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# Microbial and Processing Criteria for Production of Probiotics: A Review

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**Summary**

The use of probiotics has become widely accepted as a natural means to promote health for both humans and animals. Today, probiotics are used as health supplements in food and feeds and they are replacing the use of antibiotic growth promotors or chemical supplements. Under the right conditions the claims made for probiotic preparations can be realized. The development of suitable technology for probiotic production, taking into account viability and stability, is a key area of research for industrial production. Production of probiotics should be based on the microbial criteria, and the ability to withstand stress during processing and storage of products is important. Thermophilic/thermotolerant probiotics are of great interest in this area as they can have all the desired characteristics. This review makes an overview of probiotic selection studies including new technologies for isolation/identification, adhesion and immune response. The importance of multistrain cultures is also stressed. The development of suitable probiotics in food and feed needs good proof of their efficacy and function in order to be accepted as a valuable product.

*Key words*: probiotics, thermophilic, thermotolerant, multistrain

## Introduction

Developing probiotic food and feed is a key research and development area for future functional food markets. Probiotic foods are defined as »foods containing live microorganisms which actively enhance health of consumers by improving the balance of microflora in the gut when ingested live in sufficient numbers« (*1*). In animal feeds it refers to »live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance« (*2*). The use of probiotics to improve productivity in livestock is currently generating a great deal of interest. As microbial feed additives, probiotics offer potential as an alternative to antibiotic growth promotors, the use of which the European Union wants to phase out by 2006 (*3*). They have been reported to have many beneficial effects when used in animal feeds as a means of controlling pathogen carriage, which include competitive exclusion of pathogens (*4,5*) and improved digestion and absorption of nutrients (*4,6,7*). These have a positive effect on the growth rate and feed conversion. Therefore, the use of probiotic feed additives is of interest as a cost-effective alternative to controlling animal disease and improving breeding performance (*8*).

However, there is considerable discussion on the actual measurable benefits that such system brings in food and feed products. This paper discusses the selection criteria, desirable characteristics and some new developments in the field for screening new probiotic strains with suitable biological characteristics and resistance to stress during processing, shelf life and passage through alimentary canal.

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## Selection of Probiotics for Animal Feed Production

A summary of conventional criteria that can be used for the selection of microbial strains to be used as probiotics includes the following properties (*1*,*8*):

- *Biosafety*: the strains of microorganisms should be Generally Recognized As Safe (GRAS microorganisms), for example, *Lactobacillus* species or some *Bifidobacterium* and *Streptococcus* (*Enterococcus*) species.
- *The choice of the origin of the strain*: probiotics should preferentially originate from the target animal microflora. This choice is determined by the specific purpose of the application of the probiotics (*e.g.* location specificity or requirement for colonization). The strains should be properly isolated and identified before use.
- *Resistance to in vivo/vitro conditions*: after administration of the probiotic, the microorganisms should not be killed by the defense mechanisms of the host and they should be resistant to the specific conditions occurring in the body. They should be resistant to the pH, bile and pancreatic juice conditions.
- *Adherence and colonization of intestinal epithelium/tissue*: factors that affect colonization should be considered. These should include the resistance of bacteria themselves, the effect of gastrointestinal environment (ingredient, pH, bile, salt, *etc*.) on colonization, the existing microbes that exert interacting factors (probiotics-host-microflora interactions), *etc*.
- *Antimicrobial activity/antagonisms to pathogens*: lactic acid bacteria, which are frequently used as probiotics, have a number of antagonistic properties which operate by decreasing pH by the production of lactic acid, consumption of available nutrients, decreasing the redox potential, production of hydrogen peroxide under aerobic conditions, production of specific inhibitory components, such as bacteriocines, *etc*., and which would help to protect against pathogenic organisms. This is important for the probiotics to be effective.
- *Stimulation of immune response*
- *Viability/survival and resistance during processing (e.g. heat tolerance or storage)*

The choices of mono or multi strains, beneficial systematic effects (*e.g.* prebiotic-synbiotic system) and other properties such as oxygen tolerance, selective stimulation of beneficial bacteria and suppression of harmful bacteria are also considered (*9*).

