International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 13 (3), pp. 001-005, March, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Investigation of lactobacilli from mother's breast milk who were placed on probiotic diet

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Accepted 19 January, 2019

Human milk contains a group of bacteria with potential to be used as probiotics but the origin of these bacteria is debated. This study was aimed to identify and isolate lactobacilli in breast milk and investigate the role of consuming probiotic supplements on the lactobacilli microflora of breast milk. Ten out of twenty mothers were placed on the probiotic diet and the others were considered as controls. The breast milks of these mothers were cultured on MRS. The whole genome of 16s ribosomal DNA of different colonies was amplified using polymerase chain reaction for identification down to the strain level. Finally *Lactobacillus rhamnosus* LC705 was isolated just from four mothers who were placed on probiotic diet. Since this strain was dominant bacteria in capsules which used as a source of probiotic diets, it may imply that modulation of the mother's intestinal microflora can have an effect on the health of infants and therefore, would open new perspectives for using breast milk as a source of probiotic bacteria with bacteriotherapy approach.

Key words: Probiotic capsules, lactobacilli, breast milk, polymerase chain reaction.

INTRODUCTION

The establishment of the gut microflora is a complex process influenced by microbial and host interactions and by external and internal factors. Extrinsic factors include the bacterial load of the environment, the composition of the maternal microbiota, diet, the mode of delivery and medication. In this process, human milk plays an important role (Olivares et al., 2006).

During the earlier months after delivery, human milk is a major factor in the initiation and development of neonatal gut microbiota, because it acts as a continuous source of microorganisms to the infant gut. It is estimated that an infant consuming approximately 800 ml/day will ingest about 1×105-1×107 commensal bacteria while suckling (Martin et al., 2003). Analysis of the bacterial composition of fecal flora of the breast-fed infants confirms this fact (Heikkila and Saris, 2003).

On the other hand the major differences between the microbiological composition of breast milk and infant formula are probably the main factor responsible for the differences repeatedly observed between the gut microbiota of breast-fed and formula- fed infants (Benno et al., 1984; Favier et al., 2002; Harmsen et al., 2000; Mackie et al., 1999; Yoshioka et al., 1983). Also a main reason why the gut microbiota of breast-fed infants is composed of a relatively narrow spectrum of Gram-positive bacterial species and why a more diverse microbiota develops only after the progressive withdrawal of breast milk is the presence of a few predominant bacterial species in breast milk (Favier et al., 2002; Hall et al., 1990; Harmsen et al., 2000). Such bacteria in the infant gut, they may play a protective effect against infectious diseases (Lopez-Alarcon et al., 1997).

Most researches on the microbiology of breast milk

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have been restricted to pathogenic bacteria, mainly related to clinical cases of mastitis or infant infection. There is limited study about analysis of commensal or potential probiotic bacteria from milk of healthy women (Abrahamsson et al., 2009; Heikkila and Saris, 2003; Martin et al., 2003, 2004, 2005; Olivares et al., 2006; Sinkiewicz and Nordstrom, 2005). Commonly bacteria isolated from this substrate are staphylococci, streptococci, micrococci, lactobacilli and enterococci (Heikkila and Saris, 2003; Martin et al., 2003; West et al., 1979). Additionally, it has recently been reported that breast milk contains different species of lactobacilli, lactococci and other lactic acid bacteria with probiotic potential (Heikkila and Saris, 2003; Martin et al., 2003, 2005).

The origin of the bacteria found in human milk is debated and it is suggested that, at least, some species may be endogenously delivered from the maternal gut to the mammary gland (Martin et al., 2007). Although such hypothesis can be a subject of controversy, it should stimulate further research in this fascinating area. From a microbiological point of view, the recent application of culture-independent molecular techniques, and particularly those based on 16S ribosomal DNA genes, has led to a more complete assessment of the infant gut microbiota (Taheri et al., 2009). The main objective of the present work was to identify and isolate lactobacilli in breast milk of healthy women by using the 16S rDNA (1500 bp) sequencing method. Also the role of consuming probiotic supplements on the lactobacilli microfelora of breast milk was investigated.

