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Effect of different microencapsulating materials on survivability of *Streptococcus thermophilus* under simulated food processing and gastrointestinal conditions

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ABSTRACT

Different biopolymeric materials including skim milk (Sm), dextrin (Dex), chitosan (Ch) and denatured whey protein (DWP) separately combined with sodium alginate (Alg), were evaluated to maintain the viability of *Streptococcus thermophilus* during simulated food processing and gastrointestinal conditions. The results revealed that all the encapsulating materials were efficient in protecting *S. thermophilus* under the pasteurization temperature (65 °C for 30 min). *S. thermophilus* entrapped within Alg-Dex significantly showed the highest survivability during the refrigerated storage (≈8 °C) for one month. However, Alg-DWP seemed to be the most efficient in protecting *S. thermophilus* during freezing storage up to 3 months. All the microencapsulated *S. thermophilus* tolerated NaCl concentrations up to 5%. Tolerance of the microencapsulated cells toward organic acids was varied depending on the type of the organic acid. Alg-Ch and Alg-DWP were the most efficient in protecting *S. thermophilus* under simulated gastric juice, while Alg-DWP offer a good survival for *S. thermophilus* under simulated intestinal condition. Generally, Alg-DWP proved to be the most promising encapsulating material that maintain the survivability of *S. thermophilus* under almost all the stress conditions adopted in the current study.

Key words: Microencapsulation, *Streptococcus thermophilus*, Sodium alginate, Gastrointestinal juice

Introduction

Lactic acid bacteria (LAB) are generally recognized as safe microorganisms, they are incorporated in many industrial applications. However, they possess a golden role in production of many fermented food products (Arena *et al.*, 2017). One of the most important industrial LAB is *S. thermophilus*. It largely engaged as a starter culture for production of many fermented dairy products. It is considered as multifunctional LAB because it possesses number of functional activities including production of exopolysaccharides, bacteriocins and vitamins such as folate. In addition, *S. thermophilus* has potential as a probiotic due to its various health effects and moderate adherence in the gastrointestinal tract (Iyer *et al.*, 2010; Tidona *et al.,* 2016).

Maintenance of the viability and functionality of the starter culture during food manufacturing is a key challenge in the industrial applications. In the recent years, many publications have been investigated the role of microencapsulation technology in preserving the bacterial viability under different harsh conditions. Food fermentation using encapsulated starter culture, offers various advantages compared to traditional cultivations, e.g., rapid fermentation, higher cell density, preservation of the cells from the harsh environmental conditions such as high acidity, freezing and heating processes or bacteriophage attack, as well as facilitating the handling of cells and allowing a controlled dosage (Rokka and Rantamäki, 2010; Haffner *et al.*, 2016; Kavitake *et al.*, 2018).

Microencapsulation process involves entrapment of the microbial cell within a biopolymeric material in order to keep the viability and the functionality of the entrapped cells under different detrimental conditions (Krasaekoopt *et al.*, 2003). Several encapsulating materials have been investigated for encapsulation of LAB such as polysaccharide (alginate, chitosan, gellan gum, xanthan gum, pullan gum, k-Carrageenan), protein (whey protein, soy protein, pea protein) and lipid. Most adopted encapsulation materials are alginate-based and protein-based materials. The objective of this study is to evaluate the stability of the microencapsulated *S. thermophilus* using alginate-based

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material combined with different biopolymeric materials including skim milk, dextrin, chitosan and denatured whey protein during simulated food processing and gastrointestinal conditions.

Materials and Methods

Materials

Streptococcus thermophilus CH-1 was purchased from Chr. Hansen's Lab. (Denmark). Sodium alginate was purchased from Loba Chemie (Mumbai, India). Fresh skim milk was purchased from Faculty of Agriculture, Cairo University (Egypt). Dextrin was purchased from Merck (Darmstadt, Germany). Chitosan (Deacetylation 93%) was purchased from Oxford Lab Chem (Thane, Maharashtra, India). Whey protein concentrate 80% was purchased from milkiland Intermarket (Poland). Pepsin (1:3,000) was purchased from Science Lab (Texas, USA). Pancreatin from hog pancreas (5× USP specifications) and bile salt were purchased from BIOBASIC INC (Canada).

Microbial strains and growth condition

S. thermophilus was grown on M17 broth and incubated for 24 h at 37 ^{o}C. The strain was activated two or three times in order to obtain high biomasses in the stationary phase then the cell pellets were harvested by centrifugation at 4000 rpm, for 20 min at 4 ^{o}C. The pellets were washed by sterile saline solution (0.9% (w/v) NaCl) and recovered under the same centrifugation conditions then dissolved with an equal volume of sterile saline solution and stored at ≈8 ^{o}C till be encapsulated.

