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Research Article

Stability and Survivability of Alginate Gum-Coated *Lactobacillus rhamnosus* GG in Simulated Gastrointestinal Conditions and Probiotic Juice Development

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Survivability of probiotics is severely affected by harsh gastrointestinal conditions. In the present study, microbeads of *Lactobacillus rhamnosus* GG were formulated using alginate (1.5% w/v) and combination of alginate (1.5% w/v) with xanthan gum (0.5% w/v) through an emulsion technique to improve bacterial viability in low pH orange juice and in gastrointestinal conditions. The microbeads were tested for encapsulation efficiency, survivability in bile salt, SGF (simulated gastric juice), SIF (simulated intestinal fluid), and storage stability. Probiotic orange juice was formulated and tested for physicochemical parameters (pH, titratable acidity, and total sugars) and sensorial properties during storage. Gum-coated alginate microbeads (T_3) showed higher encapsulation efficiency, i.e., 95.2% compared to alginate microbeads (T_2), i.e., 86.85%. Similarly, T_3 showed the highest resistance against bile salt (8.50 log CFU/g), SGF (7.95 log CFU/g), and SIF (8.0 log CFU/g) during 80 min exposure compared to T_2 and free cells. The viability of gum-coated alginate beads (T_3) remained above 10^7 CFU/g in gastrointestinal conditions and at the end of 21 days storage (8.3 log CFU/mL). All physicochemical parameters of probiotic juice were significantly ($p \leq 0.05$) decreased with respect to storage except acidity. In addition, minimal changes in physicochemical parameters were observed in T_3 compared to other treatments. Treatment had no significant impact on the sensory characteristics of juice, but storage had a significant effect ($p \leq 0.05$) on the sensory characteristics of juice. The alginate gum microbeads improve the survivability of probiotics for targeted delivery. Hence, encapsulated probiotics can be used for functional beverage development to take advantage of their therapeutic benefits.

1. Introduction

Probiotics are living microbes that provide many health benefits including immunomodulation, supporting the gut flora, lowering body cholesterol, anticancer activity, effective against viral infection, and reduce lactose intolerance [1]. The consumption of beneficial bacteria should be more than 10^6 CFU/day to get health benefits [2, 3]. The products which contain probiotic bacteria need to be refrigerated to maintain their viable population which increases their storage cost [4]. There are some important factors that affect the viability of probiotics, including probiotic bacterial strain, association between the species, pH, and organic acid produced by the bacteria. In addition to this, homogenization temperature, pressure, time, incubation period, and storage conditions are other factors that affect bacterial viability [5]. Factors such as pH, dissolved oxygen, hydrogen peroxide, and buffing capacity also had a considerable impact on their viability [6].

Probiotics should have certain characteristics for the provision of health benefits. First, they must have strong resistance to acid and bile, adherence to mucosal and epithelial lining, and the ability to colonize. Second, to combat bacteriocins and bile salt, they should have antimicrobial and hydrolase activity. Finally, they should not pose a threat to humans and animals with respect to carcinogenic effects [7]. The main types of probiotics are Lactobacilli, Bifidobacteria, and some yeast, e.g., *Saccharomyces boulardii* [8]. The *Lactobacillus* bacteria are most commonly recognized and safely used by people. It includes many species including *Lactobacillus casei*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, and many others [9]. There are some other lactic acid bacteria such as *Lactococcus lactis*, *Leuconostoc mesenteriods*, *Streptococcus thermophiles*, and *Enterococcus*. The use of probiotics for the wellbeing of humans has a long history. Lactic acid bacteria are divided into two groups (homofermentative and heterofermentative). The bacteria which ferment the sugar and produce a single byproduct, i.e., lactic acid are known as homofermentative. It includes *Lactobacillus plantarum*, *Lactobacillus casei*, and *Leuconostic mesenteriods*. Bacteria which ferment sugar and give rise to lactic acid along with production of another acid, i.e., CH_3COOH and colorless gas, i.e., CO_2 as a byproduct known as heterofermentative. It includes *Lactobacillus rhamnosus* and *Lactobacillus fermentum* [10].

Lactobacillus rhamnosus provides many health benefits to human, and it has been utilized for the removal of toxins from milk through bio films [11, 12]. *L. rhamnosus* is able to stick to the epithelial lining and provide health benefits which mainly depend upon their viability ratio that should be in the range of 10^7–10^9 CFU/mL. Recent research has emphasized the vital role of the gut microbiome in regulating human health and disease. The technique used for effectively capturing bacterial species into a resistant and gelling material to form microbeads that have different sizes (from nm to mm) is acknowledged as an encapsulation [13]. The purpose of encapsulating probiotics is to enhance their viability during harsh conditions. Another benefit of encapsulation is the retention of sensory characteristics of food products [12]. During the passage through the gastrointestinal tract, encapsulated probiotic cells remain active and play their beneficial role in the body [14].

Sodium alginate, zein, gums (carrageenan, xanthan, and gum arabic), dextran, cellulose, chitosan, protein (like albumin and casein), and maltodextrin are frequently used to protect the bacteria from unfavorable conditions. The size of beads produced as a result of encapsulation is an important parameter and should be adjusted to regulate the sensory and textural properties of food products. The encapsulated probiotic also facilitates in enhancing the shelf life of food products by controlling their growth [15]. Among various techniques of encapsulation, emulsion is a simple technique used to encapsulate probiotics. Emulsion is a simple, adaptable, and least expensive procedure for the coating of bacteria in which both bacteria and coating medium are integrated into oil [16].

Alginate is an edible polymer and extensively used as an encapsulating material for probiotics (*L. rhamnosus*, *L. acidophilus*, *L. lactis*, *L. casei*, and *L. plantarum*) [17]. Alginate can withstand the severe acidic environment even at low pH (below 2), degenerate the matrix, and release its constituents [18]. Previous studies have revealed that alginate alone cannot withstand in a low pH environment, i.e., stomach. This problem can be overcome by coating the alginate beads with an edible hydrophobic polymer that is resistant to gastric fluid [19]. Another food additive, xanthan gum, also provides protection against harsh acidic environments to probiotics. It is widely used in the food industry due to its stabilizing, thickening, gelling, and dispersing properties [20, 21]. In this study, we have used a combination of alginate and xanthan gum for the encapsulation of *Lactobacillus rhamnosus* GG to take advantage of their desirable properties. Previously, no study was conducted to investigate the suitability of alginate and xanthan gum combination for the encapsulation of *Lactobacillus rhamnosus* GG.

In previous studies, dairy products have been developed with the addition of probiotic bacteria. These products are not suitable to those people who are suffering from lactose intolerance. Fruit juices have not been extensively explored in previous studies. Hence, it is the need of time to develop a sufficiently stable system that has the capability of protecting bacteria from low pH (such as fruit juices) and the low pH of food products significantly affects the growth and stability of the lactic acid bacteria (LAB). Juices are potential candidates for the addition of encapsulated probiotics without affecting the sensory characteristics of food products [22].

