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Process and Storage Stability of *Bacillus coagulans* LBSC in Food Matrices and Appraisal of Calorific Restriction

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Abstract

Background and Objective: Probiotic bacteria as food additives have led to a significant growth in functional food levels. Functional foods present multiple challenges to probiotic viability and stability. In the present study, *Bacillus coagulans* LBSC DSM 17654, a probiotic strain, was incorporated into various foods to assess its stability during processing and storage and ability to decrease food calorie contents.

Material and Methods: *Bacillus coagulans* LBSC was used to prepare various beverages and foods such as hot and cold non-alcoholic beverages, breakfast cereals, oral rehydration salts, confections, frostings, convenience foods, frozen dairy desserts, condiments, relishes, fermented milk beverages and cough syrups. The bacterial process and storage stabilities were assessed using relative viability estimation. Stability of *Bacillus coagulans* LBSC was assessed in aqueous suspensions following ICH guidelines [Q1A (R2)] under various temperatures (0-100 ºC). Strain was assessed for its *in vitro* calorie restriction capabilities when incorporated into foods.

Results and Conclusion: *Bacillus coagulans* LBSC survived well during food processing (relative viability of 99.46% ±0.49) and storage (relative viability of 99.22% ±0.51) conditions. The bacterium was stable in aqueous suspension and tolerated high temperatures well (relative viabilities of 99.56% ±0.21 and 97.59% ±0.01 at 80 and 90 ºC, respectively). *Bacillus coagulans* LBSC showed significant *in vitro* calorie decreases in probiotic supplemented foods, compared to foods with no supplementations ($p < 0.05$). In conclusion, *Bacillus coagulans* LBSC exhibited good stability in aqueous media at high temperatures. *Bacillus coagulans* LBSC was not only stable in a wide spectrum of food categories, it could grow on foods to decrease food calorie under *in vitro* conditions; suggesting its uses as a functional food ingredient for better management of obesity and ageing and their associated health risks.
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1. Introduction

Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) have defined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit for the hosts" [1]. Regular consumption of probiotics is reported to strengthen immune system, exhibit anti-allergy effects, decrease cancer risks, lower cholesterol, prevent digestive disorders and decrease systemic gastrointestinal infections [2]. Based on empirical evidence, frequently used probiotic species belong to the genera of Lactobacillus, Bifidobacterium, Bacillus, Enterococcus, Lactococcus, Pediococcus and Saccharomyces. However, health benefits, therapeutic effectiveness and technological competencies are specific to probiotic strains [3]. Probiotics are majorly delivered through oral routes either in the form of functional foods (fermented or non-fermented) or dietary supplements (powder, capsule or tablet) [4]. Functional foods exhibit one or more health benefits beyond the basic nutritions [3]. Functional foods containing probiotics represent significant proportions of food products as their demands increase following increased awareness of the consumers globally [5]. Dairy probiotic foods such as milks (normal, fermented

and flavoured), buttermilks, whey-based beverages, sour creams, milk powders, cheesees, frozen dairy desserts and baby foods have been quite popular. Considering increased lactose-intolerant populations, various probiotic products from non-dairy sources such as baked, cereal-based and baby foods, confectionaries, fruit juices and beverages have been developed recently [4,6-8].

Probiotic foods must appropriately deliver live probiotic microorganisms in adequate numbers at the time of consumption. However, most of the probiotic strains show low viable activities during manufacturing and storage of the functional foods because of exposure to high temperatures, moisture, atmospheric oxygen and other intrinsic and extrinsic processing factors. One of the popular methods to protect probiotic bacteria from such adverse conditions is encapsulation of the bacteria to preserve their viability during various stages of the product manufacturing and long-term storage and under harsh conditions of the stomach [9,10]. Encapsulation increases production costs of the probiotic supplemented functional foods, making their production technically challenging and economically unviable. From economic and technological points of view, it is imperative to include probiotic strains, befitting large-scale industrial productions and exhibiting stable properties to incorporate into food matrices with no compromising of viability, functionality and producing unpleasant flavors or textures in products [11,12]. Therefore, use of spore-forming microorganisms such as non-pathogenic bacilli as probiotics has received much attentions. Owing to their spore forming ability, probiotic bacteria include superiority over other bacteria, showing better survival and stability. As a result, these bacteria have been identified as ideal choices for the development of functional foods [2].

Recent studies have advanced understandings toward roles of probiotics and gastrointestinal microbiota in food calorie management. Caloric restriction (CR) is described as decreeses in energy intakes below the number of calories consumed ad libitum. It is a dietary regimen that decreases calorie intake without eliminating essential nutrients and preserves energy homeostasis. Usually, 10% or more decreases in calories in humans are considered as calorie restriction [13]. Since long times, calorie restriction is followed to improve health statuses and prevent metabolism and age-linked ailments; thus, this prolongs the life span [14,15]. Spore forming *Bacillus* (*B.*) *coagulans* can survive in adverse processing and storage conditions, enabling it to use in various food products for its health benefits. The *B. coagulans* is a Gram-positive, facultative anaerobic, non-pathogenic, spore-forming (terminal spores) lactic acid-producing bacteria. There are reports on good thermal tolerance of the bacteria. The optimum growth temperature and pH for range 35-50 °C and 5.5-6.5, respectively [2]. In fact, *B. coagulans* has been considered as generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA). European Food Safety Authority (EFSA) has included the bacteria in qualified presumption of safety (QPS), the lists of safe microorganisms [16]. The *B. coagulans* LBSC DSM17654, an extensively studied safe probiotic strain [17], is clinically proven as safe and is effective in alleviating gastrointestinal infections by microbiome modulation [18]. In the present study, *B. coagulans* LBSC spore preparation was used in various foods and beverages and its stability was assessed. Stability of the spore preparation was assessed in aqueous media at various temperatures. Additionally, calorie restriction property of *B. coagulans* LBSC in selected high calorie foods and beverages was assessed in vitro.

2. Materials and Methods

All the analytical-grade chemicals and reagents were purchased from Sigma-Aldrich, India, and microbiological media from HiMedia Labs, India.

