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Microencapsulation to enhance the storage stability of *Lactobacillus rhamnosus GG*

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Abstract
Probiotics are the microorganism which when administered in sufficient amount confers health benefits. The major challenge encountered is the viability during administration and storage stability. In order to overcome these limitation *Lactobacillus rhamnosus GG* were encapsulated by ion gelation method using Pectin and sodium carboxymethyl cellulose (NaCMC) separately. The developed encapsulated cells were coated using two different polymers, Eudragit S 100 and cellulose acetate phthalate (CAP). Encapsulated cells were stored at (30 ± 2 °C/65 ± 5 % RH) for 6 months known as intermediate term testing conditions (ITC) and at (40 ± 2 °C/75 ± 5 % RH) for 6 months for accelerated testing conditions as per ICH Guideline Q1A. The encapsulated *Lactobacillus rhamnosus GG* have shown better viability after long term stability study compare with the free cells. Thus, encapsulation could be a potential system for storage and administration of probiotics.

Keywords: Ion gelation, microencapsulation, probiotics, stability

Introduction
According to World Health Organization (WHO), probiotics are the live microorganism which when administered orally in an appropriate amount (>10^7 CFU/g of the finished product or be eaten in sufficient amounts to yield a daily intake of 10^8 CFU) results into health benefits [1]. Probiotics offer various benefits, such as it keeps the gut microflora healthy, relieving constipation, enhance immune system, inhibit the growth of negative bacteria, helps in vitamin synthesis, control of lactose intolerance symptoms, reduction of blood cholesterol levels and improve absorption of calcium [2]. The challenges encountered while administering probiotic orally which limits their beneficial effects are low pH in the stomach, effect of hot immune system and competition with other pathogenic bacterias [3]. Today, encapsulated lyophilized probiotics are used commercially to protect the viability of the bacteria from the external environment [4]. However, the effectiveness of these encapsulated lyophilized probiotics in improving probiotic effect in the host is still under study.

In order to exert its positive effect, the probiotic must survive during its passage through the oesophagus and acidic environment of the stomach and during storage. The potential of microencapsulation in improving probiotic viability during their gastrointestinal transit and storage have been mentioned in various reviews [5, 6, 7]. To date, various techniques have been used for probiotic microencapsulation, such as emulsion [8, 9], Extrusion [10], spray-dried [11] and ionic gelation technique 12(Chun *et al.* 2014). Recently, microencapsulation of probiotic was studied, using pectin [12], whey protein [14], chitosan-alginate [15, 16] alginate and whey protein and isomaltooligosaccharide [17].

Compared to free or unencapsulated probiotic, the encapsulated probiotic showed an increase in the probiotic viability in the gastric environment. Studies have shown that the coating of the probiotic-loaded microencapsulated formulatons showed a significant increase in the probiotic viability. In addition, a coating of encapsulated cells also enhances the mechanical strength of the polymer carriers during product development and long storage [18]. This will ultimately be helpful in protecting the probiotics

In the present investigation, *Lactobacillus rhamnosus* GG-loaded pectin and sodium carboxymethyl cellulose (NaCMC) based encapsulating systems were developed and characterized. The encapsulated cells were coated using cellulose acetate phthalate (CAP) and eudragit S 100. The stability studies were performed to investigate the role of the coated encapsulated cells in protecting the viability of the probiotic.

Material and Method

Encapsulation of Lactobacillus rhamnosus GG

Encapsulating system of *Lactobacillus rhamnosus GG* by pectin and NaCMC were developed via ionotropic gelation technique [2]. Briefly, separate solutions of pectin and sodium carboxymethylcellulose (Na CMC) were prepared using distilled water (50 ml) with continuous stirring at 100 rpm. Calculated quantities of *Lactobacillus rhamnosus* GG were dispersed in the above-prepared solutions (1:3).The above solution was then added dropwise with the help of syringe (10 ml, 24 G needle) into the beaker filled with solution (100 ml) containing calcium chloride (5 % w/v) for cross-linking pectin and $AlCl_3$ (20%w/v) solution for NaCMC respectively. The gelling solution was stirred gently (100 rpm) and the developed encapsulating systems were allowed to harden for 30 min in gelling solution.

