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Survivability of freeze-dried probiotic *Pediococcus pentosaceus* strains GS4, GS17 and *Lactobacillus gasseri* (ATCC 19992) during storage with commonly used pharmaceutical excipients within a period of 120 days

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ABSTRACT

Objective: To examine the survivability and stability of probiotic strains in presence and absence of pharmaceutical excipients for a long period of time at (4 ± 1) °C.
Methods: The survival rates of probiotic strains, *Pediococcus pentosaceus* GS4 (MTCC12683) (NCBI HM044322), GS17 (NCBI KJ608061) and *Lactobacillus gasseri* (ATCC 19992), were evaluated. Probiotic strains were lyophilized individually and in combination of excipients (sorbitol, ascorbic acid, fructose and skim milk). The preparation was monitored for 120 d storing at (4 ± 1) °C. During storage, all the preparations were evaluated for viability and stability of probiotic properties like lactic acid production, antimicrobial effect, water activity, and adherence to epithelial cells.
Results: Sorbitol, ascorbic acid and skim milk favoured the viability of freeze-dried cells and sustained probiotic properties during storage. Without excipients (control group), strains showed percentage of survivability not more than 70% while strains with excipients survived for 73%–93% for a long period of time.
Conclusions: Commonly used excipients can be considered as a vehicle for delivering active principle in probiotic formulation and for sustaining the viability and stability of probiotic strains for a period of 120 d.

1. Introduction

Delivery of probiotics for mitigation or treatment of diseases in a viable form without altering the sensory characteristics of the formulation is challenging. Probiotic microorganisms are defined as 'living microorganisms that, when ingested in certain amount, can exert health benefits beyond inherent basic nutrition' [1]; play important roles in promoting and maintaining human health [2]. Consequently, a wide variety of species and genera are evaluated as potential probiotics and considerable interest is being stimulated in the incorporation of these microorganisms into functional foods and pharmaceutical products [3–5].

The Scientific Committee on Food of the European Commission has recommended that the content of viable bacteria in formulae with long shelf life should be 10^6–10^8 colony forming units (CFU) per gram of formula prepared as ready for consumption in order to exert beneficial effects and be known as a probiotic product [6]. Among the most important characteristics are the survival of these microorganisms during storage and rapid growth during manufacture, which means that maximum survival of these bacteria in starters during processing and subsequent storage is of vital technological importance and cost-effectiveness [7,8]. Subsequently during the period of the preparation of culture concentrates with these microorganisms, production and maintenance techniques must be established which maximize the storage stability, viability, and probiotic activity of the bacterial cells [2,9]. The industrial application of lactic acid bacteria depends on the concentration and preservation technologies that are required to guarantee long-term stability of cultures in terms of viability and functional activity [10]. Freeze-dried preparations exhibit more advantages

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than preparations made with other techniques, in terms of long-term preservation, coupled with convenience in handling, storage, marketing and application. It is the most suitable and successful method of preserving probiotic bacteria, yeasts and sporulating fungi by reducing water activity on water removal [11]; however, water content is an important parameter for the stability of dried cultures. Over-drying may diminish the viability and stability of microorganisms [12]. Stability of probiotic microorganisms during freeze-drying and storage may be enhanced by addition of cryoprotectants. Cryoprotectants are used to maintain the vitality of cells during freeze-drying and subsequent storage. Generally, cryoprotectants contain non-reducing disaccharides, sugar alcohols, polysaccharides, amino acids, proteins, adonitol, betaine, glycerol, lactose, skim milk and dimethyl sulphoxide [13]. Several studies reported that skim milk, glycerol, various sugars and some probiotic substances exhibit the highest protection among diversely examined cryoprotectants media [6]. It is a challenging task to maintain higher viability in a stable probiotic potential since probiotic preparations (both food and pharmaceutical formulations) include 'active substance' (the probiotic culture). Therefore different additives or excipients are added either as emulsifiers, antioxidants or preservatives. It would be technologically and economically valuable to evaluate the effect of these compounds on the physiology of the selected probiotic strains [14,15].

Research showed that the antagonistic effect of *Pediococcus* against the pathogenic microbes like *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Listeria monocytogenes* and *Staphylococcus aureus* (*S. aureus*) resembled their intrinsic properties primarily due to secretion of lactic acid and bacteriocin [16–18]. Our research group has previously isolated and identified *Pediococcus pentosaceus* (*P. pentosaceus*) GS4 from the well-known Indian fermented food Khadi [19]. The same strain has been deposited in Microbial Type Culture Collection (MTCC), India and Gene Bank with accession number, MTCC12683. This lactic acid bacteria has probiotic potential such as acid tolerant, bile salt tolerant, lactic acid homofermenter, beta-galactosidase producer, vancomycin resistant, cholesterol assimilating, anti-oxidative and also exerts its antimicrobial effect by secreting bacteriocin like pediocin [19,20]. *P. pentosaceus* GS4 has bio-hydrogenation property which can produce conjugated linoleic acid [21]. Furthermore, it plays an important role in the mitigation of induced toxicity in liver, kidney and intestine as safe and nontoxic probiotic [22], and it is good to control the induced colon carcinogenesis in mice which caused apoptosis [5].

Recent study on viability of *P. pentosaceus* GS4 in simulated gastric condition showed maximum survivability and resistance to processing stress, and further viability and stability may be achieved in presence of protective agents such as lactose, ascorbic acid and inulin [23]. From our experimental observation, it foresees the possible therapeutic application of probiotic strains, however, it needs to understand the survival capabilities of our probiotic strains with probiotic potentials in question.

