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Research Article

SURVIVABILITY STUDY OF PROBIOTICS DURING GASTROINTESTINAL TRANSIT AFTER MICROENCAPSULATION BY SPRAY DRYING

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Abstract- Now a day's fortified foods with probiotics are more attractive due to their health benefits in human beings. Microencapsulation technique is a process to entrap a substance in a suitable material in order to generate particles with diameters of a few micrometers. The method of microencapsulation and concentration of coating material have a significant impact on the probiotic survivability and size of powder particle obtained. Milk protein (casein) used as coating material with spray drying process gave minimum powder particle size 347.7 d.nm (6% casein) in addition to obtain maximum survivability of 61.28% with 8% casein after 12 h exposure to gastric juice at pH 3.0 and in simulated bile salts at 1.0% with survivability of 85.64% (6% casein) in *in vitro* condition. The microencapsulated powders containing *L. fermentum* MTCC 8711 were stored at refrigerator (4°C) and room temperature (37°C) up to 30 days. The survivability of *L. fermentum* MTCC 8711 was found better at 4°C storage conditions which indicate that microencapsulated powder can be stored at refrigerated condition up to 20 days and storage up to 10 days at 37°C. However, they were remained in the level of recommendation probiotic cell population of 8.8×10^6 (4°C) & 4.7×10^5 (37°C) cfu/g at the end of 30 days, which was recommended for probiotic formulations.

Keywords- *Microencapsulation, Probiotics, Spray Drying, Milk Proteins, Survivability*

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Introduction

Lifestyle and dietary patterns are more important features for the general fitness of healthy people. Probiotic is a term that means "for life" and it was described by the Food and Agriculture Organization (FAO) of the United Nations and World Health Organization [1] which is defined as "live microorganisms which, when administered in sufficient amounts to confer a health benefit on the host". They are reported to take part in a therapeutic function by modulating immunity, improving lactose tolerance, lowering cholesterol, prevention of some cancers as well [2]. To generate these beneficial effects for health, probiotic have to be able to stay alive and multiply in the host. With this regard, it should be active, metabolically stable in the product or item, stay alive through the stomach and reach the digestive tract in bulky amount [3]. Certain species of lactobacilli and bifido bacteria are majorly used as probiotics [4]. However, other species such as, *Lactococcus lactis* ssp. lactis, *Lactococcus lactis* ssp. cremoris, *Enterococcus faecium*, *Leuconostoc mesenteroides* ssp. dextranicum as well as *Pediococcus acidilactici* have also been used to attain the same purposes, some yeast mainly *Saccharomyces cerevisiae* also used as probiotics [5]. The probiotic bacteria recommended in food should be in the range of 10^7- 10^9 colony forming unit per gram (cfu/g) [6]. In the improvement of safe and effective encapsulated product, it should be necessary to maintain the sufficient amount of viable cells, the shelf life of the food items or products and also during the gastrointestinal tract (GIT) transit after utilization [7]. Different parameters must be examined during the addition of probiotics in food such as, type of bacterial culture used, addition level required to have a physiological impact, survival to the process parameters, different protectants, stability during storage in addition to impact on the sensory properties [8]. In many functional foods, *Lactobacillus fermentum* MTCC 8711 is utilized as a probiotic strain. Because it has capability to avoid the adhesion of Methicillin-resistant *Staphylococcus aureus* (MRSA) to human colon adenocarcinoma cells [9].

Different strains of *L. fermentum* have shown highest cell surface hydrophobicity, high survivability up to four hours [10] and efficient riboflavin producing bacterium showed 2.29 mg/l of riboflavin within media (chemically defined) after 24 h [11].

The viability of probiotic organisms is the most important parameter as the sensitivity of probiotic bacteria affected severely in harsh conditions in GIT environment. Moreover, several factors have an effect on survivability of probiotic cultures in foods and supplements such as, dissolved oxygen, temperature, concentration of acids (lactic and acetic acid), bile salt, hydrogen peroxide (H_2O_2), buffers and digestive enzymes [12-14]. Thus, there is a need to develop technological applications for protecting the probiotic cells from such kind of intrinsic and extrinsic factors during processing and storage of foods as well as to improve survivability in human GI tract as control release mechanism.

Microencapsulation appears to be a promising technique because this technique relies on applications and parameters such as, particle size, control release mechanisms, physicochemical properties of coating materials and the core as well as process cost [15]. This technique could potentially stimulate the survival rate of probiotic bacteria within food systems and increasing the scope of applications. In this process, incorporation of probiotic cells into coating materials or an encapsulating material that can keep the cells from ruination by the harmful factors and release at controlled rates under specific conditions [16].

The main purpose of microencapsulation is to protect probiotics from bile salts, low pH and other constituents that meet during GIT [17]. Moreover, several benefits of microencapsulation of probiotic bacteria comprise of; they are protected from freezing or chilling [18], protection from intrinsic factors (*i.e*., pH, organic acids, water activity), bacteriophages, protection from oxygen (O_2) [19], storage condition [20] and also acidic condition in GIT [21] in addition to converting probiotic cells into a powder form that easy to use, as it enhances their uniform delivery throughout the end product [22].

