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Research Article Araştırma Makalesi

Biofilm Formation and Prevention of Bacteria Isolated from Fish and Fish Stalls

Balık ve Balık Tezgahlarından İzole Edilen Bakterilerin Biyofilm Oluşumu ve Önlenmesi

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Abstract: Biofilms are structures formed by bacteria in the presence of convenient media. Bacteria protect themselves from chemicals such as ozone, heat, light, and chlorine with a biofilm structure. Fish is an important food item and it is an environment where bacteria can easily reproduce. Therefore, it is also appropriate for biofilm formation. Biofilm formation in fish and fish stalls is a threat to human health. This study aims to identify bacteria isolated from fish and fish stalls and to determine their biofilm-forming ability. In addition, it investigates the antibacterial effect of rock salt, lemon juice, and vinegar against biofilm-forming bacteria by the disk diffusion method. Forty-seven bacteria were isolated and identified from fish and fish stalls. The biofilm-forming abilities of the identified bacteria were determined by qualitative and quantitative analyzes. According to the analysis results, it was determined that 36 bacterial isolates formed a biofilm. Vinegar and lemon juice, which are natural products, have been shown to strongly inhibit the growth of biofilm-forming bacteria. However, no significant effect of rock salt was found against biofilm-forming bacteria.

Keywords
- Biofilm
- Natural products
- Antibacterial effect
- Fish

Özet: Biyofilm, bakterilerin uygun besiortamları varlığında oluşturdukları yapılardır. Biyofilm yapısı ile bakteriler kendilerini ozon, ısı, ışık, klor gibi kimyasal maddelerden korumaktadır. Balık önemli bir gıda maddesidir ve bakterilerin kolayca üreyebildiği bir ortamdır. Bu nedenle biyofilm oluşumu için de uygundur. Balık ve balık tezgahlarında biyofilm oluşumu insan sağlığını tehdit eden bir durumdur. Bu çalışma balık ve balık tezgahlarından izole edilen bakterileri tanılamayı ve onların biyofilm oluşturma kabiliyetlerini saptamayı amaçlar. Ayrıca kaya tuzu, limon suyu, sirkenin biyofilm oluşturan bakterilere karşı disk difüzyon metot ile antibakteriyal etkisini araştırır. Balık ve balık tezgahlarından 47 bakteri izole edilmiş ve tanılanmıştır. Tanılanan bakterilerin biyofilm oluşturma kabiliyetleri kalitatif ve kantitatif analizler ile saptanmıştır. Analiz sonuçlarına göre, 36 bakteri izolatının biyofilm oluşturduğu saptanmıştır. Doğal ürünler olan sirke ve limon suyunun, biyofilm oluşturan bakterilerin büyümesini güçlü bir şekilde engellediği görülmüştür. Ancak kaya tuzunun biyofilm oluşturan bakterilere karşı önemli bir etkisi bulunmamıştır.

Anahtar kelimeler
- Biyofilm
- Doğal ürünler
- Antibakteriyal etki
- Balık

1. INTRODUCTION

People should pay attention to nutritional elements to lead a healthy and quality life. However, sometimes undesirable situations may occur in foods due to physical, chemical, and biological reasons. Microbial developments that may occur on food or in the environment where food is present are biological factors. Microbial developments in foods appear as foodborne infections or food poisoning. Therefore, food safety has become an important issue in terms of public health all over the

world at present. Microbial growth, biotoxins, mycotoxins, and chemical contaminants that can be seen in foods can become threatening to human health. For this reason, the emergence of foodborne diseases and their turn into epidemics affect society in terms of health, economic and social aspects (Erkmen, 2010).

The factor that promotes microbial spoilage in fish is microorganisms transmitted from the environment while the fish is alive or during processing. Environmental temperature accelerates the growth of microorganisms (Koutsomanis and Nychas, 2000). Bacteria multiply rapidly on the surface of the fish or on the stalls where the fish are stored, forming a biofilm as an extracellular structure. This structure formed by the clustering of microorganisms is a biofilm. Biofilm occurs when food hygiene is not provided and threatens human health. In cases where hygiene is poor, biofilm formation may occur on the surface of fish and in the storage stalls after the fish is caught (Nurcan and Kubilay, 2016). Many chemical preservatives are used on foods to prevent biofilm formation. However, they threaten human health since most of these chemical substances have a carcinogenic effect. For this reason, the use of natural products is preferred to prevent the formation of biofilms in foods (Boğa and Binokay, 2010).

In this study, bacteria that can be found on the surface of fish and fish stalls were identified and those that formed biofilms were determined. In addition, the effects of natural products rock salt, lemon salt, vinegar, and their mixture on biofilm formation were compared.

2. MATERIALS AND METHODS

2.1. Materials

Samples were taken from five different fish species as *Sparus aurata*, *Dicentrarchus labrax*, *Mullus barbatus*, *Sardina pilchardus*, *Engraulis encrasicolus*, and fish stalls.

