LWT - Food Science and Technology 153 (2022) 112394

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Impact of protectant uptake on the shelf-life of dried *Lacticaseibacillus rhamnosus*

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ARTICLE INFO

Keywords:
Probiotic
Nano DSC
Freeze-drying
Lactic acid bacteria
Sugars

ABSTRACT

To improve the survival of dried probiotics, it is advised to expose the bacteria to protectants prior to processing, allowing equilibration of internal solutes. However, optimal conditions for this exposure remain unclear.

This study examined solute uptake by *Lacticaseibacillus rhamnosus* HN001 (formally known as *Lactobacillus rhamnosus HN001)* at 4 °C and 20 °C, over exposure times of 0–240 min. The cells were exposed to hyperosmotic solutions of glucose and sucrose, two potential protective sugars, which are metabolisable and have different molecular weights. Sugar uptake was analysed through HPLC, while the impact on cell viability after freeze-drying was examined at 30 °C and 40 °C. The interactions between cell biomolecules and sugars were examined using Nano DSC.

Results showed that the sugars were rapidly taken up by the cells, independent of temperature. At 20 °C, glucose was readily metabolised, eventually resulting in loss of cell viability during storage. Conversely, the Nano DSC study revealed interactions between the cells and sucrose, potentially providing some explanation as to the stability of the cells.

In conclusion, sugar type and exposure temperature were shown to exert a significant effect on the viability of *Lacticaseibacillus rhamnosus*. Nano DSC is a promising technique to understand the protectant and cells' interactions.

1. Introduction

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit to the host (Sanders, 2008). In recent years, probiotic bacteria have been added to an even wider range of food products with studies showing that they can impact the brain via the gut to improve health outcomes in areas such as cognition, memory and even stress resilience (Kim, Yun, Oh, & Choi, 2018). However, ensuring probiotic stability throughout the shelf-life of the product is thought to be key for a positive effect in vivo and this remains a challenge for the food industry. Freeze drying is one technique that in the past has the resulted in improved stability for probiotics, however, the detrimental impact on the cell and their internal components during the process was noted in a recent review (Dianawati, Mishra, & Shah, 2016).

The uptake of protectants prior to drying has been suggested as having the potential to extended longer term storage of probiotics. Three potential explanations for how these protectants may work have been described in the literature (Santivarangkna, Higl, & Foerst, 2008). Firstly, water plays a critical structural role in the cell which means that in the hydrated state, biomolecules, such as lipids, protein and DNA are in their native form, however once the water is removed, interactions within and between biomolecules can occur which may be detrimental to the cells. Protectants can fill these spaces and maintain these biomolecules in their native form (Santivarangkna, Naumann, Kulozik, & Foerst, 2010). Secondly, the protectants increase the free energy of water, thus maintaining water in the surrounding area of the biomolecules (Meneghel, Passot, Dupont, & Fonseca, 2017). The hydrated biomolecules therefore remain in their native form, even though the environment is around them has been dried. Finally, protectants may form a glassy matrix, protecting the whole cells by immobilisation and slowing down any reaction that could be detrimental to the cells (Santivarangkna, Aschenbrenner, Kulozik, & Foerst, 2011). These mechanisms imply that protectants should be present both inside and outside

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https://doi.org/10.1016/j.lwt.2021.112394
Received 9 December 2020; Received in revised form 8 August 2021; Accepted 30 August 2021
Available online 13 September 2021

the cells in order to impart their protective effect.

Numerous studies have shown that incubation of the bacterial cells with the solution containing the protectants can improve the solute penetration into the cell (H. Chen, Chen, Chen, Wu, & Shu, 2015; Saarela et al., 2005). However, optimal conditions under which this incubation should take place vary with no general consensus. Leslie, Israeli, Lighthart, Crowe, and Crowe (1995) showed that trehalose would not enter *B thuringiensis* cells at temperatures above 15 °C. They suggested that the cell membrane becomes leakier in cold solutions as it goes through phase transition; thus, carbohydrates can passively diffuse through membrane, down their concentration gradient. However, contrary to this, a number of stability studies have involved solute equilibration at room temperature (Carvalho et al., 2002; Conrad, Miller, Cielenski, & de Pablo, 2000; Strasser, Neureiter, Geppl, Braun, & Danner, 2009), or even at higher temperature (Chen, Wang, Luo, & Shu, 2013). At these temperatures, cells are still active, and may uptake actively solutes as well as metabolise them. In a study by Strasser et al. (2009), the protective effects of glucose, maltodextrin, trehalose and sucrose were compared to each other. Interestingly, glucose elicited the least protection in both strains studied. One could argue that at room temperature, sugars would be taken up actively; however, cells may catabolise glucose. In a review on cryoprotectants, Hubálek (2003) explained that the ideal protectant equilibration time and temperature depend on the actual protective agent as well as on the organism. However, even though the ex-*Lactobacillus* species has been at the forefront of the probiotic studies for at least a decade now, to the best of our knowledge no study has looked at optimisation of the temperature and time of exposure for the cells prior to drying.

Therefore, this study examined the solute uptake by *Lacticaseibacillus rhamnosus* HN001 at 4 and 20 °C over 240 min. Glucose and sucrose were chosen as model sugars as they have been shown to have a protective effect on dried *L. rhamnosus* species (Saarela, Virkajarvi, Nohynek, Vaari, & Matto, 2006; Zhu, Ying, Sanguansri, Tang, & Augustin, 2013). In addition, they can both be utilised by *L. rhamnosus* HN001 (Ceapa et al., 2015), and the cells possess active transport systems for both sugars (Kankainen et al., 2009). However, given these sugars are of different molecular weights, they may diffuse through the membrane at different rates when exposed to cold conditions.

