SIV-4

POTENTIAL APPLICATION OF PREBIOTICS AND PROBIOTICS

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Abstract

The importance of probiotics and prebiotics in health-promoting effect on the host is increasing. Recent studies on gastrointestinal system have contributed to further understanding of the mechanisms involved in the interaction between probiotics and prebiotics. In this presentation, the beneficial effects of probiotics and prebiotics, and applications of microencapsulation technique for the application of prebiotics and probiotics are described.

INTRODUCTION

Probiotic is a live microbial adjuvant that beneficially affects the host by modulating microbial balance in the intestinal tract (1). Among 500 microflora in gastrointestinal tract of human, lactic acid bacteria (LAB) has been known as major beneficial organisms which effectively suppress colonization and subsequent proliferation of pathogenic bacteria in the intestine (2). The gastrointestinal tract of a healthy human provides a harsh environment for microorganisms because of gastric juices, digestive enzymes, and bile acids. Since these conditions as well as low surface tension and immune response in the gastrointestinal tract impose a significant threat to probiotic, the strains should have ability to survive during digestive process and adhere to gastrointestinal mucosa to be effective. In addition to factors affecting colonization, bacteriocin production, cholesterol lowering effect, production of CLA (conjugated linoleic acid), and immunomodulation among others have been reported as added beneficial effects by the strains of *Lactobacillus acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. casei*, *L. fermentum*, *L. plantarum*, and *L. reuteri*, and *Bifidobacterium* species (3).

Prebiotic is defined as "non-digestible food ingredient that beneficially affect the host by selectively stimulating the growth and/or activity of one or limited number of bacteria in the colon"(4-5). Thus, intake of prebiotics can significantly modulate the colonic microflora by increasing number of specific bacteria. These nondigestable-carbohydrates including sugar alcohols, disaccharides, oligosaccharides, and polysaccharides can be predominantly utilized by the desirable microorganisms and metabolized into short-chain fatty acids and organic acids such as acetic acid, propionic acid, butyric acid and lactic acid. Although the exact mechanism of prebiotic for health enhancing effect is

not clearly demonstrated several oligosaccharides including fructooligosaccharides (FOS), galactooligosaccharide (GOS), transgalactosyloligosaccharides (TOS) and soybean oligosaccharide (raffinose and stachyose), and inulin are commercially produced and claimed to have growth-promoting effects on bifidobacteria and lactic acid bacteria (5). The concept of synbiotics (mixture of probiotic and prebiotic) is also suggested to improve the beneficial effects of probiotics but the validity of this concept still need to be studied further.

Microencapsulation is a physical process that forms a wall or shell around the core material to control the release of the core content under specific conditions (6). The microencapsulation of probiotic cultures may provide protections against heat, moisture, acid, oxygen, and gastric enzymes, thereby extending their viability during storage, processing and digestion. Although numerous methods have been developed to encapsulate food ingredients, spray drying still remains dominant in commercial prospect (7). However, spray-drying may not be appropriate for the encapsulation of heat-sensitive probiotic cultures. As an alternative, a hybridization technique by surface modification might have advantage for the encapsulation over spray drying. The lyophilised cultures are hybridized with coating materials by friction and collision while unwanted rise of temperature is prevented by a cooling unit. Some pharmaceutical powders have been encapsulated by the technique (8). The application of the technique in the production of probiotic cultures is being evaluated by the author.

MATERIALS AND METHODS

Bacterial strains

Lactic acid bacteria were grown for 18 hrs at 37°C in MRS broth (Difco, Detroit, MI) and the strains were subcultured three times before use. The culture was stored at –80°C in the 10% skim milk medium supplemented with 30% glycerol.

Acid tolerance

The strains were incubated at 37°C for 18 hrs and then centrifuged at 5000 ×g for 20 min at 4°C. The collected cells were resuspended in sterile saline (0.85% NaCl). The cells were inoculated at *ca* 10^6 CFU/ml in MRS broth adjusted to pH 2.5 with 1*N* HCl for 1 h. The viable cell count was determined after 0 and 3 h of incubation at 37°C. Aliquots (1ml) of acid exposed and untreated cells were diluted in sterile peptone dilution blank and plated onto MRS media containing 2% β-glycerophosphate and allowed to be incubated at 37°C for 2 days before enumeration. Acid tolerance was expressed as the difference in log viable cells between treatment and control.

