



# Effect of sub-lethal heat stress on viability of *Lacticaseibacillus casei* N in spray-dried powders

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## ABSTRACT

The potential of thermal adaptation enabling a probiotic *Lacticaseibacillus casei* N (N) to withstand heat stress and spray drying was investigated. Among the encapsulating agents used (maltodextrin, corn starch (CS), and acacia gum) for lactobacillus, CS had shown the highest survivability of 95.6% after spray drying. Further, the slurry prepared using *L. casei* (N), and CS as carrier material was subjected to sub-lethal heat stress at 45 and 50 °C for 60 min before spray drying. After spray drying, survivability was increased by 0.26 and 0.19 log in N + CS 45 and N + CS 50, respectively, compared to a control that was not treated to sub-lethal stress (N + CS). During storage studies for 90 days, the sub-lethal heat-stressed probiotic powders had shown better survivability at 4 °C. The survivability of *L. casei* (N) in spray-dried powders in simulated gastric fluid revealed that N + CS 45 had 3.06 log cycles higher survivability than N + CS. The SDS PAGE and FTIR analysis of intracellular proteins of heat-stressed *L. casei* (N) cells revealed overexpression of heat shock proteins indicating a change in protein structure. The study suggests that the sub-lethal heat stress before spray drying increases the viability of probiotic cultures, which have potential food applications.

## 1. Introduction

Probiotics are generally microbial dietary supplements that have a beneficial effect on the host when consumed in sufficient quantities. The probiotics are familiar as lactic acid bacteria (LAB), which are consumed as fermented foods (Parvez, Malik, Ah Kang, & Kim, 2006). However, fermented dairy products have a short life span and also require refrigeration conditions for transportation and storage, which increases the cost of incorporating probiotics. Commercially, spray drying and freeze-drying are the two most widely used tools for producing probiotic powders. Spray drying is a low-cost technology with high-yield and is commonly used in the food industry to produce large quantities of dried cells of probiotics (Ainsley Reid et al., 2005; Muthukumarasamy, Allan-Wojtas, & A Holley, 2006) in contrast to freeze-drying which is a costly and time-consuming process (Gardiner et al., 2000).

However, in spray drying, viability loss can occur in probiotic cultures, as the cells are exposed to high temperatures and rapid dehydration. From the past few decades, researchers have made various attempts to enhance the viability of probiotics in food products during their production, processing, and storage. Therefore, many recent studies have focused on developing different approaches to improve the

survival of probiotics, such as screening resistant strains, adding extra nutrients, microencapsulation, and inducing the cellular stress response (Tripathi & Giri, 2014). Among these approaches, pre-adaptive tolerance responses have been observed in lactobacilli strains, which help ameliorate the adverse effects and improve the survivability of the cells in a subsequent lethal challenge (Ricciardi et al., 2012). It is also reported that prior exposure of microorganisms to sub-lethal stresses increases its resistance to the drying process and storage by inducing the production of cellular components, which protects the cell under stress conditions (Broadbent & Lin, 1999). Furthermore, some researchers reported that pre-adaptation to sub-lethal treatments such as oxidative, acid, bile salt, and osmotic stresses can also enhance the heat tolerance in lactobacilli culture (Ma et al., 2021).

Microencapsulation is a useful technique to maintain the viability of cells and stabilize the cells by protecting them against the harsh gastrointestinal environment, delivering viable cells into foods, and controlling the release of cells in the colon. The microcapsule is a thin, spherical, semipermeable, or nonpermeable and strong membrane surrounding a liquid core and solid with a small diameter that varies a few microns to 1 mm. The carrier materials or encapsulating agents are usually regarded as safe (GRAS) ingredients to use in food applications

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(Abd El-Salam & El-Shibiny, 2012). Sugars like glucose, sucrose, and oligosaccharides (Zhang, Lin, & Zhong, 2016); polysaccharides like starch, alginate, cellulose, cyclodextrin, maltodextrin, and pectin; gums like gum arabic, carrageenan, and guar; proteins like whey proteins, skim milk, egg proteins, zein, gelatin, and soy proteins and their mixtures are widely used as encapsulation agents. Maltodextrin (MDX) is the most often used encapsulating agent in the food sector due to its low cost and availability (Bhagwat, Bhushette, & Annapure, 2020). Gum acacia (GA) is a natural composite of proteins and polysaccharides with properties like excellent emulsification and high solubility (Gul & Atalar, 2019). The use of corn-starch as an encapsulating ingredient in probiotic microencapsulation has solved a number of technological challenges, including thermal stability, controlled release of bioactive molecules, and longer shelf life (Archacka, Celińska, & Białas, 2020).

The primary objective of this study was to evaluate the effect of different encapsulating agents on *L. casei* (N) survival during spray drying and to investigate the influence of sub-lethal heat stress on the survivability of *Lactocaseibacillus casei* N (N) during spray drying and its storage at 4 °C for 90 days. The ability of heat-stressed LAB to survive in simulated gastrointestinal conditions after storage was also evaluated. The effect of heat stress on intracellular proteins of *L. casei* (N) was studied after exposing the free cells to higher temperatures.

## 2. Materials and methods

### 2.1. Raw materials

The carrier materials (Maltodextrin- DE ≤ 20 and Gum acacia-95%) and De Man Rogosa Sharpe media (MRS-HiVeg) were procured from Himedia India Pvt. Ltd, Mumbai, India. Corn starch (95.3%) was obtained from the local market. *Lactocaseibacillus casei* N (N) a lactobacillus strain, was obtained from Microbiology and Fermentation Technology department, CSIR-CFTRI (Central Food Technological Research Institute)- Mysuru, India. The genome of this strain has been deposited with the primary accession number CP077759 in GenBank (NCBI).

### 2.2. Sub-lethal heat stress and encapsulation of lactobacillus by spray drying

#### 2.2.1. Feed preparation

The lyophilized *L. casei* (N) was activated in MRS broth and incubated at 37 °C for 24 h under static conditions. The activated cells were stored in glycerol stocks at -20 °C for future use. An aliquot of the activated culture was transferred, recultured, and incubated at 37 °C for 18 h. The cells were harvested by centrifuging at 8000×g for 20 min at 4 °C, and the pellet was washed two times in 0.85% sodium chloride (NaCl) solution. The cells were stored at 4 °C until further use.

The feed or slurry for spray drying was prepared according to Chandralekha et al., 2016 by adding (10% w/v) quantity of carrier materials (maltodextrin, acacia gum, and corn starch) along with 5% (w/v) microbial cells in 400 mL of distilled water. The contents were mixed using a tabletop magnetic stirrer to get a uniform solution.

#### 2.2.2. Exposure of *L. casei* (N) to sub-lethal heat stress

The slurry or feed for spray drying was prepared as described in 2.2.1. Then it was subjected to 45 °C and 50 °C (sub-lethal heat stress) separately for 60 min. The slurry was immediately cooled to room temperature by keeping the slurry in water and constant stirring to prevent heat stress. The samples were withdrawn at 0 min and after 60 min for plating on MRS agar plates to check the viability. Serial dilutions were transferred to sterile Petri plates and pour plated using MRS agar, and then the plates were incubated for 48 h at 37 °C.

#### 2.2.3. Spray drying

Spray drying of the prepared slurry was performed in a lab-scale spray dryer (LabPlant SD-05, 1050 × 620 × 500 mm (H x W x D),

Chelmsford, UK) with a 2-fluid nozzle type atomizer. The parameters adopted for spray drying were as follows-inlet air temperature (120 ± 2 °C) and outlet air temperature (65 ± 5 °C). At first, distilled water was fed to the spray dryer until the steady-state and thermal equilibrium conditions were reached and then changed to the probiotic-containing feed solution. The feed solution was continuously stirred using a magnetic stirrer to avoid phase separation. The yield of spray-dried powder was calculated by the following equation.

$$\text{Dry cell powder yield (\%, w/w)} = (P_w / T_s) \times 100$$

'P<sub>w</sub>' is the cell powder weight collected after spray drying, and 'T<sub>s</sub>' is the total soluble solid content in the slurry. The spray-dried microparticles were collected from the cyclone base and stored in low-density polyethylene bags (LDPE) at 4 °C for further analysis.

### 2.2.4. Efficiency of encapsulation

The efficiency of encapsulation (EE) was calculated as the survival rate of the microorganisms immediately after spray drying during the microencapsulation process (Nunes et al., 2018). The EE % was calculated using the formula

$$\text{EE\%} = (N_0 / N_f) \times 100$$

Where 'N<sub>0</sub>' is the number of viable cells (log CFU/g) from the spray-dried microparticles, and 'N<sub>f</sub>' is the number of viable cells (log CFU/g) in the slurry before the spray drying.

