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Pistacia atlantica (Bene) Oleoresin Gum: The Antioxidant and Antimicrobial Activity, and Potential Encapsulation of Saccharomyces boulardii and Lactobacillus acidophilus in Kefir with Sodium Alginate/Bene Gum Polymer

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Abstract

One of the main products obtained from the *Pistacia atlantica* (Bene) tree, in addition to the fruit, is oleoresin gum which is rich in polysaccharides and polyphenolic compounds. This study was conducted to evaluate the antioxidant and antimicrobial activity of Bene gum and its potential application with alginate in probiotic microencapsulation. Chemical analysis showed that the most abundant monosaccharides in Bene gum were arabinose, galactose, and galacturonic acid, and the most representative volatile compound was α-pinene. Bene extract was rich in phenolic compounds and showed good antioxidant activity. The results showed that the gum was effective against all bacteria tested at a concentration of 50 µg/ml and Gram-negative bacteria showed higher resistance than Gram-positive bacteria. Encapsulation of *Saccharomyces boulardii* and *Lactobacillus acidophilus* with sodium alginate and Bene gum biopolymers gave an encapsulation yield of 80%. The survival rate of encapsulated probiotics compared to the free form was significantly increased after exposure to simulated gastrointestinal conditions. The viability results of probiotics in kefir were very similar to results obtained from simulated gastrointestinal conditions, while the encapsulated form of probiotics in kefir showed a consistent increase in viability over 28 days of storage. Overall, products encapsulated with a high percentage of gum showed better taste acceptance than uncoated and alginate-coated samples. Likewise, samples containing microencapsulated probiotics also generally had good overall acceptability. Based on these results, it can be concluded that Bene gum is an excellent candidate for the preparation of various biocomposites and nanoparticles.

Introduction

Pistacia atlantica is a species of pistachio tree known as Persian turpentine and called Bene or wild pistachio tree in Iran. *Pistacia atlantica*, a tree from the *Anacardiaceae* family with three subspecies (*kurdica*, *mutica,* and *cabulica*), is overlapped with Pistacia vera in some areas of Iran, Turkey, and Iraq. More than one million hectares mainly Zagros mountains (western Iran) and less in the central and eastern part of Iran are covered by *kurdica* and *mutica* subspecies [1]. This tree usually has a high resistance to unfavorable and harsh climatic conditions, which is considered a special feature for it. *P. atlantica* is a moderately growing deciduous tree that can reach a height of 7 m (23 ft) with branches that spread and erect to form a dense crown [2]. The fruit and gum of the Bene tree (turpentine sap) have many edible, industrial, and medicinal uses and are still important commodities. There are some reports in ancient literature, including traditional medicine and especially Avicenna's Canon of medicine, that highlight the therapeutic effects of Bene gum such as peptic ulcer treatment, mouth freshener, antiseptic, gum tissue strengthener, appetizer, astringent, diuretic, gastrointestinal disorder treatment, etc. [3, 4].

Scientific *findings* have also revealed that different parts of this plant exhibit broad pharmacological activities, such as antioxidant properties [5], antimicrobial [6], antiviral [7], anticholinesterase [8], anti-inflammatory [9], antinociceptive [10], antidiabetic [11], antitumor [12], antihyperlipidemic [13], and hepatoprotective activities [14], and also their beneficial effects in gastrointestinal disorders [3, 9].

One of the main products obtained from the *P. atlantica tree*, in addition to the fruit, is oleoresin gum which is obtained from the trunk of the tree by piercing it with a special tool and collecting it in a clay bowl embedded in the trunk (Fig. 1) [15]. This resin also called “Saqez” is rich in phytochemicals and antioxidants, with good antifungal and antimicrobial activities [16, 17], which makes it an excellent candidate for the preparation of various biocomposites and nanoparticles [18]. Given the richness of polysaccharides and polyphenolic compounds in this gum and the lack of studies on its use as a food-grade biopolymer, this study was conducted to evaluate its antioxidant and antimicrobial activity and potential application in probiotic microencapsulation. For this purpose, encapsulation of *Saccharomyces boulardii* as a probiotic yeast and *Lactobacillus acidophilus* as a probiotic bacterium using alginate and Bene gum was done and the survivability of encapsulated strains in a simulated gastrointestinal (GI) environment and in kefir as a food model was evaluated.

Materials And Methods

Materials, Chemicals, and Microorganisms

The oleoresin gum of Bene (*Pistacia atlantica* subsp. *kurdica*) was prepared in Kurdistan province, west of Iran. Sodium alginate was purchased from Sigma-Aldrich Chemical Co. (Oakville, ON, USA). *Lactobacillus acidophilus* La-5 was obtained from Chr. Hansen A/S, Hørsholm, Denmark, and cultured in de Man, Rogosa, and Sharpe (MRS) broth (Merck, Darmstadt, Germany) at 37 °C for 48 hours. *Saccharomyces boulardii* CNCM I-745 (Yomogi®) were inoculated on Yeast Extract–Peptone–Dextrose media (YPD, DIFCO, Detroit, MI) and incubated at 30 °C with moderate shaking for a total period of 96 h. After centrifugation at 3220 ×g at 4 °C for 10 min and washing twice with sterile phosphate-buffered saline (PBS), the inoculum of each probiotic was standardized in a final concentration of 9 log CFU/mL PBS for future use.

Chemical Analysis of Gum

Chemical analysis of Bene oleo-resin gum, including moisture, ash, protein, fat, and crude fiber, was determined using methods approved by AOAC [19]. Monosaccharide determination was performed according to our previous method [20]. Briefly, the gum was hydrolyzed by acids (0.5 M sulphuric acid, 80 °C, 18 h) and hydrolysates were neutralized with NaOH solution after centrifuging (1500g for 10 min), the supernatant was filtered through a 0.45 µm filter membrane. Then, the composition of the monosaccharides was determined by an HPLC system with an RI detector (Knauer, Germany), Eurokat H carbohydrate analysis column (300×8 mm, Knauer, Germany), and flow rate of 0.4 Ml/min^{-1} with a column temperature of 75 °C.

Chromatographic analysis of volatile components of gums was carried out on Shimadzu QP-2010 GC-MS system equipped with DB-5 ms capillary column (length, 30 m; column i.d., 0.25 mm; film thickness, 0.25 mm) and connected to a flame ionization detector. The ultrasound-assisted extraction of Mirahmadi et al. [21].

