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UNVEILING NON-CHOLERIC VIBRIO SPECIES IN CRUSTACEANS AND AQUATIC SNAILS: A COMPREHENSIVE STUDY IN THE SOUTH AND SOUTHEASTERN ROMANIA'S HYDROGRAPHIC SYSTEM

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Abstract

This study investigated the prevalence of non-choleric *Vibrio* bacteria in aquatic crustaceans and snails across south and southeast Romania, from the Danube Delta to the Black Sea coastline. Spanning 1997 to 2019, 11,916 crustacean and 4,844 snail samples underwent bacteriological analysis. Among crustaceans, 194 non-choleric *Vibrio* strains were isolated (collecting frequency: 1.532), including *V. alginolyticus* (74 strains), *V. parahaemolyticus* (59 strains), *V. vulnificus* (47 strains), *V. mimicus* (9 strains) and *V. furnissii* (8 strains). Snails yielded 31 strains (harvesting frequency: 0.64), primarily *V. alginolyticus* (14 strains) and *V. parahaemolyticus* (16 strains), with one *V. vulnificus* strain. Occurrences were higher in the Danube Delta and surroundings, particularly in marine environments. Notably, a correlation suggested higher trophic-level species had more non-choleric vibrios. Understanding these dynamics is vital for ecosystem and public health. Importantly, this research highlights the potential for pharmaceutical applications, emphasizing the significance of investigating non-choleric *Vibrio* species in crustaceans and snails in the pharmaceutical industry.

Rezumat

Studiul a investigat prevalența bacteriilor *Vibrio* non-colerice în crustacee și melci acvatici din sudul și sud-estul României, din Delta Dunării până la țărmul Mării Negre. În perioada 1997 - 2019, 11.916 probe de crustacee și 4.844 de melci au fost supuse analizei bacteriologice. În rândul crustaceelor, au fost izolate 194 de tulpini de *Vibrio* non-coleric (frecvență de colectare: 1.532), inclusiv *V. alginolyticus* (74 de tulpini), *V. parahaemolyticus* (59 de tulpini), *V. vulnificus* (47 de tulpini), *V. mimicus* (9 tulpini) și *V. furnissii* (8 tulpini). Melcii au prezentat 31 de tulpini (frecvență de recoltare: 0,64), în principal *V. alginolyticus* (14 tulpini) și *V. parahaemolyticus* (16 tulpini), cu o tulpină *V. vulnificus*. Prezența acestor specii a fost mai mare în Delta Dunării și în împrejurimi, în special în mediile marine. Studiul sugerează că speciile de nivel trofic superior au avut mai mulți vibrioni non-colerici. Înțelegerea acestei dinamici este deosebit de importantă pentru ecosistem și pentru sănătatea publică. Astfel, cercetarea speciilor de crustace și melci non-colerici evidențiază potențialul pentru aplicații farmaceutice, subliniind importanța investigării acestor specii pentru industria farmaceutică.

Keywords: *Vibrio* species, contamination, prevalence, chitosan source, pharmaceuticals

Introduction

Non-choleric strains of *Vibrio*, despite being commonly regarded as non-pathogenic, can still have a considerable impact on human health [1, 2]. As stated by Xie *et al*., these strains serve as reservoirs for well-known virulence genes, and the transmission of these genes can transform a non-pathogenic strain into a pathogenic one [3]. According to Castillo *et al*., prophages, which are found in environmental vibrios, including non-pathogenic strains, can encode virulence factors, such as bacterial toxins, and enhance the pathogenicity of these strains [4]. Bacteriophages can transfer virulence factors to bacteria *via* a phenomenon called lysogenic conversion, thereby enabling non-pathogenic strains to acquire virulence or enhance their existing virulence [5].

Research has indicated that *Vibrio* species, even strains that do not cause cholera, might carry virulence genes that are often linked with *Vibrio cholerae*, the pathogenic bacterium responsible for the development of cholera. Sechi *et al*. discovered that virulence genes of *V. cholerae* have been identified in various other *Vibrio* species, indicating the possibility of these strains causing pathogenicity [6]. In addition, the evolutionary mechanism of *Vibrio cholerae* involves the incorporation of new virulence factors by horizontal gene transfer. This leads to the emergence of previously non-toxigenic strains that can produce severe symptoms of infection in humans.

