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The determination of antimicrobial and antibiofilm activities of foodborne lactic acid bacteria against *Enterobacter cloacae* isolates

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Abstract: The aim of this study was to investigate the antimicrobial and antibiofilm activities of 10 different lactic acid bacteria (LAB) strains isolated from local food sources of animal origin against 4 *Enterobacter cloacae* isolates obtained from clinical cases and determine their adhesion potentials to intestinal epithelial cells. In this study, all *Enterobacter cloacae* isolates (P3, P4, P5, P7) identified with the BD Phoenix automation system were detected to form biofilm with both Congo red agar and Microtiter plate methods. Amoxicillin-clavulanate, cefuroxime, and ampicillin resistance was determined in all isolates. It was determined that LAB strains producing exopolysaccharide (EPS) were able to colonize intestinal epithelial cells. It is noteworthy that LAB extracts were effective to inhibit the biofilm formation of P3Ec, which had higher antibiotic resistance than those of other isolates. Antimicrobial effect of LAB extracts on *Enterobacter cloacae* were also detected by both agar disc diffusion and well diffusion tests. In this study, all of the isolated LAB strains (especially *L. lactis*, *L. fermentum*, and *L. casei*) are good candidates for controlling *Enterobacter cloacae* biofilm formation. These findings indicate that *L. lactis*, *L. fermentum*, and *L. casei* can potentially be developed as novel antibiofilm agents.

Key words: *Enterobacter cloacae*, antimicrobial resistance, biofilm, lactic acid bacteria

1. Introduction

Biofilm refers to complex aggregate microorganism communities that are bound to a surface, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Some bacteria are tightly embedded in the extracellular matrix to form a biofilm. Biofilm not only makes microorganisms resistant to adverse environmental conditions, but also protects them from phagocytes and complement systems [1,2]. Therefore, biofilm-forming microorganisms are considered as the main cause of persistent hospital infections, especially in immunocompromised individuals [2]. Biofilm increases resistance to antibiotics by about 1000 times, making treatment more difficult [3]. Some bacteria, including the genus *Enterobacter*, can move actively due to the flagella they have. Motility helps food intake and colony formation in bacteria. Bacteria including the genus *Enterobacter*, are encapsulated lactose-fermenting mobile bacteria that cause pneumonia and urinary tract infections, especially with the use of contaminated devices such as catheters and probes [4].

Diarrhea is an important factor in the formation of many gastrointestinal tract pathologies such as irritable bowel syndrome and chronic inflammation by causing intestinal microflora imbalance [5]. One of the reasons for intestinal microbiota imbalance is the unnecessary use of antibiotics. Due to the biofilm formation of pathogenic bacteria, the effectiveness of antibiotics in the treatment of human and animal infections is a concern [6]. Currently, the increase in the resistance of the members of the family *Enterobacteriaceae* against antibiotics is one of the major problems. One of the factors that cause bacterial resistance is the biofilm generated by *Enterobacter* strains [4]. The World Health Organization (WHO) describes probiotics as live microorganisms that benefit the health of the host when consumed in sufficient quantities. Lactic acid bacteria (LAB) are the main source of probiotics in nutrients. Probiotics must survive under stressful conditions of the gastrointestinal tract by tolerating acid, bile, and gastric enzymes and should be colonized by binding into the intestinal epithelial cells. Furthermore,

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probiotics must have antimicrobial effects against pathogenic microorganisms [7].

Studies have shown that LAB support the digestion and assimilation of nutrients [8], modulate the immune system [9], remove toxic substances, and prevent the reproduction or invasion of parasites and pathogenic bacteria to prevent gastrointestinal infections [10]. Recently, the use of probiotics has been considered as a natural alternative to antibiotic supplements [7]. However, studies on enteropathogenic bacteria inhibited by LAB are very few. Furthermore, Cui et al. [7] have reported that studies on the impact of LAB on enteropathogenic bacterial biofilm have been neglected.

The aim of this study is to determine the antimicrobial and antibiofilm effect of LAB isolated from local food sources against *Enterobacter cloacae* isolated from animals and determine their adhesion potential to intestinal epithelial cells.

2. Materials and methods

2.1. Microorganisms used in the study

Ten LAB isolates were defined in terms of species with universal primers and 16S rRNA sequence analysis previously from local meat and dairy products. The LAB were obtained from the culture collection of Afyon Kocatepe University, Technical Vocational School of Bayat (Table 1).

