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Enhancing the viability of Lactobacillus rhamnosus GG after spray drying and during storage

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Graphical abstract



Abstract

Increasing knowledge about the human microbiome has led to a growing awareness of the potential of applying probiotics to improve our health. The pharmaceutical industry shows an emerging interest in

pharmaceutical formulations containing these beneficial microbes, the so-called pharmabiotics. An important manufacturing step is the drying of the probiotics, as this can increase the stability and shelf life of the finished pharmabiotic product. Unfortunately, drying also puts stress on microbial cells, thus causing a decrease in viability. We aimed to examine the effect of different drying media and protective excipients on the viability of the prototype probiotic strain *Lactobacillus rhamnosus* GG after spray drying and during subsequent storage for 28 weeks. The presence of phosphates in the drying medium showed to have a superior protective effect, especially during long-term storage at room temperature. Addition of lactose or trehalose resulted in significantly improved survival rates after drying as well as during long-term storage for the tested excipients. Both disaccharides are characterized by a high glass transition temperature. Maltodextrin showed less protective capacities compared to lactose and trehalose in all tested conditions. The usage of mannitol or dextran resulted in sticky powders and low yields, so further testing was not possible. In addition to optimizing the viability, future research will also explore the functionality of cellular probiotic components after spray drying in order to safeguard the probiotic activity of the formulated pharmabiotics.

Key words: spray drying, Lactobacillus rhamnosus GG, protection, viability, stability, saccharides

1. Introduction

The increasing knowledge about the human microbiome, creates more awareness of the important role micro-organisms play in our health and immune system. Growing evidence emerges on the involvement of an imbalance between pathogenic and beneficial bacteria in the pathogenesis of diseases like 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). Several of these diseases are commonly treated with antibiotics. However, these antibiotic treatments often also affect beneficial bacteria, impairing the restoration towards a healthy and balanced microbiome. Furthermore, with antimicrobial resistance rapidly spreading worldwide and the slow

development of new antibiotic products during the last decade, the search for alternative therapies is accelerating, among them the use of probiotic therapies. Probiotics are defined as 'live micro-organisms which, when applied in adequate amounts, confer a health benefit to the host' (FAO/WHO, 2006).

Bacterial probiotic cultures are grown in a liquid medium, however, liquid formulations are bulky and the bacterial viability rapidly decreases, especially when handled at room temperature (Schoug, 2009). One way of preserving the viability of probiotics can be achieved by reducing the available water content. Drying of bacterial cultures is preferred above freezing. Although drying often causes detrimental cell damage, frozen concentrates require sub-zero temperatures during handling and transportation and therefore greatly limit usability and consumer convenience (Broeckx et al., 2016). Nowadays, freeze drying is the most common way to dry probiotics. Although it is a well-known and well-described process, it still has several drawbacks. It is a time-consuming and costly batch process in which the end product is a cake. To obtain a dry particulate powder, additional processing steps are necessary (Broeckx et al., 2016; Maltesen and van de Weert, 2008; Santivarangkna et al., 2008). Therefore, the use of spray drying emerges as a promising alternative in drying probiotic cells. Spray drying is a cheap, continuous, and fast process, where the end product consists of individual powder particles, containing the probiotics. However, the key challenge in drying probiotics is the maintenance of their viability during/after the process and subsequent long-term storage. During spray drying the bacteria encounter several stresses, such as dehydration and high temperatures, next to atomization, osmotic and oxidative stress. These stresses generally lead to damage to cellular structures (Ananta and Knorr, 2004; Gardiner et al., 2000; Golowczyc et al., 2011; Sunny-Roberts and Knorr, 2009; Teixeira et al., 1997), resulting in lower survival rates after drying. Several protection strategies can be envisaged to enhance bacterial viability, such as addition of protective agents, adapting the process parameters, or pre-conditioning the bacterial cells with a sublethal stress factor (reviewed in Broeckx et al., 2016).

