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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Antibacterial Activity and In vitro Probiotic Properties of *Lactococcus lactis* Isolated from Sea Bass (*Dicentrarchus labrax*)

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Abstract: This study aimed to screen the antibacterial effect of *Lactococcus lactis* against selected pathogens and to characterize its probiotic properties in vitro. The whole-cell product and cell-free supernatant of *L. lactis* were tested and the antibacterial effect of the whole-cell compound was found to be greater than that of the cell-free supernatant. *L. lactis* exhibited the greatest inhibitory effect against *A. veronii* from which it was isolated from the same environment. *L. lactis* showed observable growth at 4-37 °C, 1-4% NaCl, 0.3% ox gall and pH 2-3. The cells had 89.3 % hydrophobicity. Of the 19 enzymes tested in the API-ZYM system, only 7 were evident for the strain. *L. lactis* was resistant to streptomycin and sulfadiazine but was susceptible to 7 other antibiotics commonly used in aquaculture. It was γ-hemolytic. The results demonstrated that *L. lactis* exhibited probiotic properties such as being able to survive in a wide temperature and salinity range, growing in acidic and bile salt environments, and producing enzymes that can support digestion. According to these findings, *L. lactis* may have the potential to be used as a probiotic supplement in aquaculture.

Keywords: Antibacterial activity, *Lactococcus lactis*, probiotic.

Levrekten (*Dicentrarchus labrax*) İzole Edilen *Lactococcus lactis*'in Antibakteriyel Aktivitesi ve In vitro Probiyotik Özellikleri

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Öz: Bu çalışmanın amacı, *Lactococcus lactis*'in seçilmiş patojenlere karşı antibakteriyel etkisini taramak ve probiyotik özelliklerini laboratuvar ortamında karakterize etmektir. Çalışmada, *L. lactis*'in tam-hücre ürünü ve hücresiz süpernatantı test edilmiştir ve tam hücreli bileşiğin antibakteriyel etkisinin, hücresiz süpernatantınkinden daha fazla olduğu tespit edilmiştir. *L. lactis* en büyük inhibitör etkiyi test edilen bakteriler arasında, aynı ortamdan izole edilen *A. veronii*'ye karşı göstermiştir. *L. lactis*, 4-37 °C, %1-4 NaCl, %0,3 oxgall ve pH 2-3'te gözlemlenebilir büyüme sergilemiştir. Hücreler %89,3 oranında hidrofobik özellik göstermiştir. API-ZYM sisteminde test edilen 19 enzimden sadece 7'sinin *L. lactis* için belirgin olduğu tespit edilmiştir. *L. lactis*'in, streptomisin ve sülfadiazin'e dirençli, su ürünleri yetiştiriciliğinde yaygın olarak kullanılan diğer 7 antibiyotiğe karşı ise duyarlı olduğu belirlenmiştir. İzolat γ-hemolitiktir. Sonuçlar, *L. lactis*'in geniş bir sıcaklık ve tuzluluk aralığında hayatta kalabilme, asidik ve safra tuzlu ortamlarda gelişebilme ve sindirimi destekleyebilen enzimler üretebilme gibi probiyotik özellikler sergilediğini göstermiştir. Bu bulgulara dayanarak *L. lactis*'in su ürünleri yetiştiriciliğinde probiyotik takviyesi olarak kullanılma potansiyeli olduğu öngörülmektedir.

Anahtar kelimeler: Antibakteriyel aktivite, *Lactococcus lactis*, probiyotik.

INTRODUCTION

Disease outbreaks are a major problem in the aquaculture industry. Among the diseases, bacterial fish diseases are considered to cause the most mortality in fish (Gomez-Gil et al., 2000; Balta, 2020). Antibiotics and their derivatives are widely used for the control and management of bacterial diseases (Midhun et al., 2017; Balta & Tekin 2021). The excessive and uncontrolled use of antibiotics causes the development of antibiotic-resistant bacteria, as well as the deterioration of the beneficial microbiota in the intestines (Resende et al., 2012). Probiotic supplements can be used as alternative biocontrol applications to reduce these risk factors and develop environmentally friendly disease management (Verschuere et al., 2000).