Animal-derived pathogen strains were obtained from the culture collection consisting of samples from the field brought to the Animal Hospital and Research Centre and Necropsy Laboratory at the Faculty of Veterinary Medicine of Afyon Kocatepe University. They included cow mastitis (P7), horse runny nose (P3, 5), and canine abdominal swab (P4) and they were identified in the Medical Microbiology Bacteriology Laboratories of the Eskişehir Osmangazi University Faculty of Medicine using the BD Phoenix automation system.

2.2. Detection of biofilm formation in pathogen isolates

2.2.1. Congo red agar method (qualitative method)

The isolates were firstly kept in Congo red agar medium [11] at 37 °C for 24 h, then they were incubated at 25 °C for 48 h. Red, black, rough, dry, and transparent colonies in the medium were evaluated as biofilm (slime)-positive while pinkish red, flat, and central dark colonies were evaluated as biofilm-negative [12].

2.2.2. Quantitative detection of biofilm formation

Enterobacter strains were incubated at 37 °C for 24 h in nutrient broth (NB). Subsequently, microorganism culture of 150 µL was transferred to a 96-well microtiter plate. These plates were reincubated at 37 °C for 24 h. After incubation, the liquid medium was poured, the wells were washed 3 times with distilled water, and crystal violet solution of 150 µL [0.5% (v/v)] was added into the wells. After being kept in ambient temperature for 45 min the wells were washed again with distilled water 3 times. Then 150 µL of ethanol and acetic acid (95:5) was added and left to stand for 10 min to dissolve the dye. After this step, 100 µL was taken from each well and transferred to a new microtiter plate. The absorbance values of each well at 570 nm were determined using ELISA (Thermo Multiskan Go). *P. aeruginosa* ATCC 11778 strain, which is known to generate biofilm, was used as a positive control and microorganism-free medium was used as a negative control. Excessive absorbance values compared to the negative control indicate that microorganisms can form biofilm [13,14].

Table 1. LAB 16S rRNA results.

Isolates*	16S rRNA analysis results	Number of compared bases	(%) Similarity
L1	*Lactococcus lactis* subsp. *lactis* strain CAU9932 (Sequence ID: MF098094.1)	1413	98%
L2	*Lactobacillus fermentum* strain CAU:3341[Sequence ID: MF354239.1]	1402	98%
L3	*Enterococcus faecalis* strain FC1377 [Sequence ID: MG871229.1]	1522	99%
L4	*Lactobacillus casei* strain 090 [Sequence ID: JN560917.1]	1443	99%
L5	*Lactobacillus plantarum* strain Lb17 [Sequence ID: MG825687.1]	1373	100%
L6	*Enterococcus faecium* strain CAU2799 [Sequence ID: MF425224.1]	1335	99%
L7	*Lactobacillus curvatus* strain 1TP06-BL06 [Sequence ID: MG031211.1]	1470	99%
L8	*Enterococcus durans* strain CAU6145 [Sequence ID: MF424830.1]	1407	99%
L9	*Lactococcus garvieae* strain CAU6586 [Sequence ID: MF108375.1]	1396	94%
L10	*Enterococcus faecium* strain CAU10244 [Sequence ID: MF429017.1]	1377	99%

*Lactic acid bacteria were isolated in project numbers AKÜ BAP 17. MYO. 07 and AKÜ BAP 17.SAĞ.BİL.06.

2.3. Swimming, swarming, and twitching motilities of *Enterobacter* spp.

In order to determine the motility status in pathogenic strains, swarming tests on NB medium according to the method used by Rashid and Kornberg [15] and swimming and twitching motility tests according to Deziel et al. [16] were carried out.

2.4. Determination of EPS production of LAB

To produce the EPS of LAB, the method developed by Marshall and Rawson [17] was used. For this, LAB isolates were activated by incubation in nutrient broth for 24 h at 37 °C and subsequently equal amounts of the samples brought to 0.5 McFarland turbidity (approximately 1 to 4 × 108 CFU/mL) were transferred into NB medium of 5 mL and incubated for 20 h at 37 °C. After incubation, cultures of 1 mL were taken and distributed into Eppendorf tubes, which were incubated in a water bath at 100 °C for 10–15 min. Trichloroacetic acid (TCA; 85%) at a rate of 0.17% was added to the samples that were cooled at room temperature and were centrifuged. The supernatant obtained was placed into another Eppendorf tube and the same amount of ethanol was added, and the content was centrifuged. After repeating this process, EPS production was determined with phenol sulfuric acid method and by reading the absorbance values at 490 nm. This test was repeated 3 times. The results were evaluated according to the established glucose standard curve.

