ISSN 1330-9862
(FTB-3575)

original scientific paper

Cultivable Bacteria from Milk from Slovenian Breastfeeding Mothers

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Received: November 30, 2013
Accepted: March 5, 2014

Summary

The human milk microbiota plays an important role in the development of infant´s intestinal microbiota and in the protection of infants against pathogenic microorganisms. The aim of this study is to investigate the microbial composition of human milk from 47 breastfeeding mothers, sampled separately from the left (L) and the right (R) breast, on the 30th day after giving birth. We quantified some major bacterial groups in human milk, compared the cultivable bacteria from the left and the right breast and identified strain diversity of lactobacilli. The results revealed that human milk contains lactic acid bacteria, bifidobacteria and mesophilic aerobic bacteria, of which the last were the most abundant group. Although the microbial composition of human milk in L and R breast samples was comparable, the concentration of bacteria in the two samples from the same mother might vary, therefore milk sample taken from one breast only does not reflect the average microbial composition. Using random amplified polymorphic DNA (RAPD), 86 presumptive isolates of lactobacilli from representative samples of human milk from 11 mothers were classified into 11 groups. Moreover, representatives of different RAPD groups were identified using 16S rDNA sequencing. Out of 11 RAPD groups, 4 groups (21 % of all isolates) belonged to the species *Lactobacillus gasseri*. The most representative RAPD profile (48 % of isolates) was found to belong to the species *Lactobacillus fermentum*. Other RAPD groups were associated with *L. salivarius*, *L. reuteri*, *Enterococcus faecium*, *Staphylococcus epidermidis* and *Bifidobacterium breve* species.

Key words: human milk, cultivable microbiota, RAPD PCR, *Lactobacillus* sp., 16S rDNA sequencing

Introduction

Human milk is a complex biological fluid adapted to fulfil the nutritional requirements of the rapidly growing infant. It contains the right balance of nutrients for the growth and development of newborns, and also contains many bioactive substances that benefit neonates' immune system (*1*,*2*). The bioactive substances present in colostrum, transitional as well as in mature human milk include immunoglobulins, immunocompetent cells, antimicrobial fatty acids, polyamines, oligosaccharides, lysozyme, lactoferrin, glycoproteins and antimicrobial peptides (*3*,*4*).

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Human neonates are born with an immature immune system, and many of the innate components of mucosal immunity are not yet fully developed (*2*). After birth, neonates are continuously exposed to new microbes that enter the gastrointestinal tract from the environment (food, air, skin, *etc.*) (*5*). This begins with human milk, which may contain up to 10^5 CFU of commensal bacteria per mL (*6–8*).

Frequently isolated bacterial groups include genera *Staphylococcus, Streptococcus, Lactococcus, Leuconostoc, Weissella, Enterococcus, Propionibacterium, Lactobacillus* and *Bifidobacterium,* which should be considered as components of the natural microbiota of human milk, rather than as mere contaminant bacteria (*9*). These commensal bacteria may originate from the nipple and surrounding skin as well as from the milk ducts in the breast. Different studies also suggest that some bacteria present in the maternal gut could reach the mammary gland during late pregnancy and lactation through a mechanism involving gut monocytes (*10,11*). The recent culture-independent studies of bacterial community based on pyrosequencing of the 16S rRNA gene revealed much higher diversity of human milk microbiome than could be concluded from the previous results obtained by culture-dependent methods (*12,13*).

It is now well known that human milk bacteria may play several roles in the infant gut. First of all, they can contribute to the reduction of the incidence and severity of infections in the breast-fed infant by different mechanisms, such as competitive exclusion, production of antimicrobial compounds, or improvement of the intestinal barrier function by increasing mucine production and reducing intestinal permeability (*10,14*).

The greatest difference between the microbiota of breast-fed and formula-fed infants lies in numbers and species composition of bifidobacteria. Breast-fed infants show a predominance of bifidobacteria and lactobacilli, whereas formula-fed infants develop a mixed microbiota with a lower number of bifidobacteria (*7,15*). Besides streptococci, which are very frequently isolated from human milk, viable bifidobacteria and lactobacilli are also commonly found (*16*). The isolates of lactic acid bacteria and bifidobacteria from human milk are attractive candidates for use in infant formulas due to their positive effects on health and safety.

