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# Co-effect of microencapsulation and prebiotics on the survivability of some lactic acid bacteria in simulating gastrointestinal tract and storage conditions

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Abstract

Probiotics lose their viability during formulation, processing, and storage. The current work investigates the co-effect of three different combinations of encapsulation and prebiotics on the Survival of *L. rhamnosus, L. acidophilus, and B. adolescentis* under different conditions. In simulating gastric juice solution, the free cells survivability ranged between 36.5% to 40.5% for *B. adolescentis and L. rhamnosus*, after 2 hr, respectively. However, the encapsulated bacteria survival, ranged between 54.5% to 78.5% for *B. adolescentis* and *L. rhamnosus*, respectively. The encapsulated bacteria exhibited the highest survival rates, between 78.5%, and 76.5% for *L. rhamnosus, and L. acidophilus*, respectively, and 68.7% for *B. adolescentis* against the enzymatic gastric juice. In the simulating intestinal juice solution, cells encapsulated with resistant starch (ARs) and oligosaccharides (ARsG or ARsF) significantly enhanced survival over bacteria encapsulated with alginate alone and free cells, where the survivability was 104.4% for *L. rhamnosus*, 103.4% for L. acidophilus and 103.6% for *B. adolescentis* . A highly significant difference in survival rates was found between encapsulated and non-encapsulated bacteria when stored at 4 $^{o}$C and 25 $^{o}$C for 30 days. Survivability between 31.5% to 77.1% was apparent for *L. acidophilus* and *L. rhamnosus*, respectively, after 30 days at 4 $^{o}$C. In contrast, free bacterial cells recorded a 29.1% to 31.5% survivability. After 30 days, the survivability of microencapsulated bacteria at 25$^{o}$ C ranged between 15.6% and 63.6%, while the survival rate of free bacteria declined between 10.9% and 13.5%. Overall, microencapsulation of the tested strains enhanced bacteria tolerance, survival, and storage periods, especially at 4 $^{o}$C.

## 1. Introduction

Quinoa (*Chenopodium quinoa* Willd.) is a pseudocereal of the Amaranthace family which originated from the Andes of South America where it has been cultivated since more than 5,000 years ago [1]. Quinoa is an allotetraploid genus (2n=4x=36) thus exhibits disomic inheritance for most qualitative traits [2]. *Chenopodium quinoa* Willd., commonly known as quinoa, is a native crop of South America, which has been traditionally used as a staple food source by ancestral populations along the Andes region [3].

Every year, consumers become more aware of the importance of a healthy diet in the proper functioning of the body, which drives the demand for functional foods. Probiotics are live microorganisms with healthy and beneficial effects, such as antimicrobial activities, immune-stimulants, and anti-carcinogenic effects, on animals and humans upon ingestion in viable sufficient amounts [1,2]. Probiotics must be viable, metabolically active inside the gastrointestinal tract, and safe to have a beneficial effect [3]. Consequently, probiotic bacterial cells must withstand food storage and processing time and sustain harsh environments within the GIT to efficiently deliver viable probiotics to the large intestine. Probiotics reach the site of action in a very small number, as evidenced by high bacterial mortality during manufacturing and passage through the GIT[4]. Microencapsulation has been proposed to improve probiotic survivability in foods during storage and after consumption[5]. Microencapsulation, which is a method of encapsulation of live probiotics cells, is a promising technique of encapsulation due to their successful oral delivery and its resistance to the hard-conditions, such as high pH, a highly acidic environment, and it can protect the probiotic bacteria from the hydrolytic and proteolytic actions of the digestive enzymes which are present at the stomach and the upper portion of the small intestine [6]. Microencapsulation has been studied *in vitro* and *in vivo* environments [7]. The microbeads encapsulation improved the growth and viability of *S. boulardii and E. faecium* compared to non-encapsulated controls under high temperature and high humidity[8]. The presence of resistant starch at a concentration of 0.5% improved the Entrapment efficiency (EE) compared to the formula of Alginate alone. Furthermore, the mixture of resistant starch with Alginate also prolonged the gradual release of nisin and its activity during storage [9]. Similarly, Probiotic growth and activity are promoted by nondigestible oligosaccharides and polysaccharides, known as prebiotics [1]. Prebiotics are oligosaccharides and polysaccharides, resistant to gastric acidity, hydrolysis by mammalian enzyme hydrolysis, and (GIT) absorption [10]. Accordingly, it is highly recommended to formulate a precise and optimum combination of prebiotics and

probiotics, providing specific health benefits via synergistic action. It has been reported that due to the significant impact of prebiotics on probiotics' vitality and growth-promoting, choosing the appropriate prebiotic compounds to generate effective foods comprising a mixture of probiotics and prebiotics is essential [11].

Therefore, the present study aimed to investigate further the role of the combined efficacy of microencapsulation with the addition of certain prebiotic substances in different combinations on enhancing the properties of selected Lactobacillus bacteria and Bifidobacteria through the simulating GIT and under different storage conditions.

