

PROBIOTICS
YOUR FRIENDLY GUT
BACTERIA



PROFESSOR DR. MOHD. YAZID BIN ABD. MANAP

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ABSTRACT

The functional food concept has in recent years, moved progressively towards the development of dietary supplements that may stimulate gut microbial composition and activities. The rationale behind these advances is consequent to the realization that gut microflora has profound influence on the host's health. The human gastrointestinal tract (GIT) represents an ecosystem of the highest complexity and is very dynamic in composition. The micro biota exists in a commensal, symbiotic or an antagonist microbial relationship. Among more than 400 species of bacteria present in the GIT of an adult human being, bifidobacteria and lactobacillus are considered to be the most beneficial to human health. Members of these genera are thought to enhance digestion, adsorption of nutrients, prevention of colonization by pathogens, decreasing serum cholesterol and stimulation of immune responses. The ability of these bifidobacteria and lactobacilli to ferment non-digestible oligosaccharides may be an important characteristic which enables them to establish themselves in the colon. Studies were undertaken by researchers in our laboratory to elucidate the probiotic characteristics and effects of the bifidobacteria species isolated from the human GIT and to propose screening, cultivation and preservation and delivery techniques for this bacterium.

Keywords: Probiotic, Bifidobacteria, Identification, Health effects, Food Supplement.

INTRODUCTION

Probiotics are defined as live microbial food supplements that have beneficial effects on human health. The concept of probiotics evolved at the turn of the 20th century from a hypothesis first proposed by Nobel Prize winning Russian scientist, Elie Metchnikoff, who suggested that the long, healthy life of Bulgarian peasants resulted from their considerable consumption of fermented milk products. He believed that, when consumed, the fermenting lactobacilli from fermented dairy products positively influenced the microflora of the colon towards a healthy balance, subsequently decreasing toxic microbial activities.

The use of micro-organisms in food is not a recent invention. Long before there was refrigeration, freezing and canning, man used a technique of food preservation that exploited micro-organisms. This process is known as fermentation. Fermentation is essentially the use of micro-organisms to modify food characteristics, in such a way that the growth of pathogenic and spoilage microbes is inhibited, nutritional digestibility is improved, and consumer acceptance of the final product is encouraged. In general, food poisoning outbreaks are not commonly associated with the consumption of fermented foods.

The historical association of probiotics with fermented dairy products still holds true today. Investigations in the field of probiotics during the past several decades have expanded beyond bacteria isolated from fermented dairy products, to bacteria of intestinal origin. The probiotic bacteria most commonly studied include members of the genera *Bifidobacterium*, *Lactobacillus*, and *Lactococcus*. The genus *Bifidobacterium* has however received exceptional attention and has been marketed widely throughout the world as probiotic starter culture preparations.

The genus *Bifidobacterium* includes Gram-positive, non-acid fast, non-spore forming and non-motile. They are strict anaerobes. However, some species can tolerate oxygen only in the presence of carbon dioxide. They are present in rods

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of various shapes such as short, regular, thin cells with pointed ends, long cells with slight bends or protuberances or with a large variety of branching (Figure 1).

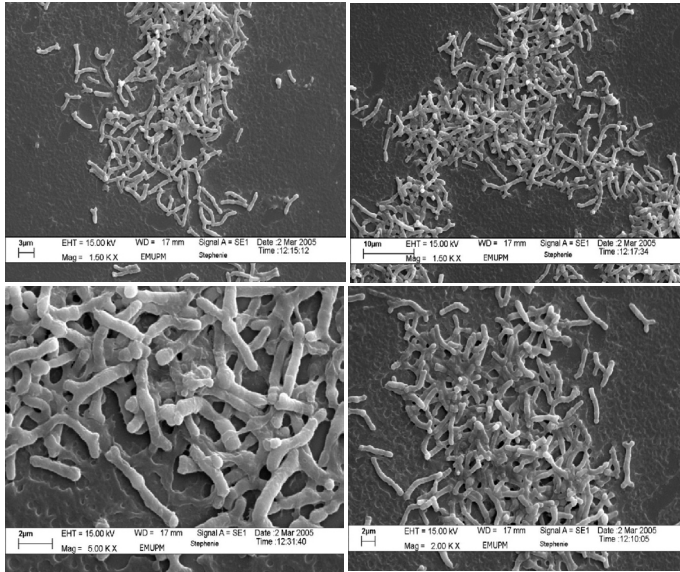


Figure 1 The morphology of *Bifidobacterium pseudocatenulatum* G4 under SEM microscope

The genus *Bifidobacterium* is the most dominant organisms in the gastrointestinal tract of breast-fed infants and the major organisms in the colonic flora of healthy children and adults. Their dominance in the faeces of breast-fed infants is thought to provide early protection against infection.

Bifidobacteria play an important role in the health of humans through several mechanisms such as competitive exclusion of pathogenic and putrefactive bacteria, deconjugation of bile salts, immune stimulations, control of intestinal functions and enhancement of mineral absorption. The administration of bifidobacteria fortified infant formulas, can be particularly beneficial for premature

infants and helps to quickly establish the desired intestinal flora. Increasing the number of intestinal bifidobacteria promotes intestinal acidification and reduces both ammonia concentration and urease production in adults. Additional benefits associated with the consumption of bifidobacteria include reduction in constipation in the elderly, reduction of diarrhoea and stimulation of IgA production.

The above statements make bifidobacteria a popular choice as a probiotic for human consumption. However, the probiotic effects of this genus are strain specific. Hence, new strains are needed to improve the bio-therapeutic action of the bifidobacteria included in several foods and pharmaceutical products. Consequently, a proper identification procedure for new strains is required. In this regard, the use of molecular methods appears to be the method of choice, since the pattern of sugar fermentation could lead to confusion in species identification. Scientific evidence showed that strains belonging to *Bifidobacterium animalis*, on the basis of their sugar fermentation profiles, were identified as *Bifidobacterium longum* by DNA homology, and had difficulties in distinguishing *Bifidobacterium adolescentis* from *Bifidobacterium pseudocatenulatum*. For these reasons, more reliable techniques such as DNA and RNA analysis coupled with conventional biochemical tests for bacterial identification would be preferable.

A research team in the Probiotic Research Laboratory, Faculty of Food Science & Technology, UPM embarked on a systematic study to isolate, characterize and screen the genus *Bifidobacterium* that has been isolated from healthy infant stool and to evaluate their probiotic characteristics, and to establish the possible mechanisms of their probiotic health effects. Attempts at mass cultivation, coating technology and preservation and delivery techniques were also undertaken. The main aim of our investigations was to exploit the potentials of bifidobacteria as probiotics in various industrial applications. The technology maps shown in Table 1 are used as a guide by our researchers to establish our research direction.

Table 1 Benefit of probiotic, industrial application and technology mapping for development of new probiotic strain

Benefit of probiotic	Industrial application	Technology map
Inhibiting harmful bacteria and increasing intestinal balance blood	Pharmaceutical industry (For intestinal disorders, harmful bacteria) Decreasing cholesterol Level in (Health, function, general food)	<pre> graph TD MC([Mother Culture]) --> S([Screening]) S --> I[Isolation technology] </pre>
Increasing immunity	Livestock farms (Medicine for animal and feed)	<pre> graph TD S([Screening]) --> E[Evaluation] E --> ST[Screening technology] </pre>
Anticancer effect	Dairy industry (Functional yogurt)	<pre> graph TD C([Cultivation]) --> O[Optimization] O --> CT[Cultivation technology] </pre>
Inhibiting intrinsic infections	Cosmetic industry (Functional cosmetics)	<pre> graph TD CO([Coating]) --> SS[Securing stability] SS --> COT[Coating technology] </pre>
Anti-aging effect	Confectionary (Bread, chocolates, gums, biscuits)	<pre> graph TD FSD([Freeze/Spray Drying]) --> FWC[Free water clearance] FWC --> FDT[Freeze-drying technology] </pre>
Increasing nutritional value		<pre> graph TD F([Formulation]) --> FT[Formulation technology] </pre>
Skincare effect		

PROBIOTIC RESEARCH ACTIVITIES

Table 2 Research activities by the Probiotic Research Group in the Faculty of Food Science & Technology, UPM

-
1. Production of Labneh (fresh fermented milk) using combined bacteria-yeast fermentation technique.
 2. Fermentation of prebiotic inulin using Simulated Human Intestinal Microbial Ecosystem (SHIME) model.
 3. Utilization of bifidobacteria in brown rice beverage technology for the enhancement of therapeutic benefits.
 4. Molecular analysis of bacteria in the ileum and cecum of broiler chicken fed with prebiotic-probiotic supplemented diet under stress conditions.
 5. Evaluation of technological and functional properties of *Bifidobacterium pseudocatenulatum* G4 as a potential probiotic adjunct in the production of Cheddar Cheese.
 6. Utilization of tropical fruit by-products as a source for non-digestible dietary fibre and its health-promoting properties.
 7. Development of clarified and inulin fortified banana juice.
 8. Molecular characterisation of bifidobacteria.
 9. Influence of bile salt deconjugation activity by *Bifidobacterium* spp on cholesterol assimilation in in-vitro and in-vivo conditions.
 10. Characterization and Molecular Studies of Fructose-6-Phosphate Phosphoketolase (F6PPK) in Bifidobacteria.
 11. The relationships between probiotics and the alleviation of colon cancer
 12. Indigenous probiotic starter cultures of traditional fermented foods of Iran.
 13. Control of mutagenic secondary bile salts by probiotic bacteria.
 14. Biochemical and molecular characterization of inulinase from endophytic bacterium A5).

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15. Rapid detection and enumeration of *Bifidobacterium longum* by real-time PCR targeting of the bile salt hydrolase (*bsh*) gene.
16. Prebiotic potential of oligosaccharides from *Ganoderma lucidum*.
17. Deconjugation of glycosides by selected *Bifidobacterium* species.
18. Cloning and expression of inulinase genes from endophytic fungi.
19. Large-scale cell mass production of *Bifidobacterium pseudocatenulatum*
20. Rapid detection of intestinal bacteria using real-time PCR.
21. Rapid detection and enumeration of bacteria in caecum of chicken with real-time PCR.
22. The storage stability of probiotic bifidobacteria in BHA and AHA fortified infant formulations.
23. Identification and evaluation of non-dietary fibre from Malaysian tubers.
24. Clinical trials. Effect of Inulin on colonic calcium absorption in children.
25. Development of nutritious fermented spray dried medida, the Sudanese cereal porridge from malted brown rice flour with *Bifidobacterium longum* BB536
26. Bacterial Profile of Infants' Diarrhoea.
27. Alleviation of faecal Malodour using probiotics and prebiotics.
28. Pharmacokinetics of bifidobacteria.
29. Development of Bio therapeutic Agents for Infants' Diarrhoea.
30. Antagonistic Effects of Bifidobacteria in Synbiotic Systems.
31. Development of Soft Serve Synbiotic Ice Cream.
32. Symbiotic Relationship between Selected Probiotic Bacteria in Chemostat Culture.
33. *In vitro* Inhibitory Activities of Bifidobacterium spp on *Campylobacter jejuni* isolated from Chicken.
34. Detection and identification of bifidobacteria based on polyclonal antibody – cell wall protein interaction.
35. The Approach of Antigen-Antibody (indirect ELISA) for the Detection of Bifidobacteria.

36. Isolation and Characterisation of *Bifidobacterium* Spp. from Infant Stool.
 37. Perencatan Terhadap Pertumbuhan. *Escherichia coli* 2520 (USCC) oleh Laktoferin dan *Bifidobacterium asteroidis*.
 38. Probiotic Characteristics of *Bifidobacterium* Spp. by *in vitro* assessment.
-

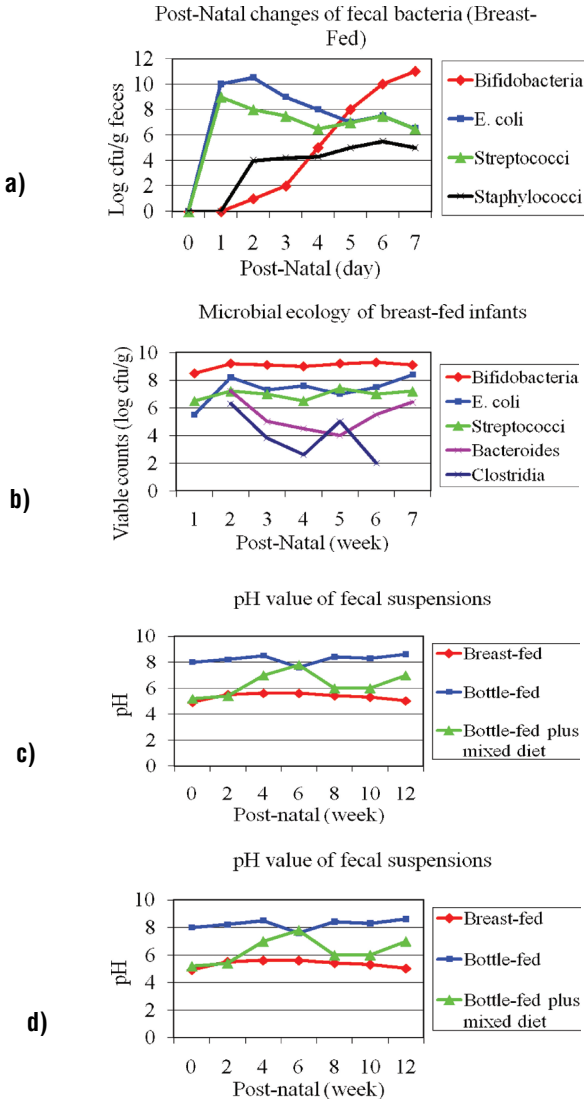
PROBIOTIC CHARACTERISTICS

Bifidobacteria are Gram-positive bacteria inhabiting the intestinal tract of humans and animals. They are one of the most dominant organisms in the colonic flora of breast-fed infants and healthy children or adults (Figure 2). Their dominance in the faeces of breast-fed infants is thought to provide protection against infection (Mitsuoka, 1996). *Bifidobacterium* spp have received increasing attention for their use as probiotics. Probiotics are described as beneficial live microorganisms that, when administered orally, help to promote the growth of 'friendly' bacteria in the gut that would subsequently prevent colonization of pathogens, regulate intestinal motility, reduce risk of carcinogenesis in the intestine, boost the immune system and alleviate lactose intolerance. In order for probiotics to exert health benefits on the host, the ingested organism must first survive the harsh conditions during gastric transit, such as the acidic environment of the stomach, and then persist in the gut (Yazid *et al.*, 1998).