2.5. Determination of antibacterial effects of lactic acid bacteria

2.5.1. Preparation of lactic acid culture filtrates

In order to determine the antimicrobial effects of LAB isolates, an extract from each isolate was planted in De Man-Rogosa-Sharpe (MRS) broth medium and incubated at 37 °C for 24 h, after which the plasma was centrifuged at 8000 rpm for 10 min (4 °C). Culture supernatants were collected into sterile flacon tubes. The supernatants obtained were drawn with sterile injectors and filtered through a sterile membrane filter with a 0.2-µm pore diameter, and the filtrates were used to determine the antimicrobial activity [18].

2.5.2. Determination of antimicrobial activity of lactic acid culture filtrates

The antimicrobial effect of culture filtrates was investigated by using the agar well diffusion test and agar disc diffusion method. Suspensions were prepared from the 24-h cultures of *Enterobacteriaceae* strains used in the study in the agar medium, equivalent to 0.5 McFarland turbidity in distilled water, for the agar diffusion test. The bacterial suspensions were dispersed into the NA medium using a sterile swab stick. The wells were drilled with a sterile agar drill of 6 mm in diameter. Lactic acid culture filtrates of 100 µL were added to the wells [18]. For the agar disc diffusion method, Mueller Hilton agar (MHA) was applied according to the standard method by impregnating empty antibiotic discs with a filtrate of 20 µL [19]. In both methods, after 24 h of incubation at 37 °C, the impact of antimicrobial activity was evaluated according to the presence of a zone of inhibition.

2.6. Determination of antibiofilm effect of LAB extracts

The isolates were planted on appropriate media and incubated at 37 °C for 24 h. Subsequently LAB extracts were added to the cultures and transferred to ELISA plates, which were incubated at 37 °C for 24 h.

The effect of LAB on antibiofilm was determined according to the method by Thenmozhi et al. [20]. Briefly, the LAB were incubated in MRS media at 37 °C for 48 h and then centrifuged at 4000 rpm, and filtered through a membrane filter. Cell-free supernatant (CFS) was extracted twice with the same volume of ethyl acetate [21]. Equal amounts of extracts were dissolved in distilled water and diluted to one half of the previous concentration 3 times, and each dilution was used to detect the effect of antibiofilm. The isolates were planted on appropriate media and incubated at 37 °C for 24 h. Subsequently LAB extracts were added to the cultures and transferred to ELISA plates, which were incubated at 37 °C for 24 h. After incubation, the liquid medium was poured and the wells were washed 3 times with distilled water. Crystal violet solution at 0.5% was dispersed into the wells and incubated at room temperature. The wells were assayed in ELISA at 570 nm and biofilm inhibitory effects were determined [13,14]. The antibiofilm activity of the extracts was calculated with the percent reduction formula.

% Inhibition = (A control – A sample / A control) × 100

A control: Absorbance value containing only *Enterobacter* strains

A sample: Absorbance value with LAB extracts (LAB + *Enterobacter*) added

3. Results

According to the BDPhoenix bacterial identification and antibiogram sensitivity test, isolates P3, P4, P5, and P7 were defined as *Enterobacter cloacae*. The sensitivity test results are given in Table 2.

All isolates were resistant to amoxicillin-clavulanate and ampicillin. The highest antibiotic resistance was found in strain P3. This strain is resistant to amoxicillin-clavulanate, ampicillin, cefepime, ceftriaxone, cefuroxime, and ertapenem antibiotics, and moderately sensitive to meropenem and tigecycline antibiotics.

3.1. Detection of biofilm formation in *Enterobacter cloacae* isolates

In both methods, it was determined that *Enterobacter cloacae* isolates formed biofilm (Figure 1 and Table 3). The

Table 2. The results of antibiotic susceptibility testing of *E. cloacae*.