2.2. Collection of samples

Samples were collected from Kuşadası, Güzelçamlı, Söke, and İncirliova districts of Aydın province in December 2016. Samples taken from fish and fish stall surfaces were taken into 0.85% physiological saline water (FTS) using a sterile swap and stored. The collected samples were brought to the Microbiology Laboratory of the Biology Department of Aydın Adnan Menderes University and stored at +4^{o}C.

2.3. Isolation and identification of bacterial strains

For enrichment, 1 mL of the samples in the FTS tubes was inoculated into Tryptic Soy Broth (TSB) medium tubes and incubated at 37^{o}C for 24 hours. At the end of the incubation, a serial dilution was made from 10^{-1} to 10^{-6}. 0.1 µL of 10^{-4}, 10^{-5}, 10^{-6} dilutions were taken and inoculated into Tryptic Soy Agar (TSA) media. All Petri dishes were incubated at 37^{o}C for 24 hours. Colony selection was made at the end of the incubation and the purification process was applied. Pure cultures were taken in 20% Skim Milk and stored at -20^{o}C (Törün et al., 2017; Tekin and Çoban, 2021; Çoban and Barışık, 2021).

For molecular identification, total genomic DNA was isolated from the broth culture of bacterial strains (De Boer and Ward, 1995). Amplification of 16S rDNA of bacterial strains was performed by using universal primers (Lane, 1991). PCR reactions were performed at 94ºC 3 min, 94ºC 40 sec, 60ºC 40 sec, 72ºC 40 sec with 35 cycles, respectively. Amplification products were sent GATC BioTech, Germany for sequence analysis. The phylogenetic tree was created using the Maximum Likelihood method (Tamura and Nei, 1993). The sequence samples were compared using BLASTn software in GENBank. ClustalW program (MEGA 7.0 software) was used for sequences (Kumar et al., 2016).

2.4. Analysis of biofilm-forming

The biofilm formation ability of bacterial isolates was been examined qualitatively and quantitatively. Qualitative biofilm formation was investigated on congo red agar media (Freeman et

al., 1989). The bacteria strains have inoculated the plates and Petri dishes were incubated at 37^{o}C for 24 hours. As a result of incubation, strains that formed dry crystallized rough black colonies were evaluated as biofilm positive, while strains that formed red or pink smooth colonies were evaluated as biofilm negative (Melo et al., 2013; Shrestha et al., 2018).

Quantitative biofilm formation was examined in the Elisa Plate (Christensen et al., 1982). Bacterial strains were inoculated in TSB medium and incubated overnight at 37^{o}C. After, 150 µL of the activated cultures were added to the individual wells of 96-well Elisa Plate and incubated at 37^{o}C for 3 days. Later, samples from the Elisa Plate were poured and washed 3 times with sterile dH_2O and dried. And then, 150 µL of 0.1% crystal violet was put in the wells and left for 45 minutes, washed, and air-dried again. Next, 200 µL ethanol: acetic acid (v/v) was added to the wells and waited for 10 minutes. From here, 100 µL of it was taken into a new Elisa Plate and measured in a spectrophotometer at OD 570 nm (Mathur et al., 2006; Melo et al., 2013).

TSB medium was used as negative control and *Staphylococcus aureus* ATCC 25923 was used as a positive control. Strains with optical density values ≥ 0.240 were considered strong adhesion, strains with optical density values 0.120-0.240 were considered moderate adhesion, and strains ≤ 0.120 were considered weak/negative (Mathur et al., 2006; Demir and İnanç, 2015).

2.5. Preparation of natural substances

Rock salt, grape vinegar, and lemon juice used as natural products were purchased commercially from the market. Rock salt (10 mg/mL) was prepared with sterile water. The total acidity rate of the grape vinegar (pH 3.52) used is 4g/100 mL. The titration acidity value of the lemon juice (pH 4.5) used is 45g/L. Mixtures of rock salt, vinegar, and lemon juice 1:1:1 (v/v) were prepared and its effect was tested against biofilm-forming bacteria.

2.6. The effect of natural substances against biofilm-forming bacteria

Biofilm-forming bacteria were determined by qualitative and quantitative analysis. The effects of natural substances against biofilm-forming bacteria were determined using the agar well diffusion method (CLSI, 2015; EUCAST, 2019). The tested microorganisms were incubated at 30-37^{o}C for 24 h and then, cell number was regulated as 1×10^8 cells/mL according to 0.5 McFarland standard tube (Çoban et al., 2021; Şahin et al., 2021; Koseoglu et al., 2022). Mueller Hinton Agar media (MHA) (20 mL) were prepared in Petri dishes for analyzing the effect of natural substances. The microorganism cultures (100 µL) were inoculated on plates. Wells with a diameter of 6 mm were created on the medium with a sterile stick. After, the wells were filled with 50 µL of rock salt, vinegar, lemon juice, and their mixtures. Distilled water was used as a positive control. Next, all Petri dishes were incubated at 30-37°C for 24 h (Çoban et al., 2021; Şahin et al., 2021; Koseoglu et al., 2022).

