Inhibitory Effect of Probiotics Lactobacillus Supernatants Against Streptococcus Mutans and Preventing Biofilm Formation

Tuğba Demir¹,a,*, Hakan Demir²,b

¹Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Sivas Cumhuriyet University, 58140 Sivas, Turkey
²Department of Prosthodontics, Faculty of Dentistry, Sivas Cumhuriyet University, 58140 Sivas, Turkey

*Corresponding author

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Abstract

Probiotic microorganisms can release bioactive substances that can inhibit the growth and biofilm formation of pathogenic microorganisms such as *Streptococcus mutans*. Dental caries is a multifactorial chronic infection disease, which starts with bacterial biofilm formation caused mainly by *S. mutans*. This study investigated the characteristics of *Lactobacillus* spp. strains as an oral probiotic. Twelve *Lactobacillus* spp. species obtained from fermented milk and dairy products were identified. Antimicrobial activity was determined with the Minimum Inhibition Concentration against *S. mutans* as an oral pathogen. Biofilm formation capabilities of the identified *Lactobacillus* strains in supernatant and culture media were determined. In addition, their ability to α-amylase tolerance and pH values (24h-48h) were determined. *L. plantarum* showed the highest antimicrobial activity compared to other *Lactobacillus* strains. Also, *L. plantarum* inhibited biofilm formation.

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Probiyotik Lactobacillus Süpernatantlarının Streptococcus Mutans'a Karşı İnhibitör Etkisi ve Biyofilm Oluşumunu Önele

MAKALE BİLGİSİ

ÖZ

Probiyotik mikroorganizmaların ürettiği biyoaktif maddeler, *Streptococcus mutans* gibi patojenik mikroorganizmaların büyüməsini ve biyofilm oluşumunu engelləyən dəyərək olub. Düşə çıxaları, olaraq *S. mutans*in nədən olduğu bakteriyel biyofilm oluşumu şəxsindən çox faktorlə rəqəmsal bir qəza tiyəşinə nəzarət etməkləridir. Bu qəzə uçaqda oral probiyotik olaraq *Lactobacillus* spp. suşlarının karakteristrik oqaqlarını araşdırılmasının məqsədi. On iki *Lactobacillus* spp. türü fermente süt və süt ürünleri ilə əlavə edilmişdir. *S. mutans* suş karışı antimikrobiyal aktivite, Minimum İnhibisyon Konsantrasyonu ilə belirlənmişdir. Tespit edilən *Lactobacillus* suşlarının süpernant və kultür ortamında biyofilm oluşturma yetenəkləri araşdırılmış, ayrıca α-amilaz tolerans və pH dəyişim (24h-48h) kəbiliyyətlərini incələnmişdir. *L. plantarum*, digər *Lactobacillus* suşlarına kıyaslə en yüksək antimikrobiyal aktivitəsi göstərmiş, ayrıca *L. plantarum*, biyofilm oluşumunu inhibe etmişdir.

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Introduction

Probiotics are included in the literature as living microorganisms that can provide a health benefit to the host by balancing microorganisms (Meurman, 2005). Studies conducted in the field of characterization of probiotic bacteria have shown their anti-cancer, anti-oxidant, anti-bacterial and anti-fungal effects (Lee and Kim, 2014; Li et al., 2019). Probiotics can be found in different functional and traditional foods (yoghurt, types of cheese and fermented foods, etc.) (Lodi et al., 2010; Taha et al., 2017). Lactic acid bacteria are the most common group to obtain probiotics. Lactobacillus spp. and Bifidobacterium spp are the main ones treated intestinal dys-function and dys-biosis. (Meurman and Stamatova, 2007).

Probiotic bacteria aid in periodontitis by stabilizing the oral microbial-flora. Lactobacillus spp suppress growth of periodontal pathogens and inhibited them. In recent studies showed application of advantageous bacteria, as an assistant to inhibit re-colonization of periodonto pathogens and in overall pocket depth reduction. Probiotic microorganisms can adhere to dental tissues as part of the biofilm. These microorganisms with functional properties can compete with the growth of cariogenic bacteria or periodontal pathogens such as S. mutans. (Balakrishnan et al., 2000; Penala et al., 2016; Higuchi et al., 2019).