There are number of techniques for microencapsulation, but spray drying is one of the advances, most common and low-cost method to form microencapsulated food materials through which a liquid item is atomized in a hot gas current to promptly obtain powder form. The energy utilization during spray drying is 6 to 10 times lesser than freeze drying and producing a good quality end product. In comparison to freeze-drying, the expense of spray-drying process is 30-50 times lower [23]. Particles size of 2 to 3 microns was acquired at higher atomization gas flow rates, representing initial bead size of 4 to 7 microns at most whereas lower flow rates gave significantly bigger particles with wider size distribution [24].

Different types of coating materials used for microencapsulation are generally recognized as safe (GRAS) ingredients that can be utilized in food applications [25]. There are various food grade materials which have been used for microencapsulation of probiotic for example, soluble starch, polydextroses, maltodextrins, pectin, cellulose, acacia gum or xanthan gum, alginate, carrageenan and among proteins *i.e*., casein, gelatin, skim milk as well as whey protein [26-27]. Milk proteins have excellent functional properties and act as good quality covering material for microencapsulation by spray-drying. Furthermore, proteins have high binding characteristics for the flavor components [28]. Gelatin and milk proteins (Casein or Whey) are most common proteins used as encapsulating food ingredients via spray-drying. Casein protein is used as a coating agent for hyprol encapsulation because of the advantages to select food protein matrices contain their abundant renewable sources, high nutritional value in addition to acceptable as a naturally arising food constituent which can degrade by some enzymes [29]. So, the present study is an attempt to explore the spray drying for microencapsulation of probiotics with milk proteins to improve survivability during gastrointestinal transit in *in vitro* condition.

Materials and Methods

Revival of probiotic culture

Freeze dried culture of *L. fermentum* MTCC 8711 was inoculated in MRS broth and incubated at 37°C for 24 h. The fresh culture was prepared by adding 1% inoculums to MRS broth and grow again under the similar conditions for 24 h. In late exponential phase the culture was separated by centrifugation at 10,000 rpm for 15 min. The cell pellet was washed twice with 0.90 % (w/v) sodium chloride (NaCl). *L. fermentum* MTCC 8711 culture stock were kept in 10% glycerol and lyophilized vial at -20°C for further study.

Feed solution preparation for spray drying

For preparation of feed solution for spray drying, initially overnight culture of *L. fermentum* MTCC 8711 was cultured into MRS broth and incubated at 37°C in anaerobic condition for 24 h to 48 h. Separate out the probiotic cells by using centrifuge at 4°C then rinse with sterile saline solution for further used for microencapsulation by spray drying with casein proteins as a coating material.

Preparation of casein solution

In present study, casein milk protein was also used as coating material which was procured from Himedia Laboratories Pvt. Ltd. (Mumbai, India) and *L. fermentum* MTCC 8711 culture was used as probiotic strain. Three types of feed solutions were prepared with different concentration of casein such as, 6% casein, 8% casein and 10% casein. The mixture of casein was previously sterilized in vertical autoclave at 121°C for 15 min and then the pH was adjusted to 8.0 ± 0.1 using 1.0M sodium hydroxide (NaOH) solution under constant mechanical agitation to dissolve it properly [30]. The fresh probiotic culture of *L. fermentum* MTCC 8711 (3% w/v) was added to the casein mixture and all these feed solutions were directly used for spray drying for microencapsulation.

Microencapsulation of probiotic *L. fermentum* MTCC 8711 by spray drying

Microencapsulation of *L. fermentum* MTCC 8711 was carried out using lab scale spray dryer SPD-P-111 (Technosearch instruments, Thane, India). The spray drying system consisted of drying chamber, blower, air heater, scrubber, feed pump, cone, collection bottle and two cyclones (Primary and secondary). The inlet air, heated to 105°C by an electrical heater, flow rate was maintained at 1.5 mL/min and drying chamber with an outlet temperature of 80°C. Feed solution was delivered by a peristaltic pump into a fluid stainless steel atomizer. The spray dried powder was collected at the bottom of a cyclone, packed in polythene terephthalate bags by using modified atmospheric packaging machine (MAP 430-GS, mfg. by Elixir technologies), sealed and wrapped with aluminum foil and stored at 4°C & 37°C. Spray drying of *L. fermentum* MTCC 8711 was carried out with different feed solutions (6%, 8%, 10% casein) as per parameters indicated in [Table-1].

Table-1 Different parameters for spray drying system

SN	Different Parameters	Different coating materials Casein
1	Inlet temperature (°C)	90
2	Outlet temperature (°C)	60
3	Temperature of plate (°C)	35
4	Cooling temperature (°C)	50
5	Aspiration flow rate (Nm^3/h)	70
6	Feed pump flow rate (ml/min)	1.5
7	Stirrer speed (rpm)	15

Morphological study of microencapsulated powder

Morphology analysis under Scanning Electron Microscope (SEM)

All the microencapsulated powder samples (6% casein, 8% casein and 10% casein) were carried out for size and surface morphology analysis with the help of scanning electron microscope. Small amount (~2 mg) of powder samples were used for analysis. In this process the sample was coated with gold with the help of vacuum sputtering machine EMITECH SC 7620 sputter coater at 500 kv for 4 min and pressure currant was 10mA. Individual powder sample was put in sample holder (aluminium stub). Microscopic analysis of individual powder samples was carried out by using ZEISS EVO-18 SEM having acceleration voltage of 15 kW. An individual powder sample was fixed on aluminum stub with double-sided adhesive tape. SEM image data of powder was collected over a selected area of the powder samples and 2D image was visualized that display properties comprise shape, size and texture of powder samples [31].

