THE EFFECT OF ENCAPSULATION ON SOME PROPERITES *OF LACTOBACILLUS PLANTRAUM*

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ABSTRACT

The objective of the study was to enhance the survivability of *Lactobacillus plantarum* (ATCC 29521) and investigate their resistance to some extreme conditions following its microencapsulation with alginate, chitosan, and olibanum gum. The study's findings showed that the use of olibanum gum in the first and second layers with alginate and chitosan protected the bacteria against various forms of pasteurization, as well as against extremely acidic conditions and high bile salt concentrations. After 28 days of storage at 4°C, the bacteria coated with the using of olibanum gum in the first and second layer demonstrated a great superiority in maintaining high numbers and better viability. The use of gum also contributed to increase the ability of the bacteria to withstand freezing storage at -18°C and re-thawing. The results also showed that olibanum gum was a superior cryoprotectant during freeze-drying process and storage for six months at 22 ℃. Also scanning electron microscope images showed that beads encapsulated with using of olibanum gum loaded a higher number of *Lb. plantarum*.

Keywords: pasteurization, olibanum gum, extrusion, stress tolerance, storage stability, food safety

تاثير التغليف على بعض خصائص *Lactobacillus plantarum*

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المستخلص

هدفت الدراسة الى تعزيز قدرة بقاء *Lb. plantarum* (ATCC 29521) ودراسة مقاومتها لبعض الظروف المتطرفة بعد التغليف الدقيق بالالجينات والكيتوسان وصمغ الاوليبانوم وأثبت النتائج التي تم الحصول عليها في هذه الدراسة إلى أن استخدام صمغ الاوليبانوم في الطبقة الأولى والثانية مع الالجينات والكيتوسان عمل على حماية البكتريا عند مواجهة عدة انواع من البسترة وكذلك في مواجهة الظروف الحامضية المتطرفة والتراكيز العالية من املاح الصفراء. أظهرت البكتريا التي استخدم في تغليفها صمغ الاوليبانوم تفوق ملحوظ بالاحتفاظ بأعداد عالية وبعيوشية أفضل عند الخزن بدرجة 4 م لمدة 28 يوم و كما ساهم استخدام الصمغ بزيادة قدرة البكتريا على تحمل الخزن بالتجميد −18 م وإعادة الذوبان .كما اظهرت النتائج ان صمغ الاوليبانوم كان بمثابة عامل حماية من البرودة أثناء عملية التجفيف بالتجميد والخزن لمدة ستة أشهر عند درجة حرارة 22 م. كما اظهرت صورالمجهر الالكتروني الماسح ان الحبيبات المغلفة باستخدام صمغ الاوليبانوم تحمل عدد اكبر من *Lb. plantarum*.

الكلمات المفتاحية: البسترة، صمغ الاوليبانوم، البثق، تحمل الاجهاد، ثباتية الخزن، سلامة الغذاء

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INTRODUCTION

Probiotics are "live microorganisms that impact health advantages on the host when provided at suitable levels" (12). Probiotics (typically lactobacilli and bifidobacteria) have been demonstrated to be useful in preventing certain medical disorders (4). These microbial dietary preparations may have beneficial effects on the physiological systems of humans and animals by modulating systemic and mucosal immunity, increasing nutrient absorption and utilization, restoring nutritional balance and improving their health condition by restoring the balance of the gut microbiota (3) , and increasing the antagonistic action of beneficial microorganisms against diarrhea-causing bacteria, especially *Lb. plantarum* has the ability to colonize GIT and play a role in protective mechanisms against pathogens like Shiga toxins (1,5). Also, *Lb. plantarum* produce biosurfactant which have antimicrobial activity against some microorganisms such as *S. aureus* and *P. aeruginosa* (46). When compared to other starter cultures of bacteria, *Lb. Plantarum* is the most effective in producing exopolysaccharides, which serves to improve the viscosity and strength of the probiotic-fermented milk product. Changes in oxygen exposure, relative humidity (RH), temperature, osmotic pressure, and pH are the key environmental factors that affect probiotic viability during food production, storage, and transportation. Most probiotics are destroyed by high levels of oxygen, relative humidity, osmotic pressure, and temperature (21, 37). Also, during transit through the gastrointestinal tract, factors such as high ionic strength, enzyme activity (pepsin, lipases, proteases, amylases, and so on), bile acids, and mechanical churning are other potentially adverse circumstances in the stomach that have been reported to affect the viability of various probiotics (44). The most difficult aspect of working with probiotic microbes is maintaining their viability during food processing and storage, even at low and high temperatures. A number of technologies have been developed to increase or improve probiotic viability in various food matrixes; the encapsulation technique has been proven to be the most efficient. Microencapsulation is the technique of filling, covering, or coating a single liquid or solid (core) droplet or molecules with a continuous polymeric (shell) surface to form capsules (22). Probiotics have been effectively encapsulated in chitosan-based polymers (e.g., alginate–chitosan, gelatin, inulin, and xanthan gum, alginate-based materials (e.g., calcium alginate, sodium alginate, alginate, and animal proteins (like collagen or vegetable proteins, maltodextrin, gum arabic, cellulose derivatives, carrageenan, and milk protein-based materials (e.g., whey protein isolates, sodium caseinate gelled with transglutaminase) (18,35,17). For the first time, olibanum gum was used to encapsulate *Lactobacillus plantarum* which consists of 30-60 % resin and 5-10% essential oil (39). The aims of the study were to determine the resistance of microencapsulated *Lactobacillus plantraum* by using olibanum gum as the first and second layer in combination with alginate and chitosan to some extreme conditions, such as high temperature, low temperature, low pH levels and high bile salts concentrations, and study the shape and size of microcapsules as well the number of bacteria loaded in it.

