Journal of Food Technology 3 (4): 479-490, 2005

Effect of Stress on Cells of *Lactobacillus delbrueckii* sp. *Bulgaricus*

Joana Silva, Ana Sofia Carvalho, Paula Teixeira and Paul A. Gibbs
Escola Superior de Biotecnologia, Universidade Católica Portuguesa
Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal

Abstract: Cultures of *Lactobacillus delbrueckii* sp. *bulgaricus* play an important role in the production of fermented foods and are frequently used as starter cultures for dairy fermentations combined with other species. It These cultures is are particularly used in the industrial production of yoghurt and cheeses. Large scale Pproduction methods of dried *L. bulgaricus* powders, for inoculating the production vat directly, involve treatments that stress cells in such a way that they lose some of their original activity. containing viable and active organisms which are long-term preserved during storage in the dried state, areis presented. This review covers the environmental stress responses in cells of *L. bulgaricus* which have been investigated. The responses of *L. bulgaricus* cells to heat, cold, acid, osmotic, oxygen, starvation, drying and during storage in the dried state are described. Attempts to improve the survival of *L. bulgaricus* during drying and subsequent storage in the dried state are also discussed in this review.

Key words: *L. bulgaricus*, stress, survival, industrial production

INTRODUCTION

Lactobacillus delbrueckii sp. *bulgaricus* hereafter termed *L. bulgaricus*, is a homofermentative Lactic Acid Bacterium (LAB) used as a starter culture in important dairy fermentations especially in yoghurt and in other fermented milk productiosn[1]. such as yoghurt, cheese, milk, meat, fruit, vegetables and bread. Fermented dairy products have been a major part of the diet of people around the world and the health benefits associated with these products have been reported[1,2]. *Lactobacillus delbrueckii* sp. *bulgaricus* is economically very important especially in yoghurt manufacturing and in fermented milk for human consumption[1]. Despite the industrial interest in *L. bulgaricus* however, little is known about its physiology and genetics[3].

In the industrial production of yoghurt, The usual industrial practice is to add *Streptococcus thermophilus* and *L. bulgaricus* to produce yoghurt are usually in order toused together in order to reduce the fermentation time. *L. bulgaricus* produces essential aminoacids from milk proteins owing to its proteolytic nature and the symbiotic relationship of *L. bulgaricus* and *S. thermophilus* is well known; the formerprevious organism produces amino nitrogen for the latter organism and it continues toalso convert lactose to lactic acid during refrigerated storage, known as postacidification which is essential for food product fermentation and stability. On the other hand, Exopolysaccharides (EPS) produced by *L. bulgaricus* and *S. thermophilus* play an important role in improving the texture and stability of yoghurt and preventing syneresis[4]. Since the addition of stabilizers of animal or plant origin to natural yoghurts is prohibited and since there is a growing popularity for food products without additions, the utilization of EPS-producing LAB starter cultures has been gained popularity[5,6]. At present, starter cultures have become an integral part of a successful industry; their relevance is reflected by the economic value of their end products.

From a commercial point of view it is clear that the starter culture must, contain the maximum number of viable organisms, be highly active under production conditions, and be free of contaminants. The development of concentrated cultures, for inoculating bulk starters or the production vat directly, has eliminated many of the problems traditionally involved in the preparing and maintaining of starter cultures. The most common technologies used for concentrating/preserving *L. bulgaricus* cells are fluidized bed dryingzation, freezing, freeze-drying and spray-drying. The choice depends on the technological, views, but also on the economical and on the practical limitations.

It is important to understand the behaviour of *L. bulgaricus* when facing dairy processes and during commercial starter preparation such as heating, drying, rehydration and storage in the dried state. The

Corresponding Author: Paula Teixeira, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal
Tel: 351 225580045, Fax: 351 225090351

development of concentrated cultures, for inoculating bulk starters or the production vat directly, has eliminated many of the problems traditionally involved in the preparing and maintaining of starter cultures. The most common technologies used for preserving *L. bulgaricus* cells are fluidization, freezing, freeze-drying and spray-drying. The choice depends on the technological views, but also on the economical and practical limitation. During these processesstarter cultures preparation and during fermentation, *L. bulgaricus* cells are exposed to stressful conditions such as high and low temperatures, high osmolarity/dehydration, oxidative stress, to nutrient starvation and especially to acidic stress since during fermentation high levels of lactic acid areis produced resulting in some loss of activity/viability[7-14] resulting in can some losst some of activity/viability. including, changes in morphology[15], exposure to high osmolarity/dehydration[13], to oxidative stress[8], to carbohydrate starvation and especially to acidic stress since during fermentation high levels of lactic acid is produced[9,10,12,14].From an industrial point of view,Therefore, the development of protocols for the preparation of starter cultures containing highly active, viable and tolerant to adverse conditions LAB would be very useful[6]. A number of environmental parameters environmental parameters can be manipulated during growth and/or starters production, to in order to increase cellularincrease cellular control survival/activity *L. bulgaricus* without changing the safety , and the nutritive value and sensorial characteristics ofcharacteristics of the final fermented products. Despite the industrial interest in *L. bulgaricus* however, a lot still remains unknown about its physiology and genetics[3] especially under Therefore, it is important to understand the favourable growth and drying conditions; the response of the organism to heating, drying and during storage in the dried state and the resistance occurred during the fermentation processes. stress conditions.

In this study an extensive review of the actual knowledge on the adaptation of *L. bulgaricus* to the stress factors encountered during starter/fermented foods production and/or preservation is presented.

STRESS

Heat stress/shock response: Despite the industrial applications, knowledge of physiological adaptations by lactic acid bacteria to dairy processing conditions such as heating remains limited. Temperature is one of the most important parameters affecting the growth and survival of bacteria. Optimum growth temperatureThe Heat Shock (HS) response refers tois defined as an abrupt increase in to a non- lethal temperature causing the expression of an adaptive response characterized by the transient induction of specific proteins known as Heat Shock Proteins (HSPs). These protect heat-stressed cells by their ability to recognize nascent polypeptides, unstructured regions of proteins and exposed hydrophobic stretches of amino acids, preventing their irreversible aggregation with other proteins[16,18]. The regulation of the HS response has been studied extensively in *E. coli* and *B. subtilis*. HSPs, also called *stress proteins*, are a group of proteins that are present in all cells in all life forms[19].

Gram-positive bacteria have diverse regulatory strategies depending on the specific heat shock genes and specific bacteria. The heat shock genes can be divided on the basis of *cis*-elements and the transcriptional activators or repressors used: - Class 1 genes, encode major chaperones DnaK-DnaJ-GrpE and GroEL-GroES; -Class 2 genes is a large group of genes that are positively controlled by a general stress; Class 3 genes encode some of the highly conserved proteins and are important in the virulence and survival of several pathogens; Class IV includes the rest of the heat-inducible genes controlled by as yet undefined mechanisms[19].