Determination of particle size of microencapsulated powder

Particle size analyzer were used for the size and distribution of the microencapsulated powder (Zetasizer Nano-series ZS90, Malvern Panalytical Ltd., UK). Powder samples having different concentrations (6% casein, 8% casein and 10% casein) were analyzed by dissolving in distilled water. Distilled water used as a dispersing medium and taken as a reference having refractive index-1.33, viscosity-0.7920 and dielectric constant-76.75. Disposable four- side plain cuvettes were used under an operating temperature of 30°C. The average particle size was carried out in triplicate for each solution.

Purity of *L. fermentum* MTCC 8711 in microencapsulated powder

The purity and probiotic property of microencapsulated powders were confirmed using Gram reaction, catalase test and motility test.

Survival of microencapsulated *L. fermentum* MTCC 8711 cells in Gastrointestinal Transit (GIT) condition

Survival in simulated gastric juice (SGJ) condition

As per method given by Krasaekoopt *et al*. (2004) [33] was used with some modifications. Simulated gastric juice condition was prepared (*in vitro*) by taking 10 ml MRS broth with different pH (3.0, 5.0 and 7.0) adjusting by using 0.5N hydrochloric acid and sterilized it at 121°C for 15 min. 0.5g of microencapsulated powders were dissolved in 9.5 mL of sterile simulated gastric juice and incubated at 37°C for 12 h with constant agitation at 50 rpm in incubator with shaker (Make: REMI Elecktrotechnik). Then after, 0.1mL culture was spread on MRS agar plate for observation of gastric juice tolerance capacity, if bacterial cells are grown in plate or tubes which considered as gastric juice tolerance.

Survival in simulated intestinal fluids (SIF) condition

To evaluate the intestinal fluids tolerance capacity of *L. fermentum* MTCC 8711, method suggested by Krasaekoopt *et al*. (2004) was used with some modification. In this process, simulated intestinal fluid condition (*in vitro*) was prepared by dissolving bile salt (Hi Media Laboratories, Mumbai, India) in 10 mL MRS broth

with different concentrations (0.5%, 1.0% and 1.5%) by adjusting the pH 8.0 with 0.1 N NaOH and sterilized at 121°C for 15 min. 0.5g of microencapsulated powders were dissolved in 9.5 mL of sterile simulated intestinal fluids and incubated at 37°C for 12 h with constant agitation at 50 rpm in a incubator with shaker (Make: REMI Elecktrotechnik). Then after, 0.1mL culture was spread on MRS agar plate for observation of intestinal fluids tolerance capacity, if bacterial cells are grown in plate or tubes which considered as intestinal fluids tolerance.

Water activity and moisture content of microencapsulated powder

After spray drying, the water activity of microencapsulated powder samples *i.e*., 6% casein, 8% casein and 10% casein was measured by using a water activity meter at 27°C (novasina, Lab swift aw, Switzerland).

The average moisture content of microencapsulated powder samples were determined by oven drying at 102°C for 2h. Moisture content was analyzed by determination of the difference in weight before and after drying, expressed as a percentage of the initial powder weight [34]. In this method, known mass of sample (0.5 g) was taken in aluminum dish and dried in a hot air oven (Make: Macro scientific works Pvt. Ltd.) at 102 ± 2°C until it reaches constant weight.

Moisture content (%) = (weight of samples before drying–weight of samples after drying) × 100

Survivability of microencapsulated probiotic cells at different storage time

For the enumeration of viable counts, take 1.0 gm of microencapsulated powder samples and added to 9 mL of saline sterile solution, the serial dilutions were prepared before spreading on to the MRS agar plate. This sample solution was stirred on the magnetic stirrer to dissolve the powder and release of entrapped probiotic bacteria. The plates were incubated for 24 - 48 h at 37°C and the microencapsulated probiotic cells were enumerated with the help of digital colony counter.

Microencapsulated powders were packed in polyethylene terephthalate bags with the help of modified atmospheric packaging machine and kept at 4 °C & 37°C for the time interval of 0 day, 10 days, 20 days and 30 days. The survivability of *L. fermentum* MTCC 8711 was determined for their stability at given specific time intervals.

Statistical Analysis

The collected data were subjected to statistical analysis. Data were analyzed by analysis of variance (ANOVA) and critical difference test at 5% level of significance ($P<0.05$) to compare the different treatments means with the help of SPSS software version 26.0 (SPSS, Inc., Chicago, IL, USA).

Results and Discussion

Microencapsulation of *L. fermentum* MTCC 8711

Microencapsulation of *L. fermentum* MTCC 8711 was carried out using lab scale spray dryer SPD-P-111 (Technosearch instruments, Thane, India). In this exploration, the prepared feed solutions were directly utilized for microencapsulation process and the process of microencapsulation is depicted in section, materials and methods with different parameters of spray drying like inlet temperature, feed flow rate and outlet temperature. Then microencapsulated powders were examined and analyzed for further analysis such as surface morphology study by scanning electron microscope, powder particle size by particle size analyzer, survivability in gastrointestinal tract and during storage, water activity and moisture content determination.