The present study was designed to evaluate the resistance of free and encapsulated bacteria in orange juice having low pH and in gastrointestinal conditions. The bacterial species *Lactobacillus rhamnosus* GG was encapsulated with two resistant materials (alginate and xanthan gum) through an emulsion method (using canola oil). The beads were tested for survival in acidic conditions (SIF, SGF, and bile salt), encapsulation efficiency, storage stability, and stability in low pH orange juice.

2. Materials and Methods

2.1. Materials. Fresh oranges and canola oil were purchased from the local market of Faisalabad. The oranges were properly stored at room temperature (25°C) with a relative humidity of 85–90%. Sodium alginate (E-401) and xanthan gum (E-415) were purchased from Sigma-Aldrich (Sigma-Aldrich, Tokyo, Japan) while calcium chloride was purchased from Carlo-Erba (Milano, Italy). Growth media (De Man, Rogosa and Sharpe agar) for the bacteria was acquired from Difco (Sparks, USA).

2.2. Culture Preparation of Lactobacillus rhamnosus. The frozen pure culture of *Lactobacillus rhamnosus* GG (ACCT 53103) was obtained from the Department of Microbiology, University of Agriculture, Faisalabad. After the preparation

and sterilization of MRS agar media, bacterial cells were inoculated on Petri plates by the streak plate method and placed at 37°C for 48 hours. Gram staining and catalase test was performed to determine the purity of culture. Before experimental use, *L. rhamnosus* GG was reactivated in MRS broth and incubated at 37°C for 48 hours. The cells were harvested by centrifugation at 3000 × *g* for 20 min (Thermo Fisher Scientific Inc.; 75005286 EA) and washed with distilled water. The washing procedure was repeated three times to achieve the final concentration of bacterial cells 10^8-10^9 CFU/g.

2.3. Encapsulation Procedure and Bead Formation. The encapsulation was carried out by following the method of Afzaal et al. [23] with slight modifications (Table 1). The solution of hydrogels containing sodium alginate (1.5% w/v) and xanthan gum (0.5% w/v) was used for the encapsulation of *Lactobacillus rhamnosus* GG and to form microbeads. Better gelling characteristics were attained by developing the solution one day before encapsulation. An emulsion-based method was used for the preparation of beads. Sodium alginate (1.5% w/v) and xanthan gum (0.5% w/v) solutions were sterilized at 121°C for 15 min. After that, 1 mL of cell suspension (harvested by centrifugation 3000 × *g* for 20 min) was mixed separately with 20 mL sterilized alginate and SA-X (sodium alginate + xanthan) solution [19]. The whole mixture was continuously stirred with the help of a magnetic stirrer, and 100 mL of canola oil was added along with an emulsifier (span 80) into the beaker. The whole solution containing SA (sodium alginate) and SA-X (sodium alginate + xanthan) with bacterial cell suspension was gently added into 100 mL canola oil to form the emulsion by gentle shaking at 200 rpm for 15 min [24]. 0.1 M calcium chloride (100 mL) was moderately added into the stable emulsion for the hardening of beads. After 5 minutes, the stable emulsion was broken down and alginate microbeads (T_2) and AL-X (alginate with xanthan gum, T_3) beads were collected. Filter paper (Whatman No. 4, Fisher Scientific) was used for the washing of microbeads and stored at refrigeration temperature 4°C for further use [19]. Plain juice (no addition of probiotics) was used as a control sample (T_0), while free probiotics (T_1) were added to investigate their viable count against encapsulated ones.

2.3.1. Efficiency of Encapsulation. The total viable cells of *L. rhamnosus* GG were determined before and after encapsulation by following the method described by Azam et al. [12]. Efficiency of encapsulation was investigated by dissolving the microcapsules into 9.0 mL sodium citrate solution (2% w/v). The pH of this solution was adjusted to 7.0. The probiotic cells of *L. rhamnosus* GG were released. After that, 10 serial dilutions were prepared and poured onto MRS agar plates for the enumeration of bacteria. Incubation was done at 37°C for 48 hours in anaerobic chamber (Bactron SHEL LAB, anaerobic Chamber, USA) to prevent any damage to bacterial colonies. Bacterial colonies were calculated using the colony counter (model 870) [25, 26]. The following equation was used to determine the encapsulation efficiency of the system:

Table 1: Treatment plan for encapsulation of *Lactobacillus rhamnosus* GG.

Treatments	Sodium alginate	Xanthan gum
T_0 (control)	—	—
T_1	Free form	Free form
T_2	1.5%	—
T_3	1.5%	0.5%

T_0 = control, plain juice; T_1 = free *L. rhamnosus*; T_2 = sodium alginate (1.5%)-encapsulated *L. rhamnosus*; T_3 = sodium alginate (1.5%)- and xanthan gum (0.5%)-encapsulated *L. rhamnosus*.

$$\mathrm{EE}\,\frac{\mathrm{N}}{\mathrm{N_o}} \times 100. \quad (1)$$

EE shows encapsulation efficiency, N represents the no. of cells released from microspheres, and N_o represents the free cells during the production of microcapsules.

2.3.2. Bead Size. The diameter of microbeads was measured with the help of light microscopy having the magnification power of 4X or 10X (Olympus BX51) as described by Azam et al. [27].

2.4. In Vitro Gastrointestinal Assay

2.4.1. Survivability of Free and Microencapsulated Lactobacillus rhamnosus in Bile Solution. The survivability of free and encapsulated bacteria was demonstrated by following the method of Xiao et al. [20] with slight modifications. The survivability of *L. rhamnosus* was determined through the utilization of bile salt solution (2.5% w/v) with adjusted pH 4.0. Specific volume of bile salt solution (4.5 mL) was taken into 2 separate test tubes for both free and microencapsulated bacteria. After that, the suspension of free (0.5 mL) and encapsulated *L. rhamnosus* GG (0.5 g) was transferred into both test tubes. Incubation was carried out with constant agitation at 100 rpm and growth was investigated after fixed intervals (0, 20, 40, 60, and 80 min). Saline solution (NaCl 0.8%) was used for making the serial dilution of free cells. In addition, an 80 μL aliquot was used for the growth of encapsulated bacteria on MRS agar media using the pour plate method [25].

2.4.2. Survivability of Free and Microencapsulated Lactobacillus rhamnosus in Simulated Gastric Fluid (SGF). The viability of both free and encapsulated bacteria was determined by following the method of Yasmin et al. [25] with slight modifications. For this purpose, 4.5 mL simulated gastric fluid (SGF) was used. The SGF was prepared by using NaCl (2.0 g/L) and saline water whose pH was adjusted to 1.2 ± 0.1 by using 1 M HCl [28]. Microencapsulated probiotic cells (0.50 g) were transferred to SGF and activated in MRS broth by incubating at 37°C. The incubation was carried out at different time intervals (0, 20, 40, 60, and 80 min) to analyze the bacterial growth. The enumeration was carried out after washing and harvesting the beads through centrifugation (4000 rpm for 10 min), whereas free

cells (0.50 mL) were diluted with 4.5 mL SGF. After 2 hours stay, 1.0 mL of SGF carrying free *L. rhamnosus* was poured onto MRS agar plates and incubated. The final results were recorded in log CFU/mL [29].

