Impact of environmental factors on viability and stability and high pressure pretreatment on stress tolerance of *Lactobacillus rhamnosus* GG (ATCC 53103) during spray drying

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## KURZFASSUNG

Es ist aus der Literatur bisher bekannt, dass die gesundheitsfördernde Wirkung von probiotischen Bakterien (u.a. Wiederherstellung und Stabilisierung einer ausgewogenen Darmflora, Verdrängung von Krankheitserregern und unerwünschten Bakterien im Darm, Beeinflussung des Immunsystems und Verbesserung der natürlichen Abwehrkräfte des Körpers, Senkung der Konzentration gesundheitsschädigender Stoffwechselprodukte und krebsfördernder Enzyme im Dickdarm, Verhinderung bzw. Verkürzung von Durchfallerkrankungen, usw.) nur mit lebenden Bakterien zu erzielen ist. Vorgeschlagen wird eine tägliche Dosis von mindestens $10^8$ lebenden Zellen, um einen gesundheitsrelevanten Effekt beim Verzehr probiotischen Produkts zu bewirken. Damit diese Lebendkeimzahl eingehalten werden kann, ist die Fähigkeit der probiotischen Bakterien in Lebensmitteln sowie bei technologischen Verarbeitungsprozessen bis hin zum Ende der Mindesthaltbarkeitsfrist sicherzustellen.

In dieser Hinsicht ist die Trocknung von probiotischen Bakterien zur Herstellung vom probiotischen Pulver (als Endprodukt oder als Halbfabrikat), die üblicherweise durch die Gefriertrocknung erfolgt, als ein kritischer Verarbeitungsschritt anzusehen. Da diese als sehr schonend geltende Trocknungsart allerdings zeitaufwendig und relativ kostenintensivr Prozess ist, wird in der letzten Zeit vermehrt untersucht, ob dieses Trocknungsverfahren durch Sprühtrocknung ersetzt werden kann.

Diese Arbeit befasst sich in erster Linie mit der Evaluierung der Anwendbarkeit von Sprühtrocknung als eine alternative Methode zur Herstellung von Probiotika enthaltenden Trockenpräparaten. Dazu wurden optimale Trocknungsbedingungen ermittelt und die verwendeten Schutzmedien auf thermo-physikalische Beschaffenheit und Wechselwirkung mit einem synthetischen Membransystem untersucht. Eine Trocknungstemperatur von 80 °C wurde als ein annehmbarer Kompromiss ermittelt. Bei dieser Temperatur ließ sich eine probiotische Pulverzubereitung auf Milchbasis mit einer Restfeuchte von 4 Prozent und einem Keimgehalt von ca. $10^9$ Zellen pro g herstellen. Der Auswahl des Trägermediums wurde eine große Rolle zugeschrieben, da dies das Überlebensverhalten während der Lagerzeit determiniert, was letztlich das Qualitätsmerkmal der probiotischen Produkte und somit die gesundheitsfördernde Wirkung vom Probiotikaverzehr beeinflußt. Obwohl partieller Austausch der Magermilch durch kommerzielle präbiotische Substanzen unter Beibehaltung einer konstanten Trägerstoffkonzentrationen keinen negativen Einfluss auf die unmittelbare Überlebensrate von *Lactobacillus rhamnosus* GG bei der Sprühtrocknung hatte, fand im Vergleich zu Magermilch eine stärkere Keimzahlreduktion in den beiden präbiotischen Kombinationspräparaten während der Lagerung bei 37°C statt. Desweiteren wurde festgestellt, dass das Vorliegen des Glaszustandes in dem Trägermaterial nur eine untergeordnete Rolle bei der Gewährleistung der Lagerstabilität der darin befindlichen Keime spielt. Die unterschiedliche Schutzwirkung der verwendeten Trägermaterialien müssen

vielmehr in der räumlichen bzw. chemischen Struktur der Schutzmoleküle liegen, die wiederum Einfluss nehmen auf die Art und Weise, wie sie mit Zellmembranen bzw. ihre Bestandteile in Wechselwirkung treten. Diese Sicht wurde durch weitere Arbeit mit der Trocknung von den als Modellsystem für Doppellipidmembran dienenden Liposomen im Beisein von Zuckermolekülen bestätigt. Ferner wurde zur weiteren Klärung über den herausragenden Schutzeffekt von Magermilch auf die Lagerstabilität getrockneter Bakterien der Einfluss von Milchproteinen untersucht. Es zeigte sich, dass bei der Lagerung die Keimzahlreduktion bei Bakterien in proteolytisch behandelter Magermilch wesentlich schneller erfolgte als die in nativer Magermilch.

Unterstützend wurde an einer analytischen Methode zur Charakterisierung der Zellschädigung während der Sprühtrocknung gearbeitet. Dazu wurde die durchflußzytometrische Analysenmethode verwendet. Dies benötigt entsprechende Färbungs- und Messungsstrategie, die im Vorfeld etabliert werden musste. Bei der Etablierungsphase wurde die Methode eingesetzt zur Beurteilung der Art und des Ausmaßes der zellulären Schädigung, die unter Anwendung verschiedener physikalischer Behandlung, wie beispielsweise Hochdruck-, Hitze-, oder Ultraschallbehandlung an den Zellen von *L. rhamnosus* GG herbeigeführt wurde. Dafür wurde die Farbstoffkombination Carboxyfluorescein-diacetat und Propidiumiodid angewandt. Es zeigt sich, dass während bei der Hitzebehandlung oberhalb 60°C der Verlust der Membranintegrität als primäre Ursache des hitzebedingten Zelltodes ausgemacht werden konnte, führte offensichtlich die irreversible Schädigung der membrangebundenen Enzymsysteme zum hochdruckinduzierten Zelltod. Anders als die hitzegetöteten Zellen bestand die Population der druckinaktivierten Zellen hauptsächlich aus Zellen, die noch intakte Membranen und aktives intrazellüläres Enzym besitzen. Hinsichtlich der Inaktivierung von *L. rhamnosus* GG während Sprühtrocknung führten den Ergebnissen zufolge höhere Temperaturen während der Trocknung zu stärkeren Schäden in der Zellmembran, welche als die Hauptursache für die beobachtete Inaktivierung identifiziert werden kann.

Letztlich wurde die Eignung der Hochdruckvorbehandlung zur Verbesserung der Hitzestabilität von *L. rhamnosus* GG überprüft. Perspektivisch ließe sich diese Art von Vorbehandlung einsetzen, um die probiotischen Keime mit Hilfe ihrer induzierbaren Abwehrmechanismen gegenüber der Hitze- und Dehydrationsstress bei der Sprühtrocknung resistenter zu machen. Es zeigte sich, dass durch Druckvorbehandlung die Keime kurzzeitig gegenüber letaler Hitze resistenter waren. Es wurde ebenfalls festgestellt, dass offensichtlich die als Reaktion auf die Druckvorbehandlung synthetisierten Stressproteine an der erhöhten Hitzeresistenz beteiligt sind. Anhand durchflusszytometrischer Analyse war es ersichtlich, dass diese Metaboliten die bakteriellen Membranen einen erhöhten Schutz vor hitzebedingter Schädigung verliehen.

## ABSTRACT

Current literature data suggest that the health-promoting effect of probiotic bacteria (re-establishment and stabilization of a balanced gut flora, prevention of pathogens outgrowth in the intestine, impact on the immune system, reduction of the concentration of toxic metabolites and carcinogenic enzymes in the large intestine, prevention of gastro intestinal diseases, etc.) is only achieveable upon consumption of living bacteria. A daily dose of at least $10^8$ living cells has been suggested to assure health-relevant effects following the consumption of probiotic product. To maintain this proposed number of living bacteria, the survivability of probiotic bacteria during processing as well as in food has to be guaranteed up to the end of the shelf life.

In this respect the drying process for the production of probiotic powder (as final or as semifinished product), which is generally performed by freeze drying can be considered as a critical processing step. However, since freeze drying is a time and cost intensive process, there is an growing interest on the application of spray drying.

This work deals primarily with the evaluation of the applicability of spray drying as an alternative method for the production of probiotic powder. Optimal drying conditions were determined and the protective media were characterized in terms of their thermophysical properties and capability of direct interaction with a synthetic membrane system. Spray drying at temperature of 80 °C allowed production of probiotic powder on skim milk basis with a residual moisture of 4% and a bacterial load of $10^9$ cells per g. The selection of the carrier medium is regarded crucial, since this medium determines the survival behavior during the storage, which in turn affects the quality criteria of the probiotic products as well as the declared health-promoting effect. Partial exchange of the skim milk solids with commercial prebiotic compounds did not have any detrimental effect upon spray drying. However, the inactivation of spray dried *Lactobacillus rhamnosus* GG during storage at 37°C was more pronounced when the bacteria were dried in prebiotic preparations compared to the ones dried in skim milk. Furthermore it was found that the formation of a glassy state contributed only little to the maintenance of storage stability. The different protective effect of the drying media applied was thought to be governed by the spatial and/or chemical structure of the protective compounds, which could facilitate a direct interaction with cell membranes and/or their components. This view was confirmed by further work regarding the drying of model phospholipid bilayers, i.e. liposomes in the presence of different types of sugar molecules. The influence of milk proteins was examined to further clarify the outstanding protection of bacteria dried in skim milk. It was shown that probiotic bacteria dried in proteolytically treated skim milk were inactivated faster than the one dried in native skim milk during storage.

Furthermore, work on flow cytometric analysis was conducted in order to allow the characterization of cell damage during spray drying. This required appropriate staining and

measurement strategy, which had to be established first. Initially, this method was applied to characterize cellular damage as affected by different physical treatments, including high pressure, heat, or ultrasound. It was shown that during heat treatment beyond 60°C the loss of the membrane integrity could be identified as the primary cause of heat-induced cell death. In contrast, the irreversible damage of the membrane-bound enzyme systems was responsible for high pressure-induced cell death. Compared to the heat-killed cells the population of the pressure-inactivated cells consisted mainly of cells, which still had intact membranes. With help of flow cytometric analysis it was demonstrated that upon spray drying, the higher level of inactivation of *L. rhamnosus* GG at higher temperatures was closely related to increased damage in the cell membrane.

The application of sub-lethal high pressure pretreatment was examined in terms of evaluating possible approaches for the improvement of heat stability of *L rhamnosus* GG. The rationale of applying this pretreatment is to take advantage of the inducible defense mechanisms of the organisms to make them more resistant against stresses related to spray drying, i.e. stress due to thermal exposure and dehydration. It was shown that pressure pretreated bacteria showed higher thermotolerance as compared to unadapted ones. Furthermore, it was found that apparently the stress proteins synthesized as response to the pressure pretreatment are involved in the increased heat tolerance. On the basis of flow cytometric analysis it was demonstrated that these proteins may have a protective effect on the bacteria membranes, which led to an increased protection against heat-induced damage.

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# 1 GENERAL INTRODUCTION

Technological aspects of the production of probiotic containing food products

## 1.1 Probiotic products

Probiotic products represent a strong growth area within the functional foods group and intense research efforts are under way to develop dairy and non-dairy products into which probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* species are incorporated. These bacteria represent a unique group of lactic acid bacteria which is claimed to benefit human health upon consumption, by improving the endogenous micro-flora of the gut, provided that a sufficiently high number of viable and highly functional cells are consumed regularly [1, 2]. Moreover, a general consensus regarding the importance of high levels of live microorganisms for probiotic products has already been highlighted [3]. The use of the word probiotic is therefore restricted to products which contain live microorganisms in an adequate dose in order to exert the desirable effects [4].

Microorganism, which is regarded to exert probiotic effect, is not exclusively lactic acid bacteria. For instance, the application of yeast species *Saccharomyces boullardii* as probiotic microorganism was regarded to be promising [5]. Moreover, spore forming bacteria, primarily of the genus *Bacillus* have also been studied and commercialized as probiotics for human and animal use [6]. Several species from the genus *Enterococcus* are also used as probiotics. However, in recent years safety concerns have been raised on their use as probiotics, since they have been associated with some serious infective diseases and with multi-resistance to antibiotics – especially concerning the transmission of these resistances to other strains [7].

When the application of probiotic is characterized in terms of the type of food products in which they are harboured in, yoghurt is certainly one of the mostly used food vehicle for probiotic consumption. Probiotic yoghurt was reported to account for 13% of all yoghurt sold in Germany [8], where in year 2003 as many as 1,5 million tons of yoghurt were produced and a yoghurt consumption per head of 15 kg was observed [9]. In Europe, the probiotic yoghurt market alone was estimated to be worth a value in the region of € 900 million [10] and made up approximately 65% of the total European functional food market [8].

Apart from yoghurt as the classical food vehicle, during recent years probiotics have been increasingly incorporated into non-dairy food system. Based on the water activity of the food products, where probiotics are incorporated in, there are two major food ecosystems, i.e. the ecosystem of dehydrated products with low aw-value and the one of products with intermediate to high aw-values. Trials already conducted to incorporate probiotic in different food systems are documented in Table 1.

**Table 1.**

Overview of works dealing with incorporation of probiotics into dairy or non-dairy products

## Low $a_w$ food product

| Food product | Organism | Reference |
|---|---|---|
| Oat-based cereal bar | *Bifidobacterium lactis Bb12* | [11] |
| Freeze-dried yoghurt | *L. acidophilus, B. bifidum, B. longum* | [12] |
| Spray dried skim milk powder with or without prebiotics | *L. paracasei, L. salivarius, L. rhamnosus* | [13, 14] |
| Dry sausage | *L. acidophilus, L. crispatus, L. amylovorus, L. gasseri, L. johnsonii, L. gallinarum, L. rhamnosus* | [15-18] |
| Dried fruits | *L. casei* | [19] |

## Intermediate to high $a_w$ food products

| Food product | Organism | Reference |
|---|---|---|
| Yoghurt | *L. acidophilus, B. bifidum, L. casei, B. breve, B. longum* | [12, 20-24] |
| Skim milk with prebiotics | *Bifidobacterium* | [25] |
| Soymilk | *B. infantis, B. longum* | [26, 27] |
| Low fat quark cheese | *L. acidophilus* | [28] |
| Infant formula | *B. bifidum, B. breve, B. infantis, B. longum* | [29, 30] |
| Tomato juice | *L. acidophilus, L. plantarum, L. casei* | [31] |
| Mayonnaise | *B. bifidum, B. infantis* | [32] |
| Cheese | *B. lactis, L. acidophilus* | [33-36] |
| Cheese-based dip | *L. acidophilus, L. paracasei, L. rhamnosus, B. animalis* | [37] |
| Ice cream | *B. longum, B. brevi, B. infantis, L. acidophilus, B. bifidum* | [38-43] |
| Frozen yoghurt | *L. acidophilus, B. bifidum* | [44-46] |
| Chocolate | *L. reuteri* | [47] |

### 1.2 Impact of probiotic on consumer's health – Mode of action and selection criteria

The probiotic concept was worked upon by Metchnikoff at the beginning of the century, who tried to link the health and longevity of Bulgarian peasant, who consumed large quantity of fermented milk, with the composition of their internal flora [48]. As a consequence, he suggested to manipulate the enteric flora in a beneficial way, i.e. by replacing the harmful microbes by useful microbes, so as to achieve health benefits in the host.

A huge number of reviews have summarized the health benefits in the ingestion of probiotics along with their potential and established modes of action in maintaining intestinal and urogenital health [1, 49-53]:

1. Aid in lactose malabsorption due to bacterial lactase activity
2. Protection against gastro-intestinal infections such as traveller's diarrhoea, infantile diarrhea, antibiotic-induced diarrhoea, inflammatory bowel diseases, *Helicobacter pylori* associated infections by
   - Competition for nutrients and adhesion sites on intestinal mucosa, which facilitate preferential colonization
   - Secretion of antimicrobial substances such as organic acids, hydrogen peroxide, bacteriocins, antibiotics and deconjugated bile acids, which prevent the outgrowth of pathogens
   - Formation of short-chain fatty-acids (e.g. butyric acid, propionic acid), which reduce gut pH and simultaneously serve as a substrate for colonic mucosa through
   - Attenuation of virulence
   - Blocking of toxin receptor sites
   - Stimulation of mucosal and systemic host immunity, either general (increased levels of cytokines, IgA, $\gamma$-interferon, increased phagocytic activity) or specific response (specific antibodies to certain pathogens)
   - Suppression of toxin production
3. Suppression of cancer by
   - Mutagen binding
   - Deactivation of carcinogen and/or procarcinogen
   - Inhibition of carcinogen-producing enzymes of colonic microbes
   - Inhibition of tumour formation and proliferation
4. Reduction of the risk of coronary heart disease as a consequence of
   - Interference with cholesterol absorption from the gut
   - Direct assimilation of cholesterol
   - Production of metabolites that affect the systemic levels of blood lipids
5. Prevention of urogenital infections by
   - Adhesion to urinary and vaginal tract cells

- Colonization resistance
- Production of inhibitors ($H_2O_2$, biosurfactant)

6. Alleviation of constipation
7. Improvement of the nutritional value of foods

As documented by the aforementioned overview, the types of health-associated effects upon probiotic consumption are relatively broad. Furthermore, apart from the strain dependency of probiotic effect, the efficacy of probiotic consumption for prophylactic or therapeutic use can be different, if other characteristics of the consumer (e.g. age, ethnic group, diet behaviour) – which determine the composition of gut microflora – are also taken into account.

Thus, in order to facilitate harmonization the scientific evidences on the safety, efficacy and effectiveness of probiotic consumption as well as to further consolidate the acceptance of probiotics, it is important to generate guidelines and recommend criteria and methodology for the evaluation of probiotics, and to identify and define what data need to be available to accurately substantiate health claims. A working group was convened by FAO/WHO to identify and outline the minimum requirements needed for probiotic status, as schematically shown in Figure 1 [4].

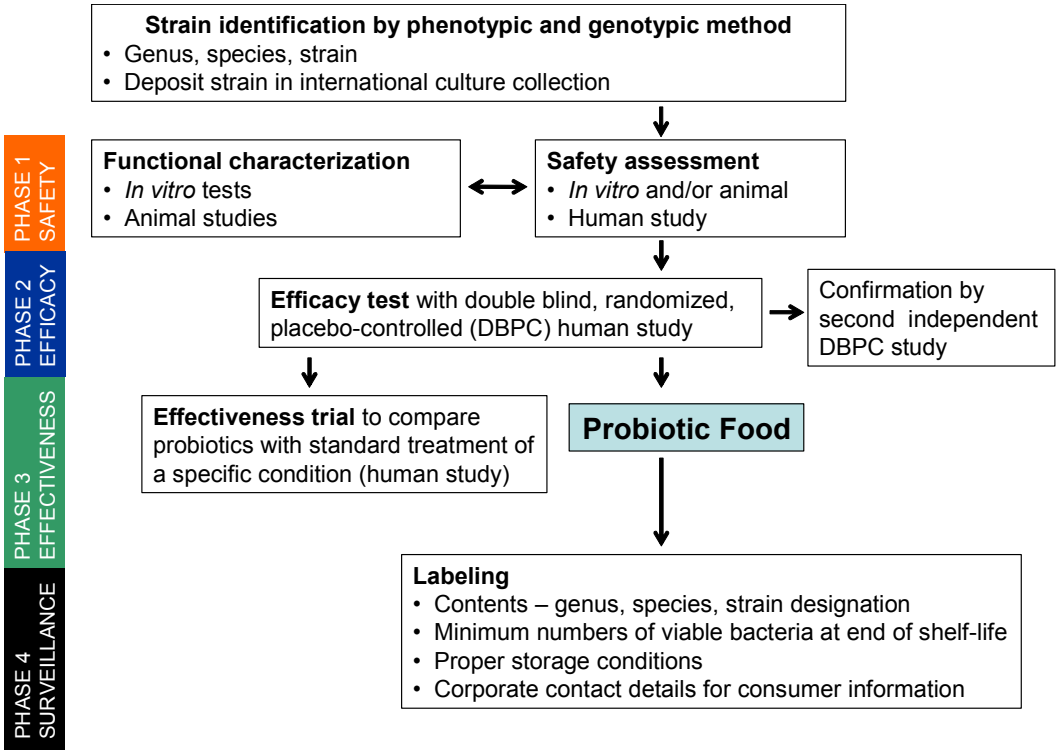


**Figure 1**

Proposed guidelines for the evaluation of probiotics for food use

The efficacy of therapeutic or prophylactic application of probiotic is highly dependent on some functional characteristics, which in turn serve as selection criteria. Some in vitro tests such as resistance to gastric acidity, bile acid resistance, adherence to mucus and/or human epithelial cells and cell lines, antimicrobial activity against potentially pathogenic bacteria, reduction of pathogen adhesion to surfaces, bile salt hydrolase activity, resistance to spermicides (applicable to probiotics for vaginal use) were broadly applied [4].

### 1.3 Technological approaches for improvement of viability retention

According to the proposed general concept on the efficacy of probiotic application probiotic function is only obtained with living cultures [4, 54, 55]. The probiotic bacteria must therefore be viable at the time of consumption and maintain their viability throughout the gastrointestinal tract. Recommendations for the minimum suggested level for probiotics in the food to attain this viability are quite variable [56]. In general, a level of $10^6$ CFU $g^{-1}$ at the time of consumption is required [3, 57], although in some cases a minimal level of $10^5$ cfu per gram till the end of best before used period was considered as sufficient [21, 58, 59]. Official standards requiring a minimum of $10^6$-$10^7$ CFU $g^{-1}$ have been introduced by several food organizations worldwide. In Japan, a guideline has been developed by the Fermented Milks and Lactic Acid Bacteria Beverages Association which requires a minimum of $10^7$ viable bifidobacteria cells $mL^{-1}$ to be present in dairy products [60]. The Federation Internationale de Laiterie/International Dairy Federation (FIL/IDF) requires $10^7$ CFU of *L. acidophilus* in products such as acidophilus milk and $10^6$ CFU $g^{-1}$ of bifidobacteria in fermented milks containing bifidobacteria at the time of sale [61]. Likewise the Swiss Food Regulation as well as the MERCOSOR regulations requires a minimum of $10^6$ CFU of viable bifidobacteria in similar products [62].

Rather than to achieve a specific health effect in humans, all these standards were primarily adopted to provide bacterial concentrations that were technologically attainable and cost-effective [63]. More critical than the concentration of the probiotic bacteria in the food, however, is the minimal daily intake of the probiotic bacteria necessary to attain a therapeutic effect. A daily dose of at least $10^8$ cells was proposed to elicit the health promoting effect on consumers health [51]. These high numbers – achievable by consuming 100 g or mL of food products containing a minimal level of $10^6$ CFU $g^{-1}$ or $mL^{-1}$ – have been suggested to appropriately compensate for the possible loss in the numbers of probiotic organisms during passage through the stomach and intestine.

The criteria regarding critical concentration of probiotics in food must be considered in the production of probiotic containing food products throughout the whole processing line, in order to achieve and maintain high level of viability under retention of the health-related

functionality. Additional technological issue addressed to probiotic bacteria is that they are not creating unpleasant flavours or textures upon when incorporated into food products [55].

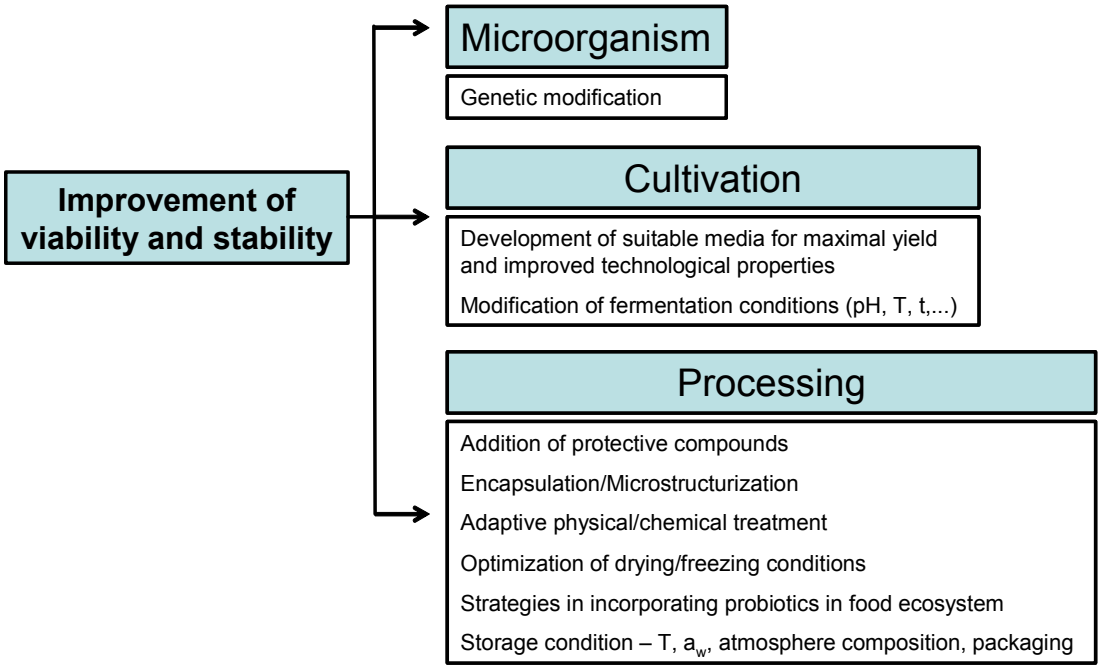


**Figure 2**

Relevant target sites where improvements on the stability of probiotic bacteria can be achieved

Figure 2 outlines some feasible approaches addressed to the improvement of the viability and stability of probiotic cells. Modifications to improve these crucial technological traits can either be aimed on genetic level by using genetic engineering or on physiological level in upstream (pre-harvesting phase) and downstream processing steps (post-harvesting phase).

### *1.3.1 Genetic engineering of microorganism*

Since technological properties are genetically determined [64], it is of interest to make use of already existing database on bacterial genes being activated under certain stressful conditions or genes differentially expressed in resistant mutant in order to create food-grade mutants [65-70]. Modifications can be performed by applying food-grade plasmids, integration of foreign DNA in the chromosome of target bacteria and application of regulating system [71, 72]. With help of this strategy it is expected that technologically sensitive but highly effective probiotic bacteria can potentially be manipulated to become more robust for survival under harsh conditions, such as food product development and gastrointestinal transit.

For instance, recombinant sucrose-6-phosphate synthase (SpsA) was synthesized in *Escherichia coli* by using the spsA gene of the cyanobacterium [73]. Transformants exhibited

a 10,000-fold increase in survival compared to wild-type cells following either freeze-drying, air drying, or desiccation over phosphorus pentoxide. Trials have also been made to produce mutants which overexpress protective stress metabolites, i.e. heat shock proteins GroEL and GroES, thereby improving tolerance against abusive heat treatments [74]. Increasing the available GroES and GroEL concentration prior to the stresses associated with freezing, lyophilization, or spray-drying may offer additional protection against protein denaturation and produce a more viable and physiologically active product [66]. Attempts had been made to convert *Saccharomyces cerevisae* into a yeast preferring growth under high hydrostatic pressure (piezophile) by manipulating the genome and by introducing genes that control high pressure growth in yeast so as to allow unique microbial biotransformation [75]. Another possible approach in terms of reducing detrimental effect of freezing dealt with the introduction of ice nucleation gene into yeast cells [76]. The mutants were able to synthesize ice nucleating proteins, which were able to trigger ice formation at temperatures, where ice nucleation has not occurred and thus – due to a higher $\Delta T$ – potentially increase freezing rate.

Apart from the context of viability enhancement, genetically modified dairy starter culture had already been developed for cheese production [77]. With help of the genetic engineering it also seems possible to improve acidification, flavouring and texturizing properties of yogurt and cheese starters, which can lead to the removal of chemical additives from the formulation [71, 78]. For instance, works have been performed to transfer genes coding for production of exocellular polysaccharide (EPS), which increases the viscosity of yogurt and decreases susceptibility to syneresis [78]. Furthermore, research is under way to develop strains of starter culture bacteria that are resistant to bacteriophage infection or to equip them with beneficial traits, such as antimicrobial production [79]. Ultimately, genetic manipulation could also be made to improve specific health-related effects of probiotic bacteria [78].

However, within the current legislative situation, i.e. Novel Food Regulations, which sets a high standard of safety precautions as well as the current rejective behaviour of consumer on the application and presence of genetically modified microorganism in food [79], this approach seems not likely to be applicable in the near future.

### *1.3.2 Cultivation*

Another approach is dealing with the improvement of cultivation techniques. Since the growth condition is also known to dictate the robustness of industrial microbial strains, efforts are continuously done by modifying the composition of fermentation media or by altering fermentation conditions. The addition of Tween 80 or calcium in fermentation media was reported to positively affect the survival characteristics during freezing [80-82]. Similarly, the

type of base used to control pH was found to account for good cryotolerance [82]. Moreover, harvesting time, growth temperature and pH of the fermentation broth are considered as crucial operating factors during fermentation, which need to be adjusted properly, since they also determined survival properties during freezing and freeze-drying [83-89]. However, *Lactobacillus acidophilus* grown in free-pH fermentation runs (final pH 4.5) tended to be resistant to low acidity, high ethanol concentration, freezing/thawing cycle, $H_2O_2$, and lyophilization, whereas cells from cultures under controlled pH (pH = 6.0) were very sensitive [90]. This findings questions the validity of growing cultures near neutrality under controlled pH to ensure maximum biomass and active cells, since growth under achievement of low final pH assured a better survival during the industrial processes and gastro-intestinal transit.

### *1.3.3 Downstream processing*

**Inclusion of protective compounds**

Addition of protective compounds to prevent cell death during drying and freezing is one of the most feasible approach to confer protection [91-94]. The additives include a variety of simple or more complex chemical compounds. Cryoprotective chemicals can be divided in permeating cryoprotectants, e.g. dimethyl sulphoxide (DMSO), glycerol, which can pass through cell membranes and non-permeating cryoprotectants, e.g. hydroxyethyl starch, various sugars, which cannot enter cells [95]. Permeating cryoprotectants were found to reduce harmful concentrations of solute/electrolytes in the cell, stabilize cell proteins, stabilize plasma membrane by electrostatic interaction and prevent intracellular ice formation by lowering the intracellular freezing point. High concentration of extra- and intracellular cryoprotectants is reported to facilitate vitrification due to dramatic reduction of ice nucleation and crystal growth [95]. Furthermore, in the presence of cryoprotectants, the effect of extracellular solution changes can be minimized [96]. The effect of different protective agents differs according to the microorganism used, but the selection made was rather empirical than based on mechanistic background.

In the vast majority of probiotic products the protective agent is restricted to milk-based carrier category. Although decent protection without including milk-based additives and/or ingredients seems to be very difficult to achieve [97-99], technological improvements are important when aiming at diversified application of probiotic in novel and non-traditional products. Especially the incorporation of probiotics in non-dairy products stored at room temperature, such as cereal products and chocolate can create an overwhelming challenge for their stability [2, 55].

**Encapsulation**

Another practicable approach is by means of encapsulating the cells into a protective matrix, through which their survival in harmful environmental conditions can be enhanced [100]. A lot of works have evidenced, that encapsulated cells had improved survival properties under *in vitro* gastrointestinal condition as compared to control population [24, 32, 101-109]. Similarly, they protective coatings or matrices can facilitate better survival in food products [32, 43, 110-112]. Trials have already been made to evaluate the contribution of encapsulation to enhancement of survival during drying and storage [99, 107, 113-116]. In many cases it was not clearly validated, whether the cells were fully immobilized in an external protective matrix or they merely adhered to the protective compounds.

**Adaptive treatment for stress induction**

Furthermore, the properties of the cells themselves can be improved by induction of their protective mechanisms. Particularly, this approach utilize their stress adaptation and cross-protection responses, which could enhance the survival of probiotics in stressful conditions and to improve their technological properties [69, 117, 118]. The importance of inducing beneficial stress responses of probiotic microorganism have only recently gained increasing research interest. Studies have been performed in assessing homologous or heterologous stress responses on probiotic lactobacilli and bifidobacteria to increase tolerance against acid [119, 120], bile [118, 120], sodium chloride [118, 121], freeze-thawing cycle [65, 68, 122, 123], heat [68, 118, 120, 124-128], spray drying [115, 129, 130], fluidized bed drying with subsequent storage [131], desiccation [132] and high hydrostatic pressure [133]. The applied stress inducers for the pre-treatment step are either of physical or chemical nature. This adaptive response is characterized by physiological changes so that the bacteria become more tolerant to adverse conditions following exposure to mild, non-lethal stress conditions. Physiological changes reported in bacteria include the transient induction of general or specific proteins. With respect to heat shock response a set of conserved heat shock proteins are generally overexpressed [134]. Classical heat shock proteins are the molecular chaperones (e.g. DnaK, GroEL, etc.) or ATP-dependent proteases (ClpP). These proteins play roles in protein folding, assembly, and repair and prevention of aggregation under stress and nonstress conditions [117, 135]. Moreover, the accumulation of compatible solute, such as betaine and trehalose during osmotic stress, was accounted for enhancement of viability upon drying [132, 136, 137]. Compatible solutes can counterbalance the external osmotic conditions without adversely affecting the structure of proteins and other macromolecules within cells [138]. Bacteria could adapt to environmental stress conditions such as cold temperature by modifying the fatty acid composition of the cellular membrane, in particular by increasing the proportion of unsaturated fatty acid residues [81].

Most of the studies on adaptive response were performed with cultures from exponential growth phase, since bacteria that enter into stationary phase had already developed resistance against various types of environmental stress. However, data on stress response studies with culture from stationary growth phase revealed the potential of pretreatment of cells in this particular growth stage to improve survival during subsequent treatment [90, 120, 123, 131].

**Optimization of drying or freezing conditions**

Optimization of drying or freezing conditions is a multi-disciplinary task, where innovative engineering solutions can be expected since this step not only determines survival behaviour considerably, but potentially also the whole processing line, energy consumption and the resulted product characteristics.

*Freezing* is performed either to produce frozen bacterial culture as an end product or to prepare intermediate product for subsequent freeze-drying process. Moreover, there are also some end products harbouring frozen microorganism such as frozen dough containing baker's yeast with conserved desirable metabolical features [139].

However, freezing exerts different injury effects on lactic acid bacteria. In particular, freezing rate seems to be crucial in achieving high level of survival [82]; however the effect of freezing rate was different for the different microbes [89]. It was proposed, that freezing rate principally influences the size of ice crystals and the site, in which ice nucleation and crystal growth occur, which ultimately determine the type of cellular damage experienced by frozen cells, or whether vitrification takes place or not [95, 140]. Rapid freezing (achieved by freezing at –196°C) was reported to have a better effect on microbial survivability and storage stability [141, 142] although other studies suggested that high cooling rate did not improve viability retention [143] or might even have detrimental effects on cells [123].

A general explanation of the effect of freezing rate on biological cells was proposed by Mazur et al, who introduced the two-factor hypothesis of freezing damage, according to which there are two independent mechanisms of damage during freezing, one active at low freezing rates, the other at high freezing rates [140]. Although the optimal cooling rate can vary by orders of magnitude for different cell types, the qualitative behaviour appears to be universal. In both cases, mechanical damage, linked to the interaction between cells and ice crystals, highly impact the integrity of the cell structure [144].

At low freezing rates, cellular damages were related to the exposure of highly concentrated intra- and extracellular solution [95]. The removal of water (as ice crystallizes) in the surrounding medium results in increased extracellular solute concentration [96]. On the other hand, osmotic-driven migration of water from the cell increases intracellular solute concentration. Although this dehydration can reduce the probability of intracellular ice

formation, the solute concentration might reach a detrimental level. In contrast, at high freezing rates, cell injury is attributed to the mechanical forces as a consequence of the formation of intracellular ice. Membrane rupture due to osmotic fluxes might also contribute to cell damage [145]. However, there were some cases reported, in which the intracellular ice *per se* did not cause cell death but even improve survival [146, 147]. In both cases, mechanical damage, linked to the interaction between cells and ice crystals, may affect the integrity of the cell structure [144].

By using very high cooling rates at low temperatures molecular motion is arrested prior to crystal formation [96]. In particular, the increased viscosity of the solution reduces the molecular diffusion and the rate of ice nucleation as well as crystal growth, so that ice formation decreases and all phase changes can be inhibited [95]. The unfrozen solution remains in a metastable state, with an amorphous, non crystalline structure.

Despite of the general notion of an optimal rate for freezing of biological cells [140], operating at higher freezing rates seems to be more attractive when economical value of the freezing process such as reduced processing time is taken into account. Moreover, the possibility to reach the vitrified state, where the dangers of intracellular ice formation and injury by concentrated solutes can be avoided, can only be realized by rapid freezing.

An increase of the freezing rate can also be achieved by increasing surface area per unit weight; thus accelerating the removal of crystallization heat. This process improvement could be realized by generating droplets of bacterial suspension along with protective agent, which are then immediately immersed in liquid nitrogen at –196°C. Another possibility to have a sufficiently high freezing rate is by producing a controlled spray of high surface-to-mass ratio droplets (size of approximately 5 to 30 µm) in air blast freezer.

*Drying* is also widely used as a means of preservation of bacterial cells, although the process itself and subsequent storage are known to be lethal to a large fraction of the dried population. Compared to frozen concentrated bacterial preparation, the use of dried bacterial population do not require cryogenic shipment and storage. In addition, owing to removal of water the weight of the product can be markedly reduced. However, it was reported that the time lag before acidification begins is longer for the dried than for the frozen cultures [91]. Viability loss during drying was related to damage to the cell wall and cytoplasmic membrane, so that the dried cells became more sensitive to NaCl [13, 14, 148]. Damage on cell membrane could be detected by increased permeability of ß-galactosidase substrate, higher diffusion rate of DNase into cells and by leakage of UV-absorbing materials from the cells [148-150]. Following drying changes were reported to take place in the unsaturated:saturated fatty acid ratio, a loss of ΔpH and a decrease in the activity of membrane bound $H^+$-ATPase [151]. Damage of membrane bound $H^+$-ATPase, which is responsible for pH homeostasis in acidic environment by discharging $H^+$ from the cell reduce

the ability to tolerate acidic conditions [152]. Protective compounds, primarily saccharides protect membrane and proteins from dehydration damage, most likely by hydrogen bonding to polar residues in the dry macromolecules, as described by the water replacement hypothesis [153, 154]. Protective effects of saccharides are also related with the ability of sugar to form a high viscous glassy matrix during dehydration [155].

Bacteria can be dried either with freeze drying, vacuum drying, spray drying, or fluidized bed drying [91, 129, 156-158]. Most of dried bacterial preparations are currently produced with freeze-drying due to the possibility to operate at mild conditions, so that the degree of injury could be minimized. However, some drawbacks of freeze drying process, such as long processing time and high energy consumption, led to efforts in evaluating alternative drying processes. Spray drying is one of the promising process for production of dry probiotic preparations [13, 14, 115, 130, 159], since under optimized conditions it allows high processing rates and low operating costs under maintaining a high degree of survivability.

**Storage condition**

Furthermore, the storage conditions (packaging material, temperature, humidity etc.) can greatly affect the stability of the probiotic product. In general frozen and dried product should preferably be stored at low temperature to ensure viability retention.

Storage temperatures lower than –80°C are found to be sufficient in maintaining high level of viability [82, 95, 143] and shelf-life can be dramatically increased as the storage temperature is reduced. At –196°C it was reported that there is insufficient thermal energy for deteriorative chemical reaction [160].

When probiotic dried preparation is subjected to prolonged storage, low relative humidity (11 – 22%) was found to enhance bacterial stability [161]. Storage at zero humidity seems to be disadvantageous due to the apparent increased rate of lipid oxidation [162], particularly on bacterial membranes. Membrane deterioration as a result of exposure to oxygen was based on the oxidation of unsaturated fatty acid in cellular membrane [163]. Oxidation process is reported to be activated by an increase in the residual humidity [161] and could be effectively counteracted by storage in the absence of oxygen [164, 165]. Enhancement of shelf life of dried probiotic bacteria could be also achieved when storage temperature was lowered [166, 167]. Similar to chemical reactions in general, the effect of storage temperature on the rate of viability loss could be described by Arrhenius equation.

The packaging material can be considered as a critical factor during storage. Compared to storage polyester (PET) bottles, skim milk-encapsulated bifidobacteria stored in glass bottles showed a relatively low viability reduction during prolonged storage at 4°C, regardless of the presence of oxygen scavenger and desiccant [99]. This difference was attributed to the relatively high oxygen permeability of PET bottles [60].

**Strategies to incorporate probiotics in cultured food ecosystem**

Futhermore, when cultured dairy products are considered as food vehicle for probiotic consumption, there are numerous challenges related to the instability of some strains of probiotic bacteria in fermented milk products. Careful selection of yoghurt starter is prerequisite in order to maintain high viability level of probiotic, since yoghurt culture is capable of creating environments that inhibit not only undesirable microbial contaminants but also the co-existing probiotic bacteria. This inhibitory activity is attributed to several factors, including production of lactic and other organic acids, hydrogen peroxide, and bacteriocins, as well as reduced availability of nutrients [168-170]. To enhance the growth and high viability level of probiotics in yoghurt some practical solutions such as the use of higher inocula of probiotics, the addition of growth promoting factors such as amino acids, peptides and other micronutrients as well as the addition of ascorbic acid or cysteine, which decrease redox potential, were proposed [171-173]. Apart from addition of growth-promoting substrates the conditions in the manufacture and storage of yoghurt could be manipulated in order to increase probiotic survival [174]. The methods applied encompassed termination of fermentation at pH>5.0, reduction of storage temperature to less than 3-4°C, addition of pH buffering agents such as whey protein concentrates, heat shock for prevention of excessive acid production, reduction of incubation temperature in favour of bifidobacterial growth, mechanical rupture of yoghurt bacteria and attenuation of yoghurt bacteria by high pressure treatment.

## 1.4 The EU-Project PROTECH - QLK1-CT-2000-30042

The present PhD thesis is performed in TU Berlin, Department of Food Biotechnology and Food Process Engineering, within the frame of an EU funded project PROTECH (Nutritional enhancement of probiotics and prebiotics: Technology aspects on microbial viability, stability, functionality and on prebiotic function, QLK1-CT-2000-30042), in which the impact of processing technologies on probiotic and/or prebiotic based functional food is evaluated. Figure 3 illustrated the objects of investigation and how the project partners are interlinked in the multitude of tasks [175]. In brief, development of a fermentation medium as well as optimization of harvesting time with respect to post-harvesting stability were pursued. In the drying study freeze-drying and spray-drying were evaluated, in terms of identification of suitable processing regimes, performance of different protectants in offering high survival during drying and storage, factors governing storage stability during storage, etc. The use of sub-lethal stress to improve technological behaviours (i.e. heat and oxygen tolerance) as well as to enhance resistance against extreme conditions in gastro intestinal tract (bile tolerance) was evaluated. Proteomic approach was applied in order to examine the specific response of the cells. The survival of probiotics in yoghurt was assessed to determine critical factors

governing survival in well-established food system and how modifications on this system could be made to reduce cell death, especially by incorporating prebiotics. The possibility of enzymatically modifying prebiotics into a more complex structure, thus making them less fermentable, was investigated. Feeding trials of commercial prebiotics supplemented with probiotics were performed in order to assess whether these properties could induce beneficial changes on the composition of short chain fatty acid in different parts of rat colon.

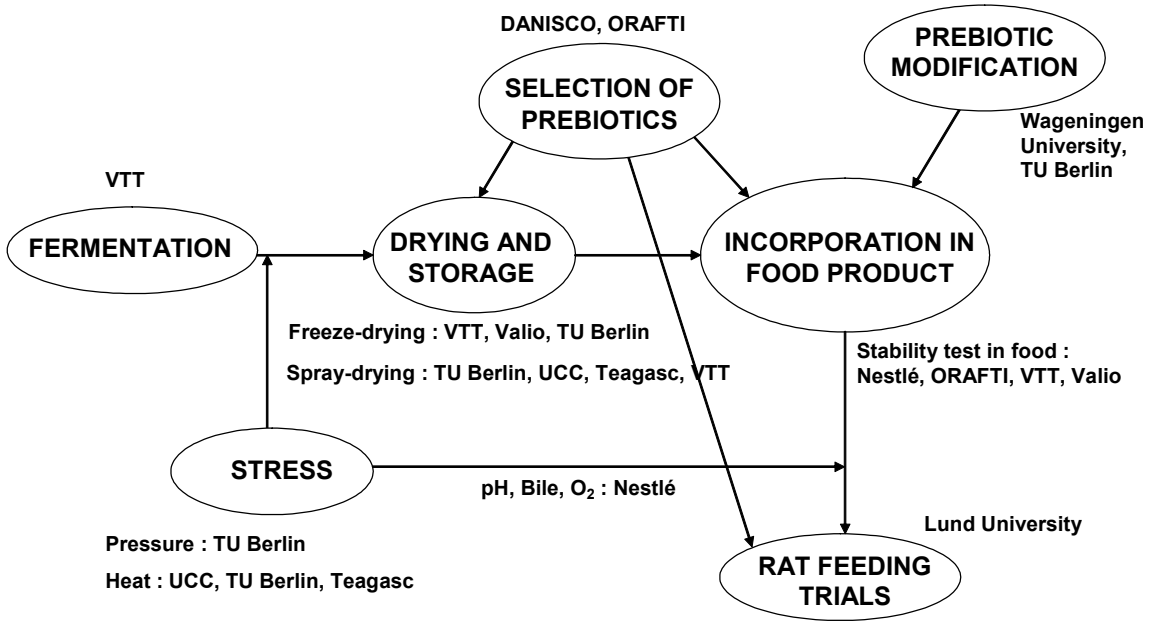


**Figure 3**

General and specific objects of investigation, which were accomplished by partners participating in PROTECH project along with the network of interaction among partners.

## 1.5 Structure of PhD thesis

The work is focused on the exploration of spray drying as an alternative processing method to produce dried probiotic preparation. In this context investigations were performed on studying the mechanism leading to cell damage during drying and the role of physical state of the drying matrix and the interaction of protective compounds with cellular membrane in dehydration tolerance. Moreover, a pre-adaptation step under sub lethal high pressure level was assessed on its potential in increasing heat tolerance. The linkage between the separate works were shown in Figure 1.4.

In the first chapter flow cytometric analysis was applied to evaluate the impact of physical and chemical treatments on viability state of probiotic microorganism. Particularly, the effect of heat treatment and high hydrostatic pressure treatment as well as the combined effect of both treatments was assessed on *Lactobacillus rhamnosus* GG (ATCC 53103). The impact of low pH was studied in order to draw conclusion about survival mechanism of probiotic. A multiple staining strategy using fluorescent dyes carboxyfluoresceindiacetate (cFDA) and propidium iodide (PI) was applied.

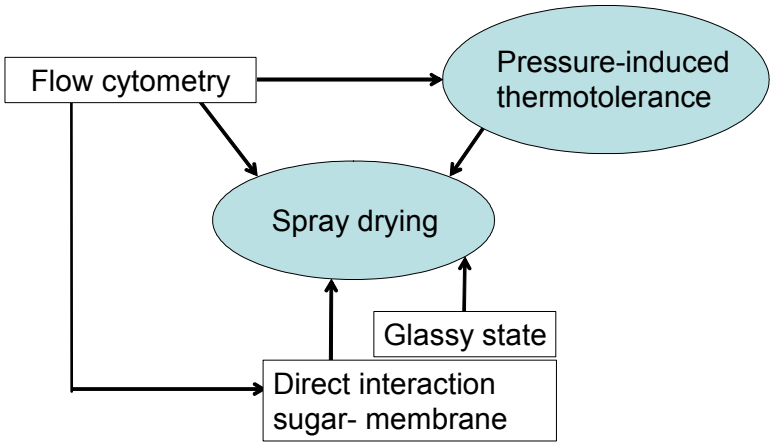


**Figure 1.4**

Structure of the works performed within this PhD thesis

In the chapter dealing with spray drying, the works comprised of the selection of optimal processing condition, in which a compromise has to be taken in order to achieve powder with an appreciable level of viable probiotic bacteria and sufficiently low residual moisture to enable stable storage. The nature of drying induced cellular injury was also a subject of investigation and therefore evaluated with flow cytometric analysis previously established. Differential Scanning Calorimetry (DSC) was applied to investigate the role of physical state, in particular the presence of drying medium in glassy state, on survival behaviour during storage. The work with liposome as a simple model for bacterial membrane was done due to the fact that storage stability was found to be governed not only by physical state of the extracellular matrix, but also by direct interaction between saccharides, which constituted the protective compound, with cellular membrane.

The work on the application of pressure pre-treatment to induce beneficial stress response was done to evaluate whether and to which extent heat resistance of *L. rhamnosus* is affected by mild pressure treatments prior to exposure to lethal temperature. Apart from identifying the optimal combinations of pressure, temperature and treatment time for the pre-treatment steps, it was attempted to elucidate the possible cellular mechanisms involved in the acquisition of heat tolerance with help of flow cytometry technique.

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# 2 FLOW CYTOMETRIC ANALYSIS FOR INACTIVATION STUDIES

Application of flow cytometric analysis to evaluate the mechanism of inactivation by physical treatment methods

## 2.1 Introduction

### *2.1.1 Effect of physical inactivation treatments on microorganisms*

***Heat***

Reduction or inactivation of microbial populations by thermal processes is a common process of food preservation in use nowadays. Cells contain several targets for the action of heat. The basal heat resistance of microorganism is related to the intrinsic heat stability of the essential macromolecules, including ribosomes, nucleic acids, enzymes and proteins inside the cells and the membrane [1, 2]. During exposure to heat structural changes in these critical cellular components may lead to cell death, for instance loss of specific secondary and tertiary structure of ribosomal subunit, protein coagulation, degradation of RNA and membrane damage. However, the exact prime cause for cell death upon heat exposure is still not clearly understood [3]. Heat treatment at temperatures in the vicinity of 60°C was reported to cause damage in the cytoplasmic membrane of *Lactobacillus bulgaricus*, whereas for temperature of 65°C and immediately above, ribosomes and/or proteins denaturation as well as cell wall damage may be responsible for thermal death [4]. Electron microscopy study on heat treated *Bacillus cereus* showed that following exposure to 62°C for 2 min or 15 min or to 100°C for 5 min the appearance of membrane changed: it developed holes and fractures [5]. Concentric rings appeared inside the cytoplasm and the ribosomes disappeared. After heating to higher temperature, dense areas of precipitation owing to protein coagulation appeared in the cytoplasm [6].

***High pressure***

To meet the requirement of producing high quality food under appropriate minimization of microbial contamination, processing concepts based on the use of emerging technologies have been developed, into which high hydrostatic pressure treatment could be classified. The efficacy of this novel processing in inactivating different types of microorganisms is well documented [7-10]. Moreover, the potential of high pressure technology as an alternative tool on modifying macromolecules (proteins, polysaccharides, etc.), as well as to assist and/or to substitute conventional freezing methods, has been reviewed [11-13].

Many studies had been initiated to provide improved knowledge on the mode of action of high pressure on microorganisms.

Hydrostatic pressure was reported to affect the intracellular pH of microorganisms by enhancing the dissociation of weak organic acid, increasing the permeability of the cytoplasmic membrane and inactivation of enzymes required for pH homeostasis [14-16]. The major constituents of this crucial cellular function are located in cellular membrane. The regulation of a fairly constant internal pH ($pH_{in}$) was considered as crucial for maintaining

microbial viability, and consequently a substantial pressure-induced loss of this transport functionality would reduce the ability of microorganisms to survive harsh environment during and after pressure treatments [17].

Lactic acid bacteria, which do not possess an electron transport chain, maintain a pH gradient by proton-translocating activity of $F_0F_1$ ATPase. This membrane-bound enzyme appears to be a possible target for pressure induced inactivation of microorganisms. This multimeric enzyme can either synthesize ATP using protons or conversely expulse protons out of the cell with the energy provided by the ATP hydrolysis [18]. In lactic acid bacteria, the latter activity increases at low pH and is crucial to maintain the ΔpH [19]. Damage of membrane bound $H^+$-ATPase, which is responsible for pH homeostasis in acidic environment by discharging $H^+$ from the cell, can reduce the ability to tolerate acidic conditions [20, 21].

Early study on the effect of relatively low pressure (50 MPa) on the $F_0F_1$ ATPase of the isolated membranes of *Streptococcus faecalis* showed that under the investigated condition the proton-translocation step, and not the ATP hydrolytic step was inhibited by pressure [22]. Upon pressurization of *Lactobacillus plantarum* at 250 MPa, the activity of $F_0F_1$ ATPase was decreased [23]. Along with that, acid efflux was impaired and the regulation of $pH_{in}$ was hampered. Upon observing the ATP pool and acid efflux it was also noted that the glycolysis (ATP generating system) was less sensitive to pressure than $F_0F_1$ ATPase (ATP utilizing system). Exposure to high pressure (at 200 or 300 MPa) on lactic acid bacteria suspended in acidic environment resulted in a decrease of the $pH_{in}$ to the extracellular pH value and the capacity to restore the $pH_{in}$ was totally lost [17].

Furthermore, not only pH homeostasis mechanisms were affected by pressure, but also other critical membrane-bound transporters, particularly multi drug resistance (MDR) transport system. It is a group of integral membrane proteins that transport hydrophobic drugs and lipids across the cell membrane. MDR transporters can be divided into two classes based on their source of energy: Secondary transporters, which use proton gradients to facilitate an antiporter mechanism, and ATP binding cassette (ABC) transporters that couple the efflux of substrate across the cell membrane with energy derived from ATP hydrolysis. ABC transporters belong to one of the largest superfamilies of proteins and that either import or export a broad range of substrates that include amino acids, ions, sugars, lipids, and drugs [24].

Pressure as high as 200 MPa was reported to inactivate HorA of *L. plantarum* [15]. This functional molecule is a membrane-bound, ATP-dependent MDR enzyme, which confer hop resistance on beer spoilage bacteria and has high homology to other bacterial ATP-binding cassette-type multidrug transporters. Following exposure to a sub-lethal level of pressure, which is sufficient to inactivate HorA, cells of *L. plantarum* failed to survive during subsequent storage in media containing hop extract [25].

Furthermore, high pressure studies on LmrP activity of *L. lactis* revealed that the loss of viability after pressure treatment correlated with reduced LmrP activity and the loss of the ability to restore a ∆pH after pressure treatment [26]. This enzyme is one MDR transport enzyme in *L. lactis*, which is involved in drug/toxin extrusion in a proton motive-dependent (∆pH dependent) manner. High pressure impaired the activity of the enzyme and not the proton motive force.

The composition and the phase behaviour of cytoplasmic membranes were also implicated in irreversible pressure denaturation of membrane-bound proteins, such as HorA [27]. It is known that the function of membrane-integral proteins is not only influenced by its three dimensional structure but also to the composition and the phase behaviour of the cytoplasmic membrane.

Elevated pressure was reported to reduce the activity of transmembrane enzyme $Na^+/K^+$-ATPase either by induced dissociation and/or unfolding of protein subunits of the enzyme or by alterations of membrane fluidity which hinders conformational transition of the protein required for the reaction [28]. Similarly, other research group came to the conclusion that the inhibition of this enzyme, which couples the chemical energy from hydrolysis of ATP to dynamic gradients of $Na^+$ and $K^+$ across the plasma membrane, was due to pressure induced ordering of the acyl-chains of lipid matrix or due to subunit dissociation [29]. At least three stages of damage was proposed to take place upon pressurizing this enzyme, which was embedded in phospholipids bilayer. Pressure of 100 MPa or lower induces a decrease in the fluidity of the liposome's lipid bilayer and reversible conformational changes of the transmembrane protein, resulting in the functional disorder of the enzyme $Na^+/K^+$-ATPase [30]. Pressure of 100 to 220 MPa causes a reversible phase transition of the lipid bilayer and the dissociation of protein subunits. These changes bring about the separation of protein and the lipid bilayer, producing transmembrane tunnels. Pressure of 220 MPa or higher is accompanied by irreversible protein unfolding and fragmentation of the lipid bilayer, thus destroying the gross membrane structure. However, due to the presence of cytoskelett and other constituents of cell envelope, which confer higher mechanical or structural stability to microbial membranes, critical pressure level in inducing irreversible damage on microbial membrane should be higher compared to the one required to irreversibly degrade artificial membrane.

As already mentioned before, pressure upshift induced in one-component phospholipids bilayer a decrease in the membrane fluidity, which is as a result of phase transition from the liquid crystalline state to the gel phase. It was previously suggested that the fluidity of the bacterial membrane had a direct role in the pressure resistance of bacteria [31]. FT-IR spectroscopy of the bacterial membrane revealed that the addition of sucrose can reduce the melting temperature ($T_m$) of the gel-liquid crystalline phase transition [26]. At higher

pressures the $T_m$ of bacterial membranes were lower in the presence of sucrose than in its absence so that the pressure required to induce a phase transition from the liquid crystalline phase to gel phase is increased when sucrose is present. For a given pressure level addition of sucrose can prevent bacterial membranes from experiencing phase transition and thus maintained in them a more fluid state during pressure treatment and partly contribute to enhanced resistance of bacteria to pressure [26].

Irreversible degradation of intracellular components was also deemed responsible for viability loss. A correlation was observed between loss of cell viability and decrease in ribosome-associated enthalpy in cells subjected to pressures of 50-250 MPa for 20 min [32]. Cell death and ribosome damage were therefore closely related phenomena. Transmission electron microscopy (TEM) micrographs showed that pressure induced denaturation of ribosomes may be manifested by the presence of dense compacted interior regions of the cytoplasm after pressure treatment at 500 MPa [33]. The authors of the latter study also observed a reduction of the area of the ribosomal peak as a function of pressure, which emphasized the implication of damage on the ribosomal subunit in cell death.

In addition, a study on the electrophoretic mobility of pressure treated intracellular enzymes of *L. monocytogenes* strains was made to relate the cell death of these organisms to the pressure induced conformational modifications of those enzymes [34]. Although no positive correlation was found between the overall pressure resistance of the organisms and the pressure resistance of the investigated enzymes (13 types), a wider range of metabolic enzymes should be assayed in order to elucidate the contribution of intracellular enzyme to overall pressure resistance.

The role of certain proteins on bacterial inactivation was emphasized by Ludwig *et al* (1996) and Perier-Cornet *et al* (2005), who based their ideas on the similarity of elliptic form of the p,T-isokinetic stability diagram of *E. coli* with the elliptic character of protein denaturation diagram [35, 36].

Studies on changes on cell morphology following pressure treatment revealed that despite the clear evidence on pressure induced damage on this membrane-bound enzyme no direct morphological changes on the membrane of *L. plantarum* could be observed [23]. Scanning electron micrograph of *L. monocytogenes* showed that no morphological changes were observed on cells inactivated by pressure as high as 345 MPa [37]. Pressure induced damage on cell morphology was more pronounced in exponential growth phase as evidenced by observations made with transmission electron microscopy on *L. lactis* or *E.coli*, which stressed that exposure to high pressure caused cell envelope damage [38, 39].

***Ultrasound***

The application of ultrasound processing in the food industry for preservation purposes has received increasing attentions. Briefly, it is broadly accepted that ultrasound alone is not effective enough to inactivate bacteria in food [40]. However, improvements could be made by coupling of ultrasound with heat treatment. The concept of ultrasound assisted thermal processing (thermosonication), which is based on the synergy between ultrasound and heat for bacterial inactivation, has been proven to be of potential interest in food preservation, especially to enhance the lethal effect of conventional thermal treatments. Several research groups extensively studied the bactericidal effect of ultrasound and particularly its synergistic potential when applied simultaneously with heat [41-43]. Recent investigations have shown the influence of amplitude, external static pressure and temperature as well as pH and composition of treatment medium as the key processing variables [44, 45]. Generally, the mechanism underlying microbial inactivation during ultrasound treatment in liquid medium was related to physical disruption (shear stress, localized heating) and chemical reactions (production of free radicals) within the microorganisms' cell [40]. These degradative events occurred as a consequence of the microscopic shock waves, which were generated upon implosion of gas bubbles [46]. The implosion itself resulted from the pulsation of the gas bubbles, which underwent regions of alternating compression and expansion within the propagated longitudinal waves [44].