Survival of *Bifidobacterium Pseudocatenulatum* Strains Isolated from Breast-fed Infants in Simulated Gastric pH Environment

On average, the human stomach secretes 1–2 L of gastric juice per day stimulated by the act of eating and the presence of food in the stomach. The germicidal effect of gastric juice is mainly attributed to the low pH of hydrochloric acid (Yazid *et al.*, 1999). However, gastric pH does not remain constant over time. After administration of a meal, the pH of gastric juices can reach above 6, and then decline gradually to the fasted state value of pH 1-2 . In the fasted state,

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Figures 2 a) Post-Natal changes of fecal bacteria (Breast-Fed) b) Microbial ecology of breast-fed infants c) pH value of fecal suspensions d) Microbial ecology of bottle-fed infant.

Source: Arezou (unpublished)

the median gastric pH is 1.7. After food intake, the median gastric pH increases to pH 6.7, then declines back to the fasted state value within 2 hours. Different ranges of pH values, from pH 1 to 3 have been used by various researchers for the screening of acid tolerance of potential probiotic strains *in vitro* (Yazid *et al.*, 2000). Hence, for successful establishment of ingested probiotics, bacteria must be able to survive low gastric pH.

The genus *Bifidobacterium* is grouped into 28 different species (Table 3). Seven species are of human origin namely; *B. breve* and *B. infantis*, isolated mainly from infant, and *B. adolescents*, *B. longum*, *B. bifidum* and *B. catenulatum* and *B. pseudocatenulatum*, isolated from healthy free living adults.

Table 3 List of species in the genera of *Bifidobacterium*

<i>B. adolescentis</i> ^a	<i>B. cuniculi</i>	<i>B. merycicum</i>
<i>B. angulatum</i> ^a	<i>B. dentium</i> ^a	<i>B. minimum</i>
<i>B. animalis</i>	<i>B. gallicum</i>	<i>B. pseudocatenulatum</i> ^a
<i>B. asteroides</i>	<i>B. gallinarum</i>	<i>B. pseudolongum</i>
<i>B. bifidum</i> ^a	<i>B. globosum</i> ^a	<i>B. pullorum</i>
<i>B. boum</i>	<i>B. indicum</i>	<i>B. ruminantium</i>
<i>B. breve</i> ^a	<i>B. infantis</i> ^a	<i>B. saeculare</i>
<i>B. catenulatum</i> ^a	<i>B. lactis</i>	<i>B. subtile</i>
<i>B. choerinum</i>	<i>B. longum</i> ^a	<i>B. suis</i>
<i>B. coryneforme</i>	<i>B. magnum</i>	<i>B. thermophilum</i>

^aSpecies isolated from human sources

Source: Shuhaimi *et al* (1999)

One commonly found species in the faeces of breast-fed infants are *Bifidobacterium pseudocatenulatum* (Shuhaimi *et al.*, 2002), which are largely unexplored as probiotics. Extensive investigations were performed on this species which include: generation of genomic DNA fingerprints for *B. pseudocatenulatum* isolated by RAPD (Shuhaimi *et al.*, 2001a), deconjugation of

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bile acids (Rezaei *et al.*, 2004); antibacterial activity, antimicrobial susceptibility and adherence properties (Yazid *et al.*, 2000). Investigations on the survival of several *B. pseudocatenulatum* strains in acidic conditions, mimicking the pH of the human stomach, and their ability to inhibit specific food borne pathogens were undertaken by using the Simulated Human Intestinal Microbial Ecosystem (SHIME) technique using continuous flow fermented system (Figure 3).



Figure 3 Simulated Human Intestinal Microbial Ecosystem (SHIME) bioreactor system

Source : Anas (unpublished)

exposed this bacterium to HCl acid for 3 hours by adjusting the pH to 1.0, 2.0 and 3.0. At pH 1.0, three strains, D19, F91 and G4 survived a 1 hour exposure, with a decline in viability of about 4-5 log cfu ml⁻¹. After 2 hours, no viable cells could be detected for all isolates. At pH 2.0, strains G4, D19, C20 and A50 were able to endure and maintain a high number of viable cells following a 3 hour exposure. The other strains showed progressive reduction in viability during the 2 and 3 hour incubation. Strain F91, which survived pH 1.0 for 1 hour could not tolerate pH 2.0 after 2 hours. At pH 3.0, all strains exhibited high endurance with more than 5 log cfu ml⁻¹ after 3 hours. High survival rate was observed at pH 6.5 (control), with little or no decrease in cell count. Among all the strains tested, *B. pseudocatenulatum* G4 and D19 demonstrated the best tolerance to low pH, thus suggesting potential as probiotic candidates for industrial application.

Deconjugation of Bile Acids by *Bifidobacterium Pseudocatenulatum* Isolates

Mammalian bile acids are C₂₄ steroid carboxylic acid molecules, which are synthesized in the liver as free bile acids and then conjugated to either glycine or taurine via an amide bond. These conjugated bile acids are then released into the small intestine where they act primarily as emulsifiers of dietary lipids. Most conjugated bile salts excreted (about 97%) are reabsorbed from the small intestine and returned to the liver through the hepatic-portal circulation (Figure 4). Several genera of intestinal bacteria, including *Enterococcus*, *Peptostreptococcus*, *Bifidobacterium*, *Fusobacterium*, *Clostridium*, *Bacteroides* and *Lactobacillus* (Chateau *et al.*, 1994), yield bile salts hydrolases (BSH) which cleave the amino acid moieties from conjugated bile acids. The majority of bacteria capable of deconjugation are strictly anaerobes. They can biotransform bile acids into a number of different metabolites. Normal human faeces may contain more than 20 different bile acids that have been formed from primary bile acids, cholic acid and chenodeoxycholic acid (Setchell *et al.*, 1983). The

resulting free bile acids from deconjugation process are less efficiently recycled by the enterohepatic circulation (Chikai *et al.*, 1987). Furthermore, deconjugated bile salts are known to co-precipitate cholesterol at pH values lower than 5.5 and bind to bacterial cells and dietary fiber, which enhance their fecal excretion (Rezaei *et al.*, 2004).

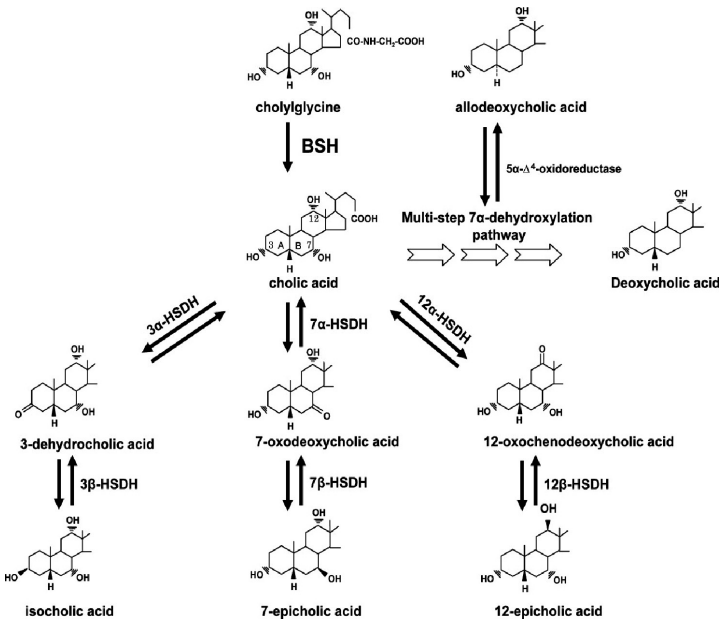


Figure 4 Bacterial bile salt-biotransforming reactions in the human intestinal tract

Deconjugation or dehydrogenation of 3 α -, 7 α - and 12 α -OH groups and 7 α -dehydroxylation are the principal and important types of biochemical reactions brought about by intestinal microflora on bile acids (Hayakawa, 1973). Ferrari *et al.* (1980) further confirmed the ability of bifidobacteria strains to deconjugate taurocholic and glycocholic acids when they found that 48 out of the 52 isolates of *Bifidobacterium* spp. studied acted on both the conjugated bile acids. They

concluded that the ability of deconjugation taurocholic and glycocholic acids is widespread among members of *Bifidobacterium*. Thus, it may be assumed that bifidobacteria, one of the main bacterial groups in the gut, have an important role in the transformation of bile acids in the intestine.

We have demonstrated that *Bifidobacterium* spp. is capable of splitting the amide bond of both taurocholic and glycocholic acids. The survival and growth rate of twenty eight isolates of bifidobacteria in bile were evaluated (Rezaei *et al.* 2004). Among 28 isolates, 25 were tolerant towards 2% concentration of bile while 14 isolates were tolerant towards 4% concentration of bile after 12 hours of exposure. Six isolates of bifidobacteria with higher tolerance to 4% concentration of bile were further evaluated for their ability to deconjugate different types of bile acids, namely taurocholic acid (TC), glycocholic acid (GC), taurochenodeoxycholic acid (TCDC), glycochenodeoxycholic acid (GCDC), taurodeoxycholic acid (TDC) and glycodeoxycholic acid (GDC). Three *Bifidobacterium infantis* isolates (D22, F117 and G4) were found to exhibit similar deconjugation activity and were able to deconjugate 78.6 – 84.6% (TC), 98.9 – 99.9% (GC), 87.9 – 97.5% (TCDC), 91.1 – 100.0% (GCDC), 83.7 – 87.8% (TDC) and 96.5 – 99.0% (GDC).

Antibacterial Effect of Probiotics and Antimicrobial Susceptibility of Bifidobacteria

The antibacterial effect of probiotics is an important prerequisite criterion for selecting an effective probiotic strain. Several investigators suggested that probiotics have inhibitory effects due to the production of short chain fatty acids. Most probiotics have antagonistic interaction with many pathogens including: *Candida albicans*, *Clostridium difficile*, enterotoxigenic *E. coli*, *Klebsiella* spp., *Salmonella* spp., *Vibrio* spp. and *Helicobacter pylori*. Probiotics produce low molecular weight metabolites (such as hydrogen peroxide, lactic and acetic acid and other aromatic compounds) and secondary metabolites with wide inhibitory

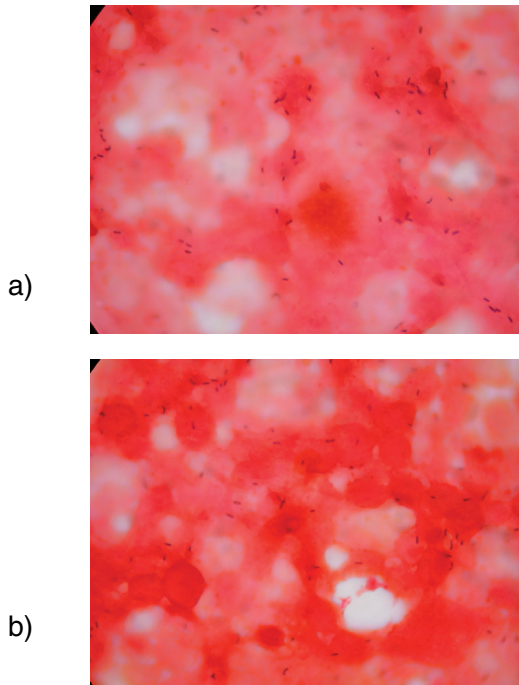
spectrum against many harmful organisms such as *Salmonella*, *Escherichia coli*, *Clostridium* and *Helicobacter* (Niku-Paavola *et al.*, 1999).

The *L. rhamnosus* strain GG produces low molecular weight antimicrobials, possibly short chain fatty acids but distinct from lactic and acetic acids, with inhibitory activity against anaerobes such as *Clostridium*, *Bacteroides* and *Bifidobacterium* and against *Enterobacteriaceae*, *Pseudomonas*, *Staphylococcus* and *Streptococcus*. Among probiotic metabolic products, antibiotic like substances (bacteriocins) have been shown to exert an inhibitory effect against sporeformers such as *Bacillus* or *Clostridium*. Other metabolites of nonbacteriocin antibacterial like compounds were produced by strains such as the *L. acidophilus* (johnsonii) strain LA1. It 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*. (Niku-Paavola *et al.*, 1999).

An *in vivo* evaluation revealed that the possible mechanisms responsible for the antagonism between two bacteria in the intestinal ecosystem include competition for either nutrients or adhesion sites and production of either bacteriocins or volatile fatty acids. It has been considered that the LEE type III secretion system of EHEC is important for bacterial adhesion to a host's intestinal cells during the early steps of infection. It is possible that binding of probiotics to Caco-2 cells might interfere with the adhesion of pathogens (Figure 5).

