Turk. J. Fish.& Aquat. Sci. 27(2), TRJFAS28832
https://doi.org/10.4194/TRJFAS28832

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RESEARCH PAPER

Turkish Journal of FISHERIES and AQUATIC SCIENCES

Assessment of Microbial Contamination, Antibiotic Resistance in *Enterobacter* Species and Biofilm Formation in Mussels (*Mytilus galloprovincialis*) from the Lapseki Coast of the Çanakkale Strait (Türkiye)

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How to Cite

Özcan Ateş, G., Soyiç, M.H., Hanedan Angı, T. (2027). Assessment of Microbial Contamination, Antibiotic Resistance in *Enterobacter* Species and Biofilm Formation in Mussels (*Mytilus galloprovincialis*) from the Lapseki Coast of the Çanakkale Strait (Türkiye). *Turkish Journal of Fisheries and Aquatic Sciences*, *27(2), TRJFAS28832.* https://doi.org/10.4194/TRJFAS28832

Article History
Received 18 July 2025
Accepted 15 September 2026
First Online 22 September 2026

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Keywords
Enterobacter spp.
Mussels
Antibiotic resistance
Biofilm formation
One health

Abstract

Global warming also impacts the aquatic ecosystem, leading to changes in the reproduction and development of both beneficial and harmful organisms. In 2021, a significant mucilage layer was observed in the straits, and mucilage formation recurred in 2022, though to a lesser extent. Global warming accelerates the growth and spread of pathogenic microorganisms and toxic microalgae, particularly in marine environments and among bivalve mollusks. Since humans consume bivalve mollusks, their infection with pathogenic microorganisms poses health risks that vary with the type of microorganism. Thus, monitoring microbiological contamination in these foods is essential. This study aimed to examine microbial contamination of mussels along the Lapseki coast of the Çanakkale Strait, considering the presence of mussel farms and naturally harvested and consumed mussels. The results showed high microbial counts, mold contamination, and the presence of antibiotic-resistant *Enterobacter* species in the collected samples. Some isolates have been determined to have the potential to produce broad-spectrum beta-lactamases (ESBL). Only one isolate was identified as a moderate producer of biofilm. These findings highlight the public health risks associated with mussels and underscore the need for environmental monitoring of antibiotic resistance.

Introduction

The phylum Mollusca, class Bivalvia, which includes various bivalve mollusks, plays a crucial role in fisheries and aquaculture. This class is also vital for maintaining ecosystem complexity and functionality. Furthermore, marine bivalves, such as oysters, scallops, and mussels, provide a rich source of protein for human diets. They also hold significant economic value (Smaal et al., 2019; FAO, 2020; Zgouridou et al., 2022).

Bivalve mollusks feed by filtering water, allowing them to accumulate a significant quantity of microorganisms, some of which can be pathogenic to higher vertebrates and humans. In recent years, increasing human activities, particularly urban waste discharges in coastal areas, ship traffic, agricultural runoff, and uncontrolled aquaculture, have become significant sources of microbial pollution in aquatic ecosystems. Furthermore, certain microorganisms can infect these mollusks, creating risks for aquaculture. This issue results in both economic losses and public health concerns (Zannella et al., 2017; Carella et al., 2018). Key bacterial infection agents include genera such as *Acinetobacter*, *Aeromonas*, *Bacillus, Micrococcus*, *Moraxella*, *Photobacterium*, *Pseudomonas*, and *Vibrio* (Zannella et al., 2017; Zgouridou et al., 2022).

In modeling the effects of global warming on the climate, it is suggested that sea surface temperatures (SSTs) and precipitation will increase, particularly. It is estimated that sea surface salinity (SST), especially in coastal waters, will decrease due to increased precipitation and high freshwater inflow. These factors are said to lead to structural changes in the microbial communities of the seas, affecting both species and components. Additionally, it is thought that they will play a significant role in the proliferation of harmful algae. Therefore, both environmental changes and shifts in the microbial community suggest a high risk of infection (Turner et al., 2016). Furthermore, many bivalve species inhabit coastal waters and estuaries. These locations are particularly prone to pollution due to urbanization. Thus, urbanization and pollution, combined with both SST and pathogenic infectious agents, may heighten the risk of pathogenic microorganisms in bivalve mollusks (Moullec et al., 2019; Zgouridou et al., 2022).

Global warming also impacts the aquatic ecosystem, causing changes in the reproduction and development of both beneficial and harmful organisms. A serious mucilage density was experienced in the straits in 2021, and mucilage formation was repeated in 2022, albeit with less intensity. Global warming accelerates the development and spread of pathogenic microorganisms and toxic microalgae, especially in marine habitats and bivalve mollusks. Since bivalve mollusks are consumed as human food, they can be infected with pathogenic organisms, which can affect human health differently depending on the type of microorganism. Therefore, microbiological contamination of these foods should be monitored.

