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Effect of different microencapsulation materials on stability of *Lactobacillus plantarum* DSM 20174

Lulwah Y. Al-Furaih[1], Ibtisam M. Ababutain[1]*, Azzat B. Abd-El-Khalek[2] and Ahmed M. Abdel-Salam[3]

[1]Department of Biology, College of Science, University of Dammam, P.O. Box 1982, Dammam 31441, Saudi Arabia.
[2]Dairy Science Department, National Research Centre, Dokki, Cairo, Egypt.
[3]Food Science and Human Nutrition Department, College of Agriculture and Veterinary Medicine, Qassim University, P.O. Box 6622 Buraidah 51452 Al-Qassim, Saudi Arabia.

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The aim of this work was to investigate the effect of different microencapsulation materials on the stability of probiotic bacterium (*Lactobacillus plantarum* DSM 20174). Microencapsulation methods with alginates were carried out using sodium chloride, canola oil, olive oil, and chitosan. The recorded data showed that the encapsulated probiotic bacterium was more stable compared with free cells. Olive oil capsules recorded the highest stability at pH 2 after incubation period of 24 h with stability up to 0.00004%. Olive oil and chitosan capsules showed stability with high concentration of bile salts (0.5%) with stability percent of 82 and 65% respectively, after 2 h of incubation. Sodium chloride and chitosan capsules gave the best stability percent of 0.026 and 0.00005%, respectively, at heat treatment up to 65°C for 30 min. Storage treatment at 4°C for 17 days reduced the stability of all capsule types, whereas sodium chloride and chitosan capsule showed stability percent up to 59 and 56%, respectively.

Key words: Microencapsulation, *Lactobacillus plantarum,* olive oil and alginate.

INTRODUCTION

Probiotic bacteria are described by the World Health Organization (WHO) as “live organism, which when administered in adequate amounts confer health benefits to the host” (FAO/WHO, 2002). Probiotic can provide beneficial effects on the human body by keeping the healthy gut microflora, inhibiting the growth of pathogenic bacteria, relieving constipation, stimulating the immune system, synthesizing vitamins working as antimicrobial agents, and improving the absorption of calcium, when there are enough probiotic in colon (Rokka and Rantamäki, 2010). To produce such beneficial effects, probiotics have to be able to survive and multiply in the host. In this regard, probiotics should be metabolically stable and active in the product, survive passage through the stomach and reach the intestine in large amounts (Laparra and Sanz, 2010). In fact, there are still, a number of problems related to the low survival of probiotic bacteria under gastrointestinal conditions, pH, hydrogen peroxide, oxygen and storage (Martín et al., 2015).

Recent research indicates that the microencapsulation

*Corresponding author. E-mail: Iababutain@uod.edu.sa or Dr.king2007@hotmail.com.

of probiotic cells presents one of the most promising and efficient techniques for the enhancement of probiotic survival (Chávarri et al., 2008). Microencapsulation can be defined as a process in which cells are retained within an encapsulated membrane to reduce cell injury or cell lose, in a way that results in appropriate microorganism release in the gut (Sultana et al., 2000).

To enhance the efficacy of probiotic, micro-encapsulation has been introduced by entrapping cells into a polymer matrix (Dianawati et al., 2013; Dong et al., 2013). Alginate has been widely used as a micro-encapsulation material as it is cheap, non- toxic and compatible with most other materials. It is also able to absorb water quickly (Rowley et al., 1999), which assists in gel formation. Sodium alginate is a generally regarded as safe (GRAS) material certified by FDA (George and Abraham, 2006). Coating alginate capsules containing probiotic with chitosan has been shown to promote cell protection and enhance the effectiveness of encapsulation either in food and beverages (Nualkaekul et al., 2012; Brinques and Ayub, 2011; Krasaekoopt et al., 2006).

Whereas probiotic are living cells, the conditions for implementation of this technology are designed to maintain cell viability. In fact, selecting the encapsulation technology is very important. Whereas probiotic are living cells, the conditions for implementation of this technology are designed to maintain cell viability. Therefore, the main aim of this work was to study the effect of different microencapsulation materials on stability and survival of *Lactobacillus plantarum* DSM 20174 during cold storage, low pH, bile salt and temperature.

MATERIALS AND METHODS

Preparation of bacterial culture

L. plantarum DSM 20174 obtained from Deutsche Sammlung von Mikroorganismen DSM und Zellkultturen-GmbH, Germany, was reactivated on De Man, Rogosa, Sharpe broth (MRS) for 2 times at 30°C for 24 h before use. The cells were harvested by centrifugation (Optima L-100XP, Beckman Preparative Ultra-centrifuge, Shanghai, China) at 5,000 rpm for 20 min, at 4°C. Then bacterial cells were washed twice using 1.0% peptone solution and re-suspended in 5 ml of 0.1% peptone solution.

Microencapsulation technique

The microencapsulation technique of *L. plantarum* DSM 2017 with alginates was carried out using sodium chloride, canola oil, olive oil and chitosan.