### *2.1.2 Flow cytometry*

The need to have a quasi real-time assessment microbiological method in order to describe the viability state of bacteria in a more precise manner triggers the development of microbiological rapid methods or improvement of already existing ones. Flow cytometry is regarded as one versatile tool for research in microbiology, which exhibits three unique technical properties of high potential to be used in various microbiological studies including assessment of microbial viability or metabolic activity, monitoring of gene expression system, as well as identification and enumeration of microorganisms [47, 48] :

(i) its tremendous velocity to obtain and process data; allowing analyses to be performed at a flow rate of 10 – 100 µL $min^{-1}$ and detection of up to 10000 events $s^{-1}$, when the microbial concentration in the processed sample is sufficiently high;

(ii) high-speed multiparametric data acquisition and multivariate data analysis, which combine direct and rapid assays to determine numbers, cell size distribution and additional biochemical and physiological characteristics of individual cells, thus revealing the heterogeneity present in a population; and

(iii) the sorting capacity of some cytometers, which allows the physical separation or transfer of specific populations or even single cells into tubes, onto slides or onto agar plates, thus allowing further physical, chemical, biological or molecular analysis and establishing a link between the reproductive viability and the staining pattern of bacteria.

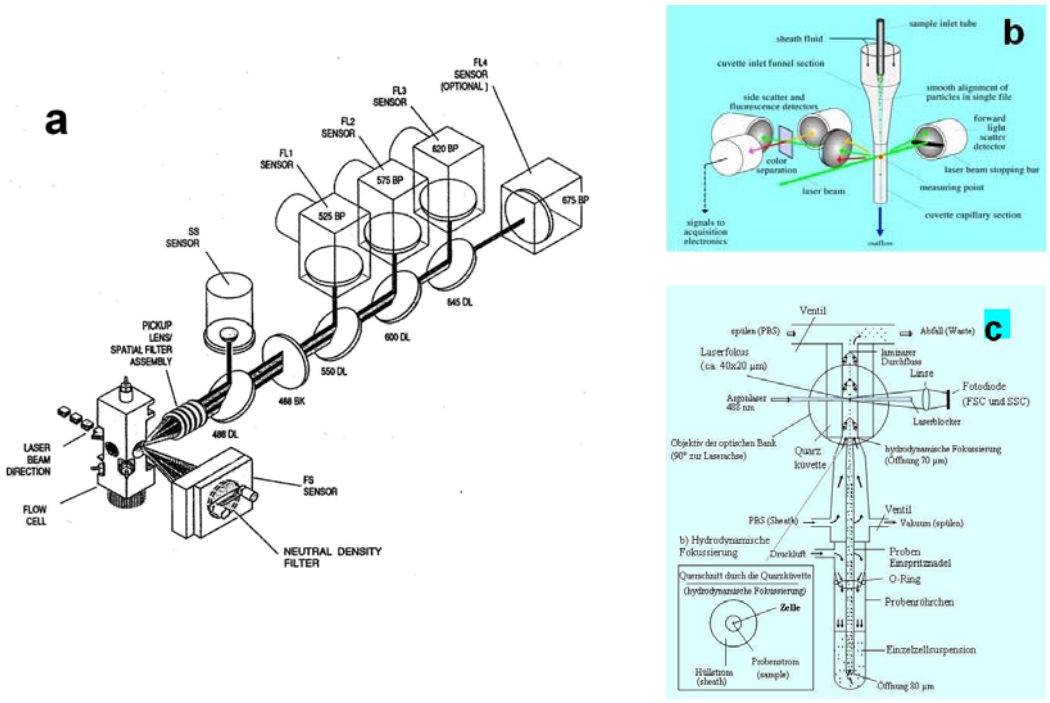


**Figure 1**

(a) Schematic diagram of the basic components of a Beckman Coulter flow cytometer (BeckmanCoulter Inc., Miami, Florida, USA)

(b) More detailed view of the interaction between cells and light in the illumination zone as well as the types of signals detected by the sensors.
Forward angle light scatter is measured with FS sensor at low angle with a photodiode. Right angle light scatter (detected by SS sensor) and fluorescence (detected by FL1 to FL3 sensors) are collected at 90°, split by a series of dichroic mirrors and filters in different colors, and measured by photomultiplier tubes. Graph was taken from http://www.cytobuoy.com/index.html

(c) Hydrodynamic focusing of the sample stream through flow cell by regulating the pressure of the sheath fluid against the cell suspension in order to align the particles in single file. Graph was taken from http://www.facslab.toxikologie.uni-mainz.de/zytometrie.jsp

Figure 1 shows the basic components of a flow cytometer, which is constituted of a flow cell, a light source, optics, detectors, electronics and computer. In addition, a flow cytometer can be equipped wit a cell-sorting device. The working principle of a flow cytometer can be briefly described as follows: Microbial cells in suspension flow in single-file through a laser-illuminated zone where they scatter light and emit fluorescence that are collected, filtered, amplified and converted to digital values that are stored in a list mode data files on a

computer, where each event (i.e. presence of a microbial cell) with the corresponding data for each parameter is recorded sequentially. The magnitudes of the parameter measured (SS, FS, or fluorescence) are sorted electronically into 'bins' or 'channels'. As a result, a set of rapid, multiparametric measurements could be performed on each single cell of interest.

The delivery of the cells in a single file modus, i.e. one after another through a focused laser beam (usually 488 nm blue laser) is ensured by hydrodynamic focusing using a sheath fluid, normally a saline solution (Fig. 1c).

The scatter parameters measured by flow cytometer are known as forward scatter (FS, measured in forward angle direction) and side scatter (SS, measured in the right angle direction), which provide information about physical characteristics of a cell such as its size and granularity, respectively [49]. The measurement of FS/SS parameters can be used to distinguish cells in a mixed sample according to their morphological properties, thus allowing exclusion of background from the cell of interest. More interesting for microbiological analysis is the possibility to stain cells with fluorescent dyes or fluorogenic substrates, which then allow the analysis of structural properties or biological activities as well as taxonomic analysis on single cell level. As can be seen in Figure 2 the fluorescent dyes usually used may be [50-52]:

(i) stains which bind to (or react with) particular molecules such as DNA, RNA or protein,

(ii) fluorogenic substrates which reveal distributions in enzymatic activity,

(iii) indicators which change their property as a function of $pH_{in}$ or which are taken up in response to the state of membrane polarization, or

(iv) antibodies or oligonucleotides tagged with a fluorescent probe.

For visualization purposes, data are displayed either as a frequency distribution where the magnitude of the parameter measured (SS, FS, or fluorescence) is plotted against the number of cells. Alternatively, the data can be represented by two- or three-parameter density plots of light scattering *versus* fluorescence or – in case of dual staining procedure – the fluorescence from a DNA stain *versus* the fluorescence owing to microbial enzyme activity. Thus, an impression about the distribution of a variety of properties of interest amongst the cells in the population as a whole can be gained.

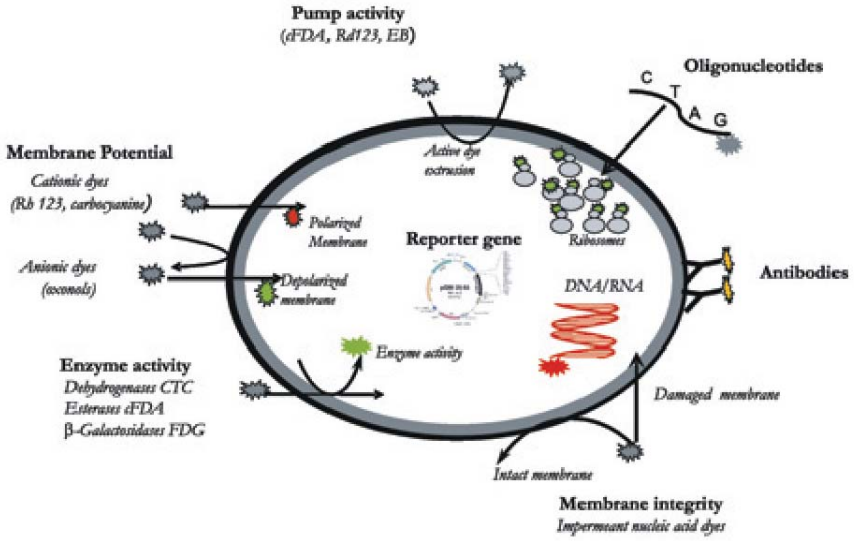


**Figure 2**

Different cellular target sites for physiological and taxonomical probes used in combination with flow cytometric analysis. Figure is taken from [52].

### *2.1.3 Determination of viability status of microorganism with fluororescence probes*

The determination of the impact of industrial treatment on microorganisms – either during cultivation, inactivation or preservation steps – basically relies on the use of classical plate count methods, which is still regarded as the benchmark method for the determination of viability. According to this method viability is defined as the ability to reproduce and form a visible colony, which typically contains at least $10^6$ cells [53]. Reproductive growth or culturability is regarded as the highest level of physiological fitness [51], for which some requirements need to be fulfilled: an intact cytoplasmic membrane which functions as a barrier between the cytoplasma and the extracellular environment, DNA transcription, RNA translation, generation of energy for maintenance of cell metabolism, biosynthesis of essential molecules such as proteins, nucleic acids, polysaccharides, as well as growth and multiplication [54].

However, classic culture technique has some drawbacks related to the fact that it could not give insights about population heterogeneities or the physiology of individual organism [53]. Besides, long determination time which principally arises from the application of this method leads to significant limitations in anticipating abnormalities in growth or surviving behaviour of bacteria during industrial processes. Moreover, culturing technique may underestimate the numbers of truly viable bacteria. It is known that under stress conditions or limitation of nutrient availability or due to imposed sub-lethal injury, some cells can enter a non-culturable state, yet they can still exhibit metabolic activity [55, 56]. In natural environments such as soil or seawater or gastro-intestinal tract bacteria from this viable but not culturable (VBNC) state can be encountered as well [57-60]. Reports on bacteriological quality of different types of

water revealed that the ratio of colony forming unit to total bacterial number were less than 2% [61] or even 2-4 log orders of magnitude lower [62]. Apparently, in this VBNC state bacteria undergo metabolic change leading to the production of cells that no longer actively form colonies on solid media, but retain other indicators of cell viability, such as active membrane potential, maintenance of cellular integrity and the capacity for metabolic activity [63]. It was thought that the risk of the residual metabolic activities of such bacteria not detected by standard culturing technique (or non-growing) could lead to food spoilage or accumulation of toxins due to retention of gene encoding virulence and resuscitation of nonculturable cells, so that nonculturable pathogens may still pose a hazard to public health [47, 63, 64].

The application of flow cytometry analysis permits simultaneous evaluation of multiple cellular parameters, both structural and functional on single cell level. This approach would then allow an extended description of bacterial viability state beyond the one based on reproductive capacity as well as identification of heterogeneities within population with regard to structure and function [48, 65].

In the following the application of some physiological probes as well as possible combined application among them, which are relevant for use in combination with flow cytometry, are briefly reviewed.

***Esterase activity***

Esterases are present in all living organisms [66]. The fluorogenic substrate cFDA (carboxyfluorescein-diacetate) is used primarily for the evaluation of cellular enzymatic activity. It is a lipophilic, non-fluorescent precursor that readily diffuses across the cell membranes [67]. In the intracellular compartment it undergoes hydrolysis of diacetate groups by unspecific esterases into a polar, membrane-impermeant fluorescent compound cF (carboxyfluorescein). Carboxyfluorescein is a derivative of fluorescein which are more negatively charged at physiological pH, and thus less likely to leak from the cells [54]. The substrate cleaving reaction of intracellular esterase is typically not energy dependent [64] and the enzyme will remain functional in cells as long as it is retained by the intact membrane and protected from the environment [48]. Consequently, the cells only remain fluorescent if their membranes are intact and cF are unable to diffuse out; thus for cells to be associated as viable, this probe requires both active intracellular enzymes and intact membranes [68].

Many works had been performed to enumerate viable bacteria with cFDA [58, 62, 68-72]. cFDA was proved to be more effective at labelling Gram positive bacteria than Gram negative bacteria [58]. Several studies had been conducted in order to evaluate the application of other esterase substrates such as Calcein acetoxy methyl ester (calcein AM)

and carboxyfluorescein diacetate-succinimidyl ester (cFDA-SE) on bacteria. Although calcein AM has greater fluorescence intensity, reduced bleaching of fluorescence, reduced leaching from cells and is insensitive to pH changes between pH 5.5 and 10, it was found to be less reliable for viability assessment of yeasts and bacteria [73]. cFDA-SE is another fluorogenic esterified substrate similar to cFDA but differing by the presence of a succinimidyl ester (SE) group that can bind strongly to free amines [67]. cFDA-SE is also cell permeant and the DA groups are hydrolysed intracellularly by nonspecific esterases, resulting in a highly fluorescent amine reactive fluorophore (cF-SE). This molecule can react with amine containing residues of intracellular proteins, forming highly stable dye–protein adducts. However, it was reported that cFDA-SE is a poor marker of bacterial activity due to the occurrence of non-specific labelling of all cells, irrespective of their metabolic state [68]. Apart from identification of intracellular esterase activity, the pH-sensitivity of some esterase substrates such as BCECF (Bis-carboxyethyl-carboxyfluorescein) and cFDA-SE lead to their application in determine bacterial intracellular pH [17, 74].

The major drawback in using esterase substrate to identify the bacterial viability status is particularly based on the fact that intracellular fluorescein accumulation owing to enzyme-substrate reactions in cells exposed to cFDA is not energy-dependent. As a consequence, it may be expected that this staining technique does not reflect the energetic status of a cell very directly and will not therefore adequately distinguish degrees of cell viability reflecting a generalised physiological or energetic capacity [60].

In line with the proposed drawback it was reported that even cells killed by $H_2O_2$, $\gamma$-irradiation, and heat still showed esterase activity and cF accumulation [48, 75]. On the other hand it is also possible that cells with damaged membranes contain active esterases; they just can not retain the products. Irreversible membrane permeabilization in absence of esterase inactivation was achieved by applying pulsed electric fields on LGG (data not shown), where following the pulsed electric fields treatment at 35 kV/cm the cells were stained by PI and simultaneously extracellular cF fluorescence was observed.

The enzymatic conversion proceeds approximately as rapidly in cells treated with inhibitors of energy metabolism as in control cells (Shapiro, H., personal communication in Cytometry Mailing List). As typically used, the conversion of cFDA into cF discriminates between cells with intact membranes, which retain the dye, and cells with damaged membranes, from which dye leaks out much more rapidly. Thus, at the end, the information obtained from cF retention (dye retention assay) and from exclusion of nucleic-acid excluded dyes such as PI (dye exclusion assay) is basically the same, i.e. that the membrane is intact [64]. In conclusion, the occurrence of esterase activity and intracellular accumulation of cF does not necessarily reflect crucial metabolic activities which are involved in the maintenance of reproductive growth [48]. Metabolic activity should be better demonstrated using dyes

responsive to energy metabolism, for example, indicators of membrane potential. The measurement of membrane potential allows a more accurate evaluation of functional cell integrity, because it corresponds to the energetic state of the membrane and the cell's capacity to synthesize ATP [76].

***Pump activity***

Bacteria have a very efficient efflux pump, which result in rapid efflux of dye, thus hampering interaction with the target molecule and is regarded as a major obstacle for multiparametric measurement. On the other hand dye efflux can serve as an additional measure of cell viability [77]. For instance, extrusion of carboxyfluorescein (cF) from intact cells of *L. lactis* [78] and *S. cerevisae* [75] was found to take place in an energy dependent manner. The efflux experiments showed an excellent correlation between the viability of *S. cerevisiae* cells and the ability to translocate cF [75]. Labelling of *L. lactis* with cF combined with the ATP-driven efflux of cF was proven to be suitable as an additional indicator of metabolic performance, i.e. reproduction and acidification of the stressed cells [79]. With help of the cF-efflux assay population's heterogeneity following treatment could be resolved, i.e. cells that are capable of performing glycolytic activity and getting energized upon sugar addition are distinguished from the cells that are not [51].

Active dye extrusion from energized cells as described for rhodamine 123 and or fluorescein may be linked to existing multidrug resistant pumps [66]. Proton gradient could be detected by observing the efflux of ethidium bromide (EB). This nucleic acid dye can cross the intact cytoplasmic membrane but is actively pumped out of the cells via a non-specific proton antiport transport system [80]. Active exclusion of EB was reported to correlate with metabolic activity [47].

***Membrane integrity***

The presence of an intact membrane is prerequisite for maintaining the capability of metabolic activity. Cells can recover from a transient permeabilization, but if the membrane is irreversibly compromised the cell is doomed to die. Without an intact membrane a cell can not maintain electrochemical gradients so it will loose its membrane potential and pH gradient. As the intracellular compartment is no longer separated from the environment, components leak out of the cell and potentially toxic chemicals from the environment diffuse freely into the cell. Under this circumstance breakdown of cell components and finally the degradation of the whole cell occur [51]. However, whether or not the criterion of membrane integrity is a reliable indicator of viability is a matter of controversy; basically due to the presence of a significant proportion of bacteria after heat inactivation trials, which was poorly stained by propidium iodide (PI) [79, 81]. PI is most commonly applied for the determination

of membrane integrity [54, 66]. It is a membrane-impermeant, nucleotide-binding probe which is excluded from cells with intact membrane. Following loss of membrane integrity PI diffuses into the cells and intercalates into the double stranded helical structure of nucleic acids (DNA or RNA) forming a red-fluorescent complex. Dye exclusion assay using PI has been considered as the most reliable stain as PI positive cells have not yet been shown to grow upon sorting [47]. This supports the hypothesis that with the breakdown of cell wall integrity, irreversible damage is achieved, thus it remains the best indicator for cell death. Other impermeant dyes which can only enter cells with sufficient membrane damage and are excluded by cells with intact membrane are the cyanic nucleic acid dyes TOTO-1, TO-PRO-3, Sytox Green™ [67]. Compared to PI these new DNA probes are highly fluorescent [82] and allow better discrimination of intact and membrane-compromised cells [83].
EB, which is a homolog of PI is not suitable for use as membrane integrity indicator since it is actually taken up by bacteria and is rapidly removed by an efflux pump [84]. Compared to EB PI carries an additional positive charge over ethidium and is therefore more likely to remain excluded from membrane-intact cells [76]. Thus, it is more appropriate to use EB as supravital or total cell stain in combination with a mixture of sodium azide and Tween-20, which could overcome the dye extrusion of EB without compromising membrane integrity [47].

***Membrane potential***

Membrane potential plays a critical role in bacterial physiology. As a component of the proton motive force, it is intimately involved in the generation of ATP, in the bacterial autolysis, in glucose transport, in chemotaxis as well as in survival at low pH [85]. The transmembrane electrical potential gradient in metabolically active bacteria is typically 100 mV, with the interior negative, originates from selective permeability and the active transport of ions across the cytoplasmic membrane, which causes differences in the concentrations of ions on opposite sides of the cell membrane [54, 64]. The magnitude of membrane potential is reduced to zero in dead cells, particularly when the integrity of membrane is destroyed by physical or chemical agents or by certain classes of antimicrobial drugs [85]. Alternatively, collapse in the membrane potential can be due to treatment with proton ionophores by eliminating the proton gradient across membrane. Any treatment that reduces the magnitude of membrane potential is said to depolarize the cell.
To analyze the membrane potential, distributional probes are usually applied. These are lyphopilic dyes that can readily pass the cell membrane and accumulate according to their charge [64]. Positively charged lipophilic rhodamine 123 and cyanine dyes ($DiOC_6$) can pass cell membranes, but are only retained in cells with a polarized cytoplasmic membrane. The fluorescence level is determined by the magnitude of membrane potential. $DiOC_6$ was used

to detect decrease of membrane potential in *Listeria* cells in response to bacteriocin, which reflects the mode of action of these antimicrobial peptides in inducing pore formation leading to ionic leakage [86].

However, cationic membrane potential probe is not quite suitable for Gram negative bacteria, since they frequently do not take up cationic stains unless the outer membrane of the cells is permeabilized [81, 87]. Furthermore, the measurement of membrane potential with rhodamine 123 is complicated due to the presence of active transport system, which pump out the fluorescence stain [66, 88].

On the other hand, anionic oxonol dyes such as $DiBAC_4(3)$, which is a negatively charged molecule, can enter depolarized cells or is excluded if a membrane potential is present. In depolarized cells this dye binds to lipid-rich intracellular components, resulting in bright green fluorescence. The aforementioned problems with cationic dyes are not an issue with these anionic dyes because they only enter the cell once the active transport system have ceased and the membrane potential is lost [87]. The use of this dye allow a clear discrimination between viable and depolarized/dead cells of *E. coli* after various treatments [81, 89, 90] or of *B. lactis* cells after exposure to bile salts [91]. Moreover, a strong relationship was found between the percentages of depolarized cells stained with $DiBAC_4(3)$ and the degree of cell membrane damage in dried yeast as measured by the more traditional method od leakage of intracellular compounds [92].

It was noted that fluorescence signals for both cationic and anionic lipophilic dyes are strongly dependent on cell size [53]. Thus, for precise and accurate estimation of membrane potential using $DiOC_2(3)$ a ratiometric technique is developed. With help of this approach the influence of cell size or size variations on fluorescence signal from this dye could be decoupled [85]. The numerical value of membrane potential is described as the ratio of size but potential independent green fluorescence to red fluorescence, which is both dependent on size and potential [53].

***Multiple physiological probes***

Multiple physiological probes, which are used in multiple assay or multi-staining approach, could facilitate acquisition of information about various cell properties and improved dissection of several sub-populations based on their differential dyes uptake. Care has to be taken in selecting appropriate combination of dyes with regard to excitation and emission wavelength so as to allow distinction of each probe in the presence of other [60]. Software compensation is sometimes necessary when spectral overlap between the emitted fluorescence of stain mixtures occurs.

The multiple staining strategy using fluorescent dyes cFDA and PI was broadly applied. This staining strategy was successfully used to characterize the effect of heat treatment on

*Lactobacillus plantarum* [66], the permeabilizing effect of bile salt and acid on *Lactococcus lactis* [83], the effect of ethanol stress on *Oenococcus oeni* [93], and the response of *Bifidobacterium lactis* towards bile stress [91]. Moreover, cFDA/PI staining had also been applied for the determination of viability of *Trichomonas vaginalis* [94] and for the analysis of bacterial activity in the aquatic environment [61, 95].

The applicability of the commercially available LIVE/DEAD®BacLight™ bacterial viability kit has been evaluated on a wide spectrum of bacteria [62, 69, 96-100]. This kit was developed to differentiate live and dead bacteria based on plasma membrane permeability. The staining mechanism using LIVE/DEAD kit on bacterial cells is based on the attachment of the non-fluorescent agents on nucleic acids [67]. Once the DNA-dye complex is built fluorescence could be measured. This kit is constituted of two fluorochromes, which have distinct fluorescent behaviour in terms of emission wavelengths and membrane permeability. The first component is the membrane-permeant stain SYTO9®, which stains all cells and can be used to distinguish particles from cells. Due to membrane damage, the second dye, the membrane-impermeant dye propidium iodide (PI) penetrates into cells and quenches the green SYTO9® fluorescence. When used in combination, intact cells are labeled green and cells with damaged membranes are labeled red.

Simultaneous staining with membrane impermeant and supravital DNA stains such as PI and EB combined with azide as decouplers for EB-efflux transporter was reported to be useful to differentiate between dead and potentially viable bacteria [47].

A triple fluorochrome staining procedure involving EB, PI and $DiBAC_4(3)$ was developed to differentiate starved cells of *Salmonella typhimurium* according to their dye uptake behavior [56, 64]. Compared to the triple staining method using rhodamine 123 as membrane potential dye the staining method with $DiBAC_4(3)$ has a wider range of application since rhodamine 123 staining does not work on pumping cells, i.e cells with an active efflux mechanism [64]. The sub-populations observed were categorized as metabolic active (actively excluding EB), deenergized but with a polarized cell membrane (uptake of EB but exclusion of $DiBAC_4(3)$), depolarized (uptake of both dyes) and permeabilized (uptake of PI). In combination with sorting and reproductive growth assay of separate sub-populations on agar it was found that most polarized cells could be recovered as well as a significant fraction of the depolarized cells. The latter result indicate that cells without membrane potential are not necessarily non viable [54, 81]. Pump activity was found to be a more sensitive indicator of cell stress since this activity already ceases prior to electrical depolarisation [64]. The triple staining technique with EB, PI and $DiBAC_4(3)$ was also applied to examine cell physiology of *S. cerevisae* and *E. coli* under various fermentation conditions [56, 87]. Considerable drop in cell viability, which correlated with cytoplasmic membrane depolarisation and increase of permeability,

was observed in the latter stages of fed-batch fermentations, at which the cells were exposed stress associated with glucose limitation [101].

These examples emphasize the potential of using multiple probes in giving single-cell based information on the physiological condition of bacteria and the use for monitoring changes following imposed stress or environmental changes as well as for investigating heterogeneities.

### *2.1.4 Objective*

The objective of this study was to characterize the physiological/metabolic behaviour of *Lactobacillus rhamnosus* GG population following exposure to heat, high hydrostatic pressure and high intensity ultrasound with help of flow cytometric analysis. The investigated organism served as a model system prior to further investigations with typical food spoilage bacteria. Flow cytometric analysis was performed under application of double staining method with cFDA (carboxyfluorescein diacetate) and PI (propidium iodide). With this dye combination the treatment effects on bacterial intracellular enzymatic activity and integrity of cytoplasmic membrane could be determined. Furthermore, the ability of *Lactobacillus rhamnosus* GG to extrude accumulated cF upon energization with glucose represents a physiological characteristic, which could be ascertained as an additional vitality marker. In conjunction to conventional cultivation assays the application of this rapid, fluorescence-based method along with a suitable measurement strategies allow the mechanism of bacterial inactivation by means of physical treatments to be better characterized and considered during optimization of food decontamination processes.

## 2.2 Material and methods

### *2.2.1 Test organism*

*Lactobacillus rhamnosus* GG (ATCC 53103) – thereafter abbreviated with LGG – was obtained from Valio R&D (Helsinki, FI). This probiotic strain is of human origin. The beneficial effects of LGG have been shown in many types of intestinal disturbances caused by pathogenic bacteria and viruses, as well as in prophylactic use [102]. For long-term maintenance LGG was stored as glass bead cultures (Roti®-Store, Carl-Roth, Karlsruhe, D) in a -80°C freezer (U101, New Brunswick Scientific, Nürtingen, D).

### *2.2.2 Inactivation treatments and microbiological analysis*

One bead from deep-frozen culture was transferred into MRS broth (Oxoid, Basingstoke, UK) and incubated overnight at 37°C. This broth was then used to inoculate the final broth under adjustment of $N_0 \sim 10^3$ CFU $mL^{-1}$. The culture was incubated at 37°C up to stationary growth

phase for 24 h. Cells were harvested by centrifugation at 2700 X g for 10 min at 10°C, washed twice with 10 mM sterile phosphate-buffered saline (PBS) at pH 7.0, and finally resuspended in 50 mM PBS (pH 7.0) to an $OD_{600nm}$ of 10 (corresponding to a cell concentration of $10^9$ CFU $mL^{-1}$).

For *thermal treatment* two hundred µL of cell suspension were transferred into glass vials and immersed in water bath at 60, 68 or 75°C for different exposure time.

For *high pressure treatment* the suspension was filled into 1.8 mL - cryovials (Type 375299, Nunc, Roskilde, DK). Pressure treatment was performed with a multi-vessel high pressure unit (Type U111, Unipress, Warsaw, PL). This unit consists of five pressure chambers, which are separated from each other via high pressure valves (Fig. 3). All chambers are immersed in a water bath equipped with a thermostat, which allows a simultaneous treatment of five different samples in one pressure build-up step at close to isothermal conditions. High pressure treatments at different pressure levels were conducted at 37°C for 10 min.

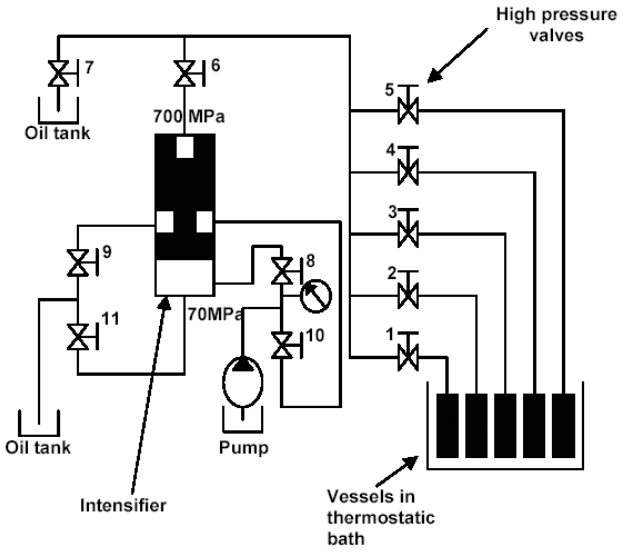


**Figure 3**

Schematic hydraulic diagram of multivessel high pressure apparatus U111. The intensifier is connected with the pressure vessels through high pressure valves (1-5). The multiplication factor (~11) of the intensifier leads to a maximum pressure of 700 MPa. Valves 6-11 are used for loading and unloading the pressure medium (silicon oil).

*High-intensity ultrasound treatments* were carried out using a Sonopuls HD 2070 homogenizer (Bandelin Electronic, Berlin, Germany). This unit was composed of a frequency generator, a piezoelectric transducer UW70 and an amplifying horn SH70 with a sonotrode KE76. The electrical energy, which produces oscillations of the piezoelements in the

transducer, is transformed by the horn and the sonotrode. The sonotrode surface vibrates at a frequency of 20 kHz.
For all experiments a wave amplitude of 160 µm (equivalent to a power input of 17.6 W) was applied. In detail, approximately 10 mL of microorganism suspension was filled into a glass beaker. Then the sonotrode was immersed into the suspension. Samples for microbiological analysis were taken periodically during the ultrasound application. The temperature changes during ultrasound treatments were monitored with a K-type thermocouple.

After treatments the samples were rapidly cooled on ice, diluted in Ringer's solution (No. 15525, Merck, Darmstadt, DE) and drop plated in duplicate on MRS agar (Oxoid, Basingstoke, UK). Plates were placed in an anaerobic jar under anaerobic atmosphere, generated by an anaerobic kit (Anaerocult®A, Merck, Darmstadt, DE). The viable cell numbers were determined after 48 h of incubation at 37°C.
All inactivation data were expressed as logarithm of the relative survivor fraction ($\log N/N_0$). N refers to the bacterial count following exposure to a particular treatment, whereas $N_0$ represents the initial count prior to the treatment. All experiments were performed at least in triplicate.

### *2.2.3 Staining procedure and measurement strategies*

Control or treated cells were initially incubated with 50 µM cFDA (Molecular Probes, Inc. Leiden, The Netherland) at 37°C for 10 min to allow intracellular enzymatic conversion of cFDA into cF. Immediately after this labelling, the cells were spun down and resuspended in 50 mM PBS-buffer (pH 7.0). This step is the followed by addition of 30 µM PI (Molecular Probes, Inc. Leiden, The Netherland). The cell suspension was kept in ice bath for 10 min to allow labelling of membrane-compromised cells prior to measurement in flow cytometer. To measure the performance of treated cells in extruding intracellular accumulated cF activity, cF-stained cells were incubated together with glucose 20 mM for a fixed holding time of 20 min at 37°C, as adapted from previous studies [75, 79]. The kinetics of cF-release from glucose energized cells were monitored by incubating cF-labeled cells at 37°C in the presence of glucose 20 mM and measuring the progress of cF-extrusion every 5 min.

### *2.2.4 Flow cytometric measurement*

Flow cytometric analysis was performed on a Coulter®EPICS®XL-MCL flow cytometer (BeckmanCoulter Inc., Miami, Florida, USA) equipped with a 15 mW 488 nm air-cooled argon laser. All the parameters were collected as logarithmic signals. Green fluorescence of cells stained with cF was collected in the FL1 channel (525 ± 20 nm), whereas red fluorescence of cells labelled with PI was collected in the FL3 channel (620 ± 15 nm).

Spectral overlap between the emitted fluorescence of stain mixtures was eliminated by appropriate software compensation. Acquisition of fluorescence data was performed by pre-setting a gate in the forward-angle light scatter (FS) vs. sideward scatter (SS) plot, with help of which bacterial cells of interest and artefacts could be discriminated. The flow rate was set at typical values of 300-600 bacterial cells per s. The software package Expo32 ADC (BeckmanCoulter Inc., Miami, Florida, USA) was used to analyse flow cytometry data. All detectors were calibrated with FlowCheck™ Fluorospheres (BeckmanCoulter Inc., Miami-FL, USA). Other specific settings of flow cytometer for this measurement are listed in Annex 2.

### *2.2.5 Analysis of flow cytometric data*

Density plot analysis of green fluorescence (FL1) *versus* red fluorescence (FL3) was applied to resolve the fluorescence properties of the population measured by flow cytometer (Fig. 1 and Fig. 2). With this graph the population was able to be graphically differentiated according to their fluorescence behaviours. Table 1 describes the quadrant designation of stained cells.

**Table 1**

Quadrant designation of cells stained with cF and PI

| Quadrant | Labelling behaviour | Cellular mechanism involved |
|---|---|---|
| #1 | $cF^-$ and $PI^+$ | cF-accumulation as a result of esterase activity not detectable |
| | | Membrane compromised |
| #2 | $cF^+$ and $PI^+$ | Active esterase |
| | | Membrane minimally damaged |
| #3 | cF- and $PI^-$ | Esterase activity not detected or cF extruded out of the cells |
| | | Intact membrane |
| #4 | $cF^+$ and $PI^-$ | Active esterase |
| | | Intact membrane |

Residual cF-accumulation activity following pressure treatments was calculated using Equation 1, in which the post-pressure activity of the population framed in quadrants #2 and #4 was set in relation to the activity of untreated cells, which were encountered in quadrant #4.

Based on the shift of cF-stained population upon glucose addition from quadrant #4 into quadrant #3 after a 20 min incubation period cF-extrusion in response to glucose energization was able to be monitored (Fig. 2) . Equation 2 calculated the performance of dye extruding mechanism of pressure treated cells. Similar to aforementioned staining strategy, glucose energized cells were measured every 5 min to follow the kinetics of cF-

extrusion of pressure treated cells. In Equation 3 the relative number of population losing intracellular accumulated cF could be followed over time.

$$EA\ [\%] = \left( \frac{Q4_{p}}{Q4_{Ctrl}} \right) \cdot 100 \qquad \text{Equation 1}$$

EA : Residual enzymatic activity in response to a particular pressure treatment

$Q4_{p}$ : Percentage of population in quadrant A4 following pressure treatment

$Q4_{Ctrl}$ : Percentage of population in quadrant A4 prior to pressure treatment

$$cFA\ [\%] = \left( 1 - \frac{Q4_{Glu}}{Q4} \right) \cdot 100 \qquad \text{Equation 2}$$

cFA : Measure of performance in extruding cF

$Q4_{Glu}$ : Percentage of population in quadrant #4 following glucose addition and 20 min incubation

Q#4 : Percentage of population in quadrant #4 prior to glucose addition

$$RcF\ [\%] = \left( \frac{Q4_{t\_Glu}}{Q4_{t=0}} \right) \cdot 100 \qquad \text{Equation 3}$$

RcF : Relative number of cells still stained with cF in quadrant #4 following glucose addition

$Q4_{t\_Glu}$: Percentage of cells still stained with cF in quadrant #4 following glucose addition and incubation for t min

$Q4_{t=0}$: Percentage of cells still stained with cF in quadrant A4 prior to glucose addition

### *2.2.6 Statistical analysis*

Statistical significance of the effect of pressure treatments on cell viability and pressure-induced changes on physiological status of LGG was examined using one-way ANOVA test. Differences were considered significant at the $p<0.05$ level of probability. All statistical analysis were performed with Origin7 software package (OriginLab, Northhampton, MA, USA)

## 2.3 Results and discussion

### *2.3.1 Basic pattern*

To differentiate bacterial population based on their fluorescence properties, the dual-parameter density plot of the green fluorescence (x-axis) and the red fluorescence (y-axis)

was used (Fig. 4). Each dot, which constitutes the cell cloud, represents one single cell, which is plotted as a co-ordinate of its green and red fluorescence value. Principally, the effect of physical treatments on LGG was evaluated as the ability to accumulate and retain cF as an indicator of membrane integrity and enzyme activity and the uptake of PI to assess membrane damage.

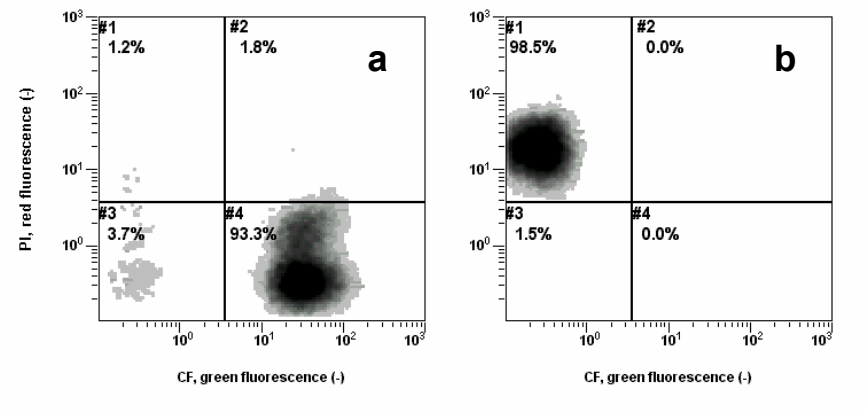


**Figure 4**

Basic fluorescence density plots (cF, green fluorescence vs PI, red fluorescence) of intact and heat treated cells of *L. rhamnosus* GG (a and b, respectively) following staining with cFDA (carboxyfluorescein diacetate) and PI (propidium iodide). Heat treatment at 75°C for 30 sec was performed to yield dead, membrane-compromised cells. The figures (in %) following the quadrant number are associated with the percentage of the population in the corresponding quadrant.

The quadrants arrangement on the dot plot were set so that viable cells of LGG with intact membranes were framed in quadrant #4 (Fig. 4a). Within this quadrant only the population, which both actively accumulated cF and excluded PI, thus showing high green fluorescence and low red fluorescence, was encountered. Prior to the treatment, all LGG cells were encountered in quadrant #4 (Fig. 4a). Upon heat-induced rupture of cell membrane and loss of cF-accumulation capacity the cells are not capable of excluding PI. This particular population, which was solely labeled by PI, showed low green fluorescence and high red fluorescence. Membrane damaged population was thus encountered in quadrant #1 (Fig. 4b).

### *2.3.2 Inactivation mechanisms by heat treatment*

Figure 5 shows a sequence of fluorescence density plots; each of them showing the result of cFDA/PI labeling on LGG after different holding time at 60°C. Prior to heat challenge, cells were found in quadrant #4, which indicated that they were solely stained by cF (Fig. 5a). Exposure to 60°C at increasing holding time resulted in a gradual increase of cells framed in quadrant #3, while simultaneously the number of cells framed in quadrant #4 was decreasing

(Fig. 5b to 5f). It was also observed, that practically no cells are found in quadrant #1. After 300 s of thermal treatment the majority of the cells are encountered in quadrant #3 (Fig. 5f). According to the quadrant designation described in Table 1, the occurrence of cells in quadrant #3 indicates that these cells were labeled neither by cF nor by PI. The increasing occurrence of a sub-population in this quadrant indicated that due to heat challenge the energy-independent accumulation of cF – which is a physiological feature of untreated cells – was considerably reduced. The absence of PI labeled population suggests that the membrane integrity was still relatively high, thus not permitting PI penetration across the membrane to intercalate with nucleic acids. Although the treatment time was prolonged to 300 s, only max. 15% of the population was showing positive PI fluorescence, suggesting that the majority of the treated cells had still high membrane integrity (Fig. 5g). The absence of PI labeling accompanied by increasing loss of cF-accumulating activity suggested that in the cells exclusively heat inactivation of esterase occurred, whereas membrane remained unaffected. Furthermore, when data from plate count method were taken into account (Tab. 2), it was evident, that even after only 120 s of exposure to 60°C 99.99999% of all bacteria were inactivated (log $N/N_0$ ~ -7). However, only as many as 4.5% of the whole population were positively stained with PI (Fig. 5g).

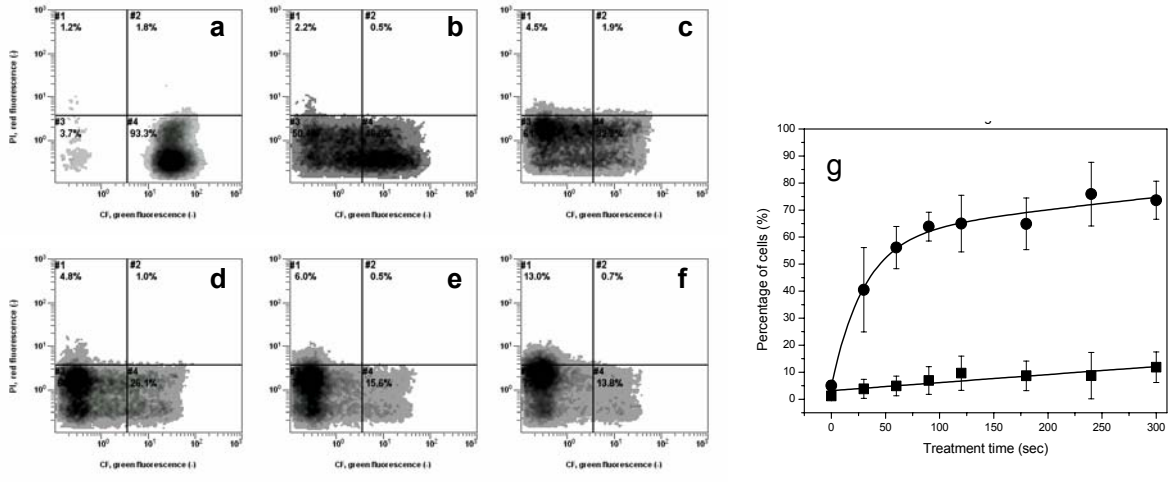


**Figure 5**

Fluorescence density plots of *L. rhamnosus* GG in response to staining with cFDA and PI after heat challenge at 60°C for different exposure time: 0 (a), 30 s (b), 60 s (c), 120 s (d), 240 s (e) and 300 s (f). The figures (in %) following the quadrant number are associated with the percentage of the cells in the corresponding quadrant.

(g) Kinetics of the increase in the percentage of *L. rhamnosus* GG cells in quadrants #1 and #3 after heat treatment at 60°C, as derived from the sequence of density plots Fig. 2a to 2f. Cells encountered in quadrant #3 (●) are neither labeled with cF nor with PI ($cF^-$ and $PI^-$), whereas in quadrant #1 (■) only the sub-population solely stained by PI ($cF^-$ and $PI^+$) are framed. The results are means based on data from three or more independent experiments with error bars indicating standard deviations.

This findings suggests that thermal death could be achieved in absence of membrane degradation, as emphasized by flow cytometric fluorescence pattern of cells killed at 60°C. Lievense *et al* (1994) who attempted to distinguish between dehydration and thermally induced damage on *L. plantarum* concluded, that drying at 5°C resulted in membrane damage and cell death, as manifested by increased penetration of Dnase into cells, whereas cell inactivation could be achieved upon exposure to 60°C in absence of membrane rupture [103]. Studies conducted by Jepras *et al* (1995) revealed the occurrence of a significant fraction of heat killed *E. coli* cells (65°C for 30 min) which was poorly stained by PI [81]. Similar findings has also been reported by Bunthof *et al* (1999), who demonstrated that from *L. lactis* cells treated at 60°C for 90 s, as much as 69% were not PI labeled although these cells could not be recovered on plates [79]. Alternatively, the cytoplasmic membrane of cells killed by heat at 60°C might be ruptured, but the pore size induced might be too small to allow proper diffusion of PI into cell interior. As a result, PI staining in such cells was not observed within the incubation period after PI addition, i.e. 10 min in ice, as described in the standard incubation protocol. A possible approach to confirm the delayed penetration of PI would be a prolonged contact time with PI. Data on the delayed diffusion of PI into lymphocytes that had been made necrotic by high pressure indicated that this is due to

gelatinised cytoplasmic proteins and steady-state level PI fluorescence was achievable only after a contact time of 300 min [104]. A general concern which is often put forward upon using PI, i.e. the low extinction coefficient and hence the relatively low fluorescence of this probe [54], did not hold true, since a relatively high PI fluorescence could indeed be detected on cells heat killed at 75°C (Fig. 4b).

A different fluorescence behavior was obtained upon heat kill of LGG at higher treatment temperatures. When the fluorescence density plots of cells treated for a fixed holding time (90 s) were compared, it is evident, that at temperatures of 68 and 75°C cells were exclusively found in quadrant #1, suggesting that PI stained cells predominated (Fig. 6b and 6c), whereas only minor population was encountered in quadrant #1 after treatment at 60°C (Fig. 6a).

In Fig. 5d the kinetics of change in the percentage of PI stained cells upon heat treatment at various temperature is shown. Following treatment at 60°C only max. 10% of the population was found in quadrant #1. In contrast, PI fluorescence occurred in more than 90% of the cells even after only exposing them to 75°C for 30 s. Similarly, at 68°C after a holding time of 90 s more that 85% of the cells were exhibiting PI fluorescence. Taking a treatment time of 90 s as a base for comparison, it is clear that according to D-values obtained from heat inactivation trials at these three temperatures (Tab. 2) 99.9999% of the initial population could not resume growth on agar.

**Table 2**

Inactivation rates (k) and decimal reduction time (D-value) of *L. rhamnosus* GG treated in sterile 50 mM phosphate-buffered saline (pH 7.0) at different temperatures. Data were calculated from two or more replicate heat inactivation experiments.

| Treatment temperature (°C) | k ± SD ($s^{-1}$) | $R^2$ ± SD | D- value = 1/k (s) |
|---|---|---|---|
| 60 | 0.061 ± 0.0068 | 0.978 ± 0.016 | 16.3 |
| 68 | 0.205 ± 0.071 | 0.949 ± 0.072 | 4.9 |
| 75 | 0.325 ± 0.148 | 0.951 ± 0.064 | 3.1 |

$R^2$ : correlation coefficient

SD : Standard deviation

The findings demonstrates that the response of the heat killed cells towards PI labelling could be differentiated according to the temperature levels. Furthermore, the difference in the PI staining capacity might reflect the difference in target sites of heat inactivation depending on the temperature level used. The possibility to inactivate intracellular esterase without seriously compromising cellular membrane might give evidence of an alternative inactivation pathway preceding or apart from heat-induced membrane damage. Inactivation of esterase, which is not involved in the maintenance of viability, is therefore indicative for a substantial

defect in an unknown, heat-sensitive cellular component, which ultimately led to cell death. In addition, this result also emphasizes the general importance of exposing vegetative cells to temperatures above 60°C, when the inactivation of intracellular esterase and membrane damage were primarily aimed.

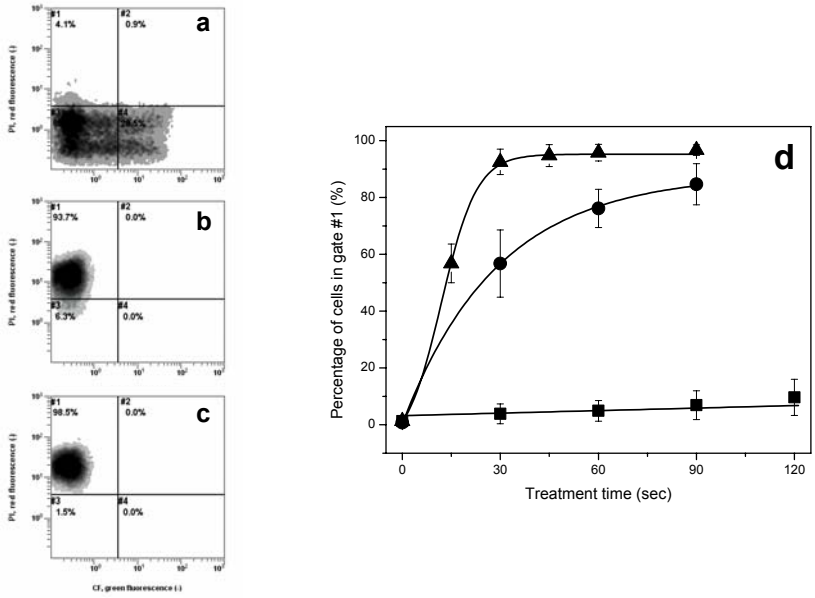


**Figure 6**

Fluorescence density plots of cFDA/PI labeled *L. rhamnosus* GG cells after exposure to 60°C (a), 68°C (b) and 75°C (c) for 90 s. The figures (in %) following the quadrant number are associated with the percentage of the cells in the corresponding quadrant.

(d) Kinetics of the increase in the number of PI labeled population (cells framed in quadrant #1) of *L. rhamnosus* GG after heat treatment at 60°C (■), 68°C (●), and 75°C (▲). The results are means based on data from three or more independent experiments with error bars indicating standard deviations.

Another cellular constituent which was reported to be implicated in thermal induced cell inactivation is ribosome [2]. A strong relationship was observed between thermal death of bacteria and the first major peak in DSC thermograms (temperature between 60 to 80°C) which is attributed to ribosomal melting [4]. Viability loss of *L. plantarum* cells occurred when the microorganisms were subjected to heat treatment in the range of 55 to 70°C for 60 s prior to DSC scan [105]. The viability loss is related to the irreversible change of apparent enthalpy and in the shapes or position of peak temperatures associated with ribosome subunits, which was much more evident the higher the applied challenge temperature was. The authors also found no apparent influence on the thermally induced transitions of other cellular structures. This findings suggest that heat induced cell death can possibly occur in absence of membrane deterioration due to ribosomal denaturation. In the latter work, the

temperature range, where ribosome denaturation was detected by DSC measurement, was found qualitatively similar to the treatment temperature used in this study (60°C), in which cells of LGG were inactivated without significant loss of membrane integrity.

In contrast, intracellular DNA denaturation was most likely not involved in the thermal inactivation at temperatures evaluated in this study, i.e. between 60 and 75°C. According to the results of previous thermal analysis studies irreversible denaturation of cellular DNA required temperatures well above the temperature of cell inactivation [106]. Irreversible DNA denaturation on *L. plantarum* was only observed after the cells were preheated to 100°C [105]. At temperatures that cause ribosome denaturation, the DNA transition is reversible [107].

### *2.3.3 Inactivation mechanisms by high hydrostatic pressure*

***Fluorescence properties of pressure treated LGG in response to cFDA/PI staining***

It is shown in Figure 7b, 7c and 7d that cells exposed for 10 min to 200, 400, and 600 MPa still possessed residual esterase activity, respectively. This is well documented by the presence of the greater part of pressure-treated population in quadrant #2 and #4, in which – similar to untreated sample (Fig. 7a) – cells with high cF fluorescence and thus high green fluorescence value were encountered. The cells accumulating cF are further separated into two sub-populations: cells solely labelled by cF in quadrant #4 and a sub-population in quadrant #2, where in consequence of a certain intensity of the applied pressure treatment, part of the population was double stained by cF and PI. This unique sub-population was observed especially following pressure treatment at pressures beyond 400 MPa (Fig. 7c and 7d). Furthermore, another sub-population which exhibited only PI fluorescence (located in quadrant #1) was found upon application of pressures in excess of 400 MPa.

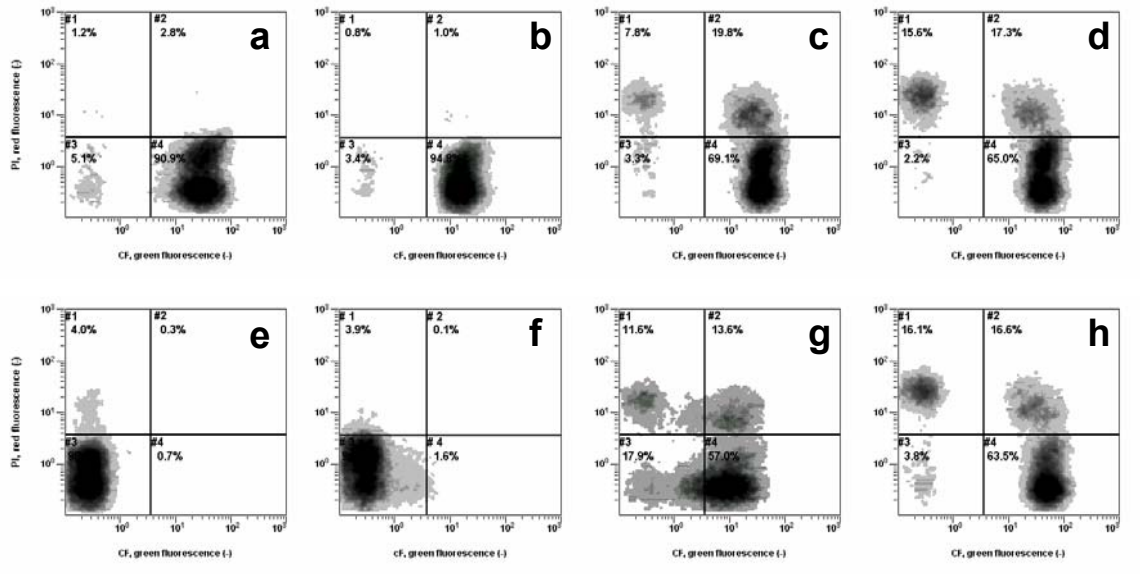


**Figure 7**

Flow cytometric fluorescence dot-plots of *L. rhamnosus* GG treated with various pressure levels in response to cFDA/PI staining (upper figures, a: control, b: 200 MPa, c: 400 MPa, d: 600 MPa) and after glucose energization assay to determine the activity of the cells in extruding the intracellular accumulated cF (lower figures, e: control, f: 200 MPa, g: 400 MPa, h: 600 MPa ).

As already mentioned before, at pressures higher than 400 MPa three sub-populations could be identified based on their differential uptake of cF and PI: cF-stained (quadrant #4), cF and PI double stained (quadrant #2), and PI stained population (quadrant #1). In the special case of the double stained population, it was reported that the occurrence of a sub-population with this unique staining characteristics was also observed in bile salt stressed bifidobacterial cells and in ethanol stressed malolactic starter cultures [91, 93, 108]. Double stained population could be regarded as an intermediate state of membrane damage. The cell membranes of these cells seemed to be irreversibly damaged to a low extent, under which relatively big molecule PI (molecular weight: 668 $g \cdot mol^{-1}$) could get into the cells, whereas simultaneously cF was still intracellularly retained. The fact that cF (molecular weight: 376 $g \cdot mol^{-1}$) which is far smaller than PI did not passively diffuse out of the cells, is surprising. Compared to the original non-fluorogenic substrate cFDA (molecular weight: 460 $g \cdot mol^{-1}$), which is moderately permeant to cell membrane, the presence of additional negative charges on cF at physiological pH may possibly inhibit its leakage out of the cells [54, 67], unless a certain degree of membrane degradation was exceeded. Strong intracellular binding of cF was documented by the retention of cF in high pressure killed cells incubated for 120 min at 37°C (Fig. 10).

The occurrence of double stained cell population indicated the presence of a threshold pressure level for irreversible membrane rupture. In excess of this critical level, which obviously settled at 200 MPa and which is marked by a drastic increase of the percentage of

PI labelled cells (Fig. 8d), PI could penetrate some cells, while for some cells cF could still be retained but for some other not. The threshold level seemed to be non-homogeneously distributed within the population, since major part of the population did not accumulate PI and consequently was still encountered in quadrant #4 (Fig. 7). Up to 600 MPa only a small part of the whole population was stained by PI (Fig. 8d); thus membrane rupture occurred only on the most sensitive populations.

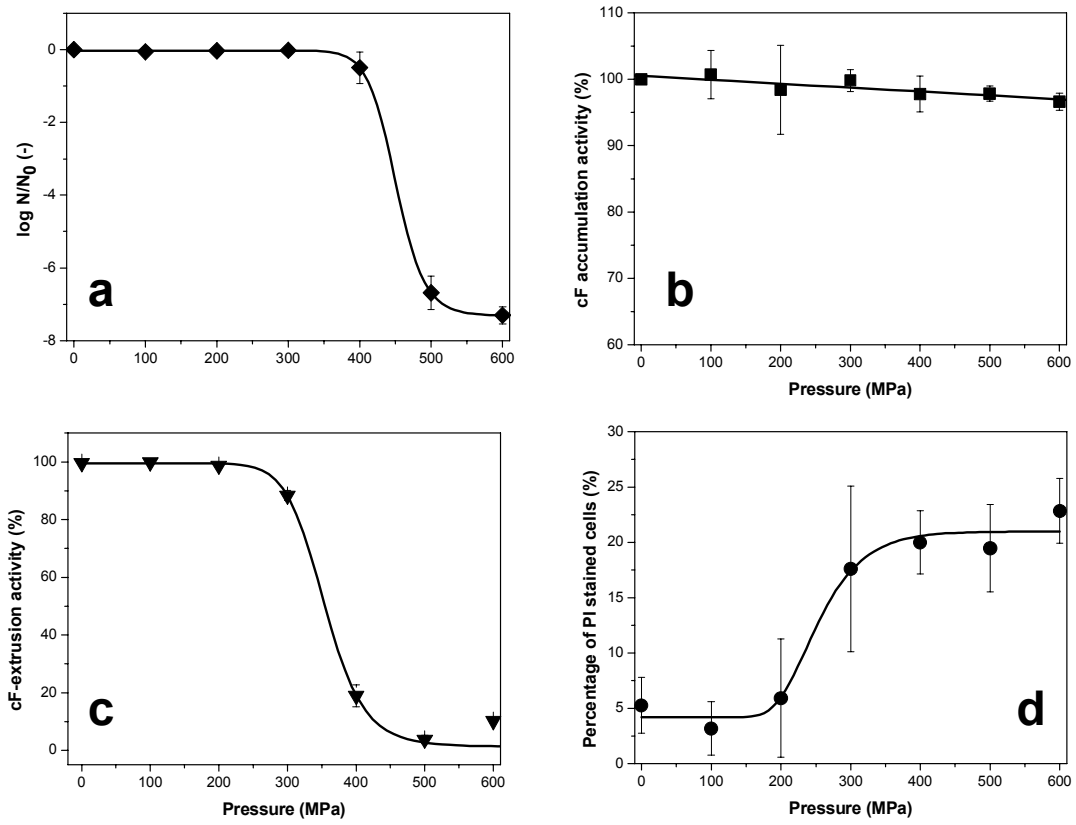


**Figure 8**

Impact of pressure treatment at 37°C for 10 min on several viability indicators of *L. rhamnosus* GG: survival ratio as determined by plate count on MRS agar (◆), and metabolic activities as derived from flow cytometric analysis including cF-accumulation activity (■), cF-extrusion activity (▼) and number of cells with damaged membrane (●). The calculation of metabolic activities was based on the analysis of fluorescence density plots in Fig. 6.

