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The survival of *Lactococcus lactis* in a convective air drying environment: The role of protectant solids, oxygen injury and mechanism of protection

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Abstract

The effect of protectant solids (lactose, sodium caseinate and their mixture) on the survival of *Lactococcus lactis* subsp. *cremoris* was studied in a single-drop convective air drying environment. The extent of death caused by oxygen injury was also studied by drying the bacteria in the presence and absence of an oxygen scavenger (sodium ascorbate). The effect of glassy matrix on bacterial survival was evaluated by calculating the evolution of glass transition temperature in the drying process. It was found that the protective efficacy of lactose and sodium caseinate was comparable at the concentrations tested. The protective efficacy of the mixed protectant matrix (lactose: sodium caseinate =3:1) was much higher compared to that of lactose or sodium caseinate alone at the same total solids level, indicating synergistic enhancement in protection by the mixed protectants. The amide-II bond of the bacterial cells was found to be unaffected during drying when protectants were present indicating the important role of hydrogen bonding in bacterial survival. Protectants significantly enhanced bacterial survival by moderating the drying rate, facilitating the formation of glassy matrix earlier in the drying process and by minimising the exposure of bacterial cells to oxygen. The presence of sodium ascorbate improved the survival of bacteria in mixed protectant solids, indicating that the death of bacteria during the convective drying process was in significant part due to oxygen injury.

Keywords: *Lactococcus*, single droplet drying, survival kinetics, oxygen injury, mechanisms of protection, oxygen scavenger, glass transition, hydrogen bonding

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1. Introduction

Lactic acid bacterial cultures used for direct vat inoculation in the making of dairy products such as cheese, yogurt and sour cream are commonly supplied in frozen or freeze dried forms (Chávez and Ledebøer, 2007; Powell et al., 2011; Santivarangkna et al., 2007). Although frozen starter concentrates are cheaper to produce and show excellent initial survival, they have drawbacks of bulkiness (larger volume than dried cultures), high transportation and storage costs (typically stored and transported at temperatures below -40°C) and short shelf life. Furthermore, post-production formulation of mixed cultures with the desired component ratios and functionality cannot easily be achieved using frozen-block cultures, although this limitation can be overcome by freezing the culture components separately in pelletized form.

The volume of dried cultures is considerably reduced and high transportation and storage costs can be avoided. The viability and vitality (activity, fermentation efficiency) of the bacterial cultures are two important prerequisites for their acceptance in commercial application. To achieve desired fermentation efficiency the cellular structure and the physiological function of dried cultures have to remain intact in prevailing processing and storage conditions. Freeze drying is preferred over spray drying for thermally sensitive bacteria as it keeps their survival to a reasonably high level. For example, To and Etzel (1997) found that the survival of three different species of bacteria (*Lactococcus lactis* subsp. *cremoris* D11, *Lactobacillus casei* subsp. *pseudoplantarum* UL137 and *Streptococcus thermophilus* CH3TH) varied between 60-70% and 0.35-34% during freeze drying and spray drying, respectively. One of the main causes of bacterial death during freeze drying is the freezing of the cultures before they are dried (Uzunova-Donev and Donev, 2000). In the absence of a suitably formulated cryoprotectant matrix, the death of bacterial cultures during freeze drying is also quite substantial. Furthermore, freeze drying is a batch process with a considerably long drying time and is also expensive due to high energy requirements (Santivarangkna et al., 2007).

Spray drying is an extensively used drying method in the dairy and pharmaceutical industries. Compared to freeze drying, spray drying is cost effective, energy efficient and high throughput, and is an equally hygienic process. It has been reported that the operational and capital costs of spray drying are one sixth and one ninth compared to freeze drying, respectively (Chávez and Ledebøer, 2007). However, the spray drying environment can be

severe for bacterial starter cultures. Hence, the effect of the drying parameters (inlet and outlet air temperatures, air flow rate, relative humidity, residence time and the presence of protective solids) on the survival and fermentation activity of resultant bacterial starter culture powder has to be understood to a considerable depth. The drying process causes damage to the cell wall and other cellular components, especially the cytoplasmic membrane and proteins (Crowe et al., 1988; Teixeira et al., 1995). This cellular injury leads to cell inactivation and negatively impacts the vitality of the final product (Volkert et al., 2008).

