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The application of multi-particulate microcapsule containing probiotic bacteria and inulin nanoparticles in enhancing the probiotic survivability in yoghurt

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ABSTRACT

The potential application for the multiparticulate synbiotic microcapsule in yoghurt production was presented in the current study. Initially, three different formulae were fabricated to investigate the mechanism of action. This was followed by characterization the size of both of the nanoparticles and the final multiparticulate microcapsule. The yoghurt was then formed and the formulae were added and subjected to 4 weeks storage period. The viability of *Bifidobacterium* mixed cultures was monitored weekly. The results showed the viability of *Bifidobacterium* cultures was enhanced when double coated microcapsule was applied compared to the free one. The enhancement was further boosted when inulin was incorporated in either free form or in the form of nanoparticles. Scanning electron microscope image showed the final multiparticulate microcapsule has size of 700 μm while the laser diffraction particle size analyzer revealed the nanoparticles size is 118.1±4 nm. It was concluded that both of the double coat layer covering the multiparticulate microcapsule and the entrapped inulin, significantly enhanced the survivability of the encapsulated *Bifidobacterium* cultures in yoghurt during the shelf period.

1. Introduction

Yoghurt is the mainly accepted fermented dairy product which provides senior level of protein, carbohydrate, calcium and B vitamins. *Lactobacillus delbrueckii* subsp *bulgaricus* and *Streptococcus thermophilus* bacteria, take part of its acid lactic fermentation which have to be in a 1:1 relation for an effectual synbiotic action (Salvatierra et al., 2004). Probiotic yoghurt can be a gorgeous to customers, for the reason that totaling of some probiotic bacteria can increase its therapeutic value and assist them to consume dietary foods with supplementary benefits to health (Hekmat and Reid, 2006). There are several microorganisms used like probiotics including *Bifidobacterium, Bacillus, Streptococcus, Saccharomyces, Aspergillus, Enterococcus, Pediococcus* and, the most used, *Lactobacillus* genre.

Moreover, to progress the probiotic bacteria in different fermented food like yoghurt, it should be supplemented with prebiotics. Prebiotics are non-digestible food ingredients that pass during the upper gut unaltered and selectively fermented by colonic bacteria (Ramirez-Farias et al., 2009). This leads to alter the composition and the activity of the gut microbiota that confers benefits upon host health (Cummings et al., 2001). Inulin in nature occurs in numerous plant foods including onion, garlic, chicory, artichoke and leek (Gibson et al., 2004). Some of the useful property of inulin in the gastro-intestinal tract include stimulating of microbial fermentation, lowering fat and cholesterol absorption, and pH decrease; these hence have a straight effect on reducing intestinal disturbances, constipation, hyperglycemia and intestinal cancer (Ziemer and Gibson, 1998; Kaur and Gupta, 2002).

The health benefits are not only reliant on the selection of microorganism with specific therapeutic properties, but it is also necessary that these live microorganisms are consumed in adequate quantities to display the preferred positive health effects. Numerous authors have recommended that the minimum imperative concentration at the instant of intake is 10^6–10^8 viable cells g^{-1} in the product (Gomes et al., 1995; Shafiee et al., 2010).

Microencapsulation is a hopeful procedure to provide physical protection and enhancement the stability of probiotic organisms in functional food products (Kailasapathy, 2006; Homayouni et al., 2008; Brinques and Ayub, 2011).

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Calcium alginate and Arabic gum are favorite materials for the encapsulation of lactic acid bacteria (LAB) because of the simplicity, non-toxicity, biocompatibility, biodegradability and low cost of these gels (Ogawa et al., 2002; Krasaekoopt et al., 2003).

Chen et al. (2007) studied probiotics that used alginate as the coating material for microencapsulation and established that the totaling of fructo-oligosaccharides (FOS), isomalto-oligosaccharides (IMO) and peptides into the materials of probiotic microcapsules provided superior protection of the organisms. In-addition, Fayed et al. (2018) investigated the microencapsulated *Bifidobacterium* within alginate and Arabic gum and loaded with inulin nanoparticles together. Found that the survivability of the encapsulated *Bifidobacterium* in the simulated gastric solution significantly was higher than the survivability of the free bacteria. Additionally, both of Cook et al. (2014) and Fayed et al. (2018) confirmed the significance of applying multiparticulate microcapsule for encapsulating prebiotic and probiotic together in the same formula.