## Materials and Methods

*Probiotic strains*

*Lactobacillus rhamanosus, L. acidophilus* and *Bifidobacteriuam adolecentis* were previously isolated from Egyptian natural fermented dairy products, molecular identified and subjected to probiotic properties tests [12 13].

*Encapsulation materials*

For the encapsulation process, Resistant starch (Levis Starch products, Canada) and Sodium Alginate (Sigma Aldrich) were used in the current work.

*Prebiotics*

Commercially available prebiotic formulations, namely Galactooligosaccharide (GOS) and Fructooligosaccharide (FOS) (Wako, Japan), were used in the current study.

*Microencapsulation procedures*

The extrusion method was used as previously described [14]. The aforementioned probiotic strains were microencapsulated with the previously mentioned prebiotic substances. Three distinct combinations of (AL1%), (AL 1%+ RS 1%) and (AL1%+ RS 1% + GOS 1%) were employed for Lactobacillus strains in the encapsulation of probiotic cells. For Bifidobacteria, the GOS was replaced by FOS as follows: (AL 1%+ RS 1%+ FOS 1%). Two ml of the LAB suspension at a high concentration ($10^9$ CFU/ml) were mixed with 18 ml of each sterile 4 different combination solutions. The combination was then injected into the Encapsulator BIOTECH (the upgraded version of the IE-50R) with a nozzle size of 500 µm. The resultant microbeads were received into a sterilized 5% (w/v) CaCl2 solution and collected by filtration after 30 minutes of hardening, washed twice with 0.9% (w/v) germ-free NaCl saline solution. Then they were kept in 0.1% (w/v) sterile peptone solution at 4 $^oC$ and 25 $^oC$ until use. As a standard, free cells from the same strains were used (as control).

*Testing the encapsulation efficiency*

The effectiveness of the process was assessed immediately after encapsulation using the same technique described by [15 16]. Briefly, one gram of microbeads from each probiotic strain was suspended in 9 ml of 0.1 M sterilized phosphate buffer saline (PBS) (pH 7.0) and then homogenized for 30 to 50 mins. By plating 0.1 mL of the appropriate diluted bacterial cultures on MRS agar plates and cultivating under anaerobic conditions for 48 Hrs, the viable cells were identified and expressed as (CFU/g), which is a measurement combining the efficacy of entrapment and the viability of living cells during the encapsulation process, was determined using the following equation. Encapsulation efficiency yield %= (N / No) $\times 100$ [17]; N is the number of viable LAB cells (log CFU/g) loaded in the gel beads, and No is the initial number of viable cells (log CFU/g) added into the mixture during the formation of the microcapsules.

*Morphology of the capsule*

The diameters of the beads were ascertained by applying the microscope caliper. The morphology of the unfilled alginate beads and the alginate beads holding bacterial strains were assessed immediately using a light microscope with a 40x magnification power. A Microscope Olympus BX51 (Center Valley, PA, USA, software cellSens) was used to take macro images of the exterior bead structure.

*Survival of microencapsulated LAB in simulated gastric juice (SGJ)*

Acid tolerance of the encapsulated probiotics and the control (free cells) was conducted [17 18] with some modifications. Briefly, one gram of the microencapsulated bacteria was added to 9 ml of SGJ (NaCl 9 g/L, KCl 0.8946 g/L, $NaH_2PO_4$ 0.8878 g/L, $NaHCO_3$ 1.680 g/L, $CO(NH_2)_2$ 0.1981 g/L, and the Pepsin enzyme 3 g/L, and adjusted to pH $3 \pm 0.2$) and incubated for 120 min. Then the encapsulated probiotic bacteria were released from the capsules by sequestering calcium ions with Phosphate buffer solution (PBS) at pH 7. Acid and enzyme tolerance were measured by comparing the final plate count after 120 min exposure with the initial plate count at zero time. For the free cell enumeration (the control), approximately 1ml of $10^9$ CFU/mL of each probiotic strain was inoculated into SGJS and incubated at 37$^\circ$ C for up to 120 min. Samples were taken initially and after 120 min for plate count. Probiotic bacteria can form chains or clusters; thus, the cells were sonicated for five seconds to disperse before serial dilutions were performed. Subsequent serial dilutions were vortexed for 30 s individually before inoculation to MRS agar plates. Plates were incubated at 37 $^oC$ for 24-48 hrs. in an anaerobic jar with an anaerobic gas generating kit (Oxoid™ AnaeroGen™ 2.5L Sachet). All tests were repeated three times to estimate means and standard deviations.

