Department of Pharmaceutical Technology and Biopharmaceutics[1], University of Vienna, Austria; Department of Drug Analysis and Pharmaceutical Technology[2], Faculty of Medicine, University of Prishtina, Kosova; Research Center Pharmaceutical Engineering GmbH[3], Graz, Austria

Prebiotics as excipients for enhancement of stability and functionality of *Bifidobacterium longum* ssp. *infantis* with potential application as symbiotics in food and pharmaceuticals

M. Basholli-Salihu[1,2], T. L. Kryeziu[2], D. Nebija[2], S. Salar-Behzadi[3], H. Viernstein[1], M. Mueller[1,*]

Received January 15, 2019, accepted March 8, 2019

**Corresponding author: Monika Müller, Department of Pharmaceutical Technology and Biopharmaceutics, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria*
monika.mueller@univie.ac.at

Pharmazie 74: 326-333 (2019) *doi: 10.1691/ph.2019.9007*

Objective: Formulations containing probiotics are promoted due to health benefits. During lyophilization and subsequent storage in the gastrointestinal tract, bacteria are exposed to stress conditions that can lead to impairment and loss of viability. *Methods*: The suitability of various excipients for enhancing the stability and functionality of *Bifidobacterium longum* subsp. *infantis* during storage as freeze-dried powder and through exposure to acid and bile was investigated. Cells were lyophilized in the presence of sucrose, trehalose, lactose, cellobiose and fructooligosaccharide (FOS) and stored at 4 ˚C or 25 ˚C. The effect of diverse protectants on the persistence after exposure to acid and bile environment was examined through determination of the colony forming units, the β-glucosidase and β-galactosidase activity and the membrane integrity changes. *Results*: Cells freeze-dried in the presence of cryoprotectants had comparable survivability during storage at 4 °C whereas the survival rate at 25 °C of cells protected by cellobiose and FOS was higher than for those protected with sucrose and trehalose. Furthermore, the respective excipients used as cryoprotectants enhanced the stability of cells exposed to simulated gastric and small intestinal medium. Stabilization may be achieved through different mechanism of action such as protecting the membrane integrity and as metabolizable substrates. Overall, prebiotic and thus metabolizable protectants including cellobiose and FOS were superior to other protectants used. *Conclusion:* In symbiotic formulas with *B. infantis,* these sugars might serve as prebiotics and stabilizers of this probiotic strain during lyophilization, storage and in gastrointestinal conditions simultaneously, potentially increasing its health-promoting effects.

1. Introduction

Probiotic formulations are promoted worldwide due to health benefits associated to their use (Hempel et al. 2012; Ruan et al. 2015). Freeze-drying is a common technique used for the conservation of probiotics, permitting low-cost delivery and management. Nevertheless, drying of probiotics remains a challenge since it might have side effects on their viability and metabolic activity (Meng et al. 2008; Broeckx et al. 2016). This may lead to the impairment of the cell membrane's integrity and fluidity, protein denaturation, DNA damage and water crystallization (Carvalho et al. 2003).

Furthermore, retention of the probiotic functionality such as the enzymatic activity is of crucial interest. In fact, the combination of probiotics and polyphenol-rich products has increased due to the activation of polyphenol glycoside by probiotics (Roncaglia 2011; Otieno et al. 2006). Bifidobacteria possess various enzymes, which enable them to hydrolyze isoflavone glycosides, anthocyanin glycosides and lignin-glycosides (Ávila et al. 2009; Basholli-Salihu et al. 2016; Marotti et al. 2007; Otieno et al. 2006; Roncaglia et al. 2011). The β-galactosidase activity also enables them to hydrolyze lactose to glucose and galactose (Osman et al. 2014).

Sucrose, trehalose and lactose are common protective excipients used for preservation of probiotics throughout freeze-drying (Li et al. 2011; Stummer et al. 2012; Vinderola et al. 2012). Supplementary potential protectants are prebiotics which are oligo- or polysaccharides, selectively fermented by probiotic bacteria and thus stimulate their development (Vijaya Kumar et al. 2005; Rivière et al. 2016). One example is fructooligosaccharide (FOS) which displayed decent protective effects throughout freeze-drying of *Lactobacillus (L.) reuteri* (Schwab et al. 2007). Previously, we presented the suitability of cellobiose as a novel prebiotic lyoprotectant to preserve the viability and β-glucosidase activity in *B. infantis* (Basholli-Salihu et al. 2014). Further studies established the protective effect of disaccharides on β-galactosidase activity as purified enzyme or from cell extract of *L. delbruecki* (Heljo et al. 2011; Vasiljevic and Jelen 2003).

Maintaining the viability not only during freeze-drying but also during subsequent storage is a critical challenge as the abilities to survive during freeze-drying and storage are not always associated (Saarela et al. 2005). Critical factors (storage temperature, water activity, exposure to oxygen) that might affect the survival of dried probiotics during storage need to be considered (Celik and O'Sullivan 2013). Additional concerns occur during ingesting, as soon as probiotics have to overcome the physiological barriers in the upper part of the gastrointestinal part, such as low pH present in stomach and bile in the intestine (Montel Mendoza et al. 2014; Stadler and Viernstein 2003). Previous studies showed that the acid and bile tolerance are strain depended. The role of glucose as a metabolizable sugar on the enhancement of probiotic survival in acidic environments has been reported beforehand (Corcoran et al. 2005). There are insufficient studies regarding the role of prebiotics, as protective excipients on the survival of bifidobacteria in acidic and bile environment. (Dianawati et al. 2016). To the best