The prevalence of non-choleric *Vibrio* strains in snails and crustaceans holds significant implications for human health. *Vibrio* species, which encompass non-choleric

strains, are common in aquatic habitats such as estuarine, marine and coastal environments [7, 8]. The presence of these bacteria has been seen in both the water column and in association with diverse species, such as snails and crustaceans [9, 10]. Huq *et al*. reported that snails and crustaceans, such as copepods and copepod egg sacs, have been shown to host *Vibrio* species, including *Vibrio cholerae* [11]. These organisms can adhere to the surfaces of live copepods and persist in their presence. The significance of *Vibrio* species adhesion is proposed to play a crucial role in the ecological dynamics of these bacteria within aquatic ecosystems as well as in the transmission patterns of cholera [11]. In addition, it has been observed that *Vibrio* species, including the non-choleric strains, have been identified in the gastrointestinal tracts of fish. According to Senderovich *et al*., fish can serve as reservoirs and vectors of *Vibrio cholerae*, which may have implications for the dissemination of these bacteria within aquatic ecosystems [12].

The existence of non-choleric *Vibrio* strains in snails, crustaceans and fish gives rise to apprehensions over the possible transfer of these bacteria to humans. The ingestion of seafood that is contaminated, such as snails, crabs, or fish that are consumed raw or under-cooked, has the potential to result in *Vibrio* infections, as highlighted by Letchumanan *et al*. *Vibrio* species, inclusive of non-choleric strains, have the potential to induce gastroenteritis and other infections in humans, especially those with pre-existing medical disorders or impaired immune systems [7].

According to Froelich *et al*., it is estimated that approximately 84,000 individuals in the United States get foodborne *Vibrio* infections annually, leading to hospitalizations and fatalities. Most of these illnesses and accompanying fatalities are attributed to *Vibrio parahaemolyticus* and *Vibrio vulnificus* [13].

Shellfish, such as clams and oysters, have been recognised as possible reservoirs of *Vibrio* infections. According to Froelich *et al*., filter-feeding shellfish can accumulate pathogenic *Vibrio* bacteria, such as *V. vulnificus* and *V. parahaemolyticus*, from their surrounding aquatic environment. When these shellfish are consumed in a raw or undercooked state, they can potentially act as a means for these bacteria to enter the human body. The investigation of *Vibrio* species prevalence in shellfish, specifically clams and oysters, has been the subject of academic research, emphasising the necessity for precise abundance models and an enhanced comprehension of the dynamics between shellfish and *Vibrio* bacteria [13]. In addition, Wang *et al*. suggested that the observed increase in seawater temperature in recent years may be a contributing factor to the growth of *Vibrio* species, hence potentially elevating the probability of foodborne infections arising from seafood contamination [14]. Various countries, such as China and South Korea, have made efforts to assess the incidence of foodborne gastroenteritis caused by *Vibrio* species. The research utilised population surveys and surveillance methods to estimate the occurrence of *Vibrio* infections and evaluate the impact of the disease [15, 16].

The investigation of non-choleric *Vibrio* species inhabiting crustaceans and aquatic snails within the hydrographic system of South and Southeastern Romania holds immense promise for the pharmaceutical industry. These *Vibrio* species are known reservoirs of a rich diversity of bioactive compounds. For instance, certain *Vibrio* strains have been found to produce unique antimicrobial peptides with promising applications in combating drug-resistant bacterial infections [17]. These bioactive substances, originating from non-choleric *Vibrio* species, present tangible opportunities for pharmaceutical research and development. The identification and harnessing of such compounds could lead to the formulation of cutting-edge drugs, addressing critical gaps in current therapeutic options and providing innovative solutions to pharmaceutical challenges. By delving into the specific bioactive molecules produced by these *Vibrio* species, this comprehensive study offers a targeted approach to advancing drug discovery within the pharmaceutical sector [18, 19].

In this regard, the aim of this study builds upon previous research efforts, as aforementioned, by conducting a thorough investigation into the prevalence and distribution of non-choleric *Vibrio* bacterial species within aquatic crustaceans and snails across diverse aquatic environments in Southern and Southeastern Romania.

Materials and Methods

Samples of aquatic crustaceans were systematically collected over a 23-year period, spanning from 1997 to 2019, from various aquatic ecosystems in the Southern and Southeastern regions of Romania. These collection sites included the Danube Delta, the confluence of the Danube with the Black Sea, the Black Sea coast-line, lakes in the Razelm area, as well as different river basins. Our sampling strategy focused on capturing representative species of aquatic crustaceans indigenous to these specific habitats.