Probiotics are defined as live microorganisms that are non-pathogenic and can live in the gastrointestinal tract to benefit the host (de Vrese & Schrezenmeir, 2008). Probiotic intake improves the intestinal microbiota, provides resistance to diseases, and increases growth performance (Olmos et al., 2020). Therefore, probiotic applications play an important role in aquaculture industries. Probiotics commonly used in aquaculture are *Alteromonas, Arthrobacter, Bifidobacterium, Bacillus, Pseudoalteromonas, Rhodosporidium, Roseobacter, Streptomyces, Enterococcus, Lactobacillus, Lactococcus, Carnobacterium* and *Weissella* species (Irianto & Austin 2002; Yaylacı, 2021).

Lactic acid bacteria (LAB) are widely used in aquaculture for their ability to improve the gastrointestinal tract, digestive function, and immunity, promote growth and increase host disease resistance (Ringø et al., 2018). LAB are Gram-positive, non-motile, and non-spore-forming microorganisms that are "generally recognized as safe" (Salminen et al., 1998). LAB produces various bacteriocins and organic acids that inhibit the growth of some fish pathogens (Planas et al., 2004).

There are many probiotic strains currently in commercial use, but there is still growing interest in new strains with probiotic properties (Kim & Austin, 2008). In vitro and in vivo tests are used to select probiotic strains. In vitro tests are quite different from in vivo tests, but they are essential because they provide useful information in the characterization of potential probiotics (Jacobsen et al., 1999; Uzun Yaylacı, 2021).

The study aimed to test the inhibitory activity of *Lactococcus lactis* against selected pathogens and to characterize its probiotic properties, in vitro. The inhibitory effect was tested against pathogenic bacteria isolated from sea bass (*Dicentrarchus labrax*) showing disease symptoms. The probiotic features of *L. lactis* were investigated by assays for survivability at different temperature and salinity ranges, acid and bile salt resistance, and hydrophobicity. The enzyme profile of the isolate was defined, and its safety was determined by hemolytic activity and antibiotic susceptibility tests.

MATERIAL AND METHOD

Bacterial strains: *Lactococcus lactis* (ON564732) isolated from sea bass *(D. labrax)* (Uzun Yaylacı, 2019) was evaluated in terms of its inhibitory effect and probiotic properties. Indicator microorganisms were *Vibrio vulnificus* (KF443056), *V. harveyi* (KF443058), *V. rotiferianus* (KF443057), *Photobacterium damselae* subsp. *damselae* (KF443048) and *Aeromonas veronii* (KF443053) which were isolated from sea bass with disease symptoms and confirmed previously by Uzun and Ogut (2015). *A. sobria* (ATCC 43979), *A. hydrophila* (ATCC 7966), *Escherichia coli* (ATCC 25922), *Listeria monocytogenes* (ATCC 43251), *Salmonella enterica* (ATCC 13076) and *Staphylococcus aureus* (ATCC 25923) were also tested. The stock cultures were maintained with 10% (v/v) glycerol at -70 °C.

Antibacterial activity: The whole-cell product and the cell-free supernatant of *L. lactis* were evaluated for antibacterial activity against the indicator strains mentioned above. *L. lactis* was grown in deMan Rogosa Sharpe (MRS) broth at 30 °C for 24 h. After centrifugation (10000 × g, 10 min, 4 °C), the pellets and supernatant were separated from each other. The pellets were washed twice and resuspended in phosphate-buffered saline (PBS) (pH 7.2). The cell-free supernatant was filtered through 0.22 µm-pore size filters.

The agar well diffusion method was carried out to screen the antibacterial activity of *L. lactis* according to Tagg et al. (1976) with minor modification. The samples (30 µL) were added to wells cut in tryptic soy agar (TSA) plates previously inoculated with indicator bacteria. Wells filled with 30 µl of PBS (pH7.2) served as controls. The plates were incubated at 30 °C for 24 h. The experiments were repeated three times and the diameter of the inhibition zone was measured.

Growth at different temperatures and salt concentrations: To observe visible growth at different salt concentrations, the overnight culture (100 µl) of *L. lactis* inoculated on MRS agar plates supplemented with 1%, 2%, 3%, 4%, 5%, and 6% of 1 M NaCl and incubated at 30 °C for 48 h (Mohamad et al., 2020). To observe visible growth at different temperatures, *L. lactis* was prepared under the same conditions, inoculated on MRS agar plates, and incubated at 4, 10, 25, 35, 37, 40, and 44 °C for 48 h. The assays were performed in triplicate.