MATERIALS AND METHODS

probiotic bacteria: *Lactobacillus plantarum* (ATCC 29521) was obtained from the Iranian Research
Organization for Science and Technology's microbial collection. The bacterial cells activated at 37 °C for 24 h in Man, Ro-gosa & Sharpe (MRS) broth. The cells were then sub cultured two times at the same conditions. After that, the cells were collected by centrifugation at 4000 rpm for 20 minutes at 4 °C. The bacterial cells were washed two times with sterile saline solution (0.8% (w/v) NaCl) and then dissolved in sterile saline solution. The number of bacteria was adjusted to 4.9 $\times 10^{11}$ cfu/ml according to the standard curve that correlate the absorbance and colony forming unit (cfu) (26). The cell suspension was then applied for microencapsulation.

Encapsulating agent composition and preparation

Preparation of a first layer solution: 3% (w/v) sodium alginate solution: 3g of sodium alginate was dissolved in 97 ml of distilled water, then heated until boiling for 1 minutes

and sterilized at 121 °C and pressure of 15 psi for 15 minutes (8).

3% (w/v) sodium alginate with 0.5% (w/v) olibanum gum solution: %3 sodium alginate with 0.5% olibanum gum was prepared according to (29) with some modifications as follows: 0.5 g of olibanum gum was weighted in an empty beaker, after that 97 ml distilled water was added and beaker was placed on a hot plate at 45 ℃. Then 3 g of sodium alginate was gradually added to the mixture by stirring at 45 ℃ to dissolve the olibanum gum and sodium alginate and to make cross - linking between alginate and olibanum gum. Finally, the mixture was sterilized at 121 ℃ at 15 Psi for 15 min.

Preparation of a second layer solution

Preparation of 0.5 % (w/v) chitosan solution

Chitosan solution was prepared at a concentration of 0.5 % (w/ v) according to the method reported by Ali *et al*. (2) with some modifications. Chitosan is dissolved in glacial acetic acid at a concentration of 1 % with constant stirring for 3 hours at 45 ℃ until it melts. The pH was adjusted to (5.7–6) with 1N NaOH. After filtering out the undissolved portion with a polyester towel, the chitosan solution was sterilized at 121 °C and pressure of 15 psi for 15 minutes.

Preparation of 0.5 % (w/v) chitosan with 0.5% (w/v) olibanum gum solution: This layer was prepared by dissolving 0.5 g of olibanum gum with 99.5 ml of 1% glacial acetic acid at 45 ℃ with constant stirring until the gum completely dissolved, then adding 0.5 g of chitosan gradually at 45 ℃ with stirring for 4-5 h. The pH was adjusted to (5.7- 6) with 1N NaOH. After filtering out the undissolved portion with a polyester towel, the chitosan solution was sterilized at 121 °C and pressure of 15 psi for 15 minutes.

Microencapsulation of *Lactobacillus plantarum* by extrusion method

First - layer microencapsulation

Lactobacillus plantarum was encapsulated using the extrusion technique according to (15) with some modifications. 10 ml of pre-syringe (25 G, 0.5 mm, from a height of 10 cm). After the completion of the extrusion process, the beads are stirred in a hardening solution for 10 minutes. Then microencapsulated beads were left in a hardening solution for 2 hours at room temperature. The beads were filtered with Whatman TM 40 (125 Mm) filter paper and washed two times with sufficient 0.1% peptone water. After that, the beads were kept for next experiments at 4 °C in 0.1% peptone water. This treatment is named as T1. For the first layer encapsulation of treatments T2 and T3, 10 ml of pre-cultured bacteria 4.9×10^{11} cfu/ml was added to the solution of Alg 3% and olibanum gum 0.5%. The microencapsulated cells were obtained as mentioned in treatment T1.

Second - layer microencapsulation

15 g of microencapsulated cell was submerged in 100 ml of second-layer solution of 0.5% chitosan and mixed well for 45 min. After the mixing process, 100 ml of hardening solution 0.1N $CaCl_2$ was added with constant stirring for 45 min. After that, the beads were washed with sufficient sterilized peptone water and dried, next the beads were stored in peptone water 0.1% at 4 ℃ and ready for future tests. This was named T2. The same steps mentioned above were used for the preparation of treatment T3 but this time the second layer solution consisted of (0.5% chitosan and 0.5 % olibanum gum).

Microencapsulation of *Lactobacillus plantarum* by freeze-drying method

Lb. plantarum was encapsulated using freeze-drying according to the method described by (11) with some modifications. 2 g of chitosan and 0.5 g of olibanum gum (w/v) was added to 98 ml of acetic acid 1% with gentle stirring for 4-5 h at 45 ℃, the pH was adjusted to (5.7-6) by using 1N NaOH. The pre-cultured *Lb. plantarum* (4.9×10^{11} cfu/ml) were transferred to the mixture under regular mechanical agitation. The appropriately prepared and well-mixed samples were frozen at a temperature of −18 ℃ for 24 h, lyophilized by a laboratory-scale lyophilization machine (Alpha 1 – 2 Freeze Dryer, Germany) at the following conditions of operation: Temperature of the condenser: − 60 ℃, pressure: 1- 0.1 kPa, drying period: 72 h and ultimate temperature: 25 ℃ (9). The freeze-dried microencapsulated *Lb. plantarum* were ground manually under aseptic conditions, transferred into sterilized vials. The samples were stored in glass desiccators containing saturated salt of $CaCl_2$. Desiccators were stored at room temperature

22 ±3 ℃ for 6 months to determine the viability of microencapsulated bacteria in comparing with lyophilized free cells of *Lb. plantarum* during the storage period (32).

