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Isolation and Characterization of Lactic Acid Bacteria from Human Milk and Newborn Feces

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Breast milk is a rich sou0000rce of nutrients. It helps to establish a good intestinal flora of infants. In this context, we have isolated and characterized lactic acid bacteria from human milk and infant feces of the northwest Algerian population (Oran). In the present study, 87 bacterial strains were isolated from breast milk and infant feces samples from 20 mother-infant pairs. Isolates were identified by 16S rDNA sequencing analysis. The results of 16S rDNA sequencing analysis showed that the strains isolated from milk and faecal samples are part of 12 different species belonging to the genera: *Enterococcus, Staphylococcus, Streptococcus* and *Lactobacillus*. A homogeneous distribution was found for the four genera in the two media. Also, several mother/infant pairs shared some bacterial species belonging to different bacterial genera. Comparing the intestinal flora of vaginally delivered infants and those born by Caesarean section, a significant difference was found for the genus Staphylococcus (P-value <0.05) with a high rate among infants born by cesarean section. These results indicate that breast milk and delivery mode may contribute to the infant gut colonization.

Keywords: Lactic acid bacteria; human milk; infant feces; 16S rDNA sequencing analysis; vaginally delivery; cesarean section.

Breastfeeding is the best way to provide newborns with the nutrients that they need for healthy growth and development[1]. The World Health Organization (WHO) and The United Nations Children's Fund (*UNICEF)* recommended exclusive breastfeeding for the first six months of life, and its pursuit up to two years associated with appropriate complementary feeding[2, 3]. Human milk educates the infant immune system and confers protection against gastrointestinal infections[4, 5], respiratory infections[4, 6], allergic diseases[7] and it is also associated with a reduced long term risk of diseases such as inflammatory bowel disease (IBD), obesity or diabetes as reviewed by the American Academy of Pediatrics[8]. The protective role of breast milk seems to be the result of the

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synergistic action of several bioactive molecules such as carbohydrates, oligosaccharides, nucleotides, fatty acids, immunoglobulins, cytokines, immune cells, lysozyme, lactoferrin and other immunomodulating factors[9, 10]. Human milk oligosaccharides (HMOs) are nondigestible carbohydrates that are fermented in the colon, stimulating the growth and/or activity of specific fecal bacteria (including bifidobacteria) that impact health positively in infants receiving breast milk[11].

Human milk is an important factor in the initiation, development, and composition of the neonatal gut microbiota. It has been shown that an infant takes approximately 10^5-10^7 bacteria daily via consuming 800 mL of breast milk[1, 12, 13].

In the last years, some studies have shown 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 breastfed 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[14].

Breast milk bacteria may also participate in the correct maturation of the infant immune system since some strains are able to modulate both natural and acquired immune responses in mice and humans[15].

In relation to allergic conditions, the human milk strain *Lactobacillus gasseri* CECT 5714 reduced the incidence and severity of the allergic response in an animal model of cow's milk protein allergy[16].

Finally, human milk bacteria have a remarkable potential to play metabolic roles in the infant. The glycobiome of some lactobacilli and bifidobacteria, including that of species that have been isolated from human milk, may help to create a specific "healthy" microbiota in the infant gut[17, 18]. These microorganisms might also contribute to infant digestion through the breakdown of sugars and proteins; this possibility is particularly attractive having in account that transit of food through the gastrointestinal tract is shorter in infants than in adults and, also, that the pH of the infant's stomach is higher than that of the adult. In this context, human milk lactobacilli strains are metabolically active in the infant gut and increase the production of functional metabolites such as butyrate, which is the main energy source for colonocytes and a relevant compound in the modulation of intestinal function. As a result, they improve the intestinal habit, with an increase in fecal moisture, and in stool frequency and volume[19, 20].

During the first years of life, the intestinal microbiota of newborns is still relatively dynamic[21]. It has been postulated that environmental factors, such as delivery mode, may influence the composition of the human microbiota throughout life[22].