Resistance to low Ph: The acid tolerance of *L. lactis* was determined according to Maragkoudakis et al. (2006). The overnight culture was centrifuged (10000 × *g*, 10 min, 4 °C) and washed twice with PBS buffer (pH 7.2). The pellets were resuspended in PBS adjusted to pH 2.0 or pH 3.0 with 0.5 N HCl and incubated at 30 °C for 3 h. PBS adjusted with pH 7.2 was used as a control. During the cultivation, samples were withdrawn at 0, 1.5, and 3 h and serially diluted in PBS (pH 7.2). MRS agar plates inoculated with 100 µl suspension were incubated at 30 °C for 48 h. Viable colonies were enumerated using the pour plate method. The assays were performed in triplicate.

Bile salt tolerance*:* The bile salt tolerance of *L. lactis* was determined by following the procedure described by Mohamad et al. (2020) with minor modifications. The overnight culture was centrifuged (10000 × *g*, 10 min, 4 °C) and washed twice with PBS buffer (pH 7.2). The pellets were resuspended in PBS containing 0.3% (w/v) ox gall (Sigma) and incubated at 30 °C for 6 h. During the cultivation, samples were withdrawn at 0, 3, and 6 h and serially diluted in PBS (pH 7.2). MRS agar plates were inoculated with 100 µl of suspension and incubated at 30 °C for 48 h. Viable colonies were enumerated using the pour plate method. The assays were performed in triplicate.

Hydrophobicity: The hydrophobicity of *L. lactis* was evaluated according to the method of Vinderola et al. (2004) with minor modifications. The overnight culture was centrifuged at 10 000 × *g* for 10 min at 4 °C and washed twice with PBS buffer (pH 7.2) and adjusted to the optical density at 600 nm (OD_{600}) (H_0). *n*-hexadecane was used as a solvent. The bacterial suspension was mixed with 20% (w/v) n-hexadecane vortexed for 2 min. After the mixture was separated into two phases (30 min, 30 °C), the OD of the aqueous phase was measured at OD_{600} (H_1). The hydrophobicity was calculated according to the following formulae:

$$\text{Hydrophobicity (\%)} = [(H_0 - H_1) / H_0] \times 100$$

Hemolytic activity: *L. lactis* was streaked onto an agar plate containing 7% sheep blood. After incubation at 30 °C for 48 h, the plates were evaluated for hemolytic activity.

Enzyme profile: The enzyme profile of *L. lactis* was performed using the API ZYM kit (BioMerieux, USA) according to the manufacturer's instructions.

Antibiotic susceptibility: The antibiotic susceptibility of *L. lactis* was determined by using the disc diffusion method described by Bauer et al. (1966). Antibiotic discs containing ampicillin (10 µg $disc^{-1}$), rifampicin (10 µg $disc^{-1}$), amoxicillin (10 µg $disc^{-1}$), penicillin G (10 units), vancomycin (30 µg $disc^{-1}$), streptomycin (10 µg $disc^{-1}$), chloramphenicol (30 µg $disc^{-1}$), oxytetracycline (30 µg $disc^{-1}$) and sulphadiazine (300 µg $disc^{-1}$) were placed on the Mueller Hinton agar plates and incubated at 30 °C for 24 h. The experiments were repeated three times and the clear zones (including disc diameter) were measured.

Statistical analysis: The data were presented as means ± standard deviation. The results were analyzed using a one-way analysis of variance (ANOVA) using MINITAB 19 software. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Antibacterial activity: The antimicrobial effect of the whole-cell compound and cell-free supernatant of *L. lactis* was evaluated against twelve pathogens by the agar well diffusion method. Four indicator bacteria were inhibited by the whole-cell compound or cell-free supernatant of *L. lactis.* Moreover, the inhibition effect of the whole-cell compound was found to be greater than that of the cell-free supernatant (Table 1). This result can be explained by the fact that similar to the report of Liu et al. (2015), the cultured cells of probiotic bacteria may contain antimicrobial compounds that are not present in their cell-free supernatant.

