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Antibacterial Effects of Microencapsulated Probiotic and Synbiotics

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Abstract

Determination of the effect of the microencapsulation process on the antibacterial activity of probiotics is an important issue. The present study aims to evaluate the inhibition effect of microencapsulated probiotic culture with prebiotics against two different pathogen enterococci strain. *Lactobacillus rhamnosus* was used as probiotic, inulin and fructooligosaccharide (FOS) were used as prebiotics. Microencapsulation maintained with three different combinations as the only probiotic culture, probiotic culture with inulin and probiotic culture with FOS. In addition, the effect of inulin and FOS on the survival of *L. rhamnosus* was evaluated. *L. rhamnosus* was microencapsulated by the extrusion technique and its antibacterial effect on clinical vancomycin-susceptible *Enterococcus faecalis* (VSEF) and clinical vancomycin-resistant *Enterococcus faecium* (VREF) growth was determined. According to the obtained results, microencapsulated probiotic culture with prebiotic addition showed an inhibition effect on VREF growth. Furthermore, it was found that the survival rate of the probiotic culture cells varied depend on the prebiotic. It was determined that inulin was more efficient on *L. rhamnosus* cell viability than FOS.

Keywords: *Enterococcus*, *Lactobacillus rhamnosus*, probiotic, synbiotic

Mikroenkapsüle Probiyotik ve Sinbiyotiklerin Antibakteriyel Etkisi

Özet

Mikroenkapsülasyon işleminin probiyotiklerin antibakteriyel aktiviteleri üzerine etkisinin belirlenmesi oldukça önemlidir. Bu çalışmada, prebiyotiklerle birlikte mikroenkapsüle edilen probiyotik kültürün iki farklı patojen enterokok gelişimi üzerine inhibe edici etkisi araştırılmıştır. Probiyotik olarak *Lactobacillus rhamnosus* (*L. rhamnosus*), prebiyotik olarak inulin ve fruktooligosakkarit (FOS) kullanılmıştır. Mikroenkapsülasyon, sadece probiyotik kültür, probiyotik kültür ile inülin, probiyotik kültür ile FOS olmak üzere 3 farklı kombinasyonda gerçekleştirilmiştir. Ayrıca, FOS ve inülinin *L. rhamnosus* canlılığı üzerine etkisi belirlenmiştir. *L. rhamnosus* ekstrüzyon tekniği ile mikroenkapsüle edilmiş ve vankomisin-duyarlı *Enterococcus faecalis* (VSEF) ile vankomisin-dirençli *Enterococcus faecium* (VREF) üzerine antibakteriyel etkisi araştırılmıştır. Elde edilen sonuçlara göre, prebiyotik ilavesi ile mikroenkapsüle edilen probiyotik kültür VREF üzerine inhibe edici etki göstermiştir. Probiyotik hücrelerin canlılığının prebiyotik çeşidinden etkilendiği gözlenmiştir. *L. rhamnosus* canlılığı üzerinde inülinin FOS'a göre daha etkili olduğu tespit edilmiştir.

Anahtar kelimeler: *Enterococcus*, *Lactobacillus rhamnosus*, probiyotik, sinbiyotik

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Introduction

Probiotics are defined as live microorganisms that have a beneficial health effect on consumer through creating and improving gastrointestinal microflora when ingested in adequate amounts (Gonzalez-Aguilar et al., 2010; Fadhil and Akın, 2016; Karri et al., 2016). It is reported that regular consumption of probiotics strengthens the immune system through lowering cancer risk, blood cholesterol and digestive troubles, having an anti-allergic effect, preventing gastrointestinal system infections (Coman et al., 2014; Göçer et al., 2016; Praepanitchai et al., 2019). Probiotics have been known as being able to inhibit the growth of pathogens by several mechanisms or pathways. These are;

- Decreasing the pH of the lumen by producing lactic acid,
- Producing antimicrobial metabolites such as hydrogen peroxide, diacetyl, acetoin, organic acid, antibiotics, bacteriocins and free radicals,
- Clinging to receptors and compete for food sources,
- Stimulating the formation of protective mucin,
- Stimulating the production of secretory IgA (Yılmaz, 2004; Kıran and Osmanağaoğlu, 2012; Singh et al., 2014; Swetwiwathana and Visessanguan, 2015).