	Microorganism							
	P3		P4		P5		P7	
Antibiotic	MIC		MIC		MIC		MIC	
Amikacin	≤4	S	≤4	S	≤4	S	≤4	S
Amoxicillin-clavulanate	>32/2	R	>32/2	R	>32/2	R	>32/2	R
Ampicillin	>8	R	>8	R	>8	R	>8	R
Aztreonam	≤1	S	≤1	S	≤1	S	≤1	S
Cefepime	>8	R	≤1	S	≤1	S	≤1	S
Ceftazidime	≤0.5	S	≤0.5	S	≤0.5	S	≤0.5	S
Ceftriaxone	>4	R	≤0.5	S	≤0.5	S	≤0.5	S
Cefuroxime	>8	R	8	R	>8	R	>8	R
Ciprofloxacin	≤0.125	S	≤0.125	S	≤0.125	S	≤0.125	S
Ertapenem	>1	R	≤0.25	S	≤0.25	S	≤0.25	S
Gentamicin	≤1	S	≤1	S	≤1	S	≤1	S
Imipenem	1	S	≤0.25	S	≤0.25	S	≤0.25	S
Meropenem	4	I	≤0.125	S	≤0.125	S	≤0.125	S
Netilmicin	1	S	1	S	1	S	1	S
Piperacillin	≤4	S	≤4	S	≤4	S	≤4	S
Piperacillin-tazobactam	≤4/4	S	≤4/4	S	≤4/4	S	≤4/4	S
Tigecycline	2	I	1	S	1	S	1	S
Trimethoprim-sulfamethoxazole	≤ 1/19	S	≤1/19	S	≤1/19	S	≤1/19	S

MIC: Minimum inhibition concentration, S: Susceptible, I: Intermediate, R: Resistant.

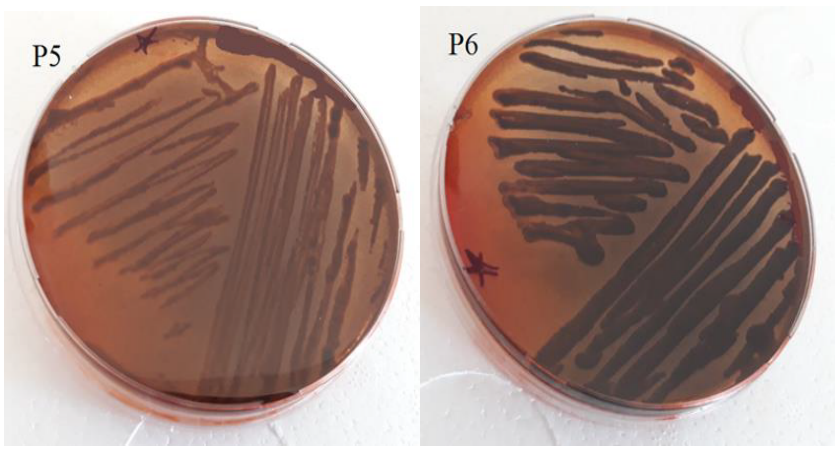

Figure 1. Biofilm-positive P5 and P6 isolates with Congo red agar method.

data obtained as a result of the microtitration plate method are compared with the negative control in Table 3.

The highest absorbance was found for P3 as 1.047. This was followed by P4 (0.895), which we had determined previously to be highly antibiotic resistant. The results indicate that biofilm formation may be responsible for the antibiotic resistance of P3. The formation of biofilm in the isolates and motility test results show that the isolates can swim, swarm, and twitch (Table 3).

3.2. EPS production of lactic acid isolates

A glucose standard curve was prepared by using a glucose solution of 1.25–100 mg/mL to calculate the EPS amounts

generated by LAB (Figure 2). The equation y = 0.0482x + 0.4242 was used to express the EPS amount (mg/mL) corresponding to the absorbance (Table 4).

The highest EPS production (49.31 mg/mL) was found in the *Enterococcus faecalis* (L3) strain, followed by *Lactobacillus plantarum* (L5) with EPS of 34.02 mg/mL and *Lactobacillus fermentum* (L2) with EPS of 33.77 mg/mL.

3.3. Antimicrobial effect of LAB extracts

The results of both agar disc diffusion and well diffusion tests indicate that the tested amounts of the extracts have antimicrobial effect (Table 5). It was determined that L1, L4, L6, and L10 isolates had antimicrobial effects by both agar disk diffusion test and agar well diffusion test on *Enterobacter cloacae* isolates.

3.4. Antibiofilm effect of LAB extracts

The inhibition percentage values of LAB extracts on *Enterobacter cloacae* isolates are shown in Table 6.

