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Research Article

Inactivation Effect of Probiotic Biofilms on Growth of *Listeria monocytogenes*

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Abstract

Probiotic lactic acid bacteria and their biofilms have antagonistic activity against food spoilage organisms and pathogenic bacteria. Recently, researchers focused on the use of probiotic biofilms for inhibition of pathogenic bacteria. The aim of this research is to improve probiotic biofilms with optimal prebiotic concentration and to determine their inactivation effect on both planktonic cells and biofilm growth of *Listeria monocytogenes.* Biofilm formations were detected by using microplate method. Prebiotic ingredients were used to form biofilm with highest viable probiotic cell counts and optimal concentrations of prebiotic ingredients were determined according to the response surface method. Biofilm produced by *Lactobacillus casei* Shirota and *Lactobacillus rhamnosus* contained 9.46 and 9.66 log cfu/mL viable cell counts, respectively. Optimal prebiotic concentrations were found 3% casein peptone-0% fructo-oligosaccharides (FOS) for biofilm formation with highest viable cell counts by *L. casei* Shirota and 1.5% casein peptone-1.5% FOS for biofilm formation with highest viable cell counts by *L. rhamnosus.* Probiotic biofilms exhibited inactivation against growth of *L. monocytogenes* and caused a reduction of 0.66- 2.01 log cfu/mL for planktonic *L. monocytogenes* and 0.40-1.69 log cfu/mL for *L. monocytogenes* biofilm. Planktonic cells of *L. monocytogenes* were observed to be more susceptible to probiotic biofilms than biofilm of *L. monocytogenes.* Biofilm of *L. rhamnosus* showed higher inhibition effect on *L. monocytogenes* growth than *L. casei* Shirota. These findings showed that biofilms of probiotic *Lactobacillus* strains used in this study may be excellent candidate for controlling of pathogenic bacteria.

Keywords: *Biofilm, Probiotic, L. monocytogenes, Inhibition of pathogens*

Listeria monocytogenes'in Gelişimi Üzerine Probiyotik Biyofilmlerin İnaktivasyon Etkisi

Özet

Probiyotik laktik asit bakterilerinin ve biyofilmlerinin gıdaları bozucu organizmalara ve patojen bakterilere karşı antagonistik etkileri bulunmaktadır. Son zamanlarda araştırmacılar patojen bakterilerin inhibisyonu için probiyotik biyofilmlerin kullanımı üzerine yoğunlaşmışlardır. Bu çalışmanın amacı, ideal prebiyotik konsantrasyonuyla probiyotik biyofilmleri geliştirmek ve *Listeria monocytogenes*'in hem planktonik hücrelerinin hem de biyofilmleri üzerine probiyotik biyofilmlerin inaktivasyon etkisini belirlemektir. Biyofilm oluşumları mikroplak yöntemi kullanılarak uygulanmıştır. Prebiyotik katkılar en yüksek canlı probiyotik hücre sayılı biyofilmleri oluşturmak için kullanılmış ve prebiyotik katkıların ideal konsantrasyonları cevap yüzey tekniğine göre belirlenmiştir. *Lactobacillus casei* Shirota ve *Lactobacillus rhamnosus* tarafından üretilen biyofilmler sırasıyla 9.46 log kob/ml ve 9.66 log kob/mL canlı hücre sayısı içermiştir. *L. casei* Shirota ve *L. rhamnosus* tarafından en yüksek canlı hücre sayılı biyofilm oluşumu için ideal prebiyotik konsantrasyonları sırasıyla %3 kazein pepton-%0 FOS ve %1.5 kazein pepton-%1.5 FOS olarak bulunmuştur. Probiyotik biyofilmler *L. monocytogenes* gelişimine karşı inaktivasyon sergilemiştir ve *L. monocytogenes*'in planktonik hücrelerinde 0.66-2.01 log kob/mL'lik ve biyofilmlerinde 0.40-1.69 log kob/mL'lik bir azalışa neden olmuştur. *L. monocytogenes*'in planktonik hücreleri probiyotik biyofilmlerine *L. monocytogenes*'in biyofilmlerinden daha duyarlı bulunmuştur. *L. rhamnosus* biyofilmi *L. monocytogenes* gelişimi üzerine *L. casei* Shirota'dan daha yüksek bir inhibisyon etkisi göstermiştir. Bu bulgular bu çalışmada kullanılan probiyotik türlerin biyofilmlerinin patojen bakterilerin kontrolünde çok iyi birer aday olabileceğini göstermiştir.