Teixeira *et al.*[20] showed that cells of *L. bulgaricus* exposed to a sublethal heat treatment acquire the ability to withstand subsequent lethal heat challenges, a phenomenon known as thermotolerance. Heat-induced thermotolerance has been observed in several other LAB such as as *L. bulgaricus*[20], *Lactococcus. lactis*[21], *Enterococcus faecalis*[22] and *Streptococcus thermophilus*[10]. Nevertheless, the HS response is also invoked by various types of environmental stresses and conditions such as drying[23], osmotic[9] and acid shock [24], oxidative[8] and starvation conditions[25]. The HS response and thermotolerance of *L. bulgaricus* has been investigated by several other authors. Lim *et al.*[24] also studied *L. bulgaricus* adaptation to an acid stress. In exponential growth phase, adapted cells (30 min at pH 4.75) of *L. bulgaricus* were more tolerant to lethal acid stress (30 min at pH 3.6). Under acidic conditions three highly induced proteins were observed identified by N-terminal amino acid sequence as GroES, GroEL and DnaK; however, a different induction mechanism was observed for GroES and GroEL suggesting post-transcriptional regulation mechanisms. Previously, Lim *et al.*[26] had demonstrated that exponential *L. bulgaricus* cells under acidic conditions (pH 4.75) showed an increased expression of GroES, GroEL and DnaK. Rechinger *et al.*[15] monitored the *L. bulgaricus* proteome by [^{35}S]-methionine and observed a higher expression of “early” proteins, GroEL and GroES in frozen cells (15.7 and 4.8,

respectively) compared to cells re-suspended in milk (0.04 and 0.9% w/v). Gouesbet *et al.*[27] demonstrated that *L. bulgaricus* acquired enhanced thermotolerance after moderate heat shock. Gouesbet *et al.*[1] reported that *L. bulgaricus* after 10 min at 65°C showed an over-expression of HSPs (DnaK and GroEL). They also analysed the thermoadaptation mechanisms involved in heat resistance and found that above a certain threshold, cells reach a maximum level of protection that cannot be easily exceeded. They also compared the protein patterns of the original strains with the selected thermoresistant variants and showed that the variants were able to induce more rapidly their adaptative mechanisms than the parent strains. The degree of thermotolerance conferred on *L. bulgaricus*, by an heat shock, when cells were grown in controlled and non-controlled pH conditions, during exponential and stationary growth phases, was evaluated by Silva *et al.*[28,29]. For exponential phase cells, thermotolerance was enhanced by submitting the cells to prior HS independently of the pH of growth conditions. Stationary phase cells were significantly more resistant to heating than cells in the exponential phase but only when growth occurred under non-controlled pH. Induced thermotolerance in stationary phase cells, was only observed for cells grown under controlled pH. Except for cells in the stationary growth phase grown under non-controlled pH, the shock treatment resulted in an intracellular increase in the GroES and GroEL.

According to Silva *et al.*,[14,28] GroES and GroEL chaperones play a crucial role in the *L. bulgaricus* stress response. The relationship between the pH of growth of *L.actobacillus delbrueckii* spp. *bulgaricus* and thermotolerance, survival through spray drying and during storage of the dried cells, was evaluated. Cells grown under acidic conditionsin (non-controlled pH) were more resistant to heat stress, spray drying and storage in the dried state than cells from cultures grown under controlled pH (pH=6.5). It was also observed by this research team that tThe intracellular levels of GroES, GroEL and Hsp70 were were enhanced when after cells were growing the cellsn under non-controlled pH conditions. These authors also determined theThe effect of the growth phase, and the pH growth conditions and heat shock on the resistance of *L. bulgaricus* to stress conditions such as heating, drying and storage in the dried state[28] was evaluated. The heat shock proteins, GroES and GroEL, were involved in both shock responses (acid development and heat shock). Stationary phase cells grown under non-controlled pH conditions were significantly more resistant to heat than cells in the exponential phase and cells grown under controlled pH in both growth phases. Again, except for cells in the stationary growth phase and under non-controlled pH, the heat shock treatment resulted in an intracellular increase in heat shock proteins (GroES and GroEL).

Heat-induced thermotolerance has been observed in other LAB such as *Lactococcus lactis*[21], *Enterococcus faecalis*[22] and *S. thermophilus*[10]. Nevertheless, the HS response is also invoked by various types of environmental stresses and conditions such as drying[23], osmotic [9]and acid shock[29] and starvation conditions[25].

Cold stress/shock response: When exposed to abrupt decreases in temperature, microorganisms undergo severe physiological disturbances such as reduction in membrane fluidity, changes in the level of DNA supercoiling, and the formation of stable secondary structures in DNA and RNA that impair replication, transcription and protein synthesis. The usual liquid crystalline nature of membrane is changed to a gel-phase state upon cold shock. After the temperature downshift, the proportion of unsaturated fatty acids in the membrane lipids increases contributing to a greater degree of flexibility. In response to these effects and to ensure cellular activity, various bacteria develop an adaptative response to cold shock, which permits adaptation of the cells to the new temperature[6,30]. In comparison to heat, cold shock responses have been studied less, however, this response has been characterized by the decrease of the synthesis of most proteins and induction of some specific proteins known as Cold Shock Proteins (CSPs cspCSPs (cold shock proteins)), resulting in a lag period of growth. Cold shock response is of vital importance for the survival of *L. bulgaricus* during the preparation of frozen or freeze-dried starter cultures and storage of yoghurt prior to consumption. Freezing causes complex stress conditions since cells can be damaged by the formation of ice crystals and by the high osmolarity due to freezing out of water and subsequent high concentrations of internal solutes which determines the survival of cells after freezing and during the period of storage in the freeze-dried state[6,31,32]. Several authors have reported that when the temperature decreases, the proportion of unsaturated fatty acids in membrane lipids increases[13,33]. Survival during and after freezing depends on the species being analysed, the growth conditions, the age of the cultures and the suspending medium[6,34]. Serror *et al.* [35] reported the adaptation of *L. bulgaricus* cells to sub-optimal temperatures and characterized the molecular adaptation regulated by the *csp* gene.characterized the two csp genes of *L. bulgaricus* after adaptation to sub-optimal temperatures, i.e. 42 to 25 or 15°C. They found that the ccspA gene was demonstrated to encodes a canonical CspA-like protein whereas the cspB gene encodess a less

conserved protein compared to the characterized prokaryotic CspCSP. However, these authors claimed that only cspA appeared to be cold inducible with a maximal induction at 25°C since;. On the other hand, cCspB might play a major role during active growth at normal temperature.