Bile tolerance

Bile tolerance was determined by inoculating (*ca* 10^6 CFU/ml) MRS broth containing 0.3% oxgall (Difco, Detroit, MI) inoculated with a resuspended culture grown at 37°C for 18 hrs. The bacteria were plated onto MRS media and enumerated after incubation for 24 hrs at 37°C. The bile tolerance was expressed as follows: (Final log number of viable cells) - (Initial log number of viable cells).

Bacteriocin production and activity

Bacteriocin activity was determined by spotting 20µl aliquots of two-fold serial dilutions of the culture supernatant that was adjusted to pH 6.5 using 10N NaOH onto the surface of MRS agar. The spotted agar was then overlayed with 0.8% MRS agar inoculated with 1% of the indicator strain. The plates were incubated at 37°C for 24 h. The bacteriocin activity was expressed as a reciprocal of the highest dilution showing a clear inhibition zone on the MRS agar.

Measurement of cholesterol assimilation

The amount of cholesterol in the cell free spent broth was determined by the method of Rudel and Morris (9). A 0.5 ml aliquot of the sample was added to 3ml of 95% ethanol and 2 ml of 50% KOH followed by heating at 60°C for 10 min. Five ml of hexane was added to the cooled samples and mixed well for 30 sec. A measured aliquot (2.5 ml) of the hexane layer was dried under a flow of N_2 gas. Color developed by the addition of *o*-phthalaldehyde and concentrated sulfuric acid was measured at a wavelength of 550 nm using a spectrophotometer (Beckman DU Series 600, Beckman Instruments Inc., USA). The amount of cholesterol was calculated from a standard curve using 0, 10, 20, 40 and 80 µg/ml cholesterol (Sigma Chemical Co. St. Louis, MO).

CLA analysis

Cells grown to an O.D. = 1 were used as an inoculum in the experiments. Skim milk (11% w/v) with filter sterilized (0.22 µm, Milipore, Millipore Corp., Bedford, MA) linoleic acid solution (0.1g/L final) was used to test CLA production. Samples were extracted and methylated as previously described by Kim and Liu (1999). Fatty acid methyl esters were analyzed by GC (HP5890, Hewlett Packard, USA) on the Supelcowax-10 fused silica capillary column (Supelco. Inc, USA). Heptadecanoic acid ($C_{17:0}$; Sigma Chemical Co. St. Louis, MO) was used as an internal standard. Cis-9, trans-11 octadecadienoic acid (<99% cis-9, trans-11 isomer; Matreya Inc., USA) was used as the CLA standard.

Effect of prebiotics on the growth of the probiotics

Prebiotics (sorbitol, lactulose, mannitol, raffinose, inulin and fructooligosaccharide) were added to basal medium with fresh cultures and incubated at 37C for 48 h. The viable cells of *L. acidophilus* were enumerated on BCP agar.

Encapsulation of probiotic

The impact of processing conditions on the viability of probiotic cultures was examined. The lyophilised cultures was ground by A-10 mill (IKA Labortechnik, Germany) and the particle size was controlled between 53 and 106 μm prior to the surface modification. To optimize the process in a hybridizer system (Model NSH-0, Nara Machinery Co. Ltd., Japan) viability of the cultures were determined at different rotor speed. The encapsulaion was processed in two steps. The cultures were coated with different prebiotics including fructooligosaccharide, lactulose and inulin followed by a second surface coating using Sureteric^{R}, an enteric coating material, to enhance encapsulation efficiency. During the process, temperature of hybridization chamber was maintained not to exceed 30°C by circulating chilled water in a jacket.

Scanning electron microscopy of encapsulated cultures

The surface of encapsulated probiotic was observed with a scanning electron microscope (Hitachi S-2380, Ltd., Japan). The samples were coated for 60 s with gold-palladium in an E-1010 ion sputter coater (Hitachi Ltd., Japan) and the topography of the particles was observed at 15 Kv.

RESULTS AND DISCUSSION

Comparison of probiotic characteristics

The acid tolerance of LAB is dependent upon the pH profile of H^{+}-ATPase and the composition of the cytoplasmic membrane, which is largely influenced by the type of bacteria, type of growth media and the incubation conditions. Strains of *L. acidophilus* as well as the strains of *L. casei* and *L. plantarum* have been shown to survive at pH 3.0 or less. As shown in Table 1, a relatively high population of *L. rhamnosus* 7469 and *L. casei* survived under acidic conditions while *L. acidophilus* GP2A and GP1B were the most sensitive in the acid environment.