In this research, potential optimization of the spray drying process of probiotic bacteria was evaluated by the addition of protective agents. As long-term stability data of this drying technique are still not commonly available, the survival rates were followed during long-term storage at refrigerated and room temperature. First, the effect of salts such as sodium chloride and sodium phosphates on the bacterial

viability was assessed by using different drying media, i.e. the feed solution to be spray dried. The presence of 0.75%-0.90% (m/V) saline can provide an isotonic medium to maintain cell integrity and viability. A phosphate buffer offers a physiological pH value, which may be important to maintain bacterial viability. In the case of lactic acid bacteria, such as Lactobacillus rhamnosus GG, the sodium phosphate buffer can also counteract the lactic acid, produced by the bacteria during growth and metabolism, limiting the negative influence of acidification of the surrounding medium. To evaluate this effect of isotonicity and buffer capacity, the influence on the bacterial viability of demineralized water (no salts, no buffer) was compared with a phosphate buffer (PB), saline, and phosphate buffered saline (PBS). Furthermore the effect of different saccharide solutions in PBS on the viability of L. rhamnosus GG was examined. The tested excipients included lactose and trehalose - a reducing and non-reducing disaccharide, respectively-, mannitol - a polyol -, and dextran and maltodextrin - two polymers with an average MW of 78.5 kDa and 3 kDa, respectively. Saccharides can protect bacterial cells during dehydration by substituting for the removed water molecules, known as the water replacement theory (Crowe et al., 1998; Golovina et al., 2009; Grasmeijer et al., 2013; Vehring, 2008). As water molecules evaporate, they are replaced by the saccharide's hydroxyl-groups, which can interact with the polar groups of the phospholipid bilayers of the bacterial cell membrane, thus maintaining the membrane integrity. Moreover, some sugars can act as glass formers and will form a protective matrix in which the probiotics are embedded, referred to as the glass transformation theory (Grasmeijer et al., 2013). In a glassy matrix all diffusion-controlled processes are slowed down, such as bacterial metabolism as well as deteriorative reactions from the environment, thus enhancing bacterial viability during long-term storage. To investigate long-term protective effects of all excipients, each drying medium was combined with each of the screened saccharides. The obtained powders were then stored for several weeks at a relative humidity (RH) of 11% at two storage temperatures (4°C and room temperature).

2. Experimental Procedures

<u>Bacteria and growth conditions:</u> *Lactobacillus rhamnosus* GG (ATCC53103) is a probiotic strain that was originally isolated from human fecal samples (Doron et al., 2005; Segers and Lebeer, 2014). For long-term storage, this bacterial strain was kept in a 1:1 50% aqueous glycerol/MRS (overnight culture)

solution at -80°C. For each experiment, fresh bacterial cell cultures were obtained as follows: a frozen culture of *L. rhamnosus* GG was transferred onto a de Man, Rogosa and Sharpe (MRS) agar plate (Carl Roth, Mühlburg, Germany) and incubated for two days at 37°C. A single colony of *L. rhamnosus* GG was picked up from the plate and inoculated in 10 ml liquid MRS broth (Carl Roth) and incubated at 37°C without shaking for 24 hours. Afterwards, a 1% (v/v) dilution was made in liquid MRS broth and *L. rhamnosus* GG was incubated at 37°C without shaking for another 16 hours, which was the experimentally obtained timepoint at which *L. rhamnosus* GG obtained the stationary phase of its growth curve.

<u>Media composition</u>: Sodium chloride (NaCl) was purchased from Carl Roth (Mühlburg, Germany), sodium dihydrogen phosphate dihydrate (NaH₂PO₄.2H₂O) and disodium hydrogen phosphate dihydrate (Na₂HPO₄.2H₂O) of analytical grade were acquired from Merck (Darmstadt, Germany) and were used to prepare phosphate buffer (PB, pH 7.2) and phosphate buffered saline solution (PBS, pH 7.2). PB is composed of 0.3 g/l NaH₂PO₄.2H₂O, 1.54 g/l Na₂HPO₄.2H₂O and demineralized water. PBS is made by the addition of 8.2 g/L NaCl to PB. The saccharides tested, included lactose monohydrate (Fagron, Waregem, Belgium), trehalose dihydrate (provided by Nagase GmbH, Dusseldorf, Germany), mannitol (Merck, Darmstadt, Germany), dextran (MW: 78.5 kDa; Sigma-Aldrich, Steinheim, Germany,) and maltodextrin (dextrose-equivalent (DE) = 6, provided by Roquette, Lestrem, France).