In the United States the rate of cesarean delivery (CD) has risen 48% since 1996, reaching a level of 31.8% in 2007[23]. This trend is reflected in many parts of the world, with the most populous country in the world, China, approaching 50%[24] and some private clinics in Brazil approaching 80%[25]. In Algeria the rate is approximately 40%[26]. This may incur several risks for the child like depression due to general anesthesia, fetal injury during hysterotomy and/or delivery, increased likelihood of respiratory distress even at term, and breastfeeding complications. Concurrent with the trend of increasing CD, there has been an epidemic of both autoimmune diseases such as type 1 diabetes, Crohn's disease, and multiple sclerosis and allergic diseases, such as asthma, allergic rhinitis, and atopic dermatitis[27, 28].

In this context, the purpose of this study is to characterize lactic acid bacteria isolated from breast milk and newborn feces. This to investigate the relationship between breast milk and infant feces also the difference between the intestinal flora of infants vaginally delivered and those born by Caesarean section in the population from Oran (Algeria).

MATERIALS AND METHODS

Subjects and sample collection

Breast milk samples and feces of breast-fed infants were selected to achieve isolation of their microbial flora. These samples were collected from twenty pairs of women and their respective infants aged up to six months. The subjects were of Algerian origin from Oran. The samples were recruited at the maternity service of the University Hospital of Oran. Nine of these infants were

vaginally delivered, while eleven were born by caesarean section. All participants were healthy and without prenatal problems. The sampling was performed under sterile conditions. The milk samples were collected in a sterile tube by manual expression using sterile gloves. Previously nipples and mammary areola had been cleaned with sterile water and then wiped with sterile gauze containing an antiseptic gel Aniosgel 85 NPC (Anios, France), the first drops were discarded after the milk was collected in sterile tubes. In addition, feces samples were collected with sterile swabs. The samples were kept at 4°C until delivery to the laboratory and immediately processed.

Count and Isolation of bacterial strains

Once diluted in saline water (NaCl, 0.9%), the samples were cultured in triplicate on de Man-Rogosa-Sharpe agar (MRS, Oxoid, United Kingdom) supplemented with L-cysteine (0.5 g/L) (MRS-Cys) and Columbia blood agar (CBA, Biomerieux, France). The agar plates were incubated at 37° C under anaerobic conditions using Genbox anaer (Biomerieux, France) for 48h-72h. Bacterial counts were performed on MRS-Cys agar plates. About 1 g of freshly feces and 1 mL of milk samples were transferred into flasks containing 9 mL of salt solution (NaCl, 0.9 %) and the suspension was homogenized for 2 min. After, tenfold serial dilutions were done and samples (0.1 mL) were plated in 3 Petri dishes for each dilution chosen. Colonies with different morphologies were purified and tested for catalase reaction, Gram staining and microscopic examination. Subsequently, 87 isolates were randomly selected for further studies and stored at -80 ° C in MRS broth with 30% glycerol.

DNA extraction

Genomic DNA was directly isolated from overnight broth culture using glass beads and a FastPrep-24 instrument (MP Biomedicals, France) in the presence of 400 µl of a phenol/chloroform/isoamyl alcohol mixture (25:24:1 v/v/v) and 400 µl of water. The aqueous phase was collected after centrifugation (16 000 g, 10 min) and the DNA was precipitated by incubation for 30-60 min at -20°C with 800 µl of ethanol and 120 µl of 3M sodium acetate. The pellet was centrifuged (16 000 g, 15 min), washed with 70% ethanol and resuspended in 50 µl of distillated water.