Based on these predetermined criteria and specific properties that are desired, it should be possible to select the best commercially available probiotics that can be helpful therapeutically and nutritionally. However, the criteria for probiotics in feed need not be as specific as those required for food applications.

## Viability of Probiotic Organisms

Viability and stability of probiotics has been both a marketing and technological challenge for industrial producers. For the probiotics to be functional they have to be viable and in sufficient dosage levels (*10*). The production of probiotic supplements for food/feed requires that the strains maintain a suitable level of viable cell count during the processing of the product and its shelf life. Once a desirable culture is selected, the technological demands placed on probiotic strains are great and new manufacturing process and formulation technologies may often be required to retain viability and hence functional health properties. Before probiotic strains can be delivered to food/feed products, they should survive processing and gastrointestinal stress factors and maintain their biological function within the host (*11*). All of these criteria need to be taken into account in the choice of a probiotic strain.

Microencapsulation is an available technology which allows probiotics to be formulated into food systems and helps them preserve their viability to be delivered into the gastrointestinal tract. Thermal processing for obtaining a storable form of the probiotics, and the choice of proper feed matrix suitable for retaining the viability of the probiotic during the production of the pellet need to be appropriate.

Thermophilic/thermotolerant organisms have an advantage in that they withstand higher temperature during processing and storage (*12*,*13*). They have a better chance of remaining viable during the drying process required for prolonged storage and they lead to a distinctly effective product. Attempts are thus being made to select or isolate thermophilic/thermotolerant probiotics from the available sources and examine the survival of these strains during spray drying and to define the optimal processing parameters for obtaining the best products.

To achieve health benefits, probiotic bacteria must be viable and available at a high concentration, typically $10^6$–$10^7$ CFU/g of product (*14*). In different countries specific requirements vary depending on products. Despite the importance of viability, surveys conducted to validate viability claims have shown low populations of probiotic bacteria in probiotic foods (*15*). Several factors have been claimed to be responsible for the loss of viability of probiotic organisms: acidity of products, acid produced during refrigerated storage (post acidification), level of oxygen in products, oxygen permeation through the package, sensitivity to antimicrobial substances produced by bacteria (*16*). Strategies to improve viability of probiotic organisms are appropriate selection of acid and bile resistant strains, use of oxygen impermeable containers, two-step fermentation, microencapsulation, stress adaptation, and incorporation of micronutrients such as peptides and amino acids. The survival of the strains during the passage through the stomach can be studied *in vitro* in gastric juice. Study showed that, among several strains of *L. acidophilus* and *Bifidobacterium* spp., only a few strains survived under the acidic conditions and bile concentrations normally encountered in fermented products or in the gastrointestinal tract (*15*). Once a desirable culture, which is resistant to acid and bile conditions, is selected, it must be stable with regard to viability and level of activity during production and storage. Most food/feed manufacturers use strains of probiotic that can retain their viability (*i.e.* remain alive and grow well to high number) through the manufacturing pro-

cess, storage conditions, the product's shelf life, and the distribution times.

## Thermophilic LAB (T-LAB)

Thermophilic lactic acid bacteria (T-LAB) are well-known for their biotechnological importance in the production of cheeses and fermented milk, which all require incubation of milk or curd at a relatively high temperature (45 °C or above) during their production process. Thermophilic bacteria that are mainly used as dairy starters belong to three species: *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Lactobacillus delbrueckii* ssp. *bulgaricus* or *lactis* (*17*,*18*). *L. casei* and *L. plantarum* are also grouped in thermophiles and used in dairy products. Delcour *et al*. (*19*) have reviewed the genetic and other important characteristics required to understand T-LAB. The manner in which they exploit milk protein and sugar, adapt to stress, and horizontally exchange genetic information which may help in screening the T-LAB from the available sources is described in the following paragraphs.

