Thermotolerance, Survival, and Stability of Lactic Acid Bacteria After Spray Drying as Affected by the Increase of Growth Temperature

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ORIGINAL RESEARCH

Thermotolerance, Survival, and Stability of Lactic Acid Bacteria After Spray Drying as Affected by the Increase of Growth Temperature

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Abstract

Microencapsulation of lactic acid bacteria (LAB) via spray drying differs from that of common bioactive substances in that the intrinsic stress tolerance of cells can be modulated to improve cell survival. In this study, elevated growth temperatures that were 3–5 °C above the standard conditions were used to culture *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus rhamnosus* GG (LGG), and *Lactobacillus acidophilus* for spray drying. The heat-adapted cultures showed lower bacterial population than the controls by 0.45 log at stationary growth phase and produced lactobacilli cells with elongated shape, while their metabolic activities were maintained similar to the controls. Heat-adapted *L. cremoris* and LGG demonstrated increases in survival by 0.7–1.5 log and 0.3 log, respectively, after heat treatment at 60 °C. The thermotolerance of *L. acidophilus* grown at 42 °C was dependent on growth phase, and the culture entered death phase within 24 h of incubation. The survival of heat-adapted *L. cremoris* and *L. acidophilus* after spray drying was increased by 21.0% and 13.7%, respectively, whereas the increase shown by LGG was relatively insignificant (9.9%). Spray-dried powders containing heat-adapted cells showed substantial reduction of viability at the first week of storage, reaching 1.03–1.23 log, compared to 0.87–0.90 log of reduction shown by the controls. The findings demonstrated that strain-specific cellular response toward variations in growth conditions is crucial to the intrinsic properties of LAB and to cell survival during spray drying and storage. Controlling cellular response is one of the key factors in developing a viable spray drying scheme for active LAB.

Keywords Active dry probiotics · *Lactobacillus rhamnosus* GG · Pre-adaptation · Probiotics powder · Storage stability · Stress tolerance

Introduction

Several lactic acid bacteria (LAB) strains are reputed probiotics when orally administrated in a viable form with adequate amount (Forster and Lawley 2015; Vasiljevic and Shah 2008). These microorganisms have attracted growing attention in recent years both as functional foods and as potential pharmaceutics (Bansal and Garg 2008; Kavitake et al. 2018). In food and pharmaceutical applications, dry bacteria powder is a convenient product form for transportation and incorporation into other processing operations such as mixing and tableting (Fu et al. 2018; Peighambardoust et al. 2011). Compared to liquid product, it has advantages in the long-term maintenance of the activity of bio-active components, which can effectively prolong the shelf-life of products (Rabaioli Rama et al. 2020; Schutyser et al. 2012).

Spray drying can rapidly convert liquid feed to powder, with a high production rate up to several tonnes per hour (Jin and Chen 2010). It is capable of processing heat-sensitive material, as the temperature of atomized droplets could be controlled at a relatively low level by evaporative cooling effect (Fu et al. 2011). It is a common approach utilized for the microencapsulation of bio-active substances to protect them from environmental stresses (Fu et al. 2020; Oliveira et al. 2018). Spray drying has been actively

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investigated for its potential in producing active probiotics powder, in which bacterial cells are encapsulated by protectant matrix (Kavitake et al. 2018; Schutyser et al. 2012). In such a process, the processing procedure must be carefully controlled to minimize the loss of bacterial viability. Unoptimized spray drying conditions may expose bacterial cells to high temperature and rapid dehydration, causing modifications of the structure of sensitive proteins as well as alterations of the physical state of lipids on cellular membrane (Fritzen-Freire et al. 2013; Gul and Atalar 2019; Leuenberger et al. 2017). To protect the viability of LAB cells during spray drying, various approaches have been proposed. Microcapsules with novel structure have been designed to improve the stability of cells toward heat treatment, in vitro digestion, and storage (Arslan-Tontul and Erbas 2017; Zhao et al. 2018). The formulation of protectant proves to be a crucial factor influencing the survival of spray-dried cells (Liu et al. 2018b; Su et al. 2019), and the underlying protective mechanisms for a variety of reagents have been explored for developing high-performance protectant formulation.

As living microorganisms, LAB cells differ from other bioactive substances such as vitamins and fatty acids, in that they can respond to environmental stresses, and can be injured and repair the injury to continue multiplying. Previous studies showed that the intrinsic stress tolerance of a given LAB strain may vary depending on growth conditions and specific treatments of cells. Adding sucrose to growth medium could improve the thermotolerance of cells and increase the survival of cells in subsequent vacuum drying (Silva et al. 2004; Tymczyszyn et al. 2007). Subjecting cells to a sublethal heat treatment after the standard cultivation procedure not only enhanced the thermotolerance of cells but also exerted cross-protective effects toward other stresses, such as salt and bile (Desmond et al. 2001). Huang et al. (2016a) found that culturing LAB strains with concentrated sweet whey effectively improved the survival of spray-dried cells. The mechanism was attributed to LAB cells acquiring tolerance to multiple stresses under the hyperosmotic growth condition, by the expression of stress proteins and the intracellular accumulation of compatible solutes (Huang et al. 2016b).

With regard to the industrial production of LAB powder, the induction of cell stress tolerance by altering growth conditions is more cost-effective than incorporating a pre-adaptation procedure after the cultivation process of cells. It saves a processing step, and also avoids the possible loss of cell viability during the sublethal treatment. To induce the adaptive response of cells, culturing lactobacilli under a variety of sublethal conditions has been attempted, including the use of high and low temperature, the variation of pH, oxygenation and osmotic pressure, as well as the addition of inhibitory substances such as bile salts (Dijkstra et al. 2014; Gaucher et al. 2020; Settachaimongkon et al. 2015). Increasing the growth temperature may be a straightforward approach to enhance the thermotolerance of cells. Dijkstra et al., (2014) reported that *Lactococcus lactis* grown at an elevated temperature exhibited the expression of stress genes, which were positively correlated with the increased robustness of cells toward heat and oxidative stresses. Cebrián et al. (2019) showed that a high growth temperature was associated with an increase in the rigidity of cellular membrane of *Staphylococcus aureus*, contributing to improved heat stability. As heat stress is generally acknowledged as a major stress during spray drying (Liu et al. 2018a; Perdana et al. 2013), culturing LAB cells at elevated growth temperatures may be a useful processing strategy to increase the survival of spray dried LAB. However, the influence of growth temperature on LAB cells could vary among individual strains; the extent of the effect of elevated growth temperature on LAB survival during spray drying warrants detailed investigation.

In this study, three LAB strains, namely *Lactococcus lactis* ssp. *cremoris*, *Lactobacillus acidophilus* NCFM, and *Lactobacillus rhamnosus* GG were cultured both under standard growth conditions and under increased growth temperatures by 3–5 °C. The objective was to examine the capability of the modified cultivation approach to improve the viability retention of LAB cells during both spray drying and storage. The protectant used in the spray drying experiments was reconstituted skim milk (RSM) at 30 wt%, which was one of the most reputed protectants for spray drying of LAB (Dimitrellou et al. 2016; Gong et al. 2019; Gul and Atalar 2019). The activity of fresh culture, the thermotolerance of cells, and the stability of spray-dried cells were investigated.

Materials and Methods

Microorganisms

Lactococcus lactis subsp. *cremoris* (CGMCC 1.3991 or ATCC 19257) was cultured on M17 agar plates composed of M17 broth (Oxoid CM0817, Oxoid Ltd., UK) and 12.0 g/L agar. Incubation was carried out at 30 °C for 48 h. *Lactobacillus acidophilus* NCFM and *Lactobacillus rhamnosus* GG (LGG) were cultured on MRS agar plates (Oxoid CM1175) at 37 °C for 48 h. The cultures were stored at 4 °C. Subculture to fresh medium was performed every 7 days.