The main action of probiotic bifidobacteria strains may be on the induction of protective factors in epithelial cells. *In vitro* studies evaluating adhesion of probiotic bacteria to epithelial cells described a protective induction of mucin and the release of antibacterial products that were harmless to lactobacilli and bifidobacteria but were active against pathogens. The properties of inducing mucus secretion, preventing pathogenic bacteria adhesion and invasion into epithelial cells, have been described also for *L. acidophilus* strains that secreted

a product with antibacterial activity against Gram-positive and Gram-negative pathogens (Coconnier *et al.*, 1997).



Figures 5 a) Adhesion of *Bifidobacterium pseudocatenulatum* G4 to human epithelium cell. Adhesion scores in 20 randomized microscopic fields per coverslip were determined

b) Adhesion *Bifidobacterium pseudocatenulatum* G4 to HT-29 cell culture observed using light microscopy Gram-staining (magnification x1000).

Source: Ali (unpublished)

The effect of probiotic organisms on the micro ecology of the gut is to some extent dependent upon its ability to survive and preferably inhibit the proliferation of pathogens. In addition, various compounds produced during growth of the

probiotic have been shown to inhibit the growth of pathogens such as *Vibrio cholera* and *Bacillus cereus*. These compounds include organic acids such as lactic and acetic acids and antibiotic-like compounds such as reuterin and bacteriocin. The organic acids lower the pH thereby indirectly affecting growth of the pathogens. Numerous bacteriocins have been reported to be produced by probiotic microorganisms namely acidophilin, bifidin and nisin (Klaenhammer, 1998). They can either have very broad range activity or specifically inhibit the growth of a very limited range of closely related microbes.

Several probiotic microorganisms were tested in our laboratory for antimicrobial activity against selected food-borne pathogens namely *Escherichia coli*, *Salmonella enteritidis*, *Listeria monocytogenes* and *Bacillus cereus*. *Bifidobacterium breve* and *Streptococcus faecalis* showed maximum antibacterial activity against all target microorganisms, while *Bifidobacterium infantis* had the least antibacterial activity. The results of viable counts and Ph profile revealed that a combination of probiotic microorganisms in a mixed culture system exerts better inhibitory effects against enteropathogenic *E. coli* compared to a single probiotic dose.

Three different dosages of *Bifidobacterium pseudocatanulatum* were evaluated for their inhibitory activities against *Salmonella enteric ser. Hindmarsh 4F1* isolated from children clinically diagnosed with diarrhea. The investigations had been made by using the continuous flow culture system. Initial concentrations of bifidobacteria affects the duration of inhibitory activity against *Salmonella*. Lower dose/concentration of bifidobacteria needed shorter time to exhibit their inhibitory activities. The inhibitory activity of the bifidobacteria was due to the production of acetate and lactate. Acetate and lactate production was excessive at lower bifidobacteria concentration.

Numerous studies have documented the antagonistic activity, *in vitro* and *in vivo*, of bifidobacteria against entero-virulent bacteria involved in gastrointestinal disorders. Species belonging to the genera *Salmonella*, *Listeria*, *Campylobacter*

and *Shigella*, as well as *Vibrio cholera*, were all affected. Meanwhile, the *in vivo* study done by Saavedra et al. demonstrated that *B. bifidum* and *S. thermophilus*, when administered together, decrease the incidence of diarrhea by 31% in a group of hospitalized infants.

Mitsuoka (1996) reported that in the large intestine, bifidobacteria produces acetic and lactic acids and is thought to inhibit the proliferation of putrefactive bacteria such as escherichia, clostridia and eubacteria, and inhibit the synthesis of harmful substances. Ibrahim and Bekerovainy (1993) reported that the inhibitory effect was due to the decrease in the pH of the culture medium from acetic and/or lactic acids production by bifidobacteria.

Antimicrobial susceptibility of intestinal micro-organisms is an important criterion for selecting an organism as probiotic. The administration of antimicrobial substances can alter the intestinal microbial balance and suppress certain beneficial bacterial groups, including bifidobacteria. The altered microbial balance may result in intestinal disorders. The susceptibility of bifidobacteria to various antimicrobial agents is of interest in understanding the alteration of normal intestinal microflora when antimicrobial agents are taken.

Eighteen Bifidobacterium strains were tested in our laboratory for their susceptibility to a range of antimicrobial agents. All the strains tested were susceptible to several groups of antimicrobial agents; they were cephalosporin (cefamandole, cefazolin, cefaperazone, and cefoxitin), polypeptide (bacitracin), macrolide (erythromycin), Penicillin (amoxicillin), phenicol (chloramphenicol) and β -lactam (imipenem). The results showed that bifidobacteria are resistant to a wide range of antimicrobial agents (Yazid *et al.*, 2000)

CULTIVATION

Viability of *Bifidobacterium Pseudocatenulatum* G4 after Spray-drying and Freeze-Drying

Bifidobacterium, a predominant genus in the stools of breast-fed infants, came a long way before it was widely used as probiotic dietary adjuncts with health-benefiting properties. Now, bifidobacteria is extensively incorporated into yogurts, cultured milk drinks, cheese or as dietary supplements in the form of dried products (Ross *et al.* 2005).

For probiotics to exert health benefits to the host, viability of the strain is of utmost importance. The recommended minimum count is 10^6 live organisms per g of food at the point of consumption. The conventional technique of using stock cultures with multiple sub culturing steps for the preparation of bulk starters is time consuming with higher risk of contamination. With the advances in cell mass production technology, the development of concentrated starter cultures in the form of freeze-dried and spray-dried for direct product vat inoculation has been a better alternative.

Dehydration is a common practice to preserve biological materials that are stable in the long run. Among the many cell preservation methods, spray-drying is the most widely used technology in starter culture preservation techniques because it is economical, especially in large-scale commercial production. Despite the fact that spray-drying is more cost-effective, many microorganisms cannot tolerate the drying process due to the high heat involved. Many factors affect survival during spray-drying such as the strain, growth phase, protective medium used and outlet temperature of spray-drier and pre-adaptation treatment of the culture (Corcoran *et al.*, 2004). Heat-adaptation, also known as heat-shock treatment, has been reported to increase thermotolerance of bacterial cells during spray-drying (Kabeir, 2005). Thus, the application of heat-adaptation techniques prior to spray-drying has gained popularity.

On the other hand, freeze-drying, the sublimation of ice from frozen preparations, is a popular method for the preservation of lactic acid bacteria. Although freeze-drying is commonly used, the microorganisms are also susceptible to various stress and cell injury. Therefore, cryoprotectants are commonly added to minimize cell damage. Skim milk is a popular drying medium and cryoprotectant because it contains protein that prevents cellular injury and facilitates rehydration by creating a porous structure in the freeze-dried powder (Abadias *et al.*, 2001). Many other compounds have also been tested to improve survival of LAB during freeze-drying such as disaccharides, polyols, vitamins and proteins. Several suitable preservation methods for *B. pseudocatenulatum* have also been evaluated in different milk-based cryoprotectants and heat-adaptation treatments at various air outlet temperatures during freeze-drying and spray-drying (Table 4 and 5).

Influence of pH and Impeller Tip Speed on the Cultivation of *Bifidobacterium Pseudocatenulatum* G4 in a Milk-Based Medium

Since the suggestion that the longevity of Bulgarian peasants was related to their high consumption of fermented milk, by Metchnikoff in 1908, probiotic organisms, such as bifidobacteria, have been increasingly incorporated into dairy products such as yogurt or cultured milk drinks. As a result, probiotic dairy products have emerged as one of the most developed functional food products in the European market (Shortt *et al.*, 2004)

The mass production of a probiotic culture is commonly carried out using a milk-based medium in the controlled environment of a fermenter (Stephanie *et al.*, 2007). In addition to the importance of using a good growth medium that is capable of supporting optimal cell production, microbial growth also depends on environmental factors, such as pH and agitation rate. The optimum pH for the growth of *B. bifidum* ranges between 6.0 and 7.0.

Table 4 Viability, percent survival and moisture content of spray-dried *B. pseudocatenulatum* G4

Treatment	Outlet Temperature (°C)	Heat-adaptation Temperature (°C)	Viability before spray-drying (log ₁₀ c.f.u. g ⁻¹)	Viability after spray-drying (log ₁₀ c.f.u. g ⁻¹)	Survival (%)	Moisture content (%)
A	75 ± 2	-	9.379 ± 0.017 ^{ad}	6.787 ± 0.001 ^{ac}	26	9.17 ± 0.24 ^a
B	85 ± 2	-	9.143 ± 0.027 ^b	5.797 ± 0.006 ^b	5	6.24 ± 0.30 ^b
C	75 ± 2	45	9.424 ± 0.017 ^a	6.838 ± 0.041 ^a	26	9.27 ± 0.49 ^a
D	85 ± 2	45	9.292 ± 0.082 ^{abd}	6.838 ± 0.011 ^a	36	5.25 ± 0.35 ^b
E	75 ± 2	60	9.595 ± 0.006 ^c	6.871 ± 0.043 ^a	19	8.62 ± 0.51 ^a
F	85 ± 2	60	9.238 ± 0.046 ^{bd}	6.737 ± 0.007 ^c	32	6.31 ± 0.27 ^b

Results are shown as mean ± S.D. of 2 experiments.

Values in the same column with different superscripts, differ significantly (P < 0.05).

Source: Stephanie *et al* (2007)

Table 5 Viability, percent survival and moisture content of freeze-dried *B. pseudocatenuatum* G4.

Treatment	Protective medium	Viability before freeze-drying (\log_{10} c.f.u. g ⁻¹)	Viability after freeze-drying (\log_{10} c.f.u. g ⁻¹)	Survival (%)	Moisture content (%)
1	Distilled water	9,499 ± 0.006 ^{ab}	8,587 ± 0.158 ^a	12.68 ± 4.67 ^a	0.87 ± 0.05 ^a
2	10% skim milk	9,450 ± 0.023 ^a	9,364 ± 0.015 ^b	82.07 ± 1.54 ^b	4.41 ± 0.44 ^{bcd}
3	18% skim milk	9,510 ± 0.009 ^{ab}	9,415 ± 0.014 ^b	80.35 ± 0.74 ^b	3.72 ± 0.11 ^b
4	10% skim milk + 5% glucose	9,432 ± 0.031 ^a	9,335 ± 0.014 ^b	80.06 ± 3.06 ^b	4.20 ± 0.03 ^{bcd}
5	10% skim milk + 5% sucrose	9,558 ± 0.033 ^b	9,445 ± 0.042 ^b	77.06 ± 1.73 ^b	4.64 ± 0.18 ^{cde}
6	10% skim milk + 5% lactose	9,563 ± 0.026 ^b	9,472 ± 0.022 ^b	81.08 ± 0.78 ^b	5.10 ± 0.16 ^d
7	18% skim milk + 5% glucose	9,548 ± 0.031 ^{bc}	9,430 ± 0.008 ^b	76.41 ± 6.89 ^b	4.06 ± 0.23 ^{bc}
8	18% skim milk + 5% sucrose	9,464 ± 0.018 ^{ac}	9,319 ± 0.004 ^b	71.65 ± 3.67 ^b	4.92 ± 0.26 ^{de}
9	18% skim milk + 5% lactose	9,585 ± 0.006 ^b	9,487 ± 0.060 ^b	80.08 ± 9.97 ^b	4.21 ± 0.13 ^{bc}

Results are shown as mean ± S.D. of 2 experiments.

Values in the same column with different superscripts, differ significantly ($P < 0.05$).

Source: Stepthanie *et al.* (2007)

No growth could be observed at a pH of 5.5 or less. During fermentation of bifidobacteria, pH of the medium drastically decreases due to the accumulation of organic acids. As a result, many researchers control the probiotic fermentation processes at specific pH values. For instance, in one study, the pH of the medium was held constant at 6.2 during the cultivation of *B. longum* (Doleyres *et al.*, 2002). In other studies, a pH of 6.5 was used to grow *Lactobacillus* strains in a milk-yeast extract medium (Enfors *et al.*, 2001).

The effect of mixing is another important environmental factor that affects the growth performance of probiotic organisms. In addition to the uniform distribution of nutrients and heat in the fermenter, good mixing is also necessary to prevent cells from being subjected to fluctuations in pH due to the intermittent action of pH control. For instance, an agitation speed of 200 rev/min was used for the cultivation of *B. longum* in glucose or lactose-based media as well as for the cultivation of *L. lactis* in milk-based media (Garro *et al.*, 2004). An agitation speed of 100 rev/min was used during lactobacillus fermentation in 10% skim milk supplemented with yeast extract [6]. Her *et al.* used an agitation speed as low as 10 rev/min to cultivate *B. longum* in De Man, Rogosa and Sharpe (MRS) broth and whey-based medium (Heenan *et al.*, 2002).

With the optimization of process variables in small-scale studies, one major concern is the scale-up of fermentation processes to a larger vessel. Various factors, such as unequal mass, heat or oxygen transfer, may affect microbial growth after scale-up. The applicability of different scale-up strategies is dependent upon the process conditions. For example, scale-up based on the volumetric coefficient of oxygen transfer, $k_L a$, might be an appropriate criterion for scaling-up aerobic fermentation processes. A constant power per volume ratio can be used in almost every scale-up problem, with the exception of problems related to mixing. However, it is known that scale-up on this basis will increase the shear in the fermenter.

Mixing time, the time required to reach a particular mixing intensity at a given scale, is another option. However, this method is known to be costly because large-scale reactors require more power. A simple technique that takes the shear of the impeller tip into consideration is scale-up based on constant impeller tip speed. If scale-up is attempted based on this technique, the power consumption per unit volume will decrease (Hsu *et al.*, 2005). The amount of shear due to agitation should be kept at an appropriate level since higher shear rates may physically damage the cells, thereby affecting the microorganisms. Therefore, researchers should thoroughly consider the different aspects of the various scale-up methods for different microorganisms.