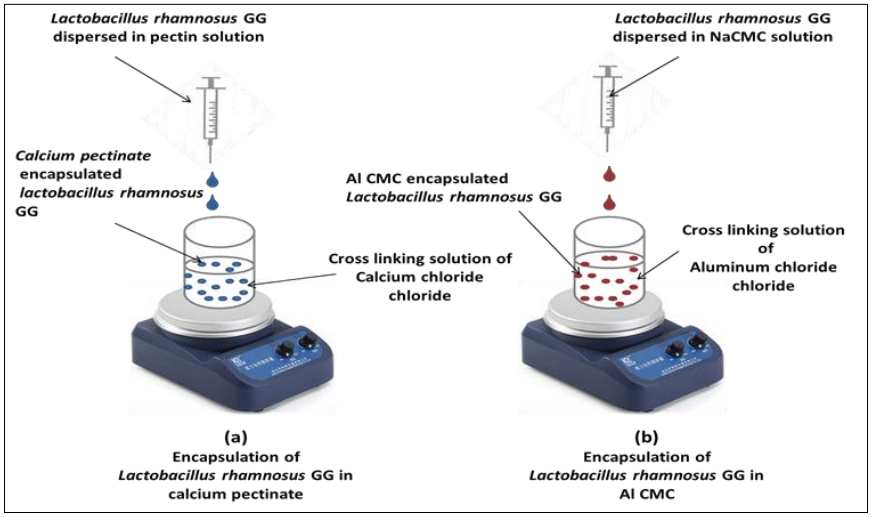

Fig 1: Encapsulation of *Lactobacillus rhamnosus* GG (a) Encapsulation of *Lactobacillus rhamnosus* GG by pectin (b) Encapsulation of *Lactobacillus rhamnosus* GG by NaCMC

Coating of encapsulated Lactobacillus rhamnosus GG:

Cellacefate (Cellulose acetate phthalate, CAP) is a pH-sensitive polymer which gets dissolved in an aqueous solution at pH above 6.2. The coating solution of CAP (5 % w/v) was prepared within phosphate buffer saline (PBS, pH 6.8). Polyethene glycol 200 (10 % dry weight of CAP) was used as a plasticizer [19]. The enteric coating solution was then filtered aseptically using a 0.2 µm membrane filter. Encapsulated cells were dispersed in the enteric coating solution and stirred at 400 rpm at room temperature for 30 min. The CAP coated encapsulated cells present in the solution were filtered using filter paper, kept on a petri dish and dried for two days at room temperature. The coated encapsulated cells were kept in desiccators for 24h, then transferred aseptically into a sterile glass vial, sealed and stored in the refrigerator for further studies. The entire process was carried out aseptically on a horizontal laminar flow bench.

Similarly, aqueous solution of Eudragit S 100 was prepared using phosphate buffer at pH 7. Five gm of Eudragit S 100 was added to 100 ml of phosphate buffer solution with continuous stirring. Prepared coating solution was filterd and the previously encapsulated beads were dispersed in the enteric coating solution at 400 rpm for 30 minutes at room temperature [20]. The coated beads were filtered with filter paper, spread on a petri dish and dried for 2 days at room temperature. Final formulations were kept in a desiccator for 24 hours, then transferred aseptically into a sterile glass vial, sealed and were stored in the refrigerator for further studies. The entire process was carried out aseptically.