MATERIALS AND METHODS

Samples

Breast milk samples (n=20) were collected from healthy lactating volunteers in their mid twenties with 1-6 months old breast fed babies. Ten out of 20 mothers were placed on the probiotic diet for 30 days. They consumed daily one capsule of probiotic complement (Complete Probiotic, London Drugs, WN Pharmaceuticals Ltd, Canada) and the other 10 mothers were considered as controls. Dairy and overall dietary intake and lifestyle were constant between treatments for removing the effect of confounding factors, they were controlled during one month. The study was approved by the Ethical Committee of the Hospital District of Mashhad (Iran) and written consent was obtained from the mothers.

Sampling

For sampling, nipple and mammary areola of the left breast were cleaned with soap and sterile water, and then chlorhexidine (Ferdowsi Laboratorios, Mashhad, Iran) was applied. The breast milk sample was collected in a sterile tube after manual expression using sterile-gloves. The first drops (approximately 2 ml) were discarded to avoid chlorhexidine contamination. Parallel, a swab from the nipple and mammary areola was obtained to assess the efficacy of the antiseptic treatment. Sterile tubes containing samples were kept in ice during transportation to the laboratory for less than two hours.

Culturing

Different amount of samples (50, 70 and 100 µl) were plated on non-selective MRS (deMan Rogosa and Sharpe) agar and incubated in anaerobic conditions at 37°C for 48 to 72 h. Anaerobic conditions and MRS medium were used to support growth of lactobacilli, which are nutritionally fastidious anaerobes. Different colonies were randomly selected from each plate and purified on MRS agar to be evaluated by catalase test, gram stain, and bacterial morphology. The bacteria samples suggested to be lactobacilli, after culturing in MRS broth and centrifuging, were suspended in 15% sucrose and lyophilized for long time storage.

Analyses of probiotic complement capsules

In order to validate bacterial stability and viability in the capsules, one capsule was thoroughly mixed in saline buffer and cultured on non-selective MRS agar. The cultured bacteria were incubated under anaerobic condition for 48 to 72 h at 37°C. Other processes such as bacterial isolation and identification were conducted using the same procedures as used for breast milk bacteria.

PCR detection

Identification of the bacteria at the subspecies level was conducted by 16s rDNA complete sequencing (1500 bp). To extract bacterial DNA, 300 µl of a solution containing 50 mM sucrose, 10 mM EDTA and 25 mM Tris-Hcl (pH-8) were added to the washed cells of bacteria and after adding 10 µl of 10 mg/ml lysosyme, cells were incubated at 37°C for 10 to 15 min, the process was followed by Bioneer kit (USA Bioneer, Inc. 1000 Atlantic Avenue, Alameda, CA 94501 USA) . For partial sequencing of 16s rDNA region, bacteria DNAs were amplified by universal primer pair consisted of the forward (5 GAG AGT TTG ATC CTG GCT CAG 3) and reverse (5 GAA AGG AGG TGA TCC AGC CG 3) (Taheri et al., 2009). Deoxyribonucleic acid amplification of the (~1,500 bases) fragment was carried out in a 20 l reaction mixture containing 2 l of 10 × PCR buffer, 1 l deoxynucleoside triphosphate mixture (10 mM), 1.2 l of $MgCl_2$ (50 mM), 1 l of each primer (100 pmol l^{-1}), 3 l of DNA, and 0.3 l of Taq DNA polymerase (5 U l^{-1}). The PCR started with heating at 95°C for 5 min and followed by 35 cycle (consisted of 1 min at 95°C, 1 min at 53°C, and 1 min at 72°C) and final segment at 73°C for 3 min. The PCR products were analyzed on agarose gel consisting of ethidium bromide and were cut out and extracted by gel extraction kit (USA Bioneer, Inc. 1000 Atlantic Avenue, Alameda, CA 94501 USA). PCR fragments were sequenced by Se ttingen, Germany) and BLAST sequence alignment program of National Center for Biotechnology Information was used for the identification of bacteria strains (http://www.ncbi,nlm.nih.gov/BLAST/).

Statistical methods

The t-test analysis was used for evaluation the differences in means between a group of who were given probiotic capsules and control.

RESULTS

Culturing breast milk and probiotic complement capsules

In this study, 309 different colonies were isolated from 20

Table 1. Properties of isolated strains and statistical analysis of difference between probiotic treated mothers and control group for the isolation of lactobacilli in their breast milk.