Since it is not yet possible to quantify the changes occurring in the bacterial cells and their survival *in situ* when they are subjected to spray drying, single droplet drying is used instead. Single droplet drying, in which a single droplet is suspended in moving and conditioned air (Adhikari et al., 2000), provides the closest resemblance to the spray drying environment. It has been shown that the droplet temperature and the water evaporation rate have a significant effect on the survival of bacterial starter cultures during spray drying (Chen and Patel, 2007; Etzel et al., 1996). Li et al. (2006) investigated the inactivation kinetics of *Bifidobacterium infantis* and *Streptococcus thermophilus* in single droplet drying at air temperatures of 70°C and 110°C (corresponding relative humidity values of 4.2% and 1.1%) using skim milk as a suspending medium. They reported that the inactivation mainly occurred at the early stage of drying when the evaporation rate was high. Yamamoto and Sano carried out single droplet drying experiments to measure the retention of enzymes incorporated in sugar solutions. They found that the air temperatures and droplet size significantly affected the inactivation rate while the effect of initial water content was insignificant (Yamamoto and Sano, 1992). The inactivation kinetics of α -amylase were studied by Meerdink and Van't Riet (1995) by incorporating the α -amylase into maltodextrin droplets in the range of 75°C-100°C (corresponding relative humidity values of air ranged from 4.87% to 1.13%). They concluded that the inactivation rate of the enzyme is more sensitive to the change in droplet temperature than to the water evaporation rate (Meerdink and Riet, 1995). The above mentioned studies do not offer a unanimous view whether the drying rate or the droplet temperature is the limiting factor of bacterial survival.

Different protective agents such as carbohydrates, proteins, amino acids, gums and skim milk are used at different concentration levels to minimise the bacterial inactivation during drying. It is reported that low molecular weight carbohydrates stabilize the cell membrane and protein conformation in a dry state by bonding with the macromolecules of

bacterial cells (Rudolph and Crowe, 1985). Buitink et al. (2000) suggested that proteins can be more effective as protective materials for bacterial cultures than sugars as they are more stable above their glass transition temperature. It is reported that the combination of different protectants (a mixture of sugar and protein) can have synergic effect on cell viability in spray drying rather than acting individually (Santivarangkna et al., 2007). Desmond et al. added 10% of gum acacia to 10% of reconstituted skim milk and found that the survival of *Lactobacillus paracasei* was increased up to 1000 fold (Desmond et al., 2002). Hubalek (2003) and Santivarangkna et al. (2007) reported that the efficacy of protectants can also depend on the strains of bacteria being used (Hubalek, 2003; Santivarangkna et al., 2007).

To our knowledge, there is no publication reporting a comparative single-drop study on the survival kinetics of lactic acid bacteria as a function of protectant solids concentration. Most importantly, there are no reports on how the exposure to an oxygen rich environment such as prevailing in standard drying processes affects bacterial survival. In addition, the mechanisms by which protectants enhance bacterial survival have not been studied through *in situ* measurement of survival kinetics. In this context, the objectives of this paper were three fold. Firstly, we aimed at measuring experimentally the drying kinetics (moisture content history, temperature history) and the survival kinetics of *L. lactis* starter cultures during a convective drying process. Secondly, we aimed at studying the effect of protectant solids concentration on the survival of these bacteria. We used sugar (lactose), protein (sodium caseinate) and their admixture at varying concentration to study both the survival and the drying kinetics. Finally, we quantified the extent of death of lactic acid bacterial cells due to oxygen injury and investigated if hydrogen bonding and glass transition of the protectant matrix were involved as mechanisms in enhancing bacterial survival.

2. Materials and methods

2.1 Bacterial culture

Lactococcus lactis subsp. *cremoris* (ASCC930119) was from the Dairy Innovation Australia culture collection (Pillidge et al., 2009). These bacteria were sub-cultured overnight in 15 ml of M17 broth at 30°C under static conditions. The resulting cultures were transferred to 1 L of M17 broth under the same conditions. Cells were harvested by centrifuging them at 2500×g for 5 minutes, then suspended at approximately 10^{10} cells/ml in peptone water

(control) or solutions of lactose, sodium caseinate or a mixture of lactose:sodium caseinate (3:1).

2.2 Solution preparation

α -Lactose monohydrate (99.8% purity; Sigma-Aldrich, Australia) and sodium caseinate (NaCas) with protein content of 90.5% (MG2972, Murray Goulburn, Australia) were used as protectant solids without further purification.

Lactose solutions (10% and 15%, w/w) were prepared with deionised water at room temperature with gentle stirring. Similarly, sodium caseinate solutions (5% and 10% w/w) were prepared by heating to $45\pm 1^\circ\text{C}$ with gentle agitation. Lactose:sodium caseinate (in dry solids ratio of 3:1, w/w) solutions were prepared with heating as above, at 10%, 25% and 35% (w/w) solids. The moisture contents of the lactose and sodium caseinate powders were determined prior to use and compensated for while making the solutions. In the samples that contained oxygen scavenger, sodium ascorbate was used at 0.7% (w/w).

2.3 Measurement of drying kinetics

Measurements of moisture content and temperature histories of the single droplets were made with a custom-built single droplet drying instrument. The details of the instrument are provided elsewhere (Adhikari et al., 2007; Ghandi et al., 2012). In brief, this instrument supplied heated air in a flat airflow pattern at 0.5 ms^{-1} . A Teflon cylinder attached to a load cell held a thin glass filament (0.2 mm diameter) and two droplets were suspended (one each end of the glass filament). Each droplet ($7\pm 0.1\text{ }\mu\text{L}$) was transferred using a $10\text{ }\mu\text{L}$ syringe. Industrial spray drying units usually use 65°C to 85°C as outlet air temperatures when drying thermally sensitive products. Therefore, we carried out drying experiments at these two temperatures.

