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Prevalence and Antimicrobial Resistance of Bacterial Foodborne Pathogens Isolated from Oysters and Mussels Collected from The Lagoon of Bizerte, Tunisia

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Abstract

Mediterranean lagoons, like the Bizerte Lagoon in northern Tunisia, are ecosystems highly susceptible to eutrophication and pollution. The hydrobiological conditions of these lagoons are directly affected by the volume and quality of water that flows into them. Organisms residing in this environment, such as bivalves that accumulate various toxic compounds, are constantly subjected to changes and disturbances, often leading to resistance and development of adaptive behaviors. Simultaneously, the increasing global demand for bivalve mollusks due to their health benefits has raised concerns about potential contamination by bacterial pathogens. This study aims to detect pathogenic bacteria in raw oysters and mussels from the Bizerte lagoon in Tunisia, as well as evaluate their resistance to antibiotics. A total of 79 isolates were collected, with 57 originating from 37 oyster samples and 23 from 12 mussel samples. The most common bacterial genera identified were Psychrobacter (32.8%), Vibrio (20.9%), Pseudoalteromonas (14.9%), and Acinetobacter (10.4%) among Gram-negative bacteria, and Staphylococcus (44.4%), Bacillus (33.3%), and Cytobacillus (22.2%) among Gram-positive bacteria. Observations revealed high levels of resistance to cephalosporins, penicillin, and carbapenems, while chloramphenicol and trimethoprim-sulfamethoxazole showed common susceptibility. It is worth noting that 86% of isolates were derived from oysters, and 87% from mussels exhibited patterns of resistance to multiple antibiotics. Hemolysis assays demonstrated alpha-hemolytic activity in 30 isolates. Oyster samples isolates displayed hydrolytic enzyme activity, including amylase (49.1%), DNase (47.4%), lecithinase (43.9%), cellulase (36.8%), lipase (35.1%), gelatinase (26.3%), and chitinase (19.3%). On the other hand, mussel samples isolates showed production of lipase (60.9%), gelatinase (52.2%), lecithinase (43.5%), amylase (39.1%), DNase (26.1%), and cellulase (17.4%), but no production of chitinase was detected. These findings highlight the risk of contamination and antimicrobial resistance carrying associated with the consumption of raw bivalve mollusks and the potential environmental damage and its transmission to humans via the general food chain.

Keywords: Bacterial Foodborne Pathogens; Antimicrobial resistance; Food safety; Shellfish; Bivalve Mollusks; Oysters; Mussels

Abbreviations: ARB: Antibiotic-Resistant Bacteria; AMR: Antimicrobial Resistance; MGES: Mobile Genomic Elements; ICES: Integrative and Conjugative Elements; MDR: Multi-Drug Resistant; Its: Internal Transcribed Spacer; NRRL; The Northern Regional Research Laboratory; BRD: Brown Ring Disease: EPA: Eicosatetraenoic Acid

Introduction

The Mediterranean lagoons are extremely vulnerable ecosystems to pollution, leading to phenomena such as eutrophication and microbiological contamination. The hydro-biological conditions prevailing in these lagoons are directly influenced by the quantity and quality of discharged effluents, which increasingly and continuously disrupt the ecosystem, the trophic level of the environment, and especially the habitat of living organisms. These bio-physico-chemical disturbances are characterized by recurrent harmful microalgae blooms, often foul-smelling emissions, as well as the development of dissolved and particulate organic-mineral load, resulting in a general degradation of the lagoon ecosystem and the surrounding coastal systems. The Bizerte lagoon, in northern Tunisia, is a hub for

numerous urban, agricultural, industrial, and port activities, which generate multiple contaminations affecting its biodiversity and aquaculture productivity [1].

For this purpose, the discharge of treated or untreated wastewater containing organic micropollutants such as various antimicrobials and pharmaceutical compounds, agricultural fertilizers, biocides, etc. resulting from several wastewater treatment and agricultural activities prompts consideration of the long-term future of these compounds in the lagoon, as the Bizerte lagoon serves as a northern watershed receiving discharges from surrounding cities. Among the compounds that may be discharged into this important lagoon receptor, medicinal and pharmaceutical compounds such as endocrine disruptors, fungicides, antibiotics, and their residues can be particularly mentioned.

These specific residues promote the development of microbial resistance and adaptation to these molecules, often with negative consequences on the overall ecosystem of the lagoon and its microbial inhabitants, algae, and plants. The interest in medicinal residues, especially antibiotics, has drawn the attention of ecologists in recent years. Therefore, the World Health Organization has emphasized that the spread of antibiotic-resistant bacteria (ARBs) in the food chain and the environment has become a serious problem, as antimicrobial resistance (AMR) affects both humans and animals [2]. There is limited data on antibiotic resistance in seafood. However, current AMR surveillance programs and risk assessment studies on food as a potential AMR transmission channel focus on terrestrial food-producing animals. However, terrestrial bacteria, including those that cause AMR, can enter aquatic ecosystems via sewage systems, land runoff, and the feces of wildlife and birds [2,3].

The use of antimicrobials in aquaculture can also directly lead to the spread of AMR, but antibiotic residues in rivers can promote the persistence of ARB [4]. It is known that the use of other chemicals such as biocides, which are often used in seafood production, lead to antibiotic resistance [5-7]. As a result, natural ecosystems serve as a possible source of ARGs, but can also spread horizontally via mobile genomic elements (MGEs) in addition to vertical transmission via insertion sequences, transposons, integrons, plasmids, and integrative and conjugative elements (ICEs) that enable the transmission of ARGs to others bacterial cells and promote the spread of antibiotic resistance among bacteria that coexist in the same ecological niche [8-10].

The presence of ARBs in seafood threatens human health and facilitates the transmission of resistance traits to other medically significant microorganisms. Besides Vibrio bacteria, which are frequently found in the marine environment, seafood is generally safe from causing infections in humans. However, there is a possibility of human enteric infections arising from seafood contaminated by water sources during shellfish harvesting or through contamination during post-harvest handling processes [11]. The dangerously high antibiotic resistance patterns of clinical isolates of enteric bacteria have led to increasing worry about their rapid spread through the food chain and water. This concern is further complicated by multi-drug resistant (MDR) bacteria among enteric pathogens in seafood. Several studies conducted recently have highlighted MDR bacteria in seafood [12,13].

The model organism chosen is the mussel, which acts as a filterer and concentrator of various pollutant compounds. Our research focused on the presence of antibiotic resistance and residues in the tissue of mussels isolated from the Bizerte Lagoon, since the mussels typically hosted a high microbial load in their tissues, making them a risk category for consumers. Also, they can serve as excellent indicators of fecal contamination, reflecting the bacterial load present at a particular location in the water column [14]. Consequently, mussels can be valuable objects for the study of bacteria carrying some antibiotic resistance factors, and could be a good tool for transmitting these potential toxics and deleterious residual antibiotic compounds to human consumers.

The primary objective of this research was to ascertain the range of antimicrobial resistance and examine the occurrence of antibiotic-resistant bacteria in recently harvested shellfish from the Bizerte lagoon. The survey also aimed to assess the potential risk of these mollusks serving as vectors for antibiotic resistance factors in human organisms.

Materials and Methods

Sample Collection, Bacterial Isolation, Characterization and Identification

Fresh seafood was gathered from the lagoon of Bizerte in Tunisia from March 2021 to March 2022 (Figure 1). A total of forty-nine samples were collected during this period, with thirty-seven being oysters and twelve mussels. The samples were placed in sterile collection bags with ice, immediately transported to the laboratory, and processed for bacterial isolation through selective enrichment and plating. Upon removal of shell debris and algae, the bivalves were dried, disinfected with 70% ethanol, opened using a sterilized scalpel, and then incubated in a sterile bag with peptone salt broth for 24 hours at 24 °C.

