18 Manufacture of Probiotic Bacteria

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18.1 Introduction

Lactic acid bacteria (LAB) have been used for many years as natural biopreservatives in fermented foods. A small group of LAB are also believed to have beneficial health effects on the host, so called probiotic bacteria. Probiotics have emerged from the niche industry from Asia into European and American markets. Functional foods are one of the fastest growing markets today, with estimated growth to 20 billion dollars worldwide by 2010 (GIA, 2008). The increasing demand for probiotics and the new food markets where probiotics are introduced, challenges the industry to produce high quantities of probiotic cultures in a viable and stable form. Dried concentrated probiotic cultures are the most convenient form for incorporation into functional foods, given the ease of storage, handling and transport, especially for shelf-stable functional products. This chapter will discuss various aspects of the challenges associated with the manufacturing of probiotic cultures.

18.2 Selection of Strains

For a strain to be considered probiotic, it should adhere to certain criteria as follows. Preferably the microbes should have GRAS (Generally Regarded As Safe) status, have a long history of safe use in foods, be non-pathogenic, and acid and bile tolerant (Morgensen et al., 2002). Probiotics are described as "live microorganisms which, when administrated in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). However, there is no general consensus as to whether probiotics should be viable in all cases to exert a health benefit, with some studies demonstrating that non-viable probiotic bacteria can have a beneficial effect on the host (Ouwehand and Salminen, 1998; Salminen et al. 1999). While most probiotic products are developed for the dairy industry, they are also used in non-dairy foods, such as energy bars, dietary supplements and pet food.

Selection of the right probiotic strain is crucial and in general probiotic bacteria need to be viable in the product at the end of shelf-life for a product to be commercially successful. This requirement poses challenges during processing and following ingestion. For example, the bacteria are exposed to different technological stresses, such as acid, osmotic, cold, heat and drying stress. Probiotic strains are generally of the genera *Lactobacillus* and *Bifidobacterium*, and to a lesser extent *Pediococcus, Propionibacterium, Enterococcus, Bacillus, Streptococcus* and *Saccharomyces* (Champagne and Møllgaard, 2008).

Selecting the appropriate strain for a particular food can be divided in four categories:

- Performance in the gastrointestinal tract (GIT)
- Industrial production
- Safety of the microorganisms
- Health benefit

This chapter will focus on the first two categories. The remaining categories are described elsewhere.

18.2.1 Performance in the Gastrointestinal Tract

Following ingestion, probiotics pass through the stomach before they reach the small intestine. The acidity of the stomach is known to fluctuate, from pH 1.5 to 6.0 after food intake (Waterman and Small, 1998). In the stomach, exposure to gastric acid and the proteolytic activity of pepsin can result in viability losses. Furthermore, bile acids and pancreatin in the small intestine present further challenges to viability of probiotics during transit through the gastrointestinal tract (GIT) (Hofmann et al., 1983; Lee and Salminen, 1995).

In vitro methods have been developed to select for strains that can withstand the extreme conditions in the stomach. While there has been no consensus on the pH range that needs to be analyzed for the selection of potential probiotics, values between 1 and 5 have been studied extensively. For instance, *L. acidophilus* challenged at pH 3 showed higher acid tolerance when challenged in broth compared with phosphate buffer (Hood and Zoitola, 1988). Similarly, LAB survived better at low pH in milk than in buffered saline, and furthermore survival of LAB increased in the stomach (*in vivo*) when administered with milk (Conway et al., 1987). Collado and Sanz (2006) selected for acid resistant

bifidobacteria by prolonged exposure of human feces to low pH, and subsequently exposed the isolated pH tolerant bacteria to high bile salt concentrations and NaCl. This selection method proved very successful for the isolation of potential probiotics. Others screened *L. casei* strains for probiotic properties, such as acid and bile tolerance, adhesion to epithelial cells, antimicrobial effects and cholesterol reduction and showed there was great variation between strains (Mishra and Prasad, 2005). Similar variation was also observed in a study where thirteen spore-forming bacilli belonging to *Sporolactobacillus, Bacillus lavolacticus, Bacillus racemilacticus* and *Bacillus coagulan*s species were screened for resistance to acidic conditions and bile. Only five bacteria were capable of growth in MRS adjusted to pH 5, and all *Bacillus coagulans* and *racemilacticus* tolerated bile concentrations over 0.3% (Hyronimus et al., 2000). Propionibacteria have been increasingly used in functional foods. *In vitro* assays for the transit tolerance of these bacteria showed strain specific resistance for acid (pH 2.0–4.0). All propionibacteria tested survived simulated small intestinal conditions (Huang and Adams, 2004). For simulating the small intestine, bile salt concentrations between 0.15 and 0.3% have been recommended (Goldin and Gorbach, 1992), which agrees with the physiological concentration in the GIT. Following initial acid tolerance screening in modified MRS (pH 3.0), it was reported that one *Bifidobacterium breve* strain showed better survival capabilities when exposed to 0.5% pepsin, 1% pancreatic, and better adhesion properties out of 35 bifidobacteria strains tested, including *B. infantis, B. longum, B. bifidum, B. adolescentis, B. breve, B. animalis, B. asteroids, B. globosum* and *B. pseudocatenulatum* species (Liu et al., 2007).

In addition to overcoming the stresses encountered in the stomach and small intestine, adherence to epithelial cells is considered a desirable probiotic trait (Guarner and Schaafsma, 1998). Several studies have shown that probiotics can adhere to epithelial cells *in vitro*, with the most common cell lines used for these experiments being HT-29, HT-29MTX, Caco-2 and Int-407 lines (Bernet et al., 1994; Fernandez et al., 2003; Sarem et al., 1996). HT-29, Caco-2 and Int-407 cell lines have the characteristics (morphology and physiological) of normal human intestinal cells. HT-29MTX is a mucus excreting form of HT-29. These cell lines have been cultured for the analysis of the adhesion of enteropathogens, and also for the analysis of the adherence of probiotics. Although adherence is deemed an important probiotic trait, not all probiotics have been proven to colonize the intestine *in vivo*. *Lactobacillus rhamnosus* GG has been reported to remain in the human GIT for approximately 1 week after administration is discontinued (Alander et al., 1999; Goldin and Gorbach, 1992).

It has been shown that some probiotics do not have to be viable to prevent adherence of pathogens to epithelial cells. For example, heat killed *Lactobacillus acidophilus* inhibits the adherence of diarrheagenic *Escherichia coli* to Caco-2 cells *in vitro*, by competitive exclusion (Chauviere et al., 1992). Several studies have shown that probiotics, mainly lactobacilli and bifidobacteria, can prevent or minimize adhesion of pathogens (Bernet et al., 1994; Neeser et al., 1989).

Other approaches for selection of probiotics are screening for antimicrobial activity and ability to stimulate the host immune response. Animal studies have shown that the immune response is up-regulated when probiotics are consumed (Galdeano and Perdigon, 2006). These assays are very useful, but time consuming and costly to perform as a screening method. On the other hand, selecting antimicrobial activity against a certain pathogen, using common microbiological methods is a more feasible selection method. The relatively low cost of genome sequencing has opened the way to functional genomic studies of a variety of probiotics, providing insight into the molecular basis for probiotic traits as production of antimicrobial compounds, adhesion or adaptation to the environment. These developments could lead to novel probiotic screening methods on a genomic level (Dellaglio et al., 2005; Schell et al., 2002).