*Survival of microencapsulated LAB in simulating intestinal juice (SIJ)*

*The* methods used were according to [17 18] with some modifications. Briefly, one gram of each of the microencapsulated probiotic bacteria was added to 9ml of adjusted to pH 6.8 ± 0.2 SIJS containing (NaCl 9 g/L, KCl 0.8946 g/L, $NaH_2PO_4$ 0.8878 g/L, $NaHCO_3$ 1.680 g/L, $CO(NH_2)_2$ 0.1981 g/L, bile salt 3 g/L, and pancreatin enzyme 1 g/L) and incubated for 240 min. For the free cells (the control), about 1ml of $10^9$ CFU/mL of each strain was inoculated into the SIJS and incubated at 37 ◦C for up to 120 min (2 hr.). Microbial growth was recorded by evaluating the viable cell counts at 0 times and after 120 min on MRS agar in an anaerobic condition. The bile and the tolerance of pancreatin of each strain were

measured by comparing the count after 120 min of exposure with the initial count at zero point. The survival measurements were done by counting colonies on solid media in triplicate: serial dilutions (100 µL in 1000 µL) in peptone water from $10^{-1}$ to $10^{-6}$, then spread on MRS agar plates, and finally, incubation for 24-48h at 37°C.

*Long-term refrigeration (4°C) and room temperature (25 °C) storage stability of microencapsulated LAB strains*

The survival of microencapsulated LAB cells for long-term refrigeration storage was investigated in comparison with free cells by adopting the approach of [19]. Samples of the beads and free cells were stored in the refrigerator at 4° C and room temperature at approximately 25° C and were taken over 4 weeks on days 0 (initial), 2, 4, 7, 15, 23, and 30. They were used for determining the live bacterial cell counts after disintegration in 0.1 M sterile phosphate buffer. Briefly, microencapsulated LAB cells (1 g) and free cells (1 ml) were suspended in 9 ml of 0.1% (w/v) sterile peptone solution for plate count.

The survival percentages at the end of the test were calculated as follows:

% survival = (log No. of CFU/ml after 30 days/ log No. of CFU/ml at zero time) x 100

*Statistical Analysis*

All measurements were conducted three times, and the data were presented as means of those three replicates. Then, the obtained data were subjected to statistical analysis using the F test and Duncan's multiple- multiple range test to compare the means [20].

**Results and discussion**

*Effect of the microencapsulation on the morphology and size of the capsules (microbeads)*

A digital microscope was used to analyze the morphological characters of the obtained microbeads and the trapped bacterial cells. Fig.1 .It was obvious that the microbeads for all the different materials used in this process were approximately spherical. Besides, microencapsulation provided the encapsulated bacterial cells with a relatively intact physical barrier.

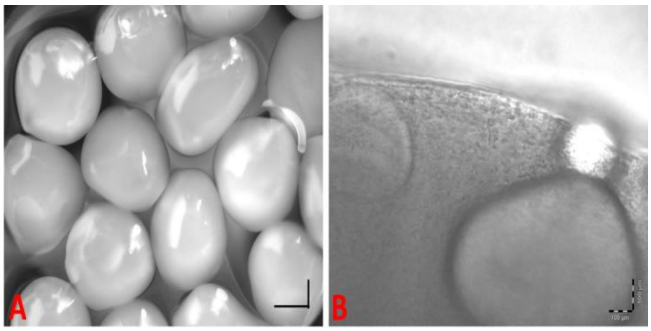


**Figure (1a):** Digital stereomicroscopy images of: A- filled bead with bacterial cells (15.6X) Scale bars above represent 1 mm. B- trapped bacterial cells inside the beads (100x 1.40) scale bars.

The results of encapsulated *L. rhamnosus* given in Fig. 2 and Table (1) showed that the microcapsules had an approximately spherical shape without cracks and were of uniform size. The mean size of the capsules was recorded as the diameter of the encapsulated beads, and the data (Table 1) showed that the beads prepared with sodium alginate had a mean diameter of 358.35 µm and the mean diameter increased with the addition of prebiotics ( 495.21 µm for Alginate with resistant starch beads). The mean diameter for Alginate with resistant starch and oligosaccharide beads was 543.72 µm. Thus, encapsulation efficiency is a measure that combines the efficiency of entrapment and the viability of living bacterial cells during the encapsulation process. Table 1 also showed that the efficiency % of oligosaccharide encapsulated bacterial cells was higher than that of sodium alginate alone (A) encapsulated cells (75.6 %). Furthermore, the efficacy of encapsulated cells differed according to the encapsulating agent. The efficiency of ARs encapsulated cells (85.5%) and ARsG / ARsF cells (89.2%) were higher. Those observations agree with the previous studies by [9], who found that the average size of capsules was 300 ± 20 µm and resistant starch particles were visible inside the capsules. A similar result for the diameter of the encapsulated beads was also recorded earlier, and data showed that the beads prepared with sodium alginate had a mean size of 715 µm. In comparison, the carrageenan beads had an average diameter of 727 µm [21 22].

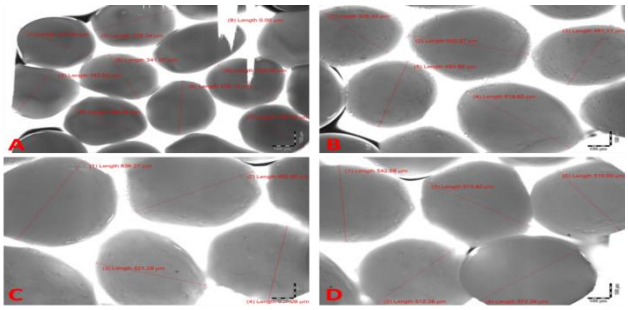


**Figure (1b):** Diameter of the encapsulated bacterial beads of different combinations: A- beads with Alginate alone B- beads with alginate and resistant starch C- beads with alginate, resistant starch, and Galactooligosaccharide D- beads with alginate, resistant starch, and Fructooligosaccharide.

