Atomization gas type, device configuration and storage conditions strongly influence survival of Lactobacillus casei after spray drying

Atomisation gas type, device configuration and storage conditions strongly influence survival of *Lactobacillus casei* after spray drying

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Abstract

Many pathologies are characterised by an imbalance between the beneficial bacteria and potentially pathogenic microbes, i.e. dysregulated host/microbial interactions. Probiotic bacteria are increasingly explored for regulation of immune responses and restoring this imbalance. The application of probiotics most commonly involves drying in formulation development, that can potentially reduce their viability and functionality, hence different protective strategies are needed. Here, spray drying was used for probiotic preservation and effects of device configuration, oxygen injury and encapsulation with increasing skim milk concentrations, were studied on the survival of *L. casei* AMBR2. Quantitative analysis showed no significant differences on bacterial viability for spray drying in an open versus closed system whilst spray drying in a co-current gas flow. Addition of the anti-oxidant, ascorbic acid, to the drying medium adversely impacted viability. Also, no benefits of nitrogen spray drying were observed, implying that oxygen-induced damage is not significant in this setup, at least for the specific tested strain. Additionally, this work shows how storage conditions and total feed solid content significantly alter spray drying viability outcomes. Spray drying was able to retain the high viability of cells of 10^9 CFU/g and higher after 52 weeks at refrigerated storage, whereas 10^6 CFU/g were maintained at room temperature. This study favours air over nitrogen-based spray drying and confirms the method suitability for high viability retention over a year of shelf-life.

Key words: spray drying, nitrogen, processing, stability, inert loop, skim milk, ascorbic acid

1. Introduction

Lactic acid bacteria (LAB) are a group of widely studied beneficial microorganisms, extensively used in food industry, pharmaceutics, for agricultural purposes. Among the LAB, members of the *Lactobacillus casei* group, recently revised as the *Lacticaseibacillus* genus, are perhaps most commonly applied with significant commercial value [1, 2]. The focus on the group's members and their significance is particularly in context of functional foods and pharmabiotics [2]. Recently, a novel human derived strain, *L. casei* AMBR2, was isolated and characterised for its potential application in various spheres as a probiotic [3, 4]. Probiotics are "live microorganisms that, when administered in adequate amounts confer a health benefit on the host" [5]. To exert a health benefit on the host, probiotics typically need to be applied in the amount of 10^7-10^8 CFU/mL, although the required dose can be strain, formulation and application dependent, with applications outside the gut generally requiring lower doses [6]. In order to meet these standards, an economically feasible production tailored to the specific strain and targeted application is needed. Therefore, spray drying is an attractive low-cost, flexible alternative to conventional freeze-drying for the probiotic product manufacturing at many different scales. It is being increasingly explored for the formulation of probiotics and other bioactives for multiple applications, also outside the gastro-intestinal tract where spray-dried powder can have superior characteristics compared to freeze-dried powder [7–11].

Loss of bacterial viability after spray drying has been many times documented [12–16]. Cell injury is attributed primarily to inlet and outlet heat, feed moisture content, dehydration and osmotic stress, but also atomisation and oxidative damage [17, 18]. Additionally, various strategies such as adjustment of cell number inputs, bacterial growth phase in the moment of processing, modelling approach, studying drying kinetics and kinetics of cell death etc. have been proposed for the optimisation of process parameters towards the retention of probiotic properties and better probiotic survival [17–22]. The impact of atomisation gas type and loop

configuration influence upon bacterial viability, powder characteristics, and process scalability for an industrial application are usually overlooked. Laboratory scale spray dryers are commonly used in an open loop system, meaning that additional optimisation for the industry size production is needed in terms of cost, efficiency and environmental issues [23]. Moreover, atomisation gas type may influence partitioning of sample feed (feed to be dried) into droplets, regarding droplet size, droplet velocity and consequently affect the properties of the end product - powder [23, 24]. Typically, for aqueous feed suspensions, air is used, but for non-aqueous feed, inert gases such as nitrogen and helium, are employed. Depending on the feed solution/suspension composition, the aimed level of crystallinity of the final powder, switching drying gas from air to nitrogen or other gases could provide a significant improvement [24]. Moreover, depletion of oxygen from spray drying process could limit probiotic cell damage originating from reactive oxygen species (ROS) [25]. This is potentially dependent on the inherent oxidative stress resistance capacities of the probiotic strains used, because some strains express catalase or super oxide dismutase enzymes that can mitigate ROS [3], especially considering that viability and activity after spray drying are vastly strain-specific [7].

