



Evaluation of *Lactobacillus plantarum* encapsulated with *Eleutherine americana* oligosaccharide extract as food additive in yoghurt

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Abstract

This study aimed to determine the survival and antibacterial activity of *Lactobacillus plantarum* TISTR1465 encapsulated with *Eleutherine americana* oligosaccharide extract. Capsules were stored at 4 °C for 0, 2, and 4 weeks. The encapsulated cells were evaluated for their survival after sequential exposure to simulated gastric and intestinal juices, then evaluated in terms of their antibacterial activity. Survival of the encapsulated cells was higher than that of free cells at weeks 2 and 4. Highest levels of viable cells were observed with encapsulation in *E. americana* oligosaccharide extract. No surviving free cells were found in week 4. Yoghurt prepared with encapsulated cells showed less acidification than with free cells. Antibacterial activity of *L. plantarum* TISTR1465 before pH neutralisation against *Clostridium perfringens* ATCC13124, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Salmonella* Typhimurium ATCC13311 was higher than after pH neutralisation. Encapsulation by extrusion enhanced antibacterial activity of the cells against enteropathogenic bacteria. The antibacterial activity of encapsulated cells against Gram-positive bacteria was higher than that against Gram-negative bacteria. Results indicates that *L. plantarum* TISTR1465 encapsulated with *E. americana* oligosaccharide extract showed potential for application as a functional food additive.

Keywords Antibacterial activity · Encapsulation · *Lactobacillus plantarum* · Oligosaccharide extract · Survival

Introduction

Functional foods provide health benefits beyond traditional nutrients. Probiotics and/or prebiotics are natural additions that can enhance the functionality of food products. The market share of probiotics has been 30% of the worldwide functional market, which is estimated to be resulting in a \$50 billion market [1]. Probiotics are live microorganisms which, when ingested in adequate numbers, confer beneficial effects to the host. Many

lactobacilli fall into the category of Generally Regarded as Safe (GRAS) bacteria, and are used in probiotic foods. Moreover, lactobacilli produce substances that inhibit pathogens [2]. It is known that there are various factors that affect the viable number of probiotics, including gastrointestinal conditions, the food processing, and the storage temperature. International Dairy Federation (IDF) has suggested that the survival of probiotics should be at least 10⁷ CFU/g (of the food product) at the time of consumption [3]. There is an increasing demand for probiotic-containing dairy products, and there are many studies of incorporating probiotics in yoghurt [4, 5] or in cheese [6, 7]. Yoghurts are the most popular dairy foods for the delivery of probiotics, due to their excellent nutritional values. However, the production of lactic acid by *Lactobacillus delbrueckii* subsp. *bulgaricus* or by *Streptococcus thermophilus* is the major factor reducing survival of probiotics in yoghurt [3].

Encapsulation by extrusion has been developed to protect and maintain live probiotics in food products and during gastrointestinal transit. Sodium alginate is the most commonly used encapsulating agent for probiotics. However, the effectiveness of sodium alginate is limited due to its porous nature that allows diffusion of other substrates into the beads. To overcome these

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problems, a combination of sodium alginate with prebiotic oligosaccharides may better protect probiotics in adverse conditions due to synbiosis [8]. Prebiotics refer to non-digestible oligosaccharides that improve the host health by increasing the number of bacteria in the colon [9]. Many commercial prebiotic oligosaccharides are synthesised by chemical and/or enzyme reactions. The potential use of oligosaccharides from *Eleutherine americana* bulb instead of commercial oligosaccharides is of high interest, since most consumers tend to prefer new prebiotics from natural sources [10]. *E. americana* bulb extracts have been used in foods [11, 12]. Moreover, an *E. americana* oligosaccharide extract has been used as a prebiotic that stimulates growth of the intestinal microbiota [10]. *E. americana* oligosaccharide extract has demonstrated resistance to low pH and partial tolerance to human α -amylase [13]. It has been previously found that encapsulation of *Bifidobacterium longum* with *E. americana* oligosaccharide extract increased its survival in simulated gastrointestinal conditions [13], in fresh milk tofu, and in pineapple juice [14]. It has been documented that encapsulation of probiotic lactobacilli with chitosan [15], pectin and whey protein [16], or inulin [17] increases survival of those probiotics in gastrointestinal conditions and in yoghurt. Adding probiotic lactobacilli encapsulated with calcium alginate or chitosan increased antibacterial activity significantly more than by adding free cells [18, 19]. However, there has been no prior study on both survival and antibacterial activity of *L. plantarum* TISTR1465 encapsulated with a prebiotic oligosaccharide extract.

Therefore, the objectives of this study were to evaluate survival and antibacterial activity of *L. plantarum* TISTR1465 encapsulated with *E. americana* oligosaccharide extract.

Materials and methods

Probiotic bacteria

Lactobacillus plantarum TISTR1465 (Thailand Institute of Scientific and Technological Research) was cultivated in 50 mL of MRS broth (Merck, Darmstadt, Germany) and incubated at 37 °C for 24 h. The test samples were centrifuged at 10,000×g for 10 min, at 4 °C, and the pellets were washed with 0.85% (w/v) normal saline solution. The cell suspensions were adjusted to a final concentration of approximately 1×10^{10} CFU/mL in 10 mL of 0.1% (w/v) peptone. These samples were divided into two groups: one was used for encapsulation and the other as free cells (non-encapsulated *L. plantarum* TISTR1465).

