

Optimization of Entrapment Substances for Microencapsulation of *Lactobacillus plantarum* and *Lactobacillus casei* Shirota Against Gastric Conditions

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Abstract

Microencapsulation is a promising method that has considerable effects on protection of probiotic viability. A variety of coating materials have been utilized to enhance the stability of probiotic microorganisms during the transition through gastrointestinal tract. The aim of this research was to determine optimum coating material combinations for probiotic microencapsulation against gastric conditions. Fructooligosaccharides, peptide, sodium alginate, gelatin and gellan gum were used as entrapment substances to microencapsulate *Lactobacillus plantarum* and *Lactobacillus casei* Shirota with extrusion technique. The response surface technique was applied to detect the optimum proportion of encapsulation substances against gastric condition. Microencapsulation protected probiotic cultures against stress factors such as simulated gastric juice and bile-salt solution. Optimum rate of encapsulation substances varied according to the type of probiotic bacteria. Test results showed that *L. plantarum* should be coated with 1.5% alginate, 0.92% gellan gum, 0.18% gelatin, 0.36% peptide and 1.31% fructooligosaccharides for highest protection. *L. casei* Shirota should also be coated with 2% alginate, 0.98% gellan gum, 0.51% gelatin, 0.86% peptide and 1.98% fructooligosaccharides for highest protection. This research concluded that microencapsulation with encapsulation materials at optimum concentration provided improved protection for the probiotics.

Keywords: *Lactobacillus casei* Shirota, *Lactobacillus plantarum*, Microencapsulation, Response surface method, Extrusion

Lactobacillus plantarum ve *Lactobacillus casei* Shirota'nın Gastrik Koşullara Karşı Mikroenkapsülasyonu İçin Kaplama Materyallerinin Optimizasyonu

Öz

Mikroenkapsülasyon, probiyotik canlılığının korunması üzerinde önemli etkileri olan umut verici bir yöntemdir. Probiyotik mikroorganizmaların gastrik koşullara karşı dayanımını arttırmak için çeşitli kaplama materyallerinden yararlanılmıştır. Bu çalışmanın amacı gastrik koşullara karşı probiyotik mikroenkapsülasyonu için ideal kaplama materyali kombinasyonunu belirlemektir. Fruktooligosakkarit, peptit, sodyum aljinat, jelatin ve gellan gam ekstrüzyon tekniği ile *Lactobacillus plantarum* ve *Lactobacillus casei* Shirota'yı mikroenkapsüle etmek için tutuklayıcı maddeler olarak kullanılmıştır. Gastrik koşullara karşı enkapsülasyon materyallerinin ideal oranları cevap yüzey tekniği ile elde edilmiştir. Mikroenkapsülasyon işlemi yapay gastrik su ve safra tuzu çözeltisi gibi stres faktörlerine karşı probiyotik kültürleri korumuştur. Kaplama materyallerinin ideal oranları probiyotik bakteri türüne göre değişmiştir. Test sonuçları yüksek düzeyde koruma için *L. plantarum*'un %1.5 aljinat, %0.92 gellan gam, %0.18 jelatin, %0.36 peptit ve %1.31 FOS ile kaplanması gerektiğini göstermiştir. Yüksek düzeyde koruma için *L. casei* Shirota ise %2 aljinat, %0.98 gellan gam, %0.51 jelatin, %0.86 peptit ve %1.98 FOS ile kaplanmalıdır. Bu araştırma, en uygun konsantrasyonda kaplama materyalleri ile mikroenkapsülasyonun, probiyotiklerin canlılığını iyileştirdiği sonucunu çıkarmıştır.

Anahtar sözcükler: *Lactobacillus casei* Shirota, *Lactobacillus plantarum*, Mikroenkapsülasyon, Cevap yüzey tekniği, Ekstrüzyon

INTRODUCTION

Probiotics have numerous useful properties on human

health. Because of their beneficial effects, probiotic cultures often used in several functional food products ^[1]. As a matter of fact, there is a recent trend towards consumption



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of functional foods worldwide [2-4]. World Health Organization (WHO) reported that probiotics are live microorganisms that, "when administered in sufficient amounts, confer a health benefit on the host" [5]. *Lactobacillus* and *Bifidobacterium* species have been the most commonly known probiotics and play an important role in the function and integrity of the intestinal ecosystem and the immune system [6-8]. Especially, *L. plantarum* and *L. casei* Shirota are the most popular and often performed probiotics in food production.