**Table (1):** Diameters and encapsulation efficiency (%) of microencapsulated probiotic bacteria with 3 different materials.

| Beads properties | Mean diameter (µm) | Encapsulation efficiency % |
|---|---|---|
| (A): Alginate 1% | 358.35 | 75.6 |
| (ARs): Alginate 1% + Resistant starch 1% | 495.21 | 82.5 |
| (ARsG/F): Alginate 1%+ Resistant starch 1% + GOS 1% or FOS 1% | 543.72 | 89.2 |

*Survival of microencapsulated cells of 3 probiotic strains in simulated gastric juice (SGJ)*

The results given in Table (2) showed that the viable count for the free cells of *L. rhamnosus* highly decreased markedly from 8.92 to 3.62 log CFU/ml, followed by the encapsulated cells with Alginate alone 1%, which showed a somewhat high decrease also from 7.43 to 4.31 within 2 hrs., meanwhile,

blending two encapsulation substances as in the encapsulated cells with (ARs) gave higher protection to the bacterium, where the viable cells decreased somewhat slightly from 8.21 to 5.64. The highest viable count was recorded for the encapsulated cells with two encapsulation materials plus a prebiotic substance (ARsG), which exhibited the slightest decrease after incubation in the acidic enzymatic gastric environment for 120 min (from 8.83 to 6.94 log CFU/g). The survival percentage of the bacterial cells in the treatment of ARsG encapsulation (78.5%) was almost 2-fold higher than that of the free-cells treatment (40.5%) (Tab1e 2). In the case of strain *L. acidophilus* (Table 2), similar results were obtained, where the free cells had the highest decline in viable count from 8.81 to 3.26 log CFU/ g., followed by the encapsulated cells with Alginate 1% (A). Then came the treatment o encapsulation with two materials (ARs), which decreased less from 8.12 to 5.33 log CFU/g. Again, the encapsulated cells with ARsG gave the lowest rate of decline in cell survival from 8.71 to 6.66 log CFU/g beads under the same conditions. Similarly, in Table (2) also, the survival of bacterial cells in the ARsG encapsulation treatment (76.5%) was almost 2-fold higher (highly significantly higher at a 1% level of probability) than that of free-cell treatment (37 %) . As for *B. adolescentis* strain, the data exhibited a similar trend as presented in Table (2), where the treatment of the free cells had recorded the highest decline in mean viable count from 8.61 to 3.14 log CFU/ g, followed by the encapsulated cells with Alginate 1% (A) which was almost similar in count decline from 7.11 to 3.88 log CFU/ml. Similarly, the number of viable cells encapsulated with ARs showed a high decrease from 7.81 to 4.91 log CFU/g. Again, however, the encapsulated cells with ARsF had the lowest rate of decline, which declined to 5.86 log CFU/g after 120 min exposure to the same conditions. Also, it should be noticed that the encapsulation with ARsG for the two *Lactobacillus* strains seemed to be much more efficient (survival rates ranged from 78.5 – 76.5 %) (Tables 2) surpassed the encapsulation with ARsF of strain *B. adolescentis*, which showed slightly less survival of 68.7% within 2 hrs in SGJ.

In general, it was obvious that blending two encapsulation substances (A + Rs) increased the survivability of the bacteria. In addition, the survival percentage of the encapsulated cells of all three strains was higher than that of non-encapsulated (free cells) after 120 min, as shown in Table. (2). The survivability for the free cells ranged between 36.5% to 40.5% for *B. adolescentis* and *L. rhamnosus*, respectively. However, the encapsulated bacteria survival, in general, ranged between 54.5% to 78.5% for *B. adolescentis* and *L. rhamnosus*, respectively.

**Table (2):** Survivability of free cells and microencapsulated probiotic bacteria in simulated gastric juice for 120 minutes.

| Probiotic strain | Incubation time<br>Treatment<br>Isolates | Zero time | 1 hr. | 2 hr. | Survival % |
|---|---|---|---|---|---|
| *L. rhamnosus* | **Free cells** | 8.92 a | 6.89 c | 3.26 d | 40.5 |
| | **A.** | 7.43 c | 6.43 d | 4.31 c | 58 |
| | **ARs.** | 8.21 b | 7.27 b | 5.64 b | 68.6 |
| | **ARsG.** | 8.83 a | 8.34 a | 6.94 a | 78.5 |
| *L. acidophilus* | **Free cells** | 8.81 a | 6.55 c | 3.26 d | 37 |
| | **A.** | 7.33 c | 6.11 d | 4.19 c | 57.2 |
| | **ARs.** | 8.12 b | 7.07 b | 5.33 b | 65.6 |
| | **ARsG.** | 8.71 a | 8.04 a | 6.66 a | 76.5 |
| *B. adolescentis* | **Free cells** | 8.61 a | 6.35 b | 3.14 c | 36.5 |
| | **A.** | 7.11 c | 5.34 c | 3.88 c | 54.5 |
| | **ARs.** | 7.81 b | 6.73 b | 4.91 b | 62.8 |
| | **ARsF.** | 8.52 a | 8.01 a | 5.86 a | 68.7 |