### 2.3. Cell survival

The survival of probiotic cells in spray-dried powders was assessed using the plate count method. In 0.9 mL of phosphate-buffered saline solution (PBS pH 7.0), 100 mg of spray-dried powder was rehydrated. The rehydrated samples were mixed thoroughly using a vortex mixer to get the homogenized suspension. Then 0.5 mL of the serially diluted solution was plated using the pour plate method, and the plates were incubated at 37 °C for 48 h.

### 2.4. Storage studies of spray-dried powders

Spray-dried powders were sealed in low-density polyethylene bags (LDPE) and were kept under refrigerated conditions (4 °C) for 90 days (Wang, Ho, Chen, & Chen, 2015). During storage studies, samples were withdrawn every 30 days, serially diluted, pour plated, and checked for cell survival.

### 2.5. Spray-dried powder properties

#### 2.5.1. Moisture content and water activity

The moisture content of the spray-dried samples was calculated using an IR moisture meter (Aczet 129, Mumbai, India). Two grams of sample were placed in an aluminium pan and heated to 102 ± 2 °C until a constant weight was obtained. The results were expressed in terms of moisture content (%) on a dry basis of the sample. The water activity (a<sub>w</sub>) is defined as the partial vapor pressure of water in a solution divided by the standard state partial vapor pressure of water. A Pawkit water activity meter (METER Group Inc. Pullman, WA, USA) was used to determine the water activity (a<sub>w</sub>) of spray-dried powders.

#### 2.5.2. Particle size analysis and scanning electron microscopy

The particle size was measured using a Microtac Turbo trac dry powder dispersion system (BLUEWAVE, USA) with a particle size analyzer (S3500 Series, USA). It works on the principle of laser dispersion, which can measure the particle size over a range of 0.25–3000 μm. For each measurement, 1 g of sample was loaded, and all the measurements were done in triplicates.

The morphology of spray-dried probiotic powder with different

carrier materials was studied using scanning electron microscopy SEM (Leo 435 Vp, Leo Electronics Systems, Cambridge, UK). For sample preparation before analysis, the dry powder was put on copper grids and subsequently coated with gold.

### 2.5.3. Powder flow properties

Tap density was used to analyze the flow parameters of spray-dried probiotic powder samples. The following formulae were used to calculate Carr's index (CI) and Hausner ratio (HR) based on the volume occupied by powder before and after tapping (Chandralekha et al., 2016).

$$CI (\%) = [(T_v - B_v)/T_v] \times 100$$

The tapped volume is 'T<sub>v</sub>' while the bulk volume of the spray-dried probiotic powder is 'B<sub>v</sub>'.

$$HR = 100 / (100 - CI)$$

## 2.6. Survival of lactobacillus in spray-dried powders under simulated gastrointestinal conditions

Simulated gastric juice (SGJ) of pH 2.5 was prepared by dissolving 0.125 mol/L sodium chloride (NaCl), 0.007 mol/L potassium chloride (KCl), 0.045 mol/L sodium bicarbonate (NaHCO<sub>3</sub>), and 3 g/L pepsin (Himedia, Mumbai, India) as described by Archer & Halami, 2015 and gastric juice of pH 7.0 were used as a control. One gram of spray-dried probiotic powder was mixed with 9 mL of simulated gastric juice and incubated at 37 °C with constant agitation at 150 rpm. At 0, 1, 2, and 3 h of incubation, cell counts on MRS agar plates were taken.

Simulated intestinal juice (SIJ) was prepared by dissolving 0.1% of pancreatin in 0.15% of ox-bile (Himedia, Mumbai, India) solution with pH adjusted to 8.0 and control pH 7.0. One gram of probiotic powder was mixed with 9 mL of SIJ, incubated at 37 °C with the agitation of 150 rpm, and survivability was determined by plating on MRS agar at 0, 1, 2, 3, and 6 h.

## 2.7. Effect of heat stress on intracellular proteins of *L. casei* (N) cells

*L. casei* (N) grown in test tubes for 18 h, was subjected to different temperatures like 45, 50, 55, and 60 °C for 60 min separately. The samples were then immediately cooled in water and further incubated at 37 °C for 60 min to allow the culture to repair any damage caused due to heat stress at the molecular level. A control experiment was performed by incubating the culture at 37 °C. All the heat-stressed samples were checked for their viability on MRS agar plates.

*L. casei* (N) culture subjected to the treatment mentioned above was centrifuged at 4000×g for 10 min, and the obtained pellet was washed three times using PBS (pH 7.0). The cell pellet was taken to a prechilled mortar and pestle along with acid-washed glass beads (210–300 μm, Sigma) and crushed using 1–2 mL of PBS. The solution was centrifuged at 3000×g for 10 min, and the supernatant was transferred to the fresh Eppendorf and stored at 4 °C for further analysis.

SDS-PAGE analysis of extracted intracellular proteins was carried out according to Laemmli, 1970, in a 10% polyacrylamide gel gradient in standard Tris/glycine chamber buffer (pH 8.30). For separating gel 30% acrylamide solution, 1.5 M Tris/HCl (pH 8.8) and for stacking gel 1.0 M Tris/HCl (pH 6.8) was used along with 10% w/v sodium dodecyl sulphate (SDS), 10% w/v ammonium persulphate (APS) and 10 μL v/v N,N,N',N'-Tetramethylethane-1,2-diamine (TEMED) (Himedia, Mumbai, India). Broad range protein marker (PageRuler™ prestained protein ladder, 10–180 kDa, Thermo Fisher Scientific, Bangalore, India) and 40 μg of protein from each sample were loaded into each well. The gel was run at 60–70 V for 5–6 h in a vertical mini gel system (Bionova Biologics Pvt. Ltd, Bangalore, India).

Silver staining of the gel was performed according to Chevallet, Luche, & Rabilloud, 2006. The gel was kept overnight in a fixative solution (50% v/v methanol, 12% v/v acetic acid, formaldehyde 0.075% v/v - Merck, Bangalore, India), and the gel was washed with 50%, 30% ethanol, and water subsequently for 20 min each. The gel was sensitized (0.02% w/v sodium thiosulphate – Himedia, Mumbai, India) for 60 s and washed three times using water. Impregnator solution (0.2% w/v silver nitrate- Sigma, Bangalore, India and 0.05% v/v formaldehyde- Merck, Bangalore, India) was added to the gel and kept in the dark conditions for 20 min. The gel was washed with water three times to remove traces of the impregnator. Finally, a developer solution (6% w/v sodium carbonate – Himedia, Mumbai, India and 0.05% formaldehyde) was added to view the bands. Once the bands appeared, a stopping solution (50% v/v methanol and 12% v/v acetic acid) was added to stop the reaction. Then the gel was visualized under electronic gel documentation equipment (Biobee Tech, Bangalore, India).

The spectrum of each protein sample was recorded using an FTIR Spectrometer (SENSOR II, Bruker, Germany), with platinum attenuated total reflection (ATR) crystal and operated in the spectral range of 400–4000 cm<sup>-1</sup>. Spectra were recorded with a resolution of 4 cm<sup>-1</sup> co-adding 15 scans to improve the signal-to-noise ratio. Operation and data acquisition was carried out using OPUS software. The spectra were collected per sample using the appropriate background spectrum subtraction. All the samples were placed on ATR crystal before measurements. The crystal was cleaned using propanol and distilled water after each sample analysis.

## 2.8. Statistical analysis

All the tests were done in triplicates, and the results were given as mean ± standard deviations. GraphPad Prism 9.0.0 was used to analyze the data using two-way ANOVA (analysis of variance) consisting of categorical variables, i.e., carrier materials and time period for the resultant survivability of *L. casei* (N). Multiple comparisons were evaluated by Tukey's test differences with a significant level of  $p \leq 0.05$ .

## 3. Results and discussion

### 3.1. The survival rate of *L. casei* (N) during spray drying with various encapsulating materials

In this study, we evaluated the effects of different encapsulating agents on the survivability of *L. casei* (N) during spray drying. The results showed that the addition of carrier agents like maltodextrin (MDX), corn starch (CS), and gum acacia (GA) to probiotic cells influence their survivability during spray-drying. After spray drying, lactobacillus with a survival of more than or equal to 85% is considered to have a good encapsulation efficiency (Bradford et al., 2019). Among the encapsulating matrices studied, corn starch (N + CS) yielded the best viability of  $11.50 \pm 0.09$  log CFU g<sup>-1</sup> of survivability (95.6%), whereas N + MDX and N + GA showed  $11.15 \pm 0.11$  (93.0%) and  $11.18 \pm 0.32$  (93.2%) log CFU g<sup>-1</sup> respectively (Fig. 1). Ying et al., 2016 investigated several combinations of spray-drying encapsulating agents (Glucose, corn starch, protein, and oil) to improve the protection of *L. rhamnosus* GG and found that a combination of corn starch was more efficient in protecting the microorganism during stress conditions. Generally, starch is widely used as encapsulating material in spray drying (Domian, Brynda-Kopytowska, Cenker, & Świrzydow, 2015; Krishnan, Bhosale, & Singhal, 2005). In this current study, corn starch has shown maximum viability after spray drying. Hence, corn starch was used as an encapsulating agent in our further studies. Nunes et al., 2018 also reported that resistant starch (hi-maize) was found to have significant viability of 94.26% among the encapsulating matrices of inulin, resistant starch, trehalose, and gum arabic used to make spray-dried powders of *Lactobacillus acidophilus*.