Surface morphology study of microencapsulated powder with Scanning Electron Microscope

Different microencapsulated powder was characterized for surface morphology by scanning electron microscopy (SEM) and it has also evaluated the micro structure of different coating materials (*i.e*., 6% casein, 8% casein, 10% casein). The size of microencapsulated powder particle was obtained in the range of 1.070µm to 4.9450µm. The casein protein, the minimum size of microencapsulated powder particle was 1.070µm with 6% Casein [Fig. 1A] and maximum size of microencapsulated powder particle was 4.9450µm with 10% Casein (Fig. 1C). SEM images which shown in [Fig-1] highlighted that probiotic cells were not observable in microencapsulated powder and it indicate that the coating materials used in present study entirely encapsulated the probiotic bacterial cells.

The surface morphology of microencapsulated powder with casein was found spherical shape with concavities and no cracks on the wall material which was similar to Oliveira *et al*. (2007) in which they used casein/pectin complex as the wall material to encapsulate Bifidobacterium lactis and Lactobacillus acidophilus. Moreover, it can lower permeability of air that gives superior protection of probiotic cells and enhance survivability during storage.

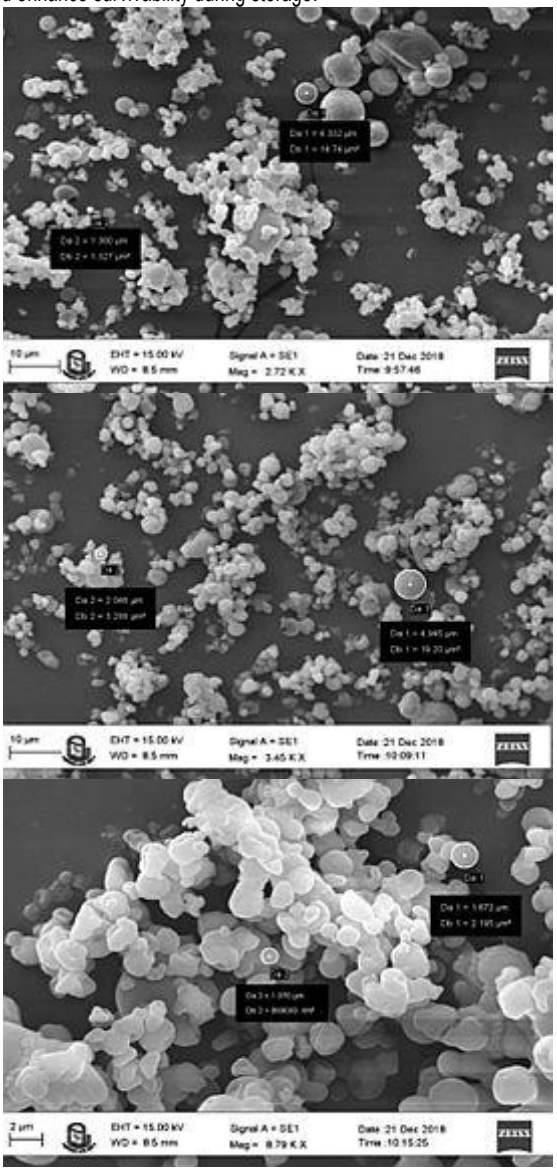

Fig-1 SEM photomicrography of microencapsulated powder containing L. Fermentum MTCC 8711 with different coating materials (A) 6% casein, (B) 8% casein and (C) 10% casein

Sheu and Rosenberg (1998) [35] reported that surface of microcapsules was entirely smooth without any cracks shows the smooth surface texture of whey protein and it also prevents loss of coating material. In this study, they observed as concentration of coating material increased, it generates concavities in microencapsulated powder. Our results are similar to them.

Particle size study of microencapsulated powder

Particle size and distribution are very important physical characteristics which has direct impact for its successful utilization in food fortification. The particle size was analyzed through particle size analyzer (Zetasizer Nano-series ZS90, Malvern Panalytical Ltd., UK). In this study, size of microencapsulated powders particle with casein protein, the size of microencapsulated powder particle was exhibit in the range of 340.0 d.nm to 550.0 d.nm.

The minimum size of microencapsulated powder particle was 347.7 d.nm with 6% casein (Fig. 2A) and maximum size of microencapsulated powder particle was 541.4 d.nm with 10% casein (Fig. 2C).

Table-2 Particle size of microencapsulated powders using different coating materials

Microencapsulated powder with different coating material		Particle size (d.nm)
Casein	A. 6%	347.7
	B. 8%	519.8
	C. 10%	541.4

[Table-2] summarizes the size of particles of different microencapsulated powder samples using different coating materials. In general, microencapsulated powder with large particle size gives additional protection to probiotic bacteria as compared to microencapsulated powder particles with small size, because it contains low concentration of coating material.

