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Impact of Freeze and Spray Drying on the Retention of Probiotic Properties of *Lactobacillus fermentum*: An *in vitro* Evaluation Model

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Abstract: Lactic acid bacteria (LAB) isolated from *Kanjika* was phenotypically and genotypically characterized as *Lactobacillus fermentum* and its probiotic properties were evaluated *in vitro*. Preparation of concentrated starter cultures *via* freeze drying and spray drying is of practical importance to dairy and food industries. This work involved the study of the effects of these techniques on the viability and retention of the key probiotic properties like acid and bile tolerance, cholesterol assimilation of *L. fermentum.* Maximum of 94% survivability was achieved in *L. fermentum* freeze dried with non-fat skimmed milk (NFSM) in comparison with 89% survivability of cells freeze dried with maltodextrin (MDX). Percentage survival was significantly higher ($P \leq 0.05$) when 1% cells (w/v) of *L. fermentum* was spray dried either with MDX (86.5%) or NFSM (88.1%), in comparison with 3% cells spray dried either with MDX (81.9%) or NFSM (85.7%). Key probiotic properties like pH tolerance, bile tolerance, antimicrobial activity and cholesterol assimilation behavior were found to be retained to a significant extent after freeze and spray drying. The present study highlights that freeze drying and spray drying of *L. fermentum* with MDX as a carrier may be a cost effective way of producing large quantities of stable preparations retaining functional properties for nutraceutical application.

Key words: Probiotics · Nutraceuticals · Kanjika · Lactobacillus fermentum · Freeze drying · Spray drying

INTRODUCTION

Probiotic microorganisms play an important role in promoting and maintaining health [1] which has stimulated considerable interest in incorporating these into functional foods and pharmaceutical products. According to the currently adopted definition by FAO/WHO, probiotics are 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host' [2] and it is recommended that probiotic products contain at least 10^7 live microorganisms per g or per ml [3]. *Lactobacillus* species have attracted much attention as probiotics due to their natural inhabitance in the human gastrointestinal system and the proposed roles they play as a part of normal human microflora.

Lactic acid bacteria (LAB) comprise a diverse group of Gram-positive, non-spore forming bacteria [4]. They generally lack catalase, although in rare cases pseudocatalase can be found [5]. It has been reported that the basic recommendation for LAB strains to be used as commercial probiotics include the following criteria: (1) should be generally recognized as safe (GRAS), (2) should be tolerant to low pH of gut and bile, (3) should be able to adhere to the intestinal epithelium of the hosts, (4) demonstrate antagonistic activity against pathogenic bacteria and (5) retain their viability during processing and storage [6 - 8]. In view of the mounting data on the beneficial effects of probiotics in maintaining and strengthening human immunity, they comprise one of the most potential categories of functional foods.

In general, *in vitro* data alone is not sufficient to describe LAB strains as probiotics, but they still remain valuable and can provide scientific insight into characteristics of probiotic organisms. The selection of suitable strains may be further refined by undertaking a series of *in vivo* experiments [9]. But, *in vitro* tests are still recommended by FAO/WHO [10] for the initial screening of potential probiotic bacteria. These tests are important since probiotics consist of a wide variety of species and

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sub-species and the ability to adhere, colonize and modulate the human gastrointestinal system is not a universal property. With this knowledge, optimal probiotic strains may be developed for commercial use.

From a commercial point of view, an inexpensive method for large-scale production of cultures containing high levels of viable probiotic cells in a form suitable for product applications is highly desirable. Dehydration is a commonly used preservation method to stabilize probiotics for their ease of storage, handling, transport and subsequent use in food and pharma industry. Freeze drying and spray drying are the most common down stream processes used for the preparation of dried stable probiotic cultures. The importance of starters in dairy industry is apparent from the industrial point of view [11]. Freeze-drying is the most widespread technique for dehydration of probiotic and dairy cultures, while spray drying has been applied to the dehydration of a limited number of probiotic cultures [12].

The present study was undertaken to characterize and compare the effects of freeze drying and spray drying on survival and retention of probiotic properties of *Lactobacillus fermentum,* a probiotic strain isolated during the preparation of *Kanjika*, an ayurvedic lactic acid fermented product. The strain was identified by partial sequencing of 16S rRNA gene.

