

PREPARATION OF ORANGE JUICE FORTIFIED WITH PROBIOTICS ISOLATED LOCALLY FROM YOGURT

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Article info	Abstract
Received: 2023-09-21 Accepted: 2023-10-25 Published: 2023-12-31	The current study was carried out to isolation and diagnosis the isolates of bacteria as probiotics which were isolated from local yogurts in Erbil city markets, The probiotic bacteria identified in the present study were <i>Lactobacillus lactis</i> , <i>Lactobacillus plantarum</i> , and <i>Lactobacillus acidophilus</i> in selective De Man-Rogosa-sharpe (MRS) agar in micro aerobic conditions. The results showed the bacteria were; Gram positive, catalase and oxidase were negative as well as all the isolated bacteria fermented the glucose, while <i>L. lactis</i> fermented lactose and glucose only. <i>L. acidophilus</i> vary in the sugar fermentation. All the bacteria were able to survive in 0.3% bile salt concentration. and with regular decreasing of the viable cells in 1.0%. <i>Lactobacillus plantarum</i> and <i>L. acidophilus</i> had the ability to withstand the increased acidity and could grow in pH ranges 3-5. The results showed antibacterial activity of the cell free supernatant of the lactic acid bacteria against <i>Staphylococcus aureus</i> and the inhibition zones were 12, 14 and 16 mm of <i>L. lactis</i> , <i>L. plantarum</i> and <i>L. acidophilus</i> respectively, and 16, 18 and 20 mm against <i>E. coli</i> respectively, these probiotic bacteria were used in supplemented orange juice. The properties of the orange juices were determined as pH, Brix, and free surviving encapsulated probiotic bacteria. The probiotic bacteria were injected either free or microencapsulated into orange juice, Brix and pH were monitored as well
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as the total number of surviving probiotics was counted weekly for two months. The results showed that the encapsulated probiotic bacteria maintained their viability during the storage period compared to the unencapsulated probiotic bacteria, where they lost their viability. The encapsulated probiotics were found to be more stable than those containing free probiotics.

Keywords: Probiotic, Encapsulate, Supplement, Lactic acid Bacteria, Orange juice.

تحضير عصير البرتقال المدعوم بالمعززات الحيوية المعزولة محليا من اللبن الرائب

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الخلاصة

أجريت الدراسة الحالية لعزل وتشخيص عزلات البكتيريا التي تم عزلها من الزبادي المحلي في مدينة اربيل باعتبارها معززات حيوية وكانت البكتيريا التي تم تشخيصها في هذه الدراسة هي *Lactobacillus lactis*، *Lactobacillus plantarum*، و *Lactobacillus acidophilus* والتي نمت باستعمال الوسط الانتقائي De Man Rogosa Sharpe (MRS) تحت الظروف الهوائية النزرة. وظهرت النتائج ان البكتريا المعزولة موجبة لصبغة جرام وسالبة لفحصي الكاتالاز والأكسيداز كذلك جميع البكتريا المعزولة مخمره لسكر الجلوكوز، بينما عزلات *L. lactis* قد خمرت اللاكتوز والجلوكوز فقط. تباينت عزلات ال *Lactobacillus acidophilus* في تخمير السكريات المختبرة. وكانت جميع البكتيريا قادرة على البقاء حية في تركيز 0.3% من املاح الصفراء ومع التناقص المنتظم للخلايا القابلة للحياة في تركيز 1.0%. *Lactobacillus plantarum* و *L. acidophilus* كان لهما القدرة على تحمل الحموضة العالية ويمكن أن ينمو في درجة حموضة تراوحت بين 3-5. بالنسبة للتأثير المضاد للبكتيريا لطاف الحر للخلايا لبكتيريا حمض اللاكتيك، أظهرت النتيجة أن النواتج الايضية له فعالية مضادة للجراثيم. وكان التأثير ضد المكورات العنقودية الذهبية *Staphylococcus aureus* تراوحت منطقة التثبيط 12، 14 و16 ملم لكل من *L. lactis*، *L. plantarum* and *L. acidophilus*. و16، 18 و ضد بكتريا *E. coli* 20 على التوالي، والتي تم استخدامها في عصير البرتقال المدعوم. تم تحديد خصائص عصائر البرتقال مثل الرقم الهيدروجيني والبريكس والبكتيريا المعززات الحيوية المغلفة والحر. وتم تقدير مستوى السكر والأس الهيدروجيني، بالإضافة إلى حساب العدد الكلي للمعززات الحيوية الحية الباقية

أسبوعياً لمدة شهرين. وأظهرت النتائج أن بكتيريا المعززات الحيوية المغلفة حافظت على حيويتها خلال فترة التخزين مقارنة ببكتيريا غير المغلفة، حيث فقدت حيويتها. وجد أن عصير الفواكه الحاوي على عزلات البروبيوتيك المغلفة قد حافظ على خواصه طوال فترة الخزن بالمقارنة مع العصير الذي لم يحتوي البروبيوتيك.