Initially, the uptake and catabolism of the sugars were assessed before studying how the stability of the freeze-dried cells was affected. In order to try to understand the nature of the interactions between the protectants and the cell biomolecules, a novel technique, Nano Differential Scanning Calorimetry (Nano DSC) was used. DSC is used to study the heat transfer occurring during physical or chemical change, such as protein denaturation, lipid melting or crystallisation. Conventional DSC has been used to measure the stability of cells and their resistance to heat or antimicrobial agents (Brannan, Whelan, Cole, & Booth, 2015; Mackey, Miles, Parsons, & Seymour, 1991; Miles, Mackey, & Parsons, 1986; Tunick, Novak, Bayles, Lee, & Kaletunç, 2009). However, the sensitivity of a conventional DSC is relatively low. Thus, it may be difficult to distinguish all thermal events occurring when heating up a cell sample. MicroCal MC-DSC is an alternative that has been used to follow melting of DNA (Duguid, Bloomfield, Benevides, & Thomas, 1996). However, to the best of our knowledge no study used the Nano DSC for such study. Interaction of the biomolecule with its environment will be seen as a shift in the melting or denaturation peak, or as a change in the enthalpy of the event, relative to the values for the sugar-free environment.

2. Materials and methods

2.1. Materials

Analytical grade glucose and sucrose were obtained from Thermo Fisher Scientific. MRS broth and MRS agar were bought from Fort Richard. Inulin (Frutafit TEX, degree of polymerisation (DP) $\geq$ 22) was obtained from IMCD New Zealand Ltd., Auckland, New Zealand. Frozen stock of *Lacticaseibacillus rhamnosus* HN001 was provided by Fonterra Ltd., Palmerston North, New Zealand.

2.2. Bacterial strain and growth

Bacteria were maintained by monthly inoculation on agar slopes, and stored at 4 °C. MRS broth was initially inoculated from the slopes and incubated for 18 h at 37 °C. This culture was then inoculated into a new MRS broth (1:9 ratio). The culture was grown for 16 h at 37 °C and under slow agitation (110 rpm). At this stage, the bacterial culture had reached its stationary phase, determined by reading the absorbance (OD_{610}) on a SPECTROstar® Nano (BMG Labtech).

2.3. Preparation of sugar solutions and phosphate buffer

Sugar solutions consisted of glucose or sucrose, which was first dissolved in 0.1 M phosphate buffer (pH 7.4, prepared in milliQ water) to reach a sugar concentration of 0.3 M (about 580 mOsmol/L) before being filtered through a sterile 0.45 μm filter. Sugar solutions and phosphate buffer were freshly prepared the day prior to each experiment and maintained either at 4 or 20 °C depending upon the temperature of the study. Phosphate buffer and glassware were autoclaved with an Astell chamber steriliser (model AVS490D) at 121 °C for 15 min.

2.4. Harvesting of the cells and exposure to the solutions

Bacteria were harvested at the start of the stationary phase by centrifugation (4000 *g* for 5 min). Centrifugation prior to the addition of sugar was performed at the temperature under study.

Bacteria cells were washed twice with phosphate buffer and then maintained at the temperature of the study. For the HPLC analysis, the second washing was done with one tenth of the volume in order to concentrate the cells and thus increase the accuracy of the study. The sugar solution was added to the cell pellets before homogenisation. Samples were either then kept on ice or left at room temperature.

For the HPLC analysis, the time of exposure varied between 1 min and 90 min. The control sample (time 0 min) consisted of cells homogenised with phosphate buffer. At each time point, cold (−20 °C) quenching solution was added in a 1:3 ratio with samples being treated in triplicate. The samples were then centrifuged, supernatants were collected, and cells were washed with the same volume of quenching solution.

The quenching solution consisted of 60% (v/v) methanol with 1.7% (w/v) ammonium carbonate in milliQ water. This was adapted from the solutions proposed by Faijes, Mars, and Smid (2007): ammonium carbonate concentration was increased in order to match the osmotic pressure applied by the sucrose solution (580 mOsmol/L) and to avoid leakage of the cells.

Cell pellets were placed at −80 °C prior to extraction, while collecting the remaining samples.

Bacteria were extracted by first adding cold (−80 °C) absolute methanol and glass beads (diameter 300–355 μm). After a first homogenisation, milliQ water was added at 1:1 ratio before being vortexed for 2 min. Four freeze-thaw cycles were performed to improve cell extraction (from −80 to 4 °C). The suspensions were also vortexed for at least 30 s after each thawing. Cell extracts were centrifuged for 10 min at 16,000 *g* and 0 °C to remove any cell fragments.

For the stability study, the exposure time varied between 1 min and 240 min. The time was increased to obtain greater variation between the results. At each time point, the slurry was placed at −40 °C and left overnight until freeze-drying the next day. All samples were prepared in triplicates.

Finally, for the Nano DSC study, only time 0 and 240 min were used, as the duration of the measurement did not allow to run another sample with an intermediate exposure time. The control consisted of cells

suspended in phosphate buffer. All samples were prepared in triplicates.

2.5. HPLC analyses

Concentrations of sugars and lactic acid in supernatants and cell extracts were analysed by HPLC using a HPX-87H column (Biorad). The runs were performed at 25 °C, preventing sucrose inversion. The mobile phase consisted of 5 mM of H_2SO_4 at a flow rate of 0.5 mL/min.

All samples were filtered through a 0.22 μm filter prior to analysis. Serial dilutions of L-lactic acid, sucrose and glucose were used to calculate concentrations in samples. Standards were freshly run prior to each set of analyses.

2.6. Shelf-life study

The sugar solutions for the study of the bacterial stability after drying were similarly prepared the day prior to harvesting the cells, and in phosphate buffer (0.1 M, pH 7.4). The composition of the solutions was adjusted in order to obtain a constant concentration of bacterial cells after drying. They consisted of 12.5% (w/v) of glucose or sucrose and 12.5% (w/v) of inulin. Inulin was used as a carrier, to ensure proper drying of the slurry. The inulin was first added to the phosphate buffer under stirring and low heat until complete dissolution (about 10 min). Then, sucrose or glucose was added to the solution under stirring. Solutions were either placed at 4 °C or maintained at room temperature (20 °C), depending upon the temperature under study.