Anahtar sözcükler: *Biyofilm, Probiyotik, L. monocytogenes, Patojen inhibisyonu*

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INTRODUCTION

Prebiotics are chemical food ingredients, which support colonization of probiotics and in recent years there has been considerable interest in the usage of prebiotics due to beneficial effects on human health and food industry [1-3]. Several fermented food products contain lactic acid bacteria with probiotic properties and are accepted as safe due to protective role of probiotics [4]. Probiotic bacteria produce antimicrobial compounds against various pathogens and thus might form a natural barrier against pathogen in the gastrointestinal tract or preserve food [5]. Probiotic *Lactobacillus* are also able to adhere to various surfaces. Adhesion of probiotic *Lactobacillus* species prevents colonization of pathogenic bacteria and plays an important role as a protective barrier [3,6,7]. Furthermore the effectiveness of probiotics is strain-specific [8].

Listeria monocytogenes, a foodborne pathogen, has been a great concern due to its capacity to survive and grow in a wide variety of food substrates and environmental conditions [5,9-11]. The prevalence of *L. monocytogenes* varies mostly depending on the product and processing environments [8,12,13]. *L. monocytogenes* can form biofilms and produce extracellular polymeric substances on various food contact materials. The ability of *L. monocytogenes* to form biofilm on different surfaces poses a major concern for food industry because biofilms show more resistance to antimicrobial compounds [9,14,15]. *L. monocytogenes* has good adhesion ability and requires only a short contact time for attachments. The adhesion of *L. monocytogenes* to various surfaces such as stainless steel, plastic, glass and rubber cause to the decrease of its sensitivity to disinfectants [5,16].

Biofilm is the sessile form of microbial life, characterized by adhesion of microorganisms to biotic or abiotic surfaces, with consequent production of extracellular polymeric substances [9,17]. Microbial biofilm may be unfavorable or favorable and undesirable or desirable in food plant and human gastrointestinal systems. For example, biofilms formed by probiotic *Lactobacillus* strains in the gastrointestinal tracts may have a protective role and valuable characteristic for host with competitive inhibition of pathogen colonization. Probiotic biofilms can favor beneficial bacterial colonization. Biofilm or adherent structured microbial communities of *L. monocytogenes* in gastrointestinal tracts or food processing environments has negative effect on human health and product quality [18,19]. In several studies it was reported that adherence of lactic acid bacteria to the surface may prevent the adherence and the biofilm formation of *L. monocytogenes*. The use of probiotic biofilms can be considered as an alternative approach for reducing growth of pathogenic bacteria as regards human health and food safety [4]. However, the optimal functionality and expression of health-promoting physiological functions of probiotics is dependent on survivability and colonization in gastrointestinal tract and fermented foods [20]. Transition from planktonic cells to biofilm of the most bacteria depends on bacterial community and environmental conditions [21]. Some factors affecting the biofilm formation are equipment, temperature, nutrients and water. Presence of prebiotics in growth medium of probiotic microorganisms improves the formation of probiotic biofilms [12].

There are many studies focused on the biofilm formation of the pathogenic bacteria and the inactivation of pathogens with chemical compounds. However, biofilm formation was not studied extensively in nonpathogenic bacteria such as different *Lactobacillus* species [19]. Additionally, only a few studies have focused on the use of probiotic biofilms to inhibit the growth of *L. monocytogenes* [11]. The objective of the present study was to determine optimal prebiotic concentration for the formation of probiotic biofilms with highest viable cell concentration and to evaluate inactivation capacities of two different biofilms produced by *L. casei* Shirota and *L. rhamnosus* on growth of *L. monocytogenes*.