The maximum powder yield (67.50%, w/w) was obtained in the case

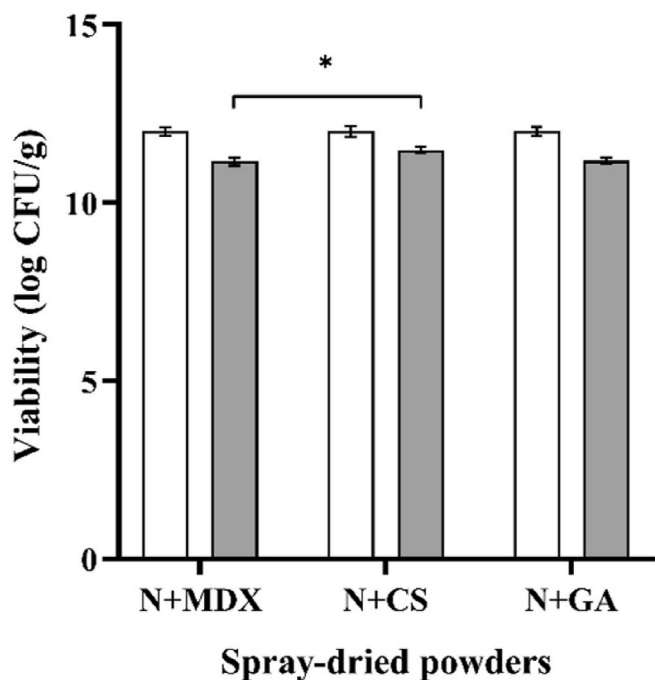


Fig. 1. Survivability (log CFU g<sup>-1</sup>) of *L. casei* (N) before spray drying (□) and after (■) spray drying with different carrier materials, MDX-maltodextrin, CS-corn starch and GA-gum acacia. \*(p < 0.05).

of gum acacia in spray-drying trials, but with a low encapsulation efficiency of 93.21%, this can be attributed to the low viscosity of its solution (Chandrasekhar et al., 2016). In contrast, the lowest powder yield was observed in the case of maltodextrin (48.23%) due to its hygroscopic nature. The sub-lethal heat-stressed samples N + CS 45 showed 56.03% powder yield with a higher encapsulation efficiency of 98.78% due to the maximum adherence of bacterial cells to corn starch particles. The results are depicted in Table 1.

### 3.2. Effect of sub-lethal heat stress on viability of lactobacillus during spray drying and storage

From our preliminary studies, we had found that there was no growth when cells were exposed to 55 °C and 60 °C. Hence 45 °C and 50 °C were considered as sub-lethal stress temperatures for *L. casei* (N). In order to improve *L. casei* (N) viability during spray drying and subsequent storage, the prepared slurry was subjected to different sub-lethal heat stress conditions (45 °C and 50 °C) for 60 min before spray drying. When lactobacilli cells were subjected to heat pre-treatment at 50 °C before the lethal temperature challenge, the viability of the cells increased (Gouesbet, Jan, & Boyaval, 2002).

As shown in Fig. 2, there is an increase in survivability of *L. casei* (N) after spray drying compared to control (N + CS) when the slurry was subjected to prior heat stress at 45 °C (N + CS 45). A 0.26 log cycles increase in viability difference was observed between the N + CS and N

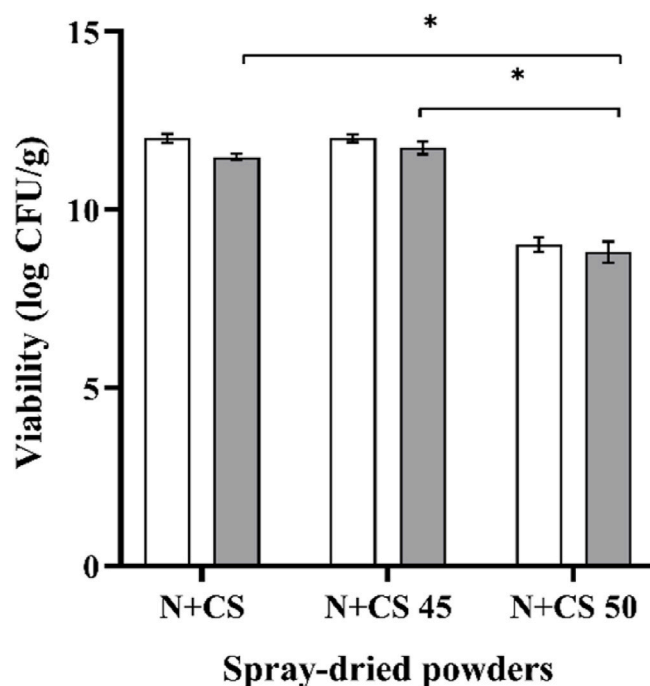


Fig. 2. Effect of sub-lethal heat stress on the viability of *L. casei* (N) encapsulated with corn starch before (□) and after (■) spray drying. N + CS is *L. casei* cells encapsulated with corn starch without any heat stress, N + CS 45 with sub-lethal heat stress at 45 °C and N + CS 50 with sub-lethal heat stress of 50 °C for 60 min. All values are mean ± standard deviation (n = 3) \*(p < 0.001).

+ CS 45. In N + CS 50, there was a 3 log cycles reduction in viability before spray drying due to high-temperature treatment, but it showed 98% of survivability after spray drying (Fig. 2). According to Desmond, Ross, O'callaghan, Fitzgerald, & Stanton, 2002, the preliminary adaptation of *L. casei* Nad and *L. plantarum* 8329 cultures to heat stress had increased the survivability by enhancing cell resistance to spray drying. Similarly, spray drying has been shown to improve the survivability of sub-lethal stressed lactobacilli cultures (Paéz et al., 2012).

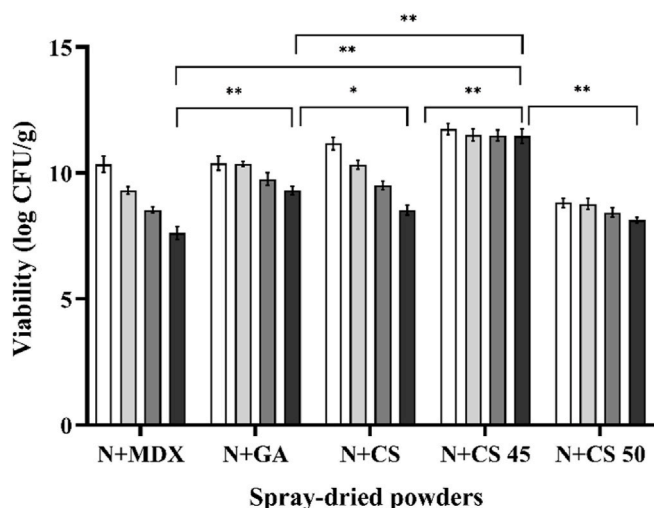
The literature studies have shown that lower temperatures improve the rates of cell viability due to the reduction of possible chemical reactions that are detrimental to microorganisms (Wang et al., 2015). Nunes et al., 2018 showed that hi-maize and trehalose presented greater viability with no significant difference (p < 0.05) in loss of viability after 120 days of storage at low temperatures. Hence, all spray-dried probiotic powders were stored at 4 °C for 90 days during shelf-life studies. Fig. 3 depicts the survival of microencapsulated cells during their storage. The *L. casei* (N) strain, when pre-treated to sub-lethal heat stress at 45 °C (N + CS 45), had shown significantly higher survivability (p < 0.01) during its storage at 4 °C for 90 days compared to other spray-dried powders. The viability of N + CS 45 on the 0<sup>th</sup> day was 11.74 ± 0.22 log CFU g<sup>-1</sup>, and on the 90<sup>th</sup> day, it was 11.46 ± 0.28 log CFU g<sup>-1</sup> with the log reduction of 0.28.

The higher log reduction of 2.72 was shown by N + MDX followed by

Table 1  
Powder yield, encapsulation efficiency, physical and flow properties of spray-dried powders.

