Critical water activity and amorphous state for optimal preservation of lyophilised 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×*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 °C/min to initiate the sublimation phase. After 40 hours of sublimation, the shelf temperature was increased to 25°C at 0.25 °C/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

105 The lyophilized sample of lactic acid bacteria were reduced in powder in a chamber of
106 very low relative humidity (around 5%) and then put in the containers used for the
107 measurement of water activity. The containers were placed in hermetic glass box containing
108 P_2O_5 or saturated salt solutions with a_w = 0.06 (LiBr), a_w = 0.11 (LiCl), a_w = 0.22
109 (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 =
110 0.75 (NaCl), a_w = 0.84 (KCl). After one week of equilibration at 25°C, the samples reached a
111 constant weight and were packed under vacuum in aluminium bags and stored at 25°C for
112 different storage times. For each relative humidity condition, three samples were prepared: the
113 first one was used for measuring water activity and water content, the second one for
114 measuring water activity and glass transition temperature and the third one for measuring
115 water activity and biological activity of lactic acid bacteria (viability and acidification
116 activity).
117
118 *2.3. Water activity and water content measurements*
119 The moisture content of the samples was measured by the Karl Fisher titration method
120 using a Metrohom KF 756 apparatus (Herisau, Switzerland). At least 20 mg of powder were
121 mixed with 2 mL of dry methanol and titrated with Riedel-deHaen reagent (Seelze, Germany)
122 until the end point was reached. The water activity of the samples was measured at 25°C
123 using an a_w meter labMaster-aw (Novasina, Precisa, Poissy, France).
124
125 *2.4. Glass transition temperature measurement*
126 Differential scanning calorimetry (DSC) measurements were performed on two different
127 power compensation DSC equipments (Perkin Elmer LLC, Norwalk, CT, USA) depending on
128 the moisture content of the samples: a Pyris 1 equipped with a mechanical cooling system for
129 the low moisture content samples exhibiting thermal events at the higher temperatures (>0°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-octodecane (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°C min^{-1} were used. The characteristic glass transition temperature (Tg) 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 (Tg) 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 Tg 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):

GAB equation: $$m = \frac{M_M C_{G/B} K a_w}{(1 - a_w)(1 - K a_w + C_{G/B} K a_w)}$$ 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 Tg of the lyophilized bacterial matrix:

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

Where Tg_m, Tg_s, and Tg_w, 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 $Tg_w = -135$°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.

179 Using the relationships between water activity, water content and glass transition
180 temperature, the physical storage stability of the lyophilized product can be predicted.
181 Referring to the critical Tg of 25°C, corresponding to storage at ambient temperature, the co-
182 lyophilized matrix of *Lb bulgaricus* CFL1 and sucrose showed a critical value of water
183 activity of 0.241 corresponding to a critical value of water content of 3.9%. This critical a_w
184 value is in accordance with previous work reported on LAB freeze-dried in sugar matrix
185 (around 0.25) and slightly higher that the critical a_w value of pure sucrose (0.235). This small
186 effect of bacteria was previously observed by (Fonseca et al., 2001). The low value observed
187 for the matrix LAB + sucrose + Md 12 (0.145) may be ascribed to the presence of
188 maltodextrin resulting in changes in sorption properties.
189 The parameters, M_M and C_B, of the BET equation have both physical significance: the
190 amount of water needed to achieve monolayer coverage and the energy term related to overall
191 energy of absorption, respectively. For *Lb bulgaricus* CFL1 co-lyophilized with sucrose or
192 fermented medium composed of various sugars, M_M tended to be lower than that expected
193 value based on contributions of the pure bacteria and protective medium. This deviation
194 suggests that the interaction of amorphous sugars and bacteria in the solid state reduces the
195 availability of water-binding sites. The M_M value, lower than expected, may also be
196 considered as evidence of the water replacement mechanism proposed for preservation of
197 dehydrated biological systems: i.e. hydrogen bonding between the sugar and the
198 biomolecules, especially the membrane proteins, when water is removed during drying
199 (Carpenter & Crowe, 1989; Costantino et al., 1998; Crowe et al., 1988; Prestrelski et al.,
200 1993). Furthermore, a number of physicochemical properties change at the monolayer water
201 coverage: heat capacity, protein conformational state, etc (Lechuga-Ballesteros et al., 2002).
202 The mobility of water is restricted below M_M and water molecules are tightly bound to others
203 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:

