Afslutningsrapport

Vitalitet af probiotiske bakterier i tørrede mejeriprodukter

Betydning af indkapslingsmatrice og tørringsmetode

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Resume af det samlede projekt

Gennem det seneste årti er flere af de mange potentielt positive helbredseffekter af probiotiske bakterier blevet eftervist, og forbrugernes efterspørgsel efter probiotiske produkter er stigende. Efter fermentering bliver probiotiske bakterier sædvanligvis tilsat til en suspension af matrixkomponenter, som herefter tørres således at der opnås en tør bakteriekultur, hvor bakterierne er indkapslet i en beskyttende matrix. I bedste fald kan bakterierne i disse tørrede kulturer overleve i en hvilende tilstand og efter rehydrering stadig være i stand til at udøve deres positive effekter, når de indtages. Oftest sker der dog et signifikant tab af bakteriernes vitalitet, når de har været igennem en tørrings-, distributions- og lagringsperiode. For industrien er det derfor en udfordring at afklare, hvordan bakteriernes vitalitet bevares bedst muligt, og i det nærværende projekt har hovedformålene været:

- at undersøge betydningen af og interaktionen mellem beskyttende matrixkomponenter og procesparametre (frysetørring og vakuumtørring)

- at definere og forstå de kemiske og fysiske ændringer, som fører til tab af vitalitet under lagring af tørrede bakteriekulturer.

Ved tørring af bakterier uden beskyttende matrixkomponenter blev overlevelsen fundet at afhænge betydeligt af procesbetingelserne. Den optimale frysetørringsproces viste sig at have høj tørringshastighed og relativ høj produkttemperatur. Ved vakuumtørring sås en optimal produkttemperatur tæt på vands trippelpunkt. En tilsætning af beskyttende matrixkomponenter til tørringsmatricerne betød, at tørringsbetingelserne fik mindre betydning for bakteriernes overlevelse. Der blev fundet et kritisk rest-vandindhold for bakteriekulturerne, hvorunder vitaliteten falder drastisk, hvis der fjernes mere vand. Dette vandindhold er 15-20 procent, og har især betydning, når der tørres uden beskyttende matrixkomponenter.

Det blev vist, at en glastilstand i bakteriekulturerne under frysetørring ikke er afgørende for bakteriernes overlevelse. På samme måde blev det for lagring af bakteriekulturerne vist, at en lagringstemperatur under glasovergangstemperaturen ikke nødvendigvis sikrer en optimal overlevelse. Der blev dog for bakterieoverlevelsen vist en tydelig afhængighed af lagrings- temperaturen såvel som af vandaktiviteten. Lagringstemperatur og vandaktivitet skal således betragtes som uafhængige parametre, der kan forårsage forskellige typer (og niveauer) af ødelæggende reaktioner i bakteriekulturerne.

Kemiske studier har endvidere vist en høj kompleksitet af fysiske og kemiske processer i bakteriekulturerne, og både reaktioner inde i bakteriecellen og i den beskyttende matrix har betydning. To overordnede reaktionstyper har været nærmere undersøgt: Oxidations- og bruningsreaktioner. Typerne og hastigheden af disse reaktioner afhænger af vandaktiviteten under lagring, og lagring ved lave a_w (under 0,22) forbedrer overlevelsen væsentligt.

Ødelæggelse (oxidation) og et muligt tab af DNA fra bakteriecellerne er sammen med koblingen mellem radikaldannelse og bruningsreaktioner nye perspektiver, som fortjener videre opmærksomhed med henblik på at forstå og forbedre vitalitetstab i tørrede bakteriekulturer.

English Summary

During the last decade various potential health effects of probiotic bacteria have been documented, and the consumer demand for probiotics is increasing. After fermentation of probiotic bacteria they are usually added to a suspension of matrix components and dried to form a dry bacteria culture, where the bacteria are encapsulated by protective matrix com-pounds. In the optimal case, the bacteria in these dried cultures will survive in a dormant state and be able to exert their positive effects upon rehydration. This is however not always what happens, and a significant loss of bacterial vitality is observed during drying, distribution and storage. The industry therefore face the challenge of defining how the vitality is better preserved, and in the current project the following main objectives have been focused on:

- Investigation of the role and interaction of protective matrix compounds and process parameters (freeze drying and vacuum drying).

- Defining and understanding the chemical and physical changes in the dried cultures leading to vitality loss of the bacteria during storage

When dried without protective compounds in the drying matrix, the survival of bacteria was found to depend on the process conditions. Accordingly, the optimal freeze drying process of cells without protective solutes exhibits a high drying rate and relatively high product temperatures. For vacuum drying the optimum product temperature lies close to the triple point of water and therefore close to freeze drying conditions. Addition of protective compounds to the drying matrix led to a reduced influence on bacteria survival for both freeze drying and vacuum drying. A critical residual water content was identified, below which bacterial survival decreases very strong with further removal of water. This critical water content is in the range of 15-20 percent.

Regarding the influence of a glassy state during drying, it was found that freeze drying of bacteria at conditions below the glass transition temperature of the drying matrix (with lactose) do not enhance survival. Similarly, it was found during storage that a glassy state is not decisive for a low vitality loss. However, a clear dependence of the inactivation rate constant on temperature *per se* as well as on water activity was found. Accordingly, separate effects of temperature and water activity have to be considered, resulting in different extents and types of detrimental reactions in the dried bacteria cultures.

Further chemical studies have illustrated a high complexity of the physical and chemical processes in dried bacteria cultures, covering both reactions within the bacteria cell, within the encapsulating matrix and interactions between the bacteria and the matrix. Two main processes have been investigated, namely oxidation and non-enzymatic browning. The rate, extent and type of these reactions depend on the humidity of the bacteria cultures during storage, and storage at low water activities (below 0.22) clearly improves the storage stability.

Damage (oxidation) and a possible leak of DNA from the bacteria cells are together with the coupling between radical formation, oxidation and browning reactions new perspec-tives, which deserves further attention in order to understand and improve the vitality loss in dried bacteria cultures.

Project Background and Aim

More and more research investigations confirm positive effects on health and well-being of several bacteria strains, the so-called probiotics [Naidu et al., 1999, Santosa et al., 2006][1], and probiotics are commonly distributed as dried products with the purpose of a subsequent addition to food items. Dairy products, such as yogurts or milk-based desserts, are frequently sold as such probiotic food items, and the market is increasing [Champagne et al., 2005][2].

The nature of the drying matrix for bacteria cultures is important in relation to stability of the bacteria during drying, transportation and storage, and for the level of active bacteria after rehydration. The drying process removes water and the matrix components serve to embed and protect the bacteria in the dried state, in which the bacteria may be able to survive in a dormant state and subsequent exert their positive effects when they are rehydrated [Champagne et al., 2005][2]. The consumers of probiotics require solid documentation of a high level of vital bacteria in order to obtain the associated health benefits, and the industry therefore face the challenge of defining the best way of preserving the vitality of the probiotic bacteria during production and storage.

The purpose of the "Vitality-project" has been through technological and chemical studies of probiotic bacteria cultures to improve the understanding of how the vitality of probiotic bacteria cultures in dried formulas is best preserved. The project has been organised as a co-operation between Central Institute for Food and Nutrition, Department Technology, Technical University München (TUM) and Department of Food Science, Faculty of Life Science, Copenhagen University (LIFE), to ensure an optimal combination of technological and chemical expertise. The experimental studies were planned to include optimisation of both freeze- and vacuum drying processes, and to focus on the influence of glass transitions, radical formation and influence of various matrix components etc. on the vitality of probiotic bacteria cultures through fermentation, drying and storage. It was the aim to obtain more fundamental information on physical and chemical reactions and interactions during drying and storage, as a basis on which new applications and formulations can be developed by the dairy industry.

Activities

A. Choice of probiotic bacteria cultures.
B. Technical studies: Fermentation of bacteria and treatment with various protectants. Freeze drying. Vacuum drying. Influence of protectants.
C. Chemical studies: ESR-spectroscopy. Calorimetry. Glassy states. Formation of radicals. Oxygen and prooxidants.
D. Interaction between technological and chemical studies.

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[1] Naidu *et al*. Probiotic spectra of lactic acid bacteria (LAB). *Crit. Rev. Food Sci. Nutr.***1999**, 38, 13-126

Santosa *et al*. Probiotics and their potential health claims. *Nutr. Rev.* **2006**, 64, 265-274.

[2] Champagne *et al.* Challenges in addition of probiotic cultures to foods. *Crit. Rev. Food Sci. Nutr.* **2005**, 45, 61-84

Results and Discussion

1. Technological Studies

Fermentation of bacteria

Figure 1 shows the course of the optical density (at 600 nm) as a measure of cell growth during fermentation on different sugars as substrate. MRS was used as growth medium with different sugars as carbon source.

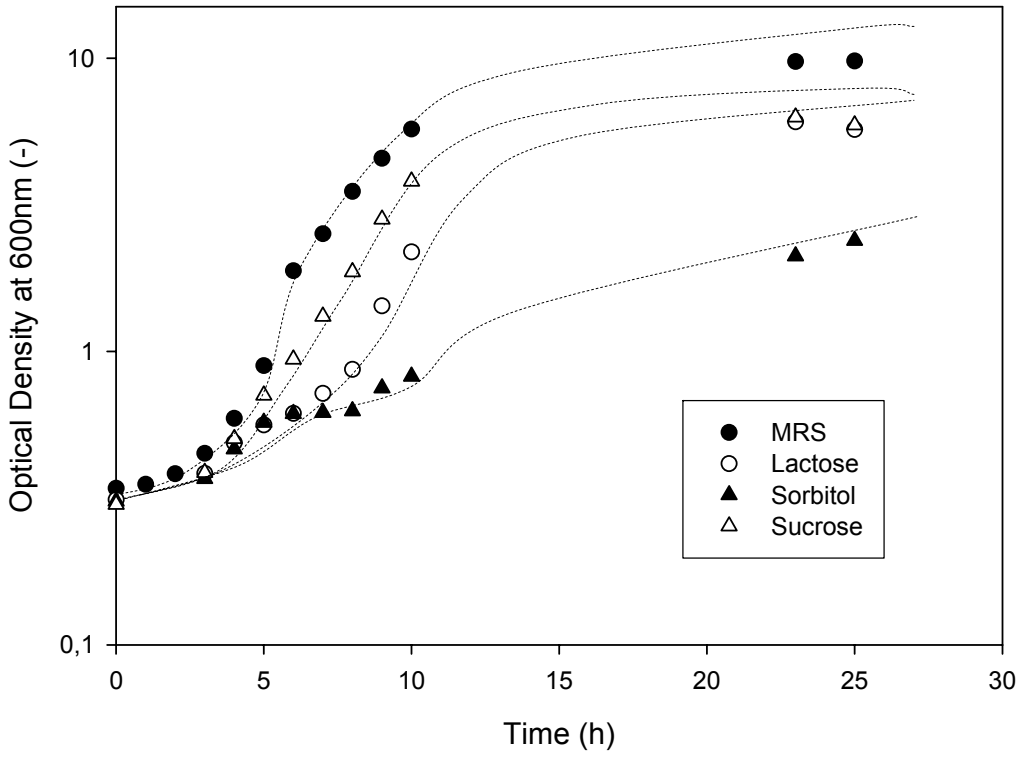

Figure 1: *Optical Density (600 nm) as a measure of cell growth during fermentation at a temperature of 37 °C with different sugars as substrate.*

It is obvious from Figure 1 that MRS with glucose as substrate leads to the fastest growth during fermentation and highest cell concentration at the end of fermentation. Furthermore, it can be seen that the exponential growth phase on MRS is finished after10 h. Next to different sugars as substrates, different growth media were investigated. It was found that the optical density in the stationary phase could be doubled (OD_{600nm}=18.8) when the MRS (with glucose as carbon source) was supplemented with additional yeast extract (10 g/l) and additional glucose (44 g/l). In order to test whether the best growth conditions also lead to the best survival rates after drying, drying experiments were carried out after fermentation with different media and process conditions. The influence of the fermentation medium on the survival with and without added protectant after vacuum drying (6 h, 15 mbar, 15 °C) is shown in Figure 2.