In view of the lack of information on the process of cultivating bifidobacteria in a milk medium, a study was carried out aimed at improving the growth performance of *B. pseudocatenulatum* in a 2-L fermenter (Table 6). The effect of impeller tip speed and strategies for controlling the pH of the medium were also investigated (Table 7 and 8). This was followed by the scale-up of the fermentation process to a 10-L fermenter based on constant impeller tip speed. Biomass production of *Bifidobacterium pseudocatenulatum* G4 in a milk-based medium was carried out in 2-L and 10-L stirred tank fermenters (Table 9). The effects of impeller tip speed (0.28, 0.56, and 0.83 m/s) and pH control (6.0, 6.5, and 7.0) on the biomass production were investigated. The growth performance in the 2-L fermenter was significantly improved when the impeller tip speed was held constant at 0.56 m/s and the pH controlled at 6.5. These conditions yielded a maximum biomass of 1.687×10^9 cfu/mL, a maximum specific growth rate of 0.504 h^{-1} , a biomass productivity of 9.240×10^7 cfu/mL-h, and a biomass yield of 9.791×10^{10} cfu/g lactose. The consumption of milk lactose resulted in the accumulation of 7.353 g/L acetic acid and 6.515 g/L lactic acid, with an acetic:lactic ratio of 1.129. Scale-up of the fermentation process to a 10-L fermenter, based on a constant impeller tip speed of 0.56 m/s, yielded reproducible results with respect to biomass production and cell viability.(Stephenie *et al.*, 2007).

Table 6 Analysis of the fermentation characteristics of *Bifidobacterium* sp
(^aKok et al., 1996)

<i>Sugar</i>	<i>Bifidobacterium</i> sp				
	<i>LW420^a</i>	<i>B. infantis^a</i>	<i>B. animalis^a</i>	<i>B. pseudocatenulatum^b</i>	<i>B. adolescentis^b</i>
D-ribose	W	+	+	+	+
L. arabinose	W	-	+	+	+
Lactose	+	+	+	+	+
Cellobiose	-	-	D	D	+
Melezitose	-	-	D	+	-
Raffinose	+	+	+	+	+
Sorbitol	-	-	-	+	+
Starch	-	-	+	+	+
Gluconate	-	-	-	-	D
Xylose	D	D	+	+	+
Mannose	-	D	D	W	-
Fructose	+	+	+	+	+
Galactose	+	+	+	+	+
Sucrose	+	+	+	+	+
Maltose	+	+	+	+	+
Trehalose	+	-	D	+	+
Melebiose	+	+	+	+	+
Mannitol	-	-	-	-	-
Inulin	+	D	-	D	-
Salicin	-	-	+	+	+

Symbols: +, positive; -, negative; W, weakly positive; D, doubt.

Table 7 Kinetic parameter values from batch fermentation of *B. pseudocatenulatum* G4 in a 2-L stirred tank fermenter at different impeller tip speed

Kinetic parameter values	Impeller tip speed (m/s)			
	0	0.28	0.56	0.83
<u>Biomass</u>				
Maximum biomass, x_{\max} (\log_{10} cfu/mL)	8.402 \pm 0.016 ^a	8.605 \pm 0.021 ^b	8.824 \pm 0.034 ^c	8.616 \pm 0.023 ^b
Time taken to reach maximum biomass, t (h)	20	20	18	18
Maximum specific growth rate, μ_{\max} (h^{-1})	0.174 \pm 0.014 ^a	0.241 \pm 0.011 ^b	0.311 \pm 0.014 ^c	0.287 \pm 0.020 ^{bc}
Biomass productivity, P_x ($\times 10^7$ cfu/mL.h)	1.145 \pm 0.021 ^a	1.913 \pm 0.096 ^b	3.590 \pm 0.293 ^c	2.181 \pm 0.122 ^b
Biomass yield, $Y_{x/s}$ ($\times 10^{10}$ cfu/g lactose)	4.791 \pm 0.704 ^a	5.825 \pm 0.117 ^a	9.146 \pm 0.607 ^{bc}	6.902 \pm 0.518 ^{ac}
<u>Organic acids</u>				
Maximum acetic acid concentration, A_{\max} (g/L)	2.018 \pm 0.229 ^a	2.490 \pm 0.123 ^{ab}	2.851 \pm 0.233 ^b	2.704 \pm 0.174 ^{ab}
Acetic acid productivity, P_A (g/L.h)	0.084 \pm 0.010 ^a	0.125 \pm 0.006 ^{bc}	0.119 \pm 0.010 ^{bc}	0.113 \pm 0.007 ^{ac}
Acetic acid yield, $Y_{A/s}$ (g acetic/g lactose)	0.307 \pm 0.023 ^a	0.357 \pm 0.025 ^a	0.346 \pm 0.022 ^a	0.434 \pm 0.090 ^a
Maximum lactic acid concentration, L_{\max} (g/L)	2.387 \pm 0.169 ^a	2.902 \pm 0.395 ^{ab}	3.850 \pm 0.207 ^b	3.487 \pm 0.307 ^{ab}
Lactic acid productivity, P_L (g/L.h)	0.099 \pm 0.007 ^a	0.145 \pm 0.020 ^{ab}	0.160 \pm 0.009 ^b	0.145 \pm 0.013 ^{ab}
Lactic acid yield, $Y_{L/s}$ (g lactic/g lactose)	0.364 \pm 0.012 ^a	0.416 \pm 0.064 ^a	0.467 \pm 0.042 ^a	0.558 \pm 0.102 ^a
Acetic: lactic ratio	0.845	0.858	0.741	0.775

Results were reported as mean \pm S.D. of two experiments.

Mean values in the same row with different letters differ significantly ($P < 0.05$).

Acetic:lactic ratios were calculated based on maximum organic acids concentration produced.

Source: Stephenie *et al* (2007)

Table 8 Kinetic parameter values from batch fermentation of *B. pseudocatenulatum* G4 in a 2-L stirred tank fermenter at different pH control

Kinetic parameter values	Culture pH			
	No pH control	6.0	6.5	7.0
<u>Biomass</u>				
Maximum biomass, X_{max} (\log_{10} cfu/mL)	8.824 ± 0.034 ^a 18	9.230 ± 0.089 ^b 20	9.227 ± 0.105 ^b 18	8.537 ± 0.075 ^a 24
Time taken to reach maximum biomass, t (h)	0.311 ± 0.014 ^a	0.486 ± 0.004 ^b	0.504 ± 0.007 ^b	0.375 ± 0.014 ^c
Maximum specific growth rate, μ_{max} (h^{-1})	3.590 ± 0.293 ^a	8.450 ± 0.099 ^b	9.240 ± 0.085 ^b	1.377 ± 0.250 ^c
Biomass productivity, P_x ($\times 10^7$ cfu/mL.h)	9.146 ± 0.607 ^a	9.589 ± 0.255 ^a	9.791 ± 0.086 ^a	3.865 ± 0.692 ^b
Biomass yield, $Y_{x/s}$ ($\times 10^{10}$ cfu/g lactose)				
<u>Organic acids</u>				
Maximum acetic acid concentration, A_{max} (g/L)	2.851 ± 0.233 ^a	7.359 ± 0.072 ^b	7.353 ± 0.182 ^b	1.892 ± 0.093 ^c
Acetic acid productivity, P_A (g/L.h)	0.119 ± 0.010 ^a	0.307 ± 0.003 ^b	0.409 ± 0.004 ^c	0.079 ± 0.004 ^d
Acetic acid yield, $Y_{A/s}$ (g acetic/g lactose)	0.346 ± 0.022 ^a	0.508 ± 0.005 ^b	0.499 ± 0.017 ^b	0.220 ± 0.011 ^c
Maximum lactic acid concentration, L_{max} (g/L)	3.850 ± 0.207 ^a	6.481 ± 0.267 ^b	6.515 ± 0.047 ^b	2.350 ± 0.098 ^c
Lactic acid productivity, P_L (g/L.h)	0.160 ± 0.009 ^a	0.270 ± 0.011 ^b	0.362 ± 0.003 ^c	0.098 ± 0.004 ^d
Lactic acid yield, $Y_{L/s}$ (g lactic/g lactose)	0.467 ± 0.042 ^a	0.447 ± 0.018 ^a	0.443 ± 0.022 ^a	0.273 ± 0.011 ^b
Acetic:lactic ratio	0.741	1.135	1.129	0.805

Results were reported as mean ± S.D. of two experiments.

Mean values in the same row with different letters differ significantly ($P < 0.05$).

Acetic:lactic ratios were calculated based on maximum organic acids concentration produced.

Source: Stephenie *et al.* (2007)

Table 9 Batch cultivation of *B. pseudocatenuatum* G4 in different fermentation scales

Kinetics parameter values	Fermentation scale	
	2-L fermenter	10-fermenter
<u>Biomass</u>		
Maximum biomass, x_{\max} (\log_{10} cfu/mL)	9.227 \pm 0.105 ^a	9.145 \pm 0.093 ^a
Time taken to reach maximum biomass, t (h)	18	18
Maximum specific growth rate, μ_{\max} (h^{-1})	0.504 \pm 0.007 ^b	0.480 \pm 0.017 ^b
Biomass productivity, P_x ($\times 10^7$ cfu/mL.h)	9.240 \pm 0.085 ^a	7.704 \pm 1.638 ^a
Biomass yield, $Y_{x/s}$ ($\times 10^{10}$ cfu/g lactose)	9.791 \pm 0.086 ^a	9.456 \pm 1.387 ^a
<u>Organic acids</u>		
Maximum acetic acid concentration, A_{\max} (g/L)	7.353 \pm 0.182 ^b	7.412 \pm 0.114 ^b
Acetic acid productivity, P_A (g/L.h)	0.409 \pm 0.004 ^b	0.412 \pm 0.006 ^b
Acetic acid yield, $Y_{A/s}$ (g acetic/g lactose)	0.499 \pm 0.017 ^b	0.509 \pm 0.026 ^b
Maximum lactic acid concentration, L_{\max} (g/L)	6.515 \pm 0.047 ^b	6.574 \pm 0.248 ^b
Lactic acid productivity, P_L (g/L.h)	0.362 \pm 0.003 ^b	0.365 \pm 0.014 ^b
Lactic acid yield, $Y_{L/s}$ (g lactic/g lactose)	0.443 \pm 0.022 ^b	0.451 \pm 0.013 ^b

Results were reported as mean \pm S.D. of two experiments.

Mean values in the same row with different letters differ significantly ($P < 0.05$).

Acetic:lactic ratios were calculated based on maximum organic acids concentration produced.

Source: Stepheie *et al* (2007)

Growth Optimization of A Probiotic Candidate, *Bifidobacterium Pseudocatenulatum* G4, in Milk Medium using Response Surface Methodology

The complex ecosystem of gut microflora plays a significant role in the gastrointestinal health of humans and animals. This has attracted worldwide interest and intensive research has been conducted on issues pertaining to gut health. Although it is desirable to have a predominance of 'good' bacteria over harmful ones, however many factors including aging, stress, diet and ingestion of antibiotics may disturb this equilibrium (Gibson and Fuller, 2000). This has paved the way for the use of probiotics, which are live beneficial microorganisms, orally administered in order to encourage them to proliferate in the intestine (Figure 6).

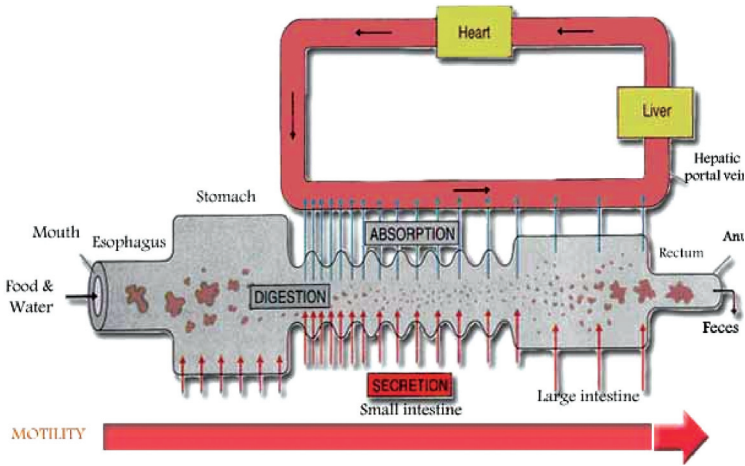


Figure 6 Illustration of GIT functions in food digestion

For a long time, probiotics have been associated with dairy products to transform yogurts, cultured milk drinks or cheese into functional foods. *Bifidobacteria*, a commonly used genus in probiotic applications, is gaining importance in the

industry due to its health benefits. Although bifidobacteria generally grow well in commercial media, these media are inappropriate for large-scale production due to the possibility of generating off-flavors in the food products. Thus, most companies opt for milk-based media for probiotic bacterial cell mass production (Gilliland *et al.*, 1985). Milk, which contains carbohydrates, fat, casein protein, vitamins and minerals, is a very nutritious growth medium for many microorganisms.

The ability of organisms to grow well in milk depends on their ability to metabolize milk protein and lactose and this ability varies considerably between strains. Generally, probiotics grow relatively slowly in milk due to a lack of proteolytic activity and therefore they require supplements of peptides and amino acids. In an effort to enhance the growth potential of probiotic strains in milk, researchers have added various concentrations of glucose as a carbon source and growth factors, such as yeast extracts. Various concentration ranges have been used: 10.0~12.0% (w/v) reconstituted skim milk; 0.2~1.0% (w/v) yeast extract; and 0.5~2.0% (w/v) glucose (Shuhaimi *et al.*, 1999). Avonts *et al.* (2004) reported that addition of yeast extract (0.3~1.0%, w/v) to 10.0% (w/v) reconstituted skim milk powder enhanced both growth and bacteriocin production of lactobacillus strains. In a different study, a yeast extract concentration as high as 6.0% was used to optimize non milk-based media for the growth of *Lactobacillus rhamnosus*.