Providing the beneficial effects expected from probiotic food is largely based on preserving the viability of probiotic microorganism present in food. Probiotic microorganism load should be at least 10^7-10^9 CFU/g in food products (Göçer et al., 2016). In this respect, the survival of probiotics in food products during storage or ripening period is an important issue (Kesenkaş et al., 2016). As a result of growing consumer demand and researcher interest, probiotic product diversity and market share are increasing day by day (Gouin, 2004; Silvi et al., 2014).

The use of probiotic bacteria in many products such as fermented dairy products (ayran, sour cream, yogurt, etc.) and baby food is quite common because of their various nutritional and therapeutic properties (Kalkan, 2016). In addition, there have been several research studies conducted on the use of probiotic microorganism in cereals, meat products, vegetables and fruit juices (Libera et al., 2015; Bağdatlı and Kundakcı, 2016; Neffe-Skocińska et al., 2016). However, there are a number of factors that limit the development and production of foods containing probiotic microorganisms (Qi et al., 2006; Anal and Singh, 2007). The decrease in the survival of probiotic microorganisms due to the environmental conditions has been one of the most important obstacles for the use of probiotics in food. In recent researches, it has been reported that microencapsulation technique is one of the new methods in order to increase the technological properties of probiotic microorganisms (Kailasapathy, 2002; Argin, 2007; Champagne and Fustier, 2007).

Microencapsulation is defined as protein and carbohydrate-based mini capsules, which are capable of keeping enzymes, cells, food components in solid, liquid or gaseous form, and other substances (Wang et al., 2009, Ünal and Erginkaya, 2015). Although there are many microencapsulation techniques have been used (such as spray drying, spray freezing, fluid bed coating, electrostatic method), the most popular microencapsulation techniques for probiotics are extrusion and emulsion (Bosnea et al., 2014; Arslan-Tontul and Erbas, 2017).

In the extrusion technique, a hydrocolloid-containing solution is prepared and mixed with microorganisms. The mixture injected through a nozzle or syringe needle into a solution that makes capsules more solid (Rokka and Rantamäki, 2010; Nazzaro et al., 2012). The calcium alginate solution has been used commonly for the microencapsulation of microorganisms. The reversion of microencapsules and releasing immobilized cells and their products in the gastrointestinal system is an advantage (Musikasang et al., 2009).

Microencapsulation of probiotic microorganisms is gaining attention with providing beneficial effects against environmental conditions and increasing survival rate of probiotics (Khalil et al., 2015; Shori, 2017). Many previous studies have shown microencapsulation techniques protect probiotics from negative conditions during preparation and passing through the digestive system (Todorov et al., 2012; Bosnea et al., 2014; Shori, 2015; Arslan-Tontul and Erbas, 2017).

Microencapsules containing antimicrobial compounds provide controlled release, ensure compound stability and inhibit inactivation of the antimicrobial compounds (Matouskova et al., 2016; Castro-Rosas et al., 2017). In a study made by Khalil et al. (2015), *Helicobacter pylori* growth was inhibited by microencapsulated probiotic bacteria (Khalil et al., 2015).

Lactobacillus rhamnosus is one of the lactic acid bacteria (LAB) that has been known to have probiotic activity (Arnold et al., 2017). *L. rhamnosus* has been shown to possess antimicrobial activity against the growth of pathogens such as *Escherichia coli* (Nikoskelainen et al., 2003; Anyogu et al., 2014; Raoult et al., 2015) *Pseudomonas aeruginosa* (Forestier et al., 2001; Coman et al., 2014; Raoult et al., 2015), *Salmonella* (Forestier et al., 2001; Coman et al., 2014; Beristain-Bauza et al., 2016), *Staphylococcus aureus* (Coman et al., 2014; Beristain-Bauza et al., 2016), *Clostridium difficile* (Forestier et al., 2001; Raoult et al., 2015), *Bacillus cereus* (Anyogu et al., 2014), *Listeria monocytogenes* (Coman et al., 2014; Raoult et al., 2015; Beristain-Bauza et al., 2016), *Shigella flexneri*, *Yersinia enterocolitica*, *Citrobacter freundi* (Jacobsen et al., 1999).

The pathogenicity of enterococci has been known for many years (Moemen et al., 2015). *Enterococcus faecium* is one of the most challenging pathogens due to obtaining antibiotic resistance genes readily (Reyes et al., 2016). Lately, vancomycin-susceptible enterococci have attracted attention due to the ability to acquire resistance genes (Thaker et al., 2015).