### *Proteolytic systems*

The ability to produce a cell wall-bound extracellular proteinases (CEP) is a very important feature of T-LAB in the hydrolysis of milk proteins (casein), providing the cells with the amino acids that are essential for growth of LAB. Cell envelope-associated proteinases are detected and characterized in various thermophilic lactobacilli (*20–23*). Genes encoding a cell wall-bound protease in T-LAB are *prtB*, *prtH*, *prtY* and *prtS*. For LAB peptidases, gene encoding are PepC, PepN, PepS and PepO. Screening of thermophilic/thermotolerant lactobacilli based on proteolytic activity is another method to identify thermophilic probiotics with the information of the cell wall-bound proteinase characteristics. Further genetic information is considered useful to improve know ledge of the functional properties of LAB and T-LAB.

### *Stress response*

During the yogurt and cheese making process, T-LAB are exposed to various changes in environmental parameters (heat, acidity, salt, cold, *etc*.) and induce adaptive responses allowing them to cope with the resulting physiological stresses (*19*). The GroES, GroEL, and DnaK proteins were found to be both heat- and acid-shock inducible in *L. delbrueckii* ssp. *bulgaricus*. A gene encoding a small 17 kDa heat-shock protein widely distributed in prokaryotic and eukaryotic organisms was reported to be plasmid-encoded in various *S. thermophilus* strains (*24*,*25*).

Other important genetic information on characteristics of T-LAB are sugar uptake and glycolysis, catabolite repression, exopolysaccharides, horizontal gene transfer, bacteriophage resistance, *etc*. (*19*). Such fundamental knowledge can be applied in the future for the improvement of T-LAB starters and in other fields of study using these microorganisms for further applications.

## Recent Studies on Processing of Thermophilic/Thermotolerant Lactic Acid Bacteria

A major challenge associated with the application of probiotic cultures in functional foods is the retention of viability during processing. Some studies have demonstrated that following spray drying, probiotics show increased sensitivity to cell wall, cell membrane and DNA damage (*26*,*27*). A number of approaches have been examined with a view of improving culture viability during spray drying. The survival of thermophilic probiotic cultures (*e.g. L. paracasei*) during cheese making and spray drying has been successful. Up to 49 % survival was obtained following spray drying at an outlet temperature of 80–85 °C (*13*). Some thermophilic lactic acid bacteria isolated and identified from dairy products (*28*) were classified as *Lactobacillus delbrueckii* ssp. *lactis* and ssp. *bulgaricus*, *L. helveticus* and *L. acidophilus*. Nitisinprasert *et al.* (*29*) successfully isolated effective thermotolerant lactobacilli which exerted broad spectrum inhibition specific (SIS) results from chicken intestine and they were classified as *Lactobacillus reuteri*. Identification of new thermotolerant species of lactic acid bacteria isolated from animal hosts like chicken has been conducted recently using molecular identification method. New isolated thermotolerant species from chicken faeces are *Lactobacillus thermotolerans* (*30*) and *Lactobacillus aminata* sp. nov., and some species belong to *Lactobacillus paraplantarum* (*31*).