Sequence analysis of the 16S rRNA gene

The sequence analysis of 16S rRNA gene was performed to identify and classify the different isolates. The 16S rRNA gene was amplified using the primers 616V (5'-AGAGTTTGATYMTGGCTCAG-3 ') and 630R (5'-CAKAAAGGAGGTGATCC-3'). The PCR mixture (50 µl) contained 10X DreamTaq buffer, 0.2 mM of each dNTP, 1 ¼M of each primer, 1 ¼g DNA template and 1.25 U DreamTaq DNA polymerase (Thermo Scientific, USA). The concentration for each reagent belongs to the final concentration in the PCR reaction. Samples were amplified in Master Cycler Personal thermocycler (Eppendorf, France) using the following program: initial denaturation at 95° C for 3 min followed by 30 cycles each consisting of 94° C for 30 s, 52° C for 30 s and 72° C for 2 min with a final extension step at 72° C for 10 min. The PCR products were purified using Nucleospin® Extract II kit (Macherey-Nagel, Düren, Germany) and sequenced by a commercial provider (Beckman Coulter Genomics, United Kingdom). The resulting sequences were used to search sequences deposited in public databases by using the BLAST algorithm. Identification to the species level is defined as a 16S rDNA sequence similarity of e" 99% with that of the prototype strain sequence in GenBank[29].

Statistical analysis

The Pearson's Ç2 test was used to compare firstly between frequencies of bacterial genera detected in breast milk and infant feces; then, between those present in feces of infants delivered vaginally and feces of those born by

Table 1. Descriptive statistics on bacterial counts (log10 CFU/mL) from breast milk and infant feces (log10 CFU/g) samples in MRS-Cys medium

	MRS-Cys medium	
	Breast milk (n=19)[a]	Infant feces (n=19)[b]
Mean	2.43	6.85
Standard deviation (SD)	0.42	0.74
Minimum	2.17	6.38
Maximum	2.69	7.32
P-value	p<0.001	p<0.001

a: lack of breast milk of the sample No. 14.
b: lack of infant feces of the sample No. 18.

caesarean section. The analysis was performed with SPSS software.

RESULTS

Bacterial counts

Bacterial counts were performed on MRS-Cys plates. The mean count values oscillated, depending on the sample, with a significant difference (P-value < 0.001), from 2.17 to 2.69 $\log_{10}$ CFU/mL for milk samples and from 6.38 to 7.32 $\log_{10}$ CFU/mL for infant feces samples (Table 1).

Isolation and identification of bacteria

A collection of 87 strains were selected as following: 49 strains from milk samples (10 rods and 39 cocci) and 38 strains from faecal samples (9

Table 2. Different species found in breast milk and infant feces samples

Mother/ infant pair	Delivery mode	Age (days/weeks)	Sample	Bacterial spacies
1	Vaginal	9 weeks	Breast milkInfant feces	*E. faecalis E. faecalis*
2	Vaginal	14 weeks	Breast milkInfant feces	*E. faecium, S. epidemidisE. faecium*
3	Vaginal	2 weeks	Breast milkInfant feces	*E. faecium, S. epidemidis E. faecium, L. plantarum*
4	Caesarean	2 weeks	Breast milkInfant feces	*E. faecalis, E. faecium, S. epidemidis E. faecalis, E. faecium*
5	Vaginal	8 weeks	Breast milkInfant feces	*E. faecalis, E. faecium, S. epidemidis, L. spE. faecalis,*
6	Vaginal	12 weeks	Breast milkInfant feces	*E. faecium, L. sp E. faecium, E. faecalis*
7	Vaginal	16 weeks	Breast milkInfant feces	*E. faecalis, L. casei, S. epidemidis E. faecalis, L. paracasei, S. epidemidis*
8	Caesarean	12 weeks	Breast milkInfant feces	*E. faecalis, L. rhamnosus, S. epidermidisE. sp, L. rhamnosus, L. fermentum*
9	Caesarean	12 weeks	Breast milkInfant feces	*E. sp, L. spE. faecalis, E. faecium, S. epidemidis*
10	Caesarean	15 weeks	Breast milkInfant feces	*E. faecalis, L. rhamnosus, S. epidemidisE. faecalis, E. malodoratus, L. fermentum, S. epidemidis*
11	Caesarean	4 days	Breast milkInfant feces	*E. faecalis, S. epidermidisE. faecalis, Strep. Salivarius, S. epidemidis*
12	Caesarean	1 day	Breast milkInfant feces	*E. faecalis, Streptomyces fimbriatus, S. epidemidisS. epidemidis*
13	Caesarean	2 days	Breast milkInfant feces	*L. rhamnosus, S. epidemidis L. rhamnosus, S. epidemidis*
14	Caesarean	2 weeks	Breast milkInfant feces	*E. faecalis, L. sp*
15	Caesarean	5 days	Breast milkInfant feces	*E. faecalis, E. faecium, L. sp, Strep. spS. epidemidis, L. sp*
16	Caesarean	1 week	Breast milkInfant feces	*S. epidermidis, Strep. mitis, L. spS. epidermidis*
17	Caesarean	2 weeks	Breast milkInfant feces	*S. epidermidisS. epidermidis, L. sp*
18	Vaginal	20 weeks	Breast milkInfant feces	*E. faecalis, Strep. salivarius, S. epidemidis, L. sp*
19	Vaginal	4 weeks	Breast milkInfant feces	*E. faecalis, S. epidemidis, Strep. salivarius, L. spL. fermentum, Strep. Salivarius*
20	Vaginal	4 weeks	Breast milkInfant feces	*S. epidemidisS. epidermidis* 87