Determination of Total Phenolic and Flavonoid Contents of Gum

The phenolic *concentration* of Bene gum was determined using the Folin–Ciocalteu reagent with gallic acid as a standard [1]. Briefly, 0.25 mL of each sample (0.25 mg added to 11.5 mL distilled water) and 0.25 mg of Folin–Ciocalteu reagent (10%) solutions were mixed for 5 min and 0.75 mL Na_2CO_3 (2%) was then added for neutralization. The absorbance was measured at 760 nm after 2 h of incubation at room temperature by using a UV-VIS spectrophotometer (2550, Shimadzu, Japan). A blank sample was used as a mixture of distilled water and reagents. The total concentration of phenolic content was expressed as gallic acid (mg) equivalent per 100-gram extract of Bene gum (mg GAE/g DM).

The total *flavonoid* contents of Bene gum were determined using the aluminum chloride ($AlCl_3$) colorimetric assay with quercetin as a standard. Briefly, 0.1 mL of sample extract (0.1 mg per 1 mL distilled water) or standard quercetin solutions (0–0.5 mg) were separately mixed with 0.05 mL of $NaNO_2$ (5%) and allowed to react for 5 min. Then 0.1 mL of aluminum chloride solution (10%) was mixed for 6 min and 0.5 mL of 1 M sodium hydroxide was added. The absorbance of the mixtures was immediately measured at 510 nm using a UV-VIS spectrophotometer (2550, Shimadzu, Japan), and total flavonoid contents were expressed as mg of quercetin equivalent per 100 gram of Bene gum extracts. All the tests were done in triplicate.

DPPH Photometric Assay of Gum

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity assay of Bene gum was performed as described by Rahman [12] with minor modification. 0.5 ml of Bene gum at various concentrations (0.1, 0.5, 1, 2, and 4 mg/mL) was added to 3.0 mL of 0.004% of DPPH in methanol. The reaction mixture was shaken vigorously and left for 45 min in the dark at room temperature. The reaction of the DPPH radical against a blank was measured at 515 nm by using a UV-Vis spectrophotometer (2550, Shimadzu, Japan). The radical scavenging activity (RSA) was calculated according to the percentage of DPPH inhibition using the following formula:

$$\text{Inhibition of DDPH (\%)} = 100 \times (A_1 - A_0)/A_0$$

Where, A_0 is the absorbance of the ascorbic acid as a positive control and A_1 is the absorbance of the sample extract. In this assay, the reference compound used was BHT.

Determination of the MIC and MBC

The MIC (minimum inhibitory concentration) and MBC (minimum bactericide concentration) of Bene gum were measured by the method involving microdilution broth (96-well microtiter plates), as described by the Clinical and Laboratory Standards Institute disk susceptibility testing [22]. Briefly, the Mueller-Hinton broth (MHB) supplemented with Tween 80 and dilutions of the samples (0.1–100 µg/mL) were dispensed into the wells of a microtiter plate, and then each well was inoculated with ca. 5×10^5 CFU/mL concentration of the bacterial suspensions including 3 gram-positive bacteria (*Listeria monocytogenes*,

Bacillus cereus, and *Staphylococcus aureus*) and 3 gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella* Typhimurium). After incubation at 35 °C for 24 h, the absorbance of wells was measured at 550 nm by a microplate reader (Bioteck Instruments, Inc. VT, USA) and the MICs were determined as the lowest concentration of Bene gum that inhibited the visible bacterial growth. Then, 10 µL from each well were subcultured onto fresh agar plates and the MBC was determined as the concentration of antimicrobial agent that causes the death of 99.9% of the initial inoculum. Each experiment was repeated three times.

Microencapsulation of Probiotics with Alginate and Bene Gum

L. acidophilus and *S. boulardii* were encapsulated in alginate and Bene gum particles through an extrusion method described by Krasaekoopt et al. [23] with some modification. Briefly, the solutions of sodium alginate with molecular weight between 80000-120000 g/mol were prepared in distilled water (3% w/v) and after gentle stirring with a magnetic stirrer at 4 °C for 12 h, sterilized by autoclave at 121 °C for 15 min. A similar process was used to produce sterile alginate concentrations (2.5, 2, and 1.5 % w/v) and Bene gum (0.5, 1, and 1.5 % w/v) solutions as other encapsulating materials (Table 1). The powder of gum was previously prepared by a vacuum oven drier at 45 °C.

Tabale 1. The designed levels of the sodium alginate and Bene oleo-resin gum used for encapsulation of *Lactobacillus acidophilus* and *Saccharomyces boulardii*.

Experiment groups	Sodium alginate (% w/v)	Bene gum (% w/v)
E1	3	0
E2	2.5	0.5
E3	2	1
E4	1.5	1.5
Control	0	0

Then, the probiotic cells with an approximate concentration of 9 log (CFU/mL) were mixed with 20 mL of each sterile encapsulating agent and incubated for 4 h at 37 °C. Finally, glycerol as a cryoprotectant (5 mL) was added to each solution and stirred at 400 rpm for 20 min. The prepared mixtures (with a flow rate of 5 mL/min) were then injected through a syringe with a 22-gauge needle equipped with a syringe pump (LEGATO® 100 syringe pmp, KD Scientific Inc., MA, USA) into 250 mL of sterile $CaCl_2$ (0.05 M) with stirring at 200 rpm and remained for 45 min for hydrogel particles formation. After filtration of solidified microcapsules with filter paper (Whatman #4), the beads were then washed twice with ultrapure water and packed in sterile glass containers. The numbers of entrapped cells were measured after adequate diluting by the surface plate count method and encapsulation yield (EY) was calculated by Equation 1 as follows:

$$EY\ (\%) = (N / N_0) \times 100 \qquad (1)$$

Where, N is the number of viable entrapped cells (CFU/mL) released from the beads, and N_0 is the number of free viable cells (CFU/mL) in a suspension containing alginate/Bene gum and bacterial culture. The bead size of the prepared microcapsules was determined at 25 ± 1 °C by a Malvern 2000 laser particle size analyzer (Model 2000, MALVERN, UK) as described by Xie et al. [24].

Viability of Encapsulated Probiotics Under Simulated Gastrointestinal Conditions

The viability of encapsulated probiotics was determined continuously in media simulating gastric juice (SGJ) by the addition of 3 g/L pepsin (Sigma-Aldrich, Steinheim, Germany) in sterile NaCl solution (9 g/L) with pH adjusted to 2.0 with hydrochloric acid. 0.2 g of microspheres with entrapped probiotics (*L. acidophilus* or S. *boulardii*) were mixed in 10 mL of simulated gastric juice and incubated for 5, 30, 60, and 120 min at 37 °C (simulating the temperature of the human body) with constant agitation at 50 rpm [25]. The survival rate (%) of encapsulated and free probiotics was calculated using the following formula:

$$\% \text{ Survival rate} = (N/ N_0) \times 100 \qquad (2)$$

Where, N_0 and N are the number of viable cells (CFU/mL) before and after exposure to gastrointestinal conditions, respectively.