MATERIAL and METHODS

All experiment was carried out three times, with duplicate samples per trial and results were expressed as average.

Microorganisms and Prebiotics

In this study, *Lactobacillus rhamnosus* (Danisco USA INC.) and *Lactobacillus casei* Shirota (Yakult-RIUM/The Netherlands) were used as probiotic cultures and *Listeria monocytogenes* (ATCC 7644-Remel/USA) was used for inactivation experiment as pathogenic bacteria. As prebiotic ingredients, casein peptone (CP) (Merck-Germany) and fructo-oligosaccharides (FOS) (Sinerji Food-Turkey) were used.

Quantification of Biofilms Produced by L. monocytogenes and Probiotic Lactobacillus Strains

The quantification of biofilm production of *L. monocytogenes* and probiotic culture were performed as described previously by Bondi et al.[22]; Kubato et al.[23] and van der Veen and Abee [24] with some modifications. Biofilm assay was performed using 12-well microtiter plates. In order to standardize the number of bacteria, overnight grown cultures were used for all experiments. Three ml of each previously obtained probiotic suspensions *(L. rhamnosus* or *L. casei* Shirota) in MRS Broth (Merck, Germany) and *L. monocytogenes* suspensions in Tryptic Soy Broth (Merck-Germany) were added into each well and microtiter plates were incubated for 48 h at 30°C to allow the adhesion and formation of mature biofilm on the well bottoms. After 48 h the suspensions were removed and the wells washed three times with 2 mL of sterile saline

solution (NaCl 0.85%) (Merck-Germany). After that each biofilm in well was resuspended in 1 mL of saline solution by pipetting rigorously and serial diluted in saline solution for quantification of the biofilm formation expressed as unit of log cfu/well. Probiotic cells from biofilm were plated on MRS Agar (Merck-Germany) and plates were anaerobically incubated for 2 days at 30°C. *L. monocytogenes* were plated on BHI Agar (Oxoid-United Kingdam) and plates were aerobically incubated for 2 days at 30°C. In addition to quantification, for confirmation of biofilm formation, attached bacteria in well were stained with 3 mL of a 0.1% (V/V) crystal violet solution (Merck, Darmstadt, Germany) for 30 min. and washed three times with 3.5 mL water to remove unbound crystal violet. After drying, attached crystal violet was dissolved in 3.5 mL of absolute ethanol (Merck, Darmstadt, Germany) and absorbance was measured at 600 nm. If absorbance is more than 0.1, result is accepted as biofilm positive.

Optimization of Prebiotic Ingredients in Probiotic Biofilms

To carry out the response surface modeling, regression was performed on the experimental results to construct mathematical models. Variables and responses were defined as prebiotic ingredients and viable cell counts in biofilms of probiotic, respectively. The response surface method was employed in a similar way to the work by Chen et al.[25].

Detection of The Inactivation Effect of Probiotic Biofilms on L. monocytogenes Growth

Three ml of tryptic soy broth containing 0.1% *L. monocytogenes*, 0.5% *L. monocytogenes*, and 0.1% mix of *L. monocytogenes* and probiotic culture (0.1% *L. rhamnosus* or 0.1% *L. casei* Shirota) were added onto attached probiotics (probiotic biofilm) in well and these microtiter plates incubated at 30°C for 24 h. After incubation, both planktonic cell enumeration and the viable count of adherent *L. monocytogenes* in probiotic biofilm were performed for inactivation test. To find inactivation of adherent *L. monocytogenes* in probiotic biofilms washed three times with saline solution and resuspended in 1 mL of saline solution by pipetting rigorously. The attached cells were serial diluted in saline solution and plated Oxford Listeria Selective Agar (Merck-Germany) incubated for 24 h at 30°C. After 24 h, both planktonic cells and adherent cells of *L. monocytogenes* were enumerated on Oxford Listeria Selective Agar (Merck-Germany) after incubation for 24 h at 30°C. Whereas to find inactivation of planktonic *L. monocytogenes* cells, 1 mL of medium was removed from each well and suspended in 1 mL of saline solution by pipetting rigorously. The planktonic cells were serial diluted in saline solution and plated on Oxford Listeria Selective Agar (Merck-Germany), then incubated for 24 h at 30°C [11].