The effect of heat shock and the induction of a stress response in *Lactobacillus* spp. have been studied for several thermophilic LAB (*13*,*32–39*). Methods of heat tolerance of mesophilic *Lactobacilli* are studied using pressure pretreatment on human origin *L. rhamnosus* GG (*40*). Cells exposed to pressure pretreatment showed higher survival than untreated cells when exposed to high temperature at 60 °C and heat adapted method showed greater thermotolerance compared to the control (*40*,*41*). Heat adaptation with cross-protection (*e.g.* heat exposure and simultaneous exposure to sublethal levels of hydrogen peroxide or bile salts or NaCl) was also studied to induce against heat stress of *L. paracasei* ssp. *paracasei* (*41*). The effect of a combination of factors (*e.g.* ethanol and pH) on heat resistance of thermophilic *L. delbrueckii* was investigated by Casadei *et al*. (*42*). Silva *et al*. (*43*) showed the induction of stress tolerance by the addition of sucrose to the growth medium as the accumulated compatible solutes during short period of drying process and storage. Saarela *et al*. (*44*) reported that sublethal treatments of acid and heat treatments of stationary phase probiotic cultures (*Lactobacilli* strains) improved the viability and enhanced their survival during lethal treatments and their ability to adapt these treatments at fermentor-scale production of probiotic cultures. Therefore, strain-specific treatments and sublethal treatments of specific growth phase probiotic cells can be utilized in the production of probiotic cultures with improved viability. The methods of pretreatment, heat adaptation and other factors that affect such investigation are very important for further studies on the viability of probiotic bacteria during heat processing.

Study of fatty acid modification in relation to the temperature is also a method to discriminate T-LAB

strains (*30*). The changes in lipid composition enable the microorganisms to maintain membrane functions in the face of environmental fluctuations. In particular, temperature-induced variations in lipid composition of bacteria are generally thought to be associated with the regulation of liquid crystalline to gel phase transition temperature for the maintenance of an ideal »functional« physiological state of cell membrane.

Interest in the stress response phenomenon of LAB species has grown (*26*,*37*). Understanding the mechanism of stress may lead to the development of cultures with improved capacity to survive and function under industrial production conditions. Examination of the increased degree of thermotolerance and suitability for spray drying conferred on probiotics by adaptation to different stresses is another challenging step being addressed. In addition, the effects of various rehydration conditions on culture performance should be taken into account, given that resuscitation of dried cultures may represent a critical control point in obtaining effective probiotic strains.

## Probiotic Selection, Isolation and Methodology Development

Since genetic stability is also required in probiotics, isolated specified target of functional strain needs to be identified. LAB belonging to the genus *Lactobacillus* have been isolated from a variety of habitats, including plant and dairy products, meat products, sewage and manure, and humans and animals. Several isolates from animal host like chicken were found to be acid and bile tolerant (*45–47*). Although several *Lactobacillus* spp. have been isolated from chicken faeces, most of the strains isolated so far have been mesophiles. However, the isolation of *Lactobacillus* spp. from chicken faeces under high temperatures has been studied and reported by Niamsup *et al*. (*30*). In their studies, they isolated a large number of LAB from various types of natural samples at relatively high temperatures, *i.e.* 40–50 °C. A recent study (*48*) used species and group-specific 16S rRNA-targeted oligonucleotide probes, dot blot hybridization (DNA-DNA hybridization) and fluorescent *in situ* hybridization (FISH) methods. The probes were designed in a way that some of the T-LAB detect novel species of *L. thermotolerans* and other LAB in chicken faeces. FISH analysis performed with group-specific 16S rRNA-targeted oligonucleotide probes was also successively used to analyze the fecal microflora of host consuming probiotic product containing specific lactobacilli (*48*).

The use of PCR assay (polymerase chain reaction) method with the 16S rRNA analysis has been studied in many works on identification of organisms isolated from chicken host, human faeces and animal feed (*49–52*). A new real-time quantitative PCR with 16S rRNA gene-targeted species/group-specific primers has recently been introduced to analyze LAB in intestine and faeces of human host (*53–55*). A real-time PCR assay for rapid and sensitive detection of a novel thermotolerant bacterium, *Lactobacillus thermotolerans*, in chicken faeces was developed and assessed by Selim *et al*. (*56*). Whole-cell protein profiling method by SDS PAGE is an additional studied method to help identify a new thermotolerant species of LAB isolated from chicken faeces (*31*) and to identify the isolates from 55 European probiotic products (*57*).

