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**Critical water activity and amorphous state for optimal preservation of lyophilized
lactic acid bacteria**

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Abstract (150 words maxi)

The aim of this study was to investigate the influence of the water activity on the stability of lyophilized lactic acid bacteria, especially in the solid glassy region. *Lactobacillus bulgaricus* CFL1 was co-lyophilized with sucrose and stored under controlled relative humidity at 25°C. Glass transition temperature (Tg), water activity, water content and loss of specific acidification activity during storage were determined. The rates of bacteria degradation were analyzed as a function of water activity and as a function of the temperature difference between storage temperature and Tg. Above Tg, the degradation rate appeared related to the physical changes of the amorphous matrix. Below Tg, the optimal stability of the lyophilized bacteria was observed in the intermediate water activity range 0.1 – 0.214. An integrated analysis of the relationships between water activity, Tg, water content and biological activity appeared as a promising approach for optimizing the freeze-drying process and predicting the storage stability.

Keywords: water activity, lactic acid bacteria, freeze-drying, glass transition, storage stability, lyophilisation, residual moisture

1. Introduction

Lactic acid bacteria (LAB) are widely used as starters for manufacturing cheeses, fermented milks, meats, vegetables and breads products. Several species have been shown to exhibit probiotic properties i.e. positive effects on human health (Naidu et al., 1999). The preparation of starter cultures requires production and maintenance techniques that maximise viability, activity and storage stability of bacterial cells. While frozen concentrates of lactic acid bacteria exhibit maximal survival in liquid nitrogen, the expense of these storage conditions limits the use of this method. Freeze-drying (or lyophilisation) appears as an alternative method for long time preservation of bacteria and yeasts.

When lactic acid bacteria are used as components of commercial starters, they are often freeze-dried in the presence of sugars and embedded in amorphous matrices (Abadias et al., 2001; Carvalho et al., 2004; Castro et al., 1997; Champagne et al., 1991; Leslie et al., 1995; Meng et al., 2008). The bacteria are so stabilized against physical and/or chemical degradation during dehydration and storage (Santivarangkna et al., 2008). The stability of bacteria in an amorphous sugar matrix is considered to depend mainly on the following two factors: the sugar it self and the physical state of the matrix. The extent of the stabilizing effect of the sugar varies with the specific sugar used (Kurtmann et al., 2009b; Miao et al., 2008; Zayed & Roos, 2004; Zhao & Zhang, 2005). For instance, lactose is currently used as protective sugar through the addition of skim milk. However, lactose as a reducing sugar reacts with milk proteins, thus inducing Maillard reaction (nonenzymatic browning). Non-reducing disaccharides such as sucrose or trehalose are reported to be among the most effective protective molecules for freeze-drying bacteria (Conrad et al., 2000; Crowe et al., 1988; Crowe et al., 1996). The other factor affecting bacteria preservation is the physical stability of the amorphous sugar matrix: when an amorphous sugar is exposed to high temperature or high humidity above the glass transition, various properties of the materials

change resulting in subsequent loss of the stabilizing effect of the amorphous sugar (Crowe et al., 1998; Patist & Zoerb, 2005; Pikal, 1999; Slade & Levine, 1991; Sun & Davidson, 1998). The most important changes are an exponential increase of molecular mobility and decrease of viscosity, which govern time-dependent structural changes such as collapse, sugar crystallisation and diffusion-controlled chemical reactions such as nonenzymatic browning (Buera & Karel, 1995; Buera et al., 2005; Jouppila & Roos, 1994a; Roos, 2002).

State diagrams have been proposed to describe the different region of the physical state of material and associated with sorption isotherms have often been related to the dried product quality and used for predicting the product stability during processing and storage (Fonseca et al., 2001; Roos, 1995). For instance, the critical water content and water activity values leading to physical changes of the amorphous material may be identified and used for process and storage design. When considering biological products such as proteins or bacteria, the effect of water on the solid state stability of glassy systems is complex, since water can play not only the role of plasticizer in a degradation process but also the role of reactant and solvent. These different roles of water suggest that progressively greater stability should be observed at lower moisture contents. The empirical rule “the drier, the better” is commonly used for designing dehydration process. However, some exceptions have been reported for dried proteins, viruses, bacteria (Breen et al., 2001; Chang et al., 2005b; Croyle et al., 2001; Greiff, 1970; Hsu et al., 1992; Pikal et al., 1992; Scott, 1958; Zayed & Roos, 2004). Moreover, very few studies have investigated the effect of residual water content on the stability of dehydrated bacteria, especially in the glassy region.

Our objective was thus to deeper investigate the effect of moisture content and water availability on the stability of freeze-dried lactic acid bacteria and to propose a useful tool combining state diagram, sorption isotherm and bacterial biological activity for designing an optimal freeze-drying process.

2. Materials and methods

2.1. Production of lyophilized lactic acid bacteria

The lactic acid bacteria strain, *Lactobacillus delbrueckii* sbsp. *bulgaricus* CFL1, was obtained from the stock culture of the Laboratoire de Génie et Microbiologie des Procédés Alimentaires (INRA, Thiverval-Grignon, France) and used for all experiments. Inocula were stored at -80°C . Cultures were grown in supplemented whey medium (60 g/L whey, 20 g/L lactose, 5 g/L yeast extract) in a 2, 15 or 75 liters fermentor at 42°C . The pH was controlled at 5.5 by addition of 1.44 M NaOH. Cells were harvested by centrifugation ($17000\times g$, 30 min, 4°C) at the end of the exponential growth phase, when the NaOH consumption rate started to decrease. After an intermediate storage period for 30 minutes at 4°C , concentrated cells were re-suspended at 4°C in a 1:2 cells/protective medium ratio. The protective medium was composed of 200 g/L of sucrose and 0.15 M of NaCl. The final protected bacterial suspensions were aliquoted into 50 mm diameter stainless steel container (15 ml filled volume). The samples were frozen at -80°C in a cold air chamber and then transferred to a pre-cooled shelf at -50°C in a SMH 90 freeze-dryer (Usifroid, Maurepas, France). After a holding step of 1 hour at -50°C , the chamber pressure was decreased to 20 Pa and the shelf temperature was increased to -20°C at $0.25^{\circ}\text{C}/\text{min}$ to initiate the sublimation phase. After 40 hours of sublimation, the shelf temperature was increased to 25°C at $0.25^{\circ}\text{C}/\text{min}$ to initiate the desorption phase. After 10 hours of desorption, the vacuum was broken by injection of air and the samples were packed under vacuum in aluminium bags and stored at -80°C until their use for storage experiments. Five fermentations were performed to generate various batches of lyophilized lactic acid bacteria.

