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Antibiotic resistant profile and some virulence factors of Çanakkale Coastline bacteria

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Abstract

Antibiotic resistance poses a significant public health concern, especially considering that microorganisms in marine environments serve as potential sources for the dissemination of resistance genes. In this study, antibiotic resistance profiles of 50 bacterial isolates obtained from coastal seawater in Çanakkale were investigated, along with the presence of tetM and vancomycin resistance genes, and various virulence factors (biofilm formation, siderophore production, enzymatic activity). Molecular identification was conducted for isolates with detected resistance genes. According to the antibiotic susceptibility testing, 90% of isolates exhibited resistance to penicillin, 76% to vancomycin, 68% to tetracycline, 54% to ampicillin, and 8% to gentamicin. Furthermore, 88% of isolates were found to have a Multidrug Resistance (MDR) index above the critical threshold; tetM was present in 30%, vanA in 10%, and both tetM and vanA in 2% of isolates. Additionally, all isolates showed high potential in terms of enzyme production, biofilm formation, and siderophore production as virulence factors. The high levels of antibiotic resistance and presence of resistance genes in these bacteria indicate uncontrolled antibiotic usage in the region, posing potential risks to public health.

Keywords: Seawater; Bacteria; Antibiotic Resistance Genes; Biofilm; Virulence

1. Introduction

The marine ecosystem, encompassing a vast diversity from the largest to microscopic organisms, plays a crucial role in global biodiversity. When assessing biodiversity in seas and oceans, microorganisms are typically classified based on their nutritional needs and transport capabilities. While deep oceans exhibit substantial biodiversity, coastal areas near shorelines generally host higher bacterial diversity, particularly enteric bacteria, which are significant components of coastal ecosystems. In areas impacted by intense human activities, where organic materials are discharged into the water, these bacteria thrive, aided by their ability to easily break down organic matter through lipolytic and proteolytic enzymes [1].

Especially gram-negative bacteria are known for their high pathogenicity due to virulence factors such as antibiotic resistance genes, enzymes, biofilm formation, and siderophores [2]. They pose significant public health concerns as agents of numerous bacterial diseases [3]. Therefore, evaluating the presence and virulence of Gram-negative bacteria along coastal areas is crucial. Antibiotics are not only used worldwide to treat infectious diseases but also as prophylactic agents to prevent infections, promote growth in agriculture and aquaculture, and preserve food. However, their intensive and uncontrolled use contributes to antibiotic resistance, one of the greatest public health challenges globally [4]. Coastal waters are considered reservoirs and pathways for the spread of antibiotic resistance [5]. Given the vastness of the marine ecosystem worldwide, studying bacterial populations, particularly along human-impacted coastal shorelines, and identifying antibiotic resistance and virulence factors are essential. Research on bacterial populations along the Turkish coastline is limited concerning the effects of antibiotics and the presence of antibiotic resistance genes.

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The focus of this study is to characterize phenotypic and genotypic antibiotic resistance profiles, enzymatic activities, biofilm formation, siderophore production, and other virulence traits of bacteria isolated from the coastal shores of Çanakkale province. The aim is to assess the bacterial pathogens' risks for public health along the Çanakkale coastline (Türkiye).

2. Materials and methods

2.1. Identification of isolates

A total of 50 bacterial isolates were obtained through isolation efforts conducted by the project team along the Çanakkale coastline (Figure 1) between 2018 and 2020. Gram staining, as well as oxidase and catalase tests, was conducted on isolates [6].