MATERIALS AND METHOD

Materials: All the chemicals used were of analytical grade. MRS (de Mann Rogosa Sharpe) broth/agar, Cholesterol and bile salts were from Himedia, India. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (iso-propyl-thio-β-D-galactopyranoside) were procured from Sigma-Aldrich, USA. Maltodextrin was obtained from Lobo chemicals, India. Nonfat skimmed milk was purchased from Karnataka milk federation, India.

Organism and Growth Conditions: Lactic acid bacteria isolated from *Kanjika*, were used in the present study [13]. The pathogenic strains used as indicators for inhibition study were *E.coli* ATCC 31075 (ATCC, Manassas, VA, USA), *E.coli* MTCC 108 (MTCC, IMTECH, Chandigarh, INDIA), *Listeria monocytogenes*, *Salmonella typhi*, *Salmonella paratyphi* and *Yersinia enterocolitica* (laboratory isolates). The cultures were maintained as 40% glycerol stock at -50°C and were periodically revived. Prior to use, the probiotic strain was transferred to MRS broth and indicator bacteria were cultured into nutrient broth and grown for two generations.

Strain Identification and Characterization: The primary identification of the strain was based on gram staining, catalase test and morphology [4]. Biochemical characterization was carried out by checking the gas production from glucose in MRS broth without citrate using inverted Durham's tubes. The pattern of carbohydrate fermentation was determined using Hicarbohydrate kit (Himedia, Mumbai, India). The isolate was also tested for its ability to grow at different temperatures ranging from 15 to 65°C at varying pH 2-10 and at varying saline concentrations up to 10% NaCl. The data obtained was matched with standard Bergey's manual [4]. Molecular characterization by 16S rRNA partial (471 nucleotide) gene sequencing was performed for strain identification.

Preparation of Feed Solutions for Freeze Drying Application: For freeze drying studies, overnight culture of *L. fermentum* was inoculated into MRS broth (1% v/v) and incubated at 37°C till the stationary phase was reached. After centrifugation (Remi cooling centrifuge, C-30, Mumbai, India) at 9000 x g for 15 min at 4°C, the freshly harvested cells were suspended in sterile distilled water (1% w/v on wet base) and vortexed to get a homogenous suspension. Individual cryoprotectants were lactose (SF1), sucrose (SF2), maltodextrin (SF3) and skimmed milk (SF4) at 20% (w/v) was added to the cell suspension and the final volume made up with distilled water to get a feed suspension of 20% (w/w) solids. The pH of the feed solution was around 4.29± 0.20. The feed suspension was stored in an ice bath for about 30 min prior to use for freeze drying.

Preparation of Feed Solutions for Spray Drying Application: For spray drying studies, two types of feed solutions were prepared. In the first type, overnight culture of *L. fermentum* was inoculated into MRS broth (1% v/v) and incubated at 37°C till the stationary phase was reached. After centrifugation at 9000 x g for 15 min at 4 °C, 1 and 3 % cells (w/v) on wet weight basis were resuspended in 10% non-fat skimmed milk solution. This type of feed solution was termed "non-fat skimmed milk (NFSM)". Similarly, in the second type, after centrifugation 1 and 3% (w/v) cells were resuspended in

10% maltodextrin solution. This type of feed solution was termed as "maltodextrin" (MDX) and pH of the feed solution was around 4.25± 0.25. These feed solutions were directly used for spray dried application.

Freeze Drying: For each of the lyophilization, flasks were filled with 100 ml of feed suspension. The feed suspension was frozen quickly by rotating the flasks in ice salt mixture and the flask was connected to a freeze drier (Heto drier) operated at 0.080 Pa and -45 °C for 18 h [14]. The freeze dried samples were analyzed immediately for the viability and stored in airtight polythene covers. The residual viability of freeze dried culture was determined in triplicates and samples were stored at 4°C ± 2.