2.2. Storage experiments

The lyophilized sample of lactic acid bacteria were reduced in powder in a chamber of very low relative humidity (around 5%) and then put in the containers used for the measurement of water activity. The containers were placed in hermetic glass box containing P_2O_5 or saturated salt solutions with $a_w = 0.06$ (LiBr), $a_w = 0.11$ (LiCl), $a_w = 0.22$ (CH_3COOK), $a_w = 0.32$ ($MgCl_2 \cdot 6H_2O$), $a_w = 0.44$ (K_2CO_3), $a_w = 0.53$ ($Mg(NO_3)_2 \cdot 6H_2O$), $a_w = 0.75$ (NaCl), $a_w = 0.84$ (KCl). After one week of equilibration at 25°C, the samples reached a constant weight and were packed under vacuum in aluminium bags and stored at 25°C for different storage times. For each relative humidity condition, three samples were prepared: the first one was used for measuring water activity and water content, the second one for measuring water activity and glass transition temperature and the third one for measuring water activity and biological activity of lactic acid bacteria (viability and acidification activity).

2.3. Water activity and water content measurements

The moisture content of the samples was measured by the Karl Fisher titration method using a Metrohom KF 756 apparatus (Herisau, Switzerland). At least 20 mg of powder were mixed with 2 mL of dry methanol and titrated with Riedel-deHaen reagent (Seelze, Germany) until the end point was reached. The water activity of the samples was measured at 25°C using an a_w meter labMaster-aw (Novasina, Precisa, Poissy, France).

2.4. Glass transition temperature measurement

Differential scanning calorimetry (DSC) measurements were performed on two different power compensation DSC equipments (Perkin Elmer LLC, Norwalk, CT, USA) depending on the moisture content of the samples: a Pyris 1 equipped with a mechanical cooling system for the low moisture content samples exhibiting thermal events at the higher temperatures ($>0^\circ C$)

and a Diamond equipped with liquid nitrogen cooling accessory (CryoFill) for the high moisture content samples (lower temperatures). Temperature calibration was done using cyclohexane (crystal-crystal transition at -87.1°C), mercury (melting point at -38.6°C) and indium (melting point at 156°C) for the Diamond; and cyclohexane (melting point at 6.5°C), n-octadecane (melting point at 27.8°C) and indium for the Pyris 1. About 10 mg of each sample was placed in 50 μl Perkin Elmer DSC sealed aluminium pans. An empty pan was used as a reference. Linear cooling and heating rates of $10^{\circ}\text{C min}^{-1}$ were used. The characteristic glass transition temperature (T_g) of samples was determined as the midpoint temperature of the heat flow step associated with glass transition with respect to the ASTM Standard Method E 1356-91. Results were obtained from at least four replicates.

2.5 Biological activity measurement

The samples were rehydrated in skim milk to the initial dry matter of the protected bacterial suspension before freeze-drying. Viability of *Lactobacillus bulgaricus* CFL1 was determined by plate assays on MRS (Biokar Diagnostics, France) agar plates. The Petri dishes were incubated under anaerobic conditions (GENbox96124, BioMérieux, Marcy l'Etoile, France) at 42°C for 48 h before counting.

The acidification activity of 100- μl samples was measured in milk at 42°C , in triplicate, using the CINAC System (Corrieu et al., 1988). The time necessary to reach the maximum acidification rate in milk (t_m , in minutes) was used to characterise the acidification activity of the bacterial suspensions. The higher the t_m , the longer the latency phase and the lower the acidification activity. The acidification activity was measured after equilibration of the samples at various relative humidity conditions and after various time of storage at 25°C of the equilibrated samples.

3. Results and discussion

3.1. Sorption isotherm and glass transition of the lyophilized bacterial matrix

Figure 1 displays the glass transition temperature (T_g) and the water content of the lyophilized *Lb bulgaricus* CFL1 in sucrose matrix as a function of water activity (a_w) at 25°C. The T_g decreased with water absorption by the matrix. The decrease was linear as water activity increased from 0.1 to 0.7, which is typical of various amorphous foods (Roos & Karel, 1991; Roos, 1987). The relationship between water content and water activity was modelled using the well-known equations of Brunauer-Emmet-Tellet (BET) and Guggenheim-Anderson-de Boer (GAB):

$$\text{GAB equation: } m = \frac{M_M C_{G/B} K a_w}{(1 - a_w)(1 - K a_w + C_{G/B} K a_w)} \quad \text{Equation 1}$$

Where m is the water content (g/g or g/100g, in dry or wet solid), M_M is the monolayer water coverage (or the moisture content at fully occupied active sorption sites with one molecule of water), $C_{G/B}$ and K are adjustable parameters. The BET equation corresponds to the equation 1 with the parameter K equal to 1.

By using the sorption isotherm, it is possible to calculate the water content value for each experimental value of glass transition temperature. The Gordon and Taylor's equation was used to model data on T_g of the lyophilized bacterial matrix:

$$T_{gm} = \frac{X_w T_{gw} + k_{GT}(1 - X_w)T_{gs}}{X_w + k_{GT}(1 - X_w)} \quad \text{Equation 2}$$

Where T_{gm} , T_{gs} , and T_{gw} , are the glass transition temperatures (K) of the mixture, of the solids and the water, respectively, X_w is the mass fraction of water, and k_{GT} is a constant. The glass transition temperature of pure water was taken as $T_{gw} = -135^\circ\text{C}$.