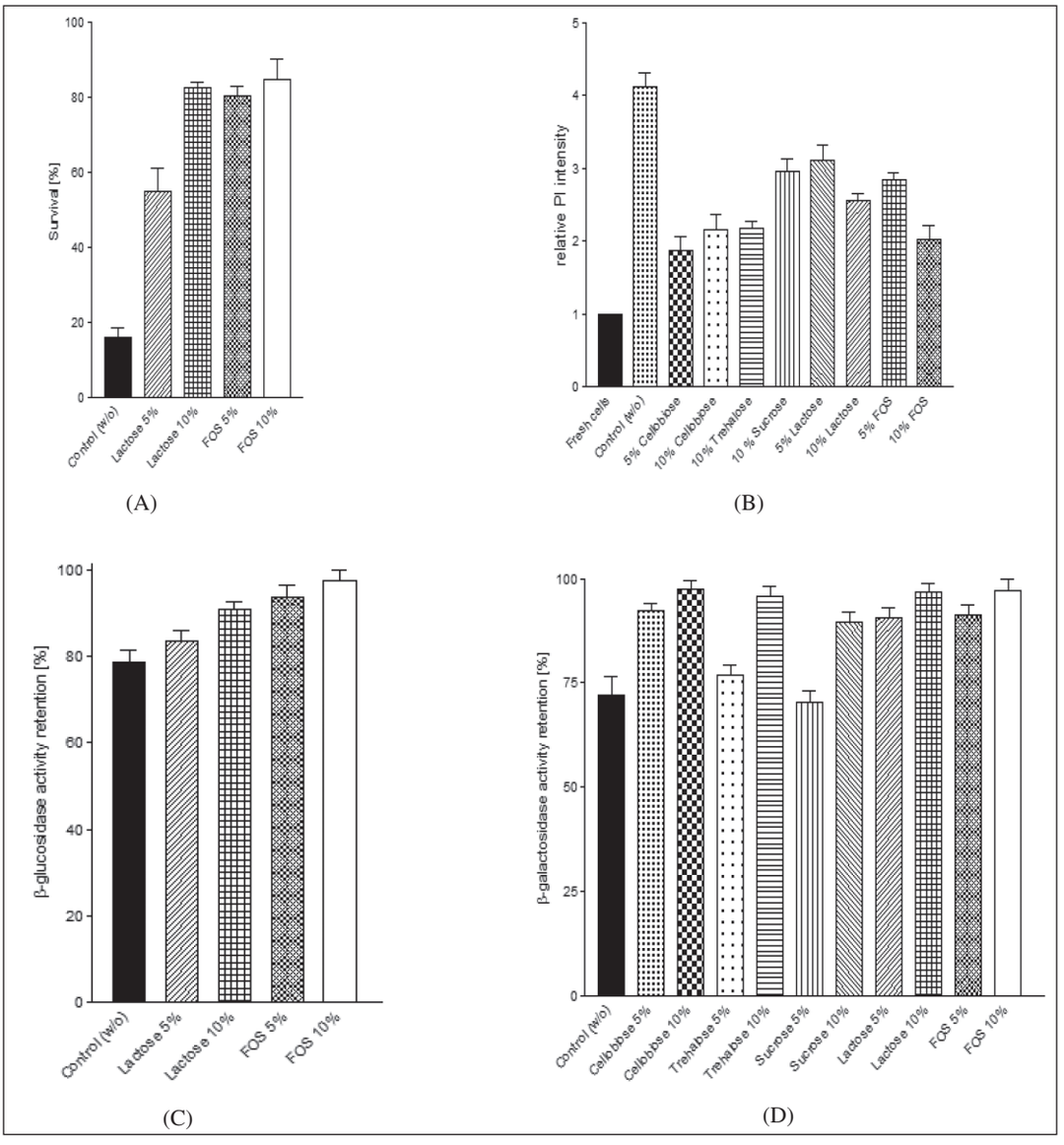

Fig. 1: (A) Survival, (B) membrane integrity (PI), (C) β-glucosidase and (D) β-galactosidase activity retention of *B. infantis* after freeze-drying in the presence of 5 or 10% of cellobiose, trehalose, sucrose, lactose and fructooligosaccharide (FOS). Control (w/o) are cells freeze-dried without any protectant. Each bar represent means ± SD, $n = 3$.

of our knowledge, no studies have been conveyed on the role of cellobiose and FOS, as protective excipients on the survival of bifidobacteria in acidic and bile environment.

The purpose of this study was to evaluate the suitability of protective excipients for application in lyophilized formula of *Bifidobacterium longum* subsp. *infantis* to improve the enzyme activity, acid and bile tolerance and overall stability during storage. Furthermore, prebiotic excipients are considered to attain symbiotic formulations.

2. Investigations and results

2.1. Viability and functionality of B. infantis after freeze-drying

Adding of protectants such as cellobiose, trehalose and sucrose improved the survival rate of *B. infantis* cells during freeze-drying as shown in our previous study (Basholli-Salihu et al. 2014). In this study we showed additionally the protective effect of FOS and lactose resulting in 86 % and 55 % survival, respectively at 5 % concentration of protectant, compared to others which ranged from 32 to 55 % and cellobiose 86 % ($p<0.001$). At 10 % (w/v) concentration no significant differences were observed between all the protectants used. The viability ranged from 81 to 89 %. The best protection was achieved by 10 % cellobiose, 10 % trehalose, 10 % FOS (Fig. 1A). The membrane integrity of the protected cells as determined by PI staining was significantly higher than that of non-protected cells. In fact, 5 and 10 % cellobiose, 10 % trehalose and 10 % FOS provided the best protection followed by 10 % lactose, 5 % FOS, 5 % lactose and 10% sucrose (Fig. 1B).

Freeze-drying reduced the remaining β-glucosidase activity in cells without any protectant to 79 % (Fig. 1C). Addition of protectants increased the remaining activity during freeze-drying depending on the protectant used. FOS (5 %) significantly ($p<0.001$) enhanced the β-glucosidase activity retention to 94 %, and significantly higher ($p<0.01$) compared to trehalose, lactose and sucrose (80 %, 84 % and 70 %, respectively). No significant differences were observed between 5 % FOS and 5 % lactose ($p>0.05$). All protectants, at 10 % concentration, cellobiose, lactose and FOS (at 5 %) were significantly more effective in protecting the β-gluco-

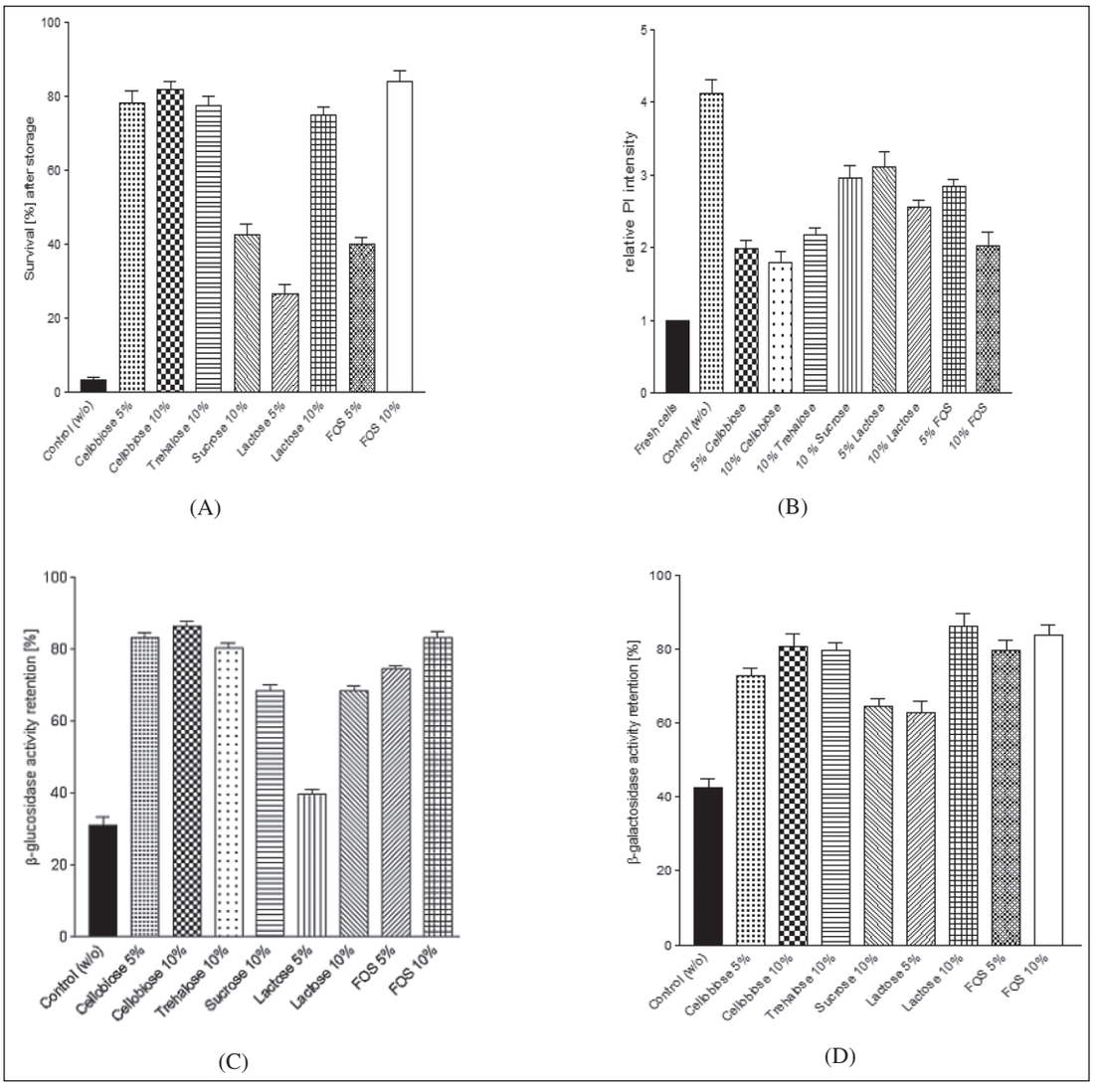

Fig. 2: (A) Survival, (B) membrane integrity (PI), (C) β-glucosidase and (D) β-galactosidase activity retention of freeze-dried *B. infantis* after 3 months of storage at 4ºC. Control (w/o) are cells freeze-dried without any protectant. Each bar represent means ± SD, $n = 3$.

sidase activity retention during freeze-drying. The best protection was achieved using FOS namely 98 % of activity retention, while no significant differences with trehalose and cellobiose used on our previous study ($p>0.05$).

