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Research Article

Improving Viability in the *in vitro* Gastrointestinal System by Encapsulation of *Lacticaseibacillus paracasei* Probiotic Bacteria on Chestnut (*Castanea sativa* Mill.) Fruit

Büşra KARKAR [1*], İlkyaz PATIR [1], Elif TÜLEK [1], Serenay EYÜBOĞLU [1], Saliha ŞAHİN [1]

[1] University of Bursa Uludag, Faculty of Science and Art, Department of Chemistry, Bursa/Türkiye

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Abstract

In this study, *Lacticaseibacillus paracasei* probiotic bacteria were encapsulated by freeze-drying (lyophilization) method using three different parts of chestnut fruit: kernel, inner, and outer shell. The encapsulation efficiencies of *Lacticaseibacillus paracasei* probiotic bacteria were determined as 85.76±0.12%, 81.21±0.02%, and 79.38±0.05%, respectively. FTIR and SEM characterized the structure and surface of the capsules. In addition, the viability of free and encapsulated bacteria was analyzed during *in vitro* gastrointestinal digestion conditions. During *in vitro* gastrointestinal digestion, free bacteria lost their viability at 90 min of gastric digestion, while encapsulated probiotic bacteria were able to reach the intestinal environment. Thus, it is assumed that new products can be developed by using *Lacticaseibacillus paracasei* loaded capsules as a natural additive in food products.

Keywords: *Lacticaseibacillus paracasei*, probiotic bacteria, encapsulation, freeze-drying, chestnut

Kestane (*Castanea sativa* Mill.) Meyvesine *Lacticaseibacillus paracasei* Probiyotik Bakterilerinin Enkapsülasyonu ile *in vitro* Gastrointestinal Sistemde Canlılığın İyileştirilmesi

ÖZ

Bu çalışmada, *Lacticaseibacillus paracasei* probiyotik bakterileri kestane meyvesinin çekirdek, iç ve dış kabuk olmak üzere üç farklı kısmı kullanılarak dondurarak kurutma (liyofilizasyon) yöntemiyle enkapsüle edilmiştir. *Lacticaseibacillus paracasei* probiyotik bakterilerinin enkapsülasyon verimlilikleri sırasıyla %85,76±0,12, %81,21±0,02 ve %79,38±0,05 olarak belirlenmiştir. FTIR ve SEM kapsüllerin yapısını ve yüzeyini karakterize etmiştir. Ayrıca, *in vitro* gastrointestinal sindirim koşulları sırasında serbest ve kapsüllenmiş bakterilerin canlılığı analiz edilmiştir. *In vitro* gastrointestinal sindirim sırasında, serbest bakteriler gastrik sindirimin 90. dakikasında canlılıklarını kaybederken, kapsüllenmiş probiyotik bakteriler bağırsak ortamına ulaşabilmiştir. Böylece *Lacticaseibacillus paracasei* yüklü kapsüllerin gıda ürünlerinde doğal bir katkı maddesi olarak kullanılmasıyla yeni ürünlerin geliştirilebileceği öngörülmektedir.
Anahtar Kelimeler: *Lacticaseibacillus paracasei*, probiyotik bakteri, enkapsülasyon, dondurup-kurutma, kestane

*Corresponding Author: busrakarkar@uludag.edu.tr
Büşra KARKAR, https://orcid.org/0000-0001-6547-5558
İlkyaz PATIR, https://orcid.org/0000-0002-3503-2550
Elif TÜLEK https://orcid.org/0009-0006-9844-3353
Serenay EYÜBOĞLU https://orcid.org/0000-0002-7809-6861
Saliha ŞAHİN https://orcid.org/0000-0003-2887-5688

1. Introduction

Interest in functional foods, defined as foods or components that contribute to body health besides essential nutrients, is rapidly increasing worldwide. Functional ingredients include various substances such as probiotics, prebiotics, phenolic substances, antioxidants, dietary fibers, oligosaccharides, vitamins, minerals, bioactive peptides, and unsaturated fatty acids such as omega-3 and phytochemicals. New functional food products are developed by adding these components to foods [1–3]. Nowadays, the most widely used functional food ingredients worldwide are i) probiotics, prebiotics, and synbiotics, ii) dietary fibers, iii) omega-3 fatty acids, oleic acid, and sterols, iv) phytoestrogens, and v) phenolic compounds [4,2]. The term probiotic is of Greek origin and means "for life." Probiotic bacteria are defined by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) as "live microorganisms that have beneficial effects on host health when consumed in sufficient amounts" [3,4]. *Lactobacillus*, and *Bifidobacterium* species are the most commonly used probiotic bacteria because they are members of the normal intestinal microbiota, and *Escherichia coli*, *Bacillus* species, *Saccharomyces boulardii* from yeasts, and *Aspergillus niger* from fungus are among the probiotic microorganisms [3,5–7]. Prebiotics are health-beneficial substrates that the intestinal microbiota selectively uses [8]. Natural sources of prebiotic components include cereals and legumes, vegetables such as chicory, onion, and garlic, and many foods such as dragon fruit and jack fruit. The primary purpose of prebiotics is to selectively stimulate the growth of probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* ssp., especially in the presence of potentially pathogenic bacterial groups. Synbiotics are defined as products that combine probiotics and prebiotics and can provide beneficial effects on health. These effects include gastrointestinal health, strengthening the immune system, inflammatory bowel diseases, obesity, diabetes, preservation of the intestinal microbiota, prevention or treatment of allergic disorders, and many other diseases [2,9].

Probiotic products are foods or dietary supplements containing microorganisms that positively affect host health. Nowadays, due to the negative perspective on drug use, the demand for the consumption of natural foods containing probiotic bacteria is rapidly increasing. Fermented dairy products are common probiotic products containing probiotic bacteria, and the natural functional properties of these products are further enhanced by probiotic bacteria [10]. For probiotic products to be effective, factors such as (i) probiotic bacteria type, (ii) daily microorganism intake, (iii) frequency of consumption, (iv) time of consumption, (v) method of consumption, and (vi) maintenance of viability in the gastrointestinal tract are important [11]. For bacteria to be applicable in foods and have the desired effect, they must survive at all stages of the food processing operation, during storage, and in the gastrointestinal tract, and can compete with the intestinal microbiota [3,6,7,12–14]. Numerous processes have been proposed to increase the viability of probiotics, including (i) selection of appropriate strains, (ii) addition of prebiotics and sugar substitutes, (iii) adjustment of fat content, (iv) inoculation rate, (v) pH adjustment, (vi) use of liquid cultures, (vii) cream fermentation level, (viii) freezing parameters, and (ix) encapsulation of probiotic culture [12,15].

Encapsulation of probiotic bacteria is a method used to enable bacteria to survive adverse environmental conditions such as low pH, bile salts, oxygen, redox potential, sugar concentration, osmotic effects, and mechanical shear and to compete with the intestinal microbiota [3,16]. For encapsulation, probiotic bacteria are loaded into capsules consisting of one or more wall materials such as alginate, starch, soy protein, and gelatin using various methods such as emulsion, extrusion, freeze-drying, and spray-drying [3,17]. Encapsulation by freeze-drying is based on freezing the product and removing ice crystals from the product by sublimation. Freeze-drying occurs in three stages. The first stage is converting water into ice crystals in freezing, shock freezing, or deep freezing. The second stage is the primary drying stage and removing ice crystals by sublimation. The third stage is the second drying stage, and the bound water is removed [18].