A milliliter of the enrichment broth was streaked on Zobell mannitol agar medium and incubated for 24-48 hours at 24 °C. Following incubation, the colonies were purified on Tryptone Soy Agar (TSA) medium. The isolates were characterized using the oxidase test, catalase test, and Gram staining. The 16S-23S rRNA internal transcribed spacer (ITS) regions were amplified with primers ITS-F (5′-GTCGTAACAAGGTAGCCGTA-3′) and ITS-R (5′-CAAGGCATCCACCGT-3′) to screen bacterial phylotype diversity. Identification of different isolates was carried out by amplifying and sequencing the 16S rRNA genes with primers F27 (5′-AGAGTTTGATCCTGGCTGGCTCAG-3′) and R1492 (5′-TACGGCTACCTTGTTACGACTT-3′). The sequences of PCR products were analyzed using the BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Hemolysis test and enzyme production tests (DNase test, lecithinase, lipase, amylase, gelatinase, chitinase, and cellulase test) were performed on the isolates [15-17].

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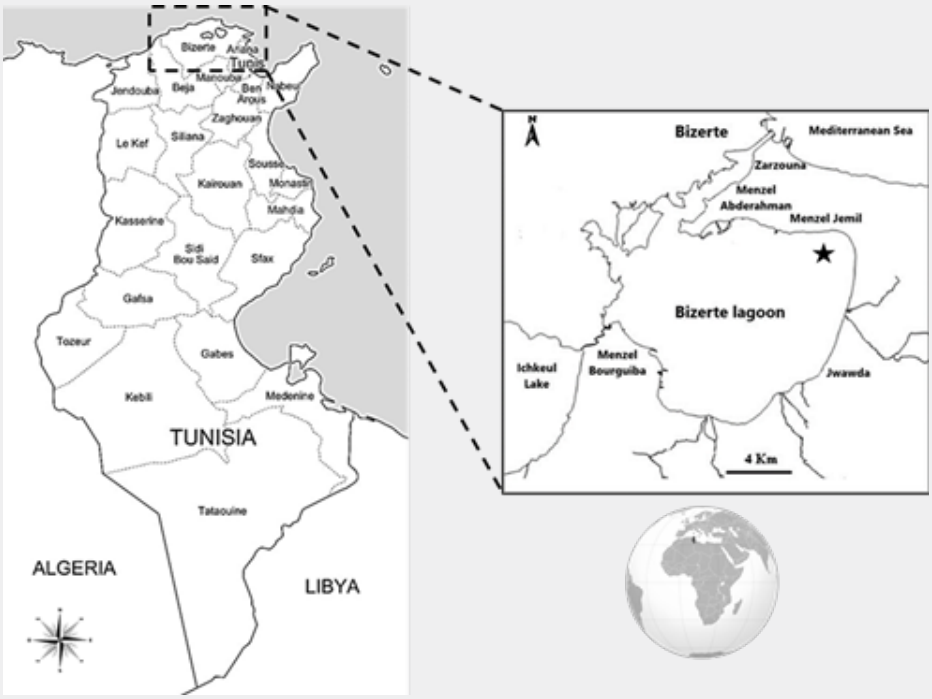

Figure 1: Map of Bizerte lagoon (Northern Tunisia). The full black star in the Northern part of the lagoon shows the sampling site location.

Antibiotic Susceptibility Tests

Antibiotic susceptibility was assessed using the disk diffusion technique on Mueller Hinton agar plates. Sixteen antibiotics were tested, such as amoxicillin-clavulanic Acid (20 + 10 µg), ampicillin (10 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cefoxitin (30 µg), chloramphenicol (30 µg), imipenem (10 µg), nalidixic acid (30 µg), streptomycin (10 µg), tetracycline (30 µg), norfloxacin (5 µg), gentamicin (10 µg), oxacillin (1 µg), tobramycin (10 µg), ciprofloxacin (5 µg), nitrofurantoin (300 µg), and trimethoprim-sulfamethoxazole (1.25 + 23.75 µg). These antibiotics were chosen due to their significance in both human and veterinary medicine. The diameter of the zones of inhibition was measured and classified as either resistant or susceptible under the guidelines provided by the Clinical and Laboratory Standards Institute [18].

Results

Bacterial Isolation, Characterization and Identification

Among the 76 colonies isolated, 22 distinct ITS-PCR profiles were identified (Figure 2). From oyster samples, three Gram-positive species were identified as Staphylococcus warneri (n=4/6; 66.7%), *Bacillus paramycoides* (n=1/6; 16.7%), and *Cytobacillus oceanisediminis* (n=1/6; 16.7%). Additionally, Gram-negative strains were identified, including *Psychrobacter nivimaris* (n=17/48; 35.4%), *Psychrobacter adeliensis* (n=1/48; 2.1%), *Psychrobacter submarinus* (n=1/48; 2.1%), *Acinetobacter schindleri* (n=7/48; 14.6%), *Pseudoalteromonas distincta* (n=5/48; 10.4%), *Pseudoalteromonas spiralis* (n=2/48; 4.2%), *Pseudomonas kilonensis* (n=1/48; 2.1%), Halomonas sulfidaeris Esulfide1 (n=2/48; 4.2%), *Vibrio chagasii* (n=2/48; 4.2%), *Vibrio alginolyticus* (n=3/48; 6.3%), *Vibrio natriegens* (n=1/48; 2.1%), *Cobetia amphilecti* (n=2/48; 4.2%), *Sphingomonas echinoides* (n=2/48; 4.2%), *Stenotrophomonas maltophilia* (n=1/48; 2.1%), and *Shewanella colwelliana* (n=1/48; 2.1%) (Table 1).

Also, we have isolated three Gram-positive bacteria from mussel samples, with two identified as *Bacillus paramycoides* (66.7%) and one as *Cytobacillus oceanisediminis* (33.3%). Out of the 19 Gram-negative isolates, we found *Vibrio alginolyticus* (26.3%), *Vibrio jasicida* (10.5%), *Vibrio natriegens* (5.3%), *Pseudoalteromonas distincta* (15.8%), *Pseudomonas kilonensis* (5.3%), *Psychrobacter nivimaris* (5.3%), *Psychrobacter piscatorii* (5.3%), *Psychrobacter submarinus* (5.3%), *Halomonas sulfidaeris Esulfide1* (5.3%), *Sphingomonas echinoides* (5.3%), *Shewanella marinintestina* (5.3%), and *Photobacterium angustum* (5.3%) (Table 1).

As for the hemolytic activity exhibited by 30 isolates, all displayed alpha hemolysis. Out of the 54 isolates obtained from oyster samples, various hydrolytic enzyme activities were screened. Positive production was observed for amylase (n=28/54; 51.9%), DNase (n=26/54; 48.1%), lecithinase (n=24/54; 44.4%), cellulase (n=21/54; 38.9%), lipase (n=20/54; 37%), gelatinase (n=15/54; 27.8%), and chitinase (n=11/54; 20.4%) (Figure 3). But the 22 isolates originating from mussels showed positive production of lipase (n=14/22; 63.6%), gelatinase (n=12/22; 54.5%), lecithinase (n=10/22; 45.5%), amylase (n=9/22; 40.9%), DNase (n=6/22; 27.3%), and cellulase (n=4/22; 18.2%). However, no production of chitinase (n=0/22; 0%) was detected (Figure 4).