The microbiological composition of human milk has not been studied in the female population in Slovenia so far. In the present study, human milk samples from 47 Slovenian mothers obtained 30 days after delivery were examined for cultivable bacteria. One of the aims was to determine whether there are significant differences in the number of selected groups of bacteria and in the strain composition between the samples of the same mother obtained from each breast, left (L) and right (R). This is important from the point of view of ensuring the representativeness of the samples intended for individual milk microbiome determination, and is to our knowledge also the first report on the comparison of microbial composition of two breasts from the same mother. Besides this, the representatives of the groups of isolates with highly similar genotypes (RAPD patterns) were identified to a species level.

Materials and Methods

Samples

The human milk samples analysed in the present study were obtained from mothers recruited in the central Slovenian region (Ljubljana) in the period from December 2010 to October 2012. The samples were manually collected from 47 mothers, separately from the left (L) and the right (R) breast, on the 30th day after giving birth. The sampling was done before breastfeeding. The nipples and mammary areolae were washed with hot water and soap, the hands were washed and soaked into antiseptic solution. The first drops of milk (approx. 0.5 mL) were discarded. All samples were collected in sterile tubes and stored at –80 °C until analyses.

Microbial plate counts

To determine the number of cultivable bacteria, milk samples were previously serially diluted in Ringer's solution (¼ strength). Non-diluted or diluted samples were plated in two parallels on different growth media as follows: de Man-Rogosa-Sharpe agar (MRS; Merck, Darmstadt, Germany) for lactic acid bacteria, brain heart infusion agar (BHI; Merck) for aerobic mesophilic bacteria, M17 agar (Merck) for lactococci and enterococci, Rogosa agar (Merck) for lactobacilli, TOS-propionate agar (Yakult Honsha Co., Ltd., Tokyo, Japan) supplemented with mupirocin for bifidobacteria, and chromogenic UriSelect 4 (URI; Bio-Rad, Marnes-La-Coquette, France) for *Escherichia coli*, *Enterococcus* sp., *Staphylococcus aureus* and *Staphylococcus epidermidis*. The plates with MRS, TOS-propionate and Rogosa agar were incubated anaerobically (Generbox anaer system; bioMérieux, Marcy l'Etoile, France) at 37 °C for 2 days. BHI, M17 and URI plates were incubated under aerobic conditions at 37 °C for 2 days. After incubation, colonies were counted.

RAPD analysis of selected isolates

Random amplified polymorphic DNA (RAPD) method was used to determine the diversity of presumptive lactobacilli in human milk on the strain level. From the milk of 11 randomly selected mothers, 10 colonies grown on Rogosa agar were selected and subcultured in MRS broth supplemented with L-cysteine (0.05 %) at 37 °C under anaerobic conditions. Bacterial cells were harvested by centrifugation and spread on Rogosa agar plates. After incubation, the bacterial genomic DNA was extracted from the colonies using DNA extraction kit Smart Helix® EZextract (Sekvenator, Ljubljana, Slovenija), following the manufacturer's instructions. DNA extracts from the colonies were used for genotyping by RAPD analysis. The amplification was performed using M13 primer (5'-GAGGGTGGCGGTTCT-3') and previously published reaction conditions (*17*). A 1000-bp ladder (Fermentas, Vilnius, Lithuania) was used as a molecular mass standard. Polymerase chain reaction (PCR) products were separated by standard gel electrophoresis, using 1.8 % agarose gel and TAE buffer. A constant voltage of 90 V was applied for 60 min. Agarose gel was stained with SYBR® Safe (Invitrogen by Life Technologies, Carlsbad, CA, USA) and images were digitized with the imaging system Syngene Chemigenius 2 (Syngene, Cambridge, UK).

Species identification of isolates by sequence analysis of 16S rDNA

Representatives of 11 different RAPD groups were identified on the species level using sequence analysis of 16S rDNA. Total genomic DNA was extracted from 18-hour MRS cultures by Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). 16S rDNA was amplified by PCR using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1495R (5′-CTACGGCTACCTTGTTACGA-3′) (*18*). PCR products were purified with the Wizard SV gel and PCR Clean-Up System (Promega). Automated sequencing of the purified products was done at Microsynth AG (Vienna, Austria). The sequences were analysed using the BLAST algorithm (*19*).

Statistical analysis

Each mother (N=47) contributed two milk samples (one from the left breast and the other from the right breast), which were analysed separately, each in parallel. The average values of two parallels were used in further analyses.