The resulting parameters of the GAB, BET and Gordon and Taylor equations are reported in Table 1. The table was completed with data from literature works on bacteria, proteins and sugars.

Using the relationships between water activity, water content and glass transition temperature, the physical storage stability of the lyophilized product can be predicted. Referring to the critical T_g of 25°C, corresponding to storage at ambient temperature, the co-lyophilized matrix of *Lb bulgaricus* CFL1 and sucrose showed a critical value of water activity of 0.241 corresponding to a critical value of water content of 3.9%. This critical a_w value is in accordance with previous work reported on LAB freeze-dried in sugar matrix (around 0.25) and slightly higher than the critical a_w value of pure sucrose (0.235). This small effect of bacteria was previously observed by (Fonseca et al., 2001). The low value observed for the matrix LAB + sucrose + Md 12 (0.145) may be ascribed to the presence of maltodextrin resulting in changes in sorption properties.

The parameters, M_M and C_B , of the BET equation have both physical significance: the amount of water needed to achieve monolayer coverage and the energy term related to overall energy of absorption, respectively. For *Lb bulgaricus* CFL1 co-lyophilized with sucrose or fermented medium composed of various sugars, M_M tended to be lower than that expected value based on contributions of the pure bacteria and protective medium. This deviation suggests that the interaction of amorphous sugars and bacteria in the solid state reduces the availability of water-binding sites. The M_M value, lower than expected, may also be considered as evidence of the water replacement mechanism proposed for preservation of dehydrated biological systems: i.e. hydrogen bonding between the sugar and the biomolecules, especially the membrane proteins, when water is removed during drying (Carpenter & Crowe, 1989; Costantino et al., 1998; Crowe et al., 1988; Prestrelski et al., 1993). Furthermore, a number of physicochemical properties change at the monolayer water coverage: heat capacity, protein conformational state, etc (Lechuga-Ballesteros et al., 2002). The mobility of water is restricted below M_M and water molecules are tightly bound to others molecules (proteins, polymers, small solutes) at such hydration levels (Lechuga-Ballesteros et

al., 2002). It has been suggested that the onset of internal protein flexibility correlated well with the attainment of monolayer coverage of water (Hageman, 1992). Thus freeze-dried proteins might exhibit increased instability above the monolayer coverage, and therefore BET monolayer water coverage appears as a useful physical property for protein formulation development (Costantino et al., 1997, 1998). Some other studies have suggested that the optimal water content for stability corresponds to the water content needed for monolayer coverage of the available surface (Hsu et al., 1992; Karel & Labuza, 1967). Concerning lactic acid bacteria, it seems interesting to verify if the relationship between M_M and the optimal water content is the same as for proteins.

3.2. Effect of water activity on the acidification activity of freeze-dried bacteria

Figure 2 displays the evolution of the acidification activity characterized by the parameter t_m as a function of the water activity of the freeze-dried bacterial suspension just after a_w equilibration of the samples, and after 7, 10 and 29 days of storage at 25°C. The lower the t_m value, the higher the acidification activity. An inversed bell-shape curve was observed with a minimal t_m value, and thus a maximal acidification activity around a value of water activity of 0.2 whatever the storage time. As expected, the t_m value increased with the storage time and that increase appeared more pronounced for the high values of water activity. The degradation of the acidification activity can be ascribed to the cell death and/or to cell membrane damages leading to higher latency phase. In order to combine the viability and the acidification activity, the specific acidification activity (t_{spe} , in min/log(CFU/ml)) was defined as the ratio of t_m to the corresponding log of cell concentration (Streit et al., 2007). Figure 3 showed the evolution of the specific acidification activity (t_{spe}) with the storage time for three relative humidity conditions. Whatever the water activity of the samples, the parameter t_{spe} increased linearly with storage time according to the following relationship:

$$t_{spe} = k_{spe} \times \text{Storage time} + A \quad \text{Equation 3}$$

Where k_{spe} is the slope of the regression line (in (min/(log(CFU/ml)))/day or t_{spe} /day) and represents the rate of loss in specific acidification activity during storage. A higher slope indicated a faster decrease of the specific acidification activity and, consequently a lower resistance to storage under various relative humidity conditions. Previous works have already described the acidification activity loss with storage time as a linear relationship for frozen lactic acid concentrates (Fonseca et al., 2000; Streit et al., 2007). The rate constants of loss of specific acidification activity k_{spe} at storage temperature of 25°C are plotted as function of water activity in Figure 4. The water activity threshold between glassy and rubbery states as well as the values of the temperature difference between the storage temperature and the glass transition temperature ($T_{storage}-T_g$) are shown. Storage of the co-lyophilized matrix of *Lb bulgaricus* CFL1 and sucrose below T_g , where the molecular mobility is sharply reduced due to the very viscosity of the amorphous state, resulted in very low rates of loss of specific acidification activity, lower than 2 t_{spe} /day. The specific acidification activity loss rate did not sharply increase with increasing water activity, as would be expected given the plasticizing effect of water on T_g and thus on mobility. The acceleration of the degradation reactions starts at water activity higher than 0.33 and $T_{storage}-T_g$ higher than 10°C. Furthermore, for water activity around 0.5-0.6, the degradation rate tends to decrease. This unpredicted event can be ascribed to a physical change in the matrix, probably related to the sugar crystallization. After this event, the rate of loss of specific acidification activity sharply increases, probably due to the H- bonding breakage between the protecting sugar and cell biomolecules (like membrane proteins and phospholipids).