2.4 Droplet mass loss measurement

Mass loss as a function of time was measured using the load cell and the data were recorded continuously to a computer. The effect of drag on the drying data was experimentally determined and compensated for. For this purpose, a blank run without any droplet was carried out and the drift due to the drag was quantified. However, the drag values were very small or negligible in all of these experiments.

2.5 Droplet temperature measurement

The droplet temperature was measured by suspending a droplet at the tip of a micro-thermocouple (0.3 mm, T-type, Omega Company, USA). Another micro-thermocouple with the same specification was used to measure the air temperature.

2.6 Glass transition temperature

The T_g values of multi-component mixtures were predicted using a mass weighted mean rule (Adhikari et al., 2009). The multi-component mixture was assumed to be composed of ‘n’ individual binary solid–water mixtures, where ‘n’ is the number of solid components. T_g for each binary solid–water mixture was calculated using the Gordon–Taylor equation (Gordon and Taylor, 1952).

$$T_{g, \text{solid-water}} = \frac{X_s T_{g,s} + X_w K_{s,w} T_{g,w}}{X_s + X_w K_{s,w}} \quad (1)$$

$T_{g, \text{solid-water}}$ is the glass transition temperature ($^{\circ}\text{C}$) of a binary mixture, X_s and X_w are the mass fractions of solid and water in solution, respectively. $T_{g,s}$ ($^{\circ}\text{C}$) is the glass transition temperature of anhydrous solid, $T_{g,w}$ ($^{\circ}\text{C}$) is the T_g of pure water and $K_{s,w}$ is a dimensionless proportionality constant that provides the moisture dependence of T_g . Finally, the T_g of the multi-component mixture was calculated as a mass weighted mean on a water free basis [Equations (2) and (3)] assuming that the solids are uniformly mixed in the system.

$$T_{g, \text{mixture}} = \sum_{i=1}^n T_{g, i-w} X_i \quad (2)$$

$$\sum_{i=1}^n X_i = 1 \quad (3)$$

$T_{g, \text{mixture}}$ is the T_g of the multi-component mixture including water. $T_{g, i-w}$ represents the T_g of binary solid–water mixtures (lactose-water, NaCas-water). X_i is the mass fraction of an individual solid component on a water free solid basis. In equation (2), the effect of water has been incorporated through the T_g relationship for each binary solid–water mixture. The T_g and $K_{s,w}$ values for pure components are listed in Table 1.

In order to ascertain the applicability of the above approach (application of equations (2) and (3) in test conditions), we measured the glass transition temperature of the spray dried

starter culture using lactose:sodium caseinate (3:1) as a protective matrix. This powder was equilibrated in saturated salt solutions with water activity or equilibrium relative humidity (in fraction) ranging from 0.11 to 0.97. Various saturated salt solutions were used and the sorption isotherm was determined at $20\pm0.5^{\circ}\text{C}$ using methods as proposed by Wolf et al. (1985). The differential scanning calorimeter (DSC) measurements were performed on a Mettler Toledo DSC (Mettler Toledo, USA). About 10 mg sample was placed in an aluminium pan (40 μl) and hermetically sealed following the protocol provided by the manufacturer. The samples were heated from 20°C to 120°C at a heating rate of $10^{\circ}\text{C min}^{-1}$ and were subsequently cooled to 20°C at a cooling rate of $10^{\circ}\text{C min}^{-1}$. The glass transition temperature (T_g) was taken as the inflection point of the increment of specific heat capacity. The DSC was pre-calibrated using the melting point (156.48°C) and melting enthalpy (28.24 J/g) of indium. Dry air (50ml/min) was used as the flushing medium.

2.7 Fourier transform infrared spectroscopy

The IR spectra of the samples were measured using a Fourier transform infrared (FTIR) spectrometer (Model 400, Perkin Elmer, Australia) using standard accessories in the wave number range of 3500cm^{-1} to 900cm^{-1} . Perkin-Elmer spectrum software (version 6.3.4) was used for FTIR analysis and second derivatives of the spectra were generated using a 49 point smoothing factor.

2.8 Measurement of cell viability

In order to measure the cellular viability, drying experiments were stopped at pre-set times and the droplets were diluted in 1 ml peptone water (1%, w/w; pH = 7 ± 0.1). Live and dead cells were counted by the fluoresceine diacetate (FDA) staining method (Lentini, 1993). The initial viable cell count was determined by using a similar droplet before it was subjected to drying. When this method was compared with the plate count method, the difference in results was within 6%.