Samples	Powder yield (% w/w)	Encapsulation efficiency (EE) (%)	Mean particle size (µm)	Moisture content (%)	Carr's Index	Hausner ratio	Water activity (a <sub>w</sub> )
N + MDX	48.23 ± 2.36 <sup>a</sup>	93.71 ± 1.69 <sup>a</sup>	8.23 ± 0.92 <sup>d</sup>	8.90 ± 0.70 <sup>a</sup>	21.74 ± 0.85 <sup>a</sup>	1.27 ± 0.08 <sup>a</sup>	0.38 ± 0.02 <sup>a</sup>
N + CS	59.24 ± 1.95 <sup>b</sup>	97.21 ± 2.04 <sup>a</sup>	19.24 ± 1.22 <sup>d</sup>	7.80 ± 0.48 <sup>b</sup>	19.20 ± 1.05 <sup>a</sup>	1.23 ± 0.05 <sup>a</sup>	0.44 ± 0.05 <sup>a</sup>
N + GA	67.50 ± 1.47 <sup>a</sup>	93.21 ± 1.32 <sup>a</sup>	7.66 ± 2.19 <sup>e</sup>	7.40 ± 0.22 <sup>b</sup>	14.25 ± 0.27 <sup>b</sup>	1.16 ± 0.06 <sup>a</sup>	0.48 ± 0.08 <sup>a</sup>
N + CS 45	56.03 ± 1.66 <sup>c</sup>	98.78 ± 2.51 <sup>a</sup>	16.03 ± 0.81 <sup>b</sup>	7.30 ± 0.62 <sup>b</sup>	18.79 ± 0.59 <sup>a</sup>	1.23 ± 0.08 <sup>a</sup>	0.43 ± 0.03 <sup>a</sup>
N + CS 50	51.02 ± 2.84 <sup>d</sup>	91.31 ± 1.88 <sup>a</sup>	11.02 ± 1.37 <sup>c</sup>	7.50 ± 0.53 <sup>b</sup>	19.13 ± 1.14 <sup>a</sup>	1.23 ± 0.02 <sup>a</sup>	0.45 ± 0.07 <sup>a</sup>

Means followed by the same letter, do not differ statistically from each other with significance level p < 0.001.



**Fig. 3.** Viability of spray-dried powders at 0<sup>th</sup> day (□), 30th day (▤), 60th day (▥) and 90th day (■) during its storage at 4 °C. N + MDX-spray dried powder of *L. casei* cells encapsulated with maltodextrin, N + GA-gum acacia and N + CS is *L. casei* cells encapsulated with corn starch, N + CS 45 with sub-lethal heat stress at 45 °C and N + CS 50 with sub-lethal heat stress of 50 °C for 60 min. All the values are given as mean ± standard deviation (n = 3) and the \*\**p* < 0.0001 and \**p* < 0.001.

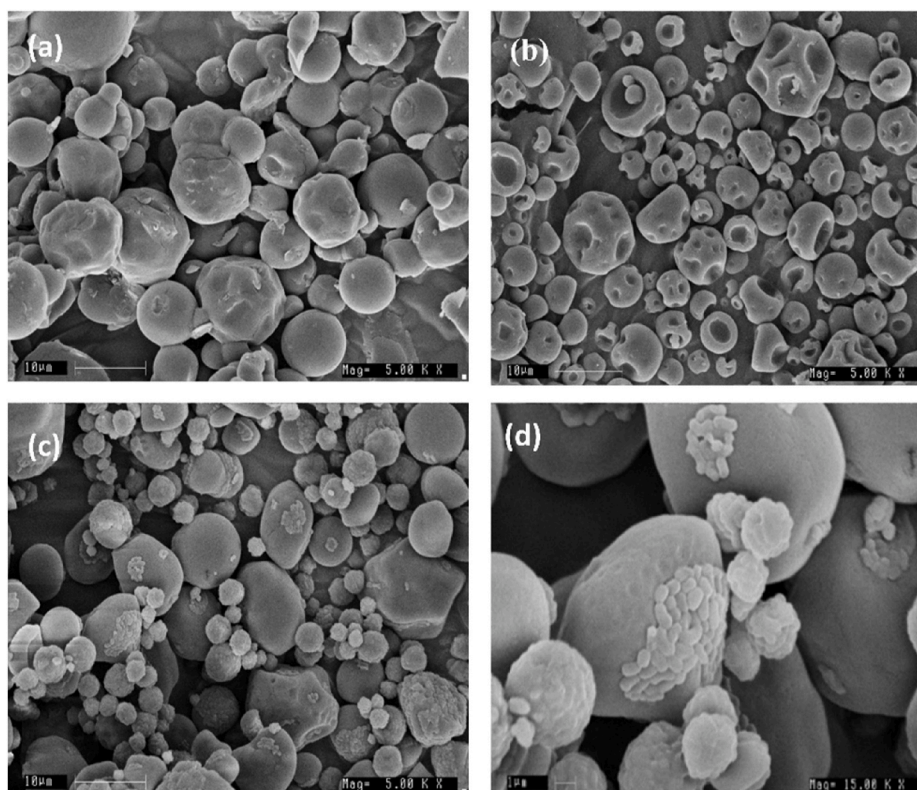
N + CS (2.63) and N + GA (1.07), whereas N + CS 50 had shown a log reduction of only 0.68 during its storage period of 90 days. Among all the encapsulating agents used, N + GA had shown higher survivability during its storage (Desmond, Ross, O'Callaghan, Fitzgerald, & Stanton, 2002), and maltodextrin had shown a poor protective effect because of its high molecular weight as it fails to replace the water molecules (Pinto et al., 2015). The spray-dried powders of N + MDX and N + GA had shown higher survival in the initial days of storage, but by the 90th day,

they had lost viability. Whereas in the sub-lethal heat-stressed powders N + CS 45 and N + CS 50, there was no significant loss of viability during 90 days of storage.

### 3.3. Morphology and particle size analysis

The microscopic images of the microparticles encapsulated with different carrier materials containing *L. casei* (N) are shown in Fig. 4. SEM revealed that the bacteria were encapsulated entirely during spray drying. Spray-dried powders of maltodextrin had a smooth surface area (Fig. 4a). The concavities were observed on the surface of microparticles with gum acacia (GA), which are typical to GA spray-dried powders (Fig. 4b) (O'Riordan, Andrews, Buckle, & Conway, 2001). There was no difference observed in the morphology of the spray-dried powder using corn starch with or without sub-lethal heat treatments. However, microparticles with corn starch had *L. casei* (N) embedded and also attached to the particle surfaces, as shown in Fig. 4c and d. Similar results were also reported by Bradford et al., 2019, with microparticles of *L. plantarum* encapsulated with hi-maize starch. There were no cracks or fractures seen on surfaces of microparticles, and the air permeability was negligible or non-existent, ensuring that probiotic microorganisms were better protected (Rajam & Anandharamakrishnan, 2015).

The microparticles were of different size ranges from 7.66 to 19.24 μm (Table 1). The smallest particle size was observed in spray-dried powders of gum acacia and the highest with corn starch. Rajam & Anandharamakrishnan, 2015 reported that larger microparticles (6.68–23.89 μm) might be attributed to various film-forming properties and gelling properties of the wall materials used for encapsulation of *L. plantarum* MTCC 5422. Similarly, 6.68–19.3 μm sized microparticles containing *L. acidophilus* La-5 were reported by Nunes et al., 2018. Smaller particle sizes are generally preferred since they ensure quality and homogeneity when used in food applications.



**Fig. 4.** SEM images of *L. casei* (N) cells encapsulated with (a) maltodextrin, (b) Gum acacia, and (c,d) Corn starch. (5000× (a,b,c) and 15000× (d) magnifications).

### 3.4. Physical properties of spray-dried powders

By increasing the solid soluble content of the feed slurry, the moisture content of the powder also increases, facilitating the strong crust formation during spray drying that restricts the diffusion of water from the interior to the surface of the droplet (Chandralekha et al., 2016). The spray-dried powders with different carrier materials showed moisture content ranging from 7.3 to 8.9% (Table 1). According to Hari, Jebitta, & Sivaraman, 2013, powders with a moisture content of less than 10% are classified as stable. All the spray-dried powders obtained in this study have moisture percentages below 10, indicating their stability during their storage for prolonged shelf-life. Thus the sub-lethal heat stress did not significantly ( $p > 0.05$ ) change the moisture content of the spray-dried probiotic powders.