Eleutherine americana oligosaccharide extract

Bulbs of *E. americana* were collected from Songkhla, Thailand. The extract was obtained by hot water extraction. Briefly, the bulbs were extracted with distilled water in ratio of 1:10 (w/v) at 80 °C for 1 h, and the filtrates were dried by

freeze dryer (Flexi Dry, Germany). *E. americana* oligosaccharide extract was obtained using *Saccharomyces cerevisiae* BCC 12652 and 80% ethanol at 4 °C for 12 h [10]. A commercial fructo-oligosaccharide preparation (Sigma-Aldrich, Steinheim, Germany) was used as a reference.

Encapsulation of *Lactobacillus plantarum* TISTR1465 with *Eleutherine americana* oligosaccharide extract

Encapsulation procedure

The encapsulation was done by extrusion as described previously by Krasaekoopt et al. [20] Briefly, two millilitres of cell suspension (1×10^{10} CFU/mL) was mixed with 16 mL of sterile 2% (w/v) sodium alginate solution (Fluka, Switzerland). Two millilitres of *E. americana* oligosaccharide extract (or of the commercial fructo-oligosaccharides) was added to the above mixtures to make the final concentration 1% (w/v). The final concentration of cells in this mixture was about 1×10^9 CFU/mL. This mixture was injected through a syringe needle size 23 G (Nipro, Japan) into sterilised 0.1 M CaCl₂ solution (Difco, Dickinson, USA) and allowed to harden for 30 min. The capsules formed were washed twice with 0.85% (w/v) normal saline solution and stored in 0.1% (w/v) peptone solution (pH 6) at 4 °C until use. The free cells were used as a control culture. The capsule diameter was measured using a vernier calliper.

Encapsulation yield (%) and cell release (%)

One gram of capsules was transferred to 9 mL of 0.1 M phosphate buffer (pH 7.4) and homogenised in a stomacher for 5 min. The samples were then centrifuged at 10,000×g for 10 min, at 4 °C, and the supernatant was plated on MRS agar. Viable cell counts after incubation are shown as log colony-forming units per gram (log CFU/g). The encapsulation yield, which is a combined measurement of the efficacy of entrapment and the survival of viable cells during encapsulation, was calculated as proposed by Chavarri et al. [21]

$$\text{Encapsulation yield (\%)} = N/\text{No} \times 100 \quad (1)$$

where N is the number of viable entrapped cells released from the capsules, and No is the number of free cells added to the biopolymer mix during formation of the capsules.

One gram of capsules was added to 9 mL of 0.1 M phosphate buffer (pH 7.4) followed by incubation at 37 °C for 1 h. The number of viable cells in the suspension was then determined by the plate count as described above [22].

$$\text{Cell release (\%)} = N/\text{No} \times 100 \quad (2)$$

where N is the number of viable cells in the suspension released from the capsules, and No is the number of free cells added to the biopolymer mix during the formation of the capsules.

Textural properties

The capsules were subjected to a texture profile analyser (TPA), Stable Micro Systems model TA-XT (UK). Thirty grams of capsules was placed on a fixed bottom glass plate under the cylindrical steel probe. Automatic detection of contact by the probe with the capsules used threshold force of 0.005 N. The textural properties of hardness, cohesiveness, and springiness were obtained using the equipment's Texture Expert Software [23].

Survival of encapsulated *Lactobacillus plantarum* TISTR1465 after sequential incubation in simulated human gastric and intestinal juices

The encapsulated *L. plantarum* TISTR1465 and the free cells were stored in a peptone solution (pH 6) at 4 °C for 0, 2, and 4 weeks, and their survival was determined after exposure to simulated gastric and intestinal juices as described by Sandoval-Castilla et al. [24]. The gastric juice was mimicked by a HCl buffer of pH 2 containing the following: NaCl, 8 g; KCl, 0.2 g; Na₂HPO₄·2H₂O, 8.25 g; NaH₂PO₄, 14.35 g; CaCl₂·2H₂O, 0.1 g; MgCl₂·6H₂O, 0.18 g; pepsin (Sigma-Aldrich), 3 g/L. The encapsulated *L. plantarum* (1 g) or the free cells (1 mL) were added to 9 mL of simulated gastric juice and incubated at 37 °C, and 150 rpm for 3 h in an orbital shaker incubator (Forma, Scientific, USA). The samples were added with 0.1 M phosphate buffer (pH 7.4), and the viable cell counts were obtained as described above. The samples after exposure to simulated gastric juice were centrifuged, washed with a 0.85% (w/v) normal saline solution, transferred in 9 mL of simulated intestinal juice (pH 7.4) containing NaCl, 6.50 g; KCl, 0.84 g; CaCl₂, 0.22 g; NaHCO₃, 1.39 g; bile salt (Difco), 3 g; and pancreatin (Sigma-Aldrich), 1 g/L, and incubated at 37 °C for 3 h in a rotary shaker. The samples were centrifuged, washed with normal saline solution, and the viable cell counts were determined as described above. Three replications of the experiment were done.

Survival of encapsulated *Lactobacillus plantarum* TISTR1465 and starter cultures in yoghurt

Preparation of yoghurt

Yoghurts were prepared following a method modified from Kailasapathy [25]. Five litres of ultra-high-temperature plain milk (UHT, Nongpho, Ratchaburi, Thailand) and skim milk powder were mixed with high-speed stirring, to make the total solids 180 g/L in yoghurt. Heating was continued at a constant 85 °C for 20 min. The sample was then cooled to 45 °C and 2% yoghurt starter culture (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) was added. Fermentation was carried out at 45 °C for 3 h until pH 4.8 was reached. After cooling to 4 °C, the mixture was divided

into four equal fractions. Then, *L. plantarum* TISTR1465 encapsulated with *E. americana* oligosaccharide extract, or with commercial fructo-oligosaccharides, free cells, or control culture (without *L. plantarum* TISTR1465) were added aseptically into the mixture. The capsules and free cells were added to concentrations of about 1 × 10⁹ CFU/g and 1 × 10⁹ CFU/mL, respectively. The ratio of capsules or free cells to yoghurt was 1:10. The yoghurt samples were distributed to sterile plastic cups and packed with sterile plastic lids.