Spray drying process: After *L. rhamnosus* GG obtained the stationary phase, the bacterial suspension was centrifuged at 3983 x g for 10 minutes at 20°C. The pH of the supernatant was measured as quality control before the supernatant was discarded. The bacterial pellet was resuspended by vortexing to its original volume in the respective spray drying medium. The spray drying media examined were demineralized water, PB, saline (0.9% (m/V) NaCl) or phosphate buffered saline (PBS). These media were either used as such, or enriched with a saccharide excipient with a 1.25%, 2.50%, or 6.25% (m/V) concentration. Spray drying was carried out with a laboratory-scale spray dryer (B-290, Büchi, Flawil, Switzerland). A co-current spray dryer configuration was used and the feed was sprayed into the drying chamber using a two-fluid nozzle. The spray drying parameters were held constant during the experiments: inlet temperature of 135° C, and feed rate of 25% (~ 7.5 ml/min), resulting in an outlet

temperature of approximately 55-60°C. The spraying air flow was set at 831 l/h and the aspirator at 80%. The spray-dried powder was collected from a single cyclone separator.

<u>Water activity</u>: Immediately after spray drying, the water activity of the powders was measured using a LabSwift water activity instrument (Novasina, AG, Lachen, Switzerland). The instrument was calibrated using Novasina humidity standards with a known relative humidity (RH), varying between Sal-T 11 with a RH of $11.0 \pm 0.3\%$ to Sal-T 84, with a RH of $84.3 \pm 0.3\%$ at 25° C.

<u>Enumeration of viable bacteria</u>: The spray-dried powder was resuspended in the used spray drying medium in a 1% (m/V) concentration, i.e. demineralized water, PB, saline or PBS. Afterwards a tenfold serial dilution (in triplicate) was prepared from the resuspended spray-dried bacteria and plated on MRS agar in duplo. The plates were then incubated at 37°C for 48 hours, whereafter the colony forming units (CFU) per plate were enumerated. CFU counts before and after spray drying were compared to obtain the bacterial survival rate. Each spray drying experiment was conducted in triplicate.

<u>Storage of spray-dried powder</u>: The spray-dried powder was analyzed for viability at predefined time intervals. The initial survival rate immediately after spray drying was compared to the viability after 2, 4, 8, 12 and 28 weeks. For each time interval, a definite amount of powder was placed in an Eppendorf tube (VWR International Europe, Leuven, Belgium), which was sealed with Parafilm® and stored in triple layered LamiPouch PET/ALU/LDPE aluminum bags (DaklaPack®, Lelystad, The Netherlands) to protect from moisture and oxygen. Afterwards the bags were placed in a desiccator brought to a constant RH of 11% with a saturated LiCl solution (Carl Roth). The desiccators were then stored at 4°C and at room temperature. During storage, the temperature and the RH were monitored with an EBI 20 TH1 temperature/humidity logger (Gullimex, Bornem, Belgium).

<u>Modulated differential scanning calorimetry</u>: To determine the crystallinity of the excipients, modulated differential scanning calorimetry (MDSC) was carried out to assess the glass transition temperature (T_g) or melting point (T_m), using a Q2000 system (TA Instruments, Leatherhead, UK). The DSC was calibrated for temperature and enthalpy using an indium standard and the Tzero calibration was performed using sapphire. Samples with a mass between 7 and 15 mg were put in Tzero pans (TA

Instruments, Zellik, Belgium). An empty Tzero aluminum pan was used as a reference. A heat run was applied for each sample between temperatures of -20°C and 130°C, using a heat rate of 2°C/min. The modulation period and amplitude were set at 1 min and 0.318°C, respectively. T_m was analyzed in the heat flow signals and T_g was analyzed in the reversed heat flow curves. The midpoint in its deflection was taken as the glass transition temperature. The initial crystallinity of the material was determined by MDSC on the non-processed saccharide powders. To assess any changes occurring in the material over time, MDSC was again performed immediately after spray drying, and after 28 and 100 weeks of storage, at both room temperature and 4°C. The obtained data were analyzed using Universal Analysis 2000 (TA Instruments).