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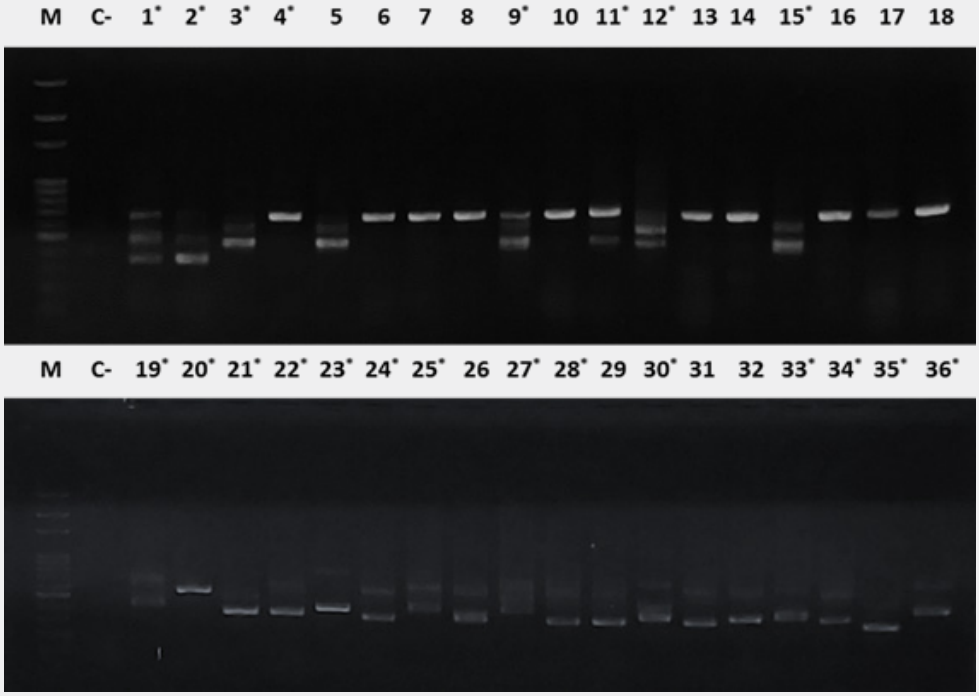

Figure 2: Examples of PCR-ITS analysis performed on 36 out of 76 bacterial colonies isolated from the oyster and the mussel samples. * Indicates example of ITS-PCR profiles whose colonies were further analyzed by 16S rDNA sequencing.

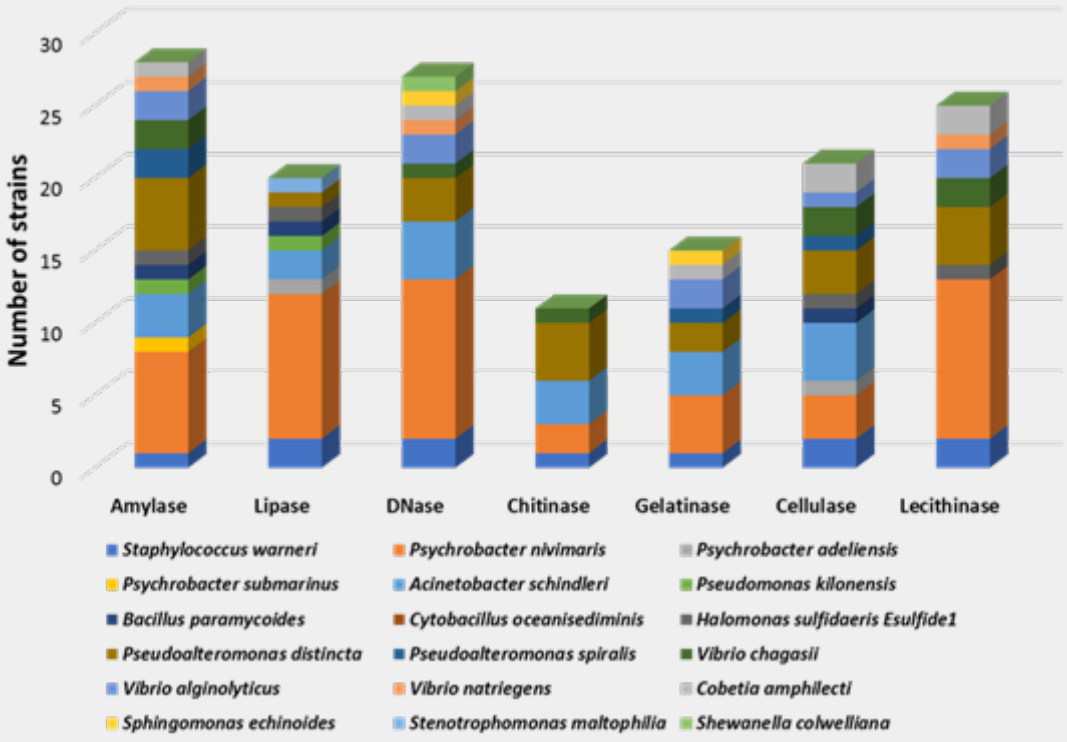

Figure 3: Hydrolytic enzyme activities among all the isolates from the oyster samples.

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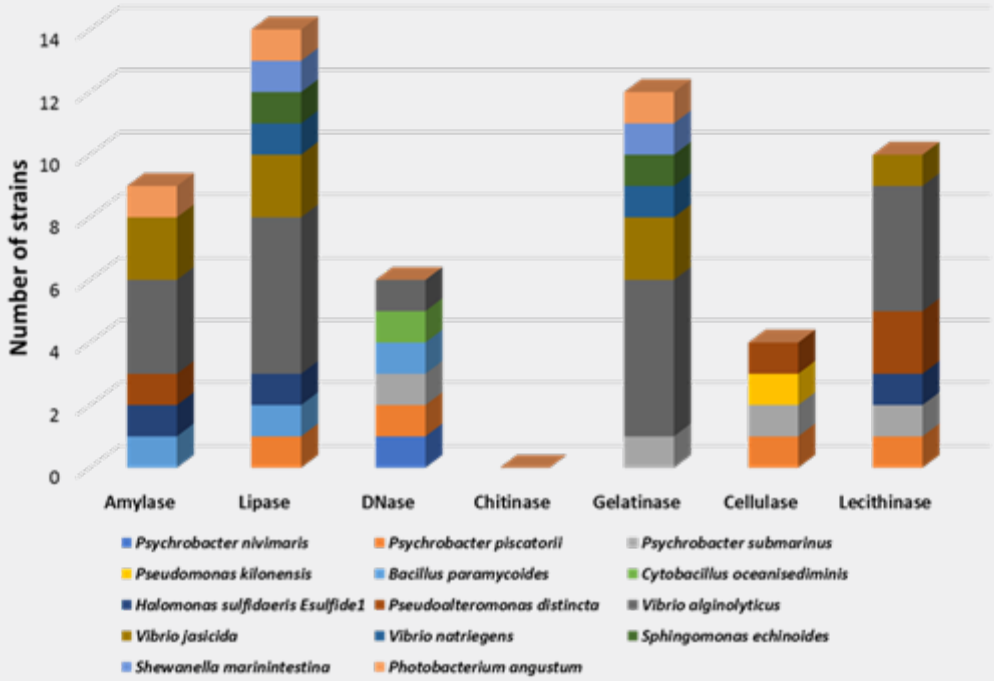

Figure 4: Hydrolytic enzyme activities among all the isolates from the mussel samples.