Various studies on probiotic preservation by air spray drying for food and pharmaceutical application are available, however a little is known about the use of the inert loop spray drying system and inert gases as carriers [26–29]. Therefore, this study aimed to compare the impact of inert loop spray drying and inert gases as carriers on the bacterial viability and powder properties of the model respiratory tract probiotic *Lacticaseibacillus casei* AMBR2 [7]. Next, the influence of the system configuration as well as the addition of antioxidants were tested on bacterial viability. Additionally, enhancing the viability of the chosen bacterial strain for more than a year using skim milk (SM) as a model excipient was investigated. Although milk by-products are widely used as good protective agents for spray drying of probiotics [30, 31], there is currently a lack of data on long-term stability of spray-

dried probiotic powders. Lastly, variation in storage viability in relation to the solid content - excipient amount, and storage conditions was evaluated via four different formulations.

2. Materials and methods

2.1 Cultivation conditions and media composition

L. casei AMBR2 LMG P-30039 was used in this study [4]. The strain was isolated from the upper respiratory tract (URT) of a healthy individual and has demonstrated properties of a potential (URT) probiotic. The documented presence of effector molecules, such as fimbriae, and its good adhesive properties to respiratory and gastro-intestinal epithelial cells could qualify it for applications besides URT. Stock cultures of AMBR2 were locally maintained in 25% (v/v) aqueous glycerol at -80 °C. Cultivation was performed in the de Man, Rogosa and Sharpe (MRS) broth (Carl Roth, Mühlburg, Germany) till stationary phase of growth was reached when cells were harvested by centrifugation for 12 minutes at 3983 x g at 20 °C (Sigma 3-16PK, Sigma Zentrifugen, Germany). To test the direct effects of device configuration demineralised water with or without addition of 0.05% (w/V) ascorbic acid (Fagron, Belgium NV.) was used. Next, water-based feed media such as 1%, 10% and 20% (w/V) SM (Oxoid, Thermo Fischer Scientific, Massachusetts, USA) including unprotected bacterial suspension (0%), as a control, were employed in shelf-life and method comparison tests. Briefly, SM was dispersed in demineralised water under magnetic stirring, until complete dissolution, under aseptic conditions. The centrifuged bacterial cells were re-suspended in prepared SM solutions.

2.2 Spray drying

Spray drying procedure was based on our previous findings [7, 20]. Laboratory-scale spray driers, B-290 and B-295 advanced inert loop containing oxygen sensor to detect oxygen levels in the system and dehumidifier (Büchi, Flawil, Switzerland) were used to process samples at constant processing parameters (135 °C inlet temperature, ~55 °C outlet

temperature, 7.5 mL/min flow and 32.5 m^3/h gas flow rate). Two different gas carriers were evaluated, atmospheric air in both, open system and closed system, and nitrogen in the inert loop – also closed system. When air was used in a closed system, oxygen sensor within the inert loop was turned off to allow performance. Spray dryers had a co-current configuration and the same two-fluid nozzle was used (orifice diameter of 1.4 mm). After 15 minutes of spray drying the powder samples were collected directly from single cyclone separators.

2.3 Long-term storage stability

Dried powders of each formulation were stored in Eppendorf tubes (VWR International Europe, Leuven, Belgium), sealed with Parafilm® and kept at room temperature with and without light exposure and refrigerated conditions (4-8 °C) at an ambient relative humidity (RH). The cells' stability was evaluated after predefined intervals of 1, 4, 28 and 52 weeks.