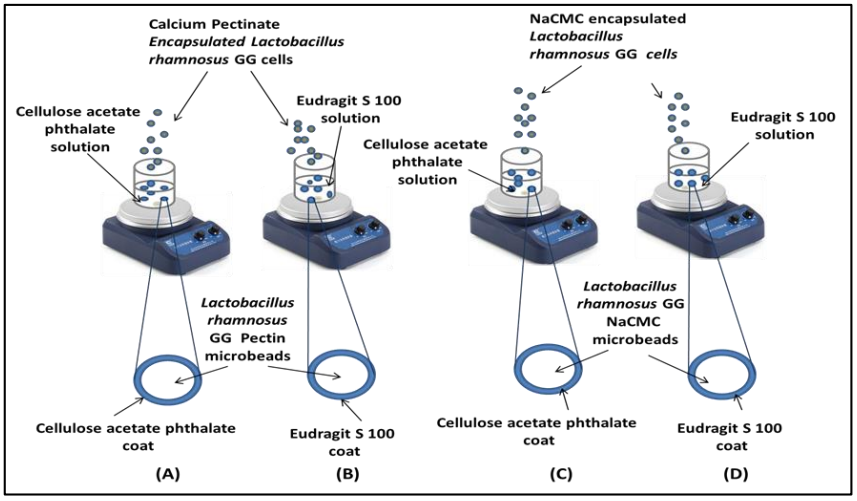

Fig 2: Coating of *Lactobacillus rhamnosus* GG encapsulated cells (A) Cellulose acetate phthalate coated *Lactobacillus rhamnosus* GG encapsulated cells (B) Eudragit S 100coated *Lactobacillus rhamnosus* GG encapsulated cells (C) Cellulose acetate phthalate coated *Lactobacillus rhamnosus* GG NaCMC encapsulated cells (D) Eudragit S 100 coated *Lactobacillus rhamnosus* GG NaCMC encapsulated cells.

Stability Study: Stability studies of coated encapsulated *Lactobacillus rhamnosus* GG cells were performed to check the effect of storage conditions on *Lactobacillus rhamnosus* GG viability as per the method described in ICH Guideline Q1A [21]. The formulated encapsulated cells were stored at (30 ± 2 °C/65 ± 5 % RH) for 6 months known as intermediate term testing conditions (ITC) and at (40 ± 2 °C/75 ± 5 % RH) for 6 months as accelerated testing conditions. Thereafter the encapsulated cells were assessed for viable *Lactobacillus rhamnosus* GG cell count, color and texture analysis at 2 weeks interval up to 2 month followed by 1 month interval up to 6 months. The results were compared with viability of control sample (viability of *Lactobacillus rhamnosus* GG prior to stability study). The control samples were kept at 2-8 °C in refrigerator.

Plating & Counting of Probiotics

The standard agar medium was used for the preparation of agar plates. Standard media were placed in a water bath at 45 °C which convert media into liquid state. One ml of sample of test medium with proper dilution was transferred into each sterile petri dish. Immediately after this molten agar media was added to the test media and mixed properly, then allowed agar to completely gel without disturbing it, and then incubating in an inverted position at 37 °C for 48 hours after solidification. Total viable cell count per plate was calculated [22]

The number of CFU per unit of the sample was calculated by following equation:

$$\text{Number of CFU} = \frac{\text{Average number of colonies counted per plate}}{\text{Dilution factor}}$$

Results

Stability Study: The stability studies results exhibited adequate stability of encapsulated *Lactobacillus rhamnosus* GG at intermediate term testing conditions (ITC) for 6 months but statistically significant reduction in viability was observed by 90 days, whereas inadequate stability was observed at accelerated testing conditions (ATC). On the other hand, stability of encapsulated cells after storage at 2-8 °C for 6 months was found to be appropriate as viability of encapsulated cells was comparable to initial viable cell count (Figure 5). The results revealed that encapsulated *Lactobacillus rhamnosus* GG exhibit adequate stability at the storage conditions of 30 ± 2 °C/65 ± 5 % RH and 2-8 °C for 6 months, as the change in color, texture and the statistically significant decrease in viable cell content with respect to initial viable cell count value was not observed (Figure 3). Contrary, at the storage condition (ATC) of 40 ± 2 °C/75 ± 5 % RH, statistically significant decrease in viable cell content with respect to initial viable cell count value was observed, indicating product may get unstable at 40 ± 2 °C/75 ± 5 % RH storage conditions (Figure 4).

