Drying techniques of probiotic bacteria as an important step towards the development of novel pharmabiotics

Drying techniques of probiotic bacteria as an important step towards the development of novel pharmabiotics

Géraldine Broeckx[a,b#], Dieter Vandenheuvel[b#], Ingmar J.J. Claes[b], Sarah Lebeer[b], Filip Kiekens[a,*]

[a] University of Antwerp, Department of Pharmaceutical, Biomedical and Veterinary Sciences; Laboratory of Pharmaceutical Technology and Biopharmacy, Universiteitsplein 1, B-2610 Wilrijk, Belgium

[b] University of Antwerp, Department of Bioscience Engineering, Research Group Environmental Ecology and Applied Microbiology, Groenenborgerlaan 171, B-2020 Antwerp, Belgium

# equal contribution as first authors

Géraldine Broeckx: geraldine.broeckx@uantwerpen.be
Dieter Vandenheuvel: dieter.vandenheuvel@uantwerpen.be
Ingmar J.J. Claes: ingmar.claes@uantwerpen.be
Sarah Lebeer: sarah.lebeer@uantwerpen.be
Filip Kiekens: filip.kiekens@uantwerpen.be

* corresponding author: Filip Kiekens, filip.kiekens@uantwerpen.be, +3232652687
address: University of Antwerp, Department of Pharmaceutical, Biomedical and Veterinary Sciences; Laboratory of Pharmaceutical Technology and Biopharmacy, Universiteitsplein 1, B-2610 Wilrijk, Belgium

Abstract

The increasing knowledge about the human microbiome leads to the awareness of how important probiotics can be for our health. Although further substantiation is required, it appears that several pathologies could be treated or prevented by the administration of pharmaceutical formulations containing such live health-beneficial bacteria. These pharmabiotics need to provide their effects until the end of shelf life, which can be optimally achieved by drying them before further formulation. However, drying processes, including spray-, freeze-, vacuum- and fluidized bed drying, induce stress on probiotics, thus decreasing their viability. Several protection strategies can be envisaged to enhance their viability, including addition of protective agents, controlling the process parameters and prestressing the probiotics prior to drying. Moreover, probiotic viability needs to be maintained during long-term storage. Overall, lower storage temperature and low moisture content result in good survival rates. Attention should also be given to the rehydration conditions of the dried probiotics, as this can exert an important effect on their revival. By describing not only the characteristics, but also the viability results obtained by the most relevant drying techniques in the probiotic industry, we hope to facilitate the deliberate choice of drying process and protection strategy for specific probiotic and pharmabiotic applications.

Keywords

Spray drying, freeze drying, fluid bed drying, vacuum drying, probiotics

1. Introduction

Our human body appears to consist of ten times more bacterial cells than human cells, which are collectively called the human microbiome. These bacterial cells play an important role in our health. Many pathologies such as inflammatory bowel syndrome (Sartor and Mazmanian, 2012;), acute otitis media (Hilty et al., 2012), irritable bowel syndrome (Kennedy, 2014) and chronic lung diseases (Dickson and Huffnagle, 2015) seem to be associated with dysbiosis or an imbalance of the human microbiome. Such imbalances could be restored by the addition of a sufficient number of beneficial microbes, the so-called probiotics. While they are well-known in the food industry, the interest in probiotics has also spiked recently in the pharmaceutical industry. The reasons for this increased interest cannot be pinpointed easily, but they include an increased interest in the human microbiome and potential applications (Claes et al., 2015), an increased awareness for the urgent need for alternatives for antibiotics (Andersson and Hughes, 2011; Oliphant and Eroschenko, 2015), and novel European and international legislation requiring more rigorous documentation of postulated health claims of probiotics in functional food (Foligné et al., 2013; Sanders and Levy, 2011).

In 2001 the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) defined probiotics as "live microorganisms, which when administered in adequate amounts confer a health benefit on the host". In the last decade, an increase could be observed in the commercial availability of probiotic products, as well in the drug, cosmetic and food industry as in the animal feed industry. However, although probiotic applications are rising, the regulatory framework is still lacking clarity (as reviewed by: Degnan, 2008; Hill et al., 2014; Hoffman et al., 2008). For regulatory issues, important differences exist between food and drug-related applications of probiotics, but the interest of the pharmaceutical industry in probiotics in more pharmaceutical formulations is certainly emerging. Formulations containing micro-organisms with positive health effects are sometimes defined as pharmabiotics.

Given that the definition highlights that they should be live micro-organisms, one of the major issues concerning the formulation of probiotics and pharmabiotics is their loss in viability after processing and during storage. Many factors, such as temperature, oxygen level, moisture level, pressure and dosage formulation have an influence on the viability of probiotics and thus their health-promoting activity. Although some immune-related effects can also be achieved by non-living variants of probiotics or their isolated compounds (Lebeer et al., 2010), the presence of a sufficient amount of viable micro-organisms in the probiotic formulation is considered to be an essential criterion for health claims (Hill et al., 2014). To enhance long-term stability of probiotics, it is important to preserve them in a dry form, in which the water content is reduced (Vesterlund et al., 2012). Many technologies can be used to dry probiotics. During these processes several factors can influence the probiotic viability. Well-designed strategies to protect probiotics during these manufacturing processes and to maintain their viability are therefore crucial for successful application of the specific drying technologies as reviewed here, with a focus on probiotic bacteria from the genera *Lactobacillus* and *Bifidobacterium.*

2. Drying technologies

Cryopreservation can be seen as one of the standard methods to preserve bacterial starter cultures over a long period of time. However, from a commercial point of view, it has several disadvantages such as the need for subzero transportation and storage temperatures, and thus high energy costs. Moreover, cellular damage to the bacterial cells can be caused by freezing and thawing. Therefore, drying of probiotics is preferred. Several methods can be distinguished, including spray drying, freeze drying, vacuum drying and fluidized bed drying. Table 1 summarizes the main characteristics of the different drying technologies, discussed in the following paragraphs.

2.1 Spray drying

2.1.1 Principle of the process

Spray drying is a rapid and cost-efficient drying method that can produce dry spherical powder particles with desired properties, like good flowability, specific residual moisture content, and uniform size distribution and shape (Sosnik and Seremeta, 2015; Vandenheuvel et al., 2013). The process can be divided in four stages (Figure 1A). Firstly, the liquid feed is atomized into a spray of little droplets (1A.a). Then, the atomized spray comes in contact with a heated gas in the drying chamber (1A.b). Here three spraying flow patterns can be applied depending on the direction in which the air and liquid enter the drying chamber: co-current, counter-current and mixed flow. As probiotics are heat sensitive organisms, it is important to apply the co-current set-up. In this pattern, the wettest droplets come in contact with the highest temperature and the driest particles with the lowest temperature, thus minimizing the risk of heat damage to the probiotics. The third phase of the spray drying process involves the drying of the droplets and the particle formation (1A.c). Finally, the solid particles are separated from the drying air. In general, coarse and heavy particles are separated at the base of the drying chamber by gravitational force. Fine particles are separated using cyclones and bag filters (1A.d). The powder collected in the collecting vessel is a ready-to-sell or ready-to-use powder (Masters, 1991). The dried particles are formed in several phases (Figure 1B). In the first place, an equilibrium state between the droplets and the drying air is reached. After contact with the hot air, water immediately starts to evaporate (1B.a). As surface saturation of the droplet can be maintained, this evaporation is characterized firstly by a period of a constant drying rate, where the temperature of the particles is defined by the wet bulb temperature (1B.b). Once saturation conditions on the droplet surface can no longer be maintained, the second drying period with falling drying rate begins. As the droplet shrinks, a crust is formed by crystallization of materials dissolved or suspended in the feed (1B.c). The evaporation rate after this crust formation depends on the moisture diffusion through the dry crust. With increasing evaporation, the crust will become thicker and the evaporation rate will become slower. The temperature of the product increases and will resemble the temperature of the drying air (1B.d). In this last stage the probiotic cells are prone to heat inactivation (Golman and Julklang, 2013; Masters, 1991; Peighambardoust et al., 2011).

Spray drying appears to provide great advantages in comparison to other drying techniques (Peighambardoust et al., 2011; Sosnik and Seremeta, 2015). It is a rapid, continuous and

economic process that can handle large amounts of liquid feed cultures in a relatively short time. The main advantage is that the powder characteristics can be easily controlled. Moreover, spray drying is cheaper to scale up in regards to equipment, utilities and cycle time. During spray drying, however, the probiotic cells encounter different stresses, which can impair their viability.

2.1.2 Key stress factors affecting viability of probiotic bacteria during spray drying

During spray drying, probiotics experience various stresses that include thermal stress, dehydration, shear stress, osmotic and oxidative stress (as also reviewed in more detail by Santivarangkna et al., 2008b) Hereby, heat stress and dehydration are believed to be the two principal mechanisms leading to inactivation and loss of viability of probiotics (Janning and in t Veld, 1994; Perdana et al., 2013). Nevertheless, the inactivation mechanisms of probiotic bacteria during spray drying are not yet fully understood. Generalization of the conclusions of the published studies is difficult because results do not only vary between different bacterial genera and species, but they are also strain-specific. Even for the same strain, the stress tolerance will be different depending on growth conditions and/or growth stage (Fu and Chen, 2011).

2.1.2.1 Heat stress

The atomized feed is exposed to high temperatures in the drying chamber. While drying temperatures can be as high as 200 °C, the probiotic cells are not constantly subjected to such high temperatures. As mentioned before, during the constant drying phase, the temperature of the probiotics is limited to the wet bulb temperature. Therefore, heat inactivation is more likely to occur during the phase of the falling drying rate. At this stage, probiotic cells can reach the temperature of the surrounding drying air. Because the dried particles often reside in the machine until the end of the drying cycle, they can be heated to the outlet temperature, making this a critical parameter influencing the viability (Behboudi-Jobbehdar et al., 2013; Fávaro-Trindade and Grosso, 2002; Ghandi et al., 2012). High temperatures can cause denaturation of proteins and destabilize membranes, possibly leading to bacterial cell death. However, it is important to note that the moisture content and water activity (a_w) of the obtained powder is linked to the outlet temperature. The a_w plays an important role during storage of probiotics. Higher temperatures, result in lower a_w and thus generally an increased stability during storage. Therefore, it is important to find an optimal outlet temperature, high enough to obtain a low a_w for incresed stability, but not to high to avoid lethal cell damage.

2.1.2.2 Dehydration

Inactivation caused by dehydration often occurs simultaneously with heat damage. During dehydration water molecules are removed from the cells and their environment, thus limiting chemical reactions and metabolic activities (Ananta, 2005). As water molecules play an important role in the stabilization of several cell components, its removal will have physiological implications on cell integrity and structure. The lipid bilayer of the cell membrane is stabilized by a balance between van der Waals attraction and hydration repulsion. This stability is weakened by dehydration, causing leakage of the intracellular

compounds, possibly causing cell death (Ananta, 2005; Oliver et al., 1998). Perdana et al. (2013) performed single droplet drying experiments on *Lactobacillus plantarum* WCFS1 and evaluated the effect of temperature and dehydration on the inactivation of probiotics. Their results showed that with the use of temperatures below 45 °C, inactivation by dehydration was predominant. This was in contrast to temperatures higher than 45 °C, where inactivation by both dehydration and heat stress were present (Perdana et al., 2013). They also proposed that the longer the drying time, the more probiotic cells are prone to dehydration stress. When extrapolating their results to a spray dryer, the researchers suggested that the major stress factor affecting viability during spray drying is heat inactivation since the drying time takes just a few seconds to milliseconds (Perdana et al., 2013).

2.1.2.3 Shear stress

During the first step in the spray drying process when the liquid is atomized, the probiotic cells can also be damaged due to shear forces. In general, it is expected that a higher atomizing pressure on the liquid feed will result in higher shear forces, which will negatively influence bacterial survival. This was demonstrated by the increased viability of a strain of *Lactobacillus bulgaricus* (Lievense and van 't Riet, 1994) and *Lactococcus lactis* subsp. *cremoris* ASCC930119 (Ghandi et al., 2012) after spray drying using lower spray pressures. However, the exact effect of atomizing pressure is difficult to assess, and will greatly depend on the processing conditions, as shown by two studies using variable atomizing pressures (Pispan et al., 2013; Riveros et al., 2009). In one study, lowering the atomization pressure from 100 to 50 kPa increased the viability of a vaginal *Lactobacillus acidophilus* strain by one log unit (Riveros et al., 2009), while another study showed no significant difference in viability of *L. acidophilus* NCIMB 70225 using atomization pressures of 415 or 275 kPa (Pispan et al. 2013). It should be noticed that in the latter study, the atomization test was performed without the spray drying process, meaning that the probiotic feed was solely put through the atomizing nozzle without heating the chamber. Indeed, in a similar set-up, a strain of *L. lactis* subsp. *lactis* was atomized into a sterile beaker (without heating) and also no significant difference was found in viability before and after atomization (Fu and Etzel, 1995). The authors concluded that no lethal damage was caused by atomization. However, they also suggested that the shear forces could have broken the bacterial clusters into smaller chains, thus obscuring the injury caused by atomization. So, it appears that the atomizing pressure as such does not exert great influence on the viability. However, when probiotics already encounter other stresses, such as heat stress, shear forces can have a negative impact on their viability, as demonstrated by the studies mentioned above (Ghandi et al., 2012; Lievense and van't Riet, 1994; Riveros et al., 2009). Therefore when spray drying probiotics, with heat stress inherent to the process, it is recommended to use low atomization forces to enhance survival after spray drying.

2.1.2.4 Osmotic stress

During dehydration, water is removed from the probiotic cells to the extracellular environment, resulting in an increase in intracellular molarity that compromises essential cell functions (Poolman, 2002). This efflux of water also causes a decrease in cytoplasmic volume. Cell turgor is lost and the cells plasmolyze, which can lead to bacterial cell death.