Regarding the viability status of this double stained population, sorting of the population of stressed *B. lactis*, which were double stained with cFDA and PI, showed that a significant part could resume growth on plates after resuscitation [91]. This physiological status was considered as a transient phase in the progressive change towards cell dying, however, death is not irreversible and double stained cells may still recover [51]

An alternative view to explain the cause of double stained population was suggested by Shapiro (2001), who speculated that this particular population was resulted from clumps

containing bacteria with opposing physiological status [53]. Some events associated with both high TO-PRO-3 fluorescence and high DiIC1(3) fluorescence, which are nucleic acid stain and membrane potential stain, respectively might represent clumps containing viable and dead cells of *S. aureus*. The viable cells have normal membrane potential and do not take up TO-PRO-3. The dead, membrane-damaged cells have zero membrane potential and do take up TO-PRO-3.

Furthermore, flow cytometric analysis combined with cFDA/PI staining strategy was also applied to evaluate the effect of high pressure on *Listeria innocua* at low temperatures, in which phase change between water and different ice modifications might occur [109]. Following pressurization at 300 MPa in liquid state (0°C), with which reduction by 3 log cycles was achieved, three different sub-populations, i.e. $cF^+PI^-$, $cF^+PI^+$, and $cF^-PI^+$, as encountered in the present study, were observed. In contrast, when phase change was induced (ice I to ice II) by means of pressurization at 300 MPa and –45°C the majority of the population was solely stained by PI. The difference in the cF/PI uptake properties might indicate different mode of action of pressure on bacterial cells depending on the physical state of water.

***Comparison of viability status as determined by cellular response to cFDA/PI labelling and standard culturability assay***

Based on these fluorescence density plots in response to cF/PI uptake, the effect of different levels of pressure on cF accumulation activity could be further quantified and compared with standard viability assessment, i.e. culturing method on MRS agar (Fig. 8a and 8b).

The results of the latter method showed that pressures lower than 400 MPa were considered to be non-lethal, whereas treatment at pressures higher than 500 MPa led to bacterial reduction by more than 7 log cycles (Fig. 8a). In contrast, it was observed that even after pressurization at pressures higher than 500 MPa, cells which were scored as dead by cultivation method still showed a high level cF accumulation capacity similar to untreated sample (Fig. 8b). These observations suggested that the cF accumulation capacity of the cell did not correlate with culturability, i.e. ability to reproduce and form visible colonies on MRS agar. Apparently the retention of intracellular esterase activity was not crucial in the maintenance of reproducibility/culturabilty. This result is further supported by other studies revealing considerable esterase functionality in cells killed by $H_2O_2$, $\gamma$-irradiation, and heat [48, 75, 79].

As previously mentioned, upon exceeding a threshold pressure level of 200 MPa bacterial membranes were compromised, thus leading to PI labelling of parts of the treated population. Nevertheless, the disruption of membranes alone could not be made responsible for loss of reproductive capacity, since upon pressure treatments at 300 and 400 MPa no significant

inactivation (Fig. 8a) was achieved although the magnitude of membrane ruptures at these pressures, i.e. the total percentage of PI stained cells, was as high as the one at higher, lethal pressure levels (Fig. 8d). Consequently, exclusion of PI from cell or the absence of PI labelling could not give profound evidence about their reproductive capacity.

Likewise, it was reported that pressure treated cells of *L. plantarum*, which were not recoverable by plate counts, were not stained by PI either [15]. Membrane damage was observed with the PI assay only for treatments resulting in greater than 5-log reductions of viable cells. The authors concluded that membrane damage owing to pressure treatment as determined with PI staining was observed later than cell death. In line with the latter observation a study which compared the type of cellular damage induced by high pressure at different growth phases showed that the inactivation of stationary-phase cells by high pressure may occur by a mechanism other than the permanent loss of membrane integrity [39]. The authors observed high pressure induced morphological and physiological changes in *E. coli* cells using lipophilic dye, which preferentially binds to cytoplasmic membrane. It was found that while in exponential-phase cells the loss of viability is always accompanied by a loss of the physical integrity of the membrane, the cell membrane in stationary-phase cells have a cytoplasmic membrane that is robust enough to withstand pressurization up to very intense treatments (600 MPa) and thus remain physically intact. It was further reported, that after high pressure treatment at 400 MPa, which was effective to kill *L. monocytogenes* and *S. aureus* by 6 to 7 log cycles, only ca. 75% of the population was positively labelled by PI [110]. Similarly, the fact that pressure inactivation is not based on membrane rupture is supported by the data on flow cytometric analysis of stationary phase cell of *L. monocytogenes*, which were fully inactivated using pressure of 400 MPa [111]. The authors demonstrated that following PI labelling there was a significant sub-population which did not take up PI, suggesting that their membranes were not seriously damaged, whereas another part of the population appeared to have been stained by PI, like heat killed cells (121°C, 15 min). Thus, the response of cells towards PI labelling, which reflects their status of membrane integrity, could not be regarded as a reliable indicator of cell viability [54]

***Fluorescence properties of pressure inactivated cells in comparison to that of heat killed cells – Difference in the inactivation pathway***

In correlation to the results of classical culturing method, the fact that pressure killed cells could still accumulate cF may put forward new considerations regarding the difference of mechanism of cell inactivation by both heat and pressure. According to Figure 8a, pressures as high as 500 or 600 MPa at 37°C gave approximately similar plate count results, i.e. more than 7 log-cycle reduction (Fig. 8a), as heat inactivation at 75°C in ambient atmosphere (Tab. 2). However, the inactivation pathway by pressure and heat differed pronouncedly, since

deviations were observed in the fluorescent profiles of the pressure (Fig. 7d) and heat killed cells (Fig. 6b). Basically, the fluorescence density plot patterns demonstrated different magnitude of membrane degradation as a result of exposure to these physical treatments. For cells inactivated by heat it was shown that the membranes of all cells were ruptured, leading to the occurrence of cells solely labelled by PI throughout the population; therefore framed in quadrant #1 (Fig. 6b). In contrast, only as many as 22% of pressure inactivated population was PI stained (Fig. 8d). This number is constituted of cells solely stained by PI (quadrant #1) and cells double-stained with cF and PI (quadrant #2). This comparison study revealed that that membrane integrity and esterase activity at the greater part of the pressure inactivated cells were not completely diminished. Compared to degradative effect of heat on cell membrane pressure induced cellular injury on bacteria, which ultimately lead to inactivation, is therefore not governed by the loss of membrane permeability.

***Activity of extruding intracellular accumulated cF in response to glucose addition***

Apart from the ability to accumulate cF and to exclude PI, which characterize intact cells of LGG (Fig. 4a), the ability to extrude accumulated cF upon energization using fermentable sugar, could also be ascertained as an additional vitality marker in order to study the mode of action of pressure on bacteria. This pump activity is most likely mediated by an ATP-driven transport system, since ATP production and rapid extrusion of cF upon energizing was observed despite dissipation of proton motive force by addition of ionophores valinomycin and nigericin [77, 79].

At physiological pH, cF has predominantly a threefold negative charge and can thus be considered practically membrane impermeable, or leaks very slowly [112]. An extensive extrusion of intracellular cF upon energization with 20 mM glucose could be followed by apparent shift of initially stained population from quadrant #4 (cF-stained) to quadrant #3 (unstained) owing to the loss of intracellular cF fluorescence (Fig. 7e to 7h). Based on the extent of population shift after 20 min incubation in presence of glucose the degree of injury on this cellular pump activity was able to be determined. Data on kinetic study of cF efflux on *L. lactis* an incubation time of 20 min in the presence of lactose was regarded as sufficient to effectively remove cF out of the cells [79].

This dye extrusion mechanism was found to be substrate specific, since upon addition of lactose, the extrusion did not take place (Fig 11a). It has been evaluated before (Annex 1), that LGG could not utilize lactose [102].

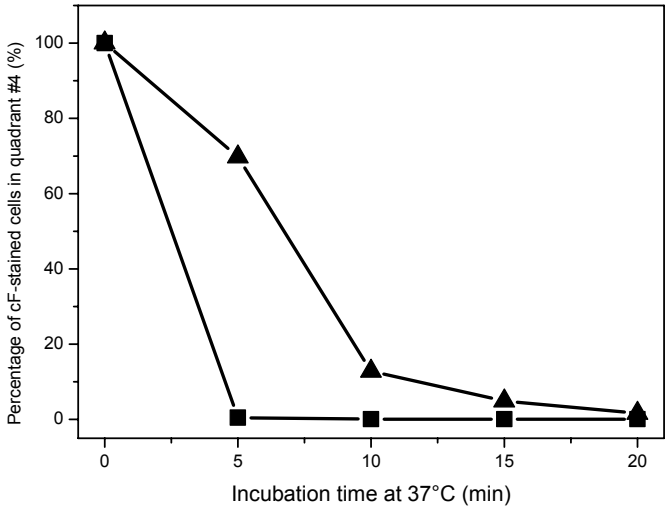


**Figure 9**

Kinetics of cF extrusion from *L. rhamnosus* GG cells, represented by the decrease in the percentage of cF-stained population in the presence of 20 mM glucose. The performance of this metabolic activity was measured on control population (■) and on cells treated at 200 MPa for 10 min at 37°C (▲).

It was observed that this performance of transporting cF out of the cells was more pressure sensitive in contrast to cF accumulation activity. After pressurizing the cells at pressures up to 200 MPa cells were able to survive and cF-efflux performance was as effective as control sample at a fixed glucose incubation period of 20 min (Fig. 7e and 7f). Following Fig. 8, no significant differences ($p>0.05$) could be observed between cells treated at 200 MPa and control group regarding the reproductive capacity, esterase activity and cF-extrusion performance. In order to profoundly elucidate cellular damage affected by incubation under elevated pressure, cF-extrusion performance was not determined at steady-state level, achievable after 20 min, but was continuously measured upon incubation at 37°C in presence of glucose (Fig. 9). It was shown from the kinetics of the migration of cF-stained cells from quadrant #4 to quadrant #3, that indeed the cF-extrusion of cells treated at 200 MPa was not as effective as untreated population, in which nearly all cells had already extrude intracellular accumulated dye within the first 5 min, whereas in the case of the pressure treated sample, the same level of dye extrusion was reached after 20 min.

According to culturing method, cells pressurized at 400 MPa were still able to recover on agar with a slight loss of viability ($\log N/N_0 < -0.5$). In contrast, flow cytometric data showed massive loss of efflux activity (Fig. 7g). Only 20% of initially cF-stained populations were becoming unstained upon glucose addition and thus separated in quadrant #3 (Fig. 8c). The results of these two viability assessments suggest, that this considerable disturbance of extrusion activity might not be detrimental for the reproductive capacity of LGG. Alternatively, the cells might be able to cope with or repair the defected and/or reduced functionality during

cultivation on MRS agar, thus not leaving it as a growth-limiting factor. When incubation period at 37°C for glucose energization assay was extended up to 120 min, it was observed that the percentage of population in quadrant #4 decreased, indicating that cells exposed to 400 MPa were still able to extrude intracellular accumulated cF (Fig. 10). The fact that compared to untreated sample (Fig. 9) the cF efflux activity of cells treated with 400 MPa was markedly reduced suggests that the occurrence of sub-lethal injury on this energy dependent dye extruding system.

At higher pressures of 500 or 600 MPa, not more than 10% of cF stained population in succeeded to transport intracellular accumulated cF across the cell membranes (Fig 8c). Passive efflux of cF owing to leakage during incubation with glucose could be excluded, since cF staining was still observed on cells previously inactivated by 600 MPa even after incubating them in the presence of glucose for 120 min (Fig. 10a) or 24 h (data not shown). According to plate count method cells treated with pressures higher than 500 MPa lost their reproductive capacity/culturability (Fig. 8a). Consequently, cF-efflux activity and culturability seemed to be strongly correlated at lethal pressure levels beyond 500 MPa. Cells which completely lost the ability to extrude dye were not able to be cultured and thus scored as dead, though still possessing enzymatic activity and intact membrane, which allow them to accumulate cF. Thus, the lethal effect of pressure (at pressures higher than 400 MPa) could was based on the irreversible perturbation of ATP-mediated dye extrusion system (Fig. 10b), without of which cells could not resume growth even on nonselective media. An irreversible rupture in this transport system did not allow the bacteria to reproduce and form colonies. In line with this observation, it was also noted that in most cases pressure induced structural changes in membrane proteins, in particular membrane-bound enzymes, generally occurred at pressures > 400 MPa [113]. The fact that efflux of fluorescence compound can give information about crucial transport mechanism located in membrane of cells of lactic acid bacteria was shown upon using ethidium bromide as a reporter probe for HorA and LmrP activity [15, 26].

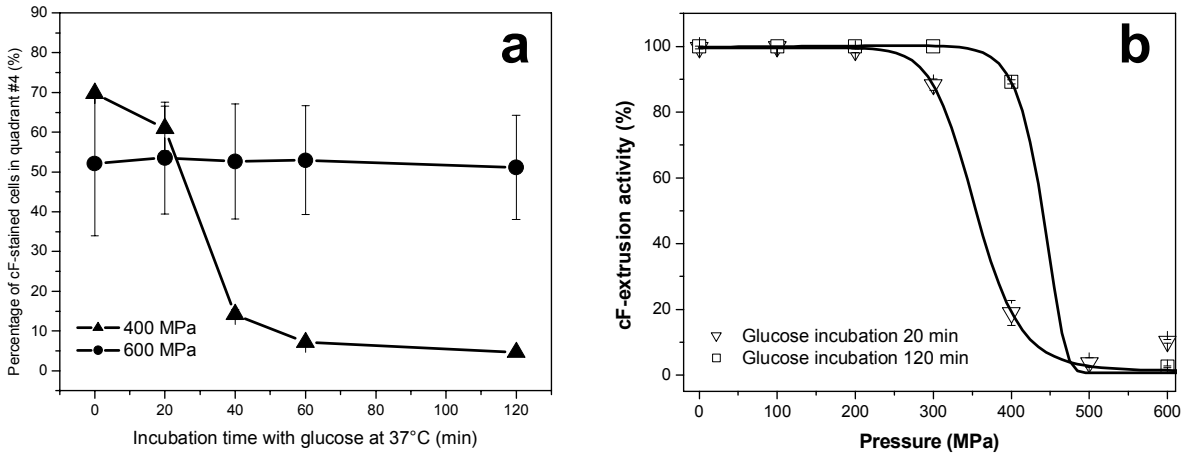


**Figure 10**

(a) Kinetics of cF extrusion from high pressure treated *L. rhamnosus* GG cells, represented by the decrease in the percentage of cF-stained population in the presence of 20 mM glucose during incubation up to 120 min. Similar to previous pressure treatments cells were subjected to elevated pressure conditions at 37°C for 10 min.

(b) Summary of pressure induced changes in cF extrusion activity of *L. rhamnosus* GG after glucose incubation for 20 min or 120 min

Membrane-bound enzymes and cytoplasmic membrane are regarded as a major target for pressure mediated sub-lethal injury or cell death [114, 115]. When bacterial cells are subjected to pressure treatment, membrane-bound enzymes, such as HorA [15, 27], LmrP [26] and $F_0F_1$-ATPase [23], are irreversibly inactivated prior to cell death. Defect in this crucial transport mechanisms must not necessarily lead to cell death, if cells are transferred to nonselective media, where they can initiate *de novo* protein synthesis or refolding of integral membrane proteins [26]. However, sub-lethally injured cells were rapidly inactivated in additional stresses exposed to them after pressure treatment due to the absence of cellular homeostasis mechanism. Pressure treatment in range between 300 to 400 MPa, which was identified as the appropriate pressure level in inducing sub-lethal injury to pressure resistant mutants of enterohemorrhagic *E. coli* to resulted in an accelerated low-pH inactivation (pH 3 to 4) during subsequent storage [116]. After treatment at a relatively moderate pressure of 300 MPa, which sufficient to inactivate the hop resistance protein HorA, sub-lethally injured *L. plantarum* cells could not survive in model beer containing 50 ppm hop extract [25]. With this regards, the authors of the latter work considered that pressure inactivation is a two-stage process involving initial sub-lethal damage on cytoplasmic membrane or membrane-associated transport systems in the first step, which is then followed by cell death as a result of adverse environmental conditions.

Further characterization of a putative ATP-dependent transport mechanism on LGG in response to glucose energization was observed upon exposing LGG to pH 3.0 at 37°C in the presence or absence of glucose. Exposure of LGG to pH 3.0 was found to be non-lethal up to an exposure time of 180 min (data not shown). According to the cF-fluorescence histograms in Figure 11a, which basically shows the response of pH stressed cells towards cFDA staining, cells which were incubated in the presence of glucose had higher cF fluorescence values compared to the ones without glucose. The cF-fluorescence values of acid stressed LGG were still low despite addition of lactose, which can not be utilized by this strain. Since cF fluorescence intensity is reported to be highly pH dependent (Fig. 11b), this findings may emphasize the role of an ATP-dependent proton extrusion system in maintaining intracellular physiological pH, which in turn results in high intracellular fluorescence intensity of cF. In absence of fermentable sugar proton gradient could not be maintained so that intracellular pH value reached levels in the vicinity of extracellular pH value, resulting in reduced cF fluorescence intensity of LGG. Exposure to pH 3.0 itself and decrease of intracellular pH did not affect viability, but an irreversible impairment on this proton translocating activity by means of pressure was reported to reduce the overall physiological fitness of the cell, especially in their ability to restore $\Delta$pH [17]. Apart from inactivating ATP dependent dye extrusion system pressure has a deleterious effect on this energy dependent proton extrusion machinery, which then restrict the pH range tolerated by bacteria [117]. Previous work already showed the implication of pressure induced damage on the proton translocating activity of $F_0F_1$ ATPase in *Lactobacillus plantarum*, leading to impairment of acid efflux and maintenance of pH gradient. This event already took place before cell death [23].

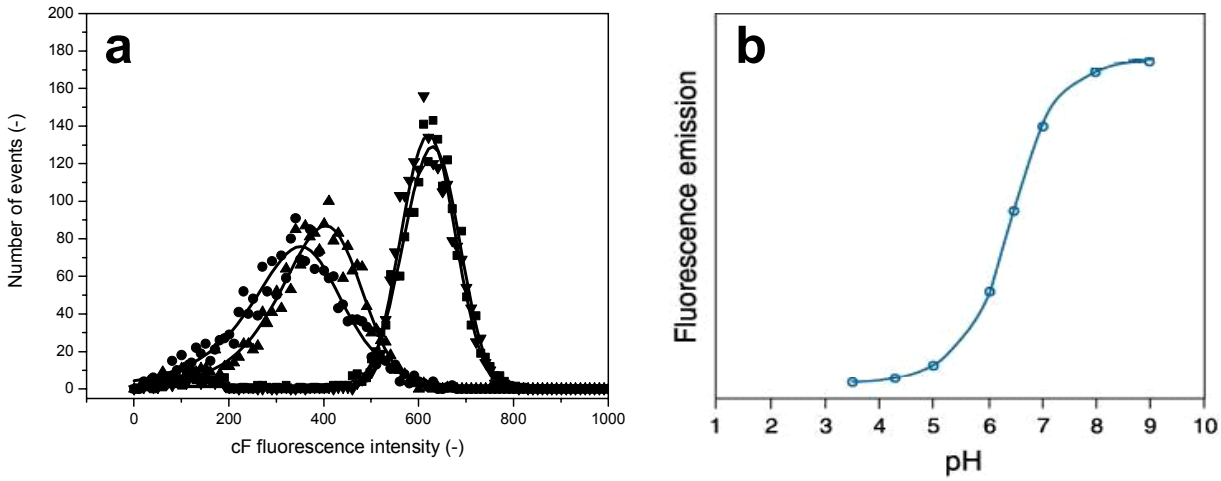


**Figure 11**

(a) Flow cytometry data on frequency distribution of the cF-fluorescence values of *L. rhamnosus* GG before (▼) and after exposure to pH 3 at 37°C for 1 h in the presence of glucose (■), lactose (●) or without any carbon source (▲).

(b) pH-dependent fluorescence of carboxyfluorescein (cF) fluorophores. Fluorescence intensities were measured for equal concentrations of the three dyes using excitation/emission at 490/520 nm. Source: http://www.probes.com/handbook/figures/0495.html

### *2.3.4 Combined application of heat and pressure*

The heat stability of intracellular esterase under high pressures was assessed by determining cell response toward cFDA/PI staining following pressure treatment at elevated temperature, in which esterase already inactivated and membrane damage occurred. As already discussed in Section 1.3.2. a temperature of 60°C was found to be effective in selectively inactivating esterase without affecting membrane integrity. Combined application of pressure and temperature processing was thought to have a synergistic effect on the inactivation processes by specifically affecting different cellular target sites, i.e. esterase by heat and membrane-bound enzymes by pressure.

In order to achieve constant temperature of 60°C throughout the whole pressurization phase bath temperature was kept at 65°C. Assisted by this experimental setup adiabatic heating generated during compression phase up to 600 MPa could elevate the temperature of the cell suspension (initial sample temperature in the centre ~ 35°C) to 60°C (Fig. 12bb). Pressure was released after a holding period of 300 s.

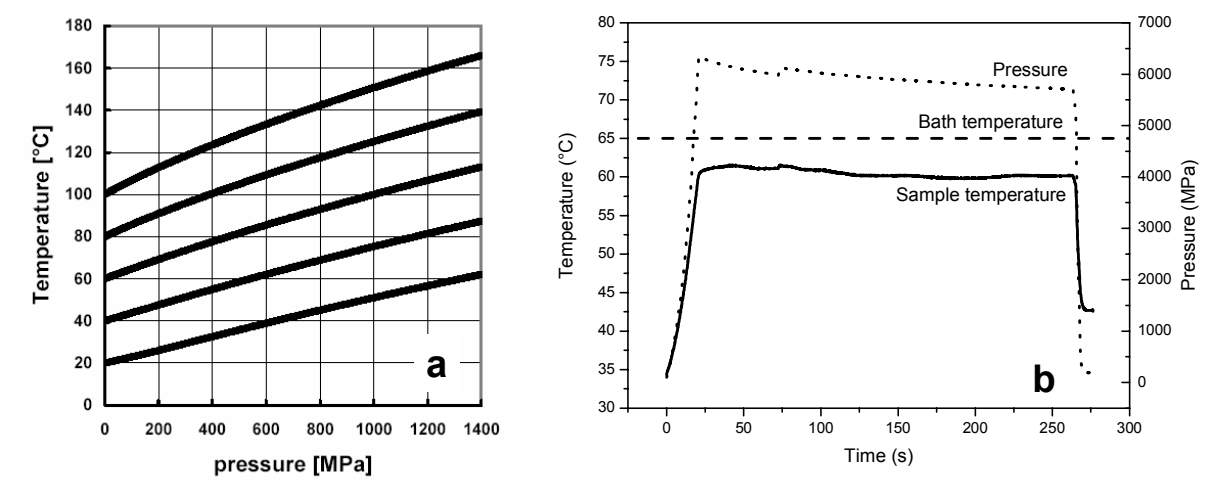


**Figure 12**

(a) Thermal behavior of water as a function of pressure and initial temperature which occurs during compression

(b) Pressure and temperature profiles recorded in the sample (suspension of LGG in PBS buffer, pH 7.0) during the high pressure processing cycle

In contrast to the fluorescence pattern of LGG treated at 60°C in ambient pressure (Fig. 13a), where due to esterase inactivation practically only $cF^{-}PI^{-}$ population was identified, there is a higher degree of heterogeneities in the response of LGG towards cFDA/PI labelling after combined pressure-heat treatment (Fig 13b).

A striking evidence of the positive effect of heat treatment at elevated pressure is that the major part of the treated population was PI stained. Membrane damage, which did not occur on cells treated at 60°C and ambient pressure, could be induced in a significant proportion of the population (single stained by PI or double stained population) by elevating treatment.

However, following pressurization at 600 MPa and 60°C a significant part of the LGG population could still accumulate cF, either with or without PI uptake (Fig. 13b). Obviously, in the cF accumulating fraction thermal damage of esterase and p-T induced degradation of membrane took place in a reduced rate at higher pressure; resulting in the enzymatic conversion of cFDA to cF and retention of the dye in the cell (single stained by cF or double stained population).

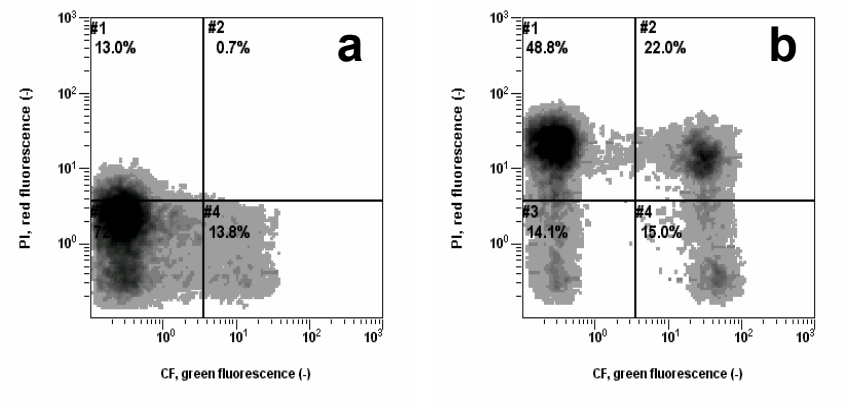


**Figure 13**

Fluorescence density plots of *L. rhamnosus* following cFDA/PI staining on (a) heat treated cells (60°C, 300 s) and (b) pressure treated cells (600 MPa, 60°C, 300 s).

In line with the latter result, it was notable that a significant number of proteins (DNA polymerases, hydrogenases, etc.) shows improved heat stability under pressure [118]. It was also found that upon elevating pressure the heat stability and *in situ* substrate conversion rate of some glycolytic enzymes could be enhanced at temperatures, at which those enzymes are degraded under ambient pressure [119]. Studying the properties of proteins potentially adaptable to high temperature by means of pressure may further open the possibility to identify processing or environmental conditions that allow heat sensitive proteins to remain stable and microorganisms viable, so as to improve biotransformation processes or to allow novel catalytic reactions to take place.

Furthermore, the results of high pressure inactivation at higher temperatures suggest that under high pressure the heterogeneity of the cells in terms of the stability of the cellular components are more pronounced and thus can be elucidated more effectively. Whereas at ambient pressure the lethal effect of heat was experienced in a nearly uniform manner throughout the population treated, the presence of more than one population differing in the types of damage induced by pressure or by combined application of pressure and temperature might open a discussion about the safety of the process, with which some of the treated cells were still capable of accumulating certain – potentially problematic – metabolites.

### *2.3.5 Inactivation mechanism by high-intensity ultrasound*

The propagation of ultrasound waves in the liquid medium is always accompanied with heat generation (Fig. 14). Since it is of importance to sufficiently exclude thermal effect in order to exclusively assess ultrasound effect on bacteria, temperature of the medium was controlled by placing the sample container in an ice bath. With help of this procedure heat dissipated upon generation of ultrasound waves could be instantly removed; thus leaving only an initial

temperature rise of approximately 10°C. Temperature equilibrium was achieved after 2 min and the temperature never exceeded 20°C (Fig. 14). Since the temperature never exceeded 20°C, thermal effect could be excluded, and the inactivation could exclusively be attributed to ultrasound effect.

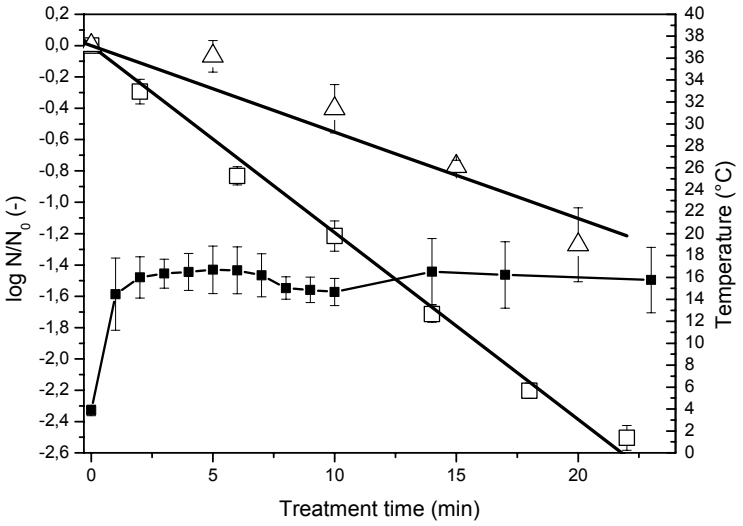


**Figure 14**

Ultrasound inactivation kinetics of *Lactobacillus rhamnosus* (Δ) and *Escherichia coli* (□) in phosphate buffer at pH 7.0 along with the temperature increase (■) in the treatment medium during propagation of ultrasonic waves. Data are the means of three replicate ultrasound treatments; the error bars represent the standard deviations of the mean.

It was evident that, the LGG was more resistant against lethal effect of ultrasound in comparison to the *E. coli* (Fig. 14). When the decimal reduction time (D-value) of ultrasound death kinetics was calculated, the D-value of LGG was more than two times higher than that of *E. coli* (18.8 min and 8.3 min, respectively). Gram negative bacteria were known to be more susceptible towards ultrasound compared to Gram positive ones [120, 121]. Gram positive bacteria were less sensitive to ultrasound since they usually have a thicker and a more tightly adherent layer of peptidoglycans than Gram negative bacteria [122].

Following exposure to ultrasound up to 20 min the major part of the LGG cells (more than 80%) were still encountered in quadrant #4 (Fig. 15) This fluorescence behaviour indicated that the majority of the treated cells was still able to retain cF and was not stained by PI. In this particular sub-population the integrity of cytoplasmic membrane was not seriously affected by ultrasound. From Figure 16a it is also evident, that by applying ultrasound for 20 min only maximal 7% of the cell population showed membrane damage, which allowed them to be labelled with PI. In contrast, according to the results of cultivation method only 8% of

the initial population could resume growth on agar following ultrasound propagation at the same condition (Fig. 16a).

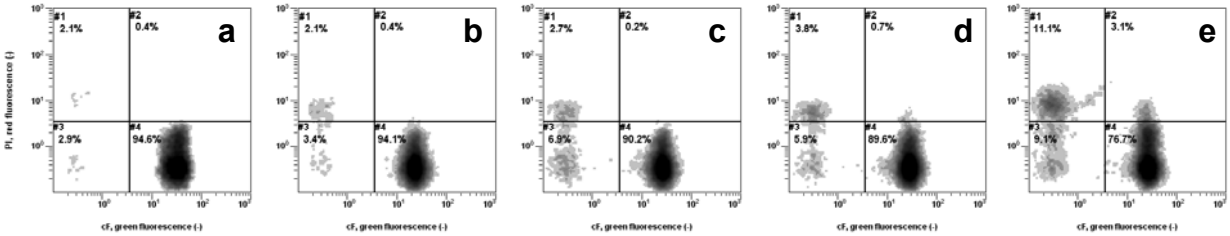


**Figure 15**

Fluorescence density plots of *L. rhamnosus* GG in response to staining with cFDA and PI after ultrasound treatment at different exposure time. Duration of treatment was 0 min (a), 5 min (b), 10 min (c), 15 min (d) or 20 min (e).

Bacterial membranes are considered as an ultimate requirement for the retention of viability. Cells which irreversibly lost their membrane integrity could neither maintain any of the electrochemical gradients necessary to remain functional nor have the potential to give rise to metabolism or proliferation due to the absence of selective permeability [48]. Therefore, such cells can be classified as dead cells, which lost their capacity to form colonies on agar [64]. In agreement with this classification, the PI labelled cells, which are encountered in quadrant #1, were considered as dead. However, the percentage of PI labelled cells (max. 7% of the whole population) are far below the magnitude of cells not able to grow on agar (92% of the initial population lost their growth capacity on agar) following ultrasound treatment. Thus, the metabolically active population in quadrant #4 (80% of the whole population) was most likely constituted of cells able to resume growth on MRS agar (viable) and the ones not able to resume growth (dead); with dead cells as the major constituent. These findings led to believe, that due to ultrasound effect cell death could even occur without any severe damage of membranes, since despite of the absence of growth on agar, the major population of ultrasound killed cells was still emitting green fluorescence as a consequence of the retention of their enzymatic activity and membrane integrity. Moreover, the presence of at least two sub-populations with regards to their fluorescence behaviour after ultrasound treatment indicated the presence of heterogeneity within the population in their capacity of resisting the deteriorating impact of ultrasound.

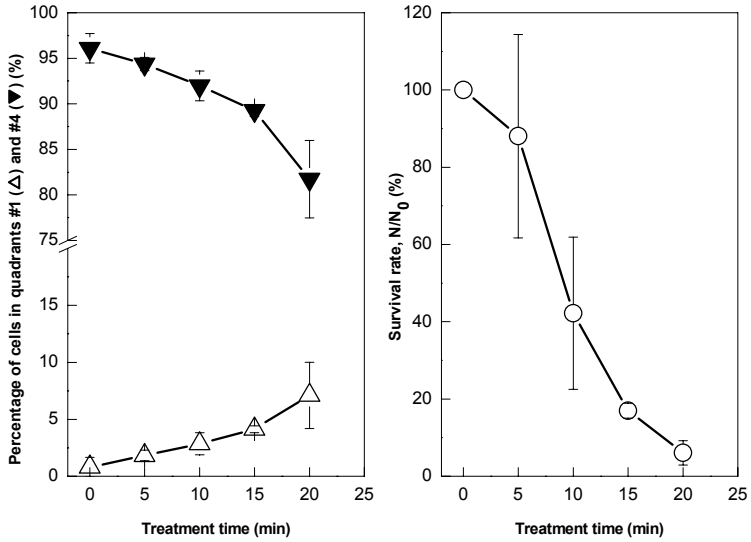


**Figure 16**

a. Changes in the percentage of *Lactobacillus rhamnosus* cells encountered in quadrant #1 (△) and #4 (▼) in response to ultrasound propagation. PI labelled cells are framed in quadrant #1, whereas the ones accumulating cF are to be found in quadrant #4. Data for this figure were derived from the density plots shown Figure 15. Data were means of three replicates of ultrasound treatments; error bars indicate the standard deviations of the mean.

b. Kinetic of viability loss upon ultrasound exposure (○) as determined by plating cells on MRS agar. Data were means of at least two replicates of ultrasound treatments; error bars indicate the standard deviations of the mean.

In conclusion, the major action site of ultrasound on inducing lethal effect was not necessarily the cytoplasmic membrane, since it was observed, that the majority of the ultrasound treated population was still able to accumulate cF and did not allow PI penetration. Thus, the degradative effect of ultrasound on the cytoplasmic membrane was less pronounced. This observation is in contrast to the suggestion found in the literature, which proposed that the target of ultrasound damage might be the inner (cytoplasmic) membrane consisting of a lipoprotein bilayer [122]. Although the power input of the ultrasound applied with regards to bactericidal efficacy was sufficient, as assessed previously [43], the treatment temperature (below 20°C) applied was possibly too low to synergistically induce membrane deterioration. Cell death which was observed upon applying high-intensity ultrasound seemed to result from non-membrane related degradation.

## 2.4 Conclusion

The present work deals with the application of flow cytometric analysis to evaluate the mechanism of microbial inactivation with LGG as model organism by means of physical

treatments. A well-described multiple staining strategy, which is composed of physiological dyes carboxyfluoresceindiacetate (cFDA) and propidium iodide (PI) was applied to examine specific cellular metabolic activities and their relative changes following inactivation treatments. It was expected that additional insights on process-induced changes in cellular integrity or metabolic activities, which were not explicitly assessable by culture techniques, could be achieved using this measurement technique. Furthermore, it is also noteworthy to differentiate the mechanisms of microbial inactivation occurred during different treatments, in order to allow problematic contaminants to be injured or inactivated more effectively as well as to effectively combine different treatments, which have different cellular target sites. Figure 17 shows the fluorescence density plots of LGG after being exposed to thermal treatment (Fig. 17b and 17c), supercritical $CO_2$ treatment (Fig. 17d), pulsed electric fields treatment (Fig. 17e), high-intensity ultrasound treatment (Fig. 17f), high pressure treatment at moderate temperature (Fig. 17g), and high pressure treatment at elevated temperature (Fig. 17h). From this overview it is obvious that different treatments led to different response of the cell to cFDA/PI labelling, indicating that the applied treatments differed in the cellular sites being primarily affected, although the survival rates according to the plate counts result were in the same range.

Regarding the heat induced damage on LGG, when these cells were exposed to 60°C at ambient pressure the cF-accumulation activity was considerably reduced without significant loss of membrane intactness. This particular physiological state left cells stained neither with cF nor with PI, even when the cells were heat treated up to 300 s. Thermal-induced death could therefore be achieved in absence of membrane degradation. In contrast, when cells were subjected to temperatures above 65°C, PI uptake already occurred in the first 90 s. From this staining behaviour it was concluded that at higher temperatures the primary target of lethal effect of heat is the bacterial cytoplasmic membrane. The findings demonstrates the differences in the target sites of heat inactivation depending on the temperature level used. Furthermore, this result also emphasizes the general importance of treating vegetative cells at temperatures above 60°C, when the inactivation of intracellular esterase and membrane damage were aimed.

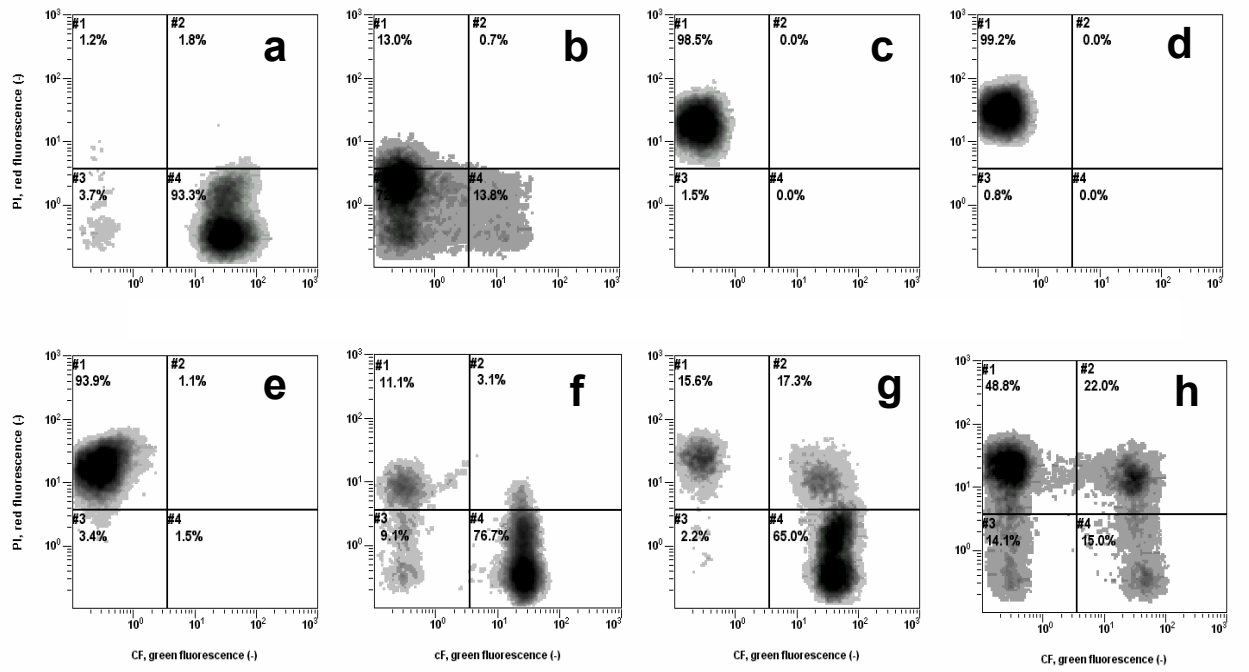


**Figure 17**

Flow cytometric fluorescence density plots of *L. rhamnosus* GG subjected to different treatment methods: a) untreated sample (log $N/N_0$ = 0); b) heat treatment at 60°C for 300 s (log $N/N_0$ = -6.8); c) heat treatment 75°C for 30 s (log $N/N_0$ = -7.5), d) supercritical $CO_2$ treatment at 40 MPa and 40°C for 10 min (log $N/N_0$ = -7.3); e) pulsed electric fields treatment with field strength of 35 kV/cm and specific energy input of 300 kJ/kg at 30°C (log $N/N_0$ = -1.2); f) high-intensity ultrasound 17.6 W for 20 min (log $N/N_0$ = -1.2); high pressure treatment at 600 MPa and 37°C for 10 min (log $N/N_0$ = -7.3) and high pressure treatment at 600 MPa and 60°C for 5 min (log $N/N_0$ ~ -7). Treatment media were phosphate buffer saline (pH 7.0)

In contrast to heat killed cells which resulted in homogeneous population with regards to their response to cFDA/PI staining, three populations differing in their behaviour upon cFDA/PI uptake were observed when high pressure killed cells were analysed. The major population of high pressure inactivated cells of LGG could accumulate fluorescent molecule carboxyfluorescein (cF), which indicated that some of the dead cells were still enzymatically active and not severely membrane compromised. The fact, that pressure inactivated bacteria could perform enzymatic conversion of cFDA into cF needs further attention, since the presence of such metabolically active, but dead bacteria in food might be critical in terms of their potential activity on excreting toxic or food spoiling metabolites. It is also obvious that the heterogeneity among the population of pressure killed cells towards cFDA/PI staining is more pronounced compared to heat killed cells. This findings suggested that the distribution in the tolerance towards deteriorative effect of pressure on membrane or intracellular enzyme – which in turn might reflect the overall resistance of the organism and their capacity of

repairing the imposed injuries – might be more pronounced in comparison to the corresponding distribution against lethal effect of heat.

Moreover, according to plate count method pressures up to 400 MPa was regarded as non-lethal on LGG. However, below this threshold level differences observed in the physiological activity of pressure-treated cells, especially in the reduced rate of dye extrusion upon glucose energization, indicated that they were sub-lethally injured. Pressure induced sub-lethal injury, which was observable up to 400 MPa, seemed to correlate more profoundly with perturbation of cellular transport mechanism rather than with the occurrence of double stained population, since the diffusion of PI in cF stained cells not only occurred on cells lethally injured by pressure as high as 600 MPa but also on cells surviving pressure treatment (for instance after treatment at 300 MPa).

Measurement of cF-extrusion activity in response to glucose energization indicated that the lethal effect of pressure (at pressures higher than 400 MPa) was related to the irreversible perturbation of dye extrusion machinery, which is most likely mediated by an ATP-driven transport system. Accordingly, dead cells, which lost the capacity to reproduce themselves and grow on agar, are the one which were not able to extrude cF, although the membrane was still intact and esterase remained active. This findings underlines the results from previous works on pressure induced damage on other ATP-dependent, membrane bound enzymes, which are crucial in maintaining viability [15, 17, 23, 26, 27].

Cell death occurred upon exposure of high intensity ultrasound under continuous removal of generated heat to LGG. In absence of thermal effect it could be shown that only a small population was labelled by propidium iodide (PI) following exposure to ultrasound up to 20 min. Within the experimental conditions investigated ultrasound did not considerably affect the cytoplasmic membrane, although according to plate count results viability loss occurred. It could be concluded that, cell death which was observed upon applying high-intensity ultrasound seemed to result from non-membrane related degradation.

Taken together the results of flow cytometric measurement on cFDA/PI labelled bacteria following exposure to heat, pressure and ultrasound one could elucidate the mode of action of these physical stressors on cellular activities or integrity. Ultimately, the use of this technique might lead to an improved design of inactivation processes, i.e. one could then choose a certain type of cellular damage preferred and then select the type of treatments required to achieve this goal. Tailor-made non-viable bacteria might be an interesting research object in the field of probiotic research, where the importance of the ingestion of viable bacteria in eliciting health effect is sometimes questioned, since non-viable bacteria were reported to be effective as well [123-125]. Non-viable probiotic cells are of interest due to easy-handling and longer shelf life [126]. However, systematic studies on the relationship between probiotic effect and the type of inactivation treatments used to produce non-viable

bacteria are still lacking. In this context, high pressure killed cells might be one of the promising candidate to be investigated, since the fluorescence pattern of pressure inactivated cells – which is indicative for a lower extent of damage on metabolic activity and on membrane – is quite similar to the one of viable cells.

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# 3 SPRAY DRYING OF PROBIOTIC BACTERIA

Effect of processing conditions and drying media on survival characteristics during drying and storage at non refrigerated conditions

## 3.1 Introduction

### *3.1.1 Drying of microorganism*

The industrial manufacture of fermented dairy products relies on the use of bacterial starter culture for the fermentation of yoghurt, cheese, sour cream, etc. Furthermore, growing interest is clearly observed in the use of probiotic bacteria, either in dairy or non-dairy products. These bacteria are commonly preserved and distributed either in frozen or dried form before they are inoculated in the fermentation tank or incorporated in the food product. Bacteria stored in frozen or dried form may be maintained for a long-term storage period. Besides, the possibility to work with frozen or dried concentrate also meets the expanding interest in the application of ready-to-use culture concentrates for direct inoculation of milk vats, which is – compared to previous practice using sub-culturing method – less prone to bacteriophage attack and contamination with other problematic microbes. Preservation of bacteria in dried form is preferred, since frozen cultures occupy a large volume, heavy and require storage at subzero temperatures, all of which results in high costs for storage, shipping and energy [1].

However, it is generally anticipated, that the transfer of bacteria into dried form, which involves physical removal of water, may have unexpected side effect; primarily loss of activity and/or ultimately loss of viability. The loss of these crucial bacterial properties has to be minimized or effectively controlled since it finally affects the productivity of the fermentation process and also the characteristics of food products containing living bacteria cells. Unexpected deviation in the functional behaviours or in the number of viable cells is usually compensated by adding higher concentration of bacteria into the fermentation broth or into the final product. Since the suggested correction procedure would result in higher production cost, it is of utmost importance to know the nature of cellular damages as induced during water removal and to find ways how to effectively protect the crucial sites in order to minimize viability loss not only during drying but also during subsequent storage.

***Cellular damage observed in bacteria during dehydration***

Dehydration decreases water availability inside or in the vicinity of the dried cells such that they reach a dormant state during which the metabolism is slowed down and even stopped completely [2]. Viability and activity loss occurred on bacteria during drying was reported to be closely related to damage to the cell wall, cytoplasmic membrane and the DNA [3, 4]. Cytoplasmic membrane damage could be detected by increased sensitivity to NaCl [4-7]. Moreover, either increased permeability of ß-galactosidase substrate [5] or increased leakage of ß-galactosidase in the supernatant fluids [4] were indicative for the loss of membrane integrity. Similarly, evidences of membrane disruption could be found in the higher diffusion rate of DNase into cells and by leakage of UV-absorbing materials or

potassium ions from the cells [1, 4, 5, 8]. Although antagonic activity of bacteriocin producing bacteria was still retained after spray drying [6, 9], it was also reported that plasmic loss occurred [10], presumably due to increased membrane permeability, leading to an absence of a specific inhibitory activity mediated by the lost plasmid.

Since permeability control is mainly associated with cell membrane, it seemed probable to relate damage in the dried cells with changes in the profile of cellular lipids, which primarily constitute the cytoplasmic membrane. Previous work on drying of *Lactobacillus bulgaricus* showed a decreased ratio of unsaturated/saturated fatty acids compared to normal cells, indicating that spray drying induced lesions in the cellular lipid-containing structures [11]. During subsequent storage in air further decrease of the unsaturated/saturated fatty acids could be observed [11, 12]. It is possible that two different mechanisms of phospholipids degradation were involved: oxidation of unsaturated fatty acid and lipolysis [12]. Furthermore, it was suggested that products from lipid peroxidation might be involved in DNA damage [11]. However, another study on the comparison of the effect of different drying methods documented that the fatty acid composition of freeze dried cells did not vary greatly from the normal cells, whereas in vacuum dried cells, the fatty acid spectra shifted towards shorter chain length [5].

Apart from affecting the cellular lipids drying induced damage could also occur on cellular proteins, either membrane-bound or cytosolic proteins [13]. It was reported that drying resulted in damage of membrane-bound proton translocating ATPase of *L. bulgaricus* [12, 14]. Following either vacuum or freeze drying the attachment of a 46 kDa surface layer protein of *Lactobacillus acidophilus* to the cell wall was destabilized, resulting in partial loss of surface proteins or making the protein easily extractable with 0.1% sodium dodecylsulfate [5]. Drying was reported to be deleterious on the functional integrity of membrane-bound proteins indirectly by either disrupting the bilayer integrity, which resulted in the displacement of the transporting proteins, or by directly denaturing these proteins [14-16].

In order to improve understanding of the drying induced damage on crucial cellular components, such as cytoplasmic membranes and proteins, and relate the damage on these macromolecules to the overall viability of the microorganism as well as to suggests approaches to effectively protect this complex biological system during drying, a lot of systematic studies have been dedicated to evaluate and differentiate the mode of action of dehydration on model systems, encompassing model membranes and proteins.

***Dehydration damage occurred on model membranes***

The effect of drying on membranes has been thoroughly evaluated on liposomes, which are artificial vesicles composed of concentric lipid bilayers separated by water compartments.

The typical characteristic of bilayer-forming lipids is their amphiphilic nature: a polar headgroup covalently attached to one or two hydrophobic hydrocarbon tails. When these lipids are exposed to an aqueous environment, interactions between themselves (hydrophilic interactions between polar headgroups and van der Waal's interactions between hydrocarbon chains) and with water (hydrophilic interactions, hydrophobic effect) lead to spontaneous formation of closed bilayers.

Liposomes can differ in size: they can range from the smallest vesicle (SUV) obtainable on theoretical grounds (diameter ~20 nm) to liposomes which are visible under the light microscope, with a diameter of 1 μm or greater (LUV), equal to the dimensions of living cells (Fig. 1). They can also differ in terms of lipid composition and structural organization, corresponding to uni-, oligo- or multi-lamellar vesicles (MUV o MVV). Liposomes are built in such a way that the solute can be encapsulated in the aqueous compartment (polar solutes) or embedded in the lipid bilayers (lipophilic or amphiphilic solutes).

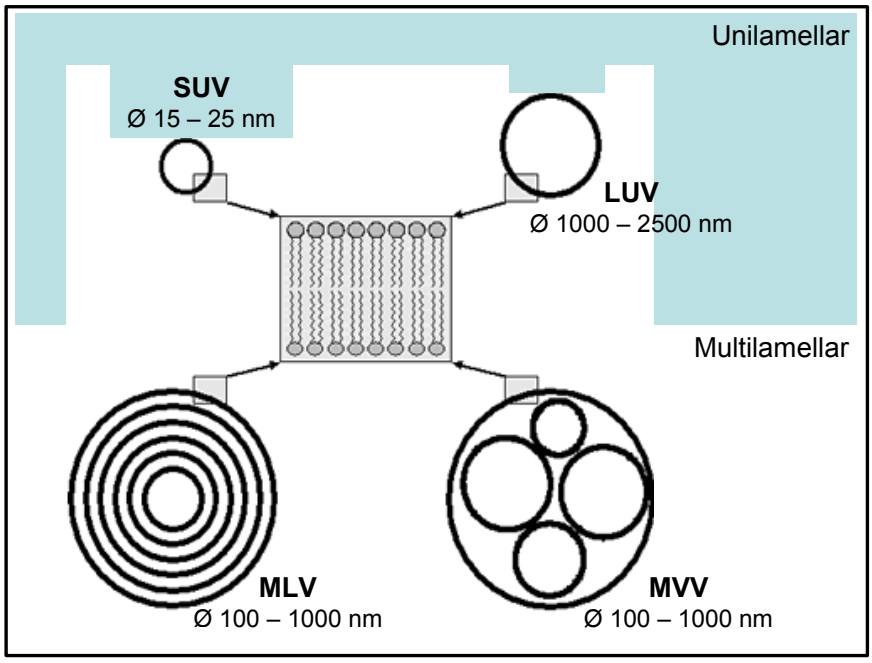


**Figure 1**

Schematic view of the liposomes of different sizes and lamellar structure. SUV: Small unilamellar vesicle; LUV: Large unilamellar vesicle; MLV: Multilamellar vesicle; MVV: Multivesicular vesicle

The properties of liposomes and their subsequent applicability depend on the physical and physico-chemical characteristics of the liposomal membrane. Usually, a zwitterionic or non-ionic lipid is used as the basic lipid for the preparation of liposomes. The net surface charge of liposomes can be modified by the incorporation of positively charged lipids, such as stearylamine, or negatively charged lipids, such as dicetylphosphate, phosphatidyl glycerol or phosphatidyl serine [17].

The fluidity of the liposomal bilayer, made from a single lipid, depends on the lipid phase transition temperature ($T_m$). As can be seen in Figure 2, when the liposome is brought to $T_m$, the membrane passes from a solid gel phase, where the lipid hydrocarbon chains are in an ordered state, to a fluid liquid-crystal phase, a disordered state, where molecules have more freedom of movement [18]. Hence, depending on lipid $T_m$, different membranes composed of distinct lipids can exhibit different fluidity levels at the same temperature. Membrane permeability is highest at the phase transition temperature [13, 19], and is lower in the gel phase than in the fluid phase [17]. A general sequence of hydrophilic solute permeability is: water > small non-electrolytes > anions > cations ≅ large non-electrolytes > large polyelectrolytes [18].

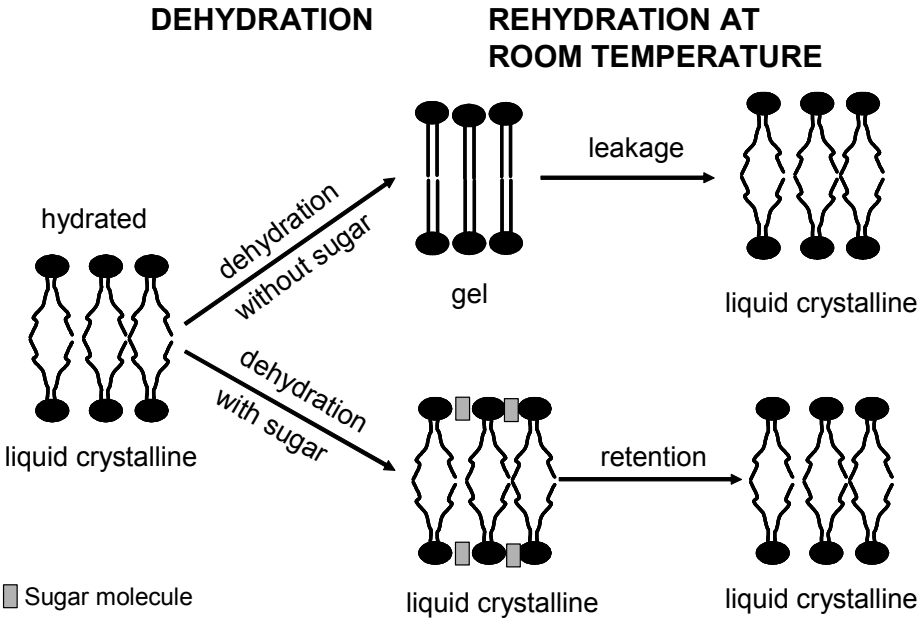


**Figure 2**

A diagrammatic representation of the effect of sugar on leakage of trapped solute when phospholipid bilayers are dried and rehydrated at room temperature, as adapted from [20]. Note that this scheme only valid for lipids having $T_m$ lower than ambient temperature.

Drying experiments with liposomes showed that the removal of water, which profoundly affects the stability of biological membranes, alters the physical properties of membrane phospholipids and leads to destructive events including fusion between adjacent vesicles and lipid phase transition from liquid crystalline to gel (Fig. 2) [21]. The destruction of dried biological membranes is manifested upon rehydration, i.e. by increased permeability of entrapped water soluble substance and lateral phase separation of membrane constituents [16, 22].

In detail, if a lipid bilayer is dried then as the water molecules are removed, the phospholipids headgroups are forced closed together. These water molecules spatially separate the phospholipids headgroups in the hydrated state. The close approach of lipid molecules leads to increased van der Waal's interactions between the fatty acyl chains. Consequently, the lipid would be more likely to undergo transition from liquid crystalline into gel phase [22]. This event appears as a shift in the gel-to-liquid crystalline phase transition toward higher temperatures, i.e. the dry phospholipids exist in a gel phase at temperatures, at which they would be in liquid crystalline phase if they were in the hydrated state [20, 22]. When these gel-phase lipids are rehydrated at room temperature, they undergo another phase transition to the liquid crystalline phase (Fig. 2). However, during membrane phase transitions, there regions of gel phase and liquid crystalline phase might coexist or there are packing defects in some regions or non-bilayer phases are formed. As a result, the membranes do not provide adequate barrier properties leading to transient leakage, which is thought from mismatch between molecules in the gel and those in the liquid crystalline state. Catastrophic membrane leakage, when occurred in biological cells, may ultimately lead to cell death [23].
In addition to a transition from the liquid crystal to the gel phase, certain phospholipids can undergo a transition from liquid crystal to hexagonal II phase as water is removed [24]. Hexagonal II phase is produced by an increased interaction between adjacent phospholipid headgroups as the separating water is removed. This type of transition is especially common in membranes high in phosphatidylethanolamine (DOPE; $T_m$ ~ -5°C [25]), such as the inner membrane of *E. coli*, and may play a role in the cell mortality.

***Dehydration damage occurred on proteins***

Most studies to elucidate the effect of drying on functionality of proteins were not conducted on complex systems such as membrane-bound proteins, since the implication of protein-lipid interaction may complicate the interpretation [16]. Instead, extensive studies have been made on soluble proteins including ß-galactosidase [26-28], trypsinogen [29], trypsin [30, 31], alkaline phosphatase [32], amylase [33], restriction enzymes [34-36], invertase [37], dehydrogenase [38], lysozyme [39] and bovine serum albumin [40, 41].
The biological function of proteins depends on their three-dimensional structure, which is determined largely by water. Loss of water, which form the hydration shell of the protein, can lead to loss of native structure, resulting in loss of biological function upon rehydration [42]. For instance, enzyme inactivation as a result of drying may involve a conformational change in the active site, which can be due to protein denaturation (unfolding and/or inter- or intraprotein hydrophobic aggregation) or to blockage of specific active groups by the formation of covalent links (i.e. condensation of amino groups of proteins with carbonyl

compounds) that modify the active site of the enzyme or make it inaccessible to its substrate [28].

### *Protection against drying induced injury on membranes*

#### Water replacement hypothesis

Certain sugars may confer protection to liposomes and isolated biological membranes against dehydration damage [15, 16, 20, 22]. The protective effect of sugar relies on a direct physical interaction between the hydroxyl groups of the sugars and the polar residues of the phospholipids head groups in dehydrated state, as described by the water replacement hypothesis [21, 43, 44]. Accordingly, sugar molecules are thought to substitute for water molecules between the lipid headgroups during dehydration and sugar molecules need to be present on both sides of liposome membrane [20] (Fig. 2). Liposomes dried without internal sugar did not remain intact and released their contents. The aforementioned hypothesis is primarily evidenced by infrared spectroscopy measurements, which showed that in the presence of sugar, the phosphate group in dry phospholipids behaves as in it is fully hydrated and *vice versa*, OH stretching bands in sugar are strongly affected by the presence of phospholipids, which suggests that the phosphate of the phospholipid and the OH group of the sugar are interacting in the dry state, possibly by hydrogen bonding [43, 45]. Adequate spacing between the lipid headgroups owing to the insertion of sugar is deemed responsible for the substantial depression of liquid-crystalline-to-gel phase transition temperatures ($T_m$), resulting in the preservation of membrane in a liquid-crystalline state, even when dry (Fig. 2). Consequently, the membrane would not pass through a phase transition during rehydration and leakage of entrapped aqueous solution could be prevented.