Chestnut (*Castanea sativa* Mill.) is a plant belonging to the Fagaceae family and an important species cultivated in Turkey, and grows widely in cool and rainy regions of the northern hemisphere. Chestnut fruit has a moisture content ranging from 40% to 64% of its fresh weight, low fat (1-10%) and cholesterol content, and does not contain gluten [20]. Studies show this fruit contains high amounts of dietary fiber, carbohydrates (40-45%), sugar, protein (3-10%), minerals, and vitamins [21]. Chestnut is a rich source of antioxidants in terms of phenolic compounds such as L-ascorbic acid, carotenoids, gallic acid, ellagic acid, and due to this content, it exhibits antimicrobial, anti-inflammatory, anti-diabetic and prebiotic properties [23–25]. Thanks to its prebiotic properties, it can be a food source for probiotic bacteria. All these properties indicate that chestnuts may benefit health and attract consumers' attention.

This study is aimed to encapsulated the probiotic bacteria *Lacticaseibacillus paracasei* LP by freeze-drying method. The encapsulation process involved using three parts of the kernel, inner shell, and outer shell of chestnut fruit as coating materials. The encapsulation efficiency of the LP capsules was determined for each coating material and additionally, conducted structure, and size analyses of the capsules using FT–IR, and SEM. Also, the viability of the capsules and free bacteria was compared during *in vitro* gastrointestinal digestion conditions. It is thought that new products can be developed using the obtained LP-chestnut fruit capsules as natural additives in food products.

2. Material and Methods

2.1. Material

Lacticaseibacillus paracasei (LP) was purchased from DSMZ (Braunschweig, Germany). MRS Agar, and MRS Broth were purchased from Biokar Diagnostics (Beauvais, France). Sodium chloride, pancreatin, and pepsin were supplied by Sigma-Aldrich (St. Louis, MO, USA). Dipotassium hydrogen phosphate was purchased from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate was purchased from Isolab (Wertheim, Germany). Soy lecithin was purchased from a local market in Bursa, Turkey. Bile salt was purchased from Edukim (Ankara, Turkey).

2.2. Methods

2.2.1. Preparation of Chestnut (*Castanea sativa* Mill.) Samples

The kernel (CFK), inner shell (CFIS), and outer shell (CFOS) of the chestnut fruit to be used as coating material in the encapsulation process were separated and dried under room conditions. The samples were then separated into small pieces with a grinder and homogenized. The ground CFK, CFIS, and CFOS coating materials were sterilized by autoclaving for just 1 min at 121°C to prevent significant changes in their nutritional values [26] (**Figure 1**). All other materials and mediums used, except for the coating materials, were sterilized at 121°C for 15 min.

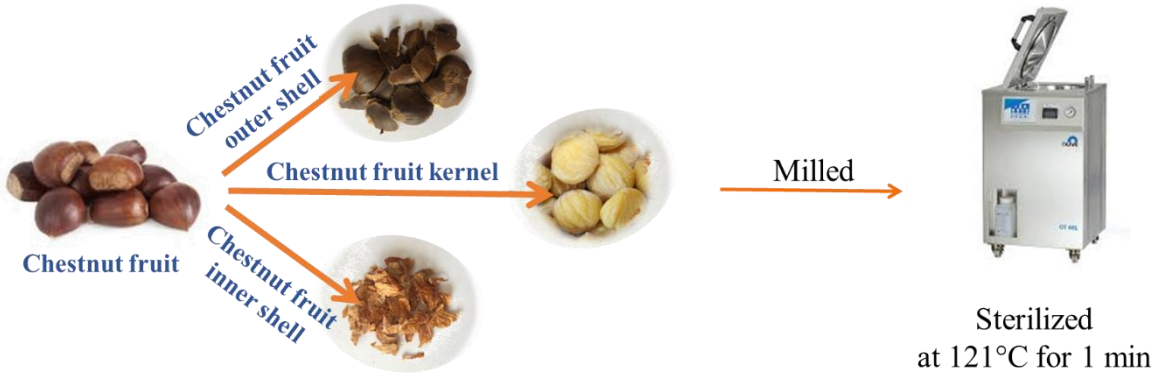

Figure 1. Preparation of coating materials

2.2.2. Activation and Development of LP Probiotic Bacteria

LP was used as probiotic bacteria. De Man Rogosa and Sharpe (MRS) liquid medium was used for activating the bacteria. Activation and development were carried out by incubating the bacterial culture in MRS-Broth medium at 37°C for 24 h for 3 at sequential time intervals, as shown in **Figure 2** [26].

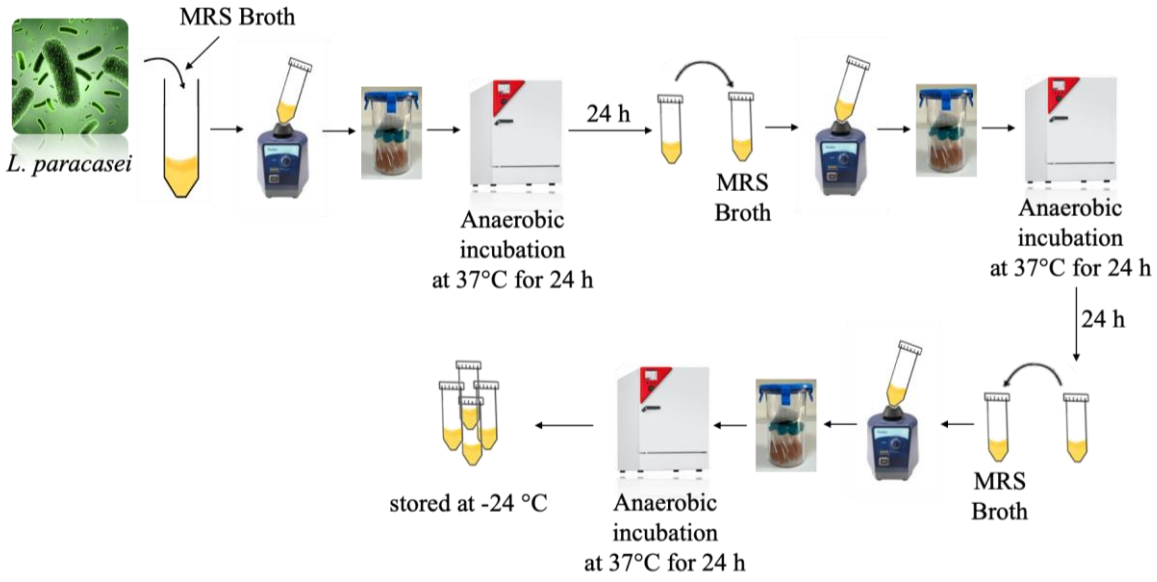

Figure 2. Development of LP probiotic bacteria by activation

2.2.3. Encapsulation of Probiotic Bacteria on Chestnut Fruit

The encapsulation of LP probiotic bacteria activated in MRS broth onto CFK, CFIS, and CFOS was conducted according to the analysis scheme in Figure 3. The encapsulation process of probiotic bacteria on CFK, CFIS, and CFOS was carried out using the freeze-drying method (Labconco, FreeZone, 2.5 Plus, temperature: -85°C, vacuum: 0.1 mbar) [27]. As a result of freeze-drying, three bacterial capsules were obtained: 1) CFK–LP, LP encapsulated on chestnut fruit kernel, 2) CFIS–LP, LP encapsulated on chestnut fruit inner shell, 3) CFOS–LP, LP encapsulated on chestnut fruit outer shell.