2.4.3. Survivability of Free and Microencapsulated Lactobacillus rhamnosus in Simulated Intestinal Fluid (SIF). The release study of encapsulated *L. rhamnosus* was carried out by using SIF (1.39 g/L $NaHCO_3$, 0.2 g/L $CaCl_2$, 0.84 g/L KCl, 6.5 g/L NaCl, and 3.0 g/L 50 mM KH_2PO_4), and the pH was adjusted to 6.8 [30]. Both free and encapsulated cells were added into separate test tubes containing 4.5 mL SIF at the rate of 0.50 mL and 0.50 g, respectively. The incubation was accomplished at 37°C with constant agitation at 100 rpm. After the predetermined time (0, 20, 40, 60, and 80 min), an 80 μL sample was withdrawn for enumeration through the pour plate method [31].

2.4.4. Storage Stability of Free and Encapsulated L. rhamnosus. The storage stability of free and encapsulated *L. rhamnosus* was determined by following the method of Yasmin et al. [25]. The samples were stored at 4°C for 0, 7, 14, and 21 days. Free cells (0.5 mL) were serially diluted in saline solution, and 80 μL aliquots were poured on MRS agar plates for viable count determination. Microcapsules loaded with *L. rhamnosus* was dissolved in 4.5 mL sodium citrate solution (50 mM) at pH 7.5, and the released bacterial count was determined by shifting 80 μL aliquots on agar plates.

2.5. Probiotic Juice Development. First of all, the oranges were subjected to tap water (2-3 min) for washing to loosen the dust from skin. The orange fruit was peeled, sliced, and a juice extractor (Walita, Joinville, Brazil) was used for extraction of juice from slices. The juice was pasteurized at 80°C for 15 min in a water bath (Marconi, Piracicaba, Brazil) to kill the vegetative forms of microbes, i.e., *Clostridium* and *Bacillus* [33, 34]. Later on, the pasteurized juice was cool down to room temperature and inoculated with 7.0 log CFU/mL probiotics (1 mL of MRS broth and 9.0 CFU/mL of probiotics) and mixed thoroughly [34, 35]. Both free and encapsulated bacteria were added into the juice and the pH was adjusted to 3.3 before and after the addition of probiotics [36].

2.6. Product Analysis. The product was stored at refrigeration temperature (4°C) for 3 weeks and the following analyses were carried out after one week interval.

2.6.1. pH, Total Sugars, and Titratable Acidity. pH, total sugars, and titratable acidity were calculated using the methods of AOAC [37]. pH and total sugars were measured by using a digital pH meter (Iino-Lab720, Germany) and a refractometer (Atago-Master, USA), respectively. A simple titration procedure was adopted for the calculation of titratable acidity. Titration was performed with NaOH and titratable acidity was calculated by using the following formula:

$$\text{Titratable acidity} = \frac{\text{Vol. of NaOH used} \times 0.1\text{N} \times 0.067}{\text{Vol. of sample}} \times 100. \quad (2)$$

2.6.2. Enumeration of Probiotic Bacteria. Enumeration of nonencapsulated or encapsulated probiotic bacteria (*Lactobacillus rhamnosus* GG) was determined by the method as described by Afzaal et al. [31]. The probiotics were released from the calcium alginate and alginate-gum microbeads. The sterilized MRS agar was used for their growth. The samples were plated and incubated at a prescribed temperature of 37°C. After 48 hours, total bacterial colonies were counted through a colony counter (model 870) and the results were presented in CFU/g [38].

2.7. Sensory Evaluation. The sensory evaluation of the final product was performed by the panellist at the National Institute of Food Science and Technology, University of Agriculture, Faisalabad. Product evaluation was performed through the proper scoring system known as 9-point hedonic scale. The product was evaluated for its flavor, taste, color, aroma, and overall acceptance during different periods (0, 7, 14, and 21 days) of storage. The study was reviewed and approved by University IRB, and informed consent was obtained from each subject to sensory evaluation performa for their participation in the study.

2.8. Statistical Analysis. The acquired data were analyzed using two factor factorial design under completely randomized design (CRD) and relative comparison of treatments was done by LSD test ($p \leq 0.05$). Statistix 8.1 (Analytical Software, Tallahassee, FI, USA) software was applied for application of statistical parameters. All trials were conducted in triplicate, and the experimental results are reported as the standard deviation and mean values [39].

3. Results and Discussion

In the present study, the survivability as well as the viable ratio of *Lactobacillus rhamnosus* GG was assessed in low pH orange juice under simulated gastrointestinal conditions. An aseptic environment was sustained throughout the experiments.

3.1. Analysis of Microbeads. The mean diameter for alginate and alginate with xanthan gum beads was 760 ± 3.42 μm and 350 ± 2.03 μm, respectively (Table 2). It was observed that alginate beads have larger diameter compared to alginate-xanthan coated beads. The size of the beads is mainly dependent upon the encapsulating material as well as the method used for encapsulation of probiotics. In the present study, medium size beads were observed and it has been concluded that medium sized beads have no adverse effect on the structure and texture of orange juice. Similar results had been reported by Nami et al. [40] who demonstrated

Table 2: Encapsulation efficiency of alginate and alginate gum-coated microbeads.

Encapsulation materials	Cells before encapsulation (N_0)	Cells after encapsulation (N)	Efficiency (%)	Diameter of beads (μm)
Sodium alginate	8.98 ± 0.10^{a}	7.80 ± 0.06^{b}	86.85 ± 1.30^{b}	760.00 ± 3.42^{a}
Sodium alginate + xanthan gum	8.50 ± 0.09^{b}	8.10 ± 0.07^{a}	95.20 ± 1.40^{a}	350.00 ± 2.03^{b}

Mean values ± S.D. in a column having similar lettering are statistically nonsignificant at 5% confidence interval.

that alginate and gum coated beads have smaller diameter (340–370 μm) compared to alginate coated beads (860–1130 μm). Many researchers have observed more smaller beads having a size range from 10 to 40 μm, but the variation in bead size may be due to the polymer used, their concentration, and the type of the encapsulating method [41, 42]. Afzaal et al. [31] have utilized alginate and WPC (whey protein concentrate) for the encapsulation of probiotics and concluded that alginate beads have diameter 716 μm while alginate and whey protein concentrate beads have a mean diameter 727 μm.