#### Formation of high viscous glassy matrix

The stabilizing properties of sugar can also be explained by the formation of glassy state by the sugar upon dehydration [46-48]. A glass is a kinetically metastable, time-dependent physical state presented in amorphous or partially crystalline materials, characterized by near absence of molecular movement [49]. The most important parameter describing the glassy state of amorphous materials is the glass transition temperature ($T_g$), below which the materials exhibit extremely high viscosities which gives them "solid-like" properties. Above the glass transition temperature, viscosity drops sharply in the "rubbery" state and the mobility of the system increases accordingly. As documented in Figure 3, glass transition temperature ($T_g$) is highly dependent on molecular weight. Furthermore, the glass transition temperatures of low molecular weight carbohydrates is specific to each anhydrous material but is extremely sensitive to water, which plasticizes the amorphous structure and reduce the $T_g$ [50]. The formation of a glassy solid results in a reduction of translational molecular motion

and rates of chemical reactions and relaxation rates for various processes in glassy matrices may be very low, especially at temperatures well below the glass transition temperature [51].

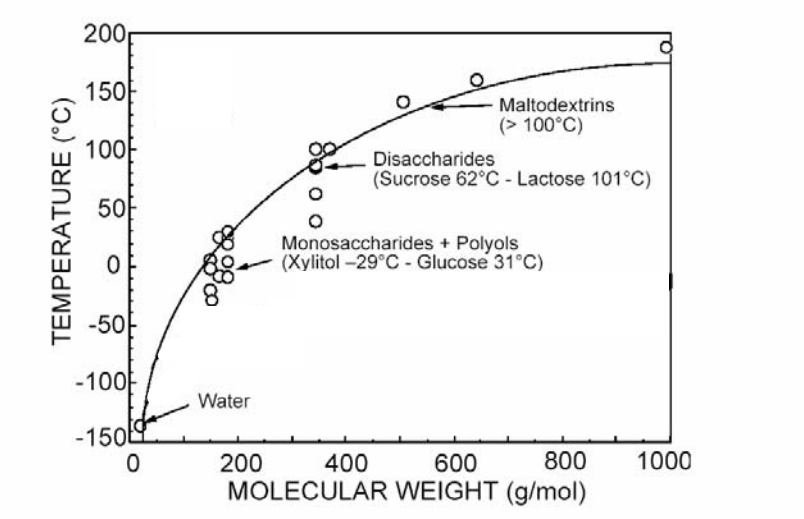


**Figure 3**

Glass transition ($T_g$) of common carbohydrates and sugars (anhydrous) as a function of molecular weight. Graph is adapted from [52].

One special dehydration damage which could be minimized upon entrapment of liposome in high viscous glassy matrix is fusion; an event which is characterized by the occurrence of large, multilamellar vesicles and leakage of the contents of the original liposomes [53, 54]. The increased stability of dry liposomes in the glassy state may be associated with elevated energy barrier for liposome fusion, which considerably reduced the mobility of molecules and facilitated physical separation of dry liposomes [47]. Alternatively, the prevention of fusion could be explained by the ability of sugar to work as a spacing matrix between liposomes [55].

It was reported that polymers, such as hydroxyethyl starch and dextran, both good glass formers which have high glass transition temperature ($T_g$) are capable of inhibiting fusion between liposomes during drying due to glass formation [46, 53, 54]. However, these polymers were not effective in preventing leakage from liposome during drying, since they did not lower $T_m$ of the lipid [45, 53]; a property which is prerequisite for prevention of leakage owing to membrane phase transition [54]. Consequently, both glass formation and decrease of $T_m$ in the dry lipids is required to retain structural integrity. Although it may be required, the thermodynamically unstable nature of the glassy state might explain why it itself is not a sufficient requirement for stabilization of dehydrated liposomes [47, 56].

A special case was observed on trehalose glass, which was found to be more superior in conferring dehydration tolerance compared to other sugar glasses. At the same water content, the $T_g$ of trehalose (100°C [57]) is much higher than sucrose (62°C [57]) or other sugars and liposome stabilized in trehalose could retain aqueous content more effectively

than the one entrapped in sucrose [58]. Apparently this remarkable effect of trehalose is resulted from its ability to form dihydrate as it absorbed water, thereby sequestering water, which might otherwise participate in lowering the $T_g$ to below ambient. Thus, trehalose could render dried biomaterials more stable when stored under high humidities and temperatures.

**Protection capacity of sugars with respect to the types of sugar moieties, types of glycosidic linkage and degree of polymerisation**

Data from a comparative study of the efficiency of sugars from different degree of polymerisation (DP) in conferring protection for liposomes against dehydration induced rupture showed that while glucose, maltose or maltotriose completely prevented the aggregation and/or fusion of liposomes during lyophilization, other malto-oligosaccharides (DP = 4 to 7) induced them due to the increase in hydrophobicity with the number of glucose residues [59].

Other polysaccharides with high $T_g$ such as dextran or hydroxyethyl starch (MW = 450000) were capable of inhibiting fusion of liposomes; however these polymers were completely ineffective in stabilizing membrane during freeze drying [45, 53], since they are thought to be sterically hindered from penetrating the bilayer in the dry state so that the gel to liquid crystalline phase transition temperature was not depressed and leakage of entrapped aqueous solution was not prevented [54]. Surprisingly, inulins of a DP ranging between 10 and 30 from chicory and dahlia could stabilize liposomes during freeze-drying and the stabilization is mediated by a direct interaction of the polysaccharides with membrane lipids despite of the proposed problem with steric hindrance [45] and even a high molecular mass bacterial levan (DP > 25000) was able to directly interact with membranes [60]. Consequently, size-related effects of steric hindrance can not fully account for the observed protective effects of oligo- or polymeric sugars. Furthermore, with increasing chain length, fructo-oligosaccharides were more effective than gluco-oligosaccharides in stabilizing dried liposomes against leakage of aqueous content after rehydration [61]. According to FTIR spectroscopy data it was observed that the ability of gluco-oligosaccharides to hydrogen bond to the head group of dry lipids decreased dramatically with increasing DP, whereas chain length hardly affected the ability of fructo-oligosaccharides to interact.

Despite the ability of both monosaccharide and disaccharide to form hydrogen-bonding with liposomes, the interaction between acyl chains is stronger in the monosaccharide system, indicating that the interaction between monosaccharide and liposome is weaker than that of disaccharide [62]. As a result, leakage of entrapped fluorescence marker was more pronounced after drying in the presence of monosaccharide, although this was capable of inhibiting fusion. In contrast, it was reported that glucose did not inhibit fusion during drying and it did not prevent leakage [54, 63]. Other study also confirmed the higher efficacy of

disaccharide to minimize drying induced damage on membrane [63, 64]. However, as already mentioned above, the efficacy of different disaccharides in protecting dried liposomes varies greatly [58]. When the ability of sucrose and trehalose to preserve a model membrane system, sarcoplasmic reticulum, were compared, it was observed that although both sugars could confer good protection, higher concentration of sucrose was required to achieve equal extent of stabilization [65]. Despite the observed superiority of trehalose over other sugars it was proposed that as freeze-drying technology improved or at ideal drying conditions the differences between disaccharides tended to disappear, and the protective effect of sugar encompasses disaccharides in general [65].

***Protection against drying induced injury on proteins***

Dehydrated proteins could also be stabilized in the presence of sugar molecules [27, 29, 39, 40]. In analogy to protective effect of sugar on dehydrated membrane stabilization of native protein state during dehydration can be explained by water replacement hypothesis. Using infrared spectroscopy method it was shown that in the presence of sugar the amide bands of the dried proteins are similar to those of hydrated proteins and the OH vibrations of sugar are altered by the protein so that they closely resemble the OH groups of the hydrated sugar [13, 39, 43]. Similarly, drying of proteins in the presence of sugar rendered the conformational state or the secondary structure of dried proteins more similar to the one of hydrated proteins, as measured with derivative infrared spectroscopy [27, 40, 66]. Sugar molecules are thought to replace water and be capable of hydrogen bonding to the polar and charged groups of the protein in the place of lost water, leading to the preservation of the native, aqueous structure in the dried state [42, 66]. Consequently, the occurrence of hydrogen bonding of sugar to the dry proteins is required for inhibition of dehydraton-induced protein unfolding [39]. Upon rehydration protein that was dried in the presence of sugar induced the refolding of protein structure, whereas the protein dried alone did not show any recovery of its native structure [40]. However, the failure of glucose to prevent lysozyme unfolding during freeze-drying showed that hydrogen bonding between carbohydrate and protein alone is not sufficient to confer protection to a protein during lyophilization [66]. The difference in the efficacy for protein preservation of different sugar despite the ability to hydrogen bond to protein – at sites which normally binds water – could be explained by the extent of interaction between protein and sugar [31]. The authors suggested that trehalose interacted more strongly with trypsin and hence it is a better preservative than sucrose because trehalose is unable to form strong hydrogen bond with other trehalose molecules in the anhydrous amorphous state. Besides, the efficacy of different sugars for protein protection could be explained by Maillard (browning) reaction between reducing sugars and proteins in the dry state, which has often been invoked as a major source of damage [28, 32, 65]. When

a freeze-dried model system was incubated with sucrose, trehalose, and glucose, the rate of browning seen with sucrose approached that of glucose – as much as 2000 times faster than that with trehalose [67]. In a comparative study on the protective effect of different types of sugars (mono-, di-, tri- or polysaccharides) it was shown that only trehalose could offer protection of dried preparation of restriction enzymes even at storage temperature up to 70°C and this superiority was thought to be based on chemical stability and non-reducing nature of trehalose [34].

The role of glassy state in the preservation of structure of native proteins have been discussed and analyzed extensively. It was hypothesized, that the glassy state is the sole important factor in long-term stabilization of stored restriction enzymes dried in a trehalose matrix because translational diffusion-limited relaxation processes taking place during protein denaturation are strongly inhibited [68]. Loss of activity of ß-galactosidase dried in lactose-containing matrix, which seemed to correlate with accumulation of the product of non-enzymatic browning reaction between lactose and enzyme, was found to be reduced by addition of maltodextrin, a polymer with high $T_g$ [28]. Addition of glass formers which increase the $T_g$ was effective in lowering sugar crystallization [28], which is known to promote water redistribution, increase the rate of diffusion-controlled reactions and affect biomolecule stability [69].

As already indicated above and also in the drying study on membrane, the entrapment of protein molecules in an amorphous, glassy matrix was not fundamental requisite for stabilization, since enzyme inactivation was observed in heated glassy matrices well below their glass transition temperature [37]. The absence of break in the Arrhenius plot for thermal inactivation of lactase in the vicinity of glass transition indicated that the formation of glassy state did not influence the deteriorative effect of heat on enzyme [26]. In addition, the restriction enzyme *Eco*RI was very stable during storage at 37/45°C in spite of the fact that sugar matrices were completely rubbery [36]. Dextran, a high molecular weight carbohydrates which remains glassy when dried, was not capable of inhibiting protein unfolding during dehydration [34, 42]. It was demonstrated in a work on drying of alkaline phosphatase that different inulin preparations with $T_g$ > 100°C gave different degree of protection during storage at 60°C and better retention of activity was achieved when inulins of relatively high DP and low content of reducing groups [32]. In line with this observation, again, non-enzymatic browning reaction, which is related to the presence of reducing sugar was proposed to be implicated in the loss of functional integrity of amino compounds present in proteins [70].

Furthermore, care has to be take in defining optimal sugar concentration added in protective matrix. One work on spray drying of trypsinogen showed that preservation of complete activity can be achieved by addition of sucrose at 1:1 mass ratio [29]. However, at high

carbohydrate concentrations, preferential sugar-sugar interactions prevailed, resulting in a phase separation within the formulation matrix. The preferential incorporation of the sucrose molecules in a sugar-rich phase reduced the actual amount of the carbohydrate available to interact with the protein and thereby decreased the number of effective protein-sucrose contacts. As a consequence, the protein could not be effectively protected during spray drying. Upon differentiating the individual stresses occurred during spray drying, i.e. heat, atomization pressure, and dehydration, it was found that dehydration was the major stress responsible for protein denaturation [29]. This observation could be confirmed by the data on high pressure study presented in Chapter 2 of this work, in which it was shown that the application of high pressure up to 400 MPa did not have any lethal effect on LGG suspended in phosphate buffer.

***Protection against drying induced injury on living cells***

A study which attempted to elucidate the impact of added trehalose on dehydration tolerance of *Saccharomyces cerevisae* and on phase transition temperature of the cell revealed that the reduction of the dry $T_m$ in the presence of sugar was deemed responsible for the high survivability of these cells after drying and rehydration [71]. The same conclusion was drawn as well upon drying of *Escherichia coli* and *Bacillus thuringiensis*, although it was also noted that sugar not only prevented drying-induced membrane phase transition but also maintained the dry proteins in their hydrated conformations [13]. The maintenance of dried proteins in a conformation similar to that of the hydrated protein is proposed to be based on the binding of sugars to the hydrophilic domains of the proteins and preventing inter- and intraprotein hydrogen bonding during drying and rehydration [72].

In contrast, added sugars (maltose, trehalose, sorbitol) did not depress $T_m$ in dry cells of *L. plantarum*, irrespective of their beneficial effect of desiccation tolerance of liposomes [73]. Therefore, it was speculated that instead of direct interaction with the polar lipid headgroups primarily the radical scavenging (antioxidative) activity of the sugar conferred dehydration protection [73], as also supported by Andersen *et al* (1999) [74]. On the other hand, it was also mentioned that the failure to provide desiccation tolerance was attributed to the fact that the sugars might not get access to the cytoplasm, since the cells were grown on glucose and fructose and thus not adapted to the uptake of the evaluated carbohydrates.

Sugar has to be present in both side of the membranes (extra- and intracellular) to allow sufficient protection of cellular components critical for viability including membranes and proteins [13, 20, 75, 76]; however the threshold level of internal sugar concentration sufficient to confer protection varies greatly depending on the cell type, drying conditions and residual moisture content. A minimum amount of sugar is needed intracellularly to hydrogen-bind to all of the lipid molecules in the plasma membrane or to form intracellular glassy state [76]. In

general, anhydrobiotic organisms have concentrations of internal sugar (especially trehalose) that range from 20 to 50% of the dry weight of the organism [56]. To allow internal accumulation of sugar, the impermeability of cell membrane to sugar has to be overcome. Some treatments have been proposed to improve diffusion of sugars across the cell membrane.

One approach made use of the membrane leakage upon temperature-induced membrane phase transition. Penetration of trehalose and sucrose into cells of *E. coli* and *B. thuringiensis* (0.43 μmol trehalose per mg dry weight) was reported to be achievable after incubation in 100 mM sugar solution at hydrated $T_m$ (at around 10°C), when membranes have greater permeability and the sugar flew down its concentration gradient and into the cells [13]. Similarly, trehalose could also be introduced into insulin-producing cells from mammalian pancreas with help of inherent leakiness of the membranes during membrane lipid phase transition at $T_m$ [19].

Furthermore, improved diffusion was achievable by long soaking time. Wolkers *et al* (2001) reported on an efficient uptake of trehalose (ca. 20 mM cytosolic concentration) into human blood platelets at 37°C in a time span of only several hours, which improved the survival rate during lyophilization [77]. Soaking of baker's yeast in 1 M trehalose solution for several days was proved sufficient to load the sugar into cells (up to 200-250 mg trehalose per g of dry cells) and it was proposed that exogeneous trehalose was incorporated by the low-affinity trehalose transport system (a facilitated diffusion process) [78].

It had been shown that osmotic induction of trehalose synthesis in *E. coli* could increase the rate of survival of desiccation [79, 80]. Drying of osmotically shocked *E. coli* (resulting in 300 mM internal trehalose prior drying) in the presence of 1 M trehalose improved their viability after drying and storage stability compared to untreated sample [80].

More sophisticated approaches on mammalian cells relied on the reversible permeabilization of plasma membranes using a switchable recombinant pore-forming hemolytic proteins α-hemolysin, which allowed introduction of trehalose into the cells and facilitated retention of the integrity of plasma membrane during drying [81, 82]. Chen *et al* (2001) reported that with this technique more than 0.1 M intracellular trehalose could be accumulated, leading to increased membrane integrity of fibroblasts after drying [76]. On the other hand, expression of recombinant genes that encode for trehalose-6-phosphate synthase and trehalose 6-phosphate phosphatase in human foreskin fibroblasts, resulted in trehalose accumulation as high as 0.3-0.4% of the dry weight of the cell and increased desiccation tolerance [83]. Furthermore, it was also shown that the electropermeabilization technique, which is widely used for introduction of DNA and other foreign molecules into cells, could be applied for the intracellular delivery of trehalose, enabling cryo- and lyopreservation of human T lymphocytes, mouse myeloma cells, and fibroblasts [84].

In the study of plant desiccation tolerance (pollen, plant seeds, resurrection plants, etc.) it was observed that mechanism of protection was related not only to the considerable accumulation of di- and oligosaccharides, as can be explained by water replacement hypothesis; the presence of compatible solutes and specific proteins, such as the late embryogenesis abundant proteins and heat shock proteins, and the accumulation of antioxidative compounds may also play a role in guaranteeing survival in dehydrated state [85].

The protective role of sugar in maintaining high survivability of dried organism may also rely on the formation of glassy state; although it was always clearly indicated whether cytoplasmic or extracellular vitrification or vitrification at both sides are mainly responsible for the limitation of dehydration-induced damage. Generally, it was proposed that immobilization by vitrification may minimize stress damage on the cellular structure and thus protect their biological capabilities during dehydration and rehydration [48]. When vitrification state is lost, free radical oxidation, phase separation and cytoplasmic crystallization would occur and impose real threat to the survival of dry organism. However, significant losses of fermentation activity of commercial dry yeast were still observed in vitrified yeast sample [86]. No break or step change was observed in the Arrhenius plot for the rates of activity loss in the vicinity of the $T_g$, indicating that the degradation took place with the same rate regardless of the thermophysical state of the matrix. Furthermore, when exposed to 70°C for 24 h yeast viability decreased with increasing molar mass of the maltodextrine, although maltodextrine is regarded as a good glass-former polymers with $T_g$ values proportional to molar mass [87]. The authors found that despite the inability of maltose to protect dried lactase this reducing sugar was more effective in ensuring high viability of dried yeast compared to maltodextrin, indicating the predominant contribution of hydrogen bonding to yeast protection. It was shown further that vitrification was not sufficient to explain protective mechanism of sugar since the viability after drying of *S. cerevisae*, which was internally loaded with 10-20% trehalose, could be dramatically improved although this amount had only a minor effect on the $T_g$ of the samples [88]. Likewise, one study on mammalian cells showed that chemical activity leading to degradation of fibroblast membrane still occurred during storage below $T_g$ [76]. It was suggested that the absence of protection in glassy state was caused by local microheterogeneities within the dried biological sample, resulting in spatial distribution of glassy and rubbery states throughout the sample volume [86].

Taking together, drying, as a preservation method of preference for living cells, induced various damages on crucial cellular components, as evidenced by many works on dehydration of membranes and proteins. This cellular injury can by chance lead to cell inactivation and therefore has to be minimized in the production of stable bioactive

preparations. Some approaches to render cells more tolerant against dehydration were investigated on model systems as well as on prokaryotic or eukaryotic cells. Besides, many studies take dehydration tolerant plants into consideration, in order to learn the mechanism of maintaining viability in dehydrated state. The superior role of sugar in conferring protection against dehydration induced cellular damage is undoubted. Although the real mechanism could not be unequivocally understood yet, the stabilizing effect of sugar relies on its ability to directly interact with membranes and proteins in dried state; thus taking over the role of water in maintaining the native or hydrated structure of these macromolecules and preventing membrane phase transition. Second, the formation of a highly viscous glassy state during dehydration in the presence of sugar could also be deemed responsible – at least partly – for the marked reduction of deleterious chemical reactions occurring in cells during water loss. Thus, both criteria have to be fulfilled by a certain sugar or a certain mixture of different types of sugar – each fulfilling one of the proposed protective mechanism mentioned above – which constitute the protective matrix, so that good survival after drying and rehydration could be ensured. Additional criteria upon selecting effective sugar as single compound or used in combination might be the chemical inertness and the inherent possibility to act as antioxidative agent. Furthermore, sufficient protection could only be achieved when direct interaction as well as formation of glassy state occurred in both sides of the membranes; thus the added sugar has to be able to penetrate cell membrane appropriately. Apart from adding sugar, incorporation of other protection-relevant compounds (e.g. antioxidant, etc.) in the drying matrix seems to be a practical approach in improving the protective capacity. Finally, the defense mechanism of the cells itself in responding to the changes in their environment during the first stages of desiccation (by accumulation of compatible solutes, synthesis of stress proteins, modified metabolic pathways, etc.) need to be explicitly studied so as to effectively utilize this cellular response in combination with an optimized protective formulation towards a better survivability.

### *3.1.2 Spray drying*

In the spray drying process the feed solution is transformed from a fluid state into a dried form by spraying the feed into a hot drying medium. The process itself involves the atomization of a liquid feedstock into a spray of droplets, which come in direct contact with electrically heated air in a drying chamber. There are three modes of contact between hot air and liquid feed: co-current, counter-current and mixed flow [89]. Figure 4 shows a schematic drawing of a lab-scale spray dryer working with in a co-current flow of drying air and feed solution.

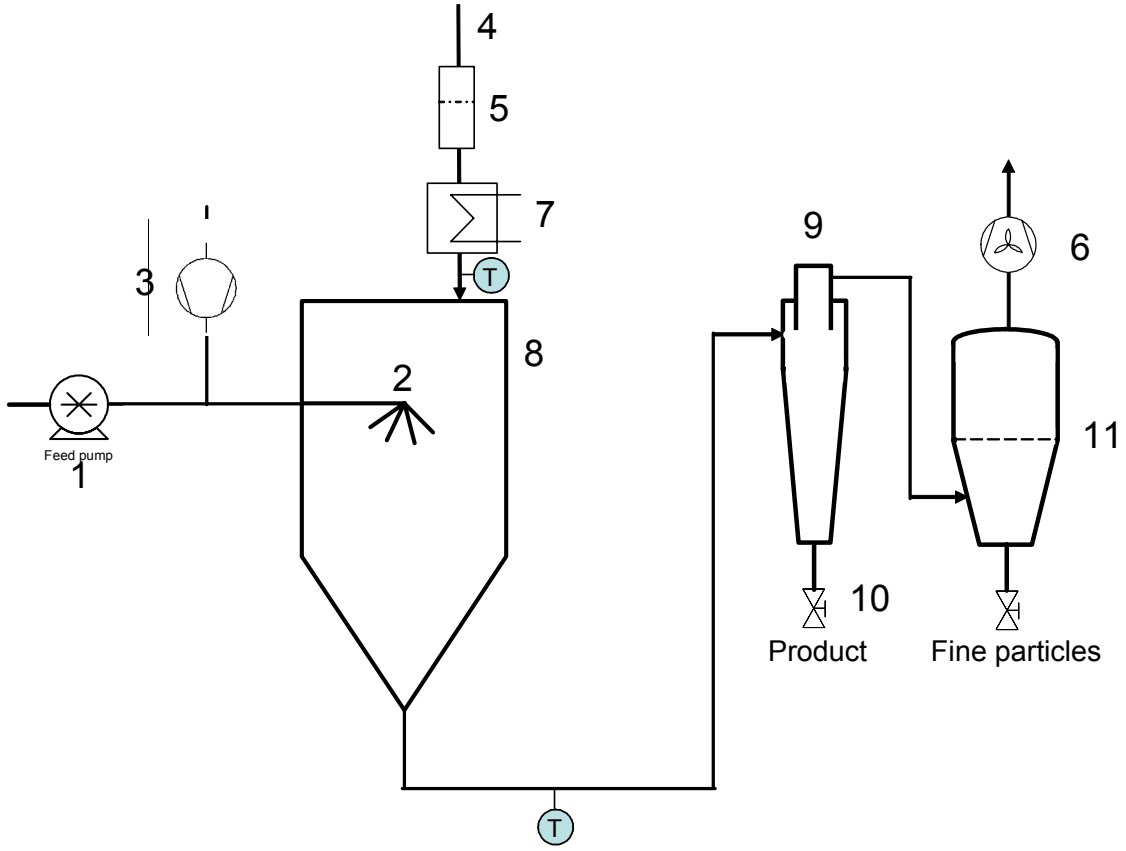


**Figure 4**

Schematic view of a spray dryer installation with its essential components. The role of each components are described in text.

The feed (1) is pumped from the product feed tank to the atomizing device which is normally located in the air disperser in the top of the drying chamber. The sprays are produced by a rotary (wheel) or nozzle atomizer (2), resulting in fine droplets of the range 2 – 150 μm diameter. When two-fluid nozzle is used to generate spray, pressurized air (3) is required. The drying air (4) is drawn from the atmosphere via a filter (5) by an aspirator (6) and is passed through the air heater (7) to the air disperser. In the drying chamber (8), The atomized droplets meet the hot air and the evaporation takes place cooling the air at the same time. Evaporation of moisture from the droplets and formation of dry particles proceed under controlled temperature and airflow conditions. The construction of the drying chamber is made under consideration of the adequate residence time and droplet trajectory distance for achieving the heat and mass transfer. After the drying of the spray in the chamber, the majority of the dried product falls to the bottom of the chamber. The dried product can either be discharged continuously from the bottom of the drying chamber or passed into a solid-gas separator (9) where the solids from the gas stream are recovered and the powder is collected at its bottom (10). The fine particles are usually collected in an outlet filter (11).

Operating conditions and dryer design are selected according to the drying characteristics of the product and powder specification, most importantly the residual moisture content of the powder and the particle size. The first variable is affected by the evaporation rate and the dryer ΔT (inlet air temperature minus the outlet air temperature), which in turn dictates the amount of drying air needed and ultimately the sizing and cost of almost all of the system components. The particle size requirement affects the choice of atomization method and can also affect the size of the dryer.

The dehydration of the atomised liquid particles proceeds from the particle surface to the inner core, resulting in the formation of protective vapour film, which surrounded the droplet and keeps the particle at the vapour saturation temperature. As long as the particle does not become completely dry, evaporation still takes place and the temperature of the solids may decrease (due to evaporative cooling) or does not approach the dryer outlet temperature [90, 91]. This is why many heat sensitive products (enzymes, microorganisms, volatile aroma compounds, etc.) can be spray dried at relatively high temperatures to produce powders with low moisture load without the danger that the product may be harmed.

### *3.1.3 Spray drying works on lactic acid bacteria*

As shown in Table 1, a lot of studies have been undertaken on evaluating the applicability of spray drying to produce lactic acid bacteria preparations for use as dairy starter (cheese or yoghurt), bacteriocin-producing and probiotic cultures.

The driving force for these studies was mainly to demonstrate the capability of spray dried cultures in replacing the usual liquid or frozen bulk starter or freeze-dried cultures in the production of fermented products. In comparison to the latter techniques of culture production, spray drying is claimed to be more cost effective and less time consuming [92]. It was reported in more detail that the energy consumption upon using spray drying is much lower (4000 – 6000 kJ/kg of evaporated water) in comparison to that of freeze drying, where as much as 100.000 kJ was required to evaporate 1 kg of water [93, 94]. Besides, freeze dried cultures often have extended lag phases since they were not only exposed to attenuating effect of freezing but they also are subjected to attenuation by dehydration effects and the destabilizing effect of freezing may increase the susceptibility of the cell to subsequent drying step [1, 95, 96].

During spray drying bacteria are faced to multiple stresses , i.e. heat (both wet and dry), oxidative, dehydration-related stresses (osmotic, accumulation of toxic compounds, etc.) acting either simultaneously or successively on bacteria, which potentially lead to cell death. Besides, as already mentioned above, the removal of water, which contributes to the stability of biological molecules, may cause irreversible changes in the structural and functional integrity of bacterial membranes and proteins. Preservation of these essential functions and

structure is crucial for the survival of bacteria and the retention of their functionality. Until now the commercial application of this preservation method for lactic acid bacteria has not won broad recognition yet. This scepticism originates mainly from the survival aspects during drying, which is suspected to be very low [4]. Besides, the storage stability and the rehydration properties of the spray dried bacteria are considered as poor. However, there is a body of evidence, which demonstrates the possibility to spray dry various strains of lactic acid bacteria without a drastic loss of viability and activity, or at least show survival rates during spray drying which are comparable to that on freeze-drying [4, 9, 10, 97].

### 3.2 Objective

The overall objective of the current study was to evaluate the feasibility of spray drying to produce dry preparations containing probiotic bacteria *Lactobacillus rhamnosus* GG. The outlet temperature, i.e. the temperature measured at between drying chamber and cyclone, which was regarded as the drying temperature, was evaluated on its effect on both the residual moisture content and the survival rate of *L. rhamnosus* GG in the resulted powder. Flow cytometric analysis in combination with carboxyfluoresceindiacetate-propidium iodide (cFDA-PI) dual staining strategy was applied to the identify the nature of spray drying-induced cellular injury as induced upon application of different drying temperatures.

Moreover, the suitability of prebiotic substances as a part of the drying medium was assessed so as to demonstrate the possibility of producing pro- and prebiotic containing preparations. The storage stability of *L. rhamnosus* GG spray dried in different carrier media was investigated with respect to their capability of forming glassy state, primarily in order to find out, whether a correlation exists between the glass forming ability of a specific carrier and the storage stability of probiotic cultures dried with it. Apart from evaluating the contribution of glassy state to dehydration tolerance, direct interaction between protective media with liposomes was assessed using flow cytometric measurement.

## Table 1

Overview of existing spray drying works performed on lactic acid bacteria

| Microorganism | Reference | Inlet Temperature (IT). | Hot air flow | Atomization pressure | Outlet Temperature (OT) | Composition of feed solution | Spray drying unit | Special remarks |
|---|---|---|---|---|---|---|---|---|
| *L. acidophilus* | [98] | 170°C | n.d. | n.d. | 75-85°C | 25 & 40% solid milk | n.d. | moisture content 3.5 - 6.1% |
| *S. salivarius subsp. thermophilus, L. delbrueckii subsp. Bulgaricus* | [97] | 140 – 180°C (160°C) | 17$m^3$/h = 0.28 $m^3$/min | 0.98 bar = 98 kPa | 60-90°C (60°C) | yoghurt, 13,63% solid non fat | n.d. | |
| *L. helveticus* | [99] | 220°C | 30$m^3$/h = 600 g/min | n.d. | 65-130°C | condensed skim milk (15-34% solid) | Lab-Plant SD-04 | OT<80°C caused fouling on the walls, Residence time 0.5 s, Particle size ~12 µm |
| *L. helveticus* | [1] | 220°C | 30$m^3$/h = 600 g/min | n.d. | 82 or 120°C | 19% maltodextrin | Lab-Plant SD-04 | OT adjustment using feed flow rate |
| *L. bulgaricus* | [3] | 200°C | n.d. | 5 bar | 62-105°C | skim milk | Niro | |
| *L. bulgaricus* | [4] | 200°C | n.d. | n.d. | 80°C | 40% maltodextrin in water or 11% milk | Niro | |
| *L. bulgaricus* | [11] | 200°C | n.d. | n.d. | 70°C | skim milk (LabM) | Niro | |
| *L. lactis, L. casei, S. thermophilus* | [100] | 220°C | n.d. | n.d. | 70-90°C | 25% (w/w) solid maltodextrin | Lab-Plant SD-04 | |
| *L. lactis* | [9] | 160 – 200°C | 28$m^3$/h = 0.47 $m^3$/min | compressed air 600 l/h | 68°C | 20% RSM | Büchi 191 | Flow rate of feed solution = 10,13,17 mL/min |
| *S. thermophilus, L. delbrueckii subsp. bulgaricus* | [101] | 180°C | n.d. | n.d. | 60-80°C | yoghurt | Luwa | water evaporating capacity : 1645 kg/h |
| *L. paracasei* NFBC338, *L. salivarius* UCC118 | [6] | 170°C | n.d. | n.d. | 60-120°C (80-85°C) | 20% RSM | Büchi B191 | moisture content in appreciable range (4%) |
| *L. paracasei* NFBC338 | [102] | 170°C | n.d. | n.d. | 85-105°C | 20% RSM | Büchi B191 | Moisture:1.7 - 3.3 % g/g<br>OT adjustment using feed flow rate |
| *Bifidobacteria* | [103] | 100°C | n.d. | 5-8 bar (Compressed air 700 l/h) | 45°C | 10% (encapsulation) | Büchi B190 | |
| *B. infantis, B. longum* | [104] | 100°C | n.d. | n.d. | 50-60°C | Gelatin, gum arabic, skim milk, soluble starch 10% (scan 2-30%) | Büchi B191 | Moisture content 7-9% |
| *L. salivarius, L. sakei* | [10] | 200°C | n.d. | 5 bar | 70°C | 11% milk | Niro | |
| *L. paracasei* NFBC338 | [105] | 170°C | n.d. | n.d. | 95-100°C or 100-105°C | 20% RSM or 10% RSM with 10% gum accacia | n.d. | Moisture: 2.5 - 3.2% g/g,<br>OT adjustment using feed flow rate |
| *L. acidophilus, B. lactis Bb12* | [106] | 130 – 190°C | n.d. | 5 kgf/$cm^2$ | 75 – 120°C | Cellulose acetate phthalate, Glycerol, Maltodextrin, Raftilose, milk | Lab-Plant SD-04 | Avg. particle size 22 µm |
| *L. rhamnosus GG, L. rhamnosus E800, L. salivarius UCC500* | [7] | 170°C | 100% | n.d. | 85-90°C | RSM 20% w/v or 10% RSM with 10% prebiotics | Büchi B191 | Moisture <4%,<br>OT adjustment using feed flow rate |

n.d. not described, RSM: reconstituted skim milk

## 3.3 Material and methods

### *3.3.1 Test organism and preparation of bacterial suspension*

*Lactobacillus rhamnosus* GG (ATCC 53103), thereafter abbreviated as LGG, was obtained from Valio R&D (Helsinki, Finland). For long-term maintenance this organism was stored as glass bead cultures (Roti®-Store, Carl-Roth, Karlsruhe, Germany) in freezer at -80°C (U101, New Brunswick Scientific, Nürtingen, Germany).

One bead of a deep-frozen culture was transferred into MRS broth (Oxoid, Basingstoke, UK) and incubated overnight at 37°C. Afterwards a final broth was inoculated with the overnight growth culture prior to another incubation period at 37°C for 24 h. Cells were centrifuged at 2000 *g* for 10 min and washed twice with PBS-buffer (phosphate-buffer-saline) pH 7.0. Following washing the pellet was resuspended in an equal volume of the final carrier solution.

### *3.3.2 Preparation of carrier solution*

Reconstituted skim milk powder, hereafter abbreviated with RSM (Oxoid, Basingstoke, UK) at a concentration of 20% (w/v) was used as the reference medium. The evaluated prebiotics were: Raftilose®P95, an oligofructose produced by partial enzymatic hydrolysis of chicory inulin (ORAFTI, Tienen, Belgium) and Polydextrose (DANISCO, Copenhagen, Denmark). Thereafter, P95 and PDX, are used as the abbreviations for Raftilose®P95 and Polydextrose, respectively. Chemical structures of these preparations are shown in Figure 5. Prebiotic media used for spray drying consisted of an equal ratio of reconstituted skim milk and each of these prebiotics (20%, w/v, total solids). The media were decontaminated by heat treatment in a water bath at 90°C for 30 min.

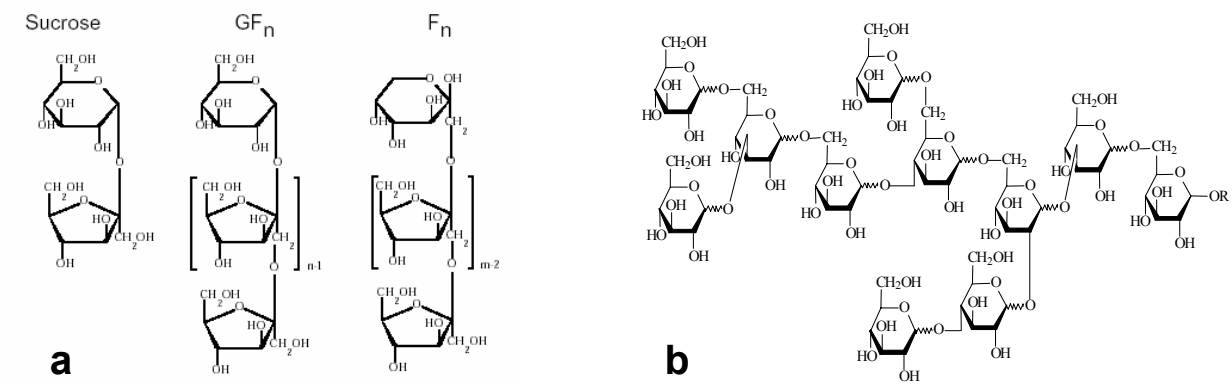


**Figure 5**

Basic chemical structure of prebiotic molecules incorporated in spray drying media (Annex 4 and 5)

(a) General structure of the two basic types of ß(2→1) fructans, which are the major constituents of the commercial oligofructose preparation Raftilose®P95. The two fructan types ($GF_n$: glucosyl type and $F_n$: fructosyl type) exist normally as a mixture. The degree of polymerisation (DP) of Raftilose®P95 ranges typically from 2 to 8.

(b) General structure of polydextrose, which is a randomly cross linked polymer of glucose with a highly branched complex 3D structure. All bonds are present as 1 – 6 and 1 – 4 linkages. In the polymer, R can be hydrogen, sorbitol-bridge or more polydextrose molecule. The commercial name of this polydextrose preparation is Litesse®.

### *3.3.3 Spray drying*

The spray-drying process of LGG in the various media was undertaken in a laboratory scale spray dryer (Büchi B-191, Flawil, Switzerland), which is schematically shown in Figure 6. The feed solution was pneumatically atomized into a vertical, co-current drying chamber using a two-fluid nozzle at a constant flow rate (5 mL $min^{-1}$). The outlet temperature was adjusted from 70 to 100°C by varying the air inlet temperature. The dried powder was collected in a product container connected in bottom part of the single cyclone separator. Once the outlet temperature stabilized, the heated glass container was disconnected and replaced with another container in order to minimize uncontrolled thermal stress on the dried bacteria.

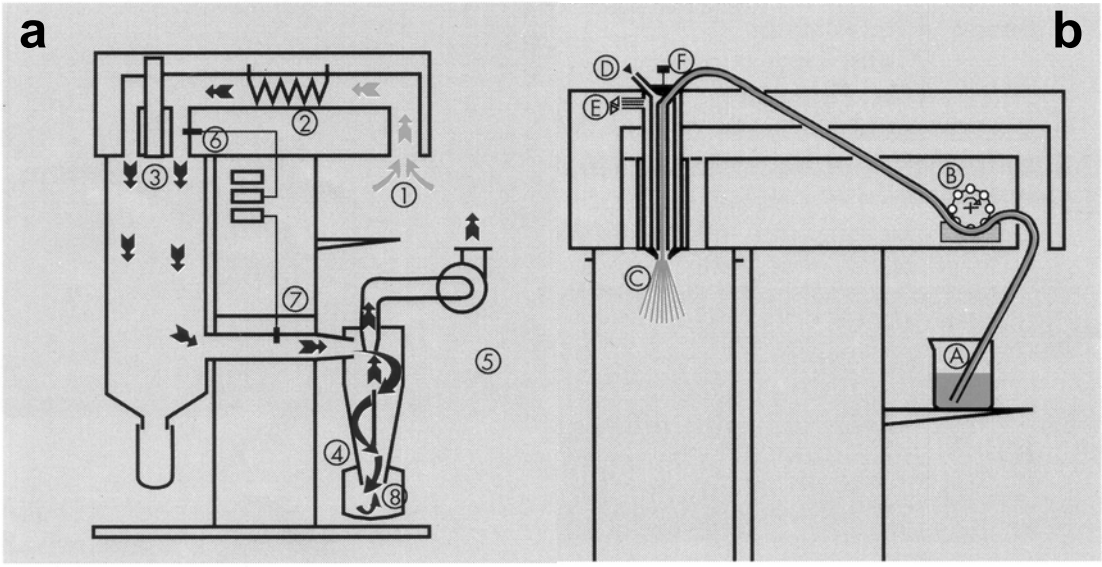


**Figure 6**

(a) Pathway of drying air in the spray dryer Büchi B191

Cold air is aspirated through the air inlet tunnel (1) and then electrically heated (2) prior to entrance in the spray cylinder, in which drying of the droplets into solid particles takes place (3). Dried powder is separated from fine particles in the cyclone (4) and collected in the glass container (8). Outlet filter is placed to remove fine particles and to prevent them from entering aspirator (5) which generates the air flow. Temperatures are measured in the entrance of spray cylinder (6, termed as air inlet temperature) and in the intermediate piece between spray cylinder and cyclone (7, termed air outlet temperature).

(b) Pathway of feed solution and pressurized air in the spray dryer Büchi B191

Feed solution (A) conveyed by peristaltic feed pump (B) and atomizing air (D, inlet) are passed separately to the nozzle head (C), where the atomization of the feed solution into fine droplets takes place. The co-current two-fluid nozzle is located at the centre of the upper part of the spray cylinder. Atomization is created by compressed air at a pressure of 0.5 to 2 bar. Nozzle diameter is 0.7 mm. Powders produced with this adjustment have particle size ranged from 5 to 15 µm on average [105].

Generally, the level of outlet temperature which is determined by the drying rate, could be adjusted by two different settings of the spray dryer. In this study, the adjustment of outlet temperature was performed by holding flow rate of the feed suspension at a constant value (25% pump capacity ~ 5 mL $min^{-1}$) for all outlet temperatures, whereas the inlet temperature was varied, as shown in Table 2. A rise in the outlet temperatures due to the fouling of the inner wall of the spray chamber can be compensated by slightly increasing the feed flow rate.

**Table 2**

Applied parameters for spray drying of *L. rhamnosus* GG

| | |
|---|---|
| **Pump capacity :** | 25 ± 2 % ~ flow rate of feed suspension 5 mL $min^{-1}$ |
| **Flow rate of drying air :** | 100% ~ 60 $m^3$ $h^{-1}$ |
| **Atomization pressure :** | 6 bar |
| **Flow rate of pressurization air :** | 700 L $h^{-1}$ |

| Aimed outlet temperature (°C) | Adjusted inlet temperature (°C) |
|---|---|
| 70 | 115 |
| 80 | 130 |
| 90 | 145 |
| 100 | 155 |

On the other hand, it is also possible to vary the flow rate of the feed solution (thereby varying the amount of water per time unit which needs to be evaporated) under holding the inlet temperature at typically high level to obtain the aimed outlet temperature, as used in previous studies (Tab. 1). This latter operational procedure is thought to be more flexible when different drying rates are frequently applied since changing the feed flow rate is faster and easier than changing inlet temperature.

### *3.3.4 Determination of moisture content in spray dried powders*

The residual moisture content of spray dried powders was determined by oven-drying at 102°C [107]. This involved determination of the difference in weight before and after overnight storage in he oven dryer. Moisture content was then expressed as a percentage of the initial powder weight.

### *3.3.5 Enumeration of probiotics after spray drying*

To determine the survival rate of the probiotic bacteria, spray dried powders were rehydrated with sterile Ringer's solution (No. 15525, Merck, Darmstadt, Germany) to obtain the same solids concentration as the initial feed solution. Afterwards, they were serially diluted and drop plated in duplicate on MRS agar (Oxoid, Basingstoke, UK). Plates were placed in an anaerobic jar (Anaerocult®A, Merck, Darmstadt, Germany) and incubated at 37°C for 48 h. Survival rate were calculated as follows: %survival = $N/N_0$ x 100, where $N_0$ represented the number of bacteria before drying and N was the number of bacteria after drying.

### *3.3.6 Staining procedure and flow cytometric assessment*

***Double staining with carboxyfluorescein diacetate (cFDA) and propidium iodide (PI)***

A 100 µL aliquot of rehydrated powder was mixed with 900 µL Ringer solution and centrifuged for 10 min at 4000 g. The pellet was resuspended in 100 µL PBS buffer 0.05 M pH 7.0 and mixed together with 100 µL of 100 µM cFDA stock solution (Molecular Probes, Inc. Leiden, The Netherlands), so that the concentration of cFDA in the reconstituted pellet suspension was 50 µM. The suspension was incubated at 37°C for 10 min to allow intracellular enzymatic conversion of cFDA into cF (Carboxyfluorescein). After excessive cFDA was removed by centrifugation, 30 µM PI (Molecular Probes, Inc. Leiden, NL) was added. The cell suspension was kept in an ice bath for 10 min to allow labelling of the membrane-compromised cells prior to flow cytometric measurement.

***Flow cytometric measurement***

All measurements were made with a Coulter®EPICS®XL-MCL flow cytometer (BeckmanCoulter Inc., Miami, USA) with 488 nm excitation from an argon-ion laser at 15 mW. The green fluorescence from carboxyfluorescein was collected through a 525 nm band-pass filter; and a 620 nm band-pass filter was used to collect red fluorescence from propidium iodide. Data were analysed with the software package Expo32 ADC (BeckmanCoulter Inc., Miami-FL, USA). Acquisition of fluorescence data was performed by pre-setting a gate in the forward-angle light scatter (FS) *versus* sideward scatter (SS) plot, which enabled bacterial cells of interest and artefacts to be discriminated. The flow rate was set at typical values of 300-600 bacterial cells per s. Further settings are listed in Annex 2.

### *3.3.7 Storage test*

The dried samples were stored at a relative humidity of 11%, which was regarded as optimal in preserving dried bacteria [108]. The relative humidity was maintained constant by storing the powder in hermetically closed jar above saturated lithium chloride solution [109]. The samples were kept at storage temperatures of 25 or 37°C. Only powders dried at an outlet temperature of 80°C were subjected to the storage test. The storage inactivation data were expressed as logarithmic value of relative survival fraction ($\log N/N_0$). N refers to the bacterial count at a particular storage period, whereas $N_0$ represents the bacterial count at the beginning of the storage. The viability loss during storage was assumed to follow first order reaction kinetics, and the first order rate constants were calculated. Three replicate storage trials were undertaken.

### *3.3.8 Differential scanning calorimetry measurement*

The glass transition temperatures ($T_g$) of spray dried preparations were determined by differential scanning calorimeter (DSC). With this technique the difference in heat flow to or from a sample, and to or from a reference material is monitored as a function of temperature, while the sample is subjected to a controlled temperature program. Simultaneously, thermogravimetric measurement (TG) was applied to monitor the change in the mass of the sample as a function of temperature. All calorimetric measurements were performed using a Netzsch STA-409C thermoanalyzer unit (Netzsch GmbH, Selb-Bayern, Germany).
All measurements were made at a linear heating rate of 20 $K \cdot min^{-1}$ using an opened platinum pan and an empty pan as a reference material. Samples of 10 – 20 mg were initially heated up linearly to 150°C to remove moisture from the sample. Prior to initiation of the second scan, the heated, water-free samples were rapidly cooled down to -30°C with nitrogen flush.

### *3.3.9 Calculation of glass transition temperatures*

The glass transition temperature ($T_g$) was determined during the second run and was defined as the midpoint value of the change in specific heat observed as an endothermic shift in the baseline of the DSC signal. Due to the aforementioned limitation, the $T_g$ of the fresh, moisture-loaded spray dried powder could not be directly determined. This value was therefore estimated by Gordon Taylor equation (Equation 1). This empirical equation is typically applied to predict the $T_g$ values of a solid substrate at various water contents. The reliability of this equation for calculating $T_g$ of various food systems has already been demonstrated in several studies [47, 58, 110-113].
For the prediction of $T_g$ in sugar/water glasses, the Gordon Taylor equation is as follows :

$$T_g = \frac{w_1 \cdot T_{g1} + k \cdot w_2 \cdot T_{g2}}{w_1 + k \cdot w_2} \qquad \text{Equation 1}$$

where $w_1$ and $w_2$ are the weight fractions of the solute and water, respectively; $T_{g1}$ is the glass transition temperature of the water-free solute; $T_{g2}$ is the glass transition temperature of water (-135°C); and k is a constant [57, 114].

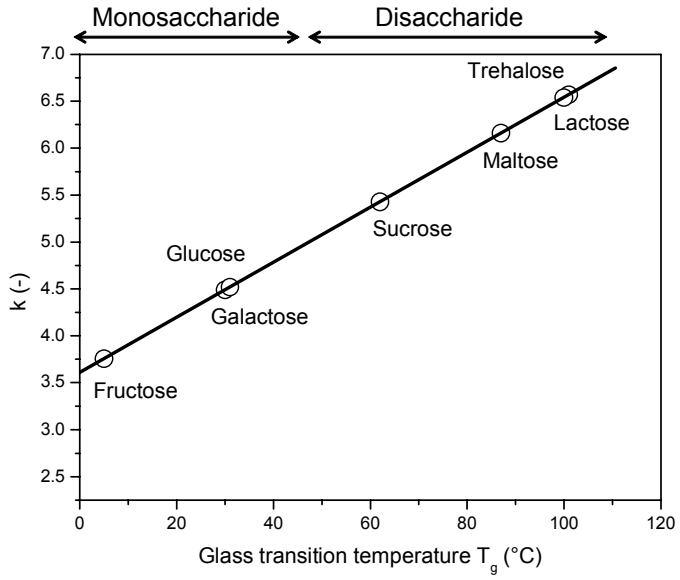


**Figure 7**

The k-values of some important mono- and disaccharides glasses (anhydrous) as a function of glass transition temperatures ($T_g$). Data were taken from [57], in which the onset of the glass transition according to DSC measurement was regarded as $T_g$. This empirical relationship allow Equation 2 to be established (see Material and Methods).

For a given sugar molecule, the $T_g$ values of several combinations of experimental weight fractions must first be determined in order to estimate k. The values of k calculated for relevant sugars (mono- and disaccharides) with $T_g$ values at several water contents are shown in Figure 7. It could be observed, that the plot of $T_g$ for the anhydrous sugars against k was linear. The regression equation obtained (Equation 2) was used in Equation 1 for the prediction of the $T_g$ value of spray drying medium used in this study at various water contents, so as to enable the generation of state diagram, which shows the glass transition temperatures over a wide moisture range.

$$k = 0.0293 \cdot T_{g1} + 3.61 \qquad \text{Equation 2}$$

### *3.3.10 Monitoring direct interaction of sugar-membranes using liposomes to determine the protective effect of sugar during drying*

***Preparation of liposome***

Egg phosphatidylcholine (EPC) was purchased from Avanti Polar Lipids (Alabaster, AL) and carboxyfluorescein (cF) was obtained from Molecular Probes (Eugene, OR)

100 µL of EPC was dried from chloroform under a stream of $N_2$ and stored under vacuum overnight to remove traces of solvent (Fig. 8). Afterwards 1 mL of 1000 µM cF 50 mM PBS

buffer (pH 7.0) was added to hydrate the dried lipids. Liposomes were prepared from these hydrated lipids using a LiposoFast-Basic hand-held extruder (Avestin Europe GmbH, Mannheim, Germany) with two layers of polycarbonate membranes with 1000 nm pores. The LiposoFast-Basic produces unilamellar liposomes by the manual extrusion of the lipid-cF suspension through a polycarbonate membrane of defined pore size, using gas-tight, glass syringes. The sample is passed through the membrane by pushing the sample back and forth between two syringes.

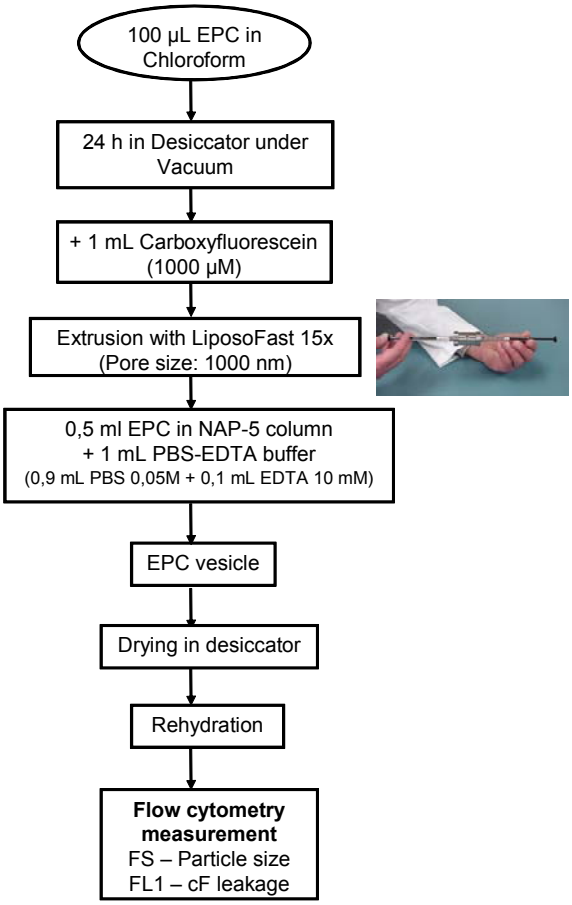


**Figure 8**

Brief overview of the procedure of preparation of liposomes made of EPC (Egg phosphatidylcholine) using LiposoFast extrusion system as well as the assessment of drying induced damage by flow cytometry.

***Leakage experiments***

For leakage experiments, 0.5 mL the vesicles obtained from extrusion was passed through a NAP-5 column (Amersham Biosciences AB, Uppsala, Sweden) and equilibrated in 1 mL PBS-EDTA buffer (50 mM PBS, 1 mM EDTA at pH 7.0), to remove the CF not entrapped by the vesicles. The eluted samples a lipid concentration of approximately 10 $mg \cdot mL^{-1}$.

Liposomes were mixed with an equal volume of concentrated solutions of sugars in PBS and filled into 1.5 mL microcentrifuge vials (Eppendorf AG, Hamburg, Germany) to reach a final lipid concentration of 5 $mg \cdot mL^{-1}$. Twenty µL was filled in each vial. The vials were then dried in desiccators at room temperature over silica gel for 24 h. Damage to the liposomes was determined in flow cytometer after rehydration with 20 µL of PBS-EDTA buffer either as leakage of the soluble marker cF or as occurrence of aggregration.

***Flow cytometric measurement and data analysis***

Assessment of liposome was performed on Coulter®EPICS®XL-MCL flow cytometer (BeckmanCoulter Inc., Miami, USA), as already described above. Exact configurations are listed in Annex 2. A separate protocol for the flow cytometric measurement of liposome was created. The application of flow cytometry in this field of research is relatively new. The most frequently applied technique to assess leakage on liposome is the fluorometer technique, which basically measures the cF-fluorescence in the suspension medium. The fluorescence of carboxyfluorescein is strongly quenched at the high concentration inside the vesicles (Concentration ≥ 100 mM) and is increased when cF is released into the medium [45]. The cF concentration in the liposomes has to be quite high so that upon leakage of a certain amount of cF into the surrounding medium measurable fluorescence signal could be detected. The total cF content of the vesicles (0% retention value) was determined after lysis of the membranes with 50 mL of 1% Triton X-100, whereas the 100% retention values were determined with freshly prepared liposomes. In contrast, using flow cytometric technique the fluorescence of cF entrapped in the liposome is measured. This method allows a considerably lower concentration of cF (Concentration ~ 1000 µM) to be used. A decrease in the fluorescence intensity as measured inside the liposome after drying and rehydration may reflect the leakage of certain amount of cF into the surrounding medium. To check this, liposomes loaded with different concentration of cF were made and measured with flow cytometer. The green fluorescence from cF was collected through a 525 nm band-pass filter. Fluorescence data was plot as frequency histogram. In general, frequency histograms can be used to display relative fluorescence or scattered light signals plotted against the number of events. With this plot the distribution in the fluorescence intensity within the detected liposomes can be observed. The mean of the fluorescence intensity of the liposomes was plotted against the corresponding cF concentration in liposomes (Fig. 9a). It is obvious, that the fluorescence intensity increased as the concentration of cF increased, indicating that the intensity of the fluorescence signals collected at 525 nm can give information about the residual cF concentration in the liposomes. A shift in the distribution towards lower fluorescence value is therefore related to the leakage of the enclosed dye in the external part of liposomes.

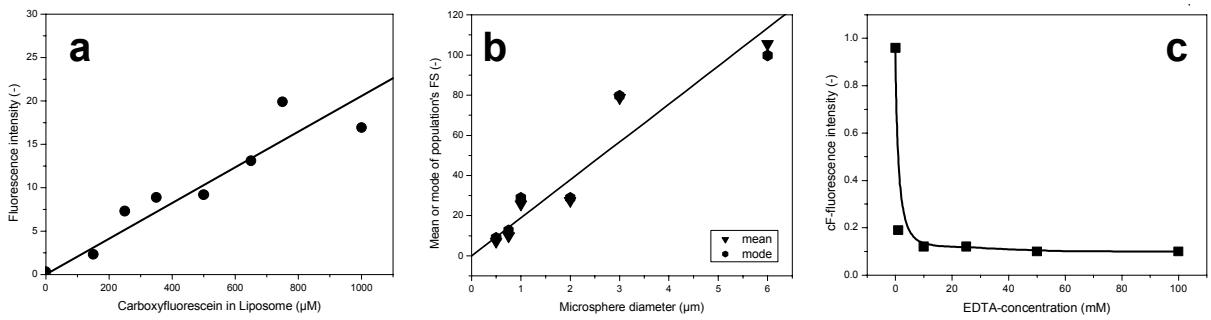


**Figure 9**

Correlation of signals measured by flow cytometer, i.e. signals measured in the 535 nm green fluorescence channel and forward scatter channel, with fluorescence intensity (Fig. 9a) and particle size of polystyrene beads (Fig. 9b), respectively. Figure 9c shows the influence of increasing concentration of EDTA on cF fluorescence intensity.

The same also apply for the determination of the distribution of particle size, which correlate with the forward scatter signal. Standard reference polystyrene beads (Polysciences Europe GmbH, Eppelheim, Germany) were used to validate a relative correlation between the intensity of forward scatter (FS) signal with particle size. It was observed that either the mean or the mode of the frequency distribution of the beads measured positively correlated with the bead size (Fig. 9b). A shift towards higher value of forward scatter is indicative for the occurrence of liposomes with increased size. This change accompanies drying of liposomes and can be related to either fusion or aggregation.

Besides, it was found that the presence of EDTA could delete cF-fluorescence, as measured by fluorescence photometer (Perkin Elmer 650-10S, Perkin-Elmer Corp., Norwalk, USA). Figure 9c demonstrates the effect of increased concentration of EDTA on the fluorescence of a 1µM cF solution. As high as 1 mM concentration of EDTA was found to be sufficient to cause maximal reduction of cF fluorescence, since at higher concentrations of EDTA no further decrease of cF fluorescence could be achieved. Thus, EDTA (end concentration 1mM) is incorporated in the PBS buffer used to dissolve sugar for leakage experiments. The addition of EDTA is thought to be necessary in order to prevent re-attachment of leaked cF on the outer phospholipid layer. It is likely, that this unexpected re-attachment would still render leaked liposomes fluorescent and thereby producing false-negative results, i.e. leaky liposomes might still be detected as intact liposome due to possible attachment of leaking cF. With EDTA outside of liposome the cF diffusing out the liposomes could be effectively sequestered, thus eliminating a potential source of erroneous fluorescence.