Spray Drying: A bench top scale dryer (JISL, Bombay, India) was used for spray drying of LAB. The inlet air, heated to 140±2°C by an electrical heater, flowed concurrently with the spray into a 12.5 L drying chamber with an outlet temperature of 40± 2°C. Feed solution was delivered by a peristaltic pump into a two fluid stainless steel atomizer. The spray dried powder was collected at the bottom of a cyclone. The moisture content of the spray dried powder was determined in triplicates by oven drying the powders at 102°C, determining the weight difference and expressing the weight loss as a percentage of the powder weight [15]. The residual viability of spray dried culture was determined and the dehydrated samples were stored at two different temperatures; 30±2° C and 4±2°C.

Cell Viability: Residual viability of freeze dried and spray dried samples was determined by the standard plate count method. The freeze dried and spray dried powder (1g) was rehydrated with 10 ml of sterile distilled water to about the same solids content as the feed solution. The rehydrated samples were kept on a shaker for 30 min to allow complete dissolution. Suitable dilutions of feed solution and rehydrated samples were prepared by serial dilution and 100 µl was plated in triplicates, using the spread plate method. Colony forming unit (cfu) was determined after incubation for 48 h at 37°C. The percentage viability of the freeze dried and spray dried sample was calculated according to Reddy *et al.* [14]

$$\% \text{ viability} = 100 \times Nr/Nf, \quad (1)$$

Where Nr was log cfu/ml of rehydrated sample and Nf was the log cfu/ml of feed solution

Determination of Acid and Bile Tolerance: The pH tolerance was tested according to Park *et al*. [16]. The enumerated spray and freeze dried LAB culture was further grown in MRS broth at 37°C and cells were inoculated into 9 ml MRS broth of pH values 2 and 2.5 adjusted with 0.1N HCl. The initial bacterial concentration was 10^7- 10^9 cfu/ml and was determined by viable cell count on MRS plates. Samples were incubated at 37 °C for 4 h. After incubation, samples were serially diluted and plated on MRS plates for determining final viable count. The survival rate was calculated as percentage of colonies grown on MRS agar after incubation in comparison to that of initial bacterial concentration. Bile tolerance of the LAB was carried out as described by Park *et al.* [16] and Reddy and Prapulla [17]. The percentage survival of test sample was calculated according to equation 2.

$$\text{Percentage assimilation} = \frac{\text{log number of viable cells after treatment (cfu/ ml)}}{\text{log number of initial viable cells before treatment (cfu/ ml)}} \times 100 \quad (2)$$

Antimicrobial Activity: Antimicrobial activity was determined using the agar well diffusion method described by Toure *et al*. [18]. Test organisms; *E. coli* ATCC 31705, *E. coli* MTCC 108, *Listeria monocytogenes*, *Salmonella typhi*, *Salmonella paratyphi, Yersinia enterocolitica* were activated for 12 h by inoculation into nutrient broth and pour plated with nutrient agar. Spent culture supernatants (SCS) of *L. fermentum* grown in MRS broth was obtained and filtered through a 0.2 µm sterile filter and 100 µl of supernatant was added into the wells on the nutrient agar drilled with sterile 7 mm diameter driller. Plates were kept in refrigerator for 2 h to diffuse the spent broth and then incubated at 37°C for 24-36 h. Diameter of zone of inhibition was measured. Antibacterial activity of SCS fractions were classified as none (-), weak (+), middle (++) and strong (+++) according to the diameter of inhibition zone of 0-9, 10-19, 20 mm and above respectively.

Antibiotic Sensitivity: Antibiotic susceptibility was determined semi quantitatively in accordance to performance standards for antimicrobial disk susceptibility tests [19] and Charteris *et al*. [20]. MRS agar plates were prepared and 100 µl of active *L. fermentum* culture was spread over the plates. Plates were incubated for 2 h at 37°C. Antibiotic discs (Himedia, Mumbai, India) were placed on the above plates and incubated for 24 h at 37°C. Zone of inhibition was measured and reported as sensitive, intermediate or resistant according to the zone size interpretative chart provided with the antibiotics.