As an exemplification, the collected specimens consisted of a wide array of species, including *Gammarus* sp., *Corophium* sp., *Daphnia* sp., *Mesomysis kowalewski* and *Artemia salina*, which were obtained from the Danube Delta up until the point of convergence with the Black Sea. Along the Black Sea coast, samples comprised crab species (*Pachygrapsus marmoratus, Macropipus holsatus* and *Carcinus mediterraneus*), various shrimp species (*Crangon crangon, Palaemon adspersus* and *Palaemon elegans*) and marine isopods commonly known as "sea fleas" (*Idothea baltica*). Additionally, *Artemia salina* samples were obtained from the Razelm Complex lakes, representing a

significant component of the local coastal fish diet. Further samples included crayfish species (*Astacus astacus*, *Astacus leptodactylus*, *Austropotamobius torrentium* and *Astacus fluviatilis*), *Daphnia* sp., *Cyclops* sp. and *Gammarus pulex*.

In parallel, aquatic snail samples were collected over an 8-year period, spanning from 2012 to 2019. The focus was primarily on the striped nerite, *Theodoxus transversalis*, a small freshwater snail with an operculum from the *Neritidae* family and the lesser ramshorn snail, *Anisus vorticulus*, an air-breathing freshwater snail from the *Planorbidae* family. The sampling locations encompassed the confluence of the Danube with the Black Sea, the Sfântul Gheorghe catchment area, Romania, and the Danube Delta.

To isolate and identify bacterial species within the *Vibrio* genus, a specialised methodology was employed. The procedures followed established standards, such as STAS ISO 8914 and FAO-OMS methods, adapted to detect all non-choleric vibrios in the samples. Many samples were processed using methods proposed by Oliver JO in 1997 or Tudor L in 1999 (often concurrently for result comparison), with confirmation through the PCR method [20-24].

The samples were prepared, and their content was homogenized and extracted. Subsequently, dilutions of 1:1 and 1:10 in alkaline buffered water (ABW) were obtained. From these dilutions, samples (0.2 g of the 1:1 dilution and 0.1 mL of the decimal dilution) were plated on agar plates containing 1% tryptone and 3% sodium chloride in duplicate.

These plates were then incubated at 37°C for 18 - 24 hours. DNA extraction involved the centrifugation of bacterial cultures and shellfish and crustacean samples at 5000 rpm for 10 minutes, followed by sediment collection using a QIAamp DNA kit. DNA quantity and purity were determined *via* spectrophotometry (260/280 nm). Concentrated DNA was evaporated under vacuum by centrifugation, and the resulting pellet was resuspended in a Tris-EDTA buffer solution (pH = 8; 10 mM Tris, 1 mM EDTA). This solution was incubated at 65°C for 10 minutes to dissolve the DNA, and concentrated DNA was stored at -20°C [23, 25].

The quantity of yearly samples gathered and analysed, together with the identified crustacean species, is provided in Table I. Notably, strains of the *Vibrio* genus were isolated from these samples, representing *V. parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, *V. mimicus* and *V. furnissii* species.

Results and Discussion

Among a comprehensive collection of 12,423 aquatic crustacean samples gathered over a span of 23 years, a total of 194 *Vibrio* strains were successfully isolated, yielding an isolation prevalence rate of 1.562% (Table I and Table II). Notably, the most frequently encountered species among these isolated strains was *V. alginolyticus*, with a count of 71 strains, followed closely by *V. parahaemolyticus*, which comprised 59 strains. Additionally, *V. vulnificus* exhibited a noteworthy presence, with 47 isolated strains. In contrast, *V. mimicus* and *V. furnissii* were less frequently isolated, with only 9 and 8 strains, respectively, observed within the collected samples.

Table II illustrates the findings of the isolation process, organized by both years and the respective sampling regions.