<u>Osmolality</u>: To measure the osmolality of the researched spray drying and rehydration media a Micro Osmometer Model 3300 (Advanced Instruments, Norwood, MA, USA) was used. The instrument was calibrated with reference standards of 50 mOsm/kg solvent and 850 mOsm/kg solvent. As verification a clinitrol solution with a known osmolality of 290 mOsm/kg solvent was measured in triplicate. The osmolality was determined to be 289.7 mOsm/kg solvent. All the osmolality measurements were carried out in triplicate.

<u>Statistical analysis</u>: Statistical significance of the differences in protective capacities of the tested spray drying media and added agents was analysed using an ANOVA-test and Tukey's multiple comparisons test. The statistical analysis was performed using GraphPad Prism, version 6.01 (GraphPad Software Inc, La Jolla, CA, USA). The statistical tests were performed at a significance level α =0.05.

3. Results and discussion

3.1. Medium optimization for spray drying

The effects of sodium chloride and sodium phosphates on the survival rate of *L. rhamnosus* GG were assessed by using different spray drying media, namely demineralized water, PB, saline and PBS. Fig. 1 shows that when saline was used as the spray drying medium, the largest decrease in cell viability was observed (1.89 log-reduction). A trend could also be observed that PB (0.93 log-reduction), and demineralized water (0.77 log-reduction) were less optimal as spray drying medium, but this was not

statistically significant compared to PBS (0.54 log units reduction in viability). As PBS has approximately the same amount of NaCl present in its composition compared to saline, the presence of the phosphates in PBS seems to have some protective effect on the bacterial viability. This could be due to the accumulation of polyphosphates, and the osmotic and pH buffering capacity of phosphates. Due to the presence of the *ppk* gene, *L. rhamnosus* GG is indeed able to accumulate polyphosphates (Alcántara et al., 2014). These linear polymers of anhydrously bound phosphates form intracellular granules which are microscopically visible in more than 95% of *L. rhamnosus* GG cells (Alcántara et al., 2014). Alcántara et al. (2014) suggested that polyphosphates are involved in stress resistance. Although this phenomenon could explain the protective effect of phosphates , it is not yet known if, under the performed research conditions, the bacteria have sufficient time to form and accumulate these polyphosphates. Furthermore, Alcántara et al. (2014) proposed the hypothesis that the phosphates could offer an extra buffering capacity to counteract cytoplasmic changes, e.g. when rehydrating the bacterial cells.

3.2. Effect of saccharides on the bacterial viability after spray drying

Different saccharides, including trehalose, lactose, mannitol, dextran and maltodextrin, were tested in different concentrations in PBS for their protective effect on the survival rate of *L. rhamnosus* GG after spray drying. The concentrations analyzed were 1.25%, 2.50%, and 6.25% (m/V). Results of the bacterial viability after spray drying are shown in Fig. 2. The line on the '1'-mark represents the survival rate of the bacterial suspensions spray dried in PBS without any saccharides added, which was used as a reference. As seen on the graph in Fig.2, addition of trehalose or lactose resulted in approximately three to four times better survival rates compared to the reference. Adding a higher concentration of these sugars to the bacterial suspension did not result in an increasing viability (Two-way ANOVA; Tukey's multiple comparisons test). These results are in line with findings by Tymczyszyn *et al.* (2006). In their study, increasing the trehalose concentration from 100 mmol/L (approximately 3.42% (m/V)) to 500 mmol/L (approximately 17.1% (m/V)) did not increase the viability of *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 after drying (Tymczyszyn et al., 2007). This indicates that the protective agents possibly exert an optimal concentration for their protective capacities, which may be linked to a maximal