## 3.4 Results and discussion

### *3.4.1 Identifying critical processing conditions*

Initial spray-drying experiments were designed to investigate the effect of process parameters, primarily different adjustment of outlet air temperatures on bacterial survival rate and residual moisture content of the powders. Taking values of critical water content for skim milk powder from literature into account, it is expected to identify optimal drying temperature, which is to be used for storage stability test.

The total solids content of the RSM as the drying medium was held constant at 20% (w/v). This solids content was frequently used and has been regarded as optimal for assuring high residual viability of different strains of lactic acid bacteria [6, 9, 99, 102]. Increasing the total solids content of the feed solution decreased the percentage of surviving bacteria [98, 99, 115], although batch-kinetics studies demonstrated that the thermoresistance increased with increasing solids content. Aside from increased osmotic stress which eventually might occur at increasing solid contents, drying conditions was expected to vary with difference in solids concentration of feed solution [98]. Increases in solids content result in increases in suspension viscosity and more concentrated feed suspensions produce larger particles [116]. These particles present relatively smaller ratios between the surface area and the volume and greater core retention, thus requiring longer drying times to achieve a given level of residual moisture; this can reduce cell viability due to the longer contact time of the particles with the hot air .

Daemen *et al* (1982) attributed the decrease in cell viability to a decrease in the drying rate for higher solids content feed solutions. They estimated that the increase in the drying time was approximately proportional to the square of particle size. This condition led to a higher moisture content at the centre of the dried particle and higher moisture contents decrease thermoresistance [115]. It was already reported that dried organisms become more resistant to heat damage [117-120]. Furthermore, spray drying conducted on spray drying of trypsinogen showed that preservation of complete activity can be achieved by addition of sucrose at 1:1 mass ratio [29]. However, at higher carbohydrate concentrations in the feed solution, preferential sugar-sugar interactions prevailed, resulting in a phase separation within the formulation matrix, which led to a reduction of the protection capacity of the sugar during spray drying.

A range of outlet temperatures between 70°C and 100°C was used in the preliminary experiments to spray dry LGG. The survival rate of LGG was inversely proportional to air outlet temperatures (Fig. 10a) and the residual moisture content increased as the air outlet temperature was reduced (Fig. 10b). It is obvious that drying should not take place at very high temperatures ($T \geq 90°C$) not only due to higher inactivation but also due to more

pronounced browning reaction [97, 101]. Low viability level due to application of high temperature was found to extend the lag phase required for complete recovery and multiplication to prior to lactic acid production [100]. On the other hand, drying at very low temperatures ($T \leq 60°C$) showed increased tendency of powder lumping due to higher moisture content [101].

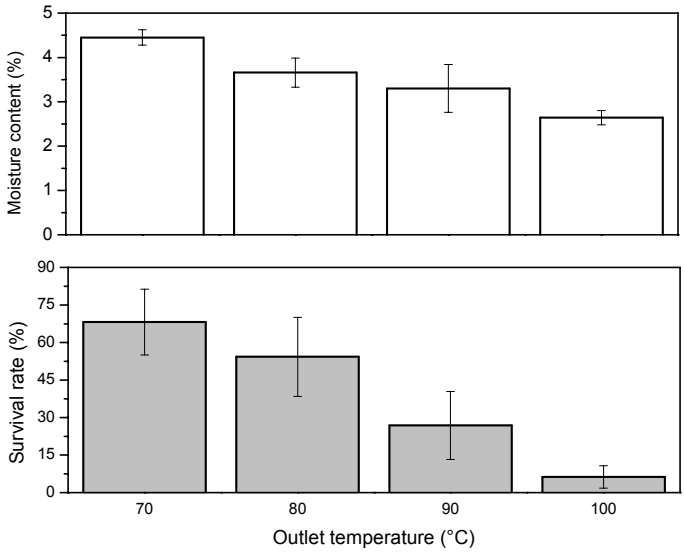


**Figure 10**

Effect of outlet temperature during spray drying on the moisture content (upper figure) and survival of *L. rhamnosus* GG (bottom figure) in probiotic powder prepared from 20% (w/v) reconstituted skim milk (RSM). Initial cell count of the feed solution was ~ $10^9$ cfu $mL^{-1}$. Data are means of two, or more, spray drying experiments, whereas the error bars represent the standard deviation of the means.

Therefore, a compromise in terms of selection of air outlet temperature is required for optimal drying results. A higher viability level, as achievable by drying at lower temperatures, is clearly preferable; however the knowledge of a critical water content should rather dictate the selection the optimal drying temperature, since a lot of technological properties of the powder are dependent on the water content. In particular, as can be seen in Figure 11a, the flowability aptitude of skim milk powders is largely influenced by water activity, $a_w$ of the powder [121]. With help of this Figure and Figure 11b, which shows the sorption isotherm of skim milk powder, the relationship between moisture content, water activity and flowability could be better examined. It is known from the literature, that a residual moisture of 4% (w/w) was regarded as a good quality parameter of dried dairy products [122]. This moisture level corresponds to an $a_w$ value of around 0.2, which in turn give a high flowability index (Fig. 11a). Residual moisture contents of around 4% (w/w) were achieved upon spray drying at an outlet temperature of 80°C. These results fit well with results made by other groups working with the same spray drying equipment as used in this study [6] or with spray dryer from

another company [1]. The range of critical moisture content as well as $a_w$ for good powder characteristics discussed here is also in line with data from literature describing desirable $a_w$ value for the survival of bacteria dried in milk-based media [123].

A different concept was followed in determining critical water content for skim milk powder. Based on the measurement of glass transition temperatures, a critical water content of 7% (w/w) was proposed for the storage of skim milk powder at 25°C (Fig. 11b) [124]. The latter critical moisture value was considerably higher than the one proposed by Masters (1985) and would theoretically allow spray drying at lower temperatures; which are less harsh for bacteria and energetically more advantageous than higher outlet temperatures. Nevertheless, a moisture content lower than 7% (w/w) after drying is preferred so as to minimize the risk of storage-related defects such as crystallization of lactose, because of the better buffering effect towards fluctuation in storage temperature during shipping or processing. Consequently, an air outlet temperature of 80°C during spray drying was used for further assessments.

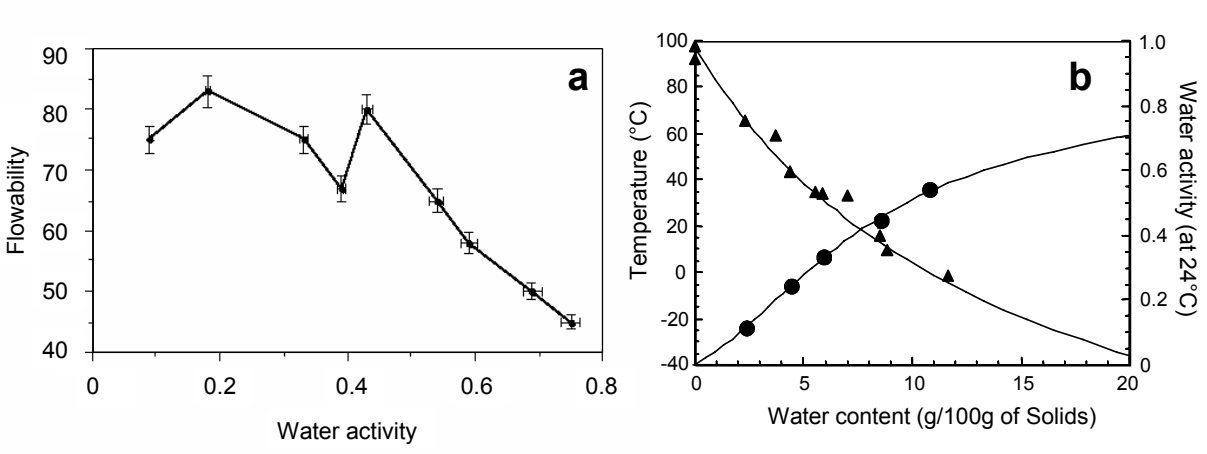


**Figure 11**

(a) Influence of water activity ($a_w$) on the flowability properties of skim milk powder [121]

(b) Glass transition temperature, $T_g$ of skim milk powder (▲) as influenced by its moisture content. Another plot shows the sorption isotherm of skim milk powder (●). Graph is adapted from [124, 125].

***The role of thermal effect in viability loss during spray drying***

It was clearly shown that the number of cells not surviving spray drying conditions increased as the air outlet temperature was elevated (Fig. 10). This observation is of qualitatively in agreement with the findings of many other studies, which examined survivability at different air outlet temperature. This tendency pointed out that thermal stress was apparently more pronounced at higher temperatures. However, during spray drying bacteria are faced not only to heat (both wet and dry), but also different other stresses, including oxidative, dehydration-related stresses (osmotic, accumulation of toxic compounds, etc.). Each of them

can potentially lead to death and it is of interest to differentiate the contribution of each of this factor to bacterial inactivation. Knowledge about the type of stress predominantly affecting bacteria or how these stresses are interrelated in inducing cellular damage during spray drying may help in identifying the processing condition with reduced lethality and effective selection of protective media. In the scope of this study the lethal effect of wet heat was evaluated. This stress was simulated by challenging LGG suspended in RSM to heat treatment at 60, 65 and 70°C. The role of heat stress was determined by analyzing kinetic parameters derived from thermal inactivation curves and comparing these with survival rates of LGG spray dried at relevant temperatures. The thermal death curves are shown in Figure 12a. From this figure it can be seen that these temperatures were found to be suitable for assessing inactivation rates in an adequate time domain. The slopes of the curves (k in $s^{-1}$) allow calculation of D-values at the treatment temperatures applied. Figure 12b was constructed upon extrapolation of the Arrhenius plot (ln k *versus* $T^{-1}$) towards higher temperatures, in order to estimate the D-values, i.e. time required to kill 90% of the initial population, within the temperature ranges similar to the air outlet temperature used for spray drying (70 to 100°C). Accordingly, the D-values for 70, 80, 90, and 100°C are approximately 6.8, 0.4, 0.03, and 0.002 s, respectively.

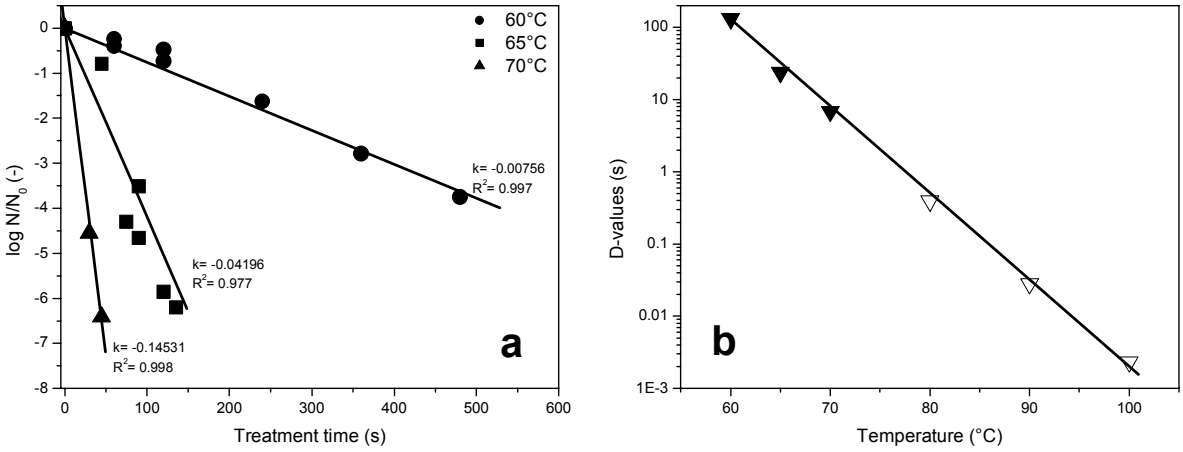


**Figure 12**

(a) Thermal inactivation curves of *L. rhamnosus* GG in reconstituted skim milk 20%. Regression lines representing first order thermal death kinetics, which were used to fit the survival data, are drawn along with the calculated inactivation rates at each temperature, k (in $s^{-1}$). The D-values at each treatment temperature are calculated as reciprocal value of k.

(b) D-values of *L. rhamnosus* GG in reconstituted skim milk 20% as a function of treatment temperature. Data points represented as closed symbols indicate D-values calculated from original data (Fig. 10a), whereas data points shown as open symbols were D-values estimated using Arrhenius equation, which in turn was constructed using original inactivation data. Accordingly, activation energy, $E_a$, of LGG inactivation in reconstituted skim milk 20% was 281.15 kJ $mol^{-1}$.

Further, when the exposure of bacteria to air outlet temperatures used during spray drying was assumed to be around 0.5 to 2 s, as already proposed in previous study [98, 99], then the percentage survival after exposure to this fixed treatment time at different treatment temperatures could be calculated using the calculated kinetic parameters as well as Arrhenius relationship. It was assumed that the total volume of the Büchi B-191 passed by the drying air was about 7 L; thus the estimated residence time at maximal aspirator capacity (60 $m^3$/h) should be ca. 0.4 s (Annex 3). Upon comparing the inactivation results obtained from spray drying and thermal kill in solution (for 0.4 s treatment time) it was obvious that – except at 70°C, where the difference in death rates is within the error range of the microbiological analysis – thermal inactivation in aqueous solution always gave higher inactivation results than spray drying (Table 3). Consequently, heat stress only played a minor role in contributing to bacterial death during spray drying, since there would be higher inactivation results during spray drying if the contribution of heat were indeed much more pronounced. Cell death during spray drying was therefore more related to non-thermal effect.

**Table 3**

Percentage survival after spray drying of *L. rhamnosus* GG at different outlet temperatures and after exposure of the bacterial solution to the treatment temperatures for 0.4 s. In both cases bacteria were suspended in reconstituted skim milk, 20% and subjected either to spray drying or to thermal treatment.

| Temperature (°C) | $N/N_0$, spray drying (%) | $N/N_0$, thermal inactivation (%) |
|---|---|---|
| 70 | 68.21298 | 87.4734 |
| 80 | 54.26882 | 9.54993* |
| 90 | 26.83412 | << 0.001* |
| 100 | 6.25322 | << 0.001* |

* estimated values

Furthermore, these data also suggested that the real temperature experienced by bacteria was much lower than the adjusted outlet temperature. It is indeed one feature of spray drying process that dehydration of the atomised liquid particles proceeds from the particle surface to the inner core, resulting in the formation of protective vapour film, which surrounds the droplet, keeps the particle surface wet and maintain the temperature at the vapour saturation temperature (wet-bulb temperature). At this drying stage, the drying rate is constant. As long as the particle does not become completely dry, thermal inactivation will be limited since evaporation still takes place. As a result, the temperature of the solids may decrease (due to

evaporative cooling) or does not approach the dryer outlet temperature [90, 91]. This is why many heat sensitive products can be spray dried at relatively high temperatures without the danger that the product may be harmed. At the subsequent drying stage, the particle surface becomes dry and the temperature may increase maximally to the dryer temperature since evaporative cooling is no longer available. Consequently, effect of temperature would be higher, but – as already mentioned above – due to the lower moisture content, the microbial cells will show higher resistance [117-120]. It is also possible microbial cell are entrapped in the solid matrix. With regard to the latter issue it was shown by confocal scanning laser microscopy (CSLM) technique, which allow cross-sectional analysis of the dried powder, that the spray dried cells were encapsulated in the milk powder particles, which may have protected the culture during spray drying [6].

Previous works on spray drying of *Salmonella* and *S. cerevisae* already indicated that thermoresistance of the bacteria (in aqueous state) and drying temperature are not the predominant factors affecting lethality [117, 126]. Therefore, extrapolation of heating death in solution is not suitable for the prediction of survival data during spray drying [3]. Likewise, Lievense *et al* (1994) already demonstrated that during dehydration at 5°C, cell death occurred due to damage in their cell membrane not related to thermal effects [8]. In contrast, they found out that exposure of cells in suspension to 60°C led to cell death but there was no indication about the occurrence of membrane damage.

### *3.4.2 Flow cytometric analysis of spray dried bacteria*

Flow cytometric method has continuously been developed and evaluated in the area of dairy industry, particularly for rapid detection, enumeration and differentiation of bacterial contaminants in milk [127, 128] as well as to analyze subpopulations of bacteria in probiotic products and dairy starters [129]. Besides, this technique was evaluated on its efficiency in determining the viability of freeze-dried bacterial cells [130] and in monitoring the cell damage and fermentation activity of dried yeast [131].

In most cases the presence of matrix in which the bacteria are embedded, such as milk proteins and lipid particles, as well constituents of protective media, might hamper the application of this technique, since fluorescence stains might bind non-specifically to proteins and lipid globule [127]. For the case of milk as matrix, clearing of milk was necessary. This can be achieved by using enzyme cocktails, which can effectively degrade milk proteins and lipid [127], as well as a special milk-clearing solution, which contains non-ionic detergent and a chelating agent as reactive ingredients [129].

In this study flow cytometric analysis was applied to evaluate cellular injury sites affected by spray drying as well as to relate data on the physiological characteristics of spray dried bacteria as obtained by flow cytometric analysis with survival data as obtained with plate

count method. To achieve these goals, LGG was stained with both cFDA and PI. In contrast to previous studies which implied the necessity of clearing prior to staining and measurement, clearing step was not applied in this study.

Briefly, the staining mechanism using cFDA and PI is based on the capability of viable to enzymatically convert non-fluorescent cFDA into a membrane-impermeant green fluorescent product cF, which can be accumulated in their cytoplasm. Thus, retention of the dye by the cells requires a high degree of membrane integrity and functional cytoplasmic enzymes. Apart from retaining cF intracellularly cells with intact membranes are able to exclude the membrane-impermeant, nucleic acid dye PI. This dye can only enter cells with compromised membranes. Intracellularly it binds the RNA or DNA; the resulted PI-nucleic acid complex emits red fluorescence upon excitation.

The dual-parameter dot plot of the green fluorescence (x-axis) and the red fluorescence (y-axis) in Figures 13a to 13f was used to differentiate bacterial populations based on their fluorescence properties in response to cFDA-PI staining. Each dot, which constitutes the cell cloud, represents one single cell, which is plotted as a co-ordinate of their green and red fluorescence value. The different intensities of the shaded area in the cell clouds signify the population density. The quadrants on the dot plot were set so that viable cells with intact membranes were in quadrant 4 (Fig 13a). This quadrant only included bacterial cells, which actively accumulated cF and excluded PI, and which therefore showed high green fluorescence and low red fluorescence. Prior to spray drying, all LGG cells were encountered in quadrant 4 (Fig. 13a). Upon rupture of the cell membrane and the concomitant loss of the CF-accumulation capacity (simulated by thermal treatment at 75°C for 90 s) the cells are not capable of excluding PI. The particular bacterial population, which was solely labelled by PI, showed low green fluorescence and high red fluorescence. Thus, the membrane damaged population was found in quadrant 1 (Fig 13b).

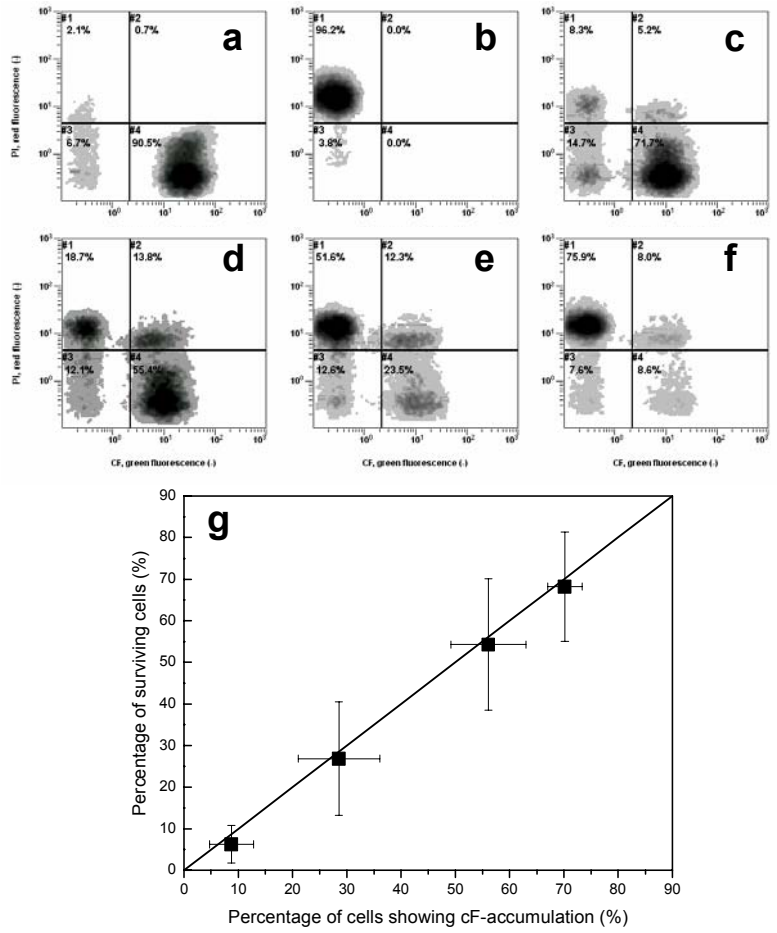


**Figure 13**

Flow cytometric fluorescence density-plot analysis (cF, green fluorescence versus PI, red fluorescence) of *L. rhamnosus* GG spray dried in reconstituted skim milk, RSM (20%, w/v) at different air outlet temperatures following staining with cFDA (carboxyfluorescein diacetate) and PI (propidium iodide). Conditions applied: viable cells prior to spray drying (a); dead, membrane compromised cells following heat treatment at 75°C for 90 s (b); and bacteria following spray drying at air outlet temperature of 70 (c), 80 (d), 90 (e), and 100°C (f). The figures (in %) following the quadrant number (top left hand corner) represent the percentage of the cells in the corresponding quadrant.

(g) Correlation between survival rates of *L. rhamnosus* GG spray dried at different air outlet temperatures in reconstituted skim milk (RSM), as determined by plating on MRS agar, and the percentage of carboxyfluorescein (cF)-accumulating population in quadrant 4 of the flow cytometry dot plots (Figure 6). The results are means based on data from three, or more, replicate experiments; error bars show the standard deviations of the means.

The fluorescence profile of the spray dried bacteria indicated that the population in quadrant 4 decreased as the air outlet temperature increased (Fig. 13c to Fig. 13f). Simultaneously, the population labelled with PI (in quadrant 1) increased. Both trends indicated that the degree of damage of cell membranes increased as the air outlet temperature increased.

When the survival rates of LGG spray dried at different air outlet temperatures, as determined by plating on MRS agar, were plotted against the percentage of cF-stained cells (in quadrant 4), a strong correlation was obtained (Fig 13g). This indicates, that cells, which were still capable of retaining cF after spray drying, were capable of forming colonies on MRS agar plates. Since the decrease in the population of cF stained bacterial cells was indicative of membrane deterioration, it is concluded that the cells suffering damage to their membranes during spray drying did not grow on MRS agar and were thus dead.

According to data obtained using confocal scanning laser microscopy it was shown that at lower spray drying temperatures (70°C) membrane intact cells predominated, whereas drying at 120°C resulted in cells with compromised membranes, which were stained by PI [6]. Similarly, Johnson *et al* (1995) also found that the loss of membrane integrity in *L. helveticus* was greater in cells spray dried at higher outlet temperature (82°C compared to 120°C), as determined by ß-galactosidase assay [1]. Evidences about increased permeability of cell membrane following drying could also be documented by increased sensitivity of dried cells to NaCl, increased leakage of potassium ions, UV-absorbing materials and ß-galactosidase in the supernatant fluids [4-7]. In terms of the exact mode of action of drying induced membrane damage it was demonstrated in a previous work on *L. bulgaricus* that spray drying induced lesions in the cellular lipid-containing structures and resulted in a reduced ratio of unsaturated/saturated fatty acids; thereby pointing out the possible implication of lipid oxidation in membrane degradation upon excessive contact with air [11]. Similarly, freeze drying is also accompanied by a decrease in the unsaturated/saturated fatty acids ratio [14].

Furthermore, it was found that membrane damage leading to increased permeability to Dnase could be achieved following dehydration in absence of heat [8]. However, as evidenced by the present study, thermal induced membrane rupture could not be fully excluded since higher magnitude of membrane damage and viability loss were obtained at higher outlet temperatures (Fig. 13). Taken together, it seems probable to conclude that damage on cell membrane occurs during spray drying and a massive injury of this cellular component (i.e. increase of permeability) results in cell death. Membrane rupture occurring during spray drying is most likely caused by synergistic effect of thermal and non-thermal (dehydration, oxidative) stresses.

In addition, on the latter stages of the work it was found that the current sampling method for the determination of the initial count of the bacteria prior to spray drying has to be re-assessed. This conclusion was triggered by the fact that in some cases the bacterial count after spray drying was higher than before, especially when low outlet temperatures were applied. This observation might be caused by the chain formation of lactic acid bacteria, which is frequently observed. Upon plating on agar, both several viable bacteria in aggregate or one single cell form one colony. It was proposed that following spray drying the chains are

broken due to shear stress, resulting in higher amounts of viable single cells; each of them are capable of forming a visible colony on agar. Eventually, in particular when the lethal effect of drying is low, the amount of single, viable cells might be higher than the initial count taken prior to drying owing to the aggregation of many cells in the latter case. A practical way to solve this problematic was suggested: instead of taking the initial count prior to drying it was proposed to consider the initial count from bacteria suspension sprayed through the two-fluid-nozzle with help of pressurized air in absence of drying air. Using this approach the same shear stress experienced by spray dried samples is also applied on the initial count. As a result, the bacteria count after atomizing through the nozzle was 2.50 ± 0.16 times more than initial count without atomization. Taking this result into account the survival rates ($N/N_0$ in percent) determined in this and many other works should therefore be less than currently detected. However, this approach was still not yet used in the present study but should be considered in further investigations in order to obtain the real magnitude of viability loss during spray drying.

### *3.4.3 Incorporation of prebiotics in the spray drying medium*

As already concluded by some authors, survival of bacteria during drying is highly dependent not only on the processing conditions but also on the type of drying media regardless of the type of drying method used [7, 132-134]. With regards to the mass and heat transfer, which extensively take place during the passage of the dried bacteria in the dryer, Lian *et al* (2002) suggested that thermal properties of the drying medium, such as thermal conductivity and diffusivity can affect survival of spray dried probiotics [104].

The interest in supplementing food with prebiotic substances is increasing. By definition, prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves the health of host [135]. The prebiotics developed so far are the non-digestible oligosaccharides and non-digestible fructans [136]. Furthermore, when in a single product both probiotic bacteria and prebiotic compounds exist, the product is called synbiotic. It is defined as a mixture of probiotic and prebiotic that beneficially affects the host by improving the survival and the implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, including the ones in the synbiotic mixture [136].

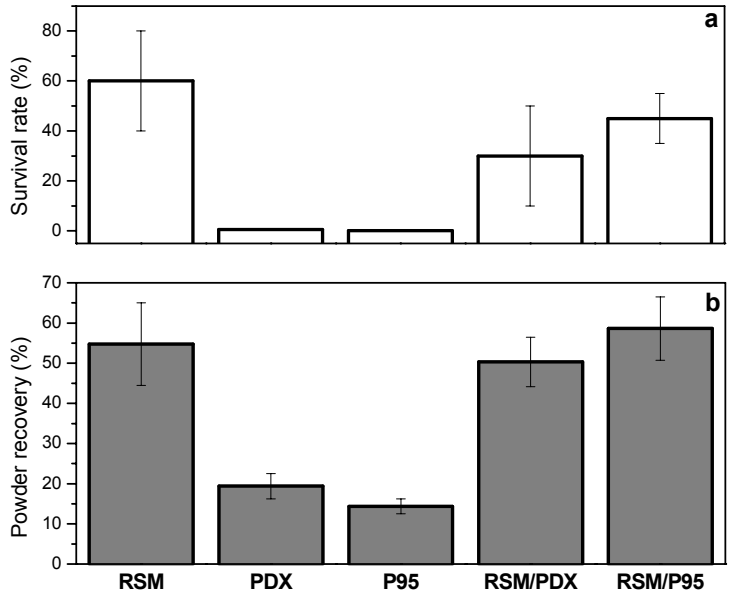


**Figure 14**

The effect of total or partial substitution of reference drying medium, i.e reconstituted skim milk (RSM) by prebiotic substances Polydextrose or Raftilose®P95, on survival rate of *L. rhamnosus* GG (Fig 12a) and on powder recovery (Fig. 12b). Figure 12a was adapted from [7].

The media were RSM, reconstituted skim milk powder (20%, w/v, total solids); Polydextrose (20%, w/v, total solids); Raftilose®P95 (20%, w/v, total solids); RSM/Polydextrose, a 50:50 (v/v) blend of RSM and reconstituted Polydextrose (both 20%, w/v, total solids) and RSM/Raftilose®P95, a 50:50 (v/v) blend of RSM and reconstituted Raftilose®P95 (both 20%, w/v, total solids). Data are the means of three, or more, replicate spray drying trials; the error bars represent the standard deviations of the mean.

As already mentioned above, fructan is considered as a good prebiotic compound and it is known from the literature that this oligosaccharide is the one specifically accumulated by various plants upon dehydration [45, 61]. Based on these positive characteristics of prebiotics, it seems probable to evaluate the possibility to use commercial non-digestible prebiotic preparation, especially the one containing fructan, as the drying medium for LGG so as to allow a one-step production of dried synbiotic preparation. The prebiotic compounds used in this study are Polydextrose (Danisco, Copenhagen, Denmark) and Raftilose®P95 (Orafti, Tienen, Belgium). Similar to the spray drying study using RSM as drying medium, the total solids content of the spray drying medium was held constant at 20% (w/v).

Unfortunately, as can be seen in Figure 14, full substitution of RSM by any of the prebiotics investigated resulted in poor survival characteristics (Fig. 14a, redrawn from [7]) and low powder recovery (Fig. 14b). Such spray dried bacteria were reported to experience considerable damage on cell membrane during spray-drying, as evidenced by the higher

sensitivity to 5% NaCl [7]. Compared to spray drying using reconstituted skim milk as carrier, the survival of LGG in polydextrose or Raftilose®P95 was more than 100 times lower (Fig. 14a). Lian *et al* (2002) also found that inclusion of prebiotic as the sole carriers during spray drying did not afford protection to cells compared with RSM [104]. In contrast, a partial (50%) substitution of milk solids by any of the prebiotics tested resulted in survival rates slightly lower than the one obtained with RSM. Furthermore, the presence of skim milk solids in spray drying media were proven to be essential in maintaining high powder recovery. Partial substitution of milk solids by prebiotic brought about a powder recovery level of ca. 50%. This yield resembles the one achieved when RSM was used as drying medium.

The poor powder yield during the production of prebiotic powder was caused by the higher stickiness of these products on the walls of drying chamber and aerocyclone, which in turn is highly influenced by the thermoplastic and hygroscopic nature of these materials. When the feed solution is being atomized into the drying chamber at any axial position of along the wall of drying chamber when the temperature becomes high enough a sticky material forms and deposits there [137]. This problem can be reduced by building large diameter dryers to keep hot parts of the wall outside the path of the sprayed particles. Alternatively, the application of a wide-body drying chamber is suggested [90]. In this system, air goes in at the top of the dryer and is discharged out the top after passing down the centre of the dryer and back up the walls. This design is suggested to provide maximum flexibility in keeping walls of the dryer cool and keeping the dry products off the walls, since the walls are relatively cold. In addition, it has been proposed to modify Büchi spray dryer to a scraped surface chamber to continuously remove deposits from the walls [137]. The authors also suggest the substituting wall material (borosilicate glass – standard in Büchi spray dryer) with cast iron. The latter material is capable of absorbing about twice and conducting about 50 times more energy than borosilicate glass, which then render a more stable and uniform wall temperature. Apart from these constructional options to increase the yield, the presence of skim milk in the drying medium is critical in maintaining acceptable level of powder recovery. The pronounced presence of sticking on drier wall when using prebiotics is thought to be influenced by their glass transition temperatures, since stickiness and caking can be prevented when the surface of the dried particle did not reach 10 to 20°C above glass transition temperatures [91].

Own data on comparing the effect of drying media on survival rate and moisture content during spray drying are shown in Figure 15. Accordingly, no substantial differences between the evaluated media (RSM, RSM:Polydextrose 50:50, or RSM:Raftilose®P95 50:50) could be observed regarding their protection capacity against dehydration at an outlet temperature of 80°C. These experimental data demonstrated that both prebiotic substances could be

incorporated in the spray drying medium without any adverse impact on the survivability and the residual moisture content.

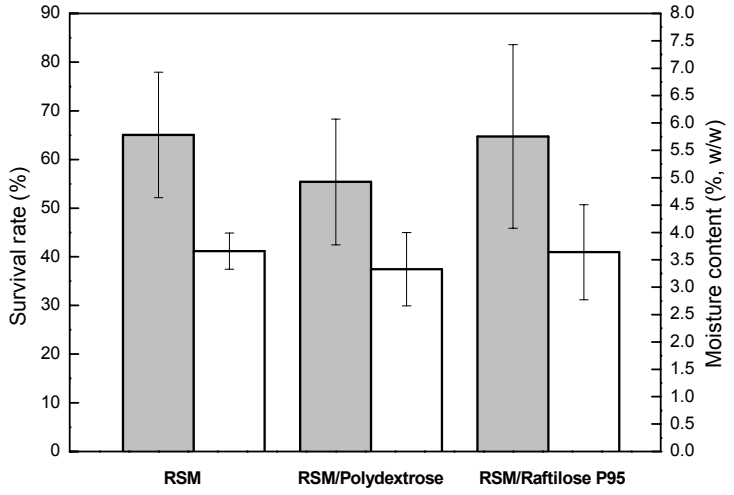


**Figure 15**

Effect of media type on the survival of *L. rhamnosus* GG (grey bars) and moisture content of powder (open bars) prepared by spray drying various media at an outlet temperature of 80°C.

The media were RSM, reconstituted skim milk powder (20%, w/v, total solids); RSM/Polydextrose, a 50:50 (v/v) blend of RSM and reconstituted Polydextrose (both 20%, w/v, total solids) and RSM/Raftilose®P95, a 50:50 (v/v) blend of RSM and reconstituted Raftilose®P95 (both 20%, w/v, total solids). Data are the means of three, or more, replicate spray drying trials; the error bars represent the standard deviations of the mean.

The approach of only partially replacing milk solids against other carrier compound was also followed in a previous work on using gum acacia as a constituent of spray drying medium [105]. The authors did not give detailed explanation why they did not perform full replacement of RSM, which was regarded as reference medium. However, it was shown that probiotics dried in the presence of gum acacia showed improved storage stability and gave better protection against low pH conditions compared to the one dried with RSM alone. It was speculated that this beneficial effect may be due to encapsulation. Furthermore, apart from their prebiotic properties, gum acacia seems was also found to give good protection against $H_2O_2$-induced oxidative damage [105]. Other carrier material, starch, which was thought to be effective for microencapsulation proved to be unsuitable for use as protective matrix for Bifidobacterium strain during spray drying, storage and stress conditions [103].

### *3.4.4 Storage test at non refrigerated conditions*

Data from literature showed that storage stability was inversely proportional to storage temperature and storage at refrigerated temperature allowed higher shelf-life of spray dried

probiotic bacteria [6, 105, 138]. However, refrigerated storage is expensive to both suppliers and retailers of probiotic products and thus there is a need to produce probiotic products that are stable at ambient temperature. Taking this consideration into account it was aimed in this work to evaluate the survival characteristics of spray dried LGG upon storage at elevated temperatures, i.e. 25 and 37°C. These temperatures are also suitable for accelerated storage test, with help of which the storage effect could be determined in a feasible time frame (within 5-8 weeks).

In this study the storage test was performed without modification of atmosphere under a constant relative humidity of 11%, which was found to be optimal for the maintenance of high level of viability during the storage of dried bacteria [108]. This behaviour might be correlated with the influence of $a_w$ or relative humidity on the rates of various chemical reactions which might have deteriorative effects on cell constituents, as evidenced by Figure 16. It is obvious that within an $a_w$ range between 0.1 to 0.2 the rates of different chemical reactions (non enzymatic browning, non enzymatic hydrolysis, enzyme catalyzed reactions, fat oxidative etc.) are very low. This range also corresponds with the relative humidity values of 11% to 23%, recommended by Castro *et al* (1995) for the stable storage of dried *L. bulgaricus*. Storage at 0% humidity was found to be more detrimental for stability of dried bacteria [108], presumably due to the increased rate of oxidation on polyunsaturated fatty acids of cell membrane as hydrate shell surrounding fatty acids is lost [91, 139]. These considerations are regarded as helpful in identifying and confirming the presence of an optimal storage condition for dried bacteria with regards to relative humidity.

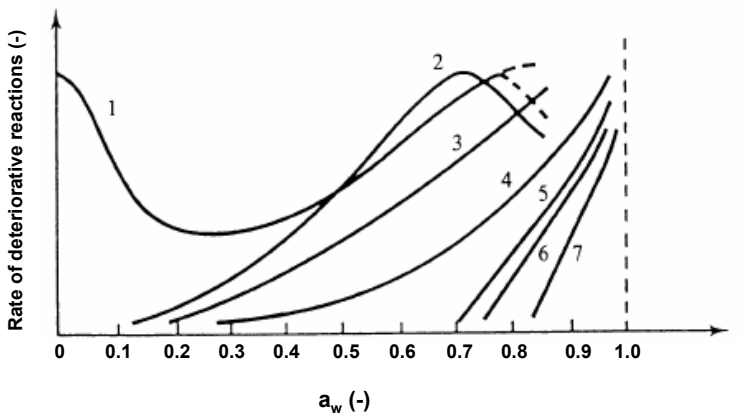


**Figure 16**

Rate of deteriorative reactions, which may lead to food spoilage, as a function of water activity [139]. The potential sources of damage on food systems are: fat oxidation (1), non-enzymatic browning (2), non-enzymatic hydrolysis (3), enzyme activity (4), growth of moulds (5), growth of yeast (6), growth of bacteria (7)

The different media, spray dried at an air outlet temperature of 80°C, were compared for the effect on the viability of LGG during prolonged storage at 25 or 37°C in a relative humidity value of 11%. Figures 17a and 17b show the loss of viability during storage at 25 and 37°C, respectively. The decline of the bacterial load was represented by the logarithmic values of the survival fractions after different storage periods. The loss of viability was accelerated at higher storage temperature. This observation is quantitatively described in Table 5, in which the inactivation rate constants (s in week$^{-1}$) of the bacteria dried in different matrices are presented. For the calculation of the inactivation rates, first order inactivation kinetics are assumed.

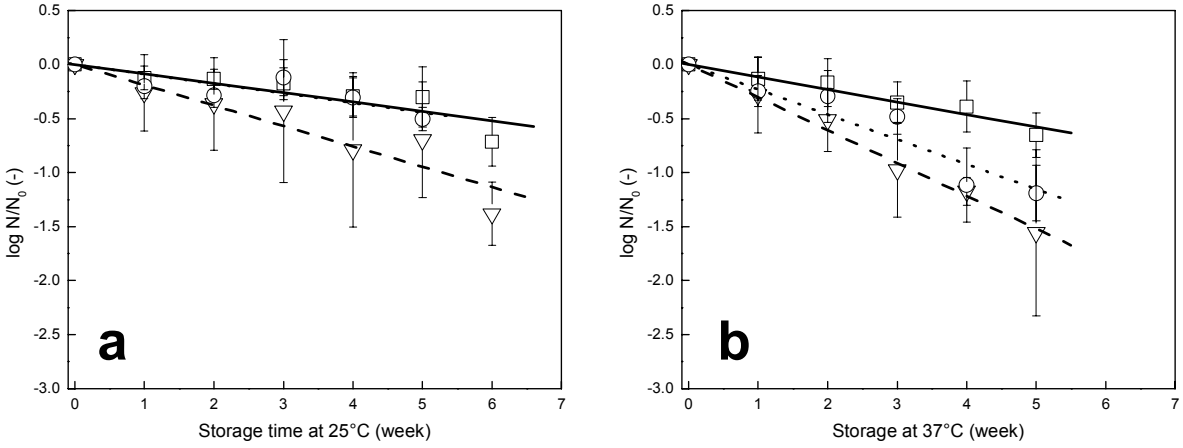


**Figure 17**

Viability loss of spray dried *L. rhamnosus* GG during storage at 25°C (a) or 37°C (b) at a constant relative humidity of 11%, which is expressed as the logarithmic values of relative survival fraction (log $N/N_0$), as described in Material and Methods. For storage stability experiments the bacteria were spray dried at an air outlet temperature of 80°C in different media, as defined in Figure 15: RSM (□), RSM/Polydextrose (○), and RSM/Raftilose®P95 (▽). Data are the means of three replicate spray drying and storage trials; the lines through the data points were fitted using linear regression of the data. The slopes of the regression lines (s) were considered as inactivation rates (in week$^{-1}$) and tabulated in Table 5 along with the corresponding correlation coefficient ($R^2$).

At both storage temperatures protective medium supplemented with Raftilose®P95 showed the poorest protection performance, whereas the one containing polydextrose was better at 37°C or even gave protection capacity equivalent to RSM at 25°C (Tab. 5). It can be concluded that replacement of milk solids with any of the prebiotics tested facilitated the degradation of crucial cell components, which ultimately led to higher viability loss during storage compared to LGG dried with RSM alone.

Taking literature data into consideration, it was documented that membrane damage occurs during storage despite the presence of skim milk solids as protectant and storage at optimal relative humidity of 11% [12, 108]. The damage in membrane was highly related to lipid

peroxidation, as expressed by a decrease in the ratio of the unsaturated to fatty acids [12]. This oxidative damage has at least two indirect consequences leading cell death: the product of lipid peroxidation may lead to DNA damage [140] and alteration of membrane lipid composition may cause dysfunction of membrane-associated enzymes, such as ATPase due to a decrease in membrane fluidity or weakening of hydrophobic interactions [12]. The implication of oxidative damage leading to membrane rupture and cell death during storage was further demonstrated to be effectively suppressed by incorporating antioxidants [138]. Alternatively, modification in the gas composition of the storage atmosphere, i.e. by replacing air with nitrogen or by applying vacuum proved to improve cell survival [108, 141]. In line with these results it could be assumed that the partial substitution of milk solids with prebiotics increased the susceptibility of fatty acids of cellular membrane to oxidative damage and that components in skim milk solids should be more effective in conferring protection against lipid oxidation. Moreover, it was found that inactivation of LGG in prebiotic containing drying media was reduced in lower storage temperature (Fig. 17a). This approach seems feasible to compensate the presumed increase of oxidation-induced viability loss upon incorporating prebiotics at cost of skim milk solids. It was suggested before that increased survival of dried bacterial culture at low temperatures might be due to a reduction of fatty acid oxidation [108].

**Table 5**

Inactivation rate constants (s in week$^{-1}$) of *L. rhamnosus* GG in different spray drying media during storage at 25 or 37°C.

| Spray drying medium | $s_{25°C}$ (week$^{-1}$)[a] | $R^{2b}$ | $s_{37°C}$ (week$^{-1}$) | $R^2$ |
|---|---|---|---|---|
| RSM | 0.087 | 0.892 | 0.115 | 0.972 |
| RSM : Raftilose®P95 (1:1) | 0.189 | 0.937 | 0.304 | 0.995 |
| RSM : Polydextrose (1:1) | 0.089 | 0.826 | 0.231 | 0.956 |

[a]The slopes of the regression lines, as shown in Figure 3, were taken as the inactivation rates. Bacteria were dried at an air outlet temperature of 80°C in reconstituted skim milk (RSM). Presented data are the means of the inactivation rates obtained from three, or more, replicate storage experiments.

[b]$R^2$: correlation coefficient

Generally, for a shelf life period of one month only a slight loss of viability occurred in spray dried skim milk (reduction of 0.25 log unit at 25°C). The high storage stability of probiotic bacteria in spray dried skim milk at non-refrigerated temperatures showed that the bacteria were sufficiently protected. This evidence justified the suitability of skim milk as a medium for the large-scale production of shelf-stable spray dried probiotic bacteria, as recommended by

Carvalho et al (2004), who suggested to use skim milk as drying medium unless a relevant information of a specific culture of lactic acid bacteria is present [141]. However, synbiotic powders showed reduced survival rate during storage at non-refrigerated temperature. The negative effect resulted from partial substitution of milk solids with the evaluated prebiotics could be most likely counteracted by storing this powder in refrigerated conditions.

### *3.4.5 The role of glassy state on bacterial storage stability*

Entrapment of a living system in a glassy matrix upon dehydration was suggested as being responsible for their long term stability [48, 142]. The glassy structure of the external matrix is a highly effective environmental barrier with an extremely low molecular mobility. With respect to the preservation of bacteria, this condition leads to a suppression of unexpected deteriorating events on bacterial membranes, which constitute the interface to the surroundings and are predominantly exposed to various environmental abuses. Lipid oxidation of membrane fatty acid was deemed responsible for cell death during storage [11]. Since translational diffusion is drastically restricted in the glassy state [68], the diffusion of oxygen, which preceded oxidative damage [74], and chemical reactions requiring diffusion [48], could most likely be limited. Other degradative events such as fusion of membranes and protein unfolding could also be prevented [56].

According to the results from storage test of spray dried probiotic (Fig. 17), it was found that the viability retention of the spray dried LGG was markedly influenced by the composition of the drying medium although a nearly identical level of viable count was determined immediately after drying (Fig. 15). As mentioned above, the formation of glassy state is thought to be essential in preventing deteriorative events during prolonged storage under a controlled condition and to ensure good stability the dried materials should be stored under their glass transition temperatures ($T_g$). Thus, it was therefore attempted to verify whether the decrease in the protection performance of prebiotic supplemented RSM-based media was related with a change in the glass forming capability as resulted from the substitution. In particular, it was hypothesized that the partial substitution of the milk solids with prebiotic led to a considerable reduction of the $T_g$ of RSM to values lower than the storage temperatures at 25 and 37°C. The $T_g$ of RSM was found to be similar to the one of lactose ($T_g$ = 101°C, according to [124]), which makes up to more than 50% of the total solids contents the skim milk. Under the hypothesized condition ($T_g < T_{storage}$) the bacteria were not entrapped in a glassy matrix but in the rubbery state, which are thought to be more susceptible to various deleterious events, as already noted above.

Figure 18 shows typical DSC- and TG-curves as obtained from thermal analysis of the constituents of the drying media. All samples were scanned twice. Between the scans the heated sample was rapidly cooled to –30°C. During the first scan, a broad endothermic peak, which is characteristic for water evaporation, was observed in all media at temperatures between 60 and 140°C. Within this particular temperature range, the thermogravimetric signal, which was simultaneously measured, showed a significant mass loss. It was therefore assumed, that moisture was removed from the sample during the first scan. This occurred in all samples. No further mass loss was observed during the second scan, when the dehydrated materials were heated up to 130°C. Moreover, it was also evident in all samples, that an endothermic shift of the baseline of the DSC signal occurred during the second scan. This latter phenomena is characteristic for glass transition. The key results of these measurements, i.e. the glass transition temperatures ($T_g$) of the investigated media, are summarized in Table 6.

**Table 6**

Glass transition temperatures ($T_g$) of the constituents of the drying media as determined by Differential Scanning Calorimetry.

| Spray drying medium | n[a] | Mean value of $T_g$ (°C) | Range of $T_g$ (°C)[b] |
|---|---|---|---|
| RSM[c] | 2 | 109 | 5.8 |
| Raftilose®P95 | 4 | 82 | 8.9 |
| Polydextrose | 4 | 102 | 11.2 |
| RSM[c]: Raftilose®P95 (1:1) | 1 | 102 | - |
| RSM[c] : Polydextrose (1:1) | 2 | 108 | 3.7 |

[a]n: Number of measurements performed on each medium to determine $T_g$

[b]Range is defined as the difference between the largest and the smallest values

[c]Reconstituted skim milk

Based on the experimental $T_g$ data of the water-free spray dried formulations, a material-specific state diagram was generated using the Gordon-Taylor equation (Equation 1). The material specific constant, k, was derived from an empirical equation (Equation 2) proposed by Roos *et al* (1993) [57]. The state diagram shows the effect of the residual water content of the dried samples on $T_g$ (Fig. 19a). It also serves as a stability map to ascertain specific combinations of water content/storage temperature in the glassy state that are suitable for stable storage. The prevalence of glassy state could only be assured as long as the samples did not absorb moisture during storage (data not shown). Moisture uptake would decrease

the $T_g$ of the system, and consequently a second-order transition of the glass towards the rubbery state (devitrification) could occur. As already noted, under this condition the entrapped bacterial samples would be more susceptible to various deteriorating reactions. To avoid moisture uptake, the bacterial samples were stored at a low relative humidity, i.e. 11%.

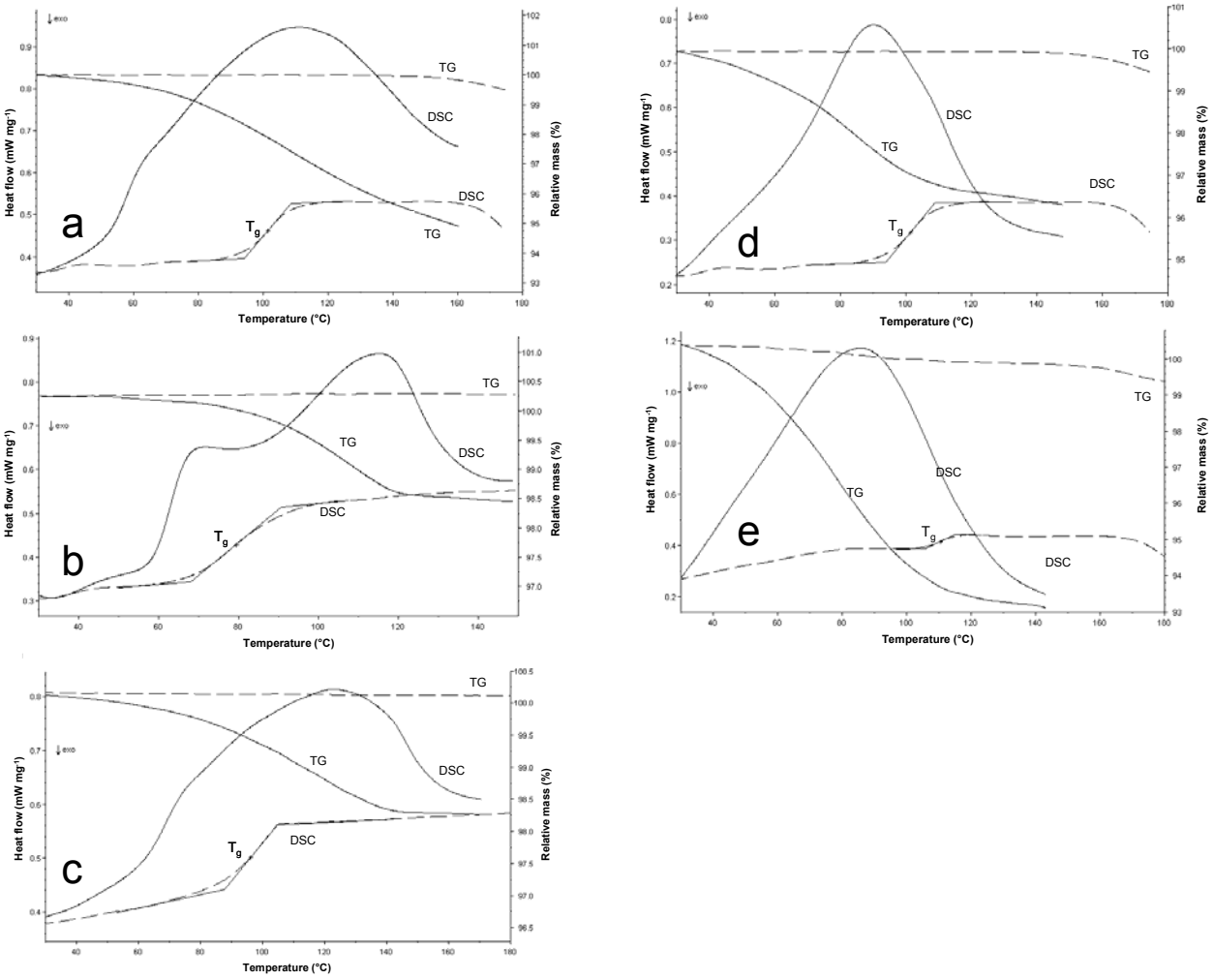


**Figure 18**

Typical thermograms showing DSC (Differential Scanning Calorimetry) and TG (Thermogravimetry) signals of the constituents in different spray dried media: (a) reconstituted skim milk (RSM); (b) Raftilose®P95; (c) Polydextrose; (d) RSM/Raftilose®P95; (f) RSM/Polydextrose.

The heat flow (DSC signals, left y-axis) and relative mass change (TG signals, right y-axis) of the samples are plotted as a function of temperature. Thermograms obtained during the first and second heating cycle are represented by the solid and dashed lines, respectively. See Material and Methods for details of glass transition temperature ($T_g$) determination.

Figure 19b shows the comparison of the $T_g$ data available for skim milk. It was found that the data from Palzer & Zürcher (2004) [113] showed a marked deviation from the values calculated with Gordon Taylor equation ($\Delta T \sim 20°C$) or from values obtained from other

works [112, 124]. Commonly, the precision of $T_g$ data for sugars, as calculated by DSC, was approximated to be in a range between ±1 and ±5°C [50]. However, large deviation of $T_g$ values on a nearly identical material has already been observed on trehalose, where the $T_g$ values compiled from different works span between 73 and 115°C [143]. The apparent discrepancies of $T_g$ values could be attributed to impurities in the material as well as residual water content [143]. Compared to $T_g$ values of skim milk powder at different moisture level as provided by Jouppila & Roos (1994) [124] and Vuataz (2002) [112], it can be observed that the $T_g$ values in this work, which were predicted with help of Gordon Taylor equation, were qualitatively in an appropriate agreement. These considerations may justify the use of this empirical equation to estimate the presence of glassy state in the spray drying media based on their anhydrous $T_g$ values, as measured by DSC (Tab. 6)

Commonly, spray drying at an air outlet temperature of 80°C results in a moisture content of no higher than 4.5%. At this residual moisture content $T_g$ values of 50.6, 49.5, and 44.5°C were calculated using Gordon-Taylor equation for RSM, RSM/PDX and RSM/P95, respectively. Since the calculated $T_g$ values are higher than the applied storage temperatures (25 and 37°C), it can be assumed that upon spray drying glassy state was achieved and thus bacteria were stored in the glassy state. It has been proposed before that when a non-crystallised milk concentrate is spray-dried, the lactose is rapidly solidified in an amorphous solid structure [144], in which milk proteins are coated [112]. Nevertheless, the glass is in a meta-stable state and will tend to convert to the crystal eventually, with a rate depending upon temperature and moisture content [114]. Since relative humidity of the storage room was held constant at 11%, there was no further uptake of moisture (moisture content at equilibrium 3.5 % w/w) according to sorption isotherm of skim milk powder [125]. Consequently, an increase of moisture content, which would lead to a depression of $T_g$, could be inhibited during the storage. Furthermore, no lactose crystallization could occur at the applied storage conditions ($T_{storage} < T_g$), since lactose can only crystallize at temperatures above $T_g$ [124]. It was reported in many studies that full crystallization of sugar led to phase separation and thus to a loss of stabilization of the entrapped proteins [33, 35], thus making the enzymes more susceptible to non enzymatic browning reactions [91].
However, when the bacterial inactivation rate constants during storage were compared, differences in the protection performance of the drying media could be ascertained. According to the data in Table 2, the protection capacity of RSM ($s_{37°C}$ 0.115 $week^{-1}$) was the highest, followed by RSM/PDX ($s_{37°C}$ 0.231 $week^{-1}$). Probiotic bacteria dried in RSM/P95 showed the lowest storage stability ($s_{37°C}$ 0.309 $week^{-1}$). A similar trend was observed at the storage temperature of 25°C, suggesting that RSM was the most effective protective media

($s_{25°C}$ 0.087 week$^{-1}$). In contrast, viability retention with RSM/P95 as drying media ($s_{25°C}$ 0.189 week$^{-1}$) was poor .

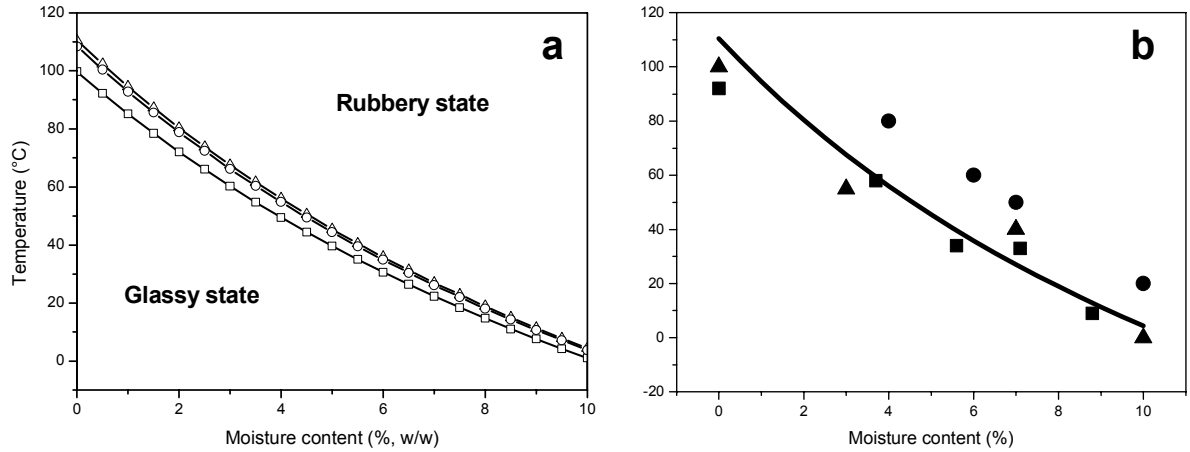


**Figure 19**

(a) Glass transition temperatures ($T_g$) of spray dried media at different moisture contents (0 to 10%, w/w): RSM (-Δ-), RSM/Polydextrose (-o-), RSM/Raftilose®P95 (-□-); the composition of the media is defined in Fig. 2. The $T_g$ values of dry, moisture-free samples (total solids content, 100% w/w) were experimentally determined and are tabulated in Table 2. Data for $T_g$ for pure water (0% total solids) were adopted from literature [57, 143]. The intermediate $T_g$ values of the dual-phase system were calculated with Gordon-Taylor equation, as defined in Material and Methods.

(b) Comparison of the $T_g$ at different moisture contents as determined by different authors: this study (solid line); Jouppila & Roos, 1994 (■) [124]; Vuataz, 2002 (▲) [112] and Palzer & Zürcher, 2004 (●) [113].

While in both storage temperatures all media should theoretically be in the glassy state (Fig. 19a), differences in protection performance between the media was observed (Fig. 17). Thus, it seems that the entrapment in a glassy matrix alone was not sufficient for maintaining stability of the spray dried bacteria during storage. If the formation of glassy state alone were indeed sufficient for preventing deleterious events from occurring, then no difference in the protective capacity during storage would be seen. However, as obvious from the incorporation of prebiotics at cost of skim milk solids the present data corroborate with previous results which showed that the type of sugar constituting the glass apparently also influenced the protection capacity conferred to the entrapped biological molecule [58].