Physicochemical parameters of encapsulated *Lactobacillus plantarum* TISTR1465 during refrigerated storage

Water holding capacity

The water holding capacity (WHC) of the yoghurt was measured by centrifugation (K241R Medium Prime Centrifuge, Chichester, UK) of at 4500 rpm for 30 min at 10 °C for 10 g yoghurt sample. The following formula for WHC [26] uses as inputs w1 = weight of co-encapsulating agents after centrifugation, and w2 = yoghurt weight.

$$\text{WHC}(\%) = \frac{1-w1}{w2} \times 100 \quad (3)$$

The water holding capacity was measured during the refrigerated storage at 0, 2, and 4 weeks.

Lactic acid determination

Production of lactic acid in yoghurt containing encapsulated *L. plantarum* TISTR1465, free cells, or control culture (without *L. plantarum* TISTR1465 and co-encapsulating agents) during the refrigerated storage for 0, 2, and 4 weeks was determined by the method of Holdeman and Moore [27] with slight modifications. The samples were centrifuged at 10,000×g for 15 min, at 4 °C, and the supernatant was subjected to high-performance liquid chromatography (Agilent 1100, Germany). The concentration of lactic acid was quantified by comparing the peak area to the standard. All experiments were performed in triplicates.

pH measurement

pH of the yoghurts was determined with a pH meter (Science analyst, Thailand) during storage at 4 °C for 0, 2, and 4 weeks.

Survival of encapsulated *Lactobacillus plantarum* TISTR1465 during refrigerated storage

Survival of encapsulated *L. plantarum* TISTR1465 and starter cultures while in refrigerated storage was studied by the

method of Brinques and Ayub with slight modifications [28]. Briefly, encapsulated *L. plantarum* TISTR1465 (10 g) or free cells (10 mL) were added to 90 mL of 0.1% (w/v) peptone solution with pH 6. The samples were stored in a refrigerator at 4 °C for 0, 2, and 4 weeks. Cell counts were determined after depolymerisation in 0.1 M phosphate buffer as described above. All the experiments were performed in triplicates.

Antibacterial activity of encapsulated *Lactobacillus plantarum* TISTR1465

Agar well diffusion method

Encapsulated *L. plantarum* TISTR1465 and the free cells were stored in a peptone solution at 4 °C for 0 and 4 weeks. Their antibacterial activity against enteropathogenic bacteria was determined using an agar well diffusion method. The experiments mainly followed from previous reports [19, 29]. Aliquots (60 µL) of encapsulated *L. plantarum* TISTR1465 (1×10^9 CFU/g), free cells (1×10^9 CFU/mL), or control culture (without *L. plantarum* TISTR1465) were placed in wells 6 mm in diameters punched in cooled MRS agar plates, and were tested against 20 µL of *Clostridium perfringens* ATCC13124 (1×10^7 CFU/mL), *Staphylococcus aureus* ATCC25923 (1×10^7 CFU/mL), *Escherichia coli* ATCC25922 (1×10^7 CFU/mL), and *Salmonella* Typhimurium ATCC13311 (1×10^7 CFU/mL). The plates were incubated at 37 °C both under aerobic and anaerobic conditions for 24 h and 48 h, respectively. The sizes of the inhibition zones were measured. The same procedure was carried out using aliquot samples after pH neutralisation. All experiments were performed in triplicates.

Broth co-culture assay

Encapsulated *L. plantarum* TISTR1465 and the free cells were kept at 4 °C for 0 and 4 weeks. Their antibacterial activities against enteropathogenic bacteria were determined by broth co-culture assay. The experiments were mainly followed from previous reports [30]. The brain–heart infusion broth (BHIB) and pre-reduced BHIB supplemented with 0.5% yeast extract were used to culture facultatively anaerobic bacteria and anaerobic bacteria, respectively. One millilitre of enteropathogenic bacteria (1×10^7 CFU/mL), namely *C. perfringens* ATCC13124, *S. aureus* ATCC25923, *E. coli* ATCC25922, and *S. Typhimurium* ATCC13311 was co-cultured with aliquots (1 mL) of encapsulated *L. plantarum* TISTR1465 (1×10^9 CFU/g), or free cells (1×10^9 CFU/mL). The samples were incubated at 37 °C in both aerobic and anaerobic conditions for 24 h and 48 h, respectively. Controls without *L. plantarum* TISTR1465 were set up under the same conditions. The same procedure was carried out using the aliquot samples after pH neutralisation. The experiments were

performed in triplicates and mean bacterial numbers in terms of colony forming units (CFU) were reported. The inhibitory effects of *L. plantarum* TISTR1465 on enteropathogenic bacteria were measured and compared with the control culture.

Statistical analysis

The data sets are reported as mean \pm standard deviation (S.D.). Differences between treatments were examined for statistical significance by analysis for variance (ANOVA). The criterion for statistical significance was $p < 0.05$.

