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Stress Response of *Lactobacillus Delbrueckii* ssp. *Bulgaricus*: The Importance of The Growth pH

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Abstract: The degree of thermotolerance conferred on *Lactobacillus delbrueckii* ssp. *bulgaricus*, by an acid shock or a heat shock, when cells were grown in controlled and non-controlled pH conditions, during exponential and stationary growth phases, was evaluated. For exponential phase cells, thermotolerance was enhanced by submitting the cells to prior heat or acid shocks 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 heat or acid shock treatments resulted in an intracellular increase in the well - known heat shock proteins, GroES and GroEL. The presence of chloramphenicol negated the heat shock response and production of GroES and GroEL, confirming that *de novo* protein synthesis was involved in cell protection. These proteins were also identified in stationary phase cells grown under non-controlled pH, suggesting their involvement in induced thermotolerance during growth at low pH. Very few studies have investigated the response of stationary phase cells. The evaluation development of the response to sub-lethal stresses as a function of the pH conditions during growth is a new insight;. In fact, it was demonstrated that the pH of growth is a crucial factor.

Key words: Heat shock protein, *L. bulgaricus,* growth phase, stress response, thermotolerance , pH

INTRODUCTION

Lactobacillus delbrueckii ssp. *bulgaricus*, hereafter termed *L. bulgaricus*, is a Lactic Acid Bacterium (LAB) used as a starter culture in important dairy fermentations, namely in yoghurt and in production of some Swiss-type cheeses[1]. The development of concentrated cultures allowing the semi-direct or direct inoculation of the production vat, has eliminated many of the problems customarily involved in preparing and maintaining starter cultures in the dairy plant. However, the price of current commercial starters and the high susceptibility of *L. bulgaricus* to the various stress conditions, including heat, acid, osmotic and oxidative stresses imposed by the preparation and storage, limits the use of these commercial starter cultures. From an industrial point of view, the development of protocols for the preparation of starter cultures containing highly active, viable cells tolerant to adverse conditions, would be advantageous[1,2].

It is well documented that when exposed to a low level of stress, most bacteria develop adaptation strategies in order to resist a subsequent exposure to a higher level of the same stress and also to a number of different stresses[3-5]. Increased thermotolerance of *L. bulgaricus* to heat and acid stresses following sub-lethal treatments has already been demonstrated[3,6-8]. Most of the previous studies, however, were done with exponential phase cells[3,7,8]. From a practical point of view, the contribution of these studies for producers theof starter cultures producers is limited. For industrial applications, cells are normally harvested at the end of the exponential or in the stationary phase, as the biomass obtained in the exponential phase of growth is less than that obtained during stationary phase. It is also accepted that exponential phase cells are more sensitive to stress treatments than stationary phase cells[6,9,10]. In fact, there are very few studies where the sub-lethal stress responses of stationary phase LAB have been investigated[6,9,11,12]. The main objective of this study was, therefore, to evaluate potential strategies that could be used to produce cells more resistant to heat stress. In order to fulfil this objective the degree of the rmotolerance conferred on stationary and

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exponential phase cells of *L. bulgaricus*, previously grown under controlled and non-controlled pH, by exposure to sub-lethal heat and acid treatments (shock treatments), was evaluated. The involvement of shock proteins in the response to these sub-lethal conditions was also investigated. The stress response of *L. bulgaricus* has already been extensively studied, but the effect of the pH conditions during growth on this response remains to be elucidated.

MATERIALS AND METHODS

Growth Conditions: *L. bulgaricus*, previously isolated from yoghurt and maintained in ESB culture collection (ESB285), was used. The reference cultures were maintained in cryogenic storage at - 80°C on glass beads. Working cultures were grown on De Man, Rogosa, Sharpe (MRS, LAB M, UK) agar as slant cultures (37°C for 48 h). Slants were maintained at 4°C and sub-cultured every month.

To prepare the inoculum, MRS broth was inoculated from the MRS agar slants and incubated at 37°C for 24 h. This cell suspension was then used to inoculate another MRS broth (1% v/v). The cultures were incubated with agitation by a magnetic stirrer bar at 37°C without or with pH controlled to pH 6.5. pH was measured by a pH electrode and meter (405-DPAS-SC-K8S/200, Mettler Toledo, Switzerland) coupled to a relay that detected pH alterations and controlled the automatic addition of 1 M sodium hydroxide by a peristaltic pump. pH changes during growth under non-controlled conditions were recorded from a pH meter (as above). *L. bulgaricus* cells were grown until the exponential and stationary growth phase, without or with pH control and harvested by centrifugation at 7000 × g for 15 min (4°C). Times of harvesting were selected on the basis of the growth curves (arrows indicated in Fig. 1). For cells grown under controlled or non-controlled pH the transition to stationary phase was not triggered by sugar exhaustion. It was demonstrated by HPLC that there was glucose remaining in the growth medium when cells entered the stationary phase of growth.