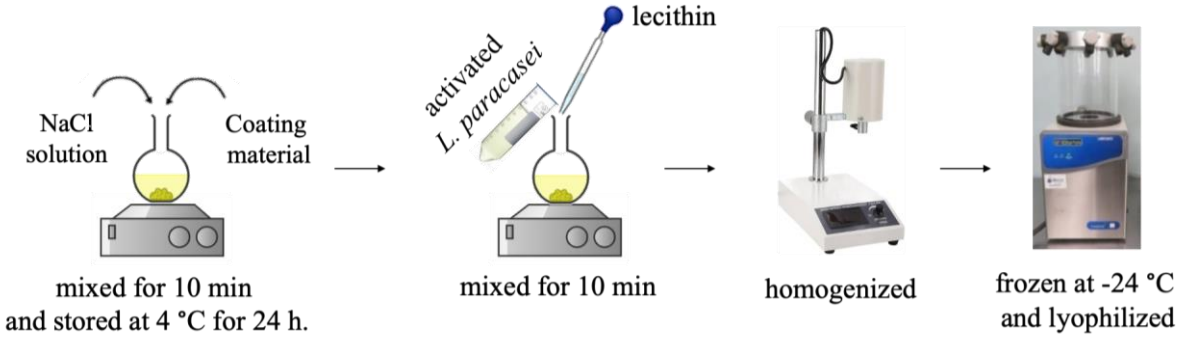

Figure 3. Encapsulation of LP probiotic bacteria on CFK, CFIS, and CFOS

2.2.4. Microbiological Analysis

Microbiological analyses of free LP (F–LP), CFK–LP, CFIS–LP, and CFOS–LP were carried out using the pour-plate method. The capsules were stirred in 0.1 M phosphate buffer (K_2HPO_4/KH_2PO_4; pH 7.0±0.2) for 30 min to open. Serial dilutions of 10^{-1}–10^{-8} of F–LP, CFK–LP, CFIS–LP, and CFOS–LP probiotic bacteria were prepared with physiological saline (FTS)

(NaCl, 0.85%; w/v). MRS-Agar liquid medium was used as medium, and after the petri dishes were placed in anaerogenic containers with AnaeroGen packages, left for anaerobic incubation at 37°C for 72 h. After incubation, colony-forming units per mL of free probiotic bacteria and per g of encapsulated probiotic bacteria were determined as log cfu/mL and log cfu/g [28,29]. Encapsulation efficiency was determined as % according to the number of viable microorganisms.

2.2.5. Characterization of capsules

FTIR and SEM analysis characterized CFK–LP, CFIS–LP, and CFOS–LP capsules. FTIR analysis of CFK–LP, CFIS–LP, and CFOS–LP capsules and their structural components (CFK, CFIS, and CFOS) was performed in the 400–4000 cm^{-1} frequency range. Scanning electron microscopy (SEM) was used to determine the size and surface morphology of CFK–LP, CFIS–LP, and CFOS–LP capsules. The samples were lyophilized before analyses.

2.2.6. *In-vitro* Gastrointestinal Digestion

To determine the viability of probiotic bacterial capsules in the *in vitro* gastrointestinal digestion system, firstly, simulated gastric fluid (SGF) and intestinal fluid (SIF) media were prepared. SGF medium was prepared by dissolving 3.2 g of pepsin in 0.2% (w/w) NaCl to make a total volume of 1 L and adjusting the pH to 2. SIF medium was prepared by dissolving 1.25 g of pancreatin, 3 g of bile salts, and 6.8 g of potassium dihydrogen phosphate in 77 mL of 0.2 N NaOH and adjusting the pH to 7 by making the total volume to 1 L [26]. *In vitro* gastrointestinal digestion of F–LP, CFK–LP, CFIS–LP, and CFOS–LP was carried out separately and simultaneously in the gastric and intestine phases (37°C, 100 rpm shaking incubator). The 9 mL of SGF and SIF medium were added separately to 1 g of CFK–LP, CFIS–LP, CFOS–LP capsules, and 1 mL of F–LP samples and incubated with mixing at 37°C for 120 min. Samples were taken from the media at the 15^{th}, 30^{th}, 45^{th}, 60^{th}, 90^{th}, and 120^{th} min, and microbiological analysis was performed. Thus, the viability of CFK–LP, CFIS–LP, CFOS–LP, and F–LP were examined separately during digestion in the simulated gastric and intestine environment. The 9 mL of SGF medium was added to 1 g of CFK–LP, CFIS–LP, CFOS–LP, and 1 mL of F–LP samples. The mixture was then digested in the gastric environment for 120 min at 37°C. Subsequently, the pH was adjusted to 7 with 1 M NaOH, and 10 mL of SIF medium was added directly to the samples. The samples were then incubated with mixing for 120 min at 37°C; after the sequential *in vitro* gastrointestinal digestion, microbiological analysis of CFK–LP, CFIS–LP, CFOS–LP, and F–LP was carried out. The number of viable microorganisms after *in vitro* gastrointestinal digestion was determined as log cfu/g for CFK–LP, CFIS–LP, CFOS–LP, and log cfu/mL for F–LP [26].

3. Results and Discussion

3.1. The viability of free and encapsulated probiotic bacteria

LP probiotic bacteria were encapsulated on CFK, CFIS, and CFOS coating materials by freeze-drying method. The viability of CFK–LP, CFIS–LP, CFOS–LP capsules, and F–LP as a result of microbiological analysis is given in **Table 1**.

Table 1. The viability of free, and encapsulated probiotic bacteria

Probiotic bacteria and capsules	Viability of probiotic bacteria
F–LP	8.78±0.03●
CFK–LP	7.53±0.02○
CFIS–LP	6.97±0.06○
CFOS–LP	7.13±0.10○

Mean ± standard deviation, F–LP: Free LP, CFK–LP: LP encapsulated with chestnut kernel CFIS–LP: LP encapsulated with chestnut inner shell, CTOS–LP: LP encapsulated with chestnut inner shell, ●: log cfu/mL, ○: log cfu/g

The viability level (7.53±0.02 log cfu/g) of CFK-LP capsules obtained using chestnut seeds followed the free LP (8.78±0.03 log cfu/g) and had the highest viability level among the capsules. Gibson and Roberfroid [30] defined prebiotics as indigestible food components that provide beneficial health effects on the host by stimulating the growth or activity of bacteria in the colon [31]. Chestnut fruit shells have high amounts of indigestible oligosaccharides and have been characterized as potent prebiotics; thus, it has been stated that they can provide modulation of microbiota [31,32]. Ozcan et al. [23] reported that chestnut flour could be used as a prebiotic for probiotic bacteria such as *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* due to its high carbohydrate and dietary fiber content. This explains that LP bacteria encapsulated with CFK (CFK–LP) showed higher viability than CFIS–LP and CFOS–LP due to the prebiotic activity of CFK.

3.2. The encapsulation efficiency of encapsulated probiotic bacteria capsules

The encapsulation efficiencies (%) of the capsules were determined using the live microorganism counts of free probiotic bacteria (F–LP) and capsules (CFK–LP, CFIS–LP, and CFOS–LP). The encapsulation efficiencies of capsules are given in **Table 2**.

