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Full Length Research Paper

Antibacterial effects of probiotics isolated from yoghurts against some common bacterial pathogens

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According to the definition of probiotics by the Food and Agriculture Organization of the United Nations/World Health Organization, "probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit on the host". Microorganisms that are probiotics in humans include *Enterococci*, *Bifidobacteria* and lactic acid bacteria, such as *Lactobacilli*, *Lactococci* and *Streptococci*. This research was conducted to determine the presence of antibacterial effects among the probiotics isolated from different bioyoghurts against some common bacterial pathogens. *Lactobacillus* sp., *Streptococcus* sp. and *Bifidobacterium* sp. from yoghurts containing probiotics were isolated and examined for their antibacterial effects against *Staphylococcus aureus, Escherichia coli, Salmonella typhi,* and *Pseudomonas aeruginosa*. The modified agar overlay method was used for determination of the presence of antibacterial effects among the isolated probiotics. Results showed the presence of antibacterial effects among the probiotics that were isolated from bioyoghurts. The spectrum of their antibacterial effects varied against the selected pathogen. Antibacterial effects are one of the most important selection criteria for probiotics, and the verified antibacterial activity of the probiotics supports the development of these functional foods as a key to the improvement of health in the consuming public.

Key words: Probiotics, bioyoghurts, *Lactobacillus* spp., *Bifidobacterium* spp., antibacterial effects.

INTRODUCTION

Probiotics are defined as non-pathogenic microorganisms, which when ingested, exert a positive influence on the host health or physiology (Fuller, 1989). Now, the definition of probiotics by the Food and Agriculture Organization of the United Nations/World Health Organization is "Live microorganisms, which when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). This definition retains the historical elements of the use of living organisms for health purposes but does not restrict the application of the term only to oral probiotics with intestinal outcomes (Reid, 2006).

Probiotics are living, health-promoting microorganisms that are incorporated into various kinds of foods. The ability of probiotics to withstand the normal acidic conditions of the gastric juices and the bactericidal properties of the bile salts, as well as the production of lactic acid that inhibits the growth of other microorganisms, allow them to be established in the intestinal tract (Catanzaro and Green, 1997).

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 found in the intestinal tract of healthy humans and in foods. The reported health benefits include: boosting of the immune system, inhibition of the growth of pathogenic microorganisms, prevention of diarrhea from various causes, prevention of cancer, reduction of the risk of inflammatory bowel movements, improvement of digestion of proteins and fats, synthesis of vitamins, and detoxification and protection from toxins (Hobbs, 2000).

Members of the genera *Lactobacillus*, *Bifidobacterium* and *Streptococcus* are the most common probiotics used in commercial fermented and non-fermented dairy products today (Heller, 2001).

Antibacterial properties are one of the most important selection criteria for probiotics (Klaenhammer and Kullen, 1999). 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 (Quwehand and Vesterlund, 2004).

A number of studies have found probiotic consumption to be useful in the treatment of many types of diarrhea, including antibiotic-associated diarrhea in adults, travelers' diarrhea, and diarrheal diseases in young children caused by rotaviruses. The most commonly studied probiotic species in these studies have been found to be *Lactobacillus GG*, *L. casei*, *B. bifidum* and *S. thermophilus* (Isolauri et al., 1991; Oksanen et al., 1990; Siitonen et al., 1990).

The aim of this study was to determine the presence of antibacterial effects among the probiotics isolated from different bioyoghurts against some common bacterial pathogens.

MATERIALS AND METHODS

Bioyoghurts, probiotics, media and pathogen strains

Probiotic bacteria were isolated from different commercially prepared bioyoghurts. Three kinds of bioyoghurts (ProFeel, Evolus and Gefilus) purchased from Helsinki supermarkets were tested. According to the product information, the bioyoghurts contain various probiotics including *Lactobacillus rhamnosus* GG (ATCC 53103), *Bifidobacterium* sp, *Streptococcus* sp. and *Lactobacillus* sp. The samples of the bioyoghurts: ProFeel, Evolus and Gefilus were shaken vigorously to suspend the bacterial contents. Then, 10 g of each bioyoghurts were separately dissolved in 50 ml (0.9%) of Normal Saline. The bioyoghurts were inoculated into M17 Agar (Merck), De Man Rogosa Sharpe Agar (MRS Agar) (Merck) and Bifidobacterium Medium (Merck). The plates were incubated anaerobically on jars using GasPak at 37°C for 72 h. The isolated bacteria were Gram stained for the study of microscopic morphology. Stock cultures of the probiotics were maintained in their MRS Agar medium at 4°C. The test pathogen bacterial isolates comprised Gram negative bacteria like *E. coli, S. typhi* and *P. aeruginosa,* and Gram positive bacteria like *S. aureus* (Chuayana Jr et al., 2003; Lim and Dond-Soon, 2009; Maia et al., 2001).

