

Original article

## Evaluation of the stability of *Bacillus coagulans* MTCC 5856 during processing and storage of functional foods

Muhammed Majeed,<sup>1</sup> Shaheen Majeed,<sup>2</sup> Kalyanam Nagabhushanam,<sup>2</sup> Sankaran Natarajan,<sup>1</sup> Arumugam Sivakumar<sup>1</sup> & Furqan Ali<sup>1\*</sup><sup>1</sup> Sami Labs Limited, 19/1& 19/2, First Main, Second Phase, Peenya Industrial Area, Bangalore, 560 058 Karnataka, India<sup>2</sup> Sabinsa Corporation, 20 Lake Drive, East Windsor, NJ 08520, USA

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**Summary** The study was aimed to find out the stability of *Bacillus coagulans* MTCC 5856 in functional foods during processing and storage. *B. coagulans* MTCC 5856 was found to be stable during baking and storage at frozen conditions of banana muffins (92% viability) and waffles (86% viability) upto 12 months. After brewing coffee at 90 °C for 2 min, there was 87% viability and retained 66% viability even after maintaining temperature at 77 °C for 4.0 h. It showed over 95% viability in chocolate fudge frosting, hot fudge toppings, peanut butter, strawberry preserve and vegetable oil at room temperature upto 12 months. It was found to be stable in apple juice upto 6 months at refrigerated condition and concentrated glucose syrup at 4 ± 3 and 25 ± 2 °C upto 24 months. *B. coagulans* MTCC 5856 showed promising stability during processing and storage of functional foods and could be an excellent probiotic ingredient to use in various food applications.

**Keywords** *Bacillus coagulans*, baked foods, beverages, functional foods, LactoSpore®.

### Introduction

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) defined probiotics as 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). The probiotic term is derived from Greek language which means 'for life.' There is potential for probiotic foods to provide health benefits and that specific strains are safe for human use as stated by FAO and WHO (Reid *et al.*, 2003). There are a number of microorganisms from different groups (e.g., *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Bacillus*, *Streptococcus* *Escherichia coli* strain Nissle 1917, enterococci and the probiotic yeast *Saccharomyces boulardii*) which are being used as probiotic food specifically fermented milk products and have been evaluated for their functional use (Cencic & Chingwaru, 2010). However, most of them lose the viability during manufacturing and storage of functional food (baked food, beverages, fruit preserves) due to either high temperature or other harsh manufacturing conditions. Hence, these probi-

otics require encapsulation via special processes to retain their viability during manufacturing, storage and exposure to harsh gastric conditions such as acid and bile (Soukoulis *et al.*, 2014; Zhang *et al.*, 2014). This special process requires additional cost to the production of functional foods and has major hurdles in making the production process economically viable. Thus, there is a need for a thermostable probiotic strain which can withstand harsh manufacturing conditions and provide sufficient and desired viable count during transportation, storage and at the time of consumption.

It is a well-known fact that *Bacillus species* has the ability to form spores when growth conditions are not in their favour and can remain in the dormant stage for many years. However, when the growth conditions are favourable such as specific nutrients, pH, temperature and moisture, it can cause spores to become vegetative and return to life through the process of germination (Nicholson *et al.*, 2000; Setlow, 2014). *Bacillus coagulans* (earlier known as *Lactobacillus sporogenes*) is a Gram positive spore bearing rod shaped, 0.9 by 3.0–5.0 µm size, microaerophilic and produces L (+) lactic acid predominantly. The safe and effective use of many *B. coagulans* strains as a probiotic in both animal and human have been well-documented with a long history worldwide (Monograph, 2002; Cutting, 2011; Jurenka,

\*Correspondent: Fax: +91 80 28373035;  
e-mail: furqan@samilabs.com

2012). The spore forming ability of *B. coagulans* helps it to survive at high temperature and to show resistance against harsh human gastric environment which enable them to deliver the probiotic benefits (Majeed & Prakash, 1998). There are a number of commercial preparations containing *B. coagulans* spores as probiotic ingredients available worldwide and reported to be stable at ambient temperature conditions which is unlikely in case of other vegetative form of probiotics (Hong *et al.*, 2005; Cutting, 2011).