18.3 List of Commercial Strains

Food products supplemented with probiotics are gaining popularity worldwide, and manufacturers are increasingly developing new probiotic products. *Table 18.1* shows a list of some commercial available probiotic cultures and their manufacturers. A list of some probiotic dairy foods and the strains they contain is given in *Table 18.2*.

18.4 Growth Media and Conditions

To produce probiotics in adequate amounts, the growth media need to be optimized for the specific strain aiming for increased biomass yield and reduction of productions costs. There are two types of fermentation media used commercially; i.e., synthetic and dairy based media. When using synthetic

Table 18.1

Manufacturers/suppliers of probiotic cultures (*Cont'd* p. 730)

Manufacturer	Probiotic Strain
Cargill (Minneapolis, USA)	*Bifidobacterium animalis* subsp. *lactis* Bf-6
	Lactobacillus johnsonii La-1
	Lactobacillus johnsonii La-19
	Lactobacillus paracasei LCV-1
Cell Biotech Europe (Copenhagen, Denmark)	*Bifidobacterium bifidum* BF2
	Bifidobacterium breve BR2
	Bifidobacterium infantis BT
	Bifidobacterium lactis BL2
	Bifidobacterium longum BG3
	Enterococcus faecium EF1
	Lactobacillus acidophilus LH5
	Lactobacillus casei LC1
	Lactobacillus rhamnosus LR3
	Lactobacillus plantarum LP1
	Lactococcus lactis SL1
	Pediococcus pentosaceus PP
	Streptococcus faecalis SFL
	Streptococcus thermophilus ST3
Cerbios-Pharma SA (Lugano, Switzerland)	*Enterococcus faecium* SF68
Chr. Hansen (Hørsholm, Denmark)	*Bacillus licheniformis* and *Bacillus subtilis* BioPlus 2B
	Bifidobacterium lactis BB12
	Lactobacillus acidophilus LA5
	Lactobacillus paracasei subsp. *paracasei* CRL-431
	Lactobacillus reuteri RC-14
	Lactobacillus rhamnosus GG
	Lactobacillus rhamnosus GR-1
	Streptococcus thermophilus TH-4
Danisco (Copenhagen, Denmark)	*Bifidobacterium animalis* subsp. *lactis* B-420
	Bifidobacterium lactis HN019
	Lactobacillus acidophilus La-145
	Lactobacillus acidophilus NCFM
	Lactobacillus rhamnosus HN001
DSM (Heerlen, The Netherlands)	*Bifidobacterium* LAFTI B94
	Lactobacillus acidophilus LAFTI L10
	Lactobacillus casei LAFTI L26

Table 18.1

Manufacturer	Probiotic Strain
Morinaga (Tokyo, Japan)	*Bifidobacterium breve* M16V
	Bifidobacterium infantis M-63
	Bifidobacterium longum BB536
	Bifidobacterium pseudolongum M-602
	Enterococcus faecium FA5
	Lactobacillus acidophilus LA5
	Lactobacillus acidophilus LAC361
	Lactobacillus gasseri LAC343
	Lactobacillus plantarum LP83
	Lactobacillus rhamnosus LCS742
Valio (Helsinki, Finland)	*Lactobacillus rhamnosus* LGG

media in the food industry, the bacteria have to be extensively washed, before addition to the product. This prevents flavor carry-over from the media, and there may be regulations in place that will prevent from adding bacteria grown on synthetic media (Abu-Taraboush et al., 1998; Ventling and Mistry, 1993). Milk or yogurt based media are more suitable for use in the food industry. An additional advantage of using a natural medium is that the probiotics do not have to be separated from the medium, while a disadvantage is that only growth promoting supplements that have no adverse effect on the final product quality can be used. Probiotics in general require a large amount of growth factors, and thus the growth media can become very complex and expensive. There is not one ideal medium for all probiotics. Even within a species, there can be differences in optimal growth conditions. When probiotics are applied in functional foods, the type of energy source used in the fermentation greatly influences the probiotic performance of the product (Carvalho et al., 2003; Mattila-Sandholm et al., 2002). It is therefore suggested to grow the starter cultures on the same sugar that will be present in the final product matrix, and thus for dairy products, lactose is the preferred energy source. This will prevent a long adaptation phase, since when bacteria have to switch from glucose to lactose as an energy source, there is the need for induction of β-galactosidase. There has been extensive research on the optimization of growth conditions for lactobacilli and bifidobacteria, and the following section details the cultivation of these strains.

Table 18.2

Commercial probiotic dairy products

Product	Manufacturer	Probiotic cultures
ABC	Sitia YOMO	*L. acidophilus, B. lactis, L. casei*
Actimel	Danone	*L. casei* DN-114 001
Active-più	Parmalat	*B. lactis*
Activia	Danone	*B. bifidum*
Activia yogurt	Danone	*B. animalis* DN173 010
Align	Procter and Gamble	*B. infantis* 35264
BEBA	Nestlé	*S. thermophilus* and *B. lactis*
Bifidus yogurt	Morinaga	*B. longum* BB536
Bio Profit	Valio	*L. rhamnosus* LC-705, *Propionibacterium freudenreichii* subsp. *shermanii* JS
Biospega	Spega	*L. acidophilus, B. lactis*
Chamyto	Nestlé	*L. paracasei*
Culturelle	Valio	*L. rhamnosus* GG
DanActive	Danone	*L. casei* DN114 001
Femdophilus	Chr. Hansens	*L. reuteri* RC-14, *L. rhamnosus* GR-1
Florastor	Biocodex	*Saccharomyces boulardii*
Fyos	Nutricia	*L. paracasei*
Gaio	MD Foods plc	*Enterococcus faecium,* K77D *L. salivarius*
Gefilac	Valio	*L. rhamnosus* GG
Good Start Natural Cultures	Nestlé	*B. lacti*
Joie	Yakult	*L. casei* Shirota
LC1	Nestlé	*L. johnsonii*
Nu Trish	Chr. Hansens	*B. lactis* Bb-12, *L. acidophilus* La5, *L. casei CRL-431*
Rolly	Snow Brand	*Bifidobacterium* subsp.
Snow yogurt + 2	Snow Brand	*L. acidophilus* SBT2062
Stonyfield Farm yogurts	Biogaia	*L. reuteri* ATCC 55730
Teddy	Fattoria Scaldasole	*B. lactis*
TopOntbijt	Coberco	*B. lactis* Bb-12 *L. acidophilus* LA5
Vifit	Campina	*L. rhamnosus* GG
Viili	Valio	*Lc. lactis* subsp. *Cremoris*
Vitality	Müller	*L. acidophilus* LA5, *B. lactis* Bb-12
Yakult	Yakult	*L. casei* Shirota, *B. breve*
Yogurt	Meiji	*L. delbrueckii* subsp. *bulgaricus* 2038
YoMi	Danisco	*B. lactis, L. acidophilus*
Yo-plus yoghurt	General Mills Inc.	*B. lactis* Bb-12

18.4.1 Bifidobacteria

Bifidobacteria are anaerobic bacteria and have strict requirements for nutrients in the media; they are however not as fastidious as lactobacilli. Bifidobacteria can be grown in semi-synthetic media containing only lactose, the free amino acids cysteine, glycine and trypthophan, several vitamins, nucleotides, and some minerals (Gomes and Malcata, 1999). In general, the media bifidobacteria contain nutritive growth supplements, and have low oxidation/reduction and pH value between 5.0 and 8.0, with the optimal pH being between 6 and 7, growth temperature between 37 and 41°C, with no growth above 45° and below 25°C (Gomes and Malcata, 1999; Kearney et al., 2008).