Results

Properties of *L. plantarum* TISTR1465 encapsulated with *Eleutherine americana* oligosaccharide extract

Encapsulation by an extrusion was applied to a combination of sodium alginate with *E. americana* oligosaccharide extract or with commercial fructo-oligosaccharides. The properties of the capsules are shown in Table 1. The capsules appeared white and round in shape. The type of co-encapsulating agents had no influence on the size of the capsules, encapsulation yield, or cell release. The diameters of the capsules made with alginate-*E. americana* oligosaccharide extract and with alginate-fructo-oligosaccharides were 1.59 and 1.47 mm, respectively. The encapsulation yields of capsules made with

Table 1 Diameter, encapsulation yield (%), cell release (%), and textural properties of *Lactobacillus plantarum* TISTR1465 encapsulated with *Eleutherine americana* oligosaccharide extract

Properties of microcapsules	Alginate-oligosaccharide extract	Alginate-commercial fructo-oligosaccharides
Diameter (mm)	1.59 \pm 0.11 ^a	1.47 \pm 0.16 ^a
Encapsulation yield (%) ¹	93.52 \pm 0.08 ^a	94.21 \pm 0.14 ^a
Cell release (%) ²	93.87 \pm 0.06 ^a	94.15 \pm 0.21 ^a
Textural properties		
Hardness (N)	2.10 \pm 0.11 ^a	0.41 \pm 0.08 ^b
Cohesiveness (ratio)	0.87 \pm 0.16 ^a	0.89 \pm 0.07 ^a
Springiness (ratio)	0.83 \pm 0.11 ^a	0.85 \pm 0.12 ^a

Values are means \pm standard deviation from triplicate determinations

Different superscripts as lowercase letters (a, b) in the same row show significant differences ($p < 0.05$)

¹ Encapsulation yield = $N/\text{No} \times 100$, where N is the number of viable entrapped cells released from the capsules, and No is the number of free cells added to the biopolymer mix during formation of the capsules

² Cell release (%) = $N/\text{No} \times 100$, where N is the number of viable cells in the suspension released from the capsules, and No is the number of free cells added to the biopolymer mix during the formation of the capsules

alginate-*E. americana* oligosaccharide extract and alginate-fructo-oligosaccharides were 93.52% and 94.21%, respectively. About 93.87–94.15% of the cells in the capsules was released within 1 h.

The textural properties of the capsules were affected by the biopolymer composition. The results show that the capsules made with alginate-*E. americana* oligosaccharide extract indicated significantly higher hardness than those made by alginate-fructo-oligosaccharides ($p < 0.05$), but these had comparable cohesiveness and springiness. The hardness of the capsules made with alginate-*E. americana* oligosaccharide extract was 2.10 N (Table 1).

Survival of *Lactobacillus plantarum* TISTR1465 encapsulated with *Eleutherine americana* oligosaccharide extract after sequential exposure to simulated human gastric and intestinal juices

Survival of *L. plantarum* TISTR1465 encapsulated with *E. americana* oligosaccharide extract, or with commercial fructo-oligosaccharides, and of free cells in peptone solution (pH 6), after sequential exposure to simulated gastric and intestinal juices, is shown in Table 2. The capsules made by extrusion were stored at 4 °C for 0, 2, and 4 weeks. Initially, the viable count of *L. plantarum* TISTR1465 was in the range 9.35–9.51 log CFU/g. The survival of encapsulated cells before and after sequential incubation in the simulated gastric and intestinal juices was higher than that of the free cells at weeks 0, 2, and 4 ($p < 0.05$).

The number of viable cells decreased with refrigerated storage for 4 weeks. The highest count of viable cells at week 4 was observed for encapsulation with *E. americana* oligosaccharide extract. The survival of *L. plantarum* TISTR1465

encapsulated with *E. americana* oligosaccharide extract declined in the peptone solution from 9.51 to 8.26 log CFU/g, respectively, when exposed to the simulated juices. However, none of the free cells survived at week 4 when exposed to the simulated juices.

Physicochemical parameters of *Lactobacillus plantarum* TISTR1465 encapsulated with *Eleutherine americana* oligosaccharide extract in yoghurt

The physicochemical parameters in yoghurt (water holding capacity, production of lactic acid, and pH) of *L. plantarum* TISTR1465 encapsulated with *E. americana* oligosaccharide extract, with commercial fructo-oligosaccharides, and of free cells are presented in Fig. 1. The capsules were kept in yoghurt at 4 °C for 0, 2, and 4 weeks. The water holding capacity (WHC) of yoghurt was in the range of 62–64% during 4 weeks of refrigerated storage. The initial concentration of lactic acid in yoghurt ranged from 1.43 to 1.50 g/L. The yoghurt containing free cells had significantly higher acidity than the samples with encapsulated *L. plantarum* TISTR1465. The amount of lactic acid in the samples containing encapsulated *L. plantarum* TISTR1465 remained constant at 4 °C for 0, 2, and 4 weeks. The highest production of lactic acid was observed in yoghurt containing free cells, increasing over the range 1.50–2.53 g/L. The concentration of lactic acid in yoghurt containing encapsulated *L. plantarum* TISTR1465 ranged from 1.43 to 1.55 g/L. The pH of yoghurt containing encapsulated *L. plantarum* TISTR1465 remained constant due to the high buffering capacity of the samples. However, pH of the samples with free cells decreased during 4 weeks of refrigerated storage.