Bile tolerance is the one of the essential criteria to be selected as a probiotic culture since bile acids have been shown to inhibit microorganisms and their inhibitory activity is greater than organic acids. LAB, which normally do not occur in the intestine, can not grow well in the media containing 0.15% oxgall. However, LAB isolated from intestinal sources such as *L. acidophilus* and *L. casei*, are capable of surviving under these same conditions due to their ability to deconjugate bile acids. Most

of the tested strains exhibited excellent bile acid tolerance. *L. rhamnosus* 7469 showed about 1.47 log increment even in media containing 0.3% oxgall after 24 hrs incubation (Table 1).

All tested strains except *L. acidophilus* NCFM produced bacteriocin or bacteriocin-like substances. The bacteriocins displayed antimicrobial activity against *Listeria monocytogenes* and spore-forming *Bacillus* species. The bacteriocins were characterized as proteins or peptides with stability over wide range of heat treatments and pHs. Some of the bacteriocins were further purified and the molecular mass were estimated in the range of 3.5 to 6.5 KDa.

Conjugated linoleic acid (CLA) occurs naturally in a wide variety of foods, especially in dairy foods that are derived from ruminant animals. CLA has been shown to possess anticarcinogenic and antiartherogenic properties which can be consumed without any apparent adverse effects on the host. *L. acidophilus* strains were capable of producing CLA in the range of 3.11 to 5.24 mg/g fat. *L. acidophilus* GP2A exhibited the highest CLA production for all the LAB strains tested, but there was no statistical difference in CLA production.

Table 1. Comparison of probiotic characteristics

	Acid tolerance[1]	Bile tolerance[2]	Bacteriocin production	CLA content (mg/g milk fat)	Cholesterol assimilation (%)
L. acidophilus 4356	-0.17±0.18	0.32±0.17	+	4.50	33.2
L. acidophilus 107A	-0.80±0.27	0.94±0.42	+	3.99	44.7
L. acidophilus 393	-1.23±0.11	0.24±0.33	+	4.25	28.4
L. acidophilus 30SC	-2.55±0.42	1.25±0.11	+	3.11	29.0
L. acidophilus A4	-1.11±0.27	0.30±0.27	+	4.65	53.0
L. acidophilus 606	-0.19±0.27	-0.01±0.36	+	4.55	50.2
L. acidophilus NCFM	-0.23±0.23	0.21±0.27		4.11	50.9
L. acidophilus 43121	-0.30±0.22	-0.89±0.24	+	3.24	57.5
L. acidophilus GP2A	-3.53±0.15	-0.20±0.30	+	5.08	56.9
L. acidophilus GP1B	-3.22±0.05	0.38±0.82	+	5.24	54.4
L. rhamnosus 7469	0.32±0.45	1.47±1.32	+		12.8
L. casei	0.29±0.61	1.37±1.12	+		86.5

[1] Differences in bacterial counts (Log CFU/ml) determined between 0 and 3 hrs of incubation at 37℃ in the media adjusted to pH 2.5. Each value represents an average from six trials.

[2] Differences in bacterial counts (Log CFU/ml) determined between 0 and 24 hrs of incubation at 37℃ in the media containing 0.3% bile salt (Oxgall). Each value represents an average from six trials.

L. acidophilus has been suggested to have a cholesterol-lowering effect in humans and animals. Among the 12 tested stains, there was some variation in cholesterol assimilation. Twelve strains can be classified into three groups (high, medium, and low) statistically, based on percent cholesterol assimilation. *L. casei, L. acidophilus* 43121, *L. acidophilus* GP2A, *L. acidophilus* GP1B, and *L. acidophilus* A4 were classified as the high assimilation group, within which cholesterol assimilation ranged from 53% to 86%. *L. acidophilus* NCFM, *L. acidophilus* 606, and *L. acidophilus* 107A, the medium assimilation group, showed from 44% to 51% of cholesterol assimilation. *L. acidophilus* 4356, *L. acidophilus 30SC*, *L. acidophilus* 393, and *L. rhamnosus* 7469 were classified as the low cholesterol group ranging from 13% to 33%.