To prepare simulated intestinal juice (SIJ), a solution of 10 g/L Oxgall (Merck) and x 10 g/L pancreatin (Sigma-Aldrich) was prepared and sterilized at 121 °C for 15 min. 0.1 mL of the bacterial cells were then exposed to SIJ and incubated at 37 °C for 5, 30, 60, and 120 min. *L. acidophilus* or *S. boulardii* were counted in MRS and YPD medium, respectively, as described in Section 2.6.

Viability of Encapsulated Probiotics in Kefir

The viability tests of encapsulated *L. acidophilus* and *S. boulardii* were performed on kefir as a model food system. For kefir production, the whole cow milk was pasteurized at 90 °C for 5 min and cooled down to 25 °C. Then, the milk was inoculated with 2% of the commercial kefir starter culture FD-DVS EXACT Kefir 2 (CHR Hansen Horsholm, Denmark), which contained *Lactococcus lactis* Spp. *cremoris*, *Lactococcus lactis* Spp. *lactis*, *Lactococcus lactis* Spp. *lactis* var. *diacetylactis*, *Leuconostoc* Spp., *Debaryomyces hansenii*, and *Streptococcus thermophilus,* and allowed to ferment at 25 °C for 12 h. The treatment groups were separated as free and encapsulated strains (*L. acidophilus* or *S. boulardii* at an initial concentration of 1.2×10^9 CFU/mL). All kefir samples were fermented at 12-14 °C for 12 h until the pH of the fermented milk reached 4.6. The curd was stirred and divided into 100 mL batches. The products were stored at 4 °C for 28 days for survival analysis [26]. MRS-Salicin-agar (MRS agar; Merck, Darmstadt, Germany) was used for the selective enumeration of *L. acidophilus*. For pure cultures, serial dilutions were prepared from kefir samples and corresponding dilutions were spread onto the surface of plates and counted after anaerobic incubation for 36 h at 37 °C. Count of *S. boulardii* from kefir samples was carried out by plating serial dilutions on Sabouraud agar medium supplemented with

chloramphenicol (50 mg/L) (Sigma-Aldrich, Steinheim, Germany) and incubating aerobically at 30 °C for at least 36 h.

Sensory Evaluation

Sensory analysis of the kefir samples was performed with 10 dairy products-trained panelists working at the Nobaveh Dairy Industries Co. (Saveh, Iran). The assessment was done using a 9-point scale hedonic procedures were ranked with an increasing format from 1 (extremely disliked) to 9 (extremely liked). All samples were stored at 4 °C for sensory analysis, so that the temperature cannot affect the organoleptic scores, and served at thirteen aliquots of kefir (10 mL each) sampled in uniform plastic cups to ensure no carry-over of sensory properties. The sensory properties evaluated included color/transparency (unusual color, fatty, nonhomogeneous, serum separation, foreign material), flavor/odor (astringent, rancid, cooked taste, high acidic, no refreshing, metallic/oxidized taste, foreign taste, yeast taste), texture (grumous, gassy, grainy, lumpy, high viscous and nonelastic), and overall acceptability attributes using reference standards [27]. During the training, feedback on attribute scoring was obtained to increase their ability to recognize slight differences in kefir samples. Panelists were given a 10-min break after every third sample to minimize sensory. The sensory analysis of kefir samples including control (without probiotic), Free *L. acidophilus* (FL), Free *S. boulardii* (FS), and designed encapsulated *L. acidophilus (EL 1-4)* and *S. boulardii (ES 1-4)*, were carried out after 1, 7, 14 and 28 days of storage. The pH and acidity of all samples were analyzed accordingly to the standard methods.

Statistical Analysis

All microbiological, chemical and sensory evaluations were performed in triplicate and repeated three times in treatment groups (E1, E2, E3, E4, and Control) examined at 4 °C during 28 days of storage. Results in this study were presented as the mean value ± standard deviation (SD) and data were subjected to one-way analysis of variance (ANOVA) and the Student's t-test was used for comparison ($p<0.05$). All analyses were performed using SPSS version 25 (SPSS Inc, Chicago, Illinois) for Windows.

Results And Discussion

Chemical Composition of Bene Gum

The general chemical compositions (moisture, protein, ash, crude fat, carbohydrate) of Bene (*Pistacia atlantica*) oleo-resin gum samples are shown in Table 2. The most abundant monosaccharides were arabinose (30.30%), galactose (18.2%), and galacturonic acid (17.69%). Our finding was in agreement with similar research by Mirahmadi et al. [21].

According to the results, *Pistacia atlantica* oleo resin gum consists of monoterpenes and monoterpenoids (96%) and trace amounts of terpenes, alcohols, and sesquiterpenes. The most representative volatile compounds of Bene were α-Pinene (79.52%), β-Pinene (6.12%), trans-Pinocarveol (1.68%), Sabinene (1.21%), and cis-Limonene oxide (1.14%) (Table 3). The same results were reported by Didehvar et al. [28],

Mirahmadi et al. [21], and Salimi et al. [29] who indicated that α-Pinene is the main compound in the ethanolic extract of Bene gum. α-Pinene is a highly bioavailable component with rapid metabolism and exhibits activity as an acetylcholinesterase inhibitor [30] and positive modulator of GABAA-benzodiazepine (BZD) receptors [31]. Some authors also have attributed the antimicrobial and anti-inflammatory activity of α-Pinene [32-34].

Table 2. The moisture, protein, ash, crude fat, carbohydrate, and monosaccharides analysis of Bene (*Pistacia atlantica*) oleoresin gum.

Compounds	Percent	Monosaccharides	Percent*
Moisture	18.52±0.02	Arabinose	30.30
Total protein	4.49±0.15	Galactose	21.81
Crude fat	0.86±0.01	Galacturonic acid	17.69
Total carbohydrate	38.73±1.52	Xylose	9.87
Total ash	0.92±0.003	Glucuronic acid	9.05
		Rhamnose	8.62
		Glucose	2.08

* The percent of monosaccharides was based on total carbohydrates.

Table 3. Volatile components of the Bene (*Pistacia atlantica*) oleoresin gum.