Bacterial responses have been reported to overcome osmotic stresses, also in probiotics, including the induction of stress proteins and the accumulation of compatible solutes (Lebeer et al., 2008; Prasad et al., 2003). Compatible solutes are small organic molecules, which are very soluble and can be accumulated to high levels in the cytoplasm of osmotically stressed cells to re-establish the osmotic balance (Carvalho et al., 2004). Other compatible solutes are electrolytes such as Na^+ and K^+ or non-electrolytes such as glycine and betaine. Osmotic stress does not only affect viability of probiotics during drying, but also plays an important role during the rehydration of the dried powders. During fast rehydration, water will be drawn into the cells caused by their high osmolarity. This implements the risk of an excess of turgor pressure that can cause the cells to lyse. The osmotic effects during rehydration will be discussed later.

2.1.2.5 Oxidative stress

Oxidative stress is caused by the oxygen content of the drying medium and dissolved oxygen in the solution (Ghandi et al., 2012). Especially most *Bifidobacterium* species require strictly anaerobic conditions, while many lactobacilli are aerotolerant. Oxidative damage is caused by reactive oxygen species (ROS) interacting with proteins, nucleic acids and lipids. As a result, denaturation and lipid oxidation can lead to cellular membrane damage and cell death (Santivarangkna et al., 2008b). Most *Lactobacillus* strains and some bifidobacteria express enzymes that can reduce the ROS molecules to less harmful molecules. Studies have shown a clear correlation between the robustness of bacterial strains under heat and oxidative stress and their survival after spray drying. For example, when comparing several strains of *L. lactis*, the phenotype of *L. lactis* subsp. *lactis* P7266 seemed to be more resistant to heat and oxidative stress compared to *L. lactis* subsp. *cremoris* AM2, (Dijkstra et al., 2014). Indeed, after an oxidative challenge (5mM H_2O_2) *L. lactis* subsp. *lactis* P7266 showed only a viability loss of 0.1 log unit, while *L. lactis* subsp. *cremoris* AM2 showed a viability loss of 4.7 log units. Another study assessed the intrinsic oxidative tolerance of several *Bifidobacterium* strains by comparing their survival rate after aerobic and anaerobic incubation conditions (Simpson et al., 2005). The best oxidative tolerance was observed for *Bifidobacterium boum* LMG 10736 and *Bifidobacterium minimum* DSMZ 10105 with survival rates of more than 30%, compared to viabilities of less than 5% for the strains that were susceptible to oxidative stress (Simpson et al., 2005).

2.1.3 Molecular mechanisms of spray drying stress on probiotics

In general, it appears that Gram-positive bacteria are more resistant to thermal and mechanical stresses than Gram-negative bacteria. This can be explained by the difference in cell wall structure, mainly the presence of a thick peptidoglycan layer in Gram-positive bacteria (40 or more layers) compared to Gram-negative cells (1-5 layers) surrounding the cell membrane (Donsì et al., 2009; Fu and Chen, 2011; Pispan et al., 2013). The cell membrane in particular appears to be damaged during spray drying. This damage can be examined with direct methods such as flow cytometric analysis with functional dyes (Ananta and Knorr, 2004) or indirect by measuring the increased sensitivity of the membrane to factors such as NaCl (Corcoran et al., 2004; Gardiner et al., 2000; Golowczyc et al., 2011b; Sunny-Roberts and

Knorr, 2009). In general, probiotics seem to have a higher sensitivity towards various stress, such as NaCl, after spray drying compared to before drying. This sensitivity appears to be strain-specific. For example, prior to spray drying, 16% of *Lactobacillus rhamnosus* GG and less than 5% of *L. rhamnosus* E800 were shown to be sensitive to the presence of NaCl (Sunny-Roberts and Knorr, 2009). However, after spray drying, more than 40% of *L. rhamnosus* GG and more than 80% of *L. rhamnosus* E800 were sensitive to NaCl. The authors also suggested that the cell membrane of *L. rhamnosus* E800 was more damaged by the spray drying process than the membrane of *L. rhamnosus* GG. Next to the cell wall and cell membrane, also ribosomes and proteins are affected by the spray drying process as demonstrated by the use of differential scanning calorimetry (DSC) experiments and chemical markers (Teixeira et al., 1997). These experiments showed that ribosomes were damaged and protein denaturation occurred by heat stress above 65 °C. Likewise, in another study, DSC-scans showed that the temperature of maximum cell death rate of *L. plantarum* ATCC 10241 corresponded with a peak in the thermograph of ribosome denaturation, indicating that ribosomes play an important role in cell damage upon spray drying (Lee, 2002). Many other studies refer to ribosomes, DNA and RNA components as the critical sites for spray drying damage (Behboudi-Jobbehdar et al., 2013; Golowczyc et al., 2011b; Santivarangkna et al., 2007; Sunny-Roberts and Knorr, 2009). However, it should be noticed that almost all of them refer to the above mentioned study done by Teixeira et al. in 1997. Therefore, the molecular impact of spray drying on specific bacterial molecules needs to be further investigated.

2.1.4. Protection strategies

While various stress factors can highly impact the survival capacity of probiotics during and after spray drying, most of these factors can, at least in part, be overcome by rational selection of appropriated protection strategies. In general, three main protection strategies can be distinguished: (i) addition of protective agents, (ii) adaptation of the process parameters and (iii) prestressing the probiotic cells prior to drying. These strategies are not only affecting probiotic viability immediately after spray drying, but also during storage. Figure 2 schematizes important examples of different protection strategies that can be used to overcome or to diminish the negative effects due to spray drying stress.

2.1.4.1. Protective agents

An overview of different protectants and/or carriers that were already studied to enhance the viability of probiotics during spray drying and storage is given in Table A.1.a. Addition of saccharides, mainly disaccharides, is one of the most commonly applied strategies to protect heat-labile products or micro-organisms, such as probiotics. To clarify the protective effect of sugars on bacterial membranes and proteins, several hypotheses can be found in the literature, including the vitrification theory, water replacement hypothesis and hydration forces hypothesis. Firstly, it is important to consider a membrane under normal physiological circumstances. Upon hydrated conditions, the bilayer is in the lamellar fluid phase. The fatty acid tails are packed in the hydrophobic region of the bilayer, while the polar head groups are aligned with water molecules (Garvey et al., 2013). During dehydration this bilayer can undergo a transition to the gel phase. In this phase, the lipid tails are pressed together in the plane of the membrane and the polar head groups are packed closer together, which can cause

package defaults upon rehydration, leading to possible leakage of intracellular components and cell death (Bryant et al., 2007). Saccharides can prevent the detrimental consequences of the loss of membrane integrity by vitrification or glass formation (Grasmeijer et al., 2013). As water is removed from the bacterial suspension the protectants in this suspension will concentrate, favoring the glassy state over the rubbery state of the cells. In the glassy state, characterized by a high viscosity (> 10^{14} Pa.s), diffusion-controlled processes and mobility are severely slowed down (Aschenbrenner et al., 2014; Bryant et al., 2007). Probiotic cells are thus embedded in a glassy matrix with enhanced chemical and physical stability. The transition from the glassy state to the rubbery state occurs at the glass transition temperature which is characteristic for each sugar (Aschenbrenner et al., 2014, 2012; Grasmeijer et al., 2013).

Another hypothesis attributed to the positive effect of sugars is the water replacement hypothesis. It states that in hydrated condition the conformation and integrity of proteins and membranes are stabilized by interaction with water molecules, mainly due to hydrogen bonding (Golovina et al., 2009). Upon water removal, the polar groups of the sugars can substitute for the water molecules. The polar head groups of the bilayer can directly interact with the OH-residues of the saccharide by hydrogen bonding. As enough space between the head groups remains during dehydration, membrane integrity can be maintained upon rehydration (Bryant et al., 2007; Clegg et al., 1982; Garvey et al., 2013; Golovina et al., 2009).

Seemingly contradicting the water replacement hypothesis, the exclusion hypothesis is also proposed frequently in literature. It describes that the addition of sugars stabilizes the phase with the smallest area. The removal of water causes the cell to contract, reducing the cell volume and bringing the bilayers into close apposition. Thus the gel phase is favored over the fluid phase (Aschenbrenner et al., 2014; Garvey et al., 2013; Golovina et al., 2009; Grasmeijer et al., 2013). The sugars are preferentially partitioned away from the polar head groups, increasing the interfacial free energy and therefore promoting the lipid phases. Andersen et al. (2011) reconciled this apparent opposing views on membrane-sugar interaction by highlighting that the effects are concentration dependent. At low sugar concentrations, direct interaction with the head groups is predominant (water replacement hypothesis). However, at higher sugar concentrations (> 0.2 M) the preferential exclusion theory is favored. Although different molecular mechanisms support these explanations, they do not exclude each other. For optimal membrane and protein stabilization it is plausible that all these mechanisms are contributing.

Some sugars such as trehalose and sucrose can also act as compatible solutes. As the drying time during laboratory spray drying is short (milliseconds to seconds) the compatible solutes should be accumulated by the probiotics before drying. Addition of these compounds to the growth media allows probiotics to take up the desired solute during their growth (Kets et al., 1996). Varying viability responses of lactic acid bacteria are observed when different compatible solutes are used. For example in a study of Sheehan et al. (2006) a fivefold higher viability after spray drying was obtained with *Lactobacillus salivarius* UCC118 overexpressing BetL (a glycine betaine transporter). This effect was attributed to the

improved ability of these organisms to take up the compatible solutes compared to the wild-type. Trehalose, also known as a compatible solute, exerts positive effects on different strains of *L. plantarum* (Lapsiri et al., 2012; Perdana et al., 2014). Also prebiotics such as inulin (Avila-Reyes et al., 2014) and fructo-oligosaccharides (Golowczyc et al., 2011a) can positively influence the viability after spray drying and during storage of various *Lactobacillus* strains, amongst which *L. rhamnosus* B442, *Lactobacillus kefir* CIDCA 8321 and *L. kefir* CIDCA 8348. Excipients such as yeast extract (Jantzen et al., 2013), dextran (Leja et al., 2009) and polydextrose (Corcoran et al., 2004) were also evaluated for their protective effect during spray drying. Addition of antioxidants, such as ascorbic acid, can be useful to protect probiotics against oxidation damage during drying as shown by Ghandi et al. (2012). When *L. lactis* subsp. *cremoris* ASCC930119 was spray dried, irrespective of the use of air or nitrogen as the atomizing gas, viability increased with addition of ascorbic acid by more than ten percent. Another excipient that has been examined for its protective effect on probiotics during drying and storage is monosodium glutamate. The addition of this compound showed a significant positive effect on the viability of *L. rhamnosus* E800, *L. rhamnosus* GG and *Lactobacillus sakei* CTC 494 after spray drying (Ferreira et al., 2005; Sunny-Roberts and Knorr, 2009). In some experiments, maltodextrin or reconstituted skim milk are also added to the feed solution, because they appear to have a positive effect on the bacterial viability after drying (Fávaro-Trindade and Grosso, 2002; Golowczyc et al., 2010; Simpson et al., 2005). The underlying mechanism of the protective effect of skim milk is still unclear, as it is a complex medium containing lactose, fat, casein, whey protein and cations such as Ca^{2+}. In a recent study, the protective effect of skim milk was attributed to the presence of Ca^{2+} and milk proteins rather than to the presence of lactose (Zheng et al., 2015). Indeed, Huang and Chen (2013) also showed that Ca^{2+} plays a role in the improvement of the heat resistance of lactic acid bacteria. Although whey proteins appear to enhance viability after spray drying, the underlying mechanisms are still under debate (Khem et al., 2015; Soukoulis et al., 2014). This indicates that also other ions and protein additives could be used as protective measures.

2.1.4.2. Process parameters

During spray drying, loss of viability occurs due to the high temperatures used in the process. Extensive research has been done on the influence of the outlet temperature. As previously explained, this is the temperature the probiotic cells are most likely to approach at the end of the drying process. This temperature cannot be individually controlled since it is dependent on the inlet temperature and the feed rate. By using higher feed rates, more liquid needs to be evaporated, and moisture content in the surrounding gas will increase. This results in a lower outlet temperature, thus preserving more viable probiotics. However, the higher the moisture content of the surrounding gas, the higher the possibility of ending up with a moist or humid product, which might by detrimental for long-term storage. This will be discussed later. Table A.1.b gives an overview of some selected studies influencing the viability of probiotics by changing the process parameters. For example, for *Streptococcus thermophilus* MK-10 and *Lactobacillus delbrueckii* subsp. *bulgaricus* 151, the viability was shown to increase from 12.7% and 8.0% to 69.5% and 22.1%, respectively, by increasing the feed rate and thereby

lowering the outlet temperature from 80 °C to 60 °C (Bielecka and Majkowska, 2000). The authors also evaluated the moisture content corresponding to each outlet temperature of the obtained powder. Powders spray dried at a lower outlet temperature had a higher moisture content than those obtained from higher outlet temperatures. This was also observed by Golowczyc et al. (2010), when spray drying *L. plantarum* CIDCA 83114 and *L. kefir* CIDCA 8348 at a constant inlet temperature of 180 °C. By increasing the feed rate, the outlet temperature lowered from 85 °C to 70 °C, resulting in a higher viability. Also Romano et al. (2014) researched the effect of the outlet temperature on the bacterial viability by varying the feed rate. They found that the best survival rates of *L. rhamnosus* GG (56.53%) and *L. rhamnosus* RBM 526 (52.63%) were obtained with an inlet temperature of 135 °C and an outlet temperature of 65 °C. To control oxidative damage, the atomizing gas can be changed. As demonstrated by Ghandi et al. (2012b), the use of nitrogen instead of air enhanced the viability of *L. lactis* subsp. *cremoris* ASCC930119 from 45.19% to 58.58% under their tested conditions. Nevertheless, further research on the effect of the drying gas on the viability of probiotics is required.