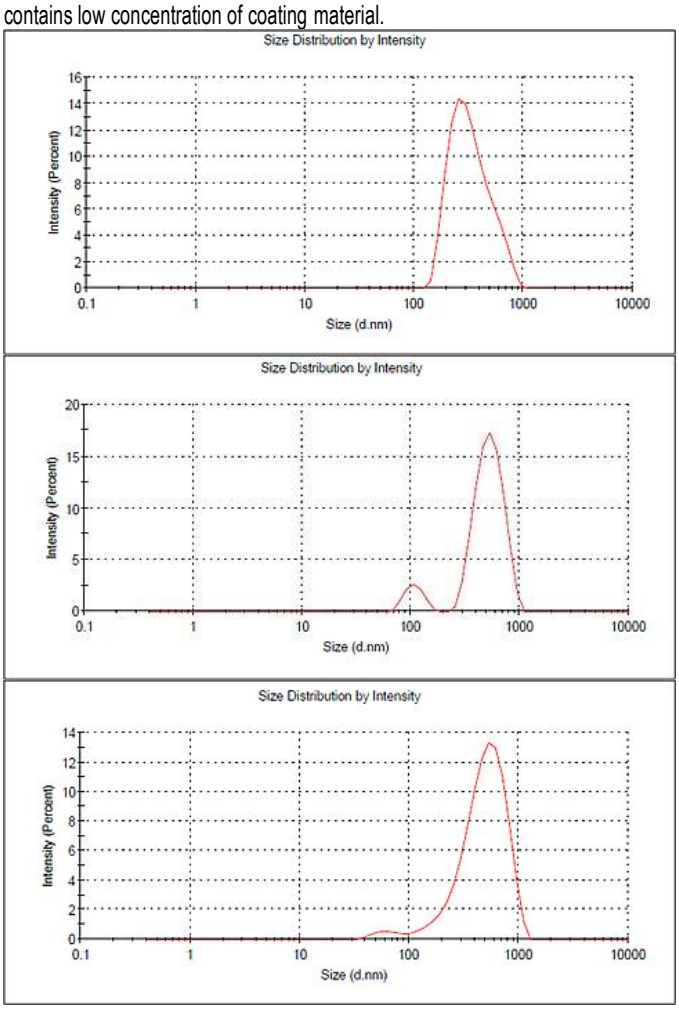

Fig-2 Particle size and size distribution by intensity of microencapsulated powder using different coating materials (A) 6% casein, (B) 8% casein and (C) 10% casein

Purity of *L. fermentum* MTCC 8711 in microencapsulated powder

To check the contamination during process and storage periods in microencapsulated powder, purity study of *L. fermentum* MTCC 8711 was carried out by three tests (Gram staining, catalase test and motility test) to confirm the presence of any other type of contaminants. After performing Gram staining, the observation of glass slide by using optical microscope (Make: Labomed) under oil immersion lens and it was observed that all cells are gram positive with rod shape and occurring in pairs. It confirmed that there was no any other contamination in the end products. In catalase test, the probiotic cells did not produce air bubbles which indicates the negative result as well as probiotic cells were found non-motile during the observation of cavity slide under 40X and after in oil immersion lens. Thus, our results found at par to Thummar and Ramani (2016) [36].

Survival of microencapsulated probiotic culture cells in Gastrointestinal Transit (GIT) condition (*in vitro* study)

Survival of probiotic cells in simulated gastric juice (SGJ)

Ability of probiotics to tolerate digestive system is one of the most significant properties for the successful incorporation of probiotic cells into functional food. For this study, survivability of microencapsulated probiotic cells was carried out in simulated gastric juice at different pH (3.0, 5.0 & 7.0) for 12 h incubation at 37°C (*in vitro* study). Invariable loss was observed in survivability of free probiotic cells which indicates the requirement of protection or encapsulation. Increasing order of percentage survivability (%) of microencapsulated *L. fermentum* MTCC 8711 with casein protein coating materials as follows:

8% casein (61.28%) > 10% casein (56.56%) > 6% casein (43.41%) > free cells (41.69%)

The survivability of *L. fermentum* MTCC 8711 was decreased after 12 h exposure to gastric juice at pH 3.0, 5.0 & 7.0 but at pH 3.0 all the samples observed precipitated and no growth found. We found maximum survivability of 61.28% with 8% casein (4.86 log cfu/g) and minimum survivability of 43.41% with 6% casein (3.41 log cfu/g) which was depicted in [Table-3] and [Fig-3]. Our results are opposition to Oliveira *et al.* (2007) who revealed that the microencapsulation of L. acidophilus and B. lactis by coacervation using casein/pectin complex followed by spray drying was helpful to protect these probiotic organisms against low acid conditions (pH-1 & 3).

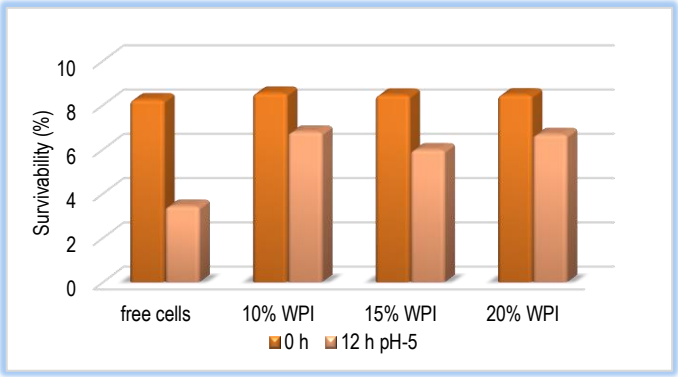

Fig-3 Survivability (log number) of microencapsulated L. fermentum MTCC 8711 during incubation period at pH-5.0 for casein with different concentration of coating materials

As per observations, if we compare both of these coating materials (casein) for microencapsulation of *L. fermentum* MTCC 8711 by spray drying it concluded that 8% casein give better protection to this probiotic at low pH.