Previous dehydration studies on microorganisms, enzymes and liposomes [26, 32, 36, 47, 86, 145] already demonstrated the occurrence of deteriorative events below $T_g$. The inappropriateness of glassy state ($T_g$) to be considered as an absolute threshold of stability may be explained by the possibility that the internal cytoplasm of the cell may not yet be a glass even though the external solution has vitrified [76]. As the rate of water transport across the plasma membrane may be less than the rate of water evaporation from the

solution, there may be too much water inside of the cells to form a glass. After the outside solution has vitrified, water transport across this vitrified layer may be extremely slow. The integrity of the plasma membrane may thus not be adequately protected by the non-vitrified internal cytoplasm and hence results in the long-term degradation of the membrane. Similarly, the cytoplasmic content may also be subject to degradation due to the absence of glassy state. Moreover, based on their investigation on instant active dry yeast Schebor *et al* (2000) also suggested that biological materials could not regarded as homogeneous materials; thus it is possible that local microheterogeneities exist [86]. This would lead to a coexistence of glassy and rubbery states within the material [26] and thus an extrapolation of the formation of glassy state to the whole volume of the biological material is not necessarily appropriate. Furthermore, there are many examples in the literature indicating that molecular motions do occur below $T_g$ [146, 147], as opposed to the general view that translational diffusion is considered to be virtually nonexistent in glassy state [68]. In the prebiotic preparations applied in this work it is possible that the presence of impurities, i.e. low molecular weights sugars might also contribute to the existence of microheterogeneities in the dried samples.

Instead of or in conjunction to their capability of forming glass upon water removal, the use of sugars as protectants of dehydrated biomaterials such as enzymes, proteins, liposomes, red blood cells and bacteria can alternatively be explained by the water replacement hypothesis [56], which envisages the function of sugars as water substitutes when the hydration shell of proteins as well as water molecules around polar residues in membrane phospholipids are removed. In terms of membrane stabilization the protective effect of sugar relies on a direct physical interaction between the hydroxyl groups of the sugars and the polar residues of the phospholipids head groups in dehydrated state so that phospholipids bilayers remain at their hydrated spacing [21, 43, 44]. Adequate spacing between the lipid headgroups owing to the insertion of sugar is deemed responsible for the substantial depression of liquid-crystalline-to-gel phase transition temperatures ($T_m$), resulting in the preservation of membrane in a liquid-crystalline state, even when dry. Consequently, the membrane would not pass through a phase transition during rehydration and leakage of entrapped aqueous solution could be prevented. Likewise, the capability of sugars to efficiently stabilize proteins during drying is attributed to the formation of hydrogen bonds with the polar and charged groups of proteins when water is removed [13, 39, 43]. This would then lead to the preservation of the native, aqueous structure in the dried state [42, 66].

Data from microbiological analysis suggested, that although partial replacement of skim milk in the carrier by prebiotic substance did not negatively impact on the spray drying survival of

probiotic bacteria (Fig. 15), the protection performance during prolonged storage was lower as the amount of skim milk solids in the carrier was reduced (Fig. 17a and 17b). In the light of the water replacement theory [56], skim milk constituents, most likely lactose, seemed to be more superior in directly interacting with the polar headgroups of membrane phospholipids, and thereby minimizing the damage on the cellular membranes during spray drying and prolonged storage. Principally, disaccharides were regarded as being effective in protecting both bacterial membranes and proteins during drying [13]. In contrast, some polysaccharides (such as dextran and hydroxyethyl starch) did not interact directly with the polar headgroups of membrane phospholipids, and thus did not protect the membranes during drying [58]. The absence of direct interaction has been attributed to the large size of the polymers, which would sterically prevent them from interacting with membrane lipids [45, 46, 54] or with proteins [148] although they commonly have high $T_g$, as also demonstrated in this study. Thus, lactose might play a dominant role in bacterial protection by means of direct interaction with sensitive biomolecules. Partial substitution of skim milk with oligosaccharides such as Raftilose®P95 (degree of polymerization, DP = 2-8) or polydextrose (DP = 12 or more) was thought to increase the amount of oligosaccharides incapable of stabilizing membrane at cost of the net quantity of lactose, which may undergo direct interaction with either bacteria membrane or proteins. As a consequence, bacterial stability during storage was adversely impacted.

In order to substantiate this hypothesis, direct interaction of different types of sugar molecules, especially the ones present in spray drying media, with phospholipid bilayers is studied on liposomes.

### *3.4.6 Monitoring direct interaction of sugar-membranes using liposomes*

#### ***Establishing the flow cytometry-based analytical procedure***

Liposomes are regarded as a suitable model system for biological membranes, on which the effect of drying can be investigated and how protective compounds, especially sugars, can protect membranes upon dehydration [23]. The effect of drying can be assessed by monitoring the leakage of aqueous fluorescent marker carboxyfluorescein (cF), which were previously enclosed in the inner part of liposomes. This can be realized by measuring the fluorescence of the suspending medium, in which cF is released using spectrofluorometry [45, 54, 61, 63], or by determining the fluorescence retained in the liposome at single particle level using flow cytometer, as applied in this study. Moreover, as drying may induce fusion or aggregation of liposomes, determination of particle size might give indication of the occurrence of this drying-related damage. This can be achieved by using particle size analyzer which operates on the principle of dynamic light scattering, thereby allowing measurement of the absolute liposome diameter [54, 59, 63]. The special case of membrane

fusion is usually determined by fluorescence resonance energy transfer method [61, 62]. Due to the possibility of simultaneous measurement of light scattering intensity using flow cytometer, this technique is applied in this study to monitor relative changes in particle size, as caused by dehydration. As already described in the section of Material and Methods, the applicability of flow cytometric analysis for assessing drying effect on liposomes was validated by using latex beads (validation of size) and liposomes with different concentration of cF (validation of fluorescence intensity).

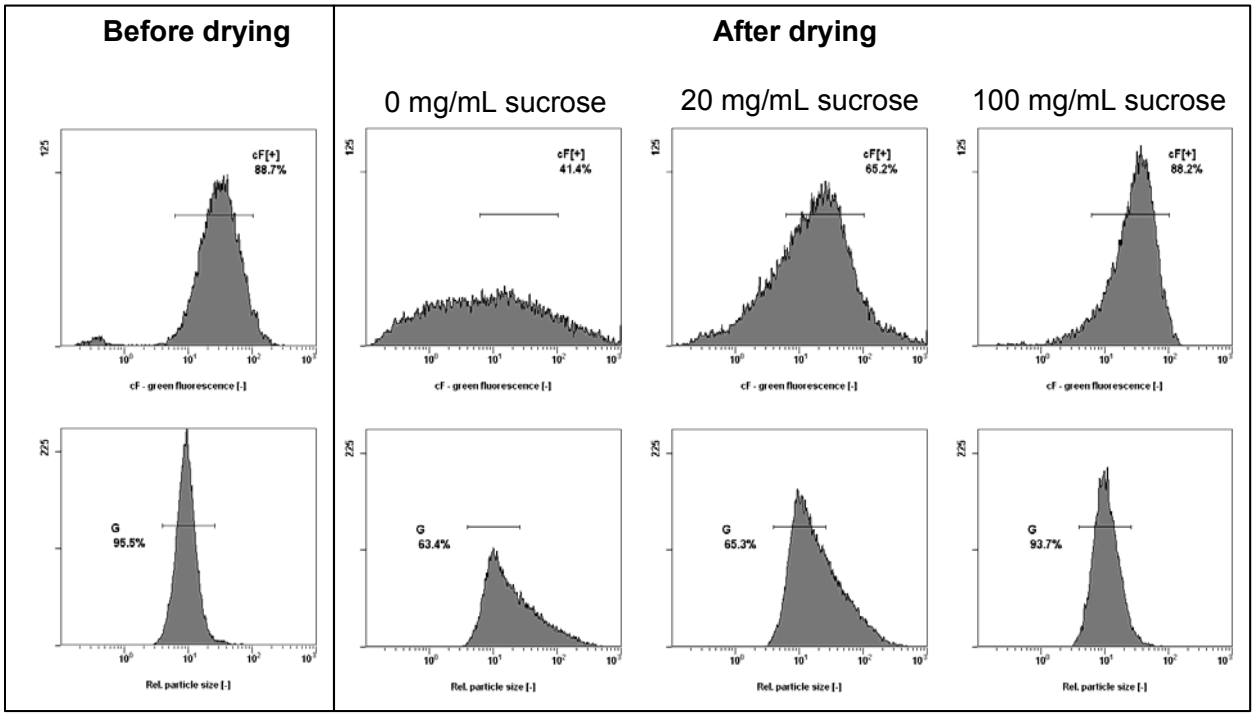


**Figure 20**

Flow cytometric fluorescence histograms showing the effect of drying of liposomes with different concentrations of sucrose on distribution of cF fluorescence intensity (upper figures) and on particle size distribution (lower figures), as examined using flow cytometric analysis. Liposomes (5 mg $mL^{-1}$) were dried in the presence of sucrose at various concentrations. The figures under the gate designations indicate the percentage of liposomes encountered in those gates with respect to their sizes or fluorescence intensities

Figure 20 visualizes the way of presenting and analyzing the data obtained from flow cytometer. The distributions of fluorescence intensity (upper figure) and particle size (bottom figure) were shown in the histograms. The manual fixation of gates (designated as “cF” and “G” in fluorescence and particle size histograms, respectively) was performed on liposomes prior to drying. The analysis software shows percentage of liposomes encountered in these gated regions. This gate analysis allows the extraction of quantitative information about the extent of damage experienced by liposomes during drying instead of qualitatively observing

the appearance of histograms. A reduction of the percentage of liposomes in any of both gates after drying and rehydration indicates that the original characteristics gated in those regions, i.e. either size distribution or cF loading, could not be restored and would thus illuminate the evidence of drying-induced damage.

As can be seen in Figure 20, marked changes were observed in both fluorescence and size distributions following drying in the absence of sucrose; not only in the shrinking and broadening of the fluorescence distribution but also a shift towards higher sizes in the histogram corresponding to liposome size. These changes indicate the leakage of cF, resulting in the marked decrease of liposomes with high fluorescence intensities and the aggregation of several liposomes, resulting in increased number of liposomes with higher size, which were previously not detectable. These indications of damage as manifested by cF leakage and size increase, are in good agreement with results from other groups, who mostly determined these changes with analytical methods other than flow cytometry [45, 61, 63]. Drying of liposomes in the presence of exogenous sugar markedly increased the retention of cF in liposomes as well as reduced the fusion/aggregation events (Fig. 20). This degree of improvement was found to be highly proportional to the amount of added sugar.

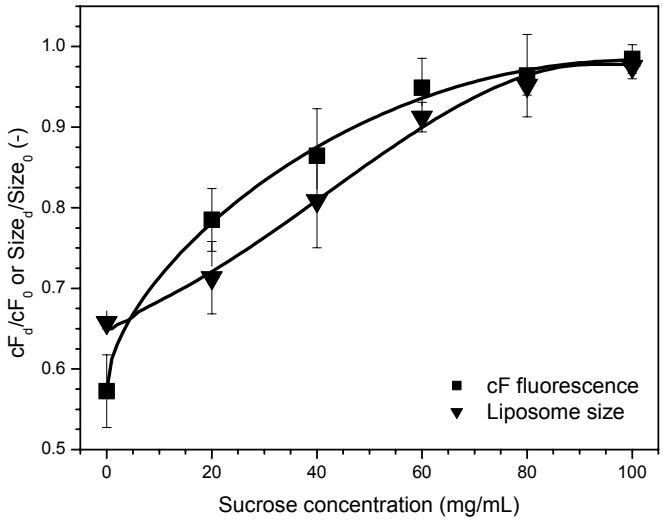


**Figure 21**

Effect of exogenously added sucrose on liposome's integrity after drying in terms of the retention of the original fluorescence distribution (■) and particle size distribution (▼) in the corresponding gates (see Figure 20). An index of 1 indicates that the size or fluorescence distribution before drying are completely restored. Liposome concentration in the liposome-sugar mixture was as high as 5 mg $mL^{-1}$. Data were means of the results from three or more independent experiments.

Furthermore, based on the results obtained from this gate analysis, the percentage of liposomes encountered in both gates "cF" or gate "G" (Fig. 20) was set in relation to the

corresponding values prior to drying in the presence of various amounts of sugar, so as to follow the magnitude of improvement in dependence on the exogenous sugar concentration (Figure 21). This plot also enables the critical sugar concentration which allows good prevention against cF leakage and/or aggregation to be determined.

It could be observed from Figure 21 that prevention of cF leakage and inhibition of liposome fusion were more pronounced the higher amount of sucrose used as drying protectant. In particular, upon addition of highest concentration of exogenous sucrose, i.e. at 100 mg $mL^{-1}$, which corresponds to a sucrose:EPC liposome mass ratio of 20:1, the original characteristics of liposomes in terms of size and fluorescence intensity could nearly be restored. Other studies reported the use of a sucrose:EPC liposome mass ratio of 11:1 [47, 53], or 17:1 [63] or 20:1 [23, 54, 149] in order to precent cF leakage and membrane fusion. The air drying procedure used in these studies reported that a residual water content of about 0.02 to 0.04 g $H_2O$ per g dry weight was achieved [23, 47, 54, 63].

The sucrose:EPC liposome ratio of 20:1 seems to be independent on the size or specific surface area of liposome. In this study the size of liposome made was 1000 nm whereas in other studies liposomes with smaller size, i.e. 100 nm [23, 47, 54, 63] were used. Tanaka *et al* (1992) also used similar concentration of 100 mg $mL^{-1}$ sucrose as used in this study to stabilize 3 mg $mL^{-1}$ sonicated EPC liposomes with diameter of 26 nm [62]. Taken together, although the specific surface area ($\mu m^2$ $g^{-1}$) of liposomes of 100 nm is estimated to be 10 times higher than that of 1000 nm, the amount of sucrose required to efficiently stabilize liposomes was in the same magnitude.

A general conclusion that can be drawn from this study is that sucrose is capable of minimizing drying induced damage. Using FTIR spectroscopy it was observed that the sugar OH groups interact directly through hydrogen bonding with the phosphate of the phospholipid headgroups of liposome [63, 64]. This direct interaction result in the reduction of gel to liquid lipid phase transition temperature $T_m$. In the absence of sugar $T_m$ of dried liposome was 40°C, whereas sucrose lowered the dried $T_m$ of liposome to 7°C, as observed by FTIR spectroscopy of the $CH_2$ symmetric stretch band of liposomes [149]. A substantial depression of $T_m$, results in the preservation of membrane in a liquid-crystalline state, even when dry [21, 43]. Consequently, the membrane would not pass through a phase transition during rehydration and leakage of entrapped aqueous solution could be prevented.

Furthermore it is obvious that fusion and leakage were closely related (Fig. 21). This close relationship was also evidenced by other studies [54, 59].

It is noteworthy to suggest that the stabilization of liposomes during drying in the presence of sucrose can be partially attributed to the ability of sucrose to form glassy state upon water removal. The formation of glassy state could also explain the requirement of a high mass ratio of sucrose to efficiently prevent drying induced leakage and fusion of liposome. The $T_g$

of air-dried liposomal sample dried with sucrose was observed between 48 and 63°C [47]. Long-term preservation of liposomes in sugar matrix could be facilitated in glassy state. Retention of cF in liposomes remained quite high after storage at temperatures below $T_g$, and decreased remarkably above $T_g$. Likewise, fusion was inhibited below $T_g$ but more pronounced above $T_g$ [47, 149].

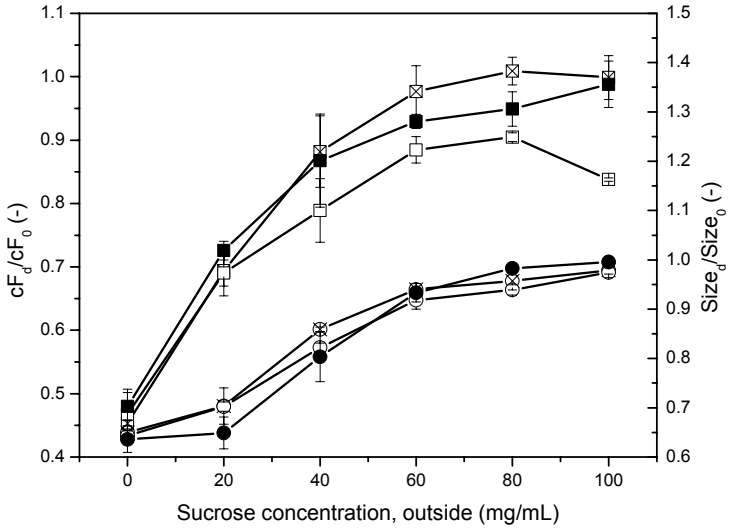


**Figure 22**

The influence of exogenously added sucrose at various concentrations on liposome's integrity in terms of the recovery of the original fluorescence distribution (squares) and size distribution (circles) in the corresponding gates (see Figure 20). Three different internal sucrose concentration were applied: 0 mg $mL^{-1}$ (filled symbols), 40 mg $mL^{-1}$ (crossed symbols), or 80 mg $mL^{-1}$ (open symbols). An index of 1 indicates that the size or fluorescence distribution before drying are completely restored. Liposome concentration in the liposome-sugar mixture was as high as 5 mg $mL^{-1}$. Data were means of the results from two independent experiments.

In addition, it was found that the presence of sucrose inside the liposomes did not have any positive effect on either cF leakage or fusion of liposomes. With regards to prevention of fusion, it was indeed expected that fusion would not be influenced by internal sugars [149]. In terms of leakage prevention, it was observed that at high concentration of internal sugar (80 mg $mL^{-1}$), the retention of cF is decreased; possibly due to harmful osmotic conditions. According to this findings, it seems that only the outer side of phospholipid layer needs to be stabilized by sugar in order to prevent the occurrence of detrimental effect as a result of drying. In addition, since stabilization of internal phospholipid layer was not required, it is possible that the inner part of liposomes was not dehydrated at all. The glassy matrix formed upon dehydration may in the outer side of liposomes may cease the diffusion of water molecule from the inner part of liposomes.

The optimal sugar/liposome ratio (Fig. 21) could be taken as a theoretical basis in predicting the critical sugar concentration required to efficiently protect bacterial membranes, for instance lactic acid bacteria. Taking a usual bacterial cell concentration of $10^9$ cFU $mL^{-1}$ into account, which corresponds to a dry matter of 10 mg $mL^{-1}$, the minimal amount of exogenous sugar required to confer protection was estimated to be 20%. This value correlates with usual sugar concentration used to protect bacteria during spray drying (Tab. 1). However, higher amounts of sugar was sometimes required. This can be explained by the fact that on bacteria not only the membrane should be protected from but also functional proteins embedded in bacterial membranes. The critical sugar concentration to maintain the integrity of liposome upon drying could therefore be effectively used to give rough estimation about the amount of protectant required to minimize drying induced damage on other organisms. With help of this simple model system labour-intensive microbiological analysis to evaluate the performance of a certain protectant as well as to determine its critical concentration could be significantly reduced.

***Stabilizing effect of sugars in the evaluated spray drying media on the integrity of liposomes during drying***

Having been establishing the analysis protocol using flow cytometry to investigate drying effect on liposomes, the sugars present in the spray drying media was evaluated on their stabilizing effect on liposomes during dehydration. Figure 23a shows the size distributions of EPC liposomes before dehydration as well as after de- and rehydration in the presence of lactose, polydextrose, Raftilose®P95 or in absence of sugar. In the case of drying without sugar, an increase in liposome size was found, as evidenced by a broadening of the histogram in the direction of higher liposome sizes. This behaviour was thought to be highly related to fusion and/or aggregation of liposomes. In contrast, using an optimized sugar : liposome ratio of 20:1, as previously determined (Fig. 21), the size distributions of liposomes dried in the presence of all evaluated sugars were nearly identical to the one before dehydration. This indicates that the addition of sugars could effectively prevent fusion and/or aggregation (Fig. 23a).

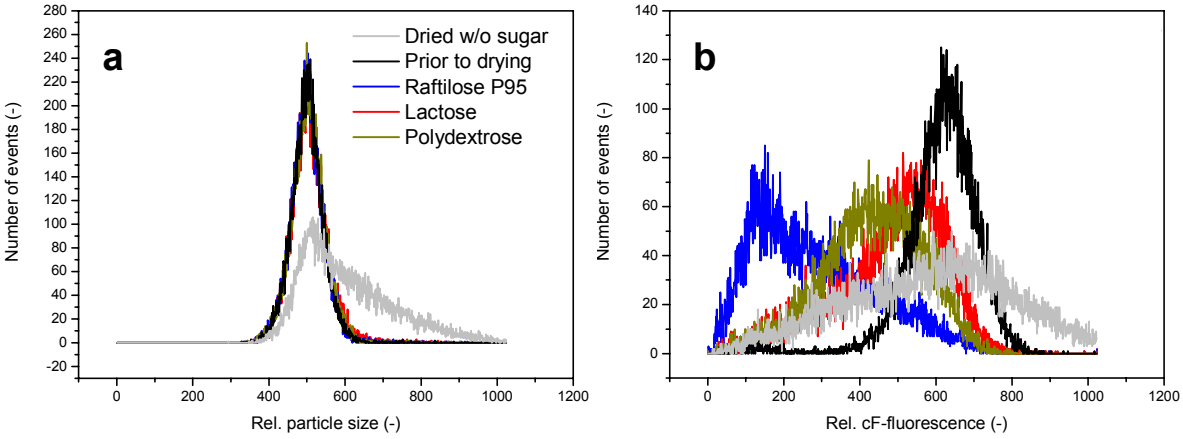


**Figure 23**

Frequency histograms obtained from flow cytometric analysis showing the effect of drying with and without different types of saccharides on important characteristics of liposomes, i.e. on particle size distribution (a) and on distribution of fluorescence intensity (b). For this experiment, liposomes (5 mg $mL^{-1}$) were dried at ambient temperature in the presence of solution of each sugar type (100 mg $mL^{-1}$) prior to rehydration and measurement.

On the other hand, not all sugars tested had the good capacity in preventing leakage of cF out of liposomes (Fig. 23b). In the absence of sugar there was only a small fraction of liposomes which still had fluorescence intensity values equivalent to that before dehydration. It is obvious that drying increased the permeability of the phospholipid bilayer, thus allowing cF to leak from the liposomes. The numerical values for the drying experiments with sugars present in spray drying media (Concentration 100 mg $mL^{-1}$) are presented in Table 7. The gating procedure described in the previous sub-section was also used to assess drying effect on the distribution of liposome size and fluorescence intensity.

**Table 7**

Effect of sugar in spray drying media on liposome characteristics after drying

| Sugar type | Rel. regain of initial fluorescence distribution ± SD | Rel. regain of initial liposome size distribution ± SD |
|---|---|---|
| Without sugar | 0.572 ± 0.0452 | 0.657 ± 0.0075 |
| Lactose | 0.686 ± 0.1618 | 0.994 ± 0.0093 |
| Raftilose®P95 | 0.379 ± 0.0977 | 0.993 ± 0.0086 |
| Polydextrose | 0.821 ± 0.1467 | 0.989 ± 0.0141 |

SD: standard deviations of three or more replicates

The capacity of the sugars evaluated here to prevent cF leakage could be clearly differentiated. Raftilose®P95 was found to be incapable of preventing loss of entrapped cF

from liposomes. The loss of cF is reflected by the apparent shift of the fluorescence distribution towards lower fluorescence values. In contrast, the stabilizing effect of either lactose or polydextrose was better than that of Raftilose®P95 in terms of cF retention. Despite good stabilizing properties the fluorescence distributions of liposomes dried together with lactose or polydextrose shifted to values lower than the one before drying. This behaviour indicates that – other than suppression of fusion/aggregation – leakage of cF could not be fully prevented by the sugars evaluated in this study in the applied concentration.

The positive effect of different types of disaccharides in minimizing drying induced damage has already been substantiated [20, 23, 54, 59]. Lactose has not been investigated yet, but the present result gives sound evidence about the positive effect of lactose to dried liposomes. In contrast, the effect of oligo- or polysaccharides is not clearly understood yet. Generally, polysaccharide, for instance hydroxyethyl starch, has elevated $T_g$. These sugars are thus capable of inhibiting fusion between liposomes during drying but it does not depress $T_m$ in the dry phospholipids so that leakage was not prevented [54]. Polysaccharides are thought to be sterically hindered from penetrating the bilayer in the dry state so that the gel to liquid crystalline phase transition temperature was not depressed and leakage of entrapped aqueous solution was not prevented [54]. However, other work reported that neither fusion nor cF leakage could be prevented by hydroxyethyl starch during freeze drying [45].

Surprisingly, other polysaccharides such as inulins of a DP ranging between 10 and 30 from chicory and dahlia could stabilize liposomes during freeze-drying and the stabilization is mediated by a direct interaction of the polysaccharides with membrane lipids despite of the proposed problem with steric hindrance [45] and even a high molecular mass bacterial levan (DP > 25000) was able to directly interact with membranes [60]. Consequently, oligo- or polymeric sugars may not be ruled out from being applied as drying protectant. It was found that with increasing chain length, fructo-oligosaccharides were more effective than gluco-oligosaccharides in stabilizing dried liposomes against leakage of aqueous content after rehydration [61]. According to FTIR spectroscopy data it was observed that the ability of gluco-oligosaccharides to hydrogen bond to the head group of dry lipids decreased dramatically with increasing DP, whereas chain length hardly affected the ability of fructo-oligosaccharides to interact. In contrast to the conclusion made by Hincha *et al* (2002), data compiled in this study revealed that polydextrose (gluco-oligosaccharides, DP = 12 or more) was more effective than Raftilose®P95 (oligofructose, DP = 2-8) in sufficiently protecting dried liposomes against fusion and cF leakage. The role of impurities, i.e. mono- or disaccharides, in polydextrose preparation, which may contribute to stabilization, could be neglected, since according to the information provided by the manufacturer the degree of

impurity is less than 10% (Annex 4 and 5), and it was shown before that high sugar to lipid ratio was needed to render liposomes stable against damage during drying.

### *3.4.7 The role of milk constituents in the protection*

It was shown that polydextrose and lactose were capable of adequately protecting liposomes against drying-induced leakage of cF and fusion/aggregation. In contrast, Raftilose®P95 could only prevent fusion/aggregation but not cF leakage, as evidenced by the shift of the distribution of cF fluorescence towards lower fluorescence values. It was noted before that sugar may protect membranes by direct interaction with polar head-groups of phospholipids [21, 43]. The maintenance of hydrated spacing upon sugar insertion leads to reduction of membrane phase transition temperature; thus preventing phase transitions and leakage during rehydration at room temperature.

The fact, that the degree of direct interaction and thus the protection performance of polydextrose, a highly branched polysaccharide, and lactose, a simple disaccharide, are similar, indicates that general concerns about the inefficiency of large sugar molecules in protecting membranes due to the steric hindrance is unfounded. In the light of this result one would expect that partial substitution of milk solids with polydextrose results in a protection capacity equivalent to RSM or at least does not bring about any detrimental effect on survivability during spray drying and subsequent storage. However, as already indicated by Figure 17, it was evident that the performance of RSM/polydextrose in protecting LGG was poorer than the one of RSM alone, especially during storage at 37°C. This result leads to further consideration, whether the reduced protection performance of RSM/polydextrose was caused by the exclusion of other milk components other than lactose. Typically the major constituents of skim milk powder are lactose at ca. 52 %, w/w, followed by milk proteins with ca. 37%, w/w [150]. The reduced availability of milk protein in RSM/polydextrose matrix was suspected as the source of the decreasing protective capacity.

To evaluate this hypothesis, RSM was treated with a proteolytic enzyme preparation Corolase®PP (ABEnyzmes, Darmstadt, Germany). The freshly prepared RSM was incubated overnight with Corolase®PP at optimal temperature of 50°C, decontaminated at 90°C and used as spray drying medium. Similarly, it was attempted to investigate the impact of enzymatically degrading lactose in RSM with ß-galactosidase (G-3665, Sigma, St. Louis, MO, USA) into glucose and galactose on protection performance of this modified RSM. It was known that hydrolysis of lactose results in a marked decrease of $T_g$ [114, 124].

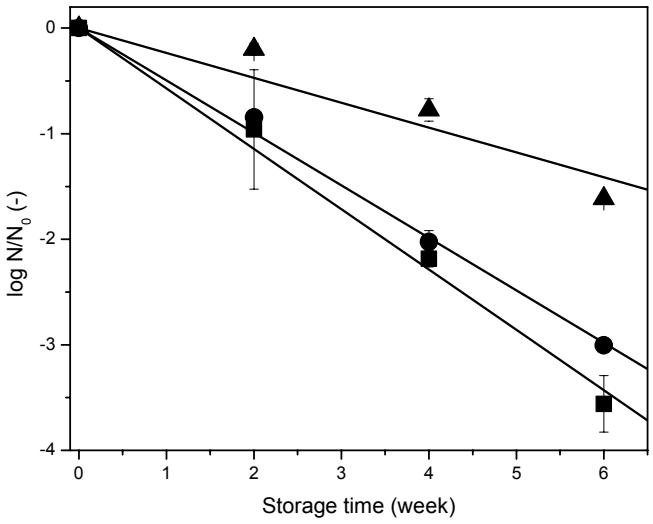


**Figure 24**

Effect of degrading skim milk components on the storage stability of *L. rhamnosus* GG spray dried with the enzymatically modified protective media: native RSM (▲), RSM pre-treated with 0.1% Corolase®PP (Annex 6) overnight at 50°C (■) or with ß-galactosidase (Annex 7) at ambient temperature for 2 h (●). Solids content was 20% (w/w). Spray drying experiment was conducted at an outlet temperature of 80°C and the dried powders were subsequently stored at 37°C and 11% relative humidity. Results are means of two independent drying and storage experiments.

It was shown in Figure 24 that the inactivation of LGG dried in proteolytic-treated RSM took place with a higher rate during storage compared to native RSM. This result gives evidence about the influence of protein degradation on the protection capacity of RSM.

A previous work had pointed out the importance of milk proteins as protective coating in stabilizing dried yeast [151]. Similarly, it was reported that milk proteins might be involved in protecting staphylococcal strains against thermal damage [115]. Positive role of milk proteins was not only demonstrated on biological materials but also on volatile aroma compound. Among the components of milk, it was reported that milk proteins seemed to have the strongest positive effect, more than lactose, on retention of diacetyl during spray drying [152]. Moreover, as already discussed before, lipid oxidation is deemed responsible for death of dried cells during storage [11, 12, 108]. Thus, the contribution of milk proteins to reduction of cellular damage of dried bacteria during storage might involve inhibition of lipid oxidation. The protective effect of milk protein was even more evident upon spray drying of LGG with trehalose (20%, w/w) as drying medium, in which compared to RSM no proteins were present. Trehalose is known to be a good drying protectant for different microorganisms [13, 71]. However survival of spray dried LGG during storage was very poor when trehalose as drying medium. After 4 weeks of storage at identical conditions, i.e. at 37°C with 11% relative humidity, a reduction by more than 5 log cycles was obtained (data not shown). From this

result it could be concluded that the application of sugar alone is most likely not sufficient to achieve an equivalent protection as obtained when skim milk is used. In addition, this findings also emphasizes the superiority of skim milk as drying protectant for lactic acid bacteria [141] and milk proteins contribute to this protective effect in a still unknown way.
Furthermore, compared to drying with native RSM the application of RSM treated with ß-galactosidase (or RSM with hydrolyzed lactose) as spray drying medium led to a increased rate of inactivation during storage. Due to the enzymatic degradation of lactose into glucose and galactose, the anhydrous $T_g$ of the RSM was reduced from 101°C to 49°C [91, 124]. At 5% water content, which is typical residual moisture for powder obtained during spray drying at 80°C, the $T_g$ of RSM with hydrolyzed lactose was –8°C [124], whereas the $T_g$ of native RSM was around 50°C (Fig. 19). Consequently, during storage at 37°C, the RSM with hydrolyzed lactose was in rubbery state, while using native RSM the bacteria were entrapped in a glassy matrix. It was concluded in sub-section 3.4.5 that the presence of bacteria in an external glassy matrix was not sufficient to ensure good storage stability (Fig. 17 and 19). However, the results of storage test of LGG in native RSM and RSM with hydrolyzed lactose (Fig. 24) demonstrated that indeed the formation of glassy state which surrounded bacteria facilitated higher survival rates. Furthermore, the higher amounts of reducing groups in sugar molecules (glucose and galactose) in RSM with hydrolyzed lactose compared to native RSM (lactose) could be regarded as an additional factor which also led to reduced stabilization of LGG in RSM with hydrolyzed lactose.


**Figure 25**

Extent of browning occurring on spray dried RSM, which were subsequently oven dried overnight at 102°C. RSM were enzymatically treated with ß-galactosidase (1), or with Corolase®PP (2), prior to spray drying at 80°C or not pre-treated (3). Solids concentration of all drying media was 20% (w/w).

It was reported the substantial decrease of the activity of dried alkaline phosphatase can be ascribed to the low $T_g$ of the used sugars in combination with the occurrence of the Maillard reaction [32]. Reducing groups can react with the amine groups of the protein and this reaction is the first of a cascade of reactions also known as the Maillard reaction. Since the enzyme is the main component containing proteins, the reducing groups in sugars used to protect the enzyme reacted with the enzyme, thus destabilizing instead of stabilizing it.

Figure 25 documents the visual evidence of Maillard reaction in RSM treated with ß-galactosidase, which was oven-dried at 102°C overnight. It was obvious that in samples containing less reducing sugar groups (native RSM and RSM treated with Corolase®PP) the browning intensity was far lower. When bacteria are dried and stored in the presence of RSM, the reducing groups of sugar may most likely react either with milk proteins, thereby modifying the physical properties of the matrix, thus destabilizing it, or with proteins embedded on cell envelope, thereby inducing cellular injury. It can be speculated that both events could lead to loss of viability during storage. The accelerated rate of inactivation of LGG in RSM treated with ß-galactosidase could therefore be attributed to the greater extent of this deteriorative reaction owing to higher amounts of reducing groups. Consequently the absence of reducing groups is another requirement to be met by sugar molecules, when they are to be applied as bacterial protective ingredient.

### *3.4.8 Role of milk constituents in conferring stability against low pH and bile acids*

For probiotic bacteria in foods to be beneficial in the host, they should be able to survive gastric transit, reach the small intestine in sufficient numbers and persist in this environment to be effective [153]. The harsh environment of the gastro-intestinal tract is mainly attributed to the low pH conditions of the stomach, in addition to the presence of bile in the small intestine. In this study, the viability of spray dried LGG was assessed following exposure to in vitro at 37°C. As already indicated above skim milk is superior in conferring protection against adverse conditions encountered during spray drying and subsequent storage. The protective effect of milk compounds was found to be related to effective direct interaction of lactose with phospholipid bilayers, high $T_g$, reduced tendency for non-enzymatic browning reaction and unknown protective action of milk.

Likewise, the specific degradation strategy was also applied to systematically evaluate the role played by skim milk constituents in conferring protection against harsh environmental conditions during passage of gastro-intestinal tract. Accordingly, RSM treated with Corolase®PP was used as spray drying medium to elucidate the role of milk proteins, whereas RSM treated with ß-galactosidase might give indication about the role of lactose.

The protocol for acid and bile stability test was adapted from the work of Saarela *et al* (2004) [154]. As a model for the performance of the dried probiotic preparations during gastric transit, the survival of the strain in each powder was investigated when exposed to PBS adjusted to pH 2.0 for 2 h. The effect of drying media on bile tolerance was examined by incubating spray dried LGG in PBS pH 7.0, which was supplemented with bile acid in an end concentration of 1%. It was found that the highest resistance of LGG to acid and bile stress was obtained when they were spray dried in native RSM (Fig. 26).

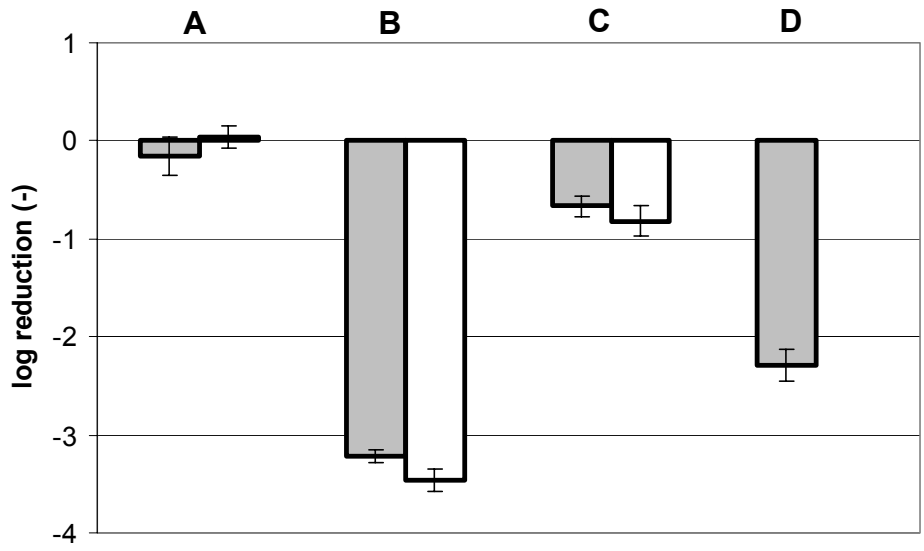


**Figure 26**

Survival rates of *L. rhamnosus* spray dried with different drying media following incubation at pH 2.0 for 2 h at 37°C (white bars) or following incubation in the presence of 1% bile acids for 3 h at 37°C (grey bars). The spray drying media evaluated were native RSM (A), RSM previously treated with Corolase®PP (C), RSM previously treated with ß-galactosidase (C), and trehalose (D). Bacteria suspended in media with 20% solids content (w/w) were spray dried at an outlet temperature of 80°C. Data are means of two independent spray drying and acid or bile challenge experiments.

When milk proteins were degraded, the acid and bile resistance were decreasing. Thus, the presence of milk proteins exerted a major effect on survival in low pH conditions and in the presence of bile. Similarly, when trehalose was used as drying medium, the bile resistance was low, pointing out the important role of milk proteins in minimizing the toxic effect of bile acids. In conclusion, it is evident that the contribution of milk proteins to acid and bile resistance was far more crucial than the one of sugar. Data from literature suggest that survival of lactic acid bacteria in human gastric juice adjusted to low pH was enhanced by the addition of skim milk [155]. Other authors proposed that milk proteins function as buffering agents *in vivo*, thereby protecting ingested bacterial strains during upper gastrointestinal transit [153].

Furthermore, there is a slight decrease of cell count (ca. 1 log cycle reduction) following acid or bile challenge when RSM with hydrolyzed lactose was used as drying medium. This observation supports the aforementioned conclusion that in RSM primarily the milk proteins and not the type of sugars rendered the bacteria more stable to acid and bile stress. With this regard it was expected that upon bile or acid challenge the survival characteristics of LGG dried in RSM with hydrolyzed lactose should be equal or even better than the one dried in native RSM due to the presence of native proteins in both media. In terms of acid tolerance, it is known, that a prerequisite for a good survival in acid environment is the presence of

fermentable sugar in the medium (R.P. Ross, personal communication). It was reported that $F_0F_1$-ATPase is involved in proton extrusion through ATP hydrolysis. Since LGG is not capable of metabolizing lactose (Annex 1) but can utilize glucose [156], it was thought that the enzyme degradation of lactose would improve the availability of fermentable sugar for LGG, resulting in maintenance of a pH and a less viability loss in the acidic environment. However, in native RSM, where no fermentable sugar was present, a higher acid tolerance was obtained. Thus, this pH-homeostasis mechanism related to $F_0F_1$-ATPase activity was most likely not involved in acid protection of LGG dried with native RSM or RSM with hydrolyzed lactose. It seems probable to conclude that in these media, native milk proteins which were still present, served as the buffering agents [153]. The viability loss of LGG dried with RSM with hydrolyzed lactose during acid or bile challenge might therefore be most likely explained by the higher amounts of reducing sugars compared to native RSM, which may undergo unexpected reaction with proteins embedded in cell wall or cell membrane of LGG during spray drying. As a result, some cells experienced cellular damage, which made them more sensitive towards acid or bile stress.

### 3.5 Conclusion

This study demonstrated the possibility of producing dry probiotic bacterial preparation using spray drying. Using reconstituted skim milk (RSM) as the drying medium, a bacterial survival rate ≥ 50% was achievable at an outlet temperature of 80°C. The powder contained more than $10^9$ cfu $g^{-1}$. Using flow cytometry, bacterial membranes were identified as the main site of injury during spray drying. The incorporation of commercial prebiotic substances such as Raftilose®P95 or polydextrose in the skim milk powder did not exert any adverse effect on bacterial survival upon spray drying. However, stability of bacteria during long term storage was impaired by partial substitution of skim milk with either of the prebiotic substances evaluated. The presence of the dried medium in the glassy state appeared to have had only little effect on bacterial stability in the spray dried powder. Although the glass transition temperatures of all media were well above the applied storage temperatures, bacteria inactivation still took place during storage; indicating the insufficiency of entrapment in glassy state in inhibiting deteriorative events involved in cell death. The decreased protection capacity of prebiotic containing media could be resulted from the reduced amount of protective compounds in skim milk solids, which could not adequately substituted by prebiotics. As a result, due to the presence of oxygen in the storage atmosphere applied in this study, related deteriorative reaction, most likely lipid oxidation, may take place at a higher rate.

Flow cytometric analysis was conducted on model membranes containing carboxyfluorescein, in order to evaluate the contribution of sugar molecule to stabilization

during drying. It was observed that oligosaccharides present in Raftilose®P95 was not capable of directly interacting with cytoplasmic membranes in dehydrated state, whereas polydextrose and lactose could effectively prevented drying induced membrane leakage. Moreover, the implication of milk proteins in stabilization of dried bacteria during storage was observed. When milk proteins were enzymatically degraded, the performance of such treated RSM-based media in conferring protection during storage was considerably reduced. In conclusion, the superiority of skim milk over the prebiotics in stabilizing dried bacteria is most likely based on the direct interaction of lactose with bacterial membranes as well as proteins and on protective effect of milk proteins. The higher susceptibility of bacteria dried in prebiotic containing matrix to deteriorative events could be reduced by storing them at low temperatures.

Apart from spray drying, another study was also conducted on the utilization of spray generation-assisted processing in subzero atmosphere. Similar to spray drying, with this approach it was envisaged to generate small droplets of bacteria suspension, where extensive heat and mass transfer occur due to large surface to volume ratio. In contrast, the process does not involve heated air to evaporate water, but due to running the process in a subzero environment the formation of small ice crystals could be achieved. These are collected in a container placed in bottom part of the freezer. A schematic drawing of the experimental unit is shown in Figure 27. The pathway of the sprayed suspension is furthermore protected with a “wave-breaker” in order to reduce turbulences resulted from the air-blast of the freezer, so that the generated droplets can freely move down instead of being circulated. Although not readily optimal, this construction already allowed reproducible production of small ice crystals, pointing out that the crystallization heat could be sufficiently removed by the cold air during the passage from the nozzle to the bottom part of the freezer. More works are required in the construction of a “freezing zone”, in analogy to the drying chamber in spray dryer, so as to realize forced, close-to-laminar flow of cold air along with the generated droplets.

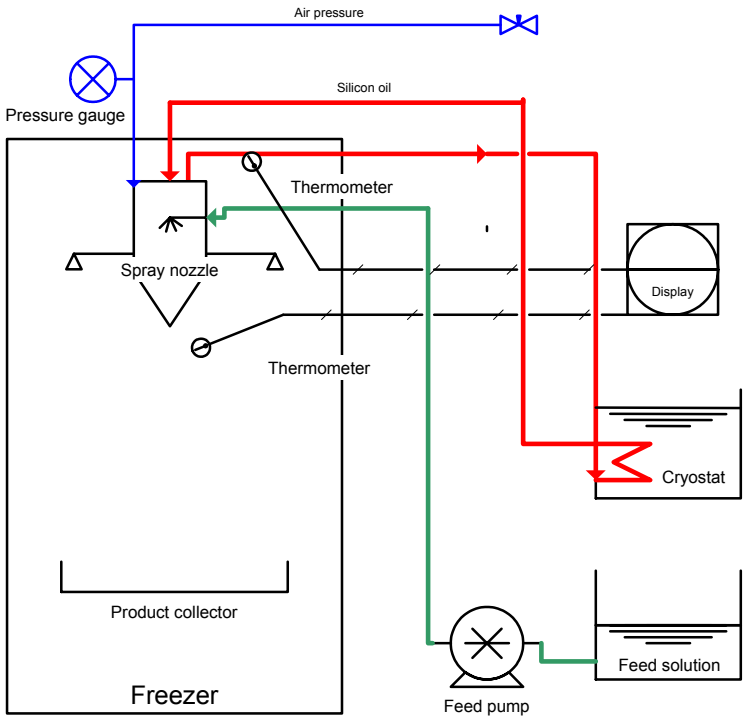


**Figure 27**

Schematic view of the spray-freezing installation used for experiments on *L. rhamnosus* GG [157]. The two-fluid nozzle from Büchi B-191 spray dryer is used to generate the spray together with pressurized air (6 bar). The nozzle was heated (T > 10°C) using silicon oil flowing through heating jacket to prevent frozen-induced clogging. The temperature of the freezer was set at –35°C.

First data on spray freezing of *L. rhamnosus* GG with the aforementioned installation have already been complied. This rapid freezing procedure was found to be less detrimental to the viability (as determined by plate count methods methods) or to the membrane integrity (as measured by flow cytometry) of probiotic bacteria than freezing an equivalent volume of bacterial suspension (data not shown). It seems that the improved survivability using spray freezing process may result from higher freezing rate [158, 159] , thus reducing the size of ice crystals and intracellular concentration effect owing to water removal from the cell [160, 161].

Based on the beneficial effects of high freezing rates on microbial viability spray freezing is therefore a good candidate to be evaluated on its feasibility as the freezing method involved in freeze-drying. In addition, it is known that in the conventional practice of freeze-drying a cake is formed, which needs to be milled in order to obtain a free-flowing dried powder. With help of the generation of small-sized frozen ice crystals the milling step may be fully circumvented or applied with a low intensity. On the other hand, it is also possible to use spray freezing to facilitate homogeneous incorporation of readily frozen probiotic bacteria into ice cream matrix. The production of probiotic ice cream typically involves addition of

suspension of probiotic bacteria in the ice cream mix prior to freezing them altogether [162, 163]. As already mentioned above the latter procedure – depending on the freezing temperature – would not allow rapid freezing to be achieved, thus increase the probability of losing a considerable amount of viable bacteria during the freezing step. These suggestions have to be investigated and substantiated more intensively in further works.

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# 4 PRESSURE INDUCED STRESS RESPONSE

Cross-adaptive stress response of pressure pre-treatment on probiotic bacteria: Characterization and importance for production processes

## 4.1 Introduction

Foods containing probiotic bacteria gain growing acceptance in broad communities. This rise is stimulated by increasing health consciousness of people in industrial countries. On the other hand due to published scientific evidences about the positive effects of probiotic bacteria on human health the consumer was becoming more convinced. This tendency was well documented by increasing market potential/market share of probiotic products among all other functional foods [1, 2].

Beneficial effects of probiotics on human health are suggested to be mainly related to the presence and activity of a high number of viable cells in the intestine. Therefore, maintenance of high viability level and retention of physiological activity during processing and storage are some of the proposed technological criteria, which need to be fulfilled by potential bacteria with probiotic traits, when they are aimed to be applied in food products [3]. Moreover it is also demanded, that the consumed bacteria persist adverse conditions throughout GI-tract without losing associated probiotic properties [4, 5].

*Technologically relevant bacterial stress response*

Stress-sensing system and defense mechanism of microorganisms were utilized to prepare themselves in withstanding either harsh conditions or sudden environmental changes. In response to these external disturbances specific metabolic processes of the treated cells (transcription rates, translation products, metabolic pathway, etc.) are altered, resulting in increased production of certain stress metabolites, which are involved in counteracting such abnormalities in their environment; thus help the bacteria survive the deleterious conditions. These survival mechanisms exhibited by bacteria when confronted to stress are generally referred to as the stress response. The exploration of bacterial stress response to adverse environmental conditions is motivated by basic scientific reasons but also by industrial and safety aspect in food microbiology [6-8].

In particular, bacterial response against stress conditions was found to be related to coordinated expression of genes which alter different cellular processes (cell division, DNA metabolism, housekeeping, membrane composition, transport, etc.) and act in concert to improve tolerance [9]. Regarding the type of external stimuli applied to induce stress response, one survival mechanism is the adaptive response, that is, when cells are exposed to a moderate level of stress, they acquire increased resistance to a subsequent exposure to a more severe level of the same stress at lethal dose (homologous agents). When cells are exposed to one stress they develop resistance, not only to that stress, but to other unrelated stresses (heterologous agents). This mechanism is known as cross-protection [10].

A specific stress that has been extensively examined is heat stress. A major group of stress metabolites which are frequently associated with bacterial response to sub-lethal dose of heat stress is referred to as heat shock proteins. An induction of genes corresponding to these proteins on transcriptional level [11, 12] as well as an up-regulation in their synthesis were observed, when the bacteria were incubated at a temperature significantly higher than their normal growth temperature [13-20]. For example, heat shock proteins in *Lactococcus lactis* subsp. *lactis* – immunologically related to DnaK and GroEL of *E. coli* – were induced when temperature was shifted from 30 to 40 or 42°C [13, 14]. Generally, these stress proteins maintain quality control of cellular proteins. They bind substrate protein in a transient non-covalent manner and function by preventing denaturation of proteins (cleaving misfolded and partially folded proteins), correctly refolding denatured proteins or removing denatured proteins before they cause damage to the cells [6, 10, 21]. In terms of viability improvement this heat inducible stress response resulted in higher survivability compared to untreated population after subsequent exposure to lethal temperatures [10, 12, 13, 16, 19, 22-24]. Because a large number of other stress conditions induce the heat shock proteins or at least the most abundant ones, this response is often termed the universal stress response [25]

The versatility of applying heat stress to exert cross-protective action against unrelated stresses has been evaluated [10, 12, 19]. With help of inducible cross-protection the stress induction procedure must not be necessarily the same as the lethal condition the bacteria have to face afterwards – a fact that allows a higher degree of freedom in selecting the type of shock inducer to be used. Although the induction of heat shock proteins by sub-lethal stresses not related to heat was regarded to be less effective as by heat [26], there is a growing body of evidence which substantiated the importance of cross-protection phenomena. For instance, a brief pre-conditioning heat shock at 55°C can trigger increased chaperone production in *Lactobacillus johnsonii* and provide significant cross-protection from the stresses imposed during the production of frozen culture concentrates [11]. Heat treatment before freezing of commercial baker's yeast in dough could also improve their freeze tolerance [27]. Heat adapted cells of *Lactobacillus plantarum* also showed increased growth at pH 5 and in the presence of 6% NaCl [19].

On the other hand, upon exposure to UV irradiation, ethanol, certain heavy metals, hydrogen peroxide and both alkaline and acidic pH conditions heat shock proteins could be induced as well [6, 28]. Preincubation at a low temperature of 8°C led also to thermotolerance to a 52°C challenge in *L. lactis* [29]. This latter study demonstrated a complex relationship between cold and heat shock. Survival characteristics of *Bifidobacterium adolescentis* during heat treatment at 55°C can not only be improved by previous adaptation at 47°C but also by pre-incubation in 1.5% NaCl [12]. Furthermore, mild osmotic stress using 0.4 M NaCl could

increase thermotolerance in *Lactobacillus delbrueckii* [23]. Pre-exposure to bile could not only help *Lactobacillus acidophilus* cells in tolerating lethal bile concentration but also against heat treatment [10]. Physiological linkage between unrelated stresses was shown by proteomic study on *L. lactis*, which revealed an overlap between the heat and salt stress responses, demonstrating that all salt stress-induced proteins, such as DnaK, GroEL and GroES were the ones, which were rapidly induced (within 10 min) by sub-lethal heat stress at 43°C [15].

The stresses associated with the production, storage, and distribution of frozen, lyophilized, or spray-dried bacterial culture concentrates can dramatically reduce their viability and activity. The triggering of bacterial stress response was proposed to be one feasible approach to maintain high viability level and retention of physiological activity during these production stages [11, 14, 18, 22, 24, 30-33].

It was reported that when exponential phase cells of *Lactobacillus bulgaricus* were heat shocked at 42°C prior to spray drying with an air outlet temperature of 80°C a significant increase of their resistance to the process as compared to control cells was observed [30]; however, it was further indicated that the survival of heat shocked cells from exponential growth phase did not reach the normal levels found with unshocked stationary phase cells. Heat pre-treated *Lactobacillus paracasei* cells were reported to possess up to 18 fold greater thermotolerance compared with control, when they were spray-dried at 95-105°C, while salt-adapted cultures exhibited 16 fold greater viability than control [24]. When a pre-adaptation step either with heat (50°C) or salt (0.6 M NaCl) was conducted prior to fluid bed drying of *Lactobacillus rhamnosus* the viability was found to be significantly improved compared with the unadapted control culture after storage at 30°C over a period of 14 weeks [18].

When frozen storage of bacteria was considered, data on pre-incubation of *L. lactis* at 8°C led to development of cryotolerance, i.e. enhanced capacity to survive exposure to freezing temperature of –20°C [29]. Cold shock at 10°C could significantly improve cryotolerance of *L. lactis* for short periods of frozen storage at –20°C, but the protective effect was less marked following longer storage period [32]. The improved cryotolerance was attributed to the presence of a cold shock protein with a molecular weight of 6.3 kDa in the pre-stressed sample. Adaptation of *Streptococcus thermophilus* at 20°C resulted in a 1000-fold increase of survival after four freeze-thawing cycles compared to control group [34]. In this organism several 7-kDa cold-induced proteins were identified as the major induced proteins after a shift to 20°C.

*Pressure stress response on bacteria*

Pressure effects on physical and biochemical processes are based on the fundamental principle of Le Chatelier (Eq. 1),

$$\left(\frac{\partial \ln k}{\partial p}\right)_T = -\frac{\Delta V^*}{RT} \qquad \text{Equation 1}$$

where k is the rate constant, p is the pressure (bar), T is the absolute temperature (Kelvin), R is the gas constant (mL bar $K^{-1}mol^{-1}$) and $\Delta V^*$ is the apparent volume change of activation (activation volume) and represents the difference in volume between the reactants and the transition state.

Analogue to Arrhenius equation which describes the temperature-dependency of reaction rate, a specific biochemical reaction might be accelerated, retarded or left unaffected depending on how the system volume changes under exposure to elevated pressure. When a reaction is accompanied by volume increase of activated complexes or end products, it is inhibited by elevated pressure. When a reaction is accompanied by a volume decrease, it is enhanced by elevated pressure. For instance, if a reaction is accompanied by a volume decrease of 300 mL $mol^{-1}$, it is enhanced more than 200 000-fold by applying a pressure of 1000 bar.

Volume changes can occur due to changes in water structure around proteins, nucleic acids, ions and enzyme substrates [35]. In particular, protein subunit dissociation (multimeric enzymes, ribosomes, cytoskeleton proteins, and proteins acting in signal transduction pathway) is facilitated by elevated pressure since upon hydration of a protein, water molecules are arranged in its vicinity more densely than in bulk water, leading to a volume reduction of the system [36, 37]. Similar to the deteriorative effect of heat, the destruction of hydrogen bonding in macromolecules was caused by decreased viscosity due to high pressure [38].

However, the overall effects of pressure on metabolic processes in living organisms are thought to be very complex. Even in the case of a well-known metabolic pathway such as glycolysis, elevated hydrostatic pressure might result in enhanced, neutral or inhibitory effects as a result of the variation in sign and size of the volume changes at each step. Thus, even when the value of ΔV* for biochemical reactions is known, it is still difficult to predict how elevated hydrostatic pressure would affect metabolic pathways or alter the pool sizes of metabolites in living organisms [39].

Most of the studies on the impact of high hydrostatic pressure on changes in microbial physiology are dedicated towards understanding of the molecular bases of life in deep-sea high pressure environments, but there is an increasing interest in the exploitation of biotechnological potential of the piezophiles (microbes preferring high pressure conditions)

and on the stress response of microorganisms not coming from high pressure environments [35, 37, 39, 40]. In terms of stress response, elevated hydrostatic pressure can strongly influence gene and protein expression in both 1 atmosphere adapted and high pressure adapted microorganism [40].

Continuous exposure of *Escherichia coli* to 55 MPa inhibited cell division but allowed the OD of the culture to increase owing to cell filamentation and the doubling of the biomass production [41]. It was speculated from the observed increase in OD following initial lag phase – in absence of an increase in viable cell number – that an overlap exists between inhibitory aspects of high pressure and other environmental stresses, for which *E. coli* has evolved adaptive response. Long filamentous *E. coli* cells with segregated nucleoids were observed after treatment at 75 MPa as resulted either by pressure induction of a putative cell division inhibitor protein or by pressure induced denaturation of FtsZ molecule, which is involved in septum formation [42]. In contrast, study performed with *Lactobacillus sanfransciscencis* showed no filament formation during incubation under elevated pressure [43].

With respect to the fact that OD can increase without any apparent increase in cell count it is also interesting to evaluate, whether under these circumstances *E. coli* might enter the viable-but-not-culturable state at elevated pressure, which is regarded as a survival strategy in coping with harsh environmental conditions [44].

The study of Welch *et al* (1997) also revealed an increase in the relative rates of a set of heat shock proteins on cells growing at elevated pressure. Proteins synthesized at increased rate with higher pressure are defined as pressure induced proteins (PIP). The alteration in the pattern of protein synthesis seemed to be important during adaptation and growth at a sub-lethal pressure level. The magnitude of PIP induction correlated with the magnitude of pressure shift (up to 100 MPa) in a barometer-like fashion. In particular, the PIP spots on 2D electrophoretic gels were identified as classical heat shock proteins acting as chaperones including GroEL, DnaK as well as proteins related to cold shock response and an unknown protein of 15.6 kDa. One unique characteristic of the proteomic profile of cells grown under elevated pressure is that high pressure induced more HSPs than most other conditions outside of those which precisely mimic a heat shock response, while also inducing more CSPs than most conditions outside of those which precisely mimic a cold shock response.

In a similar study on continuous exposure of *L. sanfranciscencis* to high pressure cold shock proteins could be identified [43]. Compared to the work of Welch *et al* (1997) pressures beyond 100 MPa were evaluated as well. Although some PIP were gradually increased with rising pressure, the induction of several other PIP occurred only at a certain pressure level. A striking difference to the pressure induced proteomic profile of *E. coli* is that the classical

stress proteins (DnaK, DnaJ, GroES) did not belong to the major PIP found in *L. sanfranciscencis*. Furthermore, the fact that the protein expression effect had a maximum after a pressure treatment of 150 MPa 1h, and was continuously decreasing upon application of higher pressures, indicated that this process was not governed by Le Chatelier principle, otherwise the effect would reach a steady-state due to the preferred volume decrease under pressure. In this respect, the implication of signal cascades which turn on the expression of proteins need to be investigated [43].

Since the alteration in the pattern of protein synthesis seemed to be important during adaptation and growth at a sub-lethal pressure level, works have been performed in order to unravel the contribution of the overexpressed proteins to pressure resistance as well as to the acquisition of cross-protection.

Molecular characterization of pressure-resistant mutants of *E. coli* and *Listeria monocytogenes* emphasized the importance of protein management, especially the role of heat shock proteins, in withstanding extremely high pressures [45, 46]. In *E. coli*, pressure resistance was found to correlate with level of dnaK expression and the heat shock proteins were suggested to prevent cellular damage and/or aid cell recovery [46].

Related study on yeast indicated that molecular chaperons hsp 104 and hsc 70 were found to confer tolerance on *S. cerevisae* towards the damage caused by hydrostatic pressure and heat, although the accumulation of trehalose was found to be more important for the acquisition of barotolerance [47-51].