A novel multiplex polymerase chain reaction (PCR) primer set for the identification of a number of probiotic *Lactobacillus* species simultaneously has been developed (*58*). It uses the primer sets comprising of specific and two conserved primers derived from the integrated sequences of 16S and 23S rRNA genes and their rRNA intergenic spacer region of each species with 93.6 % accuracy. This exceeds the accuracy of the general biochemical methods. The phylogenetic analyses, using 16S rDNA sequences of the probiotic isolates, also provided further support that the results from the multiplex PCR assay were trustworthy. The results suggested that the multiplex primer set of PCR is an efficient tool for simple, rapid and reliable identification of seven *Lactobacillus* species.

### *Adhesion/colonization*

Adhesion and colonization are important for selection and use of probiotic strains. Scanning electron microscope (SEM) has been introduced to study the density and survival of probiotics in chicken intestine after feeding chicken with the probiotic supplements (*59*). Different methods are used to study bacterial adhesion to intestinal epithelial cells, which is an important step in pathogenic infection as well as in probiotic colonization of the intestinal tract. Le Blay *et al.* (*60*) conducted comparative detection of bacterial adhesion to Caco-2 cells with ELISA, radioactivity and plate count methods. The methods tested gave similar results for the highest bacterial concentrations. However, differences among methods increased with the addition of decreased bacterial concentration due to different detection thresholds. The ELISA-based method was shown to be a good predictor for bacterial adhesion compared to the radiolabelling method when good quality specific antibodies were used. This technique is convenient and allows handling of numerous samples. Fluorescent staining of *Lactobacillus* and *Bifidobacterium* strains and a *Bifidobacterium* mixture using the viable probe carboxyfluorescein diacetate was attempted. These cells were incubated on Caco-2 monolayers and subsequent spectrofluorimetric detection following the lysis of the attached bacterial cells was done. The results were expressed as adhesion percentage (*61*), and they proved that fluorescent labelling is suitable for adhesion studies and provides a reliable and safer alternative to radioactive labelling.

### *Immune response*

Transmission electron microscopy (TEM) was used to determine how probiotic strains in contact with intestinal epithelia result in an immune response in mice (*10*). Development and validation of a new *in vitro* assay for selection of probiotic bacteria that express immune-stimulating properties in chickens *in vivo* was studied by Koenen *et al*. (*62*). They used an *in vitro* system for rapid preselection of LAB with immunomodulating properties. For T-cell proliferation following mitogenic stimulation, activation of accessory cells is required. The pre-selection assay was based on a concanavalin A (ConA)

mitogen-induced lymphocyte proliferation assay, in which enhancement or inhibition of the response was the result of the immunomodulating properties of LAB for which either T-cells or accessory cells may be sensitive.

## Multistrain Selection

According to some authors, functionality of a multistrain/multispecies probiotics could be more effective and more consistent than that of a monostrain (*63,64*). Studies on the interactions among lactic acid starter and probiotic bacteria have been investigated to establish adequate combinations of strains (*65*). A required dosage of the probiotic supplemented with normal feed should be ascertained depending on a delivery pattern and form of probiotic products when incorporated into animal feed. This review cites recent studies and methods of development of probiotics for potential functional food/feed products.