Dental caries and periodontal diseases are infectious diseases and public health problems (Coqueiro et al., 2018). Streptococcus mutans is one of the primary pathogens that responsible for tooth decay and oral cavity formation. S. mutans is among the bacteria that is early colonizer oral cavity, and responsible for formation of the biofilm in the oral cavity (Ahmed et al., 2014). S. mutans is also one of the cariogenic bacteria, and plays important role in the pathogenesis of dental caries (Balakrishnan et al., 2000).

The oral cavity is one of entrance door of the body, and is home to many microorganisms. It is stated in the literature that there are more than 700 microorganism species in the oral flora. This variety of hosts, consisting of gram-negative and gram-positive microorganisms, is in equilibrium with the body defense system. This balance could turn in favor of opportunistic microorganisms as a result of nutritional habits, saliva pH changes, and changes in body health. (Cutler and Jotwani, 2006, Wu et al., 2018). If acidic pH increases, the incidence of tooth decay increases, and as saliva pH becomes basic, the incidence of dental calculus and periodontal ailments increase (Palmer, 2014).

In dentistry, recent researches support that Lactobacillus spp. culture strains might play a role as antagonistic agents on cariogenic bacteria species inhibiting S.mutans level in saliva or pellicle (de Souza Rodrigues et al., 2020). The most commonly used species in oral probiotic preparations are Lactobacillus bulgaricus, L. acidophilus, L. casei, L. helveticus, L. lactis, L. salivarius, L. plantarum, Streptococcus thermophilus, Enterococcus faecium, E. faecalis, Bifidobacterium and Saccharomyces boulardii.

The major objective of this research is to determine the effect of Lactobacillus spp strains for preventing on tooth decaying. For this purpose, Lactobacillus spp. derived from fermented dairy product of the probiotic was investigated on the viability and in the virulence factors of S. mutans growth in supernatant culture medium, and determined their antimicrobial, biofilm and amylase tolerances.

Materials and Methods

Bacterial Strains and Sample Preparation

Lactobacillus spp. were isolated using Lactobacillus selective agar (Merck KGaA, Germany) from milk products. The selected isolates were identified according to their morphological and biochemical properties. Strains were propagated and maintained in MRS (Man-Rogosa-Sharpe) broth, at 37°C. Then, strains were cultured in MRS broth at 37°C (24h). They centrifuged for on min at 4°C, 1000 rpm to obtain the supernatant part, which were filtered and used as analysis samples. In addition, Streptococcus mutans (ATCC 25175) was incubated for 24h, 37°C and 5% CO₂ in Brain Heart Infusion (BHI) Broth.

Antimicrobial Effect of Lactobacillus Spp. Aganist S. Mutans

The Minimum Inhibitory Concentration (MIC) of samples were measured against S. mutans using 96-well-plates (Lim et al., 2020). Lactobacillus spp. (Filter-sterilized) supernatants were serial diluted using BHI broth (ranging from 100%-0.78%). The diluted samples were added into each well (10⁶ CFU/mL). The lowest sample concentration (Lactobacillus spp) that inhibited max (99%) of the inoculums were considered as the minimum inhibition concentration. Ampicillin standard antibiotic kit was used as control group in MIC experiments. (500 μg/mL- 7.8 μg/mL).

Biofilm Formation of The Culture Supernatant of Lactobacillus spp Against S. Mutans

The effect of the Lactobacillus spp supernatants on biofilm formation was evaluated as Stepanovic et al (Stepanović et al., 2000). 100μL aliquot of the supernatant was added to 100 μL BHI containing 10⁶ CFU/mL of S. mutans each, and control sample was prepared by adding 100 μL MRS medium (instead of supernatant). Each sample (200 μL) was transferred to a 96 well plate following incubation (37°C, 24h). In order to assess the extent of biofilm formation in each microplate, the culture medium was discarded, and the plate was washed with 200 μL of PBS. Adherent biofilm cells were stained with 200 μL of 0.1% crystal violet for 10 min at room temperature. The plates were rinsed with distilled water, and then the adhered dye was dissolved with acid-alcohol solutions. The absorbance was measured as 540 nm, and biofilm inhibition amount was calculated. Three replicates were prepared for each sample.