Most of the media used for the growth of bifidobacteria are derived from *Lactobacillus* media. The media described in *Table 18.3* are in general used for the growth of bifidobacteria. By supplementing the media with certain antibiotics, they can be made selective for bifidobacteria, and this has recently been reviewed by Kearney et al. (2008) and Roy (2001).

Synthetic media can be supplemented with special growth factors that increase the yield and growth performance of bifidobacteria. There is however, not one optimum medium for bifidobacteria, and therefore an optimum medium should be developed for each strain (Hartemink and Rombouts, 1999). Many approaches for the optimization of media for bifidobacteria have been taken.

Table 18.3

Culture media used for bifidobacteria, adapted from Roy (2001)

Media	Name	Additive
MRS	De Man Rogosa Sharpe	–
TPY	Tryptone Phytone Yeast	–
BL	Glucose Blood-Liver	–
CLB	Columbia	–
LCL	Liver Cysteine Lactose (Blaurock)	–
RCM	Reinforced Clostridia Medium	–
mMRS	Modified MRS	L-cysteine HCL, 0.05%
mMRS + blood	Modified MRS	L-cysteine HCL, 0.05% Sheep blood 10 ml
X-α-Gal	MRS	X-α-Gal
mBL	Modified BL without blood	L-cysteine HCL, 0.05%
mRCM	Modified RCM	Lactose 1.0% Human blood 50ml
RCPB	RCM	Prussian Blue 0.03%

Table 18.3 presents some common bifidogenic supplements used for growth promotion of bifidobacteria. Ideally, the media should have a low redox potential to cysteine, cysteine-hydrochloride or ascorbic acid are routinely used as reducing agents (Calicchia et al., 1993; Shah, 1997). Furthermore, β-glycerophosphate in combination with cysteine increased growth for *B. infantis* and *B. bifidum* in milk but had no effect on *B. longum* ATCC 15708 (Roy et al., 1990). Milk components such as whey and casein have been shown to have growth promoting capabilities. Petschow and Talbot (1990) reported increased growth of *B. bifidum serovar pennsylvanicus* and *B. longum* by addition of whey proteins (α-lactalbumin and β-lactoglobulin). Casein hydrolysates promoted growth for *B. infantis, B. breve* and *B. longum* (Proulx et al., 1994), while Rasic and Kurmann (1983) reported that extracts from potatoes, carrots and corn had an increased growth effect on bifidobacteria. Other reported growth factors include threonine, peptone, trypticase, dextrin, maltose and short chain fatty acids (Modler, 1994; Pacher and Kneifel, 1996). Yeast extracts were found to be effective growth promoters, and are generally added between 0.1 and 0.5% (v/v) (Gomes and Malcata, 1999). However, an earlier study showed that *B. infantis* did not exhibit enhanced growth when the medium was supplemented with 0.25% yeast extract, while there was more acid produced (Roy et al., 1990).

18.4.2 Lactobacilli

Lactobacilli require in general complex media containing a range of nutrients. A typical medium for *L. acidophilus* requires low oxygen tension, fermentable carbohydrate, proteins, vitamins, nucleic acid derivatives, unsaturated fatty acids and minerals such as magnesium, manganese and iron. It is reported that LAB prefer peptide bound amino acids rather than the free form (Benthin and Villadsen, 1996). By increasing the thiol groups in the media, using whey, an enriched milk flavor and increased growth of lactobacilli was obtained and furthermore, addition of peptone and trypsin promoted acid production (Kurmann, 1988). Growth can be inhibited by low pH during fermentation, and to prevent premature inhibition a buffer, such as phosphate buffer can be used to neutralize the acid production during fermentation. There is however, a limit to the amount of phosphate that can be added to the media given that high concentrations can be inhibitory because of the binding of metal ions, such as magnesium, calcium or manganese, which are essential for bacterial growth (Boyaval, 1989). Therefore, the phosphate concentration has to be adjusted to the strain used (Wright and Klaenhammer, 1983).

Experimental design was used to optimize the growth media for *L. acidophilus*, and optimal conditions were found to be pH 6.0, 30°C, 40 g/l glucose, 20 g/l peptone, 20 g/l yeast extract, 5 g/l sodium acetate and 3 g/l sodium citrate (Taillandier et al., 1996). Using response surface methodology, Liew et al. (2005) studied the combined effects of glucose, yeast extract, vitamins and pH on growth of *L. rhamnosus* ATCC7469. Optimal conditions included pH of 6.9, 1.28% vitamins, 5.0% glucose and 6.0% yeast extract. Using a similar approach, it was shown that by adjusting the inoculum size and by addition of the prebiotics, fructooligosaccharide and maltodextrin, growth and acid production of *L. casei* ASCC292 were increased (Liong and Shah, 2005).

Milk supplies the majority of the nutrients required for growth of lactobacilli. The composition of milk is typically, 87% water, 4.7% lactose, 3.8% fat, 33% protein 0.2% citrate, 0.6% minerals (Heller, 2001). Several studies have found that lactobacilli can grow up to 10^8–10^9 CFU/ml when grown in milk. Stationary phase is often reached after 24 h fermentation at 37°C, with pH between 3.9 and 4.4 (Gonzalez et al., 1993; Prajapati et al., 1987). There are several ways to improve growth of lactobacilli in milk, e.g., the milk can be supplemented with growth factors or if higher numbers are required after a certain time, the inoculum size can be increased. Supplements, such as manganese, acetate, fatty acids (e.g., oleic acid), tomato juice (Babu et al., 1992), casein powder (Miller and Puhan, 1981), whey protein (Marshall et al., 1982) or simple fermentable sugars (e.g., sucrose, fructose) (Srinivas et al., 1990) have been found to promote growth of lactobacilli. Furthermore, growth of *L. acidophilus* was optimized by adjusting skim milk media with 0.5% yeast extract and 1.0% glucose (Rana and Gandhi, 2000). Basal MRS media used for enumeration of *L. acidophilus* from yogurt can be optimized with supplements of maltose, salicin, raffinose or melibiose instead of dextrose (Hull and Roberts, 1984).

Although lactobacilli tolerate oxygen, they grow better with low oxygen concentration. Ascorbic acid was used by Dave and Shah (1998) as an oxygen scavenger and promoted growth and stability of *L. acidophilus*, while supplements of whey powder, whey protein concentrates and acid casein hydrolysates resulted in improved growth for *L. acidophilus* and bifidobacteria in yogurt.

18.4.3 Alternative Media

Probiotics are mainly grown in bovine milk, but studies have shown that they can also grow well in milk of other species, including camel (Abu-Taraboush et al., 1998), buffalo (Murad and Fathy, 1997) and goat (Gomes and Malcata, 1998).

However, Gomes and Malcata (1998) reported that *L. acidophilus* had insufficient growth in goat milk. Most of the research has been performed on bovine milk and that is the focus of this chapter.

