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Probiotic Properties of Lactic Acid Bacteria from Human Milk

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Abstract

Probiotics are preparation of live microorganisms that have beneficial effects on the health of their host. Although probiotic strains can be isolated from many sources, for human applications, the main criteria are that it must be from human origin. Milk is an important nutrient source for neonates. Previous studies have shown that human breast milk has beneficial effects on the health of neonates. One reason for these beneficial effects might be explained by the micro flora of human breast milk including beneficial lactic acid-producing bacteria. The main aim of this paper is to review best practices in isolation and characterization of lactic acid bacteria from human milk by biochemical and molecular methods and also determine the probiotic properties of lactic acid-producing bacteria. Most lactic acid-producing bacteria have effective antimicrobial activity against food-borne pathogens like Salmonella, thyphimurium CCM 5445, Escherichia coli O157:H7 NCTC 129000 and Escherichia coli NRRL B-3008. Data show that these isolates possess probiotic properties, as demonstrated by using amplification and restriction fragment length polymorphism (RFLP) of 16S ribosomal DNA (rDNA) and 16S sequencing. Therefore, it is observed that, human milk is a source of potential probiotic strains.

Keywords: Probiotics; Molecular identification; Lactic acid bacteria; Milk

Introduction

According to studies conducted by Quwehand and Fooks et al., [1,2], the word 'probiotic' comes from Greek language 'probios' which means 'for life' opposed to 'antibiotics' which means 'against life'. The history of probiotics goes way back in the history of man where fermented foods were consumed, a tradition that is well known in the Greek and Romans cultures. In 1908 a Russian researcher Ellie Metchnikoff, who has a Nobel Prize, firstly proposed the beneficial effects of probiotic microorganisms on human health. Metchnikoff hypothesized that Bulgarians are healthy and long lived people because of the consumption of fermented milk products which consists of rod shaped bacteria (Lactobacillus spp.). Therefore, these bacteria affect the gut micro flora positively and decrease the microbial toxic activity [3,4].

Parker defined 'probiotic' as 'substances and organisms which contribute to intestinal microbial balance'. In 1989, the meaning use today was improved by Fuller. Thus, probiotic is a live microbial supplement which affects host's health positively by improving its intestinal microbial balance. The probiotic concept is open to lots of different applications in a large variety of fields relevant for human and animal health. Probiotic products consist of different enzymes, vitamins, capsules or tablets and some fermented foods contain microorganisms which have beneficial effects on the health of host. The probiotic preparations use for traveller's diarrhea, antibiotic associated diarrhoea and acute diarrhoea which is showed that they have positive therapeutic effect [3]. Probiotics are used for long times in food ingredients for human and also to feed the animals without any side effects. Also probiotics are acceptable because of being naturally in intestinal tract of healthy human and in foods [3]. The probiotics which are used to feed both man and animals are shown in the Table 1.

The Effects of Probiotics on Health

According to some tests conducted by different scholars the probiotic, [3]

- Managing lactose intolerance.
- Improving immune system.
- Prevention of colon cancer.
- Reduction of cholesterol and triacylglycerol plasma concentrations (weak evidence).
- Lowering blood pressure.
- Reducing inflammation.
- Reduction of allergic symptoms.
- Beneficial effects on mineral metabolism, particularly bone density and stability.
- Reduction of Helicobacter pylori infection.
- Suppression of pathogenic microorganisms (antimicrobial effect).
- Prevention of osteoporosis.
- Prevention of urogenital infections.

Immune System and Probiotics

Studies have shown that probiotic bacteria can have positive effects on the immune system of their hosts [5]. Several researchers have studied on the effects of probiotics on immune system stimulation. Some *in vitro* and *in vivo* searches have been carried out in mice and some with human. Probiotics affect the immune system in different ways such as; producing cytokines, stimulating macrophages, increasing secretory IgA concentrations [3]. Some of these effects are related to adhesion while some of them are not [6].

Perdigon et al. feed the mice with lactobacilli or yogurt and it stimulated macrophages and increased secretory IgA concentrations.