2.1 Preparation of *B. coagulans* LBSC and viable count enumeration

Spore preparation of *B. coagulans* LBSC (5×10^{11} CFU·g^{-1}; 11.70 log CFU·g^{-1}) used in this study was carried out by Advanced Enzyme Technologies Limited, India, following a proprietary good manufacturing process. Viable spores of *B. coagulans* LBSC were enumerated based on the standard pour plate method [18]. Briefly, 1.00 ml of withdrawn samples was suspended in tween-peptone water [Composition (% w·v^{-1}): protease peptone 1.00, sodium chloride 0.50, disodium phosphate 0.35, monosodium phosphate 0.15, tween-80 0.20 and pH 7.2 ±0.2] and serially diluted. Diluted samples were heat shocked at 75 °C for 30 min using isotherm water bath, followed by immediate cooling down to below 45 °C. Then, 1 ml of the heat-treated spore suspension was dispensed in Petri plates and mixed with pre-sterilized molten GYE agar (45 ºC) [M963, HiMedia, India]. The media were set to solidify and plates were inverted and incubated at 37 °C for 48-72 h. Viable count of *B. coagulans* LBSC was expressed in logarithm of colony forming units (log CFU) using the mean of three independent analyses.

2.2 Stability of *B. coagulans* LBSC in food processing conditions

The *B. coagulans* LBSC preparation was incorporated into various foods and beverages to assess its stability under specific process conditions. Standard process parameters were used during preparation and results were recorded using calibrated equipment such as temperature probes, revolution counters, weighing balances and pH meters. Stability was investigated from the relative viability of *B. coagulans* LBSC at the specific processing time compared to initial count (zero time) under standard process conditions.

2.2.1 Non-alcoholic hot beverages

Premix powders of each masala chais (14.0 g), green teas (10.0 g), lemon teas (10.0 g) and instant coffees (14.0 g) were respectively mixed with 8.7, 3.2, 11.5 and 6.2 mg of *B. coagulans* LBSC preparation and blended. The probiotic premixes were dissolved homogeneously in 100 ml of isothermal hot water (85 °C). Samples (1.0 ml) were withdrawn at 0 and 30 min and analyzed for viable spore count of *B. coagulans* LBSC using standard pour plate method.

2.2.2 Non-alcoholic cold beverage

One serving (18.0 g) of lemon iced tea powder was mixed with 4.3 mg of *B. coagulans* LBSC preparation and blended to get homogeneous mixture. The probiotic lemon tea was mixed quickly in 200 ml of chilled water (6-8 ºC). Samples (1.0 ml) were withdrawn at 0 and 30 min and viable spores were enumerated based on the standard pour plate method.

2.2.3 Ready-to-eat breakfast cereals

Ready mix powder of Upma (thick semolina porridge) (80.0 g·serving^{-1}) was homogeneously mixed with *B. coagulans* LBSC preparation (5.69 mg). Probiotic supplemented Upma was prepared by adding hot water (85 °C) and occasional stirring. Initial (0 min) and final (30 min) samples (1.0 g) were withdrawn and viable counting were carried out based on the standard pour plate technique.

2.2.4 Ready-to-eat grain products

Briefly, 5 mg of *B. coagulans* LBSC spore preparation were mixed homogenously with sweet corn soup powders (12.0 g·serving^{-1}) and noodle cakes (17.0 g·serving^{-1}). These were processed using hot water (85 °C) following standard cooking process. Samples (1.0 ml of soup or 1.0 g of noodles) were collected at two time points (0 and 30 mins) and analyzed for viable spore count of *B. coagulans* LBSC based on the standard pour plate method.

2.3 Storage stability of *B. coagulans* food products

In general, *B. coagulans* LBSC preparation was incorporated into various foods and beverages and its stability was assessed under specific storage conditions based on guidelines from the International Conference on Harmonization [ICH, Q1A(R2)] [19]. After preparation, probiotic containing cough syrups and ice creams were respectively stored at room temperature and -20 ºC using stability chamber (Remi, India). Stability was assessed from the relative viability of *B. coagulans* LBSC at specific storage times, compared to initial counts (zero time).

2.3.1 Ready-to-drink beverages

The *B. coagulans* LBSC preparation was homogeneously mixed with various ready-to-drink (RTD) beverages such as cold coffees (5.3 mg·200 ml^{-1}), orange drinks (4.6 mg·250 ml^{-1}), mango juices (4.5 mg·180 ml^{-1}), pomegranate juices (5.4 mg·180 ml^{-1}), sports drinks (2.6 mg·500 ml^{-1}) and coconut waters (5.4 mg·250 ml^{-1}). All beverages were stored in industrial refrigerators (Remi, India) at 4 °C for specific storage periods. Samples (2.0 ml) were withdrawn at specific time intervals and viability of *B. coagulans* LBSC was analyzed based on the standard pour plate method as described previously.

2.3.2 Oral rehydration solutions/salts

Briefly, *B. coagulans* LBSC preparation (4.0 mg) was homogeneously mixed with IP-grade oral rehydration solutions/salts (ORS) (21.0 g) [composition (g·l^{-1}): sodium chloride 2.6, dextrose anhydrous 13.5, potassium chloride 1.5, trisodium citrate 2.9] and sterile distilled water (DW) to make ORS preparation and RTD ORS (980 mg·200 ml^{-1}) based on WHO recommended formula [20]. Preparations of probiotic-supplemented ORS were stored in airtight containers at room temperature (ORS preparations) and 4 °C (RTD ORS solutions). Samples (2.0 ml) were collected at specific time intervals and viable count of *B. coagulans* LBSC was assessed based on the standard pour plate method analysis.

2.3.3 Confections and frostings

The *B. coagulans* LBSC was incorporated into preparations of dark chocolate bars (11.5 mg·150 ml^{-1} molten chocolate) and polydextrose sugar syrups (6.3 mg·250 ml^{-1}) and gently mixed. Other preparation parameters such as temperature, stirring and cooling were similar to those of commercial process conditions. Probiotic chocolates and sugar syrups were stored in airtight container at 4 °C. Samples (5.0 g of probiotic-chocolates or 5.0 ml of probiotic-sugar syrups) were withdrawn at various time intervals and viable count of *B. coagulans* LBSC was analyzed based on the standard pour plate method.

2.3.4 Processed food-condiments and relishes

Briefly, *B. coagulans* LBSC was mixed homogeneously with tomato ketchups (5.6 mg·90 g^{-1}) and tomato purees (5.0 mg·200 g^{-1}) under standard process conditions and stored at 4 °C. Samples (2.0 g) were withdrawn at various time intervals and analyzed for viable count of *B. coagulans* LBSC using standard pour plate method.

2.3.5 Convenience foods and frozen dairy desserts

The *B. coagulans* LBSC preparation was incorporated into protein bars (120.5 mg·67 g^{-1}), chocolate pies (5.5 mg·7.4 g^{-1}) and ice creams (6.6 mg·43 ml^{-1}) using standard ingredients and commercial preparation processes. Packed probiotic supplemented preparations were appropriately stored at 4 °C, except for ice creams, which were stored at -20 °C. Each preparation was sampled (2.0 ml of ice creams or 2.0 g of protein bars or chocolate pies) at various time intervals for viable cell enumeration of *B. coagulans* LBSC using standard pour plate method.