2.1.4.3 Prestressing strategies

Next to addition of protective agents and controlling the process parameters, prestressing probiotics is also well known to induce protective stress responses. Table A.1.c gives an overview of the pretreatment strategies. As reviewed by Lebeer et al. (2008), several adaptation factors can be identified when probiotics are faced with harsh conditions, such as acid stress. Some of these mechanisms can elicit protection when probiotics are dried because of cross protection phenomena. One of the more vital general stress responses in probiotics is the upregulation of chaperones. These proteins intervene during protein folding, renaturation and protection of denaturated proteins (Lebeer et al., 2008). Expression of chaperones such as DnaK, GroEL and GrosES, also known as heat shock proteins, appears to be important in the protection of probiotics during spray drying, as researched by Corcoran et al. (2006). A tenfold better survival after spray drying was obtained with *Lactobacillus paracasei* NFBC 338 when overproducing GroESL in comparison to the control group. Strains of *L. delbrueckii* subsp. *bulgaricus* grown under non-controlled pH conditions were also more resistant to drying and heating than cells grown under controlled pH conditions of 6.5 (Silva et al., 2005). Under non-controlled pH conditions the pH-value decreases over time as *Lactobacillus* strains produce lactic acid. Once this weak organic acid is passed through the cell membrane, auto-acidification can occur inside the probiotic cell, imposing stress. The authors suggested that the enhanced expression of heat shock proteins, resulting from these stress conditions, could be linked with the observed protection. Cross protection was also observed when pretreating *L. rhamnosus* GG with pressure (100 MPa, 10 min, 37 °C) to enhance heat-resistance (Ananta and Knorr, 2004). Cells that were pressure-pretreated showed better heat-resistance than the untreated cells when exposed to lethal heat stress. The authors suggested a potential contribution of pressure-induced protein biosynthesis in the enhancement of bacterial heat tolerance.

Next to pH and pressure pretreatment, cells can also be osmotically stressed prior to spray drying. When *L. paracasei* NFBC 338 was exposed to mild osmotic stress (0.3 M NaCl, 30

min) the pretreated cells showed higher viability results (33.46%) compared to the control cells (8.27%) (Desmond et al., 2002). The same strain showed a similar trend when pretreated with heat (52 °C, 30 min) prior to spray drying at an outlet temperature of 95-100 °C. The viability increased from 4.3% (control group) to 24% (heat-pretreated). Paéz et al. (2012) also found higher survival rates for *Lactobacillus casei* Nad and *L. plantarum* 8329 after spray drying when they were heat-pretreated at 52 °C for 15 min. Not all studies, however, result in a positive effect of pretreating probiotics prior to spray drying. A recent study showed that mild heat stress (52 °C, 15 min) or mild acid stress (pH 4, 60 min, 37 °C) of *L. rhamnosus* 64 led to lower cell counts after spray drying than the control (Lavari et al., 2015). As results are strain-specific, it is possible that the heat stress applied before spray drying is too mild to protect this probiotic strain against an outlet temperature of 85 °C.

2.1.5. Storage conditions

From a commercial point of view, it is important to maintain the stability of the spray dried powder during shelf life. Storage temperature, moisture content, water activity, relative humidity, oxygen presence and exposure to light, amongst others, are factors that can influence the shelf life of the finished product. The impact of the storage temperature on the viability of probiotics is well-known: the higher the temperature, the lower the survival rates over time. Storage at refrigerated temperatures generally gives the best stability (Lapsiri et al., 2012; Meng et al., 2008; Schuck et al., 2013; Silva et al., 2002). As mentioned before, the moisture content or water activity of the final product needs to be brought into account, next to good viability results of the probiotics. For long-term storage of the powder, the moisture content should be preferably below 4% and the water activity around 0.1 (Behboudi-Jobbehdar et al., 2013; Dianawati et al., 2013; Shokri et al., 2015; Vesterlund et al., 2012; Ying et al., 2012). At this moisture content deteriorative reactions, such as lipid oxidation are at their minimum (Chávez and Ledeboer, 2007; Vesterlund et al., 2012). The importance of achieving powders with a low and constant final moisture content and water activity is connected with the glassy/rubbery phase transition that can occur during storage. A high water activity and moisture content result in a decrease of the glass transition temperature. When the glass transition temperature falls below the storage temperature, the product changes from a glassy state into a rubbery state, where the mobility of molecules and the rate of chemical reactions is less restricted. A higher molecular mobility destabilizes biological material and changes the powder characteristics (e.g. flowability, crystallinity) leading to loss of viability of the probiotics and a decrease of the shelf-life of the stored powder (Ghandi et al., 2012; Schutyser et al., 2012; Ying et al., 2010).

2.1.6.Rehydration

Rehydration can be considered as a critical step in the recovery of spray dried powders. Not only the rehydration solution (osmolarity, pH, composition, volume) but also the rehydration conditions (temperature, rehydration rate) affect cell viability (Figure 3). For instance, rehydrating spray dried cells of *Bifodobacterium longum* B6 and *S. thermophilus* CCRC 14085 in peptone water at a temperature between 35-50 °C seemed to result in more cell recovery compared to peptone water at a temperature of 5 to 20 °C (Wang et al., 2004). It has

also been shown that when rehydrating spray dried powder containing *B. longum* NCC3001, a solution at pH 8 gave higher viability results than at pH 4 (Muller et al., 2010). However, this pH dependency seemed to be strain-specific as it was not seen when rehydrating *Lactobacillus johnsonii* NCC533. Cell recovery also appeared to be enhanced when smaller amounts of rehydration medium were used (Muller et al., 2010). When considering the composition of the rehydration medium, the design of the study is important. This means that the effect of the rehydration medium itself is often obscured by the protective agents used during spray drying. For example, no significant differences were observed between the use of skim milk, MRS broth, deionized water or phosphate buffer as the rehydration medium (Teixeira et al., 1994). However, this study used spray dried probiotics with a maltodextrin or skim milk carrier. These carriers could mimic the protective effect seen with very rich reconstitution media, thus obscuring the differences between the rehydration media used. Also a second study showed no significant difference in viability when using different rehydration media, including maximum recovery diluent (MRD), Ringer solution and TsPlus, containing tryptone, sodium chloride and antifoam B (Muller et al., 2010). However, the investigated bacteria were obtained as commercially available dry powder, thus the presence of a protective carrier could not be excluded. Therefore, it appears that the carriers used during drying have a possible dual effect. They need to protect probiotics during drying and storage, but they can also help to revive the probiotics during rehydration. Nevertheless, in general, a good rehydration medium offers enough nutritional and buffering capacity to revive probiotics.

2.2 Freeze drying

2.2.1 Principle of the process

Since decades, freeze drying, also known as lyophilization, is the most convenient and widely used method for the removal of water to enhance storage stability of probiotics. Figure 4 represents a schematic overview of a lyophilizator and the stresses that are encountered during the process. Lyophilization can be divided in 3 steps: freezing, primary drying and secondary drying. During freezing, ice crystals are formed that can damage probiotics. The growth of the ice crystals is dependent on the freezing rate and temperature. A high freezing rate is preferred over a slow freezing rate, since it will lead to the formation of smaller ice crystals avoiding extensive cellular damage (Fowler and Toner, 2005). Not only the formation of ice crystals is detrimental to probiotics. As water crystallizes, the solutes in the remaining unfrozen fraction concentrates, which leads to chemical and osmotic damage. In the primary drying step the frozen water is removed by sublimation under vacuum, while in the secondary drying step, the unfrozen water is removed by desorption (Maltesen and van de Weert, 2008). As water plays an important role in cell integrity and stability, its removal from probiotic cells can cause extensive damage to surface proteins, the cell wall and cell membrane, thus decreasing their viability after drying (Castro et al., 1997; Teixeira et al., 1994). While it is a well-known and commonly used drying technology for probiotics, freeze drying also has several drawbacks. It is an expensive and time consuming batch process, which results in the production of a dry cake. An additional processing step is thus necessary to obtain individual powder particles (Maltesen and van de Weert, 2008; Santivarangkna et al., 2008b). Moreover,

a possible risk of cross contamination within a freeze dryer has been noted (Barbaree and Sanchez, 1982). It might be therefore advisable to pay extra attention when intermixing different bacterial species in one freeze drying chamber and to ensure proper careful use of small scale-lab freeze dryers. Nevertheless, freeze drying is a valuable technique, which is widely used and well-studied, with various strategies available for the enhancement of the survival rate of the incorporated probiotics.

2.2.2 Protection strategies

The many protection strategies that have been developed to enhance bacterial viability during freeze drying include, adding excipients to the drying medium, controlling the process parameters, prestressing the bacterial sample prior to the freeze drying and changing the fermentation conditions of the probiotics (Table A.2). However, the efficiency of these strategies is strain-dependent, because the intrinsic tolerance to the drying process varies also from strain to strain. For example, Otero et al. (2007) evaluated the effect of the addition of excipients and achieved different viability results for *Lactobacillus gasseri* CRL 1412 and *L. gasseri* CRL 1421. The latter showed to be more resistant to the freeze drying process. This strain dependency makes it difficult to draw general conclusions and guidelines.

2.2.2.1 Protective agents

Addition of cryo- and lyoprotectants to the bacterial suspension is one of the most applied protection strategies. Cryoprotectants are water soluble chemicals that lower the melting point of water. As ice crystals are formed, probiotic cells are compressed in the unfrozen fraction. Adding cryoprotectants enlarges the unfrozen fraction, giving more space to the probiotics, which leads to less cellular damage by mechanical stress or osmotic stress. In contrast, lyoprotectants protect probiotic cells during the drying steps when water is removed. The underlying mechanisms of lyoprotection are similar to the ones mentioned above during the spray drying process. Some sugars can act both as cryo- and lyoprotectant, such as sucrose and trehalose, and render positive effects on the viability of probiotics after freeze drying. For example *Lactobacillus helveticus*, originated from kefir grains, showed a viability of less than 10% when freeze dried without protectant (Chen et al., 2006). Addition of 10% (w/v) sucrose or trehalose increased the viability by 50% and 20%, respectively. Another study by Strasser et al. (2009) showed that the viability of *L. plantarum* IFA N° 278 doubled, compared to the control (16% viability), after addition of 32% (w/v) sucrose or trehalose (34% and 40% viability, respectively). The use of skim milk is also known to be a good protection strategy (Abadias et al., 2001; Castro et al., 1997; Selmer-Olsen et al., 1999). For instance, freeze drying *L. delbrueckii* subsp. *bulgaricus* DSM 20081 in the presence of 6% (w/v) skim milk resulted in a tenfold higher viability compared to freeze drying in distilled water (Jalali et al., 2012). Even more, the combination of different protectants, including skim milk, can enhance survival rates after freeze drying. For instance, freeze drying *L. paracasei* subsp. *tolerance* DSM 20258 and *L. delbrueckii* subsp. *bulgaricus* DSM 20081 in a drying media containing both skim milk and trehalose (each in a 6% and 8% (w/v) ratio, respectively), resulted in survival rates of more than 70%, whereas the viability without any protectant added was only 2% or 3%, respectively (Jalali et al., 2012). In another study, only 4% of *L. salivarius* subsp. *salivarius* UCC 500 survived after freeze drying when no protectant was added (Zayed and

Roos, 2004). Moreover, the survival rate declined with 99% or more after one week. However, after the addition of trehalose, sucrose and skim milk (each in a 4%, 4% and 18% (w/w) ratio, respectively) the viability increased by 78% and a survival rate of 85% during subsequent storage for one week was observed. Berner and Viernstein, (2006) showed that a combination of 10% (w/w) skim milk and sucrose resulted in more than 60% viability of *L. lactis* Sr. 3.54 after freeze drying, compared with 0.02% when no protectant was added. A more recent study by Jofré et al. (2015) demonstrated that the viability of *L. rhamnosus* CTC 1679 after freeze drying was 78% when a 5%/11% ratio trehalose/skim milk was used. Addition of solely 5% trehalose resulted in a survival rate of only 39%.

On the other hand, addition of cryo- and/or lyoprotectants not always seems to result in enhanced viability after freeze drying. Carvalho et al. (2002) observed no significant differences in the viability of *L. plantarum* (LR-ESB) and *L. rhamnosus* (LR-ESB) after freeze drying in the presence or absence of either inositol, sorbitol, fructose, trehalose, monosodium glutamate and propyl gallate. However, survival was higher during storage at 20 °C in the presence of these compounds. A similar observation was made by Savini et al. (2010). When freeze drying *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502, addition of inulin, glycerol, dextrin, mannitol, sorbitol or Crystalean® starch resulted in no significant difference in survival rates after drying. However, when the powders were stored at room temperature, the authors observed a significant better viability in the presence of glycerol and mannitol, compared to the control group. It should be noted that in both aforementioned studies, the drying medium of the control group consisted of (semi-) skimmed milk. Therefore, it is possible that directly after freeze drying, skimmed milk shows sufficient protective capacities to protect the probiotics during freeze drying. When the powders are stored at room temperature it appears that addition of other excipients is needed to maintain viability. These studies again showed that the effect of adding an excipient is strain-specific and that the effect of addition of protective agents can have varying effects upon the drying process itself and storage afterwards. This makes it difficult to predict the end result. Nevertheless, overall, the use of trehalose or sucrose, alone or in combinations with other protectants, results in the best viability results. Therefore addition of these compounds should be considered as the first enhancement strategy.