RESULTS

Biofilm Formation of L. monocytogenes and Probiotic Cultures

Table 1 showed that *L. monocytogenes*, *L. casei* Shirota and *L. rhamnosus* had ability of biofilm formation. Viable cell counts in biofilm of *L. rhamnosus* (9.66 log cfu/mL) were found higher than biofilm of *L. casei* Shirota (9.46 log cfu/mL). Biofilm of *L. monocytogenes* contained lowest viable cell counts (8.01 log cfu/mL).

Proportion for the Formation of Probiotic Lactobacillus Biofilms

Response surface methodology was used in the present work to develop a prediction model for establishing the optimal concentrations of prebiotics on viable cell growth in probiotic biofilms. As represented in *Table 2*, responses were obtained according to 13 combinations of prebiotics. Viable cell counts in probiotic biofilms were enumerated for each prebiotic combination and optimal prebiotic concentrations were calculated according to the obtained responses. We observed that the ability of probiotic *Lactobacillus* strains to form biofilms varied dependent on the prebiotic proportion.

In this study both of probiotic *Lactobacillus* strains utilized casein peptone. However, FOS has no effect on growth of *L. casei* Shirota. Optimal prebiotic concentration for *L. casei* Shirota were 3% peptide and 0% FOS and these results were found significant at 94% acceptable level. In this rate it is possible that *L. casei* Shirota counts in well are 9.89 log cfu/ml. Optimal prebiotic rate for *L. rhamnosus* were 1.5% casein peptone and 1.5% FOS and these results were found significant at 80% acceptable level. In this rate it is possible that *L. rhamnosus* counts in well are 10.81 log cfu/mL. The result of possibility from response surface detected that *L. rhamnosus* is more adhesive than *L. casei* Shirota in agreement with Collado et al.[3].

The Inactivation Effect of Probiotic Biofilms on L. monocytogenes Growth

In this study, inactivation efficiency of probiotic biofilms was tested against *L. monocytogenes* in different rates. For this experiment, 0.1% *L. monocytogenes*, 0.5% *L. monocytogenes*, and 0.1% mix of *L. monocytogenes* and probiotic culture (0.1% *L. rhamnosus* or 0.1% *L. casei* Shirota) were added onto probiotic biofilm and reduction

Table 1. *Biofilm formation of strains*

Strains	Biofilm Formation	Viable Cell Counts (log cfu/mL)	Absorbance
L. casei Shirota	+	9.46	0.477
L. rhamnosus	+	9.66	0.46
L. monocytogenes	+	8.01	0.44

Table 2. *The variables and responses of experiment*

Combination	Variables		Response	
	FOS (% 0-3)	Casein Peptone (% 0-3)	*L. casei* Shirota (log cfu/mL)	*L. rhamnosus* (log cfu/mL)
1	3	0	9.51	10.85
2	1.5	1.5	9.81	11.00
3	0	1.5	9.85	10.53
4	1.5	1.5	9.59	10.54
5	0	0	9.60	11.01
6	3	1.5	9.78	11.22
7	1.5	0	9.64	11.37
8	3	3	9.82	11.02
9	0	3	9.94	10.30
10	1.5	1.5	9.83	10.18
11	1.5	1.5	10.00	11.12
12	1.5	1.5	9.79	10.41
13	1.5	3	9.90	11.02