Treatment	Statistical analysis			Properties of isolated strains		
	n	Means of colonies	t-probability	Strain	Gen Bank Accession No:	% Similarity
Probiotic treatment mothers	10	0.6	2.3*	*L. rhamnosus* LC705	FM179323.1	99
Control mothers	10	0		-	-	-

*Difference was significant at P<0.05.

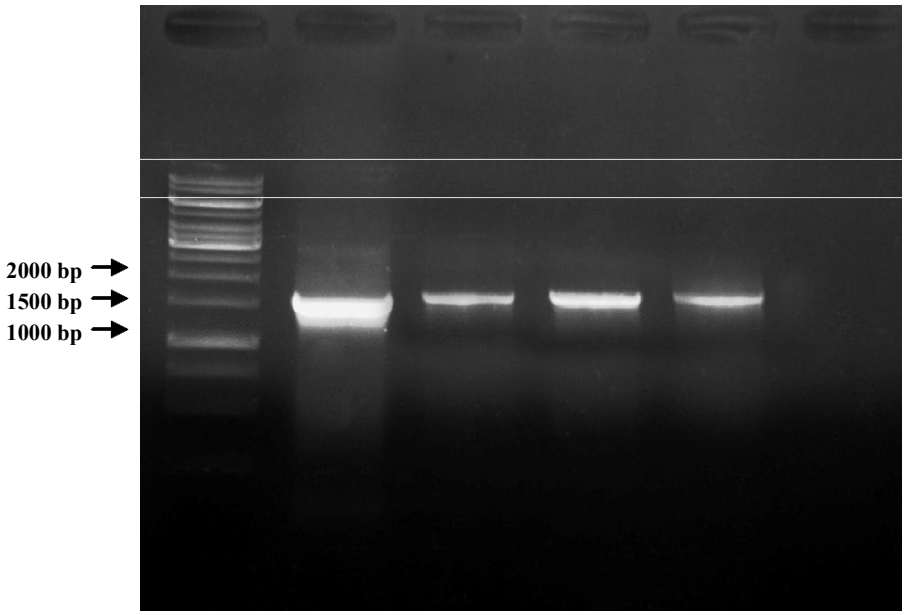

Figure 1. 16S rDNA fragments amplified by PCR from bacterial DNA.

breast milk samples. The isolates were selected according to the results of catalase test, gram stain and bacterial morphology. The results showed that 40 out of 309 were catalase negative and Gram positive stains, but just 6 out of 40 colonies showed bacillus like shape (the others were coccid form), which were isolated just from four mothers who were on probiotic diet and that difference was significant at P<0.05 (Table 1). Thirteen colonies were isolated from capsules that were catalase negative, Gram positive stains and bacillus shape.

PCR detection

The amplification of the 16S rDNA of selected strains by the PCR-based method (1500 bp) were validated on agarose gel consisting of ethidium bromide (Figure 1) and after gel extraction, followed by sequence analysis and homology search through BLAST, revealed that (99%) the strains were *Lactobacillus rhamnosus* LC705, also all 13 isolated colonies from capsules were identified as *L. rhamnosus* LC705*.* It shows that the dominant bacteria in the probiotic capsules were *L. rhamnosus* LC705*,* while *L. rhamnosus* was reported as second dominant bacteria in capsules (Table 2). In order to discard the presence of bacteria in human breast milk that was due to cross-contamination during sample collection, samples of breast skin were also collected. The absence of similarity among bacteria isolated from breast milk and from breast skin ruled out the contamination of human breast milk with breast skin and environmental bacteria.

DISCUSSION

For the first time the relation between infant mother's probiotic diet and corresponding breast milk probiotic microflora was investigated in this study. The knowledge of the commensal and/or potential probiotic bacteria that exist in milk of healthy women is very limited. It has been reported that isolated bacteria from breast milk commonly

Table 2. Bacterial composition of the capsules that the mothers consumed (each capsule contain 5 billion active cells of the probiotic bacteria).

Probiotic culture	% composition	Viable count (billion CFU*)
Lactobacillus casei	30	1.5
Lactobacillus rhamnosus	25	1.25
Lactobacillus acidophilus	15	0.75
Bifidobacterium longum	15	0.75
Bifidibacterium breve	15	0.75

*Colony forming unit.

included staphylococci, streptococci, micrococci, enterococci and lactobacilli such as *Lactobacillus gasseri, L. rhamnosus, Lactobacillus acidophilus, Lactobacillus plantarum* and *Lactobacillus fermentum* (Abrahamsson et al., 2009; Heikkila and Saris, 2003; Martin, 2004; West et al., 1979).