Determination of antibacterial effects

The selected pathogens were maintained in Brain Heart Infusion (BHI) Agar (Himedia) butt-slants in screw-capped tubes kept at 4°C. For antibacterial effects determination, the probiotics from the stock cultures were inoculated into brain heart infusion (BHI) broth (Himedia). The turbidity of the broth culture was then adjusted to equal that of 1 McFarland standard. The test pathogens from the stock cultures were subcultured in BHI broth under aerobic condition at 37°C for 18 h. The turbidity of the broth cultures was adjusted to equal that of 0.5 McFarland standards. The modified agar overlay method was used to test for the presence of antibacterial effects among the probiotics isolates. The prepared probiotics were individually inoculated into the plates by swabbing area in the center of each plate. The plates were incubated anaerobically, at 37°C for 72 h for ProFeel, Evolus and Gefilus probiotics. The growth in each plate was then overlaid with 10 ml of molten and cooled in BHI Agar previously inoculated with 1 ml of the prepared selected pathogen cultures. The agar was allowed to solidify and the plates were incubated aerobically at 37°C for 24 h. The plates were then examined for the presence of growth inhibition. To further determine whether the selected pathogens were inhibited or killed by probiotics, the growth inhibition zone was swabbed. The swab was then inoculated into BHI broths and incubated aerobically under 37°C for 24 h. The BHI broths were then checked for growth. The presence of growth in the broth was interpreted as an inhibitory property in the agar plate, while no growth was interpreted to be as a result of the bactericidal effect. Each of the tests in the determination of antibacterial effects of the probiotics was conducted in two trials, and in duplicate (Chuayana Jr et al., 2003; Lim and Dond-Soon, 2009; Maia et al., 2001; Millette et al., 2006).

RESULTS AND DISCUSSION

Macroscopic and microscopic properties of isolated probiotics

The probiotics isolates from Gefilus were cream colored, circular, convex and moist with smooth edges. Microscopic smear of Gram staining of the Colonies showed gram-positive, non-sporeforming short bacilli in pairs or in chains. The results were those expected of *Bifidobacterium* sp. found in the Gefilus bioyoghurts. Colonies of bacterial obtained from the Evolus bioyoghurts cultured produced colonies that were small, round, smooth, white and moist. The gram stained smears showed both gram-positive cocci in pairs or long chains, and also gram-positive, non-spore forming long bacilli. These are consistent with the microscopic morphology of *Streptococcus* sp. and *Lactobacillus* sp., the bacteria in Evolus bioyoghurts. The isolates from ProFeel produced yellow, round, convex and moist colonies with smooth edges. Gram stained smears showed gram-positive bacilli in pairs or chains, consistent with the morphology of *Lactobacillus* sp., which is the probiotic in ProFeel.

Antibacterial effects

Results of the modified agar overlay method showed that all the probiotic strains isolated from the different bioyoghurts were able to inhibit the growth of some, if not all of the selected pathogens. The spectrum of their antibacterial effects varied. Probiotics of ProFeel bioyoghurts inhibited the growth of all the pathogen**ic** bacteria selected against them. Evolus bioyoghurts probiotics were bactericidal for *S. aureus* and *P. aeruginosa*, but were inhibitory for *S. typhi*. Probiotics, isolated from Evolus, had no activity against *E. coli*. Although, Gefilus probiotics killed the test bacteria of

Table 1. Results of the antibacterial effects of the probiotics isolated from bioyoghurts.

Kind of bioyoghurts	Antibacterial effects against			
	Escherichia coli	***Staphylococcus aureus***	***Salmonella typhi***	***Pseudomonas aeruginosa***
ProFeel	Bacteriostatic	Bacteriostatic	Bacteriostatic	Bacteriostatic
Evolus	No activity	Bactericidal	Bacteriostatic	Bactericidal
Gefilus	Bactericidal	Bacteriostatic	Bactericidal	No Activity

E. coli and *S. typhi*, they were only inhibitory for *S. aureus* and were not active against *P. aeruginosa* (Table 1).

Results of the study showed the antibacterial effects of the probiotics isolated from the different bioyoghurts. This may be due to the production of acetic and lactic acid that lowered the pH of the media (Bezkorovainy, 2001).