occupation of binding places around the cell membrane. Mannitol did not show sufficient protective capacities after spray drying. Moreover, a higher concentration appeared to lead to a decrease in bacterial viability. Mannitol is a crystalline product and as shown by MDSC-experiments (data not shown), it again adopted a crystalline structure after spray drying. Its crystallinity can be detrimental for bacterial viability as it can damage the bacterial cell wall and membrane. Furthermore, under the tested conditions, the powders spray dried with mannitol appeared to be very sticky. A significant amount of the powder stuck to the drying chamber and the cyclone before it could be collected in a recipient. As only a limited amount of dry powder could be retained after drying to process further, we excluded mannitol from further experiments. Addition of dextran overall did not enhance bacterial viability much, with the exception of the addition of 6.25% (m/V) dextran. This concentration resulted in approximately a four times enhancement in the bacterial viability after spray drying. Even though this led to the highest survival rate after spray drying from all the tested conditions, the powder obtained after spray drying showed very low yields. As it is beneficial to have a good yield when processing the obtained powders, dextran was also excluded from further research. Maltodextrin resulted in a small, but non-significant enhancement in bacterial viability after drying, decreasing from 1.9 to 1.2 times with increasing concentration.

Overall, addition of lactose or trehalose resulted in the best viabilities and powder yields after spray drying compared to mannitol, dextran and maltodextrin. Similar findings were obtained by spray drying other biomolecules, where lactose and trehalose also gave better results (Lee, 2002; Mensink et al., 2017; Perdana et al., 2014; Soltanizadeh et al., 2014; Vandenheuvel et al., 2013). Mensink et al. (2017) suggest that this superior protective capacity of smaller molecules is due to their small size, which enables them to form better interactions and to reduce local mobility as they are less inhibited by steric hindrance in comparison to larger molecules, like maltodextrin. When looking at the concentrations added, a higher concentration did not necessarily result in significantly better survival rates. We, thus, opted to conduct the stability study with the lowest concentration of the saccharides used in this study (1.25% (m/V)), since this is more cost-effective and mostly leads to a higher CFU load per gram powder obtained.

3.3. Effect of saccharides and medium composition on bacterial viability during storage

It is important to obtain a high viability after spray drying, but even more to maintain this viability during long-term storage. A full factorial experimental design was conducted with the different drying media on the one hand and different saccharides on the other. After screening the saccharide excipients, we included trehalose, lactose and maltodextrin in the stability study. The former two are both small disaccharides with a MW of 378 and 360 Da, respectively. Maltodextrin is a polymer with an average MW of 3 kDA. This difference in MW could expose possible differences in protective capacities related to size of the protective agents. After the experimental design was conducted, the obtained powders were placed under controlled storage conditions (4°C or room temperature, each at a RH of 11%), and the viability was followed during storage of 28 weeks (Fig. 3).

It is known that storing bacterial powders at refrigerated temperatures leads to an increased stability, which is confirmed by these results. None of the stored powders at 4°C showed more than 1 log-reduction in cell viability after being stored for 28 weeks, with the exception of the spray-dried powder obtained from saline without any excipient added (log-reduction of 1.06). At this lower temperature, the kinetic energy is limited, leading to less molecular movement. Furthermore bacterial metabolism is slowed down. Bacterial cells will not only produce less waste products because of their limited metabolism, but also external detrimental reactions will be limited. This leads to better survival rates after long-term storage at 4°C.

The powders spray dried in demineralized water and saline did not show good viability results when stored at room temperature. For demineralized water all powders showed more than 5 log-reductions after storage for 28 weeks. For saline the viabilities of all the powders declined with a log-reduction ranging from 3.64 to 5.20, depending on the added saccharide.