Probiotic bacteria have to survive during gastric transit and have positive effects on health of the host. Maintenance of probiotic viability at the time of consumption and ensuring of sufficient probiotic amounts are challenges on probiotic manufacturers [9,10]. Especially stress factors such as low pH, enzymes and bile salts in gastrointestinal system lead to a negative impact on probiotic robustness and performance. Inhibitory activity of stomach acid and bile salt should be overcome to retain probiotic viability and functionality [9,11]. For the therapeutic effects of probiotics, viable cell counts should be higher than or equal to 10^7 CFU/g or mL of product and probiotic bacteria should be able to survive under gastrointestinal conditions [12,13]. In this case, microencapsulation technique is an alternative and effective strategy to protect survival of probiotics against hard conditions. The promised health benefits of probiotics were achieved with microencapsulation [14,15]. Two methods often used for microencapsulation are emulsion and extrusion. Extrusion method has many advantages that it is simple and inexpensive method with gentle operations, does not involve deleterious solvents, does not cause probiotic cell injuries and can be done under aerobic and anaerobic conditions [15,16]. In extrusion technique, probiotic bacteria are added into the hydrocolloid solution (mostly alginate) for entrapping in the gel matrix and then the cell suspension is passed through the syringe needle to form droplets, which free-fall into the solidification solution [8].

There are differences in the characteristics of probiotic strains and the right encapsulation method should be selected for each probiotic [13]. The physicochemical properties of the capsules have a significant impact on the viability of microencapsulated probiotic bacteria. Efficiency of microencapsulation can show differences depending on the kind and the concentration of the encapsulation substances, particle dimensions, initial viable cell counts and microbial strains. As a matter of fact, choice of capsule materials plays an important role in the bacterial cell protection against environmental stresses and affect release of probiotic cells as available and metabolically active state in gastrointestinal system. The appropriate encapsulation substances act as protective agent and may offer the highest robustness of the probiotics in microcapsules during transport from digestive tract of host and/or during exposure to adverse conditions from

food matrices [17]. Alginate, gelatin and gellan gum are the most often used polymers for microencapsulation of probiotic bacteria due to their simplicity, non-toxicity, biocompatibility, excellent membrane-forming ability and low cost [18-20].

The other approach utilized to achieve sufficiently high numbers of probiotics in intestinal systems is the use of "prebiotics" [21]. Prebiotics were used to refer non digestible food substances that induce the growth or activity of beneficial microorganisms in the gastrointestinal tract of host [12,22,23]. As a matter of fact, food industry and researchers showed a major concern in the use of prebiotics because of synergistic effects between probiotics and prebiotics [2]. Fructooligosaccharides (FOS) are the most commonly used prebiotics and nowadays peptides are used as growth promoter [21]. However, research on the use of prebiotic in microcapsules is scarce and more work is needed to measure the stability of these capsules system in gastric conditions.

In the present study, determination of optimum entrapment substances combinations for probiotic microencapsulation against gastric conditions and enhancement of probiotic survival were aimed.

MATERIAL and METHODS

Bacterial Strains and Culture Conditions

Probiotic strains used in this research are *L. plantarum* (Blessing-Biotech GmbH-Stuttgart/Germany) and *L. casei* Shirota (Yakult-RIUM/The Netherlands).

Probiotic cultures were grown in de Man, Rogosa Sharpe (MRS) broth (Merck, Germany) at 37°C for 24 h. After incubation, cells were removed by centrifugation (3000×g, 10 min at 4°C), washed and resuspended twice in saline solution. The final cell concentrations of probiotic cultures were adjusted to 10^{10} CFU/mL for microencapsulation.