2.7. Statistical

All the experiments were carried out in triplicate. The results were expressed as the mean value of three independent replicates ± the standard deviation using SPSS v22 program.

3. RESULT AND DISCUSSION

3.1. Isolated and identified strains

In total, 47 bacterial species were identified in this study. The DNA sequences were compared using BLASTn software in GENBank and molecular identification was performed. The bacteria species obtained are given in Table 1.

Table 1. Bacteria species isolated and identified from samples

Sample Location	Species	Accession No	% Similarity
Skin	*Enterobacter hormaechei strain FQP44*	MF144523.1	97
	Staphylococcus aureus strain K6	KX821633.1	97
	Staphylococcus aureus strain SSW20	KU922502.1	97
	Citrobacter freundii strain B25	FJ494899.1	99
	Staphylococcus aureus strain RCB1010	KT261222.1	99
	Proteus penneri strain wf-1	KT029130.1	97
	Staphylococcus aureus strain K2	KX821629.1	96
	Enterobacter cloacae strain 39	KX395979.1	98
	Staphylococcus aureus strain 5 BWI	KX456107.1	96
	Staphylococcus epidermidis strain P8	KF705248.1	98
	Lactococcus garvieae strain IMAU50143	FJ749537.1	96
	Staphylococcus aureus strain RCB1010	KT261222.1	98
	Lactococcus garvieae strain ZSJ5	KU324937.1	98
	Staphylococcus pasteuri strain AIMST.Pbst4	KM087104.1	98
	Enterococcus gallinarum	AB904770.1	95
	Staphylococcus pasteuri strain HN-35	KT003275.1	95
	Staphylococcus aureus strain SSW20	KU922502.1	99
Gill	*Serratia marcescens strain AR1*	KX343948.1	97
	Lactococcus lactis	KT633921.1	98
	Staphylococcus warneri strain STM81	KY393084.1	97
	Proteus vulgaris strain M20	KT792741.1	97
	Raoultella ornithinolytica strain JSM 05182054	KY352821.1	96
	Moellerella wisconsensis strain X	KP159747.1	98
	Proteus penneri strain ALK624	KC456589.1	96
	Staphylococcus pasteuri strain AIMST.Pbst4	KM087104.1	98
	Carnobacterium divergens strain LHICA_53_4	FJ656716.1	98
	Leuconostoc mesenteroides strain HL2	KY233187.1	95
	Proteus penneri strain NSPPN01	KT361197.1	95
	Proteus mirabilis strain T7	KJ626258.1	96
	Staphylococcus aureus strain K8	KX821635.1	99
Fish Stalls	*Citrobacter youngae strain NSKP2*	KY992522.1	95
	Staphylococcus aureus strain HN-5	KT003251.1	96
	Lactococcus garvieae strain ZSJ5	KU324937.1	98
	Leuconostoc mesenteroides strain	KF697659.1	95
	Leuconostoc mesenteroides strain 30-1	KJ477402.1	98
	Leuconostoc mesenteroides MFL24	LT853601.1	98
	Enterococcus casseliflavus	LC122272.1	96
	Enterococcus faecalis strain H50	KJ626240.1	98
	Proteus vulgaris strain BPGM7	KX156180.1	98
	Staphylococcus aureus strain K2	KX821629.1	98
	Lactococcus garvieae strain CMGB-L23	MF348235.1	98
	Staphylococcus aureus strain K5	KX821632.1	99
Collecting Plastic Pipe	*Staphylococcus capitis strain STM79*	KY393082.1	99
Boat Metal Surface	*Leuconostoc mesenteroides strain TUST005*	KC456619.1	95

Phylogenetic analysis was carried out using MEGA 6 software. Phylogenetic tree was showed in Figure 1.