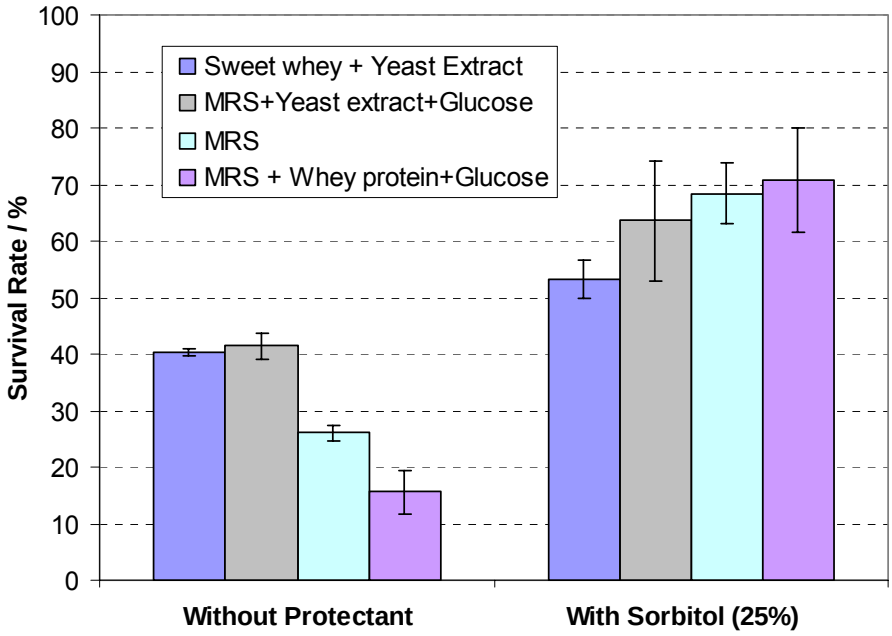

Figure 2: *Influence of fermentation medium on the survival with and without added protectant after vacuum drying (6 h, 15 mbar, 15 °C).*

It can be seen that the fermentation medium has a large influence on survival, and that the medium with the highest yield after fermentation leads to the lowest survival when no protectant is added. Furthermore, the influence of harvesting time, neutralising agent and fermentation temperature was tested with regard to survival after drying. Figure 3 shows the influence of the neutralising agent on survival after drying. Figure 4 shows the pH decrease during fermentation on MRS at 37 °C when no neutralisation is applied.

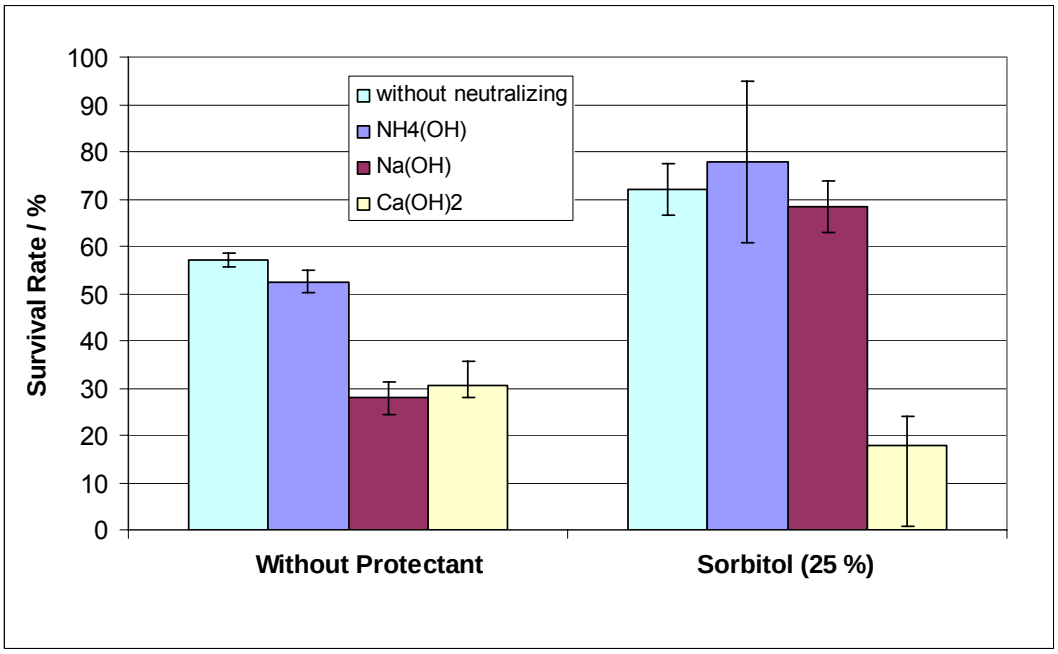

Figure 3: *Influence of neutralising agent on survival after vacuum drying (6 h, 15 °C, 15 mbar) with and without added protectant. Fermentation was carried out on MRS medium.*

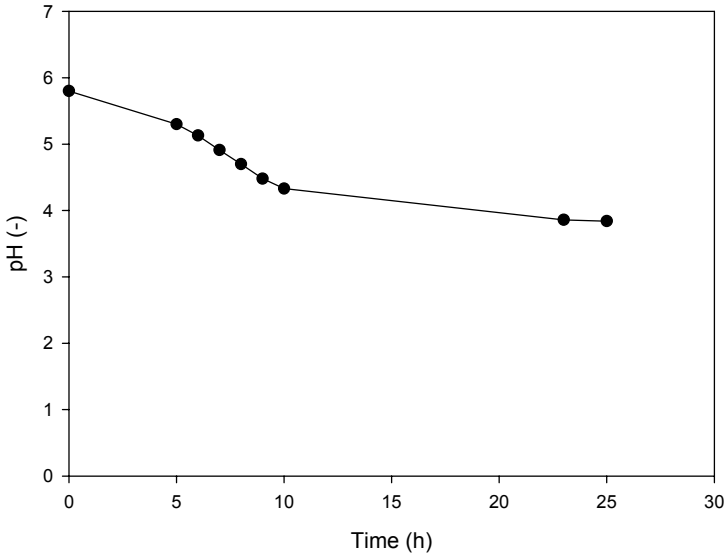

Figure 4: *pH during fermentation of Lb. paracasei ssp. paracasei at 37 °C on MRS.*

The decrease of pH is due to lactic acid production by *Lb. paracasei* ssp. *paracasei*. It can be seen that a pH of the cell suspension of 4.4 is achieved after 10 hours of fermentation. With regard to yield and survival after drying the best results were achieved on MRS when NH_4OH was used as neutralising agent or when no neutralising was applied.
Therefore, MRS was chosen as growth medium for the standard fermentation procedure for cell production. The fermentation was carried out without neutralisation and cells of the exponential growth phase were used for the drying experiments, accordingly, fermentation was stopped after 10 hours.

Freeze drying of cell suspensions with and without protective solutes and state diagram of cell / lactose suspensions

Freeze drying without protective solutes at different drying conditions

Freeze drying with and without protective solutes was carried out with the process conditions shown in Table 1.

Table 1: *Freeze drying conditions used for the freeze drying experiments (Higl et al. (2008))*

Condition / Variable	1	2	3	4	5	6	7
Chamber pressure p_K (Pa)	12	37	37	37	102	102	102
Sublimation temperature of pure water $T_{sub,w}$(°C)	-40	-30	-30	-30	-20	-20	-20
Shelf temperature T_{St} (°C)	-20	+50	+10	-10	+80	+10	-10
Term used for assigning the results $p_K / T_{sub,w} / T_{St}$	12/-40/-20	37/-30/50	37/-30/10	37/-30/-10	102/-20/80	102/-20/10	102/-20/-10

Figure 5 shows the survival of *Lb. paracasei* ssp. *paracasei* without protective solutes with dependence on residual water content X during freeze drying with different process conditions (Table 1).

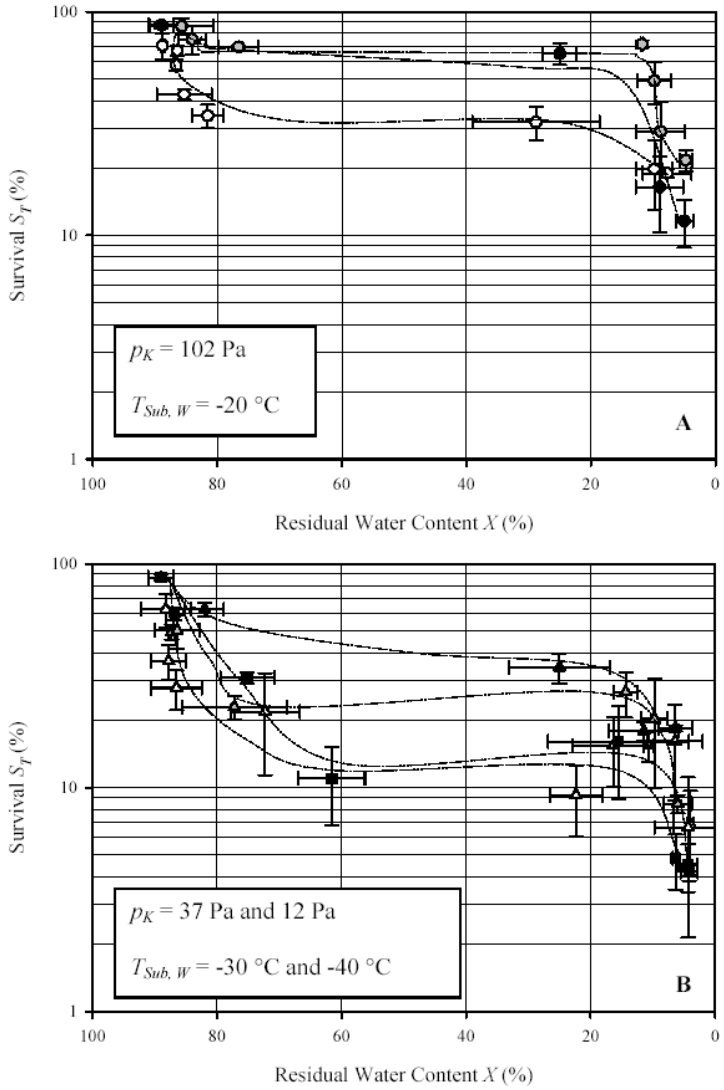

Figure 5: *Survival of Lactobacillus paracasei ssp. paracasei without protective solutes with dependence on residual water content X for different process conditions.*
A: $S_T(X)$ for process conditions 102/-20/80 (●),102/-20/10 (●),102/-20/-10 (○);
B: $S_T(X)$ for process conditions 12/-40/-20 (■), 37/-30/50 (▲), 37/-30/10 (▲), 37/-30/-10 (Δ).