First, the differences in bacterial plate counts (log CFU/mL) of particular bacterial group in the milk samples derived from the individual breasts (left: L, right: R) were calculated for each mother. The absolute values of these differences, *i.e.* $|(\log \text{CFU/mL})_R - (\log \text{CFU/mL})_L|$, were distributed in five groups on the basis of their frequency.

Second, the average bacterial plate counts were calculated from the two values obtained for two milk samples of each mother (one from the left and one from the right breast). For each bacterial group, the medians were calculated and the results presented as box-and-whisker plots representing the medians, the upper and lower extremes of the range of values and the 75th and 25th percentiles.

Results and Discussion

Human milk samples from 47 mothers were plated on MRS, BHI, M17, Rogosa, TOS-propionate or URI agar for determination of the concentration of cultivable bacteria. The average bacterial counts (CFU/mL) on different media are presented in Fig. 1. The box plots represent the median, the upper and lower extremes of the range of values and the 75th and 25th percentiles. The most represented were mesophilic aerobic bacteria ($1.1 \cdot 10^4$ CFU/mL), followed by lactococci and enterococci ($2.9 \cdot 10^3$ CFU/mL), lactic acid bacteria (LAB) grown on MRS medium ($2.0 \cdot 10^3$ CFU/mL) and *S. epidermidis* ($1.4 \cdot 10^3$ CFU/mL). Well represented was also the species *E. coli* ($5.0 \cdot 10^2$ CFU/mL). These results are in accordance with previous studies of human milk using cultivation methods (*7*,*12*,*20*).

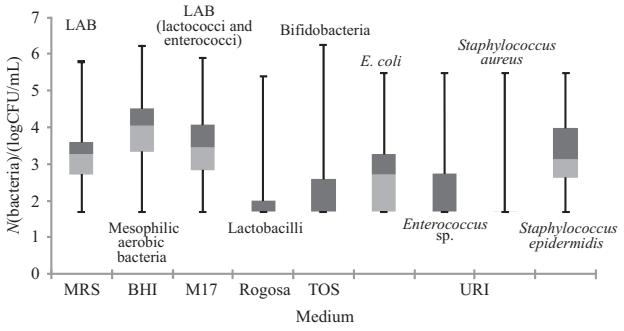

Fig. 1. Bacterial counts (CFU/mL) in breast milk (N=47) collected on the 30th day after delivery and plated on different media. The box-and-whisker plots represent the medians, the upper and lower extremes of the range of values and the 75th and 25th percentiles. LAB=lactic acid bacteria

The samples derived from the left (L) and right (R) breast of each mother were analysed separately. For the reasons of clarity, the average values of L and R samples of individual mothers were presented in Fig. 1. However, Fig. 2 represents the average differences between both counts of individual mothers' samples: $(\log \text{CFU/mL})_R - (\log \text{CFU/mL})_L$, expressed as absolute values. The results of bacterial counts of L and R samples differed less than 0.5 log units in more than 50 % of the samples, with the exception of counts on BHI agar and counts of *E. coli* on URI agar. For another half of the samples, the differences between bacterial counts in L and R samples were more than 0.5 log units, reaching even up to 3.7 log units, which is above the level that can still be considered as acceptable tolerance (0.5 log) of bacterial colony counts (*21*).

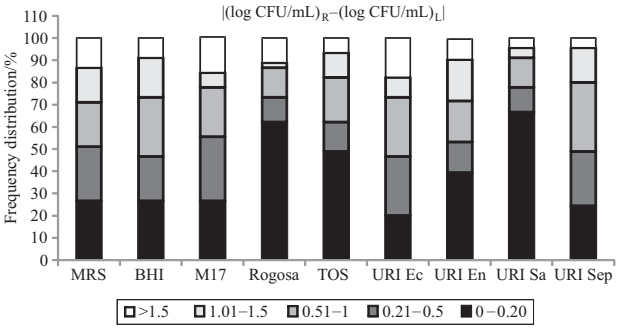

Fig. 2. Frequency distribution of the differences in the bacterial plate counts (log CFU/mL) of the milk samples from 47 breast-feeding mothers, derived from the individual breasts (left: L, right: R). The following media were used for plate counting: MRS, BHI, M17, Rogosa, TOS with mupirocin, and UriSelect 4 medium: URI Ec for *Escherichia coli*, URI En for *Enterococcus*, URI Sa for *Staphylococcus aureus*, URI Sep for *Streptococcus epidermidis*