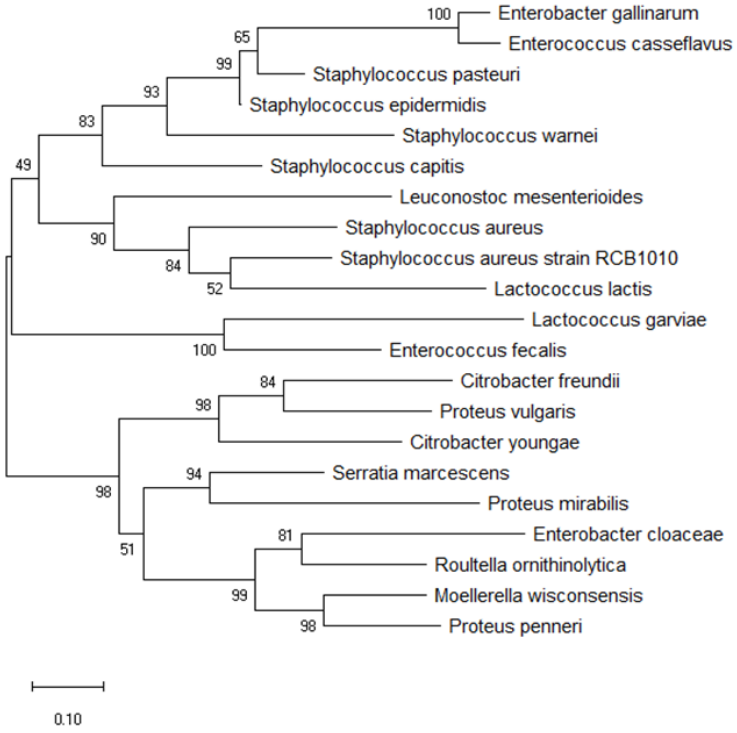

Figure 1. Phylogentic tree obtained by maximum likelihood method

3.2. Analysis of biofilm-forming

3.2.1. Qualitative determination of biofilm formation

Qualitative biofilm formation was obtained using congo red agar media. While black colony-forming bacteria are considered positive for biofilm, red-reddish colonies are considered biofilm negative on congo red agar medium (Figure 2a, b).

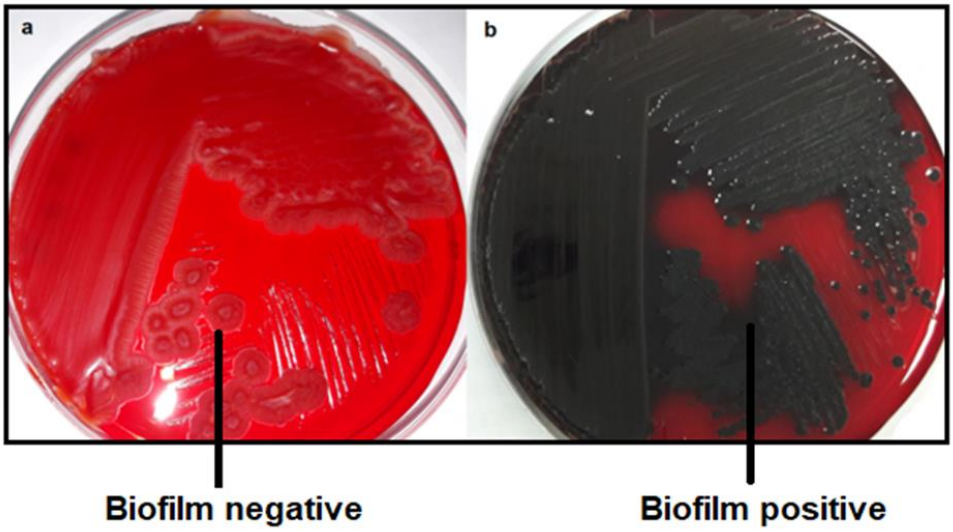

Figure 2.a. Biofilm negative in qualitative determination (*Bacillus vallismortis*) b. Biofilm positive in qualitative determination (*Staphylococcus aureus*)

3.2.2. Quantitative determination of biofilm formation

Spectrophotometer was used for the quantitative determination of biofilm formation. The results obtained in the microplate method used for the quantitative determination of biofilm formation are given in Table 2.

Table 2. Biofilm-forming bacterial species and their adherence values

	Optical Density Value			
Species	**Sample**	**Negative Control**	**Positive Control**	**Adherence**
L. mesenteroides strain MFL24	0,312	0,065	0,086	Strong
L. mesenteroides	0,459	0,065	0,086	Strong
P. penneri strain ALK624	0,399	0,065	0,086	Strong
L. garvieae strain ZSJ5	0,465	0,065	0,086	Strong
L. mesenteroides strain 30-1	0,459	0,065	0,086	Strong
S. capitis strain STM79	0,483	0,065	0,086	Strong
S. epidermidis strain P8	0,640	0,065	0,086	Strong
P. penneri strain NSPPN01	0,391	0,065	0,086	Strong
L.lactis	0,434	0,065	0,086	Strong
S. marcescens strain AR1	0,310	0,065	0,086	Strong
E. cloacae strain 39	0,123	0,065	0,086	Moderate
L. mesenteroides strain TUST005	0,123	0,065	0,086	Moderate
R. ornithinolytica strain JSM 05182054	0,160	0,065	0,086	Moderate
P.penneri strain wf-1	0,163	0,065	0,086	Moderate
S. aureus strain 5 BWI	0,225	0,065	0,086	Moderate
S. aureus strain K8	0,165	0,065	0,086	Moderate
S. pasteuri strain AIMST.Pbst4	0,175	0,065	0,086	Moderate
S. aureus strain K6	0,277	0,065	0,086	Moderate
P. mirabilis strain T7	0,286	0,065	0,086	Moderate
S. pasteuri strain HN-35	0,122	0,065	0,086	Moderate
C. youngae strain NSKP2	0,194	0,065	0,086	Moderate
P. vulgaris strain BPGM7	0,141	0,065	0,086	Moderate
S. aureus strain HN-5	0,135	0,065	0,086	Moderate
E. gallinarum	0,102	0,065	0,086	Weak
S. aureus strain SSW20	0,114	0,065	0,086	Weak
S. aureus strain K2	0,116	0,065	0,086	Weak
C. freundii strain B25	0,094	0,065	0,086	Weak
S. aureus strain RCB1010	0,054	0,065	0,086	Weak
S. warneri strain STM81	0,086	0,065	0,086	Weak
P. vulgaris strain M20	0,095	0,065	0,086	Weak
S. aureus strain K5	0,058	0,065	0,086	Weak
M. wisconsensis strain X	0,090	0,065	0,086	Weak
L. garvieae strain CMGB-L23	0,105	0,065	0,086	Weak
C. divergens strain LHICA_53_4	0,114	0,065	0,086	Weak
E. faecalis strain H50	0,081	0,065	0,086	Weak
E. casseliflavus	0,085	0,065	0,086	Weak