LactoSpore<sup>®</sup> is a commercial proprietary preparation of Sabinsa Corporation, USA which contains spores of *B. coagulans* MTCC 5856 (bearing internal reference number SBC37-01) and is marketed as a dietary supplement for over two decades worldwide. It is a shelf stable, GRAS affirmed probiotic preparation which produces the beneficial L (+) form of lactic acid in the intestines and inhibits the growth of pathogenic bacteria (Majeed & Prakash, 1998). *B. coagulans* MTCC 5856 was found to decrease clinical symptoms like bloating, vomiting, diarrhea, abdominal pain and stool frequency in a multicentre randomised clinical trial which suggested its safe and effective use in the management of diarrhoea, predominant irritable bowel syndrome (IBS) (Majeed *et al.*, 2014). Various preparations of *B. coagulans* in powder, tablet and capsule forms have been reported for the treatment of gastrointestinal disorders, vaginal infections, hypercholesterolaemia, lactose intolerance, hepatic coma and as an adjuvant to antibiotic therapy in human clinical trials (Majeed & Prakash, 1998; Monograph, 2002; Jur-enka, 2012). It has been noted that stability, safety and effectiveness of a probiotic organism is strain specific and not the species or genus specific (de Vecchi & Drago, 2006). Hence, it is essential to evaluate a probiotic strain for its application and it must be supported by adequate scientific data. There is lack of scientific data for the stability of *B. coagulans* MTCC 5856 during processing and storage of functional foods. Thus, there is a need to evaluate the survival of *B. coagulans* MTCC 5856 during manufacturing process and storage conditions of functional food such as baked foods, beverages and fruit preserves. The aim of the study was to determine the effect of manufacturing process and storage conditions of baked food, beverages and fruit preserves on the viability of *B. coagulans* MTCC 5856.

## Material and methods

### Test material and method for viable count estimation

*Bacillus coagulans* MTCC 5856 samples used in the study were manufactured by Sami Labs Limited (Bangalore, India) by following a proprietary, in-house

developed, good manufacturing process. Pure *B. coagulans* MTCC 5856 spores were spray-dried and standardised with food grade maltodextrin (Sanwa Starch Co. Ltd. Kashihara, Nara, Japan) to achieve the desired concentration of  $15 \times 10^9$  CFU g<sup>-1</sup> of powdered form for the finished product. This standardised preparation was used throughout the study until otherwise stated. In this study, all the foods supplemented with *B. coagulans* MTCC 5856 were made according to the Global Food Safety Initiative (GFSI) in a certified facility by Bureau Veritas (Food safety System Certification 22000:2011 including ISO 22000:2005, ISO/TS 22002-2009). After every time interval for the stability studies, 1.0 g of sample (baked food or other studied product containing *B. coagulans* MTCC 5856) was thoroughly mixed in sterile saline (0.9% NaCl, w/v) and then incubated in water bath for 30 min at 75 °C, followed by immediate cooling to below 45 °C. This suspension was further serially diluted in sterile saline and the viable count was enumerated by plating on glucose yeast extract agar (HiMedia, Mumbai, India) by pour plate method (Maathuis *et al.*, 2010). The plates were incubated at 37 °C for 48–72 h. Each analysis was performed in triplicate at two different occasions. Average mean of spore viable counts are expressed in log<sub>10</sub> CFU.

### Waffles

Whole wheat flour, sugar, corn flour and baking powder were mixed in a sterile bowl. Eggs, nonfat milk, fructose, soybean oil, yeast solution, melted butter, salt and *B. coagulans* MTCC 5856 (0.035%, w/w) were added to dry ingredients and whisked well to make a smooth batter. This was kept at room temperature for 8–10 min. Waffle iron was heated and brushed with butter. Prepared batter was poured in the waffle iron and spread evenly and baked for 2 min or until the waffle was golden brown. Waffles were stored in freezer (−20 °C ± 2) and the viable count of *B. coagulans* MTCC 5856 was enumerated by dissolving in sterile saline (0.9% NaCl, w/v). Samples were analysed at 0, 1, 2, 3, 6 and 12 months (ICH (International Conference on Harmonisation), 2003) by serial dilution method (Maathuis *et al.*, 2010).

### Muffin

Flour, sugar, salt and baking powder were combined in a bowl. In a separate bowl, eggs, butter, marmalade, milk and *B. coagulans* MTCC 5856 (0.092%, w/w) were combined. Dry ingredients were slowly added and stirred quickly and lightly until just mixed. It was baked for 20–25 min at 205 °C and then cooled completely and stored in the freezer (−20 °C ± 2). The viable count of *B. coagulans* MTCC 5856 was enumerated by dissolving

muffins in sterile saline (0.9% NaCl, w/v) followed by serial dilution method (Maathuis *et al.*, 2010). Samples were analysed at 0, 1, 2, 3, 6 and 12 months (ICH (International Conference on Harmonisation), 2003).