Table 2. The encapsulation efficiency of capsules

Probiotic bacteria capsules	Encapsulation efficiency (%)
CFK–LP	85.76±0.12
CFIS–LP	79.38±0.05

CFOS–LP	81.21±0.02

Mean ± standard deviation, F–LP: Free LP, CFK–LP: LP encapsulated with chestnut kernel CFIS–LP: LP encapsulated with chestnut inner shell, CTOS–LP: LP encapsulated with chestnut inner shell

The encapsulation efficiency of LP probiotic bacteria was obtained at the highest rate in CFK–LP capsules with a value of 85.76±0.12%. The lowest encapsulation efficiency, with a value of 79.38±0.05%, belonged to the capsules made with the inner shell of chestnut fruit (CFIS–LP). The encapsulation efficiency of CFOS–LP capsules was determined as 81.21±0.02%. The encapsulation efficiencies of LP probiotic bacteria encapsulated with various parts of chestnut fruit were determined to be quite high and close. However, the higher viability in CFK–LP indicates that bacteria interact with the kernel from the coating materials (CFK, CFIS, and CFOS) more than the others. Thus, the viability of bacteria is maintained by the interaction of bacteria and coating material [33]. This indicates that CFK is a better coating material for LP. Encapsulation efficiency and viability are directly related to the encapsulation of probiotic bacteria, such that the CFK–LP capsule with the highest bacterial viability has the highest encapsulation efficiency. Alfaro-Galarza et al. [34] encapsulated *Lactobacillus paracasei* subsp. *paracasei* bacteria with taro and rice starch. The encapsulation efficiency of the capsules obtained with rice starch ranged between 69.72–90.74%, and the capsules obtained with taro starch ranged between 86.06–90.33% [34]. The encapsulation efficiency (%) of LP microcapsules encapsulated with whey protein isolate and LP microcapsules encapsulated with xanthan gum was determined as 89.08±1.79%, and 86.36±1.07%, respectively by Xia et al. [35]. Accordingly, the encapsulation efficiency of CFK-LP was close and compatible with the encapsulation efficiencies reported in the literature. The main reason is the difference in the capsule wall material and the method used.

3.3. Characterization of capsules

The FTIR spectra of CFK, CFK–LP, CFIS, CFIS–LP, CFOS, and CFOS–LP capsules are given in Figure 4. The absorption band in the wavenumber range of 3600–3000 cm^{-1} represents the stretching vibrations of O–H groups resulting from the presence of hydrogen bonds. It is observed that the hydrogen bond density decreases with the encapsulation process. The bands in the 3000–2800 cm^{-1} range represent the presence of molecular and aromatic C–H [36–38]. The bands in the range of 2946–2881 cm^{-1} represent C–H (CH_2) asymmetric stretching vibrations, the bands in the range of 2881–2782 cm^{-1} represent C–H (CH_2) symmetric stretching vibrations, the bands in the range of 1486–1446 cm^{-1} represent C–H (CH_2) bending (shearing) vibrations, the bands in the range of 1382–1371 cm^{-1} represent C–H (CH_2) bending (symmetric) vibrations. The bands in the 1290–1072 cm^{-1} range show the presence of C–O (alcohol) stretching vibrations. The bands in the 1795–1677 cm^{-1} range represent C–O (ester) stretching vibrations, indicating the presence of triglyceride or functional group carbonyl ester of triglycerides [36,37,39,40]. The bands in the 1200–1000 cm^{-1} range are due to the stretching vibrations in the pyronose ring in monosaccharides (C=O, C–O–C, C–OH). The 1000–800 cm^{-1} bands indicate glycoside bonds [41]. In addition, the spectral region in the range of 1500–500 cm^{-1} represents the C–H stretching, stretching vibrations of hydrocarbons, and aromatic C=C–C vibrations [36,37]. When the spectra were examined, no major changes were observed in the

spectra before and after encapsulation. This situation shows that there was no deterioration in the structure of the chestnut fruit parts after encapsulation and that the formation of some electrostatic interactions occurred more than chemical interactions. Therefore, it can be concluded that the capsule formation is based on electrostatic force. The absence of chemical changes in the structure of the coating material during the encapsulation process is essential for the coating of probiotic bacteria and preserving their vitality.

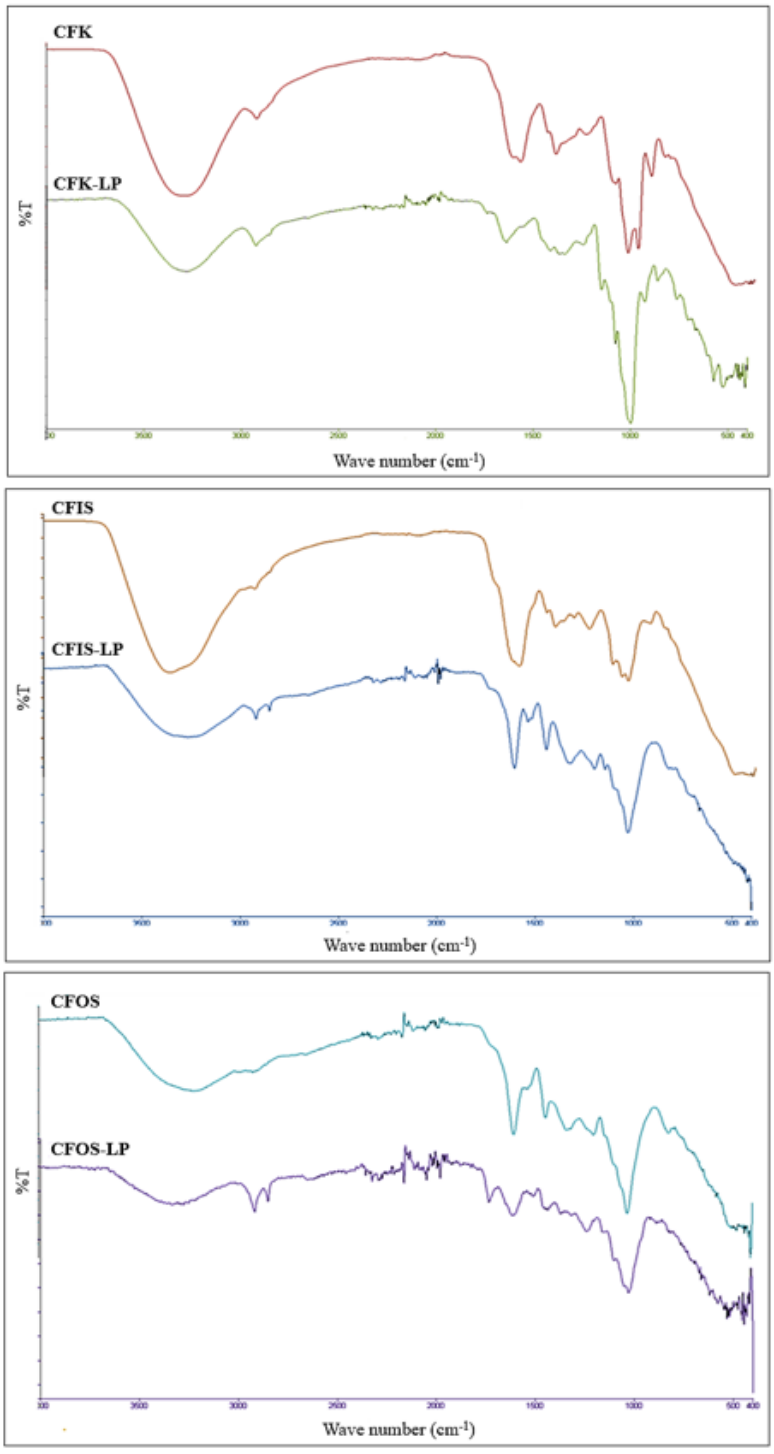

Figure 4. FTIR spectra of CFK, CFK–LP, CFIS, CFIS–LP, CFOS, and CFOS–LP.

The SEM image of CFK–LP, CFIS–LP, and CFOS–LP capsules is given in Figure 5. It is seen that the CFK–LP capsule surface has a relatively homogeneous and spherical structure, and the average capsule size is 2.210±0.130 µm. When the SEM image of CFIS–LP capsules is examined, it is seen that the capsules are both embedded in the coating material and adhere to the surface. The average dimensions of the formed CFIS–LP capsules are 5.585±0.427 µm. When the surface morphology of CFOS–LP capsules is examined, it is seen that the formed capsule sizes are not completely homogeneous, and the capsules are not entirely spherical. This situation is thought to be due to lyophilization. The average dimensions of the obtained CFOS–LP capsules are 5.764±1.004 µm.