When sodium phosphates are added to the drying medium, with or without the presence of NaCl, *L. rhamnosus* GG showed better survival rates during storage at room temperature, with log-reductions ranging from 0.83 log units to 2.73 log units after 28 weeks, depending on the added sugar. As mentioned above, phosphates could protect the bacterial cells from cytoplasmic changes (pH changes) during rehydration and upon growing. As *L. rhamnosus* GG belongs to the lactic acid bacteria (LAB), their

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cytoplasm is more alkaline than their environment. During growth and metabolism LAB produce lactic acid, a weak organic acid that acidifies the medium. At low pH the lactic acid is not ionized and can pass through the cell membrane to the cytoplasm. There, the pH is higher and the lactic acid is ionized, reducing the internal pH. The bacteria can tolerate or counteract this acidification up to a certain threshold pH value, below which cellular functions are inhibited (Kashket, 1987). Counteracting mechanisms include the use of efflux pumps which can lead to ATP depletion in the long term. When using a sodium phosphate buffer the so-called auto-acidification is limited. The buffer capacity of these phosphates limits the drop in pH value and can, thus, lead to better survival rates, as can be seen during the stability of the powders spray dried in PB or PBS. The best survival rate when stored at room temperature was observed when the bacteria were spray dried in PBS with addition of lactose (0.83 logreduction) or trehalose (1.07 log-reduction). Moreover, addition of lactose or trehalose improved the bacterial viability in all the conducted experiments, especially at 4°C. With the addition of lactose, the log-reductions of viable cells were 0.24, 0.51, 0.26 and 0.08, respectively, for demineralized water, PB, saline, and PBS. Adding trehalose to the bacterial suspension resulted in log-reductions of 0.23, 0.26, 0.14, and 0.12, respectively. This means that with addition of trehalose or lactose 31.21% to 83.54% of L. rhamnosus GG remains viable after 28 weeks of storage at 4°C.

Maltodextrin exhibited protective capacities the least of the tested saccharides. Some explanations can be suggested for this difference in protective capacity between the different saccharides. First, when looking at a bacterial cell in hydrated conditions, the water molecules can interact with the polar groups of the phospholipid bilayer of the bacterial membrane, keeping the membrane integrity intact. During dehydration, the water molecules are removed and membrane integrity is lost. Upon rehydration packing defaults in the membrane occur, which can result in cell death. Disaccharides, such as lactose and trehalose, can prevent detrimental consequences due to the loss of membrane integrity by substituting for the water molecules. When bacterial cells are dried in the presence of disaccharides, the hydroxyl residues of the sugar molecules can interact with the polar groups of the membrane bilayer, replacing the water molecules. Bacterial cell integrity will hereby be maintained and this will lead to higher survival rates. This protection strategy is called the water replacement theory. As mentioned above, maltodextrin is a polymer with a relative high MW compared

to lactose and trehalose. Thus it is possible, that this polymer encounters steric hindrance from e.g. bacterial cell wall and membrane components and cannot form sufficient hydrogen bounds with the polar residues of the bilayer to substitute for the removed water molecules (Gorska et al., 2013; Vandenheuvel et al., 2013). Therefore, the bacterial membrane cannot be stabilized, leading to cell death. This can explain why maltodextrin showed lower survival rates of the embedded bacteria after drying compared to the addition of lactose or trehalose. Another explanation for the superior protective effect of lactose and trehalose on the bacterial viability, is attributed to their glass formation capacity. To verify whether these saccharides indeed form a glassy matrix, MDSC measurements were performed (Table 1). Before spray drying, lactose and trehalose showed an endothermic peak at, respectively, 92.7°C and 137.99°C, indicating their crystalline state. However, after the drying process, they both are amorphous as indicated by the appearance of a glass transition at 64.42°C and 72.31°C for trehalose and lactose, respectively, when stored at room temperature. These findings are in line with other results found in literature where the T_g of spray-dried trehalose powders varied from 32°C to 118°C (Roe and Labuza, 2005; Simperler et al., 2006; Vandenheuvel et al., 2014; Whelan et al., 2008). The Tg of spraydried lactose powders varies in literature from 62°C to 116°C (Abbas et al., 2010; Roos, 2002). Research has shown that Tg-values can vary because of different residual water contents. The higher the water content the lower the Tg. For example Simperler et al., (2006) found that the Tg of trehalose ranged from 96°C at 0% (w/w) H₂O to 32°C at 5.3% (w/w) H₂O. Amorphous excipients seem to protect the bacterial cells better during long-term storage, as the probiotics are present in a glassy matrix. This glassy matrix restricts molecular movement and thus slows down detrimental processes, as crystallization and diffusion-controlled processes, such as deteriorative external effects, and bacterial metabolism (Broeckx et al., 2016; Roos, 2002). To validate the maintenance of a glassy matrix during storage, the DSCexperiments were repeated after 28 and 100 weeks of storage (Table 1). The powder samples with trehalose showed a Tg of 62.64°C at time point 0. When stored at room temperature for 28 weeks, we observed a similar Tg of 64.42°C. When stored at 4°C for 28 weeks the observed Tg was 53.35°C. This depression in Tg compared to time point 0 indicates the uptake of water, which acts as a plasticizer, showing the dependence of T_g on the water content. These results were reproducible between time point 28 weeks and 100 weeks, showing that the powders reached an equilibrium. A similar trend could be