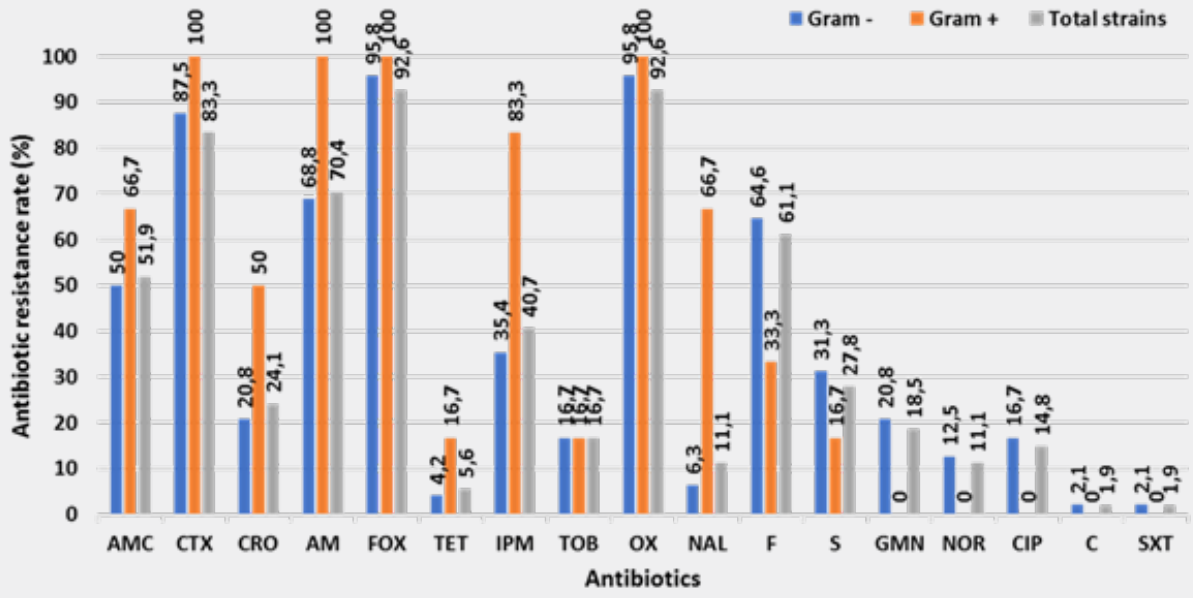

Figure 5: Antibiotic resistance of isolated strains from oyster samples.

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Table 1: Summary of the characteristics of bacterial strains recovered from oyster and mussel samples.

Strains	Data of sampling	Gram	Catalase test	Oxidase test	Enzyme production								Profiles of resistance to antibiotics	ITS haplotypes	Molecular Identification
					Amylase	Lipase	DNase	Chitinase	Gelatinase	Cellulase	Lecithinase	Hemolysis test			
Bacterial strains recovered from oyster samples															
H1	29/03/2021	+	+	-	+	+	+	+	+	+	+	+	CTX; CRO; AM; FOX; IPM; NAL; F; OX	P1	Staphylococcus warneri
H2	29/03/2021	+	+	-	–	–	–	–	–	–	+	–	AMC; CTX; AM; FOX; IPM; NAL; S; OX	P1	Staphylococcus warneri
H3	29/03/2021	+	+	-	–	+	+	–	–	+	–	–	AMC; CTX; AM; FOX; IPM; NAL; OX	P1	Staphylococcus warneri
H4	29/03/2021	+	+	–	–	–	–	–	–	–	–	–	AMC; CTX; AM; FOX; IPM; NAL; OX	P1	Staphylococcus warneri
H5	29/03/2021	-	+	-	–	+	+	–	+	–	–	–	CTX; FOX; TET; IPM; NAL; F; OX	P2	Acinetobacter schindleri
H6	29/03/2021	-	+	+	+	+	+	–	+	–	–	+	CTX; CRO; AM; FOX; F; OX	P3	Psychrobacter nivimaris
H7	29/03/2021	-	+	+	+	–	–	–	–	–	+	–	AMC; CTX; AM; FOX; F; OX	P3	Psychrobacter nivimaris
H8	29/03/2021	-	+	+	–	–	+	–	–	–	+	–	AMC; CTX; AM; FOX; F; OX	P3	Psychrobacter nivimaris

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H9	29/03/2021	-	+	+	–	+	–	–	–	–	+	–	AMC; CTX; AM; FOX; F; OX	P3	Psychro-bacter nivimaris
H10	29/03/2021	-	+	+	+	+	+	–	–	+	+	+	AMC; CTX; AM; FOX; F; OX	P3	Psychro-bacter nivimaris
H11	29/03/2021	-	+	+	–	+	+	–	–	–	+	+	AMC; CTX; AM; FOX; F; OX	P3	Psychro-bacter nivimaris
H12	29/03/2021	-	+	+	+	+	+	–	–	–	+	–	AMC; CTX; AM; FOX; GMN; F	P3	Psychro-bacter nivimaris
H13	29/03/2021	-	+	+	–	–	+	–	–	–	+	–	AMC; CTX; AM; FOX; GMN; F	P3	Psychro-bacter nivimaris
H14	29/03/2021	-	+	+	–	+	+	–	+	–	–	–	CTX; FOX; F; OX	P3	Psychro-bacter nivimaris
H15	29/03/2021	-	+	+	–	+	+	–	–	–	+	+	CTX; AM; FOX; OX	P3	Psychro-bacter nivimaris
H16	29/03/2021	-	+	+	+	+	+	–	–	–	–	–	CTX; AM; FOX; OX	P3	Psychro-bacter nivimaris
H17	29/03/2021	-	+	+	+	+	+	+	–	–	+	–	AM; FOX; OX	P3	Psychro-bacter nivimaris
H18	29/03/2021	-	+	+	–	–	–	–	+	–	–	–	OX	P3	Psychro-bacter nivimaris
H19	13/04/2021	-	+	+	+	+	–	–	–	–	–	–	AMC; CTX; CRO; AM; FOX; TET; IPM; NAL; F; S; C; OX; SXT	P4	Pseudo-monas kilonensis
H20	13/04/2021	+	+	-	+	+	–	–	–	+	–	–	AMC; CTX; CRO; AM; FOX; TET; IPM; TOB; F; OX	P5	Bacillus paramy-coides

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H21	13/04/2021	+	+	+	–	–	–	–	–	–	–	+	CTX; CRO; AM; FOX; OX	P6	Cytobacillus oceanisediminis
H22	13/04/2021	-	+	+	–	+	–	–	–	+	–	–	CTX; AM; FOX; F; OX	P7	Psychrobacter adeliensis
H23	13/04/2021	-	+	+	+	–	–	–	–	–	–	–	CTX; AM; FOX; F; OX	P8	Psychrobacter submarinus
H24	13/04/2021	-	+	+	+	+	–	–	+	–	–	–	CTX; AM; FOX; IPM; OX	P9	Halomonas sulfidaeris Esulfide1
H25	13/04/2021	-	+	+	–	–	–	–	–	–	–	–	CTX; AM; FOX; OX	P9	Halomonas sulfidaeris Esulfide1
H26	13/04/2021	-	+	+	+	–	+	–	–	–	+	–	CTX; AM; FOX; OX	P3	Psychrobacter nivimaris
H27	13/04/2021	-	+	+	–	–	–	–	–	+	–	+	CTX; FOX; F; OX	P3	Psychrobacter nivimaris
H28	16/09/2021	-	+	+	+	+	+	+	+	–	+	+	AMC; CTX; CRO; AM; FOX; IPM; TOB; GMN; NOR; CIP; F; S; OX	P10	Pseudoalteromonas distincta
H29	16/09/2021	-	+	-	–	–	+	–	–	+	+	+	AMC; CTX; CRO; AM; FOX; IPM; TOB; GMN; NOR; CIP; F; S; OX	P2	Acinetobacter schindleri
H30	16/09/2021	-	+	+	+	–	+	–	–	–	+	+	AMC; CTX; CRO; AM; FOX; IPM; TOB; GMN; NOR; CIP; F; S; OX	P10	Pseudoalteromonas distincta