2.2.2.2 Process parameters

Varying the process parameters can also largely affect the viability of probiotics during freeze drying, with the temperature at which the probiotics are frozen, being a key parameter. Various studies have shown that the lower this temperature, the better the viability results. In a first study, a strain of *Lactobacillus brevis* showed a viability of 46.4% when frozen at -20 °C, compared to a viability of 65.2% at -60 °C (Zhao and Zhang, 2005). In a second one, the viability of *L. salivarius* I24 increased from 44.35% to 65% when the temperature at which the bacteria were frozen, decreased from -30 °C to -80 °C (Ming et al., 2009). Freezing probiotic bacteria at lower temperatures corresponds to higher freezing rates and will result in smaller ice crystals, thus limiting the cellular damage, as mentioned before. However, it should be concluded that a higher freezing rate not always corresponds with the best viability results. When freezing rates were evaluated, a clear difference in viability could be noticed. Firstly, a faster temperature drop results in a better survival of the frozen probiotics, until the

optimal freezing rate is reached. After that, a further increase in freezing rate becomes again more detrimental for the preserved probiotics. However, the optimal freezing rate is dependent on the lyoprotectant used. For *L. coryniformis* Si3 the optimal freezing rate has been shown to be -2.7 °C/min in a 20% (w/v) sucrose medium, with approximately 60% viability (Schoug et al., 2006). However the authors also state that this value should not be seen as the optimal value, as there will be differences in processing conditions and tested strains.

2.2.2.3 Prestressing strategies

The pretreatment of probiotics with a stress prior to freeze drying seems to be a valid protection strategy. This includes exposing probiotics to sub-lethal temperatures. For instance, when *L. lactis* subsp. *diacetylactis* SLT6 was heat-pretreated at 45 °C for 30 min, viability increased from 21.9% for the untreated cells to 38.8% for the prestressed cells (Ziadi et al., 2005). In another study, slightly higher viability results (a ca. 0.3 log unit increase) for *Bifidobacterium bifidum* THT 0101 were obtained after exposing the cells to sub-lethal temperatures (42°C for 100-300 s) (Nguyen et al., 2014). The authors ascribe the enhanced viability to an increased exopolysaccharide (EPS) production following heat stress. The presence of a thick EPS layer is indeed positively correlated with resistance against different kinds of stresses (Alp and Aslim, 2010; Lebeer et al., 2011; Nguyen et al., 2014).

Likewise, varying the fermentation conditions, such as pH, can also affect the viability of probiotics after freeze drying. Changing the pH of the fermentation medium from 5.0 to 5.8, has for example been shown to decrease the viability of *L. rhamnosus* E800 from 48 % to 37% (Saarela et al., 2009). In addition, when lowering the pH of the culturing medium of *Lactobacillus reuteri* ATCC 55730 from 6.0 to 5.0, the viability rose from 65% to 90% (Palmfeldt and Hahn-Hägerdal, 2000).

2.2.3 Storage conditions

Probiotics need to be protected not only during the freeze drying process but also during storage. Along with the type of protectant used during freeze drying and the residual moisture content of the powder, the atmospheric oxygen level, relative humidity and temperature are important factors to take into account when storing freeze dried probiotics. Savini et al. (2010) observed differences in protection capacity of excipients used in the drying medium after drying and subsequent storage for 5 months at different storage temperatures. Glycerine and mannitol seemed to exhibit protection abilities when *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502 were stored at room temperature, whereas sorbitol, inulin, dextrin, Crystalean® did not. Overall in this work, viability decreased with increasing storage temperature. This was also observed in another study where the storage stability of *L. casei/paracasei* CTC1677, *L. casei/paracasei* CTC1678 and *L. rhamnosus* CTC1679 was remarkably higher when stored under refrigerated temperatures (4 °C), compared to room temperature (22 °C) (Jofré et al., 2015). Under the refrigerated conditions, skim milk alone or supplemented with trehalose or lactose showed the best performance, with a maximal loss of 0.9 log units after 39 weeks, whereas a maximal loss of 8 log units was observed when stored at 22 °C. In general freeze dried products need to be stored below their glass transition

temperature, where they can maintain a glassy state. Apart from temperature, the water activity of the freeze dried powder is an important factor during storage. The inactivation rate of the probiotics is higher with increasing a_w and temperature (Aschenbrenner et al., 2012; Dianawati and Shah, 2011; Higl et al., 2007; Kurtmann et al., 2009). As water can act as a plasticizer, it can decrease the glass transition temperature, thus limiting the storage stability. These results are in line with the findings of Santos et al. (2014), who showed lower survival rates of *L. delbrueckii bulgaricus* CIDCA 333 with higher relative humidities during storage. Indeed, also freeze dried *L. salivarius* subsp. *salivarius* UCC 500, stored at relative humidities of 2.8% and 5.6% resulted in higher viability results, than the powders stored at a relative humidity of 8.8% (Zayed and Roos, 2004). However, it should be noticed, that storing the probiotics at 0% moisture resulted in a decrease in cell recovery over time, compared to humidities of 2.8% and 5.6%. Therefore it can be concluded that during storage a minimal moisture content should be present to maintain viability, and "overdrying" could be harmful to probiotics (Zayed and Roos, 2004).

The oxygen level during storage also seems to affect bacterial viability. For instance, when *L. acidophilus* La-5 was stored at low oxygen levels (< 4%) the viability was better than when this strain was stored at atmospheric oxygen levels. This effect seemed to be connected with the formation of radicals, as investigated with electron spin resonance-spectroscopy (Kurtmann et al., 2009). Inclusion of the antioxidant sodium ascorbate has been shown to improve viability during storage of *L. paracasei* subsp. *tolerance* DSM 20258, *L. delbrueckii* subsp. *bulgaricus* DSM 20081 (Jalali et al., 2012), *L. acidophilus* La-5 (Kurtmann et al., 2009), *L. acidophilus* CRL 125, *L. paracasei* subsp. *paracasei* CRL 1289 and *L. salivarius* CRL 1328 (Zárate and Nader-Macias, 2006).

2.2.4 Rehydration

Upon the use of dried samples, rehydration is often needed. This hydration step is generally done right before application, and will greatly influence the final viability (Carvalho et al., 2004; Meng et al., 2008; Vega and Roos, 2006). The optimal rehydration conditions appear to be connected with the phase transition of the phospholipid cell membrane. In physiological conditions the phospholipids are present in a liquid crystalline state, allowing enough fluidity. This is crucial for the integrity and function of the membrane itself and its embedded proteins. When cells are dehydrated the lipids encounter stress. The cell membrane changes into a gel-like state at low water contents, leading to membrane packing defects. This membrane phase transition is characterized by a transition temperature T_m. Upon rehydration, the membrane can undergo the reverse phase transition from gel to liquid crystalline (Santivarangkna et al., 2008a). Since phase transitions are dependent on the T_m, higher viabilities seem to be obtained when rehydration occurs at a temperature higher than T_m. In this case, the dried membranes are already in liquid crystalline phase, making the rehydration rate less important. At temperatures lower than T_m the importance of the rehydration rate increases. Slow rehydration leads to a slow water flow through the transitioned membrane, thus preserving bacterial viability (Poirier et al., 1999; Santivarangkna et al., 2008a). However, a slow rehydration rate is not convenient for the usability of probiotics. The medium in which the

dried powders are rehydrated also influences the viability, as mentioned before. However, the effect of the reconstitution medium is strain- dependent. For instance, rehydrating a strain of *L. brevis* in distilled water resulted in lower bacterial recovery rates in comparison to rehydration in a sugar or mineral rich medium (Zhao and Zhang, 2005). On the contrary, when rehydrating *L. helveticus* strains, originating from kefir grains, no difference was observed between distilled water and 10% (w/v) skim milk. However, the authors suggested that, as kefir is made from raw milk, the dried powder already contained the milk components that protect the probiotics during rehydration (Chen et al., 2006).

2.3 Vacuum drying

2.3.1 Principle of the process

Vacuum drying (Figure 5) resembles freeze drying, with the main difference that the samples are dried through evaporation rather than sublimation. During freeze drying the samples first need to be frozen before the water is removed, whereas with vacuum drying the samples stay in liquid form. Consequently, vacuum dryers generally operate at higher temperatures and higher pressures, compared to freeze dryers. Typical pressure values for vacuum dryers are above 10 mbar, compared to generally below 10 mbar for freeze dryers. Because vacuum drying operates at higher temperatures compared to freeze drying, but at lower temperatures than spray drying, it can be seen as a more gentle process in respect to temperature (frost/heat) damage, thus limiting the viability loss of heat-sensitive probiotics. In addition the absence of oxygen during the process could limit oxidative stress, certainly when handling oxygen-sensitive probiotics such as bifidobacteria. However, dehydration stress can still cause severe viability loss. Analysis of vacuum dried cells using atomic force microscopy and Fourier transform infrared spectroscopy clearly showed that the main sites of damage are the cell wall and cell membrane (Santivarangkna et al. 2007). Therefore, it can be assumed that measures to protect the cellular membranes will be the main strategy to enhance cell viability. However, the available literature on the use of this technology for drying and preserving probiotics and insights in the dehydration stress, is currently still limited.

2.3.2 Protection strategies

The main strategies to protect probiotics against vacuum drying include the addition of protective agents or the altering of the process parameters. To the best of our knowledge, studies about pretreating cells with a sublethal stress prior to vacuum drying have not yet been published. However, as vacuum drying is similar to freeze drying, it is possible that prestressing probiotics prior to the drying process can enhance their viability.

2.3.2.1 Protective agents

Adding sugars or polyalcohols, such as trehalose and sorbitol, seems to benefit the bacterial viability. The protective effects of these compounds during dehydration are well known and the underlying mechanism is the same as previously described during spray and freeze drying (Crowe, 2007; Crowe et al., 2001; Foerst et al., 2012; Garvey et al., 2013; Gómez Zavaglia et al., 2003). For instance, when *L. paracasei* F19 was vacuum dried (15 °C, 15 mbar, 22 h) with

addition of 25% (w/w) trehalose or sorbitol, the viability increased from 29% to 70% and 54%, respectively (Foerst et al., 2012). Viability of a *L. acidophilus* strain was also enhanced from 18.9% (unprotected cells) to 37.9% by adding 20% (w/w) trehalose to the bacterial suspension (0.11 mbar, room temperature, 4 days) (Conrad et al., 2000). In another study, the addition of 1% (w/w) sorbitol to a suspension of *L. helveticus* WS1032 doubled their viability after vacuum drying (100 mbar, 43 °C, 12 h) (Santivarangkna et al., 2006). However, this study also demonstrated that not all protective agents enhance the viability after drying. For example, addition of 1% (w/w) lactose, inulin or xanthan gum showed no significant increase in survival rates compared to no addition of protectants. Moreover, increasing the amount of protectant to 10 or 100% (w/w), negatively correlated with the viability. For sorbitol, addition of 1% (w/w) enhanced the viability, but adding more sorbitol did not further increase the survival rate. In another study by the same research group, a similar trend was observed. Addition of 5 mM sorbitol increased the survival rate of *L. helveticus* WS1032 ten times, compared to no addition of a protectant (100 mbar, 43 °C, 16 h) (Santivarangkna et al., 2009). However, increasing the sorbitol concentration did not further increase the viability, indicating that there exists an optimal concentration of protective agent. Gómez Zavaglia et al. (2003) also observed an optimal concentration of trehalose when dehydrating *L. delbrueckii* subsp. *bulgaricus* CIDCA 333. Increasing the concentration from 50 to 250 mM, enhanced the probiotic viability. However, higher amounts of trehalose led to a decrease in viability. Possibly, higher concentrations of protective agents can cause an increase in osmotic gradient, and thereby negatively influence the viability.

2.3.2.2 Process parameters

The most important parameters to take into account during vacuum drying are the drying time and temperature, since they will drastically influence bacterial viability and water activity. A shorter processing time and lower temperature is preferable, as this will minimize the chance of detrimental bacterial damage. For example, when vacuum drying *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 at temperatures of 30 °C, 45 °C and 70 °C (13.3 mbar, 10 min), the membrane damage increased and water activity decreased with higher temperature (Tymczyszyn et al., 2008). Considering each temperature on its own, a longer drying time corresponded with a lower water activity and more membrane damage. A similar observation was made by another research group in two different studies that vacuum dried *L. helveticus* WS1032 (43 °C, 100 mbar) (C. Santivarangkna et al., 2007; Santivarangkna et al., 2006). Prolonging the drying time decreased water activity and viability. After a drying time of 12 hours, a sharp drop could be seen in the viability of this probiotic strain. Images obtained by atomic force microscopy showed the presence of cracks on the cell surface and lysis of this probiotic after 12 hours of drying. Another study with *L. plantarum* CIF17AN2 compared the viability of this strain in respect to different drying techniques (Hongpattarakere and Uraipan, 2015). The authors found that survival rates were higher when a shorter drying time was used (12 h, 37°C, 40 mbar compared to 5 days, room temperature, 400 mbar). It can be concluded that shorter drying times and lower temperatures are preferred in respect to viability of probiotics. However, it should be taken into account that water activity also plays an important role in viability, not only after drying, but also during storage. As mentioned before, a lower water activity is preferable for the viability. However, a minimal amount of

water should remain in the probiotic powder to be able to revive the probiotics after long-term storage.