Extrusion microencapsulation (EM)

Microencapsulation using alginate with sodium chloride

Following the procedure of Klinkenberg et al. (2001), suspension of bacterial cells was mixed with an equal volume of sodium alginate. The mixture was passed through a syringe into 60 ml sterile solution consisting of a mixture of 20 ml sodium chloride (0.5%) and 40 ml calcium chloride (0.05%) and homogenized until alginate beads were formed. The beads were left for 30 min to harden at room temperature, then washed twice with 1.0% peptone water solution and stored in a refrigerator (4°C) until further use.

Microencapsulation using alginate and vegetable oil

To form beads, an amount of bacterial cell suspension was mixed with sodium alginate solution, vegetable oil (Canola or olive oil) and Tween 80 solution at ratio 3:3:1:0.5, respectively (Klinkenberg et al., 2001). Then the mixture was dropped through a syringe into 60 ml calcium chloride solution (0.5%). The beads were left to harden at room temperature as mentioned earlier and stored for further use.

Microencapsulation using alginate and chitosan

Alginate beads previously prepared as mentioned earlier were transferred to 100 ml of Chitosan solution and mixed gently using magnetic stirrer at a speed of 100 rounds for 50 min (Krasekoopt et al., 2006). The resulting alginate coated-chitosan beads were washed twice with peptone water solution (1.0%) and stored at 4°C until further use.

Capsules morphology

Morphology of capsules was studied using photographic camera (Nikon D7000), light microscopy (Model SZ61, Olympus) following Chan et al. (2011) and scanning electron microscopy as described by Alaş et al. (2010).

Stability of the encapsulated bacterium under different conditions

Dismantling the capsules

To determine the viable count of trapped *L. plantarum* DSM 20174, cells were released from the microcapsules using the method of Kim et al. (2008). The released bacterial cells were plated on MRS agar plates using ten-fold dilutions and incubating at 30°C for 24 to 48 h. The viable population in terms of colony forming units (CFU) per gram of the sample was counted according to Stukus (1997), the experiment was conducted in triplicate.

Survival of free and encapsulated bacterium in low pH

The pH of MRS growth medium was adjusted to pH 2 and 3 according to Lo et al. (2004). The media were inoculated separately with 1% of the encapsulated and free bacterial cells. Plates were incubated at 30°C for 0 to 24 h. The encapsulated cells were periodically released from the capsules and the total CFU count per g was performed. Comparison between the released bacterial cell count and free cells were recorded. The experiment was conducted in triplicate.

Survival of free and encapsulated bacterium at high concentrations of bile salts

Resistance to bile salts was determined according Lo et al. (2004)

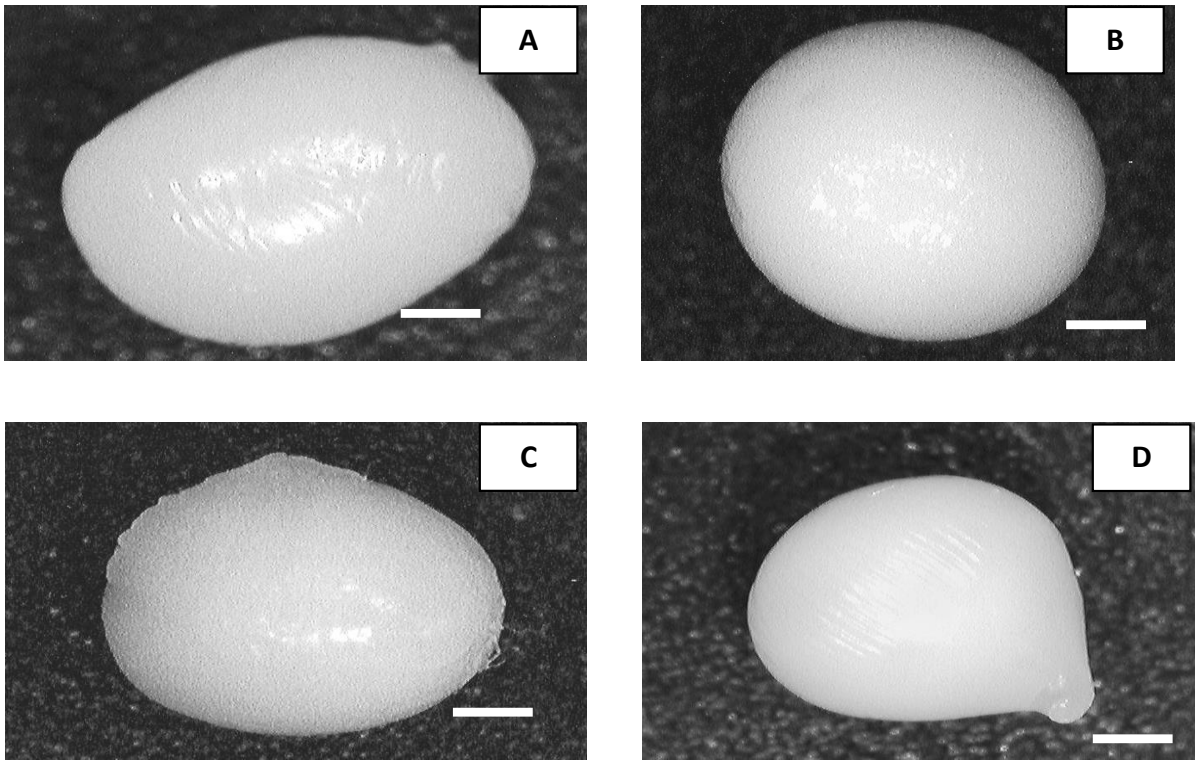

Figure 1. Photograph using Nikon D7000 Camera: (A) Alginate with sodium chloride capsule showed oval shape, (B) Alginate capsule coated with canola oil showed spherical shape with smooth surface, (C) Alginate capsule coated with olive oil showed semi spherical shape with rough surface, (D) Alginate capsule coated with Chitosan showed drop shape with Shell-like surface. Bar marker represents 2 mm.