It has been determined that diluted extracts at a ratio of 1:1 prepared from isolates of *Lactococcus lactis* (L1), *Lactobacillus casei* (L4), *Lactobacillus plantarum* (L5), *Enterococcus faecium* (L6), and *Lactobacillus curvatus* (L7) inhibit the formation of biofilm in all tested isolates. The extract of *Lactococcus lactis* (L1) diluted at a ratio of 1:1 was detected to inhibit the biofilm formation of the P3Ec isolate, which had the highest antibiotic resistance at 91.97%. Even dilution of this extract at the ratio of 1:8 inhibited biofilm formation of the same isolate at a level of 84.24%. The extracts from *Lactobacillus fermentum* (L2), *Enterococcus faecalis* (L3), and *Lactococcus garviae* (L9) isolates did not inhibit the biofilm formation of the P4Ec isolate, whereas diluted extract at a ratio of 1:1 prepared from *Lactococcus casei* (L4) reduced the biofilm formation of the same isolate by 91.91%.

It was determined that all LAB extracts among the tested isolates inhibited biofilm formation at rates ranging

Table 3. The formation of biofilm in the isolates and motility test results (mm).

Microorganism	Motility (mm)			Biofilm formation
	Swarming	Swimming	Twitching	Average absorbance (570 nm) (SD)
$P3_{Ec}$	11	10	13	1.047 (±0.53)
$P4_{Ec}$	12	13	14	0.895 (±0.22)
$P5_{Ec}$	10	11	12	0.889 (±0.25)
$P7_{Ec}$	13	12	13	0.769 (±0.28)
P. aeruginosa ATCC 11778	13	7	10	0.204 (±0.12)
NC	-	-	-	-

NC: Negative control, Ec: *E. cloacae* SD: Standard deviation.

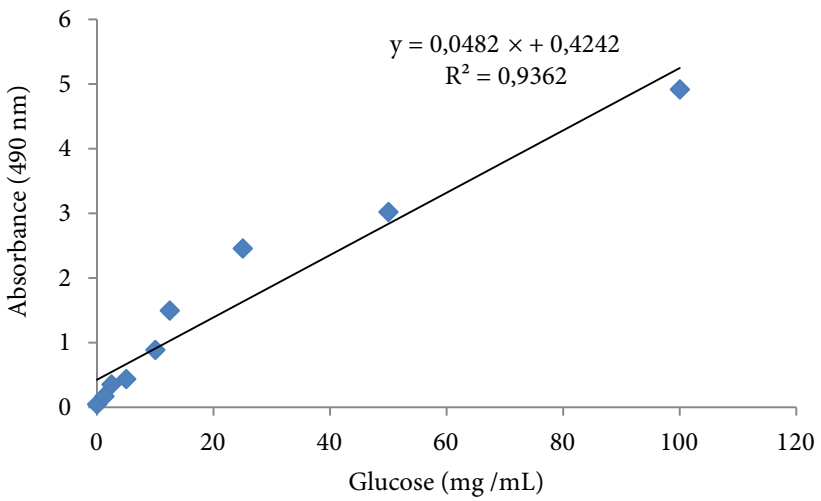

Figure 2. Glucose standard curve.

Table 4. EPS production in LAB.

Microorganism	EPS (mg/mL)
L1 (*Lactococcus lactis*)	31.44
L2 (*Lactobacillus fermentum*)	33.77
L3 (*Enterococcus faecalis*)	49.31
L4 (*Lactobacillus casei*)	26.28
L5 (*Lactobacillus plantarum*)	34.02
L6 (*Enterococcus faecium*)	14.68
L7 (*Lactobacillus curvatus*)	2.95
L8 (*Enterococcus durans*)	4.25
L9 (*Lactococcus garviae*)	7.44
L10 (*Enterococcus faecalis*)	6.15

between 17.24% and 91.97% in the tested $P3_{Ec}$ strain with the highest antibiotic resistance.

