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Do *Lactobacillus rhamnosus*-Originated Probiotic and Parabiotic Have Inhibitory Effects on Intraocular Lens Biofilm?

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ABSTRACT

Objective: Our aim was to investigate the formation of *Staphylococcus (S.) epidermidis* biofilm on hydrophobic acrylic lenses and whether the inhibition of the formed biofilm is possible with probiotic *Lactobacillus (L.) rhamnosus* 312 and parabiotic prepared from it.

Materials and Methods: The probiotic bacteria *L. rhamnosus* 312 and intercellular adhesion (ICA) gene-positive tested bacteria *S. epidermidis KA15.8* were used in the study from stock. To obtain the parabiotic the cultures were developed in De Man Rogosa and Sharpe (MRS) broth for 48 hours and autoclaved at 121ºC for 15 minutes. Biofilms on hydrophobic acrylic intraocular lenses and the antibiofilm effects of parabiotic and probiotic *L. rhamnosus* were evaluated. Scanning electron microscopy photos of biofilms produced on intraocular lenses (IOLs) were taken.

Results: Probiotic *L. rhamnosus* 312 and the parabiotic test showed antibacterial activity on test bacteria, ICA positive *S. epidermidis KA15.8*. However, the probiotic *L. rhamnosus 312* zone diameter was found to be wider. After the biofilm was formed, the addition of parabiotic inhibited the biofilm formed by *S. epidermis KA15.8* by 58.29%. The number of *S. epidermidis KA15.8* in the biofilm also decreased.

Conclusion: Parabiotic and probiotic *L. rhamnosus 312* was found effective for its antibiofilm effect. However, further studies with different concentrations are needed.

Keywords: Probiotic, parabiotic, ophthalmology, cataract, intraocular lens, biofilm

INTRODUCTION

Staphylococcus (*S.*) *epidermidis* constitute the main flora of the ocular surface and eyelids together with *Corynebacterium* species and *Propionibacterium acnes*. Also, *S. epidermidis* is the most common bacteria that causes postoperative endophthalmitis. Biofilm formation ability on abiotic surfaces contribute to the virulence of *S. epidermidis* (1-5). It is mostly accepted that *S. epidermidis* enters the intraocular area during and/or after ocular surgeries and causes endophthalmitis (3). Postoperative endophthalmitis is a devastating complication of intraocular lens (IOL) implantation after cataract surgeries. Researchers have reported the incidence rates of postoperative endophthalmitis as 0.08-0.11% after surgery (4, 6). The adhesion of bacteria and biofilm formation on IOL materials has been reported by investigators (4, 5, 7, 8).

Biofilm is a defense mechanism developed by microorganisms to avoid the bactericidal effect of an antimicrobial agent or to protect themselves against improper environmental conditions and the host's defense. The self-secreted extracellular polymeric substance (EPS) constitutes a protective environment. The formation of biofilm by ocular bacteria on intraocular lenses, contact lenses, suture material, valve implants, socket implants, orbital implants, and scleral buckles have been reported (9). It has been shown in studies that *S. epidermidis* with the intercellular adhesion (ICA) A gene adheres to intraocular

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lenses (10,11). According to the production of polysaccharide intercellular adhesion (PIA) molecules encoded by the ICA locus, biofilm formation ability may exist, especially in ICA A gene, ICA B gene, ICA C gene, and ICA D gene-positive strains (12-14).

Biofilm-producing bacteria are resistant to antibiotics. Studies on the search for new compounds to inhibit biofilms are increasing day by day. For this purpose, probiotics, postbiotics, and/or parabiotics have started to gain importance in recent years. Postbiotics and paraprobiotics are the terms that have begun to be used to identify non-vivid microorganisms or non-bacterial extracts that benefit the host by providing bioactivity (15). *In vitro* and *in vivo* studies have shown that some postbiotics and paraprobiotics exhibit bioactivities such as anti-inflammatory, anti-proliferative, antioxidant, antimicrobial and immunomodulatory (15,16).