2.9 Statistical analysis

Statistical analysis was performed using ANOVA. The mean values and standard deviations were calculated from triplicate experimental data. Differences were compared by Tukey test for all experiments and considered significant at $p<0.05$. All statistical analyses

were performed with MINITAB 15 (MINITABTM Statistical Software, MINITAB Release15 For Windows[®]).

3. Results and discussion

3.1 The drying kinetics of bacterial cells suspended in single droplets

Figure 1 (panels A, B & C) shows the experimental results obtained during the drying of single droplets of bacterial culture in peptone water and in the presence of different concentration levels of lactose, sodium caseinate and their (3:1) admixture as protectants, at set air temperature of 65°C. Similar trends in both the moisture and temperature histories were observed while drying the bacteria-containing droplets both at 45°C and 85°C (data not shown).

As can be observed from Figure 1 (Panels A and C), the rate of change of the moisture content was much smaller in the droplets containing protectant solids, which is expected as the protectant solids are capable of resisting evaporation of water due to solid-solvent interaction. This was especially noticeable when the initial concentration of the protectants was 10% (w/w) or higher. This is equally true whether the protectant solids were used individually or in a combined form.

Panels B and C show that the droplet temperature rose rapidly immediately after the start of drying (heating up period). In the absence of added protectant, the temperature initially rose quickly, then the rise slowed dramatically (temperature slowly approached the wet-bulb temperature; for example, 28.5°C±0.5 at set air temperature of 65°C), and then the temperature rapidly increased to the dry-bulb (drying air) temperature. Initially fast-evaporating droplets (droplets with no protectant) remained at lower temperatures longer than droplets with protectants, but later underwent a much faster temperature rise and approached the drying air temperature more quickly than the slow-evaporating droplets (those with protectant). Conversely, the droplet temperature of initially slow-evaporating droplets whose temperature rose comparatively faster immediately after the start of the drying process (less evaporative cooling effect) were slower to approach the drying air temperature.

Figure 1 (panel C) presents the moisture and droplet temperature histories of bacterial cultures in the presence of combined sugar (lactose) and protein (sodium caseinate) having initial solid concentration levels of 10%, 25% and 35% (w/w). The drying behaviour of lactose:sodium caseinate droplets at lower solids concentration is similar to their individual behaviour (Panels A and B) at the same low solids content (10%, w/w), but there are noticeable differences. Drying is more rapid (essentially complete by 240 s) with the blend of protectants than with either alone, and the period near the wet bulb temperature sits between the long near-plateau seen with 10% lactose and the continuing rise seen with 10% sodium caseinate. The temperature of droplets containing 25% and 35% solids (Panel C) rises quickly in the early stage of drying compared to the droplet having no protectant solids (this is expected due to evaporative cooling in the case of the latter) but is slow to approach the drying air temperature.

It has been reported that the effective moisture diffusivity which controls the outward moisture evaporation flux of solutions of low molecular weight sugars (such as lactose) and macromolecular biopolymers (such as sodium caseinate) depend on the solids concentration (Adhikari et al., 2002). The higher the solids content in the droplet the slower will be the moisture evaporation flux at the initial stage of droplet drying.

The experimental droplet temperatures versus moisture content data are presented in Figure 2 (panels A and B). This figure shows that the droplet temperatures even in the absence of protectant solids do not maintain a constant temperature regime in the vicinity of wet bulb temperature. As seen in Figure 1, the rise in the temperature of droplets containing protectants is much faster early in drying but, more clearly shown in Figure 2, low moisture levels are achieved at lower drop temperatures in the presence of protectants. Figure 2 (panel B) shows clearly that droplets having higher levels of protectant solids approach the drying air temperature later in drying than droplets containing no or low levels of protectant solids.

3.2 Survival kinetics of bacterial cells suspended in single droplets

Figure 3 and Figure 4 present the survival of bacterial cultures in the presence and absence of protectant solids as a function of moisture content at a drying air temperature of 65°C. Figure 3 (panel A) shows the effect of lactose and sodium caseinate on the survival of bacteria when the solids content is varied. In the absence of protectant solids only 16% of the bacteria survived when the moisture content of the droplet reached 0.36 kg water/kg solid.

The presence of lactose and sodium caseinate individually improved the survival of bacterial starter culture significantly ($p < 0.05$). The survival of the bacterial cultures was more than doubled (34.8%) in the presence of 5% (w/w) sodium caseinate. Similarly the presence of 10% (w/w) sodium caseinate improved the survival to 42.6% at the end of the drying. A similar trend in survival of this bacterium was observed when lactose was added. The addition of 10% (w/w) and 15% (w/w) lactose increased the survival of the bacteria to 41.7% and 48.5%, respectively. These data reveal that at identical solids concentration and prevailing drying conditions (air temperature 65°C, relative humidity 5.4% and velocity of air across the droplet 0.5 ms⁻¹), the effectiveness of 10% (w/w) lactose or sodium caseinate is almost identical (survival about 42%). Figure 3 (Panel B) extends these observations to lactose:sodium caseinate blends.