(Negative Control: Medium, Positive Control: *Staphylococcus aureus* ATCC 25923)
(Strong: ≥0.240, Moderate: 0.120-0.240, Week: ≤0.120)

3.3. Antibacterial activity of natural substances

Antibacterial activities of natural substances (rock salt, vinegar, lemon juice, and their mixtures) against biofilm-forming bacteria species were tested according to the Agar-well diffusion method (Table 3). There was no statistically significant difference in the rate of inhibition zones.

Table 3. The effect of natural substances (rock salt, vinegar, lemon juice and their mixtures) against biofilm forming bacteria species

Bacteria Species	Inhibition zones (mm)				
	1	**2**	**3**	**4**	**5**
L. lactis	12.33±0.57	11.00±1.00	_	12.33±0.5	_
L. mesenteroides	15.66±2.5	17.00±2.64	_	10.00±0.0	_
M. wisconsensis strain X	22.00±1.00	22.33±1.52	_	18.00±3.0	_
L. garvieae strain IMAU50143	17.66±0.57	13.33±1.15	10.00±1.0	10.33±1.5	_
P. mirabilis strain T7	_	_	_	_	_
S. aureus strain RCB1010	14.33±0.57	13.66±1.15	_	_	_
C. freundii strain B25	20.00±1.73	20.33±0.57	_	20.33±0.5	_
R. ornithinolytica strain JSM 05182054	11.33±1.52	15.33±2.51	_	14.33±0.5	_
S. aureus strain K2	12.66±0.57	15.33±2.3	9.33±0.57	17.66±2.5	_
P. penneri strain wf-1	15.33±2.51	21.33±0.57	_	18.66±2.0	_
L. mesenteroides strain 30-1	13.66±1.15	20.33±1.52	_	15.33±2.3	_
L. mesenteroides strain TUST005	13.66±1.15	18.00±2.64	_	15.33±2.5	_
P. penneri strain ALK624	_	_	_	_	_
S. pasteuri strain AIMST.Pbst4	18.00±1.73	20.00±2.64	_	18.66±1.1	_
C. divergens strain LHICA_53_4	17.66±0.57	14.33±1.15	_	_	_
E. cloacae strain 39	14.33±1.15	20.33±0.57	_	14.33±0.5	_
S. aureus strain HN-5	14.33±0.57	10.33±1.52	_	_	_
S. aureus strain K6	12.00±1.00	20.33±0.57	_	15.66±2.5	_
L. mesenteroides strain HL2	15.66±0.57	17.33±2.51	_	10.00±0.0	_
L. mesenteroides strain MFL2	_	_	_	_	_
P. penneri strain NSPPN01	18.33±1.15	21.66±2.08	_	16.00±1.7	_
E. casseliflavus	24.00±1.00	14.33±1.15	_	13.33±0.5	_
C. youngae strain NSKP2	_	20.00±1.00	_	15.00±2.0	_
E. gallinarum	21.66±2.08	12.33±0.57	_	_	_
S. pasteuri strain HN-35	13.33±0.57	13.33±0.57	_	10.33±0.5	_
L. garvieae strain ZSJ5	15.66±0.57	_	_	_	_
S. epidermidis strain P8	18.33±0.57	15.00±2.64	_	10.00±0.0	_
E. faecalis strain H50	14.33±0.57	18.33±1.15	_	13.33±0.5	_
S. capitis strain STM79	10.00±0.0	10.33±1.52	_	_	_
S. aureus strain SSW20	13.66±1.15	12.33±1.15	_	13.33±0.5	_
S. aureus strain 5 BWI	12.33±2.51	20.00±1.73	_	14.33±1.1	_
S. warneri strain STM81	10.66±1.15	15.66±0.57	_	_	_
P. vulgaris strain BPGM7	15.33±2.51	20.66±2.08	_	15.66±0.5	_
P. vulgaris strain M20	15.66±0.57	20.33±0.57	_	15.66±2.5	_
S. marcescens strain AR1	11.33±0.57	17.00±1.73	_	7.33±0.00	_
L. garvieae strain CMGB-L23	15.66±0.57	11.00±1.00	_	_	_
S. aureus strain SSW20	13.66±1.15	12.33±1.15	_	13.33±0.5	_
S. aureus strain K5	13.33±0.57	20.00±1.00	_	13.33±0.5	_