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Lactobacillus species Bifidobacterium	species	Others
L. acidophilus	*B. bifidum*	*Enterococcus faecalis*
L. rhamnosus	*B. animalis*	*Enterococcus faecium*
L. gasseri	*B. breve*	*Streptococcus salivarus subsp. thermophilus*
L. casei	*B. infantis*	*Lactococcuslactis* subsp. *lactis*
L. reuteri	*B. longum*	*Lactococcuslactis* subsp. *cremoris*
L. crispatus	*B. lactis*	*Propionibacterium freudenreichii*
L. plantarum	*B. adolascentis*	*Pediococcus acidilactici*
L. salivarus		*Saccharomyces boulardii*
L. johnsonii		*Leuoconostocmes enteroides*
L. gallinarum		
L.plantarum		
L. fermentum		
L. helveticus		

Table 1: Microorganisms applied in probiotic products.

Also in a human trial Halpern et al. feed human with 450 g of yogurt per day for 4 months and at the end a significant increase is observed in the production of γ-interferon [1]. Mattilla-Sandholm and Kauppila showed that Lactobacillus rhamnosus GG and Bifido bacterium lactic Bb-12 derived extracts suppress lymphocyte proliferation *in vitro*. Further evidence for immunomodulation by these two strains a children trial with severe atopic eczema resulting from food allergy. Children fed with Lactobacillus rhamnosus GG and Bifido bacterium lactis Bb-12 showed improvement in clinical symptoms compared to the placebo group.

Diarrhea

Diarrhea is many causes and many types so it is difficult to evaluate the effects of probiotics on diarrhea. But there are lots of searches and evidence that probiotics have beneficial effects on some types of diarrhea. Diarrhea is a severe reason of children death in the worldwide and rotavirus is its common cause. In the treatment of rotavirus diarrhea, Lactobacillus GG is reported really effective. The best documented probiotic effect is shortened duration of rotavirus diarrhea using Lactobacillus GG. It has been given proof in several studies around the world by some researchers like Guandalini et al., Pant et al.

Antibiotic therapy causes mild and severe outbreaks of diarrhea. The normal microflora may be suppressed during the microbial therapy and resulting with filling with pathogenic strains. The changes of microflora may also encourage the resistant strains at least Clostridium difficile which is the reason of antibiotic associated diarrhea (ADD). Probiotics which are able to restore and replace the normal flora should be used. Also they should be used in high risk patients such as old, hospitalized or immuno-compromised. Studies with *Saccharomyces boulardii* proved that Clostridium difficile concentration is decreased in the presence of *Saccharomyce boulardii* [7].

Cancer

It is thought that probiotics could reduce the risk of cancer by decreasing the bacterial enzyme activity. There are *in vitro* and *in vivo* evidences not only from animal studies but also from human studies that probiotics have beneficial effects on suppression of cancer. Oral administration of lactic acid bacteria has been shown to reduce DNA damage caused by chemical carcinogens, in gastric and colonic mucosa in rats. The consumption of lactobacilli by healthy volunteers has been demonstrated to reduce the mutagenicity of urine and feaces associated with the ingestion of carcinogens in cooked meat. When it comes to epidemiological studies, they show an association between fermented dairy products and colorectal cancer. The consumption of a large quantity of dairy products especially fermented foods like yogurt and fermented milk with containing *Lactobacillusor Bifidobacterium* may be related to a lower occurrence of colon cancer [8,9]. A number of studies have shown that predisposing factors (increases in enzyme activity that activate carcinogens, increase procarcinogenic chemicals within the colon or alter population of certain bacterial genera and species) are altered positively by consumption of certain probiotics [10].

Mechanism of probiotics

Probiotic microorganisms are considered to support the host health. However, the support mechanisms have not been explained [11]. There are studies on how probiotics work. So, many mechanisms from these studies are trying to explain how probiotics could protect the host from the intestinal disorders. These mechanisms listed below briefly [3,6,12].

1. Production of inhibitory substances: Production of some organic acids, hydrogen peroxide and bacteriocins which are inhibitory to both gram-positive and gram-negative bacteria.

2. Blocking of adhesion sites: Probiotic s and pathogenic bacteria are in a competition. Probiotics inhibit the pathogens by adhering to the intestinal epithelial surfaces by blocking the adhesion sites.