Number of bacteria
E : Enterococcus, L : Lactobacillus, S : Staphylococcus, Strep : Streptococcus

rods, 29 cocci). DNA isolation and molecular analysis were successful for 74 of the 87 strains. The amplification of the 16S rRNA gene resulted a fragment of expected size about 1500 pb. The isolates were identified to the species level by 16S rRNA gene sequencing analysis. The results showed that isolates belong to 12 different species (Table 2). Bacterial strains that we could detect in this study in breast milk are *Enterococcus faecalis, Enterococcus faecium, Lactobacillus casei, Lactobacillus rhamnosus, Staphylococcus epidemidis, Streptococcus saluvarius, Streptococcus pneumonia* and *Stremtomyces arenae.*

The bacterial flora from the faecal samples contained strains of *Enterococcus faecalis, Enterococcus faecium, Enterococcus avium, Staphylococcus epidemidis, Lactobacillus rhamnosus, Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus casei* and *Streptococcus saluvarius*. In this study S. epidermidis and E. faecalis are the predominant species in breast milk (37.5% and 28% respectively) and in feces Infant (29% and 26% respectively).

As shown in (Table 2), several mother/ infant pairs shared some bacterial species especially: *Enterococcus faecalis, Enterococcus faecium, Staphylococcus epidemidis, Lactobacillus rhamnosus and Streptococcus saluvarius.*

Statistical analysis

Comparing the flora of milk samples and faecal samples (Table 3), no statistically significant difference (*P*-value > 0.05) in colonization rates was observed of each group of bacteria enterococci (37.50% versus 42.11%), lactobacilli (22.92% versus 26.32%), streptococci (8.33% versus -5.26%) and staphylococcus (31.25% versus 26.32%).

As well, the faecal flora of infants born vaginally was compared to that of infants born by caesarean section (Table 3), enterococci represents 53.85% of bacteria present in feces of vaginally delivered infants and 36% of bacteria present in feces of those born by caesarean section, but the difference was no significant (P-value > 0.05). The same results were shown for lactobacilli (23.08 % versus 28%) and streptococci (7.69% versus 4%). Nevertheless, a significant difference was observed with staphylococci (15.38% versus 32%) with P-value < 0.05.

DISCUSSION

Human milk has been considered sterile; however, recent studies have shown that it represents a continuous supply of commensal, mutualistic and/or potentially probiotic bacteria to the infant gut [12, 30]. In the present study, 74 from 87 bacterial strains isolated were identified to the species level from breast milk and infant feces of Algerian population. The isolates belong to the genera enterococci, staphylococci, streptococci and lactobacilli. These genra are shared between breast milk and feces of newborns. No significant difference (P-value> 0.05) was observed for the four genera studied between breast milk and infants feces. This means that there is a homogeneous distribution of the four genera in these two environments. Our results are in agreement with

Table 3. Comparison of frequencies of bacterial genera detected in breast milk and infant feces as well as in feces of vaginally delivered infants and feces of those born by caesarean section

