota by culture and molecular techniques and reported the presence of *Enterococcus* and *Lactobacillus* in 9.5% and 15% of the tested samples, respectively. Soto et al. (2014) [21] using qualitative PCR analysis reported the presence of lactobacilli and bifidobacterial DNA in 67.50% and 25.62%, respectively, of the samples analyzed (160 samples). Tušar et al. (2014) [22] reported the presence of *Lactobacillus*, *Enterococcus*, *Staphylococcus*, and *Bifidobacterium* in milk collected from 47 women in Slovenia. González et al. (2013) [23] identified as the most persistent bacterial groups in milk collected from women in Mozambique as *Staphylococcus*, the species *Streptococcus* and *Lactobacillus*.

In the present study PCR techniques were utilized in a large number of human milk samples (100) and detected two species of *Bifidobacterium* and *Lactobacillus*. Moreover, the population may harbor different bacteria in different locations of the globe reflecting environmental, cultural, and nutritional differences. Several studies have reported differences in the composition and core microbiota of BM in different geographic locations [24,25]. One study suggested that women in the USA might have less *Lactobacillus* and *Bifidobacterium* in their BM compared with women in Europe [19].

Recently, a link between the infant's and adults' gut microbiome has been reported, indicating that the infant's gut is initially colonized by bacteria originating from either breast milk or the environment [26]. At the same time, human milk contains high concentrations of non-digestible complex oligosaccharides (prebiotics), which reach the colon and subsequently nourish the infant gut microbiota [8]. It has been reported that the establishment of gastrointestinal tract microbiota in infants may be critical for maintaining the health and homeostasis of animals, including humans. Breast milk is a major factor for immunological programming, metabolome, and microbiome [9,27]. However, further studies are indicated in order to highlight the correlation between human milk microbiota and the immune system stimulation in newborns, as up to now no evidence about this association has been reported [28].

Based only on the findings, we are still unable to suggest which maternal behavior serves best the infants' microbiota colonization, as it is unclear which kind of bacteria and at which concentration, serve optimally the colonization of infant gut and stimulate its immune system. Women aged less than 29 years, thinner with BMI less than 25, residing at rural areas, and consuming yogurt had a larger population of milk bacteria. As these

factors are indicators of a healthier condition of the mother, they could also be factors for a healthier infant microbiome. Soto et al. (2014) [21] suggested that the factor that exerted the strongest influence on the presence of lactobacilli or bifidobacteria was the administration of antibiotherapy to mothers during pregnancy or lactation. Cabrera-Rubio et al., (2012) [29] reported that women with high body mass indexes (BMIs) had a less diverse bacterial community in the BM microbiota with higher total bacterial loads and a higher absolute abundance *Lactobacillus* in colostrum (18 participants). Two studies (with 133 and 393 participants respectively) did not find any influence of BMI on the composition of the BM microbiota [30,31].

The comparison between colostrum and mature milk, favors colostrum as more women were positive for the identified microbes. Since colostrum milk is present only for the first three days of lactation, interesting associations rise. A suggested explanation could be that the rise of milk production and flow lead the microbial presence to diminish. What should be evaluated is whether it is beneficial for the infant as it breaks through with a rapid gut colonization in the first three days of lactation. The results of the present study agree with two studies (with 47 and 80 participants respectively) that reported higher total bacterial loads in colostrum compared to mature milk [32,33]. Finally, it is interesting that the maternal consumption of prebiotics does not influence the milk identified bacteria in this study.

5. Conclusions

The objective of the present study was to identify *Lactobacillus* spp. and *Bifidobacterium* spp. bacteria in the breast milk of healthy women in Greece by applying a realtime PCR method, and the correlation of those species with different factors. The results confirm the presence of bacterial DNA in breast milk and indicate that RT-PCR method has a significant potential in the microbiological analysis of human milk. It was also identified that factors related with higher incidence of the bacteria in breast milk were younger age, BMI < 25, residing in urban areas, and consuming yogurt regularly. Moreover, it was found that colostrum had a higher incidence of the bacteria. It remains to be proven if this microbe incidence is for the benefit of the gut colonization of the infant and if this impacts its immune robustness.

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