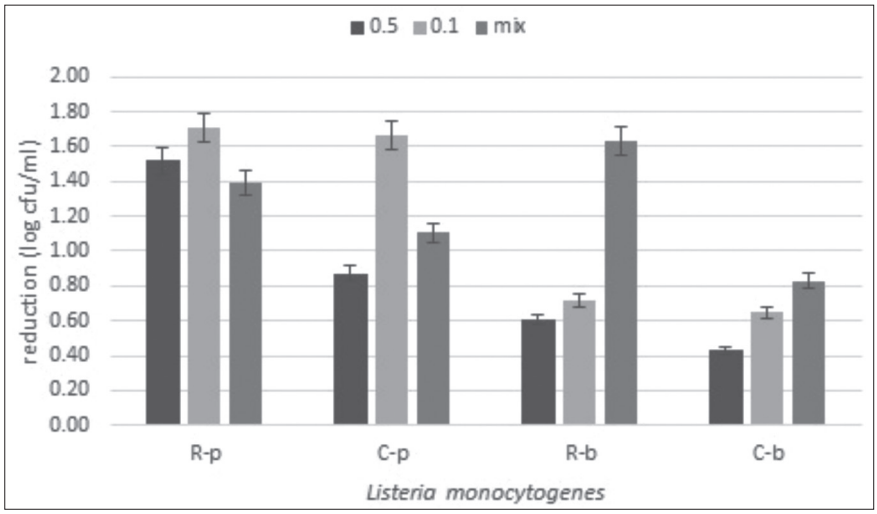

Fig 1. Reduction in viable cell counts of *L. monocytogenes*
R-p: Planktonic *L. monocytogenes* cells in well containing *L. rhamnosus* biofilm, C-p: Planktonic *L. monocytogenes* cells in well containing *L. casei* Shirota biofilm, R-b: *L. monocytogenes* attached to *L. rhamnosus* biofilm, C-b: *L. monocytogenes* attached to *L. casei* Shirota biofilm, 0.5: The effect of growth medium containing 0.5% *L. monocytogenes*, 0.1: The effect of growth medium containing 0.1% *L. monocytogenes* mix: The effect of growth medium containing 0.1% *L. monocytogenes* and 0.1% probiotic culture *(L. rhamnosus* or *L. casei* Shirota)

in viable cell counts of *L. monocytogenes* was compared. *Fig. 1* showed the inactivation effect of *L. casei* Shirota biofilm and *L. rhamnosus* biofilm on the growth of *L. monocytogenes*. As seen from our results, probiotic biofilms had not only inhibition effect on planktonic cells of *L. monocytogenes* but also biofilm of *L. monocytogenes*. Each probiotic biofilm exhibited inactivation efficiency in different levels and caused different reduction in viable cell counts of *L. monocytogenes*. The reduction in planktonic cells of *L. monocytogenes* varied from 0.66 to 2.01 log cfu/mL whereas the reduction in *L. monocytogenes* attached to biofilm changed between 0.40 and 1.69 log cfu/mL. These findings proved the hypothesis of Gomez et al.[4] that planktonic cells of *L. monocytogenes* were more susceptible than biofilm of *L. monocytogenes*. Similarly, Guerrieri et al.[11] determined that *L. plantarum* 35d biofilm, *L. plantarum* 396/1 biofilm and *Enterococcus casseliflavus* IM 416K1 biofilm caused more reduction in planktonic cells of *L. monocytogenes* than adherent cells of *L. monocytogenes*. As mentioned before in our results, *L. rhamnosus* and *L. casei* Shirota had different efficiency in terms of biofilm formation. In addition to these differences in the ability of biofilm formation of probiotics, it was detected that *L. rhamnosus* and *L. casei* Shirota showed different inactivation properties against pathogenic bacteria. Biofilm of *L. rhamnosus* showed higher inhibition effect on both planktonic cells and adherent cells of *L. monocytogenes* than biofilm of *L. casei* Shirota. These results confirmed that inhibition effect of probiotic biofilms varied according to the strains [4,11].

Additionally, 0.1% *L. monocytogenes* addition to probiotic biofilms led to the highest inactivation in planktonic cells of *L. monocytogenes,* whereas highest anti-adherence activities of probiotic *Lactobacillus* strains against biofilm formation of *L. monocytogenes* were obtained with addition of mix culture of 0.1% *L. monocytogenes* and 0.1% probiotic culture *(L. rhamnosus* or *L. casei* Shirota) to probiotic

biofilms. This situation showed that culture addition in different rates to probiotic biofilms differently affected level of inactivation. As known from literature, probiotic *Lactobacillus* strain may adhere more easily than pathogens [17]. Similarly, in our experiment with mix culture, the addition of probiotic culture plus *L. monocytogenes* caused to competition and prevented adherence of *L. monocytogenes* to probiotic biofilm. When 0.5% of *L. monocytogenes* was added to probiotic biofilm, probiotics led to 0.4 and 0.6 log cfu/mL reduction in attachment of *L. monocytogenes*. These results are considered that as addition of *L. monocytogenes* increased to probiotic biofilms, adherence of *L. monocytogenes* to probiotic biofilm reduced.