3. Competition for nutrients: Despite of the lack of studies *in vivo*, probiotics inhibit the pathogens by consuming the nutrients which pathogens need.

4. Stimulating of immunity: Stimulating of specific and nonspecific immunity may be one possible mechanism of probiotics to protect the host from intestinal disease. This mechanism is not well documented, but it is thought that specific cell wall components or cell layers may act as adjuvants and increase humoral immune response.

5. Degradation of toxin receptor: Because of the degradation of toxin receptor on the intestinal mucosa, it was shown that S. boulardii protects the host against C. difficile intestinal disease. Some other offered mechanisms are suppression of toxin production, reduction of gut pH, attenuation of virulence [7].

Antimicrobial Activity

Antimicrobial activity targets the enteric undesirables and pathogens [13]. Antimicrobial effects of lactic acid bacteria are formed by producing some substances such as organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins [14,15]. Till today there are some researches on showing that different species produce different antimicrobial substances. Here are some examples of these substances: *Lactobacillus reuterii*, which is a member of normal microflora of human and many other animals, produce a low molecular weight antimicrobial substance reuter in; subspecies of *Lactococcuslactis* produce a class I bacteriocin, nisin A; *Enterococcus feacalis* DS16 produces a class I *bacteriocincytolysin*; *Lactobacillus plantarum* produces a class II *bacteriocinplantaricin S*; *Lactobacillus acidophilus* produces a class III *bacteriocinacidophilucin* A [14]. Production of bacteriocins is highly affected by the factors of the species of microorganisms, ingredients and pH of medium, incubation temperature and time. Nisin, produced by *L. lactis subsp. Lactis* is the well-known bacteriocin and it is allowed to use in food preparations [15].

Lactobacilli and Bifidobacteria isolated from human ileum were assayed if they have antimicrobial activity against a range of indicator microorganisms, *Listeria, Bacillus, Enterococcus, Staphylococcus, Clostridium, Pseudomonas, E. coli, Lactobacillus, Streptococcus, Bifidobacterium and Lactococcus*. Antimicrobial activity of *Lactobacillus salivarus* UCC118 was counted against to these bacteria listed above. The study showed that *Lactobacillus salivarus* UCC118 is significantly capable of inhibiting *in vitro* growth of both some gram positive and some gram negative bacteria such as, *L. fermentum KLD, B. longum, B. bifidum, Bacillus subtilus, B. cereus, B.thuringiensis, E. Faecalis, E. faecium* etc. although it is not effective against some of *Lactobacillus, Lactococcus, Leuconostoc, Streptococcus* etc. species.

Some milk products were used to isolate potential probiotic bacteria and determination of their possible antimicrobial activities. *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Serratia marcescens and Candida albicans* were used as indicator microorganisms. In another study eight lactic acid bacteria strains producing bacteriocins were isolated from Burkina Faso fermented milk and they were examined for the antimicrobial activity against *Enterococcus faecalis* 103907 CIP, *Bacillus cereus* 13569LMG, *Staphylococcus aureus* ATCC25293, *Escherichia coli* 105182 CIP. The lactic acid bacteria strains were identified as *Lactobacillus fermentum, Pediococcus spp., Lactococcus spp., Leuconostocmesenteroides sub sp. mesenteroides*. The diameters of inhibition zones were obtained between 8 mm and 12 mm. Lactobacillus fermentum (S1) gave the biggest zone around 12 mm on *Enterococcus faecalis* while the smallest one is obtained from *Leuconostocme senteroides sub sp. mesenteroides* (S5) on the same strain Enterococcus faecalis.

In this study, common human pathogens *Gardnerallavaginalis, Pseudomonosaeroginosa, Proteus vulgaris, Escherichia coli, Enterobactercloacae, Streptococcus milleri, Staphylococcus aureus and Candida albicans* were used as indicator microorganisms. Six of the strains had bacteriocin activity against eight of ten different Lactobacillus species an also *S. milleri, P. vulgaris, P. aeroginosa, E. coli, E. cloacae and G. vaginalis*. But none of isolated strains showed efficiency on test organisms *S. aureus and C. albicans*. Also some characteristics of bacteriocins were obtained from the research.