The reason for this maintenance and high survivability might be that establishing a hydrogel barrier by the sodium alginate layer retarded the permeation of simulated gastric juices into the capsule to interact with the probiotic cells [26]**.** However, alginate beads were susceptible to acidic environments and their crackling and loss of mechanical stability in the lactic acid-containing environments [27]**.** Meanwhile, the alginate-starch blends render the advantage of micronutrients and metabolites diffusing through the capsules, inside and outside of the entrapped cell. In this respect, several authors concluded that blending Alginate with starch is common. It has been shown that the encapsulation effectiveness of different bacterial cells, mainly lactic acid bacteria, was improved by applying such a method [28 29 30]. Besides, Oligosaccharides, which could provide proper protection and even promote the proliferation of the cells, appeared to contribute to the growth *of L. fermentum* cells**)** [31 32]**.**

*Survival of microencapsulated cells of 3 probiotic strains in simulated intestinal juice (SIJ)*

The viability of the probiotics appears to be affected by encapsulation materials, as well as the diameter of the capsules. A reduction in diameter can remove the protective function of the encapsulation, while an increase in the diameter of the capsule reduces the digestibility of the pancreatic enzymes. Meanwhile, in a previous study, [7] mentioned that the types of encapsulated microcapsules that contained different oligosaccharides had no significant influence on the size of the beads. The sizes of beads obtained in this study were those reported by [23]. However, [24] stated that there is no "ideal particle size"; instead, the desirable average size depends on the specific application intended, and in foods, it may range from a few micrometers (less than 500 μm) to a few millimeters (up to 3 mm) Meanwhile, it should be noted that larger particles may have limited applications in food products because they often have undesirable effects on the texture properties, which is a fundamental challenge in food product development [25]**.** There was little improvement compared to the encapsulated cells with Alginate 1% alone. Similar findings by [15] showed that the yield of cells co-encapsulated with FOS was highest and reached 89.75% ± 0.6. Also, the combination of Alginate with oligosaccharides might have decreased the porosity of the gel beads and reduced the leakage of *L. fermentum* cells.

*Survival of microencapsulated cells of 3 probiotic strains in simulated gastric juice (SGJ)*

The results given in Table (2) showed that the viable count for the free cells of *L. rhamnosus* highly decreased markedly from 8.92 to 3.62 log CFU/ml, followed by the encapsulated cells with Alginate alone 1%, which showed a somewhat high decrease also from 7.43 to 4.31 within 2 hrs., meanwhile, blending two encapsulation substances as in the encapsulated cells with (ARs) gave higher protection to the bacterium, where the viable cells decreased somewhat slightly from 8.21 to 5.64. The highest viable count was recorded for the encapsulated cells with two encapsulation materials plus a prebiotic substance (ARsG), which exhibited the slightest decrease after incubation in the acidic enzymatic gastric environment for 120 min (from 8.83 to 6.94 log CFU/g). The survival percentage of the bacterial cells in the treatment of ARsG encapsulation (78.5%) was almost 2-fold higher than that of the free-cells

treatment (40.5%) (Tab1e 2). In the case of strain *L. acidophilus* (Table 2), similar results were obtained, where the free cells had the highest decline in viable count from 8.81 to 3.26 log CFU/ g., followed by the encapsulated cells with Alginate 1% (A). Then came the treatment o encapsulation with two materials (ARs), which decreased less from 8.12 to 5.33 log CFU/g. Again, the encapsulated cells with ARsG gave the lowest rate of decline in cell survival from 8.71 to 6.66 log CFU/g beads under the same conditions. Similarly, in Table (2) also, the survival of bacterial cells in the ARsG encapsulation treatment (76.5%) was almost 2-fold higher (highly significantly higher at a 1% level of probability) than that of free-cell treatment (37 %) . As for *B. adolescentis* strain, the data exhibited a similar trend as presented in Table (2), where the treatment of the free cells had recorded the highest decline in mean viable count from 8.61 to 3.14 log CFU/ g, followed by the encapsulated cells with Alginate 1% (A) which was almost similar in count decline from 7.11 to 3.88 log CFU/ml. Similarly, the number of viable cells encapsulated with ARs showed a high decrease from 7.81 to 4.91 log CFU/g. Again, however, the encapsulated cells with ARsF had the lowest rate of decline, which declined to 5.86 log CFU/g after 120 min exposure to the same conditions. Also, it should be noticed that the encapsulation with ARsG for the two *Lactobacillus* strains seemed to be much more efficient (survival rates ranged from 78.5 – 76.5 %) (Tables 2) surpassed the encapsulation with ARsF of strain *B. adolescentis*, which showed slightly less survival of 68.7% within 2 hrs in SGJ.