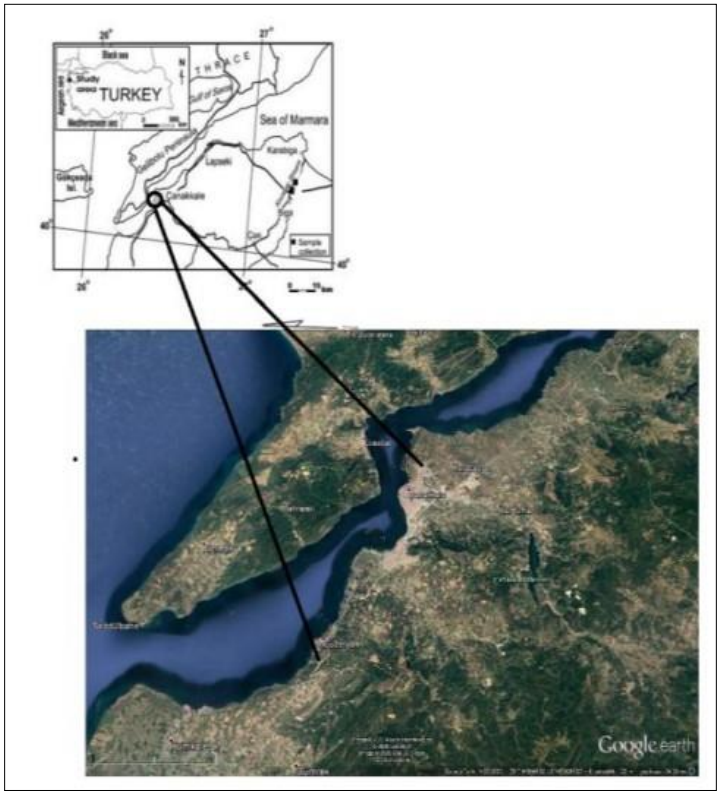

Figure 1 Çanakkale coastline (Türkiye)

2.2. Molecular identifications of isolates

Isolates identified to carry one or both antibiotic resistance genes underwent molecular identification. Species-level identification was performed through phylogenetic analysis using 16S rDNA gene sequence analyses (Table 1) as described by Yanez et al. [7]. Tools such as BLAST, GenBank, and PubMed available on NCBI were employed for this purpose.

Table 1 16S rDNA gene used in phylogenetic analysis [8].

Gene	Primers	Sequences
16S rDNA	0341f	CCTACGGGGGCGCAG
	0785r	GACTACGGGTATCTAATCC

The PCR products of these amplified genes were subjected to sequencing through service provider (MEDSANTEK) and bacterial identifications were performed. The sequence analysis results were evaluated using bioinformatics tools as mentioned in the species-level identification section.

2.3. Determining antibiotic resistance profiles

Antibiotic resistance profiles were determined using Kirby-Bauer disc diffusion method [9]. Antibiotic discs [(Ampicillin (AM10), Gentamicin (G120), Penicillin (P10), Tetracycline (TE30), Vancomycin (VA30)] were placed on Mueller Hinton Agar (MHA) plates and incubated at 37 °C for 2 days. The Multiple Antibiotic Resistance (MAR) index of the isolates was calculated according to Krumperman [10].

2.4. Potential antibiotic resistance genes

The presence of antibiotic resistance genes, [Vancomycin (VanA) and Tetracycline (TetM)], was investigated using PCR (Polymerase Chain Reaction) method (Table 2). Modifications to PCR components were made based on literature information [11] to ensure accurate detection of these genes.

Table 2 Primer sequences for antibiotic resistance genes

Antibiotic Resistant Gene	Primer sequences	Base Pair
vanA	F-5' GTA CAA TGC GGC CGT TA R-5' GGG ACA GTT ACA ATT GC	732 bp
tetM	F-5' GTT AAA TAG TGT TCT TGG AG R-5' CTA AGA TAT GGC TCT AAC AA	657 bp

2.5. Determination of enzymatic activities of isolates

DNase, hemolysis, protease, lecithinase, gelatinase, and amino acid decarboxylase [6], lipase, and amylase [12] were assessed according to standard methods reported in the literature. Siderophore production capacities of isolates involved point inoculation on Chrome Azurol S agar (MCASA). After incubated at 37 °C for 2 days, color change in the medium (formation of yellow-orange color) was recorded as a positive result [13]. Slime formation in bacteria was assessed using two different methods: Congo Red Agar (CRA): Bacteria were inoculated onto the agar medium and incubated, with results interpreted based on color changes. Standard Tube Method: Bacteria in Nutrient Broth (NB) medium (0.5 McFarland density) were incubated at 37 °C for 2 days. The liquid portion was decanted, and tubes were treated with methylene blue to assess for the presence of stained film or ring formation. The microplate method was employed to detection of biofilm formation in isolates, and results were evaluated as positive (+) or negative (-) based on spectrophotometric readings [14].