Samples were subsequently freeze dried in a Cuddon FD18 Freeze Drier (Cuddon FreezeDry, Blenheim, New Zealand) for approximately 72 h, under vacuum of approximately 1 mbar and shelf temperature reaching 20 °C. The condenser was set at −30 °C.

Once dried, the *L. rhamnosus* powders were ground using a mortar. They were then mixed with skim milk powder at 10% (w/w). Final water activity was of 0.16 ± 0.1. The powder was split in individual aluminium pouches, sealed, and stored in an incubator. Two shelf-life temperature were under study: 30 and 40 °C.

2.7. Nano DSC

The Nano DSC (TA instruments, USA) cells were conditioned the day prior to the experiment by running degassed potassium phosphate buffer in both the sample and reference cells.

After the bacteria were harvested and exposed to the sugar solution, as previously described, they were centrifuged and re-suspended in 1/10th of the same solution in order to increase the sensitivity of the measurement. The slurry was then degassed for 10 min at 4 or 20 °C before being added to the sample cell of the Nano DSC. The sample and the reference sample were heated from either 4 or 20 °C up to 130 °C at 1 °C/min. After each run, the sample cell was flushed with 1 L of milliQ water. The reference sample was kept the same for all the measurements to reduce errors.

2.8. Data analysis and statistics

The factorial design of the shelf life was done and analysed with Minitab® Statistical Software (version 18.1, © 2017 Minitab, Inc., USA). The Nano DSC thermograms were analysed using R 3.5.0 (R Core Team, 2018). As the measurements of the heat rate was given as a function of the time, the data was first resampled on a common temperature vector in order to align the signals of all samples. For comparison of samples exposed to phosphate buffer and sucrose, the region of interest (from 45 to 110 °C) was isolated, and the baseline was removed using the peak fitting method with 100 points as half width of the local window. Principal Component Analysis (PCA) was conducted on the raw thermograms (before baseline removal) for all samples, as well as after the baseline removal for cells exposed to phosphate buffer and sucrose. The peaks were identified with the first derivative. The inflexion points were obtained by deriving and smoothing the thermograms twice-over.

3. Results and discussion

3.1. Uptake and metabolism of glucose and sucrose by L. rhamnosus

The uptake and catabolism of the glucose and sucrose was examined at 4 and 20 °C. Table 1 presents the concentration of lactic acid released in the supernatant. A rapid release, and thus production, of lactic acid was observed when cells where exposed to glucose at 20 °C. After 90 min of exposure, lactic acid concentration attained about 8 g/L in the solution. However, when cells were exposed to glucose or sucrose at 4 °C or to sucrose at 20 °C, the concentration of lactic acid in the supernatant increased only slightly, with about 0.4 g/L released over the 90 min of exposure. The metabolism is, hence, slowed down at lower temperatures but not completely stopped.

Fig. 1 represents the amount of sugars and lactic acid present in the cell pellet after the exposure time. It appeared that sugars were rapidly taken up by the cells as in the first couple of minutes of exposure there was an increase from 0 g sugar taken up/g of cells (DCW) to 0.018 and 0.016 g/g of cells (DCW) for glucose at 4 and 20 °C, and to 0.031 and 0.035 g/g of cells (DCW) for sucrose at 4 and 20 °C. For both sugars, there is no clear difference between the uptake at 4 and 20 °C. Yet, it was expected that the entry at 4 °C would be facilitated because at this temperature the lipid membrane partly crystallises and thus become more permeable (Leslie et al., 1995). In the case of *L. rhamnosus*, there are several transport systems for both sugars, and this strain has shown high affinity for mono and disaccharides (Gopal, Sullivan, & Smart, 2001). Thus, active uptake might not take place at 20 °C which would explain why no differences are seen between the exposure temperatures.

3.2. Impact of the exposure time and temperature on dried L. rhamnosus

The main purpose of this study was to find the ideal time and temperature of exposure of the probiotic organism to sugar, which would lead to better stability after drying. It was hypothesised that the catabolism of the glucose could have a detrimental effect on the stability of *L. rhamnosus* as lactic acid is produced and as bacteria start a new growth phase. As previously shown, the amount of sugar interacting with the cells was constant during the 90 min of exposure. Thus, it was assumed that the time and temperature of exposure would not have any positive effect on the cell viability. The time of exposure was increased to up to 240 min in order to verify this assumption. The cells were then exposed at 4 or 20 °C to a sucrose or glucose solution and freeze-dried.

The overall stability over the storage time is presented in Fig. 2. Cells exposed to sucrose at 20 °C for 0 min and to glucose at 20 °C for 240 min were the most impacted by freeze-drying, with a viability of 9.53 and 9.56 log (CFU/g) respectively (Table 2). On the other hand, cells exposed to glucose at 4 °C for at least 60 min, and cells exposed to sucrose at 20 °C for 240 min presented a viability of about 10 log (CFU/g). Statistical analysis revealed a three-way interaction between the sugar, the temperature and the time, highlighting that the exposure settings have different impacts on the cells depending on the sugar used (Table 3). This is most likely due to the catabolism of glucose, as the release of lactic acid was occurring only for cells exposed to glucose at 20 °C. The longer time led to higher amount of lactic acid in the drying media, lowering the pH, and potentially harming the bacteria. On the other hand, mechanisms may have been different for sucrose as the longer the time of exposure time, the higher the viability at both temperatures. Thus, uptake of the solutes may have occurred over a longer period of time and helped the stabilisation of the bacteria.

Over storage, there was a substantial decrease in *L. rhamnosus* viability for cells exposed to glucose at 20 °C for 240 min, at both storage conditions (Fig. 2). This was closely followed by cells exposed to glucose at 20 °C for 120 and 60 min, when stored at 40 °C. Cells exposed to glucose at 4 °C had a similar trend, with death rate increasing with the

Table 1
Production of lactic acid in the cell supernatant after exposing the *L. rhamnosus* strain under study to glucose or sucrose at 4 or 20 °C.