Antimicrobial resistance is being tackled globally, and the WHO Bacterial Priority Pathogens List (WHO BPPL) was updated in 2024. The updated WHO list includes Acinetobacter baumannii, Enterobacterales (third-generation cephalosporin resistant), and carbapenem-resistant Enterobacterales in the critical group (WHO, 2024). *Enterobacter* species, belonging to the Enterobacteriaceae family, are facultative anaerobic Gram-negative bacilli that are widely found in nature and can cause various infections in humans as opportunistic pathogens (Davin-Regli et al., 2019). These species can be isolated from aquatic environments, food products, and living organisms; they are especially concerning due to their resistance mechanisms against antibiotics and their ability to form biofilms. The biofilm structure allows bacteria to resist environmental stresses, disinfectants, and antibiotics, making infection control complex and treatment more complicated. Many studies in the literature focus on the antibiotic resistance and biofilm formation abilities of *Enterobacter* species isolated from food, drinking water, and various seafood (Denissen et al., 2022; Oyenuga et al., 2024). However, studies focusing on *Enterobacter* species isolated from mussels that also evaluate antibiotic resistance profiles and biofilm formation are quite limited in the literature. In studies conducted along the coasts of the Marmara Sea and the Bosphorus, it was observed that strains carrying multiple antibiotic resistance genes, including those in foodborne and aquatic Enterobacteriaceae, were reported along with their biofilm formation abilities (Sivri, 2017). Studies on microbial contamination of Çanakkale mussels have been found in the literature (Künili et al., 2021; Tosun et al., 2021), but no studies have been identified on pathogenic microorganism contamination, the identification of *Enterobacter* species, antibiotic resistance, or the biofilm formation capacity of mussels after the 2021 mucilage event. Given the growing impact of global warming, regular monitoring of mussel contamination is crucial for both public health and the marine ecosystem. Therefore, this study aimed to determine the microbial contamination of mussels growing on the Lapseki coast of Çanakkale Strait and to assess the antibiotic resistance and biofilm formation capacity of the *Enterobacter* spp. isolates obtained.

Materials and Methods

Collecting Mussel Samples

A minimum of 50 mussels from each of the three regions shown in Figure 1 and Table 1 were collected on two separate occasions, 11 July 2024 and 15 July 2024, from the Çanakkale Lapseki coast. The mussels were placed in sterile containers with at least 2 liters of seawater.

Microbiological Analysis

Microbiological analyses were conducted by combining the methods described in Künili et al. (2021) and Tosun et al. (2021). The outer surfaces of the samples were cleaned with a sterile blade, and the cleaning process was completed by brushing with 70% v/v alcohol. Afterward, 50 mussels from each station were carefully opened with sterile blades, and the contents (gill, mantle, and adductor muscle tissues, and intervalvular liquid) were collected in a sterile container to create a composite sample. Two 10 g samples were taken from the composite sample collected in the sterile container for each station. Each sample was plated onto culture media in two parallel rows. Therefore, the count was calculated as four petri dishes.

Then, 10 g of mussel samples was added to 90 mL of a 1% w/v bacterial peptone (Condalab, Spain) solution containing 2.75% w/v NaCl (Isolab, Germany), then homogenized using a stomacher. The 1% bacterial peptone solution containing 2.75% NaCl was used for serial dilutions. The cultures were then carried out on the media provided in Table 2. Seawater samples were filtered using a 100 mL water cellulose acetate filter with a pore diameter of 0.45 µm (Isolab, Germany), and the filter was placed in the media described in Table 2. After incubation, colony-forming units (CFU) were counted.

Bacterial Isolation

Plate Count Agar, MacConkey Agar, Eosin Methylene Blue Agar, and Thiosulphate Citrate Bile Salts Sucrose Agar counting petri dishes were placed side-by-side, and isolates were taken from colonies with visually different colony morphologies. The isolates were purified by inoculating them into Brain Heart Infusion (BHI) Agar (Himedia, India) and then transferred to Brain Heart Infusion Broth. Afterward, they were stored at +4°C. 96 bacteria were isolated.

Evaluation of Biofilm Formation Capacity of Bacterial Isolates with Congo Red Agar

The Congo red agar (CRA) method, a qualitative screening technique for biofilm production, was employed to select which bacterial isolates to identify. To determine the biofilm formation capacity of 96 bacterial isolates, they were first incubated overnight on Brain Heart Infusion (BHI) Agar (Biolife, Spain) at 37°C. Then, they were plated on Congo Red Agar (CRA). Then, the culture was plated on CRA medium (Brain Heart Infusion Broth 37 g/L (Biolife, Spain), Sucrose 50 g/L (Isolab, Germany), Agar 10 g/L (Liofilchem, Italy), Congo red 0.8 g/L (HiMedia, India)) and incubated at 37°C for 24–48 hours. After incubation, black or purple colonies with a dry, crystal-like consistency were interpreted as positive for biofilm-producing strains, while red colonies were interpreted as negative for biofilm production (Freeman et al., 1989; Hassan et al., 2011). This analysis was performed in triplicate.