It is shown that survival during freeze drying without protective solutes is dependent on process conditions. It is influenced by product temperature and residual water content. A higher product temperature (-20 °C) during sublimation is less detrimental to cell survival and leads to a higher amount of viable cells after freeze drying.

Freeze drying with lactose as protective solute and phase-state diagram of cell/lactose suspensions

The survival of cells was investigated during freeze drying at different process conditions with lactose as protective solute. With this sugar the importance of the glassy state of the protective solute for cell stability during freeze drying was determined.

Figure 6 shows the determined phase-state diagram for cell/lactose suspensions.

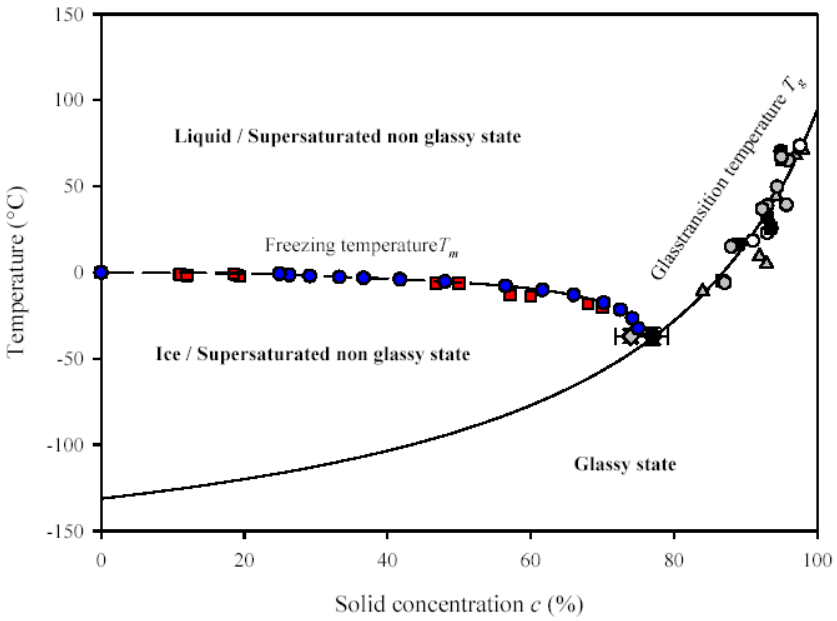

Figure 6: *Phase-State-Diagram of Lb. paracasei ssp. paracasei with lactose (0.25 related to dry matter of cells). Glass transition temperature was determined after freeze drying with conditions: 37/ -30 °C/+10 °C (▲), 102/-20 °C/+80 °C (●), 102/-20 °C/-10 °C (○), 12/-40 °C/-20 °C (■) (Higl et al. (2008)).*

Figure 7 shows the established phase-state diagram (see Figure 6) and additionally the product temperature of the cell/lactose suspensions during freeze drying with dependence on solid concentration for the process conditions 12/-40/-20 und 102/-20/80. The course of product temperature is only shown for these two process conditions as highest and lowest temperatures which were achieved during freeze drying. The product temperature achieved with all other process conditions proceeds between these curves.

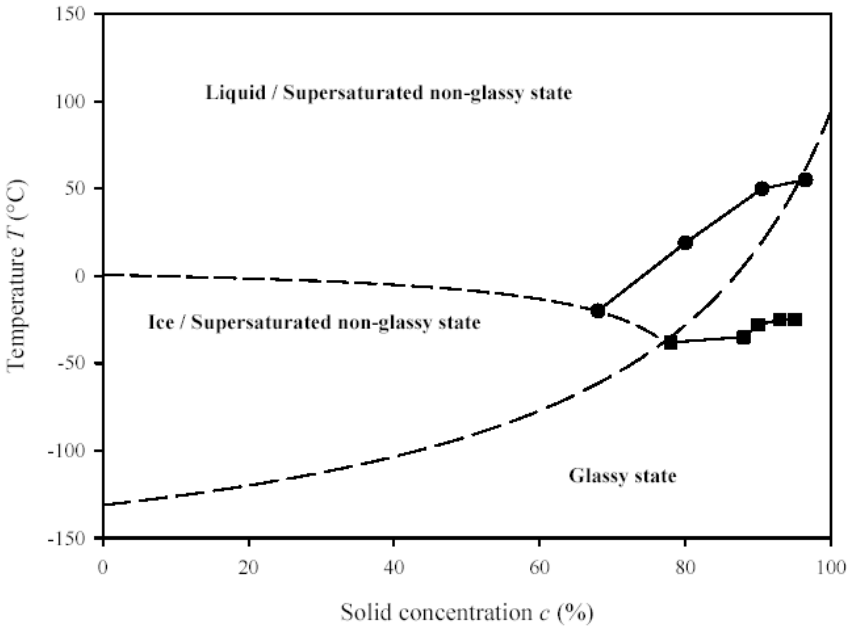

Figure 7: *Phase-State-Diagram and course of product temperature during freeze drying with drying conditions 102/-20/+80 (●) and 12/-40/-20 (■) (Higl et al. (2008)).*

It is obvious that the different process conditions lead to freeze drying of the cells in a matrix with different physical states (glassy or non glassy state).

Furthermore, survival of cells with lactose as protective solute was determined during freeze drying with different process conditions. Figure 8 shows the determined survival with dependence on residual water content *X*.

Figure 8 shows that survival with dependence on residual water content is the same for all investigated process conditions. For all process conditions survival remains at a high level until reaching a residual water content of 20 %. Below this value, survival decreases very strongly with further removal of water. Furthermore, it is obvious that freeze drying in the glassy state does not lead to higher survival of cells during and after drying.

Comparing the results of survival after freeze drying with lactose as protectant with survival after freeze drying without protective solutes, it can be concluded that survival is only increased by the addition of solutes at low product temperatures. At a high product temperature of -20 °C survival is not increased by the addition of lactose as protective solute. The same amount of viable cells after freeze drying can be achieved with and without protective solutes.

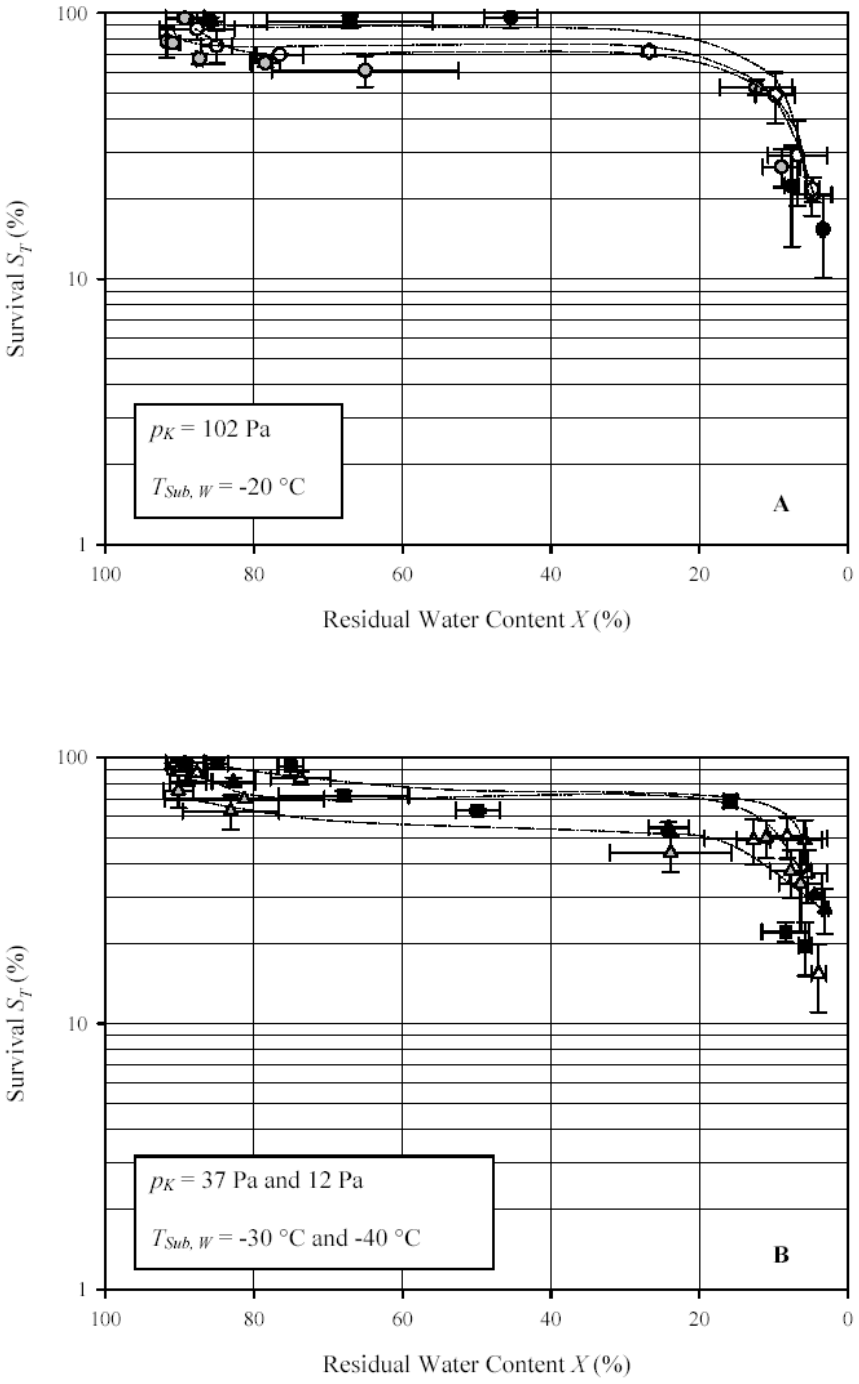

Figure 8: *Survival of Lactobacillus paracasei ssp. paracasei with lactose (0.25 related to dry matters of cells) with dependence on residual water content X for different process conditions.*
A: $S_T(X)$ for process conditions 102/-20/80 (●), 102/-20/10 (●), 102/-20/-10 (○);
B: $S_T(X)$ for process conditions 12/-40/-20 (■), 37/-30/50 (▲), 37/-30/10 (▲), 37/-30/-10 (Δ).

Vacuum drying of cell suspensions with and without protective solutes

Next to freeze drying, vacuum drying experiments were carried out in order to test the influence of an alternative drying method on survival and the role of encapsulation matrix. The following process conditions were applied (Table 2).

Table 2: *Process conditions applied for vacuum drying*

Cond.	Pressure /mbar	Temp/ °C	Drying equipment
1	15	15	Freeze dryer
2	25	30	Freeze dryer
3	25 (for 3h) then 10	25 (for 3h) then 40	Vacuum dryer

The process conditions were chosen such that the product temperature is moderate as in pre-experiments it was found that a high shelf temperature during drying (40 to 60 °C) leads to a low survival rate. Below room temperature the drying experiments had to be carried out in a freeze drying equipment (Gamma 1-20, Christ, Germany). The experiments were carried out without protectant and with sorbitol as protectant since pre-experiments revealed that sorbitol is the most effective protectant with regard to survival.
At first, the influence of concentration on survival was studied with cond. 3 as drying condition. The result is shown in Figure 9.