A-amylase Tolerance

The pH of culture supernatant was measured after incubation for 24 h and 48 h. We also compared the growth characteristics of each Lactobacillus spp. strains α-amylase tolerances were assessed as a measure of resistance to oral enzymatic stress.
Table 1. Screening of twelve *Lactobacillus spp.* bacterial strains

| Strain | Species       | α-amylase tolerance | pH 24h | pH 48h | Researches/ Results                                                                 |
|--------|---------------|---------------------|--------|--------|-------------------------------------------------------------------------------------|
| 1 LF1  | *L. fermentum*| +++                 | 6.024  | 3.24   | Antagonistic activity on the growth of S. mutans, (Strahinic et al., 2007)            |
| 2 LF2  | *L. fermentum*| ++                  | 6.104  | 4.15   | L. rhamnosus reduced S. mutans associated caries risk and initial caries development, (Cagetti et al., 2013) |
| 3 LR1  | *L. rhamnosus*| +++                 | 6.014  | 4.03   | Inhibition of biofilm formation (Lim et al., 2020)                                   |
| 4 LR2  | *L. rhamnosus*| ++                  | 5.954  | 3.88   | Hampers S. mutans growth and biofilm formation in vitro (Vuotto et al., 2014)         |
| 5 LR3  | *L. rhamnosus*| +++                 | 6.034  | 4.12   |                                                                                                                                              |
| 6 LD1  | *L. delbrueckii*| +++                | 6.03   | 4.12   |                                                                                                                                              |
| 7 LD2  | *L. delbrueckii*| ++                 | 6.103  | 3.88   |                                                                                                                                              |
| 8 LP1  | *L. plantarum*| +++                 | 6.123  | 3.92   |                                                                                                                                              |
| 9 LP2  | *L. plantarum*| +++                 | 6.137  | 3.87   |                                                                                                                                              |
| 10 LP3 | *L. plantarum*| ++                  | 6.053  | 3.95   |                                                                                                                                              |
| 11 LA1 | *L. acidophilus*| +                 | 6.023  | 3.83   |                                                                                                                                              |
| 12 LA2 | *L. acidophilus*| +                  | 6.043  | 3.91   |                                                                                                                                              |

16S rRNA gene sequence identity %; *pH 6.8 1000U/mL of enzyme, 37°C, 4h.

Table 2. Antimicrobial activity of the *Lactobacillus spp.* against *S. mutans*

| Activity (S. mutans)                          | Minimum Inhibitory Concentration (%) |
|------------------------------------------------|-------------------------------------|
| S. mutans’ + BHI broth                        | LF1 >100 LF2 >100 LR1 >100 LR2 >100 LR3 >100 LD1 >100 LD2 >100 LP1 >100 LP2 >100 LP3 >100 LA1 >100 LA2 >100 |
| S. mutans + supernatant (24h)                 | <50<50<12.5<12.5<12.5<12.5<12.5<12.5<50<50 |
| S. mutans + supernatant (48h)                 | >50>50>25<25<25<25<25<25<12.5<12.5<12.5<ND<ND |
| Ampicillin (µg/mL)                            | <0.78<0.78<0.78<0.78<0.78<0.78<0.78<0.78<0.78<0.78 |

Ampicillin was used as control and it was serially diluted from 100 µg/mL to 0.78 µg/mL. a, b, c values with different letters in the same row are significantly different (P<0.05), ND: not detected. *: 106 cfu/mL.
Figure 1. Growth of *S. mutans* in BHI mixed with the spent culture supernatant from each *Lactobacillus* spp. strains

Figure 2. Inhibitory effect of the culture medium and supernatant of *Lactobacillus* spp strains on *S. mutans* biofilm formation (P<0.05).