So far, published reports on the supplementation of milk media with sugar and growth factors have been limited to lab-scale studies that were mainly for subculturing or propagation of inoculum. There were no justifications given for the different concentrations used. Moreover, industrial media formulations are rarely revealed to maintain the company's competitive advantage.

One of the major constraints involved in designing new fermentation media is the high number of experiments involved. Response surface methodology (RSM), a combination of good experimental design, regression modeling

techniques and optimization, is a useful tool for process improvement. This methodology has been applied to the optimization of the growth of probiotic organisms in non-milk media. A central composite design is one of the most commonly used designs for response surface optimization. While rotatability is a preferred characteristic in most central composite experiments, Neter *et al.* (1996) commented that when it is physically inconvenient to extend axial points beyond the upper and lower limits of the experimental region, a face-centered central composite design (with axial distance $\alpha = 1$) provides a good alternative. In face-centered central composite design, the region of interest and the region of operability are the same. In a study to optimize microbial growth in olive juice, a face-centered design was used due to difficulty in experimenting outside the upper and lower limits of the factors.

The optimization of milk-based fermentation medium using RSM for maximum biomass production of *Bifidobacterium pseudocatenulatum* G4, expressed as \log_{10} cfu/mL, was undertaken in our laboratory. This strain was isolated from infant stools and in previous studies demonstrated the probiotic characteristics of bile tolerance and deconjugation of bile acids (Rezaei *et al.*, 2004), antibacterial activity, antimicrobial susceptibility and adherence properties (Shuhaimi *et al.*, 1999).

An initial screening study using a 2^3 full factorial design was carried out to identify the impact on biomass production of the various components of the medium which were skim milk, yeast extract and glucose. Statistical analysis suggested that yeast extract had a significant positive effect on viable cell count whereas glucose had a negative effect. Response surface methodology (RSM) was then applied to optimize the use of skim milk and yeast extract. A quadratic model was derived using a 3^2 face-centered central composite design to represent cell mass as a function of the two variables. The optimized medium composition was found to be 2.8% skim milk and 2.2% yeast extract, w/v. The optimized medium allowed a maximum biomass of $9.129 \log_{10}$ cfu/mL, 3.329

log units higher than that achieved with 10% skim milk, which is the amount commonly used. The application of RSM resulted in an improvement in the biomass production of this strain in a more cost-effective milk medium, in which skim milk use was reduced by 71.8%. (Stephenie *et al.*, 2007)

DELIVERY TECHNIQUE

The Effect of Prebiotics Supplementation on Faecal Characteristics and Microbiota of Infants

The β -(2-1)-fructans inulin and oligofructose or fructo-oligosaccharides (FOS), are natural carbohydrates, found in many foods such as leek, garlic, onion, artichoke, chicory, banana, asparagus and wheat. (Van *et al.*, 1995) (Figure 7). Based on consumption data, the average intake of inulin and FOS in a normal human diet has been estimated at about 2-10 g per day. (Roberfroid *et al.*, 2001)

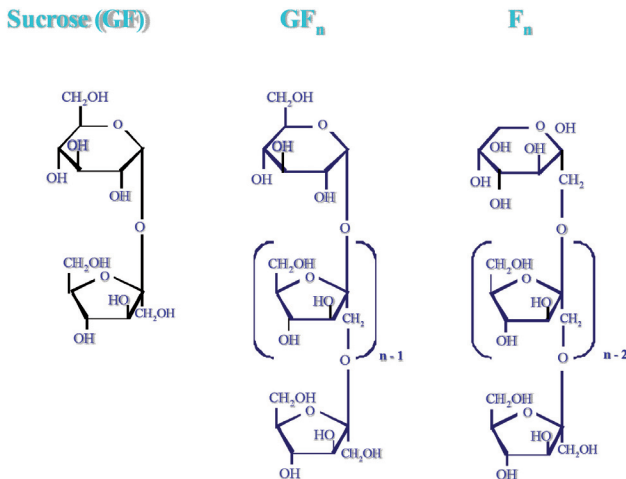


Figure 7 Chemical structure of inulin and FOS

These carbohydrates resist hydrolysis and digestion in the stomach and small intestine, and thus reach the colon intact. There they are fermented by the colonic microbiota to short chain fatty acids (SCFA) and gas. Selective fermentation of these fructans by bifidobacteria can result in an improved composition of gut microflora. This so-called prebiotic effect has been found in many human studies with adult volunteers (Gibson *et al.*, 1995) and with different types of inulin (Kolida *et al.*, 2007). *In vitro* studies also showed that oligofructose and inulin selectively stimulated the growth of bifidobacteria (Wang and Gibson, 1993).

FOS and inulin not only exert local colonic physiological effects, but also systemic effects, via the absorption of their fermentation products (SCFA) from the colon. The local effects of inulin consumption comprise the increased synthesis of vitamins, an increase in minerals, especially of calcium absorption from the colon (Meyer and Stass, 2006), and possibly, a lowered risk of colon cancer (Rafter, 2007). The systemic effects include a lowered level of serum lipids (Beylot M, 2005), an increased feeling of satiety and decreased energy intake (Cani *et al.*, 2006), or the modulation of immune functions (Seiferd and Watzl, 2007)

Most of these prebiotic studies have been carried out with adult volunteers, but the potential physiological benefits are equally important for infants. Not surprisingly therefore, prebiotics studies are also being conducted on younger people. Changes in composition of the colonic microbiota and the possible health benefits of a mixture of galactooligosaccharides and inulin in formula-fed babies have been reported (Bakker-Zierikzee *et al.*, 2005).

The effects of inulin on the microbial composition and faecal characteristics in healthy, formula-fed infants given 3 different daily dosages of native inulin (0.75 g/day, 1.00 g/day, and 1.25 g/day) were studied. At all levels of inulin consumption, a significant reduction of potential pathogenic microorganisms such as clostridia was observed. An intake of 1.25 g/day of inulin caused a

significant increase of *Bifidobacterium* spp. as well as a significant decline in Gram-positive cocci and coliform bacteria. Inulin consumption resulted in a decrease in faecal pH value and changes in faecal weight, faecal texture and colour, indicating improvement in healthy bile production and bacterial fermentation.

Survival of *Bifidobacterium Longum* BB 536 during Spray Drying of Medida Prepared from Malted Rice

Medida is a Sudanese cereal porridge consumed by adults and children of all ages. Different types of *Medida* are traditionally prepared from fermented cereal dominated by lactic acid bacteria. The fermented cereal dough is cooked by continuous stirring in a large amount of boiling water to produce the *Medida*. Previous studies on *Medida* did not go beyond the utilization of sorghum or millet flour; alpha amylase enzyme and pure lactic acid bacteria isolates from the spontaneously fermented dough. Only recently, was the first attempt to prepare fermented *Medida* from malted rice with *Bifidobacterium* reported and its physical and rheological characteristics studied (Kabeir *et al.* 2005).

Rice has several special characteristics; among which are ease of digestion, bland taste and hypoallergenic properties. It has been shown that the production of *Medida* from rice flour with *Bifidobacterium* improved its functional properties, as probiotic cultures used, and exerted beneficial effects on human hosts. The fermentation of rice with *Bifidobacterium* required amylases for saccharification of starch (Kim *et al.* 2000). Kabeir *et al.* (2005) encouraged utilizing malted flour for fermentation with *Bifidobacterium*.

In liquid fermented products *Bifidobacterium* tends to decline rapidly after manufacture and during storage (Dave and Shah 1997). Maintaining high numbers of *Bifidobacterium* (≥ 5 log CFU per gram) until the products are consumed ensures the delivery of live organisms and provides therapeutic benefits (Kurman and Rasic 1991).

Drying processes were found to extend the shelf life of the products and control their deterioration. Moreover, spray drying has been used to produce powders containing high numbers of viable bacteria from a range of genera (Etzel 1997). Nevertheless, low probiotic survival can often occur, due to cell membrane damage and affectation of ribosome and DNA at higher temperatures (Teixeira *et al.* 1997), dependent on the strain used (Kim and Bhowmik 1990). Thus, the manufacture of spray dried powder containing live probiotic bacteria at the recommended level represents a major technological challenge (Ishibashi and Shimamura 1993).

Improved microbial viability can be achieved during the spray drying process, Prasad *et al.* (2003) observed a marked improvement when *Lactobacillus rhamnosus* stationary phase cultures were heat shocked (adapted) prior to fluid bed drying. However, todate, there is little information available regarding the influence of spray drying on the survival of bifidobacteria. Studies addressing agreement between heat adaptation and spray drying for the survival of *Bifidobacterium* in cereal media such as rice, for further viability enhancement, are lacking in literature. Hence, in the present study, the effects of spray drying outlet temperatures and mild heat adaptations on the viability of *B. longum* BB536 in fermented *Medida* after spray drying were evaluated.

Safety Evaluation of Bifidobacterium Pseudocatenulatum G4 as Assessed in BALB/c Mice

The importance of intestinal microflora composition in physiological and patho-physiological processes in human gastrointestinal tracts is becoming more evident. Bifidobacteria, the normal inhabitants in the large intestines of human are considered to play an essential role in the maintenance of a healthy gut (Figure 8). They exist at high concentrations in the faeces of breast-fed infants, protecting the child against many invaders (Mitsuoka, 2000).

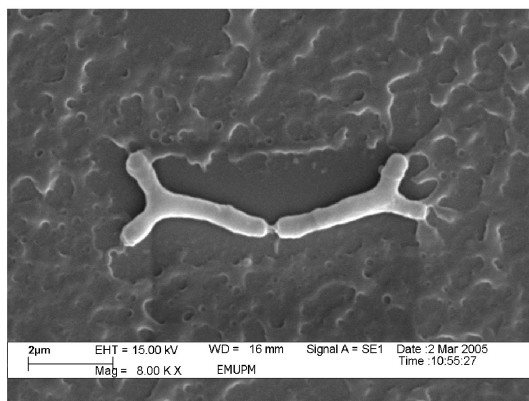


Figure 8 SEM of *Bifidobacteria pseudocatenulatum* G4

One commonly found species of bifidobacteria in breast fed infants is *Bifidobacterium pseudocatenulatum* (Shuhaimi *et al.* 2001a), which is largely unexplored as a probiotic. Earlier investigations carried out in our laboratory initiated interest in this species, which include generation of the genomic DNA fingerprint for *Bifidobacterium pseudocatenulatum* G4 by RAPD and ERIC sequence-based PCR (Shuhaimi *et al.*, 2001a; Shuhaimi *et al.*, 2001b), its potential as a probiotic candidate (Wong *et al.*, 2006) and its potential growth in food grade milk-based medium (Stephenie *et al.*, 2007).

Furthermore, it has been demonstrated that most *Lactobacillus* and *Bifidobacterium* are generally regarded as safe (GRAS) for dietary use (Saxelin *et al.* 1996). They have been consumed in commercial products without any adverse effects having been reported. However, safety studies on strains of *B. pseudocatenulatum* are limited and should be carefully assessed due to some reported clinical cases of human bacteraemia or translocation by bifidobacteria. Additionally, the new probiotic isolate might not share the safety status of commercially available strains and it is therefore imperative to confirm their *in vivo* safety status. For further establishment of the probiotic potential of this *B.*

pseudocatenulatum G4 strain, we studied the effects of 4 weeks administration of *B. pseudocatenulatum* G4 on translocation, internal organs indexes and histology changes in BALB/c mice.

Safety profiles of *Bifidobacterium pseudocatenulatum* G4 and commercial *Bifidobacterium longum* B536 were evaluated. Groups of BALB/c mice were orally administered *B. pseudocatenulatum* G4 at 2×10^4 , 1×10^8 , or 1×10^{11} CFU/day and *B. longum* BB536 at 1×10^8 CFU/day for four weeks. No abnormal clinical signs were revealed during the assessment. There were no noticeable difference in feed intake; water intake and live weight gain between treatments groups. Feeding, of the subjects with the strain G4 did not cause any changes in blood biochemistry (Albumin, Glucose, Cholesterol, and Total protein) or haematological (RBC, PCV, haemoglobin, MCV, MCHC, WBC, Neutrophils, Lymphocytes, Monocytes, and Eosinophils) measurements. Hence, the strain of *B. pseudocatenulatum* G4 evaluated in this study did not adversely affect the health of the mice and is likely to be safe and non-toxic for human consumption.

CHARACTERIZATION OF BIFIDOBACTERIUM SPECIES

Identification of the *Bifidobacterium* species is a difficult task because of phenotypic and genetic heterogeneities. Various DNA-based techniques to rapidly characterise the *Bifidobacterium* species and to support the conventional biochemical and morphological classification methods have been described. Sequencing of the 16S rRNA gene and 16S to 23S internally transcribed spacer region and comparisons with the sequence data present in the GenBank are the most popular techniques for identifying the *Bifidobacterium* species. Conserved sequences other than the 16S rRNA gene, such as the *Idh*, *recA* and *hsp60* genes, have become worthy tools for the elucidation of various taxonomic features such as genera, species and strains of *Bifidobacterium*. However, as an alternative to sequencing, which is both time consuming and technically

demanding, genus- or species-specific primers or probes were successfully designed to rapidly identify the *Bifidobacterium* species.

Several techniques such as amplified ribosomal DNA restriction analysis (ARDRA) method derived from the 16S rRNA gene has been proposed because of its rapid, reproducible and easy-to handle characteristics. Furthermore, randomly amplified polymorphic DNA (RAPD), Pulsed-Field Gel Electrophoresis (PFGE) and repetitive elements fingerprinting (Rep) are the popular methods to study genetic diversity among *Bifidobacterium* species due to their versatility.