4. Discussion

Bacteria in the genus *Enterococcus* are the causative agent of both human and veterinary sepsis [22,23]. They cause difficulties in the clinic due to the variety of antibiotic resistance manifested in veterinary medicine [24]. Recently, the prevalence of enterococci has been found to be an increasing cause of sepsis in foals during the last 30 years [22]. Furthermore, enterococci species can cause many economically significant animal diseases including bovine mastitis [25]. The incidence of enterococci as an etiologic agent of bovine mastitis was found to be as high as 21.2% [26,27]. Therefore, studies have been performed to determine the antibiotic susceptibility of enterococci, and virulence genes (*asa*1, *ccf*, *gel*E, *esp*, *Cyl*A, *ace*, and *agg*) have been determined [28]. In *Enterobacter* species, both chromosomally encoded resistance to antibiotics and resistance carried by plasmids and transferred between species have been detected. Due to the increased empirical use of beta-lactam antibiotics, the development of resistance to these antibiotics increases and multiple antibiotic-resistant strains are manifested. Although antibiotics seem to be the most effective drugs for treatment, studies are needed to investigate glycopeptides and new antimicrobial agents [28]. Among the *Enterobacter* species, *E. cloacae* is one of the most common infection agents. Many studies have been conducted to investigate the resistance status of the *Enterobacter* species.

Willis et al. [29] reported that 48% of the *Enterococcus* strains isolated from foals of 0–30 days old displayed multiple antimicrobial resistance. In another study, a total of 105 enterococci were isolated from mastitic bovine milk samples and, in general, enterococci were sensitive to ampicillin, gentamicin, and vancomycin and resistant to tetracycline, penicillin, erythromycin, cephalothin, gentamicin, and vancomycin [27].

Song et al. [30] found that 27.7% of the species belonging to the family *Enterobacteriaceae* producing 94 expanded-spectrum beta-lactamases (ESBL) were sensitive to ceftazidime, 39.4% to aztreonam, and 75.5% to cefepime. In another study, Poulou et al. [31] reported that 13.6% of the 162 isolates of *Enterobacter* spp. generating ESBL were found to be susceptible to ceftazidime, 28.4% to cefepime, and 19.8% to aztreonam.

In this study, *Enterobacter cloacae* strains were determined to be resistant to amoxicillin-clavulanate,

Table 5. The antimicrobial effect of LAB extracts.

Microorganism		Agar disk diffusion test results (mm)								
	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10
$P3_{Ec}$	11	14	9	14	9	10	-	12	12	13
$P4_{Ec}$	8	9	9	10	-	10	-	-	-	9
$P5_{Ec}$	10	10	13	11	12	10	8	9	8	9
$P7_{Ec}$	11	12	12	10	10	11	-	-	-	13
P. aeruginosa ATCC 11778	-	10	13	-	-	11	-	-	-	-
		Agar well diffusion test results (mm)								
$P3_{Ec}$	14	16	16	13	12	14	17	19	15	13
$P4_{Ec}$	14	-	-	9	14	9	16	20	-	15
$P5_{Ec}$	9	13	14	13	-	11	15	15	9	22
$P7_{Ec}$	16	14	15	14	13	17	23	25	14	16
P. aeruginosa ATCC 11778	11	13	11	10	13	13	20	19	11	15

Table 6. Inhibition (%) of biofilm of LAB extracts

Diluted LAB extract	Inhibition %			
	$P3_{Ec}$	$P4_{Ec}$	$P5_{Ec}$	$P7_{Ec}$
Lactococcus lactis (L1)				
1/1	91.97	86.48	84.02	82.31
1/2	90.44	73.29	77.72	80.36
1/4	90.16	38.10	65.69	38.36
1/8	84.24	-	30.70	-
Lactobacillus fermentum (L2)				
1/1	86.15	-	80.53	73.21
1/2	85.38	-	45.10	70.74
1/4	74.49	-	9.11	-
1/8	74.11	-	-	-
Enterococcus faecalis (L3)				
1/1	68.20	-	72.89	82.96
1/2	54.15	-	38.92	67.75
1/4	49.57	-	24.74	60.59
1/8	41.45	-	12.71	36.11
Lactobacillus casei (L4)				
1/1	80.20	91.91	79.52	81.40
1/2	79.68	42.90	48.48	80.23
1/4	38.63	10.94	17.66	60.59
1/8	17.24	-	16.64	30.65
Lactobacillus plantarum (L5)				
1/1	65.57	72.51	76.04	79.45
1/2	52.36	54.97	55	73.73
1/4	36.22	32.73	32.39	55.65
1/8	20.16	-	-	38.49
Enterococcus faecium (L6)				
1/1	51.95	72.84	81.21	81.40
1/2	43.45	48.15	37.23	76.98
1/4	18.59	20.22	21.14	56.17
1/8	-	13.96	-	29.51
Lactobacillus curvatus (L7)				
1/1	89.39	80.22	84.36	80.10
1/2	88.72	61.45	82.78	79.58
1/4	88.53	36.98	75.14	26.65
1/8	88.06	-	74.24	15.73
Enterococcus durans (L8)				
1/1	84.81	33,96	62.42	85.26
1/2	83.28	-	33.74	78.34
1/4	82.90	-	-	56.22
1/8	81.18	-	-	26.83
Lactococcus garviae (L9)				
1/1	87.58	-	34.19	17.03
1/2	87.10	-	28.34	-
1/4	84.24	-	27.44	-
1/8	81.27	-	-	-
Enterococcus faecalis (L10)				
1/1	91.21	34.18	29.80	21.45
1/2	90.06	-	24.74	-
1/4	79.56	-	22.72	-
1/8	78.03	-	12.24	-