3.2. Encapsulation Efficiency. The efficiency of encapsulation for the alginate-xanthan-coated beads was higher than the single coating of alginate beads. It is clear from Table 2 that the alginate-xanthangum-coated beads have the highest encapsulation efficiency ($95.20 \pm 1.40\%$) compared to alginate-coated beads ($86.85 \pm 1.30\%$). The main reason behind the higher encapsulation efficiency with small log reduction was the alginate-xanthan coating. The double layer coating provides more protection to bacteria from the harsh environments and encourages them to colonize into the intestine. The treatment has a significant influence on efficiency.

These findings are in compliance with Damodharan et al. [43] who encapsulated lactic acid bacteria with alginate, fenugreek, and locust bean gum and reported that the encapsulation efficiency is enhanced upto 96.8%. They reported that alginate and gum-based microbeads provide better survival rate in simulated gastric fluid and demonstrated that alginate 1%, fenugreek gum 0.5%, and locust bean gum 0.5% give the best performance for survival in gastric fluid (up to 96.44%). Another group of scientists studied the microencapsulation of probiotics with sodium caseinate and gellan gum [44]. They reported that the encapsulation efficiency was increased from 41.8% in case of free cells to 90.1% (encapsulated with sodium caseinate and gellan gum). The gums have a synergistic effect and enhance the survivability in low pH. The encapsulation efficiency also depends on the polymer concentration. The coencapsulation of probiotics with alginate, gums, and other materials enhances the efficiency upto 92–95%. The results of the present study indicated that EE (encapsulation efficiency) of microencapsulated *L. rhamnosus* is enhanced without affecting the survivability of bacteria during encapsulation by emulsion technique.

Furthermore, Yasmin et al. [25] demonstrated the encapsulation efficiency of *B. longum* by coating them with whey protein concentrate, pectin, and alginate microbeads presented good efficiency (>85%) because the wall material can act as a protective layer. Encapsulation efficiency increased with increasing concentration of polymer and directly proportional to the sphericity of microbeads. Moreover, spherical beads have more efficiency compared to other beads because they have better entanglement with bacteria and least reduction in viability.

3.3. In Vitro Gastrointestinal Assay

3.3.1. Survivability of Free and Microencapsulated L. rhamnosus in Bile Solution. In this study, the survival rate of free and microencapsulated probiotics was assessed during their 80 min exposure to bile salt solution. The encapsulation of *Lactobacillus rhamnosus* in T_2 (1.5% alginate) and T_3 (combination of 1.5% alginate and 0.5% xanthan gum) significantly improved their survival in 2.5% bile salt (pH 4.0). It is clear from Figure 1 that the encapsulated cells showed resistance to the bile salt solution compared to free bacteria. The T_3 (alginate 1.5% + xanthan 0.5%) presented the maximum survival rate compared to T_2 (alginate 1.5%). Free cells (T_1) showed maximum log reduction (7.87 log) during their exposure to bile salt ranging from 10.87 ± 0.5 log CFU/mL to 3.0 ± 0.4 log CFU/mL. However, in case of T_3 and T_2, there was only 1.7 log and 1.94 log reduction ranging from 10.20 ± 0.6 to 8.50 ± 0.4 log CFU/mL and 10.27 ± 0.4 to 8.33 ± 0.6 log CFU/mL, respectively. The results demonstrated that viable cells in beads remain above from 10^7 CFU/g which is essential for their colonization in the intestine. The high survival rate in the case of gum coated alginate beads indicated that gum prevents the diffusion of bile salt solution into the beads compared to alginate beads. Because of the low penetration of bile solution in gum coated alginate beads, T_3 has the maximum bacterial count. The bacterial cells were able to maintain their stability and viability. The results are in line with Eckert et al. [45] who investigated the stability of *L. plantarum* after coating them with WPC. They concluded that there was only 0.67 log reduction after their 4 hours exposure to bile solution. Bustamante et al. [46] determined that encapsulation enhances the survival rate of probiotics in 2% bile solution compared to free cells. Yao et al. [47] utilized alginate and gelatin for encapsulation purpose to determine the probiotics viability in 3% bile salt solution and concluded that the free cells were completely dead after their 40 min exposure to SIF while the encapsulated beads remained functional in low pH environment. Castilla et al. [48] utilized alginate and pectin for double layer coating of *L. casei* and concluded that the combination of alginate and pectin provides a better survival ratio (about 85%) to the probiotic compared to alginate alone in bile solution after 6 hours exposure. Arslan et al. [49]