3. Results and discussions

3.1. Identification results of isolates

A total of 50 isolates, 44 were identified as Gram-negative, and 6 were Gram-positive. Among these, 5 isolates were oxidase-positive (+), and 40 isolates were catalase-positive (+) (Table 3).

Table 3 Biochemical characterization results of isolates

Isolate Number	Gram	Oxidase	Catalase	Isolate Number	Gram	Oxidase	Catalase
No1	-	-	+	No26	-	-	-
No2	-	-	+	No27	-	-	+
No3	-	-	+	No28	-	-	+
No4	-	-	+	No29	+	-	+
No5	-	-	+	No30	+	-	-
No6	-	+	+	No31	-	-	+
No7	-	-	+	No32	+	-	+
No8	-	-	+	No33	-	-	-

No9	-	-	+	No34	-	-	-
No10	+	-	-	No35	-	-	-
No11	-	+	+	No36	-	-	+
No12	-	-	+	No37	-	-	+
No13	-	-	+	No38	-	-	+
No14	-	-	+	No39	-	-	-
No15	+	-	-	No40	-	-	-
No16	-	-	+	No41	-	-	+
No17	-	-	+	No42	-	-	+
No18	-	-	+	No43	-	+	+
No19	-	-	+	No44	-	+	+
No20	+	-	-	No45	-	-	+
No21	-	-	+	No46	-	-	+
No22	-	-	+	No47	-	+	+
No23	-	-	+	No48	-	-	+
No24	-	-	+	No49	-	-	+
No25	-	-	+	No50	-	-	-

A total of 19 isolates carrying the vanA gene (n=3), TetM gene (n=15), and both vanA and TetM genes (n=1) were subjected to phylogenetic identification using 16S rDNA gene sequence analysis (Table 4). These resistant gene-carrying isolates belonged to various species including *Escherichia coli* (n=5), *Enterobacter cloacae* (n=1), *Enterococcus faecalis* (n=2), *Enterobacter ludwigii* (n=1), *Enterococcus durans* (n=1), *Enterobacter hormaechei* (n=1), *Enterococcus casseliflavus* (n=2), *Enterococcus gallinarum* (n=1), *Shigella flexneri* (n=1), *Aeromonas hydrophila* (n=2), and *Citrobacter freundii* (n=2).

Table 4 Molecular İdentification results of Isolates (n=19)

Isolates no	Similarity species	Similarity Percentage	Access code
No1	*C. freundii*	82.67	gi|2485082252|CP099084.1
No3	*E. coli*	85.47	gi|1851748052|CP054363.1
No7	*C. freundii*	87.01	gi|1879651301|CP056232.1
No9	*E. coli*	90.06	gi|1829536586|CP050865.1
No10	*Enterococcus casseliflavus*	91.61	gi|2430361866|CP116026.1
No13	*Aeromonas hydrophila*	100	gi|2265458965|LR963141.1
No15	*Enterococcus gallinarum*	93.33	gi|1681051749|MN055932.1
No16	*Shigella flexneri*	89.75	gi|1860443381|CP055124.1
No17	*A. hydrophila*	100	gi|1112971476|CP018201.1
No20	*E. casseliflavus*	92.40	gi|1708149352|MN213350.1
No24	*E. coli*	91.03	gi|1829536586|CP050865.1
No27	*E. coli*	92.52	gi|2177886535|CP061264.1
No28	*Enterobacter hormaechei*	100	gi|1798302894|CP047570.1
No29	*Enterococcus durans*	96.18	gi|222142462|FJ607264.1