Composition and preparation of the encapsulating materials

Four combinations of the encapsulating materials were prepared based on alginate as principle biopolymer combined with another adjuvant biopolymer as follows (all the concentrations that used in this study were chosen after different preliminary optimization experiments):

1- Sodium alginate-skim milk (Alg-Sm) was prepared according to (Shi *et al.*, 2013) with some modifications. One part of fresh skim milk was sterilized by autoclaving at 121 ^{o}C for 5 min then mixed with 2 parts of 3% (w/v) alginate solution that was sterilized by autoclaving at 121 ^{o}C for 15 min.
2- Sodium alginate-dextrin (Alg-Dex) was prepared according to (Mirzaei *et al.*, 2012) with some modifications by dissolving 3 g (Alg) with an equal amount of (Dex) in 100 ml distilled water then the solution was sterilized by autoclaving at 121 ^{o}C for 15 min.
3- Sodium alginate-chitosan (Alg-Ch) capsule. Chitosan was prepared as the method described by (Zhou *et al.*, 1998).
4- Sodium alginate-denatured whey protein (Alg-DWP) was prepared by mixing equal volume of 10% (w/v) freshly prepared (DWP) with sterile 3% (w/v) alginate (Rajam *et al.*, 2012).

Microencapsulation procedure

Generally, the microencapsulation process was performed using the extrusion technique (Feucht and Kwak, 2013). One part of the cell suspension was mixed separately with four parts of the freshly prepared encapsulating materials with gentle stirring for 10-20 min. The mixture was then extruded into the hardening solution ($CaCl_2$, 0.2 M) through sterile syringe (25 G, 0.5 mm) with gentle stirring for 30 min to ensure complete solidification. In case of Alg-Ch, after extruding the alginate-cells mixture into the hardening solution, the harvested alginate microcapsules were then coated with chitosan by immersing (about 12 g capsules) in 100 ml of chitosan solution with gentle stirring for 40 min.

The formed microcapsules were harvested by filtration then washed by sterile saline solution and stored at ≈8 ^{o}C till be used for further analysis.

Enumeration of the microencapsulated cells

The viability of *S. thermophilus* was assessed as described by (Chávarri *et al.*, 2010). One gram of the microcapsules was dissolved in 9 ml of sterile tri-sodium citrate solution (2% w/v) and vortexed till complete dissolution then the samples were serially diluted to appreciate concentration using (0.1%) peptone and pour plated in M17 agar. The plates were incubated 24 h at 37 ºC. The viable cell number was expressed as colony forming unit per gram of microcapsule (cfu/g).

Encapsulation efficiency

Encapsulation efficiency (EE) was determined by using the following equation as described by (Fareez *et al.*, 2015):

$$EE = \frac{Log_{10}N}{Log_{10}N_o} \times 100\%$$

Where N is the number of the bacterial cells loaded inside the microcapsules and N_o is the number of the free bacterial cells added to the biopolymer mixture during the preparation of the microcapsules (before microencapsulation).

Particle size analysis

Particle size of the manufactured microcapsules was determined using static laser scattering device (Master sizer 2000, Malvern, UK). The hydrodynamic particle diameter was expressed as volume weighted mean size distribution % (d4,3).

Morphological characterization of capsules

The morphology of the microcapsules was examined using a scanning electron microscope (SEM) (model Quanta 250, high resolution field emission gun (HRFEG, Czech). Before using the SEM, the samples were immersed in buffer glutaraldehyde (0.1 M) for 2 h at 4 ºC (pH = 7.3) and were post fixed with osmium tetraoxide (0.1 M) for 1 h at 4 ºC. Samples were then consecutively dehydrated using 30, 50 and 70% ethyl alcohol for 2 mins each and remained in 100% ethyl alcohol for 30 mins at 4 ºC. After that, the samples were placed on a piece of adhesive paper and coated with gold using a vacuum sputtering coater (Edwards S150A, England).

Survivability of microencapsulated cells under simulated food processing conditions

A. Different heat treatments

Sterilized skim milk was inoculated by 10% of the microcapsules then subjected to three different heat treatments that may stimulate the potential stress that can encounter the cells during food manufacturing. The heat treatments include:

1. High incubation temperature (40 ºC for 24 h).
2. Scalding temperature of cheese production (45 ºC for 30 min).
3. Pasteurization temperature (65 ºC for 30 min).

One gram was taken from the sample and the viable cell count was determined as mentioned in the previous section.

The survivability of the encapsulated cells was determined using the following equation:

$$\text{Survivability} = \log_{10}\left(\frac{N_t}{N_i}\right)$$

Where N_i and N_t are the number of the viable cell (cfu/g) at the zero time (initial count) and at various storage time.

B. During refrigerated storage

The viability of the encapsulated cells under refrigeration was evaluated by inoculating sterilized skim milk with 10% microcapsules and kept in the refrigerator at ≈8 °C for one month. The viable cell count was determined every one week and survivability was calculated as mentioned above.

C. During freezing storage

The viability of the encapsulated cells under freezing was evaluated by inoculating 10% of the microcapsules into sterilized skim milk and kept in the ordinary freezer for three months. The viable cell count was determined every one month. One gram was taken from the sample then the viable cell count and survivability were determined as described before in the previous sections.

D. Different NaCl concentrations

Sterilized salted skim milk was prepared by adding sodium chloride at concentrations (1%, w/v), (3%, w/v) and (5%, w/v). Each of the three concentrations was inoculated by 10% of the microcapsules then incubated at 37 °C for 24 h. One gram was taken from the sample then the viable cell count and survivability were determined as described before in the previous sections.

E. Different concentrations of food-applied organic acids

Sterilized skim milk supplemented with (1%, w/v) and (2%, w/v) of lactic, citric and ascorbic acids was inoculated by 10% of the microcapsules. The samples were incubated at 37 °C for 24 h. One gram was taken from the sample then the viable cell count and survivability were determined as described before in the previous sections.