2.4 Enumeration of viable cells

Viability before spray drying, taken as the initial count, and after spray drying and storage were estimated via serial dilutions plated out onto MRS agar (Carl Roth, Mühlburg, Germany) plates in triplicate. Spray-dried powders of each batch prior serial diluting were re-suspended in adequate amounts of demineralised water, to the initial concentration before drying, at room temperature. Incubation of inoculated plates was done at 37 °C for 48h and plates containing 30 – 300 colonies were chosen for counting the viable cells.

Viability rate after spray drying was determined as a comparison between colony forming unit (CFU) counts before spray drying and CFU counts in powders rehydrated to the same solid content / water ratio as before spray drying. The data was expressed in CFU/g as mean value ± standard deviation. The same methodology was applied during the determination of bacterial viability after long-term storage under different conditions.

2.5 Powder morphology

The morphology of *L. casei* AMBR2 probiotic powders was visualised by scanning electron microscopy using a Quanta FEG250 SEM system (Thermo Fisher, Asse, Belgium). The powders were mounted on SEM stubs using conductive carbon tapes. The stubs were sputter-coated with gold (10 nm) and imaged at a voltage of 5 kV.

Particle size was measured using the laser diffraction technique (Malvern 3000, Malvern Instruments Ltd., Malvern, UK) in dry conditions in a small sample device. The size was calculated in accordance with the Mie theory of light scattering and reported as a volume equivalent sphere diameter.

2.6 Moisture content

Karl-Fisher Titration (Karl-Fisher Titrino Plus, Metrohm Germany) was used to measure water content of spray-dried powders. Analyses were carried out in 100-200 mg of spray-dried powder at room temperature with Aqualine Composite 5 (Fisher Scientific, UK) with constant stirring.

2.7 Differential scanning calorimetry

The thermal properties of the spray-dried powder samples were determined using a Discovery DSC25 equipment from TA Instrument (New Castle, DE, USA). Powder samples (5–10 mg) were analysed in Tzero aluminum pans under 50 mL/min nitrogen gas purge in modulated temperature mode. The enthalpy and temperature was calibrated using an indium standard and the heat capacity was calibrated using a sapphire standard. All samples were heated from -40 to 270 °C at a 2 °C/min heating rate with a modulation of 1.6 °C/min. Determination of the melting peak and glass transition temperature was performed using the TA Instruments TRIOS software.

2.8 Yield, bulk density and powder flowability

Yield was defined as the ratio of the amount of the dried powder vs. amount of solids in the bacterial suspension subjected to spray drying.

Yield (%)= weight of spray-dried powder (g)/ weight of solids before drying x 100

Bulk density of spray-dried probiotic powders was determined using the method described by Arepally et al. [32]. The measurements were conducted in a 10 mL graduated cylinder using the tap density tester PT-TD200 (Pharmatest, Germany). The loose and tapped bulk density of spray-dried powder was calculated using the following formulas respectively:

- Loose bulk density (g/cm^3) = weight of powder (g)/bulk powder volume (cm^3)
- Tapped bulk density (g/cm^3) = weight of powder (g)/tapped powder volume(cm^3)

Hausner ratio (HR) was used to estimate the flowability of spray-dried probiotic powders. Table1 illustrates the guidelines for the HR that were used in the result evaluation. Hausner ratio was calculated as follows:

Hausner ratio = tapped bulk density (g/cm^3)/loose bulk density (g/cm^3)

2.9 Statistical analysis

Statistical significance of the differences between obtained powders was checked with SPSS 26 software (IBM statistics, New York, USA) using two-way or one-way ANOVA-test and Tukey's multiple comparisons test. Significance level of $\alpha=0.05$ was applied. All experiments were performed at least three times. Data are expressed as means ± standard deviation or as means.

