



Effects of Cell Free Supernatants of *Lactobacillus reuteri* ATCC55730 and *Lactobacillus plantarum* FI8595 Against Selected Food-Borne Pathogens and Fish Spoilage Microorganisms

Hatice Yazgan *

*¹ Cukurova University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Adana, Turkey (ORCID:0000-0002-7520-3342),
hyazgan@cu.edu.tr

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Abstract

The effects of cell free supernatants of *Lactobacillus reuteri* ATCC55730 and *Lactobacillus plantarum* FI8595 against selected fish spoilage and food-borne pathogen microorganisms were investigated. For detection of the antimicrobial effect of cell free supernatants from two *Lactobacillus* strains, four food-borne pathogens (*Salmonella* Paratyphi A NCTC13, *Staphylococcus aureus* ATCC29213, *Klebsiella pneumoniae* ATCC700603, and *Listeria monocytogenes* ATCC19112) and three fish spoilage bacteria (*Proteus mirabilis*, *Enterococcus faecalis* and *Pseudomonas luteola*) were used. The agar well diffusion method was performed to determine the antimicrobial effects. The cell free supernatants of both *L. reuteri* and *L. plantarum* were effective on fish spoilage bacteria tested. Especially, *E. faecalis* showed high sensitivity against both CFS obtained from *L. plantarum* and *L. reuteri* with 16.75 and 22.75 mm inhibition zone diameter, respectively. The CFS of *L. reuteri* exerted a strong ability to inhibit the growth of all food-borne pathogens. The highest antibacterial effect of CFE of *L. reuteri* was observed on *S. aureus*, with the diameter of 26.50 mm inhibition zone.

Keywords: *Lactobacillus*, food-borne pathogen, fish spoilage bacteria, antimicrobial activity

Lactobacillus reuteri ATCC55730 ve *Lactobacillus plantarum* FI8595 Supernatantlarının Bazı Balık Bozucu ve Gıda Kaynaklı Patojen Bakterilerine Karşı Antimikrobiyal Etkisi

Öz

Bu çalışmada *Lactobacillus reuteri* ATCC55730 ve *Lactobacillus plantarum* FI8595 laktik asit bakterileri tarafından üretilen hücresiz supernatantın seçilen gıda kaynaklı patojen ve balık bozucu bakterilerine karşı antimikrobiyal etkisi araştırıldı. İki *Lactobacillus* suşundan elde edilen supernatantın antimikrobiyal etkisini belirlemek için, dört gıda kaynaklı patojen (*Staphylococcus aureus* ATCC29213, *Salmonella* Paratyphi A NCTC13, *Klebsiella pneumoniae* ATCC700603 ve *Listeria monocytogenes* ATCC19112) ve üç balık bozucu bakterisi (*Proteus mirabilis*, *Enterococcus faecalis*, *Pseudomonas luteola*'da) kullanıldı. Supernatantların gıda kaynaklı patojen ve balık bozucu bakterileri üzerindeki antimikrobiyal etkisi agar kuyu difüzyon yöntemine göre belirlenmiştir. *L. plantarum* ve *L. reuteri*'nin supernatantı, test edilen balık bozucu bakterilerine karşı güçlü bir antimikrobiyal etkiye sahip olmuştur. Özellikle *E. faecalis*, *L. plantarum* ve *L. reuteri*'den elde edilen supernatanta karşı sırayla 16.75 ve 22.75 mm inhibisyon zon çapı ile yüksek bir hassasiyet göstermiştir. *L. reuteri*'nin supernatantı, test edilen tüm gıda kaynaklı patojenlerin inhibisyonunda çok güçlü bir yetenek göstermiştir. Özellikle *L. reuteri*'den elde edilen supernatant, 26.50 mm inhibisyon çapı ile *S. aureus*'u inhibe etme kabiliyetinin yüksek olduğunu göstermiştir.