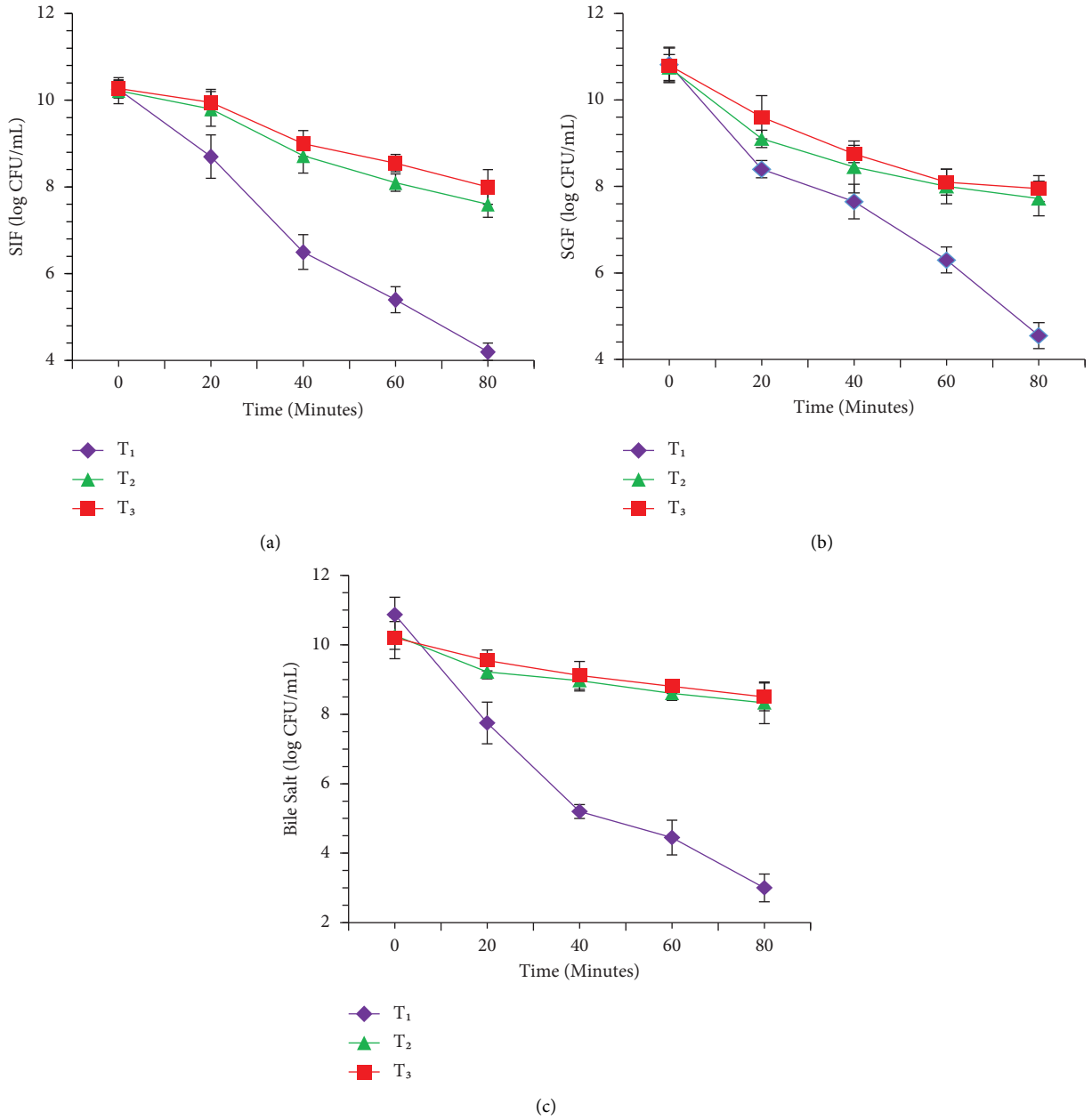

FIGURE 1: Survivability of free and encapsulated *L. rhamnosus* in (a) simulated intestinal fluid (SIF), (b) simulated gastric fluid (SGF), and (c) bile salt solution T_1 = free *L. rhamnosus*; T_2 = sodium alginate (1.5%)-encapsulated *L. rhamnosus*; T_3 = sodium alginate (1.5%)- and xanthan gum (0.5%)-encapsulated *L. rhamnosus*.

encapsulated the *Saccharomyces cerevisiae* var boulardii with different wall materials including gelatin, modified starch, gum arabic, and WPI. They demonstrated that the survival rate remains above 84.69% in acidic conditions.

3.3.2. Survivability of Free and Microencapsulated L. rhamnosus in Simulated Gastric Fluid (SGF). High survival rate of encapsulated probiotics is essential for the production of fortified or functional food items. The probiotics in food with good resistance in gastric fluid enhanced the functionality of the immune system. Microencapsulated probiotics with alginate and xanthan gum (T_3) showed a considerably good effect on the probiotic's viability during the simulated gastric treatment over time compared to the free cells. There was a notable log reduction (10.82 ± 0.4 to 4.55 ± 0.3 log CFU/mL) in T_1 (free cells) during treatment with simulated gastric fluid for 80 min. The log reduction in T_3 and T_2 was 10.80 ± 0.4 to 7.95 ± 0.3 log CFU/mL and 10.75 ± 0.3 to 7.72 ± 0.4 log CFU/mL, respectively (Figure 1). Lower reduction rate in T_3 was due to the alginate and xanthan gum encapsulation which has neutral pH due to solidification of alginate with phosphate buffer. Silva et al. [50] used alginate-gelatin and alginate-gelatin fructooligosaccharides for the microencapsulation of *L. acidophilus* in yogurt and examined the viability of microbeads in

simulated gastric and intestinal fluid. They stated that the low log reduction of microencapsulated probiotics was due to the spongy grid of gel that reduced the diffusion of fluid inside the beads of microorganisms over the period of 2 hours. Jin et al. [51] stated that the mortality rate of viable free cells in gastric fluid was due to the influence of pepsin and hydrochloric acid present in the gastric juice of human. The survival of encapsulated beads during this treatment was because of the presence of gum coating. They also reported that the structure of alginate beads after coating with gum became strengthened and dense. Chen at al. [52] demonstrated that the survival of microbeads was improved in simulated gastric fluid due to the coating with whey protein isolates (WPI). The WPI has pH buffering effect in acidic medium and protects the probiotic cells under these conditions. Qi et al. [53] stated that the survival rate of microencapsulated *S. boulardii* and *E. faecium* was improved during gastric fluid treatment for 180 minutes. They demonstrated that the survival rate of encapsulated *S. boulardii* was improved to 89.62% in SGF compared to free bacteria.

3.3.3. Survivability of Free and Microencapsulated L. rhamnosus in Simulated Intestinal Fluid (SIF). Both stomach and intestine contain different acids and pH environments. This is crucial for probiotics to being survived during gastric digestion and move into the intestine where nutrients in the food absorb. The environment of the intestine influences the mortality rate of free cells. In this study, the death rate of microencapsulated *L. rhamnosus* was determined in simulated intestinal fluid (SIF). During the treatment, free cells showed the least survivability; they decreased to 4.20 ± 0.2 log CFU/mL during the 80 minutes exposure. However, there were only 2.27 log reductions in T_3 and 2.62 log reductions in T2, ranging from 10.27 ± 0.2 to 8.00 ± 0.4 log CFU/mL and 10.22 ± 0.3 to 7.60 ± 0.3 log CFU/mL, respectively (Figure 1). The free cells showed the maximum log reduction due to free diffusion of SIF into cells. Jin et al. [51] stated that bile salt and SIF effects the viability of probiotics and increased the death rate of free cells. The bile resistance varied from strain to strain in probiotics and the viability enhanced with the coatings which acts as barriers during the diffusion process in intestinal fluid. During the determination of survival rate in xanthan coated beads, the probiotics showed improved survivability by reducing the permeability of the SIF in the cells for demolition. Mahmoud et al. [54] identified that *L. plantarum* coating with alginate-skim milk showed higher improvement during intestinal fluid exposure for 120 minutes compared to the other alginate-dextrin, alginate-DWP (denatured whey protein), and alginate-chitosan coatings. Silva et al. [50] identified that when probiotic cells remain in the intestinal fluid, they swell up and collapsed. Therefore, their functions are compromised that influence the gut health. Alginate-gelatin microbeads showed resistance to bile salt (present in the simulated intestinal fluid) by reducing the ion exchange diffusion because of the less porous structure. Shu et al. [55] studied the survival rate of free and xanthan-chitosan encapsulated *Lactobacillus acidophilus* under simulated intestinal fluid for 1 and 2 hours. The latter showed only 0.64 log reduction compared to free cells. The improvement in the viability of encapsulated cells was due to the reduction in ion exchange reaction between bile salt and microbeads.