2.3.6 Fermented milk beverages

Lassi (sweet churned yogurt) is a popular yogurt based drink in India. To prepare Lassi, *B. coagulans* LBSC preparation (4.2 mg) with the inoculum were added homogeneously to 250 mL of pasteurized full cream milk. Milk was fermented at 45 ºC overnight and stored at 4 °C up to 120 days. Samples (5.0 ml) were collected at various time intervals and analyzed for *B. coagulans* LBSC count based on the standard pour plate method. Lassi with no test strain was analyzed using *Lactobacillus* MRS agar M641 (HiMedia, India) to assess counts of native lactic acid producing bacteria.

2.3.7 Cough syrups

Generally, 100 ml of sterile cough syrups were homogeneously mixed with 4.7 mg of *B. coagulans* LBSC preparation and stored in amber bottles at room temperature. Samples (2.0 ml) were collected at various time intervals and analyzed for *B. coagulans* LBSC count using standard pour plate method.

2.4 Aqueous and thermal stabilities of *B. coagulans* LBSC

An aqueous spore suspension of *B. coagulans* LBSC (160.0 mg, 5×10^{11} CFU·g^{-1}) was prepared by mixing the bacteria with 250 ml of sterile DW (pH 7.0). Prepared spore suspensions with a viable count of 3.2×10^8 CFU·ml^{-1} were aliquoted and stored in stability chambers for ICH recommended stability testing such as real time (5 ±3 °C), intermediate (25 ±2 °C, 60% ±5% RH) and accelerated (at 40 ±2 °C, NMT 75% RH) stability [ICH (Q1A(R2)][19]. Samples (2.0 ml) were withdrawn at specific intervals and viability of *B. coagulans* LBSC was assessed based on the standard pour plate method. Similarly, thermal stability of *B. coagulans* LBSC was assessed at various temperatures (0-100 °C) for 6 h.

2.5 *In vitro* calorie restriction under simulated conditions

An isolated and axenic colony of *B. coagulans* LBSC was inoculated into 100 ml of sterile *Lactobacillus* MRS Broth (MRS Broth M369, HiMedia, India) and grown aerobically at 37 ºC overnight (< 12 h) under shaking conditions (100 rpm). Then, 10 ml of actively grown *B. coagulans* LBSC cells were inoculated and mixed homogeneously with 50 g (or ml) of food products such as sweet corn soups, chocolate pies, ice creams, noodles, biscuits, dark chocolates, Lassi, orange drinks, sports drinks and mango juices in 250 ml of simulated intestinal fluid (SIF) [21]. Inoculated suspensions with initial viable counts of 3.4 to 4.0×10^8 CFU·ml^{-1} were incubated at 37 ºC up to 120 h at 120 rpm. Samples were withdrawn at zero time and the end of incubation and dried at 70 ºC overnight. Calorific content of each dried sample (1.00 g) was estimated using bomb calorimeter (1341EE Plain Jacket Calorimeter, Parr Instrument, India). Benzoic acid was used as standard for the calibration of bomb calorimeter. Correction factor was used to compensate calorie contribution of *B. coagulans* LBSC biomass and differences in calorie contents of food samples were calculated post-incubation [22].

2.6 Statistical analysis

Relative viability (%) was calculated from viable counts of *B. coagulans* LBSC expressed in log CFU·g^{-1}/ml^{-1}/serving^{-1}. All analyses were carried out in triplicate and results were presented as the mean of three independent determinations. Differences between two means of viable counts were calculated using student's t-test. Moreover, differences between two means of calorific values were calculated using paired t-test. In general, differences were significant when $p \leq 0.05$.

3. Results and Discussion

Probiotics are needed to deliver in adequate numbers (at least 10^7-10^9 CFU) to confer their health benefits to consumers [2]. Most probiotic containing formulations are designed to deliver adequate numbers of probiotics either as direct supplements or through various food matrices. Most of the available products are dairy products such as milks, ice creams, yoghurts, cheeses and frozen desserts [23]. In addition, non-dairy foods and beverages such as soy-based drinks, fruit-based foods, fruit juices and other cereal-based products becomes promising carriers for probiotic delivery [24]. Non-dairy probiotic foods are often free of lactose and comply with vegan diets. Consumers, who have concerns about lactose and excess cholesterol consumption or need specific diets, show much interests in such products [25]. However, viability and stability of probiotic preparations during food processing, storage and consumption are still major challenges in development of probiotic-based products. In the present study, stability of *B. coagulans* LBSC was assessed in various food products by studying the bacterial viability during food processing and storage.

3.1 Stability of *Bacillus coagulans* LBSC under food processes

Stability of *B. coagulans* LBSC was assessed by assessing its relative viability in various food matrices under specific processing conditions. The *B. coagulans* LBSC showed excellent stability when spores were incorporated in ready-to-use beverage premixes, including masala chais, green teas, lemon teas and instant coffees. Necessary volumes of hot water were added to the probiotic supplemented premixes and *B. coagulans* LBSC countings were carried out after 30 min. No significant changes in the relative viability of the test strains were seen (> 98% viability; average 9.41 ±0.04 log CFU·serving^{-1}) after 30 min of processing of hot foods and beverages, compared to 0 min (9.46 ±0.04 log CFU·serving^{-1}) (Fig. 1). In green tea preparations, no statistical significant differences were seen between the zero time (9.20 ±0.01 log CFU·serving^{-1}) and

final (9.06 ±0.06 log CFU·serving^{-1}) (after 30 min) viable counts of *B. coagulans* LBSC (p > 0.05). The relative viability was minimally affected as shown by 98.48% viability post processing. Moreover, *B. coagulans* LBSC was stable (9.28 ±0.03 log CFU·serving^{-1}, relative viability of 99.46%) in cold preparation process of lemon iced teas.