Characterization of *Bifidobacterium* Species by Fructose 6-Phosphate Phosphoketolase Enzyme

Although the taxonomic position of the genus *Bifidobacterium* is unsatisfactory, the view that the bifidobacteria can be differentiated from other bacterial groups like lactobacilli, actinomycetes and anaerobic corynebacteria by the peculiar pathway employed for carbohydrate catabolism, i.e., the “fructose-6-phosphate shunt”, leading to the formation of lactic and acetic acids in the ratio 1.0: 1.5 as chief end products, is widely accepted (Scardovi *et al.*, 1971). There are two types of phosphoketolase that have been described in the genus *Bifidobacterium*: a F6P specific enzyme (F6PPK) in human species and a dual substrate xylulose 5-phosphate/fructose 6-phosphate (X5P/F6P) phosphoketolase in animal species. A preliminary study of the biochemistry, genetics and regulation of these landmark enzymes in the nutritionally important bifidobacteria in human and animal intestines was first reported by Meile *et al.* (2001). They conducted the purification of the dual substrate Xfp from *B. lactis*, a bacterium used as a probiotic supplement in fermented food. The gene (*xfp*) was identified, cloned and sequenced. However, the size and subunit composition of the Xfp protein is totally different from the $\alpha\beta$ subunit structure of a previously described F6PPK purified from *Bifidobacterium asteroides* extracts. Data from Meili *et al.* (2001) is also not conclusive as to whether the enzyme is involved

in both steps (phosphoketolase-1 and -2) of the so called *B. bifidum* pathway (Buckel, 1999).

Characterisation of *Bifidobacterium* Species by Conventional Carbohydrate Fermentation Patterns

Initial characterisation of *Bifidobacterium* species based on phenotype characteristics have been practised regularly. Carbohydrate fermentation tests leading to species differentiation were selected according to the procedure in Bergey's Manual of Systematic Bacteriology (Scardovi *et al.*, 1971). However, numerical analysis of carbohydrate fermentation patterns and morphological observation cannot differentiate some of the *Bifidobacterium* species of human origin (Bahaka *et al.*, 1993). These discrimination systems did not allow for species identification, particularly those on strains *Bifidobacterium adolescentis*, *Bifidobacterium pseudocatenulatum*, *B. infantis* and *Bifidobacterium animalis*. False declarations frequently occur among these species (Table 6). It was also reported that strains of *Bifidobacterium catenulatum* and *B. pseudocatenulatum* exhibited fermentation patterns similar to those of *B. adolescentis* (Scardovi *et al.*, 1979). Therefore, strains of *B. pseudocatenulatum* and *B. catenulatum* were, on a phenotypic basis, are barely distinguishable from *B. adolescentis*. The same situation occurs between *B. infantis* and *B. animalis*. For instance, the *Bifidobacterium* sp strain LW 420 which was originally thought to be identical to the *B. infantis* ATCC 15697 type strain, using carbohydrate fermentation patterns, does not in fact belong to the *B. infantis* strain type. Further, the homology between strain LW 420 and *B. animalis* is high (Kok *et al.*, 1996). Sakata *et al.*, (2002) reported that species of *B. longum*, *B. infantis* and *Bifidobacterium suis* showed different carbohydrate fermentation patterns. However, using the molecular methods (DNA-DNA hybridization, ribotyping and RAPD-PCR) these species could be unified as *B. longum* and divided into three biotypes, the *infantis* type, the *longum* type and the *suis* type (Sakata *et al.*, 2002).

Ribosomal RNA (rRNA) Analysis

Presently, ribosomal RNA (rRNA) is a common tool to study microbial populations. During the last two decades the gene encoding for 16S rRNA of *Escherichia coli* was sequenced, and many more 16S rRNA sequences of other bacteria have been determined. The advantages of using these genes as targets in hybridization experiments or in amplification reactions depend very much on the fact that these genes contain highly conserved regions and exist in large copy numbers within one single bacterial cell, exceeding by far the number of chromosomally encoded genes. The 16S rDNA sequences among *Bifidobacterium* species, for instance, showed similarities of over 93% (Leblond-Bourget *et al.*, 1996).

Techniques based on 16S rRNA detection have been used to detect probiotic *Bifidobacterium* species in infant faeces (Kok *et al.*, 1996). For this purpose, three strain-specific 16S rRNA gene-targeted primers have been developed. These primers allow specific detection of the organism via PCR. Specificity of the primers was determined in DNA samples isolated from single strain and mixed cultures of bifidobacteria and in heterogenous faecal samples. The feasibility of this method, for use in specific detection of probiotic strains, was investigated through addition of the *Bifidobacterium* sp. strain LW420 to infant instant milk formula (IMF) and PCR analyses of bacterial DNA isolated from faeces of 17 newborn IMF-fed infants. In faeces of all nine babies that had been fed with the probiotic IMF, the strain-specific signal could be detected. No signal was found in faeces of the eight infants that had been fed with a non-probiotic IMF, demonstrating the specificity of the PCR method. They also established a quick assay to quantitatively measure *Bifidobacterium* counts in food and faeces by dilution plating and colony hybridisation. After dilution plating of different food samples, colony lift and hybridisation with Im3, distinct dots were observed on the membrane, representing colonies of bifidobacteria. These colonies could be counted manually and CFU per gram of food sample

calculated. They also managed to detect 10^8 CFU of bifidobacteria per gram of human faeces with this method. Furthermore, a fragment of 1,498 bp of 16SrDNA was amplified and sequenced and, as expected, 16S rDNA of LW420 showed significant homology with the 16S rDNA sequences of bifidobacteria present in the databases. Relatively high homology was found with 16S rDNA of *Bifidobacterium animalis* DSM 20104.

Matsuki *et al.* (1999) developed 16S rRNA sequences for species-specific primers for the detection of *B. adolescentis*, *Bifidobacterium angulatum*, *B. bifidum*, *B. breve*, *B. pseudocatenulatum*, *B. longum* and *B. infantis*. They observed that the species-specific PCR technique, which was applied to the identification of isolated strains of *Bifidobacterium*, yielded the same results as the DNA-DNA hybridisation test. The PCR method always gave clear-cut results, even for the species that could not be clearly identified based on normal phenotypic traits. Additionally, it is possible to identify the isolate of bifidobacteria within three hours using the PCR technique. Hence, the species-specific PCR technique that they developed is a useful identification method, since it is rapid, convenient, accurate and cost-effective.

A *Bifidobacterium* genus-specific target sequence in the V9 variable region of the 16S rRNA has been elaborated and used to develop a hybridisation probe (Kaufmann *et al.*, 1997). The probe sequence was highly conserved within the genus *Bifidobacterium* and showed only one mismatch at position 1224 (*E. coli* numbering) containing either C or T. The specificity of this probe, named Im3, was used to identify all known strain types and distinguish them from other bacteria. All of the 30 strain types of *Bifidobacterium*, which are available at the German culture collection, 6 commercially available production strains and 34 closely related relevant strains (as negative controls) were tested. All tested bacteria showed distinct positive signals by colony hybridisation, whereas all negative controls showed no distinct dots except *Gardnerella vaginalis* DSM4944 and *Propionibacterium freudenreichii* subsp. *shermanii* DSM4902, which gave

slight signals. Their genus specific probe can give a good overall picture of the bifidobacterial population, but no information is obtained about the species or strain composition. Furthermore, they established a method for isolation and identification of bifidobacteria in food by using a PCR assay without prior isolation of DNA but by breaking the cells with proteinase K. Using this method, all *Bifidobacterium* strains lead to a DNA product of the expected size.

Based on the primers designed by Matsuki *et al.*, (1999) and Kaufmann *et al.*, (1997), seventeen *Bifidobacterium* strains isolated from breast-fed infant faeces, originally identified as *B. infantis* and *B. breve* using a biochemical test, were reclassified as *B.pseudocatenulatum* when identified using specific PCR and 16S rDNA sequencing (Shuhaimi *et al.*, 2002). The homology of the isolates to *B.pseudocatenulatum* JCM 1200 ranged from 98% to 99%. These PCR-based identification techniques suggest that only one species of bifidobacteria could be isolated from faecal samples using the media and growth condition described by Beerens (1990). Detection of other species of bifidobacteria was unsuccessful even though their presence in infant faeces is reported. (Matsuki *et al.*, 1999)

Specific multiplex PCR assay based on the gene amplification of the 16S rRNA by three pairs of primers in a single reaction, for specific species identification within a mixture of bifidobacteria or as a pure culture of *B. lactis*, was developed by Ventura *et al.*, (2001). They performed a multiplex PCR using oligonucleotide primers targeting a specific region of the 16S rRNA gene for the genus *Bifidobacterium* and a conserved eubacterial 16S rDNA sequence. Three pairs of primers namely Bflact2-Bflact5, PO-Lm3 and PO-338F were synthesised and used for the detection of 37 strain types of bifidobacteria. The application of the oligonucleotide pair Bflact2-Bflact5 resulted in a PCR amplicon of 680 bp only with DNA derived from *B. lactis* strains, whereas absolutely no PCR product could be detected with those primers for any other *Bifidobacterium* isolates. When a multiplex PCR was performed with this mixture of three pairs of primers in the same reaction, three major products of all expected sizes were detected

only in the presence of DNA originating from *B. lactis*. The amplification of other *Bifidobacterium* DNA species resulted in the typical pattern of only two amplicons.

Hence, the application of this multiplex PCR in the analysis of commercially available products can be a very useful tool for any rapid monitoring of the species *B. lactis*. This multiplex PCR can be considered, in terms of reduction of tedious labour time, easy application, reliability and repeatable PCR results and intrinsic controls, and low cost, a uniquely powerful tool for studying any bifidobacterial ecology and also the faecal environment of newborns, babies, toddlers and adults fed with products containing members of the genus *Bifidobacterium* in general and *B. lactis* in particular. However, such a PCR amplification that uses chromosomal DNA as a target cannot distinguish between viable and nonviable bacterial cells. Therefore, in determination of the shelf life of a product containing *B. lactis*, with regard to bacterial stability and viability, it remains essential that all detectable and viable bacterial cells are additionally plate counted. Due to the extremely short shelf life of the majority of bacterial mRNAs, it seems to be possible, and maybe more appropriate, to select mRNA as a PCR-based target to detect bacteria via PCR amplification. In particular, total RNA extraction followed by a reverse transcriptase PCR using primers Bflact2 and Bflact5 will represent a reliable tool for detection of only the viable fraction of *B. lactis* cells present in various food systems.

Leblond-Bourget *et al.*, (1996) first studied *Bifidobacterium* phylogeny by performing both 16S rRNA and 16S to 23S (16S-23S) internally transcribed spacer (ITS) sequence analysis. They determined 16S rRNA sequences of five *Bifidobacterium* strains representing four species, and compared them with the sequence available in the GenBank database, and used them to construct a distance tree and for a bootstrap analysis. Moreover, they determined the ITS sequences of 29 bifidobacterial strains representing 18 species and compared these sequences with each other. They constructed a phylogenetic

tree based on these sequence data and compared this tree with the tree based on the 16S rRNA sequence data. They found that the two trees were similar topologically; suggesting that the two types of molecules provided the same kind of phylogenetic information. However, while 16S rRNA sequences are a good tool to infer interspecific links, the 16S - 23S rDNA spacer data allowed them to determine intraspecific relationships. Each of the strains was characterized by its own ITS sequence; hence, 16S – 23S rRNA sequences are a good tool for strain identification. Moreover, comparison of the ITS sequences allowed them to estimate that the maximum level of ITS divergence between strains belonging to the same species was 13%. Their data allowed them to confirm the validity of most of the *Bifidobacterium* species which we studied and to identify some classification errors. Finally, their results showed that *Bifidobacterium* strains have no tRNA genes in the 16S – 23S spacer region.

Another way of utilizing the rRNA sequence heterogeneity in microbial ecology is the use of universal bacterial PCR primers to amplify a fragment of rRNA or ribosomal DNA (rDNA) and then separating the PCR products obtained in a sequence-specific manner in temperature gradient gel electrophoresis (TGGE) or denaturing gradient gel electrophoresis (DGGE). This technique has been successfully applied to monitor the most predominant bacterial populations in human faecal samples (Zoetendal *et al.*, 1998). Satokari *et al.*, (2001) developed and validated the method based on genus-specific PCR and DGGE to monitor the qualitative composition of the whole bifidobacterial population with merely a single PCR. They reported that even minor differences in the 16S rDNA sequence may, alter the migration behaviour of a PCR fragment in DGGE, as shown in the case of *B. adolescentis* E-981074[†]. This allows them to rapidly monitor changes occurring in the predominant members of the bifidobacterial community. The method may provide a valuable alternative to molecular typing techniques in rapidly monitoring qualitative changes in the bifidobacterial populations, although it does not allow definite discrimination

or quantification of different strains. The DGGE method has the advantage of being independent of prior time-consuming culturing of the isolates on selective medium, which may favour the growth of some strains, thereby making the results biased. The PCR approach can also, however, lead to some distortions, because some sequences may amplify better than others, and heteroduplexes can be formed during PCR (Wintzingerode *et al.*, 1997). In the PCR-DGGE approach, identification of fragments can also be done by subsequence cloning and sequencing of the PCR products, but it is hampered by the high similarity of the 16S rDNA sequences between different *Bifidobacterium* species and the inadequate sequence data quality for many of the sequences in the GenBank.

The DGGE profiles of 16S PCR amplicons of bifidobacteria showed that *B. adolescentis* was the most common species in the faeces of human adults (Satokari *et al.*, 2001). This supports the results of previous studies that stated that intestinal *Bifidobacterium* communities are host specific (Kimura *et al.*, 1997). The bifidobacterial populations were also found to be stable in composition during the 4-week study period. In general, the bifidobacterial population in the adult gut seems to be relatively stable for strain composition over several months or even a year, although some individual variations have also been detected. In contrast, in the developing gut microbiota of infants, bifidobacterial species change with time. Further studies with larger test groups are needed to make conclusions about the development and long-term stability of bifidobacterial communities.