Growth Conditions of LAB Cells

To prepare the inoculum of each strain, a single colony on the culture maintenance plate was used to inoculate fresh growth medium of 10 mL. Two bottles of M17 broth were inoculated with *L. cremoris*. Incubation was performed at the standard growth temperature of 30 °C for the first bottle and elevated growth temperature of 33 °C for the second bottle. After 12 h,

each of the inocula was transferred to fresh M17 broth at an inoculum size of 1% (v/v). The fermentation broth for biomass production was incubated at the same temperature to the corresponding inoculum for 24 h. The use of 33 °C as elevated temperature for growing *L. cremoris* was based on the results of preliminary experiments.

The inoculum medium of LGG was MRS, and incubation was performed at 37 and 42 °C, respectively. For biomass production, fresh MRS broth was inoculated with the 12-h culture, and then incubated at the same temperature to its inoculum for 24 h. Other conditions were the same to the *L. cremoris* culture. *L. acidophilus* was also cultured in MRS broth at 37 and 42 °C. All conditions were the same to the LGG culture, except that the fermentation for biomass production at 37 °C was carried out for 24 h, and that at 42 °C was carried out for 12 h, respectively.

A total of six LAB cultures were prepared, namely two *L. cremoris* cultures after incubation at 30 and 33 °C for 24 h, two LGG cultures after incubation at 37 and 42 °C for 24 h, *L. acidophilus* culture grown at 37 °C for 24 h, and *L. acidophilus* culture grown at 42 °C for 12 h. By the end of incubation, each culture was at stationary growth phase according to growth curves measured with optical density method and plate count method in preliminary experiments (Su et al. 2019; Suo et al. 2020). The heat-adapted culture and control culture of each strain were subjected to characterization analyses, heat treatment, and spray drying as shown in Fig. 1. All growth media used to culture LAB cells were sterilized at 121 °C for 15 min.

Metabolic Activity of LAB Cultures

The metabolic activity of the six LAB cultures was examined using the iodonitrotetrazolium chloride (INT) assay as described by Ulmer et al. (2000) with appropriate modifications. Each culture was centrifuged at 8000 rpm, 4 °C for 10 min to collect cells. The cell pellet was washed with phosphate buffer containing glucose (PBG, consisting of 50 mM of H_2KPO_4, 0.83 mM of $MgSO_4$, 0.33 mM of $MnSO_4$, and 22.2 mM of glucose, pH 6.5) and resuspended in PBG. The volume of PBG used to resuspend the centrifuged cell pellet was the same to the fresh culture, so the viable cell count of the resuspended culture remained the same to the fermentation broth at the stationary growth phase. INT (I6069, Macklin Biochemical Co. Ltd., Shanghai, China) was dissolved in PBG solution to prepare stock solution of 4 mM. For INT assay, LAB cell suspension was mixed with the INT stock solution at a volume ratio of 1:1. Active cells could reduce the colorless INT and generate red formazan dye as product. After reacting for 10 min, the color density of the reaction solution was read at 595 nm using a microplate reader (Molecular Devices M5, USA).

Heat Treatment Experiment

The six LAB cultures were subjected to heat treatment immediately after the incubation was terminated. Heat challenge was applied to 5 mL of culture contained in centrifuge tubes of 10 mL, using a water bath at 60 °C. The viable cell counts in the fresh culture and in cultures after different time of heat treatment were analyzed with the standard plate count method.

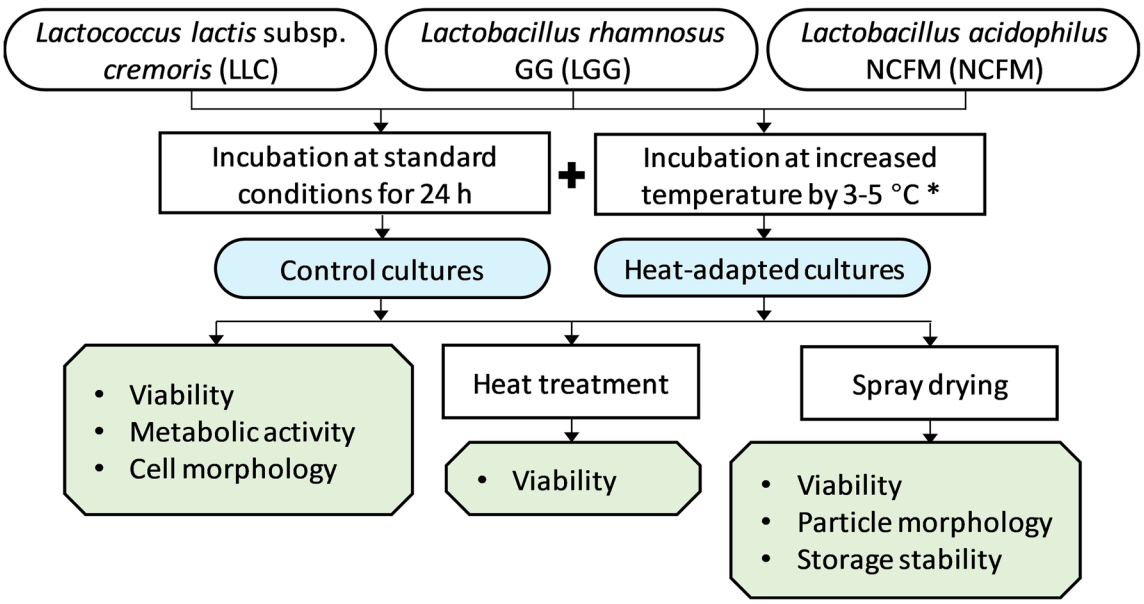


Fig. 1 Experimental procedures to prepare the heat-adapted culture and control culture of three LAB strains, and the subsequent characterization analyses, heat treatment, and spray drying. * Heat-adapted LLC and LGG cultures were grown at 33 and 42 °C, respectively, for 24 h, while heat-adapted NCFM culture was grown at 42 °C for 12 h

L. cremoris samples were heated for 2, 4, 6, 8, 10, 12, and 14 min, whereas LGG samples were heated for 6 min and *L. acidophilus* 4 min. The heated centrifuge tubes were taken out from the water bath after the designated time, and cooled with tap water for 3–4 min. Each sample was serially diluted with a dilution factor of 1/10, using 0.5% (w/v) peptone solution as diluent. At appropriate dilution factors, 0.1 mL of the diluted sample was spread onto the corresponding agar plate used to grow the strain. The spread plates were incubated at 30 °C for *L. cremoris* and 37 °C for LGG and *L. acidophilus*, respectively, for 48 h before colonies were counted.

Preparation of Bacterial Suspension for Spray Drying

Skim milk powder (Devondale, Australia) was dissolved in sterile deionized water at 30 wt% to prepare the protectant for spray drying. The reconstituted skim milk (RSM) was homogenized with a high-pressure homogenizer (ATS Engineering Inc., China) at 800 ± 50 bar for 3 passes, prior to mixing with LAB cells. Fresh LAB culture was centrifuged at 8000 rpm, 4 °C for 10 min. The cell pellet was washed with 0.5% (w/v) sterilized peptone solution and then centrifuged again at the same condition. The resulting cell pellet was re-suspended in the homogenized RSM, and the volume of RSM was controlled at 5 or 10 times the volume of the fresh culture (the ratio of culture volume to protectant volume was 1:5 or 1:10), to adjust viable cell concentration to around 1×10^8 cfu/g of dry solids mass in the feed of spray drying.