In this study, survival rates of probiotic strains of *P. pentosaceus* GS4, GS17 and *Lactobacillus gasseri* (*L. gasseri*) have been evaluated by using the method of lyophilisation and adding different excipients at (4 ± 1) °C within a period of 120 d. The effects of freeze drying and excipients on probiotic properties such as lactic acid production, antimicrobial effect, water activity, adherence to epithelial cells, growth of the probiotics and survivability were examined under the storage conditions.

2. Materials and methods

2.1. Bacterial strains

The laboratory probiotic strains *P. pentosaceus* strains GS4 (MTCC12683) and GS17 were previously isolated from Indian fermented food Khadi and identified by *16S rRNA* sequencing [accession number in Gen-Bank: NCBI HM044322 (GS4); and NCBI KJ608061 (GS17) respectively]; *L. gasseri* (ATCC 19992) was obtained from the collection of the Microbiologics, Medimark Europe, France. Three reference strains *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), and *P. aeruginosa* (ATCC 25619), which were used for the determination of antimicrobial activity, was obtained from the Microbiologics, Medimark Europe, France.

2.2. Growth conditions

Before experiments, probiotic strains were inoculated in Man, Rogosa and Sharpe (MRS) broth (HiMedia, India), and were incubated at 37 °C and sub-cultured at least twice in MRS every 12 h (log phase). Reference strains were inoculated in Luria–Bertani broth (HiMedia, India) and incubated at 37 °C for 8 h (log phase) [14].

2.3. Protective media

The excipients used in this experiment were fructose (F), skim milk powder (SM), ascorbic acid (A), and sorbitol (S) respectively. These were purchased from HiMedia, India. Protective media were prepared to protect cells during freezing which included F (8%), SM (13%), A (2.5%) and S (2.5%) while phosphate buffer saline (PBS) 0.01 M (pH 7.2) was used as a control. Before use, SM was sterilized at 115 °C for 10 min and those consisting of only sugars were sterilized by filtration using 0.45 μ filter (Merck, India).

2.4. Cell production and storage conditions

P. pentosaceus strains GS4, GS17 and *L. gasseri* at mid-exponential phase of the third subculture were inoculated at a rate of 1% (v/v) into 500 mL of MRS broth and were incubated without shaking at 37 °C for 12 h. Cells at early stationary phase were harvested by centrifugation at 15 000 *g* for 10 min at 4 °C, washed twice and resuspended in PBS. This suspension was divided into twelve aliquots of 10 mL each. Then aliquots were centrifuged, pellets were collected (about 2.5 g wet weight, with a cell density of about 5×10^9 CFU/g) and resuspended in 10 mL of PBS containing: F, 8% (w/v); SM, 13% (w/v); A, 2.5% (w/v); and S, 2.5% (w/v) individually and in combination like A + SM, F + SM, S + SM, F + S, A + S, F + A, a mixture of all excipients and a group without any excipients (control). Each suspension was transferred into sterile petridish (13 mm), frozen at −85 °C and freeze-dried for 18 h in a freeze-dryer (Thermo Fischer Micro Modulyo Freeze Dryer, USA). The lyophilized powders were stored aseptically in sterile Eppendorf tube (2 mL) at (4 ± 1) °C under the darkness.

Viability, antimicrobial activity, lactic acid production and water activity were determined at defined time intervals throughout storage period for 120 d. Assays were performed by rehydrating the lyophilized powders in 1 mL of PBS and those obtained after the first subculture in MRS broth.

2.5. Viability estimation

Viable *P. pentosaceus* GS4, GS17 and *L. gasseri* before and after freeze drying and during storage were enumerated by plate count method. Dried cells were resuspended in an appropriate volume of PBS. Cell suspension was serially diluted in sterile saline (0.85% NaCl) and plated onto MRS agar. Then viable cell counts were determined after 24 h of incubation at 37 °C [13,14]. Results were expressed as log CFU/g, of lyophilized powder using the following formula:

$$\% \text{ survival} = \frac{\text{Viable cells after freeze drying (CFU/g)}}{\text{Viable cells before freeze drying (CFU/g)}} \times 100$$

2.6. Antimicrobial activity

Antimicrobial activity was determined by agar well diffusion method [24]. The production of antimicrobial was quantified in the spent supernatants of the first subculture in MRS broth incubating at 37 °C for 12 h. The petridish containing Muller Hilton agar was seeded with *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923) and *P. aeruginosa* (ATCC 25619) in the log phase using sterile swab. A 100 µL cell free extract of probiotic strains *P. pentosaceus* GS4, GS17 and *L. gasseri* grown individually and in combination of the excipients were used in wells, with ampicillin (10 µg/100 µL) as positive control and sterile PBS as negative control. Antimicrobial activity was determined by the measurement of zone of inhibition around the wells after 24 h of incubation at 37 °C. The diameter of the zone was measured (in mm) and compared with that of positive and negative control. The experiment was performed in duplicates and each of the readings was taken by two observers and the average was calculated.