Survivability of the microencapsulated cells in simulated gastrointestinal tract conditions

A. Simulated gastric juice (SGJ)

SGJ was prepared according to the method of (Chávarri *et al.*, 2010). Saline solution (9 g/L NaCl) was adjusted to pH 2.5 with 1 N HCl and sterilized by autoclaving at 121 °C for 15 min then pepsin was suspended in the solution to final concentration 3 g/L. SGJ was inoculated with 10% of the microcapsules and incubated at 37 °C. Viable cell count was assessed after 5, 30, 60 and 120 minutes as described earlier.

B. Simulated intestinal juice (SIJ)

SIJ was prepared according to the method of (Chávarri et al., 2010; Gbassi *et al.*, 2009). A solution of 6.5 g/L NaCl, 0.835 g/L KCl, 0.22 g/L $CaCl_2$, 1.386 g/L $NaHCO_3$, and 3 g/L bile salt was adjusted to pH 7.5 and sterilized by autoclaving at 121°C for 15 min then pancreatin was suspended in the solution to final concentration 10 g/L. SIJ was inoculated with 10% of the microcapsules and incubated at 37 °C. Viable cell count was assessed after 5, 60, 90 and 120 minutes as described earlier.

Statistical Analysis

The data analysis was carried out using CoStat software (StatSoft Inc., Tulsa, USA). Analysis of variance (ANOVA) was applied to determine significant differences ($P < 0.05$) between results and LSD (Least significant difference) was used to compare means values. The data were expressed as mean ± standard error. All experiments were repeated 3 times ($n = 3$).

Results and Discussion

Encapsulation efficiency

The encapsulation efficiency of the capsules loaded with *S. thermophilus* was illustrated in Table (1)**.** According to the data in the table, there was no significant difference in the encapsulation efficiency between the different encapsulating materials loaded with *S. thermophilus* and it was ranged between 94.39% and 92.13%. This result could be regarded to the mild encapsulation method used for producing all capsules regardless to the encapsulating material (Shi *et al.*, 2013). Our result came in agreement with Shi *et al.,* (2013), who reported that the encapsulation efficiency of the encapsulated *Lactobacillus bulgaricus* using Alg-milk capsules was around 100%.

Table (1) also shows that the particle size of the four formulated capsules loaded with *S. thermophilus* was ranged between 859.72 µm and 1006.23 µm. Albadran *et al.*, (2015) also found that the Alg-Ch capsules had a diameter of around 2 mm and Fareez *et al.*, (2015) prepared capsules ranged in size between 1312.4 and 1335.7 µm. This could be regarded to the fact that extrusion technique produce capsules with size not smaller than 300 µm (Burgain *et al.*, 2011).

Table 1. Encapsulation efficiency and particle size of different microcapsules

Encapsulating material	Encapsulation Efficiency (%)	Particle size (µm)
Alg-Sm	94.39±0.94^{ab}	1006.235
Alg-Dex	92.18±1.35^{b}	859.721
Alg-Ch	92.13±0.30^{b}	920.573
Alg-DWP	92.26±2.15^{b}	968.513

Data were expressed as mean ± standard error of three replicates (n = 3).
$^{a-b}$Values in the same column with different superscript letters are significantly different (P < 0.05).

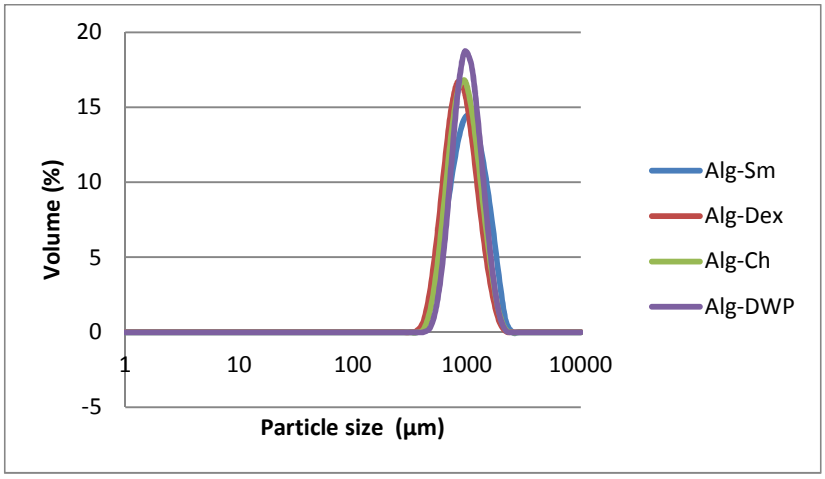

Fig. 1: Particle size distribution of the microcapsules loaded with *S. thermophilus*

Capsule morphology

The SEM images of the microcapsules loaded with *S. thermophilus* indicate that all the produced capsules were irregular in shape with rough surface and appeared as drop-like shape with small tail (Fig. 2). This observation may be attributed to the high vacuum applied during SEM analysis and the high surface tension of the used hardening solution ($CaCl_2$) that result in formation of the imperfect sphere (Maresca *et al.*, 2016).

