ABSTRACT
Prebiotics-based encapsulation aids in improving the structure of microbeads and the survivability of probiotics. The current study focused on the exploration of a prebiotic-based encapsulation system (alginate-inulin) to improve the viability of probiotics under in vitro and carrier food. Probiotic \( (L. \text{ acidophilus}) \) was encapsulated by the ionotropic gelation method. Microbeads with inulin inclusion were found to be compact and smooth with the highest encapsulation efficiency (98.87\%) among the rest of the treatments. Alginate-inulin-based microbeads showed the highest count (8.41log CFU) as compared to other treatment as well free cells under simulated gastrointestinal conditions. Furthermore, alginate-inulin encapsulation maintained recommended \( (10^7-10^8 \text{ log CFU/ml}) \) probiotic viability in carrier food throughout the storage period. Probiotic encapsulation aids in controlling the post-acidification of the carrier product (yogurt). The results of this study indicated that the alginate-inulin-based encapsulation system has promising potential to ensure the therapeutic number of probiotics in vitro as well in carrier foods.

Introduction

Beyond nutritional impacts, food can be deemed ‘functional’ if it has beneficial effects on humans, to improve health and wellbeing by reducing chronic diseases. Functional foods are gaining popularity around the world and are becoming an important constituent of people’s diets. Therefore, scientists and the food industry have turned their attention toward functional foods that contain positive health benefits beyond basic nutrition.\(^1\) Nowadays, probiotic-based functional foods are gaining popularity. Yogurt is one of the most important dairy-based products for researchers due to its higher nutritional profile especially its advantages to using as a carrier for probiotics.\(^2\)

Prebiotics were recognized for manipulating the microflora of the host and improving human health.\(^3\) The ultimate goal of the addition of prebiotics is to improve health and therefore, reduce the risk of diseases.\(^4\) Probiotics along with prebiotics play a significant role in the modulation of intestinal microbiota which exerts a beneficial effect on consumer health.\(^5\) The efficiency of probiotic bacteria depends on their viability except for their survival in the gut. Probiotics are sensitive to the harsh environment, i.e., low pH, bile salt, high temperature, etc. The incorporation of prebiotics increases the viability of bacteria that are passing through Gastrointestinal tract GIT.\(^6\)
Various approaches are tried to improve the probiotic resistance against the extreme surrounding conditions such as the use of micronutrients (amino acids, peptides, and micro-encapsulation). Providing probiotic live cells with a physical barrier contrary to harmful environments is a method of interest that is now gaining a lot of attention.\textsuperscript{[7]} Microencapsulation of probiotics also provides a physical barrier and maintains a minimum viable count of probiotics to exert positive health benefits.\textsuperscript{[8]}

Microencapsulation is an alternative technique for probiotic protection, which serves as a convenient microenvironment for the encapsulated microbes that enhance the viability of probiotics.\textsuperscript{[9]} Survival of probiotics was suppressed from various factors including acidity, pH, environmental stress, and digestive system environment.\textsuperscript{[10,11]} Alginate is the widely used encapsulation matrix, for both food-grade and non-food applications.\textsuperscript{[12]} Alginate is made up of different ratios of mannanuronic and guluronic acid units connected by glycosidic linkages, hence known as anionic polysaccharide.\textsuperscript{[13]} Lack of mechanical strength leads to poor encapsulation of alginate and lowers the effectiveness and release of encapsulated materials in stomach’s low pH are some drawbacks of the alginate hydrogel matrix.\textsuperscript{[14]} However, incorporation of prebiotic matrix along with alginate coating may result in better protection of probiotics in food systems as well as in GIT due to symbiosis.\textsuperscript{[15]}

To confirm the high level of probiotics, count for the consumers, the addition of prebiotics along with probiotics (co-encapsulation) is highly recommended. In the current study, the probiotic matrix was used to encapsulate the probiotic to prolong their viability under hostile conditions. After the encapsulation process, the encapsulated cells of probiotics were combined in the food system to further evaluate their viability and stability.

**Materials and methods**

All media and chemicals including inulin and sodium alginate used in this research study were purchased from Sigma Aldrich, Germany. The probiotic strain of *L. acidophilus* (ATTC 4356) was obtained from the National Institute for Biotechnology and Genetic Engineering (NIBGE). For yogurt preparation, raw cow milk was purchased from the local market of Faisalabad, Pakistan.