#### Brewed coffee

Roasted coffee powder was procured from the local market and blended with *B. coagulans* MTCC 5856 (3.6% w/w of  $15 \times 10^9$  CFU g<sup>-1</sup>) at room temperature with low r.p.m. for 60 min. Coffee powder containing *B. coagulans* MTCC 5856 was added to microbial free water (preheated at  $90 \pm 2$  °C) and temperature was maintained at  $90 \pm 2$  °C for 2 min using boiling water bath (Medica Instrument Mfg. Co. Mumbai, India). Samples were then cooled to  $77 \pm 2$  °C and same temperature was maintained for 4.0 h using boiling water bath (Medica Instrument Mfg. Co.). Samples were withdrawn before and after brewing followed by holding the temperature at 77 °C for 0.5, 1.0, 2.0 and 4.0 h. Samples were analysed for viable spore counts by serial dilution method (Maathuis *et al.*, 2010).

#### Chocolate fudge frosting and hot fudge topping

Chocolate fudge frosting and hot fudge toppings were procured from local market. Chocolate fudge frosting was heated at 60 °C temperature and then *B. coagulans* MTCC 5856 (0.071%, w/w) was added. This was further blended for 15 min for uniform mixing and then filled in a sterile glass jar. Similarly, hot fudge toppings was heated at 60 °C and *B. coagulans* MTCC 5856 (0.067%, w/w) was added followed by blending for 15 min and then filled in a sterile glass jar. All samples were stored at room temperature and tested at 0, 1, 2, 3, 6 and 12 months (ICH (International Conference on Harmonisation), 2003) for viable spore count by serial dilution method (Maathuis *et al.*, 2010).

#### Peanut butter and strawberry preserves

Peanut butter and strawberry preserve were procured from local market. Peanut butter was heated at 30–35 °C and *B. coagulans* MTCC 5856 (0.081%, w/w) was added followed by blending for 60 min. This was filled in airtight sterile jars and stored at room temperature. Similarly, strawberry preserve was preheated at 75 °C and *B. coagulans* MTCC 5856 (0.114%, w/w) was added to it and blended for 15 min and then filled in a sterile glass jar. All the samples were stored at room temperature and analysed at 0, 1, 2, 3, 6 and 12 months (ICH (International Conference on Harmonisation), 2003) for viable spore count by serial dilution method (Maathuis *et al.*, 2010).

#### Vegetable oil/canola oil

Pure Canola oil was obtained from local market and *B. coagulans* MTCC 5856 (0.17%, w/w) was added, mixed in blender with low r.p.m. for 30 min at room temperature. 50 mL of the Canola oil containing *B. coagulans* MTCC 5856 was filled in polypropylene tubes, sealed and stored at room temperature ( $30 \pm 2$  °C). Samples were analysed at 0, 1, 2, 3, 6 and 12 months (ICH (International Conference on Harmonisation), 2003) by serial dilution method (Maathuis *et al.*, 2010).

#### Apple juice

Apple juice containing 17% of concentrated apple juice with no added sugar, colour and preservatives was procured from local market. One milliliter of apple juice was added to 20 mL of molten (approximately 45 °C) soyabean casein digested agar (Himedia) and mixed well then quickly poured to Petri plate (Tarson Products Private Limited, Kolkata, India). After 48 h of incubation at 37 °C, no colony was observed which indicated that apple juice was free from any microbial contamination. The pH of the apple juice was measured by pH meter (Eutech Instruments, Ayer Rajah Crescent, Singapore) and recorded 3.51. One gram of *B. coagulans* MTCC 5856 (0.10% w/v) was added to 1 L of apple juice and mixed well for 30 min. Aseptically 40 mL of sample was transferred to sterile polypropylene tube and sealed with Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA). Samples were stored in refrigerated condition (4–6 °C) and tested at 0, 1, 2, 3 and 6 months of time interval for viable spore count by serial dilution method (Maathuis *et al.*, 2010).

