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Research Article

Prevalence of *Escherichia coli* and *Salmonella spp.* in mussels (*Mytilus galloprovincialis*) and serotyping of *Salmonella spp.* in Zenata and Mansouria regions of Morocco

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Abstract
Due to their immobile nature and filter-feeding habits, which enable biological particles to accumulate in their tissues, mussels are recognized as vectors of foodborne diseases. Consuming these shellfish uncooked or partly processed might result in food poisoning because of accumulated bacteria originating from the culture environment and unhygienic handling. The present study aimed to assess the presence of *Salmonella* and *Escherichia coli* as well as to biochemically and serotypically confirm *Salmonella spp.* in mussels (*Mytilus galloprovincialis*) taken from two locations in Morocco: Zenata and Mansouria. A total of 90 mussel samples were collected from October 2022 to August 2023. Two methods were employed in this study: AFNOR NF EN ISO 6579-1 (2017) for the detection of *Salmonella spp.*, and the most probable number method (MPN) using Norm ISO/TS 16649–3 (2017) for *E. coli*. The number of *E. coli* varied between 0.2/100 g and 1.7 x 10^3/100 g of mussels. The percentage of *Salmonella spp.* detected in mussel samples was 4,4%. Further analysis revealed the identification of two distinct *Salmonella* serotypes, namely *S. kentucky* (1 isolate) and *S. Typhimurium* (3 isolates). This research highlights the potential risks to public health due to the presence of pathogenic bacteria in mussels from two regions of Morocco where shellfish farming and coastal tourism are significant contributors to the local economy.

Keywords: *Escherichia* coli, Food Safety, Mussels, *Mytilus galloprovincialis*, *Salmonella*, Serotyping

INTRODUCTION

Bivalve molluscs are known for their ability to filter inorganic matter, including viruses and bacteria, from the surrounding water. However, they selectively filter phytoplankton and zooplankton for nutrition (Sferlazzo *et al*., 2018). Unfortunately, this filter-feeding behaviour can also result in the ingestion of harmful pathogens such as bacteria (e.g. *Salmonella, Arcobacter, Vibrio, Shigella*, pathogenic *Escherichia coli*), viruses (e.g.

Norovirus, Hepatitis A virus) (Ghaichat *et al*., 2023), and protozoans (*Giardia duodenales*, *Cryptosporidium spp., Toxoplasma gondii*). These pathogens can contaminate shellfish, particularly when they are grown in water polluted by human sewage or animal waste from livestock, wildlife, or agriculture (Butt *et al*., 2004 ; Campos *et al*. 2015 ; Gyawali *et al*., 2020 ; Lorenzoni *et al*., 2021 ; Marceddu *et al*., 2017 ; Mudadu *et al*., 2021 ; Polo *et al*., 2015 ; Tedde *et al*., 2019). Therefore, eating raw or undercooked shellfish can pose a threat to public health. These risks are internationally recognized and regulations exist around the world to control them (Potasman *et al*., 2002 ; Dabrowski *et al*., 2014).

Bacteria, parasites, and viruses from both humans and animals are often carried to the sea via land runoff or sewage systems. During heavy rainfall, particularly following a dry spell, there is likely to be a surge in the amount of animal faeces that makes its way to the sea (Bjørn *et al*., 2016). *E. coli* is often linked to warm-blooded animals and is widely regarded as a dependable indicator of water contamination caused by faeces from both animals and humans, which may suggest the presence of harmful bacteria known as enteric pathogens (Baylis *et al*., 2011; Jang *et al*., 2017). While *E. coli* is a common microorganism that typically resides in the mucous layer of the mammalian colon, its presence is often associated with contaminated water and suboptimal handling practices. Although many strains of *E. coli* are harmless, certain strains can lead to illnesses affecting the gastrointestinal, urinary, or central nervous systems (Kaper *et al*., 2004; Costa, 2013).

Salmonella is a major culprit in causing food-related illnesses worldwide, ranking third among bacterial pathogens responsible for foodborne diseases in humans, following *Escherichia coli* and Campylobacter, according to the World Health Organization (OMS, 2015). Presently, there are many salmonella serotypes, and over 2600 serotypes have been classified based on the reactivity of antiserum to O and H antigens (Stevens *et al*., 2009). Raw food, especially those of animal origin, is the main source of salmonellosis (Hackney and Potter, 1994). *Salmonella* is also detected in aquatic environments where it can survive for over four weeks (Roszak *et al*., 1984). The bacteria primarily inhabit the gastrointestinal tracts of animals and contaminated environments with human or animal waste (Huss, 1994). *Salmonella* is responsible for the majority of foodborne diseases or gastroenteritis cases characterized by symptoms such as diarrhea, abdominal cramps, vomiting, nausea, and fever (Sanjee and Karim, 2016).