Cells freeze-dried without any protectant showed a remaining β-galactosidase activity of 83 %. The presence of 10 % of all protectants tested during freeze-drying significantly improved the β-galactosidase activity retention ($p<0.05$, $p<0.001$) (Fig. 1D). No significant differences were observed between 10 % cellobiose, trehalose, lactose and FOS ($p>0.05$). Retention of β-galactosidase activity of cells freeze-dried in the presence of 10 % sucrose was significantly lower compared to all protectants used ($p<0.01$, $p<0.001$), namely 82 %. Addition of 5 % cellobiose, FOS or lactose significantly improved the enzyme activity retention to 92, 89 and 91 %, with no significant differences between them ($p>0.05$) (Fig. 1D). These three were significantly superior than 5 % trehalose or sucrose, namely 78 and 70 %, ($p<0.001$) which did not significantly improve the enzyme activity retention. Cellobiose, FOS and lactose showed the best protective effect at both concentrations. The effects of 5 % cellobiose and 5 % FOS protective effect are comparable to 10 % of the other protectants used.

2.2. Viability and functionality of B. infantis after storage

Survival of freeze-dried *B. infantis* after storage at 4 °C and 25 °C for 3 months is shown in Figs. 2 and 3. Cells freeze-dried without any protectant were highly sensitive to storage resulting in survival rates of 3 % and 0.3 %, respectively (Figs. 2A, 3A). Upon storage at 4 ºC, no significant decrease in the viability of freeze-dried *B. infantis* formulated with 5 or 10 % cellobiose, 10 % trehalose, 10 % FOS and 10 % lactose were found, enabling survival rates ranging between 75 and 84 % (Fig. 2A). For lactose and FOS at a concentration of 5 % and for sucrose at 10 % the protective effect was low resulting in viabilities of 27, 40 and 43 %. After storage for 3 months at 25 ºC, 5 or 10 % cellobiose, 10 % trehalose and 10 % FOS were found to retain high survival rates of *B. infantis* (51-59 %), followed by 10 % lactose (45 %) and 5 % FOS (26 %). From all protectants used, the lowest survival rates were observed for sucrose and 5 % lactose protected cells (8 and 4 %, respectively). Compared to the lyophilization process, during storage for 3 months at 4 °C, the reduction of viability for 5 % lactose, 5 % FOS, 10 % sucrose was higher. Sucrose exerted the lowest protective effect for storage of lyophilized *B. infantis* (even at 10 % concentration). FOS at both concentrations has shown a

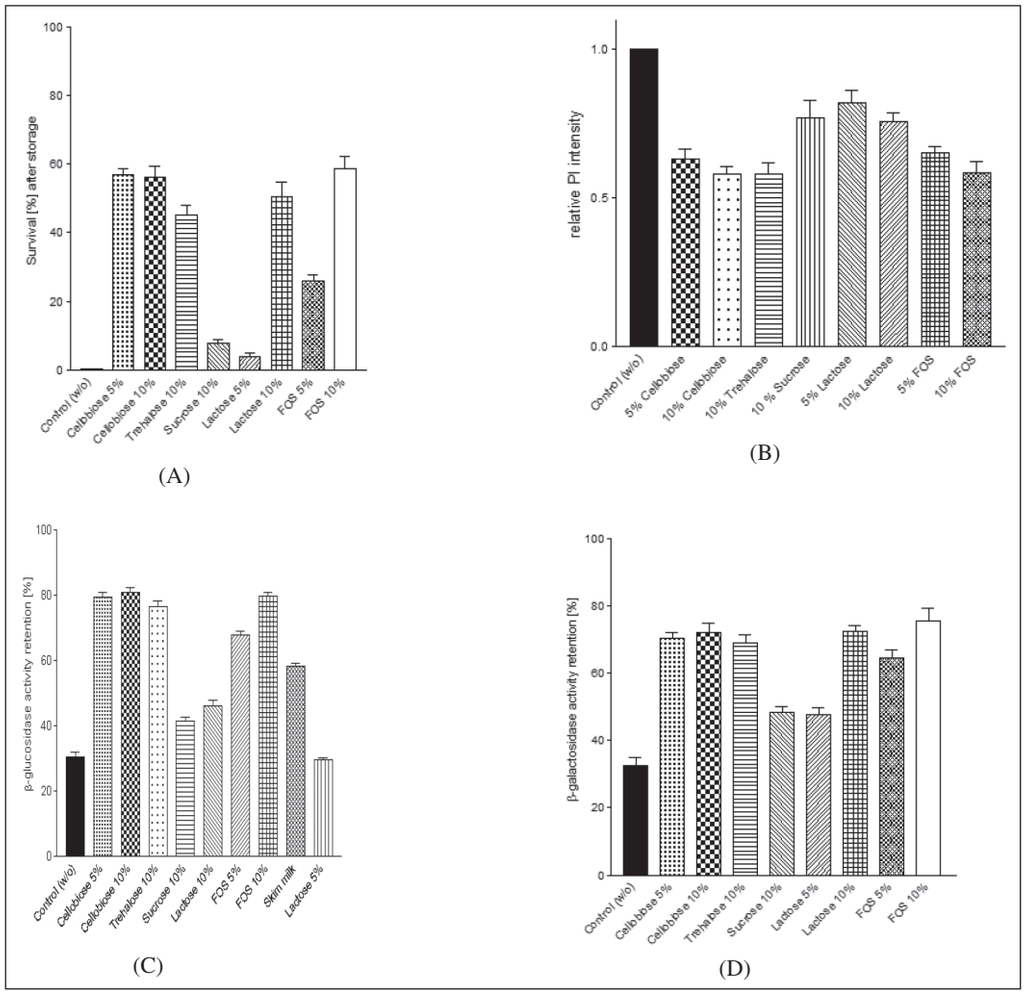

Fig. 3: (A) Survival, (B) membrane integrity (PI), (C) β-glucosidase and (D) β-galactosidase activity retention of freeze-dried *B. infantis* after 3 months of storage at 25 ºC. Control (w/o) are cells freeze-dried without any protectant. Each bar represent means ± SD, $n = 3$

good protective effect. No significant differences in the survival of *B. infantis* cells freeze-dried with 5 or 10 % cellobiose, FOS, 10 % trehalose and lactose were observed. But the differences became significant during storage, especially at 25 ºC. Cellobiose at both concentrations used (5 or 10 %), 10 % FOS and 10 % trehalose offered the best protective effect toward maintaining the viability of freeze-dried *B. infantis* during storage. After storage, the relative PI intensities increased, with significantly lower intensities after storage at 4 °C compared to storage at 25 °C (Figs. 2B, 3B).

The retention of β-glucosidase activity of freeze-dried *B. infantis* during storage after 3 months was significantly reduced in protectant-free cells, to 31 % (4 °C) and 30 % (25 °C), respectively (Figs. 2C and 3C). The presence of protectants significantly improved the stability of enzyme activity during storage ($p<0.01$). Cellobiose and FOS (10 %) were found to be superior to other protectants and concentrations used, followed by trehalose and 5 % cellobiose. The retention of β-glucosidase activities for 10 % cellobiose during storage was 86 % (at 4 ºC) and 81 % (at 25 ºC), respectively. Lactose (10 %) formulated cells offered high retention of enzyme activity during freeze-drying, but during storage at 4 ºC and especially at 25 ºC lactose could not prevent the decline in enzyme activity, in comparison to cellobiose, trehalose and FOS formulated cells. During storage at 25 °C, 10 % lactose and sucrose did not sufficiently stabilize the β-glucosidase activity resulting in 59 and 46 % remaining activity. These differences can be attributed to the differences in protecting membrane integrity.