Carr's Index (CI) in the range of 5–15 and Hausner ratio (HR) between 1 and 1.25 are values indicating excellent flowability of probiotic powders. A value indicates a good flowability between 16 and 18, a moderately good flow is indicated by a value between 19 and 21, and poor flow is indicated by a value between 22 and 35. Hausner ratios greater than 1.5 indicate weak flow (33% Carr's index) in powders with fewer free-flowing particles, such as flakes (Chandralekha et al., 2016). Our study noticed the lowest HR ( $1.16 \pm 0.06$ ) and CI ( $14.25 \pm 0.27$ ) for spray-dried powder of N + GA. Based on the scale mentioned above, this powder could be classified as excellent, thus indicating its promising potential for storage and handling. Whereas spray-dried powders using corn starch as an encapsulating agent had shown the CI in the range of 18.79–19.20 and HR of 1.23 in both control (N + CS-without sub-lethal heat stress) and sub-lethal heat-stressed N + CS 45, N + CS 50 probiotic powders indicating that these spray-dried powders have moderately good flow property. The results observed for spray-dried probiotic powders are comparable with those reported for spray-dried *L. rhamnosus* GG by flaxseed oil extract as encapsulating agents (Łopusiewicz et al., 2021).

Water activity becomes an important index for the foods prepared using spray-dried powders as it influences the shelf life of the product (Serra Costa et al., 2015). Table 1 shows the water activity ( $a_w$ ) values of spray-dried powders. The lowest water activity,  $0.38 \pm 0.02$ , was observed in N + MDX, whereas the highest water activity of  $0.48 \pm 0.08$ , was observed in N + GA. These values were not statistically significant ( $p > 0.05$ ) and were unaffected by the encapsulating material and sub-lethal heat stress. Łopusiewicz et al., 2021 reported that the water activity of lactobacillus was influenced by spray-drying temperature. Foods having a water activity of 0.6 or less are generally considered microbiologically and biochemically safe (Kumar et al., 2017).

### 3.5. Survival of spray-dried powders in simulated gastrointestinal fluids

There are reports that the lactobacilli cultures subjected to heat stress can influence the survivability of cultures under simulated gastrointestinal stress conditions (Ferrando, Quiberoni, Reinheimer, & Suárez, 2016). An increase in survivability of *L. plantarum* DPC2739 and DPC2741 was achieved under pH 5.0 when adapted to 42 °C for 24 h (De Angelis et al., 2004). The resistance to simulated gastric solutions of microcapsules produced with N + CS, N + CS 45, and N + CS 50 by spray drying is given in Table 2. The survivability rate of *L. casei* (N) in simulated gastric fluids was found to be significantly affected by sub-lethal heat stress ( $p < 0.001$ ). The spray-dried powder of N + CS 45 had shown the highest survivability of  $7.98 \pm 0.32$  log CFU  $g^{-1}$  with 3.06 log cycles more than the N + CS, whereas N + CS 50 had shown the 0.24 log cycles higher than control (N + CS). Bacterial adherence to spray-dried powder with starch improves the delivery of viable and active probiotics to the digestive tract (Anal & Singh, 2007).

The resistance of sub-lethal heat-treated spray-dried powders in the presence of simulated intestinal juice is shown in Table 2. Previous exposure to sub-lethal heat conditions influenced the survival of spray-dried powders in the simulated intestinal fluids. There was a 0.14 log

**Table 2**

Survivability of *L. casei* (N) in spray-dried probiotic powders under simulated gastrointestinal conditions.

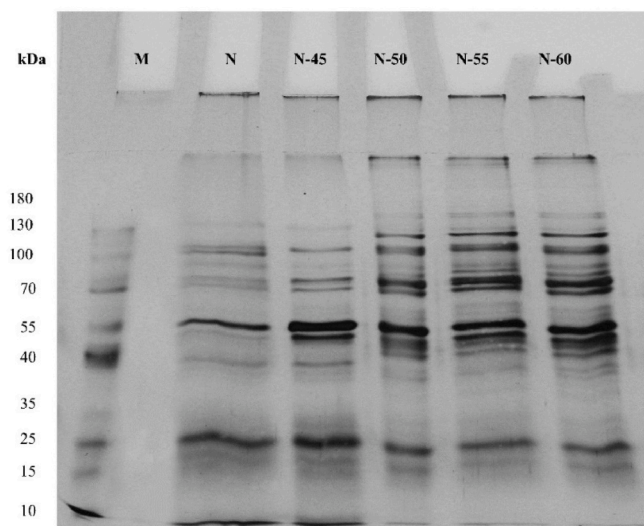
pH	Survivability (log CFU $g^{-1}$ )								
	N + MDX	N + GA	N + CS	N + CS 45	N + CS 50				
Gastric Juice	7.0	0	5.69 ± 0.18 <sup>cA</sup>	6.07 ± 0.25 <sup>bA</sup>	6.27 ± 0.35 <sup>bA</sup>	7.82 ± 0.25 <sup>aA</sup>	5.24 ± 0.21 <sup>dA</sup>		
		h	1	5.32 ± 0.24 <sup>bA</sup>	5.87 ± 0.37 <sup>bA</sup>	6.08 ± 0.28 <sup>bA</sup>	7.31 ± 0.18 <sup>aA</sup>	4.62 ± 0.24 <sup>cA</sup>	
		h	2	5.51 ± 0.21 <sup>cA</sup>	5.45 ± 0.22 <sup>cA</sup>	6.19 ± 0.22 <sup>bA</sup>	7.53 ± 0.25 <sup>aA</sup>	4.73 ± 0.29 <sup>dA</sup>	
		h	3	5.28 ± 0.15 <sup>cA</sup>	5.11 ± 0.31 <sup>cA</sup>	6.02 ± 0.36 <sup>bA</sup>	7.30 ± 0.28 <sup>aA</sup>	4.94 ± 0.31 <sup>cA</sup>	
		h	2.5	0	5.33 ± 0.16 <sup>cA</sup>	4.57 ± 0.33 <sup>dA</sup>	5.89 ± 0.36 <sup>bA</sup>	7.10 ± 0.32 <sup>aA</sup>	4.50 ± 0.35 <sup>dA</sup>
		h	1	5.07 ± 0.11 <sup>bA</sup>	4.04 ± 0.36 <sup>bA</sup>	5.25 ± 0.48 <sup>bA</sup>	7.40 ± 0.38 <sup>aA</sup>	4.66 ± 0.33 <sup>bA</sup>	
	h	2	4.48 ± 0.29 <sup>bA</sup>	3.69 ± 0.13 <sup>cA</sup>	4.87 ± 0.36 <sup>bA</sup>	7.88 ± 0.28 <sup>aA</sup>	4.99 ± 0.29 <sup>bA</sup>		
	h	3	4.56 ± 0.42 <sup>bA</sup>	3.74 ± 0.22 <sup>cA</sup>	4.92 ± 0.28 <sup>bA</sup>	7.98 ± 0.32 <sup>aA</sup>	5.16 ± 0.25 <sup>bA</sup>		
	Intestinal Juice	7.0	0	7.61 ± 0.28 <sup>aA</sup>	7.90 ± 0.41 <sup>aA</sup>	8.38 ± 0.22 <sup>aA</sup>	8.82 ± 0.19 <sup>aA</sup>	8.10 ± 0.21 <sup>aA</sup>	
			h	1	7.35 ± 0.21 <sup>aA</sup>	7.52 ± 0.29 <sup>aA</sup>	8.11 ± 0.36 <sup>aA</sup>	8.50 ± 0.18 <sup>aA</sup>	7.90 ± 0.24 <sup>aA</sup>
			h	2	7.44 ± 0.19 <sup>aA</sup>	7.68 ± 0.35 <sup>aA</sup>	8.20 ± 0.19 <sup>aA</sup>	8.55 ± 0.25 <sup>aA</sup>	7.64 ± 0.29 <sup>aA</sup>
			h	3	7.28 ± 0.09 <sup>bA</sup>	7.35 ± 0.25 <sup>bA</sup>	8.08 ± 0.28 <sup>aA</sup>	8.20 ± 0.28 <sup>aA</sup>	7.40 ± 0.31 <sup>bA</sup>
			h	6	6.86 ± 0.37 <sup>bA</sup>	6.92 ± 0.15 <sup>bA</sup>	7.66 ± 0.18 <sup>bB</sup>	7.95 ± 0.21 <sup>aA</sup>	7.00 ± 0.26 <sup>bA</sup>
			h	8.0	0	7.55 ± 0.15 <sup>aA</sup>	8.04 ± 0.18 <sup>aA</sup>	8.28 ± 0.25 <sup>aA</sup>	8.17 ± 0.22 <sup>aA</sup>
		h	1	7.25 ± 0.43 <sup>aA</sup>	7.52 ± 0.31 <sup>aA</sup>	7.56 ± 0.24 <sup>bB</sup>	7.35 ± 0.17 <sup>aB</sup>	7.88 ± 0.18 <sup>aA</sup>	
		h	2	7.11 ± 0.35 <sup>aA</sup>	7.31 ± 0.35 <sup>aA</sup>	7.69 ± 0.16 <sup>bB</sup>	7.47 ± 0.23 <sup>aB</sup>	6.97 ± 0.35 <sup>aB</sup>	
		h	3	6.67 ± 0.22 <sup>aA</sup>	7.09 ± 0.28 <sup>aA</sup>	7.56 ± 0.18 <sup>bB</sup>	7.42 ± 0.29 <sup>aB</sup>	6.42 ± 0.24 <sup>aB</sup>	
		h	6	6.13 ± 0.12 <sup>bB</sup>	6.58 ± 0.16 <sup>aA</sup>	7.16 ± 0.28 <sup>bB</sup>	7.30 ± 0.25 <sup>aB</sup>	6.10 ± 0.33 <sup>aB</sup>	