All protectants gave higher survival throughout drying, but with biphasic survival curves showing most of the cell death occurring below *ca.* 1-1.5 kg/kg moisture levels. This is the point of inflection, beyond which death per gram of water removed is much increased. Figures 1, 2 and 4 show that removal of this moisture involves more than 60-70% of the drying time and droplet temperatures above *ca.* 50°C, consistent with the interpretation that bacterial death later in drying involves extended exposure to potentially lethal temperatures.

Figure 4 shows survival through drying time. The survival of bacterial cells in the presence of different protectant solids at various concentrations was not significantly different within the first minute of single droplet drying ($p > 0.05$). This may be due to the fact that at this stage of drying the droplet temperatures were similarly low and the moisture content of the droplets was still high (14.99 and 9.49 kg water/kg solid in the case of 5% and 10% NaCas); 6.73 and 3.91 kg water/kg solid in the case of 10% and 15% lactose and 6.18, 2.13 and 1.78 kg water/kg solid in the case of 10%, 25% and 35% lactose:sodium caseinate (3:1)) leading to similar molecular mobilities and similar thermal, dehydration and oxygen-related (see section 3.2.3) stresses

Previous work (Ghandi et al., 2012) has shown that the rate of moisture evaporation and exposure to high temperatures are major causes of lactococcal death in convective drying. It appears that the survival of lactic acid bacteria is mainly negatively impacted by the following two factors: firstly, the higher moisture evaporation fluxes of water at the early stage, and secondly, the higher droplet/particle temperatures during the later stage of drying. The present study indicates that the presence of protectants improves bacterial survival in two

ways, ameliorating both causes. Firstly, the protectants moderate the drying rate in the initial stage of drying which otherwise would cause damage due to rapid outward flow of intracellular water (Ghandi et al., 2012). It is clear that death during this phase of drying is not due to thermal damage since, for example, drying without added protectants resulted in 58.2% cell death before the drop temperature reached 35°C. Secondly, protectants allow equivalent dryness to be achieved at a lower temperature (Figure 2), at least in part due to their initial higher solids (i.e. lower moisture) prior to drying. They may also have an anti-oxidative role (see section 3.2.3).

3.2.1 The role of hydrogen bonding and synergistic protection of bacteria by mixed protectants

Figure 5 (panel A) shows the infrared spectra of *L. lactis* dried in the absence and presence of different concentrations of lac:NaCas (3:1) (10%, 25% & 35%, w/w). The band at 3298 cm⁻¹ corresponds to OH stretching vibration. The CH₂ stretching vibration corresponds to the asymmetric and symmetric lipid CH₂ stretching vibration bands at 2925cm⁻¹ and 2872cm⁻¹, respectively (Oldenhof et al., 2005). The bands at 1648 cm⁻¹ and 1540 cm⁻¹ correspond to amide-I and amide-II, respectively (Leslie et al., 1995; Oldenhof et al., 2005). In the “fingerprint” region (below 1500 cm⁻¹) the absorption bands are difficult to assign but are characteristic of a sample (Oldenhof et al., 2005).

In order to investigate the effect of drying on the conformational integrity of proteins the amide-I and amide-II peaks at wave numbers between 1500 cm⁻¹ and 1650 cm⁻¹ were monitored. Carpenter and Crowe (1989) used the amide-II band to monitor the effect of drying on the structural conformation of proteins. The amide-I is the carbonyl stretch which can be used to monitor the secondary structure of proteins while the amide-II is related to the primary N-H bending and stretching (Wharton, 1986). Figure 5 (panel B) shows that the amide-II peak shifted from wave number of 1528cm⁻¹ to 1519cm⁻¹ as a consequence of drying without protectants. This shifting of the wave number to lower frequency confirms that a change in protein structure has taken place. This change in protein structure corroborates well with the 84% reduction in survival of lactic acid bacteria while drying at 65°C. However, after drying of the bacterial cells in the presence of protectants (lactose:sodium caseinate mixture) the vibration of the amide-II bond occurs at 1528cm⁻¹. This means that the presence of protectant solids during drying has prevented the shifting of the amide-II wave number to lower frequencies indicating that the change in protein

conformation was much reduced. This suggests that the greater survival of the bacteria is at least in part a consequence of maintenance of cellular protein conformation by the protectant solids during drying.