Prebiotics are nutrients that reach directly into the large intestine without digestion in the small intestine and increase the beneficial effects of probiotics on consumer health (Siro et al., 2008; Konuray and Erginkaya, 2017). Many studies have shown that inulin and fructooligosaccharide (FOS) could be utilized by LAB and improve their growth (Donalson et al., 2008; Yıldız, 2011).

Foods containing probiotic and prebiotic are described as synbiotics due to the synergistic relationship between them (Siro et al., 2008). According to the previous in vitro studies, merely an application of a synbiotic is more advantageous than prebiotic and probiotic (Gallaher and Khil, 1999).

This study proposed an in vitro model for the determination of the effect of microencapsulation on antimicrobial effect of *L. rhamnosus* against *Enterococcus* spp. In this study, *L. rhamnosus* was microencapsulated by extrusion technique with the addition of prebiotic. Effects of prebiotics on *L. rhamnosus* viability was screened. In addition, inhibition effect of microencapsulated *L. rhamnosus* on clinical vancomycin-susceptible *E. faecalis* (VSEF) and clinical vancomycin-resistant *E. faecium* (VREF) was determined.

Materials and method

Materials

Sodium alginate and gelatin were used for microencapsulation of *L. rhamnosus* (Chen et al., 2007). The coating material was prepared using distilled water. Whatman 4 filter paper was used for filtration of capsules. All mediums and chemicals were obtained from Merck (Darmstadt, Germany). FOS was acquired from Sinerji Gıda Kimya Tekstil San. Tic. Ltd. Sti. (Turkey). Inulin was acquired from Sigma-Aldrich. Phosphate buffered saline (PBS) was used to wash the cells and prepare dilutions. Anaerocult A (Merck) was used to maintain anaerobic condition for cultures. All glassware and solutions were sterilized at 121°C for 15 minutes.

Microorganisms

In this study, *L. rhamnosus* (RIUM/Holland) was used as probiotic. Clinical VSEF and clinical VREF were used as

pathogen which are provided from Cukurova University Department of Food Engineering Microbiology Laboratory.

Bacterial growth conditions

Bacterial cultures were activated by subculture on appropriate mediums. *L. rhamnosus* was grown in de Man Rogosa Sharpe (MRS) agar at 37°C for 24 h in anaerobic condition. After incubation, a single colony was inoculated into 10 mL MRS broth and incubated at 37°C for 24 h. VSEF and VREF were grown in 10 mL Nutrient Broth (NB) at 37°C for 24 h.

Microencapsulation of *L. rhamnosus* by the extrusion technique

The extrusion technique of microencapsulation was derived from Chen et al. (2007). The solution of coating material ingredients was shown in Table 1. Three different coating mixture was prepared as follows: (i) without prebiotic, containing inulin (ii), containing FOS (iii). *L. rhamnosus* was grown in MRS broth at 37°C for 24 h and centrifuged for 10 min at 6000 rpm, 4 °C. The cell pellet was washed twice with sterile PBS. Cell concentration was adjusted to 8 log CFU/mL and transferred to the sterile solution of coating material. The mixtures were injected slowly into sterile 0.05 M $CaCl_2$ through a 0.11 mm sterile needle syringe. The obtained capsules were left in solution for 30 minutes in order to provide sufficient hardness. Microencapsulated cultures were collected with filtration and stored in sterile PBS at 4°C.

Table 1. Concentration of solutions and prebiotics

Mixture no	Solution	Prebiotic
i	3% alginate and 1% gelatine	-
ii	3% alginate and 1% gelatine	1% inulin
iii	3% alginate and 1% gelatine	1% FOS

Determination of probiotic viability

In order to determine the viable cell load of microencapsulated *L. rhamnosus*, the method was derived from Krasaekoopt et al. (2004). One gram of microencapsulated *L. rhamnosus* transferred to 9 mL PBS in stomacher bag and homogenized in a stomacher (BagMixer 400 P Stomacher) for 15 min. 10-fold serial dilutions were prepared using PBS. From each dilution, 100 µL was spread-plated on MRS agar, in duplicate, and incubated anaerobically at 37 °C for 48 h.