229 $t_{spe} = k_{spe} \times \text{Storage time} + A$ Equation 3

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

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

254 Karmas et al., 1992; Lievonen et al., 2002; Roos & Himberg, 1994; Schebor et al., 1999).
255 Even if a non reducing sugar (sucrose) was used in the protective medium added to the *Lb*
256 *bulgaricus* CFL1 suspension before freeze-drying, some browning of the powder was
257 observed for the a_w values higher than 0.5. That browning could be ascribed to the residual
258 fermented medium containing reducing sugars (lactose, glucose, galactose) in the
259 concentrated bacterial suspension and/or to the hydrolysis of sucrose in acidic conditions.
260 Several authors have investigated the Maillard reaction rate in milk powders or dehydrated
261 model systems and have shown relationships between the reaction rates and the physical state
262 of the amorphous matrix (Buera & Karel, 1995; Karmas et al., 1992; Pereyra Gonzales et al.,
263 2010; Schebor et al., 1999). A large increase in the nonenzymatic browning rate was reported
264 at a range of 2°C to 40°C above Tg (Karmas et al., 1992; Lievonen et al., 1998; Pereyra
265 Gonzales et al., 2010; Roos et al., 1996). Water plasticization increases molecular mobility,
266 which may also result in the conversion of sugars such as sucrose and lactose from the
267 amorphous state to the crystalline state (Roos & Karel, 1992). In closed systems,
268 disaccharides crystallization will induce an increase in a_w due to the release of water from
269 amorphous sugar, thus accelerating deteriorative changes such as NEB (Jouppila & Roos,
270 1994a; Vuataz, 2002). Above glass transition temperature, the nonenzymatic browning rate in
271 model systems appeared to be influenced by the temperature difference (T-Tg) (Buera &
272 Karel, 1995). Furthermore, nonenzymatic browning has been showed to proceed at the slow
273 rate even well below the glass transition temperature (Karmas et al., 1992; Lievonen et al.,
274 1998; Roos & Himberg, 1994; Schebor et al., 1999), which could explain the small losses of
275 specific acidification activity observed at low moisture content ($T < Tg$). .
276 The inactivation of freeze-dried lactic acid bacteria during storage almost certainly
277 resulted not only from the nonenzymatic browning but also from other complex chemical
278 reactions such as oxidation, protein denaturation etc (Kurtmann et al., 2009a; Lai & Topp,

279 1999; Teixeira et al., 1996). The mechanism of diffusion limited chemical reaction associated
280 to the glassy state does not allows to wholly explain the complex bacteria inactivation
281 behavior. The various works on freeze-dried lactic acid bacteria revealed that the bacteria
282 inactivation rate increased with water content and that storage of the samples in the glassy
283 state led to a better survival of bacteria(Higl et al., 2007; Kurtmann et al., 2009b; Pehkonen et
284 al., 2008; Schoug et al., 2010; Selma et al., 2007). However, there is no common acceptance
285 to identify the glass transition temperature as a stability threshold. Some authors reported the
286 acceleration of the degradation rate for temperature lower than Tg and others authors for
287 temperature well above the Tg. Furthermore, the inactivation rate appears to depend on the
288 strain of the lactic acid bacteria and on the composition of the protective medium. For
289 instance, the inactivation rate of bacteria freeze-dried in a lactose matrix was reported higher
290 than the inactivation rate of bacteria freeze-dried in a sucrose matrix (Kurtmann et al., 2009b).
291 Among the various works investigating the stability of freeze-dried lactic acid bacteria
292 (Higl et al., 2007; Kurtmann et al., 2009b; Pehkonen et al., 2008; Schoug et al., 2010; Selma
293 et al., 2007; Zayed & Roos, 2004), very few have focused on the study of the inactivation rate
294 at low moisture content and below Tg. Figure 5 displays the relationships between water
295 activity, water content, glass transition temperature and the rate of specific acidification
296 activity loss. Additional sets of experimental data on bacteria stability have been included.
297 The three batches of freeze-dried bacteria differed in the physiological state of the bacteria
298 obtained after the fermentation process. The values of a_w corresponding to the monolayer
299 water coverage (M_M) and to the threshold between glassy and rubbery states ($T_{storage}$-Tg = 0)
300 are reported in the figure. Whatever the batch, very low rates of loss of specific acidification
301 activity were observed for water activities lower than the a_w value corresponding the
302 monolayer water coverage M_M (a_w = 0.214) and the rates tended to increase for a_w value
303 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 $T_{storage}$-Tg = 0°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 Tg 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