No30	*Enterococcus faecalis*	97.73	gi|1434113258|MG694608.1
No31	*Enterobacter ludwigii*	100	gi|1730123908|MN371803.1
No32	*E. faecalis*	89.93	gi|701636012|KM257650.1
No39	*Enterobacter cloacae*	94.66	gi|1226861130|CP017475.1
No43	*E. coli*	100	gi|2637717002|CP140519.1

Based on the analysis, the predominance of enteric bacteria among the bacterial species obtained suggests that the pollution along the coast of Çanakkale may be of fecal origin. Within the microbial diversity of the sea, Gram-negative bacteria are of significant importance due to their adaptation mechanisms and biochemical properties. Gram-negative bacteria inhabit various habitats such as deep sea, sea surface, and coastal areas [15]. Based on this information, our study focused specifically on isolating Gram-negative bacteria from the bacterial population along the coastal shoreline. Following our analyses, we specifically focused on isolating Gram-negative bacteria, which exhibit considerable biodiversity. Particularly, species belonging to the Enterobacteriaceae and Aeromonadaceae families were identified. Among these, *E. coli* and *C. freundii* were isolated in higher numbers compared to other species.

Numerous studies worldwide have investigated bacterial communities along different coastal regions. These studies have predominantly identified marine-origin and Gram-negative bacterial species. Bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella* spp., and others are frequently reported in these studies [16, 17].

In the marine ecosystem, various environmental factors can influence microbial activities, abundance, and enzymatic mechanisms [18]. Both biotic and abiotic factors in the oceans are known to influence the bacterial composition and abundance in the environment [19]. Our findings are consistent with the literature from around the world and Türkiye, predominantly identifying Gram-negative bacteria [17, 20, 21].

3.2. Results of antibiotic susceptibilities

It was determined that 90% were resistant to P10, 76% to VA30, 68% to TE30, 54% to AM10, and 8% to G120 antibiotics, respectively. Additionally, 78% were found to be susceptible to G120, 10% to AM10, 10% to VA30, and 6% to TE30 antibiotics. None of the isolates showed susceptibility to P10. Moreover, 34% of the isolates were classified as intermediately susceptible to AM10, 26% to TE30, 17% to VA30, 14% to G120, and 10% to P10 (Table 5).

Table 5 Antibiotic resistant profile of isolates

Isolate no	**Antibiotic**					**MAR index**	**Isolate no**	**Antibiotic**					**MAR index**
	AM10	**TE30**	**P10**	**G120**	**VA30**			**AM10**	**TE30**	**P10**	**G120**	**VA30**	
No1	R	R	R	S	S	0.6	No26	I	I	R	S	R	0.4
No2	I	R	R	S	R	0.6	No27	I	R	R	I	R	0.6
No3	I	R	R	S	I	0.4	No28	R	R	R	R	R	1
No4	S	I	R	S	I	0.2	No29	R	R	R	S	R	0.8
No5	I	R	I	S	I	0.2	No30	R	R	R	S	R	0.8
No6	I	I	R	S	R	0.4	No31	R	R	R	S	R	0.8
No7	I	I	R	S	R	0.4	No32	R	I	R	R	R-	0.8
No8	I	I	R	I	I	0.2	No33	R	R	R	S	R	0.8
No9	R	R	R	S	S	0.6	No34	I	R	R	S	R	0.6
No10	S	I	R	S	R	0.4	No35	R	R	R	S	R	0.8
No11	R	R	I	S	I	0.4	No36	R	S	R	S	R	0.6
No12	R	R	R	S	R	0.8	No37	I	I	I	S	R	0.2
No13	R	R	R	S	R	0.8	No38	R	I	R	S	R	0.6