The results of RAPD genotyping of 86 presumptive *Lactobacillus* isolates from Rogosa agar obtained from human milk of 11 mothers are shown in Fig. 3. Electrophoresis of RAPD amplification products revealed 11 RAPD patterns labelled with letters A-K. Some of the RAPD groups (A and B) included the isolates from different mothers. The pattern labelled A was, for instance, observed in the isolates from eight mothers, and pattern B in the isolates from three mothers. Other patterns were typical for the isolates from one mother only, or not more than two mothers. Often the strains derived from different breasts belonged to the same RAPD group. Considering that the isolates with the same RAPD pattern are either identical or genetically very close, it is possible to conclude that the same or very similar isolates are

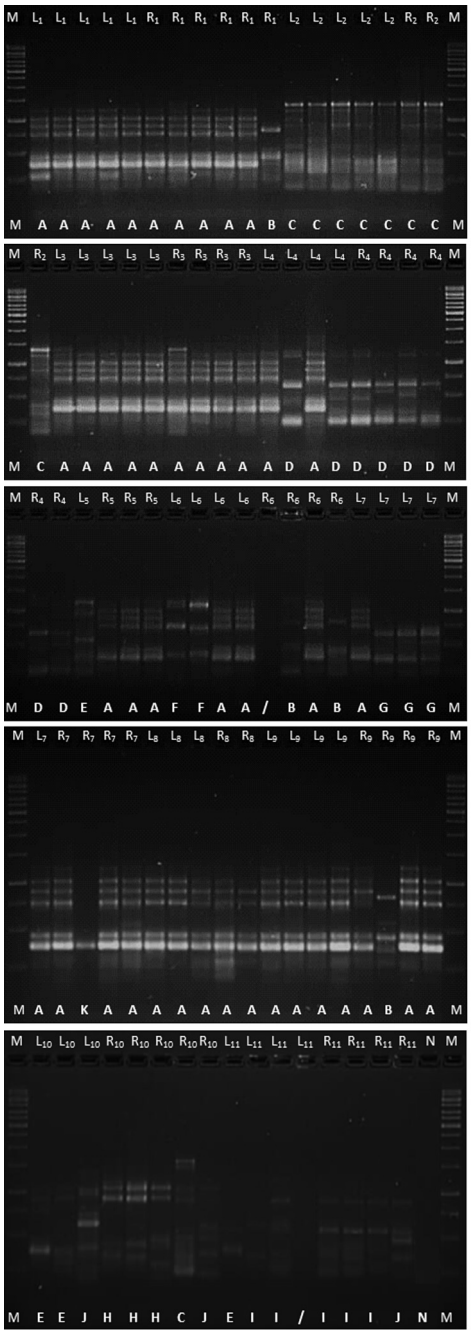

Fig. 3. Results of RAPD analysis of isolates (N=86) obtained from the milk collected from both breasts (left: L, right: R) of 11 mothers. 1–11=designation for mothers, A–K=designation for groups of isolates differing by RAPD pattern, M=molecular marker, N=negative control. One strain from each RAPD group was identified to species level by 16S rDNA sequencing. The results of identification are shown in the bottom right

usually found in both breasts. The observation of particular patterns in different mothers indicates either that certain strains are very widespread, or that RAPD has a lower discriminatory power for some species, as already reported for *L. acidophilus* (*22*). RAPD has already been successfully used for the investigation of genetic relatedness among bacterial isolates belonging to *Lactobacillus* genus, *Bifidobacterium* genus and *S. epidermidis* species from mother and child and comparison of bifidobacteria isolates from human milk or infant faeces taken at different times (*7*,*20*). While in the study of Solís *et al.* (*7*) the strains (*Bifidobacterium*) isolated from different mother–infant pairs always showed different RAPD profiles, in our study some strains (*Lactobacillus*) from different mothers shared high similarity (*7*). The presence of particular *Lactobacillus* genotypes, for instance *L. plantarum*, in different samples (milk, faeces) is, however, not so rare observation (*23*). Besides RAPD, the PCR amplification of repetitive bacterial DNA elements (rep-PCR method) is also widely used in the fingerprinting of bacteria. GTG_5 rep-PCR fingerprinting, for instance, was found useful for fast screening and grouping of vaginal lactobacilli, but combination with other methods was proposed for reliable identification (*24*). Approach including RAPD with two random primers and rep-PCR with $(GTG)_5$ primer was shown to be more discriminative for lactobacilli from different sources than rep-PCR or RAPD alone (*25*).