**Activation of *L. acidophilus***

Pure culture in freeze-dried form, *L. acidophilus* (ATTC-4356) was identified by following the method of Afzaal et al.\textsuperscript{[16]} The probiotic culture was activated under anaerobic conditions at 37°C for 24 hr in MRS (Man Rogosa Sharpe) broth. Centrifugation at 3000 g for 15 minutes using a centrifuge machine (Thermo Fisher Scientific Inc.705276 EA, USA) was done for the collection of cells of probiotics and used further for the research study.

**Encapsulation of probiotics**

*L. acidophilus* (ATTC-4356) was coated in sodium alginate and inulin and separately by ionotropic gelation (IG) method\textsuperscript{[16]} with a slight modification. Sodium alginate solution (2.0% w/v) was prepared and sterilized at 121°C for 15 min. The sterile solutions of polysaccharides (sodium alginate, sodium alginate + inulin) were combined with 2.5 mL of 10\textsuperscript{10} CFU/mL probiotic cells present in an aqueous saline solution. Afterward, 0.1 M calcium chloride in an aqueous form was added to harden the beads. Microbeads were obtained by filtration after 40–45 minutes and washed twice using distilled water. Obtained beads were stored at 4°C for further use.

**Characterization of beads**

**Encapsulation efficiency**

The encapsulation efficiency for the formed beads was calculated by using the following formula as described by Prasanna et al.\textsuperscript{[17]}
Encapsulation Efficiency = \( \frac{\text{CMC}}{\text{CAA}} \times 100 \)

where, CMC is the total number of microbeads, and CAA is the total cells added to the alginate and inulin-based microbead formulation.

**Cell size**

Determination for the size of the capsule is done by the method described by Prasanna et al.\(^{17}\) Briefly, different microbeads were observed for their size by using a Vernier caliper. For this purpose, 30–35 microbeads were selected on a random basis and their mean size was calculated.

**Morphology**

Cell morphology including surface and cross-sectional shape of the prepared beads were observed under the scanning electron microscope (SEM). Beads were mounted by using adhesive tape under a vacuum. Observations were performed by using the microscope with magnification varying from 25× – 9000 ×.\(^{18}\)

**In vitro study for artificial gastric and intestinal conditions**

The encapsulated and unencapsulated culture of *L. acidophilus* was evaluated for viability under the artificial stomach and intestinal conditions by following the methods of Atraki and Azizkhan\(^{19}\) with some minor changes. For the evaluation, under artificial gastric conditions (AGC) a solution containing aseptic saline and pepsin \((2.99 \text{ g/L})\) was dissolved and pH was adjusted to nearly 2.0 by adding 0.5 M of HCL. The solution was poured to different test tubes and encapsulated and un-encapsulated microbeads were added accordingly in a range of \(10^{10} \text{ CFU/mL}\). Tubes were examined at 0 min, 30 min, 60 min, and 120 min and the mean results were recorded as log CFU/mL.

For viability under intestinal conditions, dipotassium hydrogen phosphate \((13.5 \text{ g})\) was used along with 0.25 M NaOH solution \((150 \text{ mL})\), 18 g trypsin, and 1.5 g of bovine salt. Distilled water was added to make a solution and pH was then adjusted to 7.5 by adding NaOH. After that, the prepared solution was up to 1 L and the solution was added to the test tubes. Prepared microbeads and free probiotics are added accordingly in a range of \(10^{10} \text{ CFU/mL}\). Test tubes were examined after 30 minutes \((0 \text{ min}, 30 \text{ min}, 60 \text{ min}, \text{ and } 120 \text{ min})\) intervals and the mean results were documented.

**Product development**

Probiotic yogurt was produced by using the method adopted by Afzaal et al.\(^{16}\) Briefly, the Ultra High Temperature \((\text{UHT})\) treated milk was purchased and heated till the temperature reached 45°C. After heating, the milk was cooled to 32°C and poured in four different containers for yogurt preparation. The milk yogurt culture was inoculated with *Lactobacillus Bulgaricus* and *Streptococcus thermophilus* at 2.5% \((\text{w/v})\). Then, the sample was incubated at 42–43°C till the pH level reached at ~4.5. The milk in different containers were termed as Yo (control treatment), the next treatment was prepared with the addition of free probiotics in it along with yogurt culture and termed as Y1 (yogurt with free probiotics). Y2 is the yogurt treatment prepared in third container with probiotics encapsulated with alginate coating and Y3 is the yogurt treatment made with the addition of probiotics with alginate + prebiotic matrix. Probiotic culture \((L. acidophilus)\), free and encapsulated cells was added in it in the range of \(10^{10} \log \text{ CFU/g}\) and the samples were incubated for 6–8 hours. The yogurt was made in airtight food-grade storage cups and stored at 4°C for 21-day interval study. The sampling was carried out on weekly basis \((0, 7, 14, \text{ and } 21 \text{ days})\) for the analysis of the viable count of *L. acidophilus*.