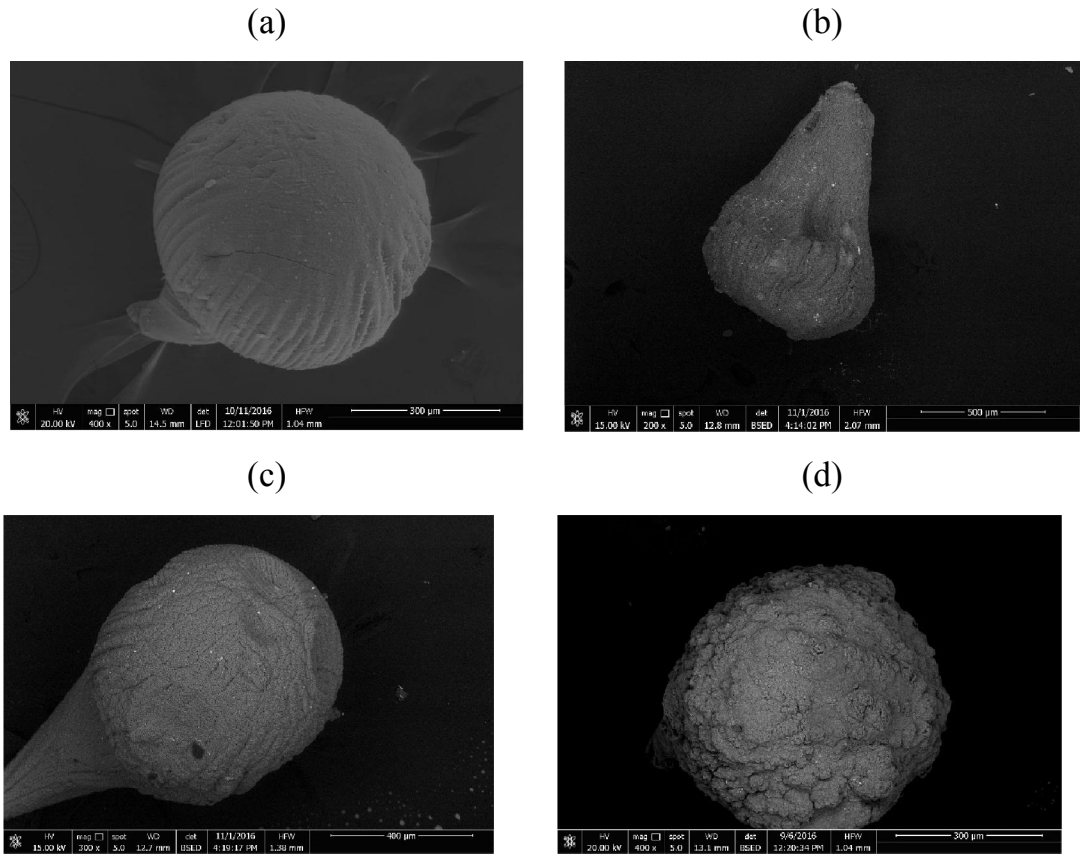

Fig. 2: Scanning electron micrograph of Alg-Sm (a); Alg-Dex (b); Alg-Ch (c) and Alg-DWP (d) capsules loaded with *S. thermophilus*.

Survivability of the microencapsulated cells under simulated food processing conditions

A. Under different heat treatments

Data recorded in (Fig. 3a, b) revealed that the viability of all the microencapsulated *S. thermophilus* increased over the initial count, which was around 10^8 cfu/g sample, after exposure to 40 ^oC for 24 h and 45 ^oC for 30 min. This may be regarded to the resistance of *S. thermophilus* to temperature reached 52 ^oC (Heller, 2001). In contrast, the viability of the encapsulated *S. thermophilus* reduced after exposure to 65 ^oC for 30 min and statistically there was no significant difference between all the tested encapsulating materials, the reduction was only 1.19 log cycle for the cells entrapped within Alg-Dex, 1.26 log cycle for Alg-Sm and Alg-Ch and 1.48 log cycle for Alg-DWP (Fig. 3c).