Several researchers have studied the viability/activity of *L. bulgaricus* during freezing and subsequent storage in the freeze-dried state. FFreezing of *L. bulgaricus* cells in the presence of specific cryoprotectants results inpromotes a decreased lower loss of viability[6,36]. Panoff *et al.*[37] reported that the tolerance of *L. delbrueckii* subsp. *bulgaricus* CIP 101027T to freezing at -20°C could be inducedwhen growth at 37°C was induced by pre-treatment with various compounds even at normal growth temperature (37°C); addition of some of these compounds have proven to have a protective effect to freezingsolutes. Rechinger *et al.*[15] monitored the *L. bulgaricus* proteome by [^{35}S]-methionine and observed a higher expression of early proteins, GroEL and GroES in cells that had been exposed to frozen temperatures. Similar studies were done byMore recently, Carvalho *et al.*[6,38,39] have been studying the effect of different compounds added to the drying and to the growth medium upon survival of *L. bulgaricus* during freezing and storage. These authors demonstrated that both drying and growth medium had a critical role upon survival subsequent to freeze drying[6]; the performance of each growth and drying medium and the selection of the rehydration and storage conditions are critical for recovery of freeze-dried cells. In order to produce and to prepare/preserve potential starter cultures such as *L. bulgaricus*, all of the above situations must be studied and optimized.In both studies some of the compounds had a greater cryoprotective effect (e.g. sucrose and adonitol) and others had a negative cryoprotective effect (e.g. glycerol,).

A better understanding of the responses to low temperatures, during and after freezing processes, may contribute to the optimization of the preparation/preservation of potential starter cultures including *L. bulgaricus*.

Acidic stress and growth phase: Many acid-tolerant fermentative bacteria have developed strategies in order to maintain a constant pH gradient rather than a constant internal pH_i. In general, a large proton gradient is disadvantageous for fermentative lactic acid bacteriaLAB, because proton translocation consumes energy, and anaerobic organisms gain significantly less energy from sugar metabolism than aerobes gain. Furthermore, a large proton gradient results in accumulation of organic acid anions in the cytosol[15]. The phenomenon of adaptation to acid stress has been studied in many numerous microorganisms[40] and differences in acid survival strategies have been found among the species including foodborne pathogens such as *Listeria monocytogenes*[41]and *Salmonella* Ttyphimurium[42].

Food fermentations are often carried out by sequential microbial populations; this occurs in dairy fermentations, such as yogurt fermentation, as well as in indigenous spontaneous fermentations of cereals and vegetables[42,43]. Indeed, lLactic acid bacteriaLAB, particularly *Lactobacillus* strains, which are considered among the most acid-tolerant bacteria, are often dominant at the end of these fermentations. *L. bulgaricus*, a homofermentative bacterium, produces lactic acid which leads to acidification of the medium to approximately pH 3.8. Therefore, *L. bulgaricus* must adapt to the increasing acidity in order to survive, the response being known as the Acid-Tolerance Response (ATR). Thus, acidity is one of the major stresses encountered by the bacterium in yogurts (pH 4.3-4.5) as well as in the stomach (pH 1.5-2) upon consumption[26]. The first published study by two-dimensional electrophoresis of the *L. bulgaricus* response to stress, was to acid stress[26]. Three heat shock proteinsHSPs (DnaK, GroES and GroEL) were found to be acid-shock inducible and were detected by ^{35}S-methionine labelled polypeptides in exponential growth phase after 15 min. at pH 4.75. These authors also reported that in exponential growth phase, adapted cells (30 min at pH 4.75) of *L. bulgaricus* were more tolerant to lethal acid stress (30 min at pH 3.6). Again, under these conditions, three highly induced proteins were observed and identified by N-terminal amino acid sequence as GroES, GroEL and DnaK; however, a different induction mechanism was observed for GroES and GroEL suggesting post-transcriptional regulation mechanisms[29].

Similar results were found by De Angelis and Gobbetti[30]. They observed that adapted cells (30 min. at pH 4.75) of *L. bulgaricus* were approximately 250-fold more tolerant to lethal acid stress (30 min at pH 3.6) than non-adapted cells.

The relationship between final pH of growth of *L. bulgaricus* and thermotolerance, survival through spray-drying and storage in the dried state was evaluated by Silva *et al.*,[28,29]. In non-controlled pH fermentation runs, cells of *L. bulgaricus* grown until the stationary growth phase the cells were found to be more resistant to heat, spray drying and during the subsequent during storage period in contrast to cells grown in controlled pH (commonly used by the starter cultures production industry).. In *L. bulgaricus*, most ATR studies have been focused on the exponential growing cells that suddenly undergo a rapid transition to low pH. R. More recently,

Silva *et al.*[29] studied cells of *L. bulgaricus* grown until exponential and stationary growth phase under controlled and un-controlled pH; they observed that cells were found to be induced .upon acidification of the culture medium during growth. Cells in exponential growth phase were more sensitive to subsequent stress treatments (heat and acid shock) than cells in the stationary growth phase. These differences were confirmed by measuring the induction of Hsp 70, GroEL and GroES. Under controlled pH conditions and in both growth phases, *L. bulgaricus* showed an over-expression of the studied heat shock proteinsHSPs after a heat (20 min. at 47°C) oran acid shock (20 min. at pH 2.0). However, under non-controlled pH conditions only exponential phase cells responded positively to the shocks, since stationary phase cells already had induced these proteins during growth. Differences between exponential and stationary-phase acid tolerance responsesATRs have been described for other organisms and it is well-accepted that exponential phase cells are more sensitive to stresses[34,42,44]. Therefore, to understand the ATR response of *L. bulgaricus* cells it is necessary to compare the differences between both growth phases with respect to the physiological and molecular mechanisms.

Drying and storage in the dried state: Dried preparations of lactic acid bacteriaLAB have the industrial advantages of long-term preservation and convenience in handling, storage, marketing and consumption[45]. The development of concentrated cultures, for inoculating the production vat directly, has eliminated many of the problems traditionally involved in the preparation and maintenance of starter cultures. Freeze- and spray-drying processes are commonly used in the dairy industry, however, there are some disadvantages associated with both techniques.

Freeze-drying is a method of preservation that depends upon the reduction of water activity by water removal by sublimation. On the other hand, spray-drying is the transformation of a solution or suspension into a dry powder in a single operation; generally heat is applied as a heated atmosphere and evaporation is promoted by spraying the liquid feed into this atmosphere. Spray-drying can be used to produce large amounts of dairy starter cultures relatively inexpensively; the spray-dried powder can be transported at low cost and can be stored in a stable form for prolonged periods[14,23]. Teixeira *et al.*[7,45]reported that no significant differences in survival were obtained when *L. bulgaricus* cells were dried by these two methods. However, if survival after drying is monitored after freeze- or spray-drying, viability may be lost during storage in the dried state[14,46]. Water activity is an important factor affecting the stability of dry and dehydrated products during storage. Controlling water activity in a dry product maintains proper product structure, texture, stability, density and rehydration properties. Water activity influences non-enzymatic browning, lipid oxidation, degradation of vitamins, enzymatic reactions and protein denaturation.