3.3.4. Storage Stability of Free and Encapsulated L. rhamnosus. The current study was conducted to demonstrate the stability of free and encapsulated *L. rhamnosus* during storage at 4°C. Figure 2 clearly indicated that microencapsulation improved bacteria stability while free bacteria lost their survivability during 21 days of storage. There was a significant ($p \leq 0.05$) higher log reduction (5.82 log) in the viability of free bacteria as compared with encapsulated bacteria, when stored for 21 days. T_3 showed the minimum log reduction (1.3 log) from 9.60 ± 0.5 to 8.30 ± 0.4 log CFU/mL, followed by T_2, in which 2.58 log reduction was observed ranging from 9.60 ± 0.3 to 7.0 ± 0.6 log CFU/mL during storage at 4°C. The utilization of gum along with alginate worked to improve the surface structure and bacterial stability. In a previous study, Yao et al. [47] reported that microencapsulated cells showed the minimum log reduction (1.7 and 2.5 log CFU/g) during the storage of 4 weeks compared to free cells. They also demonstrated that microencapsulation can work to improve the bacterial survival under adverse conditions and particularly valuable in the pharmaceutical and food industry. The results are also in line with Riaz et al. [19] who encapsulated *B. bifidum* with alginate and zein and concluded that there was only 1.82 log reduction in encapsulated cells compared to free bacteria that showed 7.72 log reduction during 32 days of storage. It has been reported that the addition of gums along with alginate for encapsulation of probiotics reduced the surface porosity of beads and enhanced their survival rate during storage [56].

3.4. Physicochemical Analysis of Probiotic Orange Juice

3.4.1. pH. A significantly ($p \leq 0.05$) decreasing trend was observed in all types of juice samples during storage. The control treatment (T_o), showed a relatively small decline in pH (2.93 ± 0.02 to 2.85 ± 0.01) at the end of the storage period. The pH of orange juice free bacteria (T_1) was decreased from 2.94 ± 0.03 to 2.58 ± 0.02 during storage (3 weeks). This reduction may be due to the production of lactic acid by free bacteria. On the other hand, the juice encapsulated having encapsulated bacteria (T_2) manifested a significant ($p \leq 0.05$) decline from 2.94 ± 0.03 to 2.76 ± 0.03. Moreover, T_3 showed a gentle decrease in pH from 2.95 ± 0.01 to 2.82 ± 0.03 during the storage period. A rapid decrease in the pH of the sample having free bacteria was observed compared to the sample having encapsulated bacteria (Table 3). The results concluded that encapsulation helps to develop the stable environment for the probiotic bacteria. It was proved that microencapsulation helps to improve the stability of orange juice by preventing the rapid acid production by the bacteria. The pH of carrier food has a significant impact on the survival and viable ratio of

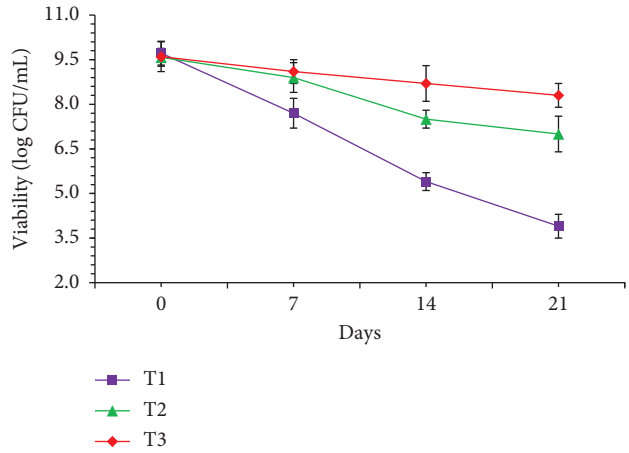

Figure 2: Storage stability of free and encapsulated *L. rhamnosus* at different intervals (0, 7, 14, and 21 days). T_1 = free *L. rhamnosus*; T_2 = sodium alginate (1.5%) encapsulated *L. rhamnosus*; T_3 = sodium alginate (1.5%)- and xanthan gum (0.5%)-encapsulated *L. rhamnosus*.

Table 3: Physicochemical analysis of free and encapsulated *L. rhamnosus*-treated orange juice.

Parameters	Treatments	0 Day	7 Day	14 Day	21 Day
pH	T_0	2.93 ± 0.02^{abc}	2.91 ± 0.03^{bcd}	2.87 ± 0.03^{def}	2.85 ± 0.01^{bcd}
	T_1	2.94 ± 0.03^{ab}	2.84 ± 0.01^{cdef}	2.70 ± 0.02^{g}	2.58 ± 0.02^{h}
	T_2	2.94 ± 0.03^{ab}	2.83 ± 0.04^{cdef}	2.80 ± 0.04^{f}	2.76 ± 0.03^{fg}
	T_3	2.95 ± 0.01^{a}	2.90 ± 0.02^{cde}	2.87 ± 0.05^{def}	2.82 ± 0.03^{ef}
Total sugars (% brix)	T_0	12.12 ± 0.05^{a}	12.00 ± 0.05^{b}	11.67 ± 0.04^{c}	11.00 ± 0.07^{de}
	T_1	12.10 ± 0.08^{ab}	10.66 ± 0.06^{f}	8.40 ± 0.06^{i}	7.90 ± 0.06^{j}
	T_2	12.11 ± 0.06^{ab}	11.04 ± 0.07^{d}	10.15 ± 0.08^{g}	9.50 ± 0.05^{h}
	T_3	12.11 ± 0.05^{a}	11.70 ± 0.09^{c}	11.07 ± 0.03^{d}	10.90 ± 0.04^{e}
Acidity (%)	T_0	0.96 ± 0.02^{hi}	1.04 ± 0.01^{efg}	1.10 ± 0.02^{cde}	1.15 ± 0.01^{c}
	T_1	0.94 ± 0.01^{i}	1.12 ± 0.04^{cd}	1.24 ± 0.03^{b}	1.35 ± 0.02^{a}
	T_2	0.95 ± 0.01^{i}	1.02 ± 0.03^{fgh}	1.07 ± 0.01^{def}	1.14 ± 0.01^{c}
	T_3	0.95 ± 0.02^{i}	1.00 ± 0.05^{ghi}	1.03 ± 0.009^{fgh}	1.05 ± 0.01^{efg}

Mean values ± S.D. in a column having similar lettering are statistically nonsignificant at 5% confidence interval.
T_0 = control, plain juice; T_1 = free *Lactobacillus rhamnosus*; T_2 = sodium alginate (1.5%)-encapsulated *L. rhamnosus*; T_3 = sodium alginate (1.5%)- and xanthan gum (0.5%)-encapsulated *L. rhamnosus*.

probiotics as the acidic pH has negative impact on the viability of probiotics. The primary reason behind the lower pH reduction in encapsulated bacteria is their low metabolic activity in juice. These results are in line with Afzaal et al. [57] who demonstrated that the pH of probiotic ice cream decreased during storage from 6.27 to 6.06 within the period of 80 days of refrigeration storage. The results regarding the pH of probiotic orange juice are completely in accordance with Nami et al. [40] who demonstrated that the pH of orange juice declines rapidly in case of free bacteria in it (2.93 to 2.51) as compared with encapsulated cells (from 2.93 to 2.70) during 6 weeks of storage. In another study, Sagdic et al. [58] proved that the addition of culture to the product caused an increase in its acidity but a reduction in its pH.