For this experiment, the pH value of the BHI broth was adjusted to 6.8, and broth was supplemented α-amylase (1000 IU/mL) (A0521; Sigma Aldrich, USA). All strains were incubated (4h, 37°C), the samples were diluted in 0.05M buffer solution (sodium phosphate), α-amylase tolerance was determined by comparing final count with the initial count. The all experiments were performed three times.

**Statistical Analysis**

All the tested sample data are reported as the mean and standard deviation. One way analysis of variance (ANOVA) with SPSS statistical software (SPSS version 19.0 software, SPSS; Chicago, IL, USA) was used to determine the significant differences. All the mean values were used for the Duncan’s multiple tests to perform posthoc verification (P<0.05).
Result and Discussion

The antimicrobial activity of the *Lactobacillus spp.* supernatants of the selected strains against *S. mutans* was interpreted by measuring the optical densities at 595 nm (Optical Density at 595nm). We selected 12 strains of *Lactobacillus spp.* that were previously isolated form the milk and milk products, including 12 strains of *L. fermentum* (LF1, LF2), 3 strains of *L. rhamnosus* (LR1, LR2, LR3), 2 strains of *L. delbrueckii* (LD1, LD2), 3 strains of *L. plantarum* (LP1, LP2, LP3) and 2 strains of *L. acidophilus* (LA1, LA2). All of the strains were screened for antimicrobial activity against *S. mutans* using cultures and MIC for that purpose we analyzed indirect effect of the *Lactobacillus spp.* using only the culture filtrate that was obtained then its growth in BHI broth for 12h and 24 h (S. mutans + Lactobacillus supernatant interaction group).

All 12 Lactobacillus strain analysis showed antimicrobial activity, only the *L. acidophilus* strains (LA1, LA2) had no inhibitory effects on *S. mutans* (Figure 1) after 24h in culture. Based on the results, it could be said that the eight strains had the highest antimicrobial activity, i.e., *S. mutans* LP1, LP2, LP3, LD1, LD2, LR1, LR2 and LR3 (Figure 1). These strains reduced *S. mutans* growth by more than 87.5% after 24h culture (Table 2). The virulence of *S. mutans* might be due to their ability to survive in acidic pH, and productability of biofilm (Krzyściak et al., 2014; Simón-Soro and Mira, 2015).

The antimicrobial activities of *Lactobacillus spp.* were investigated against *S. mutans* (Table 1). Ampicillin standard was used as control and its MICs were <0.78% for *S. mutans*. The highest MIC values of LP1, LP2, LP3 were 12.5%, 12.5%, and 12.5% against *S. mutans* at 24h. The lowest MIC values of LA1, LA2, were 50% and 50% against *S. mutans* (Table 2).

LD and LR strains showed acceptable and sensitive antimicrobial activity against *S. mutans*. These effects could be attributable to the antibacterial substances produced including organic acids, bacteriocin and biosurfactants (Lin et al., 2017). The most of oral *Lactobacillus spp.* can inhibit the growth of pathogens causing periodontitis and caries in vitro (Köll-Klais et al., 2005).

Rossoni et al. (Rossoni et al., 2018) compared the effects of twenty-two strains of *Lactobacillus* that were isolated from oral cavities of caries-free subjects. All of the *Lactobacillus spp.* showed antimicrobial activity against *S. mutans*. *L. paracasei* 25.4, *L. fermentum* 20.4, *L. paracasei* 20.3 and *L. paracasei* 11.6 observed the highest antimicrobial activity against *S. mutans*. These strains reduced *S. mutans* growth by more than 86% after 24 h in culture.

Eight *Lactobacillus spp.* strains showed different bactericidal activities in the time-kill assay. Strains LP1, LP2 and LP3 from traditionally fermented milk products showed stronger antibacterial activity compared with other *Lactobacillus spp.* (LA1, LA2) strains against *S. mutans* after incubation for 24h. The reason for this exclusion might be closely correlate to race, living environment, health status and food intake of the host.