The survival of *L. bulgaricus* during heating, spray-drying and subsequent storage in the dried state has been evaluated[14,28,29,38,45,47]. Following drying, *L. bulgaricus* cells have demonstrated an increasing sensitivity to lysozyme and NaCl, indicators of cell wall and cell membrane damage[7], DNA damage[48] and changes in fatty acids profile by the decrease in the ratio of the unsaturated to saturated fatty acids[8,38]. These authors improved this oxidation phenomenon by storing dried cultures under vacuum[36] or under controlled water activity.[7]. In *L. bulgaricus*, survival throughout drying and subsequent storage in the dried state has been reported to be dependent on the growth phase[29], on the growth conditions[14,28], on the drying medium[49] and on the rehydration conditions[6,45]. These reports demonstrated that cells of *L. bulgaricus* were more resistant to drying and subsequent storage in the dried state, during the stationary phase of growth, under non-controlled pH, and may be further protected by adding some sugars and/or protectants to the growth medium or to the drying medium.,

Some sugars and/or protectants:

Osmotic stress: To survive osmotic stress the cells need to adapt by accumulating specific non-toxic low molecular weight compounds called compatible solutes, which include sugars (e.g. sucrose, trehalose), polyols (e.g. sorbitol, inositol), amino acids (e.g. glutamate and proline) and quaternary amines (e.g. glycine betaine, carnitine)[31,50,51]. Compatible solutes are recognized as being effective in the enhancement of enzyme stability, providing protection not only against high salt but also against high temperature, rehydration, drying and subsequent storage[52]; as being essential to maintain the integrity of the cellular membrane during desiccation[53] and as allowing the cell to retain positive turgor pressure which contributes to osmotic balance with the extracellular environment[54]. The transporters involved in Ccompatible solutes, either taken up from the environment or newly synthesizetized in the cytoplasm, lead to differences in cells and , protection during storage in the dried state[3]. Most LAB strains are reported to have no possibilities to synthesize compatible solutes recovering from osmotic stress via the uptake of those molecules known as compatible solutes[52].

The addition of various compounds to the drying

medium[31,49] and to the growth medium[14,38] can modify the survival of microorganisms during drying and subsequent storage in the dried state. These compounds have been recognised as cellular protecting agents during different drying processes and subsequent storage in the dried state[14,31,53,55]; however, different LAB strains may exhibit distinct behaviours during storage in the dried state[49,56]. Stress proteins synthesis during osmotic shock has also been reported. Kilstrup *et al.*[57] demonstratedreported that *L. lactis* cells stressed with 2.5% NaCl produced the same HSPs as in cells stressed by heat (30 min at 43°C); those stressed cells were described as being more resistant to the subsequent sub-lethal treatments..

Work done by Carvalho *et al.*[58] showed that sorbitol has a strong protective effect upon survival of dried *L. bulgaricus*. The mechanisms involved in sorbitol protection were attributed to changes in the physical state of the membrane lipids and changes in the structure of sensitive proteins in the cell[32]. Also, sucrose was anfound effeicientive protectant, when added to the drying medium, during storage of dried cells of *L. bulgaricus;* however, this protection was growth medium-dependent[39]. Silva *et al.*,[14] demonstrated that when present in the growth medium, sucrose, although not fermented by *L. bulgaricus*, is accumulated and resulted in significantly enhanced survival during heating in sterile Ringer's solution and during storage in the spray dried state. The same results were obtained by Fonseca *et al.*[56] when the addition of betaine or sodium glutamate to the drying medium enhanced the survival of *L. bulgaricus* cells to freezing and frozen stporage. This improvement in the cryopreservation of *L. bulgaricus* was explained by a decrease in the number of ice crystals formed during freezing and freeze-drying storage. Similar results were obtained by these authors when betaine or salt were added to the growth medium. Indeed, during storage of spray dried *L. bulgaricus* cells, betaine or salt showed a positive effect; however, when both compounds were added together this effect was not observed Betaine has beenis proven to be an effective cell protectant for during drying processes[59] and it is thought to prevent aggregation and maintain the solubility of cellular proteins or to alter the physical properties of the cell membrane[60]. The same results were obtained by Fonseca *et al.*[56] with the addition of betaine or sodium glutamate to the drying medium. This improvement in the cryopreservation of *L. bulgaricus* was explained by a decrease in the number of ice crystals formed during freezing and freeze-drying storage.

Zavaglia *et al.* [61]showed that trehalose diminishes significantly the damage produced by dehydration both when *L. bulgaricus* cells were dried by heating or subjected to osmotic dehydration. This effect was thought to be related to the preservation of the permeability to water and to the surface potential of the bacteria. Previously, work done by de Giori *et al.*[62] demonstrated for the first time that an active transport system governs the uptake of the essential amino acid glutamate in *L. bulgaricus* CNRZ 208. Glutamate is recognized as a counterion for positively charged potassium ions to balance the intracellular charge accumulated by bacteria under osmotic stress.

Oxidative stress: Oxidative stress has been defined as a disturbance in the antioxidant balance in favor of pro-oxidants. Oxidative stress caused by increased levels of superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), or hydroxyl radical ($HO^{\cdot}$) can lead to the damage of all cellular components. For aerobically growing bacterial cells, the auto-oxidation of components of the respiratory chain is the main source of endogenous O^{2-} and H_2O_2 [63].

L. bulgaricus is an aerotolerant anaerobe that obtains most of its energy from homolactic fermentation. It does not require strict anaerobic growth conditions and tolerates the concentration of oxygen in air. Even though *L. bulgaricus* does not use oxygen in its energy metabolism, it is likely that the presence of oxygen in its environment can influence its physiology[64]. The presence of oxygen can generate partially reduced toxic intermediates of oxygen such as O^{2-} superoxide anion, H_2O_2 hydrogen peroxide and HOhydroxyl radical. The simultaneous presence of H_2O_2 hydrogen peroxide and $O2^{-}$ superoxide anions can lead further to the formation of $HO^{\cdot}$ hydroxyl radicals which are particularly harmful in *Lactobacillus* sasince they lack superoxide dismutase and arebeing unable to eliminate superoxide anionstheir elimination[65], since and they do not express conventional catalases[66]. Together, these reactive oxygen intermediates can cause severe oxidative damage such as strand breaks in DNA[67], oxidation of membrane lipids[68]and inactivation of enzymes[67]. Therefore, theits removal of these reactive oxygen intermediates isis essential to detoxify the oxidants and repair the damage.

In the literature little information is available about the effect of the oxidative stress in *L. bulgaricus*. Castro *et al.*[33]and Teixeira *et al.*,[8] suggested the involvement of membrane lipid oxidation in cellular death during storage of dried *L. bulgaricus*. Work done by Marty-Teysset *et al.*,[64] showed a difference in the growth of *L. bulgaricus* in the presence and absence of oxygen. It was also found that *L. bulgaricus* could reduce oxygen into H_2O_2hydrogen peroxide with an NADH oxidase, probably to eliminate the oxygen present. However, this detoxification of oxygen led to an overproduction of

H_2O_2 hydrogen peroxide that caused oxidative stress and triggered an early entry of the cells into stationary phase.