The present research will continue our former work done by Fayed et al. (2018). We will evaluate the potential industrial application of the synbiotic multiparticulate microcapsule in maintaining and enhancing the survivability of the probiotics in the yoghurt during the standard shelf life.

2. Materials and methods

2.1. Materials

Fresh buffalo's milk, used in the manufacture of yoghurt, was obtained from local market. Gum Arabic was obtained from local market and alginate was purchased from Lobachemie (Mumbai, India). PLGA (50:50 Lactide:Glycolide) with MW 24,000–38,000kd with obtained from Sigma-Aldrich, USA. Dichloromethane was purchased from (Merck, USA).

2.2. Bacterial strains

Lyophilized mixed cultures consist of *Bifidobacterium bifidum*, *Bifidobacterium animalis*, and *Bifidobacterium lactis* obtained from probiotics lab, National research centre, Cairo, Egypt., *Lactobacillus dulbueckii* subsp. *bulgaricus* Lb-12 DRI-VAC, obtained from Northern Regional Research Laboratory. Illinois, USA and *Streptococcus thermophilus* CH-1 obtained from Chr. Hansen's Lab., Denmark.

2.3. Methods

2.3.1. Production of nanoparticles and microcapsules

The mixed cultures of *Bifidobacterium* were sub-cultured in MRS broth supplemented with 2 gm/l sodium propionate and 3 gm/l lithium chloride prior to use. The cells were harvested and washed by centrifugation at 5.000 rpm for 15 min at 4 °C. The cell suspension (25 g) was mixed with a 100 ml of wall material sodium alginate solution (3.0% w/v).

After that, the following formulae were prepared based on the double emulsion method and extrusion method for the preparation of nanoparticle and microcapsule, respectively as described by Fayed et al. (2018):

Formula (A): Microcapsule entrapped with *Bifidobacterium* mixed cultures cells.
Formula (B): Microcapsule entrapped with *Bifidobacterium* mixed cultures cells and Inulin (1%)
Formula (C): Multiparticulate microcapsule entrapped with *Bifidobacterium* mixed cultures cells and Inulin PLGA nano-particles (1%).

2.3.2. Entrapment efficiency and particle size of inulin PLGA nanoparticles

The amount of inulin unentraped was determined by Nelson-Somogyi method (Nelson, 1944; Somogyi, 1952) then entrapment efficiency was calculated indirectly. The particle size was evaluated by Mastersizer 300 laser diffraction particle size analyzer of Malvern Instruments Ltd (Worcestershire, UK).

2.3.3. Morphological characterization of formula (C)

The morphological characters of formula (C) were 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 °C (pH = 7.3) and were post fixed with osmium tetraoxide (0.1 M) for 1 h at 4 °C. Samples were then consecutively dehydrated using 30, 50 and 70% ethyl alcohol for 2 min each and remained in 100% ethyl alcohol for 30 min at 4 °C. After that, the samples were placed on a piece of adhesive paper and coated with gold using a vacuum sputtering coater (Edwards S15).

2.3.4. Production of synbiotic yoghurt

Fresh buffalo's milk, was heated at 90 °C for 10 min, then was cooled and adjusted to 42 °C according to EL-Sayed et al. (2017). The starter cultures of yoghurt (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) were added at the concentration 2% to the milk. Each prepared formula was added to one portion of the inoculated milk at concentration of 1% while 1% free *Bifidobacterium* mixed cultures was added to a final portion as control. The samples were then transferred into plastic cups and incubated at 42 °C for 4 h until coagulation. The cups were stored at 7 °C for 30 days.

2.3.4.1. Evaluation the chemical characters of the prepared yoghurt. Fresh yoghurt samples were taken after adding the proposed formula and chemically analyzed for total solids (T.S), moisture and fat according to AOAC (2007). The pH values were measured using a digital laboratory Jenway 3510 pH meter, UK. Bibby Scientific LTD. Stone, Stafford shire, ST 15 OSA. Total protein was determined using the semi micro-Kjeldal method as mentioned by Ling (1963).

