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In-Vitro Screening of pathogenic bacteria in brown mussels *Perna perna* of Al-Mukalla coast, Gulf of Aden, Yemen

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The brown mussel *Perna perna* was harvested from two marine stations on the Al-Mukalla coast (Mouth of Khor Al-Mukalla and Broom) to detect the main load of seasonally pathogenic bacteria. 250 samples of *P. perna* were collected monthly from October 2018 until October 2019 and evaluated seasonally. A protocol was designed for the isolation, enumeration, and partial identification of coliforms, *Escherichia coli, Staphylococcus aureus, Vibrio,* and *Salmonella typhi*. The results were processed and analyzed; the means with standard deviations were calculated and then compared. Results showed that the number of isolated bacteria was generally higher in summer and lower in winter. Based on MPN, the numbers (CFU g-1of coliforms, *E. coli* and *Staph. aureus* in summer were in the range of 117.0, 131.33 and 11.13 MPN, respectively, whereas the count recorded in winter were 26.30, 46.30 and 90.37 CFU g-1), respectively. While *V. parahaemolyticus* and *S. typhi* were neglected. For Broom station, the numbers of coliform and *E. coli* were in the range of 5.60 - 9.19 and 3.15 - 4.52 MPN g^{-1}, in winter and summer, respectively. While *Staph. aureus, V. parahaemolyticus,* and *S. typhi* were absent. Significant statistical differences were found in the numbers of tested bacteria between two stations were significant at $P<0.05$, particularly in coliform numbers and *E. coli* numbers from *P. perna* mussels collected from Khor Al-Mukalla and Broom stations. No significant differences were found in *E. coli* and coliform counts, remaining within the Gulf Cooperation Council GSO acceptable range.

Keywords: coliform, marine, mussel, *Perna*, *Staphylococcus*.

INTRODUCTION

The Republic of Yemen has a coastal strip elongated through 2500 kilometers and rich in fish and marine life. Yemen has more than 150 islands; most of them are in the Red Sea. These islands are characterized by environmental conditions favorable for growth and reproduction of various species of marine life, leading to high diversity of Yemeni fishing areas.

Perna perna, a bivalve mussel that has recently invaded North America, around the Gulf of Mexico, *P. perna* is commonly known as the brown mussel. Rocky shores can be colonized naturally by the brown mussels of *P. perna,* which can also attach to submerged man-made objects such as petroleum platforms, navigation buoys, and shipwrecks (*Perna Perna (Mollusc) Gulf States Marine Fisheries Commission*, 2003). *P. perna* also lives on Yemeni coasts, where the brown mussel of the edible *P. perna* species is considered a traditional food for man in Al-Mukalla city, governorate of Hadhramout, and an important economic resource for the government, and it can be an important income for fishermen (Kasem & Al-Alimi, 2013; Sokolowski et al. 2004). Hands and scraps using knives and sharp devices must be used to collect it. Baggar, the local name of the brown mussels of *P. perna,* occurred seasonally in the intertidal zone of Al-Mukalla city.

The regions of coasts usually concentrate high population density, causing intense urban development, which is not always associated with infrastructure and sanitary policies, and the discharge of untreated sewage may be resulted directly into the sea. Consequently, the yearly increasing amounts of untreated wastewater may lead to raising the dangers of pathogenic bacteria, causing many waterborne diseases in humans (Xie et al. 2022), and thus cause public health problems.

The marine environment's sanitary conditions affect the quality of present mussels and other bivalves, and they are closely related, and the bivalves are considered a means of environmental health monitoring (Yap et al. 2021). In addition to that, improper disposal of sewage and runoff of animal and/or human feces into their habitat potentially contaminates these bivalves (Giangaspero et

al. 2014).

The consumption of raw or poorly cooked mussels may result in risks to human health. If *P. perna* or other bivalves accumulate infectious forms of microorganisms such as bacteria, viruses, or protozoa in their tissue, they can remain infective, and when the raw or poorly cooked mussels become consumed risks to human health such as gastronomy and Paralytic Shellfish Poisoning (PSP) may be resulted, so preparation of brown mussels is necessary to kill them (Carvalho et al. 2019; La Barbera-Sánchez et al. 2004; Robertson, 2007). In the natural systems, the disease problems caused by microbes have a lesser impact compared to aquaculture systems because the infection or sick organisms, such as viruses, are quickly neutralized by predators (Welsh et al. 2020). The immune system of bivalves is affected by infectious diseases (Nguyen et al. 2019). In addition to their importance as food, *P. perna* mussels have high ecological importance because they can indicate variation levels in their environment (Silva dos Santos et al. 2018).

Several bacteria species may contaminate the seafood products which when consumed as raw, undercooked, and/or poorly cooked finfish and crustaceans may cause infection for consumers (NACMCF, 2008). Contamination of seafood by *Salmonella* and *Staphylococcus aureus* may take place during processing and storage; this can be prevented by good manufacturing practices (GMPs) and hazard analysis critical control point (HACCP) (NCCDPHP, 2014; Iwamoto et al. 2010). Molluscan bivalves are filter feeders, and they tend to accumulate microorganisms in the surrounding waters, which may also contain vibrios. *V. cholerae* is of most concern because of its ability to cause cholera. *Vibrio* sp. is a well-known pathogen and has contributed to bivalve aquaculture losses (Nguyen et al. 2018). The surrounding waters may also contain harmful strains of *Escherichia coli* bacterium that is present in the gastrointestinal tracts of all warm-blooded animals (Geldreich, 1978). *E. coli* is used as a pollution indicator because if it is present, there may be more harmful microbes that are commonly associated with fecal contamination present as well (Ishii et al. 2007).

Information regarding the pathogenic bacteria species in the brown mussel *P. perna* of Hadhramout coasts is rare. So, the present study was conducted to detect the most important and occupant pathogenic bacteria contaminating the brown mussel in the marine water of Al-Mukalla coasts. This study will give an indication of the safe consumption of these mussels and the contamination degree of the Al-Mukalla marine environment with bacteria.

MATERIALS