329 hydration level, and to increase at lower and higher water contents (Labuza, 1980; Lai &
330 Topp, 1999; Pikal et al., 1991). This antioxidant effect of water has been ascribed to its
331 interaction with functional groups, which blocks these reaction sites, thereby preventing them
332 from interacting with oxygen (Lechuga-Ballesteros et al., 2002). The losses of specific
333 acidification activity observed at low a_w values may then be ascribed to oxidative membrane
334 mechanisms (Kurtmann et al., 2009a; Teixeira et al., 1996).
335
336 **4. Conclusion:**
337 The stability of lactic acid bacteria in a glass or rubbery sucrose matrix at different
338 water activity environments was analyzed. The physical properties of the matrix were
339 determined by means of state diagram and sorption isotherm. The Brunauer-Emmett-Teller
340 (BET) equation was used to describe the sorption properties and to determine the monolayer
341 water coverage M_M, reported as the optimal value of water content for product stability. When
342 plotting the loss rate of specific acidification activity of *Lb bulgaricus* CFL1 as a function of
343 water activity and positioning the a_w values corresponding to the M_M parameter ($a_w(M_M)$) and
344 to $T_{storage} - Tg = 0°C$ ($a_w(Tg)$), the $a_w(M_M)$ appears as a threshold value for bacteria stability.
345 Above $a_w(M_M)$, the degradation rate slightly increased and this increased was more
346 pronounced above $a_w(Tg)$, attributable to the physical changes of the matrix. Furthermore, a
347 slight increase of the degradation rate was also observed for very low value of a_w (<0.1),
348 probably caused by oxidative mechanisms and slow but still present Maillard reaction.
349 Our experimental results and especially the very low value of M_M compared to the
350 value of the pure sugar provide some evidences of a protective mechanism of sucrose: direct
351 interaction with the bacteria by establishing hydrogen bonding with the membrane proteins
352 and/or the lipid bilayer. Further studies are in progress to verify this mechanism and also to
353 generalize this approach with other protective medium including polymers or

354 polysaccharides. The concept of monolayer water coverage provides an interesting framework
355 for describing effects of water on the stability of glassy solids and is useful in the
356 development of freeze-dried biological products. Overdrying may be detrimental to the
357 stability of bacteria in the dried state, even when formulated with disaccharides. Combining
358 the relationships between water activity and glass transition temperature, water content and
359 biological activity appeared as a promising approach allowing a rational optimization of the
360 freeze drying process and the prediction of storage stability of lactic acid bacteria.
361

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366
367

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561

562 **Table captions**

563

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.

567

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569

570 **Figure Captions**

571

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

575

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

578

579 **Fig. 3.** Specific acidification activity (t_{spe}) of lyophilized *Lactobacillus bulgaricus* CFL1 in a
580 sucrose matrix as a function of storage time (ts) at 25°C for different values of water activity
581 (0.177; 0.326; 0.551). k_{spe}: rate of loss of specific acidification activity during storage at 25°C
582 ((min/(log(CFU/ml)))/day); t_{spe} = 0.2 x ts +40.7 (a_w = 0.177); t_{spe} = 1.5 x ts +46.4 (a_w =
583 0.326); t_{spe} = 8.1 x ts +77 (a_w = 0.551).

584

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

590

591 **Fig. 5.** Relationships between rate of loss of specific acidification activity during storage at
592 25°C (k_{spe}, in (min/(log(CFU/ml)))/day), glass transition temperature (Tg), water activity (a_w)
593 and water content (m) for bacterial suspension freeze-dried in a sucrose matrix.