The vacuum pressure used during drying is also an important drying parameter as it will influence the temperature needed for drying. A lower vacuum pressure will need lower temperatures to evaporate the solvent and in turn reduce heat-related damage (Bauer et al., 2012). By further reducing the chamber pressure, evaporative drying can occur at temperatures close to 0 °C, avoiding heat and frost damage. This method, named controlled low-temperature vacuum dehydration (CLTV), was developed firstly by King et al. (1989). King and Su (1994) compared survival rates of a *L. acidophilus* strain after freeze drying, conventional vacuum drying and CLTV. Viability results for freeze drying (52.8%) and CLTV (50%) were comparable, while conventional vacuum drying only exhibited a viability of 15.4%. Addition of glycerol enhanced viability during drying in all cases up to 73.2%, 73% and 29.5%, respectively. So, when comparing vacuum drying with freeze drying, it seems that the overall survival rates of probiotic preparations after freeze drying are higher. This could be the reason why literature on vacuum drying of probiotics is scarce. Nevertheless, since CLTV appears to be a valuable alternative for freeze drying, by obtaining comparable survival rates, this technique can be of future importance.

2.3.3 Storage

Like with freeze- and spray drying, storing probiotics at higher temperatures or in a more humid atmosphere quickly reduces their viability. For instance, when *L. paracasei* F19 was stored at 30 °C with an a_w-value of 0.33, a decrease of 7 log units after only 20 days was observed. Reducing the a_w to 0.07 resulted in higher survival rates (Foerst et al., 2012). Subsequently, the use of refrigerated temperatures led to the maintenance of the initial viability after 3 months of storage (Foerst et al., 2012). Likewise, storing *L. plantarum* CIF17AN2 at 4 °C or room temperature, resulted in a 1.36 or 2.13 log reduction in viability after 8 weeks, respectively (Hongpattarakere and Uraipan, 2015). From a commercial point of view, it is beneficial when probiotic powders can be stored at room temperature, avoiding the need for constant refrigeration or a continuous cold-chain during transportation. The addition of protectants can thus be useful to enhance storage stability at non-refrigerated temperatures. For example, the addition of 25% (w/w) sorbitol to *L. paracasei* F19 resulted in no significant loss when stored at 20 °C, while addition of trehalose did not stabilize cells during storage (Foerst et al., 2012). The authors suggested that this lack in protective effect was due to rapid crystallization of trehalose during storage, because of the reduction of the T_g signal of trehalose after 24 hours of storage. Therefore they concluded that trehalose lost its protective glassy matrix, but this remains to be experimentally documented further.

2.3.4 Rehydration

As the available literature on vacuum drying of probiotics is scarce to date, more research needs to be done on the rehydration conditions of vacuum dried probiotics. As already mentioned for spray- and freeze drying, some key points include the rehydration rate and the rehydration medium composition, with regards to osmotic and nutritional balance.

2.4 Fluidized bed drying

2.4.1 Principle of the process

Fluidized bed drying (Figure 6) is a process in which a heated gas, usually conditioned air with controlled velocity, is passed through a bed of solid particles, suspending the particles in the drying air. It should be noted that fluidized bed drying cannot be used as the sole drying technique for probiotics. Dried bacterial cells are relatively small (several μm's) and will be taken along with the drying air, ending up in the bag filters and thus leading to very low yields. Moreover, combining fluid bed drying with other drying techniques such as freeze drying or spray drying is in practice not very profitable because for example, the relatively low density of the dried particles obtained by these drying techniques, results in difficulties when trying to suspend them in the drying air. However, fluid bed drying is an important encapsulating technique and it can further reduce the residual moisture content of the probiotic formulation.

Fluidized bed drying is less time consuming than freeze drying but more than spray drying. Heat inactivation can be minimized during this process using lower drying air temperatures compared to spray drying (Barbosa-Cánovas and Juliani, 2004; Chua and Chou, 2003). When using a fluid bed dryer, the probiotics must be mixed with carriers or matrix molecules to which they can adhere. Recent studies showed the use of casein, maltodextrin, cellulose, lactose or NaCl particles as appropriate carrier particles (Bensch et al., 2014; Mille et al., 2004; Strasser et al., 2009). Usually the carrier material is brought in the fluid bed dryer firstly and then the bacterial suspension is sprayed on the fluidized carriers using a nozzle. Alternatively, the bacterial pellet can also be obtained firstly by freeze drying or spray drying, after which the particles can be encapsulated with a protective shell using a fluid bed dryer to enhance viability (Azim et al., 2012). This protective capsule can consist of fats, proteins, (poly)saccharides or other coating material to enhance the stability and/or viability of probiotics during long term storage. It can be seen as an extra layer around the probiotics that protects them against the detrimental influences of long term preservation. For example, some coating shells can minimize moisture diffusion during storage. Since the usability of fluidized bed drying of probiotics is limited, available literature is scarce. However, most studies use the first approach, with carrier particles on which the bacterial suspension is top-sprayed and dried. The main advantage of this technique is the use of larger particles, limiting the cohesive forces and thereby improving the flow characteristics of the obtained powder.

2.4.2 Protection strategies

Similar to other drying techniques, the viability is enhanced by addition of protectants, controlling the process parameters and induction of stress responses prior to drying.

2.4.2.1 Protective agents

The most commonly used protective strategies in fluidized bed drying are again the addition of protectantia, like saccharides or skim milk, or embedding the probiotics in a protective alginate matrix. For example, adding 0.5 M adonitol, reconstituted non-fat milk solids or glycerol to *L. helveticus* CNRZ 303 entrapped in calcium alginate gel beads, which were produced in advance, gave viability results of 70.7%, 56.5% and 38.6%, respectively (Selmer-Olsen et al., 1999). All these excipients enhanced the viability significantly in comparison

with the control group in Ringer's solution, where no protectant was added and where only 3.7% survived. When *Enterococcus faecium* IFA No. 278 was fluid bed dried without any protective agent the viability resulted in approximately 11% (Strasser et al., 2009). Adding 32% (w/v) glucose, trehalose, sucrose or maltodextrin enhanced their viability more than 5 times. Moreover, when drying *L. plantarum* IFA No. 278 without any protectant only 0.2% survived, while after addition of 32% (w/v) trehalose or sucrose, survival rates reached 36.9% and 36.4%, respectively (Strasser et al., 2009). However, not all studies result in positive effects of protectants. For example, when *E. faecium* M74 was sprayed on a microcrystalline cellulose carrier, addition of 100% (w/w wet cell mass) skim milk or sucrose showed no increase in survival rates compared to the cells where no protectant was added (Stummer et al., 2012).

2.4.2.2 Process parameters

Since fluidized bed drying is predominantly used as a second drying technique, it mostly influences the moisture level of the dried particles. Moreover, whenever the moisture levels are above 15% during the drying process, the drying temperature appears to have no great influence on bacterial viability (Bayrock and Ingledew, 1997). However, with lower $a_{w,}$ its influence gets more important. When spraying a bacterial suspension in a fluid bed dryer, also atomizing air pressure and spray time seemed to influence the viability. Stummer et al. (2012) showed that increasing the pressure above 1.5 bar and increasing the spray time above 30 min resulted in significant viability loss (4 log units) of *E. faecium* M74. Several mathematical and empirical models, describing heat and mass transfer in a fluid bed dryer, have been studied, taken into account the effect of the process parameters such as, loading rate, hot air humidity and temperature (Akbari et al., 2012; Debaste et al., 2008; Türker et al., 2006). Again, it seems important that every process parameter is optimized for every single probiotic strain, in order to achieve a good balance between time, temperature and a_w.

2.4.2.3 Prestressing strategies

Nag and Das (2013) compared the viability of osmotically stressed *L. casei* CRL 431 cells with unstressed cells after fluidized bed drying. Stressed cells showed better survival rates after drying and during subsequent storage at room temperature for one year. Similar results were observed when *L. rhamnosus* HN001 was prestressed with a higher temperature or osmotic pressure (Prasad et al., 2003). When the probiotics were stored at 30 °C for 14 weeks, viability reductions of 1.6 and 2 log units, respectively, were observed compared to a 7.3 log unit reduction of the unstressed cells. The authors observed that there was an upregulation in the expression of GroEL, a well-known heat-shock protein, in the prestressed bacteria. This could be the reason why they showed an enhanced viability after fluidized bed drying.

2.4.3 Storage

Lower storage temperatures promote bacterial survival, as the molecular mobility is limited at these temperatures. For instance, when the storage temperature of dried *L. casei* CRC 431 was increased from 25 °C to 37 °C, a higher log reduction was observed. Storing dried powder of *L. plantarum* IFA No 045 and *E. faecium* IFA No 278 at 4 °C, also resulted in better survival rates than when stored at higher temperatures of 22 °C and 35 °C (Strasser et al., 2009).

Another study showed that storage of *L. plantarum* DSM 20174 at -20 °C or 4 °C for 3 months preserved the viability, whereas storage at 20 °C resulted in a 99% reduction of viability (Bensch et al., 2014). Next to lowering the storage temperature, adding excipients can also enhance viability during storage. For example *L. casei* CRL 431 showed better viability after storage for 20 weeks at 25 °C when 0.5% (w/w) vitamin E was added to the bacterial suspension. Vitamin E appears to improve the storage stability by protecting the cells against oxidative damage (Nag and Das, 2013).

2.4.4 Rehydration

Regarding the rehydration conditions of fluid bed dried probiotics, it appears that higher rehydration temperatures lead to better recovery of probiotics. Dried *L. helveticus* CNRZ 303 showed, for example, better recovery rates when the rehydration medium had a temperature of 20 °C or 30 °C, whereas the viability declined at 5 °C (Selmer-Olsen et al., 1999). Likewise, *L. bulgaricus* RD 546 and *L. plantarum* RD 263 showed higher viabilities when rehydrated at higher temperatures (30-37 °C), which is in line with the theory of the membrane phase transition temperature (Mille et al., 2004).

3. Conclusion and future perspectives

The use of probiotics and pharmabiotics has become appealing for the pharmaceutical industry, as can be seen by the increasing commercially available probiotic products, the booming research and the rising patent applications. Because pharmaceutical formulations with probiotics need to be stable during long-term storage, understanding the fundamental processing steps during drying is important for the selection of the optimal drying technique and protection strategy. The most relevant drying techniques for the production of pharmabiotics are spray drying, freeze drying, and vacuum drying. Fluidized bed drying can prove useful, for example to encapsulate probiotics, but further research is still necessary to fully show the potential of this technique. Apart from the dehydration stress, each technique opposes different kinds of stress on the probiotic cells. For example, frost damage can be observed during freeze drying, whereas heat inactivation is common during spray drying. To diminish loss in probiotic viability due to these stresses, several protection strategies can be distinguished, including addition of protective agents, controlling the process parameters and prestressing probiotics prior to drying. For freeze drying, applying these protection strategies offers good viability results and high yields. However, the production of freeze-dried powders is still a time-consuming and relative expensive process. Theoretically, vacuum drying seems to be a good alternative, because more gentle process parameters are applied. In practice, however, viability results after vacuum drying are lower compared with freeze drying. A hypothesis is that slowing down the probiotic metabolism rate prior to dehydration, by using low temperatures (as with freeze drying), may have a positive effect on the viability. Therefore, controlled low-temperature vacuum dehydration (CLTV) emerges as a promising alternative to freeze drying. However, research is still rather limited. Spray drying currently appears to be the most promising alternative for freeze drying, showing several advantages. It is a rapid, continuous process which makes it cost-effective and relatively easy to scale up. But most importantly, the particle characteristics can be easily controlled, thus making it

possible to manufacture a powder with desired properties, such as moisture content, flow properties, size and shape distribution. Still the probiotic industry is hesitant in using this technique as a genuine alternative of freeze drying. This probably results from the relatively varying viability results obtained after spray drying and the lack of sufficient long-term stability data compared to freeze drying.

The different drying techniques all use similar protection strategies. In general, the addition of protectants and the adaptation of processing parameters are the most extensively studied strategies. The addition of disaccharides, such as trehalose, lactose or sucrose, is a relative simple strategy, offering promising viability results and should thus be considered as the first enhancement strategy concerning probiotic survival rates. When storing pharmabiotics for long time, powders with low residual moisture contents or water activity (values of 4% and 0.1, respectively) result in better survivability. It is therefore also advisable to store dry pharmabiotic powders in low relative humidity atmospheres, since this will limit the moisture uptake from the atmosphere by the powders..

Nevertheless, we like to point out that bacterial survival is not the only important parameter when choosing the optimal drying approach. More research is still required on the preservation of the key functional properties and probiotic activities, such as adhesion to target cells and antimicrobial activities, by the different drying techniques. The application of these drying techniques can influence the intactness of probiotic cellular structure molecules (e.g. exopolysaccharides, lipopolysaccharides, lipoteichoic acids, pili) and thereby alter the health-beneficial effects. With the current focus primarily on the survival of the probiotic bacterial cells and maintenance of high titers, the effects of the drying techniques on important cellular structures is currently greatly overlooked. With the advancement of probiotics to pharmabiotics, we foresee a growing importance of in-depth research on the effects of drying techniques on the preservation of the health benefits of probiotics.

Acknowledgments

The research of Géraldine Broeckx is funded by a PhD grant of the Agency for Innovation by Science and Technology in Flanders (IWT). This work was supported by an IOF-SBO (FFI130233) grant of the University of Antwerp and an IWT-SBO grant (ProCure, 150052) to Sarah Lebeer and Filip Kiekens.

References

Abadias, M., Benabarre, A., Teixidó, N., Usall, J., Vias, I., 2001. Effect of freeze drying and protectants on viability of the biocontrol yeast *Candida sake*. Int. J. Food Microbiol. 65, 173–182. doi:10.1016/S0168-1605(00)00513-4

Akbari, H., Karimi, K., Lundin, M., Taherzadeh, M.J., 2012. Optimization of baker's yeast drying in industrial continuous fluidized bed dryer. Food Bioprod. Process. 90, 52–57. doi:10.1016/j.fbp.2010.12.005

Alp, G., Aslim, B., 2010. Relationship between the resistance to bile salts and low pH with exopolysaccharide (EPS) production of *Bifidobacterium* spp. isolated from infants feces and breast milk. Anaerobe 16, 101–105. doi:10.1016/j.anaerobe.2009.06.006

Ananta, E., 2005. Impact of environmental factors on viability and stability and high pressure pretreatment on stress tolerance of. Technical University of Berlin.