Determination of the viability of microencapsulated *Lb. plantarum*

Probiotic bacteria were released from capsules as reported by (30) with some modifications as follows:

Releasing of the first layer

To release capsules from the first layer, 9 ml buffer mixture solution of 0.2N basic di sodium phosphate with 0.2N acidic sodium di hydrogen phosphate, pH 7 were added to 1g of the capsules.

Releasing of the second layer

The second layer of microencapsulated bacteria was released by using 0.1N tri sodium citrate with 0.1N citric acid at pH 6.3. The solution was stirred potently at (37 ℃ for 45 min) until all of the bacteria had been liberated from the capsules. The counts (cfu/g) were confirmed by plating on MRS agar plates and incubation anaerobically for 48 hours at 37 °C. The free cells were treated similarly. All samples were counted in triplicate.

Surface morphology and measurement of bead size: Scanning electron microscope (SEM) was used to analyze the shapes and surface properties of the beads using 3200 LV (Caesium^{TM}, UK) with an accelerating voltage of 20 kV, according to method described by (16) with a few modifications: The extra moisture of microencapsulated *Lb. plantarum* removed by heating beads to 50 ℃ using oven (Memmert, Germany) for 15 min. After that, they were fixed in stumps of copper tape with two sides then thinly coated in gold (180 s and 40 mA of current) and had been viewed under a microscope.

Effect of high and low temperatures on the survivability of free cell (Fc) and microencapsulated cell (MEc): High temperature treatments: The effect of pasteurization on the survivability of MEc and Fc was investigated according to the method used by (7) as follows: a nine test tubes each one contains 9 ml of 12% sterilized skim milk was inoculated with 1g of MEc and then were divided into three groups each group contain three test tubes. First group were placed in a water bath at 63 ℃ for 30 minutes, second group were placed at 72 ℃ for 15 seconds, and the third group at 80 ℃ for 5 min with constant stirring. After that the tubes were transferred to an ice bath to cool them quickly. The number of viable cells was determined. All steps and conditions mentioned above were followed for Fc.\

Low - temperature storage

One gram of MEc and 1 ml of free cell *Lb. plantarum* separately was used to inoculate 9 ml of 12% sterilized skim milk kept at 6 ± 2 °C in a refrigerator for one month and the survivability of the MEc was tested after releasing layers. The viable cells were expressed as log 10 cfu/g or ml after being counted (27).

Thawing and refreezing tolerance

The survivability of MEc against thawing and refreezing was assessed by inoculating 1 g of the microcapsules into 9 ml of sterile skim milk 12%, as well then storing them at -18 ± 2 °C for 24 hours in an ordinary freezer, then keeping them at room temperature for thawing to determine the viability of MEc and Fc. After that, the same cells were frozen for the second time and thawed, as mentioned above. The viability of thawed and refrozen cells was determined as above (27).

Evaluation of the microencapsulation

Acid tolerance: At an inoculum size of 1 ml at the number (4.9×10^{11} cfu/ml), free active cells of *Lb. plantarum* were inoculated into 9 ml of sterilized skim milk 12% that had been adjusted with 12 N HCl to 1, 2 and 3. The viability of bacteria were determined after 0, 1, 2, and 3 h of incubation at 37 ℃ according to (14). The steps mentioned were repeated with 1 g of beads and the viability was evaluated by pour plating on MRS agar. The results were expressed as log 10 cfu/g or ml. Each experiment was carried out in triplicate.

Bile salt tolerance: The method reported by Nag *et al*. (31) was modified to determine the viability of free active and microencapsulated *Lb. plantarum* against different concentrations of bile salt. One gram of microencapsulated cells was inoculated into 9 ml of skim milk that had been prepared with two bile salt concentrations 2 and 4 % (w/v) for 0, 1, 2, and 3 hours at 37 ℃ and the pour plate method on MRS agar used to determine the numbers of viable cells. Skim milk lacking bile salts was

used as a control. For free cells, the same procedure was repeated. Each experiment was carried out in triplicate.

RESULTS AND DISCUSSION

Viability of microencapsulated cells after extrusion: The determination of cell viability after the extrusion process is a very important parameter that depend on the type of encapsulation agent Table (1) clearly showed that T3 had a greater number of viable cells, reaching 11.66 log10 cfu/g. The reason for this is the use of olibanum gum in the first and second layers, which protected bacterial cells and improved cell aggregation throughout the microencapsulation process. Budianto *et al*., (8) found that using alg/ chitosan for microencapsulation of *Lb. casei* by the extrusion method showed that the number of *Lb. casei* cells before encapsulation was 12.3 log cfu/g. After extrusion, the maximum viability of *Lb. casei* was 12.26 log cfu/g. Silva *et al*. (40) report that *Lb. acidophilus* successfully microencapsulated using alginate-gelatin (AG) and alginate-gelatin-fructooligosaccharides. The (AGF) micro beads, yielding a high cell number after extrusion.

Treatments	Viability before microencapsulation	Viability after microencapsulation
T1	12.67±0.001a	10.59±0.00 d
T2	12.67±0.001a	10.65±0.00 c
T3	12.67±0.001a	11.66±0.00 b

Table 1. Effect of microencapsulation on the viability of *Lb. plantarum*

Surface morphology and bead size measurement: The SEM micrographs showing the beads with their various encapsulating matrices are presented in Fig. (1).