Table I

Non-choleric *Vibrio* strains isolated from aquatic crustacean samples

Sampling Area	Year	Total number of samples	Total number of isolated strains	Year	Total number of samples	Total number of isolated strains
Danube Delta to Black Sea Confluence	1997	92	3 strains	2009	89	3 strains
	1998	88	2 strains	2010	92	1 strain
	1999	99	3 strains	2011	79	2 strains
	2000	104	4 strains	2012	85	2 strains
	2001	82	2 strains	2013	87	1 strain
	2002	85	1 strain	2014	81	5 strains
	2003	85	2 strains	2015	84	2 strains
	2004	93	2 strains	2016	92	1 strain
	2005	97	3 strains	2017	85	3 strains
	2006	81	1 strain	2018	88	2 strains
	2007	78	4 strains	2019	91	1 strain
	2008	87	2 strains	-	-	-
	Total period		**2,024 samples - 52 strains**			
Black Sea seacoast Area	1997	185	2 strains	2009	164	2 strains
	1998	203	3 strains	2010	192	3 strains
	1999	171	4 strains	2011	188	2 strains
	2000	193	2 strains	2012	181	2 strains
	2001	197	2 strains	2013	197	5 strains
	2002	183	3 strains	2014	184	2 strains
	2003	192	2 strains	2015	179	1 strain

Sampling Area	Year	Total number of samples	Total number of isolated strains	Year	Total number of samples	Total number of isolated strains
	2004	206	2 strains	2016	167	2 strains
	2005	154	3 strains	2017	183	1 strain
	2006	162	4 strains	2018	174	3 strains
	2007	178	2 strains	2019	202	2 strains
	2008	185	1 strain	-	-	-
	Total period		**4,220 samples - 55 strains**			
Razelm Area	1997	61	3 strains	2009	68	2 strains
	1998	54	2 strains	2010	52	3 strains
	1999	59	2 strains	2011	74	2 strains
	2000	67	3 strains	2012	65	3 strains
	2001	72	2 strains	2013	66	1 strain
	2002	65	2 strains	2014	58	6 strains
	2003	62	4 strains	2015	59	3 strains
	2004	71	4 strains	2016	61	1 strain
	2005	58	3 strains	2017	67	2 strains
	2006	66	2 strains	2018	64	2 strains
	2007	57	5 strains	2019	52	0 strains
	2008	72	1 strain	-	-	-
	Total period		**1,450 samples - 58 strains**			
South and Southeast Area Rivers and Pools	1997	193	3 strains	2009	195	0 strains
	1998	220	4 strains	2010	184	1 strain
	1999	154	3 strains	2011	169	1 strain
	2000	243	2 strains	2012	187	3 strains
	2001	257	1 strain	2013	172	1 strain
	2002	247	0 strains	2014	198	0 strains
	2003	261	0 strains	2015	188	2 strains
	2004	268	2 strains	2016	173	0 strains
	2005	259	3 strains	2017	175	1 strain
	2006	238	0 strains	2018	187	1 strain
	2007	178	1 strain	2019	192	0 strains
	2008	221	0 strains	-	-	-
	Total period		**4,729 samples - 29 strains**			
Total number of samples/ entire study period			**12,423**		**194 strains**	

Table II

Yearly and total prevalence of non-choleric *Vibrio* species isolated from crustaceans

Year	4				
	Danube Delta To Black Sea Confluence	**Black Sea Seacoast Area**	**Razelm Area**	**South and Southeast Area Rivers and Pools**	**Total number of samples**
1997	3.26	1.08	4.92	1.55	2.07
1998	2.27	1.48	3.70	1.82	1.95
1999	3.03	2.34	3.39	1.95	2.48
2000	3.85	1.04	4.48	0.82	1.81
2001	2.44	1.02	2.78	0.39	1.15
2002	1.18	1.64	3.08	0	1.03
2003	2.35	1.04	6.45	0	1.33
2004	2.15	0.97	5.63	0.75	1.57
2005	3.09	1.95	5.17	1.16	2.11
2006	1.23	2.50	3.03	0	1.28
2007	5.13	1.12	8.77	0.01	2.44
2008	2.30	0.54	1.39	0	0.71
2009	3.37	1.22	2.94	0	1.36
2010	1.09	1.56	5.77	0.54	1.54
2011	2.53	1.06	2.70	0.59	1.37
2012	2.35	1.10	4.61	1.60	1.93
2013	1.15	2.54	1.51	0.58	1.53
2014	6.17	1.09	8.62	0	2.30
2015	2.38	0.56	5.08	1.06	1.57

Year	4				
	Danube Delta To Black Sea Confluence	**Black Sea Seacoast Area**	**Razelm Area**	**South and Southeast Area Rivers and Pools**	**Total number of samples**
2016	1.09	1.20	1.64	0	0.81
2017	3.53	0.55	2.98	0.57	1.37
2018	2.27	1.72	3.12	0.53	1.56
2019	1.099	0.99	0	0	0.56
Total prevalence (%)	2.57	1.30	4.00	0.61	1.562

As presented in Table II, the annual prevalence of non-choleric *Vibrio* strains in crustaceans varied across different geographical areas within the studied ecosystems. In the Danube Delta to Black Sea Confluence, the prevalence stood at 2.57%, indicating a relatively higher occurrence of these strains in this region. Moving along the Black Sea coastline, the prevalence decreased to 1.30%, still noteworthy but somewhat lower than the previous area.