3. Results

3.1 Impact of gas type, device configuration and antioxidant fortification

The viability of *L. casei* AMBR2 was examined via the nett effect of different device configurations, *i.e.* open and closed system, different gas carriers, with and without the presence of ascorbic acid (Figure 2). Interestingly, comparable viability (with 0.5 log reduction in viability) was obtained when cells were dried in an open and closed mode using atmospheric air as a carrier. Contrarily to what we expected, the addition of ascorbic acid (vitamin C) as an oxygen scavenger to reduce and prevent oxidative damage of cells, actually resulted in a significant decrease in CFU counts in both open and closed air-based systems (0.80 and 1 log reduction CFU, respectively). Furthermore, the oxygen depletion by using a 99.5% nitrogen as a gas carrier also resulted in a significantly lower CFU numbers in comparison to the air carrier systems. Vitamin C fortification of the suspensions dried in the nitrogen inert and closed system also did not result in an improved survival, yet in similar outcomes as without any additions, or a 0.85 log reduction.

3.2 Effect of excipient amount on viability and powder properties after air and nitrogen spray drying

Figure 3A shows that addition of increasing SM amounts to the feed suspension media led to an expected increase in bacterial survival in both air and nitrogen spray drying. This increase was statistically significant for all three SM concentrations when nitrogen spray drying was employed, whereas for air spray drying survival was improved significantly in 10% and 20% SM formulations, but not in 1% formulation. Our aim was to compare the two methods and their outcomes not only via viability quantification but also via powder characteristics after drying. As seen in Figure 3B, both methods resulted in powders with comparable characteristics such as drying yield, water content, flow properties, and density, in contrast to

rather different viability outcomes (Figure 3A). Drying yields were over 50% in majority of formulations, which is in alignment with the production feasibility [33]. Noticeable is low bulk density in all formulations, although its increase was recorded along with the increase in SM concentrations and consequent increase in total solid content of feed suspensions. Of note is that all formulations independently of the gas carrier had poor or very poor flow properties reflected in high Hausner's ratios. Importantly, although a desirably low water content, around 4% [15], can be observed, its further decrease was also noticed with higher solid contents. Importantly, glass transition temperatures (Tgs) were observed in thermograms of all samples except those dried without SM addition, where thermal degradation was visible, and corresponded to the literature values. However, these phase transitions events were only minor, and thus all formulations only partially amorphous, considering the presence of very sharp endothermic peaks at approx. 180 °C. These peaks correspond to the melting peak of lactose, the dominant component of SM [34] . Interestingly, a depression of Tgs, i.e. slightly lower Tgs than previously documented were observed in samples dried in the nitrogen system. Figure 3C shows particle size distributions of all tested formulations dried in both air and nitrogen indicating the increase in median particle size values (~Dx (50) (3.2 µm, 3.5 µm, 8 µm and 10 µm, for 0%, 1%, 10% and 20% SM respectively) with the increase in SM solid content. Viability results after spray drying in both systems have shown a moderate correlation with the particle size values of corresponding formulations (Pearson's coefficient r=-0.68). Next, Figure 3D visualises the particles of all formulations (0%, 1%, 10% and 20% SM) formed by spray drying in both air and nitrogen systems. Here, yet again no major differences between the two techniques were observed. The particle appearance in samples dried without SM reflected interlinked cell agglomerates of different sizes, whereas formulations with SM resulted in coating of cells, i.e. irregularly shaped partially collapsed particles. Additionally, the coating was not complete when 1% SM was used, and some free cells were still visible, but 10% and

20% SM resulted in a complete cell encapsulation. The increase in particle size with the increase of SM concentration was also recorded by SEM.