No.	Compound	Retention index	Percentage
Monoterpene hydrocarbons			
1	α-Pinene	936	79.52
2	Camphene	954	0.51
3	Sabinene	976	1.21
4	β-Pinene	983	6.12
5	Myrcene	991	0.04
6	δ-2-Carene	995	0.08
7	α-Phellandrene	1010	0.10
8	δ-3-Carene	1014	0.10
9	α-Terpinene	1021	0.28
10	ρ-Cymene	1029	0.86
11	Limonene	1033	0.11
12	1,8-Cineole	1037	0.03
13	trans-β-Ocimene	1047	0.08
14	ρ-Cymene	1096	0.20
15	trans-Pinocarveol	1149	1.68
16	Myrtenal	1206	0.63
Monoterpene oxides			
17	α-Pinene oxide	1109	0.44
18	cis-Limonene oxide	1134	1.14
Monoterpene alcohols			
19	trans-Verbenol	1153	0.11
Monoterpenoids			
20	trans-Pinocarveol	1149	1.68
21	cis-Isocitral	1166	0.32
22	Pinocamphone	1170	0.21
23	trans-Isocitral	1180	0.25

24	cis-Carveol	1227	0.09
25	Carquejol acetate	1300	0.07
Monoterpenoid phenols			
26	Carvacrol	1307	0.05
Terpenes			
27	Thuja-2,4(10)-diene	958	0.18
28	Verbenone	1218	0.44
Terpene alcohols			
29	dehydro-Linalool	1091	0.33
30	Linalool	1103	0.31
31	Neo-iso-Verbanol	1187	0.82
Terpene esters			
32	Bornyl acetate	1289	0.14
Sesquiterpenes			
33	α-Cubebene	1349	0.06
34	α-Longipinene	1354	0.02
35	α-Copaene	1379	0.09
36	trans-Caryophyllene	1410	0.02
37	β-Selinene	1495	0.08
38	δ-Cadinene	1522	0.11
39	cis-Calamenene	1528	0.03
	Total		**96.72**

Total Phenolic, Flavonoid, and DPPH Radical Scavenging of Gum

The results of the total phenolic and flavonoid contents of Bene gum are shown in Table 4. According to the results of Nachvak et al. [35], Hatamnia et al. [1], and Rahman [12], Bene extract is rich in phenolic compounds such as ferulic acid, gallic acid, quercetin, etc., and has good antioxidant and radical scavenging properties. The results also showed a positive correlation between these phenolic compounds and antioxidant activity (Table 4). This positive relation between DPPH radical scavenging activity and phenolic compounds (as potent hydrogen donors) has been reported by previous investigations [1, 35, 36]. The study findings revealed that with increasing concentrations from 0.1 to 4 mg/mL of Bene gum extract, the DPPH scavenging activity increased significantly and exhibits strong antioxidant activity

(p0.05) compared to BHT as a reference compound (86.54±0.24%). These results have been previously demonstrated for a variety of other plant-derived compounds [37-40].

Table 4. Phenol and flavonoid contents and antioxidant activities of Bene (*Pistacia atlantica*) oleoresin gum (BG).

Compound	DPPH radical scavenging (%)	Total phenol contents ($mg\ g^{-1}$) [a]	Flavonoid Contents ($mg\ g^{-1}$) [b]
Bene gum exudate	-	151.14±0.79	48.45±0.22
BG 0.1 ($mg\ ml^{-1}$)	19.23±0.69	-	-
BG 0.5 ($mg\ ml^{-1}$)	36.08±1.71	-	-
BG 1 ($mg\ ml^{-1}$)	52.24±1.09	-	-
BG 2 ($mg\ ml^{-1}$)	75.25±1.76	-	-
BG 4 ($mg\ ml^{-1}$)	88.24±1.02	-	-
BHT	86.54±0.24	-	-

[a] mg gallic acid equivalent g^{-1} extract.

[b] mg quercetin equivalent g^{-1} extract. Data are Means ± SD (n=3).

MIC and MBC of Bene Gum

The MIC and MBC of Bene oleoresin gum were determined against selected pathogens (Table 5). Results showed that gum was effective against all tested bacteria at a 50 µg/ml concentration and due to the distinctive structure and complexity of their double-layer cell membrane, Gram-negative bacteria showed higher resistance than Gram-positive bacteria [41]. Data indicated that the lowest inhibitory concentration of Bene gum was 2.50 and 3.50 µg/mL belonging to *L. monocytogenes* and *S. aureus*, respectively. The antimicrobial activity of Bene gum against some pathogens was reported by Fathollahi et al. [42] The same result was reported by Azeez and Gaphor [43] which indicated the antimicrobial activity of Bene against *Porphyromonas gingivalis*. The antimicrobial activity of Bene (*Pistacia atlantica* subsp *mutica*) essential oil against *S. aureus* and *E. coli* with MIC value ranges of 6.00-12.50 µg/mL was reported by Rezaie et al. [44]. The antimicrobial effect of Bene gum is primarily due to the presence of nonnutritive dietary components such as monoterpenes and sesquiterpenes.

Table 5. Antimicrobial activity of Bene (*Pistacia atlantica*) oleoresin gum against gram-positive and gram-negative bacterial strains (μg mL^{-1}).

Microorganisms	Bene gum	
	MIC	MBC
E. coli	17.50	25.00
S. Typhimurium	11.25	16.75
P. aeruginosa	44.50	50.00
L. monocytogenes	2.50	5.50
B. cereus	4.25	7.50
S. aureus	3.50	6.25

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

Encapsulation Yield and Bead Size

The average size and encapsulation yield of alginate and Bene gum beads are shown in Table 6. The mean size of microcapsules with alginate coating was 1.68±0.05 mm and the size of alginate capsules was slightly increased with the addition of the gum. The sizes of encapsulated probiotics with the extrusion method in previous studies ranged from 0.5 to 3 mm [45-47]. Despite to present study, Nami et al. [48] found that the presence of Persian gum significantly decreased the bead size. In accordance with our findings, Cheow et al. [49] reported that the addition of locust bean gum increased the bead size and it is due to the higher affinity of the gums for water molecules. Similar results were reported by Jouki et al. [50] and Karimi et al. [51] with alginate/quince seed gum and sodium alginate/Zedo (*Amygdalus scoparia*) gum beads, respectively.

The higher encapsulation yield of the probiotic cells belonged to 2.5% alginate and 0.5% Bene gum (E1 experimental group) and this is due to the formation of a combination of alginate with some oligosaccharides in the gum (arabinogalactan), which probably reduces the porosity of the surface of the dunks and on the other hand prevents the leakage of bacteria into the environment. The addition of Bene gum up to 1 % did not improve the efficiency of the encapsulation of probiotics (Table 6). Sultana et al. [52], demonstrated that lower amounts of starch improve the efficiency of alginate capsules, and higher amounts do not increase the encapsulation yield. In another study, the alginate-arabinoxylan composite significantly improved the encapsulation efficiency of *Lactobacillus plantarum* [53].

Table 6. Bead size and encapsulation yield (mean±SD) of microencapsulated *L. acidophilus* and *S. boulardii* with sodium alginate (SA) and Bene gum (BG).