The *B. coagulans* LBSC showed 98.96% viability in masala chais, 98.48% in green teas, 99.59% in lemon teas and 99.79% in instant coffees. Negligible decreases in viability could be attributed to inactivation of probiotic spores with tea polyphenols that have been reported to include antimicrobial potentials [26]. Additionally, pH of lemon iced teas can be as low as 2.92 due to their high acidic contents which may adversely affect viability of probiotics. However, *B. coagulans* LBSC showed an exceptional viability in acidic beverages. Majeed et al. [11] have reported high relative viabilities of *B. coagulans* MTCC 5856 (87%) in brewed coffees, which are linked to reports of the present study. The present study reported cold coffees as favorable media for *B. coagulans* LBSC as they demonstrated a high relative viability up to 99.00% on 4 months of storage at 4°C.The *B. coagulans* LBSC preserved 99.89% of the viable count (decreases of 0.01 log CFU) after 30 min of ready-to-eat (RTE) breakfast Upma preparation, compared to zero time count (9.47 ±0.10 log CFU·serving^{-1}). Furthermore, 99.79 and 99.68% of relative viability were respectively observed after 30 min of preparation of instant sweet corn soups and noodles, compared to their initial count (9.40 ±0.03 and 9.40 ±0.05 log CFU·serving^{-1}) (Fig. 1). Instant breakfast cereals such as Upma, instant soups and noodles need dry mixes with preparation in hot water (85 ºC) for nearly 5-8 min. The *B. coagulans* LBSC survived these conditions and a significant viability rate (> 99.00%) was preserved. Farmer et al. [27] reported that approximately 55% of *B. coagulans* BC30 survived manufacturing processes, while approximately 30% of the bacterial strains survived cooking processes when mixed with durum wheat semolina pasta. Differences in viability could be attributed to the temperatures, processing conditions and durations.

3.2 Storage stability of *Bacillus coagulans* LBSC food products

In this study, *B. coagulans* LBSC was incorporated into various food products and storage stability was assessed under storage conditions for a specific duration as described in Section 2.3. In fact, *B. coagulans* LBSC was stable up to 120 days with more than 99% relative viability in various RTD beverages such as cold coffees, pomegranate juices and coconut waters (9.38±0.02, 9.34±0.04 and 9.44±0.02 log CFU·serving^{-1}, respectively). The relative viability of *B. coagulans* LBSC in orange drinks (9.23±0.04 log CFU·serving^{-1} at Day 120) and sports drinks (9.02±0.04 log CFU·serving^{-1} at Day 120) included 98.61 and 99.01 %, respectively. In mango juices, 98.93% viability was preserved (9.25±0.05 log CFU·serving^{-1}) over a storage time of 120 days (Table 1).

Fruit based beverages have become further popular for probiotics supplementation. The unique physiological porous structure of the fruit pulps may protect probiotic bacteria from external stressful environments [24]. Moreover, sports drinks contain concentrated forms of sugars, electrolytes, minerals and artificial colors. The *B. coagulans* LBSC preparation showed a significant viability (> 98 %) in fruit juices for three months. Relatively, Majeed et al. [11] reported 99% viability of *B. coagulans* MTCC5856 in apple juices. In addition to bacilli, supplementation of probiotic *Lactobacillus* spp. in fruit juices has been studied extensively [23,24]. Nualkaekul and Charalampopoulos [28] reported good survival rates of *Lactobacillus* (L.) *plantarum* NCIMB 8826 in orange, blackcurrant and pineapple juices. However, the bacterial viability in cranberry and pomegranate juices were less due to the antimicrobial effects of phenolic compounds. In the current study, significant viability of *B. coagulans* LBSC (99.05%) in pomegranate juices during storage at 4 °C for 120 days was reported. Furthermore, *B. coagulans* LBSC showed more than 99% viability in fruit juices such as mango juices, coconut waters and orange drinks, which indicated excellent stability of this probiotic under various physiological conditions. Such a long-term storage of *B. coagulans* LBSC with fruit juices also did not alter their flavor and texture as well as other organoleptic profiles (unpublished data).

In the present study, stability of *B. coagulans* LBSC with ORS preparations was reported for the first time. The relative viability of *B. coagulans* LBSC in RTD ORS and ORS powder was unaffected with relative viabilities of respectively 99.58 (7.06±0.02 log CFU·ml^{-1}) and 99.29% (6.95±0.02log CFU·ml^{-1}) after four months of storage at recommended storage conditions (Table 1). These products included high concentrations of electrolyte salts, which might include inhibitory effects on bacteria [29]. In this study, *B. coagulans* LBSC showed over 99% viability in formulations with ORS after four months of storage. In fact, ORS is used in supplement therapies during dehydration and diarrhea. The *B. coagulans* LBSC has earlier been reported effective in diarrheal patients [18]. Therefore, combinations of ORS with *B. coagulans* LBSC could be more effective in patients with dehydration, diarrhea and related gastrointestinal ailments. After 90 days of storage, *B. coagulans* LBSC preserved 98.67 (9.63±0.06 log CFU·serving^{-1}) and 99.16% (9.-42±0.04 log CFU·serving^{-1}) of its relative viability in dark chocolates and sugar syrups, respectively.

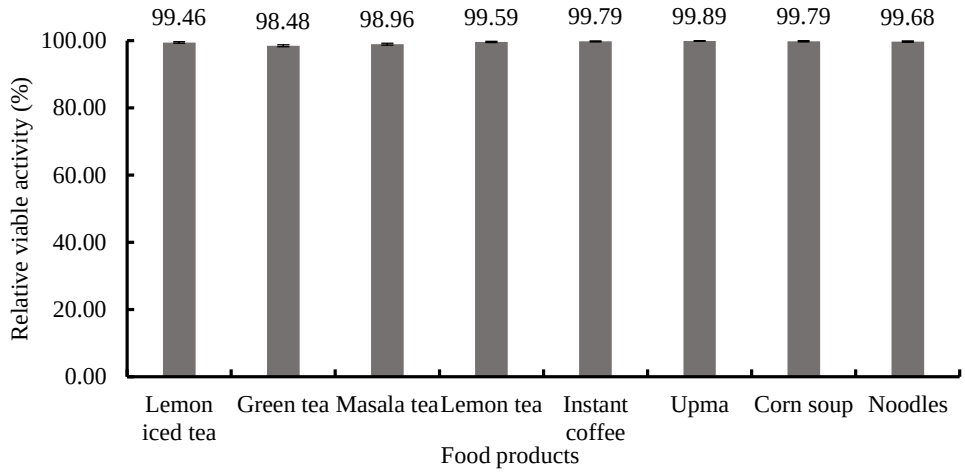

Figure 1. Relative viable activity (%) of *Bacillus coagulans* LBSC during processing of food and beverages at baseline (0 min) and 30 min.

Table 1. Storage stability of *Bacillus coagulans* LBSC when incorporated in various dairy and non-dairy food products demonstrating noticeable stability of the strain up to the maximum storage duration studied. Viable activity of *Bacillus coagulans* LBSC for cough syrup, ready to drink ORS and ORS powder is expressed as log CFU·ml^{-1}.