by inoculating 1% free and microencapsulated cells separately to MRS broth containing 0.3 and 0.5% of Oxgall bile salts. Then samples were withdrawn after incubation at 30°C for 0, 2 and 4 h to determine cell count of *L. plantarum* DSM 20174. Comparison between the released bacterial cell count and free cells were recorded. The experiment was conducted in triplicate.

Survival of free and encapsulated cells after heat treatments

Encapsulation was assessed in terms of viable cell protection efficiency during thermal processes according to Kim et al. (2008). In brief, 1 g of broth free and microencapsulated *L. plantarum* DSM 20174 were assayed for heat resistance at 65°C for different periods: 0, 15, and 30 min with MRS broth as a suspending medium. After the end of each heat treatment, samples were cooled down to room temperature (25°C) using water bath. The survived of free and encapsulated *L. plantarum* DSM 20174 were enumerated in triplicate in MRS agar plates as previously described.

Viability of free and encapsulated cells under refrigerated conditions

The viability of both encapsulated and free *L. plantarum* DSM 20174 cells were monitored by counting the CFU/ml after 17 days of storage at 4°C according to Hou et al. (2003). Survivals of free and encapsulated *L. plantarum* DSM 20174 were enumerated after 0, 4, 7, 11, 14 and 17 days. The experiments were also performed in triplicates.

Statistical analysis

The results were reported throughout the experiments as mean ± standard deviation. Statistical analysis of the data was conducted using analysis of variance (ANOVA) and t-test, Version 17 of SPSS. Values $P \geq 0.05$ were considered statistically significant.

RESULTS

Microencapsulation using alginate with sodium chloride

Capsules of alginate with sodium chloride photograph appear as oval shape (Figure 1A). Scanning electron microscopy photographs of the capsules showed smooth and uniform round declines surface (Figure 2A). Dimensions of the capsules ranged between 2120 and 2646 µm by using Optical microscope (Figure 3A) and the sphericity factor (SF) was 0.169±0.227 (Table 1).