Growth of lactobacilli and bifidobacteria is often slower in milk than in synthetic media. This is believed to be due to the low proteolytic activities of bifidobacteria (Dave and Shah, 1996; Klaver et al., 1993). Another factor affecting the growth of bifidobacteria negatively in milk is the generally low beta-galactosidase activity in bifidobacteria (Desjardins et al., 1991). The proteolytic activity can be increased by adding high proteolytic LAB to the media, however they should not outgrow the probiotic bacteria (Klaver et al., 1993). Similar to synthetic media, milk can be optimized by adding growth promoting supplements as mentioned earlier (Champagne et al., 2005; Elli et al., 1999).

Some efforts in making a media more suitable for probiotics have led to novel probiotic products based on tomato juice, peanut milk, soy milk, buffalo whey/soy milk and rice. Certain plant-extracts have been shown to benefit the growth of probiotics. For example, *L. acidophilus* was reported to grow significantly better in soy milk than bovine milk (Mital and Garg, 1992). This has also been observed in yogurt type products based on soymilk (Murti et al., 1993; Shelef et al., 1988). However, bifidobacteria do not grow well in soy milk (Macedo et al., 1998). Others have reported that certain lactobacilli can grow substantially in vegetable juices from cabbage and carrot (Savard et al., 2003).

18.4.4 Fermentation Methods

Batch or fed-batch fermentations are the preferred production processes in the dairy industry, since continuous fermentation requires costly concentration steps; however there are new developments in this fields which will be discussed later. With batch fermentation all the substrates and inoculum are mixed in a fermenter. The fermenter is temperature and pH controlled to fit the optimum growth conditions. When the desired probiotic concentration is reached, the process is stopped, the cells are harvested and the process is repeated. Depending on the quantities required batch fermenters can be as large as 10,000 L. Fed-batch fermentation allows the addition of a limiting substrate during the fermentation, and this technique is commonly used to increase bacterial concentrations. Producing probiotic cultures in fed-batch has the advantage that less exopolysaccharides are formed and thus the product is less viscous (Champagne et al., 2007). Fed-batch can also be applied to stress the bacteria at the end of fermentation to

induce a stress response (e.g., osmotic or solvent stress response) to protect them from subsequent processing steps.

Due to the difficulties in propagating a cell line on an industrial scale, starter cultures from manufactures in the form of direct vat set (DVS) cultures are preferred in the dairy industry. DVS cultures are highly concentrated freeze dried (approximately 1×10^{11} CFU/g) or deep-frozen (approximately 1×10^{10} CFU/g) cultures that can be used to directly inoculate the fermenter (Honer, 1995; Oberman and Libudzisz, 1998).

Low pH is one of the main reasons for the growth inhibition of LAB, and thus, by controlling the pH, higher biomass yields can be obtained. In batch processes, pH control is achieved by adjusting the pH with a base (e.g., ammonium or sodium hydroxide), or using a suitable buffer [e.g., N-Tris(hydroxymethyl) methyl-3-aminopropanesulfonic acid (TAPS) or phosphate buffer]. Another solution is to co-culture another microorganism that will counteract the produced acid by LAB. McCoy (1992) described a method where urease producing bacteria are co-cultured with LAB in a urea supplemented media. As both bacteria grow, urease hydrolyses urea to acid-neutralizing ammonia. Other techniques include the use of a saturated salt solution (e.g., $CaCO_3$) that gradually dissolves and neutralizes pH as the pH drops this technique is also suitable for controlling pH in agar plates. Up to 10 times more biomass can be obtained when pH control is used in fermentation. When bacteria are cultured with pH control, the specific acidification rates are lower than without pH control, which means that a higher inoculation volume or a longer fermentation time is needed (Savoie et al., 2007).

Besides traditional (fed) batch fermentation, there are other procedures available to produce high concentrated bacterial cultures. For example, Doleyres et al. (2004a) reviewed continuous fermentation and described the potential benefits. This technology could provide high cell yield and decrease the downstream processing for concentration as reported for *B. longum* ATCC15707 (Doleyres et al., 2002b). There is however, an increased contamination risk when applying this technology on an industrial scale (Lacroix and Yildirim, 2007). Taniguchi et al. (1987) reported seven times higher concentrations of *B. longum* when using a membrane bioreactor during fermentation. This reactor has a constant feed of fresh media, while the bacteria are kept in the reactor by an ultra- or microfiltration membrane. Hence, any growth inhibitory metabolites are removed from the system allowing more bacterial growth. Corre et al. (1992) also reported higher cell yield using a membrane reactor as opposed to free cell fermentation of *B. bifidum*. Schiraldi et al. (2003) reported increased cell concentration and metabolite production in a similar membrane reactor.

Another interesting development in fermentation is immobilizing the bacteria in the fermenter. Immobilized bacteria on fruit (apple and quince) were used to produce high quantities of food-grade lactic acid (Kourkoutas et al., 2005). The immobilized cells were used in subsequent batch fermentations with no significant loss in acid producing activity. Another form of immobilization can be achieved by embedding the bacteria in gel beads. This was achieved by entrapping the bacteria in spherical polymer beads with diameters ranging from 0.3 to 3.0 mm (Champagne et al., 1994; Lacroix, 2005). The active biomass is immobilized by thermal (κ-carrageenan, gellan, agarose, gelatine) or ionotropic (alginate, chitosan) gelation. Growth is observed in radial form in the beads, and this biofilm-like growth results in high cell release into the media, as a result from collision shearing forces in the reactor (Doleyres and Lacroix, 2005). Based on these techniques, several studies have shown high productivities of probiotic biomass. Ouellette et al. (1994) produced continuously fermented skim milk using immobilized *B. infantis* on κ-carrageenan/locust bean gum gel beads. Cell counts reached 2.2×10^9 CFU/ml and maximum volumetric productivity approximately 1×10^9 CFU/ml h. In another study, *B. longum* was immobilized on gellan gum gel beads (7×10^9 CFU/g) in MRS medium supplemented with whey permeate, which led to high cell production, ranging from 3.5 to 4.9×10^9 CFU/ml for *D* (Dilution rate) of 2–0.5 h^{-1} respectively (Doleyres et al., 2002b). This study also reported the highest volumetric productivity for *B. longum*, 6.9×10^9 CFU/ml/h. However, this high concentration was reached by using dilution rate of 2 h^{-1} which would lead to a cell wash-out.

In a two-stage fermentation, the effluent of one reactor flows into a second reactor. Two-stage fermentation can increase cell numbers and viability during downstream processing. Doleyres et al. (2004a) used a continuous two-stage fermentation to produce a high concentrated mixed culture (*Lactococcus lactis* subsp. *diacetylactis* and *B. longum*). In the first reactor, both strains were separately immobilized on κ-carrageenan/locust bean gum gel beads; the second reactor received free cells from the first reactor. This setup allowed for the continuous production of high concentrated cells, while the ratio of bacteria could be controlled by temperature (Doleyres et al., 2002a, 2004a). This production method also improved the stress tolerance in further downstream processing (Doleyres et al., 2004b).

A problem in growing probiotic cultures in industry is the contamination by bacteriophages, particularly when working with raw milk products, which are used in the cheese industry. Up to 1995, bifidobacteria were thought not to be perceptive to phages, but Ventura and co-workers found phage like elements in

Bifidobacterium strains (Tamime et al., 1995; Ventura et al., 2005). To prevent phage contamination two strategies are applied: (1) perform strain rotation; the disadvantage of this is that the new strain does not necessary have the same biological traits and health benefits, (2) addition of probiotics only at the final processing step; the disadvantage of this approach is that the inoculation rate has to be much higher, since there is no probiotic growth, and this would imply higher production costs. Therefore, phage resistance could be an extra preferable probiotic selection criterion (Mattila-Sandholm et al., 2002).