Identification of Bacterial Isolates That Show a Positive Reaction on Congo Red Agar using MALDI-TOF MS

Forty isolates that were positive on Congo Red agar were first revived on BHI agar medium and identified using a MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry) Microorganism Identification device (Bruker Daltonics GmbH, Bremen, Germany) according to the protocol described in Uysal et al. (2019). Scores and identification values obtained in the MALDI-TOF MS study are expressed as follows: 2.300 – 3.000 highly probable species identification (+++) (green); 2.000 – 2.299 reliable genus identification, probable species identification (++) (green); 1.700 – 1.999 probable genus identification (+) (yellow); 0.000 – 1.699 unreliable identification (-) (red).

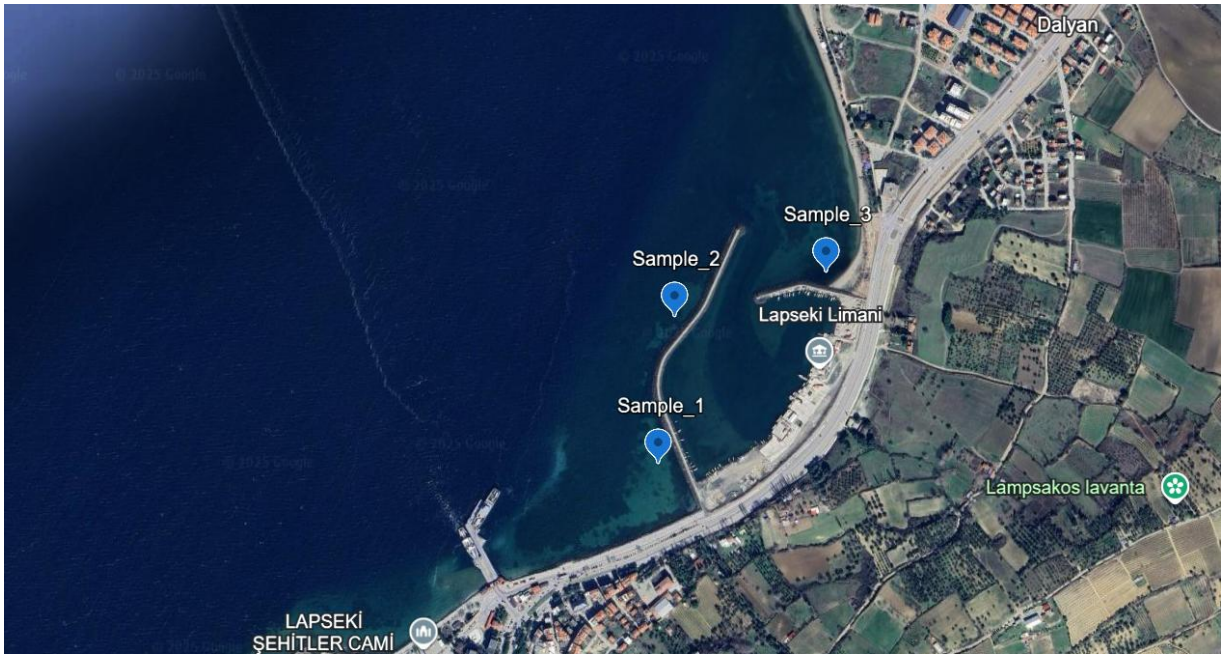


Figure 1. Sampling points (Google Earth, Access date: 20/06/2025).

Table 1. Sampling area and seawater temperature

Sampling Points	Coordinate	Sampling Date	Seawater Temperature
Sample_1	40°21`2" N 26°41`18" E	11 July 2024 15 July 2024	27.5°C 25.9°C
Smaple_2	40°21`8" N 26°41`18"E	11 July 2024 15 July 2024	27.5°C 25.9°C
Sample_3	40°21`13" N 26°41`32" E	11 July 2024 15 July 2024	27.5°C 25.9°C

Antibiotic Susceptibility Test

Antibiotic susceptibility of *Enterobacter* spp. isolates were evaluated using the Kirby-Bauer disk diffusion method. Stock cultures were revived in Tryptic Soy Agar (Biolife, Spain) at 37°C and then adjusted to 0.5 McFarland using physiological serum (PS, 0.85% w/v NaCl) and a McFarland densitometer (Biosan DEN-1, Latvia). The adjusted cell suspension was evenly spread on the Mueller-Hinton Agar surface with swabs within 15 minutes. Then, the antibiotic disks were placed within 15 minutes of the time of inoculation. In the study, amikacin (AK, 30 mcg), gentamicin (CN, 10 mcg), ciprofloxacin (CIP, 5 mcg), cefotaxime (CTX, 30 mcg), doripenem (DOR, 10 mcg), meropenem (MEM, 10 mcg), trimethoprim/sulfamethoxazole (SXT, 25 mcg), piperacillin/tazobactam (TPZ, 30/6 mcg), aztreonam (ATM, 30 mcg), cefixime (CFM, 5 mcg), ceftazidime (CAZ, 10 mcg), imipenem (IPM, 10 mcg), ceftriaxone (CRO, 30 mcg) (Bioanalyse, Türkiye) antibiotic disks were used. Antibiotic susceptibility assessment was performed following the EUCAST (The European Committee on Antimicrobial Susceptibility Testing) guideline (Version 15.0) recommendations (EUCAST, 2025). In addition, because EUCAST 15.0 lacks a breakpoint for the cefotaxime (CTX, 30 mcg) antibiotic disc against *Enterobacter* species, CLSI 2024 criteria were used to assess this value.