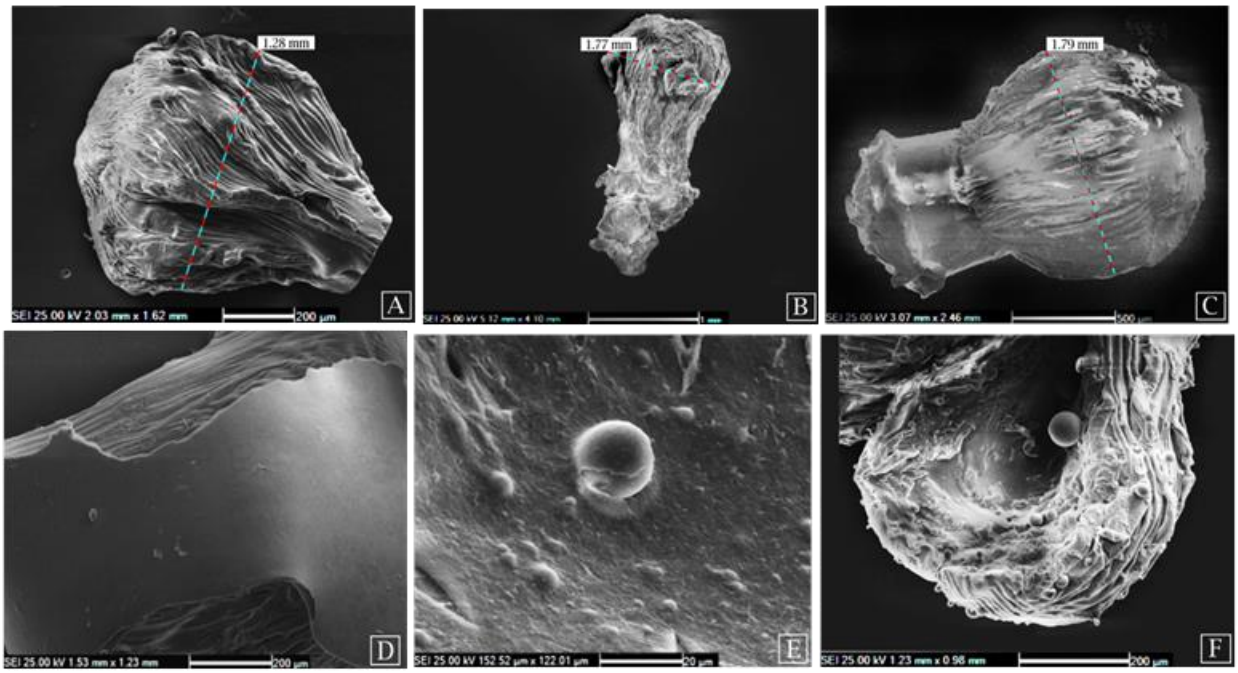

Figure 1. SEM images of encapsulated *Lb. plantarum* cells with 1000x magnification.

The surface of the beads was examined at various magnifications. Figure (1) shows the shapes of ME that were formed by the extrusion method using alginate in treatment T1, which gave spherical shapes with few deformations that were related to the high viscosity of the 3% solution of alginate that was used in this study. While the beads that formed in T2 and T3 were unusual in shape, with a rough texture and more abnormalities and had a small tail and resembled a drop due to the presence of olibanum gum in the microencapsulation solution, which leads to an increase in viscosity, and the solution does not descend through the nozzle of the syringe in a regular form. More specifically, the incorporation of olibanum gum with alginate and chitosan in the first and second layers presented a wrinkle and less spherical shape. Also, Fig. (1) showed that the lack of visible fractures on the different beads' surfaces, potentially limiting the air permeability. Nunes *et al*. (33) demonstrated that significant structural differences were identified amongst the various encapsulating agents. Several parameters, including nozzle size, polymer

composition, and concentration can influence the size and encapsulation yield of microspheres produced using the method of extrusion (41). The average size of T1 was found 1.32 mm a smaller size than T2 and T3, which reached to 1.77 and 1.79 mm respectively. The increasing particle size in treatments T3 and T2 was suggestive of the chitosan and olibanum gum deposition layers through electrolytic linkage between the amine group of chitosan and the (carboxyl) aldehyde group of D-glucuronic acid in olibanum gum. Regarding the cell number Fig.1(F) showed that T3 loaded a higher number of *Lb. plantarum* in comparison with T2 The higher number may be related to the presence of olibanum gum in the first and second layer. The mean particle size of elements affects the stability and efficiency of encapsulation (38).