Microencapsulation using alginate and vegetable oil

Alginate capsule coated with canola oil

Capsules of alginate coated with canola oil photograph

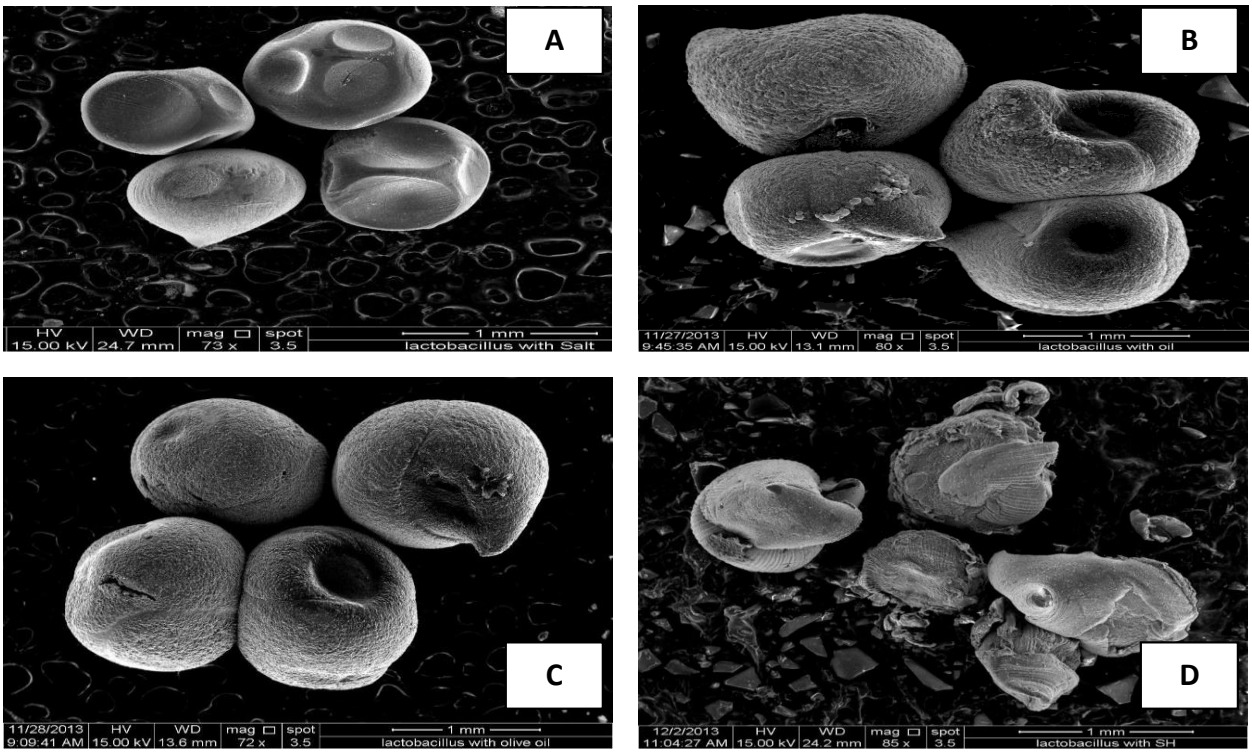

Figure 2. Scanning electron microscopy photographs showed: (A) Alginate with sodium chloride capsules size 73x, (B) Alginate capsules coated with canola oil size 80x, (C) Alginate capsule coated with olive oil size 72x, (D) alginate capsules coated with chitosan size 85x.

appear as spherical shape (Figure 1B). Scanning electron microscopy photographs of the capsules showed coarsely appears as some bacterium cells appear on the surface with a round declines from the middle (Figure 2B). Dimensions of the capsules ranged between 1991 and 2398 µm by using Optical microscope (Figure 3B) and the SF was 0.024±0.023 (Table 1).

Alginate capsules coated with olive oil

Capsules of alginate coated with olive oil photograph appear as spherical shape with curvy surface (Figure 1C). Scanning electron microscopy photographs of the capsules showed rough and round declines in the center (Figure 2C). Dimensions of the capsules ranged between 1988 and 2447 µm by using Optical microscope (Figure 3C) and the SF was 0.061±0332 (Table 1).

Microencapsulation using alginate and chitosan

Capsules of alginate coated with chitosan photograph appear as drop shape (Figure 1D). Scanning electron microscopy photographs of the capsules showed rough and shell-like broken-rough surface also shows grooves resembling shells notes lack of resistance to the capsule settings imaging electron microscope and appeared in a distorted manner (Figure 2D). Dimensions of the capsules ranged between 2214 and 1970 µm by using Optical microscope (Figure 3D) and the SF was 0.050±0.069 (Table 1).

Stability of the encapsulated *L. plantarum* DSM 20174 under different conditions

Stability at low pH

The stability of free and encapsulated *L. plantarum* DSM 20174 under low pH conditions (pH 2 and 3) is as shown in Figure 4. At pH 2 free cells of *L. plantarum* DSM 20174 and all capsules were not stable except alginate capsules coated with olive oil (Fig. 4A). While at pH 3 showed slightly effect on the stability of all samples, however, there were differences in the percentage of stability and it was in descending order: alginate with sodium chloride, alginate coated with chitosan, alginate coated with olive oil, and alginate capsules coated with canola oil (84, 63, 58, 56, and 49%), respectively.

Stability at high concentrations of bile salts

The effect of bile salt on the stability of free and encapsulated *L. plantarum* DSM 20174 is as shown in