كلمات مفتاحية: المعززات الحيوية، مغلف، المدعم، بكتيريا حمض اللاكتيك، عصير البرتقال.

Introduction

Probiotics are not a new concept, they were initially brought to the globe as a beneficial component in dairy goods, cereals, and snacks. Lactobacilli, bifidobacterium, and *Saccharomyces cerevisiae var. boulardii* are examples of probiotics. Nowadays, there are many uses of probiotics due to the health benefits that have been discovered. (8 and 12), Fruit Juice and nectars were categorized by their high nutritional fiber, plant chemical compounds, vitamins and raw materials, however the continuous development of functional foods marketing involves them in supplemented beverages with probiotic microorganisms. The variety of acidity in food ingredients can influence the existence of probiotic microorganisms owing to the high concentration of organic acids (2).

Thus, during the manufacturing process of fruit and vegetable beverages, the chosen bacterial strains must be resistant to the acidic and cold environments that are used in the process. Cultures and characterization of probiotics are required under these conditions in order to choose the one whose tolerance is maintainable for the perfect product's shelf life and to be safe for the consumer. Functional foods are foods that are consumed as part of a regular diet and have the potential to improve consumers' health or reduce their risk of disease. All types of functional foods and beverages should not be considered medicines and should only be consumed as part of a regular daily diet (24). Similarly, functional beverages, which include juices enhanced with herbs, vitamins, amino acids, and vegetables, are a fast-growing category of functional foods. Anti-inflammatory, anti-hypertensive, anticancer, antibacterial, antiviral, and antioxidant properties are among their many health advantages. (20). Some individuals who have disorders consuming milk and its products (lactose intolerance) or may favor foods with probiotics supplementation (9). A earlier study by (25); suggested that fruit juice supplemented with probiotic could be a practicable substitute for dairy-based probiotic products. Fruit juices with probiotics are only available in a few varieties on the market, mostly supplemented with *L. plantarum* or *L. rhamnosus* (14 and 17). Fruit nectars may contain substances that help probiotics survive, such as ascorbic acid, which lowers redox potential, saccharides, or organic acids that can be used as a carbon source. (14). Mango juice manufactured by the fermentation of lactic acid bacteria, (probiotic) was produced under microaerophilic conditions at 30°C for 3 days. During the fermentation phase, the bacterial counting, pH, mendable acidity and sugar were assessed and the livability of the bacteria was determined under storing conditions at 4°C for one month (4 weeks). Within 72 hours of fermentation, the pH had decreased to 3.2 (13 and 21). During the first month of storage, the bacterial count fixed not change much. (11). Previous research done by

(12 and 23) found that *L. plantarum* and *L. delbrueckii* elevated pH rapidly during the early phases of fermentation and expended more sugar than other strains. All probiotic lactic acid bacteria ingested huge amount of citric acid, which is a primary organic acid in juice. During storage, the vitality of *L. plantarum* and *L. delbrueckii* was higher. Recent studies also indicate that certain probiotics can minimize undesirable symptoms associated with anticancer therapy (5). Furthermore, (16 and 25), they developed the idea of using probiotic in fruit juices using *Lactobacillus rhamnosus*. The objective of the current study was to examine the ability of using local probiotics isolates of lactic acid bacteria, to supplemented orange juice. Upon several advantages as probiotic that have been studied, so this study aimed to examine the impact of isolated bacteria as a probiotic from local yogurt in Erbil city when adding to orange juice's characteristics.

Materials and Methods

Sample collections and isolation and identification of lactic acid bacteria: The fifty local yogurt samples were collected in sterilized containers; these samples purchase from Erbil's city markets. Serial dilution was done as 10 ml of each yogurt sample were diluted with normal saline to the 10⁻³ concentrations. The dilutions were plated on MRS agar in a volume of 0.1 ml and the cultures were incubated in microaerobic conditions for 48 hours at 37°C with 10% CO₂. Gram staining, catalase, oxidase, growth at different temperatures, sugar fermentation and motility test were done according to (18).