The retention of β-galactosidase activity of non-protected cells was significantly decreased to 42 and 32 % after storage for 3 months at 4 °C and 25 °C, respectively ($p<0.001$) (Figs. 2D and 3D). The highest remaining β-galactosidase activity was found for cells protected with 10 % lactose, 5 and 10 % FOS, 5 and 10 % cellobiose, 10 % trehalose ranging from 72 to 86 % remaining activity (at 4 °C) and 64 to 75 % remaining activity (at 25 °C). The remaining galactosidase activities for 10 % sucrose and 5 % lactose protected cells were 65 and 63%, respectively (during storage at 4 °C) and 48 and 47%, respectively (during storage at 25 °C) (Fig. 3C).

During storage, the water activity was increased (Fig. 4). The lowest increase was observed for 10 % trehalose, 5 % cellobiose and 10 % FOS.

2.3. Survival in simulated gastric medium (acid tolerance)

Different excipients are suitable to increase the acid tolerance of freeze-dried *B. infantis*. As expected, the highest loss on viability was observed for the cells freeze-dried without any protectant. Addition of cellobiose (5, 10 %), trehalose (10 %) and FOS (10 %) offered the best protective effect in simulated gastric medium ($p<0.001$), followed by 10 % lactose. The log units reduction of sucrose and 5 % lactose, was significantly higher compared to other protectants used ($p<0.05$), with more than 0.8 log units reduction. Cellobiose (5, 10 %) and FOS (10 %) were significantly superior in comparison to other protectants. No significant differences were observed between cellobiose, FOS, trehalose and 10 % lactose.

The acid tolerance of freeze-dried cells was reduced during storage for 3 months at 4 °C and 25 °C, regardless of freeze-drying formu-

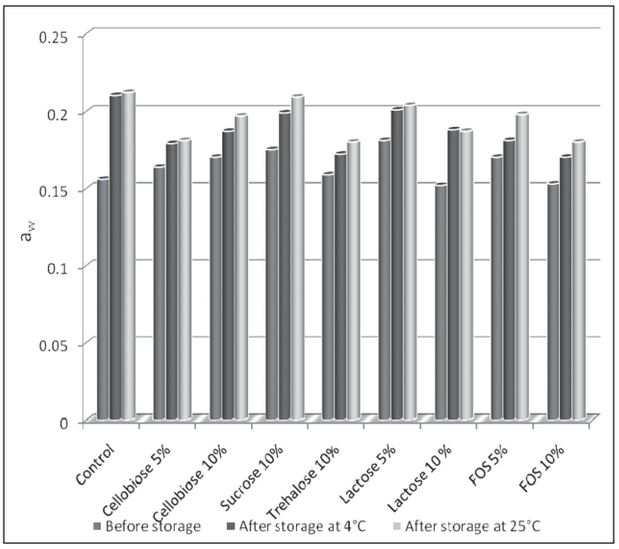

Fig. 4: Water activity (a_w) of the freeze-dried *B. infantis* cells, after storage at 25 °C and 4 °C

lations, with variations in the degree of viability loss (Table 1). No cells were detected after incubation in SGM of cells freeze-dried without any protectant. The addition of protectants significantly enhanced the acid tolerance of cells after the storage at different temperatures. Cellobiose (5 and 10 %) and FOS (10 %) formulated cells showed the best resistance to SGM after storage followed by 5 % FOS, 10 % trehalose and 10 % lactose; 10 % sucrose formulated cells exerted the lowest effect.

Table 1: Acid tolerance of freeze-dried *B. infantis* after storage for 3 months at 25 °C or 4 °C, unwashed cell suspension (protectants present in medium) and washed cells (washed twice with PBS, to remove protectants)

	Log reduction (log CFU/ml)		Log reduction (log CFU/ml)	
	Unwashed cells	Washed cells	Unwashed cells	Washed cells
Protectant	3 months storage at 25 °C		3 months storage at 4 °C	
Control (w/o)	ND	ND	ND	ND
5% Cellobiose	0.34±0.14	0.76±0.05	0.14±0.03	0.39±0.08
10% Cellobiose	0.18±0.02	0.57±0.05	0.12±0.06	0.41±0.09
10% Trehalose	0.56±0.04	0.6±0.06	0.48±0.10	0.62±0.11
10% Sucrose	2.69±0.10	3.02±0.07	1.24±0.10	1.38±0.14
10% Lactose	0.97±0.06	1.24±0.09	0.59±0.12	0.85±0.09
5% FOS	0.52±0.03	0.68±0.09	0.33±0.06	0.59±0.06
10% FOS	0.41±0.02	0.69±0.07	0.28±0.09	0.55±0.04

ND= Non-detected
Values in per cent express the relative viability (calculated as relative viability = N_t*100/N_0
N_t- number of viable cells (CFU/mL) after acidic treatment
N_0-number of viable cells (CFU/mL) before acidic treatment

To determine the influence of metabolizable excipients, the acid tolerance was determined on the unwashed and washed cells to remove the protecting excipients. The cells, formulated with the metabolizable sugars as cellobiose, lactose and FOS showed significantly better survival rates when the unwashed cells were exposed to acidic conditions compared to the washed cells ($p<0.001$) (Table 1). For trehalose, which cannot be metabolized by *B. infantis,* no significant differences were observed between washed and unwashed cells. No significant differences were observed between washed and unwashed cells for the sucrose formulated cells stored at 4 °C while the differences became significant during storage at 25 °C. No significant differences were observed between 10 % trehalose and 5 % cellobiose formulated cells when the acid tolerance was tested on the washed cells.

2.4. Survival in simulated intestinal medium (bile tolerance)

The bile tolerance of cells protected with diverse sugars is shown in Table 2. Cellobiose at both concentrations used was superior to other protectants used including FOS (10 %). Differences among washed and unwashed cells were observed specifically for cellobiose protected cells, followed by FOS and lactose. Furthermore, under these conditions, differences between washed and unwashed cells were observed also for protection using sucrose after storage at 25 °C. On no account were there any significant differences observed for washed and unwashed trehalose protected cells.

Table 2: Influence of different protectants on bile tolerance of freeze-dried *B. infantis* determined after the storage for 3 months at 25 °C or 4 °C. Viability loss (log CFU/ml ± standard deviation) after exposure for 2 h to simulated small intestinal at 37 °C

	Log reduction (log CFU/ml)		Log reduction (log CFU/ml)	
	Unwashed cells	Washed cells	Unwashed cells	Washed cells
Protectant	3 months storage at 25 °C		3 months storage at 4 °C	
Control (w/o)	ND	ND	2.78±0.02	2.95±0.05
5% Cellobiose	0.89±0.04	0.99±0.06	0.45±0.08	0.79±0.03
10% Cellobiose	0.71±0.04	0.94±0.08	0.32±0.02	0.54±0.06
10% Trehalose	1.26±0.11	1.65±0.11	0.63±0.08	0.65±0.15
10% Sucrose	2.35±0.08	2.85±0.12	1.71±0.13	2.35±0.08
10% Lactose	1.68±0.10	2.06±0.15	1.26±0.06	1.78±0.11
5% FOS	1.69±0.06	1.98±0.09	1.34±0.20	1.86±0.09
10% FOS	1.23±0.04	1.38±0.06	0.99±0.05	1.08±0.06

3. Discussion

Throughout the manufacturing process, a crucial step for development of probiotic formulation is their sustainability. Precisely, through the augmentation of it, a higher performance is provided. In addition, the hereditary features of the bacterium and the choice of excipients play a noteworthy part. The use of prebiotics for protecting viability and functionality of probiotics during freeze-drying and storage aims to obtain a stable symbiotic product. The role of cellobiose as a new potential probiotic lyoprotectant, in comparison to FOS and other common protectants on the stability and functionality of *B. infantis* was compared.