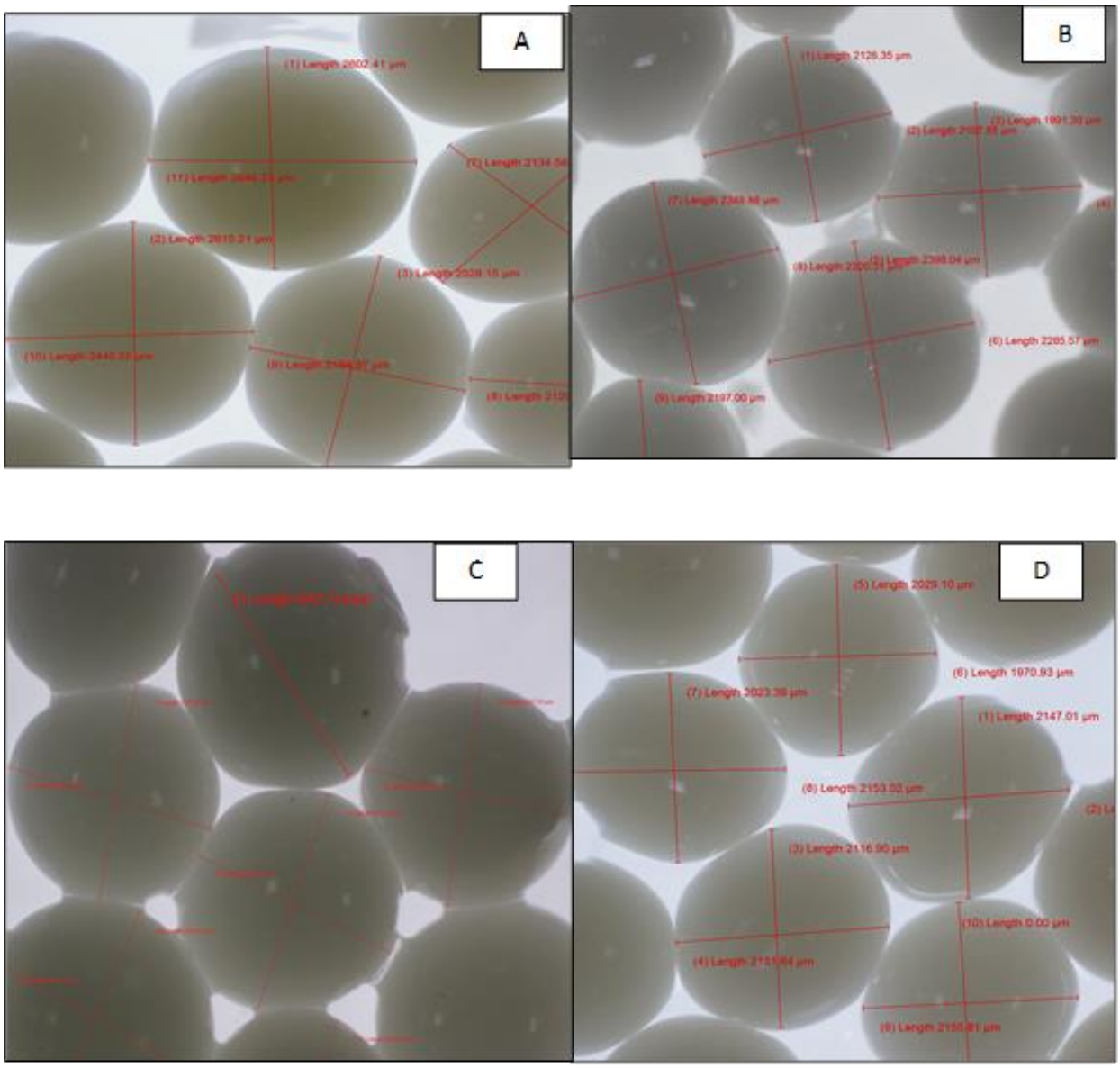

Figure 3. Optical micrograph image using Optical microscope at magnification 40X: (A) Alginate with sodium chloride capsules the dimensions ranged between 2120 and 2646 µm, (B) Alginate capsules coated with canola oil the dimensions ranged between 1991 and 2398 µm, (C) Alginate capsule coated with olive oil the dimensions ranged between 1988 and 2447µm, (D) Alginate capsule coated with chitosan the dimensions ranged between 1970 and 2214 µm.

Figure 5. The stability of encapsulated cells was significantly higher than the free cells after incubation time of 2, 4 and 24 h with 0.3 and 0.5% of bile salts concentrations. It was noted at concentration of 0.3%, bile salts slightly affect the stability of all samples after incubation for 2 h, which arranged in descending order: alginate capsules with sodium chloride, alginate capsules with olive oil, alginate capsules with chitosan, alginate with canola oil, and free cells (99, 87, 67, 54 and 50%), respectively. The results showed that the increase in the concentration of bile salts (0.5%) reduced the stability of all the samples after incubation for 2 h and the stability percentage was arranged in descending order: alginate capsules with olive oil, alginate capsules with chitosan, free cells, alginate capsules with sodium chloride, and alginate with canola oil (82, 67, 48, 43, and 32%), respectively. The results showed that the increasing of incubation period up to 4 h reduced the harmful impact of bile salts (0.3 and 0.5%). Whereas increasing the incubation period up to 24 h diminished the stability for most of the samples except the alginate with canola oil (36%), alginate with olive oil (83%) at the concentration of 0.3%, and alginate with olive oil (41%) at the concentration of 0.5%.

Table 1. Microencapsulation analysis.

Alginate capsules with:	Largest diameter (µm)	Smallest diameter (µm)	Sphericity Factor (SF)	Shape	Surface	Strength
Sodium chloride	2646	2120	*0.169±0.227	Oval	Smooth and uniform round decline	Semi-solid
Canola oil	2398	1991	*0.024±0.023	Spherical	Rough and round decline in the center	Soft
Olive oil	2447	1988	*0.061±0332	Spherical	Rough and round decline in the center	Soft
Chitosan	2214	1970	*0.050±0.069	Drop	Shell-like broken rough	Soft

*The mean difference is significant at the 0.05 level. ±Standard deviation calculated from result of five independent experiments.

Stability at heat treatment

In order to investigate the efficacy of the microcapsules in protecting *L. plantarum* DSM 20174 against heat, the stability of free and microencapsulated cells was evaluated after exposure to temperature of 65°C for 0, 15 and 30 min (Figure 6). The free cells were more sensitive to heat shock than the microencapsulated cells at 65°C, in which free cells were completely lost after 15 min of exposure to heat treatment. The microencapsulated cells varied in their stability after 15 min of incubation. The stability percentage was arranged in descending order: alginate capsules with sodium chloride (7%), alginate capsules with chitosan (0.0000048%), and finally alginate with vegetable oil (canola or olive oil) was 0.00017 and 0.00043%, respectively. Whereas alginate with sodium chloride and chitosan were resistant to heat treatment up to 30 min incubation period with stability percentage of 0.00026 and 0.0018%, respectively.