Spray Drying Experiment

Spray drying of the LAB-containing milk was performed with a micro-fluidic jet spray dryer (MFJSD, Model MDSD-III, Nantong Dong-Concept New Material Technology Ltd., Jiangsu Province, China), following experimental procedure described in previous studies (Su et al. 2019; Tian et al. 2014). The conditions were inlet temperature of 100 ± 3 °C, outlet temperature of 58 ± 3 °C, and a nozzle with orifice of 75 μm. Prior to each spray drying experiment, the spray dryer was preheated for 2.5 h to stabilize the temperature inside. The spray drying experiment of each strain was completed in 2 h after the feed was prepared, to minimize the fermentation of milk by LAB.

The moisture content of spray-dried powder and the remaining viability of LAB were analyzed on the same day of spray drying. Experimental procedures were similar to the study of Su et al. (2019), with minor modifications on the reconstitution method of the dried powder. The moisture content of the powder and the solids content of the feed were determined using a gravimetric method, whereby the samples were mixed with pre-dried sand and subjected to oven drying at 104 °C for 24 h to determine weight difference. For the plate count assay, spray-dried powder was reconstituted by dissolving 0.1 g of powder in 10 mL of peptone solution. The viable cell counts in the reconstituted milk and in the feed were enumerated following the procedure described above. The corresponding viability of LAB in spray-dried powder (N_p) and in the feed (N_0) was standardized as cfu/g dry solids mass, and the survival ratio was calculated as N_p/N_0 (Su et al. 2019). The residual powder was stored in a desiccator at 4 °C for scanning electron microscopy (SEM) analysis and storage test.

Scanning Electron Microscopy Analysis

SEM was utilized to examine the morphology of LAB cells cultured at different growth temperatures, the particle morphology of LAB powders, and the morphology of cells encapsulated in the milk matrix of the powders. For fresh LAB cultures, the cell pellet obtained by centrifugation was first washed with sterile saline solution (0.85% (w/v) NaCl solution) and then resuspended in 2.5% (w/v) glutaraldehyde. The cell suspension was kept at 4 °C for 2 h to fix the cells. After that, LAB cells were collected by centrifugation, followed by washing with sterile deionized water three times. The resulting cell pellet was dehydrated with a grade series of ethanol solution, namely 30%, 50%, 70%, 80%, 90%, and 100% (v/v), for 10 min at each grade. The dehydration was ended with repeated treatments in 100% ethanol for another three times, 15 min each time. The dehydrated cells were placed in a vacuum oven at 40 °C for 2 days to allow the ethanol to evaporate. Then, the LAB cells were carefully mounted to an aluminum stub using conducting carbon tape.

LAB powders produced by the above spray drying experiments were directly loaded to a sample stub for SEM observation. To examine the morphology of LAB cells embedded in the milk matrix, cells collected from fresh culture were spray dried with 1 wt% skim milk and the viable cell concentration in the feed was kept the same to the fresh culture (the ratio of culture volume to protectant volume was 1:1). Spray drying conditions were the same as described above. Dried powders were broken with a tiny hammer to expose inner structure, before loaded to the sample stub.

All samples were sputter-coated with platinum/gold to produce a conductive surface for SEM observation. Secondary electron SEM images for LAB particle morphology were recorded using a Hitachi SU1510 (Hitachi Ltd., Japan), and images for LAB cell morphology were recorded using a Hitachi S4700.

Stability of Spray-Dried LAB Cells During Storage

LAB powders stored at 4 °C were analyzed for the residual viability of LAB cells on days 7, 14, 45, 83, and 110 after spray drying. The powders were reconstituted, and LAB viability was analyzed with the plate count method, following the

same procedure described above. The viable cell count in cfu/mL was compared to the result on day 0, to calculate changes in LAB viability during storage.

Statistical Analysis

The heat treatment and spray drying experiments were independently carried out three or four times, with freshly prepared LAB cultures each time. The reported results are average values ± standard deviation. Metabolic activity, SEM observation, and storage tests were independently performed twice to ensure the reproducibility of the results. Statistical analyses were performed using the Student's *t* test with 95% confidence interval (Excel 2016, Microsoft Corporation, USA).

Results

The Activity and Morphology of LAB Strains Grown at Different Temperatures

The viable cell count and metabolic activity of *L. cremoris*, LGG, and *L. acidophilus* grown at the standard and the elevated temperatures are shown in Figs. 2 and 3, respectively. All cultures, including *L. acidophilus* grown at 42 °C for 12 h, reached the stationary growth phase when the incubation was terminated. *L. cremoris* and *L. acidophilus* cultures grown at the elevated temperatures showed fewer viable cells than the control cultures grown at the standard temperatures ($p < 0.05$, Fig. 2). The viable cell concentration in the heat-adapted *L. cremoris* culture and the corresponding control culture was 2.47×10^8 and 6.99×10^8 cfu/mL (8.34 and 8.84 log cfu/mL), respectively, and that of *L. acidophilus* cultures was 1.29×10^8 and 3.46×10^8 cfu/mL (8.10 and 8.53 log cfu/mL), respectively, indicating a decrease of bacterial viability by around 0.45 log when the growth temperature was increased by 3–5 °C. For LGG, the amount of viable cells after incubated at 42 °C for 24 h was consistently lower than that at 37 °C in individual experiments, with average viability of 1.98×10^9 and 2.66×10^9 cfu/mL (9.28 and 9.39 log cfu/mL), respectively. However, with triplicate experiments, the difference remained insignificant ($p > 0.1$).

The metabolic activity of each LAB strain at the two growth temperatures was similar (Fig. 3), although the bacterial population of the heat-adapted cultures tended to be lower than that of the corresponding control cultures. All six cultures quickly reduced INT to insoluble red formazan in 10 min (Fig. 3a), indicating high robustness. The results suggested that the

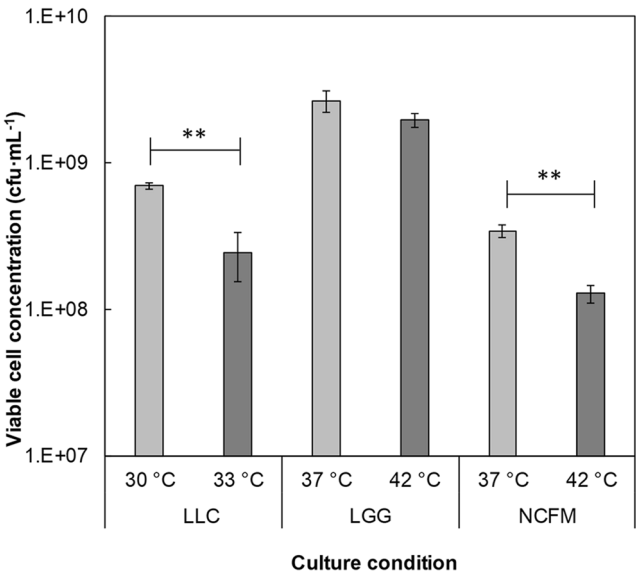


Fig. 2 The viable cell count of three LAB strains at the stationary growth phase, when incubation was carried out at the standard (30 and 37 °C) and elevated temperatures (33 and 42 °C). *LLC Lactococcus lactis* ssp. *cremoris*, *LGG Lactobacillus rhamnosus* GG, *NCFM Lactobacillus acidophilus* NCFM. ** indicate that the two values are significantly different ($p < 0.05$)