Table 2 Survival of *Lactobacillus plantarum* TISTR1465 encapsulated with *Eleutherine americana* oligosaccharide extract, after sequential exposure to simulated human gastric and intestinal juices

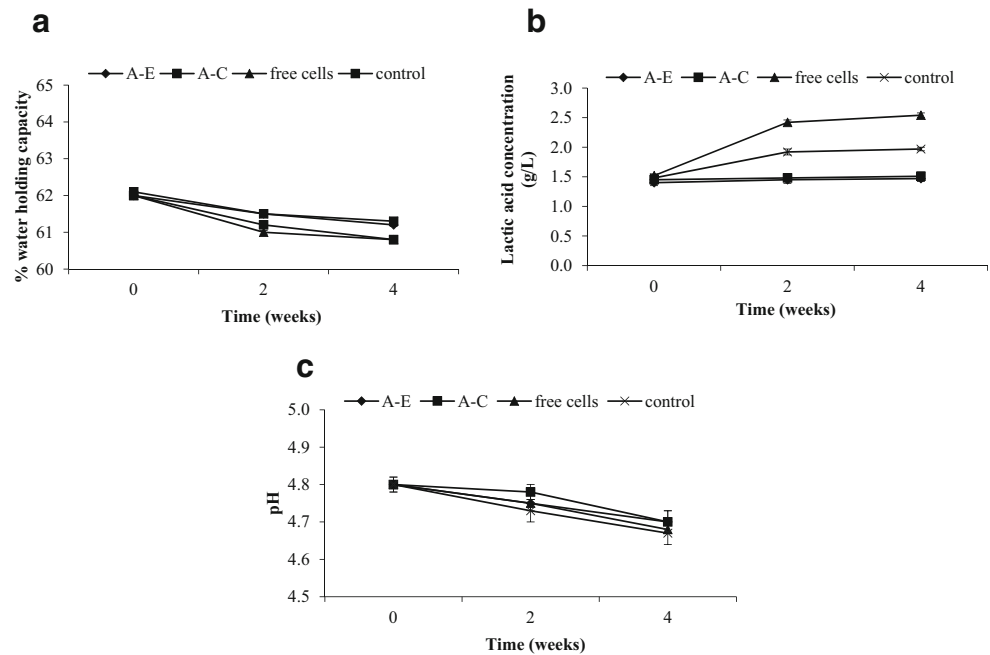
Encapsulation	Time (weeks)	Plate count (log CFU/g)		
		Pre-gastric juice	Post-gastric juice	Post-intestinal juice
Alginate- <i>E. americana</i> oligosaccharide extract	0	9.51 ± 0.11 ^{aA}	9.50 ± 0.01 ^{aA}	9.57 ± 0.11 ^{aA}
Alginate-commercial fructo-oligosaccharides		9.35 ± 0.18 ^{aA}	9.48 ± 0.05 ^{aA}	9.65 ± 0.13 ^{aA}
Free cells		9.37 ± 0.21 ^{aA}	8.54 ± 0.17 ^{bB}	8.58 ± 0.16 ^{bB}
Alginate- <i>E. americana</i> oligosaccharide extract	2	9.61 ± 0.10 ^{aA}	8.53 ± 0.21 ^{bB}	8.17 ± 0.20 ^{bB}
Alginate-commercial fructo-oligosaccharides		9.42 ± 0.16 ^{aA}	8.48 ± 0.15 ^{bB}	8.27 ± 0.07 ^{bB}
Free cells		4.81 ± 0.22 ^{cA}	3.45 ± 0.13 ^{dB}	3.51 ± 0.06 ^{dB}
Alginate- <i>E. americana</i> oligosaccharide extract	4	9.46 ± 0.10 ^{aA}	8.34 ± 0.08 ^{bB}	8.26 ± 0.25 ^{bB}
Alginate-commercial fructo-oligosaccharides		8.32 ± 0.12 ^{bA}	7.38 ± 0.16 ^{cB}	6.37 ± 0.22 ^{cC}
Free cells		0.00 ± 0.00 ^{dA}	0.00 ± 0.00 ^{eA}	0.00 ± 0.00 ^{eA}

Values are means ± standard deviation from triplicate determinations

Different superscripts as uppercase letters (A–C) in the same row show significant differences among gastrointestinal conditions ($p < 0.05$)

Different superscripts as lowercase letters (a–e) in the same column show significant time differences ($p < 0.05$)

Fig. 1 Physicochemical parameters of *Lactobacillus plantarum* TISTR1465 encapsulated with *Eleutherine americana* oligosaccharide extract in yoghurt during refrigerated storage. **(a)** Water holding capacity, **(b)** production of lactic acid, and **(c)** pH. *L. plantarum* TISTR1465 encapsulated with *E. americana* oligosaccharide extract (A-E), *L. plantarum* TISTR1465 encapsulated with commercial fructo-oligosaccharides (A-C), free cells, and control (without *L. plantarum* TISTR1465). Bars represent standard deviations of the means according to repeated experiments



Survival of *Lactobacillus plantarum* TISTR1465 encapsulated with *Eleutherine americana* oligosaccharide extract and starter cultures in yoghurt

The effects of refrigerated storage on the survival of *L. plantarum* TISTR1465 encapsulated with *E. americana* oligosaccharide extract, or with commercial fructo-oligosaccharides, the free cells, and the starter cultures are shown in Table 3. The number of starter cultures decreased during refrigerated storage for 4 weeks ($p < 0.05$). High cell viability in yoghurt (pH 4.8) in the range 9.51–9.70 log CFU/g was achieved with the encapsulation by extrusion during storage at 4 °C, for 0, 2, and 4 weeks. The viable counts of encapsulated cells were higher than those of the free cells at weeks 0,

2, and 4 ($p < 0.05$). The highest levels of viable cells were observed for encapsulation with *E. americana* oligosaccharide extract, with a decrease from 9.70 to 8.45 log CFU/g, at week 4. However, no free cells survived at week 4.