2.7. Determination of lactic acid production

A 20 mL of 12 h culture-free supernatants of *P. pentosaceus* GS4, GS17 and *L. gasseri* was used for the estimation of the lactic acid produced. A 20 mL amount of supernatant was collected by centrifuging at 10 000 × *g* for 15 min at 4 °C. As per standards provided by AOAC International [25], 1 mL of 0.1 M NaOH for neutralization of acid is equivalent to 90.08 mg of lactic acid. Amount of lactic acid produced was calculated accordingly. The titration for each supernatant was carried out in duplicates and the average value was calculated [16].

2.8. Water activity (a_w)

Water activity was measured immediately after freeze drying of the powder samples, at 25 °C, in all the conditions analysed using a Novasina water activity instrument (Switzerland).

2.9. Adherence of P. pentosaceus and L. gasseri to buccal epithelial cells

The adherence assay was performed as described previously by Johansson *et al*. [26]. Buccal epithelial cells were collected from five healthy male volunteers. Epithelial cells from the donors were obtained by buccal scraping with cotton-tipped swab and were pooled in PBS. The cells were centrifuged at 1 000 × *g* for 10 min and washed three times with PBS. The buccal epithelial cells were resuspended in PBS. In assay, each 1.0 mL of epithelial cells and bacterial suspension were mixed and incubated at 37 °C for 1 h. For control group, epithelial cell suspension was incubated with PBS instead of bacterial suspension. After incubation the mixed suspension was washed to be free from unattached bacteria by repeated differential centrifugation at 1 000 × *g* for 5 min. After washing the cells, the suspension was stained with 10% Giemsa stain. All bacteria (both indigenous and the added *P. pentosaceus* GS4 and GS17) bound to the buccal epithelial cells were counted using light microscope at 100× illumination.

2.10. Statistical analysis

All experiments were done in duplicate at regular intervals during storage. Data were analysed by the one-way analysis of variance (ANOVA) of duplicate trials. *P*-value ($P < 0.05$) was considered statistically significant.

3. Results

3.1. Survival of freeze-dried probiotic strains and L. gasseri after freeze drying and storage

Excipients used were found beneficial in supporting varied degree of survivability of lyophilised probiotic strains and the reference strain, *L. gasseri* (Figure 1 and Table 1). Without excipients (control group), strains showed percentage of survivability not more than 70% while strains with excipients survived for 73%–93%. The higher possibility of strains survivability after 90 d was found associated with the use of A alone and in combination with S and with SM, respectively (Figure 1 and Table 1). In all conditions, the strain GS4 survived better than other strains. Estimated percentages of survival of *P. pentosaceus* GS4 after 120 d were found between 77.63% ± 3.37% with F alone and 93.15% ± 0.67% with F + A respectively (Figure 1 and Table 1). *P. pentosaceus* strains GS4, GS17 and *L. gasseri* showed 1 log cycle decline up to 120 d.

No significant decline was observed after 90 d storage of GS4 and GS17 with combination of all excipients. Therefore, a mixture of all excipients was found to be the suitable storage conditions. The percent survivability was calculated and was found to be 95% after 120 d. The viable count obtained in control group showed marked decline as compared to test sample of *P. pentosaceus* GS4 with excipients. The viability of *L. gasseri* (ATCC 19992) decreased progressively to some extent in comparison to *P. pentosaceus* GS4. The GS4 could sustain its viability with 9.2 Log CFU/g in presence of F + S or A + SM (Figure 1A) while the combination of F + A in GS17 caused higher survivability (8.9 Log CFU/g) at (4 ± 1) °C ($P < 0.05$) (Figure 1B). At the 120th day retention of significant viable count was observed in *L. gasseri* (ATCC 19992) when the strain was formulated with A and S (8.7 Log CFU/g) and in mixture of all excipients (8.4 Log CFU/g) at (4 ± 1) °C ($P < 0.05$) (Figure 1C). In all the cases, mixture of all excipients served as the best suspending medium. It may be due to the presence of high solid content and higher concentration of protective components such as proteins, calcium, buffering agents like phosphate and citrate