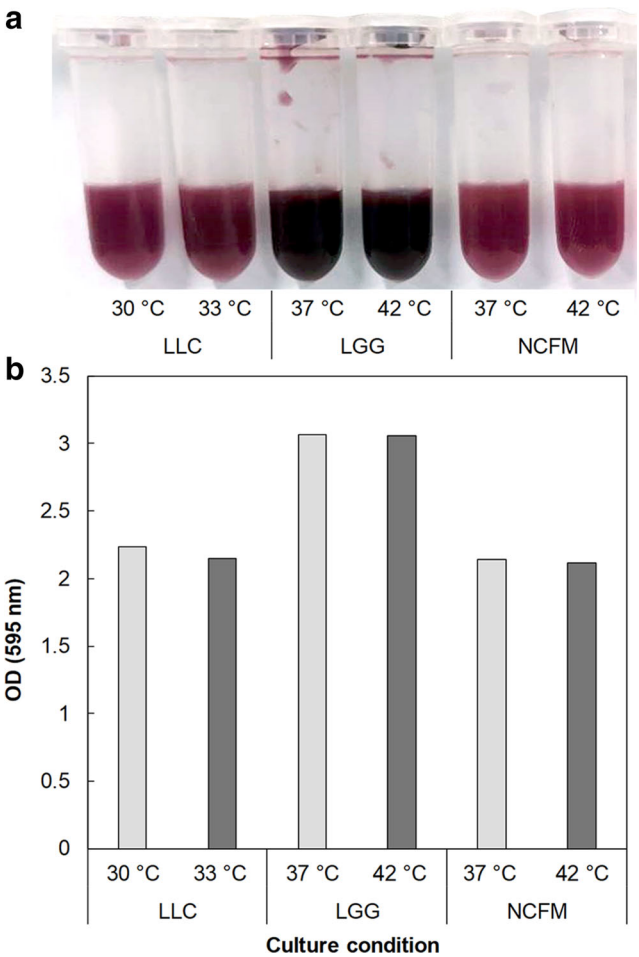


Fig. 3 The metabolic activity of three LAB strains at the stationary growth phase, when incubation was carried out at the standard (30 and 37 °C) and elevated temperatures (33 and 42 °C). **a** Color change and **b** absorbance of each culture analyzed with the iodonitrotetrazolium chloride (INT) assay. *LLC Lactococcus lactis* ssp. *cremoris*, *LGG Lactobacillus rhamnosus* GG, *NCFM Lactobacillus acidophilus* NCFM

heat-adapted cultures, albeit containing fewer cells in the fermentation broth, showed similar efficiency in generating electrons during metabolism to the control cultures. Note that among the three LAB strains, LGG showed the highest viable cell count of 1–2 × 10^9 cfu/mL (9.28–9.39 log cfu/mL; Fig. 2), and the corresponding color density after reaction with INT for 10 min was remarkably higher than that shown by *L. cremoris* and *L. acidophilus*, whose viable cell count ranged between 1 × 10^8 and 7 × 10^8 cfu/mL (8.10–8.84 log cfu/mL). The larger bacterial population may help generate higher amount of electrons per unit time, leading to better efficiency of INT reduction.

The three strains showed characteristic cellular shape and arrangement when examined with SEM (Fig. 4). *L. cremoris* was typical coccus with size perceptibly smaller than 1 μm (Fig. 4A, a). LGG was short rods, and the length of each cell was between 1 and 2 μm (Fig. 4C, c). *L. acidophilus* was also rods, but its cells were considerably longer than that of LGG (Fig. 4E, e). All three strains formed characteristic chain arrangement as expected for lactic acid bacteria. Notably, at higher growth temperature of 42 °C, both LGG and *L. acidophilus* cells were elongated. The size of heat-adapted LGG cells was increased to 2–4 μm (Fig. 4D, d), whereas for *L. acidophilus*, not only the length of individual cells was extended, but the cellular chains were also lengthened, forming fibrous structure (Fig. 4F).

Heat Treatment of LAB Strains Grown at Different Temperatures

Heat treatment experiments were performed at 60 °C to evaluate the thermotolerance of the three strains (Fig. 5). As expected, *L. cremoris* cultured at 33 °C in general showed better survival than that at 30 °C throughout the heat treatment for 14 min (Fig. 5a). The two survival curves exhibited large gaps at 4, 10, and 12 min, where the survival ratio was increased by 0.7–1.5 log with the increase in growth temperature. The survival curve of heat-adapted cells demonstrated a typical tailing effect after 8 min of heat treatment, indicating that a small portion of *L. cremoris* population (< 0.1%) had acquired excellent thermotolerance. Similarly, when the growth temperature of LGG was increased by 5 °C, the survival of cells after heat treatment for 6 min was increased by around 0.3 log (from around 12.5% to 25%, $p < 0.05$, Fig. 5b). Though the viable cell count in the fresh LGG culture at 42 °C was lower than that at 37 °C, the residual viability of the two cultures after heat treatment reached a similar level around 4 × 10^8 to 5 × 10^8 cfu/mL (8.5–8.7 log cfu/mL), demonstrating the enhanced thermotolerance of LGG when cultured at 42 °C.

The *L. acidophilus* culture at 42 °C showed a significant decrease in cell viability between 12 and 24 h of incubation, from around 1.5 × 10^8 cfu/mL to lower than 10^7 cfu/mL (8.2 compared to 6.8 log cfu/mL; Fig. 5c), indicating that the culture entered death phase. After heating at 60 °C for 4 min, cells