Anahtar Kelimeler: *Lactobacillus reuteri*, *Lactobacillus plantarum*, antimikrobiyal aktivite, gıda kaynaklı patojen bakteri, balık bozucu bakteri

* Corresponding Author: hyazgan@cu.edu.tr

1. Introduction

Lactic acid bacteria (LAB) have been used in the fermentation process as a preservation strategy since ancient times because they have the ability to produce metabolites which possess antimicrobial properties for instance, bacteriocin, organic acids, and hydrogen peroxide (Leroy & De Vuyst, 2004). These metabolites increase the food safety and shelf life by inhibition undesirable food-borne pathogens and spoilage microorganisms. They also produce several enzymes, exopolysaccharides, and aroma compounds (Jose et al., 2015). It is difficult to elucidate the precise mechanism of antimicrobial effect of different metabolic products due to the combined and synergistic interactions between these metabolic compounds (Niku-Paavola et al., 1999, Corsetti et al., 1998). Among the lactobacilli, *Lactobacillus plantarum* is one of the most adaptable species and common microorganisms found in different environments from food such as dairy, fermented meat and fish, and vegetable to the human gastrointestinal tract (Tremonte et al., 2017; Papadimitriou et al., 2015; Ricciardi et al., 2014; Basso et al., 2004; Kleerebezem et al., 2003). Moreover, *L. plantarum* strains were reported to produce different metabolite with antimicrobial properties such as bacteriocins, hydrogen peroxide, diacetyl, organic acids, and antimicrobial peptides (Cortes-Zavaleta et al., 2014; Arena et al., 2016). Various *Lactobacillus* strains containing *L. plantarum* display antimicrobial activity against spoilage and food-borne pathogen bacteria. Such antimicrobial impact was generally attributed to the production of metabolic products such as organic acids, bacteriocin, etc. (Arena et al., 2016; Rodríguez-Pazo et al., 2013; Tejero-Sariñena et al., 2012; Neal-McKinney et al., 2012; Kos et al., 2011; Tharmaraj and Shah, 2009). On the other hand, *Lactobacillus reuteri* is the most important other probiotic *Lactobacillus* species which has effective antimicrobial properties against fungi, and protozoa, Gram-positive and Gram-negative bacteria, yeast mainly because of their ability to convert glycerol into a broad spectrum antimicrobial agent, called reuterin (3-hydroxypropionaldehyde) (Bain et al., 2011; Zhao and Mutukumi 2012). They also produce lactate, ethanol, and CO₂ as end-products (Axelsson et al., 1998). Recently, researches have focused on understanding the antimicrobial mechanism of action and the usability of these compounds as antimicrobial agents in food industry and studies in this direction have increased. Therefore, there is need for safe alternative method for controlling food-borne pathogenic and spoilage microorganisms, thus reducing the risk of food-related outbreaks.

With the increasing demand for safe and high nutritional value food products, the consumption of fish and seafood has increased. However, they are considerably susceptible to chemical and microbiological spoilage throughout storage or processing (Sampels, 2015). How to control the types and amounts of pathogen and spoilage bacteria is a major issue for seafood industry (Yazgan et al., 2019; Ozogul et al., 2017). Thus, appropriate preservation approaches are required for prolonging the shelf life of such products as well as ensuring the quality, and safety (Hassoun & Karoui, 2016). Thus, the application of cell free supernatant (CFS) obtained from *Lactobacillus reuteri* and *plantarum* can be alternative sufficient method for preservation. Muhsin et al. (2015) evaluated the protective impact of *Lactococcus* cell free supernatant (CFS) and crude bacteriocin against bacterial growth and biofilm formation with the same pathogen. It has been reported that the CFS and neutralized cell free (NCFS) showed an inhibition zone when tested against

related lactic acid bacteria, food-borne pathogens, and food-spoilage microorganisms by the agar well diffusion assay (Hwanhlem et al., 2017). *Enterococcus faecalis*, *Brochothrix thermosphacta*, *Lactobacillus sakei* subsp. *sakei*, *Carnobacterium maltaromaticum*, *Staphylococcus aureus* and *Listeria ivanovii* were found to be susceptible to CFS obtained from *L. lactis* subsp. *lactis*, while only *L. sakei* subsp. *sakei*, *B. thermosphacta* and *L. ivanovii* were determined to be susceptible to NCFS obtained from same bacteria.