Although the effectiveness of lactose and sodium caseinate is almost identical under the conditions used, the mechanism with which low molecular weight sugars (such as lactose) and macromolecular proteins (such as sodium caseinate) deliver the protective function is quite different. The protective efficacy of lactose lies in the fact that it is capable of interacting with the polar group of phospholipids during dehydration. According to the 'water replacement' hypothesis, low molecular weight sugars such as lactose have greater affinity with polar moieties of bacterial cells than water when the moisture content of the cellular mass is relatively low (Crowe et al., 1988). This means that the lactose molecules occupy the sites left vacant by evaporating water molecules and by doing so they (lactose molecules) prevent the disruption of the conformational integrity of cellular structure. Furthermore, sugars such as lactose can prevent aggregation and unfolding of proteins by hydrogen bonding with polar head groups of proteins and phospholipids (Hanafusa, 1985). Water is essential for maintenance of the integrity of plasma membrane, phospholipid bi-layers and proteins. The removal of water during drying process results in a substantial decrease in the hydrogen-bonded water molecules from the head groups of phospholipid bi-layers or other cellular moieties. The decrease in the protective hydrating water layer increases the packing of head groups and forces the acyl chains to come together which subsequently increases the degree of van der Waals interactions (Crowe et al., 1985). Such interactions promote the transition of phospholipids from the liquid crystalline state to the gel phase which makes membranes leaky (Crowe et al., 1988). The low molecular weight sugars lower the transition temperature of the dry membranes and prevent the phase transition by replacing the water molecules surrounding the lipid head groups. In addition, sugars can preserve the function and structure of proteins during drying by forming hydrogen bonds with membrane proteins and help prevent protein denaturation (Carpenter and Crowe, 1988, 1989); (Ananta et al., 2005)

On the other hand, protein macromolecules such as sodium caseinate are not capable of passing through the peptidoglycan layer that covers the plasma membrane of lactic acid bacteria (Callewaert et al., 2011; Delcour et al., 1999). The proteins cannot form hydrogen bonds with the cellular components inside the cell wall and are believed to act as inactive

bulking agents. The protective coating, formed by these bulking agents (such as protein) around the cells, lowers the probability of large number of cells coming closer and fusing with each other (Champagne et al., 1991; Oldenhof et al., 2005). It appears that the protective effectiveness provided by macromolecular proteins comes through this inert bulking mechanism.

As can be seen in Figure 3 (panel B) the mixture of lactose:sodium caseinate (10% w/w total; 3:1) improved the survival of bacterial cultures significantly compared to the survival accorded by lactose and sodium caseinate individually. At the identical initial solids content of 10% (w/w) the survival of bacteria in lactose:sodium caseinate (3:1) mixed matrix was 58.5% compared to the 42% survival of bacterial cells when lactose or sodium caseinate were used individually. This higher effectiveness of the sugar:protein mixture (in this case lactose:sodium caseinate) could be attributed to the synergic effects of hydrogen bonding, water-replacement (from lactose) and non-interacting bulking (from sodium caseinate) mechanisms as well as the effects on water movement and temperature demonstrated in this study. This is in agreement with previous reports that mixed carbohydrate-protein protectant systems synergistically enhance the cell viability and prevent cellular injury during dehydration when compared with individual protectants (Buitink et al., 2000; Santivarangkna et al., 2007).

3.2.2 The role of glassy and rubbery matrix on bacterial survival

The relationships between the moisture content and the T_g were calculated using equations (1)-(3) provided in Section 2.5 of this paper and are shown in Figure 6.. The predicted line (panel A) follows the available experimental data within 4.9% experimental error ($R^2=0.98$). The interesting feature of Figure 6. (panel B) is that the T_g of the droplet containing 10% (initial) protectant is well below the droplet temperature (droplet varies from 21.16°C-63.16°C) indicating that the droplet/particle matrix is in the rubbery state. However, the T_g values of the droplets containing (initial) 25% and 35% solids (w/w) manage to cross the droplet temperature at the moisture contents of 0.15 and 0.41 (kg water/kg solid), respectively. This indicates that the matrix of the droplets containing (initial) 25% and 35% solids does attain the glassy state during drying. It can further be seen that the droplet containing 35% of solids attains the glassy state much earlier than the droplet containing 25% (initial) protectant solids.

These findings are well supported by literature which suggests that the matrix glass transition temperature of materials such as maltodextrin and whey protein isolate having higher T_g values (in their anhydrous state) attains or exceeds the droplet temperature much earlier in the drying process ((Adhikari et al., 2005). According to Buitnik et al. (2000) the proteins are capable of forming relatively stable glasses and by doing so they can be effective protective materials for bacterial cultures. This is because the protein glasses were found to resist structural collapse even at 50°C above the matrix glass transition temperature (T_g). It is also reported in literature that the structural integrity of sugar glasses only collapses about 20°C above the matrix T_g (Buitink et al., 2000).