Optimization of Entrapment Substances for Probiotic Cultures

The kind and proportion of the entrapment substances have effect on the stability of probiotic strains. The detection of entrapment substances in optimum compositions is crucial for highest protection [24]. For this reason, response surface technique was performed for optimization of entrapment substances [25]. Modelling of this experiment was based on variables (coating materials) and responses (probiotic cell viability). Modelling results from the response surface technique was detected with Design expert 6.02 software (Table 1). Alginate, gelatin, gellan gum, FOS and peptide were selected as entrapment substances. Also, responses in this experiment were based on viable cell counts of probiotics in simulated gastric fluid (SGF) and bile-salt solution (BSS) were evaluated.

Table 1. Variables of experiment

Combination	Alginate (%)	Gellan Gum (%)	Gelatin (%)	Peptide (%)	FOS (%)
1	2.00	1.00	0.00	1.00	0.00
2	2.00	0.00	0.00	1.00	2.00
3	0.50	0.00	0.00	0.00	0.00
4	0.50	0.00	1.00	1.00	2.00
5	0.50	1.00	1.00	0.00	2.00
6	1.25	0.50	0.50	0.50	1.00
7	1.25	0.50	0.50	0.50	2.00
8	0.50	1.00	0.00	1.00	2.00
9	1.25	0.50	0.50	1.00	1.00
10	1.25	1.00	0.50	0.50	1.00
11	2.00	1.00	1.00	0.00	0.00
12	1.25	0.50	0.00	0.50	1.00
13	1.25	0.50	0.50	0.50	1.00
14	2.00	0.00	1.00	1.00	0.00
15	1.25	0.50	0.50	0.00	1.00
16	0.50	1.00	1.00	1.00	0.00
17	1.25	0.00	0.50	0.50	1.00
18	1.25	0.50	0.50	0.50	0.00
19	2.00	0.00	1.00	0.00	2.00
20	1.25	0.50	1.00	0.50	1.00
21	2.00	1.00	0.00	0.00	2.00
22	0.50	0.50	0.50	0.50	1.00
23	2.00	0.50	0.50	0.50	1.00
24	1.25	0.50	0.50	0.50	1.00
25	1.25	0.50	0.50	0.50	1.00
26	1.25	0.50	0.50	0.50	1.00

Microencapsulation of Probiotic Cultures

Fructooligosaccharides and peptide are prebiotics-promoting probiotic growth and often used for synbiotic effect from synergy between probiotics and prebiotics. Previous researchers were mainly applied calcium alginate, gelatin, and gellan gum as coating materials because these entrapment substances provide better protection for probiotics in food and in the intestinal tract. The proper selection of probiotic strains, prebiotics and coating materials is crucial in obtaining a therapeutic effect [2,19,25,26]. On this sense, microencapsulation in this study were performed with entrapment substances supporting probiotic growth and protection. Probiotic cultures (*L. plantarum* and *L. casei* Shirota) were microencapsulated with entrapment substances consisting of 26 different combinations (Table 1) according to extrusion technique. As a preliminary, 26 different solutions containing sodium alginate (0.5-2%), gelatin (0-1%), gellan gum (0-1%), FOS (0-2%) and peptide (0-1%) were sterilized by autoclaving (121°C for 15 min) and cooled to 40°C. For microencapsulation by extrusion technique, probiotic cell suspension including

L. plantarum or *L. casei* Shirota (10^{10} CFU/mL) was added into 50 mL of this sterile coating material solution to yield a final concentration of 1% (V/V). This mixture was placed in a syringe with 0.11 mm needle and injected into sterilized gelling solution (0.1 M CaCl₂). The capsules, 0.5 mm in diameter were retained for 1 h for solidification and then aseptically transferred into a sterile petri dishes [19,24]. Probiotic microcapsules obtained in this study were showed in Fig. 1.

Resistance of Entrapped Probiotic Strains to SGF and BSS

A solution consisted of 0.5% sodium chloride and 0.3% pepsin was adjusted to pH 2 with 1 N HCl and was used for the determination of resistance to SGF. The microencapsulated probiotic bacteria (1 g) were added into SGF solution (10 mL) in flask and incubated in shaking water bath (100 rpm) at 25°C for 1 h. To determine the resistance to BSS, microencapsulated probiotics (1 g) were inoculated into solution of 2% ox gall powder (Sigma, USA) and incubated in shaking water bath (100 rpm) at 25°C for 1 h [24,26].