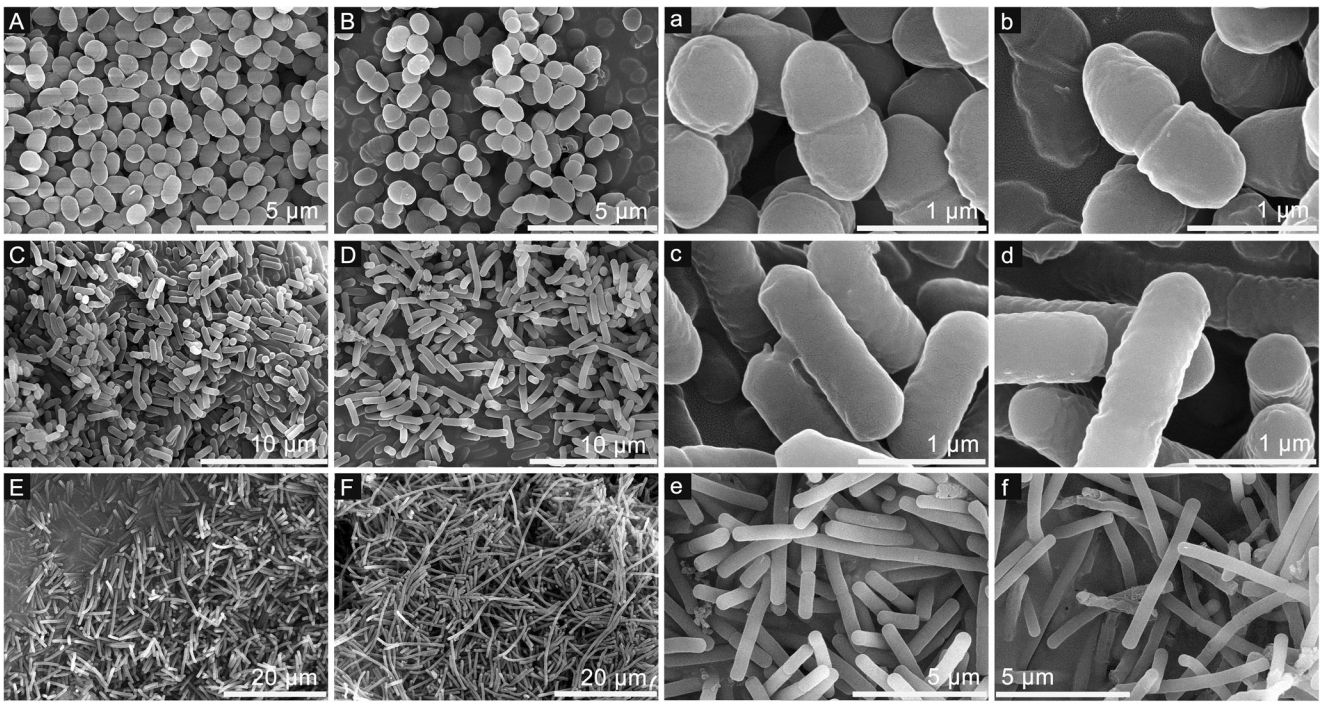
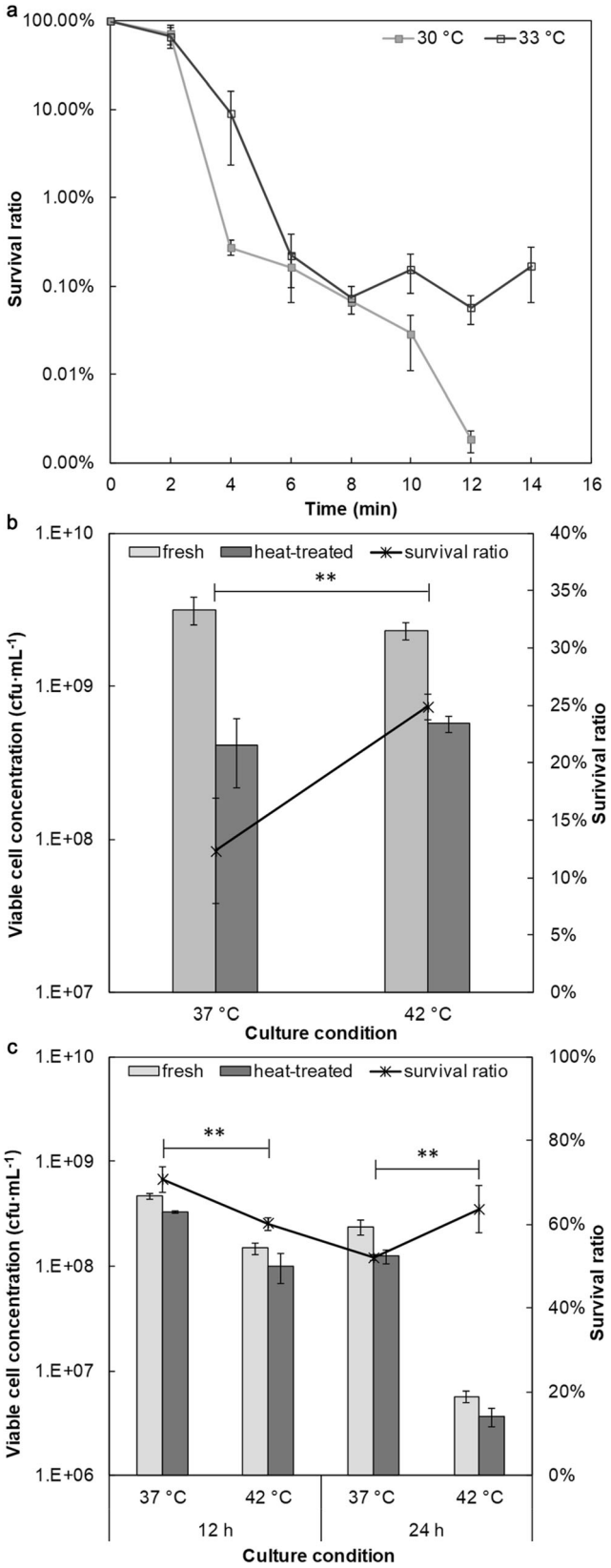


Fig. 4 The morphology of three LAB strains grown at the standard and elevated temperatures. (A, a) *Lactococcus lactis* ssp. *cremoris* grown at 30 °C, (B, b) *Lactococcus lactis* ssp. *cremoris* grown at 33 °C, (C, c) *Lactobacillus rhamnosus* GG grown at 37 °C, (D, d) *Lactobacillus rhamnosus* GG grown at 42 °C, (E, e) *Lactobacillus acidophilus* NCFM grown at 37 °C, and (F, f) *Lactobacillus acidophilus* NCFM grown at 42 °C



◀ **Fig. 5** Heat treatment of LAB cultures at 60 °C. **a** The inactivation curves of *Lactococcus lactis* ssp. *cremoris* grown at 30 and 33 °C during heat treatment for 14 min. **b** The viable cell count and survival ratio of *Lactobacillus rhamnosus* GG grown at 37 and 42 °C after heat treatment for 6 min. **c** The viable cell count and survival ratio of *Lactobacillus acidophilus* NCFM after heat treatment for 4 min, with cells grown at 37 and 42 °C for either 12 or 24 h. ** indicate that the two survival ratios are significantly different ($p < 0.05$)

grown at 42 °C for 12 h demonstrated lower survival than the control culture with the same incubation period (60.1% and 70.8%, respectively; $p < 0.05$). By contrast, for the two cultures incubated for 24 h, a higher survival ratio was observed for the heat-adapted culture (63.7% and 52.1%, respectively; $p < 0.05$), which was a similar trend to the results of *L. cremoris* and LGG. Nevertheless, because of the low viability, culturing *L. acidophilus* at 42 °C for 24 h was unsuitable for utilization in powder production. In the present study, an incubation time of 12 h was used to grow *L. acidophilus* at 42 °C.

Spray Drying of LAB Strains Grown at Different Temperatures

Spray drying experiments of the six cultures were carried out with the same protectant (30 wt% reconstituted skim milk) under similar drying conditions. As such, LAB cells in each culture had experienced similar stresses during drying, and any difference in the survival ratio after spray drying could be considered the result of the different intrinsic tolerance of cells. In the preparation of feed containing LGG and *L. acidophilus*, cells cultured at different temperatures were mixed with the protectant at the same ratio of culture volume to protectant volume (1:10). The resulting feed prepared with the control cultures grown at 37 °C showed higher viable cell counts than that with heat-adapted cultures grown at 42 °C (Fig. 6a), which was in line with the results in Fig. 2. The viable cell count in the feed containing control LGG, heat-adapted LGG, control *L. acidophilus*, and heat-adapted *L. acidophilus* was 3.97×10^8, 1.69×10^8, 1.81×10^8, and 5.77×10^7 cfu/g (8.59, 8.22, 8.16, and 7.60 log cfu/g), respectively. After spray drying, substantial viability loss was observed for all four cultures. The residual viability in spray dried powder was 8.67×10^7 and 5.03×10^7 cfu/g (7.90 and 7.69 log cfu/g) for LGG cultures at 37 and 42 °C, and 5.05×10^7 and 2.36×10^7 cfu/g (7.60 and 7.22 log cfu/g) for *L. acidophilus* cultures at 37 and 42 °C, respectively. In average, the heat-adapted lactobacilli strains tended to show higher survival ratio in spray-dried powders than the control cultures, respectively 42.3% and 28.6% for *L. acidophilus*, 30.9% and 21.0% for LGG (Fig. 6b). The difference was significant between the *L. acidophilus* cultures ($p < 0.05$) and relatively insignificant between the LGG cultures ($p = 0.06$).