2.3.4.2. Assessment the viability of the starter culture and the encapsulated Bifidobacterium mixed species in yoghurt during storage period. To assess the viability of the starter culture, M17 agar medium and MRS agar medium were used to enumerate the count of *St. thermophilus* and *Lb. bulgaricus*, respectively. The plates were incubated at 37 °C for 48 h under aerobic condition.

To determine the viability of encapsulated *Bifidobacterium* mixed cultures during storage period. 1.0 g of yoghurt sample was taken at different time interval and get dissolved in 9.0 ml 3% sodium citrate solution then stirred on a shaker to completely release the strains from the capsules. The released strains were serially diluted with saline solution and plated on MRS agar supplemented with 2 gm/l sodium propionate and 3 gm/l lithium chloride. The plates were incubated at 37 °C for 72 h under anaerobic condition.

2.3.5. Inulin release from formulae (B), and (C) during storage period

Release pattern of inulin from formulae (B), and (C) during the storage periods was determined using reducing sugar determination methods. One gm of yoghurt from each formula was mixed with 10 ml distilled water followed by dilution (according to inulin concentration in the formula). The reducing sugars were determined according to Somogyi-Nelson method (Nelson, 1944) (somogyi., 1966). Yoghurt without inulin was used as blank and all the reaction mixtures were in triplicate values.

2.4. Statistical analysis

The significance difference was tested by applying paired *t*-test using a statistical software package (Statistical Analysis System, SAS Institute Inc., Cary, NC). Differences were considered statistically significant for p value equal to or less than 0.05.

3. Results

3.1. Entrapment efficiency and particle size of inulin PLGA nanoparticles

The entrapment efficiency for the inulin in the PLGA nanoparticles was found to be 84 ± 1.5% while the particle size was found to be 118.1 ± 4 nm.

3.2. Assessment the size and the morphological characters of multiparticulate microcapsule (formula, C)

The SEM images of the microcapsule loaded with inulin PLGA nanoparticles and *Bifidobacterium* mixed cultures indicated the capsule was almost spherical in shape. The outer surface was smooth with diameter around 700 μm as shown in Fig. 1.

3.3. Evaluating the chemical properties of the yoghurt

The data shown in Table (1) indicated that there were no significant differences between control and each formula in T.S contents, moisture, fat, proteins. The values of T.S content in all formula ranged between 13.49 and 13.55. Also, the values of fat and protein ranged between 4.00 and 4.10, 3.44. and 3.60, respectively. But found the value of pH in the formula (C) significantly decreased more than the others.

3.4. Assessment the viability of starter cultures in yoghurt during storage period

The changes in the viable counts of yoghurt bacteria (*St. thermophilus* and *Lb. bulgaricus*) during storage are presented in Fig. 2. Data explained that the counts of both starter cultures for all the yoghurt under study was ranged between 7.81 & 7.07 log cfu/g and 7.71 & 7.05 log cfu/g for *St. thermophilus* and Lb. *bulgaricus*, respectively at the beginning of the storage period.

During the first three weeks, there was slight increase in both of the culture counts for all the formulae. However, the count slightly decreased at week 4 for the control yoghurt and yoghurt containing formula (A).

3.5. Assessment the viability of Bifidobacterium mixed cultures in yoghurt during storage period

The viable counts of free and encapsulated *Bifidobacterium* mixed cultures in yoghurt samples during storage are presented in Table (2). It was clear that viable counts of encapsulated mixed cultures were significantly enhanced during storage period for all the formulae compared to the free one (control) which suffered from count loss with magnitude around 0.7 log cycle at the end of storage period. However, there was variation in the magnitude of viable count enhancement for the encapsulated *Bifidobacterium* mixed cultures depending on the type of the formula prepared. The viable count increased more than 0.97 and 1.09 log cycle for the yoghurt containing formula (B) and (C), respectively at the end of storage period compared to the yoghurt containing formula (A) which the viable count increased till week 3 with 0.29 log cycle then the viable count decreased at the end of storage period.

3.6. Inulin release pattern from formulae (B) and (C) during storage period

The inulin release in the yoghurt from the formulae containing inulin (B) and (C) was examined and it was found that no inulin was released at all during storage period.