18.5 Drying Strategies

Before probiotics can be supplied to the market the bacteria need to be prepared for transport and storage. Live bacteria used in functional foods are generally stored and shipped in dried form, which is preferred over frozen form, because of the ease of long-term storage and shipping without the use of specialized refrigerated containers. However, the drying process is one of the main causes of loss in viability of probiotics. Spray and freeze drying are the two main forms of drying of probiotics. Other drying methods include vacuum drying, fluidized bed drying or a combination of drying techniques. These techniques have been extensively reviewed (Champagne et al., 1991; Meng et al., 2008; Santivarangkna et al., 2007) and this section will give an overview of the parameters involved in probiotic drying.

18.5.1 Freeze Drying

Freeze drying generally yields higher probiotic survival rates compared with spray drying (Santivarangkna et al., 2007). Freeze drying consists of three main steps, i.e.,: freezing, primary drying and secondary drying. In the freezing step, bacteria are typically frozen at −196°C in liquid nitrogen. Ice is then sublimated under high vacuum in the primary drying step by increasing the temperature. Sublimation is a phase transition, from solid to gas, that occurs at temperatures and pressures below the triple point of water. Approximately 95% of the free water is removed in this step. The hydrogen-bound water is then finally removed in the secondary drying step by desorption. Generally, drying is continued until the water contents drops below 4%, promoting long-term storage and spoilage prevention. Subsequently, the product temperature is raised to ambient

temperature. It has been reported that the temperature in the primary drying step should not be higher than the collapse temperature (T_c), which is the maximum temperature preventing the structure of the probiotics from microscopic collapse (Fonseca et al., 2004a). When collapse occurs, higher residual water content is observed, activity decreases, and it takes more time to reconstitute the powder (Fonseca et al., 2004b). Freeze drying probiotic bacteria on a commercial scale is an expensive process with low yields. The drying is batch-wise operated, which can be a limiting step if large quantities are required.

Most inactivation of the bacteria takes place in the freezing step. It has been reported that 60–70% of the cells that survive the initial freezing step will survive the dehydration step (To and Etzel, 1997). During the freezing step, extracellular ice forms, leading to a large osmotic pressure across the membrane, causing the cell to dehydrate. As the temperature drops, osmotic pressure increases and the cells dehydrate until an eutectic point is reached. Slow freezing causes more shrinkage and damage to the cell compared with fast freezing, because with slow freezing the extracellular ice is formed gradually which increases the time needed to reach the eutectic point thus allowing for more dehydration and shrinkage (Fowler and Toner, 2005; Zhao and Zhang, 2005). Baati et al. (2000) reported the opposite for *L. acidophilus*, where slow freezing and slow thawing actually increased the survival from 42 to 70%. It should be kept in mind that the damage done whilst thawing can be just as severe as freezing, since both actions apply similar stresses to the bacteria. Furthermore, there were differences in growth media, growth conditions and freezing solutions, which could explain the contradicting result (Champagne et al., 2005; Meng et al., 2008). In general, it is believed that fast freezing and slow thawing will result in the highest recovery after freeze drying. The larger the membrane surface area, the more damage is done during freeze drying, and for that reason damage during freeze-drying is higher for larger rod shaped lactobacilli than for small round enterococci (Fonseca et al., 2000). Wright and Klaenhammer (1981) also reported that the smaller bacilloid rods induced by calcium had higher survival rates than large elongated filamentous bacteria. The lipid fraction of the cell membrane is the most sensitive to damage during freeze drying. Furthermore, destabilization of RNA and DNA secondary structures results in reduced functionality of DNA replication, transcription and translation (van de Guchte et al., 2002).

A number of approaches have been used to improve the viability of bacteria during freeze drying [reviewed in Meng et al. (2008), Santivarangkna et al. (2008)]. Addition of protectants such as skim milk powder, whey protein, buttermilk, trehalose, glycerol, betaine, adonitol, sucrose, glucose, lactose, commercial

cryoprotectants (e.g., Unipectine, Satialgine) and polymers, such as dextran and polyethylene glycol (Burns et al., 2008; Capela et al., 2006) is a common way of improving stability during and after drying. The disaccharide trehalose has been reported to have a protective effect on viability during freeze drying in a number of studies. Miao et al. (2008) reported a 10% increase in viability when *L. rhamnosus* LGG was freeze dried in the presence of 15% trehalose. Similar results were reported by Zayed and Roos (2004) for freeze dried *L. salivarius* supplemented with trehalose.

18.5.2 Spray Drying

An alternative inexpensive continuous drying method with high yields is spray drying (Knorr, 1998; Zamora et al., 2006). During spray drying, the feed is pumped through a heated nozzle and is atomized into small droplets (10–200 μm) by using compressed air. The inlet nozzle temperature can reach up to 200°C. The droplets are sprayed into the drying chamber where, while falling through the chamber, co- or counter-current flowing hot air dries the droplets. The dried particles are then collected at the bottom of the chamber for further processing. The time needed for the particle to reach the bottom is referred to as "residence time." During the drying process, cells are exposed to extreme temperature, which can have detrimental effects on the integrity of the probiotic bacteria. Although the temperature is very high (130–200°C), the atomizing alone is unlikely to inactivate the cells. It has been reported that *Lc. lactis* endured no injury from shear forces or heating when atomized (Fu and Etzel, 1995). In addition, the cells undergo stress due to dehydration, oxygen exposure and osmotic pressure during spray drying (Brennan et al., 1986; Teixeira et al., 1997). Cell membrane damage has been reported to be a principal reason for cell inactivation during spray drying. Furthermore, spray dried cells were more sensitive to lysozyme, penicillin, and pyronin Y, which would indicate that besides membrane damage the cell wall, RNA and DNA are also affected (Abee and Wouters, 1999; Gardiner et al., 2000).

The outlet temperature has been inversely correlated with the survival of probiotics during spray drying. Desmond et al. (2002) showed that higher survivability during drying was achieved at lower outlet temperatures. However, lower outlet temperatures are associated with reduced residence time, leading to higher water content in the resultant powder. Water content should be below 4%, which is the maximum level required for prolonged storage, stability and spoilage prevention (Masters, 1985). Setting a correct outlet temperature alone is not

enough to achieve high viable numbers, and it is the combination of among others, outlet/inlet temperature, choice of probiotic strain and growth conditions that are important. *L. paracasei* NFBC 338 was shown to have 80% survival when spray dried in RSM at an outlet temperature of 85–90°C, and it survived significantly better than *L. salivarius* UCC118 at the same conditions (Gardiner et al., 2000). Furthermore, under similar conditions (80°C outlet temperature) *L. rhamnosus* GG survived at 60% during spray drying (Ananta and Knorr, 2003).

A number of bifidobacteria were screened for heat and oxygen tolerance, and these were subsequently spray dried, and their viability assessed during storage. It was reported that survivability was best for bacteria with high oxygen and heat tolerance. *Bifidobacterium animalis* subsp. *lactis* survived over 70% in RSM (20% w/v) at an outlet temperature of 85–90°C (Simpson et al., 2005). Furthermore, *Bifidobacterium* strains that had better heat and oxygen tolerance also exhibited better stability during storage (*Figure 18.1*).