Determination of Biofilm Formation Capacity of Enterobacter sp. Isolates by the Crystal Violet Method

Biofilm formation capacities of *Enterobacter spp*. isolates were evaluated using the crystal violet method reported in Bakht et al. (2022). In the analysis, 96-well flat-bottomed microplates and Luria Bertani (LB) Broth (Himedia, India) were used. *Pseudomonas aeruginosa* ATCC 27853 and 10145 were used as a positive control, and LB Broth medium without inoculation was used as a negative control. After incubation, the isolates revived in LB medium were seeded onto fresh LB medium, and the cell density was adjusted to 0.5 McFarland using a McFarland densitometer. Afterwards, 200 µL of the prepared suspensions were added to each well and incubated at 35-37°C for 48 hours. After incubation, the wells were washed 3 times with sterile PS. Afterward, 200 µL of 1% crystal violet dye (w/v) was added to each well and incubated at 35-37˚C for 20 minutes. Then, the wells were washed three more times with sterile PS. Finally, 200 µL of 95% ethanol was added to each well. The optical absorbance was measured at 620 nm using a microplate reader (MultiSkan FC, Thermo Fisher Scientific K.K.) (Ramos-Vivas et al., 2019). The entire study was performed in triplicate. Biofilm formation was calculated with the following formulas (Bakht et al., 2022):

If optical density (OD) < control group optical density (ODc), biofilm was not formed (negative),

If ODc < OD < 2xODc, the biofilm is weak.

If 2xODc < OD < 4xODc, the biofilm is moderate.

If 4xODc < OD, the biofilm is strong

Statistical Analysis

Data analysis was performed using IBM SPSS Statistics software (version 23.0), and results are presented as mean±standard deviation. The effect of sampling date and region on microbial load was evaluated using two-way ANOVA at a significance level of 0.05. When the homogeneity of variances was evaluated using the Levene test, it was determined that the loads of TAMB (F=2.148, P=0.087) and mold (F=0.404, P=0.842) were homogeneously distributed, whereas the loads in MacConkey agar (F=7.676, P=0.000) and EMB (F=2.843, P=0.032) media were not homogeneously distributed. ANOVA is robust to violations of assumptions such as the absence of outliers, large and uniform sample sizes, relatively equal sample variances within levels (or combinations of levels), and testing a two-way hypothesis (Tabachnick & Fidell, 2007). Therefore, we used two-way ANOVA to assess variance.

Results

Mussel samples collected on two different dates were investigated for microbial loads in various media for different microorganism groups. It was determined that the total aerobic mesophilic bacteria (TAMB) load was 2.41±0.22 and 2.99±0.18 log CFU/g in PCA medium, the probable Enterobacteriaceae load was between 3.17±0.14 and 3.48±0.21 log CFU/g in EMB medium, and

Table 2. Media, inoculation method, incubation temperature, and time used to determine microbial contamination

Microorganism	Media	Inoculation Method	Incubation Temperature and Time
Total Aerobic Mesophilic Bacteria (TAMB)	Plate Count Agar (PCA) (Neogen, UK)	Pour Plate Method	37°C 24 hours
Lactose-Fermenting-Gram-Negatives *(Salmonella, Shigella* and coliform bacteria)	MacConkey Agar (Neogen, UK)	Spreading Plate Method	37°C 24-48 hours
Enterobacteriaceae	Eosin Methylene Blue Agar (EMB) (Neogen, UK)	Spreading Plate Method	37°C 24-48 hours
Vibrio spp.	Thiosulphate Citrate Bile Salts Sucrose (TCBS) Agar (Neogen, UK)	Spreading Plate Method	25°C 72 hours
Yeast-Mold	Dichloran Rose Bengal Chloramphenicol DRBC) Agar (Biolife, Spain)	Spreading Plate Method	25°C 3-7 days

the yeast load was >6.00±0.01 log CFU/g in DRBC medium. Mold load was found to vary between 0.50±0.55 and 1.07±0.58 log CFU/g in DRBC medium. It was determined that it was 3.24±0.05 and 3.94±0.05 log CFU/g in MacConkey Agar and >6.00±0.01 log CFU/g in TCBS medium in all samples. However, a microbial load was detected in MacConkey Agar and TCBS medium used for the isolation of *Salmonella* spp. and *Vibrio* spp., and specific colony morphology could not be detected. Data on the detected loads are given in Table 3.