Many works have related the glass transition to the kinetics of diffusion-controlled chemical reaction such as Maillard reaction or nonenzymatic browning (NEB), a very important chemical reaction in foods (Bhandari & Howes, 1999; Buera & Karel, 1995;

Karmas et al., 1992; Lievonen et al., 2002; Roos & Himberg, 1994; Schebor et al., 1999). Even if a non reducing sugar (sucrose) was used in the protective medium added to the *Lb bulgaricus* CFL1 suspension before freeze-drying, some browning of the powder was observed for the a_w values higher than 0.5. That browning could be ascribed to the residual fermented medium containing reducing sugars (lactose, glucose, galactose) in the concentrated bacterial suspension and/or to the hydrolysis of sucrose in acidic conditions. Several authors have investigated the Maillard reaction rate in milk powders or dehydrated model systems and have shown relationships between the reaction rates and the physical state of the amorphous matrix (Buera & Karel, 1995; Karmas et al., 1992; Pereyra Gonzales et al., 2010; Schebor et al., 1999). A large increase in the nonenzymatic browning rate was reported at a range of 2°C to 40°C above T_g (Karmas et al., 1992; Lievonen et al., 1998; Pereyra Gonzales et al., 2010; Roos et al., 1996). Water plasticization increases molecular mobility, which may also result in the conversion of sugars such as sucrose and lactose from the amorphous state to the crystalline state (Roos & Karel, 1992). In closed systems, disaccharides crystallization will induce an increase in a_w due to the release of water from amorphous sugar, thus accelerating deteriorative changes such as NEB (Jouppila & Roos, 1994a; Vuataz, 2002). Above glass transition temperature, the nonenzymatic browning rate in model systems appeared to be influenced by the temperature difference ($T-T_g$) (Buera & Karel, 1995). Furthermore, nonenzymatic browning has been showed to proceed at the slow rate even well below the glass transition temperature (Karmas et al., 1992; Lievonen et al., 1998; Roos & Himberg, 1994; Schebor et al., 1999), which could explain the small losses of specific acidification activity observed at low moisture content ($T < T_g$).

The inactivation of freeze-dried lactic acid bacteria during storage almost certainly resulted not only from the nonenzymatic browning but also from other complex chemical reactions such as oxidation, protein denaturation etc (Kurtmann et al., 2009a; Lai & Topp,

1999; Teixeira et al., 1996). The mechanism of diffusion limited chemical reaction associated to the glassy state does not allow to wholly explain the complex bacteria inactivation behavior. The various works on freeze-dried lactic acid bacteria revealed that the bacteria inactivation rate increased with water content and that storage of the samples in the glassy state led to a better survival of bacteria (Higl et al., 2007; Kurtmann et al., 2009b; Pehkonen et al., 2008; Schoug et al., 2010; Selma et al., 2007). However, there is no common acceptance to identify the glass transition temperature as a stability threshold. Some authors reported the acceleration of the degradation rate for temperature lower than T_g and others authors for temperature well above the T_g . Furthermore, the inactivation rate appears to depend on the strain of the lactic acid bacteria and on the composition of the protective medium. For instance, the inactivation rate of bacteria freeze-dried in a lactose matrix was reported higher than the inactivation rate of bacteria freeze-dried in a sucrose matrix (Kurtmann et al., 2009b).

Among the various works investigating the stability of freeze-dried lactic acid bacteria (Higl et al., 2007; Kurtmann et al., 2009b; Pehkonen et al., 2008; Schoug et al., 2010; Selma et al., 2007; Zayed & Roos, 2004), very few have focused on the study of the inactivation rate at low moisture content and below T_g . Figure 5 displays the relationships between water activity, water content, glass transition temperature and the rate of specific acidification activity loss. Additional sets of experimental data on bacteria stability have been included. The three batches of freeze-dried bacteria differed in the physiological state of the bacteria obtained after the fermentation process. The values of a_w corresponding to the monolayer water coverage (M_M) and to the threshold between glassy and rubbery states ($T_{\text{storage}} - T_g = 0$) are reported in the figure. Whatever the batch, very low rates of loss of specific acidification activity were observed for water activities lower than the a_w value corresponding to the monolayer water coverage M_M ($a_w = 0.214$) and the rates tended to increase for a_w value lower than 0.1. The optimal range of water activity and water content for *Lb bulgaricus* CFL1

freeze-dried in sucrose matrix appears to be 0.1 – 0.241 and 2.5 – 3.7%, respectively. For values of a_w comprised between 0.214 (corresponding to M_M) and 0.241 (corresponding to $T_{\text{storage}} - T_g = 0^\circ\text{C}$), the rate of loss of specific acidification activity slightly increased. And the increase of loss rate became more pronounced for a_w values higher than 0.241. The mobility of water is restricted below M_M since water molecules are tightly bound to biomolecules surface at such hydration levels. Moreover, at constant temperature, the water mobility above M_M and below the amount of water required to depress T_g to the storage temperature is increased, but remains lower than the mobility in rubbery state.

Research works mentioning optimal storage stability at intermediate moisture content for freeze-dried biological product such as protein, viruses, gene vectors, DNA lipoplex formulation and lactic acid bacteria (*Lb bulgaricus*, *Lb rhamnosus* and *Lb salivarius*) are rare but do exist (Breen et al., 2001; Chang et al., 2005b; Croyle et al., 2001; Greiff, 1970; Hsu et al., 1992; Pehkonen et al., 2008; Pikal et al., 1992; Scott, 1958; Teixeira et al., 1995; Yu & Anchordoquy, 2009; Zayed & Roos, 2004). Teixeira et al., 1995 also reported greatest survival rate for *Lb bulgaricus* spray dried with skim milk stored at 4°C and 20°C for a_w values of 0.11 and 0.23, respectively. According to Chang et al., 2005a, optimal stability at intermediate water content appears to support the water substitute mechanism for protein stabilization. That is, the water substitute concept states that the hydrogen bonding between water and protein is critical to the thermodynamic stability of protein. At low to intermediate water level, the water may be binding to the hydrogen-bonding sites on the surface of protein which have not been occupied by the sugars. Therefore the stability can be improved with the addition of small amount of water. Furthermore, no antioxidant was added to the *Lb bulgaricus* CFL1 concentrated suspension before freeze-drying. In very dry formulations, different oxidation pathways appear to dominate protein and lipid degradation (Yu & Anchordoquy, 2009). The rate of oxidation is observed to have a minimum at the monolayer

hydration level, and to increase at lower and higher water contents (Labuza, 1980; Lai & Topp, 1999; Pikal et al., 1991). This antioxidant effect of water has been ascribed to its interaction with functional groups, which blocks these reaction sites, thereby preventing them from interacting with oxygen (Lechuga-Ballesteros et al., 2002). The losses of specific acidification activity observed at low a_w values may then be ascribed to oxidative membrane mechanisms (Kurtmann et al., 2009a; Teixeira et al., 1996).