Parameter	E1 (3% SA)	E2 (2.5% SA + 0.5 %BG)	E3 (2% SA + 1 % BG)	E4 (1.5% SA + 1.5% BG)
Size (mm)	1.68 ± 0.05^{c}	1.73 ± 0.05^{b}	1.75 ± 0.06^{b}	$1.79\pm0.04^{a*}$
Encapsulation yield	81.2 ± 1.24^{b}	88.4 ± 1.26^{a}	82.2 ± 1.03^{b}	78.3 ± 0.84^{c}

*Values with the same letter in the same row are not significantly different ($p>0.05$)

Viability of Encapsulated Probiotics Under Simulated Gastrointestinal Conditions

To be effective and exert their specific health benefit, probiotic strains must be able to survive the harsh conditions of the GI tract (particularly the acid in the stomach) and persist at high levels in the intestine (10^6 -10^8 CFU/g of intestinal content) [54]. Fig. 2 A/B shows the stability of free and encapsulated *L. acidophilus* and *S. boulardii* in simulated gastrointestinal conditions during 120 min storage. The survival rate of free *L. acidophilus* and *S. boulardii* after exposure to SGJ for 5 min was 63.8% and 72% and diminished to 3.9 and 28.8 % during 120 min treatment gastric simulated condition, respectively (Fig. 2 A/B). Encapsulation of *L. acidophilus* and *S. boulardii* with alginate improved the probiotic survival during the gastro-intestinal transition model and the survival rate of probiotics in alginate beads containing 0.5 % Bene gum (E2) was higher than that of alginate beads ($p<0.05$). The viability and the stability of free and encapsulated probiotics with alginate alone and in combination with other substances have previously been tested in simulated gastric conditions [25, 55-58], but there is no published data related to the alginate with Bene gum encapsulation procedure.

Microencapsulated *L. acidophilus* and *S. boulardii* with alginate and alginate/Bene gum were resistant to simulated intestinal juice (SIJ) and the highest resistance belonged to the E2 group (2.5% Alginate+0.5% Bene gum) with more than 10^6 CFU/mL survival after 120 min exposure. However, there was a rapid loss of free probiotics in simulated intestinal juice (SIJ), so more than 8 and 5 log CFU mL reductions in the viability of free *L. acidophilus* and *S. boulardii* were observed after exposure of 120 min, respectively (Fig. 3 A/B). The results also indicated a difference in sensitivity between *L. acidophilus* and *S. boulardii* in simulated gastrointestinal conditions during storage. Several reports have shown variations in the acid tolerance in strains of probiotic bacteria [25, 59, 60]. In accordance with our findings, Le and Trinh [61] showed that *S. boulardii* was the most acid and bile-tolerant strain, specifically free *S. boulardii* had greater viable numbers than free *L. acidophilus* possibly due to its higher tolerance to acids. The outer cell wall of *S. boulardii* is characterized by high levels of β-glucans and mannan-oligosaccharides (MOS) which possess excellent properties to resist changes in the environment such as low pH and bile salts, while bacterial cells do not have these constituents [62, 63]. Arslan et al. [64] examined the survivability of *S. boulardii* in a simulated gastric condition at different pH levels and concluded that gum Arabic (mainly contains two different polymers, glycoprotein, and polysaccharide) is the best wall material than gelatin,

whey protein concentrate, modified starch, maltodextrin, and pea protein isolate. Overall, encapsulation with alginate and Bene gum afforded better protection to both *L. acidophilus* and *S. boulardii* organisms compared to free probiotics at the same time points.

Viability of Encapsulated Probiotics in Kefir During Refrigerated Storage

The results presented in Fig. 4(A/B) show the viability of free and encapsulated *L. acidophilus* and *S. boulardii* in kefir samples during cold storage at 4 °C. In the control groups containing free *L. acidophilus*, the viable count of probiotic bacteria decreased by around 7.4 log; and this reduction rate in kefir samples inoculated with *S. boulardii* was 5 log through the 28 days of storage. Although the viable counts of encapsulated probiotics diminished throughout the storage period, the number of cells of the encapsulated *L. acidophilus* and *S. boulardii* with alginate at day 28 were 2.96 and 1.47 log cycles higher than that of free probiotics, respectively. The maximum viability was observed in kefir that was inoculated with encapsulated *S. boulardii* by 2.5% alginate and 0.5% Bene gum (E2) and only 2.57 CFU/mL reduction was observed.

Similar research for the growth of free and encapsulated *L. plantarum* with Cellulose–Chitosan in kefir was conducted by Afzaal et al. [65] and they reported an increase in the survival of encapsulated bacteria in this food model. In addition, a relevant research study was also carried out by González-Sánchez et al. [66], and the authors showed 2 and 6 log cycle losses in the number of cells of free and encapsulated *Bifidobacterium animalis* ssp. *lactis* BB12, respectively after 28 days of refrigerated storage in kefir. In the study of Yilmaz et al. [26], *Lactobacillus paracasei* was encapsulated within alginate-based electrospun nanofiber and results showed an increase in probiotic viability by nanoencapsulation of the strain in simulated gastrointestinal conditions and kefir.

The results of survivability of probiotics in kefir are very similar to results obtained from the survival of the strain under simulated gastrointestinal conditions, whereas the free form of *Saccharomyces* in kefir exhibited a steady increase in viability than *Lactobacillus* during the storage period (Fig. 4A/B). The interactions between yeasts and bacteria are very convoluted and this competition for nutrients may be beneficial or harmful to each other [67, 68]. In a study conducted by Carvalho et al. [69], the interaction of *Lactococcus lactis* and *Saccharomyces cerevisiae* in cachaça (a sugar cane spirit produced in Brazil) was evaluated and the results showed that *S. cerevisiae* was not negatively influenced by bacteria, however, a decrease in the growth of *L. lactis* was observed, which may due to alcohol production by yeast and competition for nutrients. In contrast, Paramithiotis et al. [70] reported a negative effect of LAB metabolites such as lactic and acetic acids produced in the growth rate and ethanol production of *S. cerevisiae* in sourdough. Based on the results of Moradi et al. [71] and Edwards-Ingram et al. [72], *S. boulardii* is more tolerant than other *S. cerevisiae* strains to acidic pH and can survive at pH values as low as 2.0. Moreover, in some relevant studies, the authors discovered that yeast metabolites an important role in enhancing the viability of *L. rhamnosus* GG, as bacterial populations in fermented coffee brews [73] and fermented milk [74]. Chan et al. [75] also noted that supplementation of coffees with inactivated

yeast derivatives (IYDs) enhanced the viability of *L. rhamnosus* GG and *L. paracasei* Lpc-37 during storage at 4 and 25 °C.