Antibacterial activity of encapsulated *Lactobacillus plantarum* TISTR1465

Agar well diffusion method

Aliquots of *L. plantarum* TISTR1465 encapsulated with *E. americana* oligosaccharide extract, or with commercial fructo-oligosaccharides, and free cells were tested for antibacterial activity by using an agar well diffusion method (Fig. 2). The capsules were kept in a peptone solution at 4 °C for 0 and

Table 3 Survival of *Lactobacillus plantarum* TISTR1465 encapsulated with *Eleutherine americana* oligosaccharide extract and starter cultures in yoghurt during refrigerated storage

Time (weeks)	Plate count (log CFU/g)				
	<i>Lactobacillus plantarum</i> TISTR1465			Starter culture	
	A-E	A-C	Free cells	<i>Lactobacillus delbrueckii</i>	<i>Streptococcus thermophilus</i>
0	9.70 ± 0.10 ^{aA}	9.51 ± 0.02 ^{aA}	9.62 ± 0.10 ^{aA}	9.67 ± 0.08 ^a	9.62 ± 0.10 ^a
2	8.51 ± 0.14 ^{bA}	8.27 ± 0.03 ^{bA}	3.65 ± 0.06 ^{bB}	8.52 ± 0.09 ^b	9.51 ± 0.04 ^a
4	8.45 ± 0.12 ^{bA}	7.48 ± 0.05 ^{cB}	0.00 ± 0.05 ^{cC}	7.35 ± 0.11 ^c	8.32 ± 0.07 ^b

Values are means ± standard deviation from triplicate determinations

A-E, *E. americana* oligosaccharide extract encapsulated with *L. plantarum* TISTR1465; A-C, commercial fructo-oligosaccharides encapsulated with *L. plantarum* TISTR1465

Different superscripts as uppercase letters (A–C) in the same row show significant differences among co-encapsulating agents ($p < 0.05$)

Different superscripts as lowercase letters (a–e) in the same column show significant time differences ($p < 0.05$)

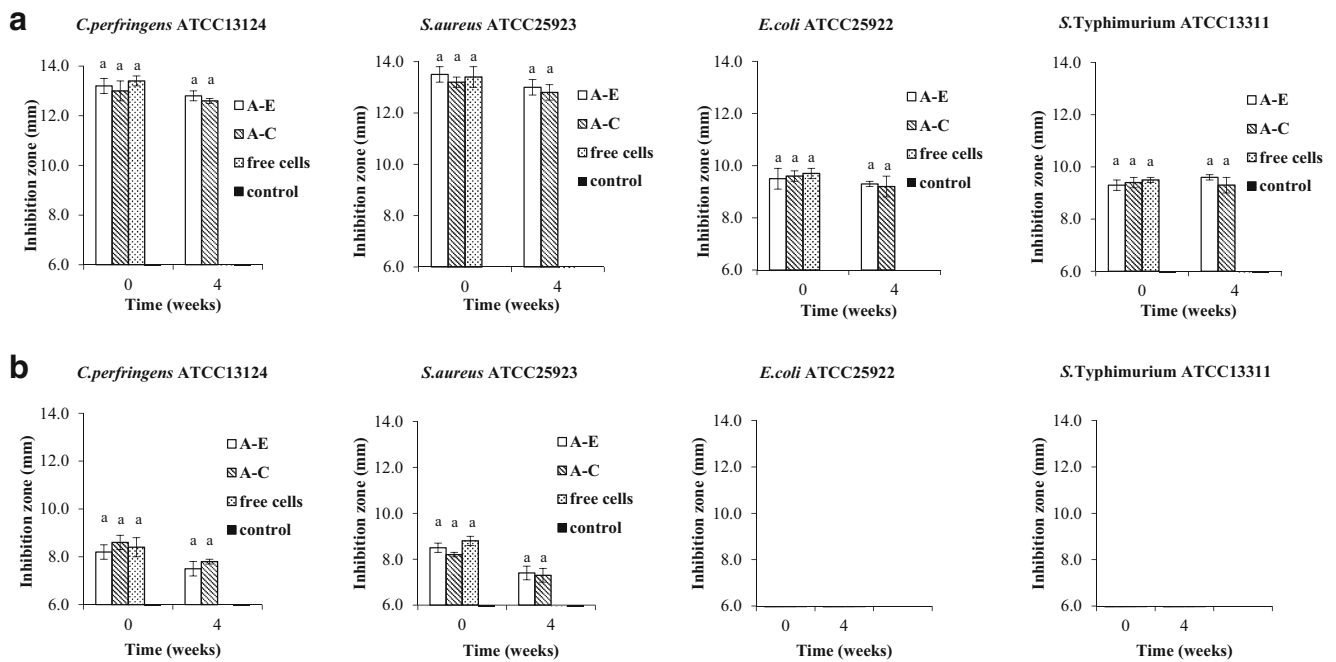


Fig. 2 Antibacterial activity of *Lactobacillus plantarum* TISTR1465 before (a) and after (b) pH neutralisation against *Clostridium perfringens* ATCC13124, *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC 25922, and *Salmonella* Typhimurium ATCC13311 by agar well diffusion method. Alginate-*E. americana* oligosaccharide extract (A-E; white bars), alginate-commercial fructo-

oligosaccharides (A-C; bar with diagonal stripes), free cells (white bar with black dots), and control (without *L. plantarum* TISTR1465; black bars). Means \pm standard deviation for triplicates are illustrated. Different lowercase letters in co-encapsulating agents indicate significant differences ($p < 0.05$)