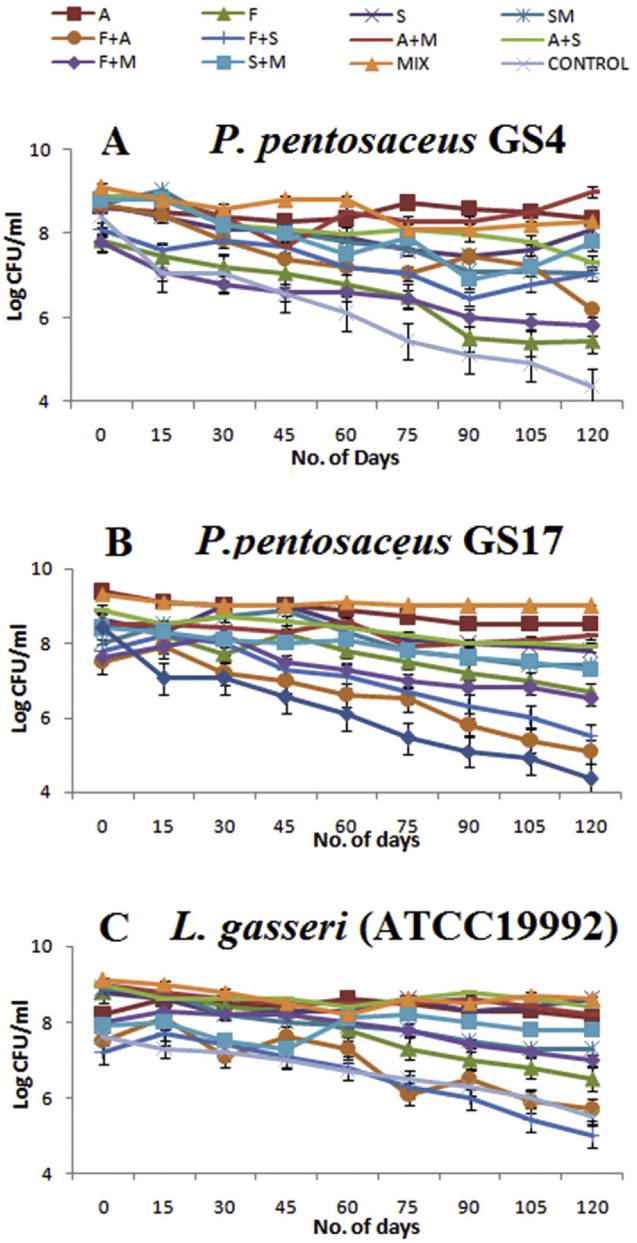

Figure 1. Survivability of probiotic strains after freeze drying with different excipients.
P. pentosaceus GS4 (A, B); *L. gasseri* (C, D); *P. pentosaceus* GS17 (E, F).

Table 1
Percent survival rate of probiotic strains after freeze drying with different excipients (mean ± SD, $n = 3$).

Strains	*P. pentosaceus* GS4	*P. pentosaceus* GS17	*L. gasseri*
SM	81.76 ± 4.05	78.30 ± 3.37	75.60 ± 5.40
S	77.63 ± 3.37	80.32 ± 6.75	85.05 ± 2.70
F	78.97 ± 2.02	72.90 ± 1.35	74.25 ± 5.40
A	91.80 ± 1.35	87.75 ± 0.67	87.10 ± 1.35
A + SM	89.10 ± 2.02	84.37 ± 1.35	86.40 ± 0.67
F + SM	88.43 ± 1.35	83.70 ± 1.35	77.63 ± 2.70
S + SM	89.77 ± 0.67	83.03 ± 1.35	83.70 ± 2.70
F + S	87.10 ± 1.35	83.10 ± 2.02	76.95 ± 3.38
A + S	87.75 ± 1.35	84.38 ± 1.35	83.10 ± 3.38
F + A	93.15 ± 0.67	85.05 ± 2.02	84.38 ± 4.05
All mix	92.48 ± 3.38	89.78 ± 4.05	83.70 ± 3.38
Control	69.53 ± 2.02	67.50 ± 3.38	69.53 ± 6.07

salts which stabilized the pH and protected the cell membrane (cell lethality) during freeze drying and storage.

3.2. Antimicrobial activity

Antimicrobial activities were evaluated before and after freeze-drying (0 d) and at specific intervals during storage (30, 60, 90 and 120 d) against *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923) and *P. aeruginosa* (ATCC 25619) (Tables 2–4). The results demonstrated that there were direct correlation between the viability of *P. pentosaceus* GS4, GS17 and *L. gasseri* (ATCC 19992) (Tables 2–4) and production of inhibitory substances (bacteriocin) in culture supernatants. *P. pentosaceus* GS4 and GS17 were found to be superior to *L. gasseri* (ATCC 19992) in all the conditions tested during storage. However *P. pentosaceus* GS4 combined with SM showed significant inhibitory spectrum against *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 25619) throughout the storage period whereas no zone of inhibitions were observed on 90th and 120th day of the storage against *S. aureus* (ATCC 25923). The significant improvement and retention of antimicrobial properties of *P. pentosaceus* GS4, GS17 and *L. gasseri* (ATCC 19992) against all the test microorganisms were observed throughout the storage period when these probiotic strains were combined with A, A + S, A + SM or with all the excipients. Control sample of both strains could not restore antimicrobial activity during the study period. This reduction of inhibitory spectrum or no antimicrobial activity might attribute to cell death due to repercussion of freeze drying.

3.3. Lactic acid production

The results showed that ability of lactic acid production of *P. pentosaceus* GS4, GS17 and *L. gasseri* (ATCC 19992) depended on the protective medium and the growth status of these microbes. In our study, the results obtained after freeze drying and the refrigerated storage of *P. pentosaceus* GS4, GS17 and *L. gasseri* for different periods of time showed that the combination of all the excipients (mixture) are the most suitable formulation for cryoprotective medium, for the growth as well as for the production of lactic acid throughout the study. Combination of all the excipients and respective addition of S and A enhanced lactic acid production ability of *P. pentosaceus* GS4 (Table 5). Lactic acid production potential of *P. pentosaceus* GS4 was significantly increased with S from initial concentration of (1.60 ± 0.10) g/20 mL to (2.33 ± 0.10) g/20 mL with loss of 0.65 log CFU/mL of viable cells when examined on 90th day of storage. *P. pentosaceus* GS17 also showed similar potential of lactic acid production (Table 6). However lower performance in lactic acid production were observed when probiotic strains were combined with F, F + A, F + SM, and F + S accordingly. The result showed significant production of lactic acid (ATCC 19992) when *L. gasseri* was combined with A as compared to S, SM, A + SM, S + SM and A and S accordingly (Table 7). Fructose used individually and in combination with other excipients had slight impact on production ability of lactic acid by *L. gasseri* (ATCC 19992). For control samples of *P. pentosaceus* GS4 and *L. gasseri* (ATCC 19992) the lactic acid production was not affected by the freeze-dried condition assayed but decreased slightly during storage and being completely abolished from 90th day in GS4 and 60th day in *L. gasseri* respectively.