1: Lemon juice, 2: Vinegar, 3: Rock salt, 4: Mixture, 5: Distilled water (The results were expressed as the mean value of three independent replicates ± the standard deviation)

4. DISCUSSION

Diseases caused by foodborne pathogens are important for public health. Biofilm formation is of great importance in the occurrence of these diseases. The formation of biofilm by microorganisms depends on the environment and the bacteria itself. Biofilms form on moist surfaces such as foods and food processing equipment. A biofilm is made up of many types of bacteria. The effect of temperature and pH is also important in the adhesion of bacteria to the surface. (Zhao et al., 2017). The structure of the bacterial cell wall (surface charge, hydrophility, surface energy, and organelles) also affects biofilm formation (Chauhan et al., 2014).

In recent years, it has been observed that the effect of microbial biofilms on fish health is important. It has been observed that *Listeria*, *Salmonella*, *Shigella*, *Vibrio*, *Bacillus* and *Aeromonas* bacteria cause fish infections and biofilm formation (Mizan and Ha, 2015). Fish pathogens can form a strong biofilm on wood, metal, fiberglass, and glass. These materials are used a lot in aquaculture units. Biofilm formation in these materials is a threat to fish and human health. Biofilm is seen on the body of the fish and the surface of many materials such as metal surfaces, freezers, and fishing nets used in facilities (Pippo et al., 2018). Lactic acid bacteria (LAB) may cause biofilms and in this sense, it represents a concern for the food industry (Winkelströter et al., 2014). *Listeria*, *Pseudomonas*, *Stenotrophomonas*, *Brochothrix*, *Serratia*, *Acinetobacter*, *Rhodococcus*, and *Chryseobacterium* were isolated from food conveyor belts (Giaouris and Simoes, 2018).

In our work; samples were taken from fish looms (plastic, mica, wooden surface), the gill and skin of the fish, as well as the metal boat storage, and plastic collection pipe. As a result of the findings, thirty-six bacteria were capable of forming biofilms of the 47 bacteria identified from these surfaces. Among the bacteria identified, it was determined that *L. mesenteroides* strain MFL24, *L. mesenteroides*, *P. penneri* strain ALK624, *L. garvieae* strain ZSJ5, *L. mesenteroides* strain 30-1, *S. capitis* STM79, strain, *P. penneri* strain NSPPN01, *L. lactis*, *S. marcescens* strain AR1 formed strong adherence. In addition, *E. cloacae* strain 39, *L. mesenteroides* strain TUST005, *R. ornithinolytica* strain JSM 05182054, *P. penneri* strain wf-1, *S. aureus* strain 5 BWI, *S. aureus* strain K8, *S. pasteuri* strain AIMST.Pbst4, *S. aureus* strain K6, *P. mirabilis* strain T7, *S. pasteuri* strain HN-35, *C. youngae* strain NSKP2, *P. vulgaris* strain BPGM7, *S. aureus* strain HN-5 demostrated moderate adherence. However, *E. gallinarum*, *S. aureus* strain SSW20, *S. aureus* strain K2, *C. freundii* strain B25, *S. aureus* strain RCB1010, *S. warneri* strain STM81, *P. vulgaris* strain M20, *S. aureus* strain K5, *M. wisconsensis* strain X, *L. garvieae* strain CMGB-L23, *C. divergens* strain LHICA 534, *E. faecalis* strain H50, *E. casseliflavus* indicated weak adherence. Giaouris et al. (2020) showed that *S. aureus*, *S. enterica* and *L. monocytogenes* which cause spoilage in foods form biofilm.

ArunKumara (2019) isolated biofilm-forming *Vibrio* spp. from fish samples. *Vibrio* species formed biofilm as strong (10 strains), moderate (17 strains), and weak (23 strains). *Vibrio*, *Serratia*, *Rhodococcus*, *Carnobacterium*, *Micrococcus*, *Morganella*, *Yersinia*, *Lactobacillus* were found on fish and seafood processing surfaces (Møretrø et al., 2016; Langsrud et al., 2016; Møretrø and Langsrud, 2017). *Pseudoalteromonas* (7 strains), *Vibrio* (7 strains), and *Halomonas* (1 strain) were isolated from sediment in fish farms (Ijima et al., 2009).