3.3 Effect of storage conditions upon shelf-life viability

The impact of different storage conditions on bacterial viability over time at ambient RH is depicted in Figure 4. Visibly higher initial cell counts per gram in 0% and 1% SM than in 10% and 20% SM formulations do not indicate better survival, but originate from a much bigger matrix (solid content) with homogenously embedded cells in the later formulations. A gradient decrease in viability was observed for all samples kept at room temperature. This decrease was especially prominent in samples kept at room temperature with daylight exposure. However, countable viability was still present in those samples but with the highest SM content at 28-week point. Regarding the samples kept without light exposure, their viability was countable at 52-week stability point only in case of 20% SM dried in both air and nitrogen. As expected for unprotected cells, their number ceased independently of the storage conditions and drying method and was not countable anymore after 28 weeks. Although, initially resulting in high viability numbers, 1% SM formulations had a steep loss of viability over time in both, room storage conditions and at refrigerated storage, where in spite of the significant loss had approx. $10^5 – 10^6$ CFU/g at the time of the last evaluation. In contrast samples containing 10 and 20% SM dried in air and nitrogen maintained very high viability at refrigerated storage for 52 weeks, $>10^9$ CFU/g of powder.

4. Discussion

In this study, we aimed to determine the importance and size of the oxidative cell injury originating from the oxygen present in the drying medium and oxygen dissolved in the suspension to be dried [35]. Removing oxygen from the drying medium and the addition of an oxygen scavenger such as ascorbic acid has been many times debated, however, experimentally

documented only in a limited number of cases [25, 36–38]. In contrast to findings of these studies, our research has shown a contradictory effect of the vitamin C feed suspension fortification (Figure 2). A potential explanation of this phenomenon can be that dehydration process itself leads to an increase in oxidative state of cells and production of ROS [35, 39] rather than ROS being introduced via drying medium. This in turn would mean that water depletion from cells during drying besides impacting DNA, RNA and proteins as a consequence has ROS generation that contributes to the additional lipid peroxidation, protein denaturation, etc. as demonstrated in eukaryotic cells [39, 40]. Also, of note is the dual nature of the ascorbic acid, on one side radical scavenger and on the other pro-oxidant that generates hydroxyl radicals that could oxidise biological molecules. Namely, its counter effect during storage stability of *Lactobacillus delbrueckii* ssp. *bulgaricus* was reported by Teixeira et al. [41] and on *Bifidobacterium longum* by Champagne et al. [42]. We extended this study and tested air spray drying in a closed system as well as nitrogen spray drying with the addition of ascorbic acid (Figure 2). Importantly, our results suggest that viability after spray drying in a closed system is equally good as in an open system, in spite of the anticipation of a slightly higher heat accumulation and different back pressures. The aspect of the device configuration is often neglected by authors or not clearly defined, although the configuration and glassware assembly type could potentially play an important role in this context as they play in mechanical forces generation and process optimisation. However, the extent of bacterial death increased when nitrogen was used as a drying gas contrasting findings of Ghandi et al. for *Lactococcus lactis* [25] and Wang et al. for *Bordetella pertussis* [43]. Underlying reasons for these outcomes are rather unclear, but could be attributed to the strain specific behaviour towards different stresses and different processing techniques. Our strain of choice should be indeed more oxidative stress resistant, but current results do not provide sufficient data on why a higher damage occurs when oxygen is diminished. Moreover, the strain's facultative

anaerobic nature would imply tolerance of both presence and absence of oxygen in any system. Combination of nitrogen and ascorbic acid has surprisingly led to a slight survival improvement in comparison to the nitrogen itself, but remained still significantly lower than survival in air-based systems (Figure 2).

Additionally, a long-term stability study with bacteria encapsulated with increasing SM solid content with varying storage conditions was conducted to further evaluate the differences between nitrogen and air spray drying (Figure 4). SM was chosen for the evaluation as milk proteins and their protective capacity in encapsulation of probiotic bacteria after spray drying and over time was documented by authors [30, 31, 44], and confirmed through our experiments. Also, the increase in SM concentrations, unlike when pure sugar protectants are used, has led to the proportional increase of particle size during encapsulation which could allow a proper evaluation of the significance of the solid content and particle size in shelf-life stability and spray drying (Figure 3C). SM beneficial effect raises from cell surface layer formation through hydrophobic reaction between cells and milk proteins resulting in a microcapsule formation [45]. Those microcapsules exhibit concavities (Figure 3D) because of processing at high temperatures that are believed to make particles stronger against fractures and solute diffusion [46] and have remained intact over time regardless of the storage condition (Supplement 1). Here, although viability results after spray drying were comparable among different SM concentrations (Figure 3A), over time the best viability was maintained with SM concentrations higher than 1%, probably due to more complete microcapsule formation as confirmed by SEM in Figure 3D. Correlation analysis has further shown a certain degree of dependency of the viability outcome and particle size, meaning that bigger particles usually led to better protection. According to Würth et al. [47] the size of SM microcapsules correlated negatively with the viability of *Lactobacillus paracasei* ssp. *paracasei* F19 due to longer retention times of bigger particles in the dryer. Yet, this was not confirmed in our study. Of note is that the