Food products	Viable activity of *B. coagulans* LBSC (log CFU·serving^{-1} or ·ml^{-1}) Time (Days)							Viabilty
	Initial 0	15	30	45	60	90	120	(%)
Chocopie	9.44±0.07^{a}	9.43±0.07^{a}	9.4±0.01^{a}	9.36±0.01^{a}	9.28±0.01^{a}	9.27±0.01^{a}	9.27±0.01^{a}	98.20
Protein bar	10.78±0.0^{a}	10.78±0.04^{a}	10.78±0.01^{a}	10.77±0.01^{a}	10.75±0.01^{a}	10.73±0.02^{a}	10.73±0.02^{a}	99.54
Dark chocolate	9.76±0.01^{a}	9.76±0.03^{a}	9.73±0.02^{a}	9.69±0.04^{a}	9.64±0.05^{a}	9.63±0.05^{a}	9.63±0.06^{a}	98.67
Orange drink	9.36±0.28^{a}	9.41±0.10^{a}	9.33±0.02^{a}	9.41±0.15^{a}	9.37±0.03^{a}	9.33±0.03^{a}	9.23±0.04^{a}	98.61
Ice-cream	9.52±0.01^{a}	9.51±0.02^{a}	9.53±0.04^{a}	9.53±0.02^{a}	9.51±0.03^{a}	9.50±0.01^{a}	9.48±0.02^{a}	99.58
Cough syrup	8.37±0.08^{a}	8.28±0.04^{a}	8.32±0.01^{a}	8.28±0.09^{a}	8.33±0.01^{a}	8.30±0.01^{a}	8.31±0.03^{a}	99.28
Lassi	9.32±0.01^{a}	9.34±0.01^{a}	9.32±0.01^{a}	9.34±0.01^{a}	9.35±0.01^{a}	9.33±0.01^{a}	9.33±0.02^{a}	100.11
Sport Drink	9.11±0.03^{a}	9.09±0.05^{a}	9.09±0.01^{a}	9.07±0.02^{a}	9.05±0.03^{a}	9.05±0.05^{a}	9.02±0.04^{a}	99.01
Cold coffee	9.42±0.05^{a}	9.43±0.03^{a}	9.44±0.05^{a}	9.42±0.02^{a}	9.42±0.02^{a}	9.38±0.01^{a}	9.38±0.02^{a}	99.58
Mango juice	9.35±0.04^{a}	9.29±0.02^{a}	9.27±0.03^{a}	9.26±0.04^{a}	9.25±0.05^{a}	9.26±0.04^{a}	9.25±0.05^{a}	98.93
Pomegranate juice	9.43±0.03^{a}	9.43±0.03^{a}	9.43±0.02^{a}	9.43±0.04^{a}	9.43±0.01^{a}	9.35±0.05^{a}	9.34±0.04^{a}	99.05
Coconut water	9.43±0.01^{a}	9.44±0.01^{a}	9.45±0.02^{a}	9.44±0.01^{a}	9.43±0.04^{a}	9.43±0.01^{a}	9.44±0.02^{a}	100.11
Ready to drink ORS	7.09±0.01^{a}	7.08±0.01^{a}	7.08±0.09^{a}	7.08±0.01^{a}	7.09±0.04^{a}	7.07±0.02^{a}	7.06±0.02^{a}	99.58
ORS powder	7.00±0.02^{a}	7.00±0.01^{a}	7.00±0.01^{a}	6.98±0.01^{a}	6.97±0.01^{a}	6.97±0.01^{a}	6.95±0.02^{a}	99.29
Sugar syrup	9.50±0.01^{a}	9.50±0.01^{a}	9.49±0.01^{a}	9.49±0.01^{a}	9.48±0.01^{a}	9.42±0.04^{a}	9.42±0.04^{a}	99.16
Tomato ketchup	9.45±0.01^{a}	9.43±0.02^{a}	9.41±0.04^{a}	9.34±0.04^{a}	9.34±0.05^{a}	9.34±0.05^{a}	9.34±0.06^{a}	98.84
Tomato puree	9.39±0.04^{a}	9.34±0.08^{a}	9.33±0.03^{a}	9.32±0.04^{a}	9.32±0.04^{a}	9.31±0.04^{a}	9.31±0.04^{a}	99.15

Means sharing the same superscript are not significantly different from each other with in a row ($p < 0.05$)

Mixing sugar confectioneries with probiotics can particularly be challe-nging because of high temperatures and complex matrices. In this study, *B. coagulans* LBSC was viable (98.7 %) up to four months when incubated with dark chocolates, suggesting its compatibility with multiple physicochemical changes such as hydrolysis, oxidation, lipid migration, water migration and sucrose inversion. Incorporation of *B. coagulans* GBI-30 6086 into milk chocolates demonstrated nearly 90% viability after storage for six months [30]. A few other studies have reported stability (70-90% viability) of probiotic lactobacilli in dark chocolates [31,32]. The higher viability (99.16%) of *B. coagulans* LBSC in sugar syrups of this study is similar to that of Majeed et al. [11]. These results reveal possibility of incorporating *B. coagulans* LBSC into foods with concentrated carbohydrate contents without compromising their viabilities. Similarly, *B. coag-ulans* LBSC preserved its relative viable count of 98.84 (9.34±0.06 log CFU·serving^{-1}) and 99.15% (9.31±0.04 log CFU·serving^{-1}) even for 120 days of storage in tomato ketchups and purees, respectively. The negligible loss of viability could be due to the vinegar contents and low pH (3.5) of these products [33,34].

In the present study, the relative viability of *B. coagulans* LBSC did not change in protein bars (99.54%, 10.73±0.02 log CFU·serving^{-1}), chocolate pies (98.20%, 9.27±0.01 log CFU·serving^{-1}) and ice creams (99.58%, 9.48±0.02 log CFU·serving^{-1}) after four months of storage. Protein bars are healthy foods, which can be easily incorporated into daily diets. Mixing probiotics with protein bars further enhances their health benefits. Furthermore, *B. coagulans* LBSC preserved its viability up to 99% within four months of storage when mixed with protein bars, chocolate pies and ice creams. Protein bars containing probiotic lactobacilli

have been assessed earlier [23,35]. Several studies reported stability of probiotics in ice creams. Salem et al. [36] reported supplementation of lactobacilli in ice creams and found that viability decreased by 2.23 log CFU·g^{-1} for *L. acidophilus*, 1.68 log CFU·g^{-1} for *B. bifidum*, 1.54 log CFU·g^{-1} for *L. reuteri*, 1.23 log CFU·g^{-1} for *L. gasseri* and 1.77 log CFU·g^{-1} for *L. rhamnosus* during 12 weeks of frozen storage.