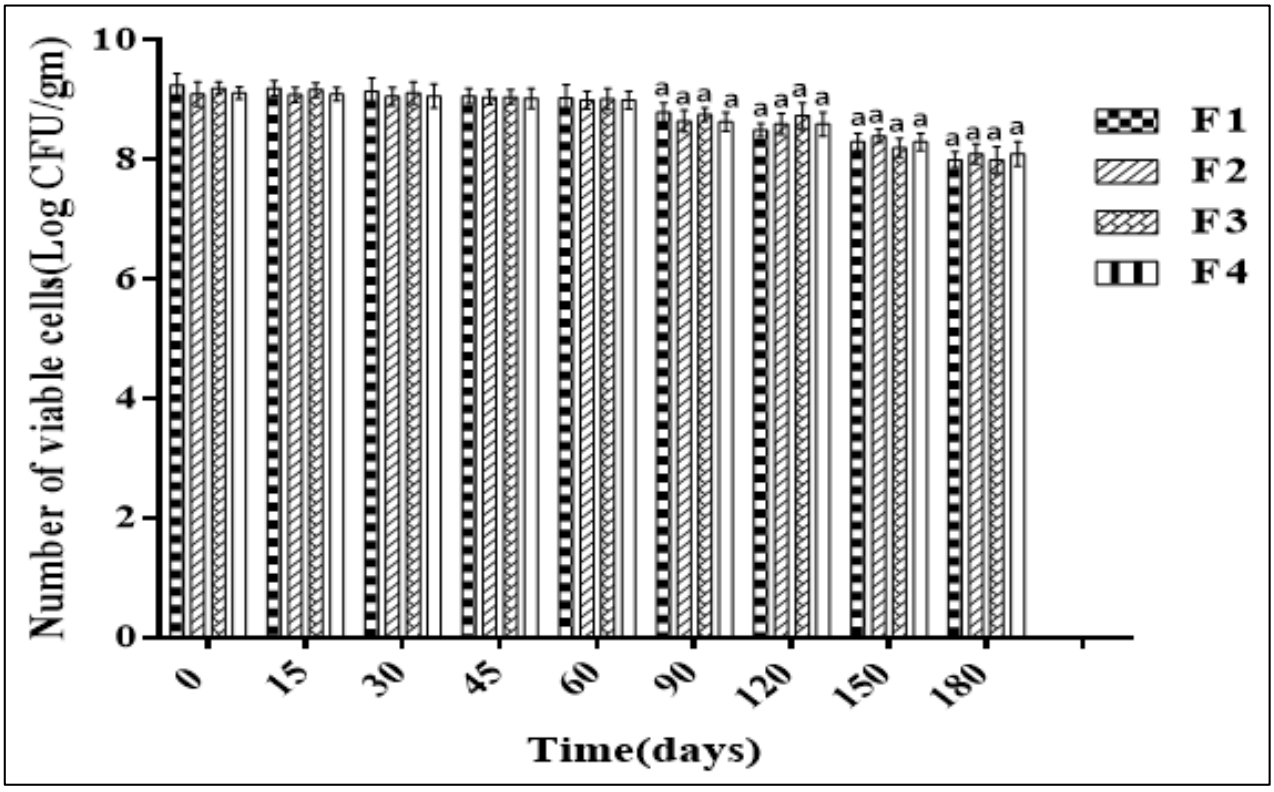

Fig 3: Stability study of encapsulated *Lactobacillus rhamnosus* GG at 30 ºC ± 2 ºC/65 ± 5% RH

Each group (n=3) represents mean ± standard deviation. Two-way ANOVA followed by Tukey's multiple comparisons test; F (3, 72) = 0.3548, P = 0.7858 for evaluating the viability of *Lactobacillus rhamnosus* GG cell from encapsulated *Lactobacillus rhamnosus* GG at different intervals. $^{a}p < 0.05$ Viability of *Lactobacillus rhamnosus* GG cells compared with viability of *Lactobacillus rhamnosus* GG at zero hour.