The present study aimed to work on samples of mussels (*Mytilus galloprovincialis*) collected in two regions of Morocco, Zenata and Mansouria, to detect the presence or absence of *Escherichia coli* and *Salmonella*, as well as to biochemically and serotypically confirm *Salmonella spp.*

MATERIALS AND METHODS

Description of the study area

The Zenata (33° 38′ 59″ N, 7° 28′ 38″ O) site is strategically located between the two cities of Casablanca and Mohammedia. The site is located on the outskirts of Casablanca, northeast of the city, in the commune of Aïn Harrouda. It covers an area of 1,830 hectares with 5 km of coastline opening onto the Atlantic Ocean (Fig.1).

Mansouria (Latitude: 33.75, Longitude: -7.3 33° 45′ 0″ North, 7° 18′ 0″ West. Altitude of El Mansouria, 12 m.) is a town in the Ben Slimane Department of Casablanca Settat, Morocco. According to the 2004 census, the population of Mansouria was 12,955 (Fig.2).

Sampling

The study was conducted from October 2022 to August 2023. Mussels were sampled twice weekly for viral and bacterial analyses (*E. coli* and *Salmonella*). A total of 90 mussels (*Mytilus galloprovincialis*) samples from two

Fig.1. *Location of mussel sampling areas from the Zenata Morocco area (https://www.google.com/maps/place/Rue+De+Zenata,+Casablanca)*

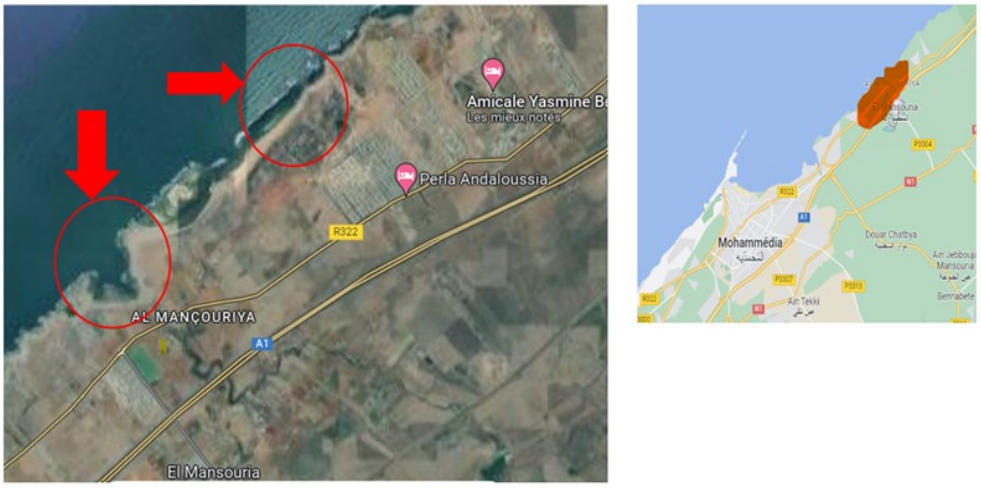

Fig. 2. *Location of mussel sampling areas from the Mansouria Morocco area (https://www.google.com/maps/place/El+Mansouria)*

different regions (Zenata and Mansouria) were examined (Fig. 1 and 2). Depending on the size of *M. galloprovincialis*, 30–60 individuals from each sample were randomly selected for analysis. All samples were sent to the National Health and Safety Administration laboratory in a refrigerated condition according to standards. Analysis began within 24 hours of sampling and was performed quickly while maintaining standards.

