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Occurrence of *Lactobacillus reuteri* in human breast milk

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Abstract

The nature and role of human milk microbiota in the early colonization and protection of infants from infection is the subject of increasing research. This study investigated the occurrence of *Lactobacillus reuteri* in milk of nursing mothers living in rural or urban areas in different geographical locations. Breast milk samples were collected from 220 mothers, 6–32 days after delivery, and analysed for the presence of total lactobacilli and *L. reuteri*. In all, 50% of mothers from rural areas in Japan and Sweden were *L. reuteri*-positive, whereas mothers from urban areas in South Africa, Israel and Denmark had very low or non-detectable levels. Overall, 15% of mothers had detectable *L. reuteri* in their milk. There were no significant differences in the prevalence of total *Lactobacillus* or *L. reuteri* in women from rural and urban habitats in the participating countries.

Key words: breast milk, lactobacilli, *Lactobacillus reuteri*, probiotics

Introduction

Human breast milk is considered to be of primary importance for the optimal health, growth and development of the infant (1). Aside from its nutritional value, it also contains several bioactive factors that may enhance the infant’s defences (1,2) and protect it against colonizing pathogens (3,4). It has been suggested that commensal and potential probiotic bacterial components present in breast milk may also be involved in the development of the infant’s gastrointestinal mucosal tissues and its acquisition of a healthy gut microbiota (2,5). Mother’s milk is a consistent source of microorganisms for the neonatal gut during several weeks after birth, and it is only after the introduction of solid food that the gut microbiota becomes more diverse and begins to resemble that of adults (5). The bacterial species generally found in healthy human breast milk belong to the genera *Staphylococcus*, *Streptococcus*, *Lactobacillus*, *Micrococcus*, *Enterococcus* and *Bifidobacterium* (4–6). Some of these genera originate from the surface of the nipple and the surrounding skin and may be adapted to survival in the milk ducts in the breast (1,5).

*Lactobacilli* make up an important part of the healthy human intestinal microbiota and are thought to be involved in the control and maintenance of the microbiota (7). *L. reuteri* is an indigenous colonizer of the gastrointestinal (GI) tract of humans and animals (7,8). *L. reuteri* has been demonstrated to have probiotic properties (7–12) and has previously been found in breast milk of Finnish women (8,13). Growing evidence that lactobacilli colonization at a very early age may protect the infant from developing atopic allergy (14) points to a possible role for a natural supply of lactobacilli in the breast milk as a beneficial factor for the suckling infant.

Little information is available in the literature concerning the presence of lactobacilli, including *L. reuteri*, in human breast milk. The present study was undertaken to investigate (i) the occurrence of *L. reuteri* in human breast milk, and (ii) the possible links between the presence of *L. reuteri* and the mothers’ geographic areas of residence.

Subjects and methods

Breast milk samples were collected from women living in urban or rural areas in Sweden, Denmark,
samples were then immediately frozen and stored at
−20°C for a maximum of 1 month. The samples
were packed with dry ice in insulated boxes (Nexpack
AB, Malmö, Sweden) and sent to a laboratory in
Sweden where they were kept at −80°C for a maximum of 1 month until analysis. The frozen
samples were thawed overnight in a refrigerator and
then maintained at room temperature for 30 min
before being diluted and plated.

Two microbiological analyses were conducted on
these samples. First, the total viable lactobacilli
present as cfu/ml sample was determined as follows.
Each sample was 10-fold serially diluted in 0.15 M
NaCl. Aliquots (100 μl) from each dilution were
directly plated on de Man Rogosa Sharpe agar plates
(MRS; Acumedia, Liusne, Sweden) and lactobacilli-
selective agar plates (LBS; Becton Dickinson
AB, Stockholm, Sweden). Plates were incubated for 48–72 h at 37°C under anaerobic conditions
(BBL® GasPak anaerobic system, Becton Dickinson
AB, Stockholm, Sweden).

Secondly, the following enrichment procedure was
used to amplify the number of L. reuteri cells present
in these samples, and thereby qualitatively assess
their presence or absence in the breast milk samples.
One ml of thoroughly mixed breast milk was added to
10 ml of freshly prepared Lactobacillus Carrying
Medium, containing 20 mM arabinose, 20 mM ribose,
1 mM MgSO4, 0.1 mM MnSO4, 5 μM FeSO4 (Merck, Lund, Sweden). The enrichment mixture was incubated at 37°C for 6–8 h. Thus
enriched, the cultures were plated onto modified
MRS agar containing 0.05 mg/ml vancomycin
(Sigma Chemical Co, St Louis, MO, USA) to
determine their L. reuteri content. Plates were
incubated for 48–72 h at 37°C under anaerobic
conditions (BBL® GasPak anaerobic system). L. re-
uteri colonies were identified and enumerated using a
method based on the L. reuteri-specific production
of reuterin from glycerol under anaerobic conditions
at 37°C and pH 6–8 (15). Seventy reuterin-positive
isolates were randomly selected to further identify
them as L. reuteri using PCR analysis.