Determination of antibacterial effect

The antibacterial effect of *L. rhamnosus* against clinical vancomycin-susceptible *E. faecalis* and clinical vancomycin-resistant *E. faecium* was evaluated using the minimal inhibitory concentration (MIC) method described by Wiegand et al. (2008) with modifications. The capsules of *L. rhamnosus*, which were prepared in three different compositions, are separately transferred into the test tubes containing sterile Mueller Hinton Broth (MHB) and 10-fold serial dilutions of these mixtures are prepared. Pathogens were incubated at 37°C for 24 h. After incubation, pathogens were inoculated with a concentration of 4 log CFU/mL in each test tube. Test tubes were incubated at 37°C for 24 h. Cultures were diluted with PBS and spread-plated on Nutrient Agar (NA) plates. Colonies were counted after incubation at 37°C for 24 h.

Results and Discussion

Determination of viable cell count

There was no difference between the capsules in terms of size and shape. Viable cell count of *L. rhamnosus*, *L. rhamnosus* + inulin and *L. rhamnosus* + FOS capsules were determined as 4.27, 8 and 5.69 log CFU/mL, respectively, which show that there is an increased survival of bacteria microencapsulated with inulin when compared to FOS. In many studies, the survival of probiotics was increased by microencapsulation (Chávarri et al., 2010; Tripathi and Giri, 2014). In the study made by Chen et al. (2007), survival of *Bifidobacterium bifidum* was increased by microencapsulation. Chandramouli et al. (2004) have shown that the alginate microencapsules could be useful to maintain the survival rate of bacteria. In addition to microencapsulation, several reports have

indicated that the viability of probiotics was increased with prebiotics. In a study made by Ambalam et al. (2015), the growth of probiotic was increased in the presence of prebiotic. Adebola et al. (2014) have shown that the growth of *L. acidophilus* NCTC 1723, *L. delbrueckii* subsp. *bulgaricus* NCTC 12712, *L. brevis* NCIMB 11973, *L. acidophilus* NCFM were supported by the addition of inulin and other prebiotics (lactulose, lactobionic acid). However, *L. reuteri* NCIMB 11951 growth was not observed with the addition of inulin. Gunenc et al. (2016) found that addition of sea-buckthorn as a prebiotic into yogurt could increase the survival of *Streptococcus thermophilus* and *L. bulgaricus* during storage. Pimentel et al. (2015) showed that addition of oligofructose improved *L. paracasei* subsp. *paracasei* viability in apple juice. In the study made by Etchepare et al. (2016), *L. acidophilus* was microencapsulated with the addition of prebiotics (resistant starch and chitosan). Survival rate was screened in a simulated gastrointestinal system and during storage. They have reported that the addition of prebiotic was beneficial for the survival of probiotic. It can be concluded that the addition of prebiotic to microencapsulation was essential for the survival of *L. rhamnosus*.

Table 2. Cell counts and antibacterial effect of *L. rhamnosus* (-: antimicrobial effect is not observed)

Mixture No	Cell counts (log CFU/mL)		
	L. rhamnosus	**VSEF**	**VREF**
i	4.27	-	-
ii	8.00	-	4.27
iii	5.69	-	3.20

Determination of antibacterial effect

Initial concentration of VSEF and VREF was set to 4 log CFU/mL. In the presence of inulin, the viable cell count of VREF was detected as 4.27 log CFU/mL whilst with FOS, the viable cell count was 3.20 log CFU/mL. Microencapsulated probiotic with FOS showed very little inhibition activity on viable cell count of VREF. Inulin addition did not inhibit the growth of VREF. In this study, the viable cell count of VSEF reduction was not observed.