Vertical/horizontal dotted lines indicate the threshold value of a_w between the glassy and the rubbery states, as well as the corresponding values of Tg and m. Vertical/horizontal bold grey lines indicate the values of a_w, Tg and m corresponding to the water monolayer coverage M_M (estimated from the BET equation). In bold under the x axis, are reported the values of the temperature difference T-Tg, (with T = 25°C) corresponding to the a_w values.

Table 1

	BET		GAB			a_w critical	Gordon Taylor		References
	M_M	C_B	M_M	C_G	K	(Tg = 25°C)	Tgs	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.,
Fermented medium			15.8	0.724	0.998	0.14	33.7	4.6	2001)
LAB[b]			8.7						(Selma et al.,
LAB[b] + M17 broth			11.8			0.083	50		2007)
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
LAB[d] + lactose + MD 12						0.228			al., 2009b)
Glucose	5.4	0.3					31/36	4.52	(Zhang &
Dextran	6.2	13.5					200		Zografi, 2000)
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 &
Skim milk	5.47	11.30	5.10	12.11	1.08	0.37	92	6.7	Roos, 1994a, , 1994b)
Isolated Soy	3.5								(Teng et al.,
Lipase	5.1								1991)
Protein	5-7								(Costantino et
Protein + trehalose	5								al., 1998)

[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: maltodextin with a dextrose equivalent of 12.
* Estimated from (Costantino et al., 1998)

Figure 1

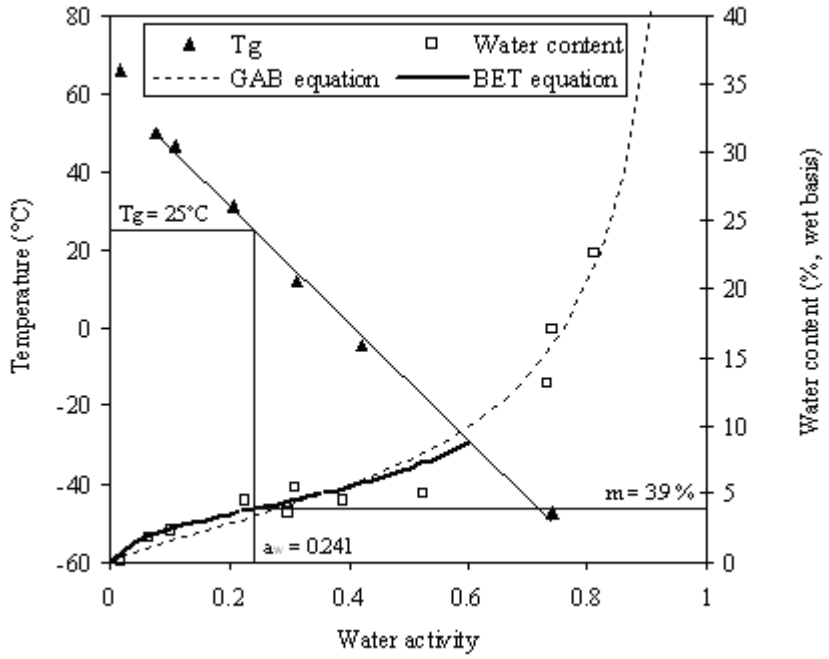

Tg
Water content
GAB equation
BET equation
Tg = 25°C
m = 39 %
aw = 0.241
Temperature (°C)
Water content (%, wet basis)
Water activity
80
60
40
20
0
-20
-40
-60
40
35
30
25
20
15
10
5
0
0
0.2
0.4
0.6
0.8
1