Cholesterol Assimilation: Cholesterol assimilation was studied by a modified Searchy and Bergquist method [21]. MRS broth containing 0.01% cholesterol and 0.3% bile and another set containing only 0.01% cholesterol were prepared. The tubes were inoculated with active culture of *L. fermentum* and incubated for 12 h at 37°C. Two MRS tubes without inoculation, one with cholesterol and bile and other with cholesterol alone were were kept as control. After incubation the samples were centrifuged at 8000 xg for 15 min. The supernatant was collected and residual cholesterol was extracted. The absorbance at 540 nm was recorded. Residual cholesterol in μg/ml and percentage assimilation of cholesterol was calculated using the formula.

$$\text{Percentage assimilation} = \frac{\text{Conc. of cholesterol in control} - \text{Conc. of cholesterol in test sample}}{\text{Conc. of cholesterol in control}} \times 100 \quad (3)$$

Presence of β- Galactosidase Activity: The method described by Karasova *et al*. [22] was used to test for the presence of β-galactosidase activity. The isolate was streaked on MRS agar containing 0.01% X-gal and 0.1 mM IPTG as an inducer. Plates were incubated at 37°C for 2 days. Colonies producing β-galactosidase appear blue in colour.

Statistical Analysis: Experiments were carried out in triplicates to evaluate the probiotic properties of the freeze dried and spray dried *L. fermentum*. The mean and standard deviation were calculated for n=3. One-way analysis of variance (ANOVA) at $P \leq 0.05$ was used to express the statistical differences between the treatment and the active cells. ANOVA was done using Origin 6 statistical software (USA).

RESULTS AND DISCUSSION

Strain Isolation and Characterization: Based on the biochemical characteristics described by Bergey's manual, the isolate was identified as *Lactobacillus fermentum.* However biochemical characterization may sometimes lead to ambiguity and also is not sufficient for establishing the taxonomic position of an organism. But for a commercial strain of LAB, which is to be used in food and pharma industries, investigation by means of molecular characterization is mandatory as per international standards. 16S rRNA partial gene sequencing for 471 nucleotides was thus carried out and indicated 98% identity with *Lactobacillus fermentum* strain (data not published). The above 16S rRNA sequence of the isolate was deposited in the gene bank and the accession number obtained is EU 263133 and the culture was deposited in the Central Food Technological Research Institute (CFTRI) Culture collection, CFTRI, Mysore, India as *L. fermentum* CFR -2192.

Probiotic Properties of Native Isolate: Earlier studies carried out in the laboratory, have clearly shown that *Kanjika* is a potent source of various LAB, possessing potent probiotic properties [13]. In the present study, the isolate *L. fermentum* was tested for low pH (2 and 2.5) and bile tolerance. These characteristics are important for probiotics, as acid tolerance is required for the bacteria to survive passage through the stomach and bile tolerance is required for survival in the small intestine [23].

According to Lin *et al*. [24] *L. fermentum* which was isolated from swine and poultry showed good acid tolerance at pH 2.5 and bile tolerance at 0.3% bile. Earlier studies on *L. fermentum* isolated from infant feces [25] have reported 60% of survivability at pH 2. In the present study the percentage survival of the isolate at pH 2 and 2.5 was found to be 88.73% and 96.41% respectively. This result indicates that the isolate shows good tolerance for low pH. When exposed to environments like mild acidification, lactic acid bacteria tend to protect themselves through the induction of a wide range of protective measures that alter cell membrane composition, extrude protons and protect macromolecules.

It is reported that certain species of Lactobacillus have the capacity to express bile salt hydrolase activity (BSHs) [26, 27]. BSHs hydrolyze the conjugated bile salt and protect the LAB from toxicity. This further helps in the survival of the strain in presence of intestinal bile (0.3%). The results obtained from the present study indicated that the percentage survival of *L. fermentum* was 93 in the presence of 0.3% bile salt. Since the isolate exhibited good acid and bile tolerance, additional probiotic properties were also studied. LAB has shown to inhibit growth of many enteric pathogens and can be used in humans to treat a broad range of gastrointestinal disorders [28].