No14	I	R	R	S	S	0.4	No39	I	R	R	S	S	0.4
No15	S	R	R	S	R	0.6	No40	R	R	R	S	R	0.8
No16	R	R	R	S	R	0.8	No41	R	I	R	S	R	0.6
No17	R	I	R	S	R	0.6	No42	R	R	R	S	R	0.8
No18	S	S	R	I	R	0.4	No43	R	R	R	S	R	0.8
No19	R	S	R	R	R	0.8	No44	I	R	I	S	R	0.4
No20	R	R	I	S	R	0.6	No45	I	R	R	R	R	0.8
No21	I	R	R	I	R	0.6	No46	S	R	R	I	R	0.6
No22	R	R	R	I	R	0.8	No47	S	R	R	S	R	0.6
No23	I	I	R	S	I	0.2	No48	R	R	R	S	R	0.8
No24	R	R	R	S	S	0.6	No49	R	R	R	S	R	0.8
No25	I	I	R	S	I	0.2	No50	R	R	R	I	R	0.8

R: Resistant; I: Intermediate; S: Sensitive

50 bacterial isolates exhibit high resistance to four clinically used antibiotics (AM10, P10, TE30, and VA30) and low resistance to the antibiotic G120. Antibiotic resistance data from marine water studies along various coasts of Turkey have been documented. High levels of resistance, exceeding 50%, to clinically used antibiotics like AM10, TE30, and VA30 among Gram-negative pathogens have been consistently reported across multiple studies [22-24]. In contrast to our findings, resistance levels to TE30 antibiotics among *Aeromonas* spp. isolated from the southern coast of Turkey were found to be low in the same study. Similarly, as in our study, the resistance rate to G120 was also found to be low [25]. Resistance against P10 has been found to be remarkably high (90%), consistent with findings from several studies [26-27]. These results support the natural resistance of Gram-negative bacteria to P10. This suggests that bacterial antibiotic resistance in coastal marine waters is likely influenced by human activities.

The high resistance observed in bacteria isolated from the sea suggests the presence of significant antibiotic carriers entering the sea through various pathways. The fact that resistance is so high in coastal waters implies a likely scenario where antibiotics accumulate in the water from various sources without sufficient degradation. In literature about antibiotic resistance genes in bacterial isolates, high resistance has generally been observed against antibiotic groups such as P10, AM10, S10, VA30, and kanamycin. Consistent with these findings, our study also found high resistance profiles against VA30, TE30, P10, and AM10. These findings provide insights into the intensity of antibiotic use in the region and the antibiotic groups frequently used in treatment processes.

Upon examining the MIC indices of the isolates, it was determined that, except for 6 isolates, the MIC index of 44 isolates was greater than 0.2. This index is commonly used to assess the level of bacterial resistance in a bacterial population exposed to multiple antimicrobial agents. The presence of isolates with high MIC indices poses a risk because infections caused by such isolates have a higher mortality rate compared to infections caused by susceptible isolates [28].

Specific PCR analyses using primers targeting the tetM gene responsible for TE30 resistance and the vanA gene responsible for VA30 resistance were performed. The tetM, vanA and both the tetM and vanA resistance genes were found in 16 isolates (No. 7, 9, 10, 13, 15, 16, 17, 20, 24, 27, 28, 29, 30, 31, 32, and 43) (Figure 2), 4 isolates (No. 1, 3, 10, and 39) (Figure 3) and 1 isolate, (No. 10), respectively. The presence of these resistance genes in the bacteria may be attributed to intense and uncontrolled antibiotic usage in the region.