Table 1. Antibacterial activity of *L. lactis* whole-cell product and cell-free supernatant.

Indicator bacteria	Inhibition zone (mm)	
	whole-product	cell-free supernatant
V. vulnificus	-	-
V. harveyi	-	-
V. rotiferianus	-	-
Photobacterium damselae subsp. *damselae*	-	-
A. veronii	21.45 ± 0.21	11.06 ± 0.36
A. sobria	11.84 ± 0.25	7.12 ± 0.11
A. hydrophila	16.25 ± 0.44	9.14 ± 0.37
S. marcescens	-	-
L. monocytogenes	-	-
S. enterica	-	-
E. coli	-	-
S. aureus	9.11 ± 0.34	-

[a] Values are the mean ± standard deviations of triplicate measurements.
-No inhibitory zone

Aeromonas spp. have been reported as opportunistic pathogens with global distribution in various aquatic environments (Burke et al., 1984). Motile (*A. hydrophila, A. veronii,* and *A. sobria*) and non-motile (*A. salmonicida*) members of *Aeromonas* may cause infections in humans and lower vertebrates, including amphibians, reptiles, and fish (Janda & Abbott, 1998). Motile *Aeromonas* infections are characterized by exophthalmia, hemorrhages, ulcerations, skin lesions, an acidic fluid, liver, and kidney lesions in fish (Garcia et al. 2007; Uzun & Ogut, 2015) and cause massive fish mortality worldwide (John et al., 2013). Among the tested bacteria, *Aeromonas* spp. were the most sensitive indicator bacteria to *L. lactis.* This result was supported by the studies of Ivanova et al. (1993), Balcazar et al. (2007), and Zhou et al. (2010), which reported that *L. lactis* could inhibit *Aeromonas* species. In the current study, the potential probiotic *L. lactis* showed the greatest inhibitory effect against *A. veronii* from which it was isolated from the same

environment. Whole-cell compound and cell-free supernatant of *L. lactis* did not show any inhibitory effect against *V. vulnificus, V. harveyi, V. rotiferianus, P. damselae* subsp. *damselae, S. marcescens, L. monocytogenes, S. enterica*, and *E. coli*.

Probiotic characteristics: The probiotic characteristics of *L. lactis* were evaluated by its NaCl, temperature, acid, and bile-salt tolerances. NaCl tolerant strains can survive in the high salt environment of the gastrointestinal tract and withstand the adverse effects of osmotic pressure (Xu et al., 2019). In this study, *L. lactis* was able to tolerate 1 to 4% NaCl but, high NaCl concentrations had an inhibitory effect on the strain. Besides, *L. lactis* was able to grow at 4 to 37 °C, but growth was not observed at high temperatures (40°C <). Low acidity and bile-rich intestinal juices in the gastrointestinal tract are effective mechanisms for the destruction of pathogens (Halim et al., 2017). A strain with probiotic potential should have acid and bile salt tolerance (Jena et al., 2013). Here, the isolate survived after incubation for 3 hat pH 2.0-3.0 (Figure 1a) and 6 h in 0.3% ox gall (Figure 1b). Similar to the report of Jawan et al. (2021), it was concluded that *L. lactis* evaluated in this study could also survive in the gastrointestinal tract and have the potential to be effective as a probiotic. Hydrophobicity can determine the adhesion capacity of probiotic bacteria to intestinal epithelial cells (Falah et al., 2019). In this study, the hydrophobicity of *L. lactis* was determined as 89.3%. Although there is no standard value for hydrophobicity, it is advantageous for probiotic bacteria, and high hydrophobicity results in better attachment to epithelial cells (de Souza et al., 2019).

Enzyme profile: The exo-enzymes of bacteria, especially amylase, protease, and lipase, can affect the digestive process of the host (Newaj-Fyzul et al., 2014). The enzymatic activities of *L. lactis* determined by the API-ZYM system are listed in Table 2. The strain showed high acid phosphatase and Naphthol-AS-BI-phosphohydrolase activities. However, the strain's lipase activities were low and negative for carbohydrase activities.