In another research, potential probiotic lactobacilli strains (*L.reuteri, L. plantarum, L.mucosae, L. rossiae strains*) (from pig feces), used as additives in pelleted feeding, were examined according to their antibacterial activity against to *Salmonella typhimurium* ATCC 27164, *E. coli* , *C. perfringens* 22G, *S. aureus* ATCC 25923, *B. megaterium F, L. innocua* DSM 20649 and *B. hyodysenteriae* ATCC 27164. Generally, the cell free extracts of lactobacilli were able to inhibit all potential pathogens except *B. hyodysenteriae* ATCC 27164. The study showed that, neutralization and treatment with catalase affect the antibacterial activity a little (D e Angelis, et al.). A similar study was conducted and in that study four Lactobacillus strains (*L. salivarus* CECT5713, *L. gasseri* CECT5714, *L.gasseri* CECT 5715 *and L.fermentum* CECT5716) isolated from human milk were investigated whether they have antimicrobial potential and for comparison *L. coryniformis* CECT5711 was used.

Molecular Identification of Probiotic Strains

Methods used for detection of probiotics in human gastrointestinal tract are identification of colony morphology, fermentation patterns, serotyping or some combination of these. Although these traditional methods have limitations they are used for identification. With the developing technology about the molecular typing it is getting more reliable to identify and differentiate bacterial strains. Classical microbiological techniques are really important for se lection, enumeration and biochemical characterization (fermentation profiles, salt-pH- temperature tolerances) but it is not efficient to classify a culture taxonomically. Molecular characterization methods are powerful even between closely related species. There are numbers of alternative taxonomic classification methods well known including hybridization with species-specific probes and generation of profile PCR applicants by species-specific primers [13]. Polymerase chain reaction based methods (PCR-RFLP, REP-PCR, PCR ribotyping and RAPD) are mainly used as molecular tools. Comparison between these methods, the most powerful and accurate one is sequencing.

Characterization of microorganisms according to their 16S rDNA regions sequencing was firstly proposed by Woese in 1987. The application of 16S or 23S rRNA-targeted oligonucleotide probes is the best and most reliable approach to identify bacteria on a phylogenetic basis. The 16S rRNA gene is nearly 1540 bases long and includes variable regions while the general structure is highly conserved. Because the probes have the broadest specificity ranging from universal to species specificity, it is possible to use 16S rRNA gene to study phylogenetic relationships between microorganisms and identify them more accurately [11,15].

In one study, the PCR-ARDRA technique was used to identify potential probiotic Lactobacillus species isolated from bovine vagina. 16S rRNA gene was amplified by PCR and products were digested with four re striction enzymes (Sau 3AI, Hinf I, Hinc II and Dra I). Most of the digestion profiles obtained from the amplified 16S rDNA gene of these strains agreed with the theoretical profile matching with *Lactobacillus fermentum*. Among all strains, four homo fermentative lactobacilli showed a restriction profile that matched with *Lactobacillus gasseri* and a facultative hetero fermentative strain was identified as *Lactobacillus rhamnosus* [16].

Restriction enzyme analysis were done by using pulsed with gel electrophoresis (REA-PFGE) and intergenic transcribed spacers (ITS)-PCR restriction fragment length polymorphism (RFLP) techniques for identification of probiotic potential strains (by sequencing of the 16S rRNA gene) isolated from koko and koko sour water (African spontaneously fermented millet porridge and drink). Taq I and Hae III restriction enzymes were used for digestion. From the result of ITS-PCR RFLP, four groups were obtained including group 1 Weisella confuse, group 2 *Lactobacillus fermentum*, and group 3 *Lactobacillus salivarus* and group 4 Pediococcus spp. At the end it was showed using for identification of these strains the ITS-PCR RFLP technique, 16S rRNA gene sequencing is very reliable [17].