Table 2
Effect of freeze drying and storage on antimicrobial activity of *P. pentosaceus* GS4.

P. pentosaceus GS4 + Excipients	*E. coli*					*S. aureus*					*P. aeruginosa*				
	0 d	30 d	60 d	90 d	120 d	0 d	30 d	60 d	90 d	120 d	0 d	30 d	60 d	90 d	120 d
SM	+++	+++	+++	++	++	+++	+++	++	–	–	+++	+++	+++	++++	++
F	++	+++	+++	+++	–	++	++	++	–	–	+++	+++	–	–	+
S	+++	++	++	–	+	+++	+++	++	++	–	+++	++	++	–	–
A	+++	+++	+++	+++	++	+++	+++	+++	+++	++	++++	+++	+++	++	–
A + SM	+++	+++	++	+++	++	+++	+++	+++	++	++	+++	++	++	+++	++
F + SM	+++	+++	++	+++	+	+++	+++	++	–	+	+++	+++	+++	–	++
S + SM	+++	++	++	+++	++	+++	+++	+++	+	++	++++	++	++	+	–
F + S	++	+++	+++	–	+	++	++	++	–	–	+++	+++	+++	++	–
A + S	+++	++++	+++	++++	+++	+++	+++	+++	++	+++	++	++++	+++	++	+
F + A	++	+++	+++	++	+	+++	++	++	+	–	+++	++	+++	+	–
All mix	+++	++++	+++	+++	+++	++++	++++	+++	++	++	+++	++++	+++	+++	++
Control	++	+	+	–	–	+++	++	++	–	–	++	++	++	–	–

++++ = 20–25 mm, +++ = 15–20 mm, ++ = 10–15 mm, + = 5–10 mm and – Not determined.

Table 3
Effect of freeze drying and storage on antimicrobial activity of *P. pentosaceus* GS17.

P. pentosaceus GS17 + Excipients	*E. coli*					*S. aureus*					*P. aeruginosa*				
	0 d	30 d	60 d	90 d	120 d	0 d	30 d	60 d	90 d	120 d	0 d	30 d	60 d	90 d	120 d
SM	+++	++++	+++	++	++	++++	+++	+++	++	++-	++++	++++	+++	++	++
F	+++	–	++	++	–	++++	++	+++	+	–	+++	+++	++	–	+
S	+++	++	++	++	+	++++	++	++	+++	+	+++	+++	+++	++	+
A	++	++++	+++	+++	++	++++	+++	+++	++	+	++++	++++	+++	+++	++
A + SM	++	+++	+++	++	+	++++	+++	++	+	–	+++	+++	+++	++	++
F + SM	++	+++	+++	+	+	++++	+++	+++	+	–	+++	++	++	+	–
S + SM	+++	++++	+++	++	+	++++	++	+++	++	++	+++	+++	++	++	+
F + S	+++	++	++	–	–	+++	+++	+++	+	+	++	+++	++	+	+
A + S	+++	+++	++	++	++	++++	+++	+++	+++	++	++	+++	++	+	++
F + A	+++	+++	+++	++	+	++++	+++	++	–	–	+++	+++	+++	++	+
All mix	+++	++++	+++	+++	++	++++	++++	+++	+++	++	++++	++++	+++	+++	++
Control	++	++	+	–	–	++++	+	–	–	–	++	++	+	–	–

++++ = 20–25 mm, +++ = 15–20 mm, ++ = 10–15 mm, + = 5–10 mm and – Not determined.

Table 4
Effect of freeze drying and storage on antimicrobial activity of *L. gasseri.*

L. gasseri + Excipients	*E. coli*					*S. aureus*					*P. aeruginosa*				
	0 d	30 d	60 d	90 d	120 d	0 d	30 d	60 d	90 d	120 d	0 d	30 d	60 d	90 d	120 d
SM	+++	++++	+++	++	++	++++	+++	+++	++	++	++++	++++	+++	++	++
F	+++	–	++	++	–	++++	++	+++	+	–	+++	+++	++	–	+
S	+++	++	++	++	+	++++	++	++	+++	+	+++	+++	+++	++	+
A	++	++++	+++	+++	++	++++	+++	+++	++	+	++++	++++	+++	+++	++
A + SM	++	+++	+++	++	+	++++	+++	++	+	–	+++	+++	+++	++	++
F + SM	++	+++	+++	+	+	++++	+++	+++	+	–	+++	++	++	+	–
S + SM	+++	++++	+++	++	+	++++	++	+++	++	++	+++	+++	++	++	+
F + S	+++	++	++	–	–	+++	+++	+++	+	+	++	+++	++	+	+
A + S	+++	+++	++	++	++	++++	+++	+++	+++	++	++	+++	++	+	++
F + A	+++	+++	+++	++	+	++++	+++	++	–	–	+++	+++	+++	++	+
All mix	+++	++++	+++	+++	++	++++	++++	+++	+++	++	++++	++++	+++	+++	++
Control	++	++	+	–	–	++++	+	–	–	–	++	++	+	–	–

++++ = 20–25 mm, +++ = 15–20 mm, ++ = 10–15 mm, + = 5–10 mm and – Not determined.