Figure 2

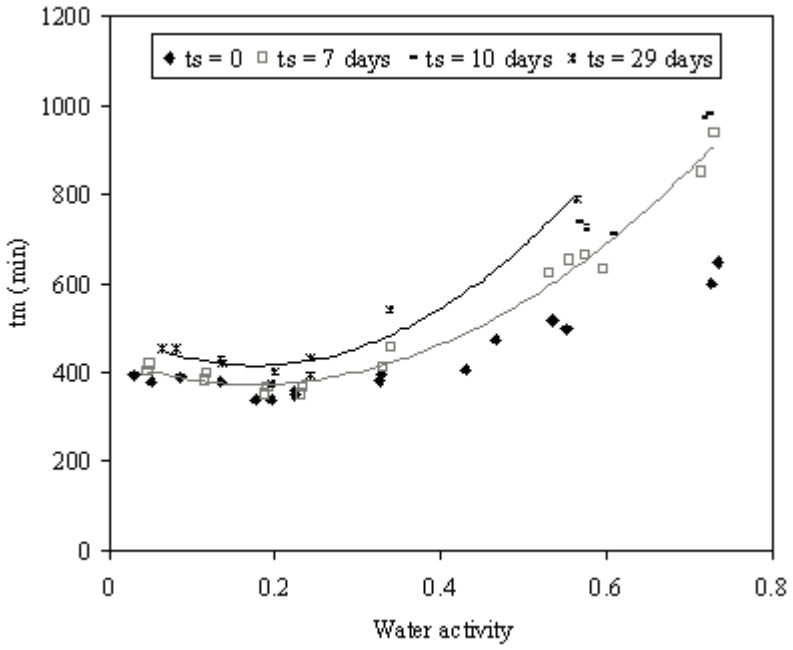

ts = 0
ts = 7 days
ts = 10 days
ts = 29 days
tm (min)
Water activity
0
200
400
600
800
1000
1200
0
0.2
0.4
0.6
0.8

Figure 3

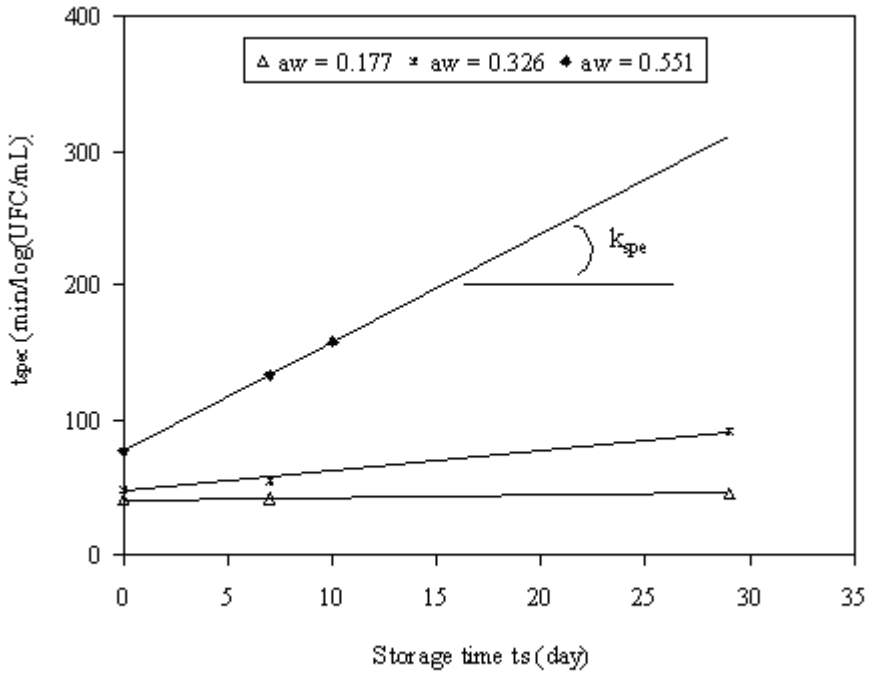

aw = 0.177
aw = 0.326
aw = 0.551
k_{spe}
tspec (min/log(UFC/mL))
Storage time ts (day)
0
100
200
300
400
0
5
10
15
20
25
30
35