Sensory Evaluation of Kefir

The fermentation process of kefir was characterized by pH value, acidity, alcohol content, color, texture, and other sensory changes. The type of culture media and time of storage of kefir had a significant effect on the sensory properties and its overall acceptability. There was a slight increase in titratable acidity (TA) and a decrease in pH during refrigerated storage for all the treatments (including a kefir batch without and with free and encapsulated *L. acidophilus* and *S. boulardii* samples). The pH results indicated that there was no significant difference ($p>0.05$) among all kefir samples. According to previous studies, the normal pH for kefir varies between 4.2-4.6 [76, 77]. The average pH value for control (no probiotic), free, and encapsulated *L. acidophilus* samples of the kefir after 28 days of refrigerated storage were 4.31, 4.48, and 4.54, respectively. The pH for free *S. boulardii* samples was slightly higher than for free *L. acidophilus* and control samples, but this increase was not significant (pH=4.57). Most studies have shown that lactic acid bacteria decrease over time and that the pH of kefir does not become more acidic [78, 79], and that the production of acetic acid is relatively slow in the presence of yeast [80].

Organoleptic stability and sensory properties of fermented probiotic food products during storage are important characteristics of consumer acceptance and preference. Therefore, we evaluated the sensory properties of kefir samples with and without free and encapsulated *L. acidophilus* and *S. boulardii* during 28 days of storage at 4 °C, and the results are shown in Fig. 5. Overall, kefir products were most accepted by evaluators on days 7, 14, 1, and 28 of storage, respectively. Additionally, products encapsulated with a high percentage of gum showed better taste acceptance than uncoated and alginate-coated samples. At this stage, it should be noted that the panelists were able to detect the taste and aromatic character of the mastic in the samples of kefir with 1.5% Bene gum (EL4, ES4), but they could not detect any difference in texture, appearance, and color of kefir samples. Likewise, samples containing microencapsulated probiotics generally also had good overall acceptability. The lowest scores for the overall acceptability were given to the kefir samples with free *S. boulardii* evaluated after 28 days of storage and it is due to the presence and growth of yeast and its effect on increasing the pH of the product as well as some change in organoleptic characteristics of the product. In the study of Hadjimbei et al. [81] which was conducted with goats' milk yogurt supplemented with *P. atlantica* resin extracts and *S. boulardii*, they revealed that there were no significant differences in flavor, body, texture, and appearance between the different yogurt samples. Schoina et al. [82] also reported an enhancement in the aromatic profile and functionality of cheese whey beverages by the incorporation of probiotic cells and *Pistacia terebinthus* resin. The results of this study showed that the use of alginate and Bene gum for microencapsulation of probiotics has functional and health-promoting properties in dairy products, as well as their effect on increasing the viability of probiotics in fermented products.

Conclusions

Bene oleoresin gum demonstrates great potential, as an ingredient or support, in the development of novel nutraceutical and functional products. This study presented the great antimicrobial and antioxidant activities of Bene gum, with its good properties in the development of a novel biopolymer for the encapsulation of probiotics in kefir. Based on these results, it can be concluded that the microencapsulation of probiotics with alginate and Bene gum plays a very important role in the protection and enhancement of these microorganisms against adverse environmental conditions, including simulated stomach and intestine conditions, as well as an increase in the viability of probiotics in kefir.

Declarations

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Conflict of interest: *The authors have no conflicts of interest to declare.*

Ethical Approval: *"not applicable"*

Consent to participate: *Informed consent was obtained from all individual participants included in the study.*

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Figures



Figure 1

Pistacia atlantica subsp. kurdica (Bene) tree with clay cup for collecting oleoresin gum and commercial chewing gum produced from the natural mastic resin.

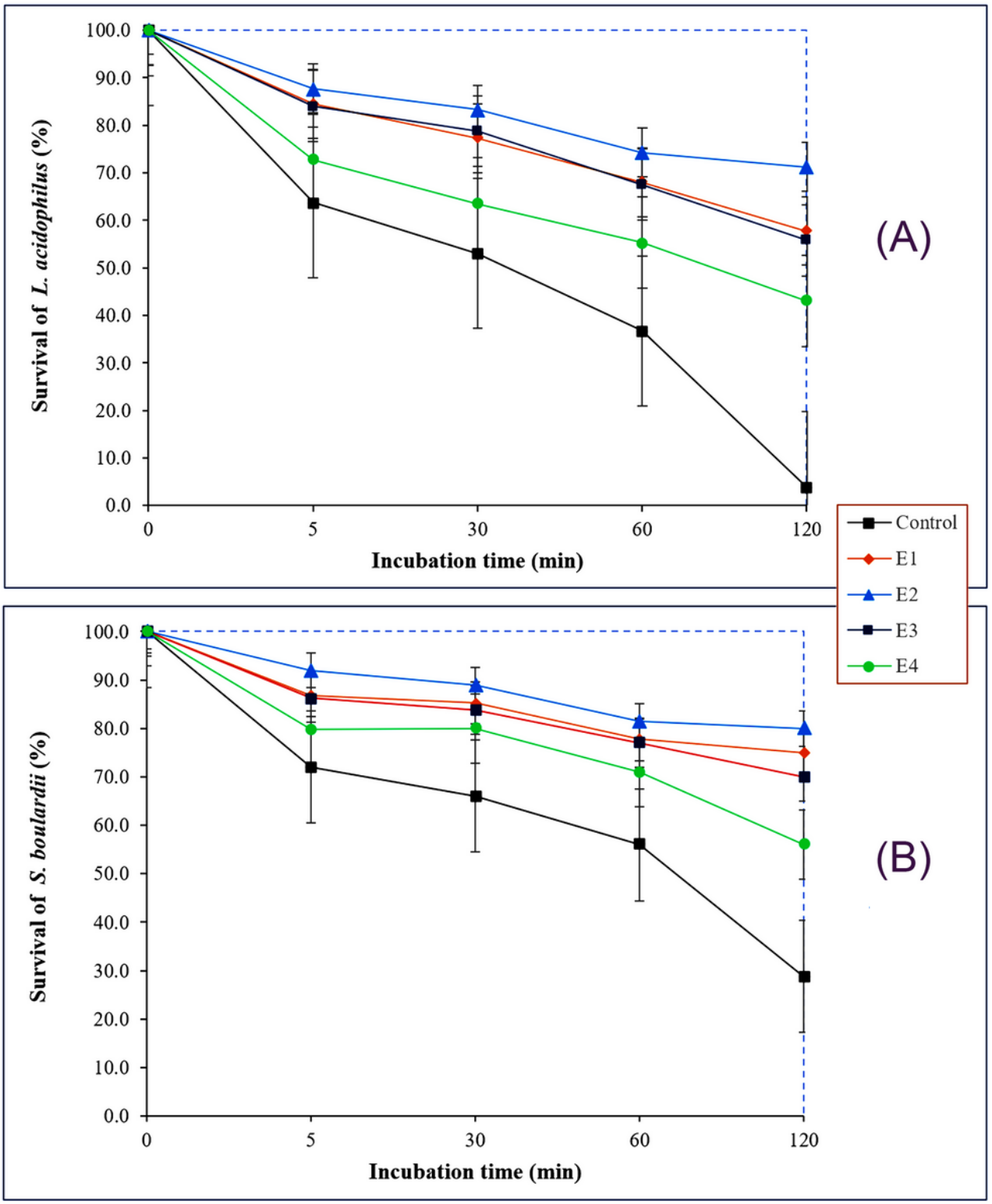


Figure 2

Survival rate (%) of free (control) and encapsulated (sodium alginate (E1), sodium alginate + Bene gum (E2-4)) *L.acidophilus* and *S. boulardii*during exposure to simulated gastric juice (pH 2.0) at 37 °C.