observed for the powder samples with lactose stored at the different storage conditions. The powder with lactose stored at 4°C for 100 weeks showed a bigger depression in T_g compared to the one stored at room temperature. Both T_g 's are lower than the initial T_g , measured at time point 0, indicating the uptake of water, which acts as a plasticizer. Furthermore, it is important that the storage temperature is below the glass transition temperature to maintain this glassy state. As a rule of thumb it is mentioned that the T_g should be 10 to 20°C higher than the storage temperature (Grasmeijer et al., 2013; Roos, 2002). If the storage temperature exceeds the glass transition temperature, a shift can be seen from the glassy to the rubbery state, where molecular mobility is less restricted, which can be detrimental for bacterial viability. The results show that the T_g is indeed high enough in all cases to support a stable amorphous structure.

Moreover, previous studies have shown that a water activity (a_w) around 0.1 or a water content around 4% is preferable because at this water activity deteriorative reactions, such as lipid oxidation, are limited (Behboudi-Jobbehdar et al., 2013; Chávez and Ledeboer, 2007; Dianawati et al., 2013; Vesterlund et al., 2012). The water activities of all powders were measured before they were stored under controlled conditions of temperature and relative humidity. None of the powders had an a_w exceeding 0.13. To maintain this low a_w of the spray-dried powders, the powders were stored at a RH of 11%. This low RH and low a_w are beneficial for long-term storage of the bacterial cells.

3.4. Osmotic stress of the spray drying medium and rehydration medium on the bacterial cells

Bacterial cells are prone to osmotic stress, not only during drying, but also during rehydration. An imbalance in the osmotic gradient can lead to an efflux or influx of water, changing the cell turgor, which can result in cell death. To enhance bacterial viability, it is important to consider the osmolality of the spray drying medium and the rehydration medium (Table 2). Thus, measuring the osmolality of the different drying and rehydration media is an important step towards optimizing the bacterial viability. We also determined the osmolality of the supernatans of the bacteria that were grown to the stationary phase.

When looking at the rehydration media, the osmolality increased from demineralized water (-1

mOsm/kg solvent), over PB and saline to PBS (287.7 mOsm/kg solvent). Indeed, the more solids present in the medium, the higher the osmotic gradient. Increasing the saccharide concentration in the drying medium, thus, also increases the osmolality. Considering the different saccharides (in demineralized water), lactose and trehalose showed similar osmolalities (34.3 and 33.7 mOsm/kg solvent, respectively), whereas maltodextrin had an osmolality of 3 mOsm/kg solvent. All the drying and rehydration media showed to have lower osmolalities compared to the supernatans of the bacterial cells (511.7 mOsm/kg solvent). In isotonic conditions no osmotic stress is put on the bacteria. Here, *L. rhamnosus* GG seemed to be able to withstand all the osmotic stress, originating from the different spray drying and rehydration media. The observed loss in survival rates, therefore, appeared to be caused by other stress factors, as mentioned above.