In general, it was obvious that blending two encapsulation substances (A + Rs) increased the survivability of the bacteria. In addition, the survival percentage of the encapsulated cells of all three strains was higher than that of non-encapsulated (free cells) after 120 min, as shown in Table. (2). The survivability for the free cells ranged between 36.5% to 40.5% for *B. adolescentis* and *L. rhamnosus*, respectively. However, the encapsulated bacteria survival, in general, ranged between 54.5% to 78.5% for *B. adolescentis* and *L. rhamnosus*, respectively.

The reason for this maintenance and high survivability might be that establishing a hydrogel barrier by the sodium alginate layer retarded the permeation of simulated gastric juices into the capsule to interact with the probiotic cells [26]**.** However, alginate beads were susceptible to acidic environments and their crackling and loss of mechanical stability in the lactic acid-containing environments [27]**.** Meanwhile, the alginate-starch blends render the advantage of micronutrients and metabolites diffusing through the capsules, inside and outside of the entrapped cell. In this respect, several authors concluded that blending Alginate with starch is common. It has been shown that the encapsulation effectiveness of different bacterial cells, mainly lactic acid bacteria, was improved by applying such a method [28 29 30]. Besides, Oligosaccharides, which could provide proper protection and even promote the proliferation of the cells, appeared to contribute to the growth *of L. fermentum* cells**)** [31 32]**.**

*Survival of microencapsulated cells of 3 probiotic strains in simulated intestinal juice (SIJ)*

Data presented in (Table 3) indicate that the viable count of the free cells of *L. rhamnosus* decreased slightly from 8.92 to 7.33 log CFU/ml, followed by the encapsulated cells with Alginate 1%. On the other hand, a positive result was recorded for the survival of the encapsulated cells with (ARs) which increased from 8.21 to 8.41, and the highest survival of bacterial cells was recorded for those encapsulated with (ARsG) , which unexpectedly showed an increase from 8.83 to 9.22 log CFU/g after 120 min. Data in Table (3) also showed a similar trend for *L. acidophilus*, where the viable count for the free cells decreased slightly from 8.81 to 7.05 log CFU/ml, followed by the encapsulated cells with Alginate 1%. Contrary to the previous results, the encapsulated cells with (ARs) increased from 8.12 to 8.21, and the highest increase in bacterial cells encapsulated with (ARsG) increased after 120 min from 8.71 to 9.01 log CFU/g. The results in Table (3) showed also a similar trend for B. *adolescentis*, where the number of the free cells also decreased somewhat slightly from 8.61 to 6.82 log CFU/ml, followed by the encapsulated cells with Alginate 1%. Meanwhile, the encapsulated cells with (ARs) increased from 7.81 to 7.93, and the highest bacterial cells increase was recorded for those encapsulated with (ARsF), which reached finally after 120 min to 8.83 log CFU/g.

In general, the survival percent of the treatments of the encapsulated cells was significantly higher than that of non-encapsulated (free cells) after 120 min, as presented in Tables (3). The survivability for the free cells ranged between 79.2% to 82.1% for *B. adolescentis* and *L. rhamnosus*, respectively. However, the encapsulated bacteria showed survivability ranging between 98.5% to 104.4% for all three strains proving that microencapsulation provided effective protection to the free cells in the intestine. Furthermore, tha addition a prebiotic substance prvided a significant degree of survivability to the encapsulated bacteria. These results are in accordance with those previously reported by [7]**.** In addition to the protection function of bacterial cells viability, oligosaccharides entrapped in the capsule wall could also have provided selective carbon sources for lactobacilli and even promoted the proliferation of the strain *L. fermentum* cells [31 32] . The effective growth-promoting and protective effect of the oligosaccharide FOS in his study could be attributed to the preference of *L. fermentum* cells entrapped to FOS encapsulated. At a deeper level,[33] revealed that microencapsulation with oligosaccharides allowed viable *L. fermentum* cells to guarantee a high level for use as a probiotic during intestinal simulation, based on the usual effective dosage of $10^7$–$10^9$ CFU/ml.

*Long-term storage at 4°C and 25°C, and stability of free cells and microencapsulated cells of three different probiotic strains*

**Table (3):** Survivability of free cells and microencapsulated probiotic bacteria in simulated intestinal juice for 120 minutes.