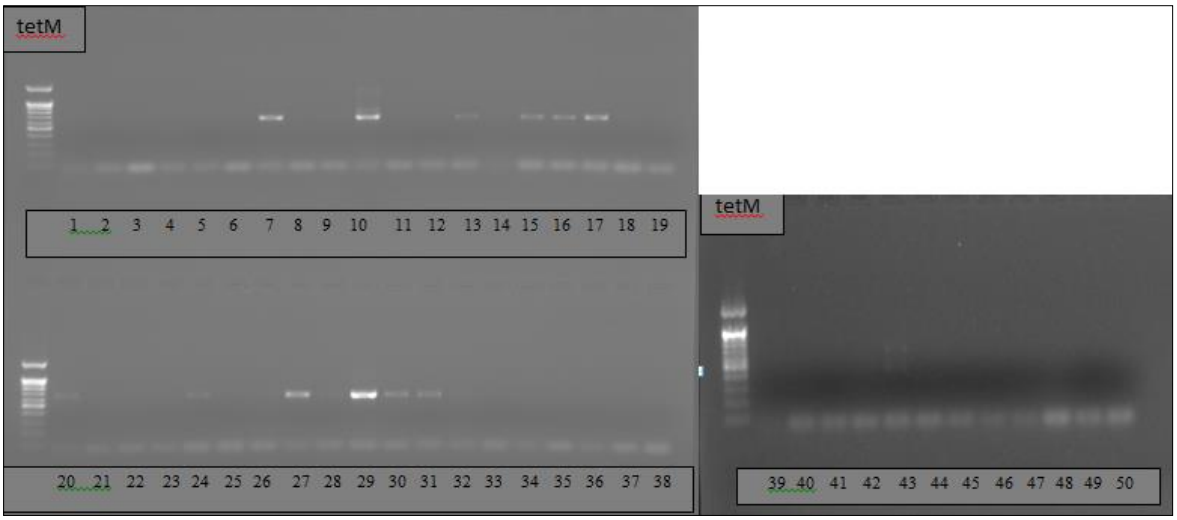

Figure 2 tetM resistance gene sequencing results of isolates

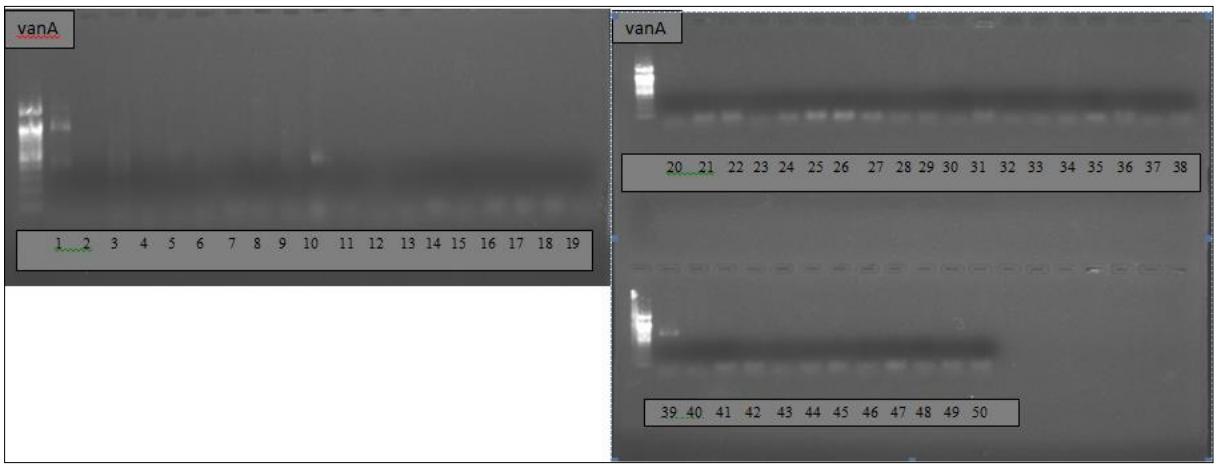

Figure 3 vanA resistance gene sequencing results of isolates

A study on antimicrobial resistance genes in Gram-negative bacteria isolated from a marine fish farm in Egypt revealed that 26.3% of the tested isolates carried at least one tet resistance determinant, with tetA being the most frequently detected gene [29]. Studies conducted on Gram-negative bacteria in fish farms have identified the presence of tet resistance genes [30 - 32]. The finding of these genes in clinical isolates from humans at nearly the same rates is of significant clinical importance. This suggests that these antimicrobial resistance genes could potentially serve as a source for their spread to humans [33]. The detection of tet resistance presence is an indicator of how widely a broad-spectrum antibiotic, commonly used in clinics and on animals, is employed.