Fig 1. Microencapsulated probiotic cells

Enumeration of Probiotic Strains in Microcapsules

One gram of microencapsulated probiotic bacteria samples were diluted with 9 mL of sterile phosphate buffer solution (0.1 M, pH 7.0) and allowed to homogenize for 15 min. Probiotic bacteria (CFU/g) were plated on de Man, Rogosa Sharpe Agar (Merck, Germany) and incubated at anaerobic conditions (Anaerocult A, Merck) for 48 h at 30°C [19,27].

RESULTS

Viable cell counts in microcapsules containing probiotic strains (*L. plantarum* or *L. casei* Shirota) were measured before and after treatment to SGF and BSS conditions and this measurement results were given in Table 2. Additionally, reduction in viable cell counts of probiotic strains after treatment of SGF and BSS for each combination of coating materials (from 1 to 26) was calculated from results in

Table 2. Responses of experiment (log CFU/g)

LP Counts Before SGF/BSS	LP Counts After SGF	LP Reduction After SGF	LP Counts After BSS	LP Reduction in BSS	LC Counts Before SGF/ BSS	LC Counts After SGF	LC Reduction in SGF	LC Counts After BSS	LC Reduction BSS
9.04	7.54	1.50	7.53	1.51	9.20	7.00	2.20	7.79	1.41
9.87	8.02	1.85	7.82	2.05	9.14	7.73	1.41	7.04	2.10
9.00	6.84	2.16	7.86	1.14	9.04	7.00	2.04	8.41	0.63
9.23	6.47	2.76	7.69	1.54	9.63	7.04	2.59	8.00	1.63
9.79	6.60	3.19	8.60	1.19	9.11	7.36	1.75	8.14	0.97
9.11	7.00	2.11	8.34	0.77	9.61	7.17	2.44	7.56	2.05
9.85	6.60	3.25	8.20	1.51	9.95	7.04	2.91	8.34	1.61
9.90	8.07	1.83	7.80	2.10	9.07	7.20	1.87	7.47	1.60
9.97	7.90	2.07	8.43	1.54	9.04	7.93	1.11	7.69	1.35
9.88	8.07	1.81	7.85	2.03	9.14	7.73	1.41	7.03	2.11
9.96	7.73	2.23	7.20	2.76	9.95	5.60	4.35	8.27	1.68
9.96	7.07	2.89	8.34	1.62	9.07	6.60	2.47	8.14	0.93
9.49	7.60	1.89	7.60	1.89	9.04	7.17	1.87	7.30	1.74
9.97	7.91	2.06	8.23	1.74	9.14	7.93	1.21	7.65	1.49
9.36	8.04	1.32	8.32	1.04	9.07	7.82	1.25	8.00	1.07
9.07	6.47	2.60	7.77	1.30	9.69	7.32	2.37	8.00	1.69
9.00	5.00	3.00	6.69	2.31	9.07	5.30	3.77	6.11	2.96
9.04	7.77	1.27	8.00	1.04	9.04	7.11	1.93	8.00	1.04
9.67	7.11	2.56	8.04	1.93	9.17	7.43	1.74	8.20	0.97
9.88	8.03	1.85	7.83	2.05	9.11	7.71	1.40	7.00	2.11
9.96	8.14	1.82	8.17	1.79	9.00	7.32	1.68	8.00	1.00
9.85	8.02	1.83	7.84	2.01	9.14	7.72	1.42	7.02	2.12
9.88	8.04	1.84	7.83	2.05	9.14	7.72	1.42	7.00	2.14
9.30	8.36	0.94	7.60	1.70	9.50	7.74	1.76	7.66	1.84
9.84	9.07	0.77	8.60	1.24	9.62	5.60	4.02	8.20	1.42
9.88	8.04	1.84	7.84	2.04	9.14	7.72	1.42	7.04	2.10

LP: *L. plantarum*, LC: *L. casei* Shirota, SGF: simulated gastric fluid, BSS: bile-salt solution, Reduction: Difference between probiotic viable cell counts before SGF or BSS and after SGF or BSS