In the present study, *B. coagulans* LBSC was stable in dairy-based beverage of Lassi without losing its viability, compared to zero-time count (100.11%, 9.33±0.02 log CFU·serving^{-1}) after four months of storage. Mixing lactobacilli probiotics with lassi has been reported earlier; however, the viability was considerably as low as 6% or less at Day 21 of storage under refrigeration conditions [37,38]. This shows superiority of spore forming *B. coagulans* LBSC over lactobacilli probiotics for use in Lassi. The *B. coagulans* LBSC preparation was stable for four months in pharmaceutical oral formulations of cough syrups by preserving its relative viable count of 99.28% (8.31 ±0.03 log CFU·ml^{-1}), compared to its initial count of 8.37 ±0.08 log CFU·ml^{-1} (Table 1). These results showed that the bacterial strain was not affected by low pH (4.0) and complex active pharmaceutical matrices of cough syrups, which usually contain chemical compounds of sedatives, cough suppressants and artificial colors [39]. Addition of probiotics can increase the effectiveness of cough syrups through its crosstalks with the lung microbiota that function via gut-lung axis [40].

3.3 Stability of *Bacillus coagulans* LBSC in aqueous media

The storage stability of aqueous spore preparations of *B. coagulans* LBSC was assessed for a period of one year under real time, intermediate and accelerated conditions [International Conference on Harmonization (ICH (Q1A-(R2)] [19]. The *B. coagulans* LBSC was stable in aqueous media with more than 98% relative viability (8.35 ±0.02 log CFU·ml^{-1}) for up to one year under the three storage conditions (Table 2). This suggests that *B. coagulans* LBSC can be mixed with packed water preparations for human consumption. Bora et al. [41] and Majeed at al. [42] have reported stability of *B. coagulans* strains at aqueous conditions, including room temperature. Direct comparison of the current experimental results with other results is not possible due to the variabilities in conditions and durations.

3.4 Thermal stability of *Bacillus coagulans* LBSC

The *B. coagulans* LBSC preparations showed dose and duration dependent responses when exposed to various temperatures up to 6 h. The bacterial strain showed 99.56% relative viability (9.02 ±0.04 log CFU·ml^{-1}) at 80 °C after 6 h, compared to its initial viability (9.06 ±0.06 log CFU·ml^{-1}) at 0 min. Decreases in viability by 0.2 and 5.8 log CFU·ml^{-1} was reported respectively at 90 (8.97 ±0.03 log CFU·ml^{-1}) and 100 °C (7.62 ±0.13 log CFU·ml^{-1}) after 2 h of exposure (Table 3). Temperature tolerance of *B. coagulans* LBSC spores is another attribute for its higher stability. Temperature causes several changes such as increases in the rate of germination; in which, spores lose their heat resistance ability due to denaturation of spore proteins. This leads to heat shock and subsequent lethal injury of the germinating spores. Such effects are frequently reported in *B. cereus* strains at temperatures higher than 80 °C [43]. In the present study, *B. coagulans* LBSC showed relative viability of 97.59% at 90 °C for up to 6 h. Several other strains of *B. coagulans* have been reported as thermo-tolerants or thermophilics. Such studies report ability of *B. coagulans* to resist thermal stress and survive [44-47]. Overall, *B. coagulans* LBSC tolerated adverse conditions of heat, low pH, antimicrobial compounds and osmotic stress during manufacturing processes and storage times of various functional foods. Such a good stability by *B. coagulans* LBSC might be attributed to its sporulating ability that is usually induced by nutrient deficiency of media and various physicochemical stresses [48,49]. The heat resistance of *Bacillus* spp. might be due to the critical water contents of spore cores, mineral ions, spore proteins and saturation of spore DNAs with α/β-type small acid-soluble proteins [50,51]. Hence, *B. coagulans* LBSC can be an excellent probiotic candidate for the production of functional foods with industrial needs of stable strains befitting large-scale productions.

3.5 Growth of *Bacillus coagulans* LBSC on food matrices *in vitro* and calorie restriction assessment

In this study, *B. coagulans* LBSC was assessed *in vitro* for its ability to grow on various food matrices and decrease calorie contents in foods. Growth experiments were carried out for 1-5 days until static calorie values were achieved in food matrices. Experiments demonstrated ability of *B. coagulans* LBSC to metabolize food matrices and decrease calorie contents of high-calorie foods.

The higher incubation period in this study was selected to achieve maximum calorie decreases and it might not represent calorie decreases under *in vivo* conditions. Supplementation of growing (metabolically active) *B. coagulans* LBSC cells showed decreases in food calories in several high-calorie foods such as soups, chocolate pies, ice creams, noodles, biscuits, dark chocolates, Lassi, orange drinks, sports drinks and mango juices.

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Table 2. Stability of *Bacillus coagulans* LBSC in aqueous medium under real time, intermediate and accelerated storage conditions for one-year duration.

Time (Days)	Viable activity (log CFU·ml^{-1}) under different storage conditions		
	Real time (5 ± 3°C)	Intermediate (25 ± 2°C/60 ± 5% RH)	Accelerated (40± 2°C/NMT 75% RH)
0	8.52±0.09^{a}	8.51±0.02^{a}	8.51 ± 0.04^{a}
15	8.50±0.10^{a}	8.51±0.01^{a}	8.50 ± 0.02^{a}
30	8.49±0.06^{a}	8.52±0.02^{a}	8.48 ± 0.11^{a}
45	8.52±0.01^{a}	8.52±0.01^{a}	8.48 ± 0.01^{a}
75	8.40±0.04^{a}	8.54±0.02^{a}	8.48 ± 0.01^{a}
105	8.40±0.02^{a}	8.47±0.02^{a}	8.48 ± 0.02^{a}
135	8.35±0.02^{a}	8.43±0.04^{a}	8.48 ± 0.07^{a}
270	8.40±0.02^{a}	8.47±0.02^{a}	8.48 ± 0.02^{a}
360	8.35±0.02^{a}	8.40±0.01^{a}	8.44 ± 0.04^{a}
Relative viability (%, on 360th day from day 0)	98.07	98.57	99.14

Means sharing the same superscript are not significantly different from each other with in the column (p<0.05).

Table 3. Thermal stability of *Bacillus coagulans* LBSC preparation when exposed to temperatures ranging from 0 to 100°C for up to 6 h duration. Relative viability (%) is expressed at specific temperature at 6 h compared to initial activity at 0°C.