The time of harvesting the cells has also been reported to have a major effect on the viability during drying and subsequent storage. It has been shown that stationary phase cells are more stable than cells harvested in exponential phase (Corcoran et al., 2004; Teixeira et al., 1995a). *L. rhamnosus* GG cells harvested at lag, log and stationary phase showed 2, 14 and 50% survival, respectively, after

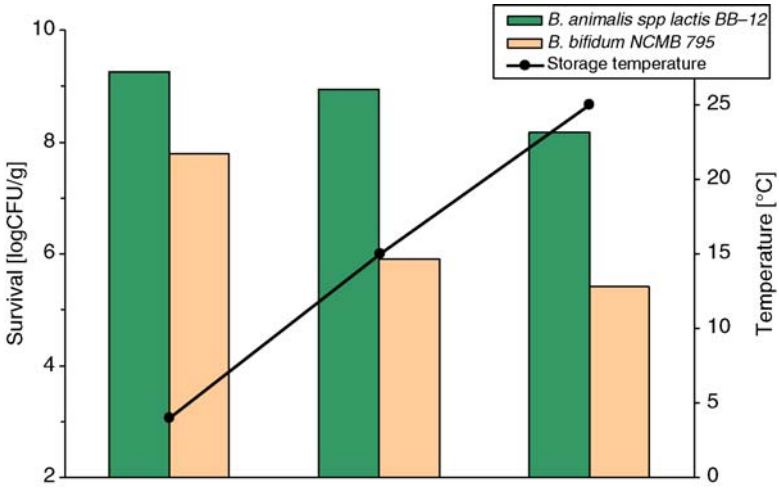

Figure 18.1

Compared storage survival of spray dried *B. animalis* subsp. *lactis* BB-12 and *B. bifidum* NCMB 795 for 30 days at three temperatures. BB-12 was more tolerant to oxygen and heat stress than *B. bifidum*. (adapted from Simpson et al. (2005)).

spray drying (Corcoran et al., 2004). Similarly, stationary phase harvested *L. delbrueckii* subsp. *bulgaricus* NCFB 1489 exhibited higher survival after spray drying compared with exponential phase harvested cells (Teixeira et al., 1995a). Prasad et al. (2003) reported that storage stability of *L. rhamnosus* HN001 was higher for stationary heat shocked bacteria than log-phase heat shocked bacteria. This could be due to certain stress mechanisms that are activated during stationary phase, and are similar to starvation conditions (e.g., glucose depletion) or acid stress.

The initial cell density can also influence cell activity after drying. Linders et al. (1998) correlated the initial cell density to the glucose fermentation activity of *L. plantarum* after spray drying. The ratio of activity before and after spray drying varied from 0.1 to 0.83 AU for initial cell concentrations of 0.025 and 0.23 g cell/g media, respectively. Therefore, a higher concentrated sample would lead to higher activity of cells after spray drying. Furthermore, it has been reported that higher initial cell concentration only marginally increased survival of *L. lactis* subsp. *lactis* after spray drying, allowing for more economical energy utilization and throughput (Fu and Etzel, 1995).

Encapsulation has proven to be an effective protection against stress endured during spray drying. By encapsulating live bacteria before drying, a protective barrier is formed around the cell, thus reducing the exposure to exterior stresses. Materials used for encapsulation include skim milk, potato starch, alginate, gum acacia, gelatine or casein. It was found that lactobacilli had increased heat tolerance when encapsulated in casein alginate beads (Selmer-Olsen et al., 1999). Lian et al. (2002) studied the survival of four encapsulated bifidobacteria in gum acacia, gelatine, and soluble starch after spray drying. Viability after spray drying was dependent on the material used for encapsulation. Besides encapsulating the bacteria in gels, spray coating the bacteria with a protective material is also an effective means of increasing viability during processing and subsequent storage. Spray-coated probiotic products survived better compared with untreated cells during their passage of the GIT, and released the biomass at predetermined sites (Siuta-Cruce and Goulet, 2001).

To further understand the processes controlling the viability during drying, genomics have been applied to investigate the role of certain genes. Desmond et al. (2004) reported that the overexpression of GroESL, a chaperone protein associated with stress response, resulted to increased heat tolerance of *L. paracasei*. Others reported that when overexpressing BetL, a betaine uptake system, the resistance to several stresses including osmo-, cryo-, baro- and chill-stress increased. Furthermore, the stability during freeze and spray drying

increased (Sheehan et al., 2006). These molecular tools are valuable for understanding the mechanisms behind stress resistance in probiotics.

18.5.3 Fluidized Bed and Vacuum Drying

Fluidized bed drying is a process where a flow of solid particles (bacteria) are dried by air that is blown through holes which causes the solid particles to be suspended and have fluid-like behavior. The particles are freely suspended in air and due to rapid heat exchange, the particles are dried (Santivarangkna et al., 2007). Operating costs of fluidized bed drying are equal to or lower than spray drying. Furthermore, the residence time can be easily extended, allowing for longer drying at lower temperatures, thus reducing the risk of heat inactivation. Yeasts have been successfully dried using fluidized bed drying (Bayrock and Ingledew, 1997a, b); it has also been applied to LAB. With fluidized bed drying, only granulated particles can be dried, therefore the bacteria must be encapsulated in support materials, such as skim milk, potato starch, alginate, or casein prior to drying. Furthermore, a fluidized bed can be used as a second drying step for the granulated particles produced during spray drying, allowing for a lower spray drying outlet temperature and higher survival of bacteria. In the fluidized bed, the powder is then dried to the desired moisture content under mild conditions.

Vacuum drying can be used to dry heat-sensitive materials, since water can be removed at low temperatures under vacuum. While freeze drying is also based on this principal, the difference is that with vacuum drying, temperatures can be kept as low as −2°C. Furthermore, since the drying takes please under vacuum, oxidation reactions can be minimized for oxygen sensitive bacteria. However, this technique has not been extensively studied for LAB. King and Su (1993) reported similar survival rates for *L. acidophilus* during freeze and vacuum drying. More recently, higher survival rates (18%) were reported, when *L. helveticus* was vacuum dried with 1% sorbitol at 43°C and 100 mbar for 12 h (Santivarangkna et al., 2006). A limitation of vacuum drying is the long drying times (10–100 h) compared with fluidized bed or spray drying, and the necessity for batch operation (Santivarangkna et al., 2007). However, this could be overcome by using continuous vacuum drying, which has been successfully applied to dehydrate materials to 1–4% moisture content in 5–10 min. Continuous vacuum dryers are available for large scale commercial product lines and are used to dry food additives, enzymes and pharmaceutical products (Hayashi et al., 1983).

18.6 Storage and Rehydration

18.6.1 Storage Conditions

The most important factors affecting survival during storage are storage temperature, oxygen, humidity and moisture content of the powder (Ananta et al., 2005; Desmond et al., 2002). High viability during processing is not correlated to high viability during storage, but the production process of probiotics does affect the storage survivability. In general, freeze dried bacteria have higher survivability when kept at low temperature and in an oxygen free environment. The survival of spray dried bacteria was also shown to be inversely correlated with storage temperature. Furthermore, by adding protective agents during growth, it has been reported that survival can be increased during storage. Selmer-Olsen et al. (1999) reported that optimal storage survival was reached when stationary phase *L. helveticus* culture was stored in non-fat milk solids or adonitol containing media. When *L. paracasei* was spray dried in milk based media containing gum acacia, over a 1000-fold increase in survival during storage for 4 weeks at 15 and 30°C, compared to milk based media alone was reported (Desmond et al., 2002). The viability of LAB during storage after freeze drying can be improved by adding supplements such as fructose, lactose, mannose, glucose or sorbitol to the growth media (Carvalho et al., 2004). However, the prebiotics inulin and polydextrose did not improve storage survival for several lactobacilli (Corcoran et al., 2004).