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H31	16/09/2021	-	+	-	+	–	–	+	–	+	–	+	AMC; CTX; CRO; AM; FOX; IPM; TOB; GMN; NOR; CIP; F; S; OX	P2	Acine-tobacter schindleri
H32	16/09/2021	-	+	-	–	–	+	+	+	–	–	+	AMC; CTX; CRO; AM; FOX; IPM; TOB; GMN; CIP; F; S; OX	P2	Acine-tobacter schindleri
H33	27/09/2021	-	+	-	–	–	–	–	–	+	+	+	AMC; CTX; CRO; AM; FOX; IPM; TOB; GMN; NOR; CIP; F; S; OX	P2	Acine-tobacter schindleri
H34	27/09/2021	-	+	+	+	–	+	+	+	+	+	+	AMC; CTX; CRO; AM; FOX; IPM; TOB; GMN; NOR; CIP; F; S; OX	P10	Pseudo-altero-monas distincta
H35	27/09/2021	-	+	-	+	+	+	–	+	–	+	–	AMC; CTX; CRO; AM; FOX; IPM; TOB; GMN; CIP; S; OX	P2	Acine-tobacter schindleri
H36	27/09/2021	-	+	-	+	–	–	+	–	+	+	–	AMC; CTX; AM; FOX; IPM; F; OX	P2	Acine-tobacter schindleri
H37	27/09/2021	-	+	+	–	+	–	–	+	+	–	–	AMC; CTX; AM; FOX; F; OX	P3	Psychro-bacter nivimaris

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H38	27/09/2021	-	+	+	–	–	–	+	–	–	+	–	AMC; CTX; AM; FOX; F; OX	P3	Psychrobacter nivimaris
H39	11/11/2021	-	+	+	+	–	–	+	–	+	+	–	AM; FOX; OX	P10	Pseudoalteromonas distincta
H40	11/11/2021	-	+	+	+	–	+	+	–	+	+	+	AM; FOX; OX	P11	Vibrio chagasii
H41	11/11/2021	-	+	+	+	–	–	+	–	+	–	–	AM; FOX; OX	P10	Pseudoalteromonas distincta
H42	6/12/2021	-	+	+	+	–	–	–	–		+	–	AMC; CTX; AM; FOX; IPM; F; OX	P12	Pseudoalteromonas spiralis
H43	6/12/2021	-	+	+	+	–	–	–	–	+	–	–	CTX; FOX; IPM; F; OX	P12	Pseudoalteromonas spiralis
H44	6/12/2021	-	+	+	+	–	–	–	–	+	+	–	AM; FOX; IPM; S; OX	P11	Vibrio chagasii
H45	6/12/2021	-	+	+	–	–	–	–	–	+	–	–	CTX; FOX; F; OX	P13	Vibrio alginolyticus
H46	6/12/2021	-	+	–	–	–	–	–	–	+	+	–	FOX; IPM; F	P14	Cobetia amphilecti
H47	6/12/2021	-	+	-	+	–	+	–	+	+	+	–	FOX	P14	Cobetia amphilecti
H48	10/3/2022	-	+	-	-	+	-	–	-	-	-	–	AMC; CTX; FOX; S; F; IPM; OX	P15	Stenotrophomonas maltophilia
H49	10/3/2022	-	+	+	+	-	+	–	+	-	+	–	AMC; CTX; FOX; S; F; OX	P13	Vibrio alginolyticus
H50	10/3/2022	-	+	+	+	-	+	–	+	-	+	–	AMC; CTX; FOX; F; OX	P13	Vibrio alginolyticus
H51	10/3/2022	-	+	+	+	-	+	–	-	-	+	–	AMC; CTX; SUL; S; OX	P16	Vibrio natriegens
H52	10/3/2022	-	-	+	-	-	-	–	-	-	-	–	CTX; SUL; FOX; S; OX	P17	Sphingomonas echinoides
H53	10/3/2022	-	-	+	-	-	+	–	+	-	-	–	CTX; SUL; F; OX	P17	Sphingomonas echinoides

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H54	10/3/2022	-	+	+	-	-	+	–	-	-	-	–	OX	P18	Shewanella colwelliana
Bacterial strains recovered from mussel samples															
M1	13/04/2021	+	+	-	+	+	–	–	–	–	–	–	AMC; CTX; CRO; AM; FOX; TET; IPM; NAL; F; OX	P5	Bacillus paramycoides
M2	13/04/2021	+	+	-	–	–	+	–	–	–	–	–	AMC; CTX; CRO; AM; FOX; TET; IPM; F; C; OX	P5	Bacillus paramycoides
M3	13/04/2021	+	+	+	–	–	+	–	–	–	–	–	CTX; CRO; AM; FOX; IPM; OX	P6	Cytobacillus oceanisediminis
M4	13/04/2021	-	+	+	–	–	+	–	–	+	+	–	CTX; CRO; AM; FOX; IPM; OX	P10	Pseudoalteromonas distincta
M5	13/04/2021	-	+	+	+	+	–	–	–	–	+	+	CTX; AM; FOX; IPM; OX	P9	Halomonas sulfidaeris Esulfide1
M6	13/04/2021	-	+	+	–	–	–	–	–	–	–	–	CTX; AM; FOX; IPM; OX	P10	Pseudoalteromonas distincta
M7	13/04/2021	-	+	+	+	–	–	–	–	–	+	–	CTX; AM; FOX; IPM; OX	P10	Pseudoalteromonas distincta
M8	13/04/2021	-	+	+	–	–	+	–	–	–	–	–	AM; FOX; F; OX	P3	Psychrobacter nivimaris
M9	13/04/2021	-	+	+	–	+	+	–	–	+	–	–	CTX; AM; FOX; F	P19	Psychrobacter piscatorii
M10	2/9/2021	-	+	+	–	–	+	–	+	+	+	–	AMC; CTX; CRO; AM; FOX; IPM; TOB; GMN; OX	P8	Psychrobacter submarinus
M11	2/9/2021	-	+	+	–	–	–	–	–	+	–	–	AM; FOX; OX	P4	Pseudomonas kilonensis

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M12	10/3/2022	-	+	+	-	+	-	–	+	-	+	–	AMC; CTX; CIP; SUL; FOX; S; F; OX	P13	Vibrio alginolyticus
M13	10/3/2022	-	+	+	-	+	+	–	+	-	-	–	AMC; CTX; FOX; S; F; IPM; OX	P13	Vibrio alginolyticus
M14	10/3/2022	-	+	+	+	+	-	–	+	-	+	–	AMC; CTX; SUL; FOX; F; OX	P20	Vibrio jasicida
M15	10/3/2022	-	+	+	+	+	-	–	+	-	+	–	AMC; CTX; FOX; S; F; OX	P13	Vibrio alginolyticus
M16	10/3/2022	-	+	+	+	+	-	–	+	-	+	–	AMC; CTX; FOX; F; OX	P13	Vibrio alginolyticus
M17	10/3/2022	-	+	+	+	+	-	–	+	-	+	–	AMC; CTX; FOX; F; OX	P13	Vibrio alginolyticus
M18	10/3/2022	-	+	+	+	+	-	–	+	-	-	–	AMC; CTX; FOX; S; OX	P20	Vibrio jasicida
M19	10/3/2022	-	-	+	-	+	-	–	+	-	+	–	CTX; SUL; FOX; S; OX	P17	Sphingomonas echinoides
M20	10/3/2022	-	+	+	-	+	-	–	+	-	-	–	CTX; SUL; S; OX	P21	Shewanella marinintestina
M21	10/3/2022	-	+	+	-	+	-	–	+	-	-	–	CTX; S; OX	P16	Vibrio natriegens
M22	10/3/2022	-	+	+	+	+	-	–	+	-	-	–	CTX; OX	P22	Photobacterium angustum

(+) positive; (-) negative; P: profile; AMX: amoxicillin-clavulanic Acid; AM: ampicillin; CTX: cefotaxime; CRO: ceftriaxone; FOX: cefoxitin; C: chloramphenicol; IPM: imipenem; NAL: nalidixic acid; S: streptomycin; TET: tetracycline; NOR: norfloxacin; GMN: gentamicin; OX: oxacillin; TOB: tobramycin; CIP: ciprofloxacin; F: nitrofurantoin; SXT: trimethoprim-sulfamethoxazole.