4. Discussion

In the current study, we continue our work done by Fayed et al. (2018) as we investigating the potential industrial application of the synbiotic multiparticulate microcapsule, particularly in yoghurt production. In our former work, we were successfully able to produce novel synbiotic multiparticulate microcapsule that manage to enhance the survivability of the probiotic in the simulated gastric juice and to extend the inulin release in the simulated intestine solution. In the current study, we investigate the ability of this multiparticulate microcapsule to maintain or even enhance the viability of the probiotic that usually added to the yoghurt along the shelf life. This will ensure that the consumer will completely benefit from the positive effect of the probiotic in the yoghurt at any time during the shelf life. The multiparticulate microcapsule under study consists of microcapsule coated with double layer (Alginate and gum agar) and nanoparticles located in the core of the capsule. The probiotic bacteria are entrapped in the microcapsule while the prebiotic inulin is entrapped within the nanoparticles. The double layer of alginate and gum agar provided protection to the probiotic bacteria from the simulated gastric pH, while the PLGA used to fabricate the nanoparticles allowed sustained release pattern for the inulin in the alkaline pH of the intestine (Fayed et al., 2018). To examine the possibility to use the mentioned capsule to achieve the former objective, we have fabricated three different types of microcapsules to carefully investigate the role of each part (layer) of the multiparticule microcapsule in maintaining/enhancing the probiotic viability in the yoghurt. Formula (A) formed from the double layer microcapsule

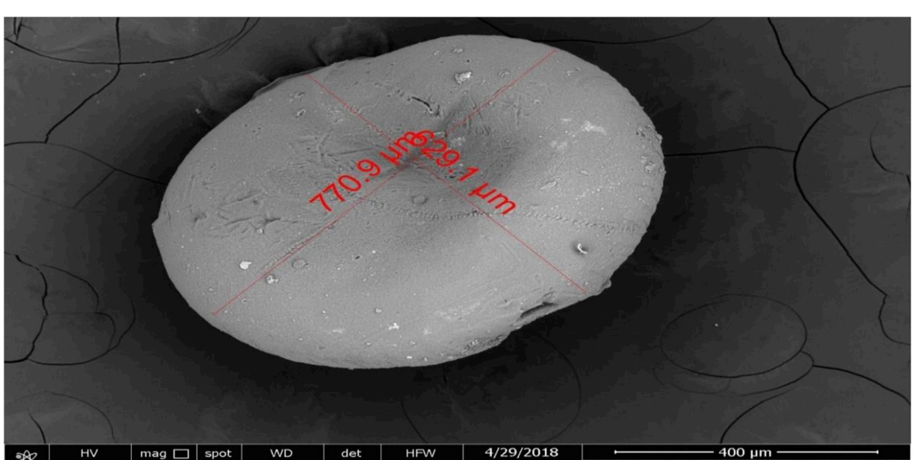

Fig. 1. Scanning electron micrograph of capsule loaded with Bifidobacterium mixed cultures and inulin PLGA nano-particles.

Table 1
The chemical composition of yoghurt containing the added formulae.

Formula	T.S%	Moisture	Fat%	Protein%	pH
Control	13.55 ± 0.032	86.45 ± 0.025	4.00 ± 0.070	3.60 ± 0.055	4.74 ± 0.024
(A)	13.50 ± 0.033	86.50 ± 0.025	4.22 ± 0.058	3.41 ± 0.049	4.55 ± 0.050
(B)	13.51 ± 0.0305	86.49 ± 0.024	4.00 ± 0.070	3.45 ± 0.046	4.50 ± 0.028
(C)	13.49 ± 0.0346	86.52 ± 0.053	4.10 ± 0.051	3.44 ± 0.050	4.42 ± 0.046

Means ± SD.