Escherichia coli analysis

To count *E. coli* in all bivalve samples, the five-tube most-probable-number (MPN) technique was used, in compliance with the European Union reference method ISO 16649-3:2017 (International Organization for Standardization, Geneva) (ISO 16649-3, 2017). A sample of 90 mussels was externally cleaned and aseptically prepared for microbiological analysis following ISO. Enumeration of *E. coli* was carried out according to the reference method ISO 16649-3 with 5 tubes and 4 dilutions of the most probable number (MPN) (ISO 16649-3, 2017). The MPN was calculated based on the number of positive tubes from 5 × 4 MPN arrays. Following a homogenous combination created by blending 30 g of mussel samples with the peptone water in a blender mixer, the mixture was added to 70 ml of peptone water in a stomacher bag. Five tubes of double-strength mineral-modified glutamate medium (Oxoid, Basingstoke, UK) were filled with 10 mL of the original solution (1/10). In addition, five tubes containing single-strength mineral-modified glutamate medium (Oxoid) were filled with aliquots of 1 mL of the 1:10 homogenate. Finally, 1 mL of additional dilutions (10^{-2}, 10^{-3}, 10^{-4}) were transferred to each of the five single-strength mineral-modified glutamate medium tubes. All tubes were incubated aerobically at 37°C for 24 h. Incubated tubes were considered positive if their color changed from purple to yellow, indicating the presence of acid. Subcultures from these tubes were plated on chromogenic tryptone bile glucuronide (TBX) agar (Oxoid) to obtain isolated colonies and confirm β-glucuronidase activity. TBX plates were incubated aerobically at 44°C for 24 h. At the end of incubation, the plates were examined for the presence of colonies with dark, light blue, or blue-green hues. This indicates the presence of putative β-glucuronidase-positive *E. coli* (Donovan *et al*., 1998).

Salmonella spp. analysis

All samples were tested for *Salmonella spp.* analyzed. Detection according to ISO 6579-1:2017 (ISO 6579-1, 2017). 25 g of each sample was added to 225 mL of buffered peptone water and incubated at 37 °C for 24 h. After incubation, 100 µL of culture (Sample + Buffered peptone water) was inoculated into 10 mL of Rappaport-Vassiliadis soy peptone concentrate broth and incubated at 41.5 °C for 24 h. At the same time, 1 ml of culture was transferred to 10 ml of Müller-Kaufman tetrathionate novobiocin broth modified with iodine and novobiocin, and 1 ml of culture was similarly transferred to 10 ml. It was mixed with cystine selenite broth and incubated at 37 °C for 24 h. After incubation, the three broths were subcultured on the surface of Xylose -lysine-deoxycholic acid agar plates, Hektoen agar, and another bismuth sulfite agar (bioMérieux, Marcy l`Etoile, France) to obtain well-separated colonies. Incubate for 24 hours at 37 °C. After incubation, the plates were examined for characteristic Salmonella-like colonies. Suspicious colonies (black centers and slightly transparent reddish areas on Xylose-lysine-deoxycholic acid agar; black colonies surrounded by a metallic sheen on bismuth agar; translucent pale green or greenish on Hectoene agar blue colonies) were identified. On each plate, identify distinctive colonies for subculturing and confirmation. On the non-selective

agar plate's surface, isolate the indicated colonies. The seeded plates should be incubated for 24 hours at 37° C on non-selective agar.

Biochemical confirmation of *Salmonella spp.*

Then, for biochemical confirmation, first place a disc of oxidase on a slide or petri dish with a loop, select a well -separated colony from the positive sample, gently rub the colony on the disc, and observe the purple color change. A negative sample for oxidase, i.e., the disc does not change color, is considered positive. After the oxidase test step, an API gallery was performed on the oxidase negative (-) samples, and the samples were incubated at 37°C for 24 hours.

Serotyping confirmation of *Salmonella spp.*

The identified biochemical isolates were analyzed using serotyping techniques, which involved the determination of somatic (O) and flagellar (H) antigens through slide agglutination tests, as described by Kauffman (1974); Grimont *et al*. (2007).

RESULTS AND DISCUSSION

Escherichia coli

During the study period, among the 90 samples examined for the presence of *E. coli*, 3 samples (3.33%) had MPN values of less than 20 colony-forming units (CFU) per 100 grams, 20 samples (22.22%) had MPN values between 20 and 230 *E. coli* 100 g^{-1} (Class A areas), 20 samples (22.22%) had MPN values between 230 and 4600 *E.coli* 100 g^{-1} (Class B areas), and 39 samples (43.33%) had MPN values greater than 4600 *E. coli* 100 g^{-1} (Class C areas). The samples from the Mansouria zone were between 0.2 to 49 *E. coli* 100 g^{-1}, which shows that the Mansouria zone was classified in class A and B and the Zenata zone was between 1.1 to 1.7 x 10^3 *E. coli* 100 g^{-1}, which shows that this area is classified in class C. The highest MPN value detected was 1.7 × 10^3 *E. coli* 100 g^{-1}, which was found in a mussel sample from the Zenata area. Table 1 provides a summary of the classification criteria for shellfish harvesting areas.