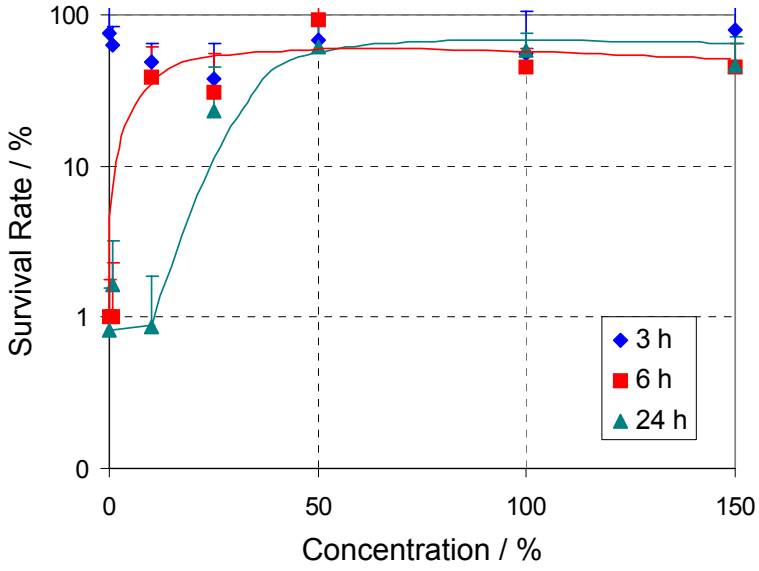

Figure 9: *Influence of sorbitol concentration on survival of L. paracasei ssp. paracasei after different vacuum drying times with cond. 3 as drying condition.*

Figure 9 shows that the effect of sorbitol is dependent on drying time and concentration. For short drying times; i.e. for high residual water contents sorbitol exerts no effect on survival. For high drying times; i.e. low residual water contents, a saturation concentration of sorbitol exists where maximum survival is achieved. The saturation concentration becomes larger when the drying time is increased. For all drying times, a concentration of 25 % is sufficient to protect the cells from drying-induced damages. Therefore in the following studies a concentration of 25 % was applied.

This result already gives a hint that the water replacement mechanism plays a role in protecting the cells from damages induced by vacuum drying. The more water is removed, the more sugar is needed to replace the hydration water around biomolecules that are essential for vitality (biomembranes and proteins). If water replacement plays the dominating role in stabilizing than the effect of sugar should mainly be visible at the end of drying when the residual water content is low and no excess water is present. The dependence of survival rate on residual water content is shown in Figure 10.

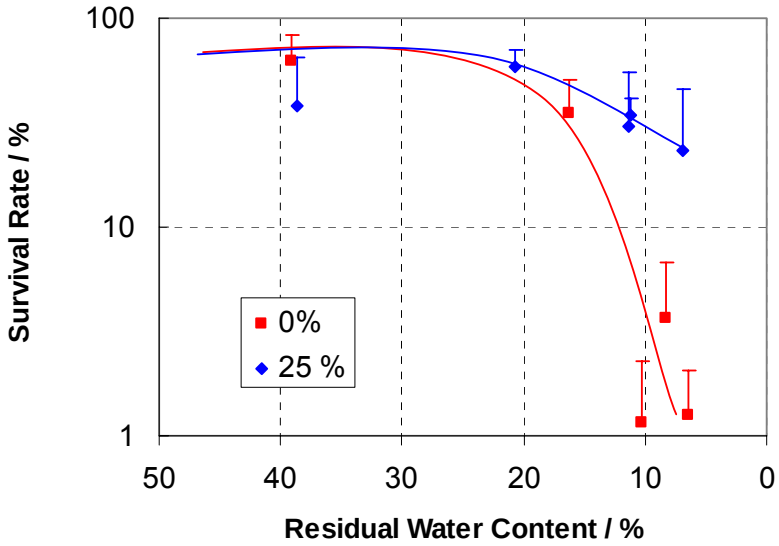

Figure 10: *Influence of residual water content on survival without added protectant (sorbitol, 25%) for process condition 3.*

Figure 10 shows that the protective effect only appears below a residual water content of about 25%. Below a RWC of about 25% the survival rate without protectant sharply decreases. The results show, that for vacuum drying water replacement seems to play a dominating role in protecting cells from drying induced damages.

The next study was to investigate the influence of drying conditions with and without added protectant on the survival. The results for the survival rate in comparison to the results achieved for freeze drying (Cond. 3 in Table 1) without protectant is shown in Figure 121.

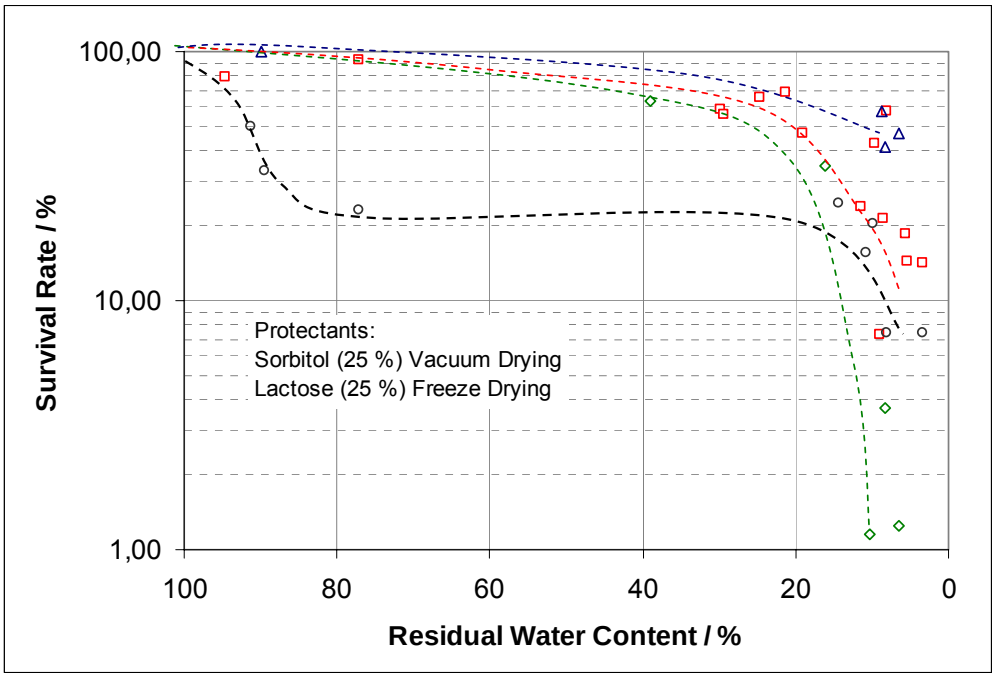

Figure 11: *Survival Rate after vacuum drying with different process conditions without protectants in comparison to freeze drying (Cond. 3 in Table 1). (Δ) Cond.1; (□) Cond. 2; (◊) Cond. 3; (○) freeze drying.*

Figure 11 shows, that without protectant the process conditions exert a large influence on survival. Like for freeze drying the survival rate is high for high residual water contents and decreases below a RWC of about 20 %. The process condition 1 (see Table 2) leads to a high survival also for low RWCs, which is even higher than the survival achieved after freeze drying. Cond. 3 leads to a sharp decrease in survival below a RWC of 20 %. This is due to the high product temperature (40 °C).

Figure 12 shows the influence of the process conditions when sorbitol as protectant is applied (filled symbols) in comparison to the survival rates without protectant (empty symbols). It is shown that the protectant plays no role for the mildest drying condition (Cond. 1) and shows the strongest influence for the most detrimental drying condition (Cond. 3). With protectant, the impact of process conditions is much weaker than without protectant. These findings are consistent with the results for freeze drying.

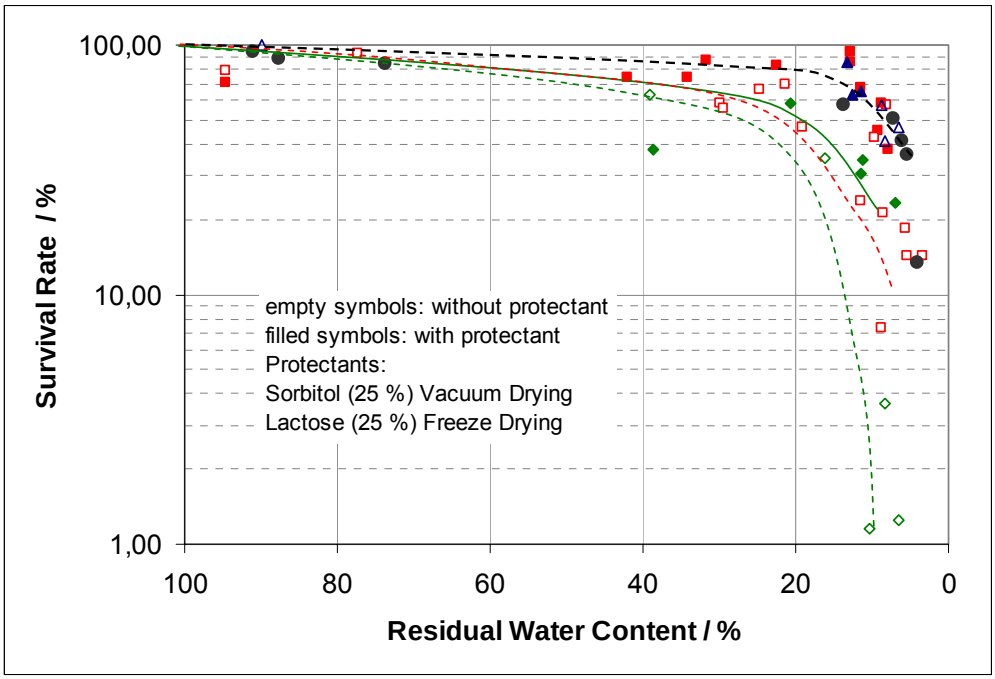

Figure 12: *Survival Rate after vacuum drying with different process conditions with protectants in comparison to freeze drying (Cond. 3 in Table 1). (▲) Cond.1; (■) Cond. 2; (♦) Cond. 3; (●) freeze drying.*

Storage stability of freeze dried *Lb. paracasei* ssp. *paracasei* in a lactose matrix at storage temperatures and a_w leading to storage in the glassy or non-glassy state

Figure 13 shows the determined glass transition temperature T_g of freeze dried cell/lactose suspensions for storage temperatures T_P of 4 °C, 20 °C and 37 °C with dependence on water activity. Furthermore, the storage conditions, for which cell stability was determined, are shown.

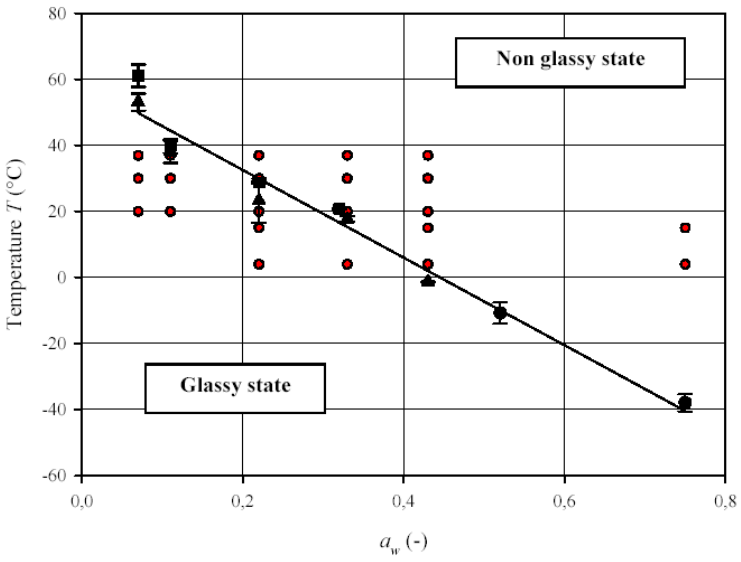

Figure 13: *Glass transition temperature with dependence on water activity of freeze dried cell/lactose preparations at the storage temperatures (●) 4 °C, (▲) 20 °C, (■) 37 °C. (●) Storage conditions T_P/a_w for investigation of cell stability (Higl et al. (2007)).*

For the different T_P/a_W conditions survival was determined during storage. It was obvious from the course of survival that the inactivation can be described by a first order reaction (Higl et al. (2007)). For comparison of cell stability at different storage conditions, the rate constant $k_{t,aw}$ of cell inactivation according to a first order reaction was determined.
The determined rate constants $k_{t,aw}$ of cell inactivation according to a first order reaction at storage temperatures of 4 °C, 15 °C, 20 °C, 30 °C and 37 °C are plotted as a function of water activity in Figure 14.