4. Conclusion:

The stability of lactic acid bacteria in a glass or rubbery sucrose matrix at different water activity environments was analyzed. The physical properties of the matrix were determined by means of state diagram and sorption isotherm. The Brunauer-Emmett-Teller (BET) equation was used to describe the sorption properties and to determine the monolayer water coverage M_M , reported as the optimal value of water content for product stability. When plotting the loss rate of specific acidification activity of *Lb bulgaricus* CFL1 as a function of water activity and positioning the a_w values corresponding to the M_M parameter ($a_w(M_M)$) and to $T_{\text{storage}} - T_g = 0^\circ\text{C}$ ($a_w(T_g)$), the $a_w(M_M)$ appears as a threshold value for bacteria stability. Above $a_w(M_M)$, the degradation rate slightly increased and this increased was more pronounced above $a_w(T_g)$, attributable to the physical changes of the matrix. Furthermore, a slight increase of the degradation rate was also observed for very low value of a_w (<0.1), probably caused by oxidative mechanisms and slow but still present Maillard reaction.

Our experimental results and especially the very low value of M_M compared to the value of the pure sugar provide some evidences of a protective mechanism of sucrose: direct interaction with the bacteria by establishing hydrogen bonding with the membrane proteins and/or the lipid bilayer. Further studies are in progress to verify this mechanism and also to generalize this approach with other protective medium including polymers or

polysaccharides. The concept of monolayer water coverage provides an interesting framework for describing effects of water on the stability of glassy solids and is useful in the development of freeze-dried biological products. Overdrying may be detrimental to the stability of bacteria in the dried state, even when formulated with disaccharides. Combining the relationships between water activity and glass transition temperature, water content and biological activity appeared as a promising approach allowing a rational optimization of the freeze drying process and the prediction of storage stability of lactic acid bacteria.

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6. References

- Abadias, M., Benabarre, A., Teixido, N., Usall, J., & Vinas, I. (2001). Effect of freeze drying and protectants on viability of the biocontrol yeast *Candida sake*. *International Journal of Food Microbiology*, 65, 3, 173-182.
- Bhandari, B. R., & Howes, T. (1999). Implication of glass transition for the drying and stability of dried foods. *Journal of Food Engineering*, 40, 71-79.
- Breen, A. D., Curley, J. G., Overcashier, D. E., Hsu, C. C., & Shire, S. J. (2001). Effect of moisture on the stability of a lyophilized humanized monoclonal antibody formulation. *Pharmaceutical Research*, 18, 9, 1345-1353.
- Buera, M. P., & Karel, M. (1995). Effect of physical changes on the rates of nonenzymic browning and related reactions. *Food Chemistry*, 52, 167-173.
- Buera, P., Schebor, C., & Elizalde, B. (2005). Effects of carbohydrate crystallization on stability of dehydrated foods and ingredient formulations. *Journal of Food Engineering*, 67, 1-2, 157-165.
- Carpenter, J. F., & Crowe, J. H. (1989). An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. *Biochemistry*, 28, 9, 3916-3922.
- Carvalho, A. S., Silva, J., Ho, P., Teixeira, P., Malcata, F. X., & Gibbs, P. (2004). Relevant factors for the preparation of freeze-dried lactic acid bacteria. *International Dairy Journal*, 14, 10, 835-847.
- Castro, H. P., Teixeira, P. M., & Kirby, R. (1997). Evidence of membrane damage in *Lactobacillus bulgaricus* following freeze-drying. *Journal of Applied Microbiology*, 82, 87-94.
- Champagne, C. P., Gardner, N., Brochu, E., & Beaulieu, Y. (1991). The freeze-drying of lactic acid bacteria. A review. *Canadian Institute of Food Science and Technology*, 24, 118-128.

393 Chang, L. Q., Shepherd, D., Sun, J., Ouellette, D., Grant, K. L., Tang, X. L., et al. (2005a).
 394 Mechanism of protein stabilization by sugars during freeze-drying and storage: Native
 395 structure preservation, specific interaction, and/or immobilization in a glassy matrix? *Journal*
 396 *of Pharmaceutical Sciences*, 94, 7, 1427-1444.

397 Chang, L. Q., Shepherd, D., Sun, J., Tang, X. L., & Pikal, M. J. (2005b). Effect of sorbitol
 398 and residual moisture on the stability of lyophilized antibodies: Implications for the
 399 mechanism of protein stabilization in the solid state. *Journal of Pharmaceutical Sciences*, 94,
 400 7, 1445-1455.

401 Conrad, P. B., Miller, D. P., Cielenski, P. R., & de Pablo, J. J. (2000). Stabilization and
 402 preservation of *Lactobacillus acidophilus* in saccharide matrices. *Cryobiology*, 41, 1, 17-24.

403 Corrieu, G., Spinnler, H. E., Picque, D., & Jomier, Y. (1988). Automated system to follow up
 404 and control the acidification activity of lactic acid starters. France: Institut National de la
 405 Recherche Agronomique.

406 Costantino, H. R., Curley, J. G., & Hsu, C. C. (1997). Determining the water sorption
 407 monolayer of lyophilized pharmaceutical proteins. *Journal of Pharmaceutical Sciences*, 86,
 408 12, 1390-1393.

409 Costantino, H. R., Curley, J. G., Wu, S., & Hsu, C. C. (1998). Water sorption behavior of
 410 lyophilized protein-sugar systems and implications for solid-state interactions. *International*
 411 *Journal of Pharmaceutics*, 166, 2, 211-221.

412 Crowe, J. H., Carpenter, J. F., & Crowe, L. M. (1998). The role of vitrification in
 413 anhydrobiosis. *Annual Review of Physiology*, 60, 73-103.