In the present study, *L. fermentum* exhibited significant antimicrobial activity against some well-known food-borne pathogens (Table 1). *L. fermentum* was further checked for the antibiotic susceptibility and was found to be resistant to most of the antibiotics tested (Table 2). Thus suggesting that the organism would not be affected by therapies using these antibiotics and might help in maintaining the natural balance of intestinal microflora during antibiotic therapy. The strain was found to assimilate cholesterol to a significant extent (80%) in presence of bile and 60% in the absence of bile,

Table 1: Antimicrobial activity of freeze dried and spray dried *L.fermentum*

Antimicrobial activity of freeze dried *L. fermentum*					
Sample	E.coli ATCC 31075	E.coli MTCC 108	Salmonella typhi	Yersinia enterocolitica	Listeria monocytogenes
Native culture	+++	+++	+++	+++	+++
SF 1	+ + +	+ + +	+ + +	+++	+ +
SF 2	+ +	+ +	+ +	++	+ +
SF 3	+ +	+ +	+ +	++	+ +
SF 4	+ +	+ +	+ +	++	+ +
Antimicrobial activity of spray dried *L.fermentum*					
1% MDX	+ +	+ + +	+ + +	+++	+ + +
3%MDX	+ +	+ +	+ +	++	+ +
1%NFSM	+ +	+ +	+ + +	++	+ +
3%NFSM	+ +	+ +	+ +	++	+ +

Zone of inhibition in mm: +, between 0 to 9mm; ++, between 10-19mm; +++, >20 mm. SF1= Lactose 20%, SF2= Sucrose 20%, SF3= Maltodextrin 20%, SF4= Skimmed milk 20%, MDX= Maltodextrin, NFSM= Non-fat skimmed milk.

Table 2: Antibiotic susceptibility of freeze and spray dried *L. fermentum*

		Interpretative zone dia (mm)[a]							
Antibiotic	Disc conc. (µg)	Native cells	SF1	SF2	SF3	1% MDX	3%MDX	1%NFSM	3%NFSM
Penicillin G	10	19(R)	30(S)	30(S)	33(S)	25(S)	23(I)	33(S)	27(S)
Chloramphenicol	30	18(R)	34 (S)	38 (S)	34(S)	34 (S)	30 (S)	27(S)	29(S)
Co- trimaxazole	25	8(R)	25(S)	29 (S)	24(S)	25(S)	29 (S)	24(S)	21(S)
Tetracycline	30	0(R)	30 (S)	35 (S)	25(S)	21 (I)	27 (S)	25(S)	26(S)
Rifampicin	5	15(R)	25 (S)	32 (S)	24(S)	25 (S)	32 (S)	23(I)	24(S)

[a]In accordance to performance standards for antimicrobial disk susceptibility tests Zone of Inhibition in mm: (R) - resistant 0-19; (I) - moderately susceptible 20-23; (S) - susceptible >24. SF1= Lactose 20%, SF2= Sucrose 20%, SF3= Maltodextrin 20%, SF4= Skimmed milk 20%, MDX= Maltodextrin, NFSM= Non-fat skimmed milk

Table 3: Change in cholesterol level of freeze dried and spray dried *L. fermentum*

Change in cholesterol level of freeze dried *L. fermentum*		
	Concentration of cholesterol (µg/ml) [reduction rate of cholesterol][a]	
Cryoprotectant used for freeze drying (g/100ml)	0.3%	0%
Control	101.24 ± 0.98 (0)	101.24 ± 1.10 (0)
Native cells	20.24 ± 1.32 (81)	35.5 ± 0.85 (66)
SF1	28.35± 1.01 (73)	47.44 ± 0.56 (54)
SF2	28.73 ± 1.22 (72)	45.32 ± 0.89 (56)
SF3	24.33 ± 0.89 (77)	41.81 ± 1.20 (59)
SF4	25.24 ± 1.13 (76)	40.5 ± 1.05 (61)
Change in cholesterol level of spray dried *L. fermentum*		
Native cells	19.03 ± 1.32 (82)	36.5 ± 0.88 (65)
1% MDX	28.35 ± 0.98 (75)	45.45 ± 0.66 (56)
3%MDX	25.20 ± 1.42 (76)	45.32 ± 0.96 (56)
1% NFSM	25.28 ± 1.31 (76)	45.76 ± 0.77 (55)
3% NFSM	24.17 ± 1.0 (77)	40.5 ± 0.87 (58)