**Physicochemical analysis of yogurt**

The pH for yogurt treatments was measured with a digital pH meter according to the method followed by Prasanna et al.\(^{20}\) pH meter (Eutech instruments pH 700) was used for pH determination of yogurt.
**Probiotic enumeration of yogurt**

The total viable probiotic count for yogurt was determined following the guidelines described by Ghasempour et al.\(^{[21]}\) with modifications. The yogurt sample was dissolved in 9 mL of peptone water and mixed thoroughly. The prepared sample solution was poured on the surface of MRS agar and incubated at 37°C and examined after every 7th day (0, 7, 14, and 21). The final colonies obtained were calculated using a colony counter.

**Sensory evaluation**

A scorecard was formed by following the guidelines of the American Daily Association, which includes the parameters as flavor, texture, appearance, and overall acceptability. According to which all the samples were organoleptically rated by using 9 points (9 = like extremely; 1 = dislike extremely). For sensory evaluation, 40 trained panelists were selected, out of which 20 were males (age between 20 and 40 years) and 20 females (20–40 between 20 and 40 years). All panelists were selected from the department of Food Science and Technology. In between the sensory evaluation, the evaluators were provided with water and unsalted crackers for neutralizing the taste buds of the evaluators.\(^{[22]}\)

**Statistical analysis**

The data obtained from all the experiments were analyzed statistically by using Statistix 8.1 software, using two-way analysis of variance (ANOVA) along with ±standard deviation at 5% the least significant difference.

**Results and discussions**

**Encapsulation efficiency**

The wall materials used for the encapsulation showed a significant effect on the encapsulation efficiency. The results shown in Table 1 illustrate that the addition of inulin along with alginate significantly improves the results regarding encapsulation efficiency. As in this study, the encapsulation efficiency for the Alginate was 92% but if we add the inulin along with alginate the encapsulation yield increases significantly to 96%. It has also been reported that the coating material helps to improve the stability of probiotics even in extreme conditions. Also, the effectiveness of the coating materials was closely related to the materials used for the coating, as the encapsulation efficiency was more in the case of inulin+ alginate matrix than with sodium alginate alone. The results of this study are in line with the findings of Wu and Zhang\(^{[23]}\) who encapsulated L. plantarum with SA and arabinobioxylan and found that the efficiency for both added wall materials increased from 85%. From this high yield, it has been shown that the beads formed as a result of the inulin + alginate matrix are compatible with the entrapment of probiotic *Bifidobacterium bifidum*. The reason behind the higher for capsules containing prebiotic matrix may be bacteria inside capsules may utilize the prebiotic matrix as its food that increases the viability of probiotic and as a result, symbiotic relation might form between probiotics and prebiotic matrix inside the capsules and resulted in higher yield as compared to other treatments.

| Cell type | Size          | Encapsulation efficiency |
|-----------|---------------|--------------------------|
| T2        | 3.51 ± 0.08 mm | 92%                      |
| T3        | 3.41 ± 0.04 mm | 96%                      |
The results varied for the case of alginate and the case of inulin + alginate matrix. While it has been cleared from Table 1 that the addition of inulin into the alginate beads results in lowering the size of the beads significantly. Moreover, it has been illustrated that the size of beads is smaller in which inulin (prebiotic) is used along with alginate as a coating material (3.41 ± 0.04 mm), however, the results for the size of alginate beads were found to be higher (3.51 ± 0.08 mm). The reason for lower capsule size is that with the addition of inulin into the alginate beads, the viscosity of the beads increased and resulted in more tightly bonded and intact structure of capsules/beads that bounded the probiotics more tightly than simple alginate coating. The results of our studies are consistent with the finding of Darjani et al., who studied that the addition of prebiotic with coating material did not increase the size of the capsules.

A cross-sectional view of the beads showed each type of bead has its distinctive structure. It had been observed that the beads with alginate showed a porous structure. While the addition of inulin into the alginate beads results in a less porous structure with comparatively low visible pores. Inulin+alginate beads are exposed to have a more compact structure; this may be due to the reason that the inulin made a compact structure with the alginate proteins. Which results in a lower size and more compact structure. The beads compact structure with milk and alginate matrix plays an important role during digestion as it protects the bacteria from being vulnerable in the harsh and extreme environmental conditions of the stomach.