The results obtained on samples from the Zenata area indicated that this zone should be classified as C. However, the Mansouria region had samples between 20 and 4600 *E. coli* 100 g− 1, classifying it as A and B. According to Regulation (EU) 2019/627 (2019), bivalve molluscs production areas are classified into three categories (A-C) based on *E. coli* levels in their flesh and intravalvular fluid. This regulation aims to safeguard human health. Consequently, the microbiological quality of bivalve mollusks sold may depend on the origin's quality and the effectiveness of post-harvest treatment (Mudado *et al*., 2022). *E. coli* was detected in Mansouria with values ranging from 0.2 to 49 MPN per 100 grams, and in Zenata with values ranging from 1.1 to 1.7 x 10^3 MPN per 100 grams. The highest *E. coli* levels were found in Zenata. Zenata's higher level of contamination is caused by the amount of industrial pollutants that are discharged into the coastline via urban sewers. The present results align with those of Mannas *et al*. (2014), who detected *E. coli* with values ranging from 1.4 x 10^2 to 1.8 x 10^3 MPN per 100 grams along the western Atlantic coast of Morocco Anza, Agadir. Another study by Lamon *et al*. (2020) found *E. coli* in 100% of samples, with a load of 39 and 37 most probable number (MPN) per 100 grams in *M. galloprovincialis* samples, respectively. The present research aligns with the findings of Ates *et al*. (2011), who detected *E. coli* in 100% of the stuffed mussel (*Mytilus galloprovincialis)* samples they examined in Ankara.

This suggests there were issues with hygiene and cross -contamination during cooking or from external sources. Additionally, Bingol and Colak (2008) analyzed stuffed mussels (*Midye Dolma*) in Istanbul and found that 31 out of the 168 samples they tested were unacceptable (18.4%). It is important to note that our samples were only collected from street vendors.

Salmonella spp.

Out of the 90 samples tested for *Salmonella spp.*, 40 were suspected to be positive based on their lack of oxidase activity and unchanged disc coloration. A colony from each plate was isolated in nutrient agar and incubated for 24 hours at 37°C. Following incubation, the API gallery was performed on the 40 suspect samples. The results of the API gallery are presented in Table 2. The API gallery results showed that of the 40 suspected samples, 4 were positive for *Salmonella spp.* (4.4%).

Table 3 indicated that out of the 4 samples tested, 3 originated from the Zenata region, while the remaining 1 sample was from the Mansouria region. From a public health perspective, eating raw shellfish carries a high risk of *Salmonella* contamination. However, there are few studies on the presence of human pathogens in live shellfish and their role in public health. Several other studies have shown lower rates of *Salmonella*. The proportion of seafood varies greatly depending on the country's development level in which the study was carried out. In this study, *Salmonella spp.* it was detected in both locations, with a prevalence of 4.4% in the Zenata and Mansouria samples. In agreement with this finding, Lozano-Leon and colleagues detected 19 *Salmonella* positive samples out of 5907 batches of mussels, showing a prevalence of 0.3%, significantly lower than the 8% previously reported (Lozano-Leon *et al*., 2019). The results also agree with Mancini *et al*., (2023) who reported that only a small number of shellfish samples (5 out of 296) are affected by Salmonella spp. 1.7% and Boutaib *et al*. (2011) detected Salmonel-

Table 1. Classification categories of shellfish

Category	E. coli level (MPN 100 g^{-1})	Treatment required
Class A	20 < E < 230	May go for direct human consumption
Class B	230 < E < 4600	Must be depurated, heat treated or relayed to meet class A requirements
Class C	< 46 000	Must be relayed for 2 months to meet class A or class B requirements – may also be heat treated

Table 2. API Gallery Results

Samples	Area	API code	API results
	Zenata	5 0 4 4 5 5 2	Esherichia coli
	Zenata	6 7 0 4 7 5 2	Salmonella spp.
	Zenata	6 7 0 4 7 5 2	Salmonella spp.
	Mansouria	3 2 0 4 5 3 2	Citrobacter freundii
	Mansouria	6 7 0 4 7 5 2	Salmonella spp.
	Zenata	3 3 0 4 5 7 3	Enterobacter cloacae
	Zenata	3 3 0 4 5 7 3	Enterobacter cloacae
	Mansouria	1 6 0 4 7 7 2	Citrobacter freundii
MUSSELS	Mansouria	3 3 0 4 5 7 3	Enterobacter cloacae
	Zenata	3 3 0 4 5 7 3	Enterobacter cloacae
	Zenata	3 3 0 4 5 7 3	Enterobacter cloacae
	Mansouria	3 3 0 4 5 7 3	Enterobacter cloacae
	Zenata	3 2 1 4 7 7 3	K. pneum. Pneumoniae
	Zenata	3 3 0 4 7 7 3	Enterobacter cloacae
	Zenata	0 3 7 4 0 0 0	Morganella morganii
	Zenata	3 3 0 4 5 7 3	Enterobacter cloacae
	Zenata	1 7 0 4 7 5 3	Citrobacter freundii
	Zenata	3 6 0 4 5 1 2	Citrobacter freundii
	Zenata	6 7 0 4 7 5 2	Salmonella spp.
	Mansouria	0 1 7 4 0 0 0	Morganella morganii
	Mansouria	3 3 0 4 7 7 3	Enterobacter sakazakii
	Mansouria	3 3 0 4 5 7 3	Enterobacter cloacae
	Mansouria	1 2 0 0 3 7 3	Pantea spp 4
	Mansouria	1 2 0 4 5 7 3	Pantea spp 2