In the Razelm area, an intriguing pattern emerged, with the prevalence spiking to 4%. This substantial increase in prevalence highlights a unique ecological dynamic or specific condition in this locale that favours the proliferation of non-choleric *Vibrio* strains.

Conversely, in the South and Southeast Area Rivers and Pools, the prevalence was notably lower at 0.61%, indicating a comparatively lower presence of these *Vibrio* strains in this aquatic environment.

These prevalence variations among different regions underscore the complex interplay of ecological factors, geographical locations and potentially distinct ecological niches within the studied ecosystems. The higher prevalence in certain areas may be influenced by factors such as temperature, salinity and the presence of specific host organisms, all of which warrant further investigation to better understand the underlying mechanisms driving these variations.

Overall, this data highlights the ecological heterogeneity within aquatic ecosystems and emphasizes the need for region-specific approaches when assessing the prevalence of non-choleric *Vibrio* strains.

Following the processing of the samples, noteworthy observations regarding the ecobiology of non-choleric *Vibrio* species have emerged. Specifically, concerning saltwater crustaceans, most *Vibrio* strains were isolated from the visceral parts, while in the case of freshwater crustaceans, most isolations were obtained from the cephalon segment. Furthermore, the prevalence of non-choleric *Vibrio* isolation was higher in saltwater crustacean samples collected from salt lakes or seawater compared to those collected from freshwater sources.

Regarding the aquatic snail samples, a total of 4,844 were collected over an eight-year period, with 31 isolated *Vibrio* strains, resulting in an isolation prevalence rate of 0.64% (Table III). Among these, *V. parahaemolyticus* was the most frequently isolated species (16 strains), followed by *V. alginolyticus* (14 strains) and *V. vulnificus* was the least commonly isolated species (1 strain) (Table III).

Table IV presents the data obtained on the yearly prevalence of non-choleric *Vibrio* strains isolated from aquatic gastropods (aquatic snails) samples.

Table III

Non-choleric *Vibrio* strains isolated from aquatic gastropods (aquatic snail) samples

Sampling Area	Year	Total number of samples	Total number of isolated strains
Danube Delta to Black Sea Confluence	2012	127	0 strains
	2013	133	1 strain
	2014	91	2 strains
	2015	116	2 strains
	2016	102	1 strain
	2017	121	0 strains
	2018	128	0 strains
	2019	108	1 strain
	Total period	**926 samples - 7 strains**	
Sfântul Gheroghe to Black Sea Confluence	2012	187	2 strains
	2013	198	4 strains
	2014	184	2 strains
	2015	179	1 strain
	2016	182	1 strain
	2017	183	0 strains
	2018	191	1 strain
	2019	186	1 strain
	Total period	**1,490 samples - 12 strains**	
Razelm Area	2012	147	3 strains
	2013	116	1 strain

Sampling Area	Year	Total number of samples	Total number of isolated strains
	2014	121	2 strains
	2015	118	1 strain
	2016	132	1 strain
	2017	128	0 strains
	2018	141	0 strains
	2019	124	1 strain
	Total period	**1,027 samples - 9 strains**	
South and Southeast Area Rivers and Pools	2012	186	0 strains
	2013	162	1 strain
	2014	158	1 strain
	2015	172	0 strains
	2016	179	0 strains
	2017	184	1 strain
	2018	178	0 strains
	2019	182	0 strains
	Total period	**1,401 samples - 3 strains**	
Total number of samples/entire study period		4,844	31 strains

Table IV

Yearly prevalence of non-choleric *Vibrio* species isolated from aquatic gastropods (aquatic snail) samples