414 Crowe, J. H., Crowe, L. M., Carpenter, J. F., Rudolph, A. S., Wistrom, C. A., Spargo, B. J., et
 415 al. (1988). Interactions of sugars with membranes. *Biochimica et Biophysica Acta*, 947, 367-
 416 384.

417 Crowe, J. H., Ried, D. S., & Crowe, L. M. (1996). Is trehalose special for preserving dry
 418 biomaterials? *Biophysical Journal*, 71, 2087-2093.

419 Croyle, M., Cheng, X., & Wilson, J. (2001). Development of formulations that enhance
 420 physical stability of viral vectors for gene therapy. *Gene Therapy*, 8, 1281-1290.

421 Fonseca, F., Beal, C., & Corrieu, G. (2000). Method of quantifying the loss of acidification
 422 activity of lactic acid starters during freezing and frozen storage. *Journal of Dairy Research*,
 423 67, 83-90.

424 Fonseca, F., Obert, J. P., Béal, C., & Marin, M. (2001). State diagrams and sorption isotherms
 425 of bacterial suspensions and fermented medium. *Thermochimica Acta*, 366, 167-182.

426 Greiff, D. (1970). Stabilities of suspensions of influenza virus dried by sublimation of ice in
 427 vacuo to different contents of residual moisture and sealed under different gases. *Applied*
 428 *Microbiology*, 20, 935-938.

429 Hageman, M. J. (1992). Water sorption and solid-state stability of proteins. In T. J. Ahern &
 430 M. C. Manning (Eds.), *Stability of protein pharmaceuticals. Part A. Chemical and physical*
 431 *pathways of protein degradation* (pp. 273-309). New York: Plenum.

432 Higl, B., Kurtmann, L., Carlsen, C. U., Ratjen, J., & Först, P. (2007). Impact of water activity
 433 and physical state on the storage stability of *Lactobacillus paracasei* ssp *paracasei* freeze-
 434 dried in a lactose matrix. *Biotechnology Progress*, 23, 4, 794-800.

435 Hsu, C. C., Ward, C. A., Pearlman, R., Nguyen, H. M., Yeung, D. A., & Curley, J. G. (1992).
 436 Determining the optimum residual moisture in lyophilized protein pharmaceuticals.
 437 *Development in Biologicals Standardization*, 74, 255-271.

438 Jouppila, K., & Roos, Y. H. (1994a). Glass transitions and crystallization in milk powders.
 439 *Journal of Dairy Science*, 77, 2907-2915.

440 Jouppila, K., & Roos, Y. H. (1994b). Water sorption and time-dependent phenomena of milk
 441 powders. *Journal of Dairy Science*, 77, 1798-1808.

442 Karel, M., & Labuza, T. P. (1967). Chemical changes in freeze-dried foods and models
443 systems. *Cryobiology*, 3, 288-296.

444 Karmas, R., Buera, M. P., & Karel, M. (1992). Effect of glass transition on rates of
445 nonenzymatic browning in food systems. *Journal of Agricultural and Food Chemistry*, 40,
446 873-879.

447 Kurtmann, L., Carlsen, C. U., Risbo, J., & Skibsted, L. H. (2009a). Storage stability of freeze-
448 dried *Lactobacillus acidophilus* (La-5) in relation to water activity and presence of oxygen
449 and ascorbate. *Cryobiology*, 58, 175-180.

450 Kurtmann, L., Carlsen, C. U., Skibsted, L. H., & Risbo, J. (2009b). Water activity-
451 temperature state diagram of freeze-dried *Lactobacillus acidophilus* (La-5): Influence of
452 physical state on bacterial survival during storage. *Biotechnology Progress*, 25, 1, 265-270.

453 Labuza, T. (1980). The effect of water activity on reaction kinetics of food deterioration.
454 *Food Technology*, 34, 4, 36-41, 59.

455 Lai, M. C., & Topp, E. M. (1999). Solid-state chemical stability of proteins and peptides.
456 *Journal of Pharmaceutical Sciences*, 88, 5, 489-500.

457 Lechuga-Ballesteros, D., Miller, D. P., & Zhang, J. (2002). Residual water in amorphous
458 solids: Measurements and effects on stability. In H. Levine (Ed.), *Progress in Amorphous*
459 *Food and Pharmaceutical Systems*. London: The Royal Society of Chemistry.

460 Leslie, S. B., Israeli, E., Lighthart, B., Crowe, J. H., & Crowe, L. M. (1995). Trehalose and
461 sucrose protect both membranes and proteins in intact bacteria during drying. *Applied and*
462 *Environmental Microbiology*, 61, 10, 3592-3597.

463 Lievonen, S. M., Laaksonen, T. J., & Roos, Y. H. (1998). Glass transition and reaction rates:
464 nonenzymatic browning in glassy and liquid systems. *Journal of Agricultural and Food*
465 *Chemistry*, 46, 2778-2784.

466 Lievonen, S. M., Laaksonen, T. J., & Roos, Y. H. (2002). Nonenzymatic Browning in Food
 467 Models in the Vicinity of the Glass Transition: Effects of Fructose, Glucose, and Xylose as
 468 Reducing Sugar. *Journal of Agricultural and Food Chemistry*, 50, 24, 7034-7041.

469 Meng, X. C., Stanton, C., Fitzgerald, G. F., Daly, C., & Ross, R. P. (2008). Anhydrobiotics:
 470 The challenges of drying probiotic cultures. *Food Chemistry*, 106, 4, 1406-1416.

471 Miao, S., Mills, S., Stanton, C., Fitzgerald, G. F., Roos, Y. H., & Ross, R. P. (2008). Effect of
 472 disaccharides on survival during storage of freeze-dried probiotics. *Dairy Science and*
 473 *Technology*, 88, 19-30.

474 Naidu, A. S., Bidlack, W. R., & Clemens, R. A. (1999). Probiotic spectra of lactic acid
 475 bacteria (LAB). *Critical Reviews in Food Science and Nutrition*, 39, 13-126.

476 Patist, A., & Zoerb, H. (2005). Preservation mechanisms of trehalose in food and biosystems.
 477 *Colloids and Surfaces B: Biointerfaces*, 40, 2, 107-113.