In the contrary, it is reported that heat shock proteins are not considered to cause barotolerance at 300 MPa in *L. sanfranciscencis*, since all kinds of stress inducers (high pressure, acidic, osmotic, cold, starvation) except for heat could induce barotolerance [52]. This observation revealed that adaptive mechanism different than the general stress response was responsible for increased barotolerance.

The contradictory findings could be explained by the fact that in comparison to the application of sub-lethal heat treatment, the signal for heat shock induction is generated only slowly by exposure to high pressure [41]. Slow or weak induction of heat shock proteins during brief exposure to growth-inhibiting level of pressure could lead to failure to induce heat or pressure resistance in *S. cerevisae* and *E. coli* by pressure shock [46, 53].

On the other hand, high hydrostatic pressure of 50-75 MPa induced synthesis of heat shock protein (hsp104) and tolerance against various stresses such as high temperature, high pressure and high concentration of ethanol [54]. Similarly, high pressure pre-treatment at 80 MPa could induce tolerance to lethal dose of heat and high pressure in *L. sanfranciscencis* [52].

Apart from the synthesis of heat shock proteins, some cold shock proteins were also induced by high pressure [41, 55]. Both low temperature and high pressure inhibit an early step of translation, and the resulted cold shock response was principally an adaptive response to facilitate gene expression in translation initiation [56]. According to the data from Wemekamp-Kamphuis *et al* (2002) cold shock proteins with a molecular size ranged at 7-kDa might be involved in adaptation to both low temperature and pressure treatment. Owing to the induction of CSPs during a cold-shock treatment at 10°C for 4 h cells of *L. monocytogenes* can better survive subsequent high pressure treatment. Furthermore, survivors of high pressure treated *Staphylococcus aureus* were approximately two log cycles higher when cells were cold shocked at a temperature of –20°C for 15 min [57].
Furthermore, the adaptive response of microorganism towards high pressure was similar to the one taking place during cold adaptation, since both high pressure and low temperature reduce the membrane fluidity, which perturbs membrane associated processes, including transmembrane ion and nutrient flux as well as DNA replication [58, 59]. To maintain proper fluidity a general shift in the lipid composition of bacterial membrane from saturated to unsaturated fatty acid was observed in response to an increase in growth pressure [60]

Genomic studies have been also conducted to identify changes in gene transcription of *Saccharomyces cerevisae* after hydrostatic pressure treatment by whole genome DNA microarray hybridization [61, 62]. The result of the hierarchical clustering analysis of genome wide expression profiles indicated that pressure shock response (180 MPa for 4 min) was highly similar to that caused by detergents, oils and freezing/thawing cycle. These kinds of stress caused damage to the membrane structure and/or cell organelles [61]. Genes being upregulated in response to the sensed damage were proposed to be involved in repair of cellular organelles or in the degradation and removal of damaged proteins. Functional classification of 274 genes affected by pressure treatment for 30 min showed that the upregulated genes were involved in stress defense and carbohydrate metabolism while most of the repressed ones were in cell cycle progression and protein synthesis categories [62]. However the upregulation of some uncharacterized genes clearly demonstrated a pressure-specific stress response pattern.

With regards to signalling pathway leading to pressure stress response Figure 1 shows schematically the commonalities of between high pressure effect and both the effect of decreases and increases in temperatures. This shared effect is of utmost importance in the signalling pathway, through which high pressure could induce the synthesis of heat shock proteins. Either high pressure, high or low temperature can destabilize the tertiary and quaternary structure of proteins. Thus, pressure-induced increases in the proportion of

misfolded proteins or dissociated subunits could induce a $\sigma^{32}$ factor dependent (in gram negative bacteria) or a CIRCE/HrcA-regulon dependent (in gram positive bacteria) heat shock response and trigger the synthesis of heat shock proteins [63, 64].

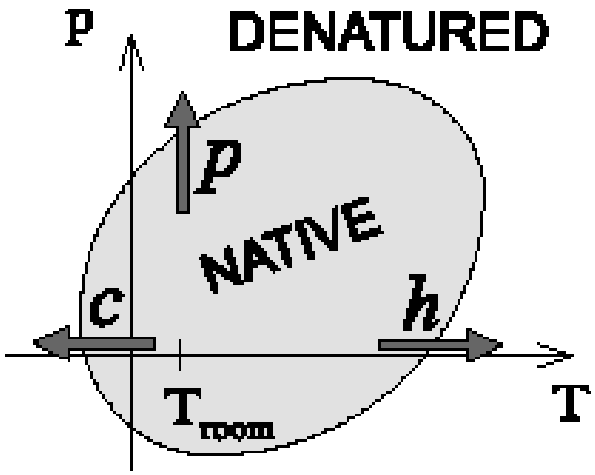


**Figure 1**

Schematic representation of the elliptic phase diagram of proteins. The arrows marked by the letters p, h, c show the specific denaturation ways known as pressure, heat and cold denaturation, respectively [65].

Alternatively, the signalling pathway was proposed to be based on its inhibitory effects on ribosome assembly or function [66]. It is known, that the prokaryotic ribosome has been implicated to act as a sensor for both heat and cold shock response networks [25]. Indeed, since cell death upon exposure to ultra high pressure

was associated with ribosome damage [67], the fact that stresses the implication of ribosome as temperature sensors, may lead to believe that cold shock and heat shock proteins play a role in stress response under exposure to elevated pressures [6].
Furthermore, it was reported that although the induction of several heat shock promoters can be induced by pressure, the genetic response of *E. coli* upon pressure treatment did not appear to be a DNA damage response usually known as SOS response [46, 68]. This is strongly different to the typical SOS response as induced by to DNA-damaging treatment.

## 4.2 Objective

This work aims to evaluate, whether and to which extent heat tolerance of *Lactobacillus rhamnosus* GG is affected by mild pressure treatments prior to exposure to lethal temperature so as to give evidence to the presence of technologically important cross-protective stress response of high pressure. Cross-protective action of pressure especially against heat needs to be extensively investigated to allow better justification regarding its applicability in assisting probiotic production, where in case of implementation of spray-drying the lethal effect of high temperature needs to be overcome.
The effect of incubation at elevated pressure was evaluated by means of monitoring their post-pressure growth characteristics. Kinetic analysis of the heat inactivation curves at 60°C of variously pre-treated cells was performed in order to identify the optimal combination of pressure and temperature required in the pre-treatment phase. The role of *de novo* protein synthesis on acquisition of pressure induced heat tolerance was evaluated. Furthermore, flow cytometric analysis combined with multiple staining strategy was applied so as to have additional insights in the physiological changes affected by pressure pre-treatment. Other cross-protective characteristics of pressure treated cells in tolerating bile acids, nisin and spray drying conditions was assessed as well.

## 4.3 Material and methods

### *4.3.1 Test organism*

*Lactobacillus rhamnosus* GG (ATCC 53103) – thereafter abbreviated with LGG – was obtained from Valio R&D, Helsinki, FL. The culture was sent in freeze-dried form in glass ampoule. For long-term maintenance LGG was stored as glass bead cultures (Roti®-Store, Carl-Roth, Karlsruhe, D) in a -80°C freezer (U101, New Brunswick Scientific, Nürtingen, D).

### *4.3.2 Preparation of bacterial suspension*

A deep-frozen culture of LGG was transferred into MRS broth (Oxoid, Basingstoke, UK) and incubated overnight at 37°C. A second MRS broth was then inoculated from the overnight

culture under adjustment of the optical density value to 0.1. determined at 600 nm (Graphicord UV-240, Shimadzu, JPN). The culture was incubated at 37°C up to until an OD value of 0.5, which corresponded to a growth stadium in the exponential phase with a cell concentration in the range of $2 \cdot 10^7 – 3 \cdot 10^7$ cfu/mL (Fig. 2).

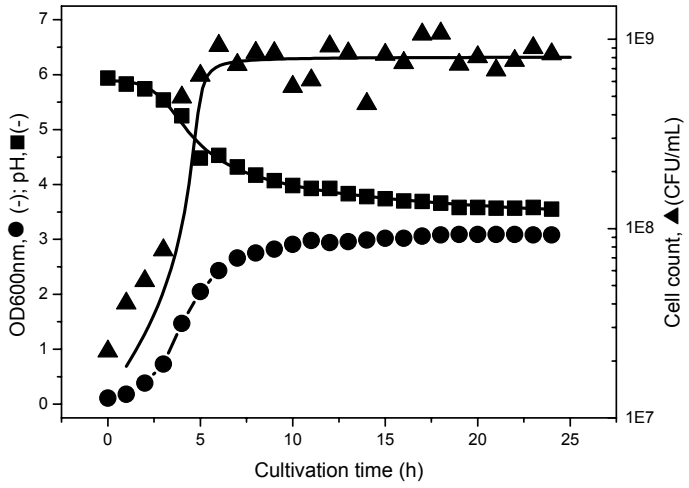


**Figure 2**

Growth behavior of *L. rhamnosus* GG as determined by 3 independent measurement methods : cell count on MRS agar, optical density (OD) of cell suspension at 600 nm, and pH of cell suspension. Cells were grown at 37°C and the initial optical density value was adjusted at 0.1.

### *4.3.3 High pressure treatment*

The high pressure unit (U111, Unipress, Warsaw, PL), as schematically drawn in Figure 3, was developed to meet the need of conducting kinetic studies up to pressures of 700 MPa and a wide temperature range between –40°C and 100°C [69]. This unit consists of five pressure chambers, which are separated from each other via high pressure valves. The chambers are immersed in a water bath equipped with a thermostat. This design allows a simultaneous treatment of five different samples in one pressure build-up step at close to isothermal conditions. Each chamber is equipped with a K-type thermocouple and a pressure sensor to monitor the temperature and pressure history of each sample during the treatment cycle.

The exponentially grown culture of LGG (OD 0.5) was filled into sample containers (Nunc Cryo Tubes Nr. 375299, Nunc A/S, Roskilde, DK) and subjected to pressure treatments at different temperatures (25 to 50°C) and various pressure level (100 to 300 MPa).

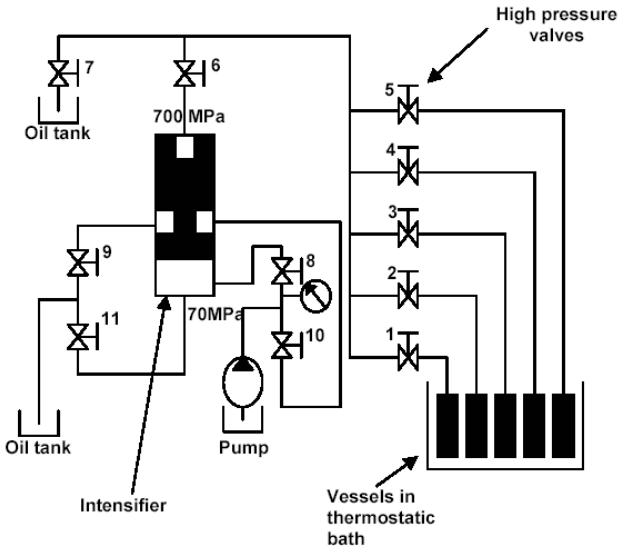


**Figure 3**

Schematic hydraulic diagram of multivessel high pressure apparatus U111. The intensifier is connected with the pressure vessels through high pressure valves (1-5). The multiplication factor (~11) of the intensifier leads to a maximum pressure of 700 MPa. Valves 6-11 are used for loading and unloading the pressure medium (silicon oil).

### *4.3.4 Assessment of growth behavior after pressure treatment*

Cells were grown according to aforementioned standard protocol to OD 0.5. The suspension was then subjected to pressure treatments for 5 min at 50, 100 and 200 MPa as well as at 100 MPa for 10 and 20 min. Only treatment temperature of 37°C was evaluated. Following pressure treatments 1 mL of treated suspension was inoculated into 50 mL of fresh MRS broth. The bottles were then incubated at 37°C. Continuous measurement of OD was performed until stationary growth phase was reached.

### *4.3.5 Lethal heat challenge at 60°C*

A pressure, temperature and time combination in the range of 100-200 MPa, 37-50°C, 5-10 min, respectively, was investigated in order to determine optimal pre-treatment conditions for the induction of heat tolerance. 0.5 mL of pre-treated and untreated sample were distributed into previously sterilized glass tubes. Cells were exposed to a temperature of 60°C in water bath for 1.5, 3, 5, 7 and 10 min. Following the heat treatment the samples were immediately stored in ice bad.

To determine the role of protein synthesis in pressure induced thermotolerance, 10 µg $mL^{-1}$ chloramphenicol was added into the treatment medium prior to pressure treatment and challenge to 60°C. In this concentration chloramphenicol is known to be not lethal to lactobacilli [23]

The pre-treatment condition was further assessed in terms of minimizing the duration of pressure treatment, so as to evaluate the possibility of pressurizing without any holding time in the manner of high pressure homogenization. Subsequent to pressure pre-treatment at with various pressure holding time (1 to 10 min) LGG cells were exposed to heat challenge at 60°C for 3 min.

### *4.3.6 Plate enumeration method*

Samples from pressure and heat inactivation experiments were serially diluted in Ringer's solution (No. 15525, MERCK, Darmstadt, D) and plated in duplicate on MRS agar without additional surface layer (Oxoid, Basingstoke, UK). Plates were placed in an anaerobic jar under anaerobic atmosphere, which was produced by an anaerobic kit (Anaerocult®A, Merck, Darmstadt, D). The viable cell numbers were determined after 48h of incubation at 37°C.
The heat inactivation data were expressed as logarithmic value of relative survivor fraction ($\log N/N_0$), which reflected the magnitude of thermal death of either pressure-treated or untreated cells at 60°C. N refers to the bacterial count following heat challenge at a particular holding time, whereas $N_0$ represents the initial count prior to the exposure to heat. Heat inactivation experiments were performed at least in two replicates. Data were analyzed and plotted with use of Origin7 software package (OriginLab, Northhampton, MA, USA).

### *4.3.7 Mathematical description of heat inactivation kinetics*

First order inactivation kinetics was used to describe the linear part of inactivation curves of heat treated LGG. The onset of the linear inactivation phase was fixed 1.5 min after the treatment was started, since inactivation-free phase during the first 1.5 min was observed. The calculated negative slope of the linear survivor curve (k) represented the contribution of a particular combination of pressure and temperature pre-treatment conditions to induction of heat tolerance. All calculations were performed using Origin7 software package (OriginLab, Northhampton, MA, USA). A three-dimensional grid of derived inactivation rate (k) for the 3D graph was constructed using *Plot It* software package (Scientific Programming Enterprise, Haslett, MI, USA). The final 3D plot describing the simultaneous dependence of inactivation rate on applied pressure levels and temperatures during pre-treatment step was drawn using TableCurve 3D software (Systat Software Inc, Richmond, CA, USA).

### *4.3.8 Staining procedure with LIVE/DEAD® BacLight™ Bacterial Viability Kit*

The LIVE/DEAD®BacLight™ bacterial viability kit (Molecular Probes Europe BV, Leiden, NL) consisted of two separate stock solutions of SYTO9® and PI. According to the information

given by manufacturer, both of them were dissolved in dimethyl sulfoxide at high concentrations: 3,34 mM SYTO9® and 20 mM PI.

Bacterial sample (15 to 20 µL portions) were added into 1 mL of distilled and previously filter-sterilized water. This bacteria suspension was then incubated for 10 min with 1,5 µL of each of SYTO9® and PI in the dark prior to the measurement.

### *4.3.9 Flow cytometric measurement and data analysis*

Analysis was performed on a Coulter®EPICS®XL-MCL flow cytometer (BeckmanCoulter Inc., Miami-FL, USA) equipped with a 488 nm laser. Cell was delivered at the low flow rate, corresponding to 400 to 600 events per s. Forward scatter (FS), sideward scatter (SS), green (FL1) and red fluorescence (FL3) of each single cell were measured, amplified, and converted into digital signals for further analysis. SYTO9® emits green fluorescence at 530 nm following excitation with laser light at 488 nm, whereas red fluorescence at 635 nm is emitted by PI-stained cells.

A set of band pass filters of 525 ± 20 nm and 620 ± 15 nm was used to collect green fluorescence (FL1) and red fluorescence (FL3), respectively. All registered signals were logarithmically amplified. A gate created in the dot-plot of FS vs SS was preset to discriminate bacteria from artefacts. Data were analysed with the software package Expo32 ADC (BeckmanCoulter Inc., Miami-FL, USA).

Dot plot analysis of FL1 vs FL3 was applied to resolve the fluorescence properties of the population measured by flow cytometer (Fig. 4). With this graph the population was able to be graphically differentiated and gated according to their fluorescence behaviours.

Two regions were created in this plot for gating cells with intact membrane and the ones with ruptured membrane.

The designation of gates according to the properties of cellular membranes was performed by means of measuring fluorescence dot plot signals of untreated cells, which were located in gate LIVE. On the other hand, cells heat treated at 95°C for 10 min were entirely encountered in the area surrounded by gate DEAD.

### *4.3.10 Statistical analysis*

The Student's t-test was applied to evaluate the impact of pressure treatment on viability. Differences were considered significant at the $p < 0.05$ level of probability. Statistical significance of the effect of different pressure treatments on post-pressure growth characteristics was examined using ANOVA-test. Statistical analysis was performed with Origin7 software package (OriginLab, Northhampton, MA, USA).

## 4.4 Results and discussion

### *4.4.1 Heat inducible thermotolerance of L. rhamnosus GG*

Prior to the trials related to pressure induced thermotolerance on LGG the heat shock response of the organism was evaluated so as to ensure that this inducible cellular defensive mechanism could be detected and quantified with the described heat challenge procedure. The machinery of heat inducible thermotolerance on LGG was investigated on exponential and stationary growth phase using MRS broth as treatment medium in order to assess its growth-phase specificity.

When cells from stationary growth phase were used, no significant differences were observed in the inactivation kinetics of pre-incubated and control cells (Fig. 4A). It is a well-known phenomena that cells in stationary growth phase have already possessed greater inherent stability against severe treatment conditions owing to either accumulation of protective compounds or exposure to various stresses in growth medium including starvation, low pH, etc. [7, 70, 71]. In this state only negligible additional heat resistance could be evoked.

With an incubation step of cells from exponential growth phase at 50°C for 10 min prior to exposure to lethal temperature at 60°C for 10 min the thermal stability of LGG could be increased in contrast to control sample (Fig. 4B). The lethal temperature of 60°C for heat challenge was adapted from studies made with other probiotic lactobacilli [10, 20, 24]. Since thermal inactivation kinetics at 60°C was followed it could be further observed, that the protective effect resulting from heat shock diminished at prolonged exposure to 60°C. No difference can be obtained in the inactivation level of pre-treated and untreated cells. This behaviour can be attributed to predominating thermal damage that was exceeding the magnitude that can be overcome by the acquired cellular repair mechanism, in which the synthesized heat shock proteins may play a major role.

A lethal temperature of 60°C seemed to be suitable to be applied for the substantiation of pressure induced thermotolerance and was applied throughout the study due to the clear tendency in differentiating untreated and pre-treated population as well as due to a sufficient reduction of cell count within 10 min of treatment.

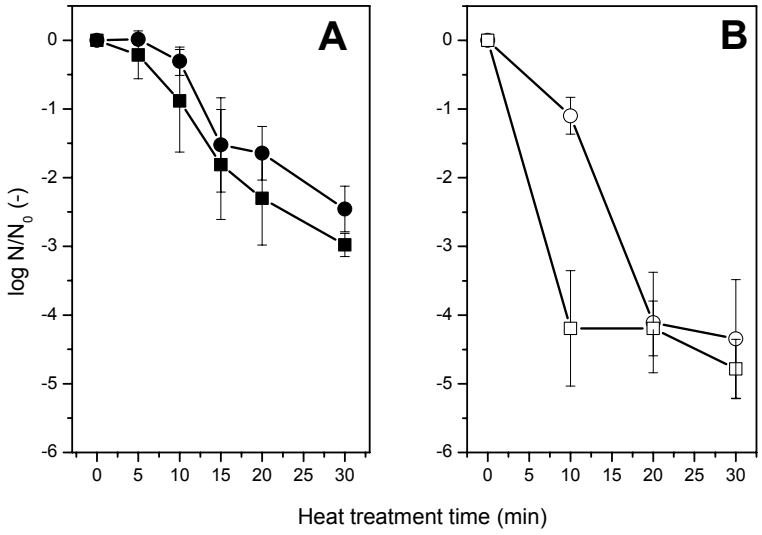


**Figure 4.**

Effect of heat shock treatment at 50°C for 10 min on the survival of *L. rhamnosus* GG from stationary (A) and exponential growth phase (B) against subsequent thermal challenge at 60°C. Treatment media were MRS broth. Heat shocked cells and untreated cells are represented by circles and squares, respectively. Data are means of at least three independent trials and error bars represent standard deviations.

### *4.4.2 Identification of non-lethal pre-treatment condition*

Cells from the exponential growth phase were pressure treated to determine the suitable pre-treatment conditions, in which stress-related thermotolerance could be evaluated. In general, the applied pre-treatment step may not exert any lethal effect on the adapted cells. Obviously, in excess of 200 MPa, inactivation of the cells occurred (Fig.5). Beneath this critical point, no significant loss ($p > 0.05$) of viability following pressurization was observed.A maximal pressure-temperature combination of 200 MPa and 50°C was then identified as upper limit. Beneath this critical point, no significant loss ($p > 0.05$) of viability following pressurization was observed. Within the range of conditions investigated the duration of pressurization (at pressures lower than 200 MPa) did not impair the viability.

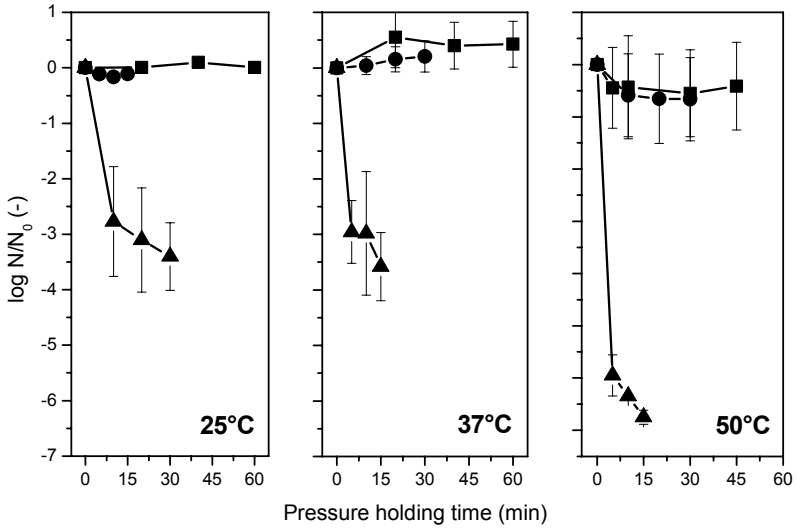


**Figure 5**

High pressure inactivation kinetics of exponentially grown *L. rhamnosus* GG cells at 100 MPa (■), 200 MPa (●), and 300 MPa (▲). For this experiment cells were growth at 37°C until OD 0.5 was reached. Treatment media were MRS broth. Initial cell count was approximately $10^7$ CFU/mL.

Pressure inactivation data were expressed as logarithmic value of relative survivor fraction (log $N/N_0$) at increasing pressure holding time. N refers to the bacterial count following pressurization at corresponding holding time, whereas $N_0$ represents the initial count prior to the exposure to pressure.

The temperature levels (25, 37 and 50°C) as indicated in the figure refer to the applied temperatures during pressure treatment. Data shown are means of at least two independent measurements.

### *4.4.3 Post treatment growth behaviour of L. rhamnosus GG*

To investigate post-pressure physiological activity, the growth behaviour of pressure treated cells was monitored by means of the measurement of the changes in the optical density of the cell suspension (Fig. 6). Pressure treatments were conducted at a constant temperature of 37°C. The effect of different pressure levels (50, 100, 200 MPa) at a constant holding time on growth behaviour was evaluated. Moreover, the impact of different pressure holding times (5, 10, 20 min) at a constant pressure level (100 MPa) on the growth behavior was characterized.

According to Fig. 6b, at low pressure level ($p \leq 100$ MPa) and short holding times ($t \leq 10$ min) the OD-values of the cells at these intensities could not be significantly differentiated from untreated population.

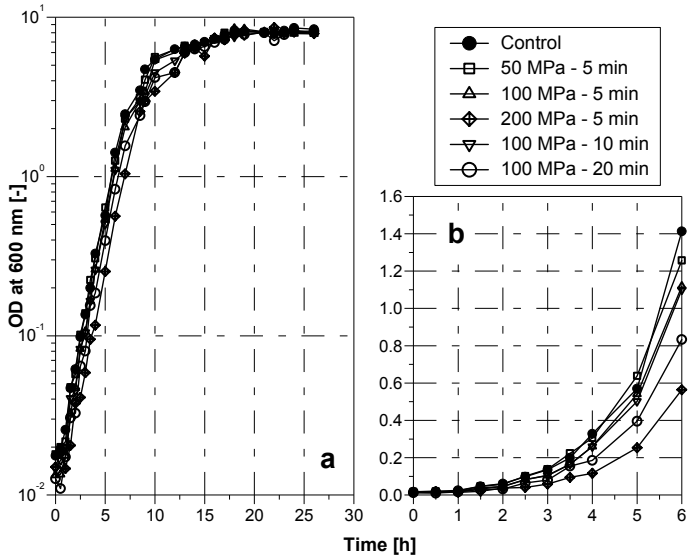


**Figure 6**

Monitoring of the growth behaviour of LGG after pressure treatment at various intensities. Parameters for pressure treatment are indicated in the legend. All treatments were performed at 37°C.

Fig. 6a showed the increase of the optical density (OD) of the pressure treated cells. In this figure the y-axis is logarithmically scaled. Fig. 6b resulted from the same experiment, but only exhibited the OD changes within the first 6 h of incubation at 37°C. The y-axis in Fig 6b is arithmetically scaled.

Detailed experimental procedure is explained in the section Material and Methods. Data shown are means of at least two independent measurements.

Apparently, at higher intensities of pressure treatments (higher pressure levels, i.e. 200 MPa, 5 min and longer duration, i.e 100 MPa, 20 min) a significant difference in the rate of OD-change to the less severely treated groups ($p < 0.05$) was obvious. Although such treated populations were not lethally affected by pressure (Fig. 5) and indeed able to resume growing and reached the stationary growth phase as the control group did, they showed a rather retarded growth at initial growth phase ($t < 6$ h).

The sigmoidal wth curves was fitted with modified Gompertz equation, which is expressed by Equation 1. With this model the characteristic parameters of the growth curves, i.e duration of lag phase and the $\mu_{max}$ (maximum change in the $OD_{600\ nm}$ ) could be derived.

$$y = A \cdot \exp\left\{-\exp\left[\frac{\mu_{\max} \cdot \exp(1)}{A} \cdot (\lambda - t) + 1\right]\right\} \qquad \text{Equation 1}$$

y: optical density at 600 nm; A: max. optical density; $\mu_{max}$: max. change in the optical density, $\lambda$: duration of lag phase

The growth parameters derived from the development of OD-values after pressure treatments using Eq.1 are listed in Table 1. From this table it is obvious that with increasing

pressure the cells required longer time to recover from the pressure-induced sub-lethal injury prior to entering the exponential growth phase. Moreover, the growth rates of pressure treated cells were generally reduced compared to the untreated ones.

**Table 1**

Growth parameters derived from modified Gompertz equation

| Pressure (MPa) | Time (min) | $\mu_{max}$ ($h^{-1}$) | $\lambda$ (h) | Fit Standard Error |
|---|---|---|---|---|
| 0 | 0 | 0.853 | 4.19 | 0.267 |
| 50 | 5 | 0.862 | 4.47 | 0.288 |
| 100 | 5 | 0.824 | 4.64 | 0.345 |
| 100 | 10 | 0.82 | 4.76 | 0.15 |
| 100 | 20 | 0.794 | 5.26 | 0.254 |
| 200 | 5 | 0.791 | 5.62 | 0.324 |

By means of flow cytometric assessment combined with LIVE/DEAD®*BacLight*™ Bacterial Viability Kit (Molecular Probes, Leiden, NL) it was possible to obtain insights in the nature of the cellular injury occurred during pressure treatment, especially when higher treatment intensities were applied. Following a pressure treatment at 200 MPa the occurrence of cell population with a higher degree of membrane damage was observed (data not shown). From these results it could be concluded that a prolonged exposure to sub-lethal stress conditions as well high pressure level may not be beneficial for the fitness of the culture. Therefore, the treatment conditions have to be optimized in a way, that increased stress tolerance could be achieved; on the other hand overprocessing which leads to higher cellular damage, has to be avoided.

### *4.4.4 Heat treatment at 60°C*

Figure 7 shows heat inactivation kinetics at 60°C of exponentially grown LGG cells. Pressure level and temperature during pre-treatment step were varied to allow better insight of the role of each process parameter on induction of thermotolerance. Upon exposure to 60°C for 5 min, cells survived better by 1.5 log-cycles in comparison to control group, when they were previously pressure treated at 100 MPa and 37°C for 10 min (Fig 7A). Induction of thermotolerance could therefore be achieved at normal growth temperature by merely elevating system pressure for 10 min. Taken the data from previous observation on post-pressure growth of cells exposed to 100 MPa for 20 min into account, it is not necessary to subject the LGG cells for a long period since a relatively short pre-treatment time can definitely confer protection against lethal effect of heat.

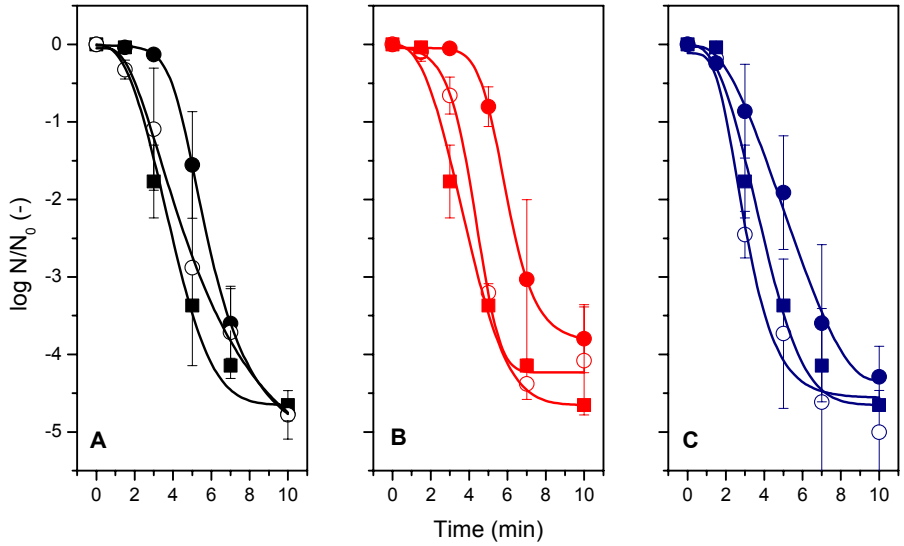


**Figure 7**

Heat inactivation curves at 60°C of LGG, which were previously pre-treated for 10 min at 100 MPa (●) and 200 MPa (○) in comparison with pressure-untreated population (■).

The denotation of the three figures with A, B, and C corresponds to the temperature levels (37, 43, and 50°C, respectively) applied during pressure pre-treatment.

In case of pressure-treated cells $N_0$ was cell count after pressure treatment. Data shown are means of at least two independent measurements.

Such improvement against lethal effect of heat could also be observed using pre-treatment at higher temperatures; however thermal reduction of bacterial load was more pronounced the higher the applied pre-treatment temperatures were (Fig. 7B and 7C). Doubling the pressure level at the adaptation step to 200 MPa was found to be less effective in provoking pressure induced thermotolerance (Fig. 7A to 7C).

To allow a better assessment of the effect of selected pre-treatment processing parameters, a mathematical modelling of the linear part of inactivation curves of heat treated LGG was performed. Figure 8a and 8b show the simultaneous dependency of heat inactivation rate (k in $min^{-1}$) at 60°C on applied pressure and temperature of adaptation step at two different pressure holding times, i.e. 5 and 10 min, respectively. The k-values were calculated according to the analysis method described in the section Material and Methods and are characteristic for each heat inactivation curves (Regression parameters in Annex 8). Furthermore, a second mathematical model based on Weibull's distribution was also used to fit the experimental inactivation data (Annex 8).

A 3D diagram was constructed from the derived inactivation rates. This plot enables a better comparison of heat protection efficacy of different pre-treatment processing parameters.

From this plot, optimal pre-treatment process conditions, which ensure minimal loss of viability during heat exposure, could be directly identified.

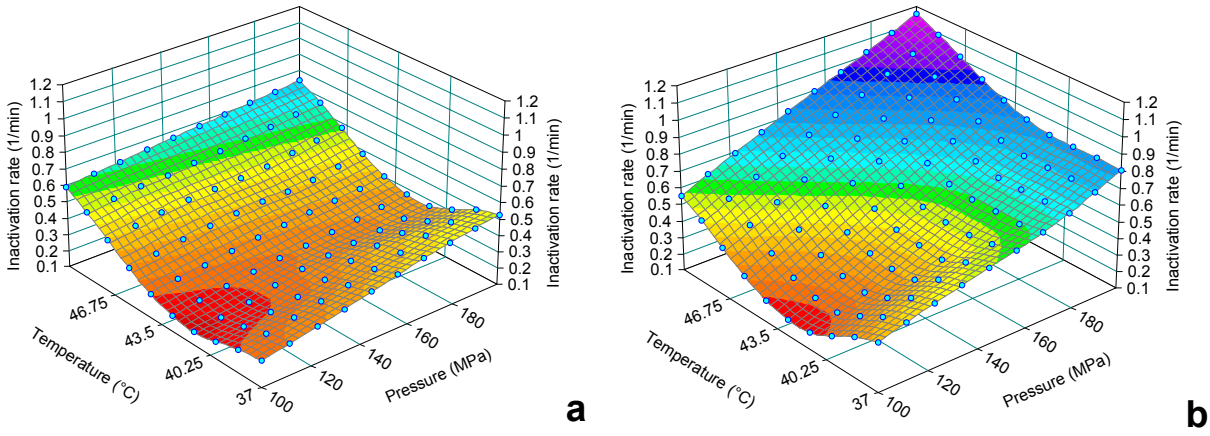


**Figure 8.**

Simultaneous dependency of heat inactivation rate of *L. rhamnosus* GG at 60°C (k in $min^{-1}$) on applied pressure and temperature in adaptation step. In Fig. 8a and 8b. the holding time during pressure application was fixed to 5 and 10 min, respectively.

Inactivation rates were obtained by calculating the slope of the linear part of the corresponding inactivation curve (see Fig 7. and explanation in the section Material and Methods).

According to the aforementioned curve analysis, the calculated k-value of the control sample was 0.996 $min^{-1}$, whereas the k-values of pre-treated samples in the applied p,T-conditions ranged between 0.199 and 1.154 $min^{-1}$. A commonality that was shared by both evaluated pressure holding times is that a local minima of heat inactivation rate was observed at pressures and temperatures as high as 100-125 MPa and 42-43°C, respectively. It was also evident from both plots, that at 200 MPa and 50°C, the protective effect of pressure treatment was at its minimum.

From this work it could be concluded that incubation of LGG at elevated pressure of 100 MPa for 5 - 10 min prior to exposure to lethal heat at 60°C led to an increasing heat resistance as compared to untreated population. Optimal working range to induce thermotolerance properly was determined at 100 MPa and 37 - 43°C. Regarding the duration of pressure holding time, pressurization up to 10 min could effectively trigger thermotolerance mechanism, when operating at these optimal conditions. These apparent optima at both pressure holding time could also be identified in the 3D plot generated under application of Weibull distribution (Annex 8).

Working at higher temperature and pressure, despite of the demonstrated possibility to induce thermotolerance, was less effective; presumably due to higher extent of injury. This

might in turn exceed the magnitude, which could still be tolerated by the cells in order to instantly regenerate after pressure release and trigger thermotolerance-conferring reactions.

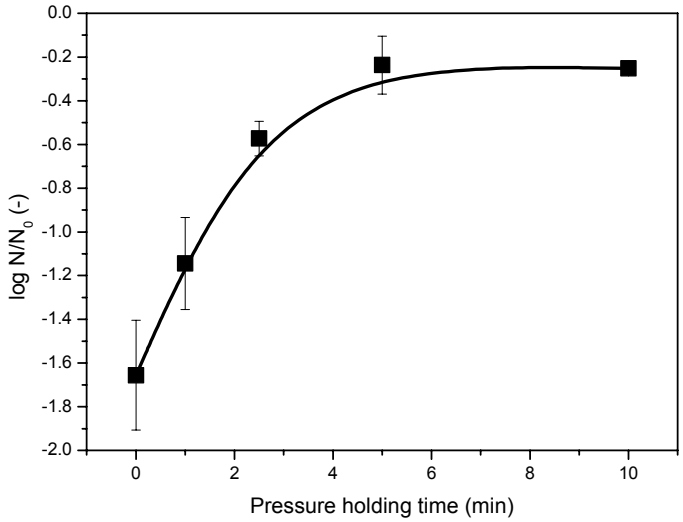


**Figure 9**

Effect of pressure holding time in the pre-treatment phase on the survival of *L. rhamnosus* GG during subsequent thermal challenge at 60°C for 3 min. Pre-treatment step was conducted at 100 MPa and 37°C. N and $N_0$ refer to cell count before and after heat treatment, respectively. Data represents means of two independent experiments.

The acquisition of thermotolerance can still be achieved by exposing the cells to high pressures for 5 min. In terms of optimizing pre-treatment step further trials were focused on the reduction of pressure pre-treatment time. In particular it is of huge interest to check whether extremely short exposure to pressure, or “flash adaptation,” could still induce tolerance in bacteria. For this evaluation the thermal challenge at lethal temperature of 60°C was not performed in form of a kinetic. Instead the cells were exposed for a fixed treatment time of 3 min, since after 3 min of holding time at 60°C good differentiation in the effect of pre-treatment on survival rate could be made (Fig. 7).

Figure 9 shows the effect of variation of pressure holding time on the resulted thermal survival rate at 60°C for 3 min. It was obvious, that there exists a threshold treatment time of 5 min, below of which the pressure-induced thermotolerance gradually decreased. Pressure-shock response, which is ultimately manifested in increase of tolerance, ranged in minutes and was relatively quick. This is similar to data on the time required to initiate heat shock response [64]. In contrast it took hours to initiate cold shock response [72]. As reviewed by Yura *et al* (2000) a modest temperature upshift (ΔT~ 12°C) elevated the synthesis of heat shock proteins almost immediately and reached a maximum induction (10- to 15-fold) within 5 min, where heat shock proteins represented over 20% of total proteins synthesized [26].

Furthermore, the rapid induction is followed by a gradual decrease, during the adaptation phase, to attain a new steady-state level (2- to 3-fold of the pre-shift level) by 20 to 30 min.
With regards to the onset of pressure shock response a proteomic study on heat shock response of *L. lactis* documented an elevated rate of synthesis of fast-induced proteins (such as DnaK, GroEL, GroES) during the first 10 min of exposure to sub-lethal heat stress [15]. Walker *et al* (1999) also reported that the maximum *groESL* transcription activity was increased following exposure to sub-lethal temperature and a 15 to 30 min exposure of log-phase cells to this temperature increased the recovery of freeze-thawed *L. johnsonii* [11]. All of these studies indicated the necessity to expose the cells to stress condition for a sufficient holding time in order to allow the cellular process of protein synthesis to be accomplished. In this context, the determination of optimum sub-lethal stress conditions (temperature, time, etc.) for RNA expression over a stress operon can further facilitate monitoring the heat shock induction of the *groESL* chaperone operon, since stress protection was found to correlate with the timing and the level of expression of the chaperone operon [11]. The idea of applying rapid pressure processing, in which cells were only subjected to a single pulse of hydrostatic pressure without any holding time, seems to be inappropriate to allow sufficient protection against heat.

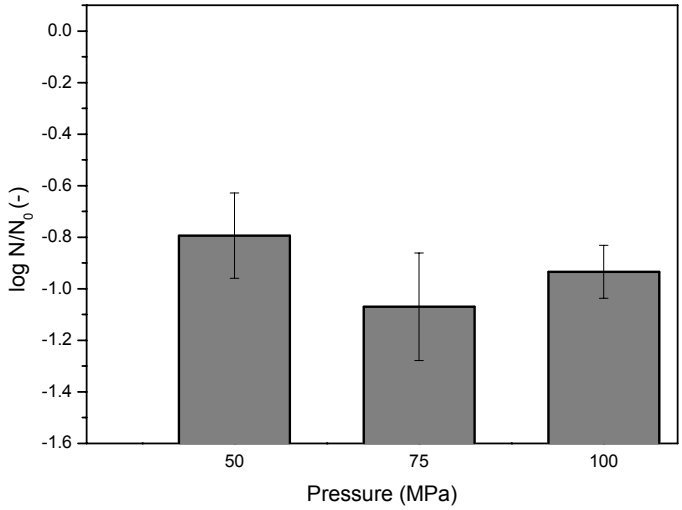


**Figure 10**

Influence of adjusted pressure levels on the survival of *L. rhamnosus GG* during high pressure homogenization process (MicronLab 40, APV Gaulin GmbH, Lübeck, Germany). Bacteria were grown to an OD of 0.5 in MRS broth and subjected to one cycle of homogenization. $N_0$ and N refer to bacterial count before and after homogenization step, respectively. Data represents means of two independent experiments.

In contrast, an extremely short exposure to sub-lethal of bile salt induced tolerance in *Enterococcus faecalis* [73]. This is the first work reporting the kinetics of induction of

tolerance against lethal concentration of bile, which could already be observed only after 5 s exposure to 0.08% bile and reached its maximum after 30 min incubation. Interestingly, the development of bile salt tolerance is not at all compromised by the blockage of protein synthesis prior to challenge in adapted *E. faecalis* cells.

Trials made on a similar rapid process, i.e. high pressure homogenization show that even during the homogenization step a reduction by one log cycle was obtained (Fig. 10). This process is commonly used for cell disruption of concentrated microbial cultures, and the subsequent recovery of intracellular metabolites. Cell death during passage through high pressure homogenizer is mostly related to turbulence, shear, cavitation – which are also the main physical causes for fat globule disruption –, and heat generation, raising the temperature by 2.5°C per 10 MPa [74].

### *4.4.5 Determination of the role of* de novo *protein synthesis in inducible heat tolerance*

Works dealing with the application of mild heat shock also demonstrated that the expression of certain proteins at higher levels were required for the acquisition of thermotolerance [16, 19, 23]. The role of protein synthesis was elucidated by incorporating a bacteriostatic concentration of chloramphenicol during adaptation, in consequence of which the tolerance against lethal heat decreased markedly. Determination of the N-terminal sequence of a series of these proteins reveals that these proteins are involved in a variety of cellular processes [16]. The majority of these proteins are homologous or immunologically related to stress proteins in other microorganisms [13, 14]. Several of the proteins identified belong to the group of molecular chaperones (e.g. DnaK, GroEL, etc.) or ATP-dependent proteases (ClpP). The induction of this type of proteins forms a highly conserved response to heat stress. These proteins are termed heat shock proteins and are involved in protein folding, assembly, and repair and prevention of aggregation under stress and normal conditions [64]. 2D-PAGE combined with densitometric analysis revealed that the chaperone protein GroEL was among the most strongly expressed proteins in the cell under heat adaptation conditions [20] and after heat shock the GroEL synthesis was reported to be 15-fold higher than the pre-shift level [18]. However, tolerance of GroESL-overproducing strains against heat, spray drying and freeze drying was not necessarily better than heat-adapted parent strain [20], since heat adaptation response that leads to an elevated state of cell resistance does not only rely on the overexpression of heat stress proteins, but may be due to the mechanisms associated with stress proteins, changes in the synthesis of some glycolytic enzymes and other stationary-phase-related proteins and regulatory factors [18].

According to Figure 11, the magnitude of cell death as a result of exposure to lethal heat could be reduced when pressure adaptation was applied on the cells prior to heat challenge.

Pressure treated cells (log $N/N_0$ ~ 0) were significantly more heat resistant than untreated populations (log $N/N_0$ ~ -1.5) after 3 min of exposure to 60°C. The inactivation-free phase was longer in the pressure pre-treated sample (3 min) compared to the control one (1.5 min). The survival curves started with a shoulder followed by a linear decline curve. Mechanistic approaches had been made to explain factors accounting for the lag phase, in which the concentration of microorganisms remained the same. Either the existence of clumps of microorganisms, the ability of the cells to resynthesize a vital component, cumulative thermal inactivation rather than instantly lethal, or multiple target sites for thermal inactivation were made responsible for the generation of lag phase [75]. Only after this limiting factor had been eradicated, the inactivation of microorganisms could follow first order kinetics in the linear phase. When the distribution of resistance within the population to be treated was considered, a distributive function combined with a first-order reaction describing subsequent linear inactivation kinetics can explain the initial transition of bacterial cells into an inactivation free, metastable intermediate state, which is reached after endogenous homeostatic mechanisms balancing displacement of equilibrium can no longer be maintained [76].

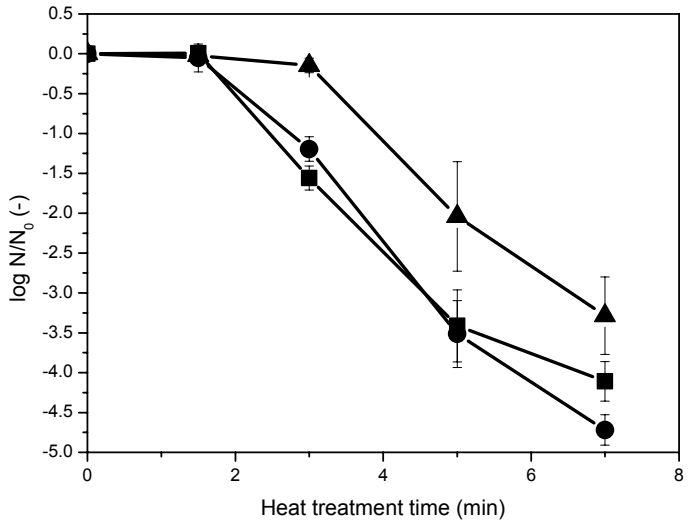


**Figure 11**

Effect of heat treatment at 60°C on the residual survival fraction (log $N/N_0$) of exponential-phase control cells (■) and that of cells pretreated for 10 min at 37°C 100 MPa in the absence (▲) and presence of 10 µg/mL chloramphenicol (●). Data are means of at least three independent experiments.

Moreover, the heat inactivation curve of pressure pre-treated LGG with chloramphenicol being incorporated in the medium resembled the one of control population (Fig. 11). This result points out that thermotolerance acquired after a mild pressure shock highly depends

on protein synthesis. The addition of chloramphenicol in the cell suspension prior to pressure adaptation blocked protein synthesis so that increased heat tolerance disappeared. Similar to heat shock response, which implicates the synthesis heat shock proteins in the induction of heat tolerance [16, 19, 23], a brief adaptation period under elevated pressure could enhance their heat tolerance as well. Indeed, it has been shown that heat shock proteins were among the ones overexpressed upon brief or continuous exposure of bacterial cells to moderate pressure levels [41, 43, 46]. This result underlines once more the universal importance of heat shock proteins in coping with various adverse conditions.

Taking the lag phase of pressure pre-treated sample prior to the linear inactivation phase was longer than that of untreated one (Fig. 11), it is most likely that the newly synthesized proteins were involved in cell repair mechanism, which temporarily led to a higher stability against heat. The induced tolerance against heat vanished after 3 min of exposure to 60°C; presumably because the rate of destruction of crucial bio-molecules exceeded the rate of repair/resynthesis of vital components [75].

### *4.4.6 Flow cytometric assessment of damaged on cellular membrane as affected by heat*

The applicability of the commercially available LIVE/DEAD®BacLight™ bacterial viability kit has been evaluated on a wide spectrum of bacteria [77-82]. This kit was developed to differentiate live and dead bacteria based on plasma membrane permeability. The staining mechanism using LIVE/DEAD kit on bacterial cells is based on the attachment of the non-fluorescent agents on nucleic acids. Once the DNA-dye complex is built fluorescence could be measured. This kit is constituted of two fluorochromes, which have distinct fluorescent behaviour in terms of emission wavelengths and membrane permeability. The first component is the membrane-permeant stain SYTO9®, which fluoresces green at 530 nm upon excitation at 488 nm. It stains all cells, thus acting as total cell stain. During cell death, accompanied with membrane damage, the second dye, the membrane-impermeant dye propidium iodide (PI) penetrates into cells and quenches the green SYTO9® fluorescence. PI is able to be excited at 488 nm as well and emits red fluorescence at 620 nm. When used in combination, intact cells are labeled green and cells with damaged membranes are labeled red.

According to flow cytometry data, exposure to 100 MPa did not affect fluorescence behavior of LGG (Fig. 12B), when compared to the one of control group (Fig. 12A). The presence of chloramphenicol in the media during pressure adaptation step did not cause detrimental effect on cellular membranes either (Fig. 12C).

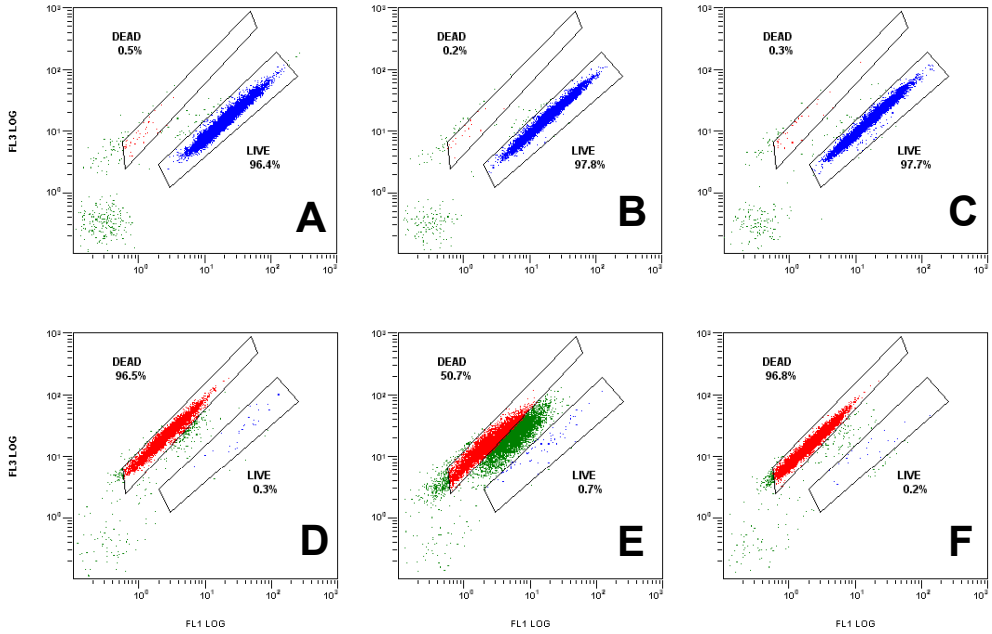


**Figure 12**

Flow cytometry dot plots of FL1 (fluorescence collected at 525 nm) vs FL3 (fluorescence collected at 620 nm) of LGG to assess the effect of heat treatment on the integrity of cellular membranes.

Two gates were fixed for discrimination of two distinct extreme states of membrane conditions, i.e. intact and completely ruptured (gate LIVE and DEAD, respectively).

Figure 12A, 12B and 12C show fluorescence behaviour of control population and population pressurized at 37°C 100 MPa for 10 min with and without chloramphenicol, respectively.

Heat treatment at 60°C for 3 min was applied to control population (D), and pressure pretreated cells (E). Pressure pretreatments at 37°C 100 MPa for 10 min were conducted in the absence and presence of chloramphenicol (E and F, respectively).

Once the cells were subjected to 60°C for 3 min a higher degree of membrane rupture was able to be detected in the dot plot FL1-FL3 (Fig. 12D to F). The onset of this cellular damage was marked by characteristic population shift from gate LIVE, where cells with intact membranes and stained by SYTO9® were located, towards gate DEAD, in which cells with completely disintegrated membranes and stained by PI were encountered. During exposure to heat structural changes in proteins and membranes may lead to cell death. Heat treatment at temperatures in the vicinity of 60°C was reported to cause damage in the cytoplasmic membrane of *L. bulgaricus*, whereas for temperature of 65°C and immediately above, ribosomes and/or proteins denaturation as well as cell wall damage may be responsible for thermal death [83]. Using fluorescence marker aimed on cells with compromised membrane the flow cytometric fluorescence data presented in this study underlined the findings of the work of Teixeira *et al* (1997) about the deteriorative action of heat at 60°C on cytoplasmic membrane. Furthermore, when Figure 12D and E are compared it is obvious that the extent

of the shift of SYTO9®-labeled, viable cells toward the population labeled with PI was reduced, when prior to exposure to lethal heat at 60°C for 3 min, the cells were incubated at 100 MPa and 37°C for 10 min (Fig 12E). Pre-treatment at elevated pressure level seemed to confer protection against destructive effect of heat on cellular membrane. The acquired membrane stabilization was documented by a less pronounced shift of the cells towards gate DEAD, which may be related to a reduced influx of PI across cytopasmic membrane. On the other hand, the presence of chloramphenicol diminished the membrane stabilization effect of pressure (Fig. 12F). The pattern of population shift from gate LIVE towards gate DEAD was found to be similar to the one of untreated population despite of application of pressure pre-treatment. Similar to the results obtained in the previous sub-section this observation is indicative for the decisive role of *de novo* protein synthesis as a consequence of pressure pre-treatment in withstanding degradative events on cell membrane upon heat treatment at 60°C.

Some works dealing with the preferential localization of heat shock proteins in cellular membrane could give some hints about their functionality. It was reported that after a sub-lethal heat treatment of cyanobacterial cells, an increase of the membrane-associated GroEL fraction was observed concomitantly with an increase in the heat stability of the photosynthetic electron transport machinery [84]. In particular, the soluble chaperonin GroEL from *E. coli* has high affinity for model lipid membranes, and the conserved C terminus of GroEL is involved in membrane binding. Apart from the role in membrane stabilization, GroEL may also function as lipochaperonin that can prevent the irreversible thermal aggregation and assist the refolding of membrane proteins. Moreover, water-soluble proteins could also be rescued by lipochaperonins under stress conditions. This suggests that, during stress, chaperonins can assume the functions of assisting the folding of both soluble and membrane-associated proteins while concomitantly stabilizing lipid membranes [85]. Similarly, it was found from a study on cellular localization of GroEL in *Clostridium difficile* using immunoelectron microscopy that after heat shock at 48 °C GroEL was distributed in a relatively uniform fashion over the bacterial surface and was partially also found to be localized within the extracellular space [86]. Furthermore, genomic expression pattern of *S. cerevisae* exposed to 200 MPa for 30 min showed the expression of genes coding proteins with molecular mass less than 20 kDa that present putative transmembrane domains [62]. An increase in the expression in the expression of small membrane binding proteins was assumed to be involved in the protection against membrane damage.

### *4.4.7 Pressure induced tolerance against nisin and bile acid*

As already mentioned in the previous sub-sections, the contribution of pressure-induced protein biosynthesis in the enhancement of bacterial heat tolerance was found to be crucial,

since the presence of chloramphenicol in the treatment medium hampered the effect of pressure adaptation on inducible tolerance against heat. Furthermore, the role of proteins expressed during pressure adaptation in withstanding the thermal degradation on cell membrane had been characterized by flow cytometric assessment.

These facts led to further question, whether pressure adapted cells also showed improved tolerance against other membrane-degrading agents, such as bile acid (B8381, Sigma-Aldrich, Munich, DE) or nisin (Nisaplin®, Danisco, Copenhagen, DK).

*Bile* is a digestive secretion that plays a major role in the emulsification and solubilization of lipids. These 'biological detergents' are synthesized in the liver from cholesterol, conjugated to either glycine or taurine, and then secreted as amino acid conjugates into the intestine where they facilitate fat absorption. Bile is primarily composed of bile acids (12% by weight), which are found as sodium salts under physiological conditions. Forming part of the body's physicochemical defense system, bile salts possess potent antimicrobial activity and have the ability to dissolve the phospholipids, cholesterol, and proteins of cell membranes. They disorganize the lipid bilayer structure of the cellular membranes, thus causing cells to lyse [87]. Analysis of *Enterococcus faecalis* susceptibility towards the bile salts gave evidence for an extremely rapid killing effect which is attributed to the solubilization of membrane components [88]. An improved bile tolerance is essential for probiotic bacteria since they need not only to survive processing conditions but also harsh environmental challenge during gastrointestinal passage. Especially to achieve high degree of gut colonization, these bacteria have to interact with inhibitory host-produced substances.

It was observed, that bacteria pressure pre-treated prior to exposure to 1%, w/v bile acid showed an improved tolerance against this antimicrobial compound (Fig.13a). This result confirmed the contributive role of protein biosynthesis during pressure adaptation in protecting cellular membranes. With regards to induced bile tolerance the pressure shock proteins can presumably be functionally grouped into bile salt hydrolases, which are known as detergent shock proteins that protect the bacteria that produce this enzyme from the toxicity of bile acids in the gastrointestinal tract [89].

*Nisin* is a protein with 34 amino acid residues which is produced by *L. lactis* subsp. *lactis*. This antimicrobial peptide has a broad activity spectrum and is active against a variety of gram-positive bacteria. It has been shown that nisin permeabilizes the cytoplasmic membrane, thereby dissipating the membrane potential, inhibiting transport of amino acids, and causing release of accumulated acids from cells and membrane vesicles from various bacteria [90]. Models for nisin/membrane interactions propose that the peptide forms poration complexes in the membrane through a multi-step process of binding, insertion, and pore formation [91].

A higher survival in the presence of 12.5 mg $L^{-1}$ nisin was obtained, when LGG were previously pressure pre-treated (Fig 13b). The applied nisin concentration was slightly lower than the one recommended by manufacturer for typical use in food. This result emphasizes once more about the structural/functional modifications in the cellular membrane of pressure adapted bacteria, which allow them to counteract the pore-forming activity of nisin and thus reduce its lethality on bacteria.

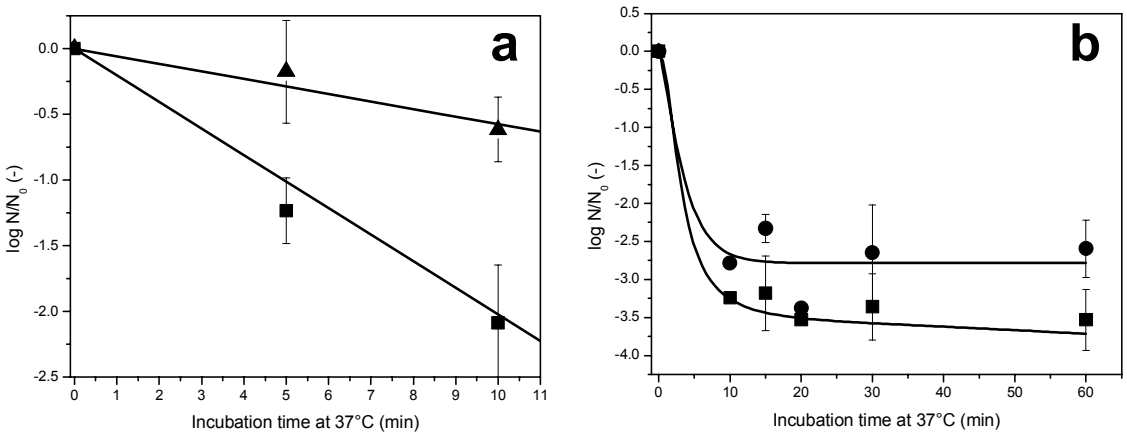


**Figure 13**

Effect of pressure pre-treatment on the survival of *L. rhamnosus* GG in the presence of bile acid (a) or nisin (b). Pressure pre-treated (▲,●) and untreated cells (■) were incubated in growth medium supplemented with bile acid (1%, w/v) or nisin (12.5 mg $L^{-1}$ ). Pressure pre-treatment condition was 100 MPa, 37°C, 10 min. Data were means of at least three independent trials and error bars represented the standard deviations of the means.