Temperature (°C)	Initial activity (log CFU·ml^{-1})	Viable activity of *B. coagulans* LBSC (log CFU·ml^{-1}) at different exposure time (h)						Relative viable activity (%)
		1	2	3	4	5	6	
0	9.02±0.07^{aA}	9.02±0.07^{aA}	9.02±0.08^{aA}	9.02±0.08^{aA}	9.02±0.05^{aA}	9.02±0.02^{aA}	9.02±0.03^{aA}	100.00
40	9.02±0.07^{aA}	9.02±0.06^{aA}	9.02±0.06^{aA}	9.04±0.05^{aA}	9.03±0.02^{aA}	9.02±0.02^{aA}	9.02±0.03^{aA}	100.00
60	9.15±0.02^{aA}	9.11±0.02^{aA}	9.11±0.04^{aA}	9.13±0.03^{aA}	9.14±0.02^{aB}	9.08±0.04^{aAC}	9.08±0.05^{aA}	99.23
80	9.06±0.06^{aA}	9.06±0.06^{aA}	9.05±0.03^{aA}	9.06±0.06^{aA}	9.05±0.03^{aAB}	9.04±0.05^{aAC}	9.02±0.04^{aA}	99.56
90	9.14±0.02^{aA}	9.02±0.07^{bA}	8.97±0.03^{bA}	8.98±0.05^{bA}	8.97±0.05^{bA}	8.95±0.03^{bAD}	8.92±0.02^{bB}	97.59
100	9.13±0.02^{aA}	9.08±0.04^{bA}	7.62±0.13^{cB}	6.78±0.05^{dB}	6.00±0.02^{eC}	4.78±0.04^{fB}	3.30±0.01^{gC}	36.14

Means sharing the same superscript are not significantly different from each other (p<0.05). Differences with in a row are shown by small letter superscript and column by capital letter superscript.

Of these foods, soups and dark chocolates included high calorific values (5750 and 6614 cal·g^{-1}, respectively) compared to other food products (4000-5000 cal·g^{-1}). The assessed (combustion method) calorific values were detected higher those claimed by food labels. This could be due the fact that the label claims generally represent metabolizable energy specific to nutritional contents [52] and do not include unabsorbed energy of non-digestible dietary ingredients, which are excreted without digestion. Several authors have reported similar differences between the laboratory assessed values of foods and those from food label claims [53-55].

Calorie restriction is considered as the difference in total calorific value in probiotic-supplemented food after growth of probiotics, compared to basic foods with no probiotic supplements. This demonstrates the extent; to which, the probiotic strain can utilize the energy content of food matrices under experimental conditions. In this study, a maximum of 62% calorie decreases was seen in soups within 24 h, followed by chocolate pies and ice creams with 39 (48 h) and 38% (48 h) decreases, respectively (Fig. 2). Overall, a 30.6% median calorie decrease was seen in selected foods. Calorie decreases of soups, noodles and dark chocolates were reported as the fastest decreses in less than 24 h, compared to those of other foods (Table 4). The viable count of *B. coagulans* LBSC in food products was at least 2.5 × 10^{9} CFU·ml^{-1} at the end of the incubation time. Thus, *B. coagulans* LBSC was assessed for calorie restriction from foods *in vitro* as an important functional probiotic strain. Growth of *B. coagulans* LBSC decreased calories from foods in simulated intestinal fluid (907-3565 calories·g^{-1}). However, these calorie decreasess were recorded in prolonged incubations under *in vitro* conditions. Lack of reports on *in vitro* food calorie restriction by other probiotics resulted in no possible comparisons with this study. It is well known that the gut microbiome absorbs up to ~30% of the host energy [56]. Jang et al. [56] reported that *L. rhamnosus* GG harvested energy sources from high fat diets and restricted their absorption by the host mice; eventually the bacteria utilized energy metabolites before excreting into feces. the current study has hypothesized that *B. coagulans* LBSC with calorie restriction ability *in vitro* might be able to accomplish/mediate energy redistribution via energy extraction in the form of metabolites such as short-chain fatty acids (SCFAs) [57,58]. Rearrangement of high-energy bonds into low energy ones might occur, decreasing the overall calorie production. Thus, differences in calories at pre and post-digestion by *B. coagulans* LBSC might be linked to production of low-calorie metabolites, volatile organic compounds (VOCs) [59], ATP expenses and Gibb's energy dissipations (in form of heat) [60]. Many bacteria spill energy, if growth media are nitrogen-limited and include excessive high-calorie carbon sources. *Streptococcus bovis*, a lactic acid bacterium, demonstrates this

phenomenon [61]. Probiotic supplementations have been reported, affecting energy homeostasis through fermentation of polysaccharides to SCFAs [62].

Probiotic strains have been supplemented with diets to restrict calorie availability. Several probiotic strains of *Lactobacillus* spp. have been incorporated in low-calorie synbiotic and probiotic diets [63] and clinically assessed in obesity, arterial hypertension [64] and weight management [65,66]. Probiotic-intervened calorie restrictions play major roles in modulation of gut microbiota, especially increased butyrate producing microbes and cometabolites, increased fatty acid catabolism, β-oxidation, glycogenolysis and gluconeogenesis at the physiological levels [15]. Nevertheless, further studies are necessary to correlate *in vitro* calorie restriction findings with *in vivo* physiological findings of similar probiotic strains. Recently, *B. coagulans* LBSC has been shown to establish healthy crosstalks by modulating healthy gut microbiome and associated metabolic normalcy in irritable bowel syndrome (IBS) [67]. In the present study, *in vitro* decreases of food calories is demonstrated using *B. coagulans* LBSC. However, association of this data under *in vivo* conditions is expected in future studies. To the best of the authors' knowledge, probiotic *Bacillus* spp. (especially *B. coagulans* LBSC) for the restriction of calorie from various foods and beverages have first been reported. This could promisingly be used in weight management, obesity and other associated metabolic syndromes.

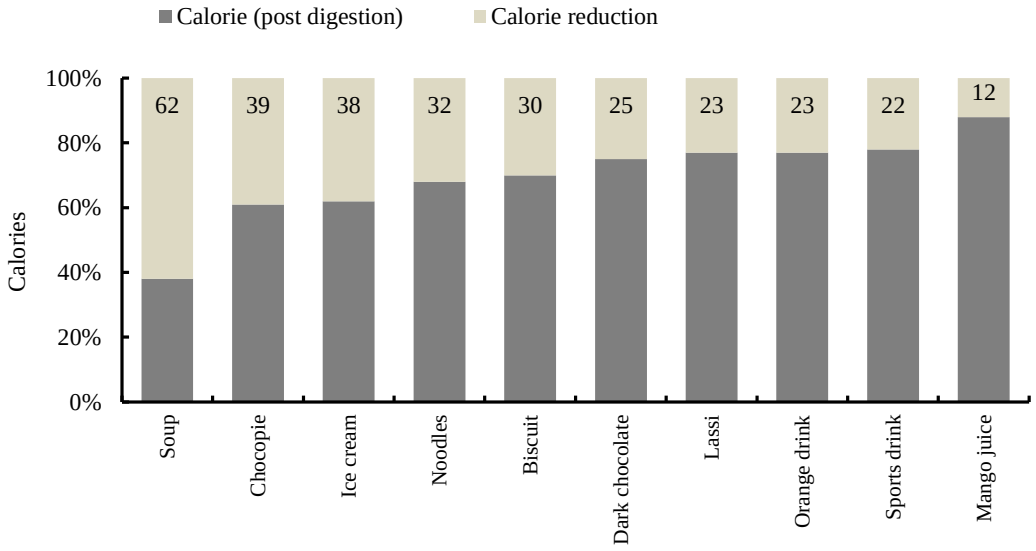

Figure 2. Reduction in calorie content (%) of various foods after digestion with *Bacillus coagulans* LBSC up to 120 h; entire bar length indicates the total calorie content while the calorie reduction (%) is highlighted with grey.