4 weeks. The antibacterial activity of the samples before pH neutralisation against *C. perfringens* ATCC 13124 and *S. aureus* ATCC25923 was better than that against *E. coli* ATCC 25922 and *S. Typhimurium* ATCC13311. The encapsulated cells had significantly larger inhibition zones than the samples with free cells ($p < 0.05$). The encapsulated cells inhibited the growth of *C. perfringens* ATCC 13124 with an inhibition zone 12.8 mm in diameter, and inhibited also *S. aureus* ATCC25923 (13.0 mm). However, the free cells did not inhibit the growth of enteropathogenic bacteria at week 4. The inhibition zones of aliquots of the encapsulated cells after pH neutralisation were smaller than before neutralisation. The encapsulated cells inhibited the growth of *C. perfringens* ATCC 13124 with inhibition zone diameter 7.5 mm and the growth of *S. aureus* ATCC25923 (7.4 mm). However, the encapsulated and free cells did not inhibit the growth of *E. coli* ATCC 25922 or *S. Typhimurium* ATCC13311 at week 4.

Broth co-culture assay

L. plantarum TISTR1465 encapsulated with *E. americana* oligosaccharide extract, or with commercial fructo-oligosaccharides, and free cells were tested for antibacterial activity by broth co-culture assay (Fig. 3). The capsules were stored in a peptone solution at 4 °C for 0 and 4 weeks. The antibacterial activity of the samples before pH neutralisation against

C. perfringens ATCC 13124 and *S. aureus* ATCC25923 was better than that against *E. coli* ATCC25922 and *S. Typhimurium* ATCC13311. The encapsulated cells had significantly higher antibacterial activity than the samples with free cells ($p < 0.05$). The encapsulated cells inhibited the growth of *C. perfringens* ATCC 13124 which was estimated by the count reduction from 8.55 to 6.40 log CFU/mL, and *S. aureus* ATCC25923 counts decreased from 9.52 to 6.47 log CFU/mL. However, the free cells did not inhibit the growth of enteropathogenic bacteria at week 4. The antibacterial activity of the samples after pH neutralisation against *C. perfringens* ATCC 13124 and *S. aureus* ATCC25923 slightly decreased, compared with control. However, free cells did not inhibit the growth of enteropathogenic bacteria at week 4.

Discussion

Encapsulation is recognised as a powerful way to improve survival of probiotic lactic acid bacteria [8]. Here, capsules were retained resistant to disintegration in simulated gastric juice (pH 2). Phosphate ions in simulated intestinal juice (pH 7.4) degrade the capsule membrane density and this enhances permeability through the outer membrane and release of the bacteria. Addition of oligosaccharide extract positively influenced the number of cells released at an appropriate time.

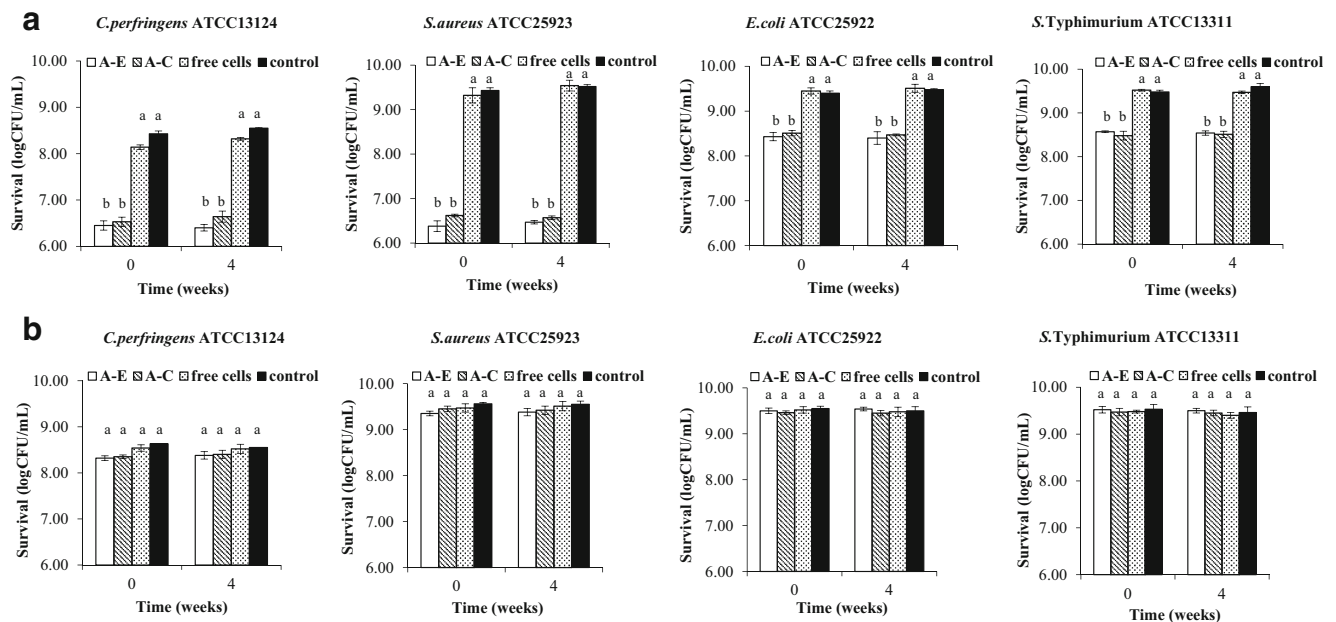


Fig. 3 Antibacterial activity of *Lactobacillus plantarum* TISTR1465 before (a) and after (b) pH neutralisation against *Clostridium perfringens* ATCC13124, *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC 25922, and *Salmonella* Typhimurium ATCC13311 by broth co-culture assay. Alginate-*E. americana* oligosaccharide extract (A-E; white bars), alginate-commercial fructo-

oligosaccharides (A-C; bar with diagonal stripes), free cells (white bar with black dots), and control (without *L. plantarum* TISTR1465; black bars). Means \pm standard deviation for triplicates are illustrated. Different lowercase letters in co-encapsulating agents indicate significant differences ($p < 0.05$)

[31] Our results indicate contrasting cross-linking reactions between alginate-*E. americana* oligosaccharide extract and alginate-fructo-oligosaccharide polymeric networks, resulting in different responses to mechanical deformation.