A large number of extracellular polysaccharides synthesized by *S. mutans* are important to the complex tridimensional structure of a dental plaque (Bowen and Koo, 2011). According to a report by Nobbs and colleagues, the formation of an early biofilm of *S. mutans* can be observed at 24 h (24 h: maturation of early-stage biofilm and 48 h: maturation of the later-stage biofilm (Nobbs et al., 2009).

In this study, we added the *L. plantarum* strains fermentation supernatant at these two time points (24 h and 48 h) to mediate the formation of the *S. mutans* biofilm. The ability of a probiotic to colonize the oral cavity is key to its function, and this ability is strain-specific. *Lactobacillus* family may effectively prevent biofilm formation, perhaps because *L. plantarum* (5D-3) had the advantage of pre-colonization of the oral cavity (Zhang et al., 2020).

The all strains that completely inhibited the growth of *S. mutans* was further investigated for effects on biofilm inhibition formation. Among to twelve strains showing antibacterial activity, LP1 exhibited the strongest antibiofilm formation activity. (Figure 2). Different studies have shown that the *L. rhamnosus* and *L. paracasei* origin consumption could reduce *S. mutans* biofilm formation (Chuang et al., 2011).

In our study, all *Lactobacillus spp.* strains prevented biofilm formation by inhibition at different levels. When our study findings are evaluated, supernatant cultures ability to inhibit biofilm formation parallels the antimicrobial activity findings. Our results related to the antimicrobial activity and biofilm formation inhibition levels of *Lactobacillus spp.* strains on *S. mutans* are close to those of previous studies. Reports have emphasized the importance of biofilm formation in the development of dental caries or oral cavity microflora (Klein et al., 2015; Salli et al., 2017). Recently, researchers are taking more interest in the use of probiotics to maintain the oral health (Rossoni et al., 2018; de Souza Rodrigues et al., 2020; Lim et al., 2020).

While both the containing culture medium and supernatant of *Lactobacillus spp.* could inhibit the *S. mutans* biofilm formation, the supernatant ingredients samples showed higher inhibitory effects (Figure 2). *L. plantarum* inhibited the *S. mutans* biofilm formation by over 80%, at the same volume, and showing it as the strongest inhibitor agents. *L. acidophilus* could inhibit the *S. mutans* biofilm formation at low rates (<30%). These biofilm mechanisms have been announced to be due to the coaggregation with *Lactobacillus spp.* resulting in physical interference and induction of exopolysaccharides production (Wu et al., 2015; Ahn et al., 2018).

Lee and Kim found that Lactobacillus rhamnosus LGG suppressed *S. mutans* biofilm formation by reducing glucan production and antimicrobial activity (Lee and Kim, 2014).

A mature dental plaque biofilm is a three-dimensional micro-ecological environment comprising various bacteria embedded in a matrix mainly composed of water-insoluble polysaccharides with a certain thickness (Featherstone, 2004). As shown in Table 1, the initial pH values also showed the α-amylase tolerance ability. The initial pH of the cultures was 6.90. After that pH value decreased to 5.95-6.13 then 24 h of incubations. The end of the incubation 45 h cultures pH of the strains was significantly lower, which ranged from 3.83-4.15 (P<0.05). Culture strains LF1, LR1, LR3, LD1, LP1, LP2 and LP3 showed the highest α-amylase resistance, with survivalibility >90%.
Conclusion

Within the limitations of this in vitro study, the following conclusions were drawn.

- Probiotics showed different levels of antimicrobial and antibiofilm activity on oral flora.
- Probiotic bacteria contained in traditional fermented milk products *S. mutans* inhibition was found at significant levels. Especially *L. plantarum* is thought to be a potent oral probiotic for oral and dental health by preventing biofilm formation.
- The identification of these *Lactobacillus* spp. strains, which naturally inhabit the oral cavity and show antimicrobial activity against *S. mutans*, contributes to the development of new probiotic agents to prevent dental caries.

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