The purpose of the current study was to evaluate the antimicrobial effect of CFS obtained from *Lactobacillus plantarum* and *Lactobacillus reuteri* and their potential use as a biopreservative in fish products.

2. Material and Method

2.1. Bacterial strains

The food-borne pathogen bacteria used to detect the antimicrobial effect of cell free supernatants from two *Lactobacillus* strains were *Staphylococcus aureus* ATCC29213, *Salmonella* Paratyphi A NCTC13, *Klebsiella pneumoniae* ATCC700603, and *Listeria monocytogenes* ATCC19112. All pathogen bacteria were purchased from the American Type Culture Collection (Rockville, MD, USA) and Spanish Type Culture Collection, CECT, Valencia, Spain. In addition, the fish spoilage bacteria were *Proteus mirabilis*, *Enterococcus faecalis* and *Pseudomonas luteola*. These strains were isolated from spoiled sea bass muscles in a previous study by Yazgan et al. (2019). Isolates were identified according to the manufacturer's instructions for the API 20E and API 20 NE strip system (BioMereux). Polymerase chain reaction (PCR) was used for rapid detection and identification of these spoilage bacteria. *Lactobacillus plantarum* FI8595 and *Lactobacillus reuteri* ATCC55730 which was used to obtain cell free supernatant were purchased from Sutcu Imam University, Kahramanmaraş, Turkey as a BGML stock culture and American Type Culture Collection (Rockville, MD, USA) respectively.

2.2. Preparation of Bacterial Cell-Free Supernatant

The cell free supernatants were prepared using the method of Hwanhlem et al. (2017). Briefly, the *L. plantarum* and *L. reuteri* were grown in two separate tubes of 50 ml including MRS broth (Merck 1.10661, Darmstadt, Germany) at 37 °C for 48 h. After that, each bacterial culture was centrifuged (Hettich 32R, Tuttlingen, Germany) at 11200xg for 10 min at 4 °C to obtain cell-free supernatants. The cell-free supernatants were filtered through membranes with pore size 0.45 µm and stored at 4 °C until use.

2.3. Determination of Antimicrobial Effect

The agar well diffusion method was performed to determine the antimicrobial effect of cell free supernatants on food-borne pathogen and fish spoilage bacteria. Briefly, Muller Hinton agar (MHA) was evenly poured into sterile petri dishes. Standardized agar wells with a diameter of 5 mm were made in the agar plate using blunt end of a sterile Pasteur pipette. One hundred µl of each pathogen and spoilage bacterial culture suspension (10⁸ cfu/ml) was spread on the agar plate. After that, each well was filled with 70 µl of two different the cell-free supernatant and MRS broth as

negative control and all plates were incubated at 37 °C for 24 hours. Then, the diameter of the inhibition circular around each well were measured and results were expressed in mm by the arithmetic mean of the diameter.

2.4. Statistical Analysis

All assays were performed in triplicate and the data were recorded as mean \pm standard deviation for measurements. The significance of differences ($p < 0.05$) was determined using one-way ANOVA with the statistical package for Social Sciences (SPSS) software (Version 20, SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

The antimicrobial effect of cell free supernatants (CFS) obtained by *L. plantarum* and *L. reuteri* against three fish spoilage bacteria including *P. mirabilis*, *E. faecalis* and *P. luteola* are presented in Table 1. The diameter of the inhibition zone generally refers to the rate of inhibition impact of the antimicrobial agent applied to the sample. It means that the antimicrobial effect is stronger in sample where a high zone diameter is detected. In the current study, significantly different ($p < 0.05$) antimicrobial activities were found in all samples. The cell free supernatants of both *L. plantarum* and *L. reuteri* had a strong antimicrobial activity against fish spoilage bacteria. Especially, *E. faecalis* displayed a high sensitivity for both of CFS obtained from *L. plantarum* and *L. reuteri* with 16.75 and 22.75 mm inhibition zone diameter, respectively. However, the lower antimicrobial activities of CFS from *L. plantarum* against this bacterium was

observed compared to that of CFS from *L. reuteri*. This inhibitory impression is probably due to higher concentration of antimicrobial metabolites produced by *L. reuteri*. Based on the results observed (Table 1), the cell-free supernatant from *L. reuteri* also showed high antimicrobial effect on *P. mirabilis* and *P. luteola* with diameter zones of 21.50 and 21.25 mm, respectively.