478 Pehkonen, K. S., Roos, Y. H., Miao, R. P., Ross, R. P., & Stanton, C. (2008). State transitions
 479 and physicochemical aspects of cryoprotection and stabilization in freeze-drying of
 480 *Lactobacillus rhamnosus* GG (LGG). *Journal of Applied Microbiology*, 104, 1732-1743.

481 Pereyra Gonzales, A. S., Naranjo, G. B., Leiva, G. E., & Malec, L. S. (2010). Maillard
 482 reaction kinetics in milk powder: Effect of water activity at mild temperatures. *International*
 483 *Dairy Journal*, 20, 40-45.

484 Pikal, M. J. (1999). Mechanisms of protein stabilization during freeze-drying and storage :
 485 The relative importance of thermodynamic stabilization and glassy state relaxation dynamics.
 486 In L. Rey & J. C. May (Eds.), *Freeze-drying / Lyophilisation of pharmaceutical and*
 487 *biological products* (Vol. 96, pp. 161-198). New York: Marcel Dekker.

488 Pikal, M. J., Dellerman, K., & Roy, M. L. (1992). Formulation and stability of freeze-dried
 489 proteins: Effects of moisture and oxygen on the stability of freeze-dried formulations of
 490 human growth hormone. *Development in Biologicals Standardization*, 74, 21-38.

491 Pikal, M. J., Dellerman, K. M., Roy, M. L., & Riggin, R. M. (1991). The effects of
 492 formulation variables on the stability of freeze-dried human growth hormone. *Pharmaceutical*
 493 *Research*, 8, 4, 427-436.

494 Prestrelski, S. J., Tedeschi, N., Arakawa, T., & Carpenter, J. F. (1993). Dehydration-induced
 495 conformational transitions in proteins and their inhibition by stabilizers. *Biophysical Journal*,
 496 65, 2, 661-671.

497 Roos, Y. (1995). Characterization of food polymers using state diagrams. *Journal of Food*
 498 *Engineering*, 24, 339-360.

499 Roos, Y., Jouppila, K., & Zielasko, B. (1996). Non-enzymatic browning-induced water
 500 plasticization. Glass transition temperature depression and reaction kinetics determination
 501 using DSC. *Journal of Thermal Analysis*, 47, 1437–1450.

502 Roos, Y., & Karel, M. (1991). Phase transitions of mixtures of amorphous polysaccharides
 503 and sugars. *Biotechnology Progress*, 7, 1, 49-53.

504 Roos, Y., & Karel, M. (1992). Crystallization of amorphous lactose. *Journal of Food Science*,
 505 57, 775–777.

506 Roos, Y. H. (1987). Effect of moisture on the thermal behavior of strawberries studied using
 507 differential scanning calorimetry. *Journal of Food Science*, 52, 146-149.

508 Roos, Y. H. (1993). Water activity and physical state effects on amorphous food stability.
 509 *Journal of Food Processing and Preservation*, 16, 6, 433-447.

510 Roos, Y. H. (1997). Frozen state transitions in relation to freeze drying. *Journal of Thermal*
 511 *analysis*, 48, 535-544.

512 Roos, Y. H. (2002). Importance of glass transition and water activity to spray drying and
 513 stability of dairy powders. *Lait*, 82, 4, 475-484.

514 Roos, Y. H., & Himberg, M. J. (1994). Nonenzymatic browning behavior, as related to glass
 515 transition, of a food model at chilling temperatures. *Journal of Agricultural and Food*
 516 *Chemistry*, 42, 893-898.

517 Santivarangkna, C., Higl, B., & Foerst, P. (2008). Protection mechanisms of sugars during
 518 different stages of preparation process of dried lactic acid starter cultures. *Food Microbiology*
 519 25, 429–441.

520 Schebor, C., Buera, M. P., Karel, M., & Chirife, J. (1999). Color formation due to non-
 521 enzymatic browning in amorphous, glassy, anhydrous, model systems. *Food Chemistry* 65
 522 (1999) 427±432, 65, 427-432.

523 Schoug, A., Mahlin, D., Jonson, M., & Hakansson, S. (2010). Differential effects of polymers
 524 PVP90 and Ficoll400 on storage stability and viability of *Lactobacillus coryniformis* Si3
 525 freeze-dried in sucrose. *Journal of Applied Microbiology*, 108, 1032-1040.

526 Scott, W. J. (1958). The effect of residual water on the survival of dried bacteria during
 527 storage. *Journal of General Microbiology*, 19, 624-633.

528 Selma, M. V., MacNaughtan, W., Mitchell, J., & Waites, W. (2007). Optimisation of
 529 production and storage stability of the starter bacteria *Streptococcus thermophilus* and
 530 *Lactobacillus plantarum*. *Journal of the Science of Food and Agriculture*, 87, 5, 765-772.

531 Slade, L., & Levine, H. (1991). Beyond water activity: recent advances based on an
 532 alternative approach to the assessment of food quality and food safety. *Critical Review in*
 533 *Food Science and Nutrition*, 30, 115-360.

534 Streit, F., Corrieu, G., & Beal, C. (2007). Acidification improves cryotolerance of
 535 *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1. *Journal of Biotechnology*, 128, 659–667.

536 Sun, W. Q., & Davidson, P. (1998). Protein inactivation in amorphous sucrose and trehalose
 537 matrices : Effects of phase separation and crystallization. *Biochimica et Biophysica Acta*,
 538 1425, 235-244.

539 Teixeira, P., Castro, H., & Kirby, R. (1996). Evidence of membrane lipid oxidation of spray-
540 dried *Lactobacillus bulgaricus* during storage. *Letters in Applied Microbiology*, 22, 34–38.

541 Teixeira, P. C., Castro, M. H., Malcata, F. X., & Kirby, R. M. (1995). Survival of
542 *Lactobacillus delbrueckii* ss *bulgaricus* following spray-drying. *Journal of Dairy Science*, 78,
543 5, 1025-1031.