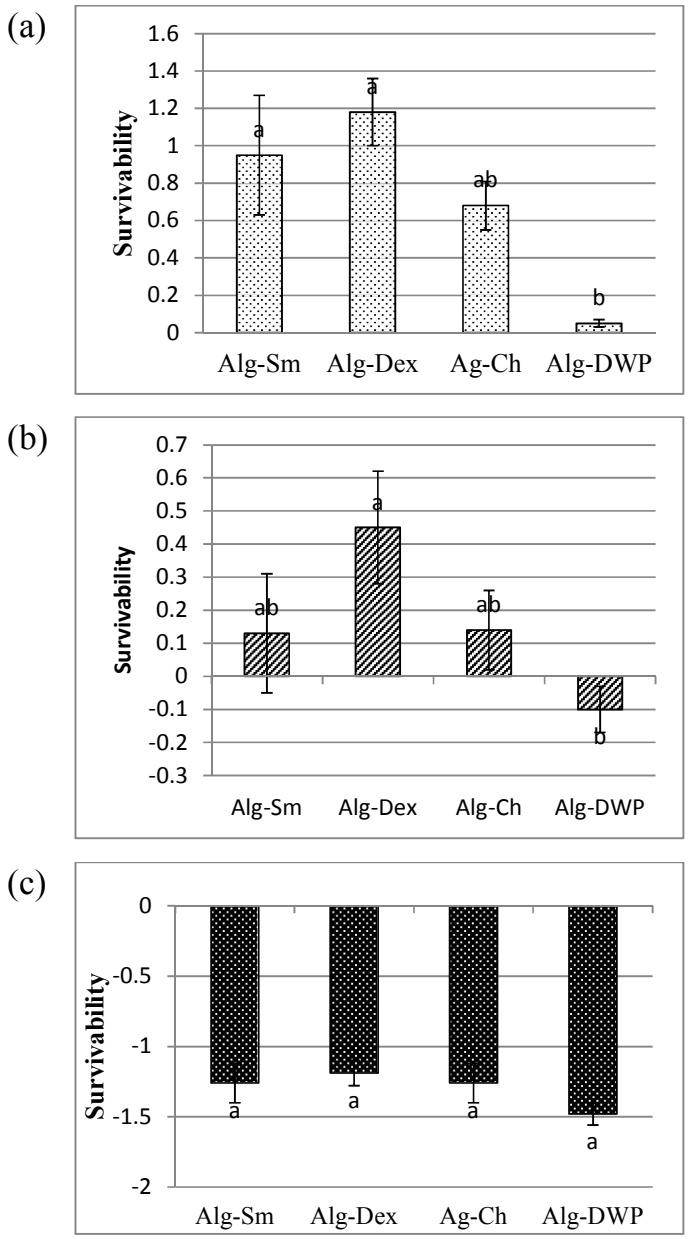

Fig. 3: Survivability of microencapsulated *S. thermophilus* after heat treatment at 40 °C for 24 hours (a); 45 °C for 30 min (b); 65 °C for 30 min (c). Error bars represent the standard error of the mean. Means for each data without a common letter differ significantly ($P < 0.05$).

B. During refrigerated storage

Results in Fig. (4) illustrate that the viability of all the encapsulated *S. thermophilus* during the refrigerated storage for one month showed little bit viable cell reduction from the initial count, which was approximately 10^9 cfu/g sample. At the end of the storage period, the cell reduction was 0.004, 0.13, 0.53 and 0.58 log cycle for the cells coated with Alg-Dex, Alg-Sm, Alg-DWP and Alg-Ch, respectively.

Since the minimum recommended dose level of the starter cultures or probiotics to perform their function is 10^6 cfu/g of the fermented product (Bilenler *et al.*, 2017), the four encapsulating materials that were used in this study were efficient in enhancing the viability of *S. thermophilus* during the refrigerated storage at ≈8 °C up to one month. However, Alg-Dex was significantly the

most appropriate encapsulating material and this could be attributed to the simulating effect of dextrin, which acts as prebiotic, on the growth behaviour of *S. thermophilus* (Effat *et al*., 2012).

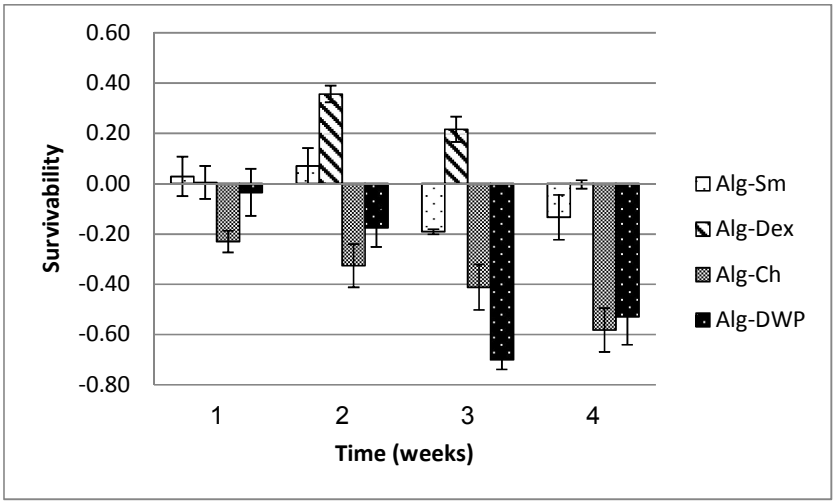

Fig. 4: Survivability of microencapsulated *S. thermophilus* during refrigerated storage for one month at ≈8 °C. Error bars represent the standard error of the mean.

C. During freezing storage

Obtained data in Fig. (5) show that the cell load within all the encapsulating materials significantly ($P < 0.05$) decreased throughout the storage period. The viability loss was only 0.89 log cycle for the cells entrapped within Alg-DWP, while the cell reduction was 1.57, 1.67 and 1.72 log cycle for the cells encapsulated with Alg-Ch, Alg-Dex and Alg-Sm, respectively. Based on the above mentioned, Alg-DWP was significantly the most efficient encapsulating material in enhancing the survivability of *S. thermophilus* under freezing conditions up to 3 months. This could be due to the adsorption of milk proteins on the cell surface that leads to a partial efflux of water from the cell, thus inhibiting the growth of ice crystals inside the cell, which subsequently reduce cell injury and cell loss (Wang *et al.*, 2015).