| Probiotic strain | Incubation time<br>Treatment<br>Isolates | Zero time | 1 hr. | 2 hr. | Survival % |
|---|---|---|---|---|---|
| *L. rhamnosus* | **Free cells** | 8.92 a | 7.64 b | 7.33 c | 82.1 |
| | **A.** | 7.43 c | 7.41 b | 7.32 c | 98.5 |
| | **ARs.** | 82.1 b | 8.52 b | 8.41 b | 102.4 |
| | **ARsG.** | 8.83 a | 9.11 a | 9.22 a | 104.4 |
| *L. acidophilus* | **Free cells** | 8.81 a | 7.38 c | 7.05 c | 80 |
| | **A.** | 7.33 c | 7.33 c | 7.31 c | 99.7 |
| | **ARs.** | 8.12 b | 8.12 b | 8.21 b | 101.1 |
| | **ARsG.** | 8.71 a | 8.82 a | 9.01 a | 103.4 |
| *B. adolescentis* | **Free cells** | 8.61 a | 7.12 c | 6.82 c | 79.2 |
| | **A.** | 7.11 c | 6.92 c | 7.13 c | 100 |
| | **ARs.** | 7.81 b | 7.82 b | 7.93 b | 101.2 |
| | **ARsF.** | 8.52 a | 8.55 a | 8.83 a | 103.6 |

Data presented in Table (4) showed that the Survival of *L. rhamnosus* strain encapsulated with ARsG stored for a month at

4°C decreased slightly from 8.83 to 6.81 log CFU/ml. Then the encapsulated cells with (ARs) exhibited a somewhat higher decrease from 8.21 to 5.42 log CFU/g, followed by the encapsulated cells with Alginate 1% alone. The most decline in viable cell count was recorded for the free cells, which were reduced from 8.92 to 2.81 log CFU/ml. Thus, the survivability at 4°C for the ARsG treatment (77.1%) and ARs treatment (66 %) proved to be significantly higher than that occurred for the free cell treatment (31.5%).

The results in Table (4) also showed a similar trend for *L. acidophilus* strain encapsulated with ARsG, which had the lowest decrease in viable count from 8.71 to 6.32 log CFU/ml, then came the encapsulated cells with (ARs) which decreased from 8.12 to 5.31 log CFU/g, followed by those encapsulated with Alginate 1% alone. The most decline in viable cell count was recorded for the free cells, which markedly declined from 8.81 to 2.64 log CFU/ml. Again, the Survival of *L. acidophilus* cells again at 4°C for the ARsG treatment (72.5 %) and ARs treatment (65.4 %) proved to be significantly higher than those recorded for the A treatment (31.5%) and free cells treatment (29.9 %). Almost the same trend also was found for *B. adolescentis* (Table 4), where encapsulation with ARsF showed the lowest decrease in viable cell count from 8.52 to 6.51 log CFU/ml, then came the encapsulated cells with (ARs) which decreased from 7.81 to 5.04 log CFU/g, followed by those encapsulated with Alginate 1% alone. The highest and most significant decline of viable cells was recorded for the free cells, which were reduced from 8.61 to 2.51 log CFU/ml. Thus, the Survival of *B. adolescentis* cells at 4°C was recorded for the ARsF treatment (76.4 %) and ARs treatment (64.5 %) , and proved to be significantly higher than those recorded for the A treatment (32.4 %) and free cells treatment (29.9 %) within one-month storage.

**Table (4) :**Storage stability of free cells and microencapsulated probiotic bacteria at 4° C for one month.

| Probiotic strain | Incubation time Treatment Isolates | Zero time | 15 days | 30 days | Survival % |
|---|---|---|---|---|---|
| *L. rhamnosus* | **Free cells** | 8.92 a | 5.82 c | 2.81 c | 31.5 |
| | **A.** | 7.43 c | 5.01 d | 2.52 c | 33.9 |
| | **ARs.** | 8.21 b | 6.82 b | 5.42 b | 66 |
| | **ARsG.** | 8.83 a | 7.82a | 6.81 a | 77.1 |
| *L. acidophilus* | **Free cells** | 8.81 a | 5.72 c | 2.64 c | 29.9 |
| | **A.** | 7.33 c | 4.91 d | 2.31 c | 31.5 |
| | **ARs.** | 8.12 b | 6.73 b | 5.31 b | 65.4 |
| | **ARsG.** | 8.71 a | 7.51 a | 6.32 a | 72.5 |
| *B. adolescentis* | **Free cells** | 8.61 a | 5.62 b | 2.51 c | 29.1 |
| | **A.** | 7.11 c | 4.73 d | 2.31 c | 32.4 |
| | **ARs.** | 7.63 b | 6.41 b | 5.04 b | 64.5 |
| | **ARsF.** | 8.52 a | 7.51 a | 6.51 a | 76.4 |

Tables (5) presents the data of long-term storage (one month) at 25°C for free cells and encapsulated cells of the same three previous probiotic strains. The obtained results showed that the cell count of *L. rhamnosus* encapsulated with ARsG partially decreased from 8.83 to 5.61 log CFU/ml within a month, followed by those encapsulated with (ARs). Then the encapsulated cells with (A) showed the highest decrease in viable cell count, remarkably reduced from 7.43 to 1.43 log CFU/ml. Then came, the free cells, which exhibited an extreme decline in viable count from 8.92 to 1.21 log CFU/ml. Generally, the survival of bacterial cells at 25° C for the ARsG treatment (63.5 %) and ARs treatment (51.6 %) was found to be significantly higher than those obtained for the A treatment and free cells treatment (19.2 and 13.5 %, respectively) within one-month storage.