3.4. Water activity (a_w)

The a_w of the *P. pentosaceus* GS4, GS17 and *L. gasseri* (ATCC 19992) varied with the suspending medium and the individual strain characteristics (Table 8). The a_w of *P. pentosaceus* GS4 freeze dried with F, F + SM and F + A were 0.813, 0.890 and 0.910, respectively. Notably, the initial viable count of this bacterium in all these three conditions was high, approximately 8.71 log CFU/mL and gradually decreased in viability with log difference of 1.31 log CFU/mL. Likely, *P. pentosaceus* GS17 represented the a_w between 4.8 and 9.6, respectively (Table 8).

The observed decrease in viability may attribute to elimination of fractions of cellular water present in the form of free, intermediate and structured water during the storage. Similar results were observed in case of *L. gasseri* (ATCC 19992) in all the three conditions studied. However, relatively high a_w were determined (ATCC 19992) when *L. gasseri was* freeze dried with S + SM, A and A + SM as compared to *P. pentosaceus* except for S (a_w-0.440). Although a_w for control samples of both

Table 5
Effect of freeze drying and storage on lactic acid production of *P. pentosaceus* GS4 (g/20 mL of 24 h culture).

Excipients	0	30	60	90	120
SM	1.68 ± 0.10	1.87 ± 0.10	1.60 ± 0.10	1.30 ± 0.10	1.11 ± 0.10
F	1.48 ± 0.10	1.33 ± 0.10	1.03 ± 0.10	0.43 ± 0.10	–
S	1.60 ± 0.10	2.88 ± 0.10	2.52 ± 0.10	2.33 ± 0.10	1.81 ± 0.10
A	2.27 ± 0.10	3.14 ± 0.10	2.63 ± 0.10	2.89 ± 0.10	0.90 ± 0.10
A + SM	1.52 ± 0.10	2.07 ± 0.10	2.54 ± 0.10	2.87 ± 0.10	1.13 ± 0.10
F + SM	1.49 ± 0.10	2.70 ± 0.10	1.21 ± 0.10	1.16 ± 0.10	0.33 ± 0.10
S + SM	1.34 ± 0.10	2.42 ± 0.10	1.74 ± 0.10	1.07 ± 0.10	1.29 ± 0.10
F + S	1.12 ± 0.10	1.60 ± 0.10	1.17 ± 0.10	1.44 ± 0.10	0.91 ± 0.10
A + S	1.50 ± 0.10	2.10 ± 0.10	2.56 ± 0.10	2.13 ± 0.10	1.13 ± 0.10
F + A	1.53 ± 0.10	2.00 ± 0.10	1.45 ± 0.10	1.14 ± 0.10	–
All mix	2.54 ± 0.10	2.87 ± 0.10	2.91 ± 0.10	1.53 ± 0.10	1.37 ± 0.10
Control	0.27 ± 0.10	1.10 ± 0.10	0.20 ± 0.10	–	–

Table 6
Effect of freeze drying and storage on lactic acid production of *P. pentosaceus* GS17 (g/20 mL of 24 h culture).

Excipients	0	30	60	90	120
SM	1.90 ± 0.10	2.87 ± 0.10	2.60 ± 0.10	1.33 ± 0.10	0.29 ± 0.10
F	0.71 ± 0.10	1.19 ± 0.10	1.73 ± 0.10	0.50 ± 0.10	–
S	2.54 ± 0.10	3.30 ± 0.10	3.11 ± 0.10	2.77 ± 0.10	1.93 ± 0.10
A	2.52 ± 0.10	3.30 ± 0.10	3.11 ± 0.10	2.54 ± 0.10	0.81 ± 0.10
A + SM	1.87 ± 0.10	2.31 ± 0.10	2.11 ± 0.10	0.80 ± 0.10	0.10 ± 0.10
F + SM	1.71 ± 0.10	2.31 ± 0.10	2.11 ± 0.10	0.76 ± 0.10	0.21 ± 0.10
S + SM	1.34 ± 0.10	2.77 ± 0.10	2.14 ± 0.10	1.91 ± 0.10	1.09 ± 0.10
F + S	1.12 ± 0.10	2.10 ± 0.10	1.57 ± 0.10	1.13 ± 0.10	0.43 ± 0.10
A + S	1.50 ± 0.10	2.81 ± 0.10	2.89 ± 0.10	2.26 ± 0.10	1.31 ± 0.10
F + A	1.53 ± 0.10	2.16 ± 0.10	1.13 ± 0.10	–	–
All mix	2.54 ± 0.10	3.49 ± 0.10	2.82 ± 0.10	2.29 ± 0.10	2.10 ± 0.10
Control	0.27 ± 0.10	1.10 ± 0.10	0.20 ± 0.10	–	–

Table 7
Effect of freeze drying and storage on lactic acid production of *L. gasseri* (g/20 mL of 24 h culture).