Statistical evaluation of the effect of sampling date and sampling area on the microbial load of mussels showed that sampling date and sampling area statistically significantly affected TAMB (F=7.974, P=0.002) and MacConkey (F=4.635, P=0.018) loads, explaining 34.7% and 23.6% of the partial eta-squared values, respectively. Sampling time, on the other hand, statistically significantly affected TAMB (F=5.298, P=0.028) and MacConkey (F=51.346, P=0.000) loads, explaining 15% and 63.1% of the partial eta-squared values, respectively. The sampling area showed statistically significant differences in the TAMB (F=16.962, P=0.000), MacConkey (F=10.119, P=0.000), and EMB (F=9.930, P=0.000) loadings, and partial eta-squared explained 53.1%, 40.3%, and 39.8% of this variance, respectively.

Regarding seawater samples, it was determined that microbial loads were greater than 6.00±0.01 log CFU/g at all sampling points and on two different dates, excluding the mold load. In contrast, mold load was 1.00±0.0 log CFU/g in DRBC medium at all sampling points and on two different dates.

Bacterial counting petri dishes were also brought together, and 96 isolates were taken from bacterial colonies of different colony morphologies. It was determined that 40 of the isolates gave positive reactions on CRA medium and were probable biofilm producers. These 40 isolates were then identified using MALDI-TOF MS. The identification information is presented in Table 4. Only one of the isolates, *Micrococcus luteus* (isolate no. 36), was obtained with a score of 2.339, and a "high species-based identification" was received. At the same time, 20 of them were identified as having a score for genus identification and probable species identification. 17 of the isolates were included in "secure genus identification and probable species identification" and were *Enterobacter* species. Seven isolates were included in "probable genus identification" and were identified as *Staphylococcus* species.

The *Enterobacter* spp. isolates, 82.25% were *Enterobacter hormaechei* and 17.65% were *Enterobacter kobei*. The susceptibility to key antibiotics, such as aminoglycosides (amikacin, gentamicin), fluoroquinolones (ciprofloxacin), carbapenems, and trimethoprim/sulfamethoxazole, is listed in Table 5. Sixteen *Enterobacter* isolates tested showed susceptibility to aminoglycosides, carbapenems (imipenem, doripenem, meropenem), penicillin inhibitors (piperacillin/tazobactam), and trimethoprim/sulfamethoxazole. Four isolates (15, 19, 24, and 30) were resistant to aztreonam (monobactam), with a resistance rate of 25%. Additionally, three isolates (15, 19, and 30) were resistant to cefotaxime (a 3rd generation cephalosporin), resulting in an 18.75% resistance rate. Among the 3rd generation cephalosporins, three isolates (15, 19, and 24) were resistant to cefixime, and four isolates (15, 19, 24, and 29) showed resistance to ceftriaxone. *Enterobacter hormaechei* isolates 15 and 19 are resistant to multiple beta-lactams, including aztreonam and cephalosporins (CTX, CFM, CRO). The observed resistance, especially to beta-lactam antibiotics, suggests that these strains may have the potential to produce extended-spectrum beta-lactamase (ESBL).

As a result of the evaluation performed using the crystal violet method, only isolate 30 was determined to have a moderate biofilm-forming capacity among the *Enterobacter* isolates included in the study. All other isolates were evaluated as negative for biofilm production.

Discussion

This study reveals that mussels from the Lapseki coast of the Çanakkale Strait are heavily contaminated with microorganisms, posing significant risks to both food safety and the environment. The microbial contamination we found aligns with regional studies, such as those by Tosun et al. (2021) and Şener et al.

Table 3. Microbial load in mussel samples in terms of log CFU/g

Sample No	S1		S2		S3	
Sampling Date	11.07.2024	15.07.024	11.07.2024	15.07.2024	11.07.2024	15.07.2024
TAMB	2.59±0.08^{aA}	2.99±0.18^{bA}	2.46±0.10^{aB}	2.41±0.22^{bB}	2.58±0.15^{aB}	2.59±0.11^{bB}
MacConkey Agar	3.24±0.05^{aA}	3.82±0.07^{bA}	3.66±0.19^{aB}	3.94±0.05^{bB}	3.60±0.29^{aB}	3.83±0.07^{bB}
EMB Agar	3.33±0.05^{aA}	3.43±0.14^{aA}	3.30±0.11^{aB}	3.17±0.14^{aB}	3.48±0.21^{aA}	3.47±0.09^{aA}
TCBS Agar	>6.00±0.01*^{aA}	>6.00±0.01^{aA}	>6.00±0.01^{aA}	>6.00±0.01^{aA}	>6.00±0.01^{aA}	>6.00±0.01^{aA}
Yeast	>6.00±0.01^{aA}	>6.00±0.01^{aA}	>6.00±0.01^{aA}	>6.00±0.01^{aA}	>6.00±0.01^{aA}	>6.00±0.01^{aA}
Mold	0.50±0.55^{aA}	0.50±0.55^{aA}	1.03±0.59^{aA}	1.07±0.58^{aA}	0.82±0.67^{aA}	0.83±0.68^{aA}

*Because more than 300 colonies were found in Petri dishes even at the -5-dilution used in the microbiological counting method, results >6.00 log were reported for dishes where the microbial load could not be counted.