The VanA resistance gene in bacteria can either be chromosomally encoded or acquired later. VA is an antibiotic used in the treatment of Gram-positive bacterial infections. Bacteria can transfer the VA resistance gene to different species and genera through mechanisms such as plasmids and transposons. This ability for horizontal gene transfer contributes to the spread of VA resistance among bacterial populations. Treating diseases caused by bacteria that have acquired resistance to VA poses significant challenges in clinical settings [34].

3.3. Results of enzymatic activities

The enzymatic activities of the isolates were determined as follows: 18% hemolysis, 20% protease, 26% amino acid decarboxylase (AAD), 40% lipase, 70% DNase, 84% lecithinase, 100% amylase, and 100% gelatinase (Figure 4).

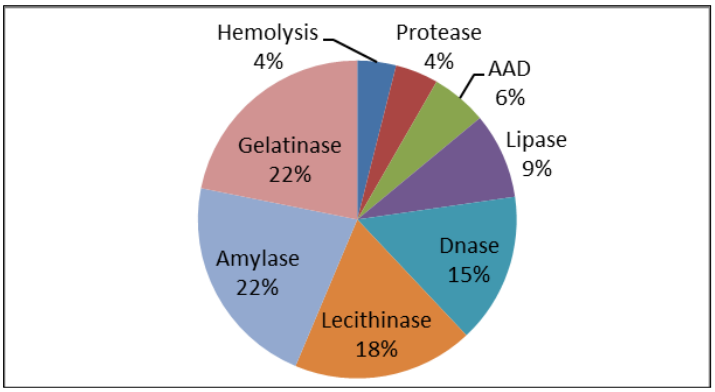

Figure 4 % enzymatic activities of the isolates

Enzyme activity determination was conducted to gain insights into the virulence of the isolates. Particularly noteworthy are the high activities of amylase, gelatinase, lecithinase, and DNase, which indicate the degree of pathogenicity. Additionally, the presence and relatively high levels of these enzymes in metabolic activities are significant. These enzymes also enhance virulence by increasing tissue invasiveness. Many pathogenic microorganisms produce toxins or hemolysins that destroy host cells and enhance their virulence. Hemolysins secreted by Gram-negative bacteria like Salmonella prevent phagocytosis, thereby increasing their virulence. Bacterial cells cause systemic infections by invading and colonizing tissues and cells both intracellular and extracellular, using their enzymatic arsenal [35]. The values obtained in our study suggest that the isolates exhibit high virulence potential.

In our study, it was concluded that 49 out of 50 isolates have the ability to form slime (Table 6). Slime formation enhances bacterial virulence by protecting them from host defenses such as phagocytosis and toxins. It is known to create a barrier that reduces antibiotic diffusion, thereby decreasing antibiotic susceptibility and complicating treatment.

Table 6 Siderophore, slime and biofilm formation result of isolates

Isolate No	Siderophore	Slime		Biofilm	Isolate No	Siderophore	Slime		Biofilm
		CRA	Test tube				CRA	Test tube	
No1	+	+	+	+	No26	+	+	+	++
No2	+	+	+	++	No27	+	+	+	+++
No3	+	+	+	++	No28	+	+	+	+
No4	+	+	+	+++	No29	+	+	+	+++
No5	+	+	+	+++	No30	+	+	+	+
No6	+	+	+	+++	No31	+	+	+	+++
No7	+	+	+	+	No32	+	+	+	+++
No8	+	+	+	+++	No33	+	+	+	+++
No9	+	+	+	++	No34	+	+	+	+++
No10	+	+	-	-	No35	+	+	+	+
No11	+	+	+	+++	No36	+	+	+	++
No12	+	+	+	++	No37	+	+	+	++
No13	+	+	+	+	No38	+	+	+	++
No14	+	+	+	+++	No39	+	+	+	+
No15	+	+	+	+++	No40	+	+	+	++
No16	+	+	+	++	No41	+	+	+	+
No17	+	+	+	+++	No42	+	+	+	+