Survival during lethal heat stress

Sub-lethal treatments: Cell pellets were resuspended to the original volume in sterile Ringer's solution with and without chloramphenicol (0.313 mM) at 47°C (heat shocked cells) or at 37°C with pH adjusted to 2.0 with lactic acid (acid shocked cells) and at 37°C at pH 6.5 (control cells) for 20 min before being submitted to the stress temperature (57°C). For both treatments no decrease of cellular viability was observed.

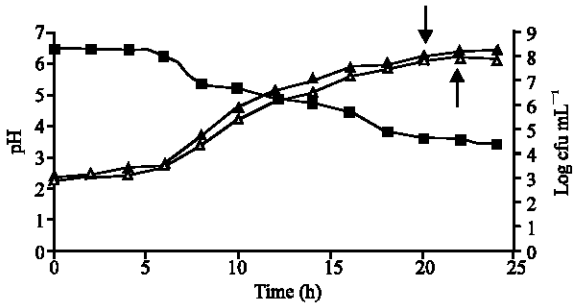

Fig.1: pH evolution (□) and cell counts during growth of *Lactobacillus bulgaricus* in MRS under controlled (▲) and non-controlled pH conditions (△)

Lethal heat treatment: Stressed Shocked and control cell suspensions were centrifuged at 7000 × g for 15 min (4°C) and re-suspended to the original volume in 11% w/v reconstituted skim milk (Oxoid, Hampshire, UK). One millilitre aliquots were transferred with vigorous mixing to 49 mL of sterile Ringer's solution already heated topreviously equilibrated to 57°C and maintained at this temperature for 30 min. At defined intervals, samples (1.0 mL) were taken from the heating medium and immediately diluted in Ringer's solution (9.0 mL) at room temperature.

Enumeration of survivors: Survivors Viable cells before each treatment and at appropriate intervals during heating at 57°C, were enumerated on MRS agar by the drop count technique[13] in two independent experiments. Three drops (20 µL each) of suitable dilutions were placed on each of 3 separate plates. Plates were incubated at 37°C for 48 hrs before enumeration.

Studies on shock proteins

Preparation of protein cell extracts: Cell pellets were prepared as previously described. Aliquots (100 mL) of the heat-shocked and the control cell suspensions were washed twice with tris-acetate-EDTA-buffer (TAE, pH 7.0) and were incubated with lysozyme (Sigma, St. Louis, USA) for 2.5 h at 37°C (40 mg lys: 0.4 mL Tris-HCl 0.064 M pH 7.0; 20µL lysozyme: 20 mL TAE). The cell suspension was then disrupted by grinding in a vortex with glass beads (150-212 μmmicrons diameter, Sigma). Between each of the 10 disruption cycles realized, cell suspensions were cooled on ice. The disrupted suspension was then centrifuged (15 min, 7000 x g for, 15 min, 4°C) and the supernatant was stored at -80°C until use. (2004). Protein concentration was determined according to Lowry *et al*[14]. Aliquots of extracts containing 15 µg protein were analysed by one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE)[15] on 15% gels using the Hoefer SE600 apparatus (Pharmacia Biotech, Buckinghamshire, UK).

Western blotting: Separated proteins from SDS-PAGE were electroblotted onto nitrocellulose membranes for 3 h at 200 mA. (2004). The blots were probed with the monoclonal Anti-GroES developed in rabbit IgG fraction of antiserum (Sigma), with the monoclonal Anti-GroEL developed in rabbit IgG fraction of antiserum (Sigma) and with the secondary antibody (mouse anti-rabbit IgG peroxidase conjugate (Sigma). The bands were visualized by treating the immunoblotts with ECL chemiluminescence reagents (Pharmacia Biotech),