**Survival of free and encapsulated L. acidophilus in simulated gastric juice (SGJ)**

The safe delivery of probiotics in the human gut is important to ensure potential health benefits. For this reason, wall materials play a very important role as they provide a safety barrier and resist environmental changes. The mean results obtained for simulated gastric juice (SGJ), showed a significant effect of wall materials which are shown in Figure 1. Maximum results were noted for encapsulated probiotics, however, the least population was calculated for free probiotics. The peak probiotic growth was noted in treatment T3 (L. acidophilus encapsulated with sodium alginate+inulin) with a minimum log reduction of 1.85 CFU/mL. However, 2.76 CFU/mL log reduction was obtained for probiotics that were encapsulated with sodium alginate after 120 minutes. The results with free probiotics reveal a rapid decline in the probiotic population with a reduction of 9.75 log CFU/mL.

The reason behind this rapid fall in the bacterial population may depend on the fact that probiotics did not survive the severe acidic conditions of the stomach and died however sodium alginate provides a barrier against these extreme conditions. As inulin is a prebiotic thus the combination of inulin with the wall coating material showed compatibility and resulted in maximum survival of L. acidophilus. Similar studies were conducted by many researchers and a study by Wu and Zhang was conducted and they discover the addition of arabinoxylan along with alginate wall material results in the better viability of L. plantarum during gastric conditions. A study by Sathyabama et al. showed positive results for coencapsulation of different prebiotic matrix along with probiotics and suggested positive role of incorporation of prebiotic into alginate matrix during encapsulation under simulated gastric conditions. It was also suggested that the probiotics encapsulation with chicoru prebiotic matrix was more stable in intestinal conditions as well. Another study by Chavarri et al. concluded that co-encapsulation of probiotics with prebiotics helps the survival and enhance the probiotic enumeration even at lower gastric conditions. The fact for higher viability might be due to the inulin long chains, owing to its self-aggregation abilities, probably forms insoluble masses inside alginate matrix which that could possibly delay the penetration of H+ ions into microcapsules by blocking the pores.

**Release of encapsulated L. rhamnosus in simulated intestinal fluid (SIF)**

For obtaining maximum health benefits from probiotics, it is essential that they pass through the gut and reach the intestinal lining of the host and colonize there. The survival rate profile of probiotics
differences according to the wall materials used in this study. The mean results for the viability of probiotics under SIF are shown in Figure 2. A significant decline was noted for all treatments when subjected to SIF. Higher results were obtained for encapsulated probiotics however a sharp decline was obtained for non-encapsulated *L. acidophilus*. Maximum survivability rate was calculated for *L. acidophilus* that was encapsulated with sodium alginate along with inulin.

A minimum of 1.85 CFU/mL log reduction was obtained for *L. acidophilus* encapsulated with sodium alginate and inulin while a log reduction of 2.31 CFU/mL was noted for encapsulated

![Figure 1](image1.png)

**Figure 1.** Viability of un-encapsulated and encapsulated (sodium alginate, sodium alginate + inulin) probiotic microgels under simulated gastric fluid conditions during storage intervals (0, 30, 60, 90, and 120 minutes) compared with control. Each line represents mean value for viability of treatments. T₁ (un-encapsulated probiotics), T₂ (*L. acidophilus* encapsulated with sodium alginate) and T₃ (*L. acidophilus* encapsulated with sodium alginate+inulin).

![Figure 2](image2.png)

**Figure 2.** Viability of un-encapsulated and encapsulated (sodium alginate, sodium alginate + inulin) probiotic microgels in simulated intestinal fluid environment during storage intervals (0, 30, 60, 90, and 120 minutes) compared with control. Each line represents mean value for viability of treatments. T₁ (un-encapsulated probiotics), T₂ (*L. acidophilus* encapsulated with sodium alginate) and T₃ (*L. acidophilus* encapsulated with sodium alginate+inulin).
L. acidophilus with sodium alginate. However, a maximum decline in the viability of free L. acidophilus was obtained and noted as 8.92 log CFU/mL at the end of 120 minutes. Rajam and Anandhamarakrishnan [29] in 2015 disclosed that the addition of Fructo-oligosaccharide (FOS) and whey protein isolate with coating materials results in better survival rates, however, FOS showed higher significant results. Silva et al. [30] proposed that the addition of probiotics with coatings such as alginate-gelatin and alginate-gelatin-FOS results in a better probiotic survival rate during intestinal digestion conditions. A study by Sathyabama et al. [26] showed positive results for co-encapsulation and suggested that the probiotics encapsulation with chicory prebiotic matrix was more stable in intestinal conditions as well. This could be attributed to the enhancement of microcapsules structure and adhesion of bifidobacteria to starch granules via surface proteins. [31] Similar findings were also reported by Chen et al. [32] that suggested that co-encapsulation of probiotics along with fructo-oligosaccharides maximizes the probiotic survival by three log cycles. The effect of inulin on the enhanced survival of probiotics in high acidic environments has also been reported by Wang et al. [33] Atia et al. [18] reported the co-encapsulation of lactobacilli along with the addition of 50 mg/g inulin enhanced the viability and survival of probiotic bacteria in gastric and intestinal.