Comparison of the species-specific PCR method with the classical culture methods revealed that some species, most frequently, *B. adolescentis*, were detected by the direct PCR method but not by culturing followed by specific PCR of the isolates (Matsuki *et al.*, 1998). In these individuals *B. adolescentis* was either not among the most numerous bifidobacteria or it failed to grow on the selective media used. Satokari *et al.*, (2001) reported that *B. adolescentis* or closely related species are numerically the most prevailing bifidobacteria in

some individuals. However, taking into consideration the possible heteroduplex formation and the fact that some species or strains may give more than one fragment in DGGE, it is difficult to give accurate estimates of the diversity of bifidobacteria in the faecal samples. The DGGE pattern shows that the bifidobacterial diversity in individual samples is quite restricted and according to sequence data, some of these strains may belong to the same species. This result is in good agreement with previous studies conducted by Mangin *et al.* (1999) and Matsuki *et al.*, (1998) where in most adults the bifidobacterial community is a combination of one to four species and several distinct strains of the same species can coexist in one community.

The development of new approaches and techniques of molecular biology would make it possible to clarify in which species the strains of bifidobacteria belong. Amplified ribosomal DNA restriction analysis (ARDRA) has excellent potential for discrimination of organisms at the species level. This technique is based on the amplification of the DNA sequence of the 16S rDNA region, followed by the digestion of PCR product with restriction enzymes (Vaneechoutte *et al.*, 1992). Roy and Sirois (2000) reported that ARDRA profiles indicate that *B. longum* and *B. infantis* can be differentiated easily. The results of the ARDRA method indicate that the subcluster containing reference and commercial strains of *B. animalis* also included the new species *B. lactis*. Ventura *et al.*, (2001) observed that the majority of the human adult gut isolates belonged to the species *B. longum*, *B. bifidum* or *B. pseudocatenulatum* when classified using the ARDRA method. Their report is in contrast to Matsuki *et al.* (1998), who reported mainly *B. adolescentis* and *B. catenulatum* isolates as being the predominant members of the human adult intestinal microflora. Furthermore these researchers reported that their specific primers were unable to distinguish between *B. longum* and *B. infantis* (Matsuki *et al.*, 1999). Although significant progress has been achieved with the application of the ARDRA method, some factors still remain to be solved. For example, *B. longum* and *B. suis* are difficult to distinguish,

although plasmid profiling for strains belonging to these species appears promising. The ARDRA technique reviewed here describes one solution for the inadequate identification methods for determining *Bifidobacterium* species and could be of help to evaluate the differences between simple and complex, dynamic or static, bifidobacterial microflora in the human gastrointestinal tract (McCartney and Tannock, 1995).

Phylogenetic Analysis of the Genus *Bifidobacterium* Based on Short Region of Certain Genes

The rapid molecular strategy utilizing targeted genes for the identification of unknown faecal strains of bifidobacteria is a novel approach to understand the diversity and phylogenetic relationship between the many different bifidobacterial isolates in the human intestine. It is much more sensitive than simply classifying at the species level using biochemical tests (Scardovi *et al.*, 1971) or species specific probes. Karlin *et al.*, (1995) has established the utility of RecA protein sequences for prokaryotic phylogenetic relationships. Like the 16S rRNA gene, the *recA* gene is considered to be universally present in bacteria, and shows a high degree of sequence conservation. Kullen *et al.* (1997) used the sequence of the *recA* gene to study the intragenic phylogeny in *Bifidobacterium*. An approximately 300 bp fragment of the *recA* gene was PCR-amplified and sequenced from six species of the genus *Bifidobacterium* namely *B. bifidum*, *B. animalis*, *B. infantis*, *B. longum*, *B. adolescentis* and *B. breve* using primers directed to two universally conserved regions of the *recA* gene. The most divergent strains, *B. bifidum* and *B. breve* showed only 82.7% identity in DNA sequence, while sequence identity between the closely related *B. longum* and *B. infantis* was found to be 96.9%. An analysis of the multiple alignment of the *recA* sequences enabled a phylogenetic tree to be constructed. The six *Bifidobacterium* spp fell into four major clusters. The cluster was in agreement with the phylogeny based on 16S rRNA gene sequences (Leblond-Bourget N

et al.,1996) with the exception of the placement of *B. adolescentis*. This *recA* sequence intrageneric, phylogenetic analysis may assist in the resolution of species designations that are a point of contention (Kullen *et al.*, 1997). It is difficult to differentiate *B. infantis* and *B. longum* by classical methodology such as by morphological analysis and carbohydrate fermentation patterns (Scardovi *et al.*, 1971). Moreover, it has been demonstrated that the 16S rRNA genes of the two species approach 99% identity (Leblond-Bourget N *et al.*,1996). In contrast, sequence information from a much shorter segment of the more divergent *recA* gene provided greater sensitivity for differentiation of *B. infantis* and *B. longum* (96.9% identity) (Kullen *et al.*, 1997). The advantage of using the *recA* gene sequence data, rather than 16S rRNA gene sequences, for intrageneric characterisation, is that *recA* is more divergent and enables classification to be achieved with a shorter segment of DNA. However, the usefulness of this approach for rapid intrageneric characterization will depend on a larger survey of the many strains from all species of *Bifidobacterium* as well as a detailed survey of other genera (Kullen *et al.*, 1997). Another universally present gene in all microorganisms (Goh SW *et al.*, 1996; Kwok *et al.*, 1999), that has been used to study the phylogeny of bifidobacteria, is the heat shock protein 60 kDA (HSP60) Jian W *et al.*, 2001, have conducted the cloning and sequencing of the *hsp60* gene from 36 *Bifidobacterium* strains representing 30 different *Bifidobacterium* species and subspecies using a pair of universal degenerate HSP60 PCR primers. They reported that the HSP60 DNA sequence similarities among bifidobacteria were 99-100% within the same species, 96% at the subspecies level and 85% at the interspecies level. The topology of the phylogenetic tree constructed using the HSP60 sequences were almost similar to that for the 16S rRNA. However, it seemed to be more accurate for species delineation and the clustering was better correlated with the DNA base composition (mol% G+C) than that of the 16S rRNA tree (Jian *et al.*, 2001).

Roy and Sirois (2000) used the conserved gene sequence other than *recA* and *hsp60*, the *ldh* gene, to characterize the *Bifidobacterium* species. Unlike the *recA* and *hsp60* genes, the *ldh* gene was only present in some genus of bacteria including bifidobacteria. Fructose-1,6-biphosphate-dependent L-lactate dehydrogenase (LDH) is a key enzyme in lactic acid fermentation by most lactic acid bacteria. The *ldh* gene sequences of 370 bp were determined for 19 *Bifidobacterium* strains including four isolates of commercial origin. Comparison of the short region of the *ldh* gene was based on the sequences comprised between the two primers LDH F1 and LDH R1 which gave a sequence of 309-312 bp (Roy and Sirois, 2000). They demonstrated that these primers are useful for the differentiation of strains belonging to *B. infantis*, *B. longum* and *B. animalis*. It is well-known that *B. infantis* and *B. longum* are difficult to distinguish using phenotypic characterization and DNA-DNA hybridization (Bahaka *et al.*, 1993). Moreover, phylogenetic data obtained from both 16S rRNA sequence analysis demonstrated that *B. longum* ATCC 15707 and *B. infantis* ATCC 15697 are closely related (Leblond-Bourget N *et al.*, 1996). However, the analysis of a short region of the *ldh* gene confirmed that it is possible to distinguish between *B. infantis* and *B. longum* but not between *B. lactis* and *B. animalis* (Roy and Sirois, 2000). The approach reviewed here showed that the *ldh* gene for the genus *Bifidobacterium* is well conserved and it is possible to use it for the identification and speciation of *Bifidobacterium* strains.

Differentiation of Bifidobacteria by Use of Pulsed-field Gel Electrophoresis and Polymerase Chain Reaction

The technique of pulsed-field gel electrophoresis (PFGE) has been exploited to successfully unravel the organisation of many bacterial genomes revealing the presence of multiple chromosomes, linear chromosomes or large plasmids in different hosts. Rare cutting enzymes employed in conjunction with PFGE have allowed species identification and strain classification within the same species and have also provided useful data for estimating genome size and for genome mapping (Smith and Condemine G., 1990). Until recently, information on genomic organisation of bifidobacteria was limited to one species of the genus which described intra-species polymorphisms between four of five *B. breve* strains examined (Bourget *et al.*, 1993). However Roy and Champagne (1996) provided a more extensive study, in which PFGE was used to compare a bank of dairy related bifidobacteria comprising culture collections and commercial strains. The method was successful in obtaining different genomic fingerprints from various *Bifidobacterium* strains. Four different genomic fingerprintings were discernible for reference strains of *B. animalis*, five for *B. bifidum*, three for *B. breve*, five for *B. infantis* and three for *B. longum*. Standard commercially-available strains of *B. animalis* are found to be identical to the reference strain of *B. animalis* ATCC 27536. On the other hand, there was more genetic heterogeneity observed from the PFGE patterns among industrial strains of *B. longum*, in that only one gave profiles similar to the *B. longum* ATCC 15707 type strain. However, a more recent study by O'Riordan and Fitzgerald (1997) showed that *B. pseudocatenulatum* 8811 and *B. bifidum* 1455 displayed identical patterns. While four strains from the species *B. infantis* were compared, all produced different patterns. It seems that intra-species relatedness did not appear to be greater than inter-species relatedness. Hence, despite its discriminatory potential, the pulsed-field gel electrophoresis technique would require a great

deal of screening to establish its taxonomic relevance. Otherwise, it would be of no value in taxonomic identification (O’Riordan and Fitzgerald, 1997).

Random Amplification of Polymorphic DNA (RAPD)

Random amplification of polymorphic DNA (RAPD) analysis is a DNA fingerprinting technique used to detect genomic polymorphisms (Williams *et al.*, 1990). In the RAPD method, genomic DNA is PCR amplified under low stringency conditions using a single, short oligonucleotide primer of arbitrary sequence. The low stringency conditions allow the primer to anneal to multiple sites on the genome, resulting in an array of amplified DNA fragments. Polymorphisms between individuals or closely related strains are detected as differences in banding patterns on an agarose or polyacrylamide gel.

RAPD analysis has been widely used in numerous applications, including gene mapping, detection of strain diversity, population analysis, epidemiology and the demonstration of phylogenetic and taxonomic relationships. Its popularity arises from its ability to quickly detect polymorphisms at a number of different loci using nanogram quantities of genomic DNA. Because it utilises arbitrary primers, RAPD analysis can be carried out on DNA from organisms for which there is little or no genomic sequence or organisation information. This makes it possible to analyse polymorphisms for virtually any organism.

Characterisation of bifidobacteria by RAPD was first demonstrated by Vincent *et al.* (1998). Five arbitrary primers were chosen in their study, on the basis of band intensity and distribution, which clearly distinguished between strains of *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. infantis* and *B. longum*. The use of five single-primer reactions under optimised conditions improved the resolution and accuracy of the RAPD method for the characterisation of dairy-related bifidobacteria. The results indicated that this method was highly reproducible in repeated analysis. Similarity between bifidobacteria strains was evaluated based on their RAPD profile. Using a set of five primers, it was

demonstrated that it could be possible to distinguish three different species of *Bifidobacterium* (*B. breve*, *B. bifidum* and *B. adolescentis*), based on similarity of the RAPD profiles to known reference strains. Later, Shuhaimi *et al.* (2001) adopted the RAPD technique in the classification of *B. pseudocatenulatum* isolated from infants' faeces. They demonstrated the ability of RAPD in differentiating strains of *B. pseudocatenulatum*. Furthermore, application of the RAPD technique may also be useful and faster than the traditional systematic technique, for placement of industrial/commercial strains into specific clusters. However, priming efficiency of PCR experiments with arbitrary primers was found to be low (Welsh and McClland, 1990). Therefore, the PCR reactions must be performed in low stringency under optimized conditions.

Short, Interspersed Repetitive DNA Sequences-based PCR for Classification of *Bifidobacterium* Species

Prokaryotic genomes contain a variety of low copy number repeated sequences, such as repetitive extragenic palindromic (REP) (Gilson *et al.*, 1984), enterobacterial repetitive intergenic consensus (ERIC) and BOXs elements (Martin *et al.*, 1992). These sequences may contribute to the evolution of chromosome structure through DNA rearrangements such as chromosomal deletions, duplication and inversions. Repeated genes also provide mechanisms to enhance bacterial virulence, such as antigenic variation in *Neisseria gonorrhoeae* and other pathogens (Haas and Meyer, 1986).

The repeated sequences, that was first described and most intensively studied is the repetitive extragenic palindrome (REP), or palindromic unit (PU) sequence, initially identified in *Salmonella typhimurium* and *E. coli* (Gilson *et al.*, 1984). The REP sequence was identified through DNA sequence comparisons of intercistronic regions of different operons. The REP sequence structure consists of a 38 nucleotides consensus sequence, which is a palindrome, and can form a stable stem-loop structure with a 50 bp variable loop in the central region of the

consensus sequence. It was estimated that there are between 500 to 1,000 copies of the REP sequence organised into clusters on the *E. coli* chromosome. These clusters could also contain other repeated elements in specific arrangements (Gilson *et al.*, 1984).