No18	+	+	+	+++	No43	+	+	+	+
No19	+	+	+	++	No44	+	+	+	++
No20	+	+	+	+	No45	+	+	+	++
No21	+	+	+	++	No46	+	+	+	+
No22	+	+	+	++	No47	+	+	+	++
No23	+	+	+	++	No48	+	+	+	+
No24	+	+	+	++	No49	+	+	+	+
No25	+	+	+	+++	No50	+	+	+	++

Congo Red Agar: CRA

It was determined that all isolates have the capacity to produce siderophores (Table 6). It has been observed that all isolates in our study have the capacity to produce siderophores. Siderophores are utilized by bacterial cells for electron transport, oxygen transport, and enzyme functions. Some bacteria use siderophores to enhance their pathogenicity, thereby considering siderophores as virulence factors. The high siderophore production potential of the isolates used in our study could suggest an increase in their virulence. Many bacteria belonging to the Enterobacteriaceae family possess siderophores specific to species such as enterobactin and aerobactin, known to enhance virulence. In Gram-negative bacteria isolated from the sea, genes responsible for siderophore function have been identified in 50% of the isolates [36]. Kokosharov and Phetisova [37] found high siderophore production in *Salmonella* bacteria. Erdem et al. [38] detected siderophores in all strains of *A. hydrophila* and *Aeromonas caviae* isolated from 120 freshwater fish, while only two strains of *Aeromonas veronii* bv. *sobria* were positive for siderophores. The ability of all isolates in our study to produce siderophores provides important insights into their pathogenicity.

In the isolates, 32% exhibited excellent, 38% very good, and 28% good capacity for biofilm formation, while biofilm formation was not detected in 2% of the isolates (Table 6). Among the biofilm-forming species, *E. coli*, *C. freundii*, *A. hydrophila*, and *S. Arizonae* were found to possess strong adherent properties, whereas an inactive isolate of *E. coli* did not exhibit biofilm-forming ability. The findings from our study are consistent with studies conducted worldwide, indicating that Gram-negative isolates exhibit a high capacity for biofilm formation [39].

4. Conclusion

Our findings demonstrate that the coastal areas of Çanakkale province are particularly vulnerable to anthropogenic pollution, especially in terms of enteric bacteria contamination. This diversity is accompanied by significantly high levels of antibiotic resistance, biofilm and siderophore formation, and enzyme activities. The high microbial pollution and pathogenicity indicate a significant threat to public health, particularly for communities using these coastal habitats. The primary reasons behind this situation are the mixing of inadequately treated wastewater and anthropogenic waste, along with antibiotic residues, in the coastal areas of Çanakkale. This has led to the presence of bacterial species with high pathogenicity and resistance profiles in the environment. Additionally, the presence and detection of resistance genes and high virulence factors among bacterial populations are important indicators of the spread of resistance genes. Therefore, the Çanakkale coastal area requires continuous monitoring for public health, ecological balance, and the sustainable use of ecosystem resources. Measures should be implemented to reduce harmful agents and ensure the health of ecosystems, especially through stricter wastewater treatment to prevent antibiotic residues from entering marine waters. Regulations should also be enforced to restrict the use of antibiotics in clinics, agriculture, aquaculture, and other activities. Further studies are needed to monitor the presence and spread of resistance genes in bacterial populations over the long term. Monitoring and controlling pathogenic bacteria in marine environments used for recreation and aquaculture are crucial for ecosystem, human, and animal welfare, as well as biodiversity conservation.

Compliance with ethical standards

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Disclosure of conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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