Maintaining the viability and functionality of probiotics during freeze-drying, subsequent storage and in the harsh conditions of the gastrointestinal tract is a critical challenge. Thus, in this study, the stabilizing effect of prebiotics (cellobiose and FOS) and lyoprotectants for subsequent storage and the acidic and bile tolerance is shown. According to our previous studies (Basholli-Salihu et al. 2014) and this study, the effect of the protectants on viability and enzyme activity of *B. infantis* during freeze-drying are comparable. In this study, we focused more on determining the role of the protective excipients during storage and after exposure to acid and bile tolerance.

The results of the present study showed that even though no significant differences were observed on the viability and enzyme activity after freeze-drying, the differences became substantially significant during storage at 4 °C and especially during storage at 25 °C and after exposure to acid and bile medium.

Among the effectiveness as good protective agent during freeze-drying the cellobiose, as new lyoprotectant and FOS has shown good protective effect during storage. According to the best of our knowledge this is the first study to show the effect of cellobiose on the protection of viability and functionality during storage of freeze-dried probiotic. For choosing the optimal lyoprotectant, its effect during storage among the protective effect during freeze-

drying should be considered. Storage conditions including oxygen, water activity and light are the main detrimental factors during storage of dried cells (Wang et al. 2004). In this study, the storage stability (4 or 25 °C for 3 months) could be significantly improved through pretreatment of cells with cellobiose (5 or 10 %), 10 % trehalose or FOS as indicated by higher remaining viabilities and membrane integrity. Pretreatment of cells with sucrose, trehalose or lactose protected well during freeze-drying but were not found to be the appropriate protectants of *B. infantis* during subsequent storage. FOS at both concentration used showed a good protective effect and a similar effectiveness like cellobiose in our previous study (Basholli-Salihu et al. 2014). Sucrose exerted the lowest protective effect for storage of lyophilized *B. infantis*. The protective effects of FOS have been reported during spray drying and during storage (Dianawati 2016). In contrary, some studies have shown that the presence of prebiotic in skim milk decreased bacterial viability during storage at 37 °C as compared to skim milk alone (Ananta et al. 2005). These contradictive results of prebiotics on viability of the probiotic during storage could be related to many factors such as bacterial strains, growth condition, drying method, storage period, proportion of prebiotic incorporated into probiotic, size of prebiotic chain, as well as the interactions of the protectant with cell envelopes. The existence of large size of polymers (such as inulin, dextran) might cause the "steric hindrance" stopping them to cooperate with dehydrated proteins and membrane lipids (Dianawati et al. 2016; Hincha et al. 2002; Broeckx et al. 2016).

Even though the lactose was significantly inferior on protecting the membrane integrity compared to cellobiose, FOS and trehalose, the protective effect on galactosidase activity was high suggesting that being a substrate for this enzyme and the ability to bind to the enzyme enables protection. It is nowadays preferable to add polymers as active protective agents during the lyophilized powder preparation process. Through them, it is likely to upsurge the stability, integrity and resistance of *Bifidobacterium* membranes, particularly during the storing process for extended shelf life. (Bagad et al. 2017; Kim et al. 2018)

Regarding the water activity, 10 % trehalose, 5 % cellobiose and 10 % FOS offered the best protection with the lowest increase. Differences can be attributed the differences in the glass transition temperature of the protectants used which were shown to be 88 °C for cellobiose, 62 °C for sucrose, 96 °C for trehalose and 108 °C for lactose (Heljo et al. 2011; Saleki-Gerhardt et al. 1994). The higher the glass transition temperature, the lower is the possibility for crystallization of sugar during storage. Primarily important, it's high Tg which amply, remains above room temperature even when exposed to high RH. Trehalose (10 %), cellobiose (5 %) and FOS (10 %) may maintain the water activity at the level necessary to preserve the cellular structures from damage in the freeze-dried microorganisms, thus allowing for an appropriate preservation and avoiding unnecessary damage during storage.

To the best of our knowledge, this is the first study to test prebiotics (cellobiose and FOS), as metabolizable sugars in comparison to other non-prebiotic disaccharides (metabolizable and non-metabolizable) on the acid and bile tolerance.

In order to determine whether the prebiotics and other sugars used in the present study, improve influence the acid tolerance as metabolizable sugars, among the protective effect on the membrane integrity, the acid tolerance was determined on the washed and unwashed cells.

The lyoprotectants, cellobiose, trehalose (10 %) and FOS (10 %) were superior also for retaining the acid tolerance of freeze-dried *B. infantis*. Our data suggests that the protective effect of protectants could be achieved by 2 different mechanisms via stabilizing the membrane integrity or as metabolizable carbon sources. The significant enhancement of *B. infantis* acid tolerance by cellobiose and FOS was achieved by their role as metabolizable sugars (substrate preference) among the protective effect on the membrane integrity. The presence of prebiotic in complex formulations has also increased acid and bile tolerance of spray-dried *Lactobacillus acidophilus* and *Bifidobacterium lactis* (Bb-12) (Fávaro-Trindade and Grosso 2002). FOS and inulin were capable of reducing the rate of cell death during storage at 4 °C of freeze-dried carrier (Capela et al. 2006).

Previously, the presence of glucose as a metabolizing sugar was shown to enhance the survival of *Lactobacillus* in acidic conditions through activating the ATP-ase which pumps protons and thus equilibrates the pH (Corcoran et al. 2005; Dianawati et al. 2013; Duary et al. 2010). The presence of prebiotic on the growth medium (GOS, lactulose) has improved the survival through the gastrointestinal tract (Hernández-Hernández et al. 2012).

In this study, cells protected by the metabolizable sugars cellobiose, lactose and FOS showed significantly better survival rates under acidic conditions when the protectants were not removed by washing. In contrast, in the presence of trehalose, which cannot be metabolized, the acid tolerance was not increased. The presence of sucrose did not protect the cells stored at 4 °C under acidic conditions while the differences became significant for cells stored at 25 °C. These results suggest that for providing stabilized probiotic strains several mechanisms of action should be considered. Our findings are consistent to a previous study in which the presence of glucose as metabolizable sugar improved the acid tolerance of the *Lactobacillus spp*. cells (Corcoran et al. 2005; Koning et al. 2001, Dianawati et al. 2016). Cellobiose enhanced the acid tolerance significantly better than other metabolizable protectants ($p<0.01$), especially after storage at 25 °C, where the metabolic activity is higher compared to 4 °C. This may be due to the metabolization of cellobiose into two molecules of glucose, thus indirectly activating the F0F1-ATP-ase and enabling the transport of cellobiose and other sugars into Bifidobacteria using ABC transporter, which has ATP-ase as a component (Wei et al. 2012; Corcoran et al. 2005; Ejby et al. 2016).

Henceforth, the energy which was obtained from glucose catabolism might possibly be used for bacterial growing (Rooj et al. 2010). Nevertheless, Jofré et al. (2014) established that freeze-dried *Lactobacillus* sp. protected with glucose was not able to survive during storage. This indicated that glucose was not suitable to be used as a protectant for dehydrated probiotic products kept at room temperature.