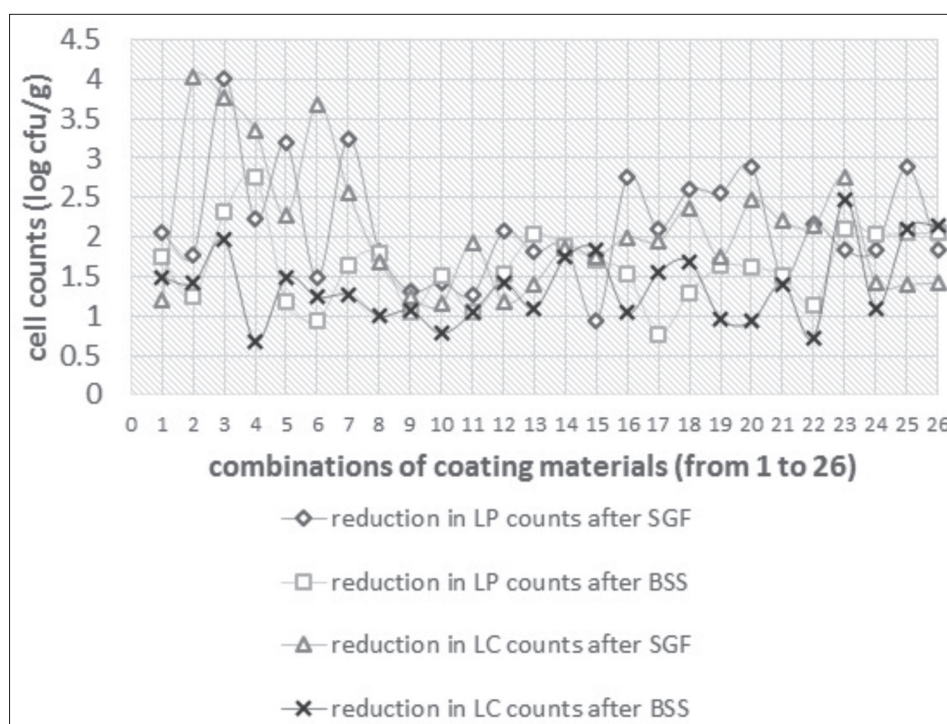


Fig 2. Reduction in viable cell counts of probiotic strains after treatment of SGF and BSS

Table 2 and this reduction was shown in Fig. 2.

As observed from results of this research, probiotic viable cell counts in microcapsules changed between 9.04 and 9.97 log CFU/g before exposure to SGF and BSS, while probiotic viable cell counts in microcapsules ranged from 5.0 to 8.60 log CFU/g after exposure to SGF and BSS.

Simulated gastric fluid conditions caused a drop from 0.77 to 3.25 log CFU/g in *L. plantarum* counts and from 1.11 to 4.35 log CFU/g in *L. casei* Shirota counts, respectively. After BSS condition, a reduction in *L. plantarum* and *L. casei* Shirota counts varied from 0.77 to 2.76 log CFU/g and from 0.63 to 2.96 log CFU/g, respectively.

Optimum concentrations of 5 different entrapment substances were predicted through the model established with response surface methodology. As seen in Table 1, concentrations of entrapment substances tested in this study were adjusted between 0.5-2% for alginate, 0-1% for gellan gum, 0-1% for gelatin, 0-1% for peptide and 0-2% for FOS. The encapsulation material composition and concentration providing the highest probiotic cell viability were calculated by using prediction model according to results obtained in Table 1. The optimum values for the obtention of microcapsules with highest probiotic robustness were found as the mix of 1.5% alginate, 0.92% gellan gum, 0.18% gelatin, 0.36% peptide and 1.31% FOS for *L. plantarum* and as the mix of 2% alginate, 0.98% gellan gum, 0.51% gelatin, 0.86% peptide and 1.98% FOS for *L. casei* Shirota.