All the values are represented as mean ± standard deviation (n = 3). Means followed by the same letter, lowercase in the row and uppercase in the column, do not differ statistically from each other by Tukey test (multiple comparisons) with significance level  $p < 0.001$ .

increase in N + CS 45 survivability in simulated intestinal fluid compared to N + CS. In contrast, N + CS 50 was not significantly ( $p > 0.05$ ) affected by sub-lethal heat exposure, and the viability loss of 1.06 log was observed compared to N + CS. These results support the findings of Barbosa, Borges & Teixeira (2015), where the sub-lethal stress did not improve the viability of lactic acid bacteria under simulated intestinal conditions. The sub-lethal heat stress of 45 °C reported in our study only effectively enhances the viability of *L. casei* (N) encapsulated cells under the gastrointestinal environment.

### 3.6. Effect of temperature on protein expression in *L. casei* (N) cells

The free cells of *L. casei* (N) were subjected to different temperatures to study the effect of heat stress on protein expression. There was no growth observed when cells were subjected to higher temperatures of 55 and 60 °C. The intracellular proteins from heat-stressed cultures were extracted, and SDS PAGE analysis was performed. Fig. 5 shows the presence of differentially expressed proteins in SDS PAGE gel, where some of the protein bands in the gel were increased, indicating the higher expression of proteins. At around 20 kDa (GrpE), an increase in protein content was observed in N-45 compared to control. Also, differences in protein content were observed approximately at 40–55 kDa, 60 kDa, and 70 kDa representing the possible overexpression of DnaJ, GroEL, and DnaK in all heat-stressed cells. There was also an increase in



**Fig. 5.** SDS PAGE (10%) analysis of intracellular proteins extracted from heat-stressed *L. casei* (N) cells. M-broad range protein marker, N – control, N-45, N-50, N-55 and N-60 – intracellular proteins extracted from *L. casei* (N) cells after subjecting them to 45, 50, 55 and 60 °C for 60 min.

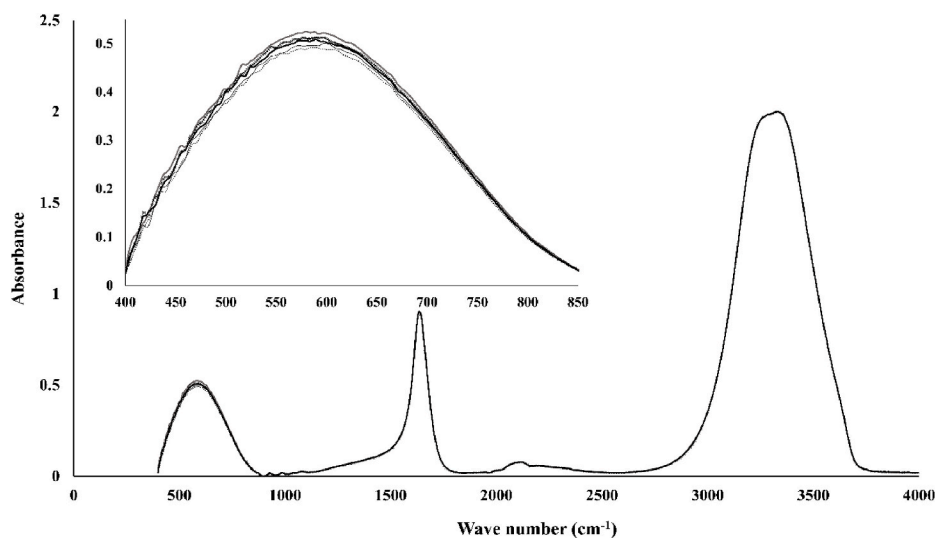
protein concentration around 100 kDa, revealing the possibility of ClpB, ClpA, ClpX proteins. It appears to be a strategy utilized mainly by lactobacillus or bacterial pathogens to generate general stress proteins in response to various environmental challenges, such as acidity, heat, and cold stresses (Gandhi & Chikindas, 2007; Guzzo et al., 2000). Under stress, this biochemical reaction reflects a cell's adaptation through modifying protein synthesis. (Fiocco, Capozzi, Goffin, Hols, & Spano, 2007). It is crucial for a cell's survival to produce heat shock proteins (HSP) in order to maintain metabolic activity and repair cell damage during heat stress.

The HSP, mainly chaperones and proteases, are responsible for refolding and degradation. GroEL (60 kDa) is a group 1 chaperonin that protects folding proteins from premature cytosolic breakdown and abnormal interactions. (Jain et al., 2017; Kramer, Ramachandiran, Horowitz, & Hardesty, 2002). DnaK (homologue to Hsp70) is the most constitutively expressed and stress-inducible chaperones in the cytosol. DnaK, together with its co-chaperone DnaJ (Homologue to Hsp40) and

regulator GrpE (Homologue to Hsp20), helps in de novo folding of proteins with the ribosome-bound chaperone Trigger factor (TF) (Caloni et al., 2012). ClpB, ClpA, and ClpX, on the other hand, are Hsp100 family members that operate as both chaperones and proteases. (Sanguini et al., 2018).

Further, the extracted intracellular proteins were subjected to FTIR analysis to observe the structural changes in the proteins expressed during heat stress. The FTIR spectra of the intracellular proteins extracted from the heat-treated *L. casei* (N) cells are represented in Fig. 6. The vibrational resonances of bonds containing hydrogen atoms, such as C–H, N–H, and O–H, observed with the first half of the FTIR spectra, which range from 3750 to 2750  $\text{cm}^{-1}$ , are correlated to the chains of several bacterial membrane phospholipids. Stretching vibrations of C=O, C=C, C–N, C–N, C–O, and C–O; bending and twisting resonance modes; and fingerprint area (1500–400  $\text{cm}^{-1}$ ) are included in the range between 1900 and 400  $\text{cm}^{-1}$  (Lozano et al., 2017). It can be seen from Fig. 6 that all the four peaks (N-45, N-50, N-55, N-60) from 400 to 800  $\text{cm}^{-1}$  are positively correlated with molecular changes in the proteins under heat stress conditions when compared to control without any heat stress (N-control). It is visible that differences in their absorbance in this region are positively correlated to the alteration of the secondary and tertiary helix structure of DNA, the generation of new gene fragments, or an increase in the transcriptional in the DNA molecule. The fingerprint region between 900 and 600  $\text{cm}^{-1}$  wavenumber indicates unique weak bands that correspond to amino acids like phenylalanine, tyrosine, tryptophan, and various nucleotides (Faghihzadeh, Anaya, Schiffman, & Oyanedel, 2016). From the spectra, the protein samples from heat-stressed cells had shown differences in absorbance compared to control. Whereas higher absorbance was observed in N-45, corresponding to changes in the protein structure.

Nine distinct IR absorption bands, amide A, B, and I-VII, result from repeating protein and polypeptide units. As shown in Fig. 6, amide IV (625–767  $\text{cm}^{-1}$ ) OCN bending, amide V (640–800  $\text{cm}^{-1}$ ) out of plane NH bending, and amide VI (537–606  $\text{cm}^{-1}$ ) out of plane C=O bending are all correlated with the vibrations as the absorbance of N-45 was higher in this region when compared other samples. These vibrational bands are very complex depending on the nature of side chains, hydrogen bonding, and force field (Kong & Yu, 2007). Also, the absorbance differences in the bands between 858  $\text{cm}^{-1}$  (deformation of C<sub>1</sub>–H and CH<sub>2</sub>) and 698  $\text{cm}^{-1}$  (C–O–C pyranose ring stretching) are assignable to changes in the mucilage polysaccharides (Łopusiewicz et al., 2021). All of these molecular changes in the cells that happened during heat stress



**Fig. 6.** FTIR spectrum of intracellular proteins of heat-stressed *L. casei* (N). N – control (—) Intracellular proteins from *L. casei* (N) cells without any heat stress, N-45 (—), N-50 (—), N-55 (—x—) and N-60 (·····) – intracellular proteins extracted from *L. casei* (N) cells after subjecting them to heat stress at 45, 50, 55 and 60 °C for 60 min.

could explain why *L. casei* (N) cells exposed to 45 °C increased their survivability after spray-drying as well as their shelf life during storage.