Effect of high and low temperatures on the survivability of Fc and MEc

High-temperature treatment:

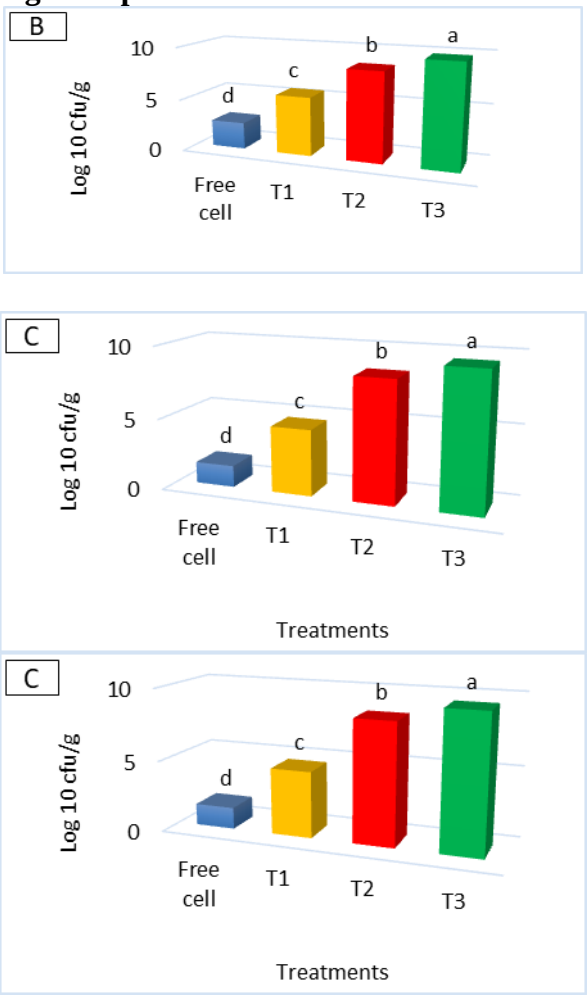

Figure 2. The survivability of free and microencapsulated Lb. plantraum (A) at LTLT 63℃/ 30 min. (B) at HTST 72℃/15 sec. (C) at 80 ℃ for 5min

The heat resistance of free and microencapsulated cells of *Lb. plantarum* was assessed by exposing them to three heat treatments. According to Fig. 2 (A, B, and C) results show that T3 was the best protective encapsulating substance for resistance of the *Lb. plantarum* to heat treatments LTLT, HTST, and 80 ℃ for 5 min, the mean value of viable cell count was found to be 9.99, 9.80, and 9.94 log 10 cfu/g respectively. The denser structure of olibanum gum used as microencapsulation material delayed the contact of probiotic bacteria with external heat and improved the survivability of *Lb. plantarum* cells against heat treatments used in the current study. Chitprasert *et al.* (10) demonstrate that the incorporation of xanthan gum (XG) into Alg beads appeared to provide better heat protection for probiotics than Alg alone, and internal coating with chitosan showed further improvement in the heat sensitivity of microencapsulated *Lb. reuteri* KUB-AC5. Other studies showed that the encapsulated *L. plantarum* NCDC201 and *L.casei* NCDC297 with two layers of alginate had greater rates of cell survival than the free cells after exposure to 75°C for 10 minutes due to the low diffusion of water into the double layer matrix of microcapsules (36). Additionally, *Lb. paracasei* FNU was protected from heating at 65 °C for 30 minutes by encapsulation using skim milk and cheese whey. In contrast to cells that are not encapsulated (20).

Low- storage temperature

All the encapsulated *Lb. plantarum* with different coating agents were able to survive well at refrigeration temperature for 28 days Table (2). In the case of free cells, the numbers reduced significantly ($p \leq 0.05$) and there was a progressive decline in the count of *Lb. plantarum* in proportion to the duration of storage time. At the end of storage period, the viable number of bacteria in a Fc, T1, T2, and T3 was decreased, and reach to 3.58, 5.66, 9.56, and 10.73 log 10 cfu/g respectively. The result suggests that combining alginate with olibanum gum and chitosan, had a positive impact ($p \leq 0.05$) on the increasing the survivability of probiotics that maybe due to create a denser structure that provide stronger defense against environmental factors, like low

temperature. Khaleel and Thaer (23) reported that the logarithm of the live numbers of *Lb. plantraum* decreased in dairy fermented from 10.53 log cfu/gm to 8.94 log cfu/gm after 10 weeks of storage at 5℃. Shi *et al*. (38) discovered that by using an extrusion method to encapsulate *Lb. bulgaricus* in alginate-milk beads, a high viability was observed while stored at 4 ℃. Wang *et al*. (43) revealed *Lb. plantarum* was more stable during refrigerator storage when it was encapsulated in skim milk-coated alginate beads containing inulin.

Table 2. Viability of free and microencapsulated *Lb. plantarum* stored at 6 ℃ for 4 weeks.

Storage time / Treatments	0 time	7 days	14 days	21 days	28 days	LSD-value
Fc(control)	10.66±0.00 b	6.82±0.021 l	5.63 ±0.020 o	4.87 ±0.012 p	3.58 ±0.023 q	0.014
T1	10.59±0.00 d	8.82±0.036 g	8.57±0.018 k	6.52±0.020 m	5.66 ±0.010 n	
T2	10.65 ±0.00 b	9.98 ±0.012 f	9.88 ±0.015 g	9.73±0.012 h	9.56±0.018 i	
T3	10.66 ±0.00 b	10.62±0.031 c	10.56 ±0.018 d	10.51 ±0.027e	10.73±0.000 a	
LSD- value					0.031	

Table 3. Survivability of free and ME *Lb. plantarum* upon repeated freezing and thawing

Treatments	Viable cell of *Lb. plantarum* FT1	FT2	LSD-value
Fc	6.74 ±0.019f	5.66 ±0.016g	0.047
T1	9.82 ±0.000 d	8.75±0.016 e	
T2	10.23 ±0.043b	10.15±0.093 c	
T3	10.97 ±0.005a	10.93 ±0.010a	
LSD-value	0.066		