Treatment	Lactic acid in supernatants						
Glucose 4 °C	Exposure time (min)	0	1.62	15.08	30	60	90
	Concentration (g/L)	0.48 ± 0.02	0.49 ± 0.03	0.61 ± 0.02	0.67 ± 0.03	0.88 ± 0.04	0.94 ± 0.06
	Production rate (mmol/min)		0.05	0.1	0.04	0.08	0.02
Glucose 20 °C	Exposure time (min)	0	1.75	15.5	30.25	60	90
	Concentration (g/L)	0.26 ± 0.05	0.37 ± 0.01	1.34 ± 0.02	2.19 ± 0.12	4.58 ± 0.14	8.05 ± 0.15
	Production rate (mmol/min)		0.72	0.78	0.64	0.89	1.28
Sucrose 4 °C	Exposure time (min)	0	1.35	15.08	30	60	90
	Concentration (g/L)	0.79 ± 0.01	0.73 ± 0.01	0.85 ± 0.03	0.96 ± 0.03	1.11 ± 0.02	1.16 ± 0.06
	Production rate (mmol/min)		−0.48	0.1	0.08	0.06	0.02
Sucrose 20 °C	Exposure time (min)	0	2	15	30.3	60.16	90
	Concentration (g/L)	0.32 ± 0.04	0.29 ± 0.02	0.46 ± 0.07	0.54 ± 0.16	0.8 ± 0.06	0.84 ± 0.06
	Production rate (mmol/min)		−0.13	0.15	0.06	0.1	0.02

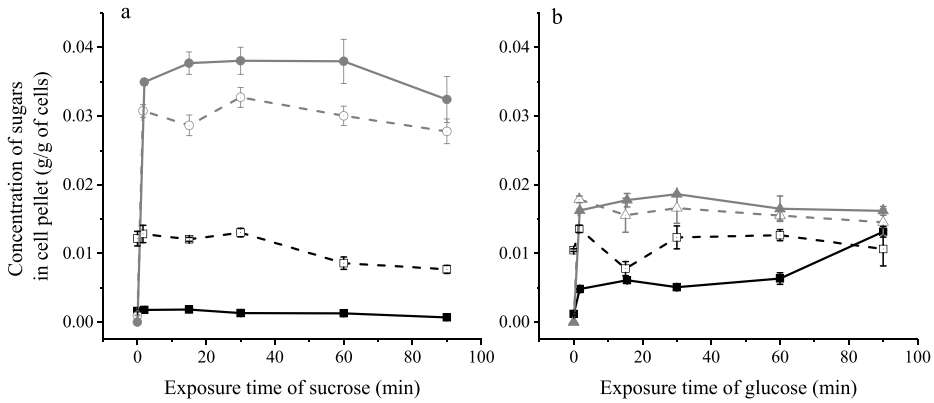

Fig. 1. Concentration of sugars (in grey) and of lactic acid (in black, squares) in cell pellets following exposure to sucrose (graph a, circles) or to glucose (graph b, triangles) at either 4 °C (in dashed line, open symbol) or 20 °C (in solid line and solid symbol). The error bars show the standard error of the values.

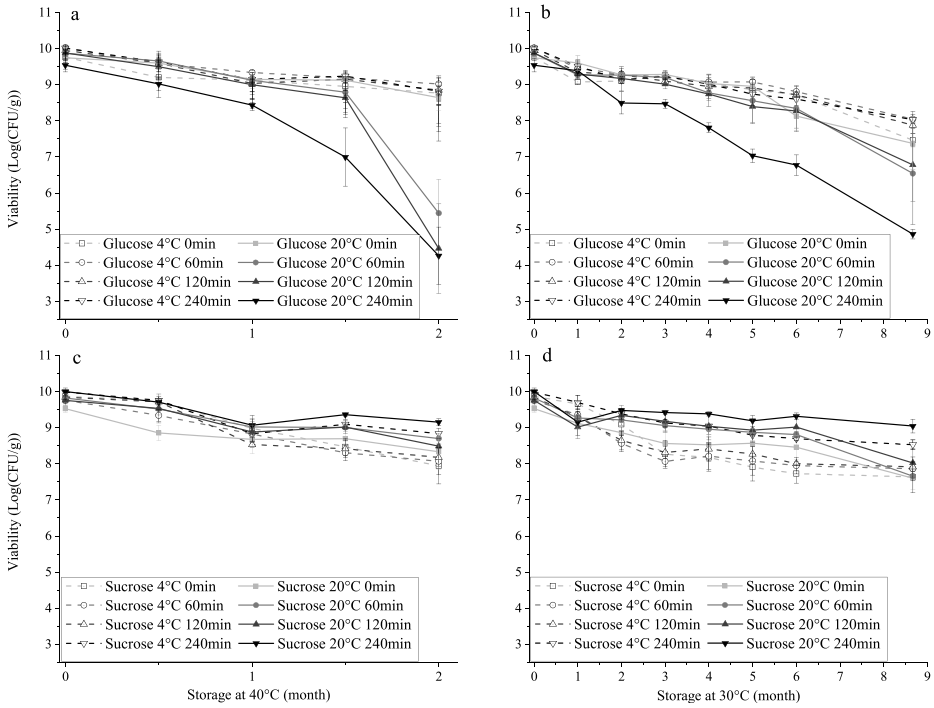

Fig. 2. Shelf life study conducted at 40 °C (graphs a and c) and 30 °C (graphs b and d) of *L. rhamnosus*. Cells exposed to glucose are presented in graphs a and b, and cells exposed to sucrose in graphs c and d. Exposure was at 20 °C (solid line) or at 4 °C (dashed line) for 0 min to 240 min. The error bars show the standard error of the values.

Table 2
Viability after drying and over storage of *L. rhamnosus* exposed to glucose or sucrose at 4 or 20 °C for up to 240 min prior to drying. Values are expressed in their arithmetic mean and their standard deviation.