Survival of probiotic cells in simulated intestinal fluids (SIF) condition

For this study, survivability of microencapsulated *L. fermentum* MTCC 8711 was carried out in simulated intestinal fluids condition (*in vitro*) with different bile salt concentration (0.5%, 1.0% & 1.5%) at 37°C for 12 h. We observed that it can provide better protection in simulated intestinal fluids condition as compared to acidic condition and can be able to liberate probiotic cells in the intestine of GIT. In this study, increasing order of percentage survivability (%) of microencapsulated *L. fermentum* MTCC 8711 with 1.0% bile salt concentration as follows:

6% casein (85.64%) > 8% casein (80.83%) > 10% casein (68.54%) > Free cells (42.42%)

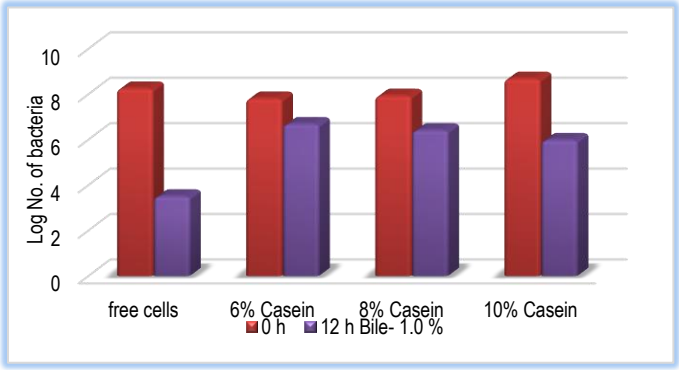

Fig-4 Survivability (log number) of microencapsulated L. fermentum MTCC 8711 during incubation period with 1.0% bile salt mixture with different concentration of coating materials

We found maximum survivability of microencapsulated *L. fermentum* MTCC 8711 of 85.64% with 6% casein (6.68 log cfu/g) coating material and minimum survivability of 68.54% in case of 10% casein (5.98 log cfu/g) coating material after 12 h exposure to 1.0% bile salt concentration [Table-3] & [Fig-4]. Dimitrellou *et al.* (2016) [37] studied survivability of microencapsulated Lactobacillus casei in bile salts condition using reconstituted skim milk as a wall material and they reported higher viability of L. casei cells in bile salts even after 6 h of incubation, our results are similar to them for this study. As per observations shown, if we compare both the coating material (casein) for survivability of microencapsulated *L. fermentum* MTCC 8711 in simulated intestinal fluids condition, coating materials give better results to survive.

Water activity and Moisture content of microencapsulated powder

In the current study, we found the water activity values of all microencapsulated powder samples in the range 0.19 to 0.31 at 0 day to 30 days storage time which shown in [Table-4]. Reyes *et al.* (2018) [38] stated that if water activity is less than 0.1, cell membrane lipids could be oxidized that lead to decrease viability of cells, our results are opposition to them. On the other hand, Manojlovi´c *et al.* (2010) [39] reported that water activity values around 0.2 have determined as an ideal value for the survival of microorganisms during storage period and for probiotics around 0.25 aw should be suggested for long-lasting storage and our results are at par to them. Fazilah *et al.* (2019) [40] studied the effect of spray drying for microencapsulation of Lactococcus lactis Gh1 with gum Arabic and *Synsepalum dulcificum* and they found aw below 0.3, our results are affirmation to them for this study. The viability of microorganisms was influenced by moisture content in dried products. In this exploration, we found the result of moisture content for different microencapsulated powder in the range of 3.86% to 5.10%. The result of sample 10% casein showed highest moisture content as compared to other sample after spray drying (0 day) [Table-4]. We observed that the moisture content of microencapsulated powder were increase from 0 day to 30 day.

In this study, our results are opposition to Rajam *et al.* (2012) [41] and Oliveira *et al.* (2007) in which they obtained the moisture content value of 2.90% to 3.60% and 9.50% to 11.0% respectively by using WPI and casein/pectin as wall material.

Survival of microencapsulated probiotic cells at different storage time

To check the survivability of microencapsulated *L. fermentum* MTCC 8711 using two different coating materials with different concentration *i.e.*, 6% casein, 8% casein, 10% casein. The viable count of microencapsulated *L. fermentum* MTCC 8711 was found more in all coating materials and remained more than 10^8 cfu/g. In this study, we have obtained the viable cell count of 6.5×10^8 cfu/g in case of casein (6% Casein) coating material after spray drying (0 day).

Similarly, viable counts for all other coating materials were mentioned in [Table-5]. Then the microencapsulated powders were packed in pre sterilized polyethylene terephthalate bags using modified atmospheric packaging (MAP) machine and stored under refrigerator condition (4°C) and room temperature (37°C). Survivability of microencapsulated cells was checked at different time interval of 0 day, 10 days, 20 days and 30 days storage shown in [Table-5] and [Fig-5 & 7].