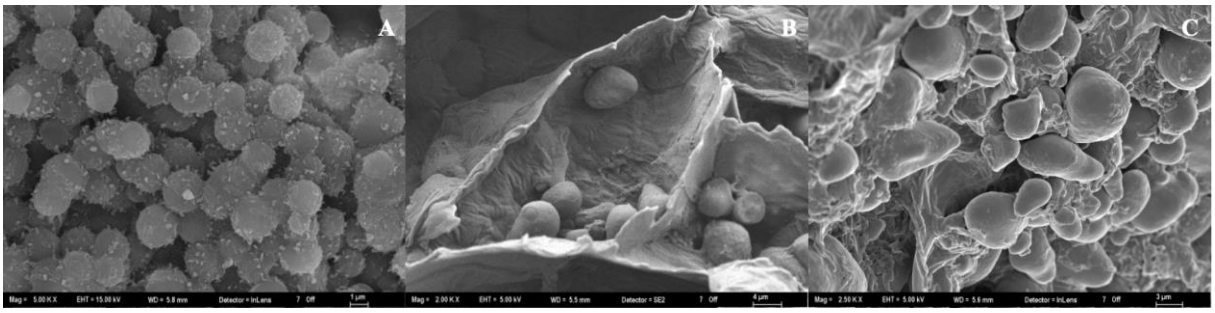

Figure 5. Surface morphology of A) CFK–LP capsules, B) CFIS–LP capsules, and C) CFOS–LP capsules

3.4. *In vitro* Gastrointestinal Digestion of LP Probiotic Bacteria

In *in vitro* gastrointestinal analyses, the digestion time in a simulated gastric environment is accepted as 90 min by some researchers, while it is accepted as 120 min by others. Similarly, the average pH range of the gastric fluid varies between 1.5 and 2.5. Release studies of free and encapsulated LP probiotic bacteria in a simulated gastric environment were carried out in a pH 2.0±0.2 environment for 120 min. The viability of LP probiotic bacteria in simulated intestinal environments was examined in a pH 7.0±0.2 environment for 120 min. The studies were carried out separately and sequentially in a simulated gastric and intestinal environment. The number of live cells (log cfu/mL-g) determined after *in vitro* gastrointestinal release analysis for free and encapsulated LP probiotic bacteria are given in Table 3.

Table 3. Viability of free, and encapsulated LP probiotic bacteria during *in vitro* gastrointestinal digestion

Medium	Digestion time (min)	Number of viable bacteria (log cfu /mL–g)			
		F–LP	CFK–LP	CFIS–LP	CFOS–LP
SGF	0	8.78±0.03	7.53±0.02	6.97±0.06	7.13±0.10
	15	6.88±0.02	7.28±0.01	4.29±0.04	6.99±0.03
	30	5.67±0.04	6.14±0.05	4.67±0.02	6.06±0.01
	45	3.04±0.01	5.64±0.09	4.64±0.10	5.10±0.06
	60	1.30±0.01	4.78±0.03	4.48±0.06	4.48±0.02

	90	nd	4.81±0.11	4.31±0.01	3.97±0.01
	120	nd	4.30±0.06	3.30±0.02	3.11±0.04
SIF	**15**	8.70±0.01	7.56±0.05	6.89±0.01	7.17±0.01
	30	8.72±0.03	7.52±0.01	6.91±0.01	7.12±0.03
	45	8.60±0.06	7.50±0.02	6.88±0.02	7.09±0.01
	60	8.58±0.01	7.47±0.03	6.93±0.06	7.11±0.05
	90	8.52±0.04	7.48±0.01	6.94±0.03	7.07±0.01
	120	8.57±0.01	7.46±0.02	6.99±0.01	7.12±0.03
SGF + SIF	**15**	6.88±0.02	7.28±0.01	4.29±0.04	6.99±0.03
	30	5.67±0.04	6.14±0.05	4.67±0.02	6.06±0.01
	45	3.04±0.01	5.64±0.09	4.64±0.10	5.10±0.06
	60	1.30±0.01	4.78±0.03	4.48±0.06	4.48±0.02
	90	nd	4.81±0.11	4.31±0.01	3.97±0.01
	120	nd	4.30±0.06	3.30±0.02	3.11±0.04
	120	nd	3.95±0.02	2.95±0.01	2.89±0.05
	150	nd	3.86±0.07	3.06±0.03	2.69±0.04
	180	nd	3.58±0.01	3.08±0.02	2.45±0.01
	210	nd	3.63±0.03	2.63±0.01	2.39±0.02
	240	nd	4.00±0.10	2.99±0.01	2.07±0.03

Mean ± standard deviation, nd: not detected, F–LP: Free LP, CFK–LP: LP encapsulated with chestnut kernel CFIS–LP: LP encapsulated with chestnut inner shell, CFOS–LP: LP encapsulated with chestnut inner shell

The viability of free and encapsulated LP probiotic bacteria in a simulated gastric environment is given in **Figure 6**. Free LP probiotic bacteria lost their viability at 90 min of digestion in the SGF medium. Encapsulated LP probiotic bacteria capsules remained highly viable throughout SGF digestion.

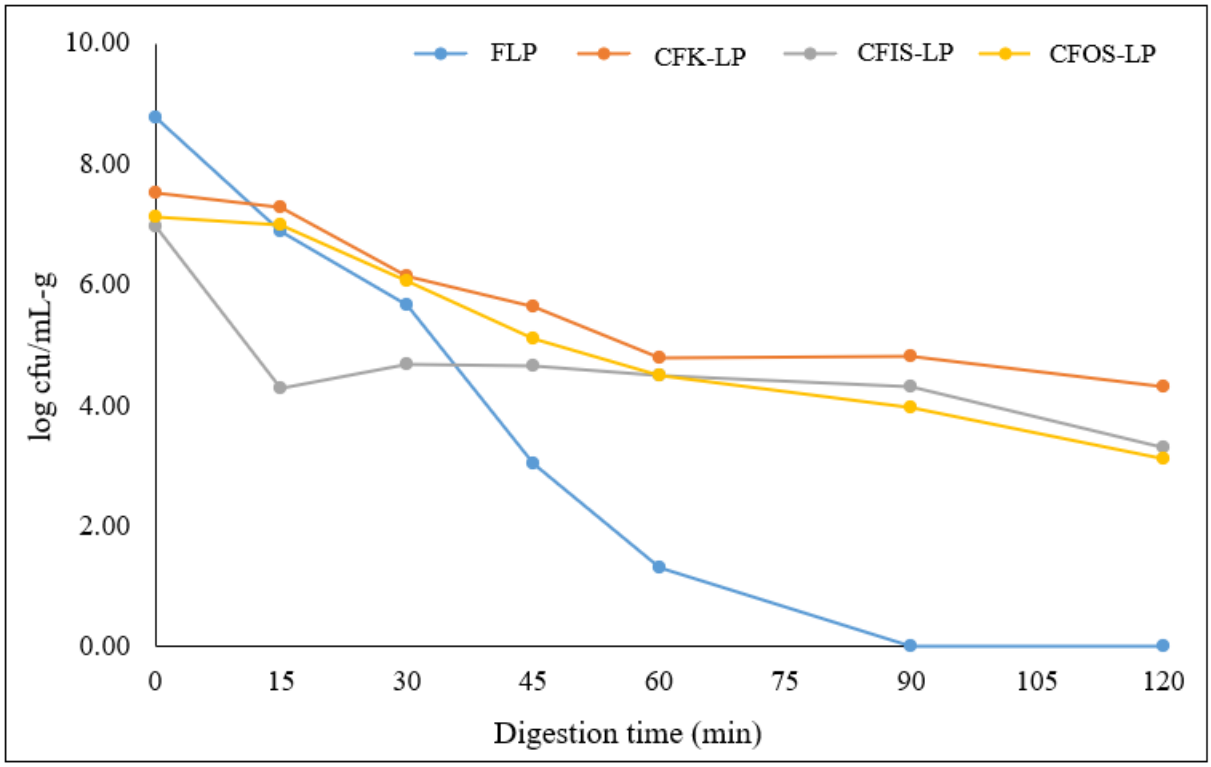

Figure 6. Viability of free, and encapsulated LP probiotic bacteria in SGF medium (F–LP: Free LP, CFK–LP: LP encapsulated with chestnut kernel CFIS–LP: LP encapsulated with chestnut inner shell, CFOS–LP: LP encapsulated with chestnut inner shell)

Figure 7 shows the viabilities of free and encapsulated LP probiotic bacteria in a simulated intestinal environment. The viabilities of free and encapsulated probiotic bacteria showed almost no change for 120 min in the SIF medium. The viability of LP probiotic bacteria was maintained in the SIF medium. Therefore, the bacteria must remain alive in the SGF medium and reach the intestinal environment.