Till today, there are some researches showing that different species produce different antimicrobial substances. Here are some examples of these substances: *Lactobacillus reuterii* (a member of normal microflora of human and many other animals) produce a low molecular weight antimicrobial substance called reuterin; subspecies of *Lactococcus lactis* produce a class I bacteriocin, known as nisin A; *Enterococcus feacalis* DS16 produces a class I bacteriocin cytolysin; *Lactobacillus plantarum* produces a class II bacteriocin plantaricin S; and *Lactobacillus acidophilus* produces a class III bacteriocin acidophilucin A. 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 its usage is allowed in food preparations (Quwehand and Vesterlund, 2004).

To have an impact on the colonic flora, it is important for probiotic strains to show antagonism against pathogenic bacteria via antimicrobial substance production or competitive exclusion. Enormous research efforts have focused on bacteriocin research. Although, probiotic strains may produce bacteriocins, their role in pathogen inhibition *in vivo* can only be limited, since traditional bacteriocins have an inhibitory effect only against closely related species such as *Lactobacillus* or on sporefomers such as *Bacillus* or *Clostridium* (Holzapfel et al., 1995). However, low molecular weight metabolites (such as hydrogen peroxide, lactic and acetic acid, and other aroma compounds) and secondary metabolites may be more important since they show wide inhibitory spectrum against many harmful organisms like *Salmonella*, *E. coli*, *Clostridium* and *Helicobacter* (Niku-Paavola et al., 1999; Skytta et al., 1992).

L. rhamnosus strain GG produces *in vitro* low molecular weight antimicrobial(s), possibly short chain fatty acid(s) but distinct from lactic and acetic acid, with inhibitory activity against bacteria such as *Clostridium*, *Bacteroides*, *Enterobacteriaceae*, *Pseudomonas*, *Staphylococcus* and *Streptococcus*, but not against other lactobacilli (Silva et al., 1987). The antagonistic activity of *L. rhamnosus* GG against enteropathogenic bacteria has also been shown *in vivo* in *S. typhimurium* infected mice (Hudault et al., 1997).

The spent culture supernatant (SCS) of *L. acidophilus* strain LB decreased the viability of *S. aureus*, *Listeria monocytogenes*, *S. typhimurium*, *Shigella flexneri*, *E. coli*, *Klebsiella pneumoniae*, *Bacillus cereus*, *P. aeruginosa*, and *Enterobacter* spp. *in vitro*. The unidentified low molecular weight antimicrobial substance(s) was independent of lactic acid production and did not affect *Lactobacillus* or *Bifidobacterium* strains tested. The antibacterial activity of *L. acidophilus* SCS towards *S. typhimurium* was also maintained *invivo* in the infected-mouse model (Coconnier et al., 1997). *L. acidophilus* strain LA1 produces nonbacteriocin antibacterial substance(s) (unidentified but distinct from lactic acid) that inhibits *in vitro* a wide range of gram-negative and gram-positive pathogens, such as *S. aureus*, *L. monocytogenes*, *S. typhimurium*, *S. flexneri*, *K. pneumoniae*, *P. aeruginosa* and *Enterobacter cloacae*. However, inhibition of lactobacilli and bifidobacteria could not be detected. Inhibitory activity of the strain LA1 towards *S. typhimurium* is also shown *in vivo* in the mouse model (Bernet-Camard et al., 1997).

The probiotic bacteria may also have competed for nutrients (Marteau et al., 1990), and simultaneously produced hydrogen peroxide and bacteriocins that acted as antibiotic agents (Wolfson, 1999). Other than bacteriocins, some are also capable of reuterine production that is known to act as an antibacterial compound (Ray, 1996).

Lactobacilli and Bifidobacteria isolated from human ileum were assayed if they have antimicrobial activity against a range of indicator microorganisms, such as *Listeria, Bacillus, Enterococcus, Staphylococcus, Clostridium, Pseudomonas, E. coli, Lactobacillus, Streptococcus, Bifidobacterium* and *Lactococcus.* Antimicrobial activity of *Lactobacillus salivarus* UCC118 was counted against these aforementioned bacteria. The study showed that *Lactobacillus salivarus* UCC118 is significantly capable of inhibiting *in vitro* growth of both some gram positive and gram negative bacteria such as, *L. fermentum, B. longum, B. bifidum, Bacillus subtilus, B. cereus, B. thuringiensis, E. faecalis, E. faecium*, etc., although it is not effective against some species of *Lactobacillus, Lactococcus, Leuconostoc, Streptococcus* etc. (Dunne et al., 1999).