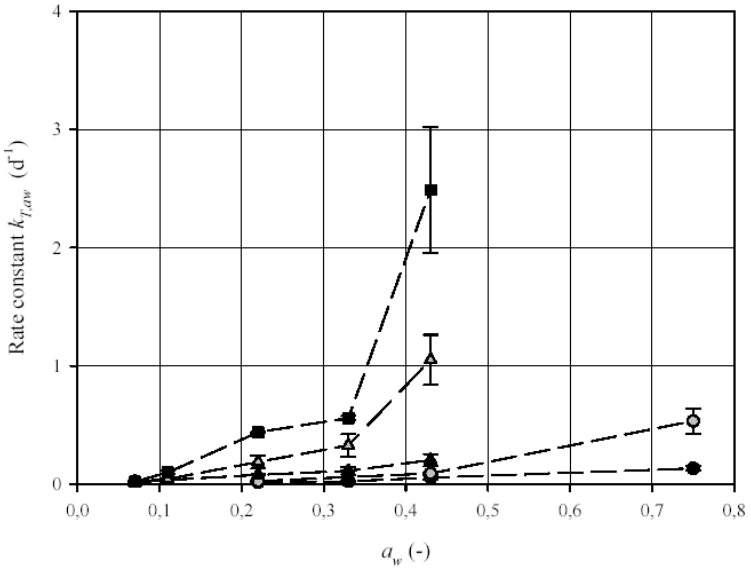

Figure 14: *Rate constant $k_{T,aw}$ for the inactivation of freeze dried Lb. paracasei ssp. paracasei in a lactose matrix with dependence on a_w for different storage temperatures per se: (●) 4 °C; (●) 15 °C; (▲) 20 °C; (▲) 30 °C; (■) 37 °C.*

Figure 15 shows the rate constant $k_{T,aw}$ as a function of the temperature difference (T_P-T_g) between temperature T_P and glass transition temperature T_g.

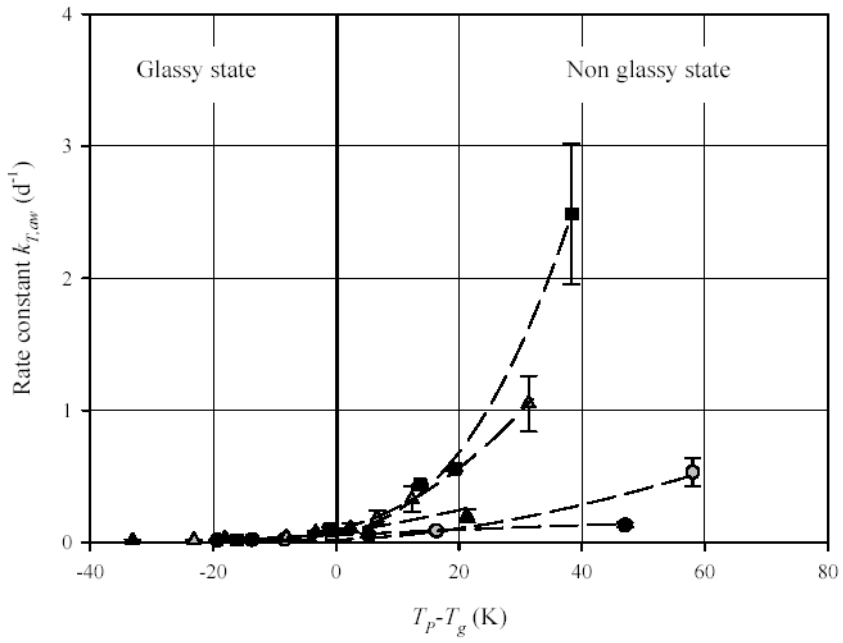

Figure 15: *Rate constant $k_{T,as}$ for the inactivation of Lb. paracasei ssp. paracasei in a lactose matrix with different a_w with dependence on temperature difference of product temperature and glass transition temperature (T_P - T_g) for different product temperatures per se: (●) 4 °C; (●) 15 °C; (▲) 20 °C; (▲) 30 °C; (■) 37 °C.*

It can be seen from Figure 14 that only slight differences between the rate constants at the different storage temperatures were observed for the two lowest water activities. For these conditions the freeze-dried system is stored in the glassy state (Figure 13). Likewise, for samples in the glassy state, inactivation is slower compared to samples in the non glassy state and only a small effect of water activity is observed for all temperatures. This is in contrast to samples in a non-glassy state where the rate constant, $k_{T,aw}$, is highly dependent on water activity at 30 °C and 37 °C, whereas at 4 °C, 15 °C and 20 °C, the water activity has only a modest effect on the inactivation rate constant. Thus, humidity has a much more deteriorative effect at higher temperatures.

In order to analyze whether the observed effect of water activity and temperature can be rationalized in terms of viscosity changes related to the glass transition is in agreement with: $\eta = f(T_P\text{-}T_g)$ and for rate constants with: $k_{T,aw} = f(T_P\text{-}T_g)$, the observed rate constants are shown as a function of (T_P-T_g) in Figure 15. If the rate constant could be rationalized by such a simple relationship, the curves corresponding to the different storage temperatures would coincide into a single master curve. This is clearly not the case for the data depicted in Figure 15. The glass-related viscosity changes can therefore not be used as an explanation for the observed changes in inactivation rate constant, $k_{T,aw}$. The glassy state is not the unifying mechanism that provides a simple explanation of the effect of water activity and temperature. Instead separate effects of temperature and water activity *per se* have to be considered.

Conclusions - Technical studies

In this study the inactivation of *Lb. paracasei* ssp. *paracasei* during lyophilisation and storage in the freeze dried state as well as after vacuum drying was investigated. It was the aim to determine the importance of process conditions and the addition of protective solutes to minimise the loss of viable cells during freeze drying and storage. Therefore, the protective effect in general with comparison to freeze and vacuum drying without protective solutes was investigated. For freeze drying, it was determined whether the glassy state is necessary for protection of cells during drying and storage. The conclusions are as follows.

Freeze Drying

Influence of process conditions on survival of bacteria during freeze drying with and without protective solutes

Inactivation of cells during freeze drying proceeds faster at low temperatures when the cells are dried without protective solutes. Low temperature during freeze drying is detrimental for cell stability. Accordingly, the optimal freeze drying process of cells without protective solutes exhibits a high drying rate and relatively high product temperatures. Inactivation of cells at low temperatures without protective solutes present can be due to a pH shift, an increased salt concentration and/or due to alleviated repulsive forces of hydrophobic groups of cell proteins. Accordingly, the cells are damaged partly previous to water removal. Therefore, dehydration has a more detrimental effect to cells at low temperatures compared to cells at higher temperatures.

The addition of lactose as protective solute leads to a reduced influence of drying conditions and a slightly higher survival at the end of drying but only for low product temperatures and low drying rates. The protective effect consists in a reduced premature damage of the cells and stabilisation of hydrated cell components (preferential hydration) at low temperatures and therefore in an increased dehydration tolerance of the cells.

With regard to the course of survival during drying with dependence on residual water content it was determined that the degree of cell loss is different in different drying stages. A critical residual water content exists below which cell survival decreases very strong with further removal of water. This critical water content is in the range of 15-20 %. The strong inactivation below this value occurs both with and without protective solutes. At this water content the structured water of biomolecules is probably removed and the used protective solute cannot replace it in order to avoid irreversible damage.

Importance of the glassy state during freeze drying and storage on cell stability

It was possible to establish a phase-state diagram for microorganisms in a lactose matrix. On the basis of this diagram process conditions for freeze drying and storage could be chosen which lead to drying and storage in the glassy or in the non glassy state.

The hypothesis that freeze drying conditions below the glass transition temperature lead to a higher survival of the cells at the end of drying cannot be confirmed by the results from these studies. It was shown that freeze drying of cells in a lactose matrix leads to identical courses of survival during freeze drying at different process conditions, independent from a product temperature above or below the glass transition temperature. The protective effect of lactose does not rely on the glassy state.

During storage it was determined that survival decreases faster with higher storage temperature and higher relative humidity. For storage conditions which lead to the glassy state the inactivation rate constant $k_{T,aw}$ was lowest. That means that the glassy state is important for cell stability during storage. However, in contrast to the classical glass theory the rate constant $k_{T,aw}$ is not only dependent on the temperature difference between product temperature and glass transition temperature, T_P-T_g, in the non glassy state according to the Williams Landel Ferry equation. A clear dependence of $k_{T,aw}$ on temperature *per se* as well as on relative humidity was found. Accordingly, the glassy state is not the unifying mechanism as explanation of the effect of water activity and temperature. Separate effects of temperature and water activity *per se* have to be considered.

Vacuum drying

It was found that sorbitol exerts a protective effect and therefore increases survival after vacuum drying. Other protectants that were investigated led to a lower survival than sorbitol (results not shown). The protective effect depends on drying time and therefore on residual water content. For all conditions investigated, a concentration of 25 % was sufficient to exert a protective effect. The protective effect only appears below a residual water content of about 25 % giving a hint that water replacement plays the dominating role in stabilizing the cells. The glassy state has not been reached during vacuum drying as sorbitol has a glass transition temperature lower than 0 °C (results not shown). Therefore, glass transition does not play a role in stabilizing the cells during vacuum drying.

The process conditions exert a strong influence on survival when no protectant is present (similar to freeze drying). Especially drying at higher temperatures (40 °C) is detrimental for the cells. With protectants the influence of process conditions becomes much weaker and the survival is on a high level (comparable to freeze drying).

2. Chemical Studies

Storage experiments with different freeze-dried bacteria cultures were initially performed with commercially available cultures in order to obtain overall information on how different external parameters influence the bacteria survival, and to implement different methods of analysis for studying chemical and physical changes during storage.

For the first storage experiments the bacteria cultures *Lactobacillus casei ssp. paracasei* (F19) and *Lactobacillus casei* (KE01) were used. Both cultures were provided by Medipharm AB. The main conclusions to be drawn from these initial experiments were the following:

- The F19 culture is very stable and therefore not optimal for fundamental studies of parameters influencing the storage stability of the dried bacteria. Only minimal losses of viability were detected in storage periods up to 12 weeks.

- The KE01 culture is much more unstable, but the received preparations were very different, with some of them being extensively moist and discoloured.