3.4.2. Total Sugars. The overall decreasing trend was observed in all juice samples during 21 days storage (Table 3). The sugar contents of orange juice having free bacteria (T_1) were decreased significantly ($p \leq 0.05$) from 12.10 ± 0.08 to 7.90 ± 0.06 during the storage (3 weeks) at 4°C. The free bacteria consumed more sugar for their metabolic activity compared to the encapsulated one. The encapsulated bacteria in T_2 and T_3 showed lower reduction in their total sugar contents ranging from 12.11 ± 0.06 to 9.50 ± 0.05 and 12.11 ± 0.05 to 10.90 ± 0.04, respectively. The double layer coating of *L. rhamnosus* with alginate and gum reduced their release of the carrier products while the free bacteria rapidly perform their metabolic activity with the utilization of sugar. Similar results were reported by Nami et al. [40] who investigated the survival of *Lactobacillus lactis* in orange juice by using alginate and persian gum for their encapsulation. They concluded that the sugar content of orange juice decreased from 11.0 to 8.00 in case of free bacteria while remaining above 10.5 in case of encapsulated bacteria after 42 days of storage. Gandomi et al. [59] demonstrated the effect of encapsulation on the survival of *Lactobacillus rhamnosus* in apple juice by using alginate and chitosan as coating materials. They concluded that bacteria undergo fermentation and utilized the sugar content of juice as substrate and these content goes on decreasing as bacteria viability increases. Among all other carbohydrates, glucose is frequently used by bacteria for the fermentation process. Due to this, the acidity increased but the carbohydrate

content of juice decreased which badly affected the product quality [60].

3.4.3. Acidity. A significantly ($p \leq 0.05$) increasing trend was observed in all treatments during storage of 21 days (Table 3). The acidity of control treatment (T_0) and juice sample having free bacteria (T_1) was significantly increased ($p \leq 0.05$) from 0.96 ± 0.02 to 1.15 ± 0.01 and 0.94 ± 0.01 to 1.35 ± 0.02, respectively during 21 days of storage. The encapsulated bacteria in T_2 and T_3 showed minimum increment in their acidity ranging from 0.95 ± 0.01 to 1.14 ± 0.01 and 0.95 ± 0.02 to 1.05 ± 0.01, respectively. The layer of xanthan gum outside the alginate layer provided double protection and helped to control the release of bacteria in the carrier products. The free bacteria continuously perform the fermentation of juice and produce lactic acid as a resultant product. Due to the increase in lactic acid content, the acidity of the juice increased during the storage. The byproducts of sugar degradation are the main cause of increased in acidity during refrigeration, i.e., lactic acid. The other reason of increased acidity during storage is that probiotic cells release an enzyme that hydrolyzes the sugars components of the juice [61]. Many researchers concluded that throughout the storage period, bacteria undergo their metabolic activity in which they utilize sugar and produce lactic acid [62, 63]. During the storage duration, the pH of juice was going on decreasing while the acidity increased because acidity has an inverse relation with pH.

3.4.4. Enumeration of Free and Encapsulated L. rhamnosus GG in Orange Juice. Both free and microencapsulated probiotics were introduced into orange juice. The bacterial count in juice was counted after one week interval during 21 days storage. A significantly ($p \leq 0.05$) decreasing trend in all types of juice samples (either containing free bacteria or encapsulated one) was observed during the storage study (Table 4). The initial bacterial count for free probiotics (T_1) was 9.50 ± 0.06 log CFU/mL which was rapidly reduced to 5.35 ± 0.04 log CFU/mL during the 3 weeks of storage interval while a lower reduction was observed for the encapsulated probiotics. The reduction in viable count of free bacteria (T_1) was maximum in the first week of storage due to temperature shock. The viable count for the probiotic that was coated with alginate (T_2) was 8.45 ± 0.05 log CFU/g, while for probiotic coated with alginate and gum (T_3) was 8.70 ± 0.05 log CFU/g at the end of the storage period. There was only 1.05 log reduction in case of alginate beads and 0.8 log reduction in the case of alginate and gum coated beads. The encapsulation materials worked for the better survival of the probiotics and protect them from acidic environment. The cell damage in free bacteria was high due to low pH and high acidity conditions. The present findings are in accordance with Afzaal et al. [31] who concluded that the total log reduction in case of alginate beads was 0.55 log and 1.13 log in case of WPC (Whey protein concentrate) beads. Kataria et al. [64] concluded that encapsulation is a beneficial technique to increase the bacterial population and for target delivery of probiotics. The similar decreasing trend for free and encapsulated bacteria was reported by Haghshenas et al. [65]. They reported that free cells of *Enterococcus durans* were reduced from 9.52–2.83 log CFU/g after month storage at 39°C.

Table 4: Probiotic count (log CFU/mL) of free and encapsulated *L. rhamnosus*-treated orange juice.

Treatments	0 Day	7 Day	14 Day	21 Day
T_1	9.50 ± 0.06^{a}	7.25 ± 0.06^{f}	6.82 ± 0.05^{g}	5.35 ± 0.04^{h}
T_2	9.48 ± 0.06^{a}	9.00 ± 0.08^{bc}	8.72 ± 0.06^{d}	8.45 ± 0.05^{e}
T_3	9.48 ± 0.05^{a}	9.07 ± 0.07^{b}	8.90 ± 0.04^{c}	8.70 ± 0.05^{d}

Mean values ± S.D. in a column having similar lettering are statistically nonsignificant at 5% confidence interval.
T_1 = free *L. rhamnosus*; T_2 = sodium alginate (1.5%)-encapsulated *L. rhamnosus* juice; T_3 = sodium alginate (1.5%)- and xanthan gum (0.5%)-encapsulated *L. rhamnosus*.