Stability at cold storage

Cell free *L. plantarum* DSM 20174 and micro-encapsulated cells were stored at refrigerator temperatures (4°C) and their stability was determined over 17 days period (Figure 7). After 14 days of storage at 4°C, the stability of alginate with chitosan was 94% and alginate with olive oil was 74%, while stability of alginate with canola oil reduced to 50%. Whereas stability of free cells and alginate capsules with sodium chloride is almost similar at 63 and 60%, respectively. Stability after 17 days decreased between 40 and 55% for all microencapsulation forms and free cells.

DISCUSSION

Microencapsulation techniques were applied to *L. plantarum* DSM 20174 and extrusion technology was used in all applications of micro-encapsulation. The present study showed the difference between the forms of capsules, size, surface and textures depending on the difference between materials used in the encapsulation process. The wet capsules were smooth texture and ranged in size between 1970 and 2646 µm, while after drying the surface became wrinkled. These results are in agreement with Kim et al. (2008) and Ma et al. (2008) who found out that dry capsules of alginate coated with chitosan were spherical in shape with a curly and wrinkle surface and explained that the drying process have direct role in the formation of curly surface.

In order to exert positive health effects, probiotic should resist the stress conditions of the stomach. The results of the current study showed a significant difference in the stability of the capsules with olive oil compared to free cells and other types of capsules in pH 2, while stability percentage varied at pH 3. In general, the alginate coated with olive oil showed a good stability under low pH conditions (pH 2 and 3) which may be considered as a new application in the field of functional foods industries. The results of this study were supported by Hou et al. (2003) who mentioned that the survival rate of bacteria encapsulated with alginate coated with sesame oil, increased up to 10^4 in pH 2 compared to free cells. Also, microencapsulation technique using alginate with three types of chitosan at different molecular weight improved the survival of *L. bulgaricus* KFRI in acidic media, despite the intensity of sensitivity of this bacterium to acids (Lee et al., 2004).

Moreover, Mokarram et al. (2009) showed that the survival of encapsulated *L. acidophilus* cells with alginate was significantly better than free cells; they explained that the multiplicity of the layers may have a role in the stability of bacterial cells.

They were found to outperform double layers' capsules followed by a single layer then free cell.

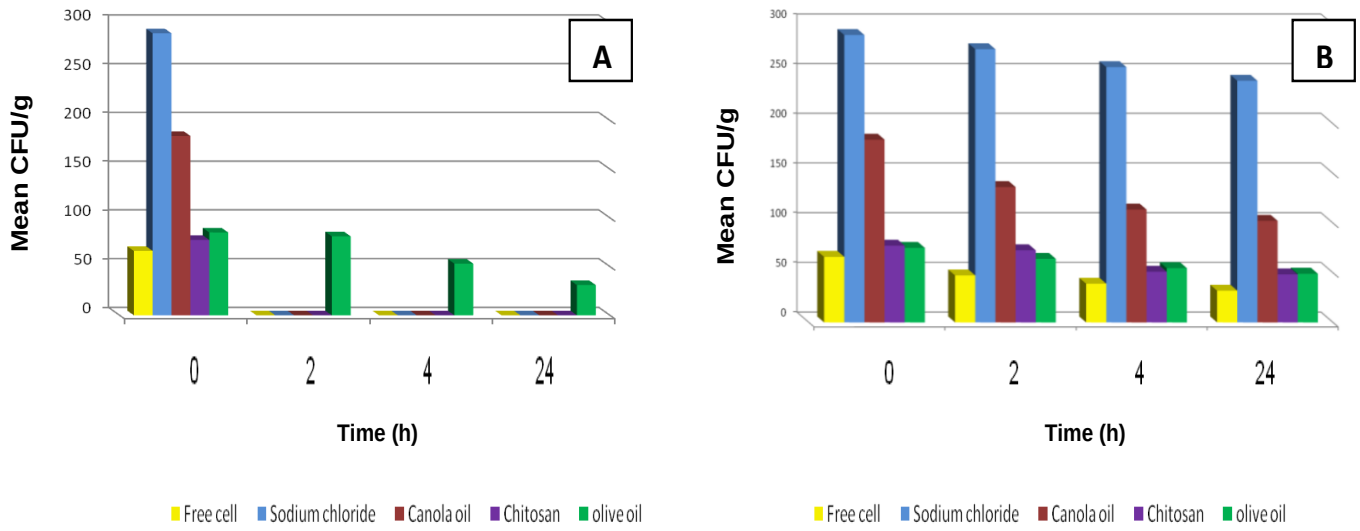

Figure 4. The stability of free and encapsulated *L. plantarum* DSM 20174 at different low pH by using pour plate count method (A) pH 2 and (B) pH 3.