In this study, it was aimed to investigate the formation of *S. epidermidis* biofilm on hydrophobic acrylic lenses and whether the inhibition of the formed biofilm is possible with probiotic *Lactobacillus* (*L.*) *rhamnosus 312* and parabiotic prepared from it.

MATERIALS AND METHODS

Bacteria

The probiotic bacteria *L. rhamnosus 312* and tested bacteria *S. epidermidis KA15.8* used in the study were obtained from Eskişehir Technical University Faculty of Science, Microbiology Department. *L. rhamnosus 312* is a probiotic bacteria of human origin. *S. epidermidis KA15.8* is a methicillin-resistant pathogen isolated from the ocular surface and has the ICA gene (8). *L. rhamnosus 312* and *S. epidermidis KA15.8* were removed from stock. *L. rhamnosus 312* was seeded in De Man Rogosa and Sharpe (MRS) broth (Merck, 110661**)** and *S. epidermidis KA 15.8* in brain-heart infusion (BHI) broth **(**Merck, 1104930500). The MRS broth tubes were incubated at 37 °C for 48 hours under 5% CO_2 conditions. BHI broth tubes were incubated at 37 °C for 24 hours. A line culture was incubated on MRS agar (Merck, 110660) and BHI agar (Merck, 1038700500) separately from the liquid media for purity control under appropriate conditions. After incubation, the purity of the cultures were checked morphologically and microscopically by Gram staining and then used in the studies.

Determination of the Antibacterial Effect of *L. rhamnosus 312* Live (probiotic) and Autoclaved Cultures (parabiotic) of the Cultures

The antibacterial activity of *L. rhamnosus 312* was determined by the well method. The *S. epidermidis* culture was transferred into sterile Mueller Hinton (MH) agar (Merck, 1038720500) at 10^6 cfu/mL and mixed well. It was then transferred to a sterile petri dish. After the agar was solidified, a 0.8 mm diameter well was opened using a sterile cork borer. Then, 80 µL of the cultures that had been developed in MRS broth for 48 hours and autoclaved at 121°C for 15 minutes and live bacterial culture in MRS broth were transferred to the well and incubated at 37°C for 24 hours. Zone diameters formed at the end of the incubation period were measured and evaluated. Petri dishes containing pathogenic bacteria were used as control.

Determination of Antibiofilm Activity of Probiotic and Parabiotic *L. rhamnosus 312* Culture on Intraocular Lenses

The experiment was carried out in two ways. In the first group, 48-hours cultures containing 10^{10} cfu/mL *L. rhamnosus 312* were used directly. In the other group, 48-hour cultures of *L. rhamnosus 312*, whose cell density was adjusted to 10^{10} cfu/mL, were used after autoclaving (paraprobiotic) for 15 minutes at 121°C. Two separate experimental sets were set up for each group, one before biofilm formation and one after biofilm formation. *S. epidermidis* was inoculated in BHI broth and incubated at 37°C for 24 hours.

Intraocular acrylic lenses were taken out of their packages, placed in the falcon containing phosphate-buffered saline (PBS), and incubated at 37 °C for 15 minutes. Then, an acrylic lens was placed in each well in a 12-well ELISA petri dish. Next, 200 µL of medium was added to the lenses to be used as a control. In order to determine the biofilm of *S. epidermidis KA15.8*, 25 µL of *S. epidermidis KA15.8* culture was transferred onto the lenses and 975 µL of tryptic soy broth (TSB) (Merck, 1054590500) containing 2% glucose was added. To determine the biofilm formed by *L. rhamnosus 312* live cells, 25 µL of *L. rhamnosus 312* culture was added to the lenses, and 975 µL of TSB containing 2% glucose was added. To determine the antibiofilm activity of *L. rhamnosus 312*, 25 µL of *S. epidermidis KA15.8* culture was added to the lenses, and 475 µL of TSB containing 2% glucose was added to 500 µL of *L. rhamnosus 312* culture. Then the prepared petri dishes were incubated at 37°C for 24 hours. All tests were prepared in triplicate. The same procedures were repeated with the autoclave *L. rhamnosus 312*.