The compacted alginate layer can enhance the permeability of hydrogels and increase the survival of encapsulated cells. In addition, probiotics encapsulated with prebiotic oligosaccharides extract that tend to decrease the pore sizes have a favourable environment for viability of the cells [32]. The layered capsules constructed by extrusion may have afforded additional physical protection against simulated gastric and intestinal juices. The results showed that the 2% sodium alginate and 1% *E. americana* oligosaccharide extract concentrations gave the maximum protection to encapsulated cells against these adverse environmental conditions. It is expected that factors such as the concentration of encapsulating agents affect the survival of encapsulated cells [33]. The good resistance of *E. americana* oligosaccharide extract to simulated gastric and intestinal juices might be due to α - and $-\beta$ glycosidic linkages in the oligosaccharide extract [10]. The acid and enzyme tolerances of probiotic cells depend on the pH profile of H^+ -ATPase in composition of cytoplasmic cell membranes [20]. Accordingly, encapsulating probiotics with hi-maize and chitosan [34], with xanthan and chitosan [35], and with inulin and chitosan [36] have improved their survival over that of free cells after exposure to simulated digestive juices.

The type of co-encapsulating agents had no influence on the survival characteristics. The WHC indicates the ability of

the proteins and fat globules to retain water within the yoghurt structure [26]. The yoghurt starter cultures with *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* could ferment lactose into lactic acid. The yoghurts are susceptible to pH decay that causes whey separation, and this also affects probiotic survival [25]. Therefore, encapsulation could decrease such problems from acidification and could maintain encapsulated probiotics during refrigerated storage. This could be due to the slow uptake of nutrients and the slow release of metabolites across the co-encapsulating agent shells of the capsules [37]. Accordingly, *Lactobacillus acidophilus* encapsulated with pectin and whey protein caused significantly less acidity in yoghurt than free cells during refrigerated storage [5, 16].

The formation of a hydrogel around the cell pellet provided probiotic protection. The diminished pore sizes hindered the interactions of bacterial cells and yoghurt during storage. At low temperatures, enzyme activity in the free cells slows and the fluidity of cell membrane is decreased, and this interferes with the transport mechanisms [38]. Moreover, hydrogen peroxide produced by probiotic bacteria, low pH, fermentation temperature, starter culture, and availability of growth factors might be affecting the survival of probiotic bacteria in yoghurt. Partially damaged capsules were broken in the yoghurt permitting the simulated juices access into the capsules [39]. Several authors have found that different probiotic strains, encapsulation techniques, and co-encapsulating agents could improve the survival rates of encapsulated probiotic bacteria

over that of free cells [16, 24, 28]. Accordingly, encapsulated probiotics with chitosan [28] and with pectin [24] have improved survival over that of free cells in yoghurt during refrigerated storage.

The survival of *L. plantarum* TISTR1465 in the simulated gastric and intestinal juices or yoghurt positively correlated with the mechanical strength of capsules. The capsules with higher mechanical strength could better resist the osmotic effects, prevent access by digestive juices, and reduce cell damage [31]. The capsules provide a favourable environment for cell growth in which higher biomass yield can be obtained as a result of substrate digestion to heat [40]. Accordingly, previous studies have demonstrated that probiotic cell survival in simulated gastric and intestinal juices was positively correlated with the mechanical properties of capsules [24, 41].

The antibacterial activity was enhanced by the cell protection that increased the production of inhibitory substances. The antimicrobial metabolites produced by probiotic lactic acid bacteria can be classified into two groups: organic acids with a broad spectrum activity, and bacteriocins with a narrow spectrum activity [29]. In this study, the antibacterial activity of aliquots of encapsulated cells before and after pH neutralisation was evaluated to determine if the inhibitory effect was due to organic acid production or due to other antimicrobial compounds. Co-encapsulating agents such as *E. americana* oligosaccharides and commercial fructo-oligosaccharides have potential use as prebiotics to produce growth stimulation of lactobacilli and increase organic acid productions. The main antibacterial activity resulted from lactic acid and acetic acid causing low pH [10]. Accordingly, lactobacilli encapsulated with sodium alginate [18], or with chitosan [19], have improved antibacterial activity against several pathogenic bacteria. The antibacterial activity was due to the production of organic acids.

There are observations on the capacity of heterofermentative probiotics lactic acid bacteria to ferment glucose via 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway. Undissociated forms of organic acids are known to penetrate and dissociate it inside the cytoplasm of pathogens, thus diminishing the intracellular with accumulation of ionised organic acids, eventually leading to the death of the pathogens [29].

In conclusion, *L. plantarum* TISTR1465 encapsulated with *E. americana* oligosaccharide extract was more efficacious than the free cells, after sequential exposure to simulated gastric and intestinal juices and refrigerated storage in yoghurt. The encapsulated cells caused less acidification during refrigerated storage than the free cells. The encapsulation with alginate and *E. americana* oligosaccharide extract improved the antibacterial activity of *L. plantarum* TISTR1465 far beyond that of the free cells. Further studies in animal models would be required before application as a functional food additive for human consumption.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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