la in 7 out of 104 samples 6.7% of *Callista chione* and *Acanthocardia tuberculatum* collected from faecal contamination in Morocco (Rabat). In contrast, Zahli *et al*. (2021) discovered a Salmonella prevalence of 19.15% in mussels obtained from Moroccan markets; similarly, Mannas *et al*. (2014) and Setti *et al*. (2009) observed a Salmonella prevalence of 15.4% and 10%, respectively, in mussel samples collected from the Atlantic coast of Morocco. Other studies, such as Salmonella was reported in 8.0% of the samples collected from Alexandria, Egypt by Bakr *et al*. (2011) and Rubini *et al*. (2018), have also detected the presence of *Salmonella* in oysters and mussels, respectively, at varying prevalence rates.

Serotyping confirmation

The results of the *Salmonella* serological tests indicated the *Salmonella* family as either *Anti-Salmonella* OMA and *Anti-Salmonella* OMB (O-Group Pool test reagent). Further analysis revealed the identification of two distinct *Salmonella* serotypes, namely *S. Kentucky* (1 isolate) and *S. typhimurium* (3 isolates). The serotyping results are presented in Table 4.

Regarding the serotyping part, two serotypes were detected in four *Salmonella* isolates. *Salmonella typhimurium* was the most commonly isolated serotype, accounting for 75% of all isolates. *S. kentucky* was isolated once each case and accounted for 25% of all isolates. The present results are consistent with those of Baudart *et al*. (2000) where, *S. typhimurium* has been shown to be one of the major serotypes isolated from humans and animals, especially bovine species. The present results are consistent with those of Mancini *et al*., (2023), involving a sole strain of *S. typhimurium* sourced from humans in the Apulia Region of Italy. Other studies have found that *S. Typhimurium* is the most common clinically important serotype of *Salmonella* present in the marine environment (Lozano-Leon *et al*.,

Table 3. Results of *Esherichia coli* and *Salmonella*

Positive samples	Esherichia coli	Salmonella
Mussel 2 (Zenata)	1700 E. coli 100 g^{-1}	Salmonella spp.
Mussel 3 (Zenata)	460 E. coli 100 g^{-1}	Salmonella spp.
Mussel 6 (Mansouria)	1,7 E. coli 100 g^{-1}	Salmonella spp.
Mussel 43 (Zenata)	310 E. coli 100 g^{-1}	Salmonella spp.

Table 4. Results of the serological test and serotyping of the 4 *Salmonella* found in mussels

Salmonella	Serological confirmation		O Antigens	H Antigens Phase 1	H Antigens Phase 2
	OMA	OMB			
S. Kentucky		OMB 08	8 , 20	i	z6
S. Typhimurium	OMA 04		1 , 4 , [5] , 12	i	1, 2

2019), with a study in Galicia's coastal waters identifying it as the second most prevalent serotype, making up 15% of isolates (Martinez-Urtaza *et al*., 2004). Consistent with this hypothesis, research conducted on the coast of Colombia found a connection between ineffective sewage treatment systems and the existence of *Salmonella* in coastal waters (Soto-Varela *et al*., 2021). *S. typhimurium* is the second highest serotype linked to human salmonellosis in the European Union, so coastal regions with high populations may face possible contamination by human pathogenic *Salmonella* serotypes (EFSA, 2017).

Conclusion

The present study found particularly high rates of *E. coli* in the Zenata region and the presence of *S. typhimurium* and *S. kentucky* in street-sold wild mussels collected in the Zenata and Mansouria regions between October 2022 and August 2023. The Zenata area was identified as the most polluted area in the study because of the large amount of industrial pollutants being released into the coastline through urban sewers. Selling ready-to-eat foods such as mussels outdoors without precautions can be a major cause of food poisoning. Thus, better controls are needed to protect these foods from contamination, and cooking and distribution conditions need to be controlled.

Conflict of interest

The authors declare that they have no conflict of interest.

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