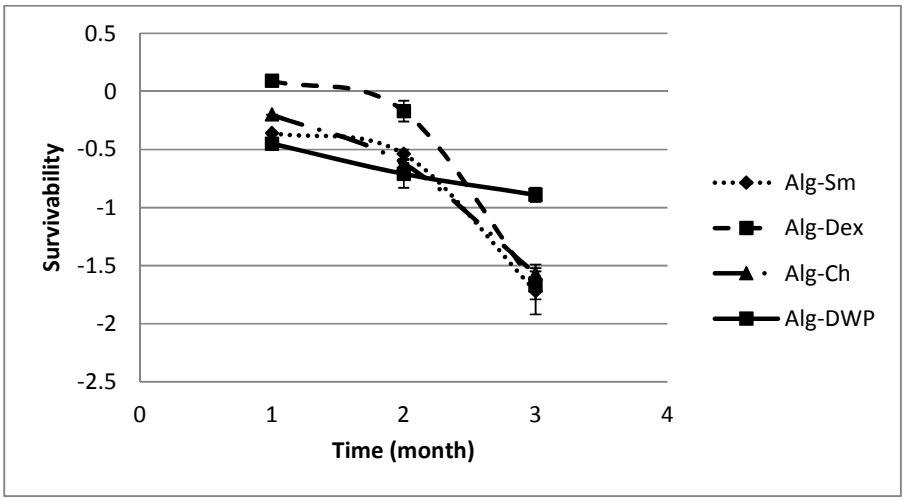

Fig. 5: Survivability of microencapsulated *S. thermophilus* during freezing storage for three months. Error bars represent the standard error of the mean.

D. Different NaCl concentrations

Results in Fig. (6) show that all the encapsulated cells were able to grow in milk salted with 1% NaCl. However, the capability of the encapsulated cells to grow in 3% and 5% NaCl decreased relative to 1% NaCl. All the encapsulated cells were able to grow in salt concentration up to 5% and there was no significant difference between using the all tested encapsulating materials. Therefore, all the encapsulating materials evaluated in this study were capable of enhancing the tolerance of *S. thermophilus* in NaCl concentrations up to 5%. Few reports investigated the tolerance of microencapsulated bacteria to high salt concentration. Bosnea *et al.*, (2014) also reported that microencapsulation using whey protein isolate and gum arabic enhanced the survival of probiotic cells after exposure to 9% NaCl for 3 h when compared with free cells.

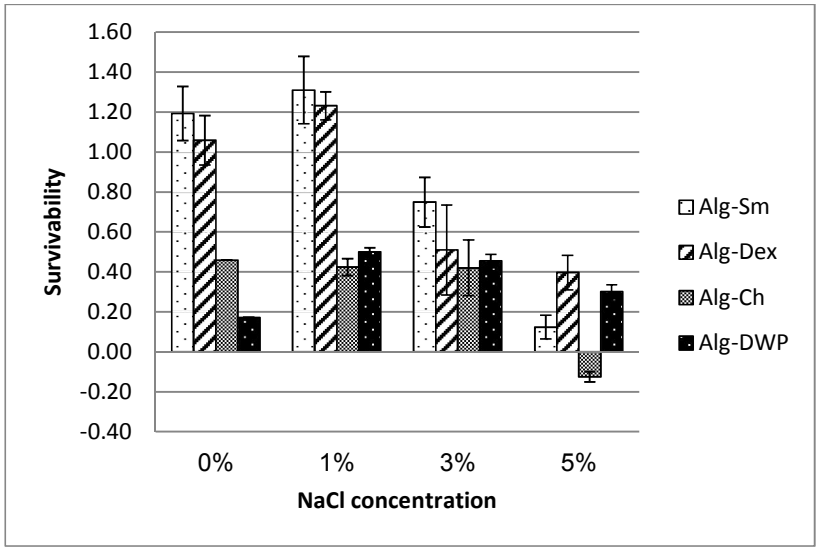

Fig. 6: Survivability of microencapsulated *S. thermophilus* in different concentrations of NaCl after incubation at 37 ^{o}C for 24 h. Error bars represent the standard error of the mean.

E. Survivability in different concentrations of food-applied organic acids

Results in Fig. (7) describe the tolerance of the encapsulated *S. thermophilus* to different organic acids (including citric, lactic and ascorbic acids) after incubation at 37 ^{o}C for 24 h.

Citric acid

In case of 1% citric acid, the cells encapsulated with Alg-Sm increased by 0.14 log cycle over the initial count, which was approximately 10^8 cfu/g sample. However, the number of cells entrapped within Alg-DWP, Alg-Dex and Alg-Ch reduced by 0.57, 1.07 and 1.93 log cycle, respectively. On the other hand, the viable cell count in all the used capsules declined in 2% citric acid. The minimum reduction was 0.37 and 0.67 log cycle for Alg-DWP and Alg-Sm, respectively. However, the maximum reduction was 2.65 and 2.78 log cycle for Alg-Dex and Alg-Ch, respectively. It is clear that Alg-DWP and Alg-Sm were the most efficient in protecting *S. thermophilus* from citric acid up to 2%. This could be due to the buffering capacity of the milk proteins (Mosilhey, 2003; Shi *et al.,* 2013).