Year	Annual prevalence (%) based on the sampling area				
	Danube Delta To Black Sea Confluence	**Sfântul Gheroghe to Black Sea Confluence**	**Razelm Area**	**South and Southeast Area Rivers and Pools**	**Total number of samples**
2012	0	1.07	2.04	0	0.78
2013	0.75	2.02	0.86	0.62	1.06
2014	2.19	1.08	1.65	0.63	1.39
2015	1.72	0.56	0.85	0	0.78
2016	0.98	0.53	0.76	0	0.57
2017	0	0	0	0.54	0.14
2018	0	0.52	0	0	0.13
2019	0.93	0.54	0.81	0	0.57
Total prevalence (%)	0.76	0.80	0.88	0.21	0.64

The annual prevalence of non-choleric *Vibrio* strains in snails exhibited variations across different geographical areas within the studied ecosystems. In the Danube Delta to Black Sea Confluence, the prevalence was recorded at 0.76%, indicating a moderate occurrence of *Vibrio* spp. in this region. In the area of Sfântul Gheroghe to the Black Sea confluence, the prevalence slightly increased to 0.80%, signifying a marginally higher presence of these strains in this specific location. The Razelm area, however, stood out with a prevalence of 0.88%, demonstrating a relatively higher occurrence of non-choleric *Vibrio* strains. This observation suggests that the ecological conditions or ecological interactions in this area may favour the proliferation of these bacteria. In contrast, the South and Southeast area of rivers and pools exhibited a lower prevalence of 0.21%, indicating a comparatively reduced presence of non-choleric *Vibrio* strains in this aquatic environment.

Additionally, within the study, it was observed that the prevalence of isolating non-choleric *Vibrio* species tends to be notably elevated within species occupying higher trophic levels of the aquatic ecosystem pyramid. This observed correlation was noted specifically in the marine ecosystem, encompassing areas such as the seacoast, the Razelm-Sinoe Complex area and the Danube Delta, where the frequency of *Vibrio* isolations surpasses that of other aquatic species at lower trophic levels.

Following the analysis of the samples, a noteworthy insight into the ecobiology of non-choleric *Vibrio* species came to light. Specifically, in the case of samples obtained from ramshorn snails, most *Vibrio* strains were found to be localised within the visceral regions. However, it's noteworthy that the isolation frequency was notably lower in comparison to the data recorded in crustaceans. Additionally, there was a discernible disparity in the prevalence of non-choleric *Vibrio* isolation between samples from aquatic snails collected in deltaic areas or the catchment regions of the Danube, in contrast to samples gathered from freshwater sources.

Although *Vibrio alginolyticus* strains have been found in crustaceans, scientific study papers do not offer definitive proof that they are the predominant *Vibrio* species in crustaceans. For example, Xie *et al.* investigated the distribution of virulence genes among *Vibrio alginolyticus* and *Vibrio parahaemolyticus* strains obtained from mariculture systems in Guangdong,

China [3]. The study detected the presence of various virulence genes in both *V. alginolyticus* and *V. parahaemolyticus* strains, but it did not provide information on the relative frequency of *V. alginolyticus* compared to other *Vibrio* species in crustaceans. In this regard, further research specifically focused on the prevalence and distribution of *Vibrio* species in crustaceans would be needed to determine the relative frequency of *V. alginolyticus* compared to other *Vibrio* species in these organisms. Additionally, even if *V. parahaemolyticus* was the most frequent strain of *Vibrio* identified in snails in the present study, a similar approach should be taken since no specific data was found in order.

Moreover, the study findings offer some insights into an intriguing ecological pattern in the context of non-choleric *Vibrio* species isolation within aquatic ecosystems. This pattern aligns with related scientific data, indicating a clear link between the trophic position of aquatic species and the frequency of non-choleric *Vibrio* isolation [20]. Specifically, species located higher up in the aquatic trophic ecosystem pyramid tend to display a relatively higher occurrence of *Vibrio* isolation. This ecological observation underscores the complex dynamics inherent in aquatic food webs, where non-choleric *Vibrio* species may play a significant role in shaping the structure of bacterial communities at higher trophic levels. On the other hand, there is limited direct information on the correlation between the trophic position of aquatic crustaceans and the occurrence of non-choleric *Vibrio* isolation. Further research specifically focused on this correlation would be needed to determine any potential relationship.

The marine ecosystem, distinguished by its diverse spectrum of habitats, served as an especially enlightening context for these findings. Notably, in regions such as the seacoast area, the Razelm-Sinoe Complex area and the Danube Delta, the study documented a heightened frequency of *Vibrio* isolations compared to aquatic species at lower trophic levels. It is noteworthy that no similar data elucidating such patterns was found in the existing literature. This observation accentuates the intricate ecological dynamics that govern bacterial prevalence, particularly in environments characterised by diverse species interactions within complex food webs. Furthermore, it is noteworthy that this correlation extends beyond the biotic components of the ecosystem.