Lowercase superscripts indicate statistically significant differences in sampling time (Tukey HSD, P<0.05). Uppercase superscripts indicate a statistically significant difference in sampling area (Tukey HSD, P<0.05).

(2013). Additionally, the high levels of Enterobacteriaceae and TAMB indicate that fecal contaminants are impacting this aquatic area.

Antibiotic-resistant Enterobacter species found in mussels are a noteworthy finding from a public health perspective, not only due to foodborne infections but also because they spread antibiotic resistance genes into the environment. Isolates with an ESBL-like phenotype and the ability to form biofilms can survive for long periods in aquatic environments and spread resistance genes. This highlights the importance of "monitoring environmentally induced antibiotic resistance," as stressed by the European Food Safety Authority (EFSA, 2023) and the World Health

Table 4. Identification data of 40 bacterial isolates

Sample	Isolate No	Best match organisms	Score values	Second best match organisms	Score values
Mussel	1	*Staphylococcus pasteuri*	1.811	*Staphylococcus pasteuri* 110706_L1 LUMC	1.613
Mussel	2	*Staphylococcus warneri*	1.792	*Staphylococcus warneri*	1.743
Mussel	3	*Enterobacter hormaechei*	2.087	*Enterobacter hormaechei*	2.085
Mussel	4	*Sphingobacterium thalpophilum* DSM 11723T HAM	1.144	*Legionella maceachernii* 28 RLT	1.126
Mussel	5	*Staphylococcus pasteuri*	1.881	*Staphylococcus pasteuri*	1.738
Mussel	6	*Staphylococcus warneri* Mb18796_1 CHB	1.641	*Limosilactobacillus reuteri* DSM 20053 DSM	1.501
Mussel	7	*Staphylococcus pasteuri* DSM 10657 DSM	1.406	*Staphylococcus aureus* spp. *aureus* DSM 3463 DSM	1.367
Mussel	8	*Aromatoleum buckelii* U120 MPB	1.388	*Limosilactobacillus gastricus* DSM 16045T DSM_2	1.380
Mussel	9	*Pseudomonas veronii* B560 UFL	1.392	*Limosilactobacillus fermentum* DSM 20391 DSM	1.355
Mussel	10	*Staphylococcus pasteuri*	1.927	*Staphylococcus pasteuri*	1.774
Mussel	11	*Enterobacter hormaechei*	2.286	*Enterobacter hormaechei*	2.264
Mussel	12	*Enterobacter hormaechei*	2.117	*Enterobacter hormaechei*	2.110
Mussel	13	*Clostridioides difficile* MB_1562_05 THL	1.426	*Stenotrophomonas maltophilia* (*Pseudomonas beteli*) LMG 978T HAM	1.377
Mussel	14	*Campylobacter jejuni* MB_7240_05 THL	1.459	*Escherichia coli* ATCC 25922 CHB	1.397
Mussel	15	*Enterobacter hormaechei*	2.204	*Enterobacter hormaechei*	2.190
Mussel	16	*Staphylococcus warneri*	1.858	*Staphylococcus warneri*	1.836
Mussel	17	*Enterobacter kobei*	2.188	*Enterobacter hormaechei*	2.100
Mussel	18	*Agrobacterium radiobacter* B170 UFL	1.326	*Cutaneotrichosporon mucoides* group ATCC 204094 THL	1.254
Mussel	19	*Enterobacter hormaechei*	2.166	*Enterobacter hormaechei*	2.148
Mussel	20	*Enterobacter hormaechei*	2.140	*Enterobacter hormaechei*	2.109
Mussel	21	*Enterobacter hormaechei*	2.058	*Enterobacter hormaechei*	2.042
Mussel	22	*Enterobacter hormaechei*	2.242	*Enterobacter hormaechei*	2.230
Mussel	23	*Enterobacter hormaechei*	2.059	*Enterobacter hormaechei*	2.052
Mussel	24	*Enterobacter hormaechei*	2.169	*Enterobacter hormaechei*	2.167
Mussel	25	*Enterobacter hormaechei*	2.255	*Enterobacter hormaechei*	2.245
Mussel	26	*Enterobacter hormaechei*	2.200	*Enterobacter hormaechei*	2.186
Mussel	27	*Enterobacter kobei*	2.018	*Enterobacter hormaechei*	2.002
Mussel	28	*Leclercia adecarboxylata*	2.124	*Leclercia adecarboxylata*	2.124
Mussel	29	*Enterobacter kobei*	2.050	*Enterobacter asburiae*	2.014
Mussel	30	*Enterobacter hormaechei*	2.280	*Enterobacter hormaechei*	2.267
Mussel	31	*Enterobacter hormaechei*	2.325	*Enterobacter hormaechei*	2.299
Mussel	32	*Pantoea agglomerans*	2.293	*Pantoea agglomerans*	2.254
Mussel	33	*Pantoea agglomerans*	1.916	*Pantoea agglomerans*	1.819
Mussel	34	*Staphylococcus warneri*	1.931	*Staphylococcus warneri*	1.765
Mussel	35	*Staphylococcus epidermidis*	1.880	*Staphylococcus epidermidis*	1.820
Mussel	36	*Micrococcus luteus*	2.339	*Micrococcus luteus*	2.278
Seawater	37	*Pantoea agglomerans*	2.010	*Pantoea agglomerans*	1.930
Seawater	38	*Aromatoleum anaerobicus* LuFRes1 MPB	1.417	*Shewanella algae* DSM 9167T HAM	1.390
Seawater	39	*Ligilactobacillus saerimneri* DSM 16049T DSM	1.390	*Liquorilactobacillus satsumensis* DSM 16230T DSM	1.378
Seawater	40	*Mycobacterium bovis* BCG 1878 PGM	1.353	*Lactobacillus plantarum* DSM 20205 DSM	1.334