For glass transition temperature based mechanisms to be effective in improving the bacterial survival, the matrix must be in a glassy state rather than the rubbery state. To maintain the preferred glassy state of the matrix, the T_g should be at least above the droplet temperature. When the matrix T_g is below the droplet temperature, it is unlikely that the particles can attain a viscosity of 10^{12} - 10^{13} (Pa.s). This level of viscosity is required for the solid matrix to become glassy (Roos and Karel, 1991) under the prevailing drying conditions. When the solids concentration of a droplet/particle is not high enough to raise the matrix T_g to higher than the particle temperature, the molecular mobility (diffusion) of water within the particle matrix during the drying process might be slowed down but remains significantly high. As a consequence the particle remains in the rubbery state, which cannot arrest the molecular mobility of water, which is very important in improving the bacterial survival. However, once the matrix T_g becomes higher than the droplet temperature (as can be achieved by judiciously selecting protectant solids and increasing their concentration), the solids matrix attains the glassy state provided that the process is rapid enough to prevent crystallization.

The calculated values (Figure 6) support the much higher survival of bacterial cells in droplets containing higher (e.g. 35%) amounts of protectant solids. This simulation also supports that higher concentration of protectant solids in the matrix has better efficacy of protecting bacterial cells as they attain the glassy state much earlier in the drying process. This means that in order to achieve higher bacterial survival during convective drying (such as spray drying) the matrix of the protectant solids should be chosen in such a way that the droplets/particles attain the glassy state much earlier in the drying process.

3.2.3 The use of oxygen scavenger to improve bacterial survival during drying

The effect of oxygen on the survival of bacterial cells during single droplet drying was studied by using two sets of droplets, one containing an oxygen scavenger (antioxidant) and the other not containing the oxygen scavenger (control). Sodium ascorbate at 0.7% (w/v) was used as the oxygen scavenger, added to the formulation just before drying. The survival of the bacteria was measured and compared with that of the control (Figure 7.).

Figure 7. shows that the presence of oxygen scavenger improved bacterial survival significantly ($p < 0.05$) at all protectant solids levels and throughout the single-drop drying history, indicating that a substantial proportion of the death is caused by oxygen damage at all stages of drying. At the end of the 10-minute drying process, the presence of ascorbate had increased the bacterial survival from 28.2% to 40.7% in the presence of 10% (w/w) protectant solids, 39.2% to 50.2% with 25% (w/w) protectants, and 55.0% to 66.9% with 35% (w/w) protectants. These survival values correspond to a reduction in bacterial death by factors of 0.83, 0.82 and 0.74, respectively.

Comparison of specific death rates (estimates calculated from survival data; not shown) showed rates to be highest early in drying and confirmed lower death rates in the presence of ascorbate throughout the drying period. At lower solids (10% and 25% w/w) the trend indicated greatest reductions in specific death rate towards the end of drying, as would be expected if the increased temperature accelerated oxidation. This trend was not seen at 35% (w/w) solids, consistent with the high solids providing sufficient intrinsic antioxidant effect, perhaps by formation of a protective encapsulating shell.

These results show that oxidative damage is a major cause of bacterial death in drying. Since oxygen exposure will increase with increasing surface:volume ratio, this damage would be greater when droplet size is smaller. In spray drying, the droplet size is typically much smaller than in this study; for example, a 10 μ m diameter droplet would have (assuming sphericity) a surface:volume ratio 240 times higher than the 2.4 mm droplets used in this study, and so the extent of oxygen damage in spray drying is expected to be much higher. Surprisingly, the effect of oxygen damage on the survival of bacteria during spray drying has historically not received its due attention.

3.3 Modelling of the survival kinetics

Generally the survival or the inactivation kinetics of bacterial cultures under constant conditions can be predicted satisfactorily by using the first order reaction kinetics model (Elizondo and Labuza, 1974):

$$\frac{dS}{dt} = -K_d S \quad (4)$$

where, S is the survival of the bacterial cultures (%), K_d is inactivation rate constant (rate of mortality) (s^{-1}). Equation (4) can be integrated using the initial load of bacterial cells in the droplet (S_0) as given by equation (5). This equation (eq. 5) was fitted to the observations by minimising the chi-square (χ^2) to calculate an apparent K_d value for each set of drying conditions (eq. 6).

$$S_t = S_0 \exp(-K_d t) \quad (5)$$

$$\chi^2 = \frac{1}{N - (p - 1)} \sum_{i=1}^N \frac{(S_i^{\text{exp}} - S_i^{\text{pred}})^2}{S_i^{\text{exp}}} \quad (6)$$

where, “exp” denotes the experimental data and “pred” refers to the predicted survival value from the model equation. N is the total number of observations and p is the number of unknown parameters. K_d is the only unknown parameter or constant in equation (5).

The application of complex models requires gathering extensive experimental data on which to base predictions. The absence of such data in many experimental and industrial situations leads to the desirability of simpler, yet usefully predictive, models. In the present study, even though the droplet temperature varied from room temperature to very close to the set drying air temperature, and variation in death rate throughout drying was observed, Figure 4 shows that the experimental survival curves could be approximated using apparent inactivation rate constants (K_d) derived by solving equation (5). The average absolute error of prediction ranged from 0.70% to 7.6% and the coefficient of determination (R^2) varied between 0.95 and 0.99, indicating likely usefulness for such a model.