To identify lactobacilli used as starter and probiotic cultures, amplified ribosomal DNA restriction analysis (ARDRA) was applied. Firstly group-specific and species-specific 16S rDNA primers were used to amplification. Cfo I, Hinf I, Tru 91 and ScrFI restriction enzymes were selected for digestion. The results revealed three groups: A, B and C. It is suggested that ARDRA by using Cfo I was reliable method for differentiation of L. delbrueckii subsp. bulgaricus and *L. delbrueckii* subsp. lactis [18].

Some researchers aimed to develop a novel multiplex PCR primer set to identify seven probiotic Lactobacillus species (*L.acidophilus, L. delbrueckii, L.casei, L.gasseri, L. plantarum, L. reuteri and L. rhamnosus*). The primer set containing seven specific and two conserved primers, was obtained from the integrated sequences of 16S and 23S rRNA genes and their rRNA intergenic spacer region of each species. 93.6%

accuracy was obtained to identify the seven target species. The study showed that the multiplex primer set is really efficient tool for simple, rapid and reliable identification of Lactobacillus species [19].

Human milk and probiotics

When it comes to the microbiological point of breast milk, human milk is really an important factor in the initiation and development and of course composition of the neonatal gut microflora since it constitutes source of microorganisms to the infant gut for several weeks after birth [20]. The composition of the gut microflora is thoroughly influenced by the diet of the infant. Thus, the presence of a few predominant Gram-positive species in breast milk may be a reason explaining why microbiota of breast-fed infants is composed of a narrow spectrum of species, and a more diverse microbiota develops after weaning [21].

The studies on the microbiology of human milk are restricted to the identification of potential pathogenic bacteria in clinical cases of mastitis or infant infections. However, it is clear that the prevention of infant from infectious diseases owing to the natural flora of human m ilk [21]. Although there is limited knowledge about the commensal or probiotic bacteria that breast milk contain, bacteria commonly isolated from this biological fluid include staphylococci, streptococci, micrococci, lactobacilli and enterococci [20-22]. Bacteria from these genera can be easily isolated from fresh milk of healthy women. So, these groups of bacteria should be considered the natural microbiota of human milk rather than mere contaminant bacteria [21,22].

From the bacteria isolated from breast milk, *Lactobacillus gasseri, Lactobacillus rhamnosus, Lactobacillus fermentum, or Enterococcus feacium* are founded and they can be regarded as potential probiotic bacteria [11,21]. Hence, breast milk, a natural source of potentially probiotic or biotherapeutic LAB, protects mother and infants against infectious diseases [21].

There are lots of studies on the effect of human milk on the health of infants and the infant diseases but surprisingly lack of studies on the microbiology of breast milk. From the few studies, it is found that human m ilk is an attractive source for potential probiotic strains. As, the bacteria implement some of the main criteria for being probiotic strains such as, human origin, survival in the gastrointestinal conditions and particularly low pH and bile, production antimicrobial compounds, adhesion to the intestinal mucosa [20].

Martin et al. aimed to investigate whether human breast milk contains potentially probiotic lactic ac id bacteria, and therefore, whether it can be considered a synbiotic food. For this purpose; they isolated lactic acid bacteria from milk, mammary areola, and breast skin of eight healthy mothers and oral swabs and feces of their respective breast-fed infants. They identified the lactic acid bacteria by RAPD-PCR analysis and 16S rDNA sequencing. From the results they identified the bacteria isolated from human milk as *Lactobacillus gasseri, Lactobacillus fermentum and Enterococcus faecium*. These species are considered among the probiotic bacteria [11] and contain strains that are used in commercial probiotic products. They concluded as their work indicates that breast milk contains lactic acid bacteria is a natural source of LAB for the newborns and may be considered a symbiotic food.

Martin et al. studied on three lactobacilli strains isolated from breast milk whether they were potential probiotic bacteria. They performed some assays to investigate some criteria need to be used as probiotic bacteria such as; survival to conditions simulating in the gastro intestinal tract, production of antimicrobial compounds, adherence to intestinal cells, production of biogenic amines, degradation of mucin, enzymatic profile and pattern of antibiotic resistance. *Lactobacillus gasseri and Lactobacillus fermentum* strains were evaluated and the results showed that the probiotic potential of lactobacilli isolated from human milk is similar to strains commonly used in commercial probiotic products.

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