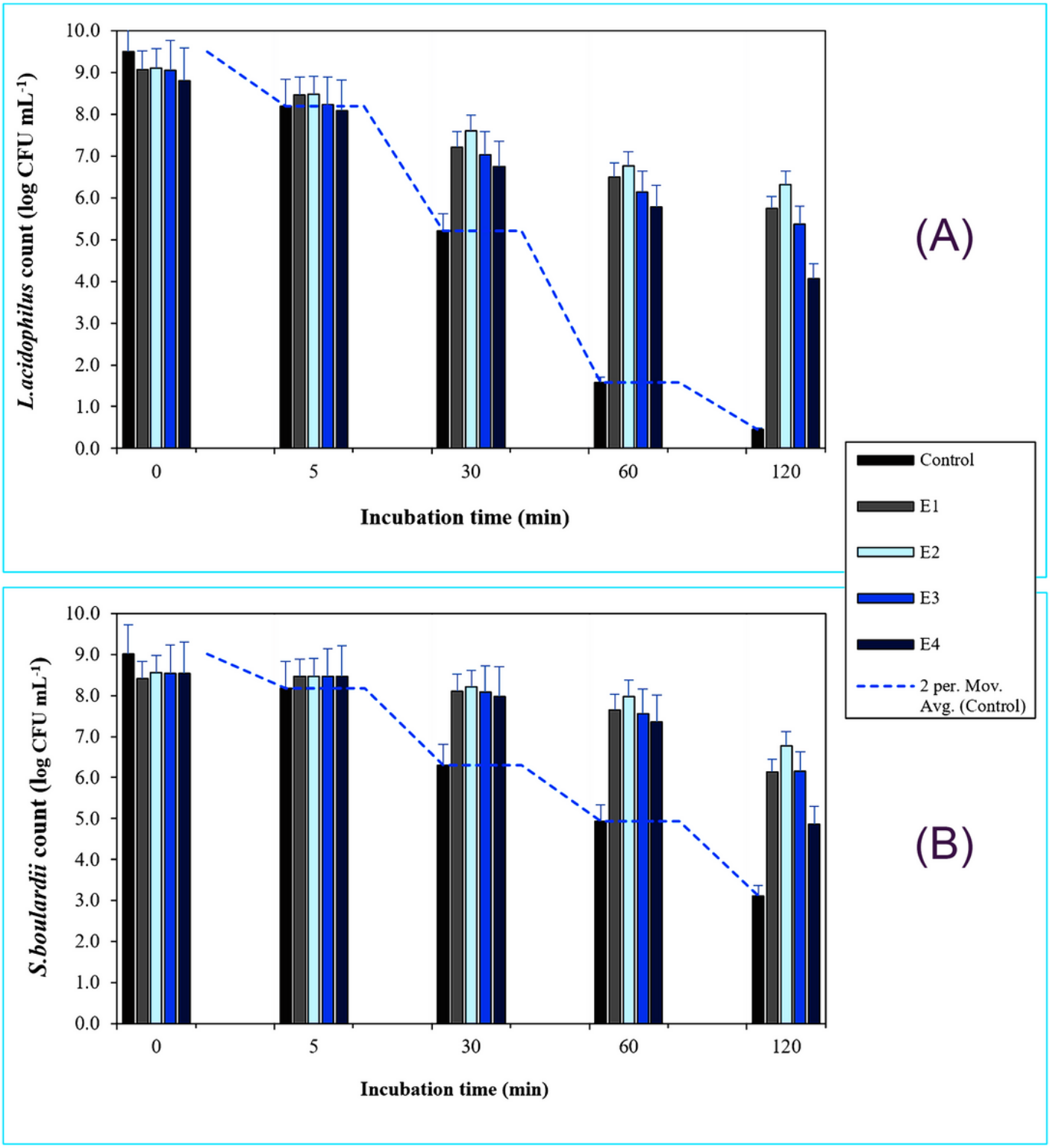


Figure 3

Stability of free (unencapsulated) and encapsulated (sodium alginate (E1), sodium alginate + Bene gum (E2-4)) *L.acidophilus* and *S. boulardii*during exposure to simulated intestinal juice at 37 °C.