Determination of probiotic properties of the local isolates of *Lactobacillus* Spp:

Determine the ability to grow in bile salt concentration: The ability of endure or surviving in bile salts concentration were examined for isolated *Lactobacilli* in (MRS) broth contained 0.3% bile salt for 24 hours under micro anaerobic conditions at 37°C. The turbidity was measured and the cultured tube more than 0.5 optical density (OD) at 560 nm were considered as bile tolerant strains. These isolates were selected for experience to broth containing higher concentration of 0.5 and 1.0 % (w/v) of bile salt. The survival range of each isolate was expressed as the percentage of viable cells in the presence of bile salt compared to the one without bile salt (7).

Acid tolerance test: The isolated *Lactobacilli* were examined for the ability to growing in adjusted pH of MRS broth to pH 2.5 for one and half hr. at 37°C. The survival rate was achieved by loop full of the growing bacteria streaked on surface of MRS agar plates; after 48 hr. of the incubation in microaerophilic condition, the grown bacteria were considered as acid tolerant. Then the growing bacteria were cultured in MRS broth with pH 2, 3, and 7 (7)

NaCl tolerance test: Twenty four hour-old young bacterial colony was incubated in a prepared MRS broth medium With different concentration of NaCl 0.1 and 0.3 % at 37°C for 24 hours, to note the presence of Growth of *Lactobacillus* isolates through the appearance of turbidity in the medium, indicative of the isolate's growth (7).

Antibacterial activity of *Lactobacilli* bacteria: The antibacterial activity of *Lactobacilli* species was determined by well diffusion methods against the growth

Staphylococcus aureus and *E. coli* isolates. Nearly 105-107 cfu/ml of the bacteria were streaked on Muller Hinton agar wells with 5 mm in diameter were done on the surface of cultured media, cell free extracts of lactic acid bacteria species (cultured in MRS broth at 37°C for 48 h.) gained by centrifugation at 6000 g for 15 min. the cell free extract sterilized through a 0.22 µm pore cellulose acetate filter, 1 ml. was added to the wells the plates kept for 2 h in room temperature after that incubated for 24 h at 37°C. The inhibition zone diameter measured in millimeter (4).

Preparation of probiotic cultures before inoculation: Three types of local probiotic bacteria were cultured individually (*Lactobacillus acidophilus*, *L. plantarum* and *L. lactis*) in 500 mL (MRS) broth for 48 h. at 37 C o. The cells were harvested and used for free and microencapsulated trials; the isolates were condensed by centrifuging at 15,000 x g for 30 minutes in 4Co, then the cells diluted by adding 25 mL of sterilized normal saline (pH 7).

Immobilization and encapsulation of lactic acid bacteria: Microencapsulate probiotic microbes were prepared according to (19); In a casing, 25 mL of local probiotic bacteria were added to 1000µl of sterilized sodium alginate 3% v/w (D3247 AJAX Chemicals Ltd., Australia). The alginate and bacteria suspension was dropped slowly into a flask contains 600 mL of olive oil plus 1 ml of tween 80. A magnetic stirrer was used to thoroughly mix this emulsion at 100 rpm. The emulsion was bothered by adding a solution of calcium chloride (0.01 M) to the side of the beaker slowly. The sodium alginate beads were detached from the aqueous phase after 30 minutes and kept cool in 4° C for 10 h. to fully solidify (19).

Preparation and fortification of orange juice: Orange juice was prepared as follow: Local oranges were purchased from a local market in Erbil city. Oranges were washed and pressed and the concentrated juice was pasteurized at 62.77 Celsius for 30 minutes. prepared ten samples of orange juice ,100 ml of each sample put in sterilized bottles, inoculated with one ml of encapsulated probiotics and one ml of 1.5X 10⁸ cfu/ ml of local probiotic bacteria, after that stored for 10 weeks at 4C°. (10).

Determination of pH and Brix: At weekly intervals, the pH and brix of supplemented juices were measured. Brix was measured with a refractometer (Atago Bellevue, Washington, USA). pH was examined at weekly intervals (Hanna Instruments, Singapore) of juices supplemented by free and encapsulated bacteria.