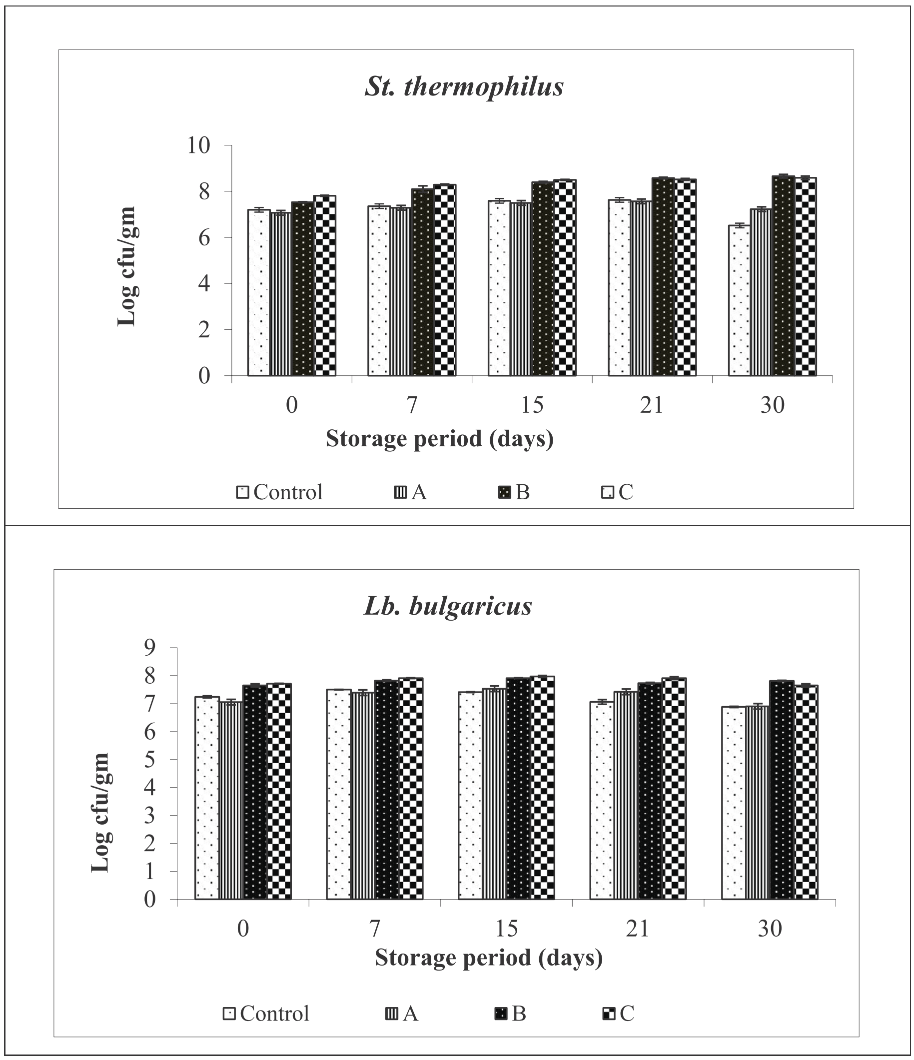

Fig. 2. Assessment the viability of starter cultures in yoghurt during storage period.

Table 2
Bifidobacterium counts (Log CFU/gm) in yoghurt samples during storage period.

Formula	Storage period (days)				
	0	7	15	21	30
Control	7.43 ± 0.049	7.53 ± 0.035	7.45 ± 0.049	7.05 ± 0.007	6.73 ± 0.035
(A)	7.81 ± 0.014	8.05 ± 0.070	8.28 ± 0.028	8.10 ± 0.141	7.64 ± 0.056
(B)	7.91 ± 0.028	8.39 ± 0.084	8.71 ± 0.127	8.95 ± 0.070	8.88 ± 0.028
(C)	7.83 ± 0.035	8.45 ± 0.070	8.72 ± 0.035	8.84 ± 0.021	8.92 ± 0.035

Means ± SD.

entrapped with the probiotic bacteria, was used to evaluate the role of the double layer to enhance the viability of the probiotic in yoghurt. To explore the role of the inulin in enhancing the viability of the probiotic bacteria, formula (B) was fabricated by entrapping the inulin together with the probiotic bacteria. Finally, formula (C) represents the final form of the synbiotic multiparticule microcapsule under the study which have major advantage over the formula (B) regarding the physical separation between the probiotic bacteria and prebiotic inulin (Cook et al., 2014)

(Fayed et al., 2018).

Both of the nanoparticles and the final multiparticule microcapsule were characterized regarding to their size, beside the assessment of the entrapment efficiency of the inulin in the nanoparticles. The size and the entrapment efficiency of nanoparticles were almost identical to the one prepared in Fayed et al. (2018) which guarantee all the advantages of the nanoparticles regarding to the bioactivity and inulin release enhancement in the intestine. For the multiparticule microcapsule under the study, the size as expected was in micro-range around 700 μm. This size range is always observed when the extrusion technique is applied to produce microcapsules (Burgain et al., 2011). Microcapsule with size larger than 300 μm can provide protection for probiotics better than the smaller one, but they are poorly distributed which require more attention during their addition to the yoghurt (Zhao et al., 2008) (Heidebach et al., 2012).