#### 4. Conclusion

We report the potential of a simple, applicable approach of sub-lethal thermal pre-treatment to increase *L. casei* (N) survival during spray drying and in simulated gastric fluid with increased shelf life. Our results highlight the increase in the survival rate of probiotics which may successfully be employed in the functional food and fermentation industries. There are not many studies on microencapsulation after being subjected to sub-lethal stress conditions. To the best of our knowledge, this is the first report on preparing probiotic powders by spray drying after subjecting the feed material containing *L. casei* (N) with corn starch as carrier material to sub-lethal heat stress. The potential of *L. casei* (N) subjected to sub-lethal heat stress and its survival at higher temperatures during spray drying was investigated. Corn starch was shown to be the best carrier material for *L. casei* (N) among the encapsulating agents studied. The slurry of *L. casei* (N) and the corn starch subjected to sub-lethal heat stress prior to spray drying increased the cell viability. Also, the sub-lethal heat-stressed spray-dried powders showed better survivability in simulated gastric conditions and improved stability after 90 days of storage at 4 °C. When free cells of *L. casei* (N) were subjected to higher temperatures, changes in protein expression were observed corresponding to heat shock proteins. Thus, the sub-lethal heat-stressed bacterial cells adapt to a higher temperature for better survival during spray drying. The same was further corroborated by FTIR analysis, where the heat-stressed cells extracted protein samples had shown differences in the absorbance corresponding to changes in molecular level (DNA), making the bacteria adapt and survive during adverse conditions. The non-lethal heat treatment of *L. casei* (N) is an excellent option for producing spray-dried probiotic powders with improved survivability, which will aid in the development of functional foods. These results indicate that probiotic powders are good candidates for use in various fruit juice products, jams, jellies, and in the development of other probiotic beverages with functional characteristics.

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#### Author contribution

**Jyothna Bommasamudram:** Conceptualization, Investigation, Data-curation, Writing, Visualization. **Arjun Muthu:** Investigation, Data-curation, Formal analysis. **Somashekar Devappa:** Conceptualization, Supervision, Writing-reviewing, and editing.

#### Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### References

- Abd El-Salam, M. H., & El-Shibiny, S. (2012). Formation and potential uses of milk proteins as nano delivery vehicles for nutraceuticals: A review. *International Journal of Dairy Technology*, 65(1), 13–21. <https://doi.org/10.1111/j.1471-0307.2011.00737.x>
- Ainsley Reid, A., Vuillemand, J. C., Britten, M., Arcand, Y., Farnworth, E., & Champagne, C. P. (2005). Microentrapment of probiotic bacteria in a Ca<sup>2+</sup>-induced whey protein gel and effects on their viability in a dynamic gastrointestinal model. *Journal of Microencapsulation*, 22(6), 603–619. <https://doi.org/10.1080/02652040500162840>
- Anal, A. K., & Singh, H. (2007). Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery. *Trends in Food Science & Technology*, 18(5), 240–251. <https://doi.org/10.1016/j.tifs.2007.01.004>
- Archacka, M., Celińska, E., & Białas, W. (2020). Techno-economic analysis for probiotics preparation production using optimized corn flour medium and spray-drying protective blends. *Food and Bioprocess Processing*, 123, 354–366. <https://doi.org/10.1016/j.fbp.2020.07.002>
- Archer, A. C., & Halami, P. M. (2015). Probiotic attributes of *Lactobacillus fermentum* isolated from human feces and dairy products. *Applied Microbiology and Biotechnology*, 99(19), 8113–8123. <https://doi.org/10.1007/s00253-015-6679-x>
- Barbosa, J., Borges, S., & Teixeira, P. (2015). Influence of sub-lethal stresses on the survival of lactic acid bacteria after spray-drying in orange juice. *Food Microbiology*, 52, 77–83. <https://doi.org/10.1016/j.fm.2015.06.010>
- Bhagwat, A., Bhushette, P., & Annature, U. S. (2020). Spray drying studies of probiotic *Enterococcus* strains encapsulated with whey protein and maltodextrin. *Beni-Suef University Journal of Basic and Applied Sciences*, 9, 33. <https://doi.org/10.1186/s43088-020-00061-z>
- Bradford, R., Reyes, V., Bonilla, F., Bueno, F., Dzandu, B., Liu, C., et al. (2019). Development of milk powder containing *Lactobacillus plantarum* NCIMB 8826 immobilized with prebiotic hi-maize starch and survival under simulated gastric and intestinal conditions. *Food Production, Processing and Nutrition*, 1(1), 1–8. <https://doi.org/10.1186/s43014-019-0011-6>
- Broadbent, J. R., & Lin, C. (1999). Effect of heat shock or cold shock treatment on the resistance of *Lactococcus lactis* to freezing and lyophilization. *Cryobiology*, 39(1), 88–102. <https://doi.org/10.1006/cryo.1999.2190>
- Calloni, G., Chen, T., Schermann, S. M., Chang, H. C., Genevoux, P., Agostini, F., et al. (2012). DnaK functions as a central hub in the *E. coli* chaperone network. *Cell Reports*, 1(3), 251–264. <https://doi.org/10.1016/j.celrep.2011.12.007>
- Chandralekha, A., Tavanandi, A. H., Amrutha, N., Hebbar, H. U., Raghavarao, K. S. M. S., & Gadre, R. (2016). Encapsulation of yeast (*Saccharomyces cerevisiae*) by spray drying for extension of shelf life. *Drying Technology*, 34(11), 1307–1318. <https://doi.org/10.1080/07373937.2015.1112808>
- Chevallet, M., Luhe, S., & Rabilloud, T. (2006). Silver staining of proteins in polyacrylamide gels. *Nature Protocols*, 1, 1852–1858. <https://doi.org/10.1038/nprot.2006.288>
- De Angelis, M., Di Cagno, R., Huet, C., Crecchio, C., Fox, P. F., & Gobetti, M. (2004). Heat shock response in *Lactobacillus plantarum*. *Applied and Environmental Microbiology*, 70(3), 1336–1346. <https://doi.org/10.1128/AEM.70.3.1336-1346.2004>
- Desmond, C., Ross, R. P., O'Callaghan, E., Fitzgerald, G., & Stanton, C. (2002). Improved survival of *Lactobacillus paracasei* NFBC 338 in spray-dried powders containing gum acacia. *Journal of Applied Microbiology*, 93(6), 1003–1011. <https://doi.org/10.1046/j.1365-2672.2002.01782.x>
- Domian, E., Brynda-Kopytowska, A., Cenker, J., & Świrydow, E. (2015). Selected properties of microencapsulated oil powders with commercial preparations of maize OSA starch and trehalose. *Journal of Food Engineering*, 152, 72–84. <https://doi.org/10.1016/j.jfoodeng.2014.09.034>
- Faghizadeh, F., Anaya, N. M., Schiffman, L. A., & Oyanedel-Craver, V. (2016). Fourier transform infrared spectroscopy to assess molecular-level changes in microorganisms exposed to nanoparticles. *Nanotechnology for Environmental Engineering*, 1(1), 1–16. <https://doi.org/10.1007/s41204-016-0001-8>
- Ferrando, V., Quiberoni, A., Reinheimer, J., & Suárez, V. (2016). Functional properties of *Lactobacillus plantarum* strains: A study in vitro of heat stress influence. *Food Microbiology*, 54, 154–161. <https://doi.org/10.1016/j.fm.2015.10.003>
- Fiocco, D., Capozzi, V., Goffin, P., Hols, P., & Spano, G. (2007). Improved adaptation to heat, cold, and solvent tolerance in *Lactobacillus plantarum*. *Applied Microbiology and Biotechnology*, 77(4), 909–915. <https://doi.org/10.1007/s00253-007-1228-x>
- Gandhi, M., & Chikindas, M. L. (2007). *Listeria*: A foodborne pathogen that knows how to survive. *International Journal of Food Microbiology*, 113(1), 1–15. <https://doi.org/10.1016/j.ijfoodmicro.2006.07.008>
- Gardiner, G. E., Sullivan, E. O., Kelly, J., Auty, M. A. E., Collins, J. K., Ross, R. P., et al. (2000). Comparative survival rates of human-derived probiotic *Lactobacillus paracasei* and *L. salivarius* strains during heat treatment and spray drying. *Applied and Environmental Microbiology*, 66(6), 2605–2612. <https://doi.org/10.1128/AEM.66.6.2605-2612.2000>
- Gouesbet, G., Jan, G., & Boyaval, P. (2002). Two-dimensional electrophoresis study of *Lactobacillus delbrueckii* subsp. *bulgaricus* thermotolerance. *Applied and Environmental Microbiology*, 68(3), 1055–1063. <https://doi.org/10.1128/AEM.68.3.1055-1063.2002>
- Gul, O., & Atalar, I. (2019). Different stress tolerance of spray and freeze dried *Lactobacillus casei* shirota microcapsules with different encapsulating agents. *Food Science and Biotechnology*, 28(3), 807–816. <https://doi.org/10.1007/s10068-018-0507-x>
- Guzzo, J., Jobin, M. P., Delmas, F., Fortier, L. C., Garmyn, D., Tourdot-Maréchal, R., et al. (2000). Regulation of stress response in *Oenococcus oeni* as a function of