DISCUSSION

It is known from literature works that free cells of probiotic

strains are more susceptible than microencapsulated cells under gastrointestinal conditions. As a matter of fact, several researchers reported that microencapsulation provided additional protection to probiotic cells with a physical barrier against stress factors in intestinal system and exhibited more robustness during gastric transit than their free cell [28-31]. Based on previous studies, there was no need to test the viability of free probiotic strains after exposure to SGF and BSS because encapsulation enhances the viability of probiotic strains. Microencapsulation technique is required to ensure survival or stability of probiotics bacteria during the passage to digestive tract of host. However, coating materials used in microencapsulation had differently effect on protection of probiotic against adverse factors [32]. In accordance with this, the present results showed that resistance in probiotic viability changed according to coating material combinations (26 different microcapsule) after SGF and BSS. The use of prebiotic materials (peptide and FOS) in addition to gelling agents such as sodium alginate and gelatin for microencapsulation provides a better protection to probiotic bacteria. These prebiotic agents may act as a supporter of probiotic viability. As a matter of fact, various coating material combinations with regard to their compositions and concentrations have caused different levels of probiotic resistance against gastric conditions according to earlier studies [18,19,26,33]. Similarly, the present study confirmed this different effect of coating material combinations on probiotic resistance. Additionally, statistical analysis showed that microencapsulation with different coating material combinations had effect on resistance of probiotic strains against SGF at the 95% confidence level.

A reduction in *L. plantarum* counts and *L. casei* Shirota counts changed approximately between 1 and 4 log CFU/g after SGF and between 1 and 2 log CFU/g after BSS. This situation detected that these probiotic bacteria were more resistant to BSS than SGF. Chen et al.^[19] reported that that probiotic strains exhibited higher resistance to acidic conditions than to bile salts. However, in another study, probiotic *L. rhamnosus* were found more resistant to bile salts than to acid^[34]. This situation considered that resistance of probiotics against SGF and BSS conditions could change according to strains.

Stimulated Gastric Fluid conditions caused a drop from 0.94 to 4 log CFU/g in *L. plantarum* counts and from 1.15 to 4.02 log CFU/g in *L. casei* Shirota counts, respectively. After BSS condition, a reduction in *L. plantarum* and *L. casei* Shirota counts varied from 0.77 to 2.76 log CFU/g and from 0.68 to 2.48 log CFU/g, respectively. As reported in Chen et al.^[25], our results indicated that coating material combinations had different effect on resistance of probiotic against SGF and BSS conditions.

As mentioned above, optimum rate of 5 different entrapment substances for microencapsulation of each probiotic cell were calculated from optimization model obtained by using response surface methodology. Concentrations of encapsulation agents changing between 0.5-2% for alginate, 0-1% for gellan gum, 0-1% for gelatin, 0-1% for peptide and 0-2% for FOS were tested. The reason for choosing these concentration ranges in this study is suggestions from previous researchers^[28]. Entrapment substances at different type and concentrations were evaluated with regard to the protection of cell viability. Probiotic microcapsules with entrapment substances at 26 different combinations were prepared according to the experimental design shown in Table 1. Formulation of optimization model from 26 coating material combinations detected optimum proportion of entrapment substances for each probiotic strain. The viability by the best combination were also tested and found higher than other combinations. These results confirmed our hypothesis that optimum combination of encapsulation materials provide highest protection against gastric conditions and give the highest cell viability^[35,36].

Concentrations of alginate used for gelling change between 1.5 and 2.5%. However when alginate was used with other gelatinization agents, concentrations of alginate were tested between 0.5 and 2%^[19]. As known from literature, peptides as nitrogen sources improve viability of probiotics^[19,25]. The present study confirmed that peptides with their prebiotic effect have synergistic activity on probiotic viability. Incorporation of microcapsules with extra coating materials supported additional protecting of the probiotic bacteria^[37,38]. Our results confirmed that. Some researcher reported that microencapsulation applications such as incorporation of different coating materials and the double emulsion

protected probiotic strains against simulated gastrointestinal tract conditions^[18,34]. Similarly, our study showed that extra coating improved survival of probiotic.

In conclusion, selection of optimum or appropriate coating materials used for microcapsules may improve the survival of probiotic strains in functional food products. Microcapsules with prebiotic may be safely used as protective delivery vehicle for the passage from gastrointestinal tract of probiotic strains. Moreover, the present study results may attract the attention of other researchers to investigate innovative entrapment substances. On this sense, further modification and improvement in microencapsulation technique is necessary for resistance of probiotics against gastric conditions.

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