DISCUSSION

Many bacteria could form biofilm by adhering to the various surfaces thanks to their aggregation ability [4]. Adherence of probiotic cultures is desirable properties for displacement of pathogens. However these beneficial effects of probiotic bacteria can be observed by having an adequate mass through aggregation. Hydrophobicity and aggregation ability of probiotics can give prediction about detection of the most useful and highly adhesive probiotic *Lactobacillus* strains [3]. Furthermore, the specific composition of the medium contribute to biofilm formation of each species [24]. As it is known that both effect of growth medium and strains were very important on biofilm formation by microorganisms. Lebeer et al.[26] reported that prebiotic may have biofilm-promoting effect. The ability of probiotic culture to metabolize prebiotics is a species-dependent feature. For this reason, the proper selection of probiotics and prebiotics for symbiotic is highly important [17]. As a matter of fact, the present treatment with prebiotics in different concentrations differently affected the biofilm formation of probiotic *Lactobacillus* strains.

In general, probiotic *Lactobacillus* strains may adhere more easily than pathogens [17]. This hypothesis proved in our study that viable cell counts in probiotic biofilms were found higher than *L. monocytogenes* biofilm *(Table 1)*. Additionally, as can be seen our results, the attachment of *L. rhamnosus* to wells (biofilm formation) were found higher than *L. casei* Shirota. In accordance with our results, Collado et al.[3] determined that *L. rhamnosus* had higher adherence abilities to well than other lactobacilli species such as *L. casei, L. acidophilus, L. plantarum* and *L. salivarus.*

Recently, researchers and industry have been focused on novel strategies using natural products to control the pathogens in food industry. The use of lactic acid bacteria showing the highest biofilm formation in food products as starter or probiotic cultures can be a very promising approach for the control of pathogenic bacteria. Especially successful results with lactobacilli biofilms were obtained to control the growth of *L. monocytogenes* [4].

As reported in the previous studies, microorganisms in biofilms are more resistant to antimicrobial agents than planktonic cells [4,11].

Inhibitory mechanisms of probiotic *Lactobacillus* strains on biofilm formation of *L. monocytogenes* based on the competition, exclusion and displacement [21]. Similarly, Aoudia et al.[20] reported that biofilm growth in probiotic *Lactobacillus* strains had an antagonistic effect against *L. monocytogenes*. Similarly, many researchers concluded that probiotic *Lactobacillus* strains or lactobacilli was capable to reduce biofilm formation of *L. monocytogenes* [4,21,27,28].

According to our results, the application of probiotic biofilms can be an alternative method to reduce the growth of pathogens. Probiotic *Lactobacillus* strains such as *L. casei* Shirota and *L. rhamnosus* might have protective role against adhesion by *L. monocytogenes* inside the gastrointestinal tract of patients and onto food contact surfaces. However the inhibition is strain-dependent and varies according to conditions in growth medium. Our data indicated that optimal probiotic adherence to surfaces made possible with prebiotics-promoting probiotic growth. Biofilm formation ability of probiotic *Lactobacillus* strains might interfere with the ability of the pathogenic species to infect the host and can prevent the colonization of food-borne pathogens. It was obtained new information about the use of potential probiotic cultures biofilms for the inactivation of *L. monocytogenes*. However, more experiments are needed to determine the efficacy of probiotic *Lactobacillus* strains in inhibiting *L. monocytogenes* when different nutrients and environmental conditions are present. Additionally, this study should be supported with *in vivo* experiments because gastrointestinal adhesiveness of certain species may be different than adhesiveness to microtiter plates.

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