In many studies, the antimicrobial effect of *L. rhamnosus* and synbiotics on several pathogens were determined. In a study made by Georgieva et al. (2015), the antimicrobial effect of *L. rhamnosus* was determined with the agar-well diffusion method. *L. rhamnosus* has shown antimicrobial activity against various pathogens (*S. aureus*, *E. coli*, *B. cereus* and *Candida albicans*). Jiang et al. (2014) reported that *L. rhamnosus* has shown strong antimicrobial activity against *C. albicans* in fructose and glucose medium. In a study made by Aryantini et al. (2017), *L. rhamnosus* strains showed inhibitory activity against *Salmonella* Typhimurium LT-2, *S. sonnei*, *L. monocytogenes* and *E. coli* O157. Rana et al. (2018) reported that antioxidant and antimicrobial peptides, which occurred through the fermentation of milk by *L. rhamnosus* C6, inhibited the growth of *E. coli* ATCC 25922. Fermentation time was found to be effective on inhibition degree. Beristain-Bauza et al. (2016) have shown that cell-free supernatant of *L. rhamnosus* NRRL B-442 has inhibition activity against *L. monocytogenes* Scott A, *S. aureus* ATCC 29413, *E. coli* ATCC 25922, or *S. enterica* serovar Typhimurium ATCC 14028 growth in whey protein isolate and calcium caseinate films. In a study made by Mpofu et al. (2016), *L. rhamnosus* yoba has shown inhibition activity against *L. monocytogenes*, *Salmonella* spp., *Campylobacter jejuni*, *E. coli* O157:H7 and *B. cereus*. Fooks and Gibson (2002) and Ambalam et al. (2015) have shown that antimicrobial activity of *Lactobacillus* spp. and *Bifidobacterium* spp. against pathogens (*C. jejuni*, *E. coli*, *S.* Enteritidis and *C. difficile*) increased in the presence of prebiotics such as starch, FOS, xylo-oligosaccharide (XOS), lactulose, galacto-oligosaccharides, isomalto-oligosaccharides. In a study made by Shokryazdan et al. (2014), *Lactobacillus* strains showed an antimicrobial effect against several pathogens (*C. albicans* (ATCC 44831), *E. faecium* (ATCC 51558), *S. epidermidis* (ATCC 12228), *Propionibacterium acnes* (ATCC 6919), *E. coli* (ATCC 29181), *S. sonnei* (ATCC

25931), *Helicobacter pylori* (ATCC 43579), *Enterobacter cloacae*, *Vibrio parahaemolyticus*, *Klebsiella pneumoniae* (K36), *S. aureus* (S244) and *L. monocytogenes*) by agar spot test. In a study made by Sim et al. (2012), LAB (*Lactobacillus*, *Lactococcus* and *Pediococcus* sp.) showed an antimicrobial effect against *S.* Typhimurium S1000, *L. monocytogenes* L55, *S. aureus* S277 and *E. coli* O157:H7. Likotrafiti et al. (2016) have shown that *L. fermentum* and *B. longum* with prebiotics (short-chain fructooligosaccharides and isomaltooligosaccharides) have an antimicrobial effect against enterohaemorrhagic *E. coli* O157:H7. Li et al. (2016) have found that *L. plantarum* showed antimicrobial activity against *S. aureus*, *L. monocytogenes*, *E. coli*, *S. enterica* and *C. albicans*.

There are also studies indicating that *L. rhamnosus* did not inhibit the growth of *E. faecalis*. Montecinas et al. (2016) studied the antibacterial effect of *L. rhamnosus* biofilm against *E. faecalis* and found no significant inhibition effect on the growth of *E. faecalis*. In a study made by Doron et al. (2015), the effect of *L. rhamnosus* GG (LGG) intake on colonization of vancomycin-resistant enterococci (VRE) was determined. No inhibition effect of LGG against VRE count was reported. Jeong and Moon (2015) have shown that *L. rhamnosus* CJNU 0519 has antimicrobial properties against LAB (*L. acidophilus*, *P. acidilactici* K10) and pathogens (*L. monocytogenes* KCTC 3569, *S. aureus* ATCC 14458) whilst *L. rhamnosus* CJNU 0519 did not show inhibitory activity against *E. faecium* MK3, *E. coli* DH5α, *Saccharomyces cerevisiae* ATCC 24858 growth.

Conclusion

In this study, we determined the viability of microencapsulated *L. rhamnosus* with inulin was higher than the viability of microencapsulated *L. rhamnosus* with FOS and microencapsulated *L. rhamnosus* without prebiotic. Microencapsulation with prebiotic addition was more efficient on *L. rhamnosus* viability than microencapsulation without prebiotic. Antibacterial effects of probiotics have been determined in most of the previous studies. However, to observe the expected antibacterial effect with the microencapsulation method, the use of appropriate microorganism and strain is important. It is thought that antibacterial effect of probiotic on *E. faecalis* may not be observed due to insufficient production of active substances such as hydrogen peroxide, organic acid, bacteriocin produced by probiotic strains or insufficiency of produced inhibitory substances. In conclusion, the study of probiotic survival rate and antimicrobial activity should be extended in order to determine the viability and inhibition effect of probiotics by different combinations of different probiotics and prebiotics, and by different combinations of pathogen cultures, in order to enhance the potential use of microencapsulated probiotics in foods.

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