The presence of lactobacilli in breast milk has been attributed to the existence of prebiotic oligosaccharides in this fluid (Kralj et al., 2002; Pridmore et al., 2004; Tieking et al., 2003; van Hijum et al., 2002). Since breast milk has been suggested as vehicle for potentially probiotic LAB, it could be considered as a natural synbiotic food that is a mixture of probiotics and prebiotics. It has also been proved that breast feeding beneficially can affect infants by improving the survival and implantation of live dietary microorganisms in the gastrointestinal tract (Martin et al., 2005).

The anti-infectious properties have been attributed to breast milk, because in breast-fed infants, the rate of infectious diseases is significantly lower compared to formula-fed infants (Fallot et al., 1980; Victora et al., 1987). Some results suggest that lactobacilli from breast milk could contribute to an anti-infective protection in neonates and would be excellent candidates for the development of infant probiotic products (Olivares et al., 2006). Therefore if bacteria with the ability to confer health benefits to human hosts were isolated from breast milk, they would be considered attractive probiotic organisms. These bacteria would fulfill some of the main criteria generally recommended for human probiotics such as human origin; a history of safe, prolonged intake by infants; and adaptation to dairy substrates (Holzapfel et al., 1998).

Among the bacteria isolated from human milk, species like *L. gasseri, L. rhamnosus* or *Enterococcus faecium* have been considered as the potential probiotic bacteria and some strains are included in a variety of commercial probiotic products (Holzapfel et al., 1998). Consequently, milk of healthy woman may be a source of potentially probiotic or biotherapeutic LAB with a role in protecting mothers and/or infants against infectious diseases (Martin et al., 2005; Olivares et al., 2006). In this study we also could isolate *L. rhamnosus* LC705 from breast milk of healthy mothers. *L. rhamnosus* LC-705 is a bioprotective LAB used to ferment dairy products (Mayra -Makinen, 1995) that has recently been studied for its probiotic properties, such as *in vitro* adhesion and effect on intestinal well being of elderly (Ouwehand et al., 2002; Tuomola and Salminen, 1998). Martin et al. (2003) showed that the source of LAB in the infant gut is originated from milk and may not be the result of contamination from the surrounding breast skin (Martin et al., 2003).

It has been demonstrated that dendritic cells can penetrate the gut epithelium to take up non-invasive bacteria directly from the gut lumen (Rescigno et al., 2001). Once vehiculated in gut-associated lymphoid tissue cells, live non-invasive bacteria can spread to other locations, because there is a circulation of lymphocytes within the mucosal-associated lymphoid tissue. Bacterial-stimulated cells move from the intestinal mucosa to colonize distant mucosal surfaces, such as those of the respiratory and genitourinary tracts, salivary and lachrymal glands and most significantly, that of the lactating mammary gland. During the lactation period, colonization of the mammary gland by cells of the immune system is a selective process regulated by the lactogenic hormone and this process is responsible for the abundance of such cells in breast milk (Bertotto et al., 1991). In this regard has been reported that *Salmonella typhimurium* DT104 transmits to infants through maternal breast milk (Qutaishat et al., 2003). Recently it has been observed that *L. reuteri* may be detected in breast milk after oral supplementation to the mother and almost in all infants after oral supplementation during the first year of life, as well as occasionally in many untreated infants (Abrahamsson et al., 2009).

Conclusion

In this study, for the first time *L. rhamnosus* LC705 was isolated from the mothers milk, who were on probiotic diet while *L. rhamnosus* was dominant bacteria in probiotic capsules which was used as a source of probiotic diets. It may imply that modulation of the mother's intestinal microflora can have a significant effect on the health of infants and this could open new perspectives for

bacteriotherapy and probiotics.

ACKNOWLEDGEMENTS

The molecular experiments of this study was performed in ImmunoBiochemistry lab in Bu- Ali research center of Mashhad University of Medical Sciences. We also appreciate the great contributions of Dr. Varasteh in this research.

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