Exposure treatment			Viability after drying	Shelf-life at 40 °C		Shelf-life at 30 °C	
				Viability after 2 months	Death rate at 40 °C	Viability after 8.5 months	Death rate at 30 °C
			Log_{10} (CFU/g)	Log_{10} (CFU/g)	/mo.	Log_{10} (CFU/g)	/mo.
Glucose	4	0	9.76 ± 0.16	8.77 ± 0.09	0.45 ± 0.07	7.46 ± 0.31	0.17 ± 0.07
Glucose	4	60	10.02 ± 0.09	9.02 ± 0.30	0.48 ± 0.08	8.05 ± 0.22	0.17 ± 0.03
Glucose	4	120	9.95 ± 0.14	8.85 ± 0.19	0.53 ± 0.13	7.88 ± 0.39	0.19 ± 0.03
Glucose	4	240	10.01 ± 0.06	8.82 ± 0.62	0.55 ± 0.20	8.03 ± 0.40	0.21 ± 0.06
Glucose	20	0	9.74 ± 0.07	8.64 ± 0.61	0.54 ± 0.29	7.38 ± 1.27	0.26 ± 0.18
Glucose	20	60	9.87 ± 0.14	5.44 ± 1.62	1.95 ± 0.57	6.54 ± 2.45	0.29 ± 0.32
Glucose	20	120	9.88 ± 0.13	4.46 ± 2.14	2.34 ± 0.80	6.78 ± 1.74	0.30 ± 0.22
Glucose	20	240	9.54 ± 0.33	4.26 ± 1.37	2.52 ± .60	4.86 ± 0.23	0.52 ± 0.08
Sucrose	4	0	9.87 ± 0.14	7.94 ± 0.87	1.02 ± 0.47	7.64 ± 0.76	0.36 ± 0.13
Sucrose	4	60	9.76 ± 0.17	8.07 ± 0.64	0.88 ± 0.41	7.86 ± 0.58	0.28 ± 0.13
Sucrose	4	120	9.85 ± 0.26	8.18 ± 0.42	0.93 ± 0.31	7.92 ± 0.21	0.27 ± 0.08
Sucrose	4	240	9.99 ± 0.01	8.84 ± 0.25	0.46 ± 0.29	8.53 ± 0.23	0.20 ± 0.03
Sucrose	20	0	9.53 ± 0.16	5.55 ± 0.84	0.51 ± 0.45	7.60 ± 0.54	0.16 ± 0.08
Sucrose	20	60	9.83 ± 0.20	8.70 ± 0.24	0.55 ± 0.15	7.66 ± 0.07	0.16 ± 0.03
Sucrose	20	120	9.76 ± 0.22	8.49 ± 0.10	0.44 ± 0.27	8.03 ± 0.71	0.09 ± 0.12
Sucrose	20	240	10.00 ± 0.17	9.16 ± 0.16	0.41 ± 0.11	9.05 ± 0.33	0.07 ± 0.01

Table 3
Analysis of the factorial design for the stability of *L. rhamnosus* after freeze-drying and over storage.

Significance of the factors	Viability after drying	Shelf-life at 40 °C		Shelf-life at 30 °C	
		Viability after 2 months	Death rate at 40 °C	Viability after 8.5 months	Death rate at 30 °C
Sugars (S)	NS	<0.0001	<0.0001	0.002	NS
Temperature (Temp)	0.013	<0.0001	<0.0001	0.019	NS
Time (T)	NS	NS	0.054	NS	NS
S*Temp	NS	<0.0001	<0.0001	0.008	<0.0001
S*T	0.057	0.002	0.005	0.042	–
Temp*T	NS	0.017	0.008	–	–
S*Temp*T	0.053	0.02	–	–	–
R^2 of the model	50.29%	83.17%	78.77%	48.4%	33.26%
Predicted R^2	0%	61.26%	58.76	17.37%	8.04%

NS: Not significant ($P < 0.05$).

exposure time. Again, statistical analysis showed a three-way interaction for the viability after the 2 months, but not for the death rate, most likely because the cell loss caused by the freeze-drying is not taken into account in the calculation of the death rate.

Cells exposed to sucrose resulted in a lower death rate with increasing exposure time at both temperature. This confirms that the optimal exposure settings for *L. rhamnosus* depend on the type of sugar. The statistical model does not show the same significance of each factor at the two different storage temperatures. While a three-way interaction explained the viability of the cells after 2 months at 40 °C, a two-way interaction explained the same result at 30 °C. The cells were more affected by the glucose-catabolism related stress when the storage temperature increased.

The steep increase in death rate with the increase in exposure time to glucose at 20 °C confirms that the catabolism occurring is detrimental for the cell stability. There are three possible explanations for this result. First, as the cells produce lactic acid, the pH of the protective solution decreases slightly – from 7.2 to 6.7, which would affect the cells over time. Second, when cells were exposed to the glucose solution at 20 °C, the rate of use was about 6 g/L of glucose per hour. Thus, after 240 min, the final concentration would be close to 10.1% (w/v), instead of 12.5% (w/v), and may not have been enough to protect the cells from dehydration and storage. Finally, as the cells use glucose over the time of the exposure, they start a new growth phase, thus becoming less resistant to the dehydration and storage stresses. Strasser et al. (2009) have reported similar results with *Enterococcus faecium* and *Lactiplantibacillus plantarum* exposed to protectants at room temperature for 1 h. The bacteria dried with glucose, had a shorter shelf-life than those dried with maltodextrin, trehalose or sucrose. As there is no comparison point in their study, it is difficult to be certain that the low stability of cells dried in glucose is caused by the activity of the cells catabolising the sugar. However, the present results might shed some further light on their results.

On the other hand, *L. rhamnosus* stability seemed to increase with increasing length of exposure time to sucrose, indicating that the sugar slowly entered or interacted with the cells, increasing its protective effect. To verify if interaction of sucrose with the cells led to the higher stability of the dried bacteria, the heat stability of the whole cells was assessed using calorimetry.