Determination the viability of probiotics isolates: The measurement of live probiotics was performed according to (6). briefly, microencapsulated probiotic bacteria were released from the microcapsules by confiscating calcium ions with a phosphate buffer pH 7.0. Once released, serial dilution was done and MRS agar, pour plate were followed, plates incubated at 37C° for 72 h with anaerobic conditions. Probiotic bacteria measurements were recorded in orange juices on a weekly for six weeks.

Results and Discussion

Isolation and identification of lactic acid bacteria: Lactobacilli were isolated from local yogurts in selective (MRS) agar in micro aerobic conditions. The results

appeared in figure1 surrounded by clear zone when cultured on the MRS contain CaCO_3 , due to produce lactic acid that utilized Calcium carbonate.



Figure 1 Growth of Lactic acid bacteria isolates on MRS plus CaCO_3 Agar.

The biochemical characteristics were shown in table 1. All the bacteria were catalase and oxidase negative as well as all the isolated bacteria fermented the glucose. *L. plantarum* isolates were fermented all tested sugar, while *L. lactis* were fermented lactose and glucose only. *L. acidophilus* vary in the sugar fermentation. The results were in agreement with the results described by (24).

Table 1 Biochemical test for *Lactobacillus* spp.

Tests	Isolates	<i>L. acidophilus</i>	<i>L. lactis</i>	<i>L. plantarum</i>
Growth at	15°C	-	-	+
	45°C	+	-	-
	Gram stain	+	+	+
	Catalase	-	-	-
	Motility	+	+	+
	Oxidase	-	-	-
Sugar Fermentation	Lactose	+	+	+
	Mannitol	-	-	+
	Raffinose	-	-	+
	galactose	+	-	+
	Sucrose	+	-	+
	Maltose	+	-	+
	Sorbitol	-	-	+
	Glucose	+	+	+

All the bacteria were able to survive in 0.3% of bile salt concentration as shown in table 2, with regular decreasing of the viable cells in 1.0%. *Lactobacillus plantarum* and *L. acidophilus* had the ability to withstand the increased acidity and could grow better in pH ranged between 3-5.

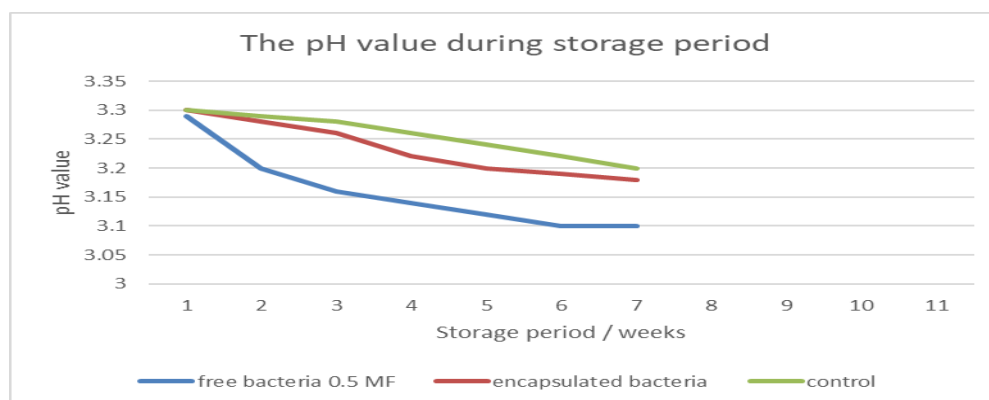
Concerning to the antibacterial activity of the cell free supernatant of the lactic acid bacteria, the result showed that the metabolite had antibacterial impact against *Staphylococcus aureus* and the inhibition zone were 12, 14 and 16 mm for *L. lactis*, *L. plantarum* and *L. acidophilus* respectively, and the effect of these bacteria against *E. coli* were 16, 18 and 20 respectively.

Table 2 Characters of the Lactic Acid Bacteria as Probiotics.