Oxygen can be lethal to strictly anaerobic bacteria, such as bifidobacteria. Membrane lipids can oxidize during storage, which changes the degree of unsaturated lipids, thus changing the passive permeability of the membrane leading to cell inactivation (In't Veld et al., 1992). Oxygen stress during storage can be minimized by removing any peroxide producing strains from the production process, and by adding antioxidants or free radical scavengers, such as ascorbic acid or monosodium glutamate to the media (Champagne et al., 2005). Packaging can also prevent oxygen from diffusing into the powders. Glass has been reported to be the best barrier against oxygen, while thick plastic or laminated pouches have also been used (Klaver et al., 1993; Wang et al., 2004). Furthermore, packaging under anaerobic conditions and purging material with inert gas (e.g., nitrogen) will limit the oxygen stress during storage for strict anaerobes.

Dried probiotics require different storage conditions than probiotics in liquid form. In the dairy industry, most of the products are liquid and have a shorter shelf-life than the dried products. Some bacteria used in the production

of yoghurts and milk based products have over-acidifying properties, which means that they acidify the product during storage. When making mixed cultures with probiotics, it is recommended to minimize over-acidifying bacteria, thereby increasing the chance of probiotic survival during storage (Kailasapathy and Rybka, 1997). Maintaining the moisture content at a certain level is crucial for the survivability of dried probiotics. It was reported that freeze dried bacteria have highest viability when relative vapor pressure (RVP) is below 11.4% during storage. At higher RVP, crystallization was observed which was detrimental to cell viability (Miao et al., 2008). In that study, disaccharides were used to improve storage stability. Similar results are reported elsewhere where the use of trehalose and lactose in combination with maltose improved survival during storage of dried bacteria (Zayed and Roos, 2004). Burns et al. (2008) reported the successful use of low cost buttermilk and whey for stabilizing probiotics during cryopreservation.

18.6.2 Rehydration

Dried probiotics require reconstitution before consumption and the reconstitution method can greatly affect the survival of the bacteria. The reconstitution process can be divided into four steps: wetting, submersion, dispersion and dissolving. Among these steps, wetting of the particles is very often the reconstitution controlling step. Other parameters such as powder quality, matrix properties (protective agents, wet-ability of powder, water activity (a_w), particle size), properties of the rehydration media (osmolarity, pH and energy source) and rehydration conditions (e.g., temperature of rehydration, (an)aerobic, duration of reconstitution and volume) may also significantly affect the rate of recovery to the viable state, and thus influence survival rates (Carvalho et al., 2003). The reconstitution media can have significant impact on the recovery of bacteria, with recovery varying up to 10-fold depending on the media used (Font de Valdez et al., 1985). Others reported that when milk was used as a drying matrix, the reconstitution solution had no significant effect on the recovery of dried bacteria (Teixeira et al., 1995a). It is postulated that the milk may have supplied all necessary nutrients to the cells, and thus masked the effect of any additional nutrients supplied with the different reconstitution media. When metabolizable sugars and salts were added to the media, improved recovery of freeze dried malolactic bacteria was observed (Zhao and Zhang, 2005). Similar increased viability was reported when injured *Serratia marcescens* and *Escherichia coli* were reconstituted in salt supplemented media (Wasserman et al., 1954;

Wasserman and Hopkins, 1957). Increased viability was observed when bacteria were rehydrated in solutions used for cryopreservation, providing a high osmotic pressure environment which could control the rate of rehydration, and thus avoid osmotic shock (Abadias et al., 2001). The rate of rehydration has been reported as an important factor for recovery of bacteria injured during the drying process (Kosanke et al., 1992; Leach and Scott, 1959). Two ways of controlling the rehydration rate are (1) by changing the amount of medium used for reconstitution and (2) by adding a protective matrix that lowers the a_w of the solution. Furthermore, particle size, porosity and wet-ability of the probiotic powder are factors impacting on the rate of rehydration. It is also suggested that the probiotics should be reconstituted at the optimal growth temperature of the bacteria. For example, freeze dried and spray dried bifidobacteria cultures that are reconstituted at 35–50°C and 20°C, respectively, showed increased survival compared with rehydration at 5–10°C (Wang et al., 2004). While pH is an important growth factor for LAB, little is known regarding the pH of reconstitution solution on the recovery of bacteria. Most reconstitution solutions used have a neutral pH using phosphate buffer. High recovery was reported for *Lactobacillus helveticus* within pH values of 6.0 to 7.0 (Selmer-Olsen et al., 1999).

Since the reconstitution conditions have a great influence on the recovery of dried probiotics and are strain specific, it should be optimized for each strain. Although most of the research on viability during storage and rehydration is concentrated on the ability of a probiotic to grow *in vitro*, it has been reported that non-culturable bacteria still retain a functional cell membrane typical of viable cells (Lahtinen et al., 2006). Therefore, it is desirable to use methods such as flow cytometry or real-time PCR in addition to plate counts to quantify viable or functional bacteria in products (Bovill and Mackey, 1997; Wai et al., 2000). Bio-assays are also useful tools to investigate the amount of active bacteria during and after reconstitution. Furthermore, the bacteria should retain their probiotic activity during and after storage. It has been reported that the cholesterol assimilation capabilities of *L. acidophilus* decreased significantly after storage for 21 days (Piston and Gilliland, 1994).

18.7 Cellular Stresses for Improving the Technological Properties of Probiotics

Improving the tolerance to environmental stresses by preconditioning of probiotics to a sublethal stress is a promising development for enhancing probiotic stability. For pathogens, such as *Salmonella typhimurium* and *Listeria*

monocytogenes, it has been shown that they survive a lethal pH when pre-exposed to a sublethal pH stress (Foster and Hall, 1990; Gahan et al., 1996). While undesirable for pathogens, this same adaptive mechanism has been the focus of many groups to improve survivability of probiotics during processing and subsequent storage (Desmond et al., 2001; Kullen and Klaenhammer, 1999; Walker et al., 1999). In general there are two types of defence mechanisms induced when bacteria are exposed to external stress. The first is a response to a sublethal stress, involving repair mechanisms, morphology changes or excretion of certain molecules from the cytoplasm, which will increase the tolerance to a homologous subsequent higher stress. The second is a more general mechanism, which protects bacteria to subsequent heterogeneous stress, also called cross tolerance (De Angelis and Gobbetti, 2004). One of these general stress responses can be observed when bacteria are exposed to heat, two chaperone protein complexes are formed, belonging to the 70 kDa DnaK and 60 kDa GroE families. These proteins are described as heat shock proteins (Hsp) but have also been associated with other stresses (Hartke et al., 1996). Chaperone proteins repair intracellular systems including refolding of polypeptides, assembly of protein complexes, degradation and translocation of proteins (Bukau and Horwich, 1998; De Angelis and Gobbetti, 2004).