Ananta, E., Knorr, D., 2004. Evidence on the role of protein biosynthesis in the induction of heat tolerance of *Lactobacillus rhamnosus* GG by pressure pre-treatment. Int. J. Food Microbiol. 96, 307–313. doi:10.1016/j.ijfoodmicro.2004.04.012

Andersen, H.D., Wang, C., Arleth, L., Peters, G.H., Westh, P., 2011. Reconciliation of opposing views on membrane-sugar interactions. Proc. Natl. Acad. Sci. U. S. A. 108, 1874–1878. doi:10.1073/pnas.1012516108

Andersson, D.I., Hughes, D., 2011. Persistence of antibiotic resistance in bacterial populations. FEMS Microbiol. Rev. 35, 901–911. doi:10.1111/j.1574-6976.2011.00289.x

Aschenbrenner, M., Grammueller, E., Kulozik, U., Foerst, P., 2014. The Contribution of the Inherent Restricted Mobility of Glassy Sugar Matrices to the Overall Stability of Freeze-Dried Bacteria Determined by Low-Resolution Solid-State 1 H-NMR. Food Bioprocess Technol. 7, 1012–1024. doi:10.1007/s11947-013-1095-7

Aschenbrenner, M., Kulozik, U., Foerst, P., 2012. Evaluation of the relevance of the glassy state as stability criterion for freeze-dried bacteria by application of the Arrhenius and WLF model. Cryobiology 65, 308–318. doi:10.1016/j.cryobiol.2012.08.005

Avila-Reyes, S.V., Garcia-Suarez, F.J., Jiménez, M.T., San Martín-Gonzalez, M.F., Bello-Perez, L.A., 2014. Protection of *L. rhamnosus* by spray-drying using two prebiotics colloids to enhance the viability. Carbohydr. Polym. 102, 423–430. doi:10.1016/j.carbpol.2013.11.033

Azim, H., Kalavathy, R., Julianto, T., Sieo, C.C., Ho, Y.W., 2012. Effect of heat, pH and coating process with stearic acid using a fluidized bed granulator on viability of probiotic *Lactobacillus reuteri* C 10. African J. Biotechnol. 11, 6857–6865. doi:10.5897/AJB11.2984

Barbaree, J.M., Sanchez, A., 1982. Cross-contamination during lyophilization. Cryobiology 19, 443–447. doi:10.1016/0011-2240(82)90173-0

Barbosa-Cánovas, G.V., Juliani, P., 2004. Adaptation of classical processes to new technical developments and quality requirements. J. Food Sci. 69, 240–250.

Bauer, S.A.W., Schneider, S., Behr, J., Kulozik, U., Foerst, P., 2012. Combined influence of fermentation and drying conditions on survival and metabolic activity of starter and probiotic cultures after low-temperature vacuum drying. J. Biotechnol. 159, 351–357. doi:10.1016/j.jbiotec.2011.06.010

Bayrock, D., Ingledew, W.M., 1997. Mechanism of viability loss during fluidized bed drying of baker's yeast. Food Res. Int. 30, 417–425. doi:10.1016/S0963-9969(97)00072-0

Behboudi-Jobbehdar, S., Soukoulis, C., Yonekura, L., Fisk, I., 2013. Optimization of Spray-Drying Process Conditions for the Production of Maximally Viable Microencapsulated *L. acidophilus* NCIMB 701748. Dry. Technol. 31, 1274–1283. doi:10.1080/07373937.2013.788509

Bensch, G., Rüger, M., Wassermann, M., Weinholz, S., Reichl, U., Cordes, C., 2014. Flow cytometric viability assessment of lactic acid bacteria starter cultures produced by fluidized bed drying. Appl. Microbiol. Biotechnol. 98, 4897–4909. doi:10.1007/s00253-014-5592-z

Berner, D., Viernstein, H., 2006. Effect of protective agents on the viability of *Lactococcus lactis* subjected to freeze-thawing and freeze-drying. Sci. Pharm. 74, 137–149. doi:10.3797/scipharm.2006.74.137

Bielecka, M., Majkowska, A., 2000. Effect of spray drying temperature of yoghurt on the survival of starter cultures, moisture content and sensoric properties of yoghurt powder. Nahrung 44, 257–260. doi:10.1002/1521-3803(20000701)44:4<257::AID-FOOD257>3.0.CO;2-E

Bryant, G., Koster, K.L., Wolfe, J., 2007. Membrane behaviour in seeds and other systems at low water content: the various effects of solutes. Seed Sci. Res. 11, 17–25. doi:10.1079/SSR200056

Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X., Gibbs, P., 2002. Survival of freeze-dried Lactobacillus plantarum and Lactobacillus rhamnosus during storage in the presence of protectants. Biotechnol. Lett. 24, 1587–1591. doi:10.1023/A:1020301614728

Castro, H.P., Teixeira, P.M., Kirby, R., 1997. Evidence of membrane damage in *Lactobacillus bulgaricus* following freeze drying. J. Appl. Microbiol. 82, 87–94. doi:10.1111/j.1365-2672.1997.tb03301.x

Chávez, B.E., Ledeboer, A.M., 2007. Drying of Probiotics: Optimization of Formulation and Process to Enhance Storage Survival. Dry. Technol. 25, 1193–1201. doi:10.1080/07373930701438576

Chen, H.C., Lin, C.W., Chen, M.J., 2006. The effects of freeze drying and rehydration on survival of microorganisms in kefir. Asian-Australasian J. Anim. Sci. 19, 126–130.

Chua, K.J., Chou, S.K., 2003. Low-cost drying methods for developing countries. Trends Food Sci. Technol. 14, 519–528. doi:10.1016/j.tifs.2003.07.003

Claes, I.J.J., Vargas García, C.E., Lebeer, S., 2015. Novel opportunities for the exploitation of host–microbiome interactions in the intestine. Curr. Opin. Biotechnol. 32, 28–34. doi:10.1016/j.copbio.2014.10.008

Clegg, J.S., Seitz, P., Seitz, W., Hazlewood, C.F., 1982. Cellular responses to extreme water loss: The water-replacement hypothesis. Cryobiology 19, 306–316. doi:10.1016/0011-2240(82)90159-6

Conrad, P.B., Miller, D.P., Cielenski, P.R., de Pablo, J.J., 2000. Stabilization and preservation of Lactobacillus acidophilus in saccharide matrices. Cryobiology 41, 17–24. doi:10.1006/cryo.2000.2260

Corcoran, B.M., Ross, R.P., Fitzgerald, G.F., Dockery, P., Stanton, C., 2006. Enhanced survival of GroESL-overproducing *Lactobacillus paracasei* NFBC 338 under stressful conditions induced by drying. Appl. Environ. Microbiol. 72, 5104–5107. doi:10.1128/AEM.02626-05

Corcoran, B.M., Ross, R.P., Fitzgerald, G.F., Stanton, C., 2004. Comparative survival of probiotic lactobacilli spray-dried in the presence of prebiotic substances. J. Appl. Microbiol. 96, 1024–1039. doi:10.1111/j.1365-2672.2004.02219.x

Crowe, J., 2007. Trehalose As a "Chemical Chaperone" Fact and Fantasy, in: Csermely, P., Vigh, L. (Eds.), Molecular Aspects of the Stress Response: Chaperones, Membranes and Networks. Springers New York, pp. 143–158. doi:10.1007/978-0-387-39975-1

Crowe, J.H., Crowe, L.M., Oliver, A.E., Tsvetkova, N., Wolkers, W., Tablin, F., 2001. The Trehalose Myth Revisited: Introduction to a Symposium on Stabilization of Cells in the Dry State. Cryobiology 43, 89–105. doi:10.1006/cryo.2001.2353

Debaste, F., Halloin, V., Bossart, L., Haut, B., 2008. A new modeling approach for the prediction of yeast drying rates in fluidized beds. J. Food Eng. 84, 335–347. doi:10.1016/j.jfoodeng.2007.05.022

Degnan, F.H., 2008. The US Food and Drug Administration and Probiotics: Regulatory Categorization. Clin. Infect. Dis. 46 Suppl 2, S133–S136; discussion S144–S151. doi:10.1086/523324

Desmond, C., Stanton, C., Fitzgerald, G.F., Collins, K., Ross, R.P., 2002. Environmental adaptation of probiotic lactobacilli towards improvement of performance during spray drying. Int. Dairy J. 12, 183–190. doi:10.1016/S0958-6946(02)00040-7

Dianawati, D., Mishra, V., Shah, N.P., 2013. Stability of microencapsulated Lactobacillus acidophilus and Lactococcus lactis ssp. cremoris during storage at room temperature at low aw. Food Res. Int. 50, 259–265. doi:10.1016/j.foodres.2012.10.023

Dianawati, D., Shah, N.P., 2011. Enzyme Stability of Microencapsulated Bifidobacterium animalis ssp. lactis Bb12 after Freeze Drying and during Storage in Low Water Activity at Room Temperature. J. Food Sci. 76, 463–471. doi:10.1111/j.1750-3841.2011.02246.x

Dickson, R.P., Huffnagle, G.B., 2015. The Lung Microbiome: New Principles for Respiratory Bacteriology in Health and Disease. PLOS Pathog. 11, e1004923. doi:10.1371/journal.ppat.1004923

Dijkstra, A.R., Setyawati, M.C., Bayjanov, J.R., Alkema, W., Van Hijum, S.A.F.T., Bron, P.A., Hugenholtz, J., 2014. Diversity in robustness of lactococcus lactis strains during heat stress, oxidative stress, and spray drying stress. Appl. Environ. Microbiol. 80, 603–611. doi:10.1128/AEM.03434-13

Donsì, F., Ferrari, G., Lenza, E., Maresca, P., 2009. Main factors regulating microbial inactivation by high-pressure homogenization: Operating parameters and scale of operation. Chem. Eng. Sci. 64, 520–532. doi:10.1016/j.ces.2008.10.002

Fávaro-Trindade, C.S., Grosso, C.R.F., 2002. Microencapsulation of L. acidophilus (La-05) and B. lactis (Bb-12) and evaluation of their survival at the pH values of the stomach and in bile. J. Microencapsul. 19, 485–494. doi:10.1080/02652040210140715

Ferreira, V., Soares, V., Santos, C., Silva, J., Gibbs, P.A., Teixeira, P., 2005. Survival of Lactobacillus sakei during heating, drying and storage in the dried state when growth has occurred in the presence of sucrose or monosodium glutamate. Biotechnol. Lett. 27, 249–252. doi:10.1007/s10529-004-8351-x

Foerst, P., Kulozik, U., Schmitt, M., Bauer, S., Santivarangkna, C., 2012. Storage stability of vacuum-dried probiotic bacterium Lactobacillus paracasei F19. Food Bioprod. Process. 90, 295–300. doi:10.1016/j.fbp.2011.06.004

Foligné, B., Daniel, C., Pot, B., 2013. Probiotics from research to market: the possibilities, risks and challenges. Curr. Opin. Microbiol. 16, 284–292. doi:10.1016/j.mib.2013.06.008

Fowler, A., Toner, M., 2005. Cryo-injury and biopreservation. Ann. N. Y. Acad. Sci. 1066, 119–135. doi:10.1196/annals.1363.010

Fu, N., Chen, X.D., 2011. Towards a maximal cell survival in convective thermal drying processes. Food Res. Int. 44, 1127–1149. doi:10.1016/j.foodres.2011.03.053

Fu, W.-Y., Etzel, M.R., 1995. Spray Drying of Lactococcus lactis ssp. lactis C2 and Cellular Injury. J. Food Sci. 60, 195–200.

Gardiner, G.E., Sullivan, E.O., Kelly, J., Auty, M.A.E., Collins, J.K., Ross, R.P., Stanton, C., 2000. Comparative Survival Rates of Human-Derived Probiotic Lactobacillus paracasei and L . salivarius Strains during Heat Treatment and Spray Drying. Appl. Environ. Microbiol. 66, 2605–2612. doi:10.1128/AEM.66.6.2605-2612.2000.Updated

Garvey, C.J., Lenné, T., Koster, K.L., Kent, B., Bryant, G., 2013. Phospholipid membrane protection by sugar molecules during dehydration-insights into molecular mechanisms using scattering techniques. Int. J. Mol. Sci. 14, 8148–8163. doi:10.3390/ijms14048148

Ghandi, A., Powell, I.B., Howes, T., Chen, X.D., Adhikari, B., 2012. Effect of shear rate and oxygen stresses on the survival of Lactococcus lactis during the atomization and drying stages of spray drying: A laboratory and pilot scale study. J. Food Eng. 113, 194–200. doi:10.1016/j.jfoodeng.2012.06.005

Golman, B., Julklang, W., 2013. Analysis of Drying Kinetics of a Slurry Droplet in the Falling Rate Period of Spray Drying 7, 344–348.