In the spray drying of *L. cremoris*, the heat-adapted culture grown at 33 °C was mixed with 30 wt% skim milk at a ratio of culture volume to protectant volume of 1:5, whereas for the control culture grown at 30 °C, a 1:10 ratio was used. The results showed that the positive effect of the enhanced thermotolerance on the survival of spray-dried LAB cells was minimally affected by the different cell:protectant ratio. The survival ratio of the heat-adapted *L. cremoris* reached 52.4%, notably higher than that of the control culture (31.4%, $p < 0.05$; Fig. 6b). The increased cell:protectant ratio resulted in a relatively high concentration of viable cells in the feed, reaching 3.68×10^8 cfu/g (8.46 log cfu/g; Fig. 6a). The residual viability of *L. cremoris* in spray-dried powder was correspondingly increased, reaching 1.68×10^8 cfu/g (8.17 log cfu/g; Fig. 6a). The increased cell density is an advantage in industrial powder production, because it allows bacterial population per unit mass of final product to be maintained at a comparatively high level, even after the powder is mixed with other excipient material.

The moisture content of the six types of LAB powders was similar, ranging between 6.8 and 8.6%. The powders also showed similar particle size around 130 μm and similar particle morphology of distorted spherical shape, as shown by the images of microparticles encapsulating *L. cremoris*, LGG and *L. acidophilus* cultured at elevated growth temperatures (Fig. 7A–C). The morphology of bacterial cells embedded in the skim milk matrix is correspondingly shown in Fig. 7a–c. At the broken surface of microparticles, LAB cells can be distinguished from milk solids by their characteristic cellular shape. The chain arrangement was observed for *L. cremoris* cells, as shown with a closed red arrow in Fig. 7a. Notably, some pits with clear cellular shape were found at the cross section of the microparticles (refer to open blue arrows in Fig. 7a, b). The pits indicated that LAB cells were once present, which might be lost under the high vacuum during sputter-coating and SEM observation.

Stability of Spray-Dried LAB Cells During Storage

The changes of LAB viability in spray-dried powders were monitored during storage at 4 °C for 110 days. The bacterial population in the six types of LAB powders after spray drying was different (Fig. 8a). To make more effective comparison between the changing trends of LAB viability during storage, the viable cell count measured at different storage periods was normalized with the value of fresh LAB powder cell count (Fig. 8b). After 110 days, the overall decrease in the viability of LGG and *L. acidophilus* was around 1 log, whereas for *L. cremoris*, a decrease of up to 2.0–2.2 log was observed. The rapid loss of LAB viability occurred during the first 2 weeks of storage, and then the curves nearly leveled off till the end of storage test (Fig. 8b). *L. cremoris* and LGG cultures grown at the elevated temperatures demonstrated higher degree of inactivation than the control cultures at the standard temperatures. After 7 days of storage, the viability of the heat-adapted cultures was decreased to a lower level than that of the control cultures, and the reduction in viability was 1.23 and 0.90 log for *L. cremoris*, and 1.03 and 0.87 log for LGG, respectively. The gap was maintained till the end of the storage trial. By contrast, the control and heat-adapted *L. acidophilus* showed similar trends of viability loss during 110 days.

All six types of LAB powders retained viable cell counts close to or higher than 10^6 cfu/g after storage for 110 days

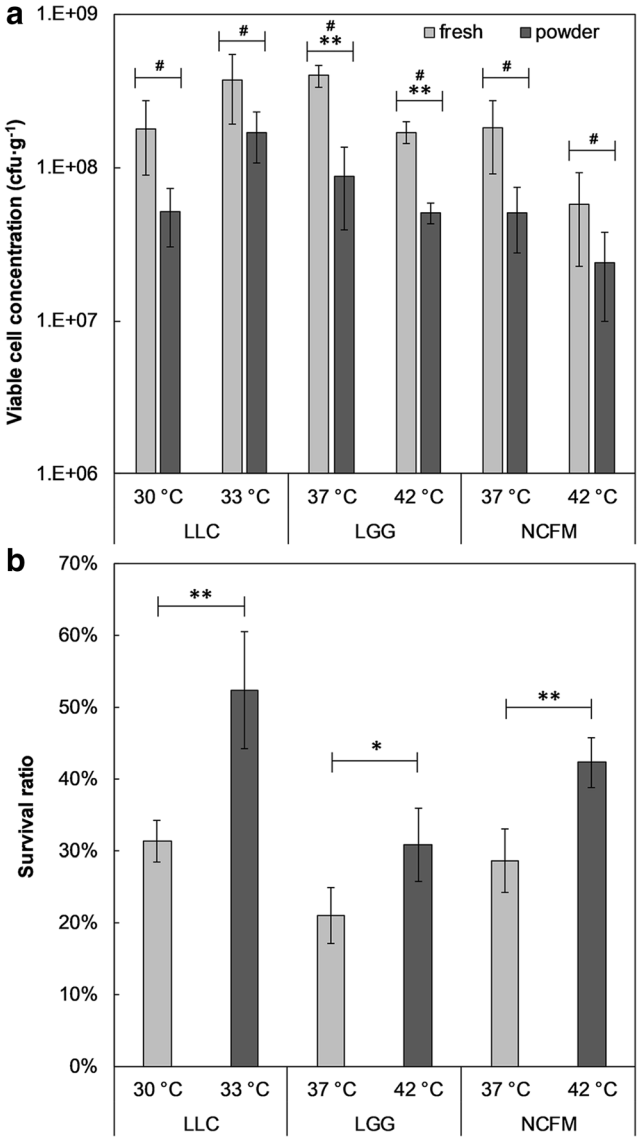


Fig. 6 Spray drying of three LAB strains grown at the standard and elevated temperatures, using 30 wt% reconstituted skim milk as protectant. **a** The viable cell counts in the feed and in freshly dried powder. **b** The survival ratio of the six cultures. *LLC Lactococcus lactis* ssp. *cremoris*, *LGG Lactobacillus rhamnosus* GG, *NCFM Lactobacillus acidophilus* NCFM. * and ** indicate that the significance of difference between the two values is $p < 0.1$ and $p < 0.05$, respectively. # in panel **a** indicates that the two viable cell counts are significantly different when analyzed in log cfu·g^{-1} form ($p < 0.05$)

(Fig. 8a). The highest viability of 5.3×10^6 cfu/g (6.72 log cfu/g) was observed with LGG cultured at 37 °C, followed by *L. acidophilus* cultured at 37 °C with a residual viability of 3.5×10^6 cfu/g (6.52 log cfu/g). *L. cremoris* cultured at 30 °C showed the lowest viability of 9.6×10^5 cfu/g (5.98 log cfu/g) among the six types of powders, which was attributed both to the substantial inactivation during storage and to the low viable cell count in freshly spray-dried powder at day 0 (Fig. 8a).