EFFECTS OF PREBIOTICS

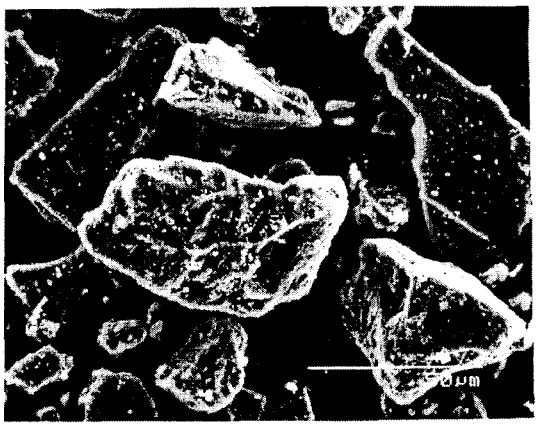
A total of 33 strains of lactic acid bacteria were tested for the growth on prebiotics including inulin, fructooligosaccharide (FOS), raffinose, sorbitol, mannitol, and lactulose. The growth patterns were vary depending on the strains of lactic acid bacteria and the prebiotics. None of the strains showed growth on inulin whereas 22 strains grew actively on lactulose when incubated 37℃ for 48 hrs. Only six, four, three and two strains exhibited growth on FOS, raffinose, sorbitol, and mannitol, respectively. Since *L. acidophilus* NCFM, *L. acidophilus* ATCC 43121, *L. casei* 911, and *L. casei* 910 were capable of utilizing more prebiotics, producing more acids during fermentation than other strains tested, they were selected for the further synbiotic studies.

Microencapsulation of probiotic by hybridization

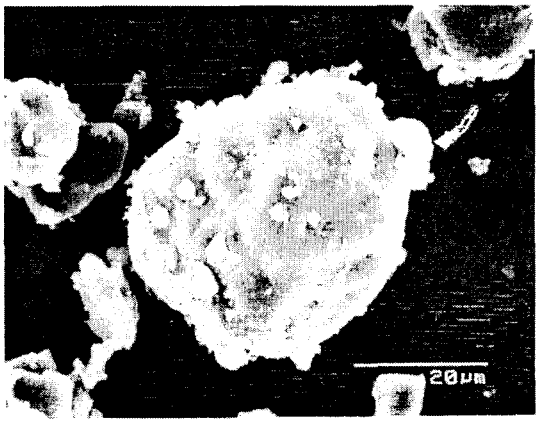
For a practical application of prebiotics, probiotics were encapsulated with selected prebiotics and then with functional coating materials. During the hybridization there was no damage on the viability of probiotic at all tested conditions (Table 2). The encapsulated probiotics formed sphere shaped particles of smooth surface with average diameter of 25 μm (Figure 1) and maintained similar levels of viability compared to that of non-coated control (Table 3). The investigations on the potential

Table 2. Effects of the hybridization conditions on the probiotic culture during microencapsulation

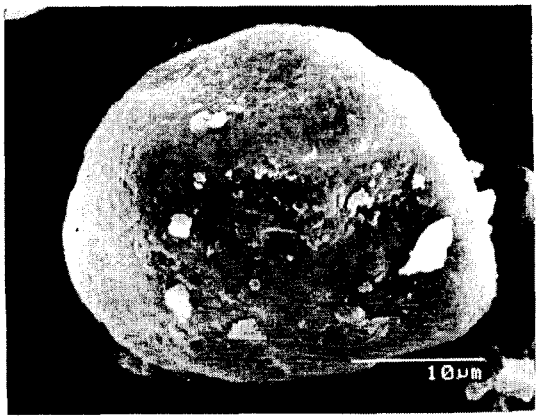
Rotor speed of hybridizer (rpm)	Viable cells (CFU/mL)
Control	2.4×10^{10}
5,000	1.7×10^{10}
7,000	1.4×10^{10}
10,000	1.0×10^{10}
15,000	1.4×10^{10}



A. Lyophilised cultures



B. First hybridization with fructooligosaccharide



C. Second hybridization with coating material (sureteric)

Figure 1. Scanning electron microscopy of the encapsulated probiotics.

Table 3. The effect of prebiotics on the viability of encapsulated-probiotics

Treatments		Viable cells (CFU/mL)
Prebiotics	Formulation ratio (probiotic : prebiotic, w/w)	
Fructooligo -saccharide	2:1	5.5×10^{9}
	4:1	7.2×10^{9}
	9:1	1.2×10^{10}
Lactulose	2:1	7.9×10^{9}
	4:1	9.1×10^{9}
	9:1	1.2×10^{10}
Inulin	2:1	8.8×10^{9}
	4:1	9.3×10^{9}
	9:1	1.0×10^{10}

benefits of encapsulated probiotic strains with functional coating agents on storage stability, growth promoting effect, acid tolerance and heat tolerance are currently under way.

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