Golovina, E.A., Golovin, A.V., Hoekstra, F.A., Faller, R., 2009. Water replacement hypothesis in atomic detail - Factors determining the structure of dehydrated bilayer stacks. Biophys. J. 97, 490–499. doi:10.1016/j.bpj.2009.05.007

Golowczyc, M.A., Gerez, C.L., Silva, J., Abraham, A.G., de Antoni, G.L., Teixeira, P., 2011a. Survival of spray-dried Lactobacillus kefir is affected by different protectants and storage conditions. Biotechnol. Lett. 33, 681–686. doi:10.1007/s10529-010-0491-6

Golowczyc, M.A., Silva, J., Abraham, A.G., De Antoni, G.L., Teixeira, P., 2010. Preservation of probiotic strains isolated from kefir by spray drying. Lett. Appl. Microbiol. 50, 7–12. doi:10.1111/j.1472-765X.2009.02759.x

Golowczyc, M.A., Silva, J., Teixeira, P., De Antoni, G.L., Abraham, A.G., 2011b. Cellular injuries of spray-dried Lactobacillus spp. isolated from kefir and their impact on probiotic properties. Int. J. Food Microbiol. 144, 556–560. doi:10.1016/j.ijfoodmicro.2010.11.005

Gómez Zavaglia, A., Tymczyszyn, E., De Antoni, G., Disalvo, E.A., 2003. Action of trehalose on the preservation of Lactobacillus delbrueckii ssp. bulgaricus by heat and osmotic dehydration. J. Appl. Microbiol. 95, 1315–1320. doi:10.1046/j.1365-2672.2003.02102.x

Grasmeijer, N., Stankovic, M., de Waard, H., Frijlink, H.W., Hinrichs, W.L.J., 2013. Unraveling protein stabilization mechanisms: Vitrification and water replacement in a glass transition temperature controlled system. Biochim. Biophys. Acta 1834, 763–769. doi:10.1016/j.bbapap.2013.01.020

Higl, B., Kurtmann, L., Carlsen, C.U., Ratjen, J., Först, P., Skibsted, L.H., Kulozik, U., Risbo, J., 2007. Impact of water activity, temperature, and physical state on the storage stability of Lactobacillus paracasei ssp. paracasei freeze-dried in a lactose matrix. Biotechnol. Prog. 23, 794–800. doi:10.1021/bp070089d

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., Calder, P.C., Sanders, M.E., 2014. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat. Rev. Gastroenterol. Hepatol. 11, 506–514. doi:10.1038/nrgastro.2014.66

Hilty, M., Qi, W., Brugger, S.D., Frei, L., Agyeman, P., Frey, P.M., Aebi, S., Mühlemann, K., 2012. Nasopharyngeal microbiota in infants with acute otitis media. J. Infect. Dis. 205, 1048–1055. doi:10.1093/infdis/jis024

Hoffman, F.A., Heimbach, J.T., Sanders, M.E., Hibberd, P.L., 2008. Executive Summary: Scientific and Regulatory Challenges of Development of Probiotics as Foods and Drugs. Clin. Infect. Dis. 46, S53–S57. doi:10.1086/523342

Hongpattarakere, T., Uraipan, S., 2015. Bifidogenic characteristic and protective effect of saba starch on survival of Lactobacillus plantarum CIF17AN2 during vacuum-drying and storage. Carbohydr. Polym. 117, 255–261. doi:10.1016/j.carbpol.2014.09.065

Huang, S., Chen, X.D., 2013. Significant effect of Ca^{2+} on improving the heat resistance of lactic acid bacteria. FEMS Microbiol. Lett. 344, 31–38. doi:10.1111/1574-6968.12151

Jalali, M., Abedi, D., Varshosaz, J., Najjarzadeh, M., Mirlohi, M., Tavakoli, N., 2012. tolerance and Lactobacillus delbrueckii subsp . bulgaricus in oral capsules 7, 31–36.

Janning, B., in t Veld, P.H., 1994. Susceptibility of bacterial strains to desiccation: A simple method to test their stability in microbiological reference materials. Anal. Chim. Acta 286, 469–476. doi:10.1016/0003-2670(94)85092-5

Jantzen, M., Göpel, A., Beermann, C., 2013. Direct spray drying and microencapsulation of probiotic Lactobacillus reuteri from slurry fermentation with whey. J. Appl. Microbiol. 115, 1029–1036. doi:10.1111/jam.12293

Jofré, A., Aymerich, T., Garriga, M., 2015. Impact of different cryoprotectants on the survival of freeze-dried Lactobacillus rhamnosus and Lactobacillus casei/paracasei during long-term storage. Benef. Microbes 6, 381–386.

Kennedy, P.J., 2014. Irritable bowel syndrome: A microbiome-gut-brain axis disorder? World J. Gastroenterol. 20, 14105–14125. doi:10.3748/wjg.v20.i39.14105

Kets, E.P.W., Teunissen, P.J.M., De Bont, J.A.M., 1996. Effect of compatible solutes on survival of lactic acid bacteria subjected to drying. Appl. Environ. Microbiol. 62, 259–261.

Khem, S., Woo, M.W., Small, D.M., Chen, X.D., May, B.K., 2015. Agent selection and protective effects during single droplet drying of bacteria. Food Chem. 166, 206–214. doi:10.1016/j.foodchem.2014.06.010

King, V.A.E., Su, J.T., 1994. Dehydration of Lactobacillus acidophilus. Process Biochem. 28, 47–52. doi:10.1016/0032-9592(94)80035-9

King, V.A.E., Zall, R.R., Ludington, D.C., 1989. Controlled Low-Temperature Vacuum Dehydration-A New Approach for Low-Temperature and Low-Pressure Food Drying. J. Food Sci. 54, 1573–1593.

Kurtmann, L., Carlsen, C.U., Risbo, J., Skibsted, L.H., 2009. Storage stability of freeze-dried Lactobacillus acidophilus (La-5) in relation to water activity and presence of oxygen and ascorbate. Cryobiology 58, 175–180. doi:10.1016/j.cryobiol.2008.12.001

Lapsiri, W., Bhandari, B., Wanchaitanawong, P., 2012. TISTR 2075 in Different Protectants during Spray Drying and Storage. Dry. Technol. 30, 1407–1412. doi:10.1080/07373937.2012.684226

Lavari, L., Ianniello, R., Páez, R., Zotta, T., Cuatrin, A., Reinheimer, J., Parente, E., Vinderola, G., 2015. Growth of Lactobacillus rhamnosus 64 in whey permeate and study

of the effect of mild stresses on survival to spray drying. LWT - Food Sci. Technol. doi:10.1016/j.lwt.2015.03.066

Lebeer, S., Claes, I.J.J., Verhoeven, T.L., Vanderleyden, J., De Keersmaecker, S.C.J., 2011. Exopolysaccharides of Latobacillus rhamnosus GG form a protective shield against innate immune factors in the intestine. Microb. Biotechnol. 4, 368–374. doi:10.1111/j.1751-7915.2010.00199.x.

Lebeer, S., Vanderleyden, J., De Keersmaecker, S.C.J., 2010. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat. Rev. Microbiol. 8, 171–184. doi:10.1038/nrmicro2297

Lebeer, S., Vanderleyden, J., De Keersmaecker, S.C.J., 2008. Genes and molecules of lactobacilli supporting probiotic action. Microbiol. Mol. Biol. Rev. 72, 728–764. doi:10.1128/MMBR.00017-08

Lee, J., 2002. Evaluation of the Heat Inactivation of Escherichia coli and Lactobacillus plantarum by Differential Scanning Calorimetry. Society 68, 5379–5386. doi:10.1128/AEM.68.11.5379

Leja, K., Bia, W., Jankowski, T., 2009. Production of dry Lactobacillus rhamnosus GG preparations by spray drying and lyophilization in aqueous two-phase systems. Acta Sci. Pol. Technol. Aliment. 8, 39–49.

Lievense, L.C., van't Riet, K., 1994. Convective drying of bacteria II. Factors influencing survival. Adv. Biochem. Eng. Biotechnol. 51, 71–89.

Maltesen, M.J., van de Weert, M., 2008. Drying methods for protein pharmaceuticals. Drug Discov. Today Technol. 5, e81–e88. doi:10.1016/j.ddtec.2008.11.001

Masters, K., 1991. Spray Drying Handbook, 5th ed. Longman Scientific and Technical, London.

Meng, X.C., Stanton, C., Fitzgerald, G.F., Daly, C., Ross, R.P., 2008. Anhydrobiotics: The challenges of drying probiotic cultures. Food Chem. 106, 1406–1416. doi:10.1016/j.foodchem.2007.04.076

Mille, Y., Obert, J.P., Beney, L., Gervais, P., 2004. New drying process for lactic bacteria based on their dehydration behavior in liquid medium. Biotechnol. Bioeng. 88, 71–76. doi:10.1002/bit.20211

Ming, L.C., Rahim, R.A., Wan, H.Y., Ariff, A.B., 2009. Formulation of protective agents for improvement of Lactobacillus salivarius I 24 survival rate subjected to freeze drying for production of live cells in powderized form. Food Bioprocess Technol. 2, 431–436. doi:10.1007/s11947-009-0184-0

Muller, J.A., Stanton, C., Sybesma, W., Fitzgerald, G.F., Ross, R.P., 2010. Reconstitution conditions for dried probiotic powders represent a critical step in determining cell viability. J. Appl. Microbiol. 108, 1369–1379. doi:10.1111/j.1365-2672.2009.04533.x

Nag, A., Das, S., 2013. Improving ambient temperature stability of probiotics with stress adaptation and fluidized bed drying. J. Funct. Foods 5, 170–177. doi:10.1016/j.jff.2012.10.001

Nguyen, H.T., Razafindralambo, H., Blecker, C., N'Yapo, C., Thonart, P., Delvigne, F., 2014. Stochastic exposure to sub-lethal high temperature enhances exopolysaccharides (EPS) excretion and improves bifidobacterium bifidum cell survival to freeze-drying. Biochem. Eng. J. 88, 85–94. doi:10.1016/j.bej.2014.04.005

Oliphant, C.M., Eroschenko, K., 2015. Antibiotic Resistance, Part 1: Gram-positive Pathogens. J. Nurse Pract. 11, 70–78. doi:10.1016/j.nurpra.2014.09.018

Oliver, A.E., Crowe, L.M., Crowe, J.H., 1998. Methods for dehydration-tolerance: Depression of the phase transition temperature in dry membranes and carbohydrate vitrification. Seed Sci. Res. 8, 211–221. doi:10.1017/S0960258500004128

Otero, M.C., Espeche, M.C., Nader-Macías, M.E., 2007. Optimization of the freeze-drying media and survival throughout storage of freeze-dried Lactobacillus gasseri and Lactobacillus delbrueckii subsp. delbrueckii for veterinarian probiotic applications. Process Biochem. 42, 1406–1411. doi:10.1016/j.procbio.2007.07.008

Paéz, R., Lavari, L., Vinderola, G., Audero, G., Cuatrin, A., Zaritzky, N., Reinheimer, J., 2012. Effect of heat treatment and spray drying on lactobacilli viability and resistance to simulated gastrointestinal digestion. Food Res. Int. 48, 748–754. doi:10.1016/j.foodres.2012.06.018

Palmfeldt, J., Hahn-Hägerdal, B., 2000. Influence of culture pH on survival of Lactobacillus reuteri subjected to freeze-drying. Int. J. Food Microbiol. 55, 235–238. doi:10.1016/S0168-1605(00)00176-8

Peighambardoust, S.H., Golshan Tafti, A., Hesari, J., 2011. Application of spray drying for preservation of lactic acid starter cultures: a review. Trends Food Sci. Technol. 22, 215–224. doi:10.1016/j.tifs.2011.01.009

Perdana, J., Bereschenko, L., Fox, M.B., Kuperus, J.H., Kleerebezem, M., Boom, R.M., Schutyser, M.A.I., 2013. Dehydration and thermal inactivation of Lactobacillus plantarum WCFS1: Comparing single droplet drying to spray and freeze drying. Food Res. Int. 54, 1351–1359. doi:10.1016/j.foodres.2013.09.043

Perdana, J., Fox, M.B., Siwei, C., Boom, R.M., Schutyser, M.A.I., 2014. Interactions between formulation and spray drying conditions related to survival of lactobacillus plantarum WCFS1. Food Res. Int. 56, 9–17. doi:10.1016/j.foodres.2013.12.007

Pispan, S., Hewitt, C.J., Stapley, A.G.F., 2013. Comparison of cell survival rates of E. coli K12 and L. acidophilus undergoing spray drying. Food Bioprod. Process. 91, 362–369. doi:10.1016/j.fbp.2013.01.005

Poirier, I., Maréchal, P. a., Richard, S., Gervais, P., 1999. Saccharomyces cerevisiae viability is strongly dependant on rehydration kinetics and the temperature of dried cells. J. Appl. Microbiol. 86, 87–92. doi:10.1046/j.1365-2672.1999.00638.x

Poolman, B., 2002. Transporters and their roles in LAB cell physiology. Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 82, 147–164. doi:10.1023/A:1020658831293

Prasad, J., Mcjarrow, P., Gopal, P., 2003. Heat and Osmotic Stress Responses of Probiotic Lactobacillus rhamnosus HN001 (DR20) in Relation to Viability after Drying Heat and Osmotic Stress. Appl. Environ. Microbiol. 69, 917–925. doi:10.1128/AEM.69.2.917

Rijkers, G.T., de Vos, W.M., Brummer, R.-J., Morelli, L., Corthier, G., Marteau, P., 2011. Health benefits and health claims of probiotics: bridging science and marketing. Br. J. Nutr. 106, 1291–1296. doi:10.1017/S000711451100287X

Riveros, B., Ferrer, J., Bórquez, R., 2009. Spray Drying of a Vaginal Probiotic Strain of *Lactobacillus acidophilus*. Dry. Technol. 27, 123–132. doi:10.1080/07373930802566002

Romano, A., Blaiotta, G., Di Cerbo, A., Coppola, R., Masi, P., Aponte, M., 2014. Spray-dried chestnut extract containing Lactobacillus rhamnosus cells as novel ingredient for a probiotic chestnut mousse. J. Appl. Microbiol. 116, 1632–1641. doi:10.1111/jam.12470

Saarela, M.H., Alakomi, H.L., Puhakka, a., Mättö, J., 2009. Effect of the fermentation pH on the storage stability of Lactobacillus rhamnosus preparations and suitability of in vitro analyses of cell physiological functions to predict it. J. Appl. Microbiol. 106, 1204–1212. doi:10.1111/j.1365-2672.2008.04089.x