544 Teng, C. D., Zarrintan, M. H., & Groves, M. J. (1991). Water vapor adsorption and desorption
545 isotherms of biologically active proteins. *Pharmaceutical Research*, 8, 2, 191-195.

546 Vuataz, G. (2002). The phase diagram of milk: a new tool for optimising. *Lait*, 82, 485–500.

547 Yu, J. X., & Anchordoquy, T. J. (2009). Effects of Moisture Content on the Storage Stability
548 of Dried Lipoplex Formulations. *Journal of Pharmaceutical Sciences*, 98, 9, 3278-3289.

549 Zayed, G., & Roos, Y. H. (2004). Influence of trehalose and moisture content on survival of
550 *Lactobacillus salivarius* subjected to freeze-drying and storage. *Process Biochemistry*, 39, 9,
551 1081-1086.

552 Zhang, J., & Zografi, G. (2000). The relationship between "BET" and "free volume"-derived
553 parameters for water vapor absorption into amorphous solids. *Journal of Pharmaceutical*
554 *Sciences*, 89, 8, 1063-1072.

555 Zhao, G., & Zhang, G. (2005). Effect of protective agents, freezing temperature, rehydration
556 media on viability of malolactic bacteria subjected to freeze-drying. *Journal of Applied*
557 *Microbiology*, 99, 2, 333-338.

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562 **Table captions**

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564 **Table 1.** Estimated values of the parameters of the BET, GAB and Gordon and Taylor
565 equations for the concentrated suspension *Lactobacillus bulgaricus* CFL1 lyophilized in a
566 sucrose matrix and for selected bacterial suspensions and pure solutes.

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Figure Captions

Fig. 1. Relationships between glass transition temperature (T_g), water activity (a_w) and water content (m) for bacterial suspension freeze-dried in a sucrose matrix. Lines indicate the location of critical T_g , a_w and m values at 25°C.

Fig. 2. Acidification activity (t_m) of lyophilized *Lactobacillus bulgaricus* CFL1 in a sucrose matrix as a function of water activity for different storage times (t_s) at 25°C.

Fig. 3. Specific acidification activity (t_{spe}) of lyophilized *Lactobacillus bulgaricus* CFL1 in a sucrose matrix as a function of storage time (t_s) at 25°C for different values of water activity (0.177; 0.326; 0.551). k_{spe} : rate of loss of specific acidification activity during storage at 25°C ((min/(log(CFU/ml)))/day); $t_{spe} = 0.2 \times t_s + 40.7$ ($a_w = 0.177$); $t_{spe} = 1.5 \times t_s + 46.4$ ($a_w = 0.326$); $t_{spe} = 8.1 \times t_s + 77$ ($a_w = 0.551$).

Fig.4. Rate of loss of specific acidification activity during storage at 25°C of lyophilized *Lactobacillus bulgaricus* CFL1 in a sucrose matrix (k_{spe} , in (min/(log(CFU/ml)))/day) as a function of water activity (a_w). A vertical line indicates the threshold value of a_w between the glassy and the rubbery states. In bold under the x axis, are reported the values of the temperature difference $T - T_g$, (with $T = 25^\circ\text{C}$) corresponding to the a_w values.

Fig. 5. Relationships between rate of loss of specific acidification activity during storage at 25°C (k_{spe} , in (min/(log(CFU/ml)))/day), glass transition temperature (T_g), water activity (a_w) and water content (m) for bacterial suspension freeze-dried in a sucrose matrix.

594 Vertical/horizontal dotted lines indicate the threshold value of a_w between the glassy and the
595 rubbery states, as well as the corresponding values of T_g and m . Vertical/horizontal bold grey
596 lines indicate the values of a_w , T_g and m corresponding to the water monolayer coverage M_M
597 (estimated from the BET equation). In bold under the x axis, are reported the values of the
598 temperature difference $T-T_g$, (with $T = 25^\circ\text{C}$) corresponding to the a_w values.
599

Table 1

	BET		GAB			a _w critical (T _g = 25°C)	Gordon Taylor		References
	M _M	C _B	M _M	C _G	K		T _{gs}	k _{GT}	
LAB ^a + sucrose	3.67	13.53	4.87	3.80	0.97	0.241	66.3	7.6	This work
LAB ^a + fermented medium			10.6	0.682	1.005	0.24	33.6	4.5	(Fonseca et al., 2001)
Fermented medium			15.8	0.724	0.998	0.14	33.7	4.6	
LAB ^b			8.7						(Selma et al., 2007)
LAB ^b + M17 broth			11.8			0.083	50		
LAB ^b + M17 broth + protective medium			5.6			0.250	64		
LAB ^b + M17 broth + protective medium + gelatine			6.9			0.283	83		
LAB ^c + lactose						0.26			(Higl et al., 2007)
LAB ^d + sucrose + MD 12						0.145			(Kurtmann et al., 2009b)
LAB ^d + lactose + MD 12						0.228			
Glucose	5.4	0.3					31/36	4.52	(Zhang & Zografis, 2000)
Dextran	6.2	13.5					200		
Trehalose	6.4	5.0					115		
Starch	6.6	17.9					225		
Sucrose	6-7*					0.236	62	5.42	(Roos, 1993, 1997)
Lactose	6.29	3.55	4.91	4.33	1.18	0.37	97	6.7	(Jouppila & Roos, 1994a, , 1994b)
Skim milk	5.47	11.30	5.10	12.11	1.08	0.37	92	6.7	
Isolated Soy	3.5								(Teng et al., 1991)
Lipase	5.1								(Costantino et al., 1998)
Protein	5-7								
Protein + trehalose	5								

^a *Lactobacillus delbrueckii* ssp. *bulgaricus* CFL1

^b *Streptococcus thermophilus* S. Bo1 ; the protective mesium was composed of skim milk, sucrose and L-ascorbic acid.

^c *Lactobacillus paracasei* ssp. *paracasei* (F19)

^d *Lactobacillus acidophilus* (La-5), MD 12: maltodextrin with a dextrose equivalent of 12.

* Estimated from (Costantino et al., 1998)

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5