Following yoghurt preparation and adding each formula as described, the chemical properties of yoghurt were evaluated to be ensure that it fellows the standard parameters and the added formula did not affect its chemical characters. Data revealed that there were no differences between control and our preparation regarding to all the chemical properties.

During storage of the yoghurt matrix the counts of starter cultures (*Lb. bulgaricus and St. thermophilus*) usually decreases during storage period due to the production of acids resulted from the metabolic activity of cultures during storage (Mehanna., 2003a) which can be seen in our study with both of the control and the yoghurt containing formula (A). This is also identical to Gustaw et al. (2011) and Yilmaz-Ersan and Kurdal (2014), whom reported that counts of starter cultures has decreased at 25th days of storage.

However, surprisingly the starter culture has improved during the storage period for the yoghurt containing formula (B) and (C). This can be attributed to the presence of inulin in the yoghurt which can encourage the growth of starter cultures. The presence of the inulin may be resulting from the adsorption of inulin on the surface of capsules during its preparation which can explain the improvement in the starter culture for the yoghurt containing formula (B) (EL-Sayed et al., 2015). Another explanation can be referred to the lactic acid produced during fermentation from the starter culture which when combines with calcium that found in alginate capsules can create weak point in the capsules that leads to release the inulin in the yoghurt matrix (El-Shefi et al., 2018a) and (Kaplan &Hutkins, 2000). This may illustrate the improvement in the starter culture for the yoghurt containing formula (C) as during the preparation of inulin PLGA nanoparticles, inulin usually adsorbed on the surface of the nanoparticles which then can be released inside the microsphere and get out through the weak point in the surface of the microsphere (Fayed et al., 2012).

Finally, monitoring the survivability of the probiotics either the free one in the control or the encapsulated one in the other samples revealed the capability of the synbiotic multiparticulate microcapsule not only to maintain the survivability of the probiotic but also to enhance it during the shelf life. As predicted, the yoghurt containing free probiotic (control) suffered from probiotic count loss upon storage for three weeks, unlike the other samples containing encapsulated probiotics. However, the probiotic count varied between the yoghurt containing formula (A) and the yoghurt containing formula (B) or (C). This can reveal the following conclusions. First, the presence of the double coating microsphere can maintain the viability of the probiotic in the yoghurt compared to the free probiotic which can be indicated from the survivability data of the control yoghurt compared to the yoghurt containing formula (A). This agrees with Jayalalitha et al.(2011) and El-Shefi et al. (2018b) who reported that encapsulated probiotic count in yoghurt was significantly greater than the free probiotic during storage period. These can be referred to the protection level provided to the probiotic by the coated capsule from the lactic acid produced during the fermentation.

The second conclusion can be observed by comparing the survivability data of the yoghurt containing formula (B) or (C) with the survivability data of the yoghurt containing formula (A). The slightly higher probiotic survivability in the yoghurt containing formula (B) or (C) compared to formula (A), indicates the possible role of the inulin presented inside the capsule with the probiotic bacteria in formula (B) or adsorbed on the surface on the nanoparticles in formula (C). The inulin is considered a prebiotic by many authors as it can encourage the growth of the probiotic during storage periods in yoghurt (Nazzaro et al., 2009; El- Batawy et al., 2014; Mehanna and Hosny, 2003; Mehanna et al., 2003b; Gibson et al., 1995; Ibrahim et al., 2004).

5. Conclusion

The present study introduced the applicability of the synbiotic multiparticulate microcapsule that contain probiotic bacteria and inulin loaded PLGA nanoparticles to enhance the survivability of the encapsulated probiotic in yoghurt for four weeks compared to the probiotic bacteria freely added to the yoghurt. In-addition, this study clarified the significance of applying the double coating layer covering the multiparticulate microcapsule and the inulin encapsulated in the PLGA-nanoparticles in enhancing the survivability of the encapsulated probiotic. Future work should include studying the effect of the synbiotic multiparticulate microcapsule produced in this study on stimulating the gut microbiota of the consumers.

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