The viable count of microencapsulated cells under refrigerated (4°C) condition remained almost stable up to 20 days and then decreased in both coating materials. Similarly, viability of probiotic cells was found stable up to 10 days at room temperature (37°C) and then gradually decreased at the end of 30 days [Fig-5]. Initially, we found viable counts of 7.2×10^8 cfu/g (0 day)after spray drying and then they were remained higher viable count *i.e.*, 8.8×10^6 cfu/g at 4°C and 4.7×10^5 cfu/g at 37°C as compared to 8% casein and 10% casein at the end of 30 days storage. Similarly, the viable cell counts at different temperature in different time interval were shown in [Table-5].

The survivability of *L. fermentum* MTCC 8711 was found better at 4°C storage conditions which indicate that microencapsulated powder can be stored at refrigerated condition up to 20 days and storage up to 10 days at room temperature. The results obtained are slightly similar to result of Thummar and Ramani (2016) who studied the viability of *L. fermentum* MTCC 8711 using soymilk maltodextrin and non-fat skim milk as an encapsulating material for microencapsulation by spray drying.

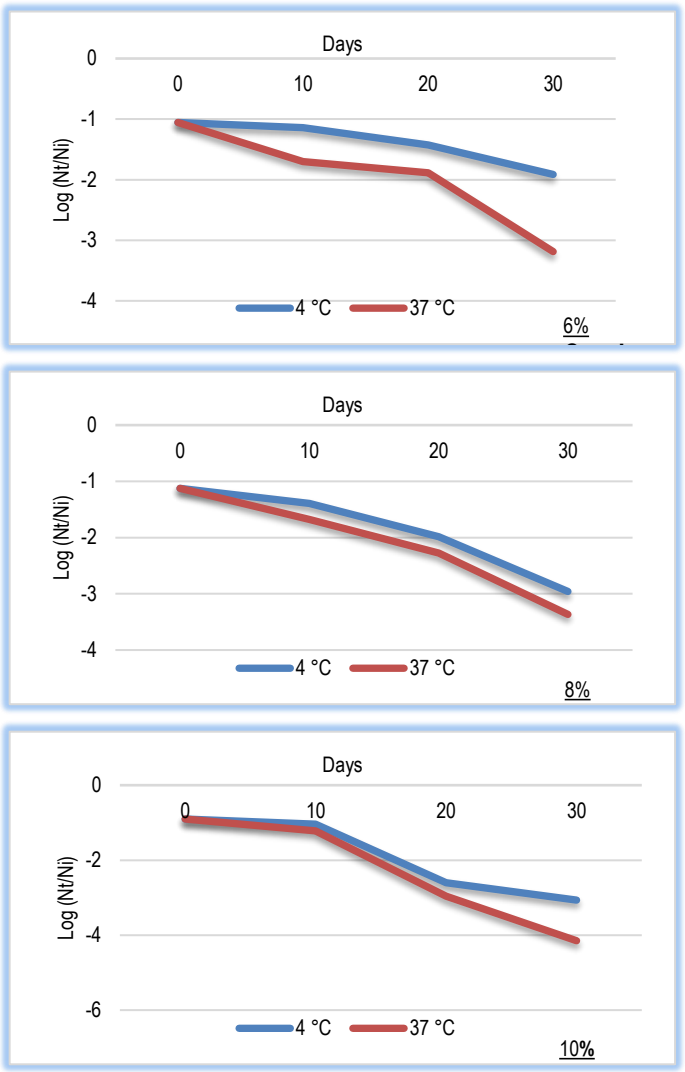

Fig-5 Survivability of microencapsulated L. fermentum MTCC 8711 at different storage time at 4°C and 37°C with 6% Casein, 8% Casein and 10% Casein

Statistical analysis

Statistical analysis was conducted using analysis of variance (ANOVA) through SPSS 26.0 software (SPSS, Inc., Chicago, IL, USA) with statistical significance difference value determined at $p \leq 0.05$. One-way analysis of variance followed by least significant difference test was used to determine significant difference in microencapsulated powder particle size. In the present exploration, various concentration of milk proteins (Casein) and microencapsulation method (*i.e.*, spray drying) are taken as independent variables while particle size is taken as dependent variables for analysis.

Effect of concentration of casein on the size of microencapsulated powder particle by ANOVA

ANOVA was also carried out to find the effect of concentration of casein on the particle size. It was found that particle size using casein as a coating material ranges from 347.8 d.nm to 351.7 d.nm with average value of 351.06 d.nm in case of 6% casein, in case of 8% casein particle size ranges from 518.2.2 d.nm to 519.8 d.nm with average value of 520.36 d.nm and in case of 10% casein it ranges from 537.2 d.nm to 541.4 d.nm with average value of 541.6 d.nm that is depicted in [Table-6]. It was observed that different concentration of casein and microencapsulation process has significant effect on the microencapsulated powder particle size. As the strength of casein concentration increase the particle size of microencapsulated powder was significantly ($p<0.05$) increase. However, the significant difference of powder particle size could be increased with casein concentration (Fig. 6). Thus, it could be concluded that particle size was directly proportional to concentration of casein.