PCR analysis
To confirm the identity of L. reuteri among the
reuterin-positive colonies, isolates were randomly
selected from the MRS medium, purified by streak
plating and subjected to sequence analysis of
16S rDNA, performed as described by Magnusson
et al. (16). Bacterial DNA was isolated from bacteria
grown in MRS broth using the DNeasy™ Tissue
Kit (Qiagen). 16S rDNA was amplified by PCR
(94°C for 30 s, 54°C for 30 s, 72°C for 80 s, step 1–3
30 cycles, 72°C for 10 min, 4°C for α) using primers
16S.S (5’-AGAGTTTGATCCTGGCTC-3’; position 8–25 in Escherichia coli 16S rRNA) and
16S.R (5’-CGGGAACGTATTCCACG-3’; position
1385–1369 in E. coli 16S rRNA). The resulting PCR

Table I. Breast milk samples obtained from urban and rural areas in seven countries.

<table>
<thead>
<tr>
<th>Country</th>
<th>Urban</th>
<th>n</th>
<th>Rural</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweden</td>
<td>Stockholm</td>
<td>19</td>
<td>Gällivare</td>
<td>10</td>
</tr>
<tr>
<td>Israel</td>
<td>Petah Tikva</td>
<td>20</td>
<td>Beersheba</td>
<td>20</td>
</tr>
<tr>
<td>South Africa</td>
<td>Cape Town</td>
<td>20</td>
<td>Suburban area of Cape Town</td>
<td>19</td>
</tr>
<tr>
<td>Japan</td>
<td>Hiroshima</td>
<td>16</td>
<td>Suburban area of Hiroshima</td>
<td>4</td>
</tr>
<tr>
<td>Peru</td>
<td>Lima</td>
<td>20</td>
<td>Huaraz, Huancayo</td>
<td>17</td>
</tr>
<tr>
<td>South Korea</td>
<td>Seoul</td>
<td>20</td>
<td>Koyang, Kimpo and Inchon</td>
<td>19</td>
</tr>
<tr>
<td>Denmark</td>
<td>Copenhagen</td>
<td>11</td>
<td>Suburban area of Copenhagen</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>126</td>
<td></td>
<td>94</td>
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</table>
product (appr. 1377 bp) was purified using the Qiagen PCR purification kit. The purified fragments were sequenced according to standard methods. The first part (about 500 bp) of the 16S rDNA sequences obtained was searched for manually and levels of similarity were analysed with the GeneBank database (http://www.ncbi.nlm.nih.gov/BLAST).

Statistical analysis
The $\chi^2$ test was used to evaluate the significant difference between geographical locations (urban and rural) and the prevalence of total Lactobacillus and L. reuteri, respectively. The Mann-Whitney U test was used to explore if significant differences existed regarding Lactobacillus counts for the above-mentioned locations. A $p$ value of <0.05 was considered statistically significant.

Ethical aspects
Mothers participating in the study gave informed consent before giving breast milk samples. Ethical approval was obtained by the regional committee at the Karolinska Institute, Stockholm, Sweden and in those countries where it was deemed necessary.

Results
Estimates of the total lactobacilli and specifically L. reuteri present in the samples obtained from mothers in each country and in different locations are shown in Figure 1a and b. L. reuteri was found in the breast milk of mothers from around the world, and of the 220 samples collected from as many mothers, 32 were found to be positive for L. reuteri, giving an overall prevalence of 15%. Breast milk from Japanese mothers showed the highest frequency of colonization with L. reuteri (11 of 20 giving positive samples). Women from other locations showed a lower prevalence of L. reuteri. Total lactobacilli were found in higher numbers in most samples examined, with 93 of 220 samples being positive (42% prevalence) in this regard. The highest prevalence was seen in South Korea and Japan. Women living in rural areas exhibited 14% of L. reuteri and 43% of total Lactobacillus in their breast milk; women living in urban areas had comparable levels of 15% and 42%, respectively.

No significant differences were observed among women from rural or urban habitats (Table II). However, women living in rural areas tended to have higher median counts of total lactobacilli in the breast milk compared with those living in urban areas ($1.3 \times 10^3$ vs $3.0 \times 10^2$ cfu/ml) (see Table III). The highest counts were found in the breast milk from mothers in Israel, South Africa, Japan and South Korea.

Discussion
This study is a first attempt to determine the presence of L. reuteri in human breast milk on a worldwide basis. Previous studies have shown that the initial flow of breast milk contains the most microbes, and that this is true even if the nipples have been washed before the milk is expressed (17). It is also known that the microbes most frequently isolated from breast milk are inhabitants of normal skin (1). Thus, it is not surprising that commensal staphylococci, micrococci and streptococci are among the predominant bacterial species in human milk (4). Staphylococcal species are thought to originate from the maternal skin during breastfeeding, and oral streptococci (e.g. Streptococcus salivar-ius) are hypothesized to be transferred from the infant mouth to the breast and from there to the milk (4).