Phenotypic resistance

A high level of resistance was observed in strains of oyster origins against various antibiotics, including cefoxitin (92.6%), oxacillin (92.6%), cefotaxime (83.3%), ampicillin (70.4%), nitrofurantoin (61.1%), amoxicillin-clavulanic acid (51.9%), imipenem (40.7%), streptomycin (27.8%), ceftriaxone (24.1%), gentamicin (18.5%), tobramycin (16.7%), ciprofloxacin (14.8%), nalidixic acid (11.1%), and norfloxacin (11.1%). But a low rate of resistance was observed against tetracycline (5.6%), chloramphenicol (1.9%), and trimethoprim-sulfamethoxazole (1.9%) (Figure 4). The majority (86%) of isolates from oysters were multidrug-resistant (Table 1). Interestingly, Gram-positive isolates from oyster origins showed no phenotypes of resistance to gentamicin, norfloxacin, ciprofloxacin, chloramphenicol, and trimethoprim-sulfamethoxazole (Figure 5).

The 22 bacterial isolates of mussel origins showed varying levels of phenotypic resistance to different antibiotics. The highest frequency of resistance was observed for oxacillin (95.5%),

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followed by cefotaxime (90.9%) and cefoxitin (86.4%). Ampicillin exhibited resistance in 50% of the isolates, while amoxicillin-clavulanic acid showed resistance in 45.5% of the isolates. Other antibiotics such as imipenem (40.9%), nitrofurantoin (40.9%), streptomycin (31.8%), ceftriaxone (22.7%), and tetracycline (9.1%) also displayed varying levels of resistance. On the other hand, a small percentage (4.5%) of isolates showed resistance to tobramycin, gentamicin, ciprofloxacin, nalidixic acid, and chloramphenicol. None of the isolates exhibited resistance to norfloxacin and trimethoprim-sulfamethoxazole. The majority of isolates from mussel origins (87%) were multidrug resistant (Figure 6 and Table 1).

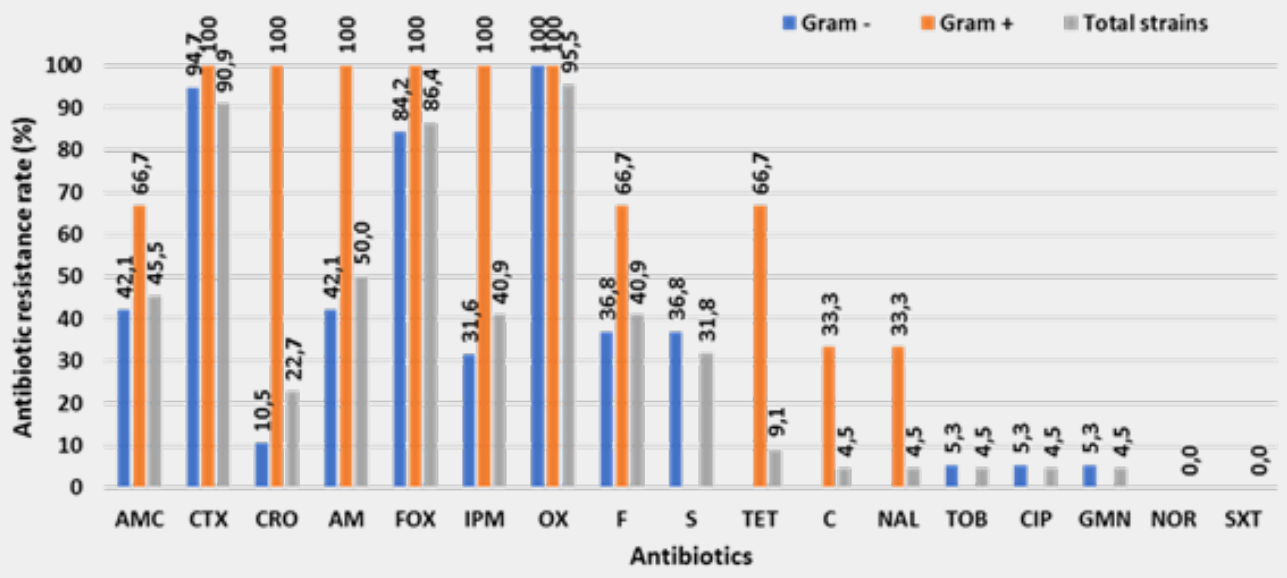

Figure 6: Antibiotic resistance of isolated strains from mussel samples.

Discussion

The release of pharmaceutical residues, especially antibiotics, into different water sources in the natural environment results in a specific microbial flora. Following the development of multidrug resistance, bacteria become the primary carriers of resistance genes and factors in aquatic and lagoon environments. Filter-feeding bivalves like mussels can accumulate a variety of bacterial species, which may pose a contamination risk to humans upon consumption. Consequently, these bivalves act as intermediaries for indirectly transmitting antimicrobial resistance genes and factors to humans. The presence of these resistant bacterial strains can disrupt other ecological niches in the environment, ultimately affecting the ecological balance of the lagoon.

The emergence of antimicrobial resistance through resistant bacteria poses a significant ecological challenge and a crucial public health concern affecting veterinary and human health, as well as the overall environment and food chain. Our research is dedicated to examining the proliferation of antibiotic-resistant bacteria and identifying pathogens in seafood products and the entire food chain of the lagoon ecosystem. A total of 79 isolates were obtained from 49 shellfish samples, with 54 and 22 isolates originating from oyster and mussel samples, respectively. Among the 54 isolates from oyster samples, tests for hydrolytic enzyme activity revealed positive results for amylase (n=28/54; 51.9%), DNase (n=26/54; 48.1%), lecithinase (n=24/54; 44.4%), cellulase (n=21/54; 38.9%), lipase (n=20/54; 37%), gelatinase (n=15/54; 27.8%), and chitinase (n=11/54; 20.4%).

Furthermore, the enzymes cellulase, gelatinase, lecithinase, amylase, and lipase were produced by the 22 isolates obtained from mussel sources. The percentages of isolates that showed positive production of these enzymes were as follows: cellulase (n=4/22; 18.2%), gelatinase (n=12/22; 54.5%), lecithinase (n=10/22; 45.5%), amylase (n=9/22; 40.9%), and lipase (n=14/22; 63.6%). However, no chitinase synthesis was detected among the 23 isolates (n=0/23; 0%).