The presence of protective excipients improved bile tolerance, apparently not only by protecting membrane integrity but also as metabolizable sugars present in the simulated medium. Cellobiose was superior compared to other protectants including FOS, thus suggesting that at pH of incubation the activity toward cellobiose was higher. Similar to the results of acid tolerance, differences between washed and unwashed cells were observed for cellobiose, FOS and lactose protected cells and for sucrose protected cells after storage at 25 °C. This finding can be explained by a higher metabolism rate of sucrose at this pH compared to SGM. No significant differences were observed for trehalose protected cells suggesting that trehalose acts only through protecting the membrane integrity. Our results agreed in some parts with previous studies, which reported that the presence of metabolizable carbohydrates in the growth medium enhance the bile resistance of the cells (De Smet et al. 1995; Perrin et al. 2000; Ruas-Madiedo et al. 2005). The presence of inulin has shown to shield *Bifidobacterium* during exposure to simulated gastric and subsequent bile setting. It has been reported that *Bifidobacterium* and *Lactobacillus spp* adapt during bile exposure, resulting in high sugar ingesting and higher ATP concentration (Sánchez et al. 2006; Burns et al. 2010; Ruiz et al. 2013; Berlec et al. 2012).

Metabolizable sugars reduce the negative effects of bile salts on the cells by their activation which is higher for oligosaccharides with higher yield of energy, compared to monosaccharides (Pessione et al. 2012; Perrin et al. 2000).

Determination of both β-glucosidase and β-galactosidase activity revealed that without protectant used, β-glucosidase is more sensitive during freeze-drying and subsequent storage than β-galactosidase. Differences in sensitivity of different enzymes during freeze-drying were reported previously (Li et al. 2011). Excipients were differently efficient in protecting the viability and β-glucosidase and β-galactosidase activity retentions. This can be attributed to the amorphous/crystalline state of the

protectants, glass transition temperature and their effect to the membrane integrity (Heljo et al. 2011; Li et al. 2011; Teekamp et al. 2017). At 5 % concentration of sucrose, trehalose and lactose the ratio amorphous/crystalline state is not seen to be enough to protect the cells and their enzymes. These results are in accordance to those reported by Vasiljevic et al. (2003). A higher retention of β-galactosidase activity during freeze-drying was observed for lactose protected cells. In fact, 5 % lactose did not show a good protective effect on viability and β-glucosidase activity, but a good protective effect for β-galactosidase activity. These data suggest that the higher retention of enzyme activity during freeze-drying is related to substrate-enzyme interaction of lactose and galactosidase and β-glucosidase and cellobiose (Schwab et al. 2007). Cellobiose was more effective in protecting both enzymes (even though was more effective for glucosidase) compared to lactose. This can be attributed the higher effectiveness of cellobiose on protection of membrane integrity.

In conclusion, freeze-drying of *B. infantis* significantly affects its enzyme activities and its survival rate during storage and under gastric and intestinal conditions. The addition of protective excipients including the prebiotics cellobiose and FOS, but also sucrose, trehalose and lactose successfully enhanced the survival and ß-glucosidase and ß-galactosidase activity retention during freeze-drying and subsequent storage and under simulated gastrointestinal conditions. Cellobiose and FOS had the highest protective effect and additionally improved the acid and bile tolerance not only as protective agents on membrane integrity but also as metabolizable substrates. Cellobiose was shown to be most effective for protecting enzyme activities and improving acid and bile tolerance, even at room temperature. A preservation of the enzyme activities is of advantage in functional food industry due to the enhancement of bioactivity and bioavailability of glycosides of secondary plant metabolites by deglycosylation. To the best of our knowledge, this is the first study to show the effect of the presence of protective prebiotics on the survival of probiotics after exposure to acidic and bile conditions. Cellobiose and FOS may be used in symbiotic formulations due to their action as prebiotic and protectant simultaneously. These findings may help to expand the applications of cellobiose and fructooligosaccharide in synbiotic products with important applications in the design of new pharmaceutical formulations.

4. Experimental

4.1. Bacterial growth conditions

The bacterial strain *Bifidobacterium longum* subsp. *infantis* (UV16PR) was obtained from Medipharm (Kågeröd, Sweden). The microorganism was activated in Reinforced Clostridial Medium (RCM, Oxoid Ltd., Hampshire, UK) and maintained in glycerol stocks at -80 ºC.

4.2. Freeze-drying of the bacterial cells

After three successive transfers in RCM, microorganisms were harvested by centrifugation (10000 x g, 4 °C, 15 min, Sorvall RC5C, Thermo Scientific, Austria), washed twice with 50 mM phosphate buffer pH 6.5 and resuspended in phosphate buffer solution containing 10 % (w/v) sucrose, lactose (Sigma-Aldrich, St. Louis, MO, USA), trehalose (Merck, Darmstadt, Germany), (Sigma-Aldrich), 5 % or 10 % cellobiose (Glycon Biochemicals, Luckenwalde, Germany), 5 % or 10 % FOS (Orafti, Town). Freeze-drying was undertaken using Heto Power Dry LL3000 freeze-dryer (Thermo Scientific, Waltham, MA, USA) according to Basholli-Salihu et al. (2014). Freeze-dried cells without protectants were prepared as control. All experiments were performed in triplicates. The dried cells were aliquoted and vacuum sealed in aluminum foil sachets for the determination of the storage stability. The viability, β-glucosidase and β-galactosidase activity were determined before and after freeze-drying.

4.3. Exposure to gastrointestinal conditions

The acid and bile tolerance of *B. infantis* after storage for 3 months at 4 °C and 25 °C was determined by exposure of cells to simulated gastric medium (SGM), (NaCl 137 mM; KCl 2.7 mM; NaH_2PO_4 10 mM; KH_2PO_4 1.8 mM and porcine pepsin 0.3 %, w/v) or simulated intestinal medium (SSIM), (NaCl 137 mM; KCl 2.7 mM; NaH_2PO_4 10 mM; KH_2PO_4 1.8 mM and bile extract 1 % (w/v)) by using the aliquots of 1 ml cell suspension. Freeze-dried cells were resuspended at initial volume (during freeze-drying) of 50 mM phosphate buffer pH 6.5 and allowed for rehydration at room temperature. Then 1 ml of the cell suspension was added to 9 ml of prewarmed simulated gastric medium, adjusted to pH 2 with HCl and incubated at 37 ºC. Aliquots were taken before incubation and after 90 min and the cell viability (CFU/ml) was determined in triplicate using the plate count method on RCA.

Bile tolerance was tested by adding 1 ml of resuspended cells to 9 ml of prewarmed simulated small intestinal medium, mixing well and incubating at 37 ºC. Aliquots were taken before incubation and after 2 h incubation at 37 °C. Cell viability (CFU/ml) was determined in triplicate using the plate count method on RCA.

Log units reduction after exposure to simulated gastric and intestinal medium was calculated as follows:

Log units reduction = $\log N_t - \log N_0$,

where N_t – Colony Forming Units (CFU/ml) after exposure to simulated gastric or intestinal medium, N_0 – CFU/ml at baseline (before treatment).

To study whether the presence on the SGM or SSIM of carbohydrates used as protectants can influence the acid and bile tolerance, the cell suspensions were prepared in two different ways:

Unwashed cell suspension: First cells were rehydrated to the initial volume (lost during freeze-drying) of 50 mM phosphate buffer pH 6.5 and after 20 min standing at room temperature the cell suspensions were added as such (1 ml) in 9 mL of SGM or SSIM. In these samples the protectant used during freeze-drying are present.

Washed cell suspension: In order to remove the protectants used during freeze-drying, after rehydration (according to the above paragraph), the cells were harvested by centrifugation (3000 x *g*, 10 min), washed with 50 mM phosphate buffer pH 6.5 and then resuspended in the same buffer. Resuspended cells (1 ml) were added to 9 ml SGM or SSIM to test the acid (or bile) tolerance.