The calculated K_d estimates are shown in Table 2. Comparing these apparent K_d values with bacterial survival data shows that this model is qualitatively consistent with trends associated with increasing temperature (higher K_d values consistent with higher observed bacterial death; experimental data not shown) and with increasing solids concentration of each protectant type. However, the trend in apparent K_d values is not consistent with the relative bacterial survival

rates seen with different protectants. Visual comparison of the survival data and predicted curves (Figure 4) suggests that this mathematical model does not adequately cope with the addition of protectants that change the profile of bacterial death at various stages of drying. Effects such as this may be behind the limited predictive ability of many models.

An estimate of relative thermal inactivation energies under different drying conditions can be derived from this apparent K_d via arbitrary selection of one temperature (*e.g.* the droplet maximum) to represent the drying profile. For example, we have derived such estimates in our own work (Ghandi et al, 2012), but we now conclude that their reliability and usefulness are compromised by the limitations of the apparent K_d estimate, by the continually-changing droplet temperature and moisture during drying, and by the significant contribution of non-thermal factors to bacterial inactivation.

As reviewed by Chen and Patel (2007), any comprehensive model of bacterial inactivation in drying (and no such model yet exists) would include thermal and dehydration inactivation parameters, in addition to considering issues such as droplet composition, water diffusion and bacterial distribution, and our results indicate that oxygen availability should be added to that list of considerations.

4. Conclusions

In this study, the drying and survival kinetics of *Lactococcus lactis* suspended in single droplets were investigated by using lactose, sodium caseinate and their admixture (3:1) at varying concentration levels and sodium ascorbate as an oxygen scavenger. The results show that the survival of these bacteria is affected by the moisture evaporation flux, the droplet temperature and oxidative damage. The presence of protectant solids was found to be very helpful in improving the survival of the bacterial cells during drying. The survival of bacteria was also enhanced by the inclusion of oxygen scavenger (sodium ascorbate), indicating that oxidative damage is a significant but historically unappreciated cause of bacterial death during convective air drying processes.

The amide-II infra-red spectroscopy peak was found to be shifted (from wave number 1528cm^{-1} to 1519cm^{-1}) when protectant solids were not used. This confirmed the protection accorded by these protectant solids to macromolecules during drying. The evolution of matrix

glass transition temperature during drying was simulated and compared with the drop temperature history. It was found that the matrix of the droplets containing 25% and 35% (w/w) protectant solids (lactose:sodium caseinate = 3:1) became glassy under prevailing drying conditions while the matrix of the droplets containing 10% (w/w) of same protectant solids remained rubbery. The bacterial survival in the case of the former was much higher than the latter indicating that the glassy matrix helps better protect the bacterial cells than the rubbery matrix.

Protectants enhanced bacterial survival by moderating the drying rate and achieving dryness at lower temperatures, by facilitating the formation of glassy matrix earlier in the drying process, and by minimising the exposure of bacterial cells to oxygen.

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NOMENCLATURE

K_d	Inactivation rate constant ($1.s^{-1}$)
S	Survival of the bacteria (%)
S_0	Initial survival of the bacteria before drying (%)
t	Time (s)
T_g	Glass transition temperature ($^{\circ}C$)
u	Moisture content (kg water/ kg solid)

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Table 1. Glass transition temperature (T_g) and its water dependence ($K_{s,w}$) of sample materials.

Samples	T_g °C	$K_{s,w}$ (dimensionless)
Water	-135	
Lactose	104	6.9
NaCas	130	8.55

Sources: (Kalicevsky et al., 1993); (Adhikari et al., 2009; Adhikari et al., 2003)

Table 2. The apparent inactivation rate constant (K_d) of *L. lactis* at different temperatures during single droplet drying.

	Temperature (°C)	K_d (s ⁻¹)
<i>L. lactis</i> without any additives	65	3.91×10^{-3}
	85	5.33×10^{-3}
<i>L. lactis</i> + 10% lactose	65	1.41×10^{-3}
	85	2.16×10^{-3}
<i>L. lactis</i> + 15% lactose	65	9.04×10^{-4}
	85	1.44×10^{-3}
<i>L. lactis</i> + 5% sodium caseinate	65	2.01×10^{-3}
	85	3.44×10^{-3}
<i>L. lactis</i> + 10% sodium caseinate	65	7.46×10^{-4}
	85	2.15×10^{-3}
<i>L. lactis</i> + 10% lactose:sodium caseinate (3:1)	65	1.59×10^{-3}
	85	3.41×10^{-3}
<i>L. lactis</i> + 25% lactose:sodium caseinate (3:1)	65	7.50×10^{-4}
	85	2.87×10^{-3}
<i>L. lactis</i> + 35% lactose:sodium caseinate (3:1)	65	4.30×10^{-5}
	85	2.01×10^{-3}

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