3.3. Understanding the interaction between the sugars and L. rhamnosus

In this study, L. rhamnosus cells were exposed to three solutions: glucose, sucrose (both prepared in phosphate buffer) and phosphate buffer alone, as a control. The buffer was at isotonic concentrations, and glucose and sucrose were at hyperosmotic concentrations. Four exposure conditions were compared: 4 °C for 0 and 240 min, and 20 °C for 0 and 240 min. The thermograms (Fig. 3) show a difference between the cells exposed to glucose and the cells exposed to sucrose or to the control. Cells exposed to glucose presented a large exothermic peak, with a maximum between 40 and 50 °C. The enthalpy of the peaks varied with the time and temperature of exposure. The smaller the peak, the less responsive the cells are to the glucose. Cells that have been exposed to the glucose for 240 min at 20 °C may utilise less glucose than other samples. During the 240 min at 20 °C, the cells have metabolised glucose and produced lactic acid as shown previously. The consequent pH drop may have caused a stress to the cells, resulting in either a loss of viable cells or lower ability to use the glucose present. The stability study showed a drop of 0.2 log (CFU/g) between freeze-dried cells exposed to glucose at 20 °C for 0 min and those exposed to glucose at 20 °C for 240 min. Even though it was not statistically significant, it still represents a loss of 37% of viable cells and could explain the difference observed in the thermograms. Up to two shoulders were present on the exothermic peak: one at 49 °C for cells exposed at 20 °C for 240 min, and one at 57 °C for all cells exposed to glucose. It is likely that endothermic events occurred at the same time as the exothermic event. One way to verify

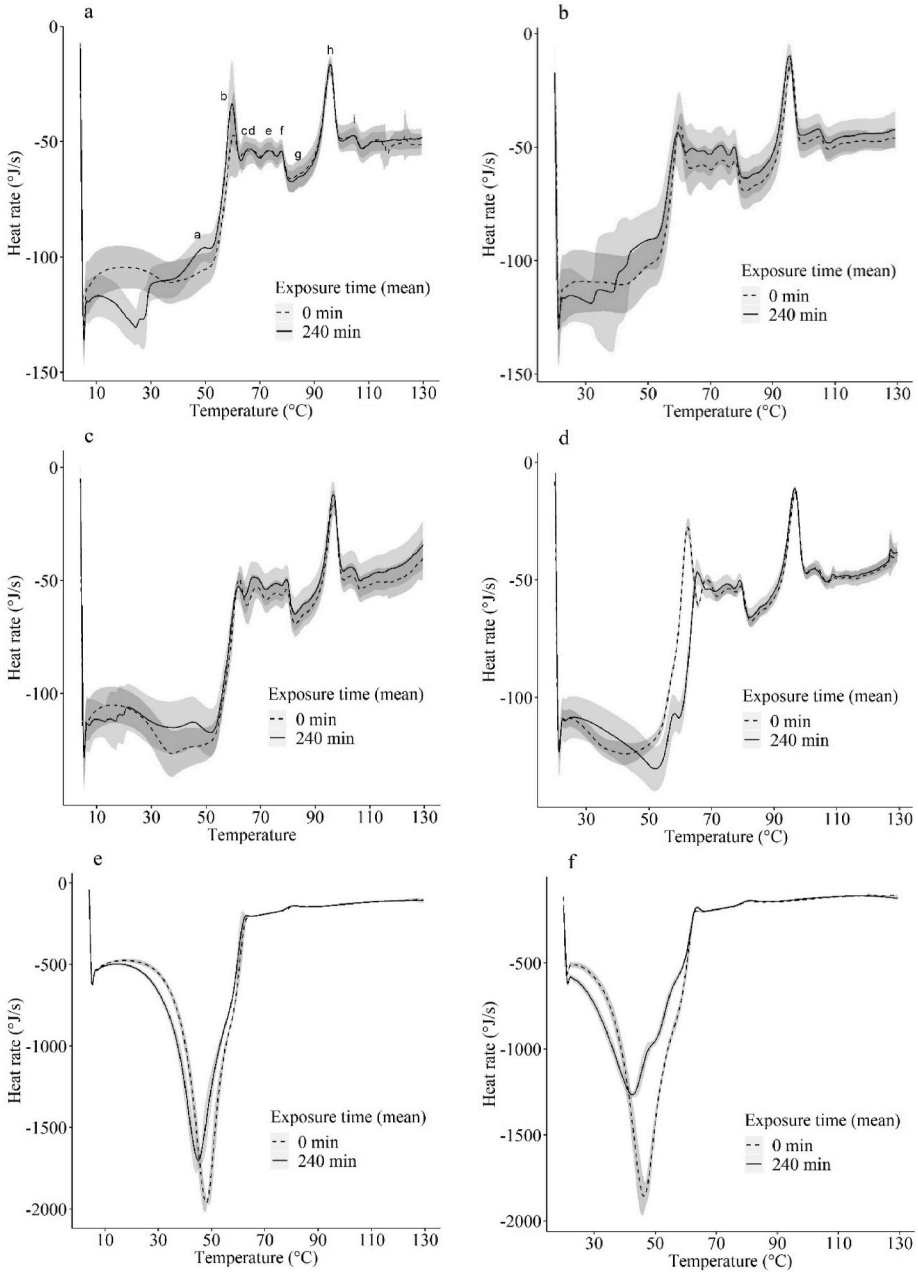

Fig. 3. Thermograms of cells exposed to phosphate buffer (control in graphs a and b), to sucrose (graphs c and d) and to glucose (graphs e and f) at 4 °C (a,c and e) or 20 °C (b,c and f) for 0 min (in dashed line) or 240 min (in solid line). The curve is the mean value of triplicates, and the standard deviation is denoted as the ribbon.

this is to look at the thermograms of cells exposed to buffer or sucrose solution, but no major endothermic events were found to occur at 49 °C for these cells. It is likely that cells metabolising glucose become less stable to heat, thus denaturation of biomolecules will occur at lower temperatures than in normal conditions. This was confirmed by the overall instability to heat for the cells exposed to glucose. Indeed, from 63 °C, the exothermic event ends and is followed by only three endothermic events: one between 63 and 63.5 °C, one at 81 °C and one around 85.5 °C. From 92 °C there are no more events, and all the cell components are thus denatured. This is a major difference between cells exposed to phosphate buffer only and to sucrose, as their last events end around 108 °C. This shows that glucose is not a good stabiliser for *L. rhamnosus* against temperature increases.