Safety

Hemolytic activity: In vitro evaluation of hemolytic activity is one of the safety prerequisites for the selection of the probiotic strain. It has been previously reported that *L. lactis* has gamma hemolytic activity (no hemolysis) (Jawan et al., 2021). Similarly, *L. lactis* showed γ-hemolysis in this study, indicating that the tested strain has the potential to be preferred as a probiotic additive.

Antibiotic susceptibility: *Determination of the antibiotic profile of L. lactis is a necessary step for its safe use as a probiotic. In the current study, L. lactis was sensitive to ampicillin, rifampicin, amoxicillin, penicillin G, vancomycin, chloramphenicol, and oxytetracycline but resistant to streptomycin and sulphadiazine (Table 3).*

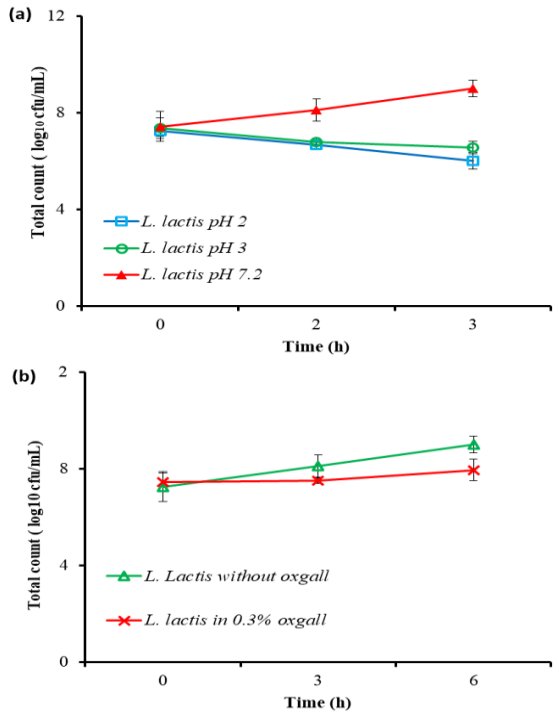

Figure 1. Survival of *L. lactis* after incubation at pH 2.0, 3.0, and 7.2 (a), in the presence of 0.3% ox gall (b). Values are means ± SD.

Table 2. Enzyme profile of *L. lactis* with API-ZYM kit

Enzyme	*L. lactis*	Enzyme	*L. lactis*
Control	-	Valine arylamidase	0
Glycosidases		Cystine arylamidase	0
α-galactosidase	0	Trypsin	0
β-galactosidase	0	α -chymotrypsin	0
β-glucuronidase	0	**Ester hydrolases**	
α-glucosidase	0	Esterase (C4)	2
β-glucosidase	0	Esterase lipase (C8)	2
N-acetyl- β-glucosaminidase	0	Lipase (C14)	1
α-mannosidase	0	**Phosphohydrolases**	
α-fucosidase	0	Alkaline phosphatase	3
Peptide hydrolases		Acid Phosphatase	4
Leucine arylamidase	3	Naphthol-AS-BI- phosphohydrolase	5

+ positive reaction; − negative reaction

Table 3. Antibiotic susceptibilities of *L. lactis*

Antibiotics	Inhibition zone (mm)
Ampicillin	31.56 ± 0.58[a]
Rifampicin	8.11 ± 0.25
Amoxicillin	35.23 ± 0.85
Penicillin G	32.51 ± 0.34
Vancomycin	16.12 ± 0.40
Streptomycin	0
Chloramphenicol	22.1 ± 0.44
Oxytetracycline	33.56 ± 1.14
Sulphadiazine	0

[a] Values are the mean ± standard deviations of triplicate measurements.

CONCLUSION

In this study, the inhibitory activity of *L. lactis* isolated from sea bass against selected pathogens was screened and its probiotic properties were characterized in vitro. The whole-cell product of *L. lactis* showed the greatest inhibitory effect against *A. veronii* isolated from the same environment. *L. lactis* showed probiotic characteristics including being able to survive in a wide temperature and salinity range, acid, and bile salt resistance, and producing extracellular enzymes that can

support digestion. Being sensitive to many antibiotics and showing gamma hemolytic activity are its positive features in terms of safety. Therefore, *L. lactis* can be used as a potential probiotic in aquaculture. Although many probiotic properties have been elucidated by in vitro tests, the strain is a good candidate for further investigation by in vivo studies.

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