Lactic acid

In case of 1% lactic acid, Alg-Ch was the only encapsulating material that allowed the growth of *S. thermophilus* and the cell count just only increased by 0.11 log cycle over the initial count, which was approximately 10^8 cfu/g sample. However, the cell count within the other encapsulating materials showed viability loss by 0.76, 1.09 and 2.72 log cycle for cells encapsulated with Alg-Sm,

Alg-Dex and Alg-DWP, respectively. In case of 2% lactic acid, the number of all the encapsulated *S. thermophilus* declined in presence of 2% lactic acid. The reduction was only 0.79 log cycle for the cells encapsulated with Alg-Ch but there was a sharp decline in case of the cells encapsulated with Alg-DWP, Alg-Dex and Alg-Sm and the viability loss was 3.53, 5.23 and 5.63 log cycle, respectively. From the above mentioned, Alg-Ch was the most appropriate encapsulating material to protect *S. thermophilus* from lactic acid containing environment up to 2%.

Ascorbic acid

Regarding ascorbic acid, Fig. (7) indicates that there was a little bit growth reduction for the cells in all encapsulating materials (relative to the initial count that was 10^8 cfu/g sample) except for the cells encapsulated with Alg-Ch, the growth of *S. thermophilus* increased by 0.34 and 0.26 log, respectively in 1 and 2% ascorbic acids. The cell reduction may be regarded to the oxygen scavenger activity of ascorbic acid that reduced the oxygen content in the growth medium and subsequently affect the growth of *S. thermophilus* (Shah, 2000). Despite that all the encapsulating materials secured 10^7 cfu/g, therefore they were efficient for maintaining *S. thermophilus* in medium containing ascorbic acid up to 2%. However, Alg-Ch appeared to be the most efficient one.

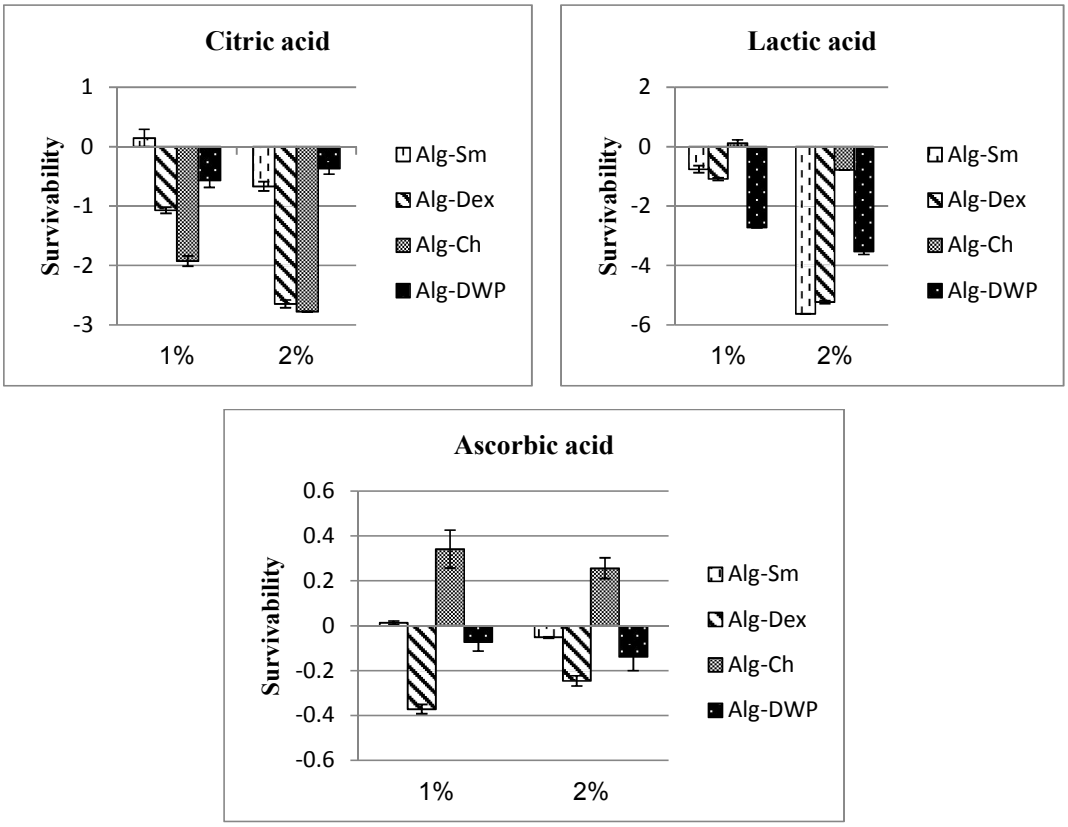

Fig. 7: Survivability of microencapsulated *S. thermophilus* in different concentrations of food-applied organic acids after incubation at 37 ^oC for 24 h. Error bars represent the standard error of the mean.

Survivability of the microencapsulated cells under simulated gastrointestinal conditions

A. Simulated gastric juice (SGJ)

Fig. (8) illustrates that there was a reduction in the population of the encapsulated *S. thermophilus* after exposure to SGJ for 120 min. However, the cell reduction was only 0.28, 0.40 and 1.35 log cycle for the cells encapsulated with Alg-Ch, Alg-DWP and Alg-Sm, respectively. On the

other hand, Alg-Dex appeared to be the less efficient encapsulating material for protecting *S. thermophilus* from the adverse simulated gastric condition since the viability loss was 2.54 log cycle. Therefore, Alg-Ch and Alg-DWP microcapsules were the most efficient encapsulating material for maintaining the viability of *S. thermophilus* under simulated gastric condition. The cationic nature of chitosan and its ability to buffer acid may limit the interaction between the cells and the acidic environment (Cook *et al.*, 2012; Chávarri *et al.*, 2010). Milk proteins can improve the survival of bacteria in simulated gastric juice due to the dense protein matrix that may reduce the diffusion rate of the acid into the microcapsules (Shi *et al.*, 2013; Shori, 2017).