cefuroxime, and ampicillin antibiotics and sensitive to amikacin, aztreonam, ceftazidime, ciprofloxacin, gentamicin, imipenem, netilmicin, piperacillin-tazobactam, trimethoprim-sulfamethoxazole antibiotics. The highest antibiotic resistance was observed in $P3_{Ec}$ (amoxicillin-clavulanate, ampicillin, cefepime, ceftriaxone, cefuroxime, ertapenem) (Table 2). Many *Enterobacter* spp. form biofilm [4,32,33]. Sabir et al. [34] investigated the biofilm formation of pathogens causing urinary tract infection and their resistance to antibiotics. They reported that 73.4% of the isolates formed biofilms and the highest biofilm production among the isolated pathogens was reported for ampicillin-resistant *Enterobacter cloacae* (87.5%).

Fighting infections caused by *Enterobacter* species is becoming increasingly difficult. The morbidity and mortality rates of resistant *Enterobacter*-induced infections are 2–3 times higher than that of normal infections [35]. Biofilm formation causes the treatment of infections to become more difficult and increases treatment costs [36]. Therefore, in recent years, researchers have started working on the advantages of some beneficial microorganisms in order to eliminate the harmful effects of biofilm. In their study Slama et al. [37] isolated probiotic *Lactobacillus* strains from fermented foods and determined that the extracts were able to eliminate the formation of *L. monocytogenes* biofilm significantly. In another study, Cui et al. [7] investigated the antibiotic activity of LAB strains which were isolated from traditional cheeses against enteropathogenic bacteria. Twelve out of 321 isolates were identified with antibiofilm activity against *Staphylococcus aureus* CMCC26003 and *Escherichia coli* CVCC230.

It was determined that *Enterobacter cloacae* isolates were mobile and formed biofilm in this study (Table 3). The highest biofilm formation was found for P3, which we determined previously to be highly antibiotic resistant. The results indicate that biofilm formation may cause the antibiotic resistance. It was observed that the biofilm formations of *E. cloacae* isolates were inhibited by all the L1, L4, L5, L6, and L7 isolates tested with the 1:1 concentration. In particular, it is noteworthy that 1:1, 2, and 3 concentration extracts of all LAB inhibited the biofilm formation of $P3_{Ec}$ which had the highest antibiotic resistance among the tested isolates. It was determined that *Lactococcus lactis* extract diluted at a rate of 1:1 was the LAB extract which inhibited the formation of biofilms more than the control (91.97% at $P3_{Ec}$). The highest levels of inhibition of biofilm for the $P5_{Ec}$ and $P7_{Ec}$ isolates were detected for the extracts of *Lactobacillus curvatus* (L7), and *Enterococcus durans* (L8) at a rate of 1:1, respectively.

It is known that LAB produce an antimicrobial peptide called bacteriocin [38]. LAB members play an important role in reducing the production of toxins in pathogenic

bacteria with the bacteriocins they produce. Therefore, there is an increase in studies on the use of LAB both in food preservation and in the prevention of pathogenic bacteria production [39,40]. Santos et al. [41] described bacteriocins as antimicrobial and antibiotic agents in their study. The antimicrobial spectrum of MccC7-C51 bacteriocin was investigated and its action against bacterial strains was noted.

In our study, both the agar disc diffusion and the well diffusion tests show that LAB extracts have an antimicrobial effect on *Enterobacter cloacae*. The differences between the results of agar disc diffusion and well diffusion tests are due to the fact that the amount of extract used in the agar disc diffusion method (20 µL) was less than that used in the well diffusion test (100 µL).