#### Concentrated glucose syrup

Concentrated glucose syrup was prepared by adding 85.0 g of glucose into 15.0 g of water and slowly heated to 70 °C for 10 min then boiled for 10 min. After boiling glucose syrup, it was cooled down to 45–50 °C and then *B. coagulans* MTCC 5856 (5.81% w/w of  $300 \times 10^9$  CFU g<sup>-1</sup>) was added and blended for 30 min at low speed. Aseptically, 50 g of the sample was transferred to sterile polypropylene bottles and sealed with Parafilm (Pechiney Plastic Packaging). Samples were stored at three different storage conditions i.e. accelerated ( $40 \pm 2$  °C,  $75 \pm 5\%$  relative humidity), room temperature ( $30 \pm 2$  °C,  $65 \pm 5\%$  relative humidity) and refrigerated ( $4 \pm 3$  °C). Samples were analysed at 0, 1, 2, 3, 6, 12, 18 and 24 months (ICH (International Conference on Harmonisation), 2003) for viable spore count by serial dilution method (Maathuis *et al.*, 2010). Average mean of triplicate samples are expressed as log<sub>10</sub> CFU g<sup>-1</sup>. Water activity was determined using a bench top water activity meter

(HygroLab, Rotronic Instrument Corp, Hauppauge, NY, USA) of  $\pm 0.003$  precision.

#### Nutritional analysis

The analysis of the proximate composition of functional foods supplemented with *B. coagulans* MTCC 5856 was carried out according to the official methods of Association of Official Analytical Chemists (AOAC International, 2003). The moisture content was determined by Karl Fischer Titrator (Spectra Lab Instruments Pvt. Ltd, Navi Mumbai, India) as per United States Pharmacopoeia method (Chapter <921>). The determination of proteins in terms of nitrogen was performed by micro Kjeldahl method as per United States Pharmacopoeia method (Chapter <461>). The nitrogen value was converted to protein by multiplying to a factor of 6.25. The ash values were obtained by heating samples at 550 °C in a muffle furnace (Heat globe, Bangalore, Karnataka, India) for 3 h. Crude fats were determined by Soxhlet apparatus using *n*-hexane as a solvent. The carbohydrate content was determined by subtracting the total protein, moisture, fat and ash content from the total dry matter. Energy value was calculated (kcal/100 g) using the equation (FAO/WHO, 2001):

$$\text{Energy value} = (9 \times \text{fat}) + (4 \times \text{carbohydrate}) + (4 \times \text{protein}).$$

#### Statistical analysis

The values were calculated as the mean of individual experiments in triplicate and viable count of *B. coagulans* MTCC 5856 was expressed in  $\log_{10}$  CFU. The data presented are the average of the three determinations. Differences between two mean values were calculated by Student's *t*-test. The chosen level of significance for all statistical tests was  $P < 0.05$ .

## Results

#### Waffles, chocolate fudge frosting, hot fudge banana muffin mix

The stability of *B. coagulans* MTCC 5856 was evaluated in waffles, hot fudge topping, chocolate fudge frosting and banana muffin mix during manufacturing process and storage at respective conditions. There was 91% survival of *B. coagulans* MTCC 5856 during the manufacturing of waffles and retained 86% viability upto 12 months of storage at frozen conditions ( $-20$  °C) (Table 1). Hot fudge topping, chocolate fudge frosting and banana muffin mix retained >95% viability during their manufacturing and storage (Table 1).

#### Brewed coffee

The viability of *B. coagulans* MTCC 5856 was evaluated in coffee powder after brewing for 2 min followed by holding temperature 77 °C for 4.0 h. There was  $0.92 \log_{10}$  CFU  $\text{mL}^{-1}$  reduction (13%) observed after brewing at 90 °C for 2 min from initial count (Fig. 1). Similarly, there was 30% and 34% viability reduction in 3.0 and 4.0 h, respectively. However, no significant reduction in the viability of *B. coagulans* MTCC 5856 was observed after brewing till 1.0 h of holding temperature (Fig. 1).

#### Peanut butter and strawberry preserves

The stability of *B. coagulans* MTCC 5856 was evaluated in peanut butter and strawberry preserves at room temperature. Peanut butter and strawberry preserves were blended with *B. coagulans* MTCC 5856 at 30–35 and 75 °C respectively. The viability of *B. coagulans* MTCC 5856 in peanut butter and strawberry preserves was retained over 97% upto 12 months of storage at room temperature (Table 2).

#### Vegetable oil (pure canola oil)

*Bacillus coagulans* MTCC 5856 was formulated in pure canola oil and stored at room temperature. There was no significant reduction in the viability of *B. coagulans* MTCC 5856 either during blending or storage. The viability of *B. coagulans* MTCC 5856 was retained more than 95% upto 12 months of storage at room temperature (Table 2).

#### Apple juice

The stability of *B. coagulans* MTCC 5856 was determined in apple juice with acidic pH (3.5) at refrigerated conditions (4–6 °C) for 6 months of storage. *B. coagulans* MTCC 5856 was found to be stable in apple juice and retained more than 99% viability upto 6 months of storage at refrigerated conditions (4–6 °C) (Table 3). There was no significant change observed in the pH of apple juice from initial to 6 months of storage (Table 3).