Sanders, M.E., Levy, D.D., 2011. The science and regulations of probiotic food and supplement product labeling. Ann. N. Y. Acad. Sci. 1219, 1–23. doi:10.1111/j.1749-6632.2010.05956.x

Santivarangkna, C., Higl, B., Foerst, P., 2008a. Protection mechanisms of sugars during different stages of preparation process of dried lactic acid starter cultures. Food Microbiol. 25, 429–441. doi:10.1016/j.fm.2007.12.004

Santivarangkna, C., Kulozik, U., Foerst, P., 2008b. Inactivation mechanisms of lactic acid starter cultures preserved by drying processes. J. Appl. Microbiol. 105, 1–13. doi:10.1111/j.1365-2672.2008.03744.x

Santivarangkna, C., Kulozik, U., Foerst, P., 2007. Alternative drying processes for the industrial preservation of lactic acid starter cultures. Biotechnol. Prog. 23, 302–15. doi:10.1021/bp060268f

Santivarangkna, C., Kulozik, U., Foerst, P., 2006. Effect of carbohydrates on the survival of Lactobacillus helveticus during vacuum drying. Lett. Appl. Microbiol. 42, 271–276. doi:10.1111/j.1472-765X.2005.01835.x

Santivarangkna, C., Kulozik, U., Kienberger, H., Foerst, P., 2009. Changes in membrane fatty acids of Lactobacillus helveticus during vacuum drying with sorbitol. Lett. Appl. Microbiol. 49, 516–521. doi:10.1111/j.1472-765X.2009.02703.x

Santivarangkna, C., Wenning, M., Foerst, P., Kulozik, U., 2007. Damage of cell envelope of Lactobacillus helveticus during vacuum drying. J. Appl. Microbiol. 102, 748–756. doi:10.1111/j.1365-2672.2006.03123.x

Santos, M.I., Araujo-Andrade, C., Esparza-Ibarra, E., Tymczyszyn, E., Gómez-Zavaglia, A., 2014. Galacto-oligosaccharides and lactulose as protectants against desiccation of *Lactobacillus delbrueckii* subsp. *bulcaricus*. Biotechnol. Prog. 30, 1231–1238. doi:10.1002/btpr.1969

Sartor, R.B., Mazmanian, S.K., 2012. Intestinal Microbes in Inflammatory Bowel Diseases. Am. J. Gastroenterol. Suppl. 1, 15–21. doi:10.1038/ajgsup.2012.4

Savini, M., Cecchini, C., Verdenelli, M.C., Silvi, S., Orpianesi, C., Cresci, A., 2010. Pilot-scale production and viability analysis of freeze-dried probiotic bacteria using different protective agents. Nutrients 2, 330–339. doi:10.3390/nu2030330

Schoug, Å., Olsson, J., Carlfors, J., Schnürer, J., Håkansson, S., 2006. Freeze-drying of Lactobacillus coryniformis Si3-effects of sucrose concentration, cell density, and freezing rate on cell survival and thermophysical properties. Cryobiology 53, 119–127. doi:10.1016/j.cryobiol.2006.04.003

Schuck, P., Dolivet, a., Méjean, S., Hervé, C., Jeantet, R., 2013. Spray drying of dairy bacteria: New opportunities to improve the viability of bacteria powders. Int. Dairy J. 31, 12–17. doi:10.1016/j.idairyj.2012.01.006

Schutyser, M. a I., Perdana, J., Boom, R.M., 2012. Single droplet drying for optimal spray drying of enzymes and probiotics. Trends Food Sci. Technol. 27, 73–82. doi:10.1016/j.tifs.2012.05.006

Selmer-Olsen, E., Sorhaug, T., Birkeland, S.-E., Pehrson, R., 1999. Survival of Lactobacillus helveticus entrapped in Ca-alginate in relation to water content, storage and rehydration. J. Ind. Microbiol. Biotechnol. 23, 79–85. doi:10.1038/sj.jim.2900693

Sheehan, V.M., Sleator, R.D., Fitzgerald, G.F., Hill, C., 2006. Heterologous expression of BetL, a betaine uptake system, enhances the stress tolerance of Lactobacillus salivarius UCC118. Appl. Environ. Microbiol. 72, 2170–2177. doi:10.1128/AEM.72.3.2170-2177.2006

Shokri, Z., Fazeli, M., Ardjmand, M., Mousavi, S., Gilani, K., 2015. Factors affecting viability of Bifidobacterium bifidum during spray drying. DARU J. Pharm. Sci. 23, 7. doi:10.1186/s40199-014-0088-z

Silva, J., Carvalho, a S., Teixeira, P., Gibbs, P. a, 2002. Bacteriocin production by spray-dried lactic acid bacteria. Lett. Appl. Microbiol. 34, 77–81. doi:10.1046/j.1472-765x.2002.01055.x

Silva, J., Carvalho, a. S., Ferreira, R., Vitorino, R., Amado, F., Domingues, P., Teixeira, P., Gibbs, P. a., 2005. Effect of the pH of growth on the survival of Lactobacillus delbrueckii subsp. bulgaricus to stress conditions during spray-drying. J. Appl. Microbiol. 98, 775–782. doi:10.1111/j.1365-2672.2004.02516.x

Simpson, P.J., Stanton, C., Fitzgerald, G.F., Ross, R.P., 2005. Intrinsic tolerance of Bifidobacterium species to heat and oxygen and survival following spray drying and storage. J. Appl. Microbiol. 99, 493–501. doi:10.1111/j.1365-2672.2005.02648.x

Sosnik, A., Seremeta, K.P., 2015. Advantages and challenges of the spray-drying technology for the production of pure drug particles and drug-loaded polymeric carriers. Adv. Colloid Interface Sci. 223, 40–54. doi:10.1016/j.cis.2015.05.003

Soukoulis, C., Behboudi-Jobbehdar, S., Yonekura, L., Parmenter, C., Fisk, I., 2014. Impact of Milk Protein Type on the Viability and Storage Stability of Microencapsulated Lactobacillus acidophilus NCIMB 701748 Using Spray Drying. Food Bioprocess Technol. 7, 1255–1268. doi:10.1007/s11947-013-1120-x

Strasser, S., Neureiter, M., Geppl, M., Braun, R., Danner, H., 2009. Influence of lyophilization, fluidized bed drying, addition of protectants, and storage on the viability of lactic acid bacteria. J. Appl. Microbiol. 107, 167–177. doi:10.1111/j.1365-2672.2009.04192.x

Stummer, S., Toegel, S., Rabenreither, M.-C., Unger, F.M., Wirth, M., Viernstein, H., Salar-Behzadi, S., 2012. Fluidized-bed drying as a feasible method for dehydration of Enterococcus faecium M74. J. Food Eng. 111, 156–165. doi:10.1016/j.jfoodeng.2012.01.005

Sunny-Roberts, E.O., Knorr, D., 2009. The protective effect of monosodium glutamate on survival of Lactobacillus rhamnosus GG and Lactobacillus rhamnosus E-97800 (E800) strains during spray-drying and storage in trehalose-containing powders. Int. Dairy J. 19, 209–214. doi:10.1016/j.idairyj.2008.10.008

Teixeira, P., Castro, H., Kirby, R., 1994. Spray drying as a method for preparing concentrated cultures of Lactobacillus bulgaricus. J. Appl. Bacteriol. 78, 456–462.

Teixeira, P., Castro, H., Mohácsi-Farkas, C., Kirby, R., 1997. Identification of sites of injury in Lactobacillus bulgaricus during heat stress. J. Appl. Microbiol. 83, 219–226.

Türker, M., Kanarya, A., Yüzgeç, U., Kapucu, H., Şenalp, Z., 2006. Drying of baker's yeast in batch fluidized bed. Chem. Eng. Process. Process Intensif. 45, 1019–1028. doi:10.1016/j.cep.2006.01.016

Tymczyszyn, E.E., Díaz, R., Pataro, A., Sandonato, N., Gómez-Zavaglia, A., Disalvo, E.A., 2008. Critical water activity for the preservation of Lactobacillus bulgaricus by vacuum drying. Int. J. Food Microbiol. 128, 342–347. doi:10.1016/j.ijfoodmicro.2008.09.009

Vandenheuvel, D., Singh, A., Vandersteegen, K., Klumpp, J., Lavigne, R., Van Den Mooter, G., 2013. Feasibility of spray drying bacteriophages into respirable powders to combat pulmonary bacterial infections. Eur. J. Pharm. Biopharm. 84, 578–582. doi:10.1016/j.ejpb.2012.12.022

Vandenplas, Y., Huys, G., Daube, G., 2015. Probiotics: an update. J. Pediatr. (Rio. J). 91, 6–21. doi:10.1016/j.jped.2014.08.005

Vega, C., Roos, Y.H., 2006. Invited review: spray-dried dairy and dairy-like emulsions--compositional considerations. J. Dairy Sci. 89, 383–401. doi:10.3168/jds.S0022-0302(06)72103-8

Venugopalan, V., Shriner, K. a., Wong-Beringer, A., 2010. Regulatory oversight and safety of probiotic use. Emerg. Infect. Dis. 16, 1661–1665. doi:10.3201/eid1611.100574

Vesterlund, S., Salminen, K., Salminen, S., 2012. Water activity in dry foods containing live probiotic bacteria should be carefully considered: A case study with Lactobacillus rhamnosus GG in flaxseed. Int. J. Food Microbiol. 157, 319–321. doi:10.1016/j.ijfoodmicro.2012.05.016

Wang, Y.C., Yu, R.C., Chou, C.C., 2004. Viability of lactic acid bacteria and bifidobacteria in fermented soymilk after drying, subsequent rehydration and storage. Int. J. Food Microbiol. 93, 209–217. doi:10.1016/j.ijfoodmicro.2003.12.001

Xavier, R.J., Podolsky, D.K., 2007. Unravelling the pathogenesis of inflammatory bowel disease. Nature 448, 427–434. doi:10.1038/nature06005

Ying, D., Sun, J., Sanguansri, L., Weerakkody, R., Augustin, M.A., 2012. Enhanced survival of spray-dried microencapsulated Lactobacillus rhamnosus GG in the presence of glucose. J. Food Eng. 109, 597–602. doi:10.1016/j.jfoodeng.2011.10.017

Ying, D.Y., Phoon, M.C., Sanguansri, L., Weerakkody, R., Burgar, I., Augustin, M.A., 2010. Microencapsulated Lactobacillus rhamnosus GG powders: relationship of powder physical properties to probiotic survival during storage. J. Food Sci. 75, E588–95. doi:10.1111/j.1750-3841.2010.01838.x

Zárate, G., Nader-Macias, M.E., 2006. Viability and biological properties of probiotic vaginal lactobacilli after lyophilization and refrigerated storage into gelatin capsules. Process Biochem. 41, 1779–1785. doi:10.1016/j.procbio.2006.03.024

Zayed, G., Roos, Y.H., 2004. Influence of trehalose and moisture content on survival of Lactobacillus salivarius subjected to freeze-drying and storage. Process Biochem. 39, 1081–1086. doi:10.1016/S0032-9592(03)00222-X

Zhao, G., Zhang, G., 2005. Effect of protective agents, freezing temperature, rehydration media on viability of malolactic bacteria subjected to freeze-drying. J. Appl. Microbiol. 99, 333–338. doi:10.1111/j.1365-2672.2005.02587.x

Zheng, X., Fu, N., Duan, M., Woo, M.W., Selomulya, C., Chen, X.D., 2015. The mechanisms of the protective effects of reconstituted skim milk during convective droplet drying of lactic acid bacteria. Food Res. Int. 76, 478–488. doi:10.1016/j.foodres.2015.07.045

Ziadi, M., Touhami, Y., Achour, M., Thonart, P., Hamdi, M., 2005. The effect of heat stress on freeze-drying and conservation of Lactococcus. Biochem. Eng. J. 24, 141–145. doi:10.1016/j.bej.2005.02.001

Captions to figures in the manuscript:

Figure 1: The spray drying process. (A) A schematic overview of a spray dryer. (B) A schematic overview of the different stages during the drying process with indication of the important heat and dehydration stresses. Black dots: bacterial cells, w: water molecules.

Figure 2: Protection strategies can be applied to protect probiotics against stresses and enhance their viability after spray drying.

Figure 3: Overview of the factors affecting the survival of dried probiotic-containing formulations after rehydration.

Figure 4.: Freeze drying. (A) Schematic overview of a freeze dryer. (B) Simplified overview of the process steps with indication of the most important stress factors.

Figure 5: Schematic overview of a vacuum dryer.

Figure 6:. Schematic overview of a fluid bed dryer.

Table 1: Overview of the main characteristics of different drying techniques.

	Spray drying	**Freeze drying**	**Vacuum drying**	**Fluidized bed drying**
Process type	continuous	batch	batch	batch/continuous
Costs*				
Fixed	12%	100%	52.2%	8.8%
Manufacturing	20%	100%	50.6%	17.9%
Control of particle charachteritics	yes	no	no	yes (to some extent)
Knowledge/ experience	increasing	well-known, well-described	limited	limited
Extra processing steps to obtain separate powder particles	no	micronization step is necessary to break up the dried cake into seperate particles	micronization step is necessary to break up the dried cake into seperate particles	granulate material is necessary
Process conditions				
a) Time	seconds-minutes	hours-days	hours-days	hours
b) Temperature	high (up to 200 °C)	low (< 0 °C)	mild	mild
c) Pressure**	limited	high vacuum (≤ 10 mbar)	low vacuum (≥ 10 mbar)	limited

* relative to the cost of freeze drying (Santivarangkna et al., 2007), ** pure vacuum = 0 mbar.