Among the 54 isolates recovered from oyster samples, six Gram-positive isolates were identified. These isolates were classified as *Staphylococcus warneri* (n=4/6; 66.7%), *Bacillus paramycoides* (n=1/6; 16.7%), and *Cytobacillus oceanisediminis* (n=1/6; 16.7%). In contrast, the 48 Gram-negative isolates obtained in this study were identified as follows: *Psychrobacter nivimaris* (n=17/48; 35.4%), *Psychrobacter adeliensis* (n=1/48; 2.1%), *Psychrobacter submarinus* (n=1/48; 2.1%), *Acinetobacter schindleri* (n=7/48; 14.6%), *Pseudoalteromonas distincta*

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(n=5/48; 10.4%), Pseudoalteromonas spiralis (n=2/48; 4.2%), *Pseudomonas kilonensis* (n=1/48; 2.1%), *Halomonas sulfidaeris Esulfide1* (n=2/48; 4.2%), *Vibrio chagasii* (n=2/48; 4.2%), *Vibrio alginolyticus* (n=3/48; 6.3%), *Vibrio natriegens* (n=1/48; 2.1%), *Cobetia amphilecti* (n=2/48; 4.2%), *Sphingomonas echinoides* (n=2/48; 4.2%), *Stenotrophomonas maltophilia* (n=1/48; 2.1%), and *Shewanella colwelliana* (n=1/48; 2.1%).

We have successfully isolated three Gram-positive isolates from mussel samples. Out of these three isolates, two were identified as *Bacillus paramycoides*, accounting for 66.7% (n=2/3), while one was identified as *Cytobacillus oceanisediminis*, accounting for 33.3% (n=1/3). Additionally, we have obtained 19 Gram-negative isolates, which were further identified as follows: *Vibrio alginolyticus* (n=5/19; 26.3%), Vibrio jasicida (n=2/19; 10.5%), *Vibrio natriegens* (n=1/19; 5.3%), *Pseudoalteromonas distincta* (n=3/19; 15.8%), *Pseudomonas kilonensis* (n=1/19; 5.3%), *Psychrobacter nivimaris* (n=1/19; 5.3%), *Psychrobacter piscatorii* (n=1/19; 5.3%), *Psychrobacter submarinus* (n=1/19; 5.3%), *Halomonas sulfidaeris Esulfide1* (n=1/19; 5.3%), *Sphingomonas echinoides* (n=1/19; 5.3%), *Shewanella marinintestina* (n=1/19; 5.3%), and *Photobacterium angustum* (n=1/19; 5.3%).

Bacillus paramycoides and *Cytobacillus oceanisediminis* have been rarely documented in Tunisia and globally. However, Jebara et al. [19] have reported the isolation of these species from samples of *Posidonia oceanica seagrass* (leaves and epiphytes) collected from the Mahdia Coast in September 2019. *Staphylococcus warneri* has previously been identified in milk from cows with clinical mastitis in Tunisia [20].

The Psychrobacter genus possesses the ability to produce chemicals and enzymes significant in industrial biotechnology, including pigments and proteins involved in heavy metal bioremediation [21]. Furthermore, *Psychrobacter submarinus* was reported by Inagaki et al. [22] as isolated from a subseafloor sediment core obtained from the southwestern part of the Sea of Okhotsk off the Shiretoko Peninsula at the eastern margin of Hokkaido, at a depth of 1,225 m. Additionally, Ettoumi et al. [23] detected *Psychrobacter submarinus* in sediment samples collected in the Tyrrhenian Sea, following a north-west to south-east direction, at depths ranging from 3430 to 3581 m.

Psychrobacter submarinus was initially isolated from sea water at a depth of 300 meters in the Pacific Ocean [24]. Recently, this bacterium has been found in the main cage aquaculture region for large yellow croaker in China, specifically in samples taken from the intestine, feed, and water in San Douai, Ningde City, Fujian Province [25]. But the first case of human infection with *Psychrobacter submarinus* was reported by Musaad et al. [26]. In 2010, *Psychrobacter piscatorii* was identified as a new species, isolated from a fish egg processing plant in Rumoi, Hokkaido [27]. More recently, Zhou et al. [28] revealed *Psychrobacter piscatorii* in a deep-sea hydrothermal vent on the East Pacific Rise.

Psychrobacter nivimaris has been found in seawater samples taken at a depth of 20 m in the southern Atlantic Ocean (Antarctica), where it was attached to organic particles. Additionally, Del Olmo et al. [29] isolated *Psychrobacter nivimaris* from various seaweed species (*Chondrus crispus, Palmaria palmata,* and *Ulva lactuca*) collected in the coastal waters of Coruña Province, North Western Spain. Furthermore, *Psychrobacter nivimaris* was recently discovered in sediments from the Walvis Ridge Ocean crest at a depth of 4,400 m [21].

On the other hand, *Psychrobacter adeliensis* was initially isolated from fast ice in the Geology Archipelago in Adelie Land, Antarctica [30]. Lytou et al. [31] and Picon et al. [32] have also identified *Psychrobacter adeliensis* in Alaria esculenta cultivated at the Port-a-Bhuiltin seaweed farm in Scotland, and in *Ulva lactuca* collected from the coastal area of Galicia (NW Spain), respectively.

Pseudoalteromonas is a widely distributed genus found in various marine environments, including oceans, sea ice, open and deep-sea waters, coastal areas, and marine sediments [33,34]. Initially classified as Alteromonas, *Pseudoalteromonas distincta* was first isolated from a marine sponge [35]. Rathgeber et al. [36] later identified Pseudoalteromonas spiralis as a metalloid-reducing bacterium isolated from deep-sea hydrothermal vents in the Juan de Fuca Ridge. Recently, Pseudoalteromonas spiralis was discovered as a Cadmium-reducing bacterium in the sediment of the Northern Coast of Indramayu, Indonesia [37,38]. But *Pseudomonas kilonensis*, although rarely detected, was first isolated from agricultural soil in Germany [39]. Additionally, *Halomonas sulfidaeris* sp. nov., a halophilic bacterium isolated from deep-sea hydrothermal vent environments, shows potential applications in various industries such as food, feed, cosmetics, pharmaceuticals, and chemicals [40,41]. Romanenko et al. [42] reported the discovery of Cobetia amphilecti for the first time, isolated from the internal tissue of the sponge *Amphilectus digitatus* in the Gulf of Alaska near Kodiak Island. This finding is of significant interest for both medical and food-related purposes [43].

Segers et al. [44] identified *Pseudomonas echinoides* from the NRRL culture collection of the Agricultural Research Service, which was later reclassified as *Sphingomonas echinoides* through a polyphasic taxonomic approach [45]. Vries et al. [46] characterized *Sphingomonas echinoides* as a biofilm-former capable of metabolizing a wide range of substrates. *Stenotrophomonas maltophilia* is a multidrug-resistant pathogen that has become an opportunistic nosocomial threat with significant morbidity and mortality rates, prevalent in various environments [47-49].

But bacterial films produced by *Shewanella colwelliana* have been shown to induce colonization of *Ostrea edulis, Crassostrea gigas,* and *C. virginica* in hatcheries [50,51]. *Vibrio spp.* have historically been linked to mortality events in mollusks and fish [52-54]. Notably, *Vibrio chagasii* and *V. alginolyticus* were isolated from carpet shell clams (*Ruditapes decussatus*) affected by Brown Ring Disease (BRD) in Tunisia. *V. jasicida*, initially described in 2012 from Atlantic salmon and flounder, has been found in marine invertebrates and vertebrates, and has recently been detected at shellfish sites in the UK [55,56]. Additionally, *V. natriegens*, known

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for its rapid growth and non-pathogenic nature, has garnered attention for its potential applications in biotechnology [57,58].