Table 4. Reduction of calorie content in selected foods after 120 h growth of *Bacillus coagulans* LBSC. Pre- and post-digestion of foods were subjected for calorie estimation using bomb calorimeter ($p < 0.05$). Instrument was calibrated using benzoic acid as calorimetric standard and yielded 6318 Cal·g^{-1}.

Sample	Pre-digestion (Cal·g^{-1})	Post-digestion (Cal·g^{-1})	Reduction in calorie (Δ Cal·g^{-1})	*p*-value	Incubation Time (h)
Soup	5750±25	2185±25	3565	0.000066	24
Chocopie	5414±12	3275±16	2136	0.000034	48
Ice cream	5919±17	3655±19	2279	0.000011	72
Noodles	5652±16	3841±10	1789	0.000040	24
Biscuit	5275±21	3668±18	1608	0.000012	120
Dark chocolate	6614±12	4945±13	1645	0.000052	24
Lassi	4233±11	3243±13	975	0.000096	120
Orange drink	4113±11	3169±12	946	0.000005	120
Sports drink	4228±21	3284±13	907	0.000395	120
Mango juice	3717±7	3268±29	446	0.001975	72

4. Conclusion

The probiotic *B. coagulans* LBSC demonstrated excellent process and storage stability in various foods and beverages. The bacterial strain well tolerated high and low temperatures and was stable in aqueous media at various temperatures. Hence, *B. coagulans* LBSC can be incurporated into functional foods with no effects on viability and functionality, conferring desired health benefits to consumers. Furthermore, *B. coagulans* LBSC is able to grow on high calorie foods to decrease food calorie *in vitro*; suggesting the bacterial use as a functional food ingredient for better management of obesity, ageing and other health risks.

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6. Conflict of Interest

The authors declare no conflict of interest.

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فرایند و پایداری انباری *باسیلوس کوآگولانس* LBSC در ماتریس‌های غذایی و ارزیابی محدودیت‌های گرمایی

پاتریک باگکار، یوجینی دیکسیت، آمیت تی واری، آنیل کومار گاپتا، چیرانجیت مایتی*

شرکت فناوری‌های پیشرفته آنزیم با مسئولیت محدود، طبقه پنجم، آ-وینگ، سان ماگنتیکا، جاده LIC سرویس، لوییس وادی، تان (w)، ۴۰۰۶۰۴، ماهاراشترا، هند.

چکیده

سابقه و هدف: باکتری‌های زیست‌یار[۱] به‌عنوان افزودنی‌های غذایی رشد قابل توجهی در سطوح غذاهای فراسودمند داشته‌اند. غذاهای فراسودمند[۲] چالش‌های متعددی دارند، پایداری و زنده‌مانی زیست‌یار. در مطالعه حاضر، *باسیلوس کوآگولانس* LBSC DSM 17654 گونه زیست‌یاری است که به‌منظور ارزیابی پایداری حین فرایند و نگهداری و توانایی کاهش میزان کالری غذاها، به مواد غذایی گوناگونی اضافه شده است.

مواد و روش ها: *باسیلوس کوآگولانس LBSC* در تهیه نوشابه‌ها و مواد غذایی گوناگونی مانند نوشابه‌های غیرالکلی سرد، غلات صبحانه‌ای، نمک های آبرسانی خوراکی، شیرینی، مواد غذایی منجمد آسان‌پز، دسرهای منجمد لبنی، چاشنی‌ها، ترکیبات خوش طعم، نوشیدنی‌های تخمیری شیری و شربت‌های سرفه مورد استفاده قرار گرفت. پایداری انباری و حین فرایند باکتری‌ها با استفاده از تخمین نسبی زنده‌مانی ارزیابی شد. پایداری *باسیلوس کوآگولانس LBSC* در تعلیق‌های[۳] آبی در درجه حرارت‌های گوناگون (°C ۱۰۰-۰) و براساس راهنمای ICH [Q1A (R2)] مورد بررسی قرار گرفت. باکتری از نظر ظرفیت محدودسازی کالری در شرایط برون‌تن[۴] هنگام افزودن به مواد غذایی بررسی شد.

یافته‌ها و نتیجه‌گیری: *باسیلوس کوآگولانس LBSC* به‌خوبی حین فرایند غذایی (زنده‌مانی نسبی ۰/۵۱ ± ۹۹/۴۶ درصد) و شرایط انبارمانی (زنده‌مانی نسبی ۰/۵۱ ± ۹۹/۲۲ درصد) زنده ماند. باکتری در تعلیق آبی پایدار بود و درجه حرارت بالا را به‌خوبی تحمل کرد (به‌ترتیب، زنده‌مانی نسبی ۰/۲۱ ± ۹۹/۵۶ درصد و ۰/۰۱ ± ۹۷/۵۹ درصد در درجه‌حرارت‌های ۸۰ و ۹۰ درجه سلسیوس). *باسیلوس کوآگولانس LBSC* کاهش معنی‌دار کالری در مکمل‌های غذایی زیست‌یار در شرایط برون‌تن، در مقایسه با موادغذایی فاقد مکمل را نشان داد ($p < 0/05$). در نتیجه، *باسیلوس کوآگولانس LBSC* پایداری خوبی در محیط‌های آبی در درجه حرارت‌های بالا را نشان داد. *باسیلوس کوآگولانس LBSC* نه تنها در محدوده وسیعی از انواع مواد غذایی پایدار بود، بلکه قادر به رشد بر روی مواد غذایی به‌منظور کاهش کالری مواد غذایی در شرایط برون‌تن می‌باشد؛ کاربرد آن به عنوان ترکیب غذایی فراسودمند برای مدیریت بهتر چاقی و سالخوردگی و مخاطرات سلامتی ایشان را می‌توان پیشنهاد داد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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واژگان کلیدی

- *باسیلوس کوآگولانس*
- محدودیت کالری
- غذاهای فراسودمند
- فرایند
- پایداری
- انبارمانی

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[۱] Probiotic bacteria
[۲] Functional
[۳] suspensions
[۴] *in vitro*