- Both the F19 and the KE01 cultures are unusable for analysis of radical formation during storage by use of ESR spectroscopy. This was most probably due to manganese residues in the cultures giving rise to high absorption in the ESR cavity and very broad and intense peaks (assigned as Mn(II) complexes) in the obtained ESR-spectra. These manganese spectra totally overlapped the (possible) ESR signals from organic radicals, and it was impossible to measure or follow any radical formation related to oxidative processes in the dried bacteria cultures.

- Time-consuming plate-counting is not readily replaced by a faster method for vitality/viability assessment. An assay for measuring metabolic activity was tested (ability of the bacteria to metabolise a tetrazolium probe), but turned out to be less sensitive and reproducible than traditional plate counting (cell proliferation). Additionally, suitable alternatives to plate counting have not been published in the literature.

For further studies it was decided to work with model cultures. These cultures were produced by using fermented F19 or La-5 (*Lactobacillus acidophilus*) added to well-defined freeze drying matrices. Such bacteria cultures were now investigated in rather simple (but time-consuming) storage experiments, where the influence on bacterial survival of storage temperature, water activity and matrix composition was evaluated. Based on these studies, a strategy for further research on dried bacteria vitality was decided on. Figure 16 visualises dried bacteria samples from a storage experiment and pinpoints many of the physical and chemical reactions and processes, which were focused on in the subsequent investigations.

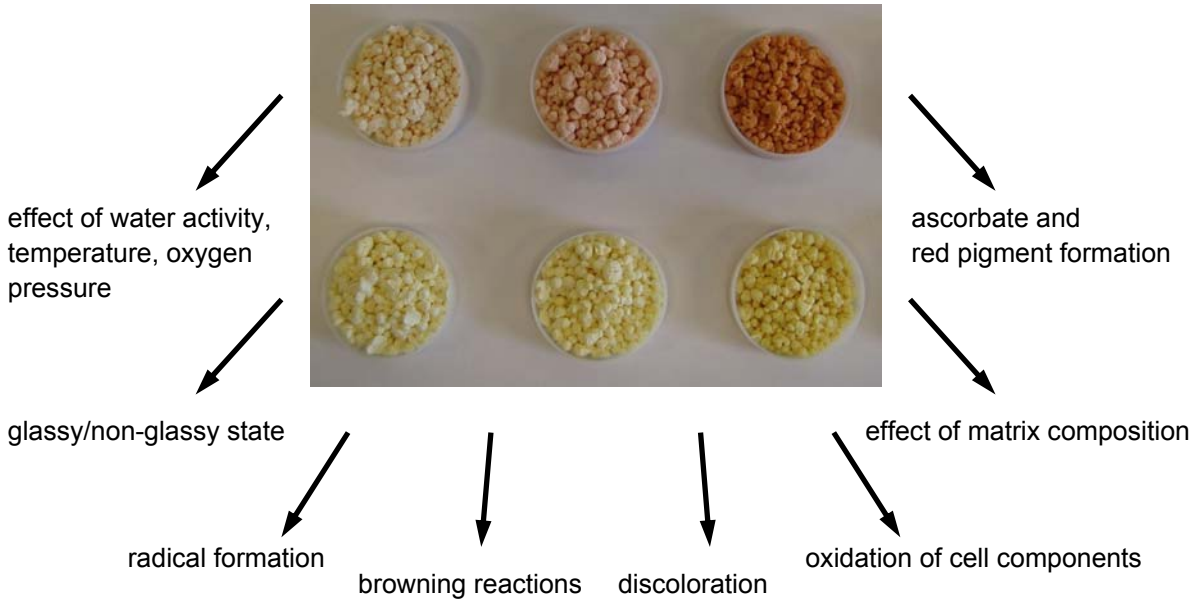

Figure 16: *Samples of Lactobacillus acidophilus freeze-dried in a matrix with sucrose and ascorbate (upper panel with ascorbate) and stored for 2 weeks at 30 °C. The water activity during the storage period was 0.11, 0.22 or 0.32 (increasing from left to right).*

Effect of water activity, temperature, oxygen pressure and ascorbate

Viability of the freeze-dried bacteria cultures was generally found to depend on both storage temperature, water activity and to a lesser degree on the matrix composition. Figure 17 shows results from a study on freeze-dried La-5, where also the effects of oxygen pressure and ascorbate as a matrix component were investigated (published in paper no. 6). Generally, increasing water activities decreased the bacteria survival as seen in Figure 17A, which shows the viability loss of freeze-dried La-5 stored at reduced oxygen level and varying water activities. It is seen that moving from storage at low moisture conditions (0.11 and 0.22) to higher moisture conditions (0.32 and 0.43) is accompanied with a significant viability loss, while from a_w = 0.32 an additional increase of a_w has only minor influence on the viability loss, and this tendency was the same for storage at atmospheric oxygen level and for samples including ascorbate in their matrices.

A clear indication of an involvement of oxygen in the destabilisation of the freeze-dried bacteria was obtained from storage at the reduced oxygen level, which significantly improved the storage stability of freeze-dried La-5 at all four levels of a_w. The detrimental effect of oxygen could, however, almost be eliminated by including ascorbate in the freeze drying medium, as it is illustrated in Figure 172B for bacteria survival during storage at 30 °C and a_w = 0.22.

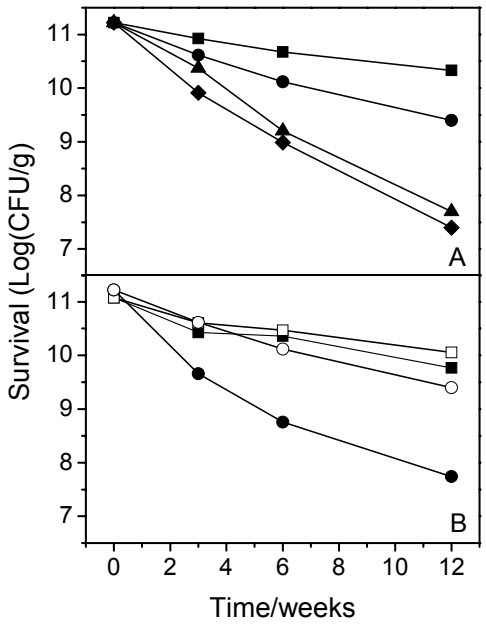

Figure 17: *(A) Survival of Lactobacillus acidophilus cultures freeze-dried in a sucrose matrix at 30 °C, < 4 % oxygen and varying water activities. (■) a_w = 0.11, (●) a_w = 0.22, (▲) a_w = 0.32, (◆) a_w = 0.43. (B) Survival of Lactobacillus acidophilus cultures freeze-dried in a sucrose/ascorbate or sucrose matrix at 30 °C, a_w = 0.22 and varying oxygen levels. (■) ascorbate, 20.9 % oxygen, (□) ascorbate, < 4 % oxygen, (●) no ascorbate, 20.9 % oxygen, (○) no ascorbate, < 4 % oxygen. From paper no. 6.*

The results show that application of La-5 to foods with elevated moisture content will be accompanied with a high and unacceptable loss of viability. Only storage at a_w 0.11 and 0.22 leads to acceptable survival levels after 12 weeks of storage, if viabilities in the order of 10^6-10^7 CFU/g are required after prolonged storage of more than 1 year (cf. Figure 17). Even at a_w 0.22 the viability loss is critical, and storage at very low a_w thus seem to be the most important issue for obtaining high storage stability of freeze-dried bacteria.

The stability at a_w 0.22 is significantly improved by excluding oxygen from interacting with the bacteria (Figure 17B), and a reduced oxygen level must be considered as being useful in cases where $a_w \sim 0.1$ or less cannot be achieved. For higher a_w 0.32 and 0.43, the positive effect of oxygen exclusion is not able to overcome the accelerated rate of bacteria viability loss, and application of freeze-dried bacteria to foods with elevated moisture content seems therefore to be a continuing challenge.

Incorporation of ascorbate in the bacterial matrix improved the storage stability of the freeze-dried bacteria, and the effect was slightly better than the effect of using reduced oxygen levels (Figure 17B). A severe limitation to the use of ascorbate in freeze-dried bacteria did, however, turn out to be pronounced discolouration of the dried bacteria samples. A pink/red colour quickly developed during storage of freeze-dried bacteria with ascorbate, even at a_w 0.11 and 0.22. This discolouration clearly hampers the application of ascorbate in commercial freeze-dried bacteria formulations, also at low moisture conditions.

Effect of glassy / non-glassy state of the freeze-dried bacteria cultures

Water activity and temperature are relatively easy to control during transport and storage, and an important step towards a deeper understanding of the complex processes leading to viability losses of dried bacteria was therefore to establish relations between water activity, storage temperature, and the physical state of the bacteria cultures.

The matrix around the individual bacteria is created by adding different compounds to the cell suspension before the drying process, and it is thus intended to protect the bacteria during drying and storage. Main components of the drying matrix are usually sugars, and thus it is possible to create an amorphous (glassy) matrix, in which the rate of chemical reactions may be limited due to a very high viscosity. As such, by creating a specific physical state, it should be possible to limit detrimental chemical reactions in the bacteria cultures during storage.

The physical state is influenced by the temperature and water activity (a_w) around the product, and the aim of the experimental set-up as published in paper no. 2 was to correlate the physical state, storage temperature and water activity to the survival of freeze-dried F19. Figure 18 shows the rate constant for inactivation of the bacteria as a function of water activity when stored at 20 or 37 °C. The a_w threshold values between glassy and non-glassy states are pointed out.

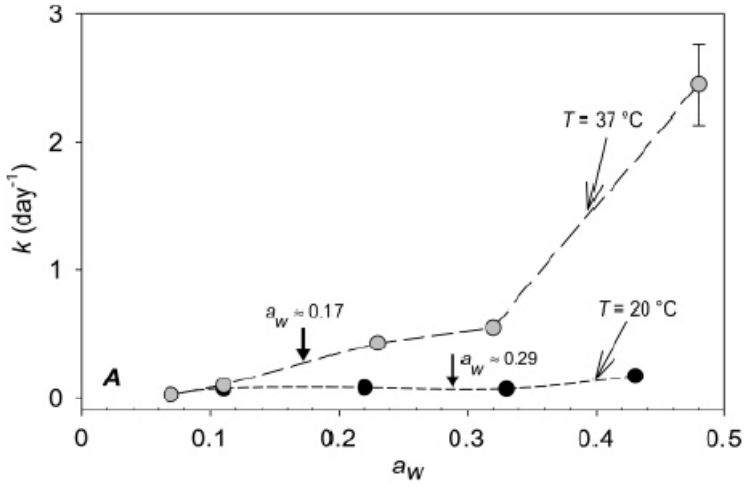

Figure 18: *Rate constant for inactivation of freeze-dried Lactobacillus casei ssp. paracasei at varying water activity and storage temperature. a_w corresponding to the temperature where the glass transition temperature (T_g) equals the storage temperature is marked by an arrow. From paper no. 2.*

As seen for the lowest a_w, there are only slight differences between the inactivation rates at 20 and 37°C. Below these a_w, the freeze dried cultures are in a glassy state. Likewise for all samples stored below the a_w threshold value, only small differences in inactivation rates are observed between the different water activities. In contrast, higher inactivation rates were observed when the bacteria culture was stored above the a_w threshold (non-glassy state), especially when both storage temperature and a_w were high.

However, as seen in Figure 18 the borderline between glassy and non-glassy state does not

fully explain the dependence of inactivation on a_w and temperature, and Tg could not be regarded as an absolute threshold of bacteria stability during storage.