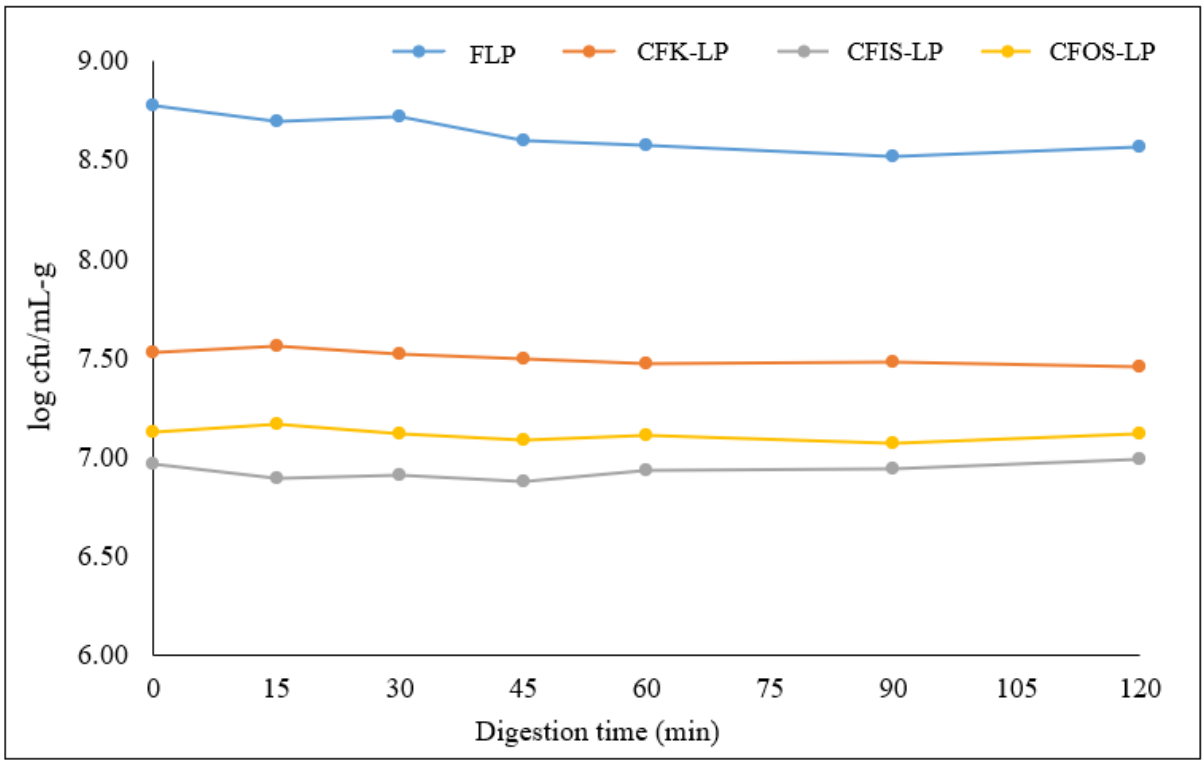

Figure 7. Viability of free, and encapsulated LP probiotic bacteria in SIF medium (F–LP: Free LP, CFK–LP: LP encapsulated with chestnut kernel CFIS–LP: LP encapsulated with chestnut inner shell, CFOS–LP: LP encapsulated with chestnut inner shell)

The viabilities of free and encapsulated LP probiotic bacteria sequentially in simulated SGF and SIF media are given in **Figure 8**. The viabilities of free, and encapsulated probiotic bacteria in SIF medium (120 min) immediately after exposure to simulated SGF medium for 120 min were compared with the initial probiotic bacteria viabilities. F–LP probiotic bacteria exposed to simulated SGF and SIF media sequentaly lost their viability completely in the SGF medium and could not reach the SIF medium alive. The numbers of viable microorganisms after 240 min of digestion of CFK–LP, CFIS–LP, and CFOS–LP capsules in SGF and SIF environments were 4.00, 2.99, and 2.07 log cfu/g, respectively.

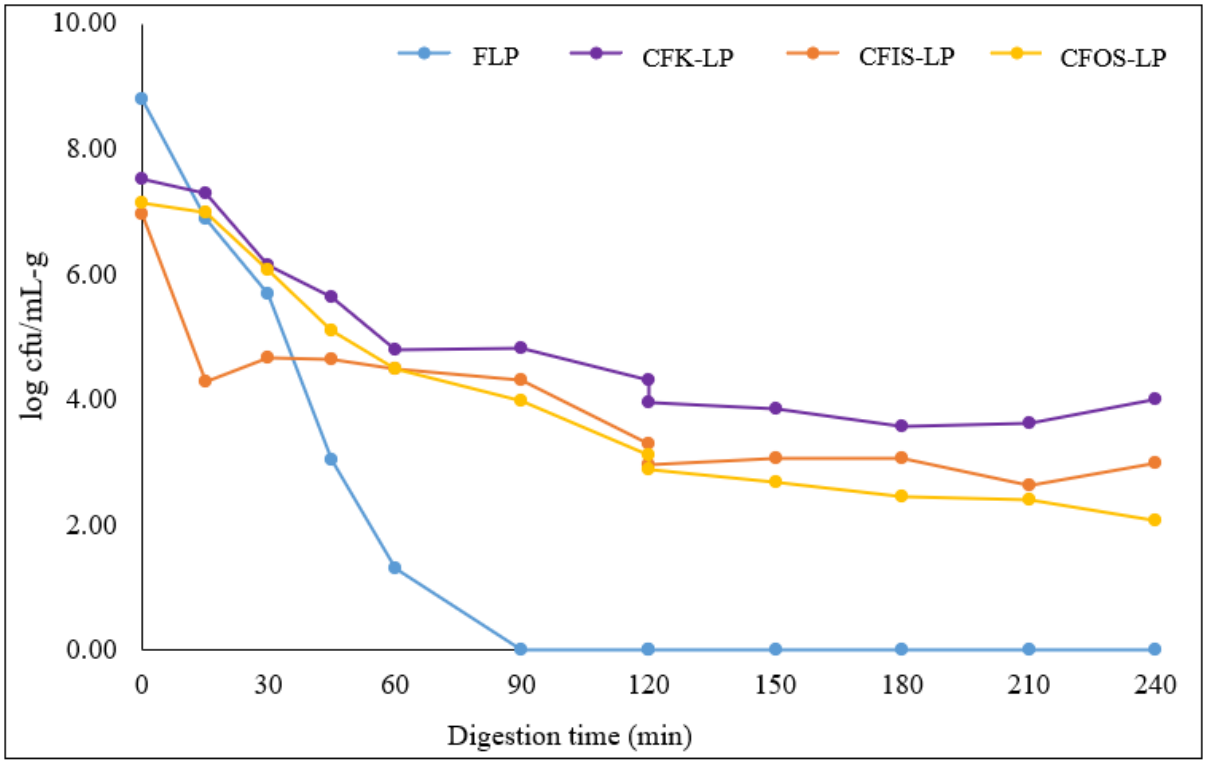

Figure 8. Viability of free, and encapsulated LP probiotic bacteria in simultaneous SGF, and SIF medium (F–LP: Free LP, CFK–LP: LP encapsulated with chestnut kernel CFIS–LP: LP encapsulated with chestnut inner shell, CFOS–LP: LP encapsulated with chestnut inner shell)