Starvation: It is well known that bacterial cells enter the stationary phase upon depletion of essential nutrients from the growth medium[69] and/or accumulation of a fermentation end product (e.g. lactic acid)[30]. Under depletion of nutrients, many groups of bacteria can exchange chemical signals to help them monitor their population densities, a phenomenon referred to as quorum sensing[70]. In the past decade, various studies indicated that many of these processes occur preferentially at high population densities and are stimulated by the exchange of chemical signals between bacteria[71]. Growing populations of bacteria, during the transition to stationary phase, release a variety of hydrolytic enzymes, produce endospores or other resting structures and antibiotics or toxins, presumably to limit inter-specific competition and obtain new sources of nutrients. (o Paul acha que podemos falar do quorum sensing) . When nutrients becoame available and cells resume growth, there is usually a lag phase before the cells start to divide and return to exponential growth. If nutrients are still available, entry into stationary phase can also be due to lactic acid dissociation intracellularly[30]. Responses to limiting conditions have been studied in bacteria; carbohydrate starvation leading to cell energy depletion; phosphate starvation which is essential for the energy supply and DNA/RNA synthesis and nitrogen starvation which results primarily in the limitation of protein synthesis[30,72,73]. Increase in the spontaneous mutation rate, induction of pathogenic genes, changes in the topology of the chromosome, changes in the fatty acid composition of the cell membranes and in the structure of the cell wall, have been reported in several starved organisms[73]. Indeed, starvation results in a morphologically distinct cell that is reported to be less active and more resistant to environmental stresses[25,74,75]. Kolter *et al.*[72] reported that starvation induces resistance to a number of stresses without prior exposure to those stresses; these authors also observed that the resistance produced by starvation is even more protective than pre-adaptation to lethal challenges of growing cells.

It is generally believed that proteins synthesized during starvation are probably involved in maintenance of cell viability and in resistance to numerous stresses [69,74,75,76]. The adaptive response during the exponential phase of growth differs from the stationary phase. Compared to exponential growth phase, starved stationary phase cells of *L. lactis* developed a strong cross-protection against heat, ethanol, acid, osmotic, freezing and oxidative stress[10,11]. Lorca *et al.*[77] reported an overexpression of 16 different proteins in stationary phase cells of *Lactobacillus acidophilus*; however only 7 of them were expressed as a result of the stationary phase while 9 were expressed as a result of the drop in pH during fermentation runs. These authors haved suggested the involvement of these proteins in cell adaptation to different stresses including those proteins produced during the stationary growth phase.

In the literature there is little information about starvation and *L. bulgaricus*, however studies comparing both growth phases have been reported by Silva *et al.* [28,29]. They showed that cells of *L. bulgaricus* in the exponential growth phase were not as resistant to the stress treatments as cells in the stationary phase. They observed that in the exponential growth phase, GroEL and GroES were induced after exposure to a heat or an acid shock. *L. bulgaricus* in the stationary growth phase and under controlled pH remained very sensitive and the induction of GroEL and GroES was only observed after exposure to stress treatments; however, during growth under non-controlled pH and since the cells have been exposure to the drop of pH during fermentation, this behaviour was not observed. Previously, Carvalho *et al.*[39] demonstrated that the effect of starvation (20 min.) in protecting dried *L. bulgaricus* cells during subsequent storage was dependent on the growth medium. Again, the starvation seemed to improve resistance to stress of stationary phase cells.

Cross protection: Many microorganisms induce a stress response consisting of an overlapping set of general stress response proteins which may confer general protection to a variety of adverse conditions.

The induction of many of the same stress proteins following exposure to a variety of different subpre-lethal stresses has been demonstrated in *L. acidophilus* CRL 639[21], in *L. lactis*[78] and in *S.* TTyphimurium[79].

Cross-protection has been described in *L. bulgaricus* by various researchers. Teixeira *et al.*[4,45] reported that survival of spray dried cells during storage was enhanced by a prior heat shock. This was only obseved for exponential phase cells.[1,27]; Gouesbet *et al.* [1] Silva *et al.*[4] demonstrated the acquisition of a cross-stress-thermotolerance by exposing cells of *L. bulgaricus* to a subpre-lethal osmotic stress. More recently, . Silva *et al.*[28] observed that cells of *L. bulgaricus* became more thermotolerant after exposure to acid and heat subpre-lethal stresses. Furthermore, these authors demonstrated

acid-induced cross protection against spray-drying and during storage in the dried state. Silva *et al.*[29] have also have observed that cells of *L. bulgaricus* in stationary phase and grown under non-controlled pH (subjected to the drop of pH during the fermentation runs) are were more resistant to several subsequent stresses including heating, drying and storage in the dried state.

INDUSTRIAL APPLICATION AND CONCLUDING REMARKSN

A greater understanding of the mechanisms underlying damage and protection by heating, drying and storage in the dried state may provide targets for improving the use of *Lactobacillus. bulgaricus* as a starter culture. In order to reach maximum biological concentration and activity, the large-scale production of commercial lactic starter cultures requires a fermenter with some controlled parameters such as temperature, pH and nutrient concentration. From an economic point of view, a significant reduction in the length of an inoculation of the vat and thereby faster and better controlled acid production in dairy products would also be of great benefit for the dairy industries. Conciliation between growth and drying conditions is essential to afford protection during heating, freezing, freeze- and spray-drying, during storage, transport and post-acidification and these should be examined experimentally, in order to maximize the stability, viability and activity of dried /concentrated starter cultures. Therefore, the use of protective agents on the survival of *L. bulgaricus* in the dried state should be determined. The addition of compatible solutes to the growth medium could help the uptake or accumulation prior to subjecting the cells to drying conditions. On the other hand, efficient induction of the stress proteins (HspSPs and/or cspCSPs) could increase heat-tolerance to lethal stresses encountered during dried starters preparation. P However, proteomic studies of *L. bulgaricus* are still being irealized and are still necessarynvestigated. A lack of genome sequences is a limiting factor for identifying stress proteins. Information on the genome, on the proteomics and on the nature of injury will lead to the identification of the genetic bases of the adaptation of this LAB to the food environment and to a better understanding and to improve characteristics of interest e.g. survival during long periods of storage at ambient temperature in the dried state. Phenomena like "quorum sensing" should be investigated in order to understand the control of population densities during the transition to stationary phase, which will be more resistant to the subsequent stresses involved in the food industry.

A lack of genome sequences is a limiting factor for identifying stress proteins. Information on the genome, on the proteomics and on the nature of injury will be needed to acquire knowledge necessary for the production of the dried starter cultures, which will be characterized by the survival during long periods of storage.

Starter cultures play an important role in the production of fermented foods from raw agricultural materials such as milk, meat, vegetables and cereals. The use of the right starter is essential, and therefore, it must be emphasised that a rigorous characterisation of starter strains is vital to ensure good performance of a culture and a high quality of the end-product. The main use of LAB starter cultures in dairy industry relies on the techniques employed to produce them, in the total economic value of fermented foods throughout the world and in the additional safety of products such as provided by probiotic and bacteriocinogenic lactic acid bacteria. Increasing consumer demand for natural food additives has focused interest on lactic starters. The optimal residual moisture of conservation of dry cells, differs in function with the culture medium composition[46], the protective compounds[14], the drying technique[6] and the conditions of conservation used.