Some biofilm-forming bacteria (*S. aureus*, *S. epidermidis*, *C. freundii*, *S. marcescens*, *E. faecalis*, *P. aeruginosa*, *L. garvieae*, *P. vulgaris*, *C. koseri*, *E. cloacae*, *P. mirabilis*, *E. gallinarum* CRL 1826) found in human skin, eyes and mucus can infect fish and fish stalls for sale in the absence of sanitation (Shin et al., 2013; Brandwein et al., 2016; Niederle et al., 2019; Raksha et al., 2019; Diriba et al., 2020). Biofilm-forming bacteria species we obtained in our study are compatible with the literature information. In this respect, contamination in fish is thought to be of human origin. Therefore, it is asserted that the employees do not act in accordance with the sanitation and hygiene rules.

The main purpose of stopping biofilm formation in the fish industry is to prevent the bacteria before they form the biofilm structure. Bacteria that cannot form a biofilm have low infectious power. It has been observed that the prevention of biofilm formation is important in the fight against fish diseases (Pippo et al., 2018). Malic acid, lysozyme, garlic oil, and oregano oil have been investigated for biofilm inhibition in the fish industry (Galie et al., 2018).

Foodborne pathogenic bacteria cause food poisoning and cause great economic losses. For this reason, it is necessary to give importance to hygiene in food processing equipment and food sales benches. Therefore, inhibition of these bacteria is important to maintain food safety and public health. In this respect, sanitation is an important rule in food and food contact surfaces (Laxmi and Sarita, 2018). Strategies for the control of biofilm formation in foods and on surfaces have been investigated by researchers (Galie et al., 2018; Bai et al., 2021; Dass and Wang, 2022). Depending on the structure of the biofilm, different methods can be used to prevent biofilms. These include mechanical cleaning, the use of antimicrobial agents, and the prevention of microbial adhesion to a surface with chemicals. To remove the biofilm, mechanical force must first be applied to the surface. Mechanical cleaning is very effective in preventing biofilm formation stages. Because cleaning with mechanical processes is

more effective than gel cleaners or low-pressure cleaning systems. However, every system is not convenient for mechanical cleaning. There are hard-to-reach points in the systems. Chemical cleaning with appropriate mineral and organic acids should be applied immediately after mechanical cleaning. The system should be cleaned with corrosion inhibitors as the acids used may cause corrosion of the metals. In recent years, different methods such as electrical fields, catalyzed modified surfaces, ultrasound, enzymes, ammonia and formaldehyde, detergents, high-pressure cleaning systems have been used to prevent biofilm formation. Enzymes provide an effect in cleaning extracellular polymers formed in the biofilm matrix. Different enzymes such as protease, α-amylase, and β-glucanase are used to remove biofilm structures formed by various microorganisms (Kartal et al. 2021; Srinivasan et al., 2021).

Apart from enzymes, chemicals such as lactic acid, sodium hypochlorite, benzalkonium chloride, hydrogen peroxide, and citric acid are mostly used for sanitation. However, most of these substances are toxic and threaten human health (Lim et al., 2017; Carrascosa et al., 2021). For this reason, in our study, natural products (lemon juice, vinegar, rock salt) that are non-toxic, do not threaten human health, and have low cost have been tried for sanitation. Therefore, antibacterial tests of lemon juice, vinegar, and rock salt as natural products were carried out against biofilm-forming bacteria. When the antibacterial effects of the products used were compared, it was observed that the most effective natural products were vinegar and lemon juice (12-24 mm inhibition zones). Lemon juice and vinegar showed high activity against all tested bacteria except *P. mirabilis strain T7*, *P. penneri strain ALK624*, *L. mesenteroides strain MFL2* bacteria. While lemon juice was only effective against *L. garvieae strain ZSJ5* bacteria (15 mm inhibition zones), vinegar was only effective against *C. youngae strain NSKP2* bacteria (20 mm inhibition zones). While rock salt was only low effective against *L. garvieae strain IMAU50143* and *S. aureus strain K2* (9-10 mm inhibition zones), it was not effective against any of the other bacteria.

Vinegar is a natural product rich in organic acids such as acetic, succinic, malic, lactic, tartaric acid, and other fermentation products. Due to the organic acids, it contains, vinegar destroys the cell wall of bacteria, inhibits macromolecule synthesis, and disrupts the intracellular osmotic balance (Chen et al., 2016). Lemon juice is a widely consumed food for health due to its vitamin C (ascorbic acid) content. In addition, it contains citric acid, phenolic compounds, flavonoids, and essential oil. Therefore, the antibacterial effect of lemon juice is also known against pathogen bacteria (Aruoma et al., 2012).