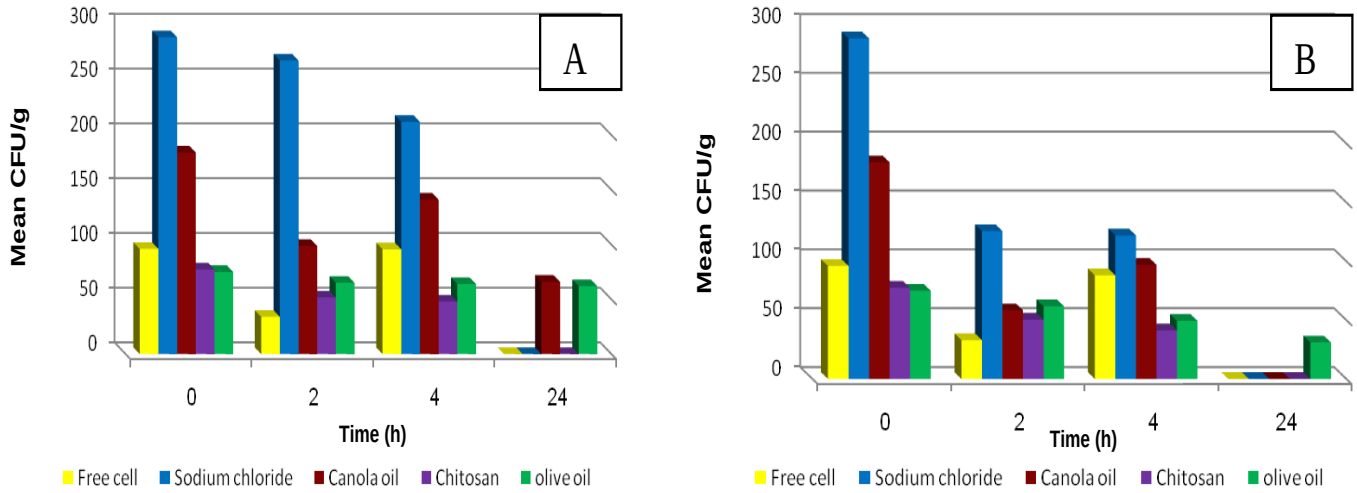

Figure 5. The stability of free and encapsulated *L. plantarum* DSM 20174 at different high concentrations of bile salts by using pour plate count method (A) 0.3% and (B) 0.5%.

Our study supported by a recent foundation of Oana (2014) who found that pH 1.5 negative impacts the stability of free cells of *L. plantarum*, when compared with encapsulated *L. plantarum* cells with alginate coated with chitosan which showed a high survival rate. Annan et al. (2008) also found that alginate capsules of *Bifidobacteria adolescentis* did not give good protection at pH 2, because gelatin were structurally unstable in simulated gastric juice due to degradation by pepsin and completely disintegrated after 45 min.

The present study has shown that there disparity in the stability of capsules at high concentration of bile salt (0.3 and 0.5%). All capsules form high stabile at 0.3 and 0.5% after 2 and 4 h, whereas diminished after 24 h except alginate coated with chitosan and alginate coated with olive oil at 0.3% and alginate coated with olive oil at 0.5%. This finding is in agreement with Kim et al. (2008) who found that alginate coated with chitosan is more stable than the free cells of *L. acidophilus* ATCC 43121 at concentrations 0.3 and 0.5% bile salt.

Due to the importance of heat treatment in the pasteurization of food for the purpose of controlling pathogenic bacteria, which will affect the efficacy of beneficial probiotic, microencapsulation has been proven

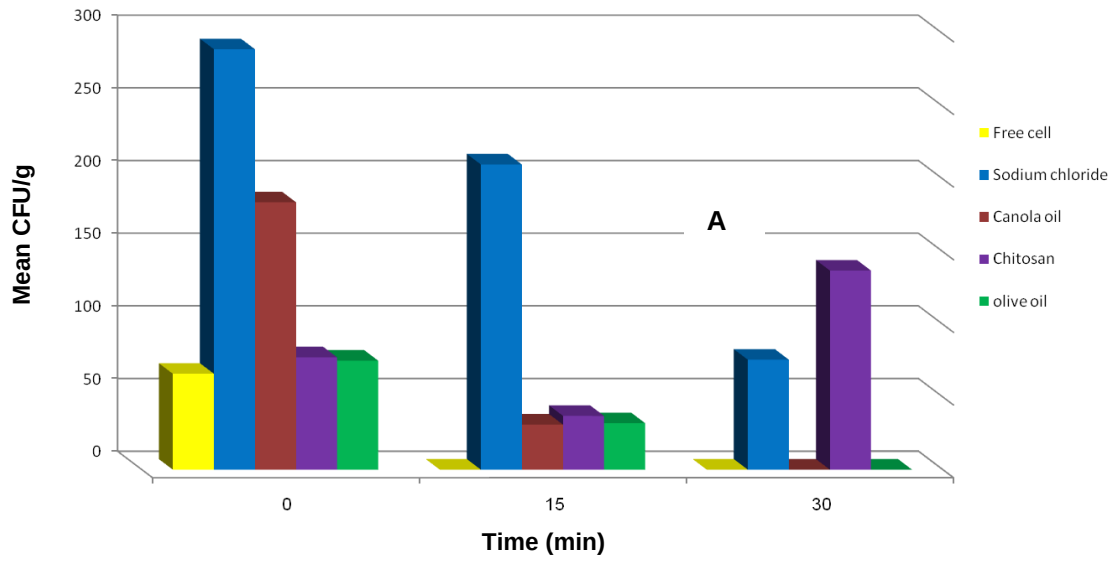

Figure 6. The stability of free and encapsulated *L. plantarum* DSM 20174 at heat treatment at (65ºC) by using pour plate count method.