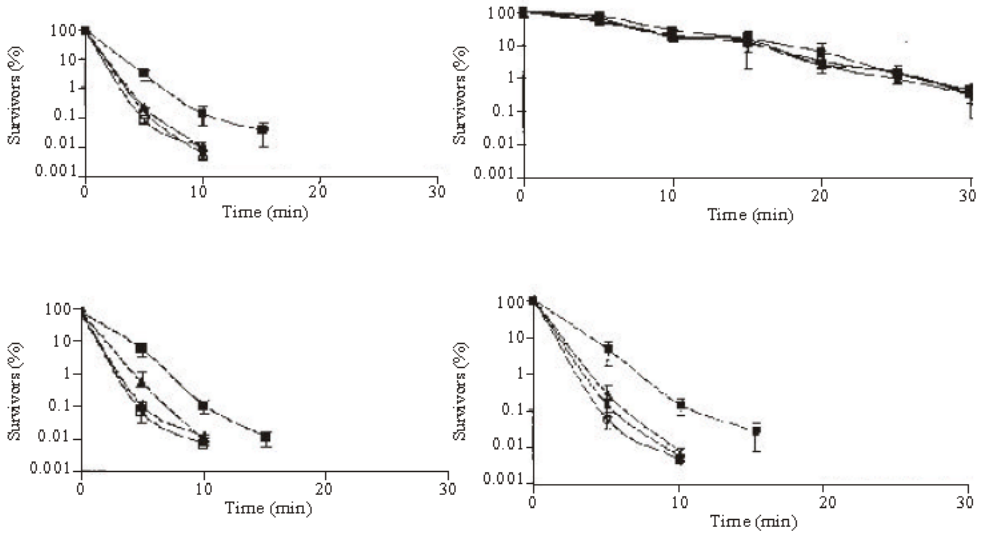

Fig.2: Effect of an acid shock at pH 2.0 for 20 min in the survival of *Lactobacillus delbrueckii* ssp. *bulgaricus* during 30 min at 57°C when cells were previously grown until exponential growth phase under non-controlled (A) and controlled (C) pH and until the stationary growth phase under non-controlled (B) and controlled (D) pH, (□, control cells; △, control cells in the presence of 0.313 mM chloramphenicol; ▲ acid shocked cells; p,□acid shocked cells in the presence of 0,,313mM chloramphenicol. Two independent experiments were realizedcarried out. The error bars on the figures indicate the mean standard deviations for the data points

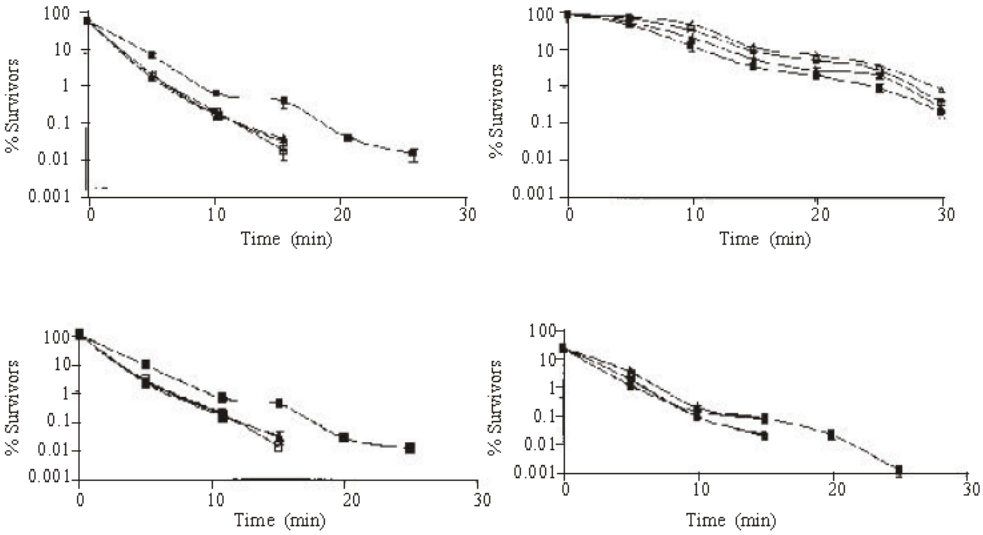

Fig.3: Effect of an heat shock at 47°C for 20 min oin the survival of *Lactobacillus delbrueckii* ssp. *bulgaricus* during 30 min at 57°C when cells were previously grown until exponential growth phase under non-controlled (A) and controlled (C) pH and until the stationary growth phase under non-controlled (B) and controlled (D) pH. (□, control cells; △, control cells in the presence of 0.313 mM chloramphenicol; |,□, heat shocked cells; p, ▲ heat shocked cells in the presence of 0.313 mM chloramphenicol. Two independent experiments were realizedcarried out. The error bars on the figures indicate the mean standard deviations for the data points

according to the supplier's instructions, followed by exposure to X-ray films (Sigma, Kodak Biomax Light Film, St. Louis, USA). After development, the films were analyzed with QuantityOne Software (BioRad, Hercules, CA, USA). Optical density results were expressed as a percentage of the total optical density.