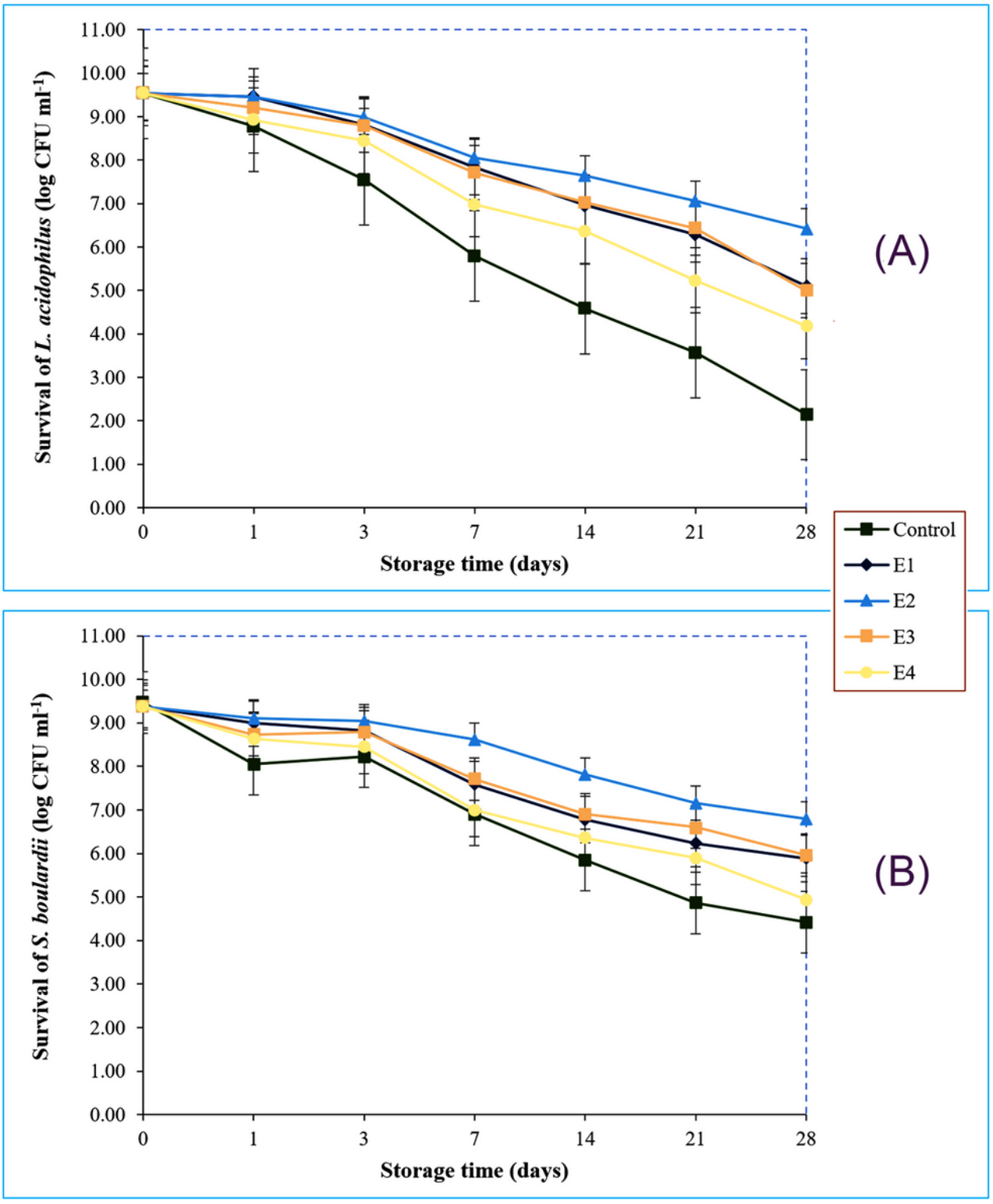


Figure 4

Survival of free (unencapsulated) and encapsulated (sodium alginate (E1), sodium alginate + Bene gum (E2-4)) *L.acidophilus* and *S. boulardii* during 28 days storage at 4 °C in kefir.

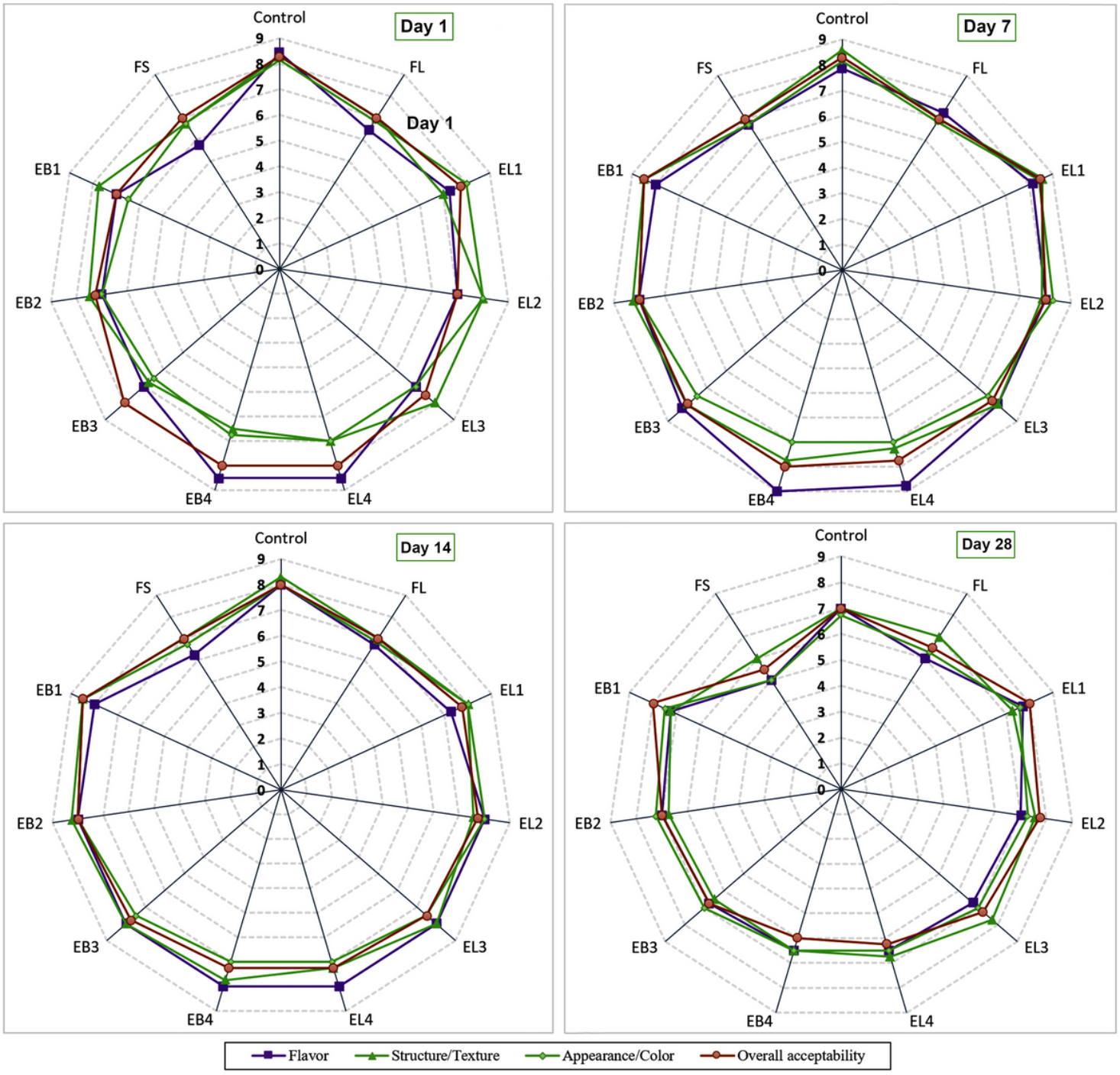


Figure 5

Sensory properties of kefir samples including control (without probiotic), Free *L. acidophilus* (FL), Free *S. boulardii* (FS), and designed encapsulated *L. acidophilus (EL 1-4)* and *S. boulardii (ES 1-4)*, after 1, 7, 14 and 28 days of storage at 4 °C.

Supplementary Files

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