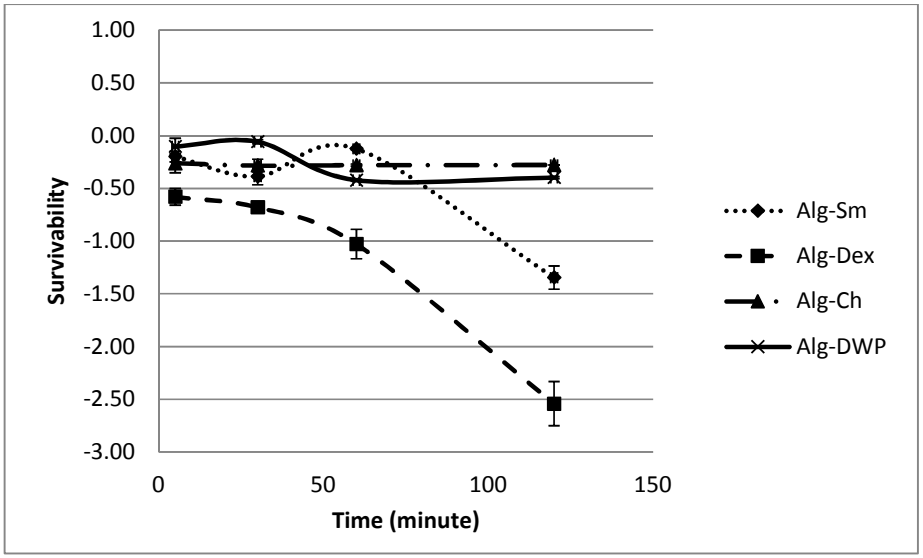

Fig. 8: Survivability of microencapsulated *S. thermophilus* after exposure to simulated gastric juice (SGJ). Error bars represent the standard error of the mean.

B. Simulated intestinal juice (SIJ)

Results in Fig. (9) illustrate that after exposure to SIJ for 120 min, the viable cell count of the encapsulated *S. thermophilus* reduced from the initial count (around 10^8 cfu/g sample). The viability loss was 0.34, 1.24, 1.35 and 1.61 log cycle for the cells encapsulated with Alg-DWP, Alg-Sm, Alg-Ch and Alg-Dex, respectively. Based on that, all the encapsulating materials secured 10^7 cfu/g and this viable cell count is within the recommended dose level to be effective as indicated before. Therefore, all capsules will take more time to be disintegrated and controlled the release of the entrapped cells, thus subsequently shorten the exposure time to the detrimental effect of the intestinal fluid (Fareez *et al.*, 2015). However, Alg-DWP microcapsules appeared to be significantly the most efficient encapsulating material for maintaining the survivability of *S. thermophilus* under simulated intestinal condition and almost all the stress conditions adopted in the present study. This could be due to that whey protein enhance the viability and the functionality of *S. thermophilus* (Zhang *et al.*, 2011).

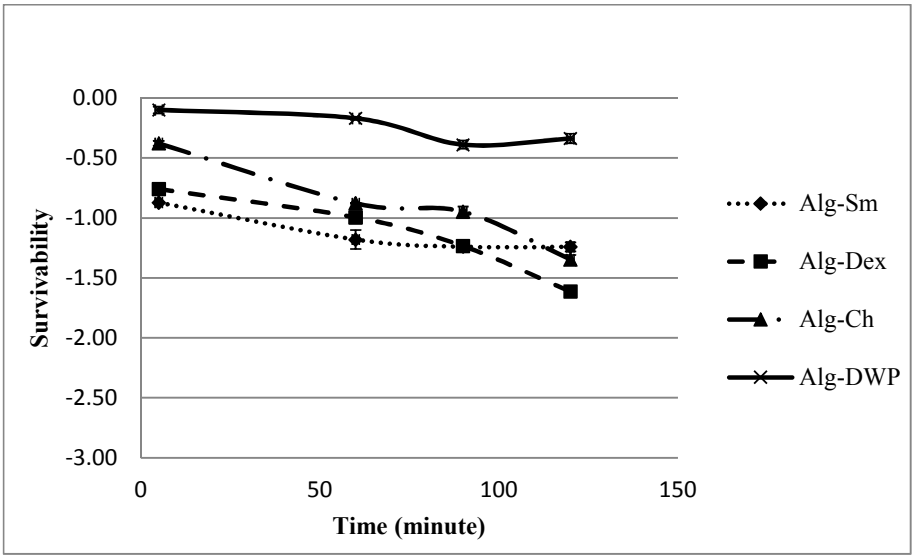

Fig. 9: Survivability of microencapsulated *S. thermophilus* after exposure to simulated intestinal juice (SIJ). Error bars represent the standard error of the mean.

Conclusion

In this context, there is a differential behavior among the different encapsulating materials toward the different stress conditions. However, Alg-DWP appeared to be the promising capsule for protection of *S. thermophilus* under most of the adopted stress conditions. Overall, the type of final application and the potential stress are the factors that will determine the appropriate type of the encapsulating material. Therefore, further studies are needed for in vivo assessment.

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