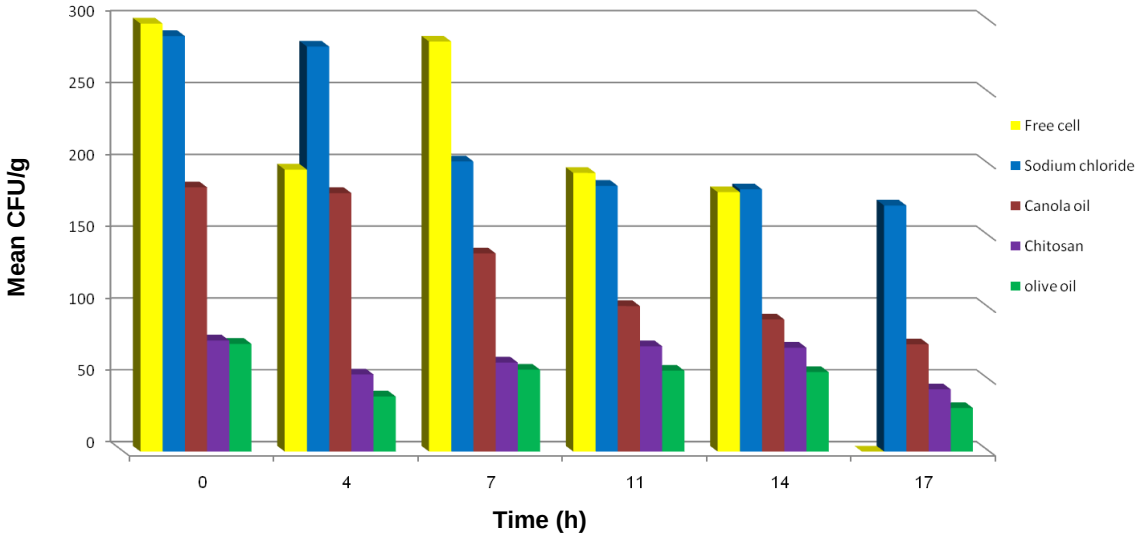

Figure 7. The stability of free and encapsulated *L. plantarum* DSM 20174 during cold storage at (4°C) by using pour plate count method.

to be one of the most efficient methods for maintaining viability and stability of probiotic, as it protects probiotic during food processing (Peck et al., 2011).

The present study showed that high temperature negatively affected free cells and completely lost during exposure to a temperature of 65°C for 15 min. The reason of short duration of thermal resistance may be due to the fact that microencapsulation methods temporarily protect bacterial cells by reducing the heat transfer from the surrounding medium to the inside of the capsule as mentioned by Ding and Shah (2008). The results of the current study are consistent with several researches who found that bacteria encapsulated more stable in heat treatment than free cells (Kim et al., 2008; Chen et al., 2007; Mandal et al., 2006).

Food storage has an important role in the preservation during transportation, and being one of the most important parameters that regulates the activities of microorganisms in food systems (Doleyres and Lacroix, 2005). Therefore, the present study was done to estimate the stability of encapsulated and free cells of *L. plantarum* DSM 20174 when exposed to 4°C for different periods. It

was found out that encapsulation has a role in increasing the stability of the bacterium cells and the results also showed that there is a difference in the stability depending on the material of microencapsulation. Our results were in agreement with Nualkaekul et al. (2012) who found that multiple layers of capsules, chitosan coated alginate, enhanced the stability of *L. plantarum* cells during storage in pomegranate juice at 4°C for 6 weeks. In the study of Woraharn et al. (2010), it was observed that encapsulation of *L. plantarum* with sodium alginate and calcium alginate provided protection after storage for 5 days at 4°C. They also found that sodium alginate capsules were better than calcium alginate as materials for encapsulation. Furthermore, our results showed that the type of oil used in the encapsulation have a direct role in the stability of the capsules under storage conditions. This observation was supported by Hou et al. (2003) who found that using sesame oil encapsulation of *L. delbrueckii bulgaricus* improved the survival of encapsulated cells when stored at a temperature of 4°C for 16 days significantly from 0.023 to 5.45% compared to the free cells. However, the results are inconsistent with the study of Lee et al. (2004) who found that both free and encapsulated cells showed similar stability within 4 weeks of storage at 4°C.

Conclusion

The encapsulation methods of *L. plantarum* DSM 20174 with alginates were carried out using sodium chloride, canola oil, olive oil, and chitosan. Materials for encapsulation impact the form of the resultant capsules which varied in terms of size, texture, shape and hardness. Data showed that the encapsulated probiotic bacterium was more stable and viable compared with free cells and alginate capsules coated with olive oil was the only one recorded stability at pH 2. This material has not been used before, which may be considered as a novel material. The alginate capsules coated with chitosan and olive oil enhanced the stability at concentration 0.5% of bile salts for 2 h of incubation. Alginate capsules with sodium chloride gave the highest stability at 65°C for 15 and 30 min. Moreover, encapsulation may enhance the survival of probiotic bacterium such as *L. plantarum* DSM 20174 in fermented food during cold storage.

Conflict of interests

The authors have not declared any conflict of interests.

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