Discussion

Owing to the biological properties of LAB as living microorganisms, the microencapsulation of LAB cells via spray drying demonstrates several unique features that are different from the processing of other bio-active substances. First of all, by dehydrating LAB cells, it is expected to inhibit their metabolic activities, so as to extend the term of preservation of cell viability (Fu and Chen 2011). Second, the response of LAB cells toward environmental stresses and protectants needs to be taken into account. Different genus, species, and strains may exhibit varying degrees of responses (Iaconelli et al. 2015; Salar-Behzadi et al. 2013) as a result of differences in gene expression, level of stress proteins, and metabolic pathways. A common criterion to examine the survival of LAB cells after spray drying is by measuring cell viability, that is, the capability of cells to multiply in rich medium (Ali et al. 2019). The result possibly includes a proportion of injured cells, which could repair the injuries and continue to multiply (Golowczyc et al. 2011; Liu et al. 2015). Furthermore, the potential beneficial effect that may be conferred by LAB cells without reproduction capability, including both inactivated cells and viable-but-nonculturable cells, remains elusive (Generoso et al. 2011; Zhang et al. 2005).

Since bacterial cells could activate stress response systems under stressful conditions, a viable approach to increase LAB survival after spray drying is to enhance the intrinsic stress tolerance of cells (Desmond et al. 2001; Gaucher et al. 2020; Huang et al. 2016b). This approach is in addition to common strategies used to improve the quality of powders that encapsulate bio-active substances, such as the optimization of microencapsulation process (Zhao et al. 2018; Zhao et al. 2019) and the use of appropriate protectants (Agudelo et al. 2017; Bustamante et al. 2017). In this study, the enhanced thermotolerance of *L. cremoris* and LGG grown at the increased temperatures (Fig. 5a, b) was in agreement with the findings in previous reports (Cebrián et al. 2019; Dijkstra et al. 2014). By contrast, *L. acidophilus* grown at 42 °C showed substantially shortened stationary phase (Fig. 5c), and its thermotolerance was largely influenced by the growth phase. At the high temperature, certain essential cellular activities might be compromised to an overly high degree that cannot be countered by cellular response system, leading to variations in cell survival after heat treatment (Wang et al. 2020).

The increase in growth temperature by 3 °C for *L. cremoris* and by 5 °C for lactobacilli exceeded the optimum temperature for culturing the individual strain. The resulting cultures

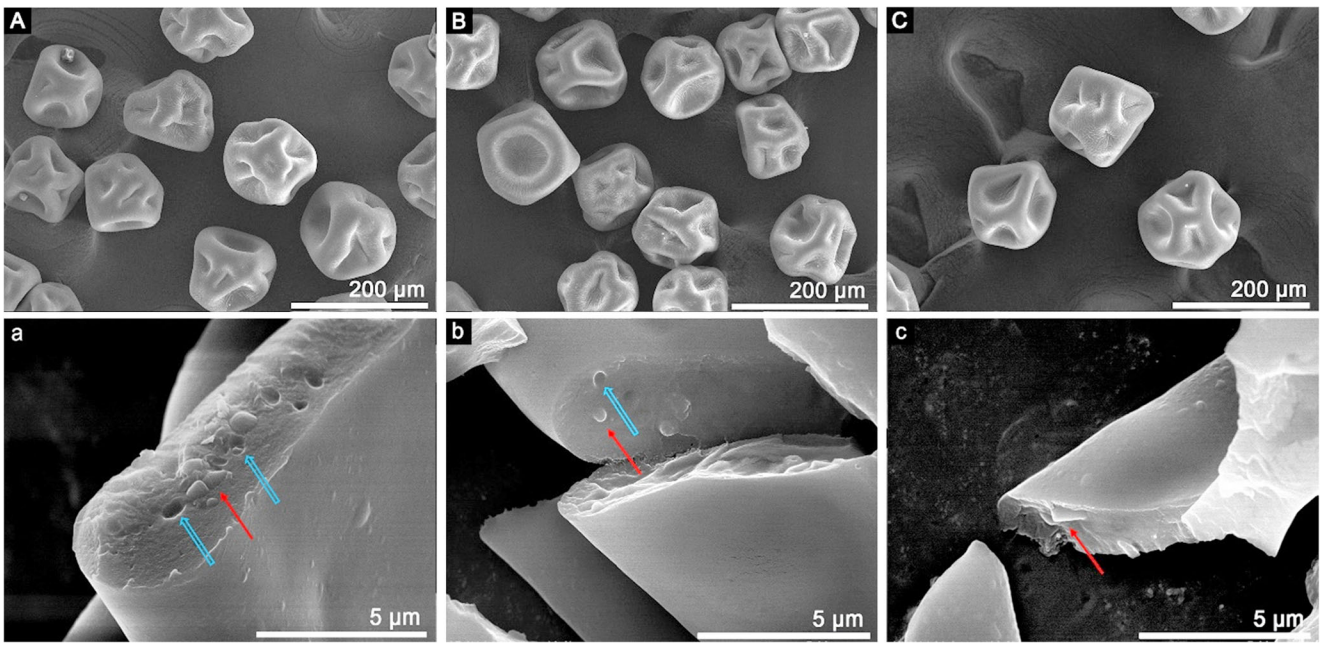


Fig. 7 Particle morphology of powders encapsulating LAB cells cultured at elevated growth temperatures, using reconstituted skim milk as protectant. (A) *Lactococcus lactis* ssp. *cremoris* grown at 33 °C, (B) *Lactobacillus rhamnosus* GG grown at 42 °C, and (C) *Lactobacillus acidophilus* NCFM grown at 42 °C, respectively. (a–c) Show the morphology of corresponding LAB cells encapsulated in the skim milk matrix. Solid red arrows indicate the encapsulated LAB cells, whereas open blue arrows indicate pits with cellular shape

tended to show lower bacterial population at the stationary phase than the control cultures (Fig. 2), indicating the negative effect of the high temperature on bacterial growth. Nevertheless, the high temperature could promote the rate of enzymatic reactions as commonly observed for most chemical reactions (Madigan and Martinko 2006). The increased rate of enzymatic reactions might compensate for the low amount of viable cells, resulting in the similar metabolic activity of the heat-adapted cultures to the controls (Fig. 3). Since enzymatic reactions play a key role in the majority of cellular activities such as energy metabolism, DNA replication, and protein synthesis (Madigan and Martinko 2006), the high reaction rate could be associated with the acceleration of cellular activities. One of the indications was the early occurrence of the stationary growth phase for *L. acidophilus* grown at 42 °C. In addition, the rapid division of cells could be partly reflected by the elongated cellular morphology of the lactobacilli strains (Fig. 4D, d, F, f).

The increased survival of heat-adapted *L. cremoris* and *L. acidophilus* after spray drying was an expected trend, because heat stress in addition to dehydration stress was considered a main factor responsible for the inactivation of LAB cells during spray drying (Ghandi et al. 2012; Perdana et al. 2013). Though the difference between the control and heat-adapted LGG cultures remained insignificant with triplicate experiments (p = 0.06, n = 3), it was possible that the difference may become significant with the increase in sample size. The inlet and outlet temperatures of the spray drying processes used in the current study were controlled at low levels (100 and 58 °C, respectively). Nevertheless, the temperature history experienced by individual droplets during drying might enter high temperature range for a short time (Rogers et al. 2012), constituting heat challenge for LAB cells. A high temperature environment above the maximum growth temperature of bacteria favored the survival of cells with enhanced thermotolerance. Previous studies showed that spray dried LAB cells exhibited higher degree of damages on lipid structure and secondary protein structures (Hlaing et al. 2017), as well as on certain functionalities such as the hydrophobicity of cellular surface (Iaconelli et al. 2015), than cells dehydrated by freeze drying, which was free of heat stress. In future studies, the temperature of spray drying needs to be further lowered to minimize the detrimental effect of heat.