Thawing and refreezing tolerance

All the encapsulated *Lb. plantarum* with different coating agents were able to survive well at freezing -18 ±2 ℃ for 24 h (FT1) and thawing and refreezing two times (FT2). Table (3) shows that T3 has the highest survivability than T2 and T1 after exposure to FT1 and FT2 conditions, the mean value of viable cell count was found 10.97 and 10.93 log10 cfu/g respectively. In the case of free cells of *Lb. plantarum*, the viability of free cells of *Lb. plantraum* lost one log cycle after exposure to FT1 and FT2 conditions, respectively. This might be due to the cell membrane's fusion and thermotropic phase changes, but during microencapsulation, their viability was kept appropriately constant. Frakolaki *et al*. (16) found that adding k-carrageen and xanthan gum to the alginate encapsulating combination showed that the survival of microencapsulated bacteria with alginate under freezing conditions was higher than free cell-only. Mohmoud *et al*. (27) stated that the encapsulating agents Alg-DWP and Alg-skim milk were notably the most effective for improving the ability to survive of *Lb. plantarum* under freezing.

Evaluation of the microencapsulation

Acid and bile salt tolerance: The results of the current study revealed that ME cells were more able to survive at pH 1, 2, and 3 in comparison with free cells. Figure (3) shows a noticeable decrease in numbers of free *Lb. plantarum* at pH 1 after 1, 2 and 3 hours and reduced about 6 log cycles/ml after 3 hours of treatment. Figure (4) shows a clear decline in viable numbers, of bacteria when exposed to acidic conditions at pH 2 after 1, 2, and 3 hours separately, as the viable numbers of bacteria decreased in the free cells treatment from 8.56 log cycles/ml before treatment to 3.49 log cycles/ml after 3 hours of treatment, while the viable numbers of ME bacteria decreased in T1, T2, and T3 from 9.54, 10.56 and 10.63 log cycles/ml before treatment to 4.54, 8.86 and 9.57 log cycles /ml after 3 hours of treatment. Various response was noted for pH 3, the reduction of free cell was less intense, and loss 6 log cycle, from 9.52 log cycles/ml before treatment to 3.62 log cycles/ml after 3 hours of treatment. Whereas MEc had a better performance, the viable numbers decreased in T1, T2, and T3 from 10.56, 10.58 and 10.65 log cycles/ml before treatment to 4.56, 8.98 and 9.73 log cycles /ml after 3 hours of treatment respectively Fig. (5).

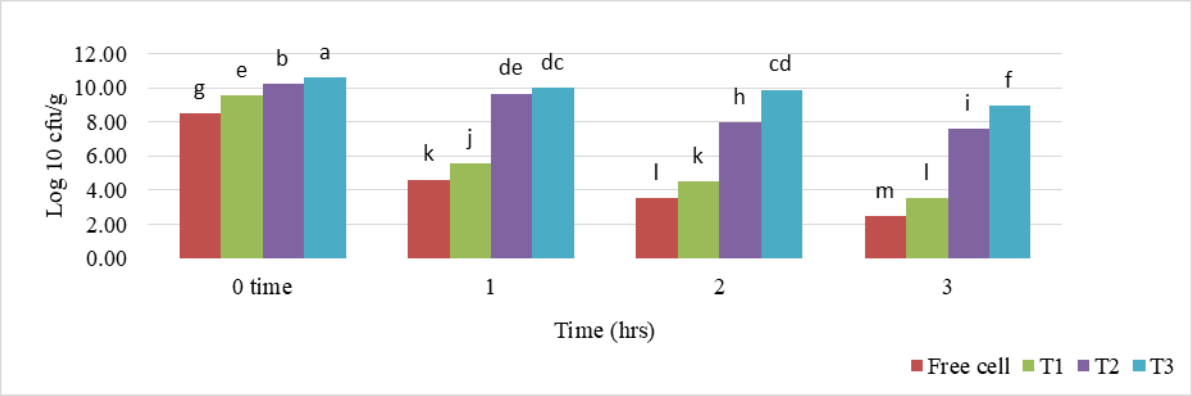

Figure 3. Effect of pH 1 on survivability of free and microencapsulated *Lb. plantarum*

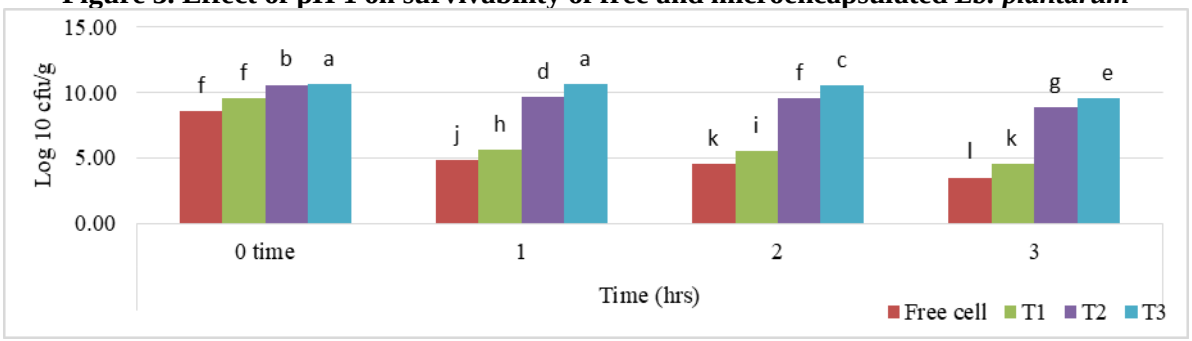

Figure 4. Effect of pH 2 on survivability of free and microencapsulated *Lb. plantarum*