Furthermore, representatives of 11 different RAPD groups found in human milk were identified by 16S rDNA sequencing and BLAST comparison. The analysis revealed that seven of them belonged to *Lactobacillus* genus. The assignment of these seven representatives to four *Lactobacillus* species was based on 99 % of identity (E=0.0), with the sequences found by BLAST search. The bacterial isolates from two most represented RAPD groups belonged to *L. fermentum* and *L. gasseri*. Assuming that the isolates with the same RAPD pattern belong to the same species, it could be estimated that 51 % of the colonies belonged to *L. fermentum* and 22 % of the colonies belonged to *L. gasseri.* In total, we identified four different species of lactobacilli, *i.e. L. fermentum, L. gasseri, L. salivarius* and *L. reuteri*. Three RAPD groups were actually found to be associated with non-*Lactobacillus* species, which probably belong to *Bifidobacterium breve* (97 % identity), *S. epidermidis* (95 % identity) and with higher probability to *Enterococcus faecalis* (99 % identity). This observation was not surprising, as the lack of selectivity of culture media for lactobacilli has been reported several times (*12*,*26*,*27*). With the exception of *E. faecalis*, the detected species are commonly isolated from human milk (*9*).

Martín *et al.* (*20*) detected representatives of *S. epidermidis*, *Staphylococcus hominis*, *Lactobacillus casei*, *L. gasseri*, *L. gastricus*, *L. fermentum*, *L. plantarum*, *L. reuteri*, *L. salivarius*, *L. vaginalis*, *B. breve* and *B. longum* in human milk by cultivation methods. It is interesting that six out of eight species isolated in the present study are the same as those reported by Solís *et al.* (*7*) and Martín *et al.* (*20*).

Conclusions

Cultivable lactic acid bacteria, bifidobacteria and mesophilic aerobic bacteria were detected in milk collected from 47 breastfeeding mothers on the 30th day after giving birth. The most represented were mesophilic aerobic bacteria grown on BHI agar medium, followed by lactococci and enterococci grown on M17 agar, and lactic acid bacteria grown on MRS agar. Presumptive *E. coli* and *S. epidermidis* species were also present in consider-

able amounts. The presence of the *Bifidobacterium* and *Lactobacillus* representatives was common, the latter being further characterised by RAPD genotyping and 16S rDNA based identification.

The concentration of bacteria between the samples obtained from the left or right breast of the same mother often varied more than 0.5 log units, leading to the conclusion that the analysis of the milk sample taken from one breast only does not reflect the representative picture of microbial composition. Therefore, either both samples (L and R) should be analysed or both samples should be mixed in equal volumes before the analysis.

In addition to agar media commonly used for plate counting of lactic acid bacteria, bifidobacteria and aerobic mesophilic bacteria, the UriSelect 4 (URI) chromogenic agar, originally developed for the isolation and counting of urinary tract pathogenic microorganisms, was found useful for the plate counting of different groups of bacteria in human milk, such as presumptive *Enterococcus* genus or *E. coli*, *S. aureus* and *S. epidermidis* species. However, it should be taken into consideration that the colour-based differentiation is not always reliable, but presents an estimation. Individual colonies, however, can be further confirmed on the species level. Rogosa agar, commonly used for isolation of lactobacilli from different media, was also found not to be perfectly selective, as was revealed by the 16S rDNA sequencing of the representative strains sharing the same RAPD patterns. Although lactobacilli prevailed among the 86 colonies isolated from ROGOSA agar, *B. breve*, *S. epidermidis* and *Enterococcus faecalis* were also found.

Besides the three non-lactobacilli species already mentioned, the representatives of different RAPD patterns turned out to belong to the species which are typical representatives of the human milk microbiota, namely *L. fermentum*, *L. gasseri*, *L. salivarius* and *L. reuteri.*

Acknowledgements

The study 'The role of human milk in development of breast-fed child's intestinal microbiota' was approved by The National Medical Ethics Committee of the Republic of Slovenia (32/07/10, 38/02/12) and registered at ClinicalTrials.gov (NCT01548313). This project was fully funded by Slovenian Research Agency – ARRS (J4-3606). Evgen Benedik and Tina Tušar received grants from Slovenian Research Agency's young researcher programme. We are also grateful to all study participants and to medical staff, especially to Janja Gržinić. Tatjana Robič and Aneta Soltirovska Šalamon also deserve special acknowledgement for their valuable contribution.

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