309 double increase in solid content from 10% to 20% SM overall neither led to a further viability
310 improvement nor to a better shelf-life meaning that there are minimum needed and maximum
311 necessary concentrations of the excipient (Figure 3A and Figure 4). When literature is browsed,
312 much higher concentrations of SM and excipients in general may be seen (over 30% w/V),
313 which is not cost-effective for an industrial scale production. On the other hand, considering
314 very good protective power of SM, differences between air and nitrogen spray drying were not
315 distinguishable as when no excipients were added. Important is to mention that powders
316 obtained by both methods had overall similar characteristics in terms of yield, flowability, low
317 bulk density, that increased with the increase in solid matter, and water content (Figure 3B).
318 As reviewed by many authors [19, 48–50] main aspects important to consider for storage
319 stability are temperature, oxygen content in packaging and moisture level of the product. Our
320 findings confirm the importance of the storage temperature in preserving high bacterial
321 viability over time where, as expected, refrigerated storage conditions resulted in the highest
322 viability over time (~1 log reduction for all formulations of 10 and 20% SM after 52 weeks)
323 (Figure 4). Similar was shown by Wanticha Lapsiri, Bhesh Bhandari and Penkhae
324 Wanchaitanawong [51] with spray-dried *L. plantarum* TISTR 2075 whose survival during
325 shelf-life was predominantly dependent on the storage temperature and RH, as well as by Reyes
326 et al. [52] and 60 day-storage stability of *L. plantarum* NRRL B-4496. It is well-known that
327 storage temperature should always be below the Tg of the product to increase its stability and
328 that moisture content plays a role in relation to it [48, 51]. However, although all of our
329 powders were kept at temperatures below their Tg the improvement was only significant at 4-
330 8 °C, whereas we do observe better viability outcomes over shelf-life in samples with lower
331 initial water content related to the increase in solid content of spray-dried suspensions. Hence,
332 Tg cannot be considered as a main inactivation point, since multiple causes underlie the storage
333 death rate and progress [31]. Next, amorphous systems are in most of the times favourable over

crystalline or partly crystalline when it comes to the viability protection of bacteria over time, because glassy matrix, such as formed with sugars like lactose or trehalose, is believed to restrict molecular movements and slow down detrimental processes [28, 53]. Contrarily, our current and unpublished results do not stress the importance of material state, but rather formulation specific reactions between excipients and bacteria. Besides, Önneby et al. [54] reported the example of freeze dried *Sphingobium* sp. where complete loss of viability happened after 4 weeks in both amorphous and crystalline sugar matrices. When it comes to storage stability, it is plausible that light exposure could have a negative effect, due to UV rays causing DNA damages [55], which is quite visible in our results (Figure 4A). Moreover, the detrimental effect of the daylight was seen already after a couple of days and resulted in a very fast and steep decrease in viability during storage. This requires careful manipulation in downstream processing as well as special packaging requirements. As concluded by Tripathi et al. [55] next to light permeability of the packaging material, other aspects such as packaging technique, type of its material, i.e. glass or different types of plastic, could result in different shelf-lives especially because of different oxygen permeability. Thus, as suggested by the same author, glass packages with a hermetic closure should lead to more stable products which could be a potential solution for our formulations kept at room temperature in dark.