### *4.4.8 Spray drying of pressure pre-treated bacteria*

The potential of pressure pre-treatment at 100 MPa was assessed on its impact on the survival of LGG during spray-drying. Pressure adaptation on LGG was done in their own growth media, whereas reconstituted skim milk (20%, w/v) was used as the drying medium. In a related study the drying medium was the one also used to grow the bacteria, i.e. reconstituted skim milk [24]. This was not possible for LGG due to their inability to ferment lactose [92]. Preliminary results showed that pressure adaptation improved the survival of LGG during spray-drying at outlet temperatures of 80 and 90°C (Fig. 14). The survival rate of pressure-treated cells was as high as 32%, whereas spray-drying of untreated population resulted in only 14% survival (unpublished data). Due to the predominant heat damage which exceeded the capacity of pressure induced proteins in conferring protection the pressure induced increase of survival rate could not be quantified during spray drying at an outlet temperature of 100°C. The level of improvement achieved in the present study was in the same range as obtained in the work of Teixeira *et al* (1995), where they showed that heat

shock at 50°C for 30 min on exponentially growing cells of *L. bulgaricus* (grown in MRS broth and heat shocked in skim milk) increased the survival by 10% during spray drying at an outlet temperature of 80°C [30]. However, the magnitude of viability enhancement during spray drying can be markedly enhanced by growing and performing heat adaptation in the final drying medium. It was reported, that the viability of heat adapted *L. paracasei* in reconstituted skim milk was enhanced 6-fold and 18-fold during spray drying at outlet temperatures of 95-100°C and 100-105°C, respectively [24]. Similar improvement of tolerance against spray drying was achieved using salt adaptation with 0.3 M NaCl. Although this osmotic pre-treatment was efficient but the presence of salt in the product could be a problem during the preservation process and/or subsequent cell uses [23]. Furthermore, heat and osmotic adaptation on both stationary-phase and log-phase were found to be also effective in improving the storage stability of dried *L. rhamnosus* [18].

In conclusion, data from this and other related works on induction of thermotolerance by physical or chemical stresses give evidence to the potential of utilizing this particular cellular auxiliary mechanism in protecting probiotic bacteria from multiple environmental stresses (including heat, osmotic and oxidative stress) encountered during spray drying as well as during subsequent storage.

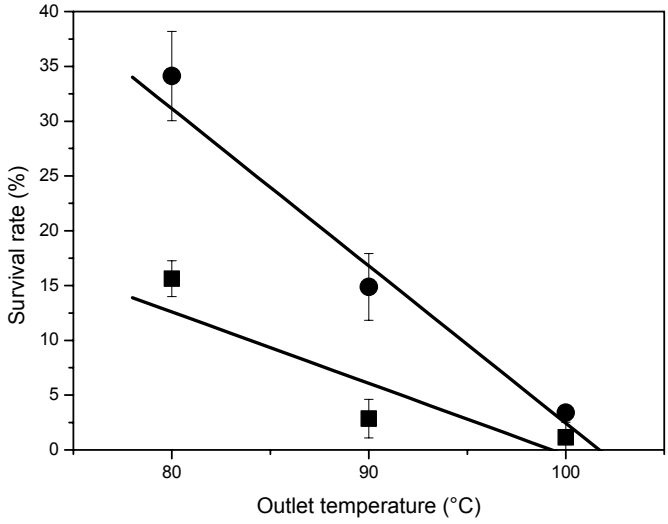


**Figure 14**

Effect of pressure pre-treatment on the survival rate of *L. rhamnosus* GG during spray drying at various outlet temperatures. Drying carrier was 20% (w/v) reconstituted skim milk (RSM). Initial cell count of the feed solution was ~ $10^7$ CFU/mL. Bacteria were grown in MRS broth until an OD value of 0.5 was reached. Pressure pre-treatment conditions were 100 MPa and 43°C for 10 min. Pressure pre-treatments were conducted on bacteria in their own growth medium. Data are means of two, or more, spray drying experiments.

### *4.4.9 Pressure induced thermotolerance on other L. rhamnosus strain*

The intrinsic thermal resistance of *L. rhamnosus* E800 was found to be higher than the one of *L. rhamnosus* GG. According to Figure 7 a reduction by almost 2 log cycles was achieved on untreated population of LGG from exponential growth phase upon exposure to 60°C for 3 min, whereas the strain E800 from the same growth stage could resist this challenge condition without loss of viability (Fig. 15). This tendency has also been confirmed by other research group that compared the thermal resistance of various probiotic lactobacilli suspended in reconstituted skim milk [93]. The thermal resistance is therefore highly strain-specific and genetically determined [94].

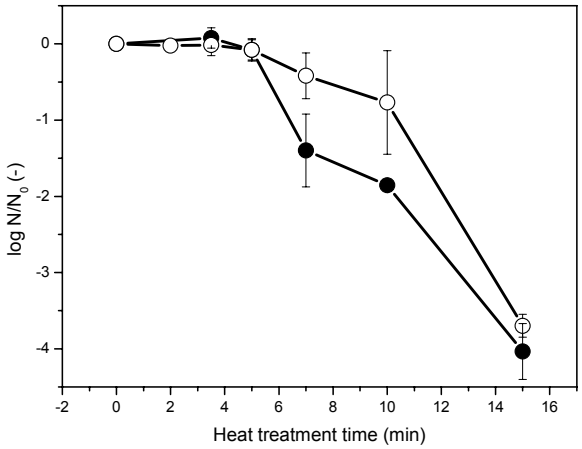


**Figure 15**

Effect of high pressure pre-treatment on the inactivation kinetics of *L. rhamnosus* strain E800 (VTT, Espoo, FI) upon exposure to a lethal temperature of 60°C. Similar to trials made with LGG, cells were grown until an OD value of 0.5 was reached. Afterwards, cells were either pre-treated with 100 MPa, 37°C for 10 min (○) or left untreated (●) prior to heat treatment at 60°C.

## 4.5 Conclusion

The effect of mild pressure pre-treatments on technological behaviour of LGG was evaluated. With respect to heat tolerance pressure pre-treated cells (at 100 MPa and 37°C) showed higher survivability than untreated ones when both were exposed to heat treatment at 60°C. Further investigation with flow cytometric analysis indicated that the acquisition of pressure-induced heat tolerance was related to membrane stabilization and protein biosynthesis. Pressure induced thermotolerance occurred as a consequence of stabilization of cellular membranes – presumably by incorporation of heat shock proteins into cytoplasmic membrane – which in turn led to an enhanced transient protection against degradative effects of heat on cell membrane. The absence of induced thermal tolerance upon addition of Chloramphenicol suggested that the proteins expressed during pressure adaptation was involved in the prevention of thermal degradation on cell membrane. Pressure pre-treated

LGG showed also an improved tolerance against chemical compounds which are able to degrade cytoplasmic membranes, such as bile acid and nisin. This result confirmed the positive role of protein synthesis during pressure adaptation in protecting this vital cellular component. The potential of utilizing pressure shock response in increasing the survivability of LGG during spray drying, which poses multiple environmental stresses (including heat, osmotic and oxidative stress) was also demonstrated.

In the production of lactic acid bacteria, where they are exposed to different types of environmental stresses, cross-protection induced by expression of adaptive response could be regarded as an effective approach in enhancing stability. Cross-protective action of pressure especially against heat was investigated in this work to evaluate its possible application in assisting probiotic production, in particular to facilitate higher bacterial survival upon spray-drying. The improvement of tolerance against various lethal stresses not related to the stress inducers is resulted from an involvement of common regulators. One advantage rising from this cross adaptive stress response is that there is a higher degree of freedom in selecting the type of stress to be applied for the induction of for instance heat tolerance. Generally, technologically relevant stress imposed to bacteria can be classified in two major groups: physical and chemical means. Use of physical stresses such as heat, UV, irradiation and pressure for the induction step would be more attractive compared to the use of chemical additives, including osmotic agents (salt or sugar), acid, bile, etc., which in many cases need to be removed from the product. With regards to acid stress response, Saarela *et al* (2004), who had conducted stress induction of probiotic bacteria in fermenter, found that in the fermenter it took 45 min to reach pH 4 [33]. Unfortunately, a feasible approach to drastically reduce pH by using a stronger acid might have had detrimental effects on cell viability because of the developing acid concentration gradient.

Due to these major drawbacks of chemical stress inducers physical methods appear to be a better choice. Exposure to sub-lethal heat could easily be done with existing equipment; however temperature increase to 47°C for heat pre-treatment took 10 min [33]. In order to accelerate heat transfer and achieve thermal equilibrium temperature gradient could easily be increased. Yet, the presence of a radial temperature field with higher temperature at the fermenter's wall would lead to an overprocessing of bacterial population at this site.

With help of the application of pressure to induce beneficial stress response on bacteria the limitations of heat transfer into product with small ratio of surface to volume could be compensated. Provided that the medium does not show pronounced inhomogeneity in compressibility the effect of pressure is uniform because of the instantaneous transmission which affects all volume elements of the confined fluid [95]. Due to adiabatic compression

pressure generation is accompanied by temperature increase of 2 to 3°C per MPa [96], which can be instantly equilibrated by temperature control.

The trials conducted in this PhD thesis to induce thermotolerance were principally performed on cells from exponential growth stage. Cells from stationary growth phase were ruled out, since bacteria that enter into stationary phase had already developed resistance against various types of environmental stress (including subsequent down-stream processing and storage). It is likely that for this reason stress treatment studies on probiotic cultures usually have been performed with log-phase cultures instead of stationary phase cultures. There are very few studies where the sublethal and lethal stress responses of stationary-phase probiotic cultures have been investigated in well-controlled manner. However, data on stress response studies with culture from stationary growth phase revealed the potential of pre-treatment of cells in this particular growth stage to improve survival during subsequent treatment [18, 33, 97, 98].

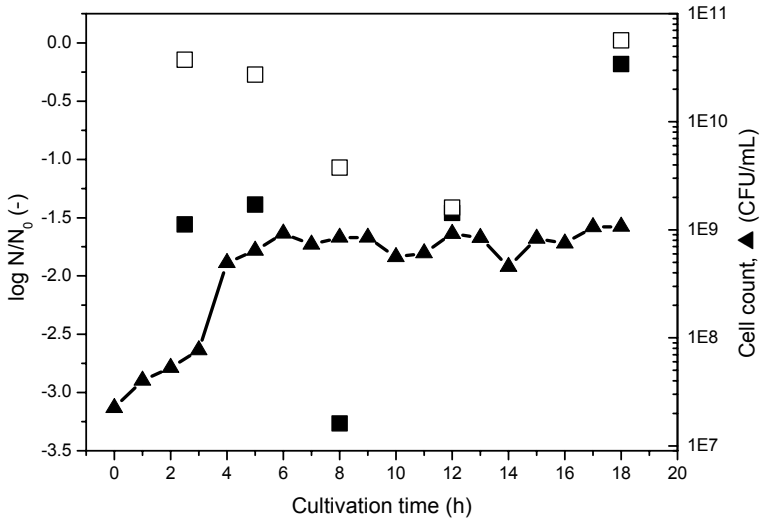


**Figure 16**

The survival rate of *L. rhamnosus* GG heat treated at 60°C for 3 min subsequent to pressure pre-treatment (□) or without any pre-treatment (■) as a function of cultivation time. Cell count (▲) is included in order to establish a correlation between growth-stage and acquired or intrinsic heat tolerance. The conditions for pressure pre-treatment: 100 MPa, 37°C for 10 min.

Own data compiled on the influence of growth phase on the level of pressure inducible thermotolerance of heat treated LGG revealed that the heat tolerance of cells from stationary growth phase could still be improved by this adaptive response mechanism (Fig 16). According to Figure 16 cells entered stationary growth phase after 5 to 6 h of cultivation in MRS broth. It was demonstrated further that cells harvested until 8 h of fermentation still showed inducible stress response leading to higher tolerance against heat. In contrast,

pressure pre-treatment on cells from mid- or late-stationary phase (after 12 or 18 h of cultivation, respectively) did not increase their heat tolerance. These data indicated the importance of further assessing the adaptive or cross-protective behaviours of cells from stationary growth phase exposed to sub-lethal stress. As already stressed by Saarela *et al* (2004) stationary-phase cells had to be studied instead of log-phase cells as during the industrial production of cultures high enough cell densities have to be reached before harvesting the cells.

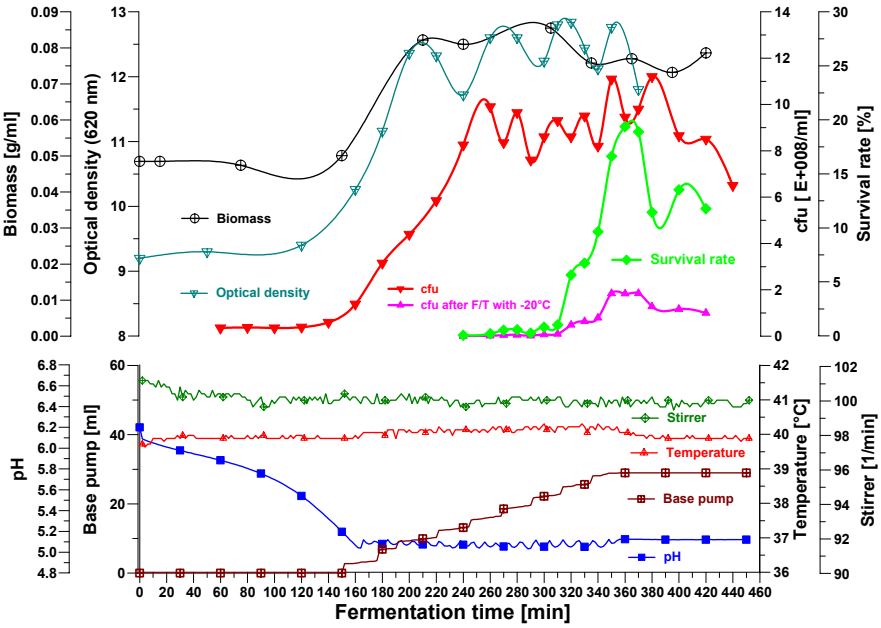


**Figure 17**

Full fermentation profile of *Lactobacillus bulgaricus* showing values measured online (bottom) and offline (upper). The fermentation was performed using a 2-liter bioreactor (Biostat M, Braun Biotech International GmbH, Melsungen, D).

Further arguments on inducing adaptive or cross-protective stress response during stationary growth phase were substantiated by Figure 17, which basically shows a profile of pH-controlled fermentation of *L. bulgaricus* [99]. Taken into account the measured offline values (i.e. cell count, biomass, optical density) which indicate the progress of the growth, it is obvious that the cells entered the stationary phase after 4 to 6 h of cultivation. However, the survival rates of the cells upon challenging them to freeze-thaw step were very poor at the first hours in the stationary phase and increased drastically after 80 min of further residence in this growth phase. There was also a certain fermentation time where the survival rate reached the highest value. This behaviour points out that there is still considerable differences in the resistance of the bacteria within the stationary growth phase. This conclusion was supported by works on *L. lactis*, in which the authors found that stationary phase is not a uniform state of cell properties [100]. They also confirmed the existence of

different “states” in the course of stationary phase. Taken together, these facts emphasize the necessity to revisit the inducible stress response within stationary phase. In particular, it is of utmost importance to evaluate whether the induction of stress response to improve the technological suitability of bacteria is possible at the beginning of stationary growth phase. Taking the scenario described in Figure 17 into consideration – when improvement in freeze/thaw survival within the first hours of entry in stationary growth phase can be achieved using sub-lethal stress, then the fermentation time in order to get viable and stable culture could considerably be reduced.

Additionaly, the results of this study on pressure induced cross adaptation point out also some precautional consideration for the use of high pressure technology for pasteurisation purposes. Due to the possibility for cells to gain protective stress response at lower pressure levels the residence time at these critical pressure levels (up to 200 MPa) has to be kept as minimal as possible, in order to minimize the acquisition of multi-stress tolerance of certain pathogens. It can be extracted from this work that a proper acquisition of thermotolerance required at least 5 min of holding time at 100 MPa. Based on this consideration a rapid compression step bears a beneficial side effect, since it is unlikely that adaptive stress response can be triggered efficiently in this highly changing environmental conditions during pressurization. Currently there is not many processing units available that utilize rapid compression step. A commercial unit of LAB50 single pressure processor (SIG Simonazzi,I) allows high compression and decompression rates: a processing pressure level of 600 MPa is generated in about 3-4 seconds, while complete release of pressure is realized in less than 1 second [101].

To ensure the safety of the processing which employ the effect of mild pressure in conferring cross-protection against heat and other membrane-destabilizing stresses one has to take the worst case scenario into account, particularly when problematic microbial contaminants such spoilage or pathogenic microbes incidentally pre-stressed and acquired tolerance against multiple stresses. Data on the effect of pressure treatment on pathogenic bacteria *E. coli* showed that sub-lethally injured bacteria could not survive in acidic environment, i.e. in fruit juices (pH 3 to 4) during cold storage [102], whereas probiotic bacteria might have better survival characteristics due to their higher acid tolerance. However, the aforementioned safety concerns emphasized that upon integration of this innovative processing concept into industrial application it is necessary to utilize existing genomic or proteomic tools in order to identify pre-treatment conditions, with help of which the beneficial shock response can be specifically aimed on microorganisms to be produced and not on bio-contaminants. The risk of the aforementioned incidence could also be controlled by applying aseptic processing line.

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# 5 SUMMARY AND OUTLOOK

Probiotic products gain growing interest in the global food markets, as documented by increasing market revenues in Europe and the USA [1]. A key factor responsible for this development is the improved awareness of consumers about the relationship between diet and health. This consumer's perception together with sound scientific evidences about the effectiveness of probiotic therapy and innovative marketing strategies promote the willingness of the consumer to buy probiotic products. Apart from using fermented milk as food vehicle for probiotic consumption there is an increased interest in the diversification of probiotic containing food products, which eventually require processing and storage conditions far beyond the ones currently applied and optimized for maintaining good survival behaviour in fermented dairy products. Regardless of the type of food vehicle the ultimate requirement for declaring probiotic products as such remains the same, i.e. that the product should contain living bacteria in a sufficient number until the end of its shelf life. The technological challenge constantly addressed to scientists working in this field is to identify critical environmental factors in a given product, which may lead to cell injury or loss of viability during processing and storage as well as to develop innovative but feasible solution to sufficiently protect probiotic bacteria in order to maintain the viability levels above the dose required for eliciting health effects.

The present work is primarily focused on the exploration of spray drying as an alternative processing method to produce dried probiotic preparations in milk based media. Most of dried bacterial preparations are currently produced by freeze-drying due to the possibility to operate at mild conditions, so that the degree of injury could be minimized. However, some drawbacks of freeze drying process, such as long processing time and high energy consumption, led to efforts in evaluating alternative drying processes [2]. Spray drying is one of the promising process for production of dry probiotic preparations, since under optimized conditions it allows high processing rates, low energy requirements and thus lower operating costs.

In this context investigations were performed to study the mechanisms leading to cell damage during drying and the role of the physical state of the drying matrix as well as the interactions of protective compounds with cellular membranes as related to dehydration tolerance. Moreover, a pre-adaptation step under sub lethal high pressure conditions was assessed regarding its potential in increasing tolerance against heat, which is considered a viability-determining hurdle for probiotic bacteria during spray drying.

To allow identification of cellular injuries occurring during spray drying of *L. rhamnosus* GG (LGG) a microbiological analysis method involving flow cytometric analysis in combination with a multiple staining strategy was prepared and established. The application of this technique to evaluate the mechanism of microbial inactivation with LGG as model organism

by means of physical treatments was discussed in Chapter 2. Physiological fluorescence dyes carboxyfluoresceindiacetate (cFDA) and propidium iodide (PI) were applied to examine process-induced changes in cellular integrity or metabolic activities, which were not explicitly assessable by culture techniques. As a result, it was possible to differentiate the mechanisms of microbial inactivation occurring during different physical treatments, in order to allow problematic contaminants to be injured or inactivated more effectively as well as to effectively combine different treatments, which have different cellular target sites. It was found that although the survival rates according to the plate counts result were in the same range, different treatments led to different responses of the cell to cFDA/PI labelling. This may indicate that the treatments applied differed in the cellular sites being primarily affected.

Regarding heat induced damage on LGG, it was demonstrated that the target sites of heat inactivation may differ depending on the temperature level used. Based on these results, it is recommended to expose vegetative cells to temperatures above 60°C, when the inactivation of intracellular esterase and membrane damage is aimed. In contrast the major population of high pressure inactivated cells of LGG could accumulate fluorescent molecule carboxyfluorescein (cF), which indicated that some of the dead cells were still enzymatically active and not severely membrane compromised. The fact, that pressure inactivated bacteria could perform enzymatic conversion of cFDA into cF needs further attention, since the presence of such metabolically active, but dead bacteria in food might be critical in terms of their potential activity on excreting toxic or food spoiling metabolites. Moreover, it was shown that the lethal effect of pressures higher than 400 MPa was related to the irreversible perturbation of dye extrusion machinery, which is most likely mediated by an ATP-driven transport system. Accordingly, dead cells, which lost the capacity to reproduce themselves and grow on agar, are the ones which were not able to extrude cF, although the membrane was still intact and esterase activity remained. These findings underline the results from previous works on pressure induced damage on other ATP-dependent, membrane bound enzymes, which are crucial in maintaining viability [3-7]. Likewise, it seems that high intensity ultrasound did not considerably affect the cytoplasmic membrane, although according to plate count results viability loss occurred. It could be concluded that, cell death which was observed upon applying high-intensity ultrasound seemed to result from non-membrane related degradation.

The importance of the ingestion of viable bacteria in eliciting health effect is sometimes questioned, since non-viable bacteria were reported to be not only as effective as viable ones; they are of interest due to easy-handling and longer shelf life [8]. However, systematic studies on the relationship between probiotic effect and the type of inactivation treatments used to produce non-viable bacteria are still lacking. In this context, high pressure killed cells might be one of the promising candidate to be investigated, since the fluorescence pattern of

pressure inactivated cells – which is indicative for a lower extent of damage on metabolic activity and on membrane – is quite similar to the one of viable cells. Moreover, probiotic bacteria may become dormant during storage, i.e. they retain a functional cell membrane typical for viable cells, but were not culturable [9]. The transformation into a non-culturable state seems to be a universal adaptive response in coping with adverse environmental conditions. Supported by this findings and taking into account that the greater amount of bacteria in the gastrointestinal tract is not necessarily culturable, the current practice in putting bacterial culturability as an absolute measure of probiotic effectiveness seems to be not necessarily appropriate and would exclude many functional but non-culturable strains, which might be more effective than culturable probiotics in eliciting health effect upon consumption.

Chapter 2 deals with the comprehensive evaluation of the spray drying process in the manufacture of probiotic powders. The investigation of bacterial stability during subsequent storage is included as well. It was found that when reconstituted skim milk (RSM) was used as the drying medium, a bacterial survival rate ≥ 50% was achievable at an outlet temperature of 80°C. The powder contained more than $10^9$ cfu $g^{-1}$ of LGG. Using flow cytometric analysis, bacterial membranes were identified as the main site of injury during spray drying. The protective media used as drying medium should therefore be targeted on protection of membrane against deteriorative effects of water removal and thermal stresses on membranes occurring during spray drying.

The incorporation of commercial prebiotic substances such as Raftilose®P95 (oligofructose) or polydextrose in the skim milk powder with the aim to produce synbiotic powder did not exert any adverse effect on bacterial survival upon spray drying. However, stability of bacteria during long term storage was impaired by partial substitution of skim milk with either of the prebiotic substances evaluated. The entrapment of bacteria in an extracellular glassy state appeared to have only little effect on their stability. Although the glass transition temperatures of all media were well above the storage temperatures applied, bacterial inactivation still took place during storage; indicating the insufficiency of entrapment in glassy state in inhibiting deteriorative events involved in cell death. The decreased protection capacity of prebiotic containing media could be resulted from the reduced amount of protective compounds in skim milk solids, which could not adequately substituted by prebiotics. As a result, due to the presence of oxygen in the storage atmosphere applied in this study, related deteriorative reaction, most likely lipid oxidation, may take place at a higher rate.

The assessment of the contribution of sugar molecule to stabilization during drying was conducted with help of flow cytometric analysis on dried model membrane. It was observed

that oligosaccharides present in Raftilose®P95 were not capable of directly interacting with cytoplasmic membranes in the dehydrated state, whereas polydextrose and lactose could effectively prevent drying induced leakage on membranes. Moreover, the effect of milk proteins in stabilization of dried bacteria during storage was observed. When milk proteins were enzymatically degraded, the performance of such treated RSM-based media in conferring protection during storage was considerably reduced. In conclusion, data compiled in this study suggest that the superiority of skim milk over prebiotics in stabilizing dried bacteria is most likely based on the direct interaction of lactose with bacterial membranes as well as proteins and on protective effect of milk proteins. The higher susceptibility of bacteria dried in prebiotic containing matrices to deteriorative events could be reduced by storing them at refrigerated temperatures.

Furthermore, in Chapter 4 the application of sub lethal high pressure pretreatment was assessed on its effectiveness in inducing adaptive responses, which may ultimately lead to an increase of the bacterial tolerance against stress related to harsh conditions encountered during spray drying. It was shown that pressure pre-treated cells showed higher survivability than untreated ones when both were exposed to heat treatment at 60°C. Further investigation with flow cytometric analysis indicated that the acquisition of pressure-induced heat tolerance was related to membrane stabilization and protein biosynthesis. Pressure induced thermotolerance occurred as a consequence of stabilization of cellular membranes – presumably by incorporation of heat shock proteins into cytoplasmic membranes – which in turn led to an enhanced transient protection against degradative effects of heat on cell membranes. The absence of induced thermal tolerance upon addition of chloramphenicol, an inhibitor of protein synthesis, suggested that the proteins expressed during pressure adaptation was involved in the prevention of thermal degradation on cell membrane. Pressure pre-treated LGG also showed an improved tolerance against chemical compounds which are able to degrade cytoplasmic membranes, such as bile acid and nisin. This result confirmed the positive role of protein synthesis during pressure adaptation in protecting this vital cellular component. The potential of utilizing pressure shock response in increasing the survivability of LGG during spray drying, which poses multiple environmental stresses (including heat, osmotic and oxidative stress) was also demonstrated. In relation to this observation it was proposed that physical methods appear to be a better choice for the induction of adaptive response. Exposure to sub-lethal heat could easily be done with existing equipment; however temperature increase to 47°C for heat pre-treatment took 10 min [10]. Yet, the presence of a radial temperature field with higher temperature at the fermenter's wall would lead to an overprocessing of bacterial population at this site. The limitations of heat transfer into product with small ratio of surface to volume could be

compensated by application of pressure, which is transmitted instantaneously throughout all volume elements of the confined fluid [11]. More studies are required to investigate the pressure stress response of bacteria from different sub-phases in the stationary growth stage, since cultures from stationary phase have higher biomass, which makes them more attractive for industrial production.

In conclusion, the maintenance of high viability level of a given probiotic bacteria in the dehydrated state is determined by both extrinsic and intrinsic factors, which create a dynamic environment in extra- or intracellular space (Fig. 1).

As shown in Figure 1, the ***processing condition*** used to spray dry bacteria not only has a direct influence on the bacterial viability but also affects the properties of drying media. With regards to the latter issue, overprocessing would result in thermal degradation of protective compounds, such as sugar (due to browning). Low drying rate would facilitate the sugar crystallization rather than entrapment of bacteria in glassy state.

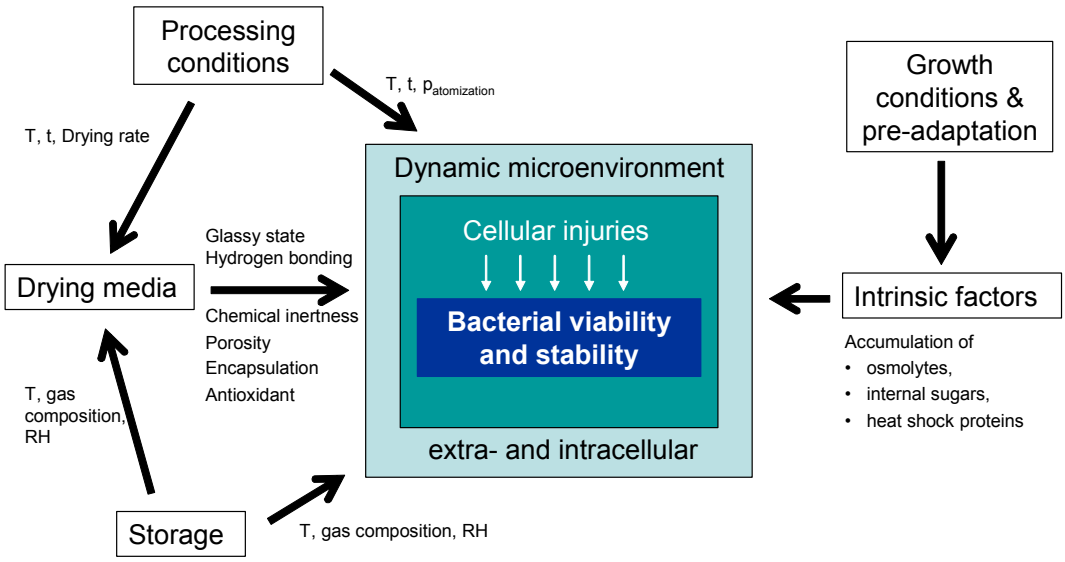


**Figure 1**

Interplay among extrinsic and intrinsic factors, which govern the viability and stability of spray dried bacteria

In terms of ***the drying media*** used to protect the bacteria, the thermophysical properties, chemical inertness, antioxidative properties, possibility of hydrogen bonding with cellular components upon water removal, etc. should be evaluated. It is expected that physico-chemical changes occurred in the protective matrix due to ***changes in the environmental conditions*** (humidity, temperature, atmosphere) or as a result of ***modification in the matrix composition*** would lead to a considerable alteration of the microstructure in the vicinity or inside the dried bacteria. This may eventually lead to higher susceptibility of protected cells towards reactive molecules which increase the potential of irreversible cellular injuries. This

situation necessitates the identification of the critical alterations and how to effectively prevent them.

Furthermore, the efforts in improving understanding on the nature of ***cellular injuries*** affected by industrial process need to be reinforced. The knowledge acquired from these microbiological analyses would allow scientific-based solutions, in terms of alteration of process or environmental parameters or selection of protective media, to be applied in order to prevent or minimize cellular injury.

It is also of interest to take advantage from the ***defense mechanism*** of the cells themselves in responding to the changes in their environment during the first stages of desiccation (by accumulation of compatible solutes, synthesis of stress proteins, modified metabolic pathways, etc.) need to be studied explicitly to effectively utilize this cellular response and identify the most important stress metabolites involved in the dehydration tolerance. It is also possible to control the growth of the probiotic bacteria in such a way that increased accumulation of protective metabolites can be achieved.

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# 6 ANNEXES

## 6.1 Annex 1 : Fermentation profile of *L. rhamnosus* GG (API 50 CHL, Bio Merieux, France)

**0 d** **1 d** **2 d**

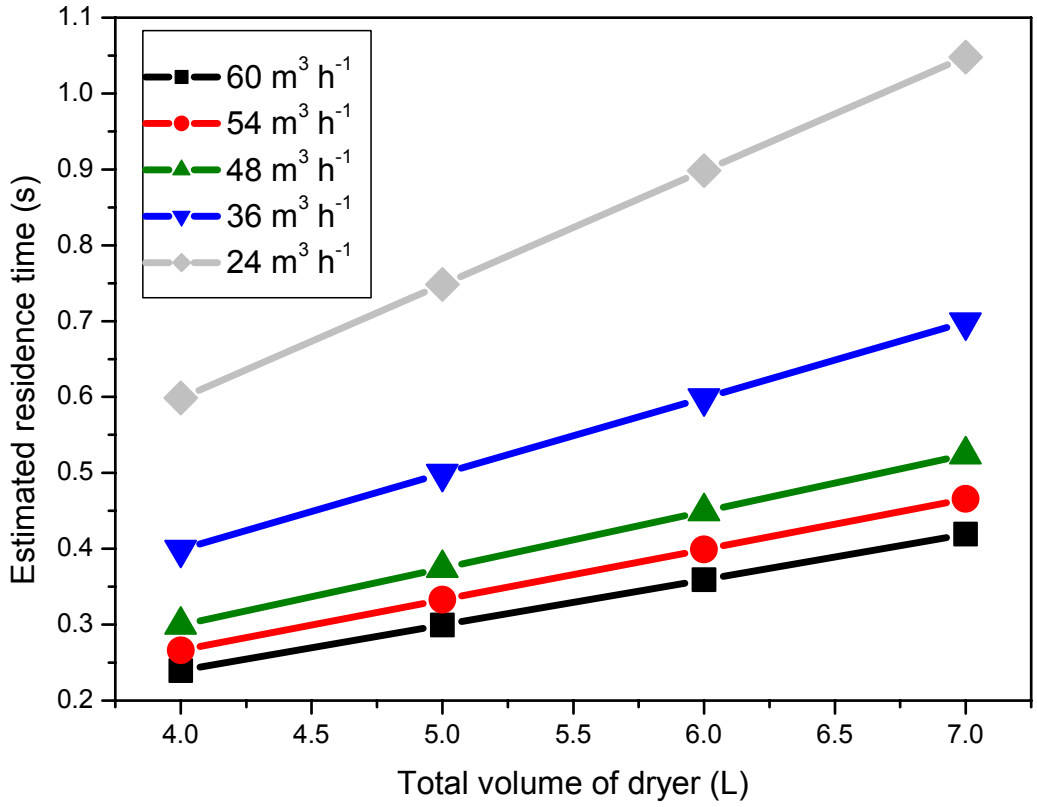
| | | Carbohydrate | Fermentation |
|---|---|---|---|
| Row 1 | 0 | Control | - |
| | 1 | Glycerol | - |
| | 2 | Erythritol | - |
| | 3 | D-arabinose | + |
| | 4 | L-arabinose | - |
| | 5 | Ribose | + |
| | 6 | D-xylose | - |
| | 7 | L-xylose | - |
| | 8 | Adonitol | - |
| | 9 | β-methylxyloside | - |
| Row 2 | 10 | Galactose | + |
| | 11 | D-glucose | + |
| | 12 | D-fructose | + |
| | 13 | D-mannose | + |
| | 14 | L-sorbose | - |
| | 15 | Rhamnose | - |
| | 16 | Dulcitol | + |
| | 17 | Inositol | + |
| | 18 | Mannitol | + |
| | 19 | Sorbitol | + |
| Row 3 | 20 | α–methyl-D-mannoside | - |
| | 21 | α–methyl-D-glucoside | - |
| | 22 | N-acetyl-glucosamine | + |
| | 23 | Amygdaline | + |
| | 24 | Arbutine | + |
| | 25 | Esculine | + |
| | 26 | Salicine | + |
| | 27 | Cellobiose | + |
| | 28 | Maltose | - |
| | 29 | Lactose | - |

| | | Carbohydrate | Fermentation |
|---|---|---|---|
| Row 4 | 30 | Melibiose | - |
| | 31 | Saccharose | - |
| | 32 | Trehalose | + |
| | 33 | Inuline | - |
| | 34 | Melezitose | + |
| | 35 | D-raffinose | - |
| | 36 | Amidon | - |
| | 37 | Glycogene | - |
| | 38 | Xylitol | - |
| | 39 | β-gentiobiose | + |
| Row 5 | 40 | D-turanose | - |
| | 41 | L-lyxose | - |
| | 42 | D-tagatose | + |
| | 43 | D-fucose | - |
| | 44 | L-fucose | + |
| | 45 | D-arabitol | - |
| | 46 | L-arabitol | - |
| | 47 | Gluconate | + |
| | 48 | 2-cetogluconate | - |
| | 49 | 5-cetogluconate | - |

## 6.2 Annex 2 : Detector's configurations for flow cytometric analysis

| Dye combination | FL1 (Volt) | FL3 (Volt) | Compensation FL1 in FL3 (%) | Discriminator | Sample |
|---|---|---|---|---|---|
| cFDA/PI | 807 | 807 | 28.2 | SS = 0 | *L. rhamnosus* GG (stationary phase) |
| LIVE/DEAD® Bac Light | 508 | 508 | Not made | FS = 8 | *L. rhamnosus* GG (exponential phase) |
| cF | 961 | Not relevant | 17.8 | FS = 3 or 4 | Liposomes |

## 6.3 Annex 3 : Estimated residence time of dried particle in spray dryer




Total volume of Büchi Spray Dryer B-191 was estimated to be 6.8 L.

Standard working condition for flow rate of drying air in Büchi B-191 60 $m^3$ $h^{-1}$ (100% power of aspirator)

## 6.4 Annex 4 : Technical specifications of Raftilose®P95 (Orafti, Tienen, Belgium)

***Description***

**Raftilose®P95**

- Is a powder containing mainly oligofructose produced by partial enzymatic hydrolysis of chicory inulins;
- Is a good food ingredient composed of oligofructose, fructose, glucose and sucrose

**Oligofructose**

is a mixture of oligosaccharides which are composed of fructose units linked together by ß(2-1) linkages. Almost every molecule is terminated by a glucose unit. The total number of fructose or glucose units (= Degree of Polymerisation or DP) of oligofructose ranges mainly between 2 and 8.

***Compositional Specifications***

All values expressed on dry matter.

| | |
|---|---|
| Oligofructose | ≥ 93.2 % |
| Glucose + fructose + sucrose | < 6.8% |
| | |
| Dry Matter (d.m.) | 97 ± 1.5 % |
| Carbohydrate content | > 99.5 % |
| Ash (sulphated) | < 0.2 % |
| Conductivity (28 Brix) | < 250 µS |
| Heavy Metals | Pb, As each <0.2 mg/kg |
| | Cd, Hg each <0.01 mg/kg |
| pH (30-50°Brix) | 5.0 – 7.0 |

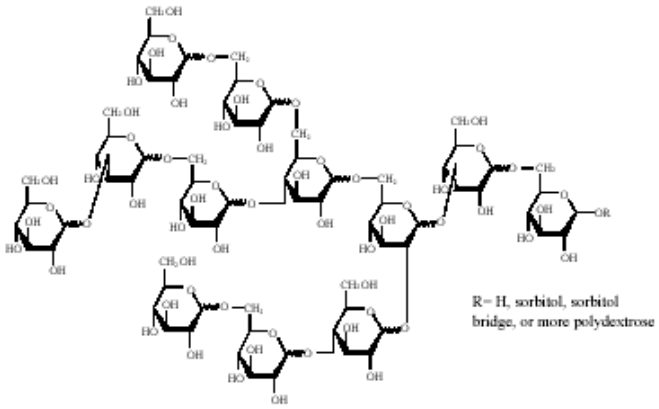
***Other information***

| | |
|---|---|
| Aspect | fine white powder |
| Behaviour | hygroscopic |
| Taste | Slightly sweet, without aftertaste |
| Solubility in water | > 750 g/L |
| Wettability in water | Excellent |
| Dispersability in water | Excellent. May require stirring |
| Density | Approx. 600 ± 70 g/L |
| Optimal storage conditions | Cool and dry, in its original airtight packaging |
| Maximum durability | minimum 18 months upon delivery |

## 6.5 Annex 5 : Technical specifications of Polydextrose (Danisco, Copenhagen, Denmark)

# LITESSE® ULTRA™

Refined Polydextrose FCC - Powder




DESCRIPTION: Litesse® Ultra™ - Powder (Refined Polydextrose FCC) is a sorbitol-terminated, randomly bonded condensation polymer of D-glucose with some bound sorbitol and a suitable acid.

SOLUBILITY: It is very soluble in water (approximately 80g/100ml @ 20°C), but only sparingly soluble to insoluble in most organic solvents.

STORAGE: Litesse® Ultra™ - Powder should be stored in tight containers in a dry place below 40°C (104°F).

| SPECIFICATIONS: | LIMITS: | METHOD: |
|---|---|---|
| Appearance | Fine White powder | Visual |
| Odour and Taste | Practically odourless with a slightly sweet taste | FCC |
| Identification** | Meets test | FCC |
| Assay, as Polydextrose* | Min. 90.0% | HPLC |
| 1,6 Anhydro-D-Glucose (Levoglucosan)* | Max. 4.0% | HPLC |
| Reducing Sugar* | Max. 0.25% | Titration (Copper Reduction) |
| Sorbitol + Reducing Sugar* | Max. 6.0% | HPLC, Titration |
| Water | Max. 4.0% | FCC |
| Molecular Weight Limit (High-Molecular-Weight Polymer [max 22,000 MW]) | Meets Test | HPLC |
| 5-Hydroxymethylfurfural* | Max. 0.1% | FCC |
| Heavy Metals** | Max. 5 ppm | FCC |
| Lead** | Max. 0.5 ppm | FCC |
| pH (10% w/v aq.) | 4.5 to 6.5 | FCC |
| Residue on Ignition (Sulfated Ash) | Max. 0.3% | FCC |
| Nickel | Max. 2 ppm | AAS-Flame |
| Acidity (Anhydrous Basis) | Max. 0.002 meq/g | Titration |

* Calculated on the anhydrous, ash-free basis.
** Data derived from in-process control testing

The information contained herein is based on data available to us and is believed to be accurate. However, no warranty is expressed or implied regarding the accuracy of this data or the results to be obtained from its use. Users should make their own investigations to determine the suitability of the information for their particular purposes. For actual values, please refer to the Certificate of Analysis.

August 22, 2002

## 6.6 Annex 6 : Technical specification of COROLASE®PP (AB Enzymes, Darmstadt, Germany)

# COROLASE® PP

**Beschreibung und Spezifikation**

**2001.09.01**
Rev. Nr. 03

### Beschreibung

COROLASE® PP ist ein Proteinasepräparat, welches neben Endo-Protease auch verschiedene Amino- und Carboxypeptidase-Aktivitäten enthält. COROLASE® PP wirkt im neutralen und schwach alkalischen pH-Bereich am besten.
Das Produkt wird aus Schweinepankreasdrüsen gewonnen.

- IUB-Nr.: 3.4.21.4
- CAS-Nr.: 9002-07-7

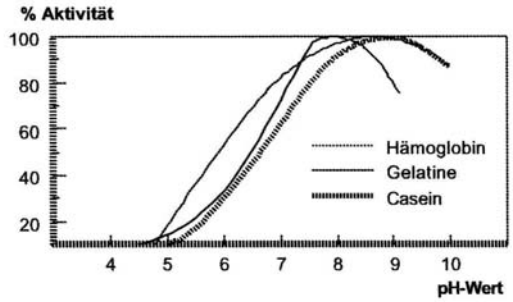
### Eigenschaften

COROLASE® PP hat folgende Merkmale:

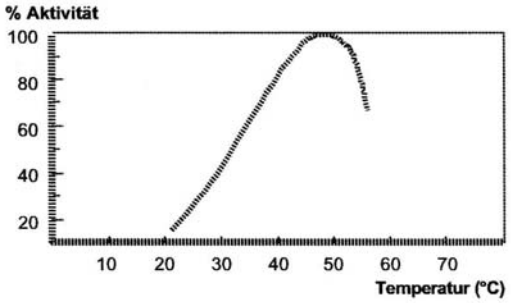
a) pulverförmiges Produkt
b) beige Farbe und charakteristischer Geruch

pH- und Temperaturoptimum:

- Abb. 1: pH-Optimum




- Abb. 2: Temperatur-Optimum




### Anwendung

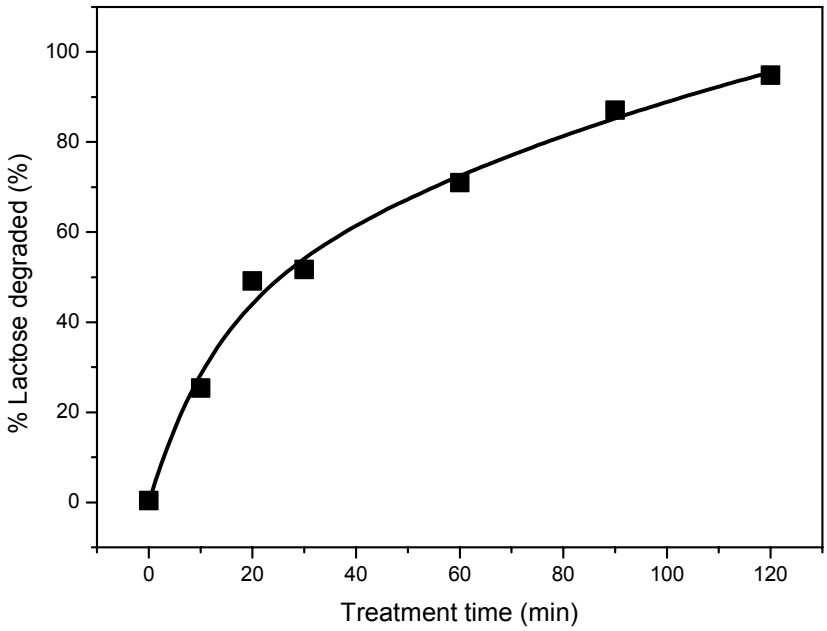
Substrate für COROLASE® PP sind alle in der Lebensmitteltechnik eingesetzten Proteine tierischer und pflanzlicher Herkunft. Besonders vorteilhaft entfaltet sich die Wirkung des Enzyms bei Substraten wie Casein, Molkenprotein oder Sojaprotein, die bereits bei geringer Hydrolyse zur Bildung von Bitterpeptiden neigen. Durch die hohe Exopeptidaseaktivität werden entstehende Bitterpeptide gezielt abgebaut und der Bitterpunkt zu höheren Hydrolysegraden verschoben.

### Dosage

Die empfohlene Dosage liegt bei 0,01-0,5 % bezogen auf den Proteingehalt des Substrates.

## 6.7 Annex 7 : Kinetic of lactose degradation using ß-galactosidase (G-3665, Sigma, St. Louis, MO)

Dosage : 0.5 mL ß-galactosidase in 100 mL RSM



## 6.8 Annex 8 : Regression parameters for heat inactivation curves (4.4.4)

(A) Model : linear regression, partial

| Pressure treatment time 5 min | | | |
|---|---|---|---|
| Pressure (Mpa) | Temperature (°C) | k ($min^{-1}$) | $R^2$ |
| 100 | 37 | 0.256 | 0.99 |
| 100 | 43 | 0.203 | 0.967 |
| 100 | 50 | 0.592 | 0.985 |
| 200 | 37 | 0.529 | 0.962 |
| 200 | 43 | 0.366 | 0.974 |
| 200 | 50 | 0.737 | 0.998 |

| Pressure treatment time 10 min | | | |
|---|---|---|---|
| Pressure (Mpa) | Temperature (°C) | k ($min^{-1}$) | $R^2$ |
| 100 | 37 | 0.388 | 0.926 |
| 100 | 43 | 0.199 | 0.878 |
| 100 | 50 | 0.55 | 0.997 |
| 200 | 37 | 0.808 | 0.989 |
| 200 | 43 | 0.842 | 0.964 |
| 200 | 50 | 1.154 | 0.971 |

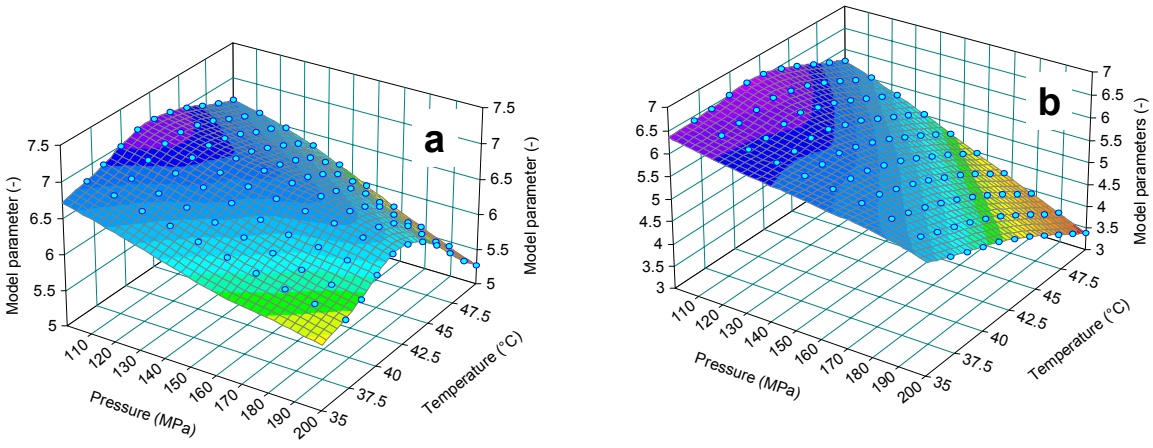
(B) Model : Weibull distribution

$$\log\left(\frac{N}{N_0}\right) = N_E \cdot e^{\left[-\left(\frac{t}{b}\right)^4 - 1\right]}$$

$N_E$: lower asymptote, b:model parameter

| Pressure treatment time 5 min | | |
|---|---|---|
| Pressure (MPa) | Temperature (°C) | Model parameter, b (-) |
| 100 | 37 | 6.8123 |
| 100 | 43 | 7.09048 |
| 100 | 50 | 6.6723 |
| 200 | 37 | 6.00561 |
| 200 | 43 | 6.35517 |
| 200 | 50 | 5.2672 |

| Pressure treatment time 10 min | | |
|---|---|---|
| Pressure (MPa) | Temperature (°C) | Model parameter, b (-) |
| 100 | 37 | 6.3918 |
| 100 | 43 | 6.5109 |
| 100 | 50 | 5.7465 |
| 200 | 37 | 5.2006 |
| 200 | 43 | 4.5051 |
| 200 | 50 | 3.3871 |




3D plots generated under application of Weibull distribution for identification of optimal pre-treatment conditions at pressure treatment time of 5 (a) or 10 min (b)

# 7 LIST OF DISSEMINATION ACTIVITIES

***Scientific articles***

Ananta, E., Heinz, V., Schlüter, O., Knorr, D.
Kinetic studies on high-pressure inactivation of *Bacillus stearothermophilus* spores suspended in food matrices
Innovative Food Science & Emerging Technologies 2 (2001) 261-272

Ananta, E. & Knorr, D.
Pressure induced thermotolerance of *Lactobacillus rhamnosus* GG.
Food Research International 36 (2003) 991-997

Ananta, E., Heinz, V. and Knorr, D.
Assessment of high pressure induced damage on *Lactobacillus rhamnosus* GG by flow cytometry
Food Microbiology 21 (2004) 567–577

Luscher, C., Balasa, A., Frohling, A., Ananta, E., and Knorr, D.
Effect of high-pressure-induced ice I-to-ice III phase transitions on inactivation of *Listeria innocua* in frozen suspension
Applied and Environmental Microbiology 70 (2004) 4021-4029

Ananta, E. et al
Processing effects on the nutritional advancement of probiotics and prebiotics
Microbial Ecology in Health and Disease 16 (2004) 113-124

Ananta, E. and Knorr, D.
Evidence on the role of protein biosynthesis in the induction of heat tolerance of *Lactobaccilus rhamnosus* GG by pressure pre-treatment
International Journal of Food Microbiology 96 (2004) 307-313

Ananta, E., Volkert, M. and Knorr, D.
Cellular injuries and storage stability of spray dried *Lactobacillus rhamnosus* GG
International Dairy Journal 15 (2005) 399-409

Ananta, E., Bauer, B., Volkert, M. und Knorr, D.
Sprühtrocknung von probiotischen Bakterien
Deutsche Molkerei Zeitung – dmz 2 (2005) 52-55

Ananta, E., Voigt, D., Zenker, M., Heinz, V. and Knorr, D.
Cellular injuries upon exposure of *Escherichia coli* and *Lactobacillus rhamnosus* to high-intensity ultrasound
Accepted for publication in Journal of Applied Microbiology

***Oral presentations***

Angersbach, A., Heinz, V., Schlueter, O., Ananta, E., Knorr, D., Bunin, V.
Sicherung der Reproduzierbarkeit von Populationszuständen bei der Untersuchungen von Mikroorganismen unter Nutzung einer elektro-optischen Messmethode
Proceedings vom GDL-Kongress Lebensmitteltechnologie 8-10 November 2001, Berlin
GDL eV, Bonn

Ananta, E., Heinz, V., and Knorr, D.
Anwendung von hohem hydrostatischen Druck zur Erhöhung der Hitzeresistenz von probiotischen Mikroorganismen.
Vortrag anlässlich der Sitzung von GVC-VDI Fachausschuss Lebensmittelverfahrenstechnik, 12-14 März 2003, D-Fresing-Weihenstephan

Ananta, E. and Knorr, D.
Pressure-induced stress response of probiotic bacteria *Lactobacillus rhamnosus* GG and its potential towards their industrial production.
Oral presentation at the conference New Functional Ingredients and Foods 2003, 9-11 April 2003, Copenhagen, Denmark

Ananta, E., Volkert, M., Gloyna, D. & Knorr, D.
Untersuchungen zur Anwendbarkeit von Sprühtrocknung in der Herstellungstechnologie von probiotischen Bakterien.
Vortrag anlässlich des Kongresses der Gesellschaft Deutscher Lebensmitteltechnologen, 25-27 September 2003, Stuttgart-Hohenheim.

Ananta, E. and Knorr, D.
Cross-adaptive stress response of pressure pre-treatment on probiotic bacteria: Characterization and importance for production processes
Oral presentation at ICEF9 International Congress on Engineering and Food, 7-11 March 2004, Montpellier, France

Ananta, E. und Knorr, D.
Zur Optimierung der Herstellung von probiotischen Mikroorganismen – Verfahren, Neue Ansätze, Methodik.
Vortrag anlässlich des Minisymposiums vom Biotechnologie Centrum (BTC) – Berlin, 9-10 Juli 2004, Berlin

Ananta, E. and Knorr, D.
Use of flow cytometric analysis for the evaluation of microbial inactivation mechanisms
Oral presentation at FoodMicro 2004, 12-16 September 2004, Portoroz, Slovenia

Ananta, E., Volkert, M. and Knorr, D.
Factors influencing survival rate and storage stability of spray dried *Lactobacillus rhamnosus* GG
Oral presentation at International Probiotic Conference, 15-19 September 2004, Kosice, Slovakia

Ananta, E., Volkert, M., Voigt, D., Gunawan, R., and Knorr, D.
The role of protective media in the viability retention of probiotic bacteria *Lactobacillus rhamnosus* GG during spray drying and storage
Oral presentation at EFFoST Conference, Food Innovations for an Expanding Europe, 26-29 October 2004, Warsaw, Poland

Ananta, E. und Knorr, D.
Ansätze zur Verbesserung der Stabilität von probiotischen Bakterien in Lebensmittelsystemen – Ergebnisse aus dem EU-Projekt PROTECH
Vortrag anlässlich des Professorentreffens vom VdF - Verband der Deutschen Fruchtsaftindustrie e.V., 3 November 2004, Berlin

***Poster presentations***

Heinz, V., Ananta, E. and Knorr, D.
Spore control in food by pressure assisted heating
Poster presentation at EUROCAFT 2001, 5-7 December 2001, Berlin

Ananta, E., Ponanti, M. and Knorr, D.
Improvement of survival rate of probiotic bacteria during spray-drying using high pressure and heat pre-treatment.
Poster presentation at IBERDESH 2002 – Symposium Drying : Process, structure and functionality, 25-27 September 2002, Valencia, Spain.

Ananta, E., Heinz, V. and Knorr, D.
Flow cytometric analysis of high pressure treated microorganism.
Poster presentation at SAFE Consortium meeting: Newly emerging pathogens, 24-25 April 2003, Brussels, Belgium.

Ananta, E., Heinz, V. and Knorr, D.
Impact of high pressure treatment on metabolic activities of lactic acid bacteria as assessed by flow cytometric analysis.
Poster presentation at European Federation of Food Science and Technology (EFFoST) meeting – Nonthermal processing workshop, 7-10 September 2003, Wageningen, The Netherlands.

Ananta, E., Voigt, D., Zenker, M. and Knorr, D.
Applicability of high-intensity ultrasound to inactivate *Escherichia coli* and *Lactobacillus rhamnosus* and to increase sensitivity against nisin
Poster presentation at SAFE Consortium meeting: Novel food-preservation technologies, 22-23 January 2004, Brussels, Belgium.

Ananta, E., Ardia, A., Heinz, V. and Knorr, D.
High pressure treatment of bacteria and spores – Monitoring pressure-induced changes in microbial metabolic activities and cellular properties using flow cytometer
Poster presentation at ICEF9 International Congress on Engineering and Food, 7-11 March 2004, Montpellier, France.

Ananta, E., Volkert, M. and Knorr, D.
Crucial aspects of the application of spray drying in the production of probiotics and prebiotics containing preparation
Poster presentation at 3rdPROEUHEALTH workshop, 15-17 March, Sitges, Spain

Luscher, C., Ananta, E. and Knorr, D.
Application of high pressure processing to frozen food systems - Influence on microorganisms
Poster presentation at FoodMicro 2004, 12-16 September 2004, Portoroz, Slovenia

# 8 CURRICULUM VITAE

**PERSONAL INFORMATION**

| | |
|---|---|
| Name | : Edwin Ananta |
| Nationality | : Indonesian |
| Date and place of birth | : Malang, Indonesia, January 23rd 1975 |
| Marital status | : Married |

**EDUCATION**

*August 1993 – September 1994*

Visit of German course and university preparatory school at Berlin University of Technology

*October 1994 – May 2000*

Dipl.-Ing in Food Technology at Berlin University of Technology

Thesis: Combined action of pressure and temperature for decontamination purposes in different stages of cocoa processing

**INTERNSHIP**

*August - September 1996*

Internship at University Potsdam, Germany

Assisting Prof. G. Muschiolik and co-workers in the production and analysis of multiple emulsions

*August - October 1997*

Industrial internship at Storck Schokolade GmbH, Berlin, Gemany

Performing routine chemical analysis in the quality control of chocolate production plant

**WORKING EXPERIENCE AS STUDENT**

*December 1997 – March 2000*

Student co-worker in Berlin University of Technology, Department of Food Biotechnology and Food Process Engineering

**WORKING EXPERIENCE**

*June 2000 – May 2005*

PhD student and research co-worker at Berlin University of Technology, Department of Food Biotechnology and Food Process Engineering

PhD Thesis: Identification of environmental factors involved in viability and stability of probiotic bacteria *Lactobacillus rhamnosus* GG during production processes and new approaches for improvement

**ORGANIZATIONAL ACTIVITIES**

*Since 1996*

Member of GDL (Society of German Food Technologists)

*Since 1999*

Member of VDI e.V. (“Verein deutscher Ingenieure” - The German Engineers’ Association)

**Danksagung**

Die Arbeiten für die vorliegende Dissertation wurden in der Zeit von 2001 bis 2005 am Institut für Lebensmittelbiotechnologie –und prozesstechnik der TU Berlin durchgeführt.

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