**Physicochemical analysis**

Mean results for the pH of yogurt shown in Table 2 indicates their storage and a significant interaction imparted between them. The highest pH value was observed at 0 days in all treatments that decreases with time. Maximum pH was observed for yogurt with free probiotic bacteria on the 21st day (3.16 ± 0.03), respectively. However, the storage study showed the least decrease of pH in yogurt containing sodium alginate along with inulin 4.57 ± 0.01 during storage. From the results, it can be concluded that microencapsulation of probiotic bacteria along with prebiotic matrix results in the formation of a more stable product that can resist pH for a longer duration of time. The results of the present study are in line with the findings of. [34]

**Microbiological assay**

Mean results regarding the viability of probiotic bacteria showed a significant decrease in the viable count of probiotics as time progresses during storage. From Figure 3, it has been shown that the viability for Y1 (yogurt with free probiotics) was found to be the lowest at the end of the storage study due to a rapid decline of probiotics while Y2 and Y3 showed higher viability rates, respectively. However, the maximum probiotic viability was observed in the Y3 yogurt treatment that had the probiotics encapsulated with alginate together with inulin.

A maximum of 8.76 log CFU/g log reduction was noted in Y1 while a 1.84 log CFU/g reduction in viability was calculated for Y3 yogurt treatment. Hence, according to this research, coating materials improve the viability of probiotics in dairy products during storage. [35] Qi et al. [36] also suggest that the polymers enhance the chance of probiotic viability under adverse and in vitro situations.

| Table 2. Effect of pH on different probiotic yogurt treatments. Values are expressed as means (n = 3) with ± standard deviation followed by the same lower-case letters on the columns that differ significantly by LSD at 5% probability. |
| Treatment | Storage days |
| Y0 | 0 day | 7th day | 14th day | 21st day |
| Y1 | 4.80 ± 0.03a | 4.76 ± 0.07ab | 4.41 ± 0.01e | 4.02 ± 0.05f |
| Y2 | 4.69 ± 0.01c | 4.17 ± 0.03g | 3.51 ± 0.02l | 3.16 ± 0.03j |
| Y3 | 4.74 ± 0.01d | 4.63 ± 0.05cd | 4.44 ± 0.03ef | 4.06 ± 0.09h |
| 3 | 4.80 ± 0.04a | 4.80 ± 0.02a | 4.51 ± 0.07de | 4.57 ± 0.01d |
**Sensory evaluation**

Figure 4 shows the mean significant results for sensory evaluation according to which it can be concluded that the yogurt sample with free probiotics showed the least acceptability in taste, flavor, sourness, and overall acceptability. However, yogurt samples with encapsulated probiotic cells showed...
higher acceptability scores from all sensory evaluators. From all the collected data, it had been observed that the sensory evaluation portrays an important role during the storage of all yogurt samples regarding taste, flavor, and texture. Over time, the carbohydrates present inside the yogurt were consumed by the bacteria which results in the production of organic acids which affects the organoleptic yogurt properties.

Conclusion

In the recent study, *L. acidophilus* was encapsulated using wall material (sodium alginate) along with prebiotic (inulin) to determine the effect of probiotic viability under simulated gastric and intestinal conditions. High encapsulation efficiency was obtained for probiotic cells having inulin with wall material with better overall structural stability. The cells showed better viability when subjected to simulated in vitro environments. Probiotic viability cells containing inulin in coating material showed better probiotic viability during storage. Hence, microencapsulation of probiotic bacteria along with prebiotic matrix is an efficient way to prolong the viability of probiotics to obtained potential health benefits.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Ethical approval

The study does not involve any human or animal testing.

Data availability statement

The data that support the findings of this study are available from the corresponding author, upon request.

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