5. Conclusions

In conclusion, we found that addition of ascorbic acid in the feed suspension medium did not improve cell survival after spray drying as well as that drying in closed systems with recirculating gas results in comparable viability results as the open-system spray drying. Nitrogen gas carrier also did not result in an improved spray drying outcomes, although resulted in powders of similar characteristics as when atmospheric air was the carrier. Therefore, at least for *L. casei* AMBR2 oxidative damage during spray drying is not the main cause of the viability loss. SM provided high long-term viability protection at refrigerated storage while it's

concentration evaluation revealed no need for very high solid amounts to protect bacteria effectively. Our research has once again highlighted the influence of storage conditions upon shelf-life probiotic viability. Although our results favour air spray drying, nitrogen can find its application for more oxygen sensitive microorganisms and their preservation.

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7. Conflict of interest

None.

8. References

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Figure 1 Device configuration, open system (A), closed system (B)

Table 1 The Hausner ratio as a scale of flowability; European Pharmacopoeia 9.8, 2.9.36 Powder Flow, 01/2010

Figure 2 Log reduction after spray drying of unprotected *L. casei* AMBR2 cells using air as a carrier: in an open system (AO), in a closed system (AC), in an open system with presence of vitamin C in feed suspension medium (AOC), in a closed system with presence of vitamin C in feed suspension medium (ACC); log reduction in viability after spray drying of unprotected cells using nitrogen as a gas carrier (N), and with vitamin C addition to feed suspension medium (NC). Stars (*) indicate the level of statistical significance (one-way ANOVA-test and Tukey's multiple comparisons test, $p<0.05$).

Figure 3 Comparison of log reduction of *L. casei* AMBR2 when dried in absence of protectants (0%), 1%, 10% and 20% (w/V) SM in both air and nitrogen (A) where stars indicate the level of statistical difference significance (two-way ANOVA-test and Tukey's multiple comparisons test, $p<0.05$). Powder characteristics of AMBR2 spray-dried formulations of unprotected cells (0%) and 1%, 10% and 20% SM dried in both, air and nitrogen gas stream (B), particle size distribution of all formulations (C), scanning electron micrographs of AMBR2 formulations (D) in absence of protectants dried in air (a) and in nitrogen (e), in 1% SM dried in air (b) and in nitrogen (f), in 10% SM dried in air (c) and in nitrogen (g), in 20% SM dried in air (d) and in nitrogen (h).

Figure 4 Shelf-life viability up to 52 weeks at room temperature with daylight exposure (A), at room temperature without daylight exposure (B) and at refrigerated conditions (C) of *L. casei* AMBR2 unprotected cells (0%) and protected with 1%, 10% and 20% (w/V) SM dried in air open system (air) and nitrogen system (nitrogen).

Supplement 1 Scanning electron micrographs of *L. casei* AMBR2 spray-dried powders after 28 weeks of shelf-life dried in air: unprotected cells kept at room temperature with daylight exposure (a1), at room temperature without daylight exposure (a2), at refrigerated conditions (a3), 1% SM formulation cells kept at room temperature with daylight exposure (b1), at room temperature without daylight exposure (b2), at refrigerated conditions (b3), 10% SM formulation cells kept at room temperature with daylight exposure (c1), at room temperature without daylight exposure (c2), at refrigerated conditions (c3), 20% SM formulation cells kept at room temperature with daylight exposure (d1), at room temperature without daylight exposure (d2), at refrigerated conditions (d3). Scanning electron micrographs of AMBR2 spray-dried powders after 28 weeks of shelf-life dried in nitrogen: unprotected cells kept at room temperature with daylight exposure (e1), at room temperature without daylight exposure (e2), at refrigerated conditions (e3), 1% SM formulation cells kept at room temperature with daylight exposure (f1), at room temperature without daylight exposure (f2), at refrigerated conditions (f3), 10% SM formulation cells kept at room temperature with daylight exposure (g1), at room temperature without daylight exposure (g2), at refrigerated conditions (g3), 20% SM formulation cells kept at room temperature with daylight exposure (h1), at room temperature without daylight exposure (h2), at refrigerated conditions (h3).