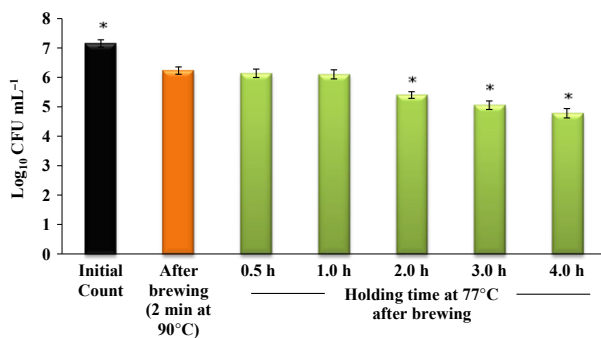
#### Concentrated glucose syrup

*Bacillus coagulans* MTCC 5856 was blended with concentrated glucose syrup at 45–50 °C and stored at accelerated ( $40 \pm 2$  °C) room temperature ( $30 \pm 2$  °C) and refrigerated conditions. At refrigerated conditions, *B. coagulans* MTCC 5856 retained 99% viability upto 24 months of storage. However, at accelerated conditions it retained 96% upto 12 months and 90% ( $0.97 \log_{10}$  CFU  $\text{g}^{-1}$  reduction) upto 24 months of

**Table 1** Viability of *B. coagulans* MTCC 5856 in waffles, hot fudge frosting, chocolate fudge frosting and banana muffin mix upto 12 months of storage at room temperature

Time (months)	Waffles		Hot fudge topping		Chocolate fudge frosting		Banana muffin mix	
	Log <sub>10</sub> CFU g <sup>-1</sup>	Viability (%)	Log <sub>10</sub> CFU g <sup>-1</sup>	Viability (%)	Log <sub>10</sub> CFU g <sup>-1</sup>	Viability (%)	Log <sub>10</sub> CFU g <sup>-1</sup>	Viability (%)
Initial count	6.720 ± 0.015 <sup>a</sup>	100	7.006 ± 0.017 <sup>b</sup>	100	7.031 ± 0.015 <sup>c</sup>	100	7.143 ± 0.014 <sup>d</sup>	100
0	6.163 ± 0.019 <sup>a</sup>	91.71	6.978 ± 0.012 <sup>b</sup>	99.60	6.930 ± 0.017 <sup>c</sup>	98.58	6.993 ± 0.017 <sup>d</sup>	97.89
1	6.123 ± 0.013 <sup>a</sup>	91.12	6.975 ± 0.019 <sup>b</sup>	99.57	6.921 ± 0.019 <sup>c</sup>	98.44	7.022 ± 0.012 <sup>d</sup>	98.31
2	6.100 ± 0.011 <sup>a</sup>	90.78	6.961 ± 0.014 <sup>b</sup>	99.37	6.919 ± 0.013 <sup>c</sup>	98.41	6.924 ± 0.016 <sup>d</sup>	96.94
3	6.113 ± 0.017 <sup>a</sup>	90.97	6.962 ± 0.013 <sup>b</sup>	99.38	6.914 ± 0.012 <sup>c</sup>	98.34	6.920 ± 0.015 <sup>d</sup>	96.87
6	5.892 ± 0.015 <sup>f</sup>	87.68	6.799 ± 0.014 <sup>b</sup>	97.06	7.043 ± 0.017 <sup>c</sup>	100.17	6.921 ± 0.014 <sup>d</sup>	96.89
12	5.792 ± 0.017 <sup>f</sup>	86.19	6.782 ± 0.015 <sup>b</sup>	96.81	6.836 ± 0.019 <sup>c</sup>	97.23	6.916 ± 0.013 <sup>d</sup>	96.82

Different letter in the same column indicate significant differences ( $P < 0.05$ ). Values in a given column which are followed by the same letter are not statistically different ( $P > 0.05$ ).



**Figure 1** Effect of brewing temperature (90 °C) and holding temperature (77 °C) on the viability of *B. coagulans* MTCC 5856. Values are average mean of triplicate performed at two different occasions and represented in log<sub>10</sub> CFU mL<sup>-1</sup>. \* $P < 0.05$ ; (Student's  $t$  test).

storage. Similarly, at room temperature it retained 96% upto 12 months and 91% (0.85 log<sub>10</sub> CFU g<sup>-1</sup> reduction) upto 24 months of storage (Fig. 2). There was no significant difference observed in the water activity of concentrated glucose syrup from initial time to 24 months of storage (Table 4).