While free bacteria lose entirely their vitality in the gastric environment and cannot reach the intestinal environment, the survival of CFK–LP, CFIS–LP, and CFOS–LP capsules throughout gastric and intestinal digestion shows the achievement of encapsulation. The highest viability after gastrointestinal digestion was for CFK–LP again, as found in the results of bacterial viability and encapsulation efficiency studies of the initial capsules. This shows that CFK protects bacteria from extreme pH conditions, salt concentration, and the presence of enzymes in the gastrointestinal tract and does not entirely lose its capsule integrity. Xiao et al. [35] also reported that free bacteria significantly lost their viability in the gastric environment and that the wall materials of LP–loaded capsules acted as a physical barrier in the gastrointestinal environment and maintained their viability by reducing the interaction of bacteria with the environment.

4. Conclusion

In this study, the inner shell (CFIS), outer shell (CFOS), and kernel (CFK) of chestnut fruit were separated and ground. CFIS, CFOS, and CFK to be used as coating material were sterilized at 121°C for 1 min and used in the encapsulation process. LP probiotic bacteria were encapsulated with sterilized CFK, CFIS, and CFOS using the freeze-drying method. The encapsulation efficiencies of CFK–LP, CFIS–LP, and CFOS–LP capsules formed by encapsulating LP probiotic bacteria with CFK, CFIS, and CFOS coating materials were determined as 85.76±0.12%, 79.38±0.05%, and 81.21±0.02%, respectively. FTIR spectra were examined for the structural analysis of the prepared CFK–LP, CFIS–LP, and CFOS–LP

capsules, and no major change was observed in the structures before and after encapsulation. This situation indicates no deterioration in the structure of the chestnut parts after encapsulation, and that capsule formation is based on electrostatic force. The absence of chemical changes in the structure of the coating material during the encapsulation process is essential for the coating of probiotic bacteria and preserving their viability. The capsule dimensions were determined by taking SEM images of the prepared capsules. It was determined that the CFK–LP capsule surface had a very homogeneous and spherical structure, and the average capsule size was 2.210±0.130 μm; the CFIS–LP capsules were both embedded in the coating material and held on the surface, and the average capsule size was 5.585±0.427 μm, the CFOS–LP capsules were not completely spherical due to lyophilization. The average capsule size was 5.764±1.004 μm. The viability of free and encapsulated LP probiotic bacteria in the *in vitro* gastrointestinal digestion system was examined. F–LP bacteria lost their viability in the 90th min of gastric digestion and could not reach the intestinal environment. However, the number of live microorganisms after 240 min of sequental digestion in the gastric and intestinal environments of CFK–LP, CFIS–LP, and CFOS–LP capsules were 4.00 log cfu/g, 2.99 log cfu/g, and 2.07 log cfu/g, respectively. Considering the analysis results, LP probiotic bacteria could not reach the intestinal environment when they were free, but they could reach the intestinal environment when they were encapsulated. LP probiotic bacteria were coated with the chestnut kernel, chestnut inner shell, and chestnut outer shell by freeze-drying method, and microcapsules that can be used as food additives to create a functional food product were obtained. It is thought that with the prepared microcapsules, new products with the taste they want in the functional milk and dairy products market can be developed for the consumers, and the country's economy can contribute by increasing the industrial use of these products.

Ethics in Publishing

There are no ethical issues regarding the publication of this study.

Author Contributions

Büşra Karkar: Conceptualization; data curation, formal analysis; investigation; visualization; methodology; validation; writing – review and editing. **İlkyaz Patır:** Investigation; visualization; writing – original draft; review and editing. **Elif Tülek:** Conceptualization; data curation, formal analysis; investigation; visualization; methodology; validation. **Serenay Eyüboğlu:** Methodology; data curation; formal analysis. **Saliha Şahin:** Conceptualization; funding acquisition; resources; software; supervision.

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References

[1] Aboulfazli, F., Shori, A. B., Baba, A. S., (2016) Effects of the replacement of cow milk with vegetable milk on probiotics and nutritional profile of fermented ice cream, LWT- Food Science and Technology, 70, 261–270.

[2] Jaddu, S., Katam, S., (2018) Hub of health: Nutraceuticals and functional foods, Journal of Pharmacognosy and Phytochemistry, 7(2), 1327–1331.

[3] Sengsaengthong, S., Oonsivilai, R., (2019) Effect of microencapsulation of *Lactobacillus sp.* 21C2-10 isolated from cassava pulp on physicochemical, sensorial and microbiological characteristics of ice cream, International Food Research Journal, 26(2), 585–594

[4] Castillo M. D., Iriondo-DeHond A., Martirosyan D. M., (2018) Are functional foods essential for sustainable health?, Annals of Nutrition and Food Science, 2(1), 1015.

[5] Açu, M., Kinik, Ö., Yerlikaya, O., (2017) Functional properties of probiotic ice cream produced from goat's milk, Carpathian Journal of Food Science and Technology, 9(4), 86–100.

[6] Homayouni, A., Azizi, A., Ehsani, M. R., Yarmand, M. S., Razavi, S. H., (2008) Effect of microencapsulation and resistant starch on the probiotic survival and sensory properties of synbiotic ice cream, Food Chemistry, 111, 50–55.

[7] Niamah, A. K., Al-Manhel, A. J., Al-Sahlany, S. T. G., (2018) Effect microencapsulation of *Saccharomyces boulardii* on viability of yeast *in vitro* and ice cream, Carpathian Journal of Food Science and Technology, 10(3), 100–107.

[8] Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., Scott, K., Stanton, C., Swanson, K. S., Cani, P. C., Verbeke, K., Reid, G., (2017) Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics, Nature Reviews Gastroenterology & Hepatology, 14(8), 491–502.

[9] Markowiak, P., Śliżewska, K., (2017) Effects of probiotics, prebiotics, and synbiotics on human health, Nutrients, 9(9), 1–30.

[10] Yılmaz-Ersan, L., Kurdal, E., (2014) The production of Set-Type-Bio-Yoghurt with commercial probiotic culture, International Journal of Chemical Engineering and Applications, 5(5), 402–408.

[11] Santiago-López, L., Hernandez-Mendoza, A., Garcia, H. S., Mata-Haro, V., Vallejo-Cordoba, B., Gonzalez-Cordova, A. F., (2015) The effects of consuming probiotic-fermented milk on the immune system: A review of scientific evidence, International Journal of Dairy Technology, 68(2), 153–303.

[12] Champagne, C. P., Raymond, Y., Guertin, N., Belanger, G., (2015) Effects of storage conditions, microencapsulation and inclusion in chocolate particles on the stability of probiotic bacteria in ice cream, International Dairy Journal, 47, 109–117.

[13] Chaikham, P., Rattanasena, P., (2017) Combined effects of low-fat ice cream supplemented with probiotics on colon microfloral communities and their metabolites during fermentation in a human gut reactor, Food Bioscience, 17, 35–41.