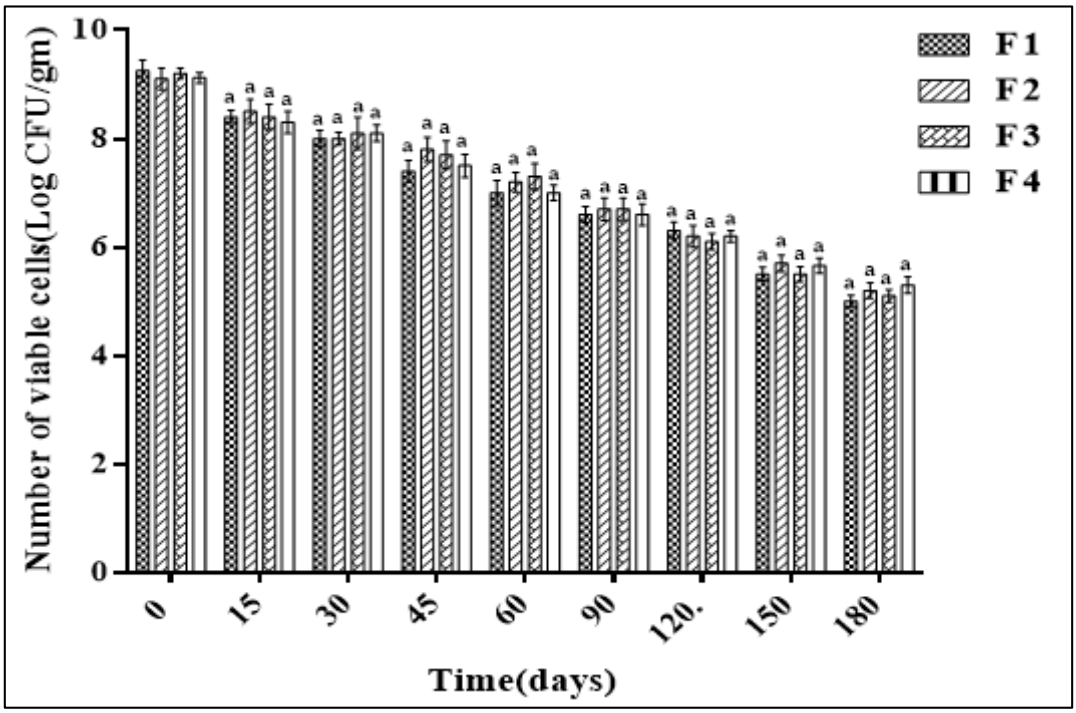

Fig 4: Stability study of encapsulated *Lactobacillus rhamnosus* GG at 40 ºC ± 2 ºC/ 75 ± 5 % RH

Each group (n=3) represents mean ± standard deviation. Two-way ANOVA followed by Tukey's multiple comparisons test; F (3, 72) = 1.735, P = 0.1675 for evaluating the viability of *Lactobacillus rhamnosus* GG cell from encapsulated *Lactobacillus rhamnosus* GG at different intervals. [a]p < 0.05 Viability of *Lactobacillus rhamnosus* GG cells compared with viability of *Lactobacillus rhamnosus* GG at zero hour.