- environmental changes and growth phase. *International Journal of Food Microbiology*, 55(1–3), 27–31. [https://doi.org/10.1016/S0168-1605\(00\)00209-9](https://doi.org/10.1016/S0168-1605(00)00209-9)
- Hari, K., Jebbitt, S. R., & Sivaraman, K. (2013). Production and characterization of sugarcane juice powder. *Journal of Sugarcane Research*, 3(1), 20–34.
- Jain, S., Smyth, D., O'Hagan, B. M. G., Heap, J. T., McMullan, G., Minton, N. P., et al. (2017). Inactivation of the *dnaK* gene in *Clostridium difficile* 630  $\Delta$ erm yields a temperature-sensitive phenotype and increases biofilm-forming ability. *Scientific Reports*, 7(1), 1–13. <https://doi.org/10.1038/s41598-017-17583-9>
- Kong, J., & Yu, S. (2007). Fourier transform infrared spectroscopic analysis of protein secondary structures. *Acta Biochimica et Biophysica Sinica*, 39(8), 549–559. <https://doi.org/10.1111/j.1745-7270.2007.00320.x>
- Kramer, G., Ramachandiran, V., Horowitz, P. M., & Hardesty, B. (2002). The molecular chaperone DnaK is not recruited to translating ribosomes that lack trigger factor. *Archives of Biochemistry and Biophysics*, 403(1), 63–70. [https://doi.org/10.1016/S0003-9861\(02\)00213-8](https://doi.org/10.1016/S0003-9861(02)00213-8)
- Krishnan, S., Bhosale, R., & Singhal, R. S. (2005). Microencapsulation of cardamom oleoresin: Evaluation of blends of gum Arabic, maltodextrin and a modified starch as wall materials. *Carbohydrate Polymers*, 61(1), 95–102. <https://doi.org/10.1016/j.carbpol.2005.02.020>
- Kumar, R. G., Lekshmi, Chatterjee, N. S., Tejpal, C. S., Vishnu, K. V., Anas, K. K., et al. (2017). Evaluation of chitosan as a wall material for microencapsulation of squalene by spray drying: Characterization and oxidative stability studies. *International Journal of Biological Macromolecules*, 104. <https://doi.org/10.1016/j.ijbiomac.2017.03.114>, 1986–1995.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680–685. <https://doi.org/10.1038/227680a0>
- Lopusiewicz, L., Bogusławska-Wąs, E., Drozłowska, E., Trocer, P., Dłubała, A., Mazurkiewicz-Zapałowicz, K., et al. (2021). The application of spray-dried and reconstituted flaxseed oil cake extract as encapsulating material and carrier for probiotic *Lactocaseibacillus rhamnosus* GG. *Materials*, 14(18). <https://doi.org/10.3390/ma14185324>
- Lozano, M., Rodríguez-Ulibarri, P., Echeverría, J. C., Beruete, M., Sorolla, M., & Beriain, M. J. (2017). Mid-Infrared spectroscopy (MIR) for simultaneous determination of fat and protein content in meat of several animal species. *Food Analytical Methods*, 10(10), 3462–3470. <https://doi.org/10.1007/s12161-017-0879-1>
- Ma, J., Xu, C., Liu, F., Hou, J., Shao, H., & Yu, W. (2021). Stress adaptation and cross-protection of *Lactobacillus plantarum* KLD5 1.0628. *CyTA - Journal of Food*, 19, 72–80. <https://doi.org/10.1080/19476337.2020.1859619>
- Muthukumarasamy, P., Allan-Wojtas, P., & Holley, R. (2006). Stability of *Lactobacillus reuteri* in different types of microcapsules. *Journal of Food Science*, 71(1), 20–24. <https://doi.org/10.1111/j.1365-2621.2006.tb12395.x>
- Nunes, G. L., Etchepare, M. de A., Cichoski, A. J., Zepka, L. Q., Jacob Lopes, E., Barin, J. S., et al. (2018). Inulin, hi-maize, and trehalose as thermal protectants for increasing viability of *Lactobacillus acidophilus* encapsulated by spray drying. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology*, 89, 128–133. <https://doi.org/10.1016/j.lwt.2017.10.032>
- O'Riordan, K., Andrews, D., Buckle, K., & Conway, P. (2001). Evaluation of microencapsulation of a Bifidobacterium strain with starch as an approach to prolonging viability during storage. *Journal of Applied Microbiology*, 91(6), 1059–1066. <https://doi.org/10.1046/j.1365-2672.2001.01472.x>
- Paéz, R., Lavari, L., Vinderola, G., Audero, G., Cuatrin, A., Zaritzky, N., et al. (2012). Effect of heat treatment and spray drying on lactobacilli viability and resistance to simulated gastrointestinal digestion. *Food Research International*, 48(2), 748–754. <https://doi.org/10.1016/j.foodres.2012.06.018>
- Parvez, S., Malik, K. A., Ah Kang, S., & Kim, H. Y. (2006). Probiotics and their fermented food products are beneficial for health. *Journal of Applied Microbiology*, 100(6), 1171–1185. <https://doi.org/10.1111/j.1365-2672.2006.02963.x>
- Pinto, S. S., Fritzen-Freire, C. B., Benedetti, S., Murakami, F. S., Petrus, J. C. C., Prudêncio, E. S., et al. (2015). Potential use of whey concentrate and prebiotics as carrier agents to protect Bifidobacterium-BB-12 microencapsulated by spray drying. *Food Research International*, 67, 400–408. <https://doi.org/10.1016/j.foodres.2014.11.038>
- Rajam, R., & Anandharamakrishnan, C. (2015). Microencapsulation of *Lactobacillus plantarum* (MTCC 5422) with fructooligosaccharide as wall material by spray drying. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology*, 60(2), 773–780. <https://doi.org/10.1016/j.lwt.2014.09.062>
- Ricciardi, A., Parente, E., Guidone, A., Ianniello, R. G., Zotta, T., Sayem, S. M. A., et al. (2012). Genotypic diversity of stress response in *Lactobacillus plantarum*, *Lactobacillus paraplantarum* and *Lactobacillus pentosus*. *International Journal of Food Microbiology*, 157, 278–285. <https://doi.org/10.1016/j.ijfoodmicro.2012.05.018>
- Sangpuii, L., Dixit, S. K., Kumawat, M., Apoorva, S., Kumar, M., Kappala, D., et al. (2018). Comparative roles of *clpA* and *clpB* in the survival of *S. typhimurium* under stress and virulence in poultry. *Scientific Reports*, 8(1), 1–12. <https://doi.org/10.1038/s41598-018-22670-6>
- Serra Costa, S., Souza Machado, B. A., Martin, A. R., Bagnara, F., Ragadalli, S. A., & Rabello Costa Alves, A. (2015). Drying by spray drying in the food industry: Microencapsulation, process parameters and main carriers used. *African Journal of Food Science*, 9(9), 462–470. <https://doi.org/10.5897/AJFS2015.1279>
- Tripathi, M. K., & Giri, S. K. (2014). Probiotic functional foods: Survival of probiotics during processing and storage. *Journal of Functional Foods*, 9, 225–241. <https://doi.org/10.1016/j.jff.2014.04.030>
- Wang, S. Y., Ho, Y. F., Chen, Y. P., & Chen, M. J. (2015). Effects of a novel encapsulating technique on the temperature tolerance and anti-colitis activity of the probiotic bacterium *Lactobacillus kefirifaciens* M1. *Food Microbiology*, 46, 494–500. <https://doi.org/10.1016/j.fm.2014.09.015>
- Ying, D. Y., Sanguansri, L., Weerakkody, R., Bull, M., Singh, T. K., & Augustin, M. A. (2016). Effect of encapsulant matrix on stability of microencapsulated probiotics. *Journal of Functional Foods*, 25, 447–458. <https://doi.org/10.1016/j.jff.2016.06.020>
- Zhang, Y., Lin, J., & Zhong, Q. (2016). Effects of media, heat adaptation, and outlet temperature on the survival of *Lactobacillus salivarius* NRRL B-30514 after spray drying and subsequent storage. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology*, 74, 441–447. <https://doi.org/10.1016/j.lwt.2016.08.008>