Functionality of a multistrain probiotic could be more effective and more consistent than that of monostrain probiotic. Colonization of an ecosystem providing a niche for more than 400 species in combination with individually determined host-factors is anticipated to be more successful with multistrain (multispecies) of probiotics than monostrain preparations (*66,67*). Interaction among lactic acid starter and probiotic bacteria has been investigated to establish adequate combinations of strains to manufacture probiotic dairy products using strains of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactococcus lactis*, *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium* spp. (*63*). The detection of bacterial interactions was carried out using well-diffusion agar assay, and the interactions found were further characterized by growth kinetics. The study of interactions by growth kinetics allowed the setting of four different kinds of behaviour between species of lactic acid starter and probiotic bacteria (stimulation, delay, complete inhibition of growth, and no effects among them). The possible interactions among the strains selected should be considered when choosing the best combinations in order to optimize their performance in the process and their survival in the products (*65*). Recently, functionality and efficacy of monostrain, multistrain and multispecies probiotics have been summarized by Timmerman *et al*. (*63*). From a wide range of work trials in different hosts, multispecies probiotics were found to be superior in treating antibiotic-associated diarrhea in children, improve growth performance and particularly mortality in broilers and showed better protected mice against *S. typhimurium* infection. Also, multispecies probiotics provided the best clearance of *E. coli* O157:H7 from lambs (*63*). Rats challenged with *S. enteritidis* showed best post-challenge mass gain when treated with multispecies probiotics. Possible mechanisms underlying the enhanced effects of probiotic mixtures are also discussed (*63*). It is emphasized that strains used in multistrain and multispecies probiotics should be compatible and preferably synergistic. The design and use of multistrain and multispecies probiotics need to be studied well before use.

## Application of Probiotics in Feed Processing

Probiotics are incorporated into animal feed as one of specific additives (including antioxidants, binding agents, preservatives, enzymes) in different ways (*e.g.* during or after processing or top-dressing feed mix). In Europe this comes under strict legislation for reared species or groups of species. Maximal authorized doses and conditions of use are specified. Feed manufacture involves pelleting, extrusion process and complementary processes, requires pressures and high temperatures which may affect the viability of probiotics applied in the feed. Typical feed for broiler chickens is processed at about 75–85 °C for 15–20 s with a moisture content of 15 % before pelleting.

In order to maintain viability and survival of probiotics during feed manufacturing, cultures (broth, dry), drying method used (spray-dry, freeze-dry), single or mixture culture (monostrain or multispecies), feed matrices (*e.g.* alginate beads, xanthan-gellan beads, fermented feed), synbiotic effect (prebiotics), *etc.* are studied (*68*).

Composite carrier matrix systems are being developed for the purpose of protecting probiotics from the stress and heat (spray-dry) treatment. Leverrier *et al*. (*69*) investigated tolerance of probiotics to stress in digestive tract with simulated gastrointestinal juice consisting of acid and bile salt by using different food matrices (alginate beads, xanthan-gellan beads, fermented milk). Tests on viability of bacteria on spray-drying in an aqueous binary mixture composed of skim milk and polyvinylpyrrolidone (PVP K90) as encapsulation matrix have also been done (*70*). Boza *et al*. (*71*) reported that dehydrated glucose syrups resulted in products with the greatest percentage of survival of probiotic cells microencapsulated in it during the spray-drying, compared to other carbohydrates (maltodextrin, gum acacia and modified starch materials).

In the freeze-drying method, glycerol and skim milk that act as cryoprotective agents appeared to preserve probiotic strain (*72*). Carvalho *et al*. (*73*) reviewed several relevant factors for the preparation and preservation of freeze-dried lactic acid bacteria, including specific effects of intrinsic factors, growth factors, sublethal treatments, drying media, storage and rehydration. Freeze-drying, however, is a costly method for large scale production of such products.

Probiotic milk-based formulations were spray-dried with various combinations of prebiotic substances in an effort to generate synbiotic powder products. The effect of growth phase and inclusion of a prebiotic substance in the feed media on probiotic viability during spray-drying was studied. It was found that *Lactobacillus rhamnosus* GG, spray-dried in stationary phases of growth in reconstituted skim milk, as well as polydextrose (PD) mixture at an outlet temperature of 85–90 °C survived best (31–50 %) in both feed media and were the most stable during powder storage at 4–37 °C during 8 weeks, with 30–140-fold reductions in cell viability at 37 °C in RSM and PD/RSM powders, respectively (*74*).