Another substance synthesized by LAB are EPSs. These synthesized EPSs protect the bacterium against incidents such as phagocytosis and protozoa breakdown, phage effect, antibiotics, and osmotic pressure [42]. Studies have been carried out to determine whether probiotic bacteria such as *Lactobacillus* spp. produce EPS or not. As an example, Tallon et al. [43] investigated the EPS production of the *Lactobacillus plantarum* EP56 strain isolated from maize and found that EPS production was 0.114 mg/mL. In their study, Looijesteijn et al. [44] reported that bacteria were protected against bacteriophages, metal ions, and various antimicrobial agents such as lysozyme by the EPSs generated by *Lactococcus lactis* subsp. *cremoris* NZ4010.

In our study, we determined the adhesion capacity and colonization of the LAB strains into intestinal epithelial cells. It was determined that *Enterococcus faecalis* (L3) produced the most EPS (49.31 mg/mL), followed by *Lactobacillus plantarum* (L5) with 34.02 mg/mL EPS and *Lactobacillus fermentum* (L2) with 33.77 mg/mL EPS. The *Lactococcus lactis* CNM81 strain isolated from raw milk was found to have a potential antibiofilm effect on *Salmonella typhimurium* SL1344 [45]. Similarly, *Lactococcus lactis* (L1) isolates in this study were found to inhibit biofilm formation in all isolates including the $P3_{Ec}$ strain, which has the highest antibiotic resistance with a 1:1 concentration. In addition, even the lowest dilution of *Lactococcus lactis* (L1) (1:8) was determined to inhibit biofilm formation of this strain at a rate of 84.24%. All *Lactobacillus casei* (L3), *Lactobacillus plantarum* (L5), *Enterococcus faecium* (L6), and *Lactobacillus curvatus* (L7) exhibited antibiofilm activity on all isolates tested with the 1:1 concentration. As the dilution rate increased, the antibiofilm effect decreased and/or was eliminated.

Enterococcus spp. LAB are among important bacteria in terms of both food microbiology and clinical microbiology [46,47]. In addition to its capacity to improve the organoleptic properties of some foods, *Enterococcus faecalis* is used as a starter culture in the maturation of some fermented milk and meat products together with other LAB because of its lipolytic and esterolytic activity, and its capacity to benefit from citrate and synthesize volatile aromatic compounds. Enterococci are used as probiotics in human and animal intestinal flora to ensure microbial balance [48]. Furthermore, some pharmaceutical products containing *Enterococcus* strains as a probiotic culture are used in the clinical treatment of humans [47]. These probiotic preparations are used to treat gastroenteritis by improving the gastrointestinal balance and prevent enteric diseases in animals [49]. Two species in the genus *Enterococcus* have been reported as having probiotic properties, namely *Enterococcus faecium* and *Enterococcus faecalis* [50]. According to our data, *Lactobacillus fermentum* (L2), *Lactobacillus casei* (L3), *Lactobacillus plantarum* (L5), and *Lactobacillus curvatus* showed similar effects with *Enterococcus faecium* (L6). EPSs produced by LAB play an important role in the food and health industries. In previous studies, it was stated that EPSs regulated the immune system, lowered cholesterol, and had antiulcer and antitumor effects [51]. EPS forms a bond between intestinal epithelial tissue and bacteria in the intestinal flora. Therefore, the strains capable of producing EPSs are capable of adhering to the epithelium at a high capacity, so the production of EPS is an important factor that enables probiotics to colonize the intestinal surface and maintain viability [52]. It was determined that the LAB isolates used in our study were able to colonize the EPS intestinal epithelial cells. Because of the resistance against antibiotics in the last years, scientists have been looking for alternative sources for treatment. The antimicrobial and antibiofilm compounds produced by the LAB used in this study can be used in the treatment of many diseases, and the use of antibiotics can be decreased this way. It was determined that the LAB isolates used in our study were able to colonize the EPS intestinal epithelial cells.

In conclusion, in this study, the antimicrobial and antibiofilm activities of foodborne LAB were investigated against *Enterobacter cloacae* strains of animal origin and their adhesion potential to the intestinal epithelial cells was determined. Based on the data obtained in this study, almost all of the LAB isolates (especially *L. lactis*, *L. fermentum*, and *L. casei*) strains are good candidates for controlling *Enterobacter cloacae* biofilm formation. These findings indicate that *L. lactis*, *L. fermentum*, and *L. casei* can potentially be developed as novel antibiofilm agents. However, further in vitro and in vivo studies of these LAB strains should be conducted.

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Conflict of interest

The authors declare no conflict of interest for the present study.

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