Characters	<i>L. acidophilus</i>	<i>L. lactis</i>	<i>L. plantarum</i>
Bile salt tolerance			
0.3%	+++	+	+++
0.1%	++	+	++
Acid tolerance			
pH 2	++	+	++
pH 3	+++	+	+++
PH 5	+++	++	+++
PH 7	++	+	++
PH 8	+	-	+
Inhibition zone diameter (mm)			
<i>Staphylococcus aureus</i>	16	12	14
<i>E. coli</i>	20	16	18

Properties of fortified Orange juice:

pH development: Figure 2 showed pH changes in orange juice containing free and encapsulated probiotic bacteria during six-weeks storage period. The orange juice fortified with probiotic bacteria inspected, a similar tendency in pH drop was observed in both free and encapsulated orange juice. pH of orange extract throughout the six weeks of storing period was higher than that of orange juice inoculated with free probiotic isolates. the results showed that after six weeks of storing, the average pH of the juice adding free probiotic bacteria reduced from 3.29 to 3.1 (figure 2); nevertheless, the pH of juice with encapsulated probiotics adding only dropped to 3.18.

**Figure 2 pH value of fortified orange juice during storage period.**

The findings showed that microencapsulating probiotic bacteria produced more stable goods over a longer storage time, as well as more steady functional foods. In a comparable study done by (4), they observed the existence of three separate local isolates of the probiotic bacteria *Lactobacillus lactis*, *Lactobacillus acidophilus* and *Lactobacillus plantarum* as free and microencapsulated probiotic bacteria in orange and apple juice. The current study revealed that the local probiotics examined bacteria showed a tendency to decrease pH in both free and encapsulated orange juice. The final pH of orange fortified juice with encapsulated probiotic bacteria after six weeks of storage was higher than that of orange juice injected with free probiotic bacteria. This finding suggests and supports the idea that probiotic bacteria in a microencapsulated, immobilized state have a more unchanging environment. It's

possible that free probiotic bacteria consumed carbohydrates and created modest amounts of lactic acid.

The Brix content during storage period of fortified juice: The results as appeared in figure 3 depicts the variations in brix in orange extract having free and encapsulated probiotics. The sugar content of the juice was lowered during the six-week trial, the final brix of orange juice fortified with encapsulated *Lactobacillus lactis*, *Lactobacillus acidophilus*, and *Lactobacillus plantarum* bacteria was greater than orange extract with free probiotic bacteria. After six weeks of storage, the average in orange extract with probiotic bacteria reduced by 0.7 gm/ml. In orange juice containing free probiotic bacteria, however, there were only 0.5gm/ml decreases in Brix. The present results in matching with the results of (4) who reported the persistence of free and microencapsulated *Lactobacillus lactis*, *Lactobacillus acidophilus*, and *Lactobacillus plantarum* bacteria in orange and apple juice. The result revealed that the orange juice initially had a lower brix value of 10.0. The number of free probiotic bacteria has decreased.

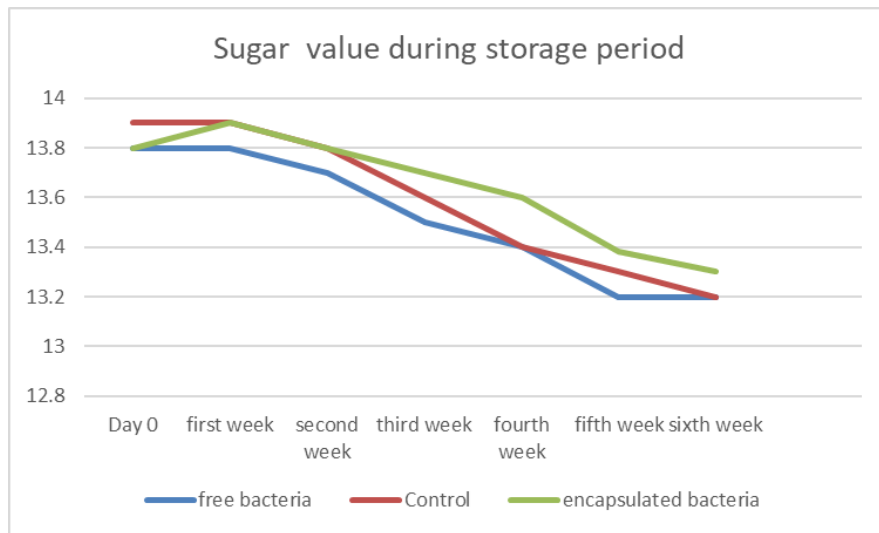


Figure 3 Brix percentage of supplemented orange extract during storing period.

According to the present results of the pH and brix value of orange juice ranges were in agreement and acceptable according to (15), as well as in agreement with previous studies established by (1 and 22).