Genera	Breast milk		Infant feces		*P*-value	Vaginal delivery		Caesarean section		*P*-value
	n	%	n	%		n	%	n	%	
Enterococci	18	37.50	16	42.11	0.283	7	53.85	9	36	0.361
Lactobacilli	11	22.92	10	26.32	0.516	3	23.08	7	28	0.463
Streptococci	4	8.33	2	5.26	0.374	1	7.69	1	4	0.811
Staphylococci	15	31.25	10	26.32	0.087	2	15.38	8	32	0.040
Total	48		38			13		25		

n:number , %: frequency, p: significance

previous studies which state that the bacterial composition of the fecal microbiota of the breast-fed infant usually reflects that of breast milk[31].

We have found that *S. epidermidis* is predominant in breast milk (37.5%) and in the infant feces (29%). Several studies showed that *S. epidermidis* was the predominant species in milk and feces of breast-fed infants while it was less prevalent in those of formula-fed infants[31, 32]. This suggests that the diet can affect the composition of the intestinal microbiota. In addition, breast milk contains not only potentially probiotic bacteria but also prebiotics which are able to stimulate growth of probiotics[11].

Recent studies have shown that breast milk contains bifidobacteria[33, 34]. Furthermore, an Algerian study has shown that the number of bifidobacteria reached 26.10^9 CFU/g in breastfed infant feces[35]. In our study, we could not isolate bifidobacteria this fact is probably due to their fastidious growth requirements. In fact, the MRS-cys medium promotes the growth of lactic acid bacteria especially *Lactobacillus*. CBA agar is a selective medium for the isolation of gram positive bacteria (especially staphylococci and streptococci). The use of dependent-culture techniques limited the capacity to explore the microbiota because most intestinal bacteria require strict anaerobic conditions for growth or have specific nutritional requirements.

Also, we have noticed that several mother / child pairs shared some bacterial species (*Enterococcus faecalis, Enterococcus faecium, Staphelococcus epidermidis et Streptococcus salivarius*). This suggests that breast milk could contribute may contribute to the infant gut colonization.

It has been postulated that delivery mode shapes the intestinal microbiota of infants[36]. Comparing the intestinal flora of vaginally delivered infants and those born by Caesarean section, we noticed that there is a homogeneous distribution of the genera *Enterococcus, Streptococcus* and *Lactobacillus* (P-value> 0.05). However, a Spanish study showed that Enterococcus is predominant in feces of infants born by caesarean section and Lactobacillus is predominant in feces of vaginally delivered infants[32]. While a US study showed that *Lactobacillus* is predominant in feces of vaginally delivered infants [36]. Regarding *Staphylococcus*, we found a significant difference (P-value < 0.05) with a high rate in feces of infants born by caesarean section. This finding is consistent with the results reported in Spanish and US populations[32, 36]. It has been reported that that vaginally delivered infants acquired bacterial communities resembling their own mother's vaginal microbiota, dominated by Lactobacillus, Prevotella, or Sneathia spp., and C-section infants harbored bacterial communities similar to those found on the skin surface, dominated by Staphylococcus, Corynebacterium, and Propionibacterium spp[36].

CONCLUSION

In the present study, we have characterized some species belonging to the breast milk and feces of breastfed infants. We found that some mother-infant pairs shared the same species. This suggests that breast milk may contribute to the establishment of the intestinal flora of infants. It would be interesting to make a comparison with non-breastfed children to see the real impact of breast milk on the composition of the intestinal flora. To complete our study, it will be necessary to make a MLST analysis for characterization of isolates at the strain level. To determine whether breast milk and infant feces share the same strains.

To compare the intestinal flora according to the delivery mode, we suggest increasing the number of samples for better results. This is the first study to compare the flora of breast milk and infants feces of the Algerian population and the effect of the delivery mode on the composition of the intestinal flora of newborns. Thus, we propose to perform other studies to explore other genera such as bifidobacteria and to characterize isolates at strain level. This to confirm or affirm our findings on the role of breast milk and the delivery mode in the establishment of the intestinal flora of newborns.

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