Excipients	0	30	60	90	120
SM	1.87 ± 0.10	2.33 ± 0.10	2.60 ± 0.10	1.44 ± 0.10	0.22 ± 0.10
F	1.44 ± 0.10	1.91 ± 0.10	1.20 ± 0.10	–	–
S	1.96 ± 0.10	2.10 ± 0.10	1.64 ± 0.10	1.33 ± 0.10	0.89 ± 0.10
A	2.10 ± 0.10	2.60 ± 0.10	2.34 ± 0.10	1.84 ± 0.10	1.77 ± 0.10
A + SM	2.21 ± 0.10	2.46 ± 0.10	1.54 ± 0.10	1.39 ± 0.10	0.50 ± 0.10
F + SM	0.33 ± 0.10	1.15 ± 0.10	0.20 ± 0.10	–	–
S + SM	1.61 ± 0.10	2.18 ± 0.10	1.72 ± 0.10	1.24 ± 0.10	0.18 ± 0.10
F + S	1.58 ± 0.10	1.90 ± 0.10	1.07 ± 0.10	0.66 ± 0.10	–
A + S	1.98 ± 0.10	2.19 ± 0.10	1.44 ± 0.10	1.09 ± 0.10	0.56 ± 0.10
F + A	1.23 ± 0.10	1.78 ± 0.10	1.19 ± 0.10	0.54 ± 0.10	–
All mix	1.92 ± 0.10	2.19 ± 0.10	1.63 ± 0.10	1.00 ± 0.10	0.40 ± 0.10
Control	0.27 ± 0.10	0.20 ± 0.10	–	–	–

Table 8
Water activity of probiotic strains after freeze drying with different excipients (mean ± SD, n = 3).

Excipients	*P. pentosaceus* GS4	*P. pentosaceus* GS17	*L. gasseri*
SM	0.454 ± 1.300	0.678 ± 0.087	0.601 ± 0.078
S	0.852 6 ± 0.070 0	0.930 9 ± 0.070 0	0.905 ± 0.070
F	0.731 ± 0.078	0.522 ± 0.078	0.470 ± 0.078
A	0.661 ± 0.070	0.574 ± 0.070	0.922 ± 0.078
A + SM	0.653 ± 0.078	0.626 ± 0.070	0.913 ± 0.078
F + SM	0.931 ± 0.070	0.687 ± 0.070	0.922 ± 0.070
S + SM	0.609 ± 0.078	0.731 ± 0.078	0.791 ± 0.078
F + S	0.809 ± 0.078	0.536 ± 0.070	0.913 ± 0.070
A + S	0.661 ± 0.070	0.748 ± 0.070	0.792 ± 0.070
F + A	0.887 ± 0.070	0.922 ± 0.078	0.913 ± 0.078
All mix	0.511 ± 0.078	0.557 ± 0.078	0.635 ± 0.078
Control	0.361 ± 0.070	0.496 ± 0.070	0.601 ± 0.070

microorganisms studied were low as compared to individual and combined excipients, there were no sustainable bacteria at the end of the study. This high death rate may be due to over drying and lack of protective agents in control.

3.5. Adherence to buccal epithelial cells

Adherence of microbes to epithelial cells is important and represents the colonization and site specificity. The binding of *P. pentosaceus* GS4, GS17 and *L. gasseri* (ATCC 19992) to buccal epithelial cells and effect of freeze drying and excipients on adhering properties of the strain were studied and examined under light microscopy. The results indicated that adherence to epithelial cells varied among the strains and excipients used. A, SM, S and combination of all excipients with *P. pentosaceus* GS4, GS17 and *L. gasseri* (ATCC 19992) adhered significantly well to buccal epithelial cells. The intermediate adhesions of bacteria were observed in the case of *P. pentosaceus* GS4 combined with F, F + A, S + A and F + S. *L. gasseri* (ATCC 19992) showed significantly lower adhesive ability in rest of the conditions tested. The results obtained did not allow any generalization since both strains indicated characteristic behaviour in all the conditions tested.

4. Discussion

The sustainability of probiotics during the production process is a crucial factor for successful development of probiotic formulation. Suitable selection of excipients for preserving survivability and inherent biological characteristics are important criteria for early product development process. Recently, we determined *in vitro* tolerance of our probiotic strains under simulated gastrointestinal conditions as well as shelf life of probiotic in freeze dried form which could be achieved by selection of compatible excipients [23,27]. In present study, we assessed the effect of individual component constituting the media and combination of all the constituents on survival and biological properties of three probiotic strains *P. pentosaceus* GS4, GS17 and *L. gasseri* (ATCC 19992). Our results indicated that the four excipients used individually and seven different combinations exerted protection during freeze drying and storage; however, these had little effect on viability of strains. These excipients can adsorb on bacterial cell membrane to form viscous layer, which inhibits intracellular formation of ice. They also can prevent injurious eutectic freezing of cell fluids by trapping salts and prevent membrane protein denaturation to inhibit membrane damage [14,23]. In the present study, none of the excipients supported *P. pentosaceus* GS4, GS17 and *L. gasseri* (ATCC 19992) from progressive cell death, however, their combination, S + A + SM + F partially protected the cells from damage and thus was identified as the most suitable conditions for storage. The membrane integrity of the bacterial cells can be maintained by addition of sorbitol during freeze drying and storage time and by increasing the ratio of unsaturated to saturated fatty acid [28]. The positive effect of sorbitol is detected due to increased permeability of bacterial cell membrane for water and stabilization of membrane protein and function thus favouring transport across membrane [12]. Zayed and Roos suggest that it is desirable to suspend bacterial cells in media that contain not only protective excipients but also buffering agents for stabilizing the pH [2]. Ascorbic acid is an antioxidant and inhibits the membrane lipid oxidation which in turn affects the survival of cells during freeze drying and shelf life on dried state [23,27]. Ascorbic acid 2.5% (w/v) used individually and in combination with other excipients are found to be a suitable protective agent for sustainability of *P. pentosaceus* GS4, GS17 and *L. gasseri* (ATCC 19992) during storage condition. The survival of bacterial cells at low temperature was achieved by using skim milk as drying medium [1]. The protective effect of skim milk with sorbitol was achieved due to presence of secondary alcoholic group in this sugar alcohol [6]. Again, combination of skim milk and ascorbic acid preserve viability by exerting protective effect through formation of protective coating around cells and stabilizing the cell membrane [7], and combination of skim milk with fructose create the porous structure in the freeze dried powder that make rehydration as well as recuperation of the cells easier [12,29]. The study results show that bacterial cells in skim milk medium in combination with other excipients has better ability to preserve viability during storage, 88.43% ± 1.35% to 89.77% ± 0.67% in *P. pentosaceus* GS4, 83.03% ± 1.35% to 84.37% ± 1.35% in GS17 and 77.63% ± 2.70% to 86.40% ± 0.67% in *L. gasseri* (ATCC 19992) respectively than in phosphate buffer saline in case of control group [69.53% ± 2.02% in *P. pentosaceus* GS4, 67.50% ± 3.38% in GS17 and 69.53% ± 6.07% in *L. gasseri* (ATCC 19992)]. The viability of both strains is found to be non-significant when mixed with fructose individually or in combination with other excipients. This loss in viability may be due to metabolism of this monosaccharide by bacterial cells [10,12,30].