3.5. Sensory Evaluation. The results regarding the flavor, taste, aroma, color, and overall acceptability are presented in Figure 3. It is clear that there was no significant ($p \geq 0.05$) difference in the sensory parameters among the different treatments. The bacteria in encapsulated form or free form have no impact on the flavor, taste, aroma, color, and overall acceptability of orange juice. While on the other hand, in case of storage a significant difference ($p \leq 0.05$) is reported. All sensory parameters showed a decreasing trend during storage. At the end of the 3rd week, a minimum score was allotted to each treatment. In the case of flavor, aroma, and taste, the maximum mean score was assigned to T_3 (7.29 ± 0.04, 7.41 ± 0.03 and 6.90 ± 0.04) followed by T_1 and T_0, respectively. While in the case of overall acceptability, the highest mean score was also gained by T3, i.e., 6.15 ± 0.05, followed by T_2 (5.78 ± 0.04) and T_0 (5.68 ± 0.2), respectively. In the case of color evaluation of probiotic juice, T_3 evaluated best by gaining the maximum sensory mean score, i.e., 7.49 ± 0.05 followed by T_0 and T_1, i.e., 7.25 ± 0.07 and 7.08 ± 0.03. The maintenance of acceptability of food products with the addition of probiotics is a very vital step as customers do not show curiosity towards functional drinks having unusual smell, taste, color, and flavor [66]. The flavor and taste score of probiotic orange juice was in between of 6 and 7 that indicate that the consumers showed moderately or slightly liked behavior. The minimum score was assigned to the juice sample having free bacteria in it because probiotic bacteria immediately carry out the fermentation process by utilizing sugars and produce sour taste while encapsulated bacteria have slow release from the beads and produce minimal changes in flavor and acceptability [41]. The dead bacteria can also increase the acidity of juice by releasing enzymes [67]. The results of the present study regarding sensory evaluation were in line with Afzaal et al. [31] who demonstrated that free and encapsulated bacteria have no impact on the sensory parameters of ice cream while a significant impact was reported in case of storage interval. Rathod et al. [68] also reported the similar trend for probiotic apple and orange juice and concluded that the organoleptic properties of probiotic beverages were decreased during storage. Costa et al. [33] also determined the similar trend for the probiotic orange juice and concluded that the

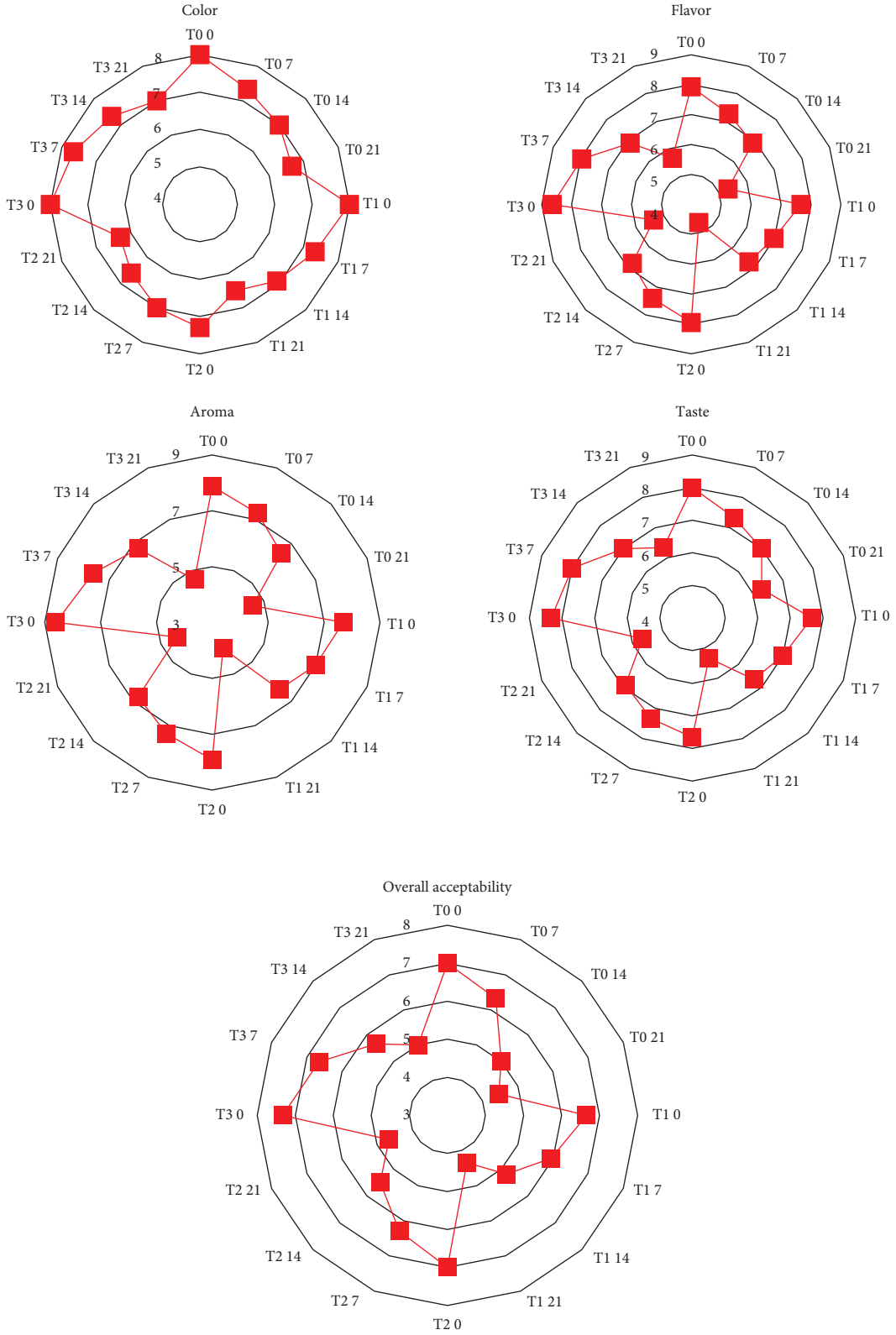

Figure 3: Sensory evaluation of free and encapsulated *L. rhamnosus*-treated probiotic orange juice at 0, 7, 14, and 21 days T_0 = control, plain juice; T_1 = free *L. rhamnosus*; T_2 = sodium alginate (1.5%)-encapsulated *L. rhamnosus*; T_3 = sodium alginate (1.5%)- and xanthan gum (0.5%)-encapsulated *L. rhamnosus*.

acceptance score was in the range of 7 according to the hedonic scale. It has been determined that probiotic juice with *Lactobacillus rhamnosus* has more taste score and acceptability compared to juices with *Lactobacillus delbrueckii* and *Lactobacillus plantarum* [69]. Taste profile is negatively affected by bacterial metabolism due to artificial taste development of juice that is not acceptable for consumers. The addition of sucrose to the juices can only improve the taste profile and overall acceptance [70]. Maillard reaction is another reason of flavor and color reduction due to temperature alteration throughout storage. The packaging of juice soon after the heat treatment can also reduce the changes in flavor, aroma, and taste of orange juice. Production of spoilage microbes in juice can also alter the taste and color profile [71].

4. Conclusion

Microencapsulation is a valuable technology for securing the therapeutic level ($\geq 10^6$ CFU/g) of beneficial microbes in any carrier product. In the current study, coating of alginate and xanthan gum provides the maximum encapsulation efficiency (95.20%) than alginate beads. Moreover, gum coated alginate beads indicated maximum viability and minimum log reduction (<3 log CFU/g) in SIF, SGF, and during storage. The probiotic count in orange juice remained above the recommended level, i.e., 8 log CFU/mL at the end of the 21 days storage period. The study suggests that gum coated alginate beads can be used for better survival of probiotics and orange juice with encapsulated probiotics may contribute a good market share due to its therapeutic benefits [72].

Data Availability

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

Conflicts of Interest

The authors declare that are no conflicts of interest.

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