**Nutritional analysis**

The proximate composition of functional foods supplemented with *B. coagulans* MTCC 5856 was analysed by following official methods of Association of Official Analytical Chemists. The content of protein and carbohydrate in functional foods supplemented with *B. coagulans* MTCC 5856 varied from 0.34% to 22.26% and 0.11% to 88.81%, respectively (Fig. S1). Similarly, the content of fat and moisture in functional foods supplemented with *B. coagulans* MTCC 5856 varied from 0.04% to 98.77% and 0.75% to 48.78%, respectively (Fig. S1). The data of the nutritional studies suggested that *B. coagulans* MTCC 5856 was found

to be stable in a variety of functional foods with different nutritional profiles. The energy values of functional foods supplemented with *B. coagulans* MTCC 5856 varied from 204.4 kcal/100 gm to 890.7 kcal/100 gm (Table S1) which also did not affect the stability of *B. coagulans* MTCC 5856.

**Discussion**

The results of *B. coagulans* MTCC 5856 viability in waffles and banana muffin mix clearly indicated that there was no significant decrease in the survival of *B. coagulans* MTCC 5856 during baking of waffles and banana muffins (Table 1). The study revealed that *B. coagulans* MTCC 5856 was stable at high temperature (baking conditions) and retained its viability upto 12 months of storage at frozen conditions (-20 °C ± 2) unlike the other strain of *B. coagulans* (Jao *et al.*, 2011). Furthermore, *B. coagulans* MTCC 5856 was found to be stable in chocolate fudge frosting and hot fudge upto 12 months of storage at room temperature (Table 1). The viability of *B. coagulans* MTCC 5856 in peanut butter, strawberry preserves and vegetable oil (pure canola oil) was retained over 97% upto 12 months of storage at room temperature (Table 2). The viability of *B. coagulans* MTCC 5856 was determined in coffee during brewing temperature (90 °C) and maintained the same at a temperature of 77 °C for 4.0 h. After brewing, 87% viability was observed and when the same coffee containing *B. coagulans* MTCC 5856 was stored at 77 °C for 4 h then the viability was 66% (Fig. 1). The study was designed by considering that coffee can be kept hot after brewing for later uses. The results of the study provided the scope to use *B. coagulans* MTCC 5856 in coffee powder with proper coverage to attain viability and dose at the time of consumption. We report for the first time the stability of *B. coagulans* MTCC 5856 in baked foods and its heat stability during coffee brew-

**Table 2** Viability of *B. coagulans* MTCC 5856 in vegetable cooking oil, peanut butter and strawberry preserve upto 12 months of storage at room temperature

Time (months)	Vegetable cooking oil		Peanut butter		Strawberry preserves	
	Log <sub>10</sub> CFU g <sup>-1</sup>	Viability (%)	Log <sub>10</sub> CFU g <sup>-1</sup>	Viability (%)	Log <sub>10</sub> CFU g <sup>-1</sup>	Viability (%)
Initial count	7.408 ± 0.012 <sup>a</sup>	100	7.085 ± 0.014 <sup>b</sup>	100	7.223 ± 0.014 <sup>c</sup>	100
0	7.317 ± 0.015 <sup>a</sup>	98.77	7.022 ± 0.019 <sup>b</sup>	99.11	7.104 ± 0.017 <sup>c</sup>	98.35
1	7.324 ± 0.013 <sup>a</sup>	98.87	7.063 ± 0.015 <sup>b</sup>	99.68	7.100 ± 0.019 <sup>c</sup>	98.30
2	7.320 ± 0.014 <sup>a</sup>	98.81	7.020 ± 0.013 <sup>b</sup>	99.08	7.080 ± 0.014 <sup>c</sup>	98.02
3	7.309 ± 0.019 <sup>a</sup>	98.67	7.002 ± 0.018 <sup>b</sup>	98.84	7.059 ± 0.016 <sup>c</sup>	97.73
6	7.315 ± 0.017 <sup>a</sup>	98.75	7.021 ± 0.015 <sup>b</sup>	99.10	7.046 ± 0.012 <sup>c</sup>	97.54
12	7.313 ± 0.011 <sup>a</sup>	98.72	7.018 ± 0.016 <sup>b</sup>	99.06	7.068 ± 0.010 <sup>c</sup>	97.85

Values in a given column which are followed by the same letter are not statistically different ( $P > 0.05$ ).