[14] Cruz, A. G., Antunes, A. E. C., Sousa, A. L. O. P., Faria, J. A. F., Saad, S. M. I., (2009) Ice-cream as a probiotic food carrier, Food Research International, 42, 1233–1239.

[15] Akalın, A. S., Erışır, D., (2008) Effects of inulin and oligofructose on the rheological characteristics and probiotic culture survival in low-fat probiotic ice cream, Journal of Food Scıence, 73(4), 184–188.

[16] Corona-Hernandez, R. I., Álvarez-Parrilla, E., Lizardi-Mendoza, J., Islas-Rubio, A. R., de la Rosa, L. A., Wall-Medrano, A., (2013) Structural stability and viability of microencapsulated probiotic bacteria: A review, Comprehensive Reviews in Food Science and Food Safety, 12(6), 614–628.

[17] Meena, K. K., Taneja, N. K., Ojha, A., Meena, S., (2023) Application of spray-drying and freeze-drying for microencapsulation of lactic acid bacteria: A review, Annals of Phytomedicine, 12(1), 706–716.

[18] Nedovic, V., Kalusevic, A., Manojlovic, V., Levic, S., Bugarski, B., (2011) An overview of encapsulation technologies for food applications, Procedia Food Science, 1, 1806–1815.

[19] López-Orozco, R., Hernández-Ceballos, M. Á., Galán, C., García-Mozo, H., (2020) Atmospheric pathways and distance range analysis of *Castanea* pollen transport in southern Spain, Forests, 11(10), 1092.

[20] Santos, M. J., Pinto, T., Vilela, A., (2022) Sweet chestnut (*Castanea sativa* Mill.) nutritional and phenolic composition interactions with chestnut flavor physiology, Foods, 11(24), 4052.

[21] Payne, J. A., Jaynes, R. A., Kays, S. J., (1983) Chinese chestnut production in the United States: Practice, problems and possible solutions, Economic Botany, 37(2), 187–200.

[22] Suna, S., Avşar, B., Koçer, S., Çopur, Ö. U., (2021) Effects of different pretreatments on the physicochemical characteristics and quality criteria of chestnut (*Castanea sativa* Mill.) pickle: A new value-added product, Journal of Food Processing and Preservation, 45(7), e15669.

[23] Ozcan, T., Yilmaz-Ersan, L., Akpinar-Bayizit, A., Delikanli, B., (2017) Antioxidant properties of probiotic fermented milk supplemented with chestnut flour (*Castanea sativa* Mill), Journal of Food Processing & Preservation, 41, 1–9.

[24] Usta-Gorgun, B., Yilmaz-Ersan, L., Sahin, S., (2022) Optimization of formulation and process conditions of chestnut-based functional beverage using response surface methodology, Journal of Food Science and Technology-Mysore, 59, 3210–3219.

[25] Yilmaz-Ersan, L., Ozcan, T., Akpinar-Bayizit, A., Usta-Gorgun, B., Keser, G., Ciniviz, M., Demiray-Teymuroglu, M., (2023) The probiotic chestnut-based dairy matrix: Influence on the metabolomic formation and the nutritional quality, International Journal of Dairy Technology, 76(4), 895–908.

[26] Karkar, B., Şahin, S., Yılmaz-Ersan, L., Akça, B., Güneş, M. E., Özakın, C., (2024) Encapsulation of *Lacticaseibacillus casei* and *Lactobacillus acidophilus* using *Elaeagnus angustifolia* L. flour as encapsulating material by emulsion method, Food Science & Nutrition, 00, 1–16.

[27] Maleki, O., Khaledabad, M. A., Amiri, S., Asl, A. K., Makouie, S., (2020) Microencapsulation of *Lactobacillus rhamnosus* ATCC 7469 in whey protein isolate-crystalline nanocellulose-inulin composite enhanced gastrointestinal survivability, LWT, 126, 109224.

[28] Chandramouli, V., Kalasapathy, K., Peiris, P., Jones, M., (2004) An improved method of microencapsulation and its evaluation to protect *Lactobacillus* spp. in simulated gastric conditions, Journal of Microbiological Methods, 56, 27–35.

[29] Tharmaraj, N., Shah, N. P., (2003) Selective enumeration of *Lactobacillus delbrueckii* ssp. *bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, Bifidobacteria, Lactobacillus casei, Lactobacillus rhamnosus* and *Propionibacteria,* Journal of Dairy Science, 86(7), 2288-2296.

[30] Gibson, G. R., Roberfroid, M. B., (1995) Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics, Journal of Nutrition, 125(6), 1401–1412.

[31] Pinto, D., de la Luz Cádiz-Gurrea, M., Vallverdú-Queralt, A., Delerue-Matos, C., Rodrigues, F., (2021) *Castanea sativa* shells: A review on phytochemical composition, bioactivity and waste management approaches for industrial valorization, Food Research International, 144, 110364.

[32] Braga, N., Rodrigues, F., Oliveira, M. B. P. P., (2015) *Castanea sativa* by-products: A review on added value and sustainable application, Natural Product Research, 29(1), 1–18.

[33] Jouki, M., Khazaei, N., Rashidi-Alavijeh, S., Ahmadi, S., (2021) Encapsulation of *Lactobacillus casei* in quince seed gum-alginate beads to produce a functional synbiotic drink powder by agro-industrial by-products and freeze-drying, Food Hydrocolloids, 120, 106895.

[34] Alfaro-Galarza, O., López-Villegas, E. O., Rivero-Perez, N., Tapia-Maruri, D., Jiménez-Aparicio, A. R., Palma-Rodríguez, H. M., Vargas-Torres, A., (2020) Protective effects of the use of taro and rice starch as wall material on the viability of encapsulated *Lactobacillus paracasei* subsp. *paracasei*, LWT, 117, 108686.

[35] Xiao, Y., Han, C., Yang, H., Liu, M., Meng, X., Liu, B., (2020) Layer (whey protein isolate)-by-layer (xanthan gum) microencapsulation enhances survivability of *L. bulgaricus* and *L. paracasei* under simulated gastrointestinal juice and thermal conditions, International Journal of Biological Macromolecules, 148, 238–247.

[36] Coates, J., (2000) Interpretation of Infrared spectra, a practical approach, in Encyclopedia of Analytical Chemistry, Meyers, R. A. (Ed.), Wiley, Chichester, UK, 10815–10837.

[37] El Mouftari, M., Mahjoubi, F. Z., Kzaiber, F., Terouzi, W., Ali, G. A. M., Souhassou, S., Oussama, A., (2021) Study of oleaster oil's falsification by ATR-FTIR and chemometrics tools, Egyptian Journal of Chemistry, 64(6), 2747–2755.

[38] Hirri, A., Gammouh, M., Gorfti, A., Kzaiber, F., Bassbasi, M., Souhassou, S., Balouki, A., Oussama, A., (2015) The use of Fourier transform mid infrared (FT-MIR) spectroscopy for detection and estimation of extra virgin olive oil adulteration with old olive oil, Sky Journal of Food Science, 4(5), 60–66.

[39] Guillén, M. D., Cabo, N., (1997) Characterization of edible oils and lard by fourier transform infrared spectroscopy. Relationships between composition and frequency of concrete bands in the fingerprint region, JAOCS, Journal of the American Oil Chemists' Society, 74(10), 1281–1286.

[40] Rohman, A., Man, Y. B. C., (2010) Fourier transform infrared (FTIR) spectroscopy for analysis of extra virgin olive oil adulterated with palm oil, Food Research International, 43(3), 886–892.

[41] Vaziri, A. S., Alemzadeh, I., Vossoughi, M., Khorasani, A. C., (2018) Co-microencapsulation of *Lactobacillus plantarum* and DHA fatty acid in alginate-pectin-gelatin biocomposites, Carbohydrate Polymers, 199, 266–275.