Table-3 Survivability (%) (log number cfu/g) of free and microencapsulated L. fermentum MTCC 8711 in simulated gastric juice using acid (in vitro study)

Samples	Conc.	Tolerance condition & Incubation time					
		0 h		After 12 h at Acid (pH- 5 for casein)		After 12 h at Bile salt (1.0%)	
		Log no.	Survivability (%)	Log no.	Survivability (%)	Log no.	Survivability (%)
Free cells	Free	8.25	100	3.44	41.69	3.5	42.42
Casein	6%	7.80	100	3.41	43.41	6.68	85.64
	8%	7.93	100	4.86	61.28	6.41	80.83
	10%	8.68	100	4.92	56.56	5.98	68.54

Table-4 Water activity (aw) and moisture content of microencapsulated powder with L. fermentum MTCC 8711 using different coating materials

Storage time (After spray drying)	Storage temperature	Different coating materials	Different Parameters	
			Water activity (a_w) (at 27 °C)	Moisture content (%)
0 day		6% Casein	0.19	3.86
		8% Casein	0.29	4.78
		10% Casein	0.26	4.96
After 30 days	4 °C	6% Casein	0.22	4.00
		8% Casein	0.30	4.9
		10% Casein	0.28	5.00
	37 °C	6% Casein	0.24	4.26
		8% Casein	0.32	4.96
		10% Casein	0.28	5.10

Table-5 Storage stability study of microencapsulated L. fermentum MTCC 8711 before, after, at 0 day, 10 days, 20 days and 30 days

Coating material		Viable cell Count (cfu/g)							
		Before spray drying	After spray drying (0 day)	After 10 days storage		After 20 days storage		After 30 days storage	
				4 °C	37 °C	4 °C	37 °C	4 °C	37 °C
Casein %	6	7.2×10^8	6.4×10^7	5.2×10^7	1.5×10^7	2.7×10^7	9.3×10^6	8.8×10^6	4.7×10^5
	8	6.4×10^9	4.8×10^8	2.6×10^8	7.6×10^8	6.7×10^7	3.4×10^7	7.1×10^6	2.8×10^5
	10	5.2×10^9	6.5×10^8	7.9×10^8	3.2×10^8	1.3×10^7	5.8×10^6	4.5×10^6	3.7×10^5

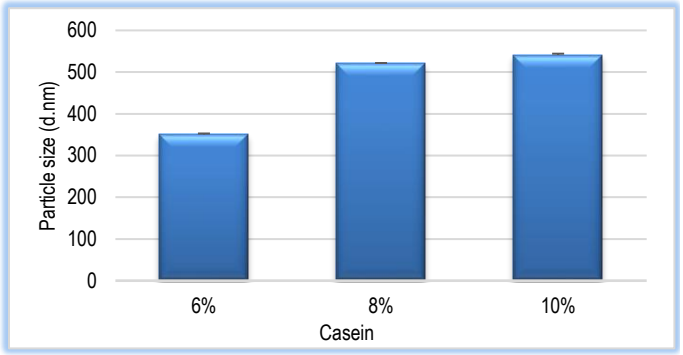

Fig-6 Effect of casein with different concentration on the size of microencapsulated powder particle

Table-6 Effect of Casein on the size of microencapsulated powder particle

Sample	Concentration	Range* (d.nm)	Average ± SE (d.nm)
Casein	6%	347.8 - 351.7	351.06 ± 1.791[a]
	8%	518.2 - 519.8	520.36 ± 1.446[b]
	10%	537.2 - 541.4	541.6 ± 2.598[c]
*Data represent mean±SE of three determinations. CD value: 7.07			

Conclusion

The purpose of this investigation was to improve the survivability of probiotic during gastrointestinal transit with different concentration of coating material *i.e.*, milk proteins and to explore the spray drying process which gives the minimum particle size and the maximum survivability. The process of microencapsulation was carried out with milk proteins as coating materials because of its excellent functional properties, high binding characteristics for the flavor components, high nutritional value. Spray drying technique was used for microencapsulation because of it decrease the transportation and storage costs, lower process cost, avoiding chances of biological & chemical degradations. Milk protein (casein) used as coating material with spray drying process gave minimum powder particle size 347.7 d.nm (6% casein) in addition to obtain maximum survivability of 61.28% with 8% casein after 12 h exposure to gastric juice at pH 3.0 and in simulated bile salts at 1.0% with survivability of 85.64% (6% casein) in *in vitro* condition. The microencapsulated powders containing *L. fermentum* MTCC 8711 were stored at refrigerator (4°C) and room temperature (37°C) up to 30 days. The survivability of *L. fermentum* MTCC 8711 was found better at 4°C storage conditions which indicate that microencapsulated powder can be stored at refrigerated condition up to 20 days and storage up to 10 days at 37°C. However, they were remained in the level of recommendation probiotic cell population of 8.8×10^6 (4°C) & 4.7×10^5 (37°C) cfu/g at the end of 30 days. The survivability was shown at refrigerator temperature than room temperature and they were remained 2.4×10^7cfu/g which was recommended for probiotic formulations. From the present exploration it is recommended to use spray drying process for microencapsulation of probiotic bacteria. Milk protein (casein) can be used to get micro particles which can be stored even at room temperature (37°C) conditions with higher survivability up to 10 days. Further research can be tackled to use this microencapsulated powder to expand various functional food products for effective intake of probiotics.

Application of research: Study of use spray drying process for microencapsulation of probiotic bacteria

Research Category: Dairy Microbiology

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Study area / Sample Collection: College of Dairy Science, Amreli, 365601

Cultivar / Variety / Breed name: Dairy cattle

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