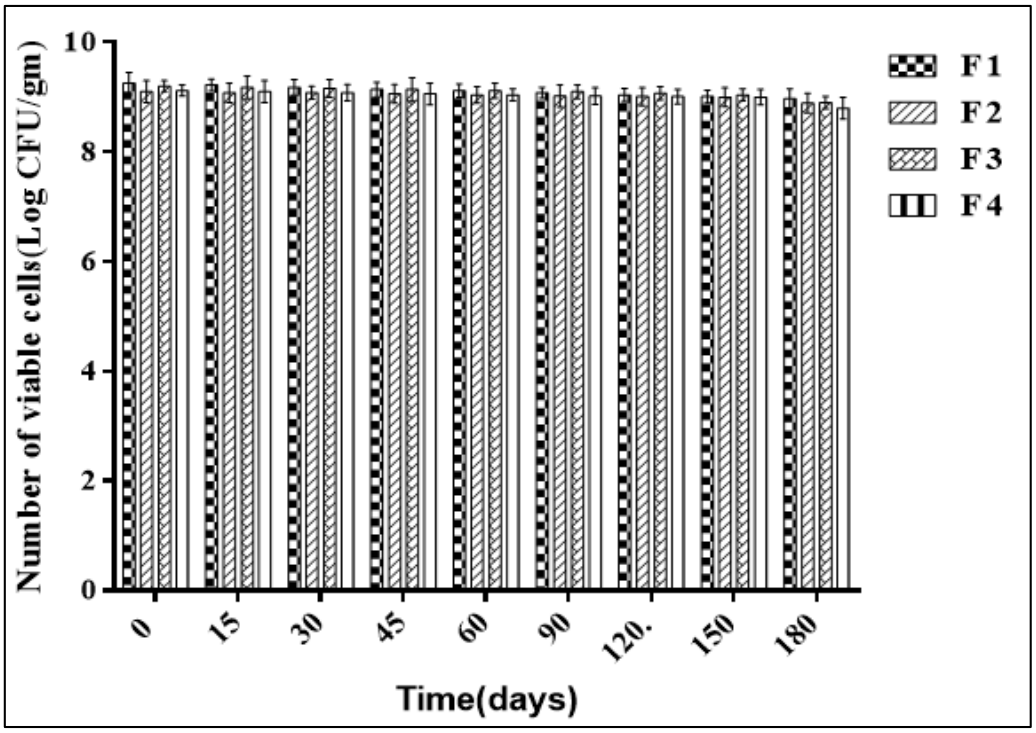

Fig 5: Stability study of encapsulated *Lactobacillus rhamnosus* GG at 2-8 ºC

Each group (n=3) represents mean ± standard deviation. Two-way ANOVA followed by Tukey's multiple comparisons test; F (3, 72) = 2.321, P = 0.0824 for evaluating the viability of *Lactobacillus rhamnosus* GG cell from encapsulated *Lactobacillus rhamnosus* GG at different intervals.

Discussion

Probiotics are live microorganisms that reside preferentially in large and also in small intestine and provide a health benefit on the host when administered in adequate amounts. The probiotics are primarily used for prevention and treatment of antibiotic associated diarrhea (AAD), *Clostridium difficile* infections (CDI) and chemotherapy induced diarrhea etc. (World Gastroenterology Organization Global Guidelines). However, the main negative factors that affect the survival of probiotics during oral administration and during storage [23].

In this study, ion gelation microencapsulation method was used to protect probiotic from gastrointestinal harsh condition and storage condition. Ionotrpic geltion method was a simple method which doesn't required any deleterious solvents an tedious steps. The principle of ion gelation method is based on polyelectrolyte complexation of polymer and crosslinking agent. The technique involves interaction of a cation (or an anion) with an ionic polymer to generate a highly cross linked structure.

The stability studies revealed that our test formulations (F1, F2, F3 and F4) were found to be adequately stable after storage at 30 ± 2°C/65 ± 5% RH and 2-8 °C for 6 months. The stability of probiotics from test formulation was found to be favorable at storage condition of 2-8 °C as no reduction in viability was observed during 120 days of viability study after storage at 2-8 °C for 6 months and found comparable to initial viable cell count, suggesting that the stability of the cells increased with decrease in temperature, since crystal rearrangement and exposure of the active ingredient are impeded at low temperatures, promoting a longer shelf life of the microcapsules. The similar results were reported by Corcoran, BM. *et al*. (2004), who evaluated the survival of *Lactobacillus rhamnosus GG* with the prebiotic (inulin) and observed that the probiotics microorganisms survived longer at low temperature [24].

Contrary, during the viability study of 120 days after storage at 30 ± 2 °C/65 ± 5% RH for 6 months, the viability of probiotics at initial 90 days was observed comparable to initial viable cells however, it was significantly decreased after 90 days. This may be due to the fact that there was greater metabolic activity of the microorganisms and results in producing metabolic acids and bacteriocins and even showing a loss of substrates during storage. Similar results were also reported by Okuro, PK *et al*. (2013) who microencapsulated *Lactobacillus acidophilus* in inulin by spray chilling, and showed that the viability remained constant for 60 days at a temperature of 22 °C [25].

Thus, results of our study suggest that our test formulations may be potential candidate for prevention of probiotics degradation and maintaining viability during storage.

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