MRS used as a negative control did not show any antimicrobial activities as expected (Table 1). The CFS from *L. plantarum* exhibited similar antimicrobial impact against *P. mirabilis* and *E. faecalis* with 16.25 and 16.75 mm inhibition zone diameter, respectively. However, cell-free supernatant from *L. plantarum* showed lower inhibition effect on *P. luteola* with the 14.88 mm inhibition zone diameter.

3.1. Antimicrobial Effect of Fish Spoilage Bacteria

Antimicrobial effect of CFS obtained from two selected *Lactobacillus* species against food-borne pathogen bacteria are showed in Table 2. According to the data obtained from current study, *L. reuteri* displayed a quite strong ability to inhibit the growth of food-borne pathogens tested. Especially, the CFS of *L. reuteri* showed the high ability to inhibit *S. aureus* with the diameter of 26.50 mm inhibition zone. Soleimani et al. (2010) found that CFS from *L. reuteri* ATCC 23272 exhibited lower antimicrobial activity (7 and 10 mm inhibition zones, respectively) on *S. aureus* ATCC25923 and *S. aureus* isolated from bovine mastitis.

Table 1. Antimicrobial activities of cell-free supernatant against fish spoilage bacteria

Fish spoilage bacteria	<i>Lb. plantarum</i>	<i>Lb. reuteri</i>	MRS broth
<i>Proteus mirabilis</i>	16.25 \pm 0.96 ^b	21.50 \pm 0.41 ^a	0.00 ^c
<i>E. faecalis</i>	16.75 \pm 0.65 ^b	22.75 \pm 0.65 ^a	0.00 ^c
<i>Pseudomonas luteola</i>	14.87 \pm 0.85 ^b	21.25 \pm 0.50 ^a	0.00 ^c

Values represents mean \pm standard deviation. The same superscript (^{a-c}) in the same row were not significantly different ($P > 0.05$)

Table 2. Antimicrobial activities of cell-free supernatant against food-borne pathogen bacteria

Food-borne pathogen bacteria	<i>Lb. plantarum</i>	<i>Lb. reuteri</i>	MRS broth
<i>Staphylococcus aureus</i>	20.38 \pm 0.85 ^b	26.50 \pm 0.82 ^a	0.00 ^c
<i>Klebsiella pneumoniae</i>	15.00 \pm 0.91 ^b	22.63 \pm 0.75 ^a	0.00 ^c
<i>Salmonella Paratyphi A</i>	21.50 \pm 0.41 ^a	18.75 \pm 0.96 ^b	0.00 ^c
<i>Listeria monocytogenes</i>	13.75 \pm 0.96 ^b	17.25 \pm 0.65 ^a	0.00 ^c

Values represents mean \pm standard deviation. The same superscript (^{a-c}) in the same row were not significantly different ($P > 0.05$)

4. Conclusions and Recommendations

The cell free supernatants of both *L. plantarum* and *L. reuteri* had a strong antimicrobial activity on fish spoilage bacteria. Especially, *E. faecalis* exerted a high sensitivity for both of CFS obtained from *L. plantarum* and *L. reuteri*. The CFS of *L. reuteri* displayed a considerably strong ability to inhibit the growth of all food-borne pathogens tested. The highest inhibition effect of CFE from *L. reuteri* was obtained against *S. aureus*. The use of CFS from *Lactobacillus* as antimicrobial agent could be innovative approach in food industry. CFS obtained from selected bacteria could be efficient in inhibiting food-borne pathogen and fish spoilage microorganisms.

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