Photobacterium angustum was found in surface coastal waters in Botany Bay, Australia, and has been identified as a psychotropic high-level histamine-producing bacteria native to tuna [59-61]. This bacterium has been extensively studied in stress investigations, such as UV radiation, revealing its resistance to long-term UVB exposure. *Shewanella marinintestina*, described by Satomi et al., [62] was isolated from a squid's intestine. Tejerina et al. [63] recently reported the discovery of *Shewanella marinintestina*, a marine bacterium found in fish guts known to produce Eicosatetraenoic acid (EPA). *Acinetobacter schindleri* was first reported by Nemec et al. [64] after being isolated from human clinical specimens. Subsequent studies have detected this bacterium in hospital settings, the microflora of grasshopper *Poecilimon tauricola*, and soil samples. Additionally, *Acinetobacter schindleri* has been found in waterfowl and their surrounding environment, including duck, goose, and soil samples [65-69].

Marine-derived foods have the potential to be contaminated by microorganisms originating from various sources, including household and agricultural wastewater, poorly managed organic waste storage, and pollution from municipal and industrial activities. The contamination level of bivalve mollusks is also significantly influenced by environmental factors [70,71]. The microbial populations in the aquatic environment and the physiological condition of the bivalve mollusks and their ability to filter and accumulate bacteria, are greatly affected by fluctuations in temperature, oxygen levels, and water salinity throughout the year [72]. The aquatic environment can be exposed to antibiotics either directly or through the presence of antibiotic metabolites from different sources, such as wastewater discharged from industries, hospitals, and breeding practices. However, 20-30% of antibiotic residues in the environment result from aquaculture activities [73,74].

Overall, the results of antibiotic susceptibility tests revealed that 86% of oyster-derived isolates and 87% of mussel-derived isolates were resistant to multiple drugs. Interestingly, none of the Gram-positive isolates derived from oysters showed resistance to gentamicin, norfloxacin, ciprofloxacin, chloramphenicol, or trimethoprim-sulfamethoxazole. Additionally, there was no resistance observed to norfloxacin and trimethoprim-sulfamethoxazole in the mussel-derived isolates.

Among the 54-oyster origin isolates, substantial resistance was discovered to cefoxitin (n=50; 92.6%), oxacillin (n=50; 92.6%), cefotaxime (n=45; 83.3%), ampicillin (n=38; 70.4%), nitrofurantoin (n=33; 61.1%), with different resistance levels were observed to amoxicillin-clavulanic acid (n=28; 51.9%), imipenem (n=22; 40.7%), streptomycin (n=15; 27.8%), ceftriaxone (n=13; 24.1%), gentamicin (n=10; 18.5%), tobramycin (n=9; 16.7%), ciprofloxacin (n=8; 14.8%), nalidixic acid (n=6; 11.1%), and norfloxacin (n=6; 11.1%). Also, a modest rate of resistance was seen against the antibiotics tetracycline (n=3; 5.6%), chloramphenicol (n=1; 1.9%), and trimethoprim-sulfamethoxazole (n=1; 1.9%).

For the mussel bacterial isolates levels resistance, the highest proportion of resistance was observed to oxacillin (n=21; 95.5%), cefotaxime (n=20; 90.9%), cefoxitin (n=19; 86.4%), and with different resistance levels towards ampicillin (n=11; 50%), amoxicillin-clavulanic acid (n=10; 45.5%), imipenem (n=9; 40.9%), nitrofurantoin (n=9; 40.9%), streptomycin (n=7; 31.8%), ceftriaxone (n=5; 22.7%), and tetracycline (n=2; 9.1%). However, tobramycin, gentamicin, ciprofloxacin, nalidixic acid, and chloramphenicol resistance was only seen in 4.5% (n=1) of isolates.

The usage of different antibiotic classes varies across different regions worldwide. In Africa, beta-lactams, tetracyclines, and quinolones are commonly used for human treatment, while in America, beta-lactams, macrolides, and tetracyclines are more prevalent. South-east Asian regions show a high usage of quinolones and cephalosporins, with Indian states having particularly high consumption of third-generation cephalosporins. In the EU, beta-lactams, macrolides, sulfonamides, and tetracyclines are frequently used, whereas the Eastern Mediterranean region reports high usage rates for beta-lactams, macrolides, and tetracyclines. The Western Pacific region, on the other hand, often uses tetracyclines, quinolones, and beta-lactams [1,74].

Moreover, in terrestrial food-producing animals, tetracyclines, penicillin, and macrolides are commonly used, while amphenicols, tetracyclines, and fluoroquinolones are prevalent in aquatic food-producing animals [75]. A study by Zhou et al. [76] revealed that aminoglycosides, beta-lactams, chloramphenicol, macrolides, nitrofurans, quinolones, sulfonamides, and tetracyclines are used in aquaculture. Xinhua and Wen [77] noted that chloramphenicol, ciprofloxacin, erythromycin, and furazolidone are specifically designed for human use, while others like amoxicillin, chlortetracycline, gentamicin S, oxytetracycline, penicillin G, streptomycin, sulfamerazine S, and sulfisoxazole are either prohibited in aquaculture or have been banned.

The resistance levels of different antibiotics in mussel bacterial isolates were examined in our study. Cephems and penicillin showed the highest resistance, followed by nitrofurans and carbapenems. However, the most commonly detected classes of antibiotic resistance were cephems and penicillin, followed by beta-lactams, carbapenems, nitrofurans, and aminoglycosides in mussel bacterial isolates. It is crucial to monitor antibiotics in the aquatic environment to prevent pollution. Due to the extensive consumption of seafood globally, it is imperative to evaluate the health risks for humans linked to antimicrobial resistant bacteria. Additionally, it is crucial to consider the impact on aquatic ecosystems, as well as the potential transmission and contamination of these bacteria to humans following consumption.

In summary, mussels serve as an indirect route for the transmission of antibiotic resistance, including genes such as carbapenem resistance, colistin resistance, and common genes typically found in commercial animal feed and aquaculture

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by-products. This is due to their filtration capacity and the accumulation of these antibiotic-resistant bacteria and their residues in their epithelium, which includes a group of powerful antibiotics vital to human health [78].

Conclusion

The current research highlights the important role of raw bivalve mollusks as potential carriers of various pathogenic bacteria, often contaminated with multiple species simultaneously. Some of these bacteria have shown resistance to commonly used antimicrobials, particularly cephems and penicillin, followed by carbapenems. However, most isolates have shown sensitivity to chloramphenicol and trimethoprim-sulfamethoxazole. Despite a significant prevalence of multi-resistant patterns, especially with 86% of isolates from oyster origins and 87% from mussel origins, it is essential to investigate antimicrobial resistance genes for a comprehensive understanding.

While some of these bacterial genera are typically considered nonpathogenic, the emergence and spread of antibiotic-resistant strains, especially against commonly used antibiotics, present a significant public health concern for human, animal microbiota, and globally the whole ecosystem. To address the development of problematic antimicrobial resistance and assess associated health risks related to seafood consumption, continuous monitoring of antibiotic residues in bivalves is crucial. Stringent oversight must protect specifically the soil and aquatic environment from antibiotic pollution.

Dedication

We would like to express our deepest gratitude and pay our respects to our co-organizer and colleague, Dr. Dorsaf ESSEBAI ELAMRI, both at the beginning and at the end. Dr. Dorsaf ESSEBAI ELAMRI played a crucial role in initiating and completing this research work. Unfortunately, in October of 2022, we received the sad news of her passing. Dr. Dorsaf ESSEBAI ELAMRI was a highly dedicated professor at the Department of Biotechnologie Bleu et Bioproduits aquatiques (B3-Aqua) in the National Institute of Science and Technology of the Sea at the University of Carthage in La Goulette, Tunisia. Her contributions were recognized through numerous awards and accolades, highlighting her excellence in both teaching and research. As we continue our work in the marine environment, particularly at the National Institute of Science and Technology of the Sea in Tunisia, Dr. Dorsaf ESSEBAI ELAMRI will always remain in our thoughts and memories.

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