An additional interspersed repetitive DNA sequence element in prokaryotic genomes has been identified in *E. coli*, *S. typhimurium* and other members of the family *Enterobacteriaceae* (Hulton *et al.* 1991). This repetitive element has alternatively been called an intergenic repeat unit (IRU) or enterobacterial repetitive intergenic consensus (ERIC) sequence. The ERIC or IRU sequence is approximately 126 bp in length. Like the REP sequence, it appears, from analysis of DNA sequence information in the data bases, that the ERIC or IRU sequence is located in non-coding transcribed regions of the chromosome, in either orientation with respect to transcription, and includes a conserved inverted repeat. The chromosomal locations of the ERIC sequence can differ in different species. In analogous locations, regardless of whether or not the IRU or ERIC sequence is present in different species, the surrounding sequence is not disturbed, suggesting either a precise deletion or insertion relative to the other species. No base pair duplication of surrounding sequence can be identified, arguing against a classic transposition mechanism for dispersion of these repetitive sequences.

Shuhaimi *et al.* (2001) reported, for the first time, that ERIC elements are present in the genome of bifidobacteria and other probiotic bacteria such as *Bacillus mesentericus*, *Lactobacillus casei* and *Streptococcus faecalis*. Their results indicate that this method is highly reproducible in repeated PCR analysis. Using a combination of 2 ERIC primers, it was possible to distinguish five different species of bifidobacteria (*B. pseudocatenuatum*, *B. infantis*, *B. longum*, *B. animalis* and *Bifidobacterium indicum*). The ERIC elements' profiles were clearly distinct among *S. faecalis*, *B. mesentericus* and *L. casei*. Recently, Ventura and Zink (2002) used the combination of 16S to 23S internally

transcribed spacer region and Eric-PCR to rapidly differentiate between *B. lactis* and *B. animalis*. Both techniques used demonstrated that *B. lactis* and *B. animalis* from two main groups and they propose that *B. lactis* should be separated from *B. animalis* at the subspecies level.

BOX primer has been used to examine DNAs of various bacteria, such as *Paenibacillus* spp. (Rosado AS *et al.*, 1998), *Bradyrhizobium japonicum* (Vinuesa *et al.*, 1998), *Azoarcus* spp. (Hurek *et al.*, 1997), *Sphingomonas* (Balkwill *et al.*, 1997) and *Xanthomonas* spp. (Louws *et al.*, 1994). Just recently, bacteria from the genus *Bifidobacterium* isolated from newborn' faeces has been characterized using BOX-PCR (Zavaglia *et al.*, 1998). The BOX-PCR method was successfully used in their study to resolve the taxonomic status of twenty-five bifidobacterial strains isolated from infant's faeces as well as reference strains from ATCC and Morinaga Milk Industry Ltd, Japan. Characteristic bands of *B. bifidum*, *B. breve*, *B. longum*, *B. infantis* and *B. adolescentis* were found in all the *Bifidobacterium* strains tested but not in lactobacilli. They also found that the predominant species among the isolated strains were *B. bifidum*, *B. longum* and *B. breve*.

CONCLUSION

Bifidobacteria are part of the rich and complex microbiota of the human GIT. They are also important in the food industry as manufacturing of some probiotic products requires a particular species. However, some of the *Bifidobacterium* species share the same phenotypic and genotypic characteristics. Hence, the characterisation of these microorganisms, to be used as probiotics, is not an easy task especially when biochemical methods are used. These conventional methods are time consuming and cannot give clear-cut results. Hence, for confirmation purposes, the application of DNA- and PCR-based methods is necessary. These methods offer fast and accurate identification of probiotic microorganisms as shown by specific PCR followed by DNA sequencing of

the 16S rRNA gene. Conserved sequences other than 16S rRNA gene such as the short region of *ldh*, *recA* and *hsp60* genes have been shown to be useful to characterize *Bifidobacterium* species. Both molecular techniques are much more sensitive and reliable than simply classifying bifidobacteria using biochemical tests. However, validation of these techniques requires a larger survey of many strains of bifidobacteria from various sources as well as a detailed survey of other genera.

PCR-based classification methods such as RAPD, ERIC, BOX and PFGE provide rapid and reproducible results in analysing genetic diversity among probiotic microorganisms. Furthermore, driven by the world-wide rapid growth of the probiotic market, DNA-based technologies for classification and identification of bifidobacteria are developing rapidly due largely to the precise nature of the DNA sequences to be detected. Hence, high throughput technology such as real time PCR should now be exploited for rapid detection and identification of bifidobacteria especially in food and pharmaceutical products. This would reduce the need for labour-intensive and time-consuming identification techniques such as multiple physiological and biochemical testing, which remain frequently unreliable, slow and expensive and also not accurate enough for the classification of bifidobacteria.

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BIOGRAPHY

Mohd Yazid bin Abd Manap, distinguished Professor of Dairy Technology and Dairy Starter Culture Technology at Universiti Putra Malaysia, Faculty of Food Science & Technology (Serdang, Malaysia), has devoted his career to studying Probiotic bacteria applied in food supplements.

He was born in Seri Menanti, Negeri Sembilan and grew up in Nyalas Melaka. His early education was at the Sekolah Kebangsaan Nyalas primary school and Jasin Secondary English School. He subsequently graduated from ITM (now UiTM) with a Diploma in Animal Health and Production in 1978.

He began his scientific career by earning his bachelor's (1982) and master's degrees (1984) in agricultural food processing at the University of Minnesota (Minneapolis, MN). Yazid then earned his doctoral degree in Dairy Technology at the University of Glasgow, Scotland, UK.

Initially, he worked as a Medical Sales Representative with Glaxo for 3 years and then with the Department of Veterinary Services Malaysia as an Assistant Veterinary Officer. He had a short stint with Grand Metropolitan Foods, Minneapolis, USA and with Nestle Switzerland for two years before joining the Faculty of Food Science & Technology in UPM where he has remained as a loyal academician ever since.

Over the years, Yazid and his colleagues at Universiti Putra Malaysia have systematically developed genetic tools for genetic characterization of lactic acid bacteria and intestinal *Bifidobacterium* species, including identification and enumeration of the cells. Recently, he focused more on preservation and delivery methods for dairy starter culture bacteria, and he and his team are interested in developing a new isolated culture, namely, *Bifidobacterium pseudocatenulatum* G4, which can be used in dietary adjuncts and dairy foods.

Yazid's research activities are enabled by his receipt of support in the form of grants from various agencies and ministries. He has benefited from receipt

of such grants from the Ministry of Science & Environment (Malaysia), National Committee School Milk Programme and SENSUS BV Holland.

He has more than 100 publications in cited refereed journals and has presented papers at national and international conferences. Yazid is also an Editorial Member of the ASEAN Food Journal. He has been given due recognition for his contributions through being awarded the Excellent Service Award on several occasions by Universiti Putra Malaysia.

Yazid has also been awarded many prizes, including Gold and Bronze medals, at Scientific, Technology and Innovation Exhibitions, National level competitions, Symposiums, global research competitions and Research Fellowships from University College Cork (Ireland).

Together with his colleagues, he has been supervising more than 50 postgraduate students. He has been serving as a member in the EXCO of PPA (Universiti Putra Malaysia), the Ministry of Health Malaysia (on the National CODEX Committee on Milk and Milk Products, National CODEX Committee on Filled Milk Products, National Committee on Food Regulations – Food Microbiology, Working Committee on Ready to Eat Foods), on Technical Committees (for Teaching and Research in Agriculture), the Putra Dairy Development Committee (University Agricultural Park, UPM), ISO 9002 Audit Committee (Faculty of Food Science & Biotechnology, UPM), Pilot Plant Working Committee (Department of Food Technology, Faculty of Food Science & Biotechnology, UPM), Curriculum Committee (Faculty of Food Science & Biotechnology, UPM) Persatuan Pegawai Akademik, Faculty's Prize and Awards Committee (Faculty of Food Science & Biotechnology, UPM) and the Bureau of Professional Services (Faculty of Food Science and Biotechnology, UPM). Yazid has a long continued commitment to several Professional Societies: Association of Analytical Chemists (USA), Bifidus Foundation (Japan), National Geographic Society (USA), Malaysian Society of Microbiology (Malaysia), Malaysian Institute of Food Technology (Malaysia) and SPSS-BI Statistical Association of Malaysia.

He is a dedicated lecturer and acts as the head of Food Service and Management Department (Faculty of Food Science & Technology, UPM), Chairman of the Faculty's Promotional Committee (Faculty of Food Science & Biotechnology, UPM), Coordinator of UPM-DUMEX Quality Development Programme, Scientific Advisor of Kelantan Biotechnology Corporation & State of Melaka and also as the instructor for Research Techniques and Scientific Methods and **Secretary of the Organizing Committee for Seminar Advances in Food Research IV** in UPM.

Despite his wide-ranging scientific accomplishments, Yazid says he is most proud of his graduate students, who, he says, continue to bring excellence to his laboratory and the university. Since 1990 his students have received recognition in Universiti Putra Malaysia. According to Yazid, working with graduate students is the most gratifying part of his job. "There is no greater experience than working with talented young people and being part of their accomplishments.

Apart from his hectic career Yazid, is also interested in farming and plantations. He spends most of his weekends and leisure time at his farm and enjoys the simple rural life. Spending his time together with caring villagers is very close to his heart. He is blessed with four children, 2 boys and 2 girls.

ACKNOWLEDGEMENT

In the name of God, the Most gracious, Most Merciful, Alhamdulillah, as with His help and consent I have embarked on this journey to explore essential areas for human progress.

Primary acknowledgement must go to the many dedicated scientists who discovered the principles of microbiology.

On a more immediate level, I express my appreciation to my parents whose dedication, love and unwavering confidence in me has eased my burdens. They deserve special mention for their unfailing support and prayers.

I must place on record my special gratitude to my graduate supervisors Professor J. D. Donker (University Minnesota) and Professor R.J.M. Crawford (University of Glasgow, Scotland, UK). Their ideals and concepts have had a remarkable influence on my entire career.

It is a pleasure to also be able to express my wholehearted gratitude to my teachers and friends for the pleasant times while working with them and for such great friendships.

Collective and individual acknowledgments are also owed to my colleagues, whose presence have perpetually refreshed me and who have been exceptionally helpful, and memorable.

I wish to gratefully acknowledged Universiti Putra Malaysia for their financial support and the provision of wonderful research facilities.

My loving thanks are also due to my extended family, Dr. Baizura, Kamal Ariffin, Mustaffa Kamel, Nurul Sofea and Yasmin, my brothers, my sisters and their families for their loving support.

LIST OF INAUGURAL LECTURES

1. Prof. Dr. Sulaiman M. Yassin
The Challenge to Communication Research in Extension
22 July 1989
2. Prof. Ir. Abang Abdullah Abang Ali
Indigenous Materials and Technology for Low Cost Housing
30 August 1990
3. Prof. Dr. Abdul Rahman Abdul Razak
Plant Parasitic Nematodes, Lesser Known Pests of Agricultural Crops
30 January 1993
4. Prof. Dr. Mohamed Suleiman
Numerical Solution of Ordinary Differential Equations: A Historical Perspective
11 December 1993
5. Prof. Dr. Mohd. Ariff Hussein
Changing Roles of Agricultural Economics
5 March 1994
6. Prof. Dr. Mohd. Ismail Ahmad
Marketing Management: Prospects and Challenges for Agriculture
6 April 1994
7. Prof. Dr. Mohamed Mahyuddin Mohd. Dahan
The Changing Demand for Livestock Products
20 April 1994
8. Prof. Dr. Ruth Kiew
Plant Taxonomy, Biodiversity and Conservation
11 May 1994
9. Prof. Ir. Dr. Mohd. Zohadie Bardaie
Engineering Technological Developments Propelling Agriculture into the 21st Century
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10. Prof. Dr. Shamsuddin Jusop
Rock, Mineral and Soil
18 June 1994

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Natural Toxicants Affecting Animal Health and Production
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Pest Control: A Challenge in Applied Ecology
9 July 1994
13. Prof. Dr. Kapt. Mohd. Ibrahim Haji Mohamed
Managing Challenges in Fisheries Development through Science and Technology
23 July 1994
14. Prof. Dr. Hj. Amat Juhari Moain
Sejarah Keagungan Bahasa Melayu
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15. Prof. Dr. Law Ah Theem
Oil Pollution in the Malaysian Seas
24 September 1994
16. Prof. Dr. Md. Nordin Hj. Lajis
Fine Chemicals from Biological Resources: The Wealth from Nature
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17. Prof. Dr. Sheikh Omar Abdul Rahman
Health, Disease and Death in Creatures Great and Small
25 February 1995
18. Prof. Dr. Mohamed Shariff Mohamed Din
Fish Health: An Odyssey through the Asia - Pacific Region
25 March 1995
19. Prof. Dr. Tengku Azmi Tengku Ibrahim
Chromosome Distribution and Production Performance of Water Buffaloes
6 May 1995
20. Prof. Dr. Abdul Hamid Mahmood
Bahasa Melayu sebagai Bahasa Ilmu- Cabaran dan Harapan
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22. Prof. Dr. Nik Muhammad Nik Abd. Majid
The Diminishing Tropical Rain Forest: Causes, Symptoms and Cure
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23. Prof. Dr. Ang Kok Jee
The Evolution of an Environmentally Friendly Hatchery Technology for Udang Galah, the King of Freshwater Prawns and a Glimpse into the Future of Aquaculture in the 21st Century
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24. Prof. Dr. Sharifuddin Haji Abdul Hamid
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Fish Processing and Preservation: Recent Advances and Future Directions
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Microbial Fermentation and Utilization of Agricultural Bioresources and Wastes in Malaysia
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29. Prof. Dr. Abdul Aziz Abdul Rahman
Sustained Growth and Sustainable Development: Is there a Trade-Off 1 or Malaysia
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Sharecropping in Perfectly Competitive Markets: A Contradiction in Terms
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