[a] Amounts of cholesterol were measured after 24 hrs incubation at 37°C, MRS broth containing cholesterol with (0.3%) or without bile salt. The results are mean of triplicates and the standard deviation. (n=3). Mean values of cholesterol assimilation of *L.fermentum* shows significant difference when compared to native cells (P = 0.05). SF1= Lactose 20%, SF2= Sucrose 20%, SF3= Maltodextrin 20%, SF4= Skimmed milk 20%, MDX= Maltodextrin, NFSM= Non-fat skimmed milk

suggesting that the strain has the potential to reduce serum cholesterol and triglyceride levels (Table 3). In addition, the strain was β-galactosidase positive and could probably be suggested for therapy application to individuals suffering from lactose intolerance [29].

Viability and Retention of Probiotic Properties of Freeze Dried *L. Fermentum*: As processing conditions associated with freeze drying are milder than spray drying, higher probiotic survival rates are typically achieved in freeze dried powders [30]. To and Etzel [31] demonstrated

that 60 to 70% of cells that survived the freeze drying conditions, can live through the dehydration step. According to Fowler and Toner [32], during freezing, gradual dehydration of the cells occur, as the ice is slowly formed outside the cell, which in turn leads to extensive cellular damage. A variety of cryoprotectants have been added to the drying media before freeze drying to protect cells against cellular damage [33]. As compatible cryoprotectants accumulate within the cells, the osmotic difference between the internal and external environments is reduced [34].

Trehalose has been shown to be an effective glass former. It is well documented that carbohydrates have protective effects for probiotic bacteria during freeze-drying, given that these cryoprotectants can raise the glass-phase transition temperature and therefore viable cells can reach the glassy phase without nucleating intracellular ice [32] and this partly explains the superior cryoprotective ability of the carbohydrates used in our studies.

In the present study four commonly used cryoprotectants viz. lactose, sucrose, MDX and NFSM have been used. The percentage survival and retention of probiotic properties of freeze dried *L. fermentum* (Fig. 1) clearly indicated that certain additives were more effective than others in their protective action. Maximum protection was achieved with skimmed milk (94%) followed by maltodextrin (89%) and lactose (82%). Minimum survival was observed in case of sucrose (76%). The results clearly indicated that freeze dried culture retained the key probiotic properties i.e. pH and bile tolerance.

Antibiotic susceptibility of freeze dried *L. fermentum* is shown in Table 2. The study revealed that resistance to some of the antibiotics was lost after freeze drying. This might be due to the damage to the cellular component of the cell wall which provided resistance to the organisms against the antibiotics. In contrast, all the combinations were able to retain the antimicrobial activity (Table 1); the ability of the freeze dried *L. fermentum* assimilate cholesterol is depicted in Table 3.

As can be seen from the results, the cholesterol assimilation property was not affected after freeze drying with any of the cryoprotectant used. However a significant ($P \leq 0.05$) reduction was found in comparison with the active cells (80% reduction) when freeze dried with MDX and NFSM (75-77% reduction). Also, *L. fermentum* exhibited better growth in the presence of cholesterol, indicating that cholesterol stimulated their growth. It is well known that probiotics are advocated for use to combat lactose intolerance. In this context it is important that the probiotic cultures retain this property after processing. We have found that the freeze dried *L. fermentum* with all the different cryoprotectants retained the β-galactosidase activity to a considerable extent. Results of this study indicated that freeze drying could be the method of choice for the preparation of stable form of *L. fermentum* used in this study.

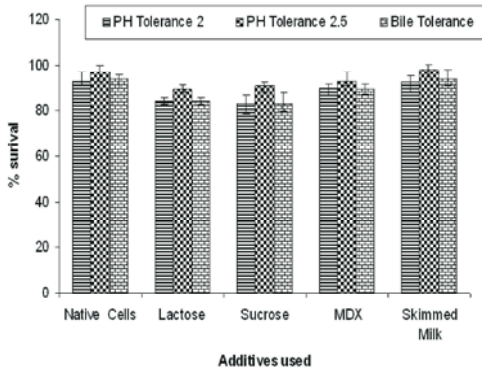

Fig.1: Effect of cryoprotectants on protection of pH tolerance (pH 2 & pH 2.5) and bile tolerance (0.3%) property of probiotic LAB after freeze drying. Results are represented as percentage of survival comparing with that of the cell concentration before and after acid and bile treatment. Values are mean for n=3 and SD are represented by error bars.