Table 5. Inhibition zone diameters (mean±standard deviation in mm) and antibiotic susceptibilities of Enterobacter isolates against antibiotic discs

Isolates No	Aminoglycosides		Fluoroquinolones	Monobactams	Cephalosporins				Carbapenems			Penicillin inhibitors	Other
	AK30	CN10	CIP5	ATM30	CTX30	CFM5	CAZ10	CRO30	IPM10	DOR10	MEM10	TPZ36	SXT25
3	32.35±1.23	21.90±0.31	36.38±0.67	32.77±1.18	23.09±0.48	24.52±0.42	27.95±0.85	21.89±0.74	23.01±1.31	31.97±0.78	24.00±2.50	21.81±0.51	28.94±0.76
11	25.34±0.40	25.29±0.30	30.90±0.73	32.82±1.58	21.40±0.89	17.45±0.37	24.15±1.48	11.81±0.53	31.16±0.58	36.19±0.85	34.95±2.32	20.33±0.37	32.06±0.80
12	25.95±1.52	23.51±0.48	32.05±1.30	28.74±1.01	21.10±1.08	18.96±0.77	26.08±1.36	13.40±0.62	32.88±0.21	31.85±1.67	37.93±1.61	16.71±1.48	30.96±1.60
15	31.29±0.98	28.21±1.96	33.11±1.21	6.00±0,01	15.51±1.81	10.27±1.67	14.76±0.51	6.00±0.01	32.36±5.38	30.96±0.91	29.67±1.43	20.63±2.75	25.90±0.39
17	25.19±0.88	21.79±0.84	35.51±0.81	31.14±1.60	25.61±0.31	26.40 ±0.53	29.94±1.86	28.53±2.41	29.43±0.51	34.49±1.66	34.32±1.34	22.71±0.65	32.34±0.29
19	25.36±1.38	29.53±0.36	30.51±1.19	6.00±0,01	19.78±0.55	15.63±1.48	26.59±1.58	21.49±0.79	49.52±3.44	47.42±3.40	45.17±2.71	18.54±1.37	29.14±1.16
20	23.08±1.20	21.08±0.87	9.19±0.52	34.46±1.76	20.33±1.02	25.30±0.79	28.55±0.92	21.46±1.25	29.13±1.27	31.70 ±1.06	31.47±0.49	20.42±1.32	30.11±1.03
21	23.78±1.18	22.42±0.69	36.78±1.29	31.25±0.85	25.95±1.43	24.17±0.49	28.58±1.41	26.14±1.25	31.62±0.65	29.29±1.02	31.35±1.22	22.96±1.28	31.86±0.67
22	23.35±0.79	24.95±0.23	35.40±1.33	32.00±0.49	27.84±0.92	24.40±0.41	29.62±1.69	17.60±0.86	27.89±0.49	22.89±1.20	33.00±1.00	22.78±0.48	31.24±0.33
23	23.82±0.28	21.87±0.37	34.46±0.70	31.26±1.32	23.70±0.61	24.06±1.06	26.44±1.87	20.08±0.69	28.52±0.98	33.18 ±1.35	32.44±1.46	21.51±0.57	27.90±1.63
24	26.73±0.76	30.17±1.64	33.43±1.11	6.00±0.01	22.37±0.43	17.68±0.57	26.23±1.40	15.08±0.67	53.48±1.22	46.47±0.88	44.74±0.70	39.50±0.80	35.14±3.91
26	23.09±0.95	22.40±0.96	36.71±1.65	31.61±1.93	25.93±0.45	25.65±1.33	27.13±1.72	11.83±0.60	29.76±1.54	34.12±2.65	33.82±1.24	22.94±0.57	33.23±1.61
27	23.65±0.48	23.15±0.51	35.33±1.36	33.13±1.19	25.45±0.41	24.97±0.47	27.84±0.84	18.56±0.33	28.67±0.87	31.00±0.75	35.90±3.81	21.62±0.44	30.19±0.69
29	22.27±0.45	21.83±0.79	35.18±0.89	32.47±1.83	24.93±1.06	25.71±0.22	29.68±1.10	12.73±1.70	7.73±0.80	30.62 ±0.53	31.04±1.18	22.07±0.56	30.44±0.40
30	24.94±0.60	24.37±1.22	37.21±1.63	6.00±0.01	13.42±1.21	26.35±0.59	29.27±1.06	25.38±0.43	33.23±0.88	32.09±1.02	33.63±0.78	25.09±0.99	32.76±0.54
31	25.00 ±0.39	23.47±0.45	6.00±0,01	34.27±0.81	19.89±0.63	24.09±0.36	29.00±1.31	19.89±0.68	31.88±1.00	34.30±3.43	36.26±0.73	21.89±0.49	35.57±2.38
EUCAST	S≥18 R<18	S≥17 R<17	S≥25 R<22	S≥26 R<21	S≥26 R<21** (CLSI, 2024)	S≥17 R<17	S≥22 R<19	S≥27 R<24	S≥22 R<19	S≥24 R<21	S≥22 R<16	S≥20 R<20	S≥14 R<11