In order to reach maximum biological concentration and activity, the large-scale production of commercial lactic starter cultures requires a round dairy processor with some controlled parameters such as temperature, pH and nutrient concentration. From an economic point of view, a significant reduction in the length of the lag phase and thereby faster and better controlled fermentation would also be of great benefit for the dairy industries[15]. Study of Silva *et al.*[14] showed that cells of *L. bulgaricus* grown under controlled pH conditions were more sensitive to heat, drying and during storage in the dried state. Therefore a conciliation between growth conditions and resistance to industrial processes such as freezing, drying, storage, transport and post-acidification should be examined experimentally, in order to maximize the storage stability, viability and activity of dried concentrated starter cultures.

ACKNOWLEDGEMENTS

This study received partial financial support via project PRAXIS/P/BIO/12147/1998 (FCT, Portugal), co-ordinated by P. aula Teixeira and P. aul Gibbs. Financial support for authors J. oana Silva and A. na S. ofia Carvalho was provided by PhD fellowships PRAXIS XXI/BD/197131/99 and PRAXISXXI/BD/18152/98, respectively (FCT, Portugal).

REFERENCES

1. Gouesbet, G., G. Jan and P. Boyaval, 2002. Two-dimensional electrophoresis study of *Lactobacillus delbrueckii* sp. *bulgaricus* thermotolerance. Applied Environ. Microbiol., 68: 1055-1063.
2. Dave, R.I. and N.P. Shah, 1998. Ingredient supplementation effects on viability of probiotic bacteria in yoghurt. J. Dairy Sci., 81: 2804-2816.
3. Chervaux, C., S D. Ehrlich and E. Maguin, 2000. Physiological study of *Lactobacillus delbruekii* Ssp. *bulgaricus* strains in a novel chemically defined medium. Applied Environ. Microbiol., 66: 5306-5311.
4. Grobben, G.J., I. C. Boels, J. Sikkema, M. R. Smith and J. A. M. De Bont, 2000. Influence of ions on growth and production of exopolysaccharides by *Lactobacillus delbrueckii* Ssp. *bulgaricus* NCBF 2772. J. Dairy Res., 67: 131-135.
5. Grobben, G.J., I. Chin-Joe, V. A. Kitzen, I. C. Boels, F. Boer, J. Sikkema, M. R. Smith, and J. A. M. De Bont, 1998. Enhancement of exopolysaccharide production by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCBF 2772 with a simplified defined medium. Applied Environ. Microbiol., 64: 1333-1337.
6. Carvalho, A.S., J. Silva, P. Ho, P. Teixeira, F. X. Malcata and P. Gibbs, 2004b. Relevant factors for the preparation of freeze-dried lactic acid bacteria. Intl. Dairy J., 14: 835-847.
7. Teixeira, P.C., M.H. Castro and R.M. Kirby, 1995c. Survival of *Lactobacillus delbrueckii* ssp. *bulgaricus* following spray drying. J. Dairy Sci., 78: 1025-1031.
8. Teixeira, P.C., M.H. Castro and R.M. Kirby, 1996. Evidence of membrane lipid oxidation of spray dried *Lactobacillus bulgaricus* during storage. Lett. Applied Microbiol., 22: 34-38.
9. Sanders, J.W., G. Venema and J. Kok, 1999. Environmental stress responses in *Lactococcus lactis*. FEMS Microbiol Rev., 23: 483-501.
10. Wouters, J.A., B. Jeynov, F.M. Rombouts, W. M. De Vos, O. P. Kuipers and T. Abee 1999a. Analysis of the role of 7 kDa cold-shock proteins of *Lactococcus lactis* MG 1363 in cryoprotection. Microbiol.,145: 3185-3194.
11. Wouters, J.A., F. M. Rombouts, W.M. De Vos, O. P. Kuipers and T. Abee, 1999b. Cold shock proteins and low-temperature response of *Streptococcus thermophilus* CNRZ302. Applied Environm. Microbiol. 65: 4436-4442.
12. Wouters, J.A., H.H. Kamphuis, J. Hugenholtz, O.P. Kuipers, W. M. De Vos and T. Abee, 2000. Changes in glycolitic activity of *Lactococcus lactis* induced by low temperature. Applied Environ. Microbiol., 66: 3686-3691.
13. Foucaud-Scheunemann, C and I. Poquet, 2003. HtrA is a key factor in the response to specific stress conditions in *Lactococcus lactis*. FEMS Microbiol. Lett., 224: 53-59.
14. Silva, J., A.S. Carvalho, H. Pereira, P. Teixeira and P. Gibbs, 2004. Induction of stress tolerance in *Lactobacillus delbrueckii* ssp. *bulgaricus* by the addition of sucrose to the growth medium. J. Dairy Res., 71: 121-125.
15. Rechinger, K.B., H. Siegumfeldt, I. Svendsen and M. Jakobsen, 2000. Early protein synthesis of *Lactobacillus delbrueckii* ssp. *bulgaricus* in milk revealed by [^{35}S] methionine labelling and two-dimensional gel electrophoresis. Electroph., 21: 2660-2669.
16. Broadbent, J.R., C.J. Oberg, H. Wang and L. Wei, 1997. Attributes of the heat shock response in three species of dairy *Lactobacillus*. System. Applied Microbiol., 20: 12-19.
17. Fenton, W.A. and A.L. Horwich, 1997. GroEL-mediated protein folding. Protein Sci., 6: 743-760.
18. Nollen, E.A.A. and R. I. Morimoto, 2002. Chaperoning signalling pathways: Molecular chaperones as stress-sensing heat shock proteins. J. Cell Sci., 115: 2809-2816.
19. Yura, T., M. Kanemori and M.T. Morita, 2000. The Heat Shock Response: Regulation and Function. In: Storz, G., R. Hengge-Aronis (Eds.), Bacterial Stress Responses, ASM Press, Washington, USA, pp. 3.
20. Teixeira, P.C., M M.H. Castro and R.M. Kirby, 1994. Inducible thermotolerance in *Lactobacillus bulgaricus*. Lett. Applied Microbiol., 18: 218-221.
21. Broadbent, J.R. and C. Lin, 1999. Effect of heat shock or cold shock treatment on the resistance of *Lactococcus lactis* to freezing and lyophilization. Cryobiol., 39: 88-102.
22. Laport, M.S., J.A. Lemos, M.M.C. Bastos, R.A. Burne, M. Giambiagi-De Marval, 2004. Transcriptional analysis of the groEL and dnaK heat-shock operons of *Enterococcus faecalis*. Res. Microbiol., 155: 252-258.
23. Gardiner, G.E., E. O`Sullivan, J. Kelly, M. A. E. Auty, G. F. Fitzgerald, J. K. Collins, R. P. Ross and C. Stanton, 2000 Comparative survival rates of human-derived probiotic *Lactobacillus paracasei* and *L. salivarius* strains during heat treatment and spray drying. Applied Environ. Microbiol., 66: 2605-2612.
25. Giliberti, G., G. Naclerio, L. Martirani, E. Ricca and M. De Felice, 2002. Alteration of cell morphology and viability in a recA mutant of *Streptococcus thermophilus* upon induction of heat shock and nutrient starvation. Gene, 295: 1-6.