**Table 3** Viability of *B. coagulans* MTCC 5856 in the apple juice upto 6 months of storage at refrigerated conditions

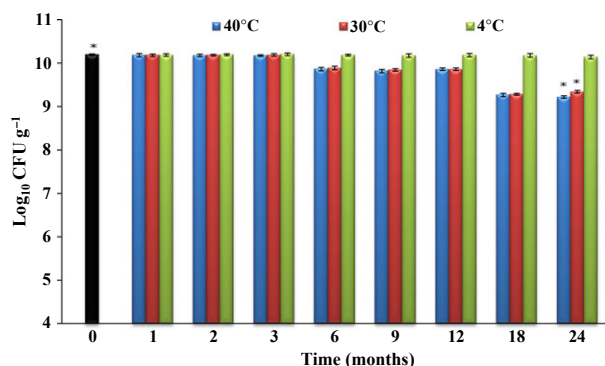
Time (months)	Apple juice		
	pH value	Log <sub>10</sub> CFU mL <sup>-1</sup>	Viability (%)
0	3.51 <sup>a</sup>	7.056 ± 0.012 <sup>b</sup>	100
1	3.54 <sup>a</sup>	7.008 ± 0.014 <sup>b</sup>	99.32
2	3.50 <sup>a</sup>	7.030 ± 0.015 <sup>b</sup>	99.63
3	3.52 <sup>a</sup>	7.025 ± 0.011 <sup>b</sup>	99.55
4	3.53 <sup>a</sup>	7.017 ± 0.013 <sup>b</sup>	99.43
5	3.56 <sup>a</sup>	7.013 ± 0.013 <sup>b</sup>	99.38
6	3.52 <sup>a</sup>	7.009 ± 0.016 <sup>b</sup>	99.33

Values in a given column which are followed by the same letter are not statistically different ( $P > 0.05$ ).

**Table 4** Water activity of concentrated glucose syrup containing *B. coagulans* MTCC 5856

Time (months)	Water activity (Aw)		
	Accelerated (40 ± 2 °C)	Room temperature (30 ± 2 °C)	Refrigerated conditions (5 ± 3 °C)
0	0.684 ± 0.011 <sup>a</sup>	0.684 ± 0.014 <sup>b</sup>	0.684 ± 0.011 <sup>c</sup>
3	0.678 ± 0.014 <sup>a</sup>	0.632 ± 0.015 <sup>b</sup>	0.659 ± 0.013 <sup>c</sup>
6	0.644 ± 0.010 <sup>a</sup>	0.665 ± 0.011 <sup>b</sup>	0.663 ± 0.015 <sup>c</sup>
12	0.598 ± 0.013 <sup>a</sup>	0.664 ± 0.010 <sup>b</sup>	0.669 ± 0.016 <sup>c</sup>
18	0.678 ± 0.014 <sup>a</sup>	0.632 ± 0.013 <sup>b</sup>	0.659 ± 0.014 <sup>c</sup>
24	0.644 ± 0.010 <sup>a</sup>	0.665 ± 0.011 <sup>b</sup>	0.663 ± 0.013 <sup>c</sup>

Water activity was determined using a bench top water activity meter and values are average mean of triplicate of each sample. There was no significant difference was observed from initial water activity upto 24 months at various storage conditions. Values in a given column which are followed by the same letter are not statistically different ( $P > 0.05$ ).

**Figure 2** Effect of storage conditions on the viability of *B. coagulans* MTCC 5856 in concentrated glucose syrup. Viable count was determined by serial dilution agar plating method. Average mean of viable counts are expressed in log<sub>10</sub> CFU g<sup>-1</sup>. \* $P < 0.05$ ; (Student's *t* test).

ing. The heat resistance property of *B. coagulans* MTCC 5856 may attribute to its dormant phase i.e. spore. Spore or endospore is a metabolically dormant phase of various *Bacillus species* and formed in process

called 'sporulation' which is usually induced by reduced levels of nutrients in the environment (Driks, 2002; Piggot & Hilbert, 2004). Spores of *Bacillus species* have been reported to have resistance against heat (wet and dry), radiation and chemicals (Nicholson *et al.*, 2000; Setlow, 2006). There are a number of mechanisms proposed for the resistance which include water content of spore core, mineral ions, spore proteins, and the saturation of spore DNA with a/b-type small acid-soluble spore proteins which protects DNA against wet heat damage (Nicholson *et al.*, 2000; Hayes & Setlow, 2001).