The PCA of the raw data (Fig. 4 a) confirmed the grouping of samples exposed to phosphate buffer and sucrose compared to the cells exposed to glucose. This difference was explained by the first PC, the second PC allows the separation of different exposure settings, following this order: cells exposed to glucose for 240 min at 20 °C, 240 min at 4 °C, 0 min at 20 °C, 0 min at 4 °C.

The thermograms of the cells exposed to phosphate buffer and to sucrose were similar to each other. Between 10 and 50 °C, and 110 °C, the thermograms baseline follow a general increase. This corresponds to an increase in heat capacity (ΔC_p) which denotes an increase in energy required to increase the temperature of the solution. As the proteins unfold during the heating process, the non-polar moieties are exposed, and the re-organisation of the water molecules around them cause the change in ΔC_p (Bruylants, Wouters, & Michaux, 2005).

Overall, samples were quite repeatable, with peaks location being maintained between replicate. However, the enthalpy of the peaks did present some variations (Fig. 3). This meant that larger peaks could hide adjacent peak(s). The PCA of the thermogram of cells exposed to phosphate buffer and to sucrose, after the removal of the baseline is

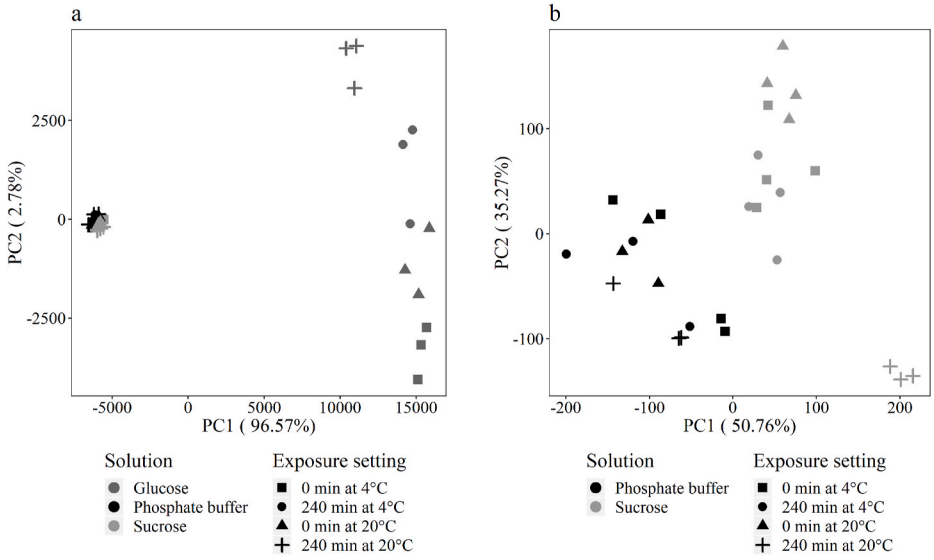

Fig. 4. First and second principal components explaining the variance between all the thermograms of cells exposed to the different solutions. The thermograms of cells exposed to the phosphate buffer, glucose or sucrose solution, before baseline removal, are presented on the left (a). Thermograms of cells exposed to sucrose and phosphate buffer, after removal of the baseline are presented on the right (b).

presented in Fig. 4 b and confirms the repeatability between samples. The samples are relatively well grouped together, with a cluster of cells exposed to the two solutions. Additionally, the cells exposed to sucrose for 240 min at 20 °C are clearly clustered together aside from all the other samples.

Nine endothermic peaks were located on the thermograms of cells exposed to phosphate buffer. They are located between 45 and 110 °C and were assigned a letter from "a" to "i" for ease of analysis (annotated on Fig. 3 a). The main characteristics of the nine peaks, such as temperature at maximum and height, after removal of the baseline, are given in Table 4. An exothermic peak is also present between 20 and 45 °C for the cells exposed to the solution for 240 min. The temperature of this peak extremes depended on the time of exposure – around 25 °C for cells exposed at 4 °C, and between 32 and 45 °C when exposed at 20 °C. The presence of such an exothermic peak was not expected. This event is related to a release of energy, so either a catabolism, either a phase change event to a more disorganised phase (e.g. melting). As this event appeared with a longer time of exposure, it could be related to the cold stress adaptation.

Cells exposed to sucrose at 4 °C had a very similar pattern to cells exposed to phosphate buffer. However, there were three small differences. Firstly, the peak b was slightly smaller than for the cells exposed to phosphate buffer. Secondly, the exothermic peak occurred at a lower temperature: between 10 and 20 °C. Finally, an additional peak appeared around 57 °C (b') in some of the samples.

Interestingly, peaks b to h presented a shift of 1 °C or more, toward higher temperatures, when cells were exposed to sucrose instead of buffer only (Table 4). The last endothermic peak (i) presented a maximum at a lower temperature when exposed to sucrose compared to phosphate buffer.

This shift to higher temperatures could be explained by two factors. Firstly, the stress induced by the increase of the osmotic pressure could lead to cells becoming more resistant to the heat. Indeed, osmotic stressed cells produce general stress response proteins that confers them overall protections, including to heat stresses (Le Marrec, 2011; Xie, Chou, Cutler, & Weimer, 2004; Zhang, Ji, Cheng, Xu, & Jin, 2018). Secondly, the presence of sucrose itself could protect the cells from heat by interacting with the different biomolecules, e.g. by maintaining the proteins in their native form (Kilimann, Doster, Vogel, Hartmann, & Gänzle, 2006). This could explain why some biomolecules have an increase in heat resistance and not others.

The shift of peaks b to h toward higher temperature, may have uncovered the additional peak b' and explains why we don't see it on samples exposed to phosphate buffer. When exposing the cells 240 min at 20 °C with sucrose, peak b' is clearly distinguishable as the peak b shifted to an even higher temperature, with an averaged maximum of 65.4 °C. It is surmised that the shift is due to an interaction of the sucrose with the biomolecule. Over the 240 min at 20 °C, the sucrose had the

Table 4
Averaged thermogram peak locations (x) and height (y) of the cells exposed to phosphate buffer or sucrose solution at 4 or 20 °C for 0 min or 240 min.