As mentioned earlier there is one way correlation between the antimicrobial activity and the viability status of the bacterial cells. The results obtained in our study for production of antimicrobial substances can not be generalized because individual strains have intrinsic biological properties and different inhibitory spectrum against pathogens. The study results showed that ascorbic acid favoured the antimicrobial activity of *P. pentosaceus* GS4, GS17 and *L. gasseri* (ATCC 19992) during storage in all the conditions tested. Significant increases in lactic acid production were determined when *P. pentosaceus* GS4, GS17 and *L. gasseri* (ATCC 19992) were combined with sorbitol and ascorbic acid. This improvement in lactic acid production was attributed to preservation of viability during storage and revival of bacterial cells when strains were resuspended in MRS broth. Thus properties of rehydration medium and constituting solutes comprising the medium acts as healing medium for injured or sub-lethally damage cells as a consequence of freeze drying [23]. Tomas *et al*. report three factors, growth medium, pH and temperature, are responsible for the production of lactic acid by *Lactobacillus acidophilus* CRL 1259 among the 134 isolates from healthy vaginal samples [31]. Production of specific lactic acid isomer (D and L-lactic acid) depends not only on gene expression for D and L-lactic acid dehydrogenase but also on the intrinsic catalytic conditions of bacterial cells which can change affinity of this enzyme for substrate [27].

Long term stability of probiotic products can be critically affected by the moisture content and storage temperature [6]. Water in bacterial cells present in three forms: free water, intermediate water and structured water [2]. Excessive freeze drying is responsible for the removal of water with consequent alteration in membrane protein leading to cell death. Abe

et al. reported that the optimum moisture content in freeze dried products varied from probiotic strains under study and composition of excipients/protective medium supplemented during freeze drying process [32]. From our study, we apprehend that moisture content in optimum concentration is required in dehydrated form for the survival and activity of bacterial cells during shelf life.

Bacterial adhesion to epithelial cells is a step forward for the colonization and persistence at target site for conferring health benefits by probiotics. Although processing and storage conditions are important determinants for adhesive properties of selected strain, source of epithelial cells play a crucial role in bacterial adherence assay [14,33]. The difference in adhesiveness of strains to buccal epithelial cells in *in vitro* model may attribute to individual differences, different cell surfaces and salivary protein coating (IgA) over buccal epithelial cells [34]. Mastromarino *et al*. report the significance of addition of excipients (polymers) which are capable of reducing surface negative electric charges in formulation for improvement of adhesive properties of bacteria [35]. The reductions of surface negative charges are important because in presence of surface negative charges, the cation activity would be considerably larger at the membrane surface than in the bulk phase. This might influence cations flux through the membrane channel and destabilized the membrane potential resulting into bacterial cell injury. Ziyadi *et al*. and Mastromarino *et al*. reported the influence of freeze drying on membrane conformation and significance of rehydration media in revival and adhesive properties of bacterial cells [6,35]. Our results indicated differences in adhesive properties of strains tested in presence of different excipients. Therefore, it is important to select the compatible excipients that maintain the viability of the bacterial cells during storage with adherence potential.

In conclusion, sorbitol, ascorbic acid and skim milk added to the probiotic suspensions before and after freeze drying are found to favour the stability of the cells during long term storage, and therefore should be considered for the production of freeze-dried cultures that could be included as active principle in formulation. Addition of polymers for stabilization of bacterial cell membrane potential and improvement of resistance of bacterial cell to process stress are found advantageous for sustainability of probiotics during storage. Since effects of lyophilization and storage are strain specific and may interfere with antagonistic property and adhesive capabilities of probiotics, these factors must be taken into consideration in product development. The probiotic strains used in this study sustain and retain adequate viable population in appropriate storage conditions with probiotic properties. The study envisages the efficacy of used excipients in supporting the long term survivability in the storage condition and hence this approach may be used for future application.

Conflict of interest statement

Authors declare that there is no conflict of interest.

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