Conclusion

The interest in probiotics and their role in our health has spiked in recent years. For the development of a pharmaceutical formulation containing probiotics, the maintenance of their viability, activity, and stability is very important and relevant. Drying is a well-known method to enhance their long-term stability and to facilitate further manufacturing such as tableting. As drying is the pivotal step in the formulation of pharmabiotics, but often detrimental to bacterial viability, this study focused on specific viability-enhancing formulation excipients used in the pharmaceutical industry. Overall in this study, the best survival rates after 28 weeks of storage (11% RH) were obtained when *L. rhamnosus* GG was spray dried in PBS with addition of 1.25% (m/V) lactose or trehalose. These are small disaccharides that could substitute for the removed water molecules during drying, and thus maintain the cell integrity. Furthermore, both disaccharides are well-known glass formers. The presence of phosphates in the drying medium, also showed to have a beneficial effect on the bacterial viability, possibly because of their buffering capacity.

As spray drying is a promising drying technique in the formulation of pharmabiotics, this research shows the importance of the composition of the drying medium and the added excipients on long-term storage stability. Future research on the preservation of key probiotic properties and activities after spray drying

are also of great importance and are currently mostly overlooked. Drying could for instance influence the intactness of some important cellular structures, such as pili, and exopolysaccharides, welldocumented probiotic factors of *L. rhamnosus* GG (Segers and Lebeer, 2014). This could alter the probiotic activity and functionality, which are related with the health-beneficial effects.

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figure legends

<u>Fig. 1:</u> Effect of different spray drying media on the viability of *L. rhamnosus* GG. The spray drying media with a statistically significant log-reduction in viability are indicated with a star (*) (One-way ANOVA, Tukey's multiple comparisons test).

Fig. 2: Addition of different excipients and their concentrations on the viability of *L. rhamnosus* GG after spray drying. The line represents the viability of *L. rhamnosus* GG after spray drying when no excipients were added (reference condition). All the viabilities were standardized to this latter survival rate. Results that are significantly different from the reference condition are indicated with a star (*) (Two-way ANOVA; Tukey's multiple comparisons test).

Fig. 3: Evolution of the survival rate of *L. rhamnosus* GG during storage after spray drying in different media, with and without the addition of different excipients. The viabilities were evaluated over a period of 28 weeks of storage at room temperature (RT, full lines) or at refrigerated temperatures (4°C, dashed lines). The spray drying media were used 'as such' (AS) or enriched with an excipient: lactose (LAC), trehalose (TRH), or maltodextrin (MD), each in a 1.25% (m/V) ratio.





Table 1.: Physicochemical properties of the tested saccharides

Sample	T _m	T _g (midpoint) (°C)	a _w
TRH ^a before SD ^c	92.70		0.414
TRH after SD			
t = 0		62.64	0.092
t = 28 weeks, RT		64.42	n.d. ^d
t = 100 weeks, RT		62.84	n.d.
$t = 28$ weeks, $4^{\circ}C$		53.35	n.d.
$t = 100$ weeks, $4^{\circ}C$		54.41	n.d.
LAC ^b before SD	137.99		0.402
LAC after SD			
t = 0		73.04	0.097
t = 28 weeks, RT		72.31	n.d.
t = 100 weeks, RT		65.90	n.d.
$t=28$ weeks, $4^{\circ}C$		*	n.d.
$t = 100$ weeks, $4^{\circ}C$		21.68	n.d.

^aTRH= trehalose, ^b LAC= lactose, ^c SD= spray drying, ^d n.d.= not determined, * failed measurement

Medium composition		Average osmolality (mOsm/kg solvent) (n=3)		
Bacterial supernatans of gr	owth medium	511.7		
L. rhamnosus GG (as such) in demineralized water	11.7		
Rehydration medium				
Demineralized water		-1		
PB		30.3		
Saline		273		
PBS		287.7		
Spray drying medium				
1.25% (m/V) lactose in der	mineralized water	34.3		
1.25% (m/V) lactose in PB	BS	320.3		
1.25 % (m/V) trehalose in	demineralized water	33.7		
1.25% (m/V) trehalose in I	PBS	318.7		
1.25% (m/V) maltodextrin	in demineralized water	3		
1.25% (m/V) maltodextrin	in PBS	288.3		
2.50% (m/V) lactose in PB	S	358		
2.50% (m/V) maltodextrin	in PBS	294		
6.25% (m/V) lactose in PB	S	466.7		
6.25% (m/V) maltodextrin	in PBS	304.3		

Table 2: Osmolality of different drying and rehydration media