Kahraman et al. (2022) researched antibacterial effect of home-made apple and grape vinegar against some food pathogenic bacteria such as *L. monocytogenes* RSK 472, *E. faecalis* ATCC 29212, *S. aureus* ATCC 43300, Methicillin-resistant *S. aureus* ATCC 25923, *B. cereus* ATCC 33019, *S. enteritidis* ATCC 13076, *S. typhimurium* ATCC 14088, *P. fluorescens* ATCC 13525, *E. coli* O157:H7 ATCC 35150. They showed that grape vinegar was more effective than apple vinegar. Kara et al. (2021) investigated the antibacterial activity of different vinegar samples against *S. aureus*, *K. pneumonia*, *E. coli* (ATB: 57), *E. coli* (ATB: 97), *P. aeruginosa* as pathogenic bacteria. While vinegar samples had a powerful effect against *S. aureus* and *P. aeruginosa* (15-32 mm inhibition zones), they had a moderate effect against *K. pneumonia*, *E. coli* (ATB: 57), *E. coli* (ATB: 97) (11-14 mm inhibition zones). In a similar study, the antibacterial effect of grape vinegar and apple vinegar was investigated against biofilm-forming bacteria. It was found that both vinegars showed high activity against *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 bacteria (21-22 mm inhibition zones) (Kahraman et al., 2021). Ousaaid et al. (2021) expressed that apple vinegar had considerable effect against *S. typhi*, *E. coli* O157:H7, *V. cholerae*, *C. albicans*, *C. tropicalis* (11-19 mm inhibition zones). Singh et al. (2020) tested the antibacterial activity of lemon juice as an antibacterial agent against *S. flexneri*, *S. epidermidis*, *Citrobacter spp.* and *Salmonella typhi*. It was shown that it had a respectable effect against *S. flexneri*, *S. epidermidis*, *Citrobacter spp*. (12-15 mm inhibition zones). Hamza et al. (2018) infered that apple, black raisin, garlic, and palm vinegar had a strong effect against *S. aureus*, *P. aeruginosa*, *Acinetobacter spp*., *E. faecalis*, *E. coli*, *K. pneumonia* (25-32 mm inhibition zones). Bakır et al. (2017) investigated the antibacterial effect of different vinegars against *S. aureus*, *S. typhimurium*, and *E. coli*. It was demonstrated that the highest effect was against *S. typhimurium* bacteria (16 mm inhibition zones). De et al. (2017) investigated the antimicrobial activity of raw

lemon against human enteric pathogens such as *K. pneumonia*, *S. typhi*, *P. vulgaris*. While the lemon juice had a high effect against *K. pneumonia*, *S. typhi* (15-21 mm inhibition zones), it had no effect against *P. vulgaris*. Oikeh et al. (2016) evaluated the antimicrobial activity of different citrus juice such as

C. tangerine (tangerine), *C. paradisi* (grape), *C. limon* (lemon), and *C. aurantifolia* (lime) against *S. aureus*, *E. faecalis*, *P. aeruginosa*, *E. coli*, *Salmonella spp.*, *C. albicans*, *A. niger*, *Penicillum spp.* While the lemon juice had a strong effect (18-24 mm inhibition zones) against *S. aureus*, *P. aeruginosa*, *C. albicans*, it had a moderate effect (10-12 mm inhibition zones) against *E. faecalis*, *E. coli*, *Salmonella spp.* bacteria. On the other hand, it had a very low effect (9 mm inhibition zones) against *A. niger*, *Penicillum spp.* In another study, the antibacterial activity of acetic acid was examined against biofilm forming pathogens on burns patients. It was determined that different concentrations of acetic acid were found to be effective against *P. aeruginosa*, *A. baumannii*, *S. aureus*, *E. faecalis*, *E. coli*, *K.pneumoniae* (Halstead et al., 2015).

It is appropriate to use vinegar and lemon juice to prevent biofilm in fish processing equipment and sales benches. In this study, it has been determined that vinegar and lemon juice can be used within the scope of food safety.

5. CONCLUSION

It is important to protect food hygiene and safety from the production to consumption of many foods such as fish, meat, and dairy products. The unsuitable storage conditions of the foods and the failure to comply with the sanitation rules of the equipment used encourage the development of bacteria that cause food spoilage and poisoning. In addition, it is also possible to contaminate food through human contact or by breathing. As a result, bacteria that develop in foods threaten human health by forming a biofilm structure. The growth of biofilm-forming bacteria in foods is due to inadequate hygiene and sanitation. According to the results of our study, it was found that the bacteria that developed in fish and fish stalls were mostly of human origin. It can be understood that the people dealing with this work do not act in accordance with the sanitation rules. Many of the isolated bacteria also appear to have strong and moderate biofilm-forming abilities. Natural products were used to prevent the growth of these bacteria and the formation of biofilm. Vinegar and lemon juice was found to be effective among natural products for hygiene and sanitation. The results obtained have brought a new perspective to the biofilm control strategy. Wiping food surfaces with vinegar or lemon juice is a precaution against biofilm-forming bacteria for sanitation. Compared to chemical disinfectants, the use of vinegar and lemon juice is an attractive practice due to its safe and inexpensive cost.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR'S CONTRIBUTIONS

The contribution of the authors is equal.

ETHICAL STATEMENT

There are no ethical issues with the publication of this manuscript.

DATA AVAILABILITY STATEMENT

Data used in this study are available from the corresponding author upon reasonable request.

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