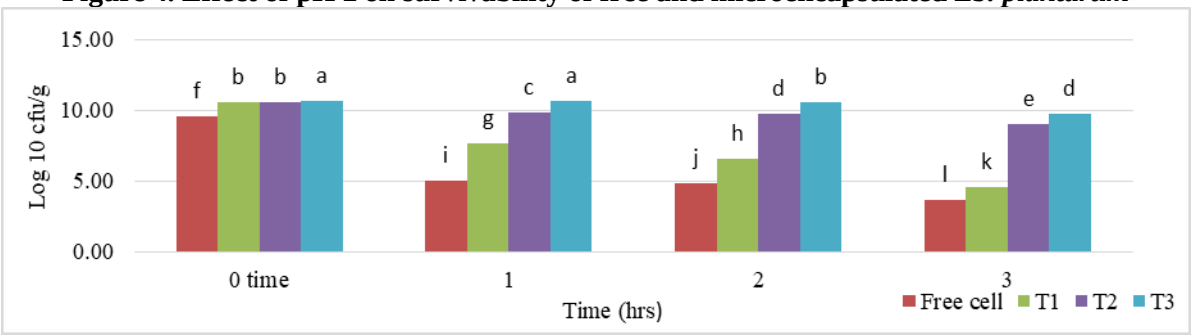

Figure 5. Effect of pH 3 on survivability of free and microencapsulated *Lb. plantarum*

Among microencapsulated treatments T3 shows the highest survivability ($p \leq 0.5$) followed by T2 at pH1, pH2, and pH3 at different incubation periods 1, 2, and 3 hours, this may be due to using of olibanum gum, in the first and second layer which is more effective in protecting bacteria against low acidic conditions. While, T1 with one layer of alginate, their numbers decreased below the required numbers, as the alginate material did not offer sufficient protection at low pH and this may be due to the decomposition of alginate in the extreme acidic environment, and thus did not preserve the bacteria and isolate them from the extreme environment led to its destruction. Resistance to bile salts, along with acidity, is an important criterion in probiotic bacteria (45). Bile salt concentrations in the small intestine are typically between 0.2-0.3%, but they can increase to 2% (w/v) depending on the physiological state of the host and the type and amount of food ingested. The probiotic bacteria must be able to tolerate at least 0.3% bile salts. As presented in Fig. (6), the microencapsulated cells were able to remain a high number at 2% and 4%, but few free cells were able to survive well at 2% and 4% bile salt. After 3 h exposure to 4% bile salt the T3 has highest viable number reached to 7.93 log10 cfu/gm and the lowest number of viable cells was in free cells which reached to 1.49 log10 cfu/ml. As a result, it will take time for all capsules to break down and release the entrapped cells, thereby reducing the time that the intestinal fluid's harmful effects are exposed (13). In another study conducted by Kowalska *et al*. (25) the number of viable bacterial cells upon exposure of alginate microcapsules to digestive fluids, ranged from 7.35 to 7.57 log cfu/ml, which was significantly greater than the number of free cells. Praepanitchai *et al*. (34) found that after

free cells of *Lactobacillus casei* NCDC-298 exposed to 1% and 2% bile salts for 12 hours, reduced their viability, dropping from 9.45 to 7.29 log cfu/ml and from 9.34 to 5.60 log cfu/ml, respectively. Hussein *et al*. (19) reported that number of viable cells of free *Lb. parabuchneri* Nu14 was decreased in the presence of bile salt 0.3 % after two hours of incubation.

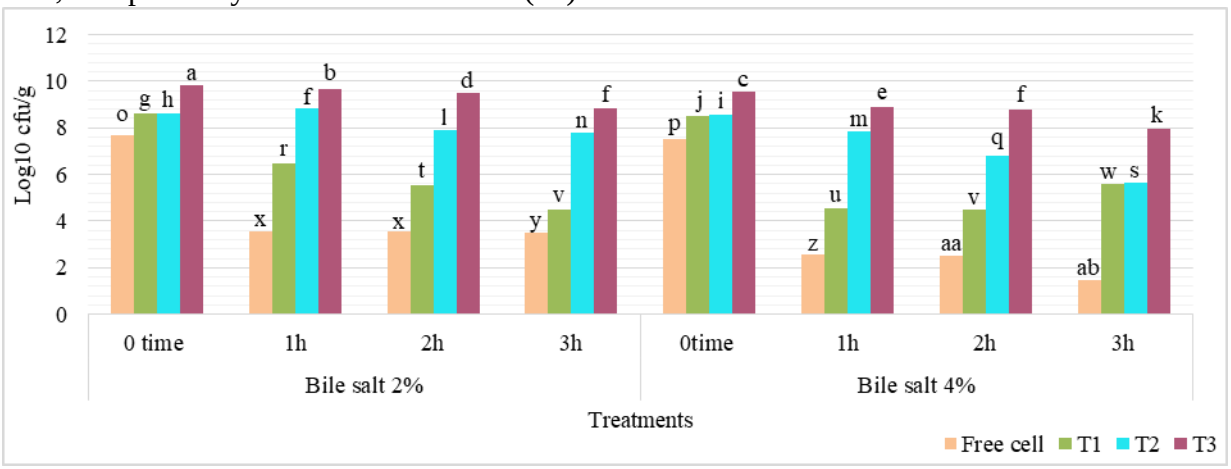

Figure 6. Effect of different concentration of bile salt on survivability of free and microencapsulated *Lb. plantarum*