	Phosphate buffer at 4 °C				Phosphate buffer at 20 °C				Sucrose at 4 °C				Sucrose at 20 °C			
	0 min		240 min		0 min		240 min		0 min		240 min		0 min		240 min	
	x	y	x	y	x	Y	x	y	x	y	x	y	x	y	x	y
a	48.9	0.4	48.6	2.2	49.9	1.3	50.0	1.2	51.0	0.2	48.1	0.8	51.5	0.2	49.4	0.3
b'	NP	NP	NP	NP	NP	NP	NP	NP	57.5	9.0	57.2	9.6	57.8	11.5	57.7	8.2
b	59.7	36.8	59.6	45.8	60.1	43.3	59.2	33.2	61.7	42.7	61.0	35.5	62.3	57.7	65.4	43.8
c	64.2	23.5	65.1	17.0	64.0	17.7	64.3	17.9	67.6	24.1	66.5	26.9	NP	NP	NP	NP
d	67.0	16.3	68.0	12.8	66.8	14.1	67.4	12.3	69.8	19.1	68.6	21.8	68.0	22.6	68.9	27.5
e	73.2	11.9	73.7	10.9	73.9	11.7	73.6	12.1	75.5	12.9	75.1	12.8	75.5	12.1	73.1	20.1
f	78.0	11.3	78.0	11.6	78.1	11.9	77.8	12.2	79.7	14.0	79.6	14.0	79.7	13.3	79.1	17.0
g	84.0	0.5	84.0	0.5	84.0	0.6	83.7	0.5	85.9	1.1	85.8	1.1	85.8	1.0	85.6	1.0
h	95.8	37.1	95.9	39.6	95.9	42.9	95.4	42.0	96.9	40.6	96.7	40.3	96.8	41.0	96.6	41.5
i	104.6	4.7	104.5	5.6	105.1	6.4	104.8	6.4	103.2	5.9	103.1	5.9	103.2	15.2	102.7	5.3

NP: Not Present

time to enter the cells, and interact with the biomolecules. This could explain why the cells exposed to sucrose at 20 °C for 240 min have better stability during freeze-drying and over storage.

These thermograms were quite similar to those found previously for *L. plantarum* (Lee & Kaletunç, 2002). The authors compared their results with a previous study on *E. coli* (Mackey et al., 1991), where they compared whole cells and cell components. They assigned the first major peak (peak b here) to the biggest ribosomal unit. The denaturation temperature in *L.plantarum* was 63 °C, which appears close to the data from the present study (between 59.6 and 60.2 °C for cells exposed to phosphate buffer and 61 and 62.3 °C when exposed to sucrose, with a maximum at 65.4 °C for cells exposed to sucrose at 20 °C for 240 min). The generally lower denaturation temperature found here could be explained by the different heating rate: in this study, the rate was at 1 °C/min, using a Nano DSC, while in the study by Lee and Kaletunç (2002) the rate was of 3 °C/min using a standard DSC. With a faster heating rate, thermal events, such as denaturation, will occur at higher temperatures (Amani, Moosavi-Movahedi, & Kurganov, 2017). They also found a smaller peak in *E.coli* at 56 °C, that was associated with the 30S ribosomal unit but this peak was not visible on the *L. plantarum* thermogram. The b' peak, which occurred around 57.5 °C, and appeared when cells were exposed to sucrose, could correspond to this peak, obscured in the thermogram of cells exposed to phosphate buffer. A peak similar to h in this study (around 95 °C), occurring at 93 °C in *L. plantarum,* was associated with DNA melting. *L. plantarum* genome has about 44.5% GC (Noda, Shiraga, Kumagai, Danshiitsoodol, & Sugiyama, 2018) compared to 46.7% for *L. rhamnosus* (Collett, Rand, Mason, & Stanton, 2008). This could explain why the melting temperature of the DNA is higher for *L. rhamnosus*. The last peak (peak i, of about 105 °C), was associated with cell wall components (Mackey et al., 1991). In order to confirm the identity of the peaks extraction would be necessary.

Thus, sucrose interacts with most biomolecules, including the ribosome and the DNA, but is not interacting with the cell wall components, or could even destabilise them, as the denaturation temperature was found lower than in the cells exposed to phosphate buffer. This could be explained by the change of osmotic pressure when adding the sucrose to the solution. When exposing the cells to sucrose for 240 min at 20 °C, the 50S ribosomal subunit was found more stable and could have led to the better stability over storage found in the shelf-life study.

4. Conclusion

In this present study we showed that, depending on the sugars employed, exposure to protectants might diminish or improve the viability of the bacterial cells after drying. The main finding derived from this study was that the exposure of protectants should be done under conditions where the cells are not metabolising sugars. The Nano DSC has proven to be a promising technique to examine the interaction between various solutes and cells. In the case of metabolisable sugars, the Nano DSC can give some information on how much energy the cells can use from a particular sugar source. In addition, this technique quickly showed interactions of the cells with protectants, i.e. sucrose. The shift of one peak toward higher melting temperatures could be related to the higher stability of the cells during storage. However, more studies are needed comparing different solutes, their impact on the cell thermograms and the resulting stability of cells.

Funding

This work was supported by Riddet Institute Research Funding [grant number C10X1003].

CRediT authorship contribution statement

Sarah Priour: Writing – original draft, Methodology, Investigation, Formal analysis. **Alan Welman:** Writing – review & editing, Conceptualization, Supervision. **Harjinder Singh:** Writing – review & editing, Funding acquisition. **Ashling Ellis:** Writing – review & editing, Conceptualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The bacterial strain used in these studies was supplied by Fonterra Co-operative limited, however, the authors declare that there is no conflict of interest.

Acknowledgment

We thank Yonatan Souid for his help on the shelf life study. Prof Geoff Jameson is acknowledged for his help and input on the Nano DSC.

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