Viability and Retention of Probiotic Properties of Spray Dried *L. Fermentum*: Spray drying is one of the predominant processing tools used in dairy industry to produce large amounts of dairy ingredients relatively inexpensive, it has been estimated that the cost of spray drying is six times lower per kilogram of water removed than the cost of freeze drying [35]. But the disadvantage is extreme cell damage during the process and hence loss of survival of the organism during spray drying and storage was observed [36]. Inlet and outlet temperature could be one of the major reasons for cell damage/ death during spray drying [37]. The optimum temperature for the growth of most of the LAB is around 40°C. Considering this factor, the experiments were carried out at a constant outlet temperature of 40°C and corresponding inlet temperature of 140°C.

Using a rifampicin resistant variant of *Lactobacillus paracasei* NFBC 338, it was shown that percentage survival (>80) was achievable during spray-drying using RSM (Reconstituted Skimmed Milk), at outlet temperatures of 85-90°C [38], while under similar conditions (outlet temperature of 80°C), Ananta and Knorr

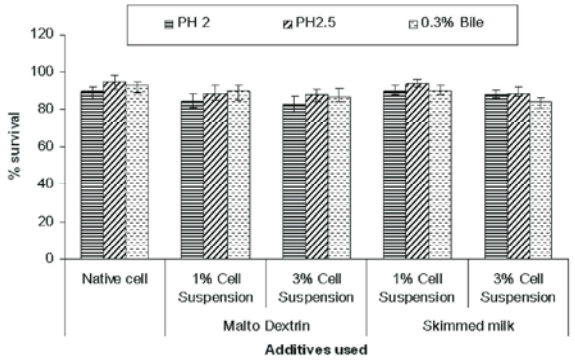

Fig. 2: Effect of cell concentration and carriers on acid tolerance (pH 2 and pH 2.5) and bile tolerance (0.3%) of spray dried *L. fermentum*: Results are represented as percentage of survival comparing with that of the cell concentration before and after acid and bile treatment. Values are mean for n=3 and SD are represented by error bars. The means of cell suspensions of both carriers MDX and NFSM shows significant difference ($P \leq 0.05$) when compared with native cells

[39] reported a percentage survival of >60 for *L. rhamnosus GG*. It has been shown that different bacterial species vary with respect to spray drying tolerance, highlighting the importance of strain selection. In this study, we have used two well known additives, MDX and NFSM at two different cell concentrations of 1% and 3%. The percentage survival was higher when 1% cells was spray dried with NFSM and MDX (88.1 and 86.5%) in comparison with that of 3% cells suspension (85.7 and 81.9%). Fig 2 depicts retention of probiotic properties in spray dried *Lactobacillus fermentum*. The probable reason for this behavior is that the ratio of additives to the cells is higher for 1% cells and thus the additive provided complete protection, whereas when 3% cell inoculum was dried with same concentration of additives, some cells might have remained unprotected and thus resulting in decreased viability. Spray drying also resulted in the loss of antibiotic resistance property against the same 5 antibiotics as discussed earlier (Table 2). The cultures retained the antimicrobial activity against all the food-borne pathogens tested as can be seen in table 1. The moisture content of the resultant spray dried powder was between 5-7%.

The ability of the spray dried *L. fermentum* in the reduction of cholesterol is depicted in Table 3. The result indicated that the reduction of cholesterol was slightly affected after spray drying with carriers, in comparison with that of native cells. However a significant ($P \leq 0.05$) reduction was found when 3% spray dried cells with maltodextrin and skimmed milk (76% reduction) in comparison with that of native cells (80% reduction).

In conclusion, the *in vitro* assessment proved the retainment of most of the key probiotic properties of *L. fermentum* after freeze and spray drying to a significant extent thus confirming it as a potent probiotic candidate for industrial applications.

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