RESULTS

Survival during lethal heat stress: Figures 2 and 3 illustrate the influence of the growth phase and pH growth conditions on the survival of *L. bulgaricus* during heat stress treatment in Ringer's solution at 57°C. Stationary phase cells grown in non-controlled pH conditions were always much more resistant to heat stress than exponential phase cells or stationary phase cells grown under controlled pH. When cultures were grown under controlled pH, exponential and stationary phase cells showed a similar heat resistance and of the same order of magnitude as that observed for exponential phase cells grown under non-controlled pH conditions.

The effect of heat or acid shocks on the increase in thermotolerance was evaluated as a function of the growth phase and pH growth conditions. As observed shown in Fig. 2C 2C, 2D, 3C and 3D, for cells grown under controlled pH conditions, heat heat or acid shock was responsible for a substantial increase in the heat resistance of cells in both exponential and stationary phases of growth. However, for cells grown under non-controlled pH conditions, this effect was only observed for cells in the exponential phase of growth (Fig. 2A, 2B, 3A, 3B). Cells grown under controlled pH and harvested in the stationary phase, were more resistant to the lethal stress than cells grown under any other condition, whether or not. For all the conditions evaluated, an increase in thermotolerance was never observed when the heat or acid shock treatment was applied in the presence of chloramphenicol.

Induction of GroES and GroEL in response to heat and acid shocks: The optical densitycell content of GroES and GroEL, measured by Western Blotting, was determined for stationary (Fig. 4B and 4D) and exponential

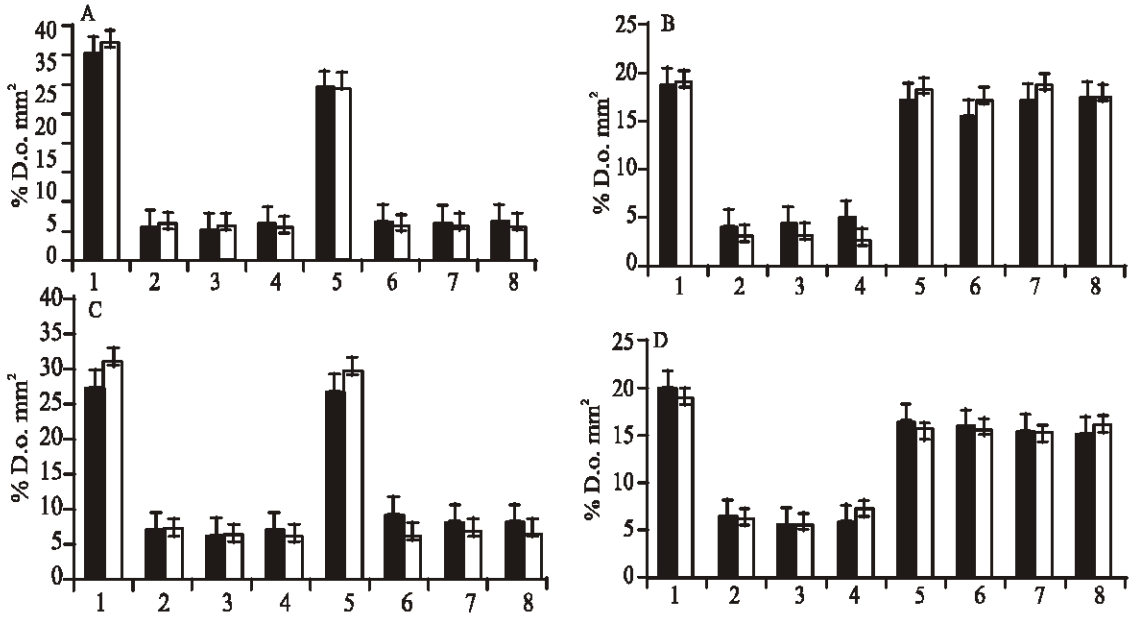

Fig. 4: Percentage of the total optical density developed by enzyme-coupled immunoblotting for shock proteins (■, GroES and □, GroEL) for *Lactobacillus delbrueckii* ssp. *bulgaricus* in exponential growth phase subjected to a heat shock (A) and an acid shock (C) and in stationary growth phase subjected to a heat shock (B) and an acid shock (D).
Cells previously grown under controlled pH: 1, shocked cells; 2, control cells; 3, control cells in the presence of 0,313mM chloramphenicol; 4, shocked cells in the presence of 0,313mM chloramphenicol
Cells previously grown under non-controlled pH: 5 shocked cells; 6, control cells; 7, control cells in the presence of 0,313mM chloramphenicol; 8, shocked cells in the presence of 0,313mM chloramphenicol. The error bars on the figures indicate the mean standard deviations for the data points of two independent experiment

phase cells (Fig. 4A and 4C) grown under both pH conditions.