Thermotolerance of lactobacilli was shown to increase by exposing bacteria to a sublethal heat shock followed by challenging the bacteria with a normally lethal heat shock (Desmond et al., 2001; Teixeira et al., 1994). Furthermore, it was reported that *L. paracasei* not only increased its thermotolerance in liquid media, but also showed 18-fold increased survival during spray drying compared to non-treated cells. Cross-protection was also studied; exposing the bacteria to sublethal levels of osmolarity and hydrogen peroxide increased the heat tolerance and spray drying stability of the bacteria, although to a lesser extent than heat-adaptation. In general, it has been shown that by pre-conditioning bacteria with a homologous sublethal stress its tolerance increases more than a heterogeneous stress. Similar results were reported for *Lc. lactis* subsp. *lactis,* which showed increased tolerance to a lethal heat treatment when pretreated with a sublethal heat shock compared to untreated cells (Boutibonnes et al., 1992). The treated cells also showed an increase of stress response proteins. However, pre-treatment of the cells with antibiotics that act on translation also induced the stress response proteins to be synthesized, but no extra heat tolerance was observed (Boutibonnes et al., 1992). This would indicate that the increased tolerance is attributed to factors other than synthesis of stress proteins. Desmond et al. (2004) showed that an overexpressing GroEL mutant of *L. paracasei* showed more heat resistance than the wild type, but less than a heat-adapted strain.

The heat shock induced production of GroESL chaperone complex has also been associated with better survival during freezing of *L. johnsonii* (Walker et al., 1999). Besides the upregulation of chaperone proteins following heat stress, it has been reported that membrane fluidity changed, and fatty acids were more saturated and elongated (Russell and Fukunaga, 1990).

Oxygen stress during the processing of probiotics is detrimental to strict anaerobes, such as bifidobacteria. Oxygen stress induced the protein Osp in an oxygen tolerant *B. longum* and changed membrane composition and morphology. The cellular fatty acids consisted of increased short chain fatty acids and cyclopropane fatty acid when compared with an oxygen intolerant *Bifidobacterium* (Ahn et al., 2001). Further understanding of these oxygen induced changes could increase bifidobacteria survival during processing. Furthermore, it is suggested that *L. acidophilus* adapts to hydrogen peroxide during yoghurt fermentation leading to improved storage survival (Hull et al., 1984). Storage stability of *L. rhamnosus* at 30°C was also increased when bacteria where pre-adapted with sublethal levels of heat and salt (Prasad et al., 2003).

High hydrostatic pressure is used as an alternative to heat treatment in the preservation of foods. High pressure causes protein denaturation and loss in membrane integrity leading to decrease in microbial activity (De Angelis and Gobbetti, 2004). This stress mechanism can be applied to induce a stress response. When 100MPa was applied to *L. rhamnosus* for 5–10 min, the survival was increased when exposed to 60°C compared to non-treated bacteria (Ananta and Knorr, 2003).

The acid tolerance of *L. acidophilus* was successfully increased by acid adaptation, increasing the viability of the *Lactobacillus* when exposed to normally lethal acidic media and yoghurt (Shah, 2000). Kullen and Klaenhammer (1999) have shown that several genes are up-regulated when *L. acidophilus* was pH challenged, including F_1F_0-ATPase, which is involved in the mechanism to stabilize the intracellular pH by removing excessive protons from the cytoplasm. Most of the research done on stress tolerance was in log phase cells, since these cells are most active and effects of external stress can be monitored better than stationary cells, which are more stable (Péter and Reichart, 2001). This late stationary phase induced stability may be due to starvation and stress response to low acidic conditions. When starvation induced stress response in *Lc. lactis* subsp. *lactis* was studied, the starved cells showed improved resistance to heat, ethanol, acid, osmotic, and oxidative challenges (Hartke et al., 1994).

Saarela et al. (2004) found improved acid resistance when lactobacilli and bifidobacteria were exposed to sublethal heat shock in stationary phase on a laboratory scale. However, when the same procedure was performed at pilot scale,

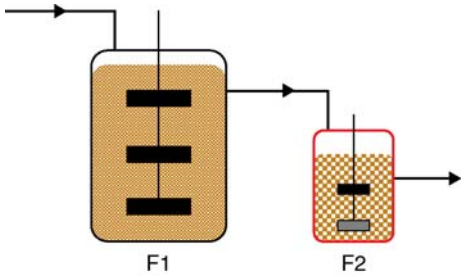

Figure 18.2
Two stage continuous fermentation setup to produce pre-conditioned bacteria with increased tolerance for downstream processing and subsequent storage. The setup consists of 2 (F1 and F2) fermenters, fermenter 1 (F1) produces a steady high concentration flow of bacteria. The effluent of F1 streams in a smaller fermenter (F2), where an external stress can be applied (e.g. heat, osmotic, acid or pressure). Since F2 is smaller, the residence time can be adjusted to provide the correct time for the stress response to be induced.

only lactobacilli showed improved resistance, indicating that up-scaling of the stress response is not straight forward. Similar cross tolerance has been reported for *L. acidophilus*. Log phase harvested cells showed increased survival when challenged with a lethal exposure to bile, NaCl or heat shock; if they were pre-conditioned with a sublethal dose of the same stress. Cross protection was also observed for different stresses tested (NaCl, heat and bile), indicating that a general defence mechanism was induced by the sub-lethal stress. In contrast to log-phase cells, stationary phase cells were inherently resistant to stress (Kim et al., 2001).

While it is not feasible to apply a heat or cold stress before drying probiotics using spray or freeze drying, respectively, since fermentation vessels are too large, there are certain fermentation set-ups that could resolve up-scaling issues. For example, a two-stage continuous fermentation can be used to induce a stress response. The first reactor produces a steady flow of high concentrated probiotics flowing into a smaller second reactor, with shorter residence time, where the stress is applied (Lacroix and Yildirim, 2007). Such developments can assist in decreasing losses during processing (*Figure 18.2*).

18.8 Summary

- The increasing demand for probiotics and the new food markets where probiotics are introduced, challenges the industry to produce high quantities of probiotic cultures in viable and stable form.

- Growth conditions have a large effect on the survivability and activity of probiotics during and after processing.
- Storage conditions, such as storage temperature, relative vapor pressure, oxygen and moisture content are very important factors to assure viable probiotics to the end of shelf-life.
- Dried probiotics are the preferred form for the ease of storage and transportation. Freeze and spray drying are the most applied drying techniques in industry.
- Reconstitution conditions such as temperature, rate of rehydration and osmolarity of the solution are vital parameters to assure resuscitation of the bacteria.
- Pre-conditioning bacteria with a sub-lethal stress can induce stress responses which can increase tolerance to subsequent stresses. This technique can be applied to increase survival during production and processing of probiotics.
- Genomics can provide insight into survival mechanisms involved during production, drying and storage of bacterial cultures, leading to the development of more efficacious probiotic products.

List of Abbreviations

a_w	Water activity
AU	Activity Unit
B.	*Bifidobacterium*
CFU	Colony Forming Units
DVS	Direct Vat Set
FAO	Food and Agriculture Organization
FOS	Fructoolisaccharides
GIT	Gastro Intestinal Tract
GRAS	Generally Regarded as Safe
L.	*Lactobacillus*
LAB	Lactic Acid Bacteria
Lc.	*Lactococcus*
MRS	de Man Rogosa Sharpe media
RCM	Reinforced Clostridium Media
RSM	Reconstituted Skimmed Milk
RVP	Relative Vapor Pressure
subsp.	subspecies
WHO	World Health Organization

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