Organization (WHO, 2015). The resistance findings are especially significant, with resistance observed against aztreonam and third-generation cephalosporins (cefotaxime, ceftriaxone, cefixime). This resistance pattern is consistent with typical phenotypes seen in ESBL-producing Enterobacteriaceae strains (Davin-Regli et al., 2019; Denissen et al., 2022). Specifically, isolates 15 and 19 were resistant to aztreonam and several beta-lactam antibiotics but remained susceptible to carbapenems — a condition that indicates a classic ESBL phenotype (WHO, 2021). However, since phenotypic confirmatory tests (such as DDST or combination antibiotic disc) were not performed in this study, this result is considered only a potential ESBL suspect. Furthermore, in areas where natural mussel populations are regularly consumed, the presence of such strains raises the risk of contamination from eating uncooked or undercooked seafood. Studies in the literature have also found ESBL-producing Enterobacteriaceae in seafood (Sanjit Singh et al., 2017; Freire et al., 2023; Suleymanoglu et al., 2024). This regional study supports the need to monitor antibiotic resistance characteristics in mussels.

When assessing biofilm formation, it is noteworthy that only *Enterobacter* isolate 30 exhibited a moderate capacity for biofilm production. The literature reports that antibiotic-resistant bacteria also tend to have increased biofilm production abilities (Bakht et al., 2022; Oyenuga et al., 2024). Biofilms are structures that microorganisms produce to survive harsh external conditions and antimicrobial agents (Rather et al., 2021). Conversely, antibiotic resistance is primarily driven by genetic mechanisms, including resistance genes and efflux systems. Therefore, a strain that produces a strong biofilm is not necessarily resistant to antimicrobial agents, and a strain resistant to antimicrobials is not necessarily a biofilm producer (Madduri et al., 2025). However, this study found that, except for one isolate, the other antibiotic-resistant isolates did not form biofilms. These findings support the idea that biofilm formation and antibiotic resistance are independent traits, and biofilm capacity alone should not be used as an indicator of antibiotic resistance.

The presence of *Enterobacter* spp. in mussels has been reported only sparingly in the literature. In this regard, this study makes a significant contribution by providing, a detailed account of the presence of *Enterobacter hormaechei* and *Enterobacter kobei* in natural mussel populations along the coasts of Türkiye, along with their resistance profiles. It also highlights the potential public health risks that seafood can pose and the need to monitor the spread of antibiotic-resistant strains from the environment. However, the biggest limitation of this study is that the generalizability of the findings from the short sampling period is restricted due to the two samples being taken 4 days apart. Therefore, it is recommended that future sampling studies be conducted over a longer period, that the analysis of genetic factors playing a role in antibiotic resistance be included, that the quantitative assessment of biofilm formation be evaluated, and that the role of biofilm formation in antibiotic resistance of isolated samples be assessed. Moreover, the phenotypic resistance profiles of Enterobacter isolates were determined in this study, and both antibiotic synergy tests and molecular mechanisms should be evaluated in future studies.

Ethical Statement

Ethics approval and consent to participate not applicable for this study.

Funding Information

This study, except for the section on determining antibiotic resistance and biofilm formation capacities, was supported by the TÜBİTAK 2209-A University Student Research Projects Support Program under project number: 1919B012337688.

Author Contribution

GÖA; Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Visualization Supervision, Resources, Writing -original draft, and Writing -review and editing

MHS; Methodology, Project Administration

THA; Methodology.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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