Effect of freeze-drying and storage on the survivability of microencapsulated and free *Lb. plantarum* : Table (4) showed that the using of (2% chitosan and 0.5% olibanum gum) enhanced the survivability of *Lb. plantarum* during freeze - drying treatment. The result shows that the free cell *Lb. plantraum* more sensitive for freeze drying, and 4 log cycle of loss was observed. This may be due to dehydration resulting to high osmotic stress and high solute concentrations in the extracellular medium (cryoconcentration). The process of cryoconcentration after freezing results in permanent harm to macromolecules, specifically cell surface proteins, which has an immediate impact on the viability of bacteria (6). From table (4) it is clear that the mean value of viable ME *Lb. plantarum* cells lose one log cycle after freeze-drying. ME *Lb. plantarum* is less affected by freeze-drying this may be due to the protective layer of olibanum gum and chitosan causing *Lb. plantarum* more survival for freeze drying. Bodzen *et al*. (6) found that the addition of cryoprotectants like chitosan, cellulose, starch, glucose, xanthan gum, carrageenan, whey, casein, and inulin was the most effective protection strategy to prevent bacteria cells from freeze drying and to decrease cell death. The reason may be attributed to the presence of olibanum gum, which has a role as a cryoprotective to protect the *Lb. plantarum* bacteria from lyophilization. Makinen *et al*. (28) demonstrated that the presence of light, a gaseous environment, a_w, temperature during storage are important factors in the loss of viability.

Table 4. Effect of freeze-drying on (log10 cfu/gm or ml) of free and microencapsulated *Lb. plantarum*

Treatments	Number of viable bacteria
Pre-culture before freeze drying	**12.67± 0.001**
Fc after freeze drying	**8.99± 0.015**
MEc after freeze drying	**11.96± 0.015**
LSD-value	**0.035**

Our results from Fig. (7) show that ME *Lb. plantarum* cells less effected by storage at 22 °C for six months compared to un-capsulated. The reduction of viable cell count in free cell *Lb. plantarum* reached 4.51 log10 cfu/ml after six months of storage and was no longer considered a probiotic, while the number of viable cells reached 9.50 log10 cfu/g at the end of storage. Wang *et al*. (42) who found that the decrease in probiotic survivability associated to water activity (a_w) during storage, and the process of moisture variation during storage

was monitored by the use of cryoprotectant supplementation. Favaro-Trindade *et al*. (14) discovered that the microencapsulated *Lb. acidophilus* population varied in counting values from 9.04 to 7.23 log cfu/g throughout freeze-dried storage for 30, 60, 90, and 120 days. The decrease in *Lb. acidophilus* after 120 days was 2.47 log cycles. Khoramnia *et al*. (24) showed that adding cryoprotectants greatly increased *Lb. reuteri* survival at both 4 and 30°C when compared to cells without cryoprotectants. Also, after six months of storage, the survival rates of cells with cryoprotectants reached 96.4% and 73.8% at 4 and 30°C, respectively**.** Based on our findings, using olibanum gum in the first and second layer with alginate and chitosan might be the most effective capsule to increase stress tolerance of *Lb. plantarum* (ATCC 29521) against high temperatures (LTLT, HTST and 80 ℃), low temperatures (4 ℃ and -18 ℃), also increase their resistance to low pH levels, and high concentrations of bile salt. Also, using 0.5% olibanum gum as a cryoprotectant, showed a higher survival number of microencapsulated *Lb. plantarum* after freeze-drying and during storage at 25 ℃ for six months.

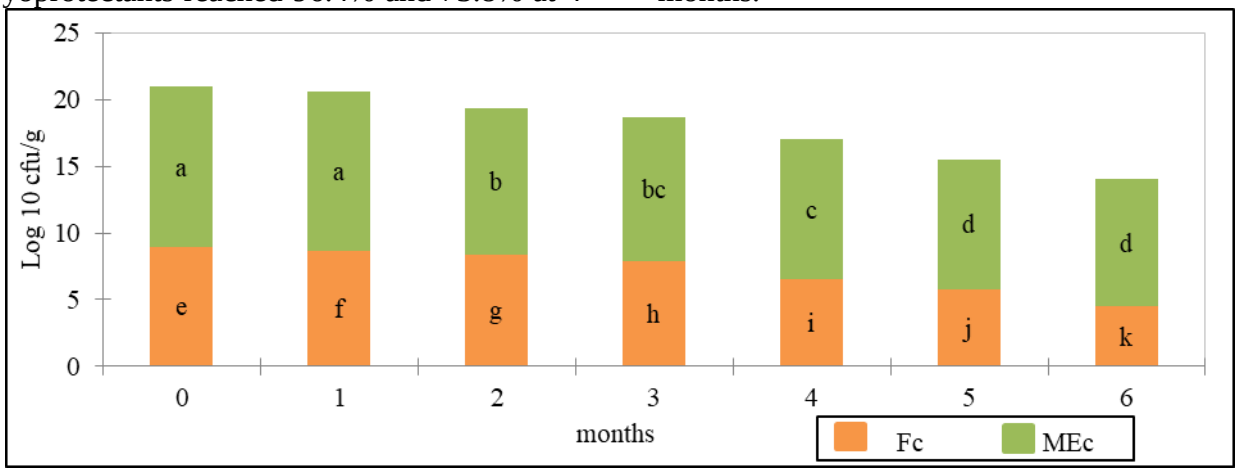

Figure 7. Effect of storage at 22 ℃ on lyophilized free and microencapsulated *Lb. plantarum*

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