Determination of Biofilm

In order to determine the biofilm, the lenses were carefully washed with PBS, and the bacteria in planktonic form were removed and then transferred to Eppendorf tubes. Next, 200 µL of 96% methanol was added to them, kept for 10 minutes, and washed with sterile PBS, and 100 µL of crystal violet was transferred to each Eppendorf tube and kept for 5 minutes. After washing them with sterile PBS, they were placed on 12 plates and 1000 µL of 33% glacial acetic acid was transferred onto them to release the cells. Then, 200 µL was transferred to 96 plates and a reading was made in the spectrophotometer at 570 nm.

Determination of the Count of Bacteria in Biofilm

The same set of experiments set up for biofilm was prepared. In order to determine the biofilm, the lenses were carefully washed with sterile PBS, and the bacteria in planktonic form were removed, and then transferred to falcons containing 1 mL of sterile PBS. To separate the cells from the biofilm matrix, they were vortexed for 1.5 seconds and sonicated for 15 minutes. Then, bacteria counts were done by using the drop plate method. Lactic acid bacteria were incubated at 37°C for 48 hours in an environment containing 5% CO_2, and pathogens were incubated at 37°C for 24 hours. Bacteria counts were done after incubation. All studies were examined in triplicated.

Scanning Electron Microscope (SEM)

Bacterial adhesion was examined by SEM with some modifications according to Okajima et al. (17). The *S. epidermidis* isolate was incubated in TSB containing 0.25% glucose for 24 hours at 37°C. After incubation, IOLs were carefully removed and washed 3 times with PBS. The IOL was fixed with 2.5% (wt/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) by keeping it for 2 hours at room temperature. It was then washed 3 times for 15 minutes in 0.1 M sodium cacodylate. After this process, the lenses were rinsed with distilled water and dehydration was performed with alcohol series (50%, 70%, 80% and 95%). After 7 minutes in each batch, they were kept in two pure alcohols for 15 minutes. Drying was carried out in a Critical Point Dryer immediately after the alcohol series. They were then covered with gold and examined in SEM.

RESULTS

Probiotic *L. rhamnosus 312* and the parabiotic test showed antibacterial activity on the tested bacteria, ICA-positive *S. epidermidis KA15.8*. However, the probiotic *L. rhamnosus 312* zone diameter was found to be wider. Zone diameters of probiotic *L. rhamnosus 312* and parabiotic were measured as 13 mm and 11 mm, respectively. Both probiotic *L. rhamnosus 312* and *S. epidermidis KA15.8* formed a biofilm on the acrylic lens (Figure 1). The addition of probiotic bacteria before biofilm formation inhibited the biofilm formation of *S. epidermidis KA15.8* by 57.55%. The viable bacteria count in the biofilm also supported this finding. The number of *S. epidermidis KA15.8* in the biofilm decreased from 7.00 Log 10/mL to 5.22 Log 10/mL. In the case of autoclaved probiotic bacteria/parabiotic addition, biofilm formation decreased by 59.61%. Due to bacterial numbers, the number of *S. epidermidis* bacteria decreased from 7.10 Log 10/mL to 3.45 Log 10/mL (Figure 1). After biofilm formation, the addition of probiotic *L. rhamnosus 312* inhibited the biofilm formation of *S. epidermis KA15.8* by 55.3%. Due to *S. epidermis KA15.8* numbers in the biofilm, it decreased from 8.40 Log 10/mL to 6.16 Log 10/mL. After the biofilm was formed, the addition of parabiotic inhibited the biofilm formed by *S. epidermis KA15.8* by 58.29%. The number of *S. epidermidis KA15.8* in the biofilm also decreased from 8.20 Log 10/mL to Log 4.65 Log 10/mL (Figure 2). SEM images also confirm the findings (Figure 3).