In a parallel study with La-5 two different carbohydrate types (sucrose/ lactose) were used in the freeze drying matrix, and hence it was possible to distinguish between an effect of water activity and an effect of glass transition temperature on the survival of the bacteria. (For the results shown in Figure 18, a_w and T_g is coupled, and could not be varied independently, since only one type of matrix compositions was used). Two water activity-temperature state diagrams were constructed by use of differential scanning calorimetry analysis – one for bacteria freeze-dried with sucrose and one for bacteria freeze dried with lactose – see Figure 19.

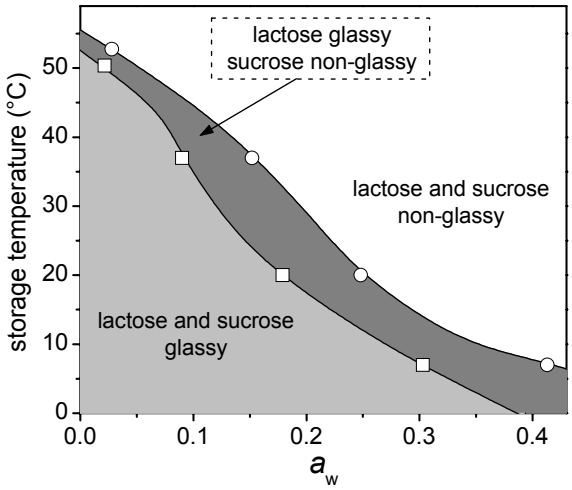

Figure 19: *Water activity-temperature state diagrams of Lactobacillus acidophilus freeze-dried in a sucrose or lactose matrix. (□) sucrose matrix, (○) lactose matrix. The experimental combinations of water activity and storage temperatures are connected to visualise the borders between glassy/non-glassy regions. From paper no. 5.*

For the bacteria samples with sucrose the border points between glassy and non-glassy state lie at even lower water activities than for the bacteria samples with lactose (see Figure 18). This result illustrates the influence of different carbohydrates on the physical state of dried bacteria cultures. To obtain a glassy state of bacteria samples with sucrose, an even lower a_w (or storage temperature) is required as compared to bacteria samples with lactose. The clear difference between the two a_w, T -state diagrams (and their deviation from the a_w, T - state diagram for pure lactose) also shows that in order to optimize production and storage conditions for different formulations of freeze-dried bacteria to obtain a glassy/non-glassy state, one single master a_w, T - state diagram for dried bacteria is not sufficient.

Nevertheless, based on the a_w, T - state diagrams established in the present study it is possible to predict the influence of the matrix compositions on the physical state, and the a_w, T - state diagrams in Figure 18, allowed us to investigate the relationship between physical state of a freeze-dried bacteria culture and the survival of the bacteria during storage. The following storage experiments showed that storage at the intermediate a_w (0.23), where the two types of samples according to the a_w, T - state diagram are in

different physical states, did not result in a protective effect of being in the glassy state. The inactivation rate of bacteria freeze-dried in a lactose matrix was higher for all the investigated a_w than was the inactivation rate of bacteria freeze-dried in a sucrose matrix.

It was concluded that the physical state of the dried samples did not have a dominating influence on the viability of the bacteria cultures, and establishment of a glassy matrix around the bacteria will not alone prevent the inactivation processes in the bacteria. Hence, obtaining a better stability of freeze-dried bacteria seems to depend on further identification of the chemical reactions leading to viability losses.

Radical formation and oxidation of specific cell components

The storage experiments with varying atmospheres showed that oxygen has a clear impact on the destabilisation of the freeze-dried bacteria (cf. Figure 17B). This detrimental effect of atmospheric oxygen was reduced by including ascorbate in the freeze drying medium, and upon analysis by ESR-spectroscopy free radicals were detected in the dried bacteria. Broad single-peak ESR spectra were obtained, with shape and g-values depending on the presence of ascorbate and the extent of browning. Ascorbyl radicals were detected in bacteria samples with ascorbate, indicating that ascorbate is involved in one-electron transfer reactions where radicals formed in the bacteria are scavenged. Such mechanism might be able to interfere with oxidation processes in the bacteria, which otherwise result in cellular damages.

A connection between oxidation processes and viability losses in dried bacteria is suggested several times in the literature. However, as only few studies have focused on which specific cellular components are oxidized, such a connection is still on its hypothesizing level. Both oxidative damages to the bacteria cell wall, the cell membrane, and DNA have been suggested.

In order to get a closer to an identification of chemical oxidation processes involved in bacteria inactivation, the use of an oxidation-sensitive fluorescent probe, C11-BODIPY$^{581/591}$, for investigation of lipid oxidation in dried biological membranes was optimised (presented in paper no. 8). Figure 20 shows detection of the probe in freeze-dried bacteria by confocal laser scanning microscopy.

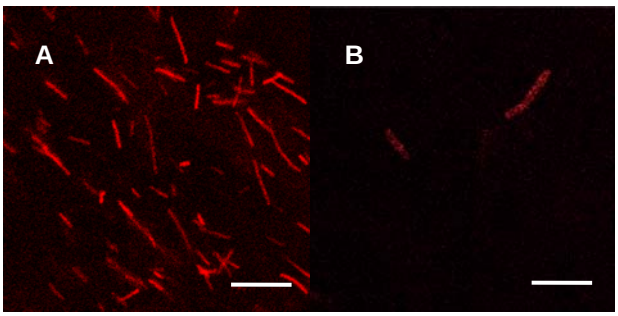

Figure 20: *Incorporation of C11-BODIPY$^{581/591}$ in freeze-dried Lactobacillus acidophilus (La-5). Incorporation is visualised in the dried state after storage at a_w 0.23 and 30 °C for 21 days. Scale bar equals 20 µm. From paper no. 8.*

The CLSM pictures show that the BODIPY probe is incorporated in the bacteria rods after the freeze drying process and storage for three weeks, and it can be visualized by analysis directly on the dried bacteria samples (A) as well as single cell imaging (B). No probe oxidation was detected after this relatively short storage, where a simultaneously loss of bacteria viability from $4.1 \cdot 10^8 \pm 0.3 \cdot 10^8$ cfu/g to $2.2 \cdot 10^8 \pm 0.3 \cdot 10^8$ cfu/g was observed.

Browning processes in dried bacteria cultures

Despite the clearly visible discoloration process in freeze-dried bacteria during storage (as seen in Figure 16), very few comments are made in the literature on the possible correlation between the browning processes and the unwanted loss of bacteria viability, which is occurring during storage. Also the specific kind of browning reactions involved in such bacteria inactivation remain unidentified.

In the current project, the discoloration was followed in various storage experiments with F19 and La-5, and it was found to increase with storage time and with increasing a_w and storage temperature. Further experiments were conducted in order to obtain more information on the origin of the discolouration, and these results are summarized and discussed in paper 11.

Figure 21 illustrates some of the results, as obtained by UV-spectroscopic analysis of supernatants from centrifuged, re-suspended, freeze-dried bacteria, and as seen the initial UV spectrum (storage time zero) had a clear absorbance maximum at ~ 260 nm, a maximum which is characteristic for nucleic acids from e.g. RNA and DNA. As the storage proceeded it was observed that the UV-absorption increased and that the spectrum is red-shifted. This shift allows several explanations, and among those both formations of HMF or oxidation mediated DNA damage are discussed in paper no 11.

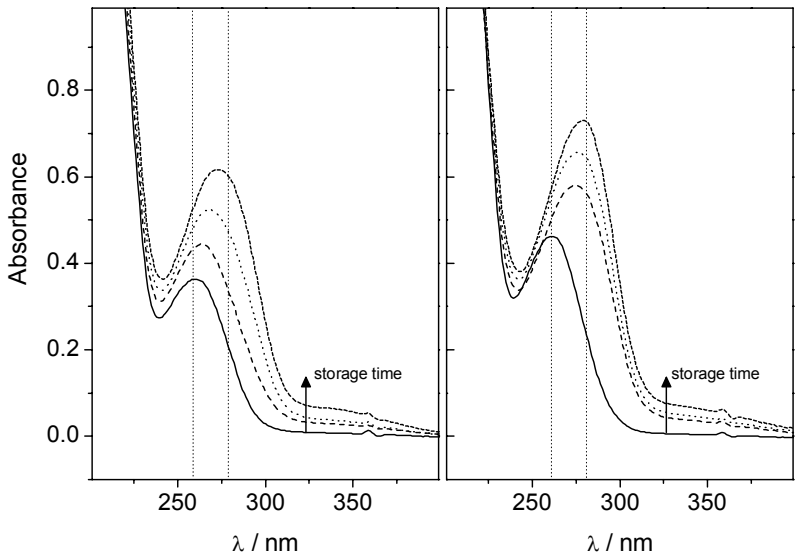

Figure 21: *UV-spectra of supernatants from re-suspensions of freeze-dried L. acidophilus in sucrose/maltodextrin matrix (left panel) or lactose/maltodextrin matrix (right panel). Samples were stored at 30 °C and a_w 0.22 and taken out for measurements at week 0, 2, 4 and 9. Vertical lines indicate λ_{260} and λ_{280} in each panel. From paper no. 11.*

At present, the conclusion on the correlation between browning processes and bacteria inactivation during storage in the dried state is that the browning of freeze-dried bacteria seems to be related to various types of browning reactions, whose dominance depend on a_w and to a lesser degree on the composition of the freeze drying matrix. The discoloration in bacteria samples is most likely related to various types of browning reactions, including carbonyl-protein (or carbonyl-DNA) interactions and carbohydrate condensation/ polymerisation (without involvement of proteins). The latter may at low a_w proceed mainly upon hydrolysis of the peptidoglycan layer in the bacteria cell wall. One single type of browning reaction can therefore not be related to bacteria cell death, and losses of viability seem to involve both oxidation reactions, browning reactions and the physical stability of the bacteria membrane/cell wall, and a further outlining of the correlation between these requires further investigations.

Peptides as antioxidants and carbonyl quenching compounds in biological membranes

A range of peptides were subjected to *in vitro* analyses with the purpose of determining their antioxidative mechanisms, and methods were selected to express antioxidative activity at different stages of the oxidation process. This set-up, covering the ability of peptides to interfere with the lipid oxidation chain reaction as well as to protect proteins from direct and indirect oxidation, provided basis for a detailed understanding of the antioxidative mechanisms of the peptides.

The study (which is presented in paper no. 10) showed that the efficiency of peptides as radical scavengers as well as their function as lipid oxidation chain-breaking antioxidants is rather low (see Figure 22), and indicated that earlier studies on antioxidative activity of peptides should have included relevant comparisons of the obtained efficiencies to those of more well-documented antioxidants. Also irrelevant high dosages of peptides, as used in many studies, may have led to misinterpretations of the antioxidative mechanisms of peptides. It was shown that the effect of peptides towards oxidation in biological membrane systems is mainly due to a protection of vital proteins from being oxidatively modified. The protection is obtained through a prevention of lipid oxidation derived carbonylation (indirect oxidation) and through interference with aqueous radical species (direct oxidation), and it is only achieved if the peptides are present in sacrificial concentrations.