In case of storage of encapsulated bacteria at 25 °C , the results in Table (5) showed a similar trend, where *L. acidophilus* encapsulated with ARsG exhibited a somewhat slight decrease from 8.71 to 5.12 log CFU/ml after 30 days , followed by those encapsulated with (ARs), showing a more considerable decline from 8.12 to 4.11 log CFU/ml. Then came the encapsulated cells with (A), which had an extreme and significant decline in viable cell count (from 7.33 to 1.31 log CFU/ml), followed by the treatment of the free cells, which declined similarly in viable count from 8.81 to 1.14 log CFU/ml.

The general survival % of *L. acidophilus* cells at 25° C within one month for the ARsG treatment (61.5 %) and ARs treatment (50.6 %) proved to be significantly higher than those obtained for the A treatment and free cells treatment (17.8 and 12.9 %, respectively). Data given in Table (13) showed similar behaviour for the strain B. *adolescentis* encapsulated with ARsF, which decreased slightly from 8.52 to 5.31 log CFU/ml, followed by those encapsulated with (ARs). Then came the encapsulated cells with (A), which showed the highest decrease in viable cell count (from 7.11 to 1.11 log CFU/ml), followed by the free cells, giving the extreme decline in viable count from 8.61 to 0.94 log CFU/ml. The behavior in survival % for all treatments of *B. adolescentis* was almost similar to those obtained for both *Lactobacillus* strains.

In conclusion, it is essential to develop more integrated microcapsules to enhance the effectiveness of microcapsule storage at different temperatures.

The survival % of the encapsulated viable cells was significantly higher than that of non-encapsulated (free cells) after 30 days. An approximate range of survivability between 31.5% to 77.1% was apparent for *L. acidophilus* and *L. rhamnosus*, respectively, after 30 days at 4° C. In contrast, lower survivability ranging from 29.1% to 31.5% was found for the free bacterial cells' treatments. Furthermore, when comparing the results of microencapsulated bacterial cells stored at 25° C, it was apparent that survivability ranged between 15.6% to 63.6% after 30 days; meanwhile, higher decreases in survival stability ranged between 10.9% to 13.5% were found for free bacterial cells. It is also worth mentioning that blending two encapsulation substances (Alginate+ Res.Starch) was more effective than using Alginate alone, and adding a prebiotic material gave the best results for encapsulation and storage.

The high survivability of the encapsulated bacteria was probably due to the formation of a hydrogel around the bacterial cell pellet that protected the encapsulated cells; hence the external solution had to permeate through the alginate layer before reaching the microencapsulated bacterial cells[34]. Given the accumulating evidence for the viability of microencapsulated probiotic cultures, it is commonly believed that beads stored in low-temperature conditions contained more viable cells for over 4 weeks, representing a significant improvement over storage at room temperature and the non-encapsulated cells [35]. A previous study found that fructo-oligosaccharides and microencapsulation were responsible for probiotic strains' survival during prolonged yoghurt storage [36] .

For example, producing chitosan-coated alginate-oligosaccharides beads would help to reduce the leakage of oligosaccharides in beads [37]. On the other hand, [38] found no significant reduction in microencapsulated cell viability after 45 days of storage at 8º C and 25º C and -18º C, with a cell count of 7.6 log CFU. This may be attributed to the formation of capsules that effectively protected cells under environmental stress, such as oxygen and moisture, and limited internal heat diffusion. At room temperature (25º C), there was a 2.52 log CFU.g-1 reduction in the viability of *L. plantarum* after 45 days of storage.

**Table (5) :** Storage stability of free cells and microencapsulated probiotic bacteria at 25º C for one month.

| Probiotic strain | Incubation time Treatment Isolates | Zero time | 15 days | 30 days | Survival % |
|---|---|---|---|---|---|
| *L. rhamnosus* | **Free cells** | 8.92 a | 5.03 c | 1.21 c | 13.5 |
| | **A.** | 7.43 a | 4.42 d | 1.43 c | 19.2 |
| | **ARs.** | 8.21 a | 6.21 b | 4.24 b | 51.6 |
| | **ARsG.** | 8.83 a | 7.21 a | 5.61 a | 63.5 |
| *L. acidophilus* | **Free cells** | 8.81 a | 4.92 c | 1.14 c | 12.9 |
| | **A.** | 7.33 a | 4.31 d | 1.31 c | 17.8 |
| | **ARs.** | 8.12 a | 6.13 b | 4.11 b | 50.6 |
| | **ARsG.** | 8.71 a | 6.92 a | 5.12 a | 61.5 |
| *B. adolescentis* | **Free cells** | 8.61 a | 4.72 c | 0.94 c | 10.9 |
| | **A.** | 7.11 c | 4,13 d | 1.11 c | 15.6 |
| | **ARs.** | 7.81 b | 5.81 b | 3.82 b | 48.9 |
| | **ARsF.** | 8.52 a | 6.93 a | 5.31 a | 62.3 |

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