Existence of Free and Encapsulated Probiotic Bacteria in orange juice: Survival of free probiotic bacteria shown in figure 4. The orange juice quickly lost its free probiotic bacteria by the fourth week, and by the fifth and sixth weeks, less viable bacteria were still alive. The three probiotic bacteria isolates had a comparable drop in viability. *L. acidophilus* showed a higher acid tolerance than *Lactobacillus lactis*, and *Lactobacillus plantarum*, which contributed to its higher viability rates. The probiotic bacteria that were enclosed and protected from the acidic environs of orange extract fixed and not lose viability as rapidly as the free probiotic bacteria after six weeks of storage, as shown in figure 5. Moreover > 10⁵ cfu/ml were present.

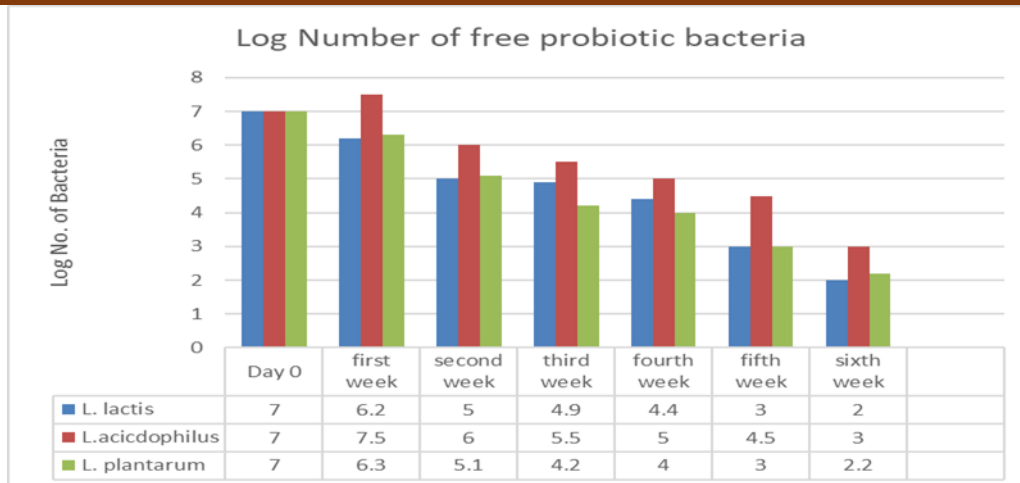


Figure 4 Free probiotic bacteria survived in orange juice during storage period.

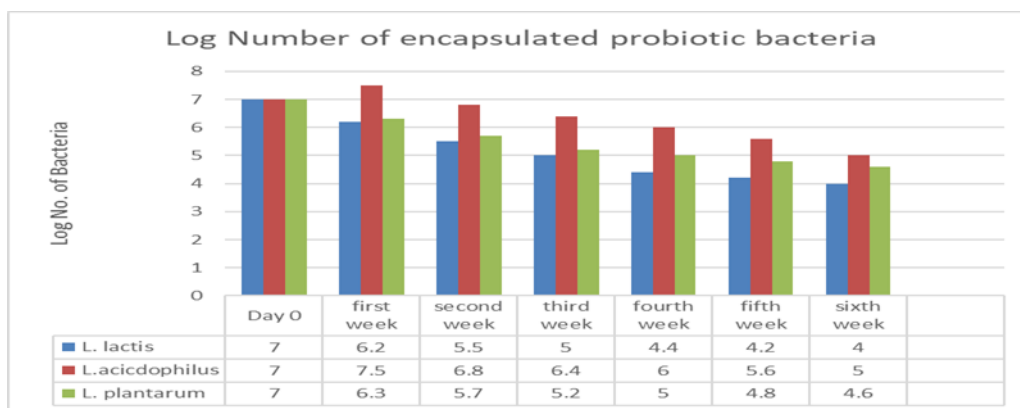


Figure 5 Encapsulated probiotic bacteria survived in orange juice during storage period.

Conclusion: According to the current study, the local isolates of *Lactobacillus lactis*, *Lactobacillus acidophilus* and *Lactobacillus plantarum* from local yogurt in Erbil city, showed beneficial probiotic properties. Additionally, these probiotics added to orange juice as encapsulated probiotic bacteria, demonstrated a greater presence in orange extract than the extract containing free probiotic bacteria. Probiotic had a reduction in pH and Brix concentration during storage.

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