Figure 4

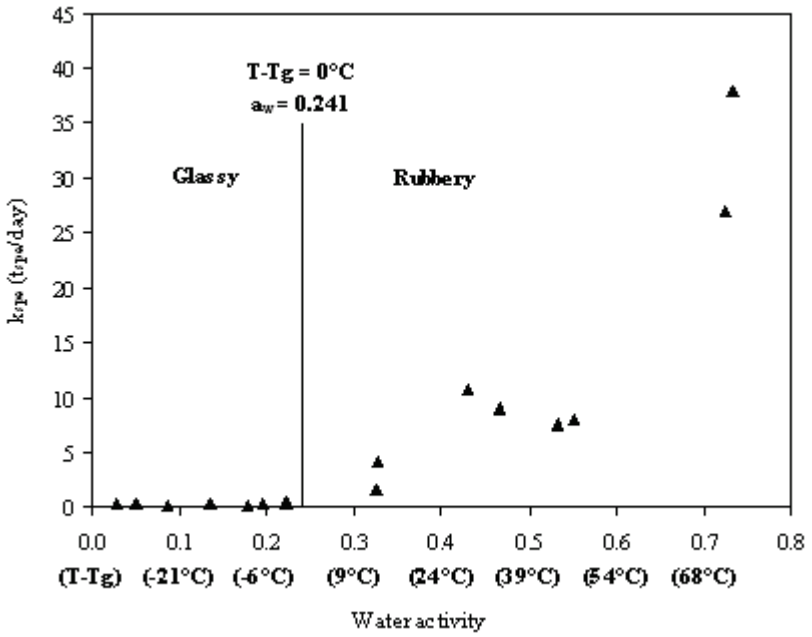
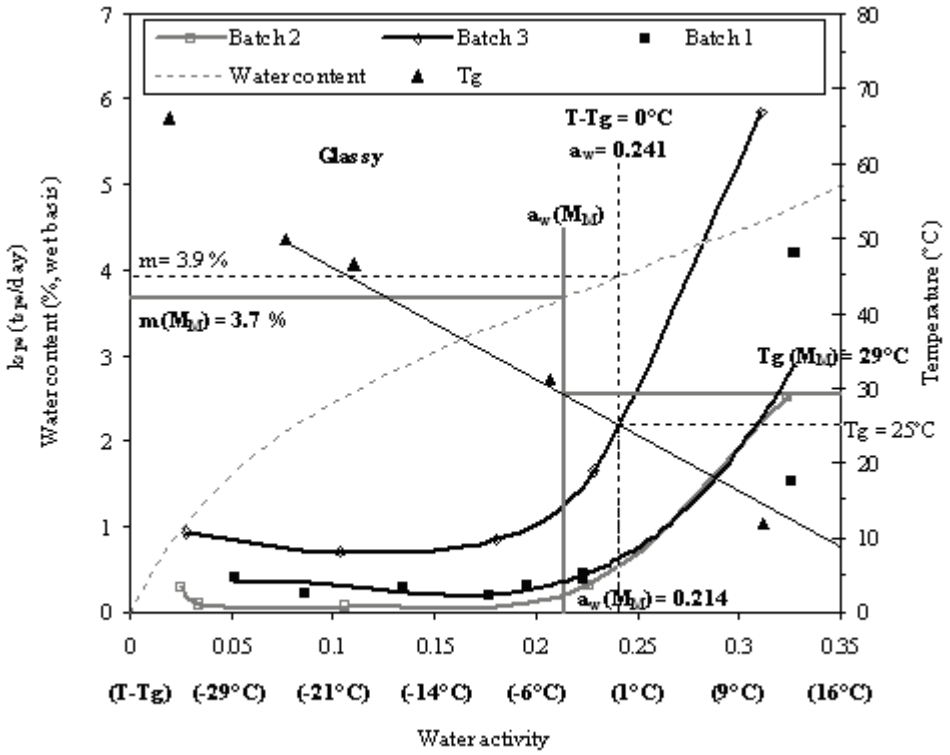

T-Tg = 0°C
a_w = 0.241
Glassy
Rubbery
k_{rpe} (t_{rpe}/day)
45
40
35
30
25
20
15
10
5
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
(T-Tg)
(-21°C)
(-6°C)
(9°C)
(24°C)
(39°C)
(54°C)
(68°C)
Water activity

Figure 5


Batch 2
Batch 3
Batch 1
Water content
Tg
Glassy
T-Tg = 0°C
a_w= 0.241
a_w ($M_{1,f}$)
m= 3.9 %
$m(M_{1,f})$ = 3.7 %
$Tg(M_{1,f})$ = 29°C
Tg = 25°C
a_w ($M_{1,f}$) = 0.214
k_{ps} (10^{-6}/day)
Water content (%, wet basis)
Temperature (°C)
Water activity
(T-Tg)
(-29°C)
(-21°C)
(-14°C)
(-6°C)
(1°C)
(9°C)
(16°C)