The researched data shows that the elevated prevalence of non-choleric *Vibrio* species is not confined to living organisms alone; it also manifests in the isolation rates observed in water and sediment samples [22-24]. In this regard, Böer *et al*. examined the temporal and spatial distribution patterns of potentially pathogenic *Vibrio* species at recreational beaches in the German North Sea. The study found that *Vibrio* spp. concentrations in sediments were up to three orders of magnitude higher than in water samples [26]. This suggests that non-choleric *Vibrio* species are not merely passive passengers within these ecosystems but play an active and dynamic role in various environmental compartments.

One of the central findings that emerged from our research underscores the importance of host specificity in the isolation of non-choleric *Vibrio* species. Specifically, in the case of samples collected from saltwater crustaceans and, with a lower frequency, from ramshorn snails, our data revealed that most *Vibrio* strains were concentrated in the visceral parts. Similar findings were presented by Rungrassamee *et al*., who characterised the intestinal bacteria in wild and domesticated adult black tiger shrimp (*Penaeus monodon*) and identified *Vibrio* as one of the bacterial genera present in the intestines of the shrimp [27]. On the other hand, none of the investigated papers directly address the research question of whether non-choleric *Vibrio* species can be identified in the visceral parts of snails or in the cephalon segment of freshwater crustaceans. Additionally, the low frequency of isolation in ramshorn snails suggests a relationship between non-choleric *Vibrio* species and their host organisms, likely influenced by host-specific factors such as physiological differences or microbial interactions.

Another central finding of this research is the pharmaceutical value of these non-choleric *Vibrio* species. The geographic setting of the area between the Danube Delta and the Black Sea creates a heightened likelihood for the dissemination of *Vibrio cholerae* species. Hence, the pharmaceutical sector places significant emphasis on closely monitoring aquatic organisms, particularly crabs and snails, as part of their exploitation efforts. Chitosan is a product obtained by removing acetyl groups from chitin. Crustaceans are commonly used as the main source for producing chitosan from chitin. Chitosan's solubility in water makes it a more convenient polymer to handle. Chitosan has found extensive applications in various sectors, including pharmaceuticals, the food industry, agriculture and medicine (such as artificial skin and orthopaedic tissue engineering) [28, 29]. These biopolymers possess features such as biocompatibility, biodegradability, nontoxicity and gel-forming ability, as well as biological activities including antibacterial, immunological and antioxidant effects. As a result, they can be regarded as a new and valuable source of functional materials. The distinctive characteristics of chitosan render it an excellent candidate for many pharmacological formulations, specifically as transporters for medicinal components [30]. Chitosan acts as a non-viral carrier for gene transfer, enabling the transmission of genetic material without the need for viruses. Moreover, the capacity of the substance to enclose and safeguard genetic material and active components is essential for maintaining their durability and effectiveness. Chitosan has a significant medicinal advantage in facilitating the slow release of medicines, which helps

to maintain sustained therapeutic effects. In addition, chitosan has played a crucial role in strengthening the precise transport of nucleic acids, improving their ability to reach specific therapeutic targets, and thereby enhancing the overall effectiveness of gene treatments and medication formulations. Chitosan is a versatile and promising substance that has the potential to enhance drug delivery systems and gene treatments in the field of pharmaceutical applications [31-33].

Conclusions

In this study, the prevalence and distribution of non-choleric *Vibrio* strains in aquatic ecosystems were investigated, revealing a prevalence of 1.532% in crustacean samples and 0.64% in snail samples, with distinct regional variations. The three most frequently isolated species, *V. alginolyticus*, *V. parahaemolyticus* and *V. vulnificus*, showed varying prevalence patterns. Importantly, the findings underscore a direct linkage between contamination by non-choleric *Vibrio* at different trophic levels, emphasising the role of invertebrates, particularly crustaceans, as vectors of contamination. Understanding these ecological dynamics is crucial for ecosystem health, food web dynamics and public health considerations, highlighting the importance of investigating non-choleric *Vibrio* species in crustaceans and snails for the pharmaceutical industry.

Conflict of interest

The authors declare no conflict of interest.

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