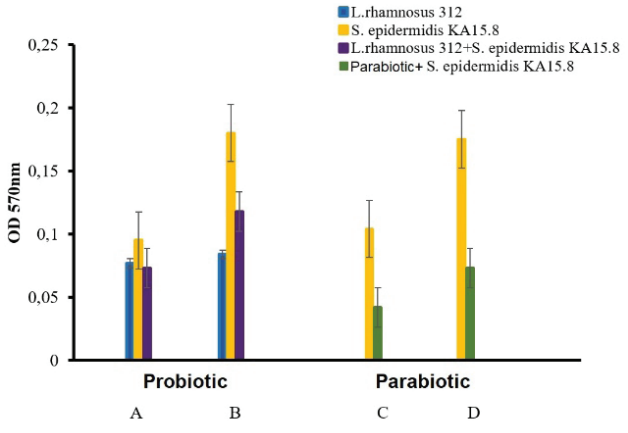

Figure 1. Antibiofilm activity of probiotic *L. rhamnosus 312* and parabiotic.

A: Addition of the probiotic before biofilm formation; B: Addition of the probiotic after biofilm formation; C: Addition of the parabiotic before biofilm formation; D: Addition of parabiotic after biofilm formation.

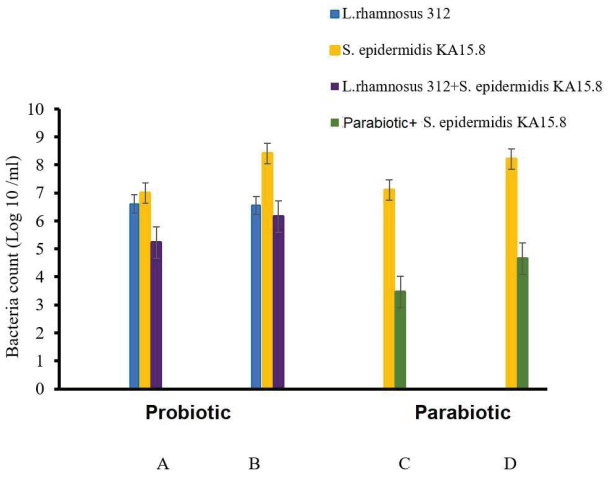

Figure 2. Bacteria counts in the biofilm.

A: The number of bacteria in the biofilm formed as a result of the addition of the probiotic before the biofilm formation; B: The number of bacteria in the biofilm when the probiotic is added after the biofilm has formed; C: The number of *S. epidermis* in the biofilm formed as a result of the addition of the parabiotic before the biofilm is formed; D: The number of *S. epidermis* with the addition of parabiotic after biofilm formation.

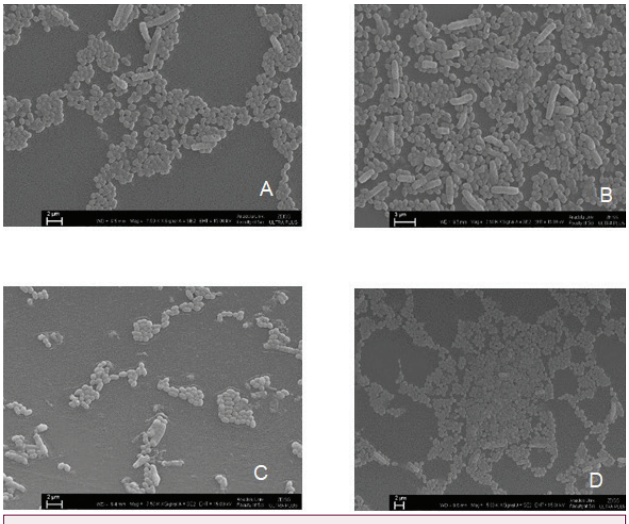

Figure 3. The effect of probiotic *L. rhamnosus 312* and parabiotic on *S. epidermidis 15.8* biofilm.

A: Probiotic applied before biofilm formation; B: Probiotic applied after biofilm formation; C: Parabiotic applied before biofilm formation; D: Parabiotic applied after biofilm formation.