For cells grown under non-controlled pH the observed optical density of both protein bands was significantly higher in the stationary phase cell extracts (Fig. 4B and 4D). and hHeat and acid shock led to a significant enhancement in the expression of both heat shock proteins, but only infor exponential phase cells (Fig. 4A and 4C). When growth occurred under controlled pH, the optical density of both protein bands was similar for exponential and stationary phase cells and both shocks treatments led to an enhancement in the expression of both heat shock proteins in both stationary and exponential phase cells. No induction of these HS proteins was observed when the shock treatments were performed in the presence of chloramphenicol.

DISCUSSION

The main objective of this work was to evaluate possible strategies that could induce in *L. bulgaricus*, a higher resistance of *L. bulgaricus* to lethal heat treatments.

The induction of thermotolerance as a response to heat and acid shocks, was evaluated for both stationary and exponential phase cells grown under controlled and non-controlled pH conditions. With the exception of stationary phase cells grown under non-controlled pHh, heat and acid shocks increased the thermotolerance of *L. bulgaricus*. This confirms that in this species suggesting thermotolerance could be was induced by environmental acidification occurring during growth. A few studies with LAB have demonstrated that even for stationary phase cells, a sub-lethal stress might improve survival during subsequent lethal stresses. A low pH-inducible stationary phase tolerance to ethanol, hydrogen peroxide, freezing and freeze drying was observed in *L. acidophilus* CRL 639. However, this effect was not observed for osmotic and heat stresses[9]. De Angelis *et al*., 2004a showed that survival of stationary phase cells of *Lactobacillus plantarum* DPC2739 at 72°C for 90s was increased by 2 $\log_{10}$ cycles if the cells were pre-adapted to 42°C for 1h. In this study, however, the final growth pH was not given. Strain dependent adaptive response to sub-lethal stresses has been reported and might, in addition to variations in the experimental methodologies, explain the differences observed in relation to other studies[3,16]. It is important to point out that in previous studies describing an increase of stress-tolerance of stationary phase bacteria, the protecting mechanisms have been associated with starvation[17].

In this study, when cells were growing under non-controlled pH, the entry into the stationary phase was probably triggered by the low external pH and concomitant reduction in internal pH [18]. The mechanism responsible for entry into the stationary phase when growth was performed under pH controlled conditions, however, is not known, but is likely to result from quorum sensing [19,20] or the accumulation of toxic metabolites such as hydrogen peroxide[21] and not starvation as glucose was present in the growth medium when cells entered the stationary phase of growth.

Protein synthesis *de novo* was demonstrated to be involved in cell protection by heat or acid shock, as the increase in thermotolerance was neverinduction when these treatments were applied in the presence of chloramphenicol. Induction of the molecular chapersons GroEL and GroES, in addition to other proteins, as a responseto sub lethal shock has been assoclated with the development of stressresistance[5,7,22-25].

From the current study, it is clear that induction of thermotolerance by increasing exposure to acidic pH conditions, rather than exposure just to increasing levels of lactate at neutral pH, is more effective than heat or acid shocks. Nevertheless, the levels of GroES and GroEL were similar to those in shocked cells. Other mechanisms (e.g., sigma factors) induced by growth in decreasing pH and permitting active growth to continue at low pH, are also reported to be involved in thermotolerance and resistance to other stresses[5,10].

The results of this study are in agreement with the general observation that stationary phase cells are better able to survive subsequent stresses[11,26], but for *L.bulgaricus*. Even so, it is important to remark that this was only observed for cells grown under conditions of non-controlled pH. These findingsThis result is are of major importance for those who produce or utilise starter cultures. With the objective of maximising the cellular biomass, in industrial processes, cells are commonly grown under controlled pH. However, as viability is an important parameter in starter cultures preparation/storage /utilization, it would therefore be advisable to optimise growth conditions in order to obtain more resistant cells. From this standpoint, further research should be carried out in order to develop strategies for increasing the tolerance of stationary phase cells to the environmental stresses normally encountered during starter cultures production and utilisation.

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