Probiotic cultures specifically heterogeneous group of lactic acid bacteria (*Lactobacillus*, *Enterococcus*, *Streptococcus* and *Bifidobacterium*) have been used to make yoghurt and fermented dairy products which is proven to deliver health benefits (Holzapfel & Schillinger, 2002; Ranadheera *et al.*, 2010; Rivera-Espinoza & Gallardo-Navarro, 2010). In recent times, fruit and

vegetable juices have attracted a lot of attention as a medium to deliver probiotic cultures because they contain essential nutrients, bioactive compounds, soluble fibres and have good taste profiles that are appealing to all age groups (Tuorila & Cardello, 2002; Yoon *et al.*, 2004; Sheehan *et al.*, 2007; Daneshi *et al.*, 2013). *B. coagulans* MTCC 5856 was found to be stable in apple juice and retained more than 99% viability upto 6 months of storage at refrigerated conditions (Table 3). There was no significant change observed in the pH of apple juice from initial to 6 months of storage (Table 3) which indicated its metabolic inactivation at refrigerated conditions. Fruit juices have complex components, such as carbohydrates and proteins which can switch on germination of *B. coagulans* MTCC 5856. Therefore, the apple juice containing *B. coagulans* MTCC 5856 spores were stored at refrigerated conditions. Additionally, no significant decrease in the viability of *B. coagulans* MTCC 5856 was found in vegetable oil at room temperature upto 12 months of storage (Table 2). The long-term stability of *B. coagulans* MTCC 5856 was conducted in concentrated glucose syrup to explore its potential in various concentrated carbohydrate based products. *B. coagulans* MTCC 5856 showed 90%, 91% and 99% viability at 40 °C, room temperature (30 °C) and refrigerated conditions upto 24 months of storage, respectively (Fig. 2). There was no significant difference observed in the water activity of concentrated glucose syrup from initial time to 24 months of storage (Table 4). To the best of our knowledge, this is the first report to provide the evidence that *B. coagulans* MTCC 5856 remain viable in glucose syrup upto 24 months of storage.

A range of functional foods with different nutritional profiles was studied to evaluate the stability of *B. coagulans* MTCC 5856. The data of the study concluded that regardless of variation in the proximate parameters such as protein, fat, carbohydrate and moisture; *B. coagulans* MTCC 5856 was found to be stable in a range of food products with different calorific values.

In conclusion, *B. coagulans* MTCC 5856 was found to be stable during processing and respective storage conditions of baked food, beverages, vegetable oil, concentrated glucose syrup and even in brewed coffee. *B. coagulans* MTCC 5856 is a shelf stable, GRAS affirmed, clinically documented probiotic strain (Majeed & Prakash, 1998; Majeed *et al.*, 2014). *B. coagulans* MTCC 5856 has a history of more than two decades of safe and effective use as dietary ingredients in various ranges of products such as capsule, tablets, frozen yoghurt, tutfuiti and recently in bread. The *B. coagulans* spore preparation is currently added to the intended foods at levels of  $10^8$  to  $2 \times 10^9$  cfu/servings (Majeed & Prakash, 1998; FDA, 2011; Majeed & Kamarei, 2012; Majeed *et al.*, 2014). For the first time, the current study

provides detailed stability evidence of *B. coagulans* MTCC 5856 in different range of functional foods (baked goods, beverages, vegetable oil, concentrated glucose syrup and brewed coffee). However, the molecular mechanism by which *B. coagulans* MTCC 5856 spores showed stability during processing and storages of functional foods needs to be evaluated.

Various dietary foods products containing probiotics have been extensively studied for different benefits for human health all over the world (Lollo *et al.*, 2012, 2013, 2015). Additionally, foods containing active culture of probiotics have been thoroughly studied for sensory profiling by quantitative descriptive analysis and acceptance test (Cruz *et al.*, 2013; Esmerino *et al.*, 2013; Morais *et al.*, 2014; Felicio *et al.*, 2016). There is a scope of carrying out additional studies to have extended benefits of foods supplemented with *B. coagulans* MTCC 5856 for the human health and sensory profiling.

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### Conflict of interests

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Proximate analysis of different functional foods supplemented with *B. coagulans* MTCC 5856. Data are the mean values of triplicate determinations. WF, waffles; HFT, hot fudge toppings; CFF, chocolate fudge frosting; MF, muffins; VO, vegetable oil; PB, peanut butter; SP, strawberry preserve; AJ, apple juice; SS, sugar syrup; BC, brewed coffee.

**Table S1.** Energy value of different functional foods supplemented with *B. coagulans* MTCC 5856.