It is well known that prokaryotic cells could activate heat shock proteins toward heat stress (De Angelis and Gobbetti 2004; Papadimitriou et al. 2016; van de Guchte et al. 2002). Although the specific cellular response mechanism varies among species and strains, the conserved chaperones such as GroES, GroEL, DnaK, and DnaJ families are often identified in LAB cells after heat shock (De Angelis and Gobbetti 2004; Sanders et al. 1999). At an increased environmental temperature, not only the expression of stress proteins is increased in LAB cells, but the capacity of chaperones binding to unfolded polypeptides also demonstrates substantial improvement (Sherman and Goldberg 1993). As such, culturing LAB cells at an elevated growth temperature might be able to maintain chaperone proteins active, leading to the improved cell thermotolerance observed in the current study. In addition, our results showed that such a strategy could effectively increase the survival of spray-dried LAB cells, which was reported for the first time.

The decreased stability shown by the heat-adapted *L. cremoris* and LGG during storage at 4 °C was an unfavorable attribute in industrial production. The compromised stability might be ascribed to two reasons. First, LAB cells grown

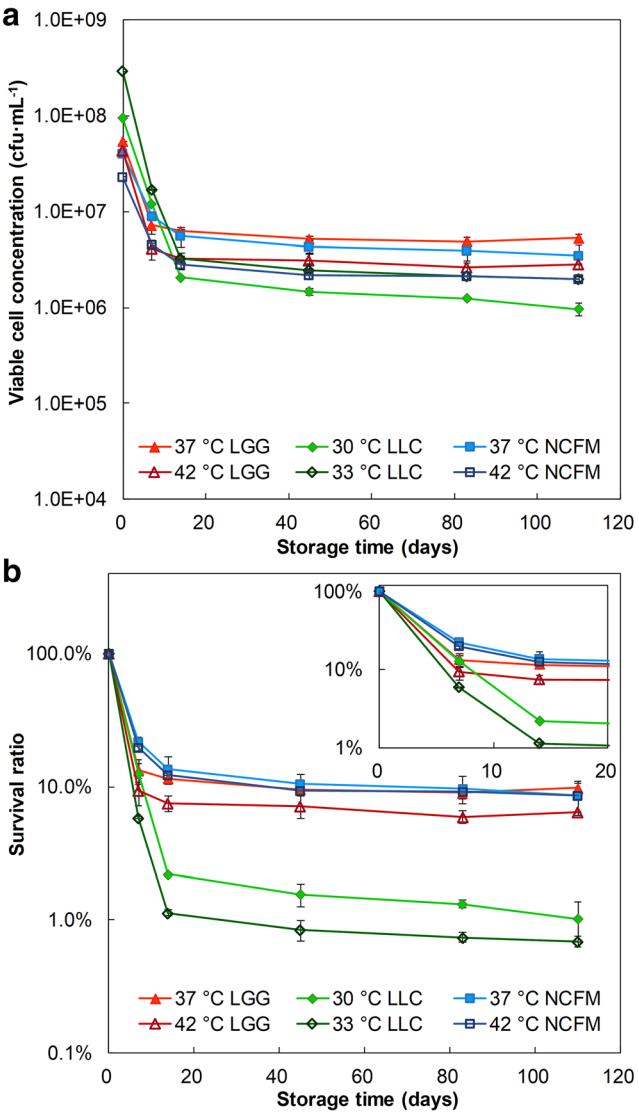


Fig. 8 Changes in cell viability in six types of LAB powders encapsulating three LAB strains grown at the standard and elevated temperatures, during storage at 4 °C. **a** Viable cell count. **b** Survival ratio. The inset in panel **b** compares the trends of viability decrease during storage for 20 days. *LLC Lactococcus lactis* ssp. *cremoris*, *LGG Lactobacillus rhamnosus* GG, *NCFM Lactobacillus acidophilus* NCFM

under the high temperature naturally tend to be instable. The instability, which could be linked to the rapid division, is in line with the low bacterial population at the stationary growth phase (Fig. 2). Second, freshly spray-dried LAB powder may contain a proportion of cells bearing sublethal injuries (Ananta et al. 2005). Cebrián et al. (2019) reported that bacterial culture grown at an elevated temperature tends to contain a larger proportion of sublethally damaged cells than the culture grown at the standard condition. When heat-adapted cultures were spray-dried and immediately analyzed for cell survival, the injured bacterial population might be able to repair the injuries and continue to multiply in the rich growth medium, particularly with the highly active chaperon proteins. As a result, high survival ratios of LAB were observed for the heat-adapted cultures after spray drying (Fig. 6b). During storage of spray-dried powders, cellular activities in the milk matrix were decreased to a low level, and the injured cells might gradually lose the capability to reproduce. As such, the higher proportion of injured cells in the heat-adapted cultures led to a higher degree of inactivation than the control cultures after the first week of storage, and the difference in viability was maintained during the following storage period (Fig. 8b).

Besides the cultivation of LAB at elevated temperature, previous studies have reported other cultivation approaches that are capable of improving the thermotolerance of cells, such as culturing cells in concentrated sweet whey of 20–30 wt% total solids (Huang et al. 2016b), or in growth medium supplemented with Ca^{2+} (Wang et al. 2020). Probiotic strains cultured in concentrated sweet whey demonstrated excellent survival after spray drying and good stability during storage (Huang et al. 2016a; Huang et al. 2017), indicating that balanced thermotolerance and storage stability may be achieved by optimizing cultivation and spray drying conditions. As shown in the current study, the enhanced thermotolerance and increased cell survival after spray drying may not directly correlate with an improved storage stability of spray-dried LAB, suggesting that the cellular response of LAB depends on the specific culturing conditions and protectant composition, and should be carefully controlled to maximize the retention of cell viability during processing and storage.

Conclusions

This study evaluated the potential of culturing LAB strains at elevated temperatures for increasing the survival of bacterial cells after spray drying. Increasing the growth temperature of *L. cremoris*, LGG, and *L. acidophilus* by 3–5 °C led to a general improvement of the thermotolerance of cells, and also impacted on the biological properties of strains, including decreased bacterial population at the stationary growth phase, increased metabolic activity of individual cells, and the elongation of lactobacilli cells. The survival ratios of *L. cremoris* and *L. acidophilus* after spray drying were significantly increased from 31.4 to 52.4% and from 28.6 to 42.3%, respectively; whereas heat-adapted LGG also showed increased survival from 21.0 to 30.9%, which was relatively insignificant. Spray-dried powders prepared with heat-adapted *L. cremoris* and LGG showed a higher degree of viability loss by 1.03–1.23 log during the first week of storage, which might be related to the proportion of injured cells in the powders. The findings demonstrated the crucial effect of cellular response on LAB viability retention during spray drying and storage. While culturing LAB strains at elevated temperatures proved to be useful in increasing the survival of cells toward heat stress, the resulting cellular response required further optimization in future studies, to yield robust cells with high activity and stability. Understanding the cellular response of LAB toward different cultivation and processing conditions facilitates a rational process design for producing active LAB powders via spray drying.

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Nomenclature cfu, Colony-forming unit; INT, Iodonitrotetrazolium chloride; LAB, Lactic acid bacteria; LGG, *Lactobacillus rhamnosus* GG; LLC, *Lactococcus lactis* subsp. *cremoris* ATCC 19257; M17 broth, Broth used to culture lactococci; MRS broth, de Man, Rogosa and Sharpe broth used to culture lactobacilli; N_0, The viability of lactic acid bacteria in the feed of spray drying (cfu/g dry solids mass); N_p, The viability of lactic acid bacteria in spray dried powder (cfu/g dry solids mass); NCFM, *Lactobacillus acidophilus* NCFM; PBG, Phosphate buffer containing glucose; RSM, Reconstituted skim milk

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