## Prebiotics

Prebiotics are nondigestible carbohydrates including lactulose, inulin, and a range of oligosaccharides that supply a source of fermentable carbohydrates for beneficial bacteria in the colon (*75*). They selectively stimulate the proliferation and/or activity of populations of desirable bacteria *in situ* (*76,77*). Prebiotics might influence the growth and survival of the probiotics by influencing the growth and metabolites of both the probiotics and the starter. Rycroft *et al.* (*78*) compared *in vitro* fermentation properties of commercial prebiotic oligosaccharides and found that particular prebiotics increase cell number of specific bacteria in the gut. Xylooligosaccharides and lactulose produced the highest increase of *Bifiodobacteria*, whereas fructooligosaccharides produced high population of lactobacilli. The study provided comparative data on the properties of commercial prebiotics, allowing targeting of dietary intervention for particular applications and blending of oligosaccharides to enhance overall functionality.

Due to the potential synergy between probiotics and prebiotics, foods containing a combination of these ingredients are often referred to as synbiotic (*76,79*). Interaction between the probiotic and the prebiotic *in vivo* might be favoured by an adaptation of the probiotic to the prebiotic substrate prior to consumption. This might result in a competitive advantage for the probiotic if it is consumed concurrently with the prebiotic (*11*). In animal models, the inclusion of resistant starches in the diet has been shown to increase the numbers of probiotics (*80,81*). The benefits of using resistant starch extend beyond traditional prebiotics, since resistant starch can be used to ensure the viability of probiotic populations from the food to the large intestine. Resistant starch offers an ideal surface for adherence of the probiotics to the starch granule during processing, storage and transit through the upper regions of the gastrointestinal tract, providing robustness and resilience to environmental stresses. Bacterial adhesion to starch may also provide advantages in new probiotic technologies to enhance delivery of viable and metabolically active probiotics to the intestinal tract (*82*). This includes the technology to encapsulate probiotics within starch granules that are then coated with amylase (*83*). Binding of adhesive strains to the resistant starch core may facilitate encapsulation of the bacteria using this technology.

## Conclusion

For successful delivery of probiotics in products to the intestine, the beneficial microorganisms must survive food processing, storage during product maturation and shelf life, as well as the stress conditions in the host gastrointestinal tract system. The selection of probiotics with all these characteristics is a technological challenge and is evaluated according to the viability and stability of probiotics in the final probiotic product. The criteria for the selection of probiotics thus also includes acid tolerance, bile tolerance, heat tolerance, and ability to metabolize prebiotics, adherence and colonization to intestinal epithelium/tissue, stimulating immune response, antimicrobial activity/antagonisms to pathogens, improving host digestion, *etc.* Fundamental knowledge of specific characteristics of proteolytic activity, stress response, *etc.* of thermophilic LAB can be applied for the improvement of T-LAB and other mesophilic probiotics. Development of isolation and identification methods requires a molecular method to confirm the genetic stability of the isolated microorganism from the sample using different methods *e.g.* species and group-specific 16S rRNA-targeted oligonucleotide probes, dot blot hybridization (DNA-DNA hybridization) and fluorescent *in situ* hybridization (FISH) method with the use of multiplex and real-time PCR assays. ELISA-based method and fluorescent labelling are suitable for adhesion studies and provide a reliable and safer alternative to radioactive labelling. Study of functionality of multistrain probiotics has been shown to be more effective and more consistent than that of monostrain probiotics. Also, factors related to feed technology for development of probiotics in animal feed are to be taken into account for the future probiotic products. Prebiotics like resistant starch can be incorporated with probiotics (synbiotic system) as a composite carrier matrix system to increase the survival of probiotics of the stress and heat (spray-dry) treatment and make the system more effective. Overall choice of suitable probiotics which can survive stresses during processing, and passage through alimentary canal followed by their proliferation and ascertaining if they carry out their biological role in food and feed is important for these probiotics to be applied successfully.

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