4.4. Cell enumeration

Viability of cells was determined before and after freeze-drying and after storage. Fresh cell suspension (1 ml) or 1 ml of rehydrated cells were serially diluted with phosphate buffered saline (PBS) and plated onto Reinforced Clostridial Agar plates, in triplicate, then incubated at 37 ºC under anaerobic conditions in an anaerobic chamber using Anaerogen kits for 48-72 h. Cell viability was expressed as colony forming units (CFU) per ml. The survival rate was expressed in per cent: Survival (%) = N/N_0, where N-viability after freeze-drying/storage and N_0-viability of cells before freeze-drying.

4.5. Determination of the enzymatic activity of probiotics

Enzymatic activity was tested in the cell-free extract which was prepared by mechanical disruption using glass beads as described previously (Basholli-Salihu et al. 2014). In brief, resuspended cells were mixed with sterile glass beads (200-300 µm) at a ratio 1:1.5 (w/w) and disrupted by vortexing for 5 cycles of 2 min with 2 min break on ice. Then suspensions were centrifuged (17000 x g, 30 min, 4 °C) in a Sorvall® RC5C centrifuge to remove the cell debris and glass beads. The supernatant was clarified using a 0.45 µm filter (Merck Millipore, Darmstadt, Germany) and used for enzyme activity assay.

The β-glucosidase and β-galactosidase activity of cell-free enzyme were determined before and after freeze-drying, and before and after storage for 2 and 3 months at 4 ºC and 25 ºC, using *p*-nitrophenyl-β-D-glucopyranoside and *o*-nitrophenyl-β- D-galactopyranoside (p-NPG and o-NPG, Sigma-Aldrich) as substrates, according to Basholli-Salihu et al. (2014). Shortly, for determination of β-glucosidase or β-galactosidase activity, 600 µl of cell-free enzyme were mixed with 150 µl of pNPG solution (5 mM) in 50 mM phosphate buffer pH 6.5 (for β-glucosidase activity) or 150 µl of oNPG solution (15 mM) in 50 mM phosphate buffer pH 6.5 (for β-galactosidase activity). The mixtures were incubated for 15 min at 37 °C. The reaction was stopped on ice by adding 375 µl of cold NaOH (0.1 M). The reaction solution was centrifuged for 10 min at 13000 rpm and the amount of p-nitrophenol or o-nitrophenol released was measured at 410 nm using Hitachi U-1100 spectrophotometer (Hitachi Ltd. Tokyo, Japan).

The enzyme activity before freeze-drying was defined as 100 % activity. The relative retention was calculated as a

$$\text{Retention, \%} = N*100/N_0$$

where: N- β-glucosidase or β-galactosidase activity after freeze-drying/storage, N_0-β-glucosidase or β-galactosidase activity before freeze-drying.

The protein concentration was determined using Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA) and Bovine Serum Albumin (Sigma-Aldrich, St. Louis, MO, USA) as standard. Determination of protein concentration was performed in order to avoid possible differences in concentrations before and after freeze-drying and subsequent storage.

4.6. Determination of water activity (a_w)

The water activity was determined using water activity analyzer (Ebro AWX 3001, Ingolstadt, Germany). The measurements were carried out after storage using 500 mg of dried cells, according to Stummer et al. (2012).

4.7. Evaluation of the membrane integrity

The changes of membrane integrity during freeze-drying and storage were determined using propidium iodide (PI) staining (Stummer et al. 2012). Cell suspensions (500 µl) were mixed with 500 µl PI solution (2.4 µg/ml) and then incubated at 37 °C for 15 min. Reaction mixture (50 µl) was pipetted on the 96 well plates and the fluorescence intensity was measured using microplate reader (Infinite®200, Tecan Austria GmbH, Austria). The results were calculated as relative PI intensity.

Acknowledgements: The authors are grateful to the Austrian Ministry of Science and Technology (Bertha von Suttner program) for providing financial support to Mimoza Basholli-Salihu.

Conflicts of interest: None reported.

References

Ananta E, Volkert M, Knorr D (2005) Cellular injuries and storage stability of spray-dried Lactobacillus rhamnosus GG. Int Dairy J 15: 399-409.

Ávila M, Hidalgo M, Sánchez-Moreno C, Pelaez C, Requena T, de Pascual-Teresa S (2009) Bioconversion of anthocyanin glycosides by Bifidobacteria and Lactobacillus. Food Res Int 42: 1453-1461.

Bagad M, Pande R, Dubey V, Ghosh AR (2017) Survivability of freeze-dried probiotic Pediococcus pentosaceus strains GS4, GS17 and Lactobacillus gasseri (ATCC 19992) during storage with commonly used pharmaceutical excipients within a period of 120 days. Asian Pac J Trop Biomed 7: 921-929.

Basholli-Salihu M, Mueller M, Salar-Behzadi S, Unger FM, Viernstein H (2014) Effect of lyoprotectants on β-glucosidase activity and viability of Bifidobacterium infantis after freeze-drying and storage in milk and low pH juices. Food Sci Technol 57: 276-282.

Basholli-Salihu M, Schuster R, Mulla D, Praznik W, Viernstein H, Mueller M (2016) Bioconversion of piceid to resveratrol by selected probiotic cell extracts. Bioprocess Biosyst Eng 39: 1879-1885.

Berlec A, Ravnikar M, Štrukelj B (2012) Lactic acid bacteria as oral delivery systems for biomolecules. Pharmazie 67: 891-898.

Broeckx G, Vandenheuvel D, Claes IJ, Lebeer S, Kiekens F (2016) Drying techniques of probiotic bacteria as an important step towards the development of novel pharmabiotics. Int J Pharm 505: 303-318.

Burns P, Sánchez B, Vinderola G, Ruas-Madiedo P, Ruiz L, Margolles A, Reinheimer J, Clara G (2010) Inside the adaptation process of Lactobacillus delbrueckii subsp. lactis to bile. Int J Food Microbiol 142: 132-141.

Capela P, Hay TKC, Shah NP (2006) Effect of cryoprotectants, prebiotics and microencapsulation on survival of probiotic organisms in yoghurt and freeze-dried yoghurt. Food Res Int 39: 203-211.

Carvalho AS, Silva J, Ho P, Teixeira P, Malcata FX, Gibbs P (2003) Effect of various growth media upon survival during storage of freeze-dried Enterococcus faecalis and Enterococcus durans. J Appl Microbiol 94: 947-952.

Celik OF, O'Sullivan DJ (2013) Factors influencing the stability of freeze-dried stress-resilient and stress-sensitive strains of bifidobacteria. J Dairy Sci 96: 3506-3516.

Corcoran BM, Stanton C, Fitzgerald GF, Ross RP (2005) Survival of probiotic lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. Appl Environ Microbiol 71: 3060-3067.

De Smet I, Van Hoorde L, Vande Woestyne M, Christiaens H, Verstraete W (1995) Significance of bile salt hydrolytic activities of lactobacilli. J Appl Bacteriol 79: 292-301.

Dianawati D, Mishra V, Shah NP (2013) Survival of Bifidobacterium longum 1941 microencapsulated with proteins and sugars after freezing and freeze drying. Food Res Int 51: 503-509.

Dianawati D, Mishra V, Shah NP (2016) Survival of microencapsulated probiotic bacteria after processing and during storage: a review. Crit Rev Food Sci Nutr 56: 1685-1716.

Duary RK, Batish VK, Grover S (2010) Expression of the atpD gene in probiotic Lactobacillus plantarum strains under in vitro acidic conditions using RT-qPCR. Res Microbiol 161: 399-405.

Ejby M, Fredslund F, Andersen JM, Žagar AV, Henriksen JR, Andresen TL, Svensson B, Slotboom DJ, Hachem MA (2016) An ATP-binding cassette transporter mediates the uptake of α-(1, 6)-linked dietary oligosaccharides in Bifidobacterium and correlates with competitive growth on these substrates. J Biol Chem jbc-M116.