Human lactobacilli and enterococci colonize mucous membranes and can be readily isolated from the gastrointestinal and the urogenital tracts (7). The infant, however, becomes colonized during the birth process during contact with the mother's vaginal microflora, the normal microbiota of the parents, and from the ambient environment transmitted to the mother's breast and hands during nursing. An endogenous route has also been described, namely,
via dendritic cells that penetrate the gut epithelium through opening the tight junctions between intestinal epithelial cells to sample bacteria directly from the gut lumen (18). Once internalized or attached to dendritic cells (or other types of lymphocytes), bacteria may spread to other locations via the bloodstream and circulation of lymphocytes within the mucosal-associated lymphoid tissue system (18). A study of pregnant mice that were orally inoculated with a genetically labelled Enterococcus faecium strain revealed that this strain could be isolated from the amniotic fluid of the inoculated animals (19). The authors suggested that this bacterium entered the uterine environment through the bloodstream (19). Others believe this cannot be the case since the mesenteric lymph nodes act as a firewall to prevent live commensals from penetrating the systemic immune compartment (20). The fact that \( L. \) reuteri and other lactobacilli are found in the milk indicates that these bacteria have the ability to colonize the mammary ducts, in agreement with previous findings (5,21). The presence of lactic acid bacteria in the breast milk of healthy mothers may be a significant source of lactic acid bacteria for colonization of the infant gut. Specific lactic acid bacteria have been reported to have effects on the symptoms of atopic eczema and allergy (14,22,23). Based on the present study, it was determined that \( L. \) reuteri was present in their breast milk. In an earlier follow-up study with Finnish mothers (from delivery to 3 months after giving birth), it was shown that lactobacilli were present in 40% of their breast milk samples, and \( L. \) reuteri was present in 20% (13). This finding is consistent with the present study. Together, they indicate that \( L. \) reuteri is a natural microbiotic component of human milk.

The levels of lactobacilli observed in breast milk from urban and rural habitats ranged from \( 2.0 \times 10^1 \) to \( 5.0 \times 10^4 \) and \( 1.0 \times 10^4 \) to \( 6.0 \times 10^4 \) cfu/ml, respectively. Information in the literature enumerating lactic acid bacteria in breast milk is limited. Martin et al. (21) have shown counts of lactic acid bacteria in breast milk to vary between \( 2.0 \times 10^4 \) and \( 1.0 \times 10^5 \) cfu/ml. That these numbers may be relatively low does not preclude their importance in the development of the infant’s microbiome. Given the likelihood that limited numbers of \( L. \) reuteri would be found in the breast milk samples provided (i.e. below the detection limit of \( < 1.0 \times 10^4 \) cfu/ml), an enrichment procedure was employed to ascertain only their presence or absence in these samples. Using this procedure it was determined that \( L. \) reuteri is present in human breast milk, and that this source may lead to colonization of the infants and subsequent growth in the developing infant gut.

The overall prevalence of lactobacilli and \( L. \) reuteri does not show any significant difference between rural and urban areas. However, there were considerable differences observed between countries and rural and urban areas, as illustrated in Figure 1. Japanese women seem to have a higher prevalence of \( L. \) reuteri colonization than women from the other countries in the study. This may be related to the wide use of functional foods, probiotics and various fermented foods as an important part of the Japanese diet (24). Dietary habits and environmental conditions are known to influence the microbial composition of the gut microbiota.

It is agreed that breastfeeding is the best way to ensure that an infant receives beneficial microbiota and optimal substrates for the growth of these bacteria. These benefits are associated with a lower risk of gastrointestinal, respiratory and other infections (2). Breast milk may be considered as a natural symbiotic (21,25), and evidence from this study suggests that \( L. \) reuteri is one of the beneficial components in this regard.

**Conclusions**

\( L. \) reuteri is found in the initial flow of human milk. It is found in a minority of nursing mothers but in countries geographically widely apart. Further studies are needed to provide a better understanding of

<table>
<thead>
<tr>
<th>Table II. Prevalence (%) of Lactobacillus and ( L. ) reuteri in rural and urban areas.</th>
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<tbody>
<tr>
<td>Bacteria</td>
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<tr>
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<tr>
<td>Lactobacillus</td>
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<tr>
<td>( L. ) reuteri</td>
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</table>

No significant (NS) difference was observed between urban and rural areas and the prevalence of Lactobacillus and \( L. \) reuteri, respectively.

<table>
<thead>
<tr>
<th>Table III. Total Lactobacillus counts in breast milk samples obtained from urban and rural areas in seven countries expressed as median values in colony-forming units (cfu) per ml breast milk for those subjects that had detectable values.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Country</td>
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<tr>
<td>Sweden</td>
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<td>Israel</td>
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<td>South Africa</td>
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<td>Japan</td>
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<td>Peru</td>
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<td>South Korea</td>
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<tr>
<td>Denmark</td>
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<td>Median</td>
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</table>

No significant (NS) difference in Lactobacillus counts was observed between urban and rural areas.
the significance of this and other lactic acid bacteria in breast milk and their relevance to the health of the infant and mother.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References