Some milk products were used to isolate potential probiotic bacteria and in determining their possible antimicrobial activities. *S. aureus, E. coli, P. aeruginosa, S. typhi, Serratia marcescens* and *Candida albicans* were used as indicator microorganisms. After the study, the results showed that Yakult and Ski D' Lite probiotics inhibited all of the test indicator microorganisms; Nestle yogurt probiotics were bactericidal for *S. aureus* and *P. aeruginosa*, but inhibitory for *S. typhi*; Neslac probiotics killed *E. coli* and *S. typhi*, while they were only inhibitory for *S. aureus* and *C. albicans*; and Gain probiotics inhibited *C. albicans* (Chuayana Jr et al., 2003). In another study, eight lactic acid bacteria strains producing bacteriocins were isolated from Burkina Faso fermented milk and were examined for the antimicrobial activity against *Enterococcus faecalis, Bacillus cereus, S. aureus and E. coli*. The lactic acid bacteria strains were identified as *Lactobacillus fermentum*, *Pediococcus* sp., *Lactococcus* sp., and *Leuconostoc mesenteroides* subsp. *mesenteroides.* The diameters of inhibition zones were obtained between 8 and 12 mm. *Lactobacillus fermentum* gave the biggest zone around 12 mm on *E. faecalis*, while the smallest one was obtained from *L. mesenteroides* subsp. *mesenteroides* on the same strain of *E. faecalis* (Lei and Jacobsen, 2004).

In a research which was aimed to test the production of bacteriocin in vaginal lactobacilli flora, characterization of this flora was also made. The first antimicrobial activity was assayed for 100 vaginal lactobacilli isolates, of which six of them were determined for the production of bacteriocin. In this study, common human pathogens such as *Gardneralla vaginalis*, *Pseudomonos aeroginosa*, *Proteus vulgaris*, *E. coli*, *Enterobacter cloacae*, *Streptococcus milleri*, *S. aureus* and *Candida albicans* were used as indicator microorganisms. Six of the strains (*S. milleri*, *P. vulgaris*, *P. aeroginosa*, *E. coli*, *E. cloacae* and *G. vaginalis*) had bacteriocin activity against eight often different *Lactobacillus* species, but none of the isolated strains showed efficiency on test organisms *S. aureus* and *C. albicans.* Also, some characteristics of bacteriocins were obtained from the research (Karaoglu et al., 2003). In another research, potential probiotic lactobacilli strains (*L. reuteri, L. plantarum, L. mucosae* and *L. rossiae* strains from pig feces), used as additives in pelleted feeding, were examined according to their antibacterial activity against *Salmonella typhimurium*, *E. coli*, *C. perfringens*, *S. aureus*, *B. megaterium*, *L. innocua* and *B. hyodysenteriae*. Generally, the cell free extracts of lactobacilli were able to inhibit all potential pathogens except *B. hyodysenteriae*. The study showed that neutralization and treatment with catalase affect the antibacterial activity a little (Angelis et al*.,* 2006). A similar study was conducted on four *Lactobacillus* strains (*L. salivarus*, *L. gasseri*, *L.gasseri* and *L. fermentum*) isolated from human milk, and in that study, an investigation was done on whether or not they have antimicrobial potential, and a comparison was made between them and *L. coryniformis*. All of the strains showed antibacterial properties against pathogenic bacteria (*Salmonella choleraesuis*, *E. coli* O157:H7, *S. aureus*, *Listeria monocytogenes* and the spoilage strain *Clostridium tyrobutyricum*). However, the antimicrobial properties of lactobacilli strains varied and *L. salivarus* revealed not only the best *in vitro* antibacterial activity, but also the highest protective effect against *Salmonella* strain in the murine infection model (Olivares et al., 2006).

Finally, the capability of the probiotics incorporated in bioyoghurts to inhibit the growth, or even kill certain selected pathogens confirms the health benefits one derives from the consumption of these yoghurts. Consuming these products can help protect one from occurrences of diarrhea, food poisoning and even systemic and enteric infections. The verified antibacterial effects of the probiotics supports the development of these functional foods as a key to the improvement of the health in the consuming public.

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