Favaro-Trindade CS, Grosso CR. (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 Microencaps 19: 485-494.

Heljo VP, Jouppila K, Hatanpää T, Juppo AM (2011) The use of disaccharides in inhibiting enzymatic activity loss and secondary structure changes in freeze-dried β-galactosidase during storage. Pharm Res 28: 540-552.

Hernández-Hernández O, Muthaiyan A, Moreno FJ, Montilla A, Sanz ML, Ricke SC (2012) Effect of prebiotic carbohydrates on the growth and tolerance of Lactobacillus. Food Microbiol 30: 355-361.

Hempel S, Newberry SJ, Maher AR, Wang Z, Miles JN, Shanman R, Johnsen B, Shekelle PG (2012) Probiotics for the prevention and treatment of antibiotic-associated diarrhea: a systematic review and meta-analysis. JAMA 307: 1959-1969.

Hincha DK (2002) Cryoprotectin: a plant lipid–transfer protein homologue that stabilizes membranes during freezing. Philos Trans R Soc B 357: 909-916.

Jofré A, Aymerich T, Garriga M (2014) Impact of different cryoprotectants on the survival of freeze-dried Lactobacillus rhamnosus and Lactobacillus casei/paracasei during long-term storage. Benefic Microbes 6: 381-386.

Kim M, Nam DG, Kim SB, Im P, Choe JS, Choi AJ (2018) Enhancement of viability, acid, and bile tolerance and accelerated stability in lyophilized Weissella cibaria JW 15 with protective agents. Food Sci Nutr 6: 1904-1913.

Koning SM, Elferink MG, Konings WN, Driessen AJ (2001) Cellobiose uptake in the hyperthermophilic archaeon Pyrococcus furiosus is mediated by an inducible, high-affinity ABC Transporter. J Bacteriol 183: 4979-4984.

Li B, Tian F, Liu X, Zhao J, Zhang H, Chen W (2011) Effects of cryoprotectants on viability of Lactobacillus reuteri CICC6226. Appl Microbiol Biotechnol 92: 609-616.

Marotti I, Bonetti A, Biavati B, Catizone P, Dinelli G (2007) Biotransformation of common bean (Phaseolus vulgaris L.) flavonoid glycosides by bifidobacterium species from human intestinal origin. J Agric Food Chem 55: 3913-3919.

Meng XC, Stanton C, Fitzgerald GF, Daly C, Ross RP (2008) Anhydrobiotics: The challenges of drying probiotic cultures. Food Chem 106: 1406-1416.

Montel Mendoza G, Pasteris SE, Otero MC, Fatima Nader-Macías ME (2014) Survival and beneficial properties of lactic acid bacteria from raniculture subjected to freeze-drying and storage. J Appl Microbiol 116: 157-166.

Osman A, Symeou S, Trisse V, Watson KA, Tzortzis G, Charalampopoulos D (2014) Synthesis of prebiotic galactooligosaccharides from lactose using bifidobacterial β-galactosidase (BbgIV) immobilised on DEAE-Cellulose, Q-Sepharose and amino-ethyl agarose. Biochem Eng J 82: 188-199.

Otieno DO, Ashton JF, Shah NP (2006) Evaluation of enzymic potential for biotransformation of isoflavone phytoestrogen in soymilk by Bifidobacterium animalis, Lactobacillus acidophilus and Lactobacillus casei. Food Res Int 39: 394-407.

Perrin S, Grill JP, Schneider F (2000) Effects of fructooligosaccharides and their monomeric components on bile salt resistance in three species of bifidobacteria. J Appl Microbiol 88: 968-974.

Pessione E (2012) Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows. Front Cell Infect Microbiol 2: 86.

Rivière A, Selak M, Lantin D, Leroy F, De Vuyst L (2016) Bifidobacteria and butyrate-producing colon bacteria: importance and strategies for their stimulation in the human gut. Front Microbiol 7: 979.

Rooj AK, Kimura Y, Buddington RK (2010) Metabolites produced by probiotic Lactobacilli rapidly increase glucose uptake by Caco-2 cells. BMC Microbiol 10: 16.

Roncaglia L, Amaretti A, Raimondi S, Leonardi A, Rossi M (2011) Role of bifidobacteria in the activation of the lignan secoisolariciresinol diglucoside. Appl Microbiol Biotechnol 92: 159-168.

Ruan Y, Sun J, He J, Chen F, Chen R, Chen H (2015) Effect of probiotics on glycemic control: a systematic review and meta-analysis of randomized, controlled trials. PLoS One 10: e0132121

Ruiz L, Margolles A, Sánchez B (2013) Bile resistance mechanisms in Lactobacillus and Bifidobacterium. Front Microbiol 4: 396.

Ruas-Madiedo P, Hernández-Barranco A, Margolles A, Clara G (2005) A bile salt-resistant derivative of Bifidobacterium animalis has an altered fermentation pattern when grown on glucose and maltose. Appl Environ Microbiol 71: 6564-6570.

Saarela M, Virkajärvi I, Alakomi HL, Mattila-Sandholm T, Vaari A, Suomalainen T, Mättö J (2005) Influence of fermentation time, cryoprotectant and neutralization of cell concentrate on freeze-drying survival, storage stability, and acid and bile exposure of Bifidobacterium animalis ssp. lactis cells produced without milk-based ingredients. J Appl Microbiol 99: 1330-1339.

Saleki-Gerhardt A, Ahlneck C, Zografi G (1994) Assessment of disorder in crystalline solids. Int J Pharm 101: 237-247.

Sánchez B, De Los Reyes-Gavilán CG, Margolles A (2006) The F1F0-ATPase of Bifidobacterium animalis is involved in bile tolerance. Environ Microbiol 8: 1825-1833.

Schwab C, Vogel R, Gänzle MG (2007) Influence of oligosaccharides on the viability and membrane properties of Lactobacillus reuteri TMW1. 106 during freeze-drying. Cryobiology 55: 108-114.

Stadler M, Viernstein H (2003) Optimization of a formulation containing viable lactic acid bacteria. Int J Pharm 256: 117-122.

Stummer S, Toegel S, Rabenreither MC, Unger FM, 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.

Teekamp N, Tian Y, Visser JC, Olinga P, Frijlink HW, Woerdenbag HJ, Hinrichs WL (2017) Addition of pullulan to trehalose glasses improves the stability of β-galactosidase at High moisture conditions. Carbohydr Polym 176: 374-380.

Vasiljevic T, Jelen P (2003) Drying and storage of crude β-galactosidase extracts from Lactobacillus delbrueckii ssp. bulgaricus 11842. Innovative Food Sci Emerging Technol 4: 319-329.

Vijaya Kumar SG, Singh SK, Goyal P, Dilbaghi N, Mishra DN (2005) Beneficial effects of probiotics and prebiotics on human health. Pharmazie 60: 163–171.

Vinderola G, Zacarías, MF, Bockelmann W, Neve H, Reinheimer J, Heller KJ (2012) Preservation of functionality of Bifidobacterium animalis subsp. lactis INL1 after incorporation of freeze-dried cells into different food matrices. Food Microbiol 30: 274-280.

Wang YC, Yu RC, Chou CC (2004) Viability of lactic acid bacteria and bifidobacteria in fermented soymilk after drying, subsequent rehydration and storage. Int J Food Microbiol 93: 209-217.

Wei X, Guo Y, Shao C, Sun Z, Zhurina D, Liu D, Liu W, Zou D, Jiang Z, Wang X, Zhao J, Shang W, Li X, Liao X, Huang L, Riedel CU, Yuan J (2012) Fructose uptake in Bifidobacterium longum NCC2705 is mediated by an ATP-binding cassette transporter. J Biol Chem 287: 357-367.