26. Lim, E.M., S.D. Ehrlich and E. Maguin, 2000. Identification of stress-inducible proteins in *Lactobacillus delbrueckii* subsp. *bulgaricus*. Electroph., 21: 2557-2561.
27. Gouesbet, G., G. Jan. and P. Boyaval, 2001. *Lactobacillus delbrueckii* subsp. *bulgaricus* thermotolerance. Le Lait, 81: 301-309.
28. Silva, J., A. S. Carvalho, R. Vitorino, R. Ferreira, P. Domingues, F. Amado, P. Teixeira and P. Gibbs, 2005a. Effect of the pH of growth on the survival of *Lactobacillus delbrueckii* Ssp. *bulgaricus* to stress conditions during spray. J. Applied Microbiol. 98: 775-782.
29. Silva, J., A. S. Carvalho, R. Ferreira, P. Teixeira and P. Gibbs, 2005b. Stress response of *Lactobacillus delbrueckii* ssp. *bulgaricus*: The importance of the growth pH, Submitted to Le Lait.
30. De Angelis, M. and M. Gobbetti, 2004. Environmental stress response in *Lactobacillus:* A review. Proteomics, 4: 106-122.
31. Linders, L.J.M., W.F. Wolkers, F.A. Hoekstra and K. Van´t Riet, 1997a. Effect of added carbohydrates on membrane phase behaviour and survival of dried *Lactobacillus plantarum*. Cryobiology, 35:31-40.
32. Linders, L.J.M., G. I. W. De Jong, G. Meeerdink and K. Van´t Riet, 1997b. Carbohydrates and the dehydration inactivation of *Lactobacillus plantarum*: The role of moisture distribution and water activity. J. Food Eng., 31: 237-250.
33. Castro, H., P. Teixeira and R. Kirby, 1996. Changes in the membrane of *Lactobacillus bulgaricus* during storage following freeze-drying. Biotechnol. Lett., 18: 99-104.
34. Derzelle, S., B. Hallet, K.P. Francis, T. Ferain, J. Delcour and P. Hols, 2000. Changes in *csp*L, *csp*P and *csp*C mRNA abundance as a function of cold shock and growth phase in *Lactobacillus plantarum*. J. Bacteriol., 182: 5105-5113.
35. Serror, P., R. Dervyn, S. D. Ehrlich and E. Maguin, 2003. csp-like genes *Lactobacillus delbrueckii* sp. *bulgaricus* and their response to cold shock. FEMS Microbiol. Lett., 226:323-330.
36. Castro, H., P. Teixeira and R. Kirby, 1997. Evidence of membrane damage in *Lactobacillus bulgaricus* following freeze-drying. J. Applied Microbiol., 82: 87-94.
37. Panoff, J.M., B. Thammavongs and M. Gueguen, 2000. Cryoprotectants lead to phenotypic adaptation to freeze-thaw stress in *Lactobacillus delbrueckii* ssp. *bulgaricus* CIP 101027T. Cryobiology, 40 : 264-269.
38. Carvalho, A.S., J. Silva, P. Ho, P. Teixeira, F.X. Malcata and P. Gibbs, 2003b. Effect of different sugars added to the growth and drying medium upon thermotolerance and survival during storage of freeze-dried *Lactobacillus delbrueckii* ssp. *bulgaricus*. Biotechnol. Progress, 20: 248-254.
39. Carvalho, A.S., J. Silva, P. Ho, P. Teixeira, F.X. Malcata and P. Gibbs, 2003d. Effects of addition of sucrose and salt and of starvation upon thermotolerance and survival during storage of freeze-dried *Lactobacillus delbrueckii* sp. *bulgaricus*. J. Food Sci., 68: 2538-2541.
40. Shabala, L., B. Budde, T. Ross, H. Siegumfeldt and T. McMeekin, 2002. Responses of *Listeria monocytogenes* to acid stress and glucose availability monitored by measurements of intracellular pH and viable counts. Intl. J. Food Microbiol., 75: 89-97.
41. ´Driscoll, B.O., C. G. M. Gahan and C. Hill, 1997. Two-dimensional polyacrylamide gel electrophoresis analysis of the axcid tolerance response in *Listeria monocytogenes* LO28. Applied Environ. Microbiol., 63: 2679-2685.
42. Bang, I.S., B. H. Kim, J. W. Foster and Y. K. Park, 2000. OMPR regulates the stationary-phase acid tolerance response of *Salmonells enterica* serovar *typhimurium*. J. Bacteriol., 182: 2245-2252.
43. Meaden, P.G., N. Arneborg, L.U. Guldfeldt, H. Siegumfeldt and M. Jakobsen, 1999. Endocytosis and vacuolar morphology in *Saccharomyces cerevisiae* are altered in response to ethanol stress or heat shock. Yeast, 15:1211-1222.
44. Pagán, R. and B. Mackey, 2000. Relationship between membrane damage and cell death in pressure-treated *Escherichia coli* cells: Differences between exponential and stationary phase cells and variation among strains. Applied Environ. Microbiol., 66: 2829-2834.
45. Teixeira, P.C., M. H. Castro and R. M. Kirby, 1995b. Spray drying as a method for preparing concentrated cultures of *Lactobacillus bulgaricus*. J. Applied Bacteriol., 78: 456-462.
46. Carvalho, A.S., J. Silva, P. Ho, P. Teixeira, F.X. Malcata and P. Gibbs, 2004a. Effects of various sugars added to growth and drying media upon thermotolerance and survival throughout storage of freeze-dried Lactobacilus delbrueckii ssp. *bulgaricus*. Biotechnol. Prog., 20: 248-254.
47. Teixeira, P.C., M. H. Castro and R. M. Kirby, 1995a. Death kinetics of *Lactobacillus bulgaricus* in a spray drying process. J. Food Prot., 57: 934-936.