DISCUSSION

The term paraprobiotics are used to refer to non-living or inactivated strains of probiotics, in their intact or fragmented form (18). However, there is no complete consensus on these definitions. It usually includes probiotic-derived metabolites or cell wall-derived materials without living microorganisms or cell structures (18). The preparations of *L. rhamnosus 312*, both alive and non-living, both prevented the biofilm formation of *S. epidermidis* and inhibited the formed biofilm on the acrylic lens.

Mohamed et al. reported that cell-free filtrates of *Lactobacillus* and *Bifidobacterium* inhibited the growth of *S. epidermidis* isolates originating from the conjunctiva (19). Researchers reported that probiotics could be alternative antimicrobials against pathogenic *Staphylococcus spp*. associated with conjunctivitis. Their findings support our results. It has been reported that the antimicrobial effect is associated with metabolic products of probiotic bacteria, such as bacteriocin, bacteriocin-like substances, hydrogen peroxide, acetic acid, and substances such as diacetyl, and lactic acid (20). Negi et al. found that the antimicrobial activity of cell-free supernatants of *Lactobacillus spp*. was dependent on the presence of an antimicrobial protein (21). Mazoteras et al. (22) reported that biofilm on IOLs can be found many years after uncomplicated cataract surgeries. Kıvanc et al. (23) and Okajima et al. (17) reported that *S. epidermidis* formed a strong biofilm on acrylic lenses, as in our findings. El-Ganiny et al. (24) reported that, contrary to our findings, *S. epidermis* isolates, isolated from soft lenses formed weak biofilms. The fact that bacteria in biofilms are more resistant to antiseptics, antibiotics, and host defenses causes problems in their inhibition. Physiological heterogeneity, complex structure, high flow expression, and the relative anaerobicity in the deeper layers of the biofilm may be responsible for this high resistance. Studies for the prevention and inhibition of biofilm formation have intensified in recent years. One of the leading methods is probiotic lactic acid bacteria.

Studies on the application of postbiotics and parabiotics to foods are increasing day by day. There are also some studies in the field of health. Mantziari et al. presented a data about the usage of post-probiotics against pathogens that cause pediatric infectious diseases. However, a study on lenses were not encountered in the literature review (25). According to our knowledge, this study is the first study on the inhibition of *S. epidermidis* biofilm in acrylic lenses by probiotic lactic acid bacteria and parabiotic biofilm. A product has been prepared and patented for the use of the supernatant of *Lactobacillus paracasei* in the treatment of conjunctivitis, especially vernal keratoconjunctivitis (VKC) (26). There are some studies on the inhibition of biofilm in soft lenses and the antibiofilm activity of some disinfectants, herbs, and solutions (24, 27, 28). Kilvington and Lonnen evaluated the ability of contact lens solutions to remove the biofilm formed on silicone hydrogel lenses. Results have been reported to be unsatisfactory when the scrubbing and rinsing steps of the lenses are skipped (29).

In recent years, it has been shown that as an alternative to the antimicrobial and antibiofilm activities of probiotic bacteria, cell-free filtrates or substrates with inhibited cells also show antimicrobial and antibiofilm activities. Postbiotics include products of microbial action such as the fermentation of carbohydrates, vitamins, various peptides, and the synthesis of enzymes. Even some structural components of bacteria, such as teichoic acid, are considered postbiotics. It has been reported that postprobiotics have different functions such as immunomodulators, antioxidants, and antimicrobials (16, 30). From this perspective, postbiotics/parabiotics prepared from lactic acid bacteria contain many metabolites used as raw concentrate (extract) or semi-purified form (18).

In conclusion, the parabiotic of *L. rhamnosus 312* was found effective for its antibiofilm effect. However, further studies are needed on this subject. Putatively, higher antibiofilm activity can be achieved with a concentration adjustment. In particular, the parabiotic product can have many advantages. *L. rhamnosus 312* is a promising isolate considering that paraprobiotics and postbiotics have significant potential for the development of biotechnological products.

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