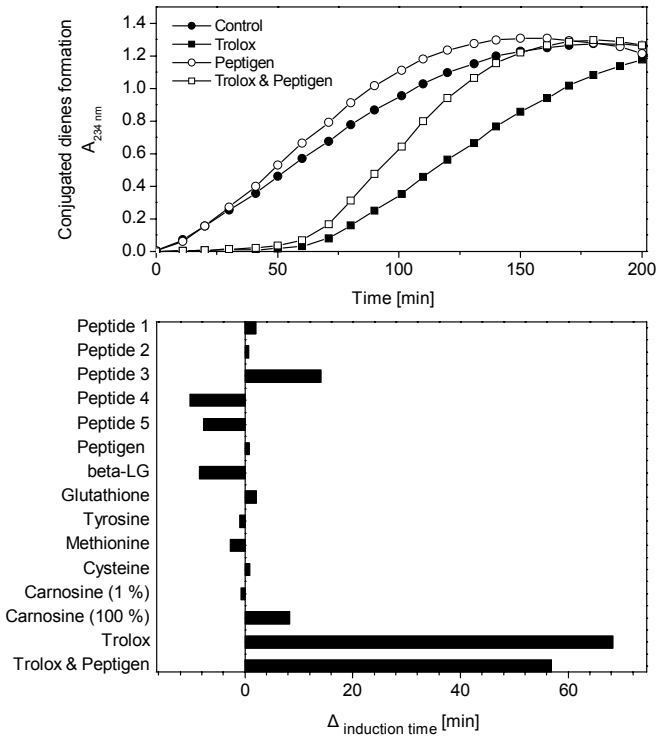

Figure 22: *Upper panel shows conjugated dienes formation in a peroxidizing liposome model system containing 150 µM lipid, 0.75 mM AAPH, and 1.5 µM of either Trolox or Peptigen, or 1.5 µM Trolox and 1.5 µM Peptigen in combination. Lower panel shows induction time for samples containing similar concentration as above and calculated as stated in eq. 4. Carnosine was additionally added in 150 µM concentration. Negative* $\Delta_{induction\ time}$ *indicates that the sample is prooxidative, meaning that the induction time is shorter than a control sample without antioxidant. From paper no. 10.*

Conclusions for the “Vitality” Project

1. Technical Studies

Inactivation of *Lb. paracasei* ssp. *paracasei* during lyophilisation and storage in the freeze dried state as well as after vacuum drying was investigated. It was the aim to determine the importance of process conditions and the addition of protective solutes to minimise the loss of viable cells during freeze drying and storage.

For both freeze and vacuum drying, survival depends on process conditions when dried without protectants. Accordingly, the optimal freeze drying process of cells without protective solutes exhibits a high drying rate and relatively high product temperatures. For vacuum drying the optimum product temperature lies close to the triple point of water and therefore close to freeze drying conditions.

The addition of protectants leads to a reduced influence of process conditions on survival for both freeze drying (lactose as protectant) and vacuum drying (sorbitol as protectant).

With regard to the course of survival during drying with dependence on residual water content it was determined that the degree of cell loss is different in different drying stages. A critical residual water content exists below which cell survival decreases very strong with further removal of water. This critical water content is in the range of 15-20 %.

Regarding the influence of the glassy state during drying, it was found out that freeze drying conditions below T_g of the lactose matrix do not enhance survival. However, the glassy state may also be important during storage of cells. In contrast to the classical glass theory the inactivation rate constant is not only dependent on the temperature difference between product temperature and glass transition temperature, T_P-T_g, according to the Williams Landel Ferry equation. A clear dependence of the inactivation rate constant on temperature *per se* as well as on relative humidity was found. Accordingly, the glassy state is not the unifying mechanism as explanation of the effect of water activity and temperature. Separate effects of temperature and water activity *per se* have to be considered. Further experiments on the impact of glassy state on basic reactions leading to the inactivation of the cells are needed.

2. Chemical Studies

It is well-documented in the literature that matrix encapsulation of probiotic bacteria during drying has a significant influence on the stability of the bacteria in the subsequent storage period. In terms of selecting specific matrix components in order to obtain the best storage stability, the literature is more inconclusive, and no superior matrix compound has been identified. The chemical studies performed as a part of this project have supported these observations. In accordance with this, an encapsulation of the bacteria in a protective glassy state (due to selection of certain matrix carbohydrates) was found not to be decisive for the vitality loss during storage.

These results point at a high complexity of the physical and chemical processes in dried bacteria cultures and on the importance of indentifying these processes in order to establish procedures for obtaining a better stability of the bacteria cultures. The chemical processes taking place include both reactions within the bacteria cell, within the encapsulating matrix and interactions between the bacteria and the matrix. The chemical studies have focused on two main processes, namely oxidation and non-enzymatic browning. The rate, extent and type of oxidation and non-enzymatic browning depend on the humidity of the bacteria cultures, and storage at low water activities (below 0.22) clearly improves the

storage stability, but also severely limits the application possibilities of the bacteria cultures. The following main observations were obtained:

- A reduced oxygen level during storage improves the storage stability, especially at a_w < 0.22, and strongly indicate that oxidation reactions are involved in bacteria destabilisation.

- Detection of membrane lipid oxidation directly on the dried bacteria samples is possible by means of incorporated fluorescent probes.

- Discoloration of bacteria cultures (clearly visible at a_w > 0.22) is due to non-enzymatic browning processes, including both carbohydrate-protein interactions and carbohydrate condensations.

- Radical formation and discoloration in the dried bacteria cultures point at oxidation and non-enzymatic browning as being correlated processes.

- Leakage and oxidation of bacterial DNA during storage is indicated, and is increasing with increasing viability losses.

- Protection against viability losses during storage by including small compounds such as ascorbate and peptides in the freeze drying matrix seems to be dosage-related, with sacrificial concentrations (1:1 mole ratio in relation to lipids) being necessary. Ascorbate will, however, lead to red discoloration of the dried cultures.

Perspectives – Significance to the Dairy Industry

Several types of products based on freeze dried lactic acid bacteria are of increasing importance for the dairy industry. These include: (i) dried milk powder mixtures to be sold in countries with lack of facilities for refrigeration, and to which water is added for over-night fermentation to produce yoghurt-type products, (ii) biscuits for long-term storage for emergency rations containing probiotic bacteria, which are becoming active upon ingestion, and (iii) various types of probiotic semi-dry foods like candy bars or powders to be added to drinks or deserts. Some of these products are high-priced, and product development is expected to result in new types of products in the years to come.

The current project has outlined some principles for optimal drying procedures and for use of the sugar alcohol, sorbitol, as addition prior to drying. Moreover, it is concluded that the glassy state as such is not securing optimal storage conditions for survival of probiotic bacteria, but that the storage temperature and water activity also have to be considered. Furthermore, it is found that a non-reducing sugar is a better protector of the bacteria than a reducing sugar with a lower glass transition temperature. Future research should accordingly look for non-reducing sugars with low glass transition temperatures to obtain optimal vitality of the dried bacteria formulations.

The project provides a wealth of information on specific effects of various additives and procedures, which readily could find practical use by the industry. Examples are the importance of reduced oxygen content in packages and the use of ascorbate as additive for products for which a red discoloration is acceptable.

Publications and Presentations

Publications in international journals

1. Först P, Kulozik U (**2007**). Validation of a novel *in-situ* weighing system during vacuum drying. *Drying Technology* 25, 767-773.

2. Higl B, Kurtman L, Carlsen CU, Ratjen J, Först P, Skibsted LH, Kulozik U, Risbo J (**2007**). Impact of water activity, temperature and physical state on the storage stability of *Lactobacillus paracasei* ssp. *paracasei* freeze-dried in a lactose matrix. *Biotechnology Progress* 23, 794-800.

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

4. Higl B, Santivarangkna C, Först P (**2008**). Bewertung und Optimierung von Gefrier- und Vakuumtrocknungsverfahren in der Herstellung von mikrobiellen Starterkulturen. CIT 80 (8), 1157-1164.

5. Kurtmann L, Carlsen CU, Skibsted LH, Risbo J (**2009**). Water activity-temperature state diagram of freeze-dried probiotic bacteria cultures (*Lactobacillus acidophilus*). Influence of physical state on bacteria survival during storage. *Biotechnology Progress* 25, 265-270.

6. Kurtmann L, Carlsen CU, Risbo J, Skibsted LH (**2009**). Storage stability of freeze-dried *Lactobacillus acidophilus* (La-5) in relation to water activity and presence of oxygen and ascorbate. *Cryobiology* 58, 175-180.

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9. Först P, Reitmaier J, Kulozik U (**2008**). The role of sorbitol for the molecular mobility and survival of vacuum dried preparations of *L. paracasei* ssp. *paracasei* (submitted).

10. Jongberg S, Skibsted LH, Carlsen CU (**2009**). Mechanisms of peptides as antioxidants and carbonyl quenchers in biological membranes (submitted).

11. Lone Kurtmann, Skibsted LH, Carlsen CU (**2009**). Browning of freeze-dried probiotic bacteria cultures and its relation to the loss of viability during storage (submitted).

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1. Carlsen CU, Skibsted LH (**2004**). Hvordan bevares vitaliteten af probiotiske bakterier i tørrede mejeriprodukter? *Mælkeritidende* 8, 193-197.

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1. Först P, Kulozik U (**2004**). Validation of an *in-situ* microbalance technique and continuous measurement of sugar solutions during vacuum drying. 2nd International Symposium on Spray Drying of Milk Products, Cork, Ireland, 19-21 October 2004.

2. Först P, Kulozik U (**2005**). Enhanced Survival of *Lb. paracasei* after vacuum drying – role of process parameters and protective compounds. IDF World Dairy Summit, Vancouver, Canada, 19-22 September 2005.

3. Higl B, Först P, Kulozik U (**2005**). Einfluss von Prozessparametern und Schutzstoffen auf die Überlebensrate von *Lactobacillus paracasei ssp. paracasei* bei der Gefriertrocknung. GVC Tagung, Fachausschuss Lebensmittelverfahrenstechnik, Berlin, Germany, 8-9 March 2005.

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6. Först P, Reitmaier J, Kulozik, U (**2006**). Importance of Drying Matrix and Mechanism of Protection of Probiotic Lactic Acid Bacteria during Vacuum Drying. In: *Drying 2006*, Farkas, I. Gödöllő: Szent István University Publisher, Vol. B, 1103-10.

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1. Ratjen J (**2006**). Physical state and storage stability of freeze dried microorganism preparations at different storage temperatures and water activities (TUM/LIFE).

2. Reitmaier J (**2006**). Untersuchungen zum Einfluss der Schutzstoffkonzentration und den Schutzmechanismen auf Überlebensrate von Lactobacillus paracasei ssp paracasei nach der Vakuumtrocknung (TUM).

3. AlBesharat R (**2006**). Investigations on the influence of fermentation conditions on the survival of probiotic cultures after drying (TUM).

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6. Dennery G. (**2008**). Assessment of the relation between the Maillard reaction and the death of freeze dried probiotic bacteria during storage (LIFE/ENITIAA, Nantes).

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2. Usinger J (**2006**). 1H-NMR Messungen an mit Schutzstoffen getrockneten Liposomen (TUM).

3. Hoff S (**2006**). Indkorporering af oxidationsfølsom fluorescerende probe i et bakteriesystem (LIFE).

4. Kamstrup MH (**2007**). Radicals in dry foods (LIFE).

5. Kisbæk M, Dedenroth SE (**2007**). Maillard reaktioner og vitalitet af frysetørrede probiotiske bakterier (LIFE).

6. Lindkvist M (**2009**). Stabilitet af frysetørrede probiotiske bakteriekulturer. Indflydelse af matrix på stabilitetsforringende reaktioner (LIFE).

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