48. Teixeira, P.C., H. Castro, C. Mohacsi-Farkas and R. M. Kirby, 1997. Identification of sites of injury in *Lactobacillus bulgaricus* during heat stress. J. Applied Microbiol., 82: 219-226.
49. Carvalho, A.S., J. Silva, P. Ho, P. Teixeira, F. X. Malcata and P. Gibbs, 2002. Effect of additives on survival of freeze-dried *Lactobacillus plantarum* and *Lactobacillus rhamnosus* during storage. Biotechnol. Lett., 24: 1587-1591.
50. Leslie, S.B., E. Israeli, B. Lighthart, J. H. Crowe and L. M. Crowe, 1995. Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. Applied Environ. Microbiol., 61: 3592-3597.
51. Carvalho, A.S., J. Silva, P. Ho, P. Teixeira, F. X. Malcata and P. Gibbs, 2003a. Effect of various growth media upon survival during storage of freeze-dried *Enterococcus faecalis* and *Enterococcus durans*. J. Applied Microbiol., 94: 947-952.
52. Poolman, B. and E. Glaasker, 1998. Regulation of compatible solute accumulation in Bacteria: Micro review. Mol. Microbiol., 29: 397-407.
53. Kets, E.P.W. and J. A. M. de Bont, 1994. Protective effects of betaine on survival of *Lactobacillus plantarum* subjected to drying. FEMS Microbiol. Lett., 116: 251-256.
54. Bremer, E. and R. Kramer, 2000. Coping with Osmotic Challenge: Osmoregulation through Accumulation and Release of Compatible Solutes in Bacteria. In: Storz, G. and R.L. Hengge-Aronis, (Eds.), Bacterial Stress Responses, Washington DC. ASM Press, pp: 79-97.
55. Kets, E.P.W., P.J.M. Teunissen and J.A.M. de Bont, 1996. Effect of compatible solutes on survival of lactic acid bacteria subjected to drying. Applied Environ. Microbiol., 62, 259-261.
56. Fonseca, F., C. Béal, F. Mihoub, M. Marin and G. Corrieu, 2003. Improvement of cryopreservation of *Lactobacillus delbrueckii* Ssp. *bulgaricus* CFL 1 with additives displaying different protective effects. Intl. Dairy J., 13: 917-926.
57. Kilstrup, M., S. Jacobsen, K. Hammer and F. K. Vogensen, 1997. Induction of heat shock proteins DnaK, GroEL and GroES by salt stress in *Lactococcus lactis*. Applied Environ. Microbiol., 63: 1826-1837.
58. Carvalho, A.S., J. Silva, P. Ho, P. Teixeira, F.X. Malcata and P. Gibbs, 2003c. Protective effect of sorbitol and monosodium glutamate during storage of freeze-dried lactic acid bacteria. Le Lait, 83: 203-210.
59. Cleland, G., P. Krader, McCree, J. Tang and D. Emerson, 2004. Glycine betaine as a cryoprotectant for prokaryotes. J. Microbial Method, 58: 31-38.
60. Rudolph, A., J. Crowe and L. Crowe, 1986. Effects of three stabilizing agents-proline, betaine and trehalose on membrane phospholipids. Arch. Biochem. Biophys., 245: 134-143.
61. G. A. Zavaglia, E. Tymczyszyn, G.De Antoni, A.E. Disalvo, 2003. Action of trehalose on the preservation of *Lactobacillus delbrueckii* ssp. *bulgaricus* by heat and osmotic dehydration. J. Applied Microbiol., 95: 1315-1320.
62. de Giori, G.S., C. Foucaud-Scheunemann, M. Ferchichi and D. Hemme, 2002. Glutamate uptake in *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 208 and its enhancement by a combination of Mn^{2+} and Mg_{2+}. Lett. Applied Microbiol., 35: 428-432.
63. González-Flecha, B. and B. Demple, 1995. Metabolic sources of hydrogen peroxide in aerobically growing *Escherichia coli*. J. Biol. Chem., 270: 13681.
64. Marty-Teysset, C., F. De La Torre and J. R. Garel, 2000. Increased production of hydrogen peroxide by *Lactobacillus delbrueckii* sp. *bulgaricus* upon aeration: Involvement of an NADH oxidase in oxidative stress. Applied Environ. Microbiol., 66: 262-267.
65. Gregory, E.M. and Fridovich, 1974. Oxygen metabolism in *Lactobacillus plantarum*. J. Bacteriol., 117: 166-169.
66. Hung, J., D. Cooper, M. S. Turner, T. Walsh and P. M. Giffard, 2003. Cystine uptake prevents production of hydrogen peroxidase by *Lactobacillus fermentum* BR11. FEMS Microbiol. Lett., 1: 93-99.
67. Amanatidou, A., E. J. Smid, M.H.J. Bennik and L. G. M. Gorris, 2001. Antioxidative properties of *Lactobacillus sake* upon exposure to elevated oxygen concentrations. FEMS Microbiol. Lett., 1: 87-94.
68. Guerzoni, M.E., R. Lanciotti and P. S. Cocconcelli, 2001. Alteration in cellular fatty acid composition as a response to salt, acid, oxidative and thermal stresses in *Lactobacillus helveticus*. Microbiology, 147: 2255-2264.
69. Siegele, D.A. and L. J. Guynn, 1996. *Escherichia coli* proteins synthesized during recovery from starvation. J. Bacteriol., 178: 6352-6356.
70. Winans, S.C. and J. Zhu, 2000. Roles of Cell-cell Communication in Confronting the Limitations and Opportunities of High Population Densities. In: Storz G. and R. Hengge-Aronis(Eds.). Bacterial Stress
71. Waldburger, C., D. González and G. H. Chambliss, 1993. Characterisation of a new sporulation factor in *Bacillus subtilis*. J. Bacteriol., 175: 6321-6327.
72. Kolter, R., D. A. Siegele and A. Tormo, 1993. The stationary phase of the bacterial life cycle. Ann. Rev. Microbiol., 47: 855-874.

73. Givskov, M., L. Eberl, S. Moller, L. K. Poulsen and S. Molin, 1994. Responses to nutrient starvation in *Pseudomonas putida* KT 2442: Analysis of general cross-protection, cell shape and macromolecular content. J. Bacteriol., 176: 7-14.
74. Rockabrand, D., T. Arthur, G. Korinek, K. Livers and P. Blum, 1995. An essential role for the *Escherichia coli* DnaK protein in starvation induced thermotolerance, H_2O_2 resistance and reductive division. J. Bacteriol., 177: 3695-3703.
75. Palumbo, E., C.F. Favier, M. Deghorain, P.S. Cocconcelli, C. Grangette, A. Mercenier, E.E. Vaughan and P. Hols, 2004. Knockout of the alanine racemase gene in *Lactococcus plantarum* results in septation defects and cell wall perforation. FEMS Microbiol. Lett., 233: 131-138.
76. Rallu, F., A. Gruss and E. Maguin, 1996. *Lactococcus lactis* and stress. Antonie Van Leeuwenhoek, 70: 243-251.
77. Rockabrand, D., K. Livers, T. Austin, R. Kaiser, D. Jensen, R. Burgess and P. Blum, 1998. Roles of DnaK and RpoS in starvation-induced thermotoilerance *of Escherichia coli*. J. Bacteriol., 180: 846-854.
78. Lorca, G.L. and F. De Valdez, 2001. A low-pH-inducble, stationary-phase acid-tolerance response to *Lactobacillus acidophilus* CRL 639. Curr. Microbiol., 42: 21-25.
79. Baik, H.S., S. Bearson, S. Dunbar and J.W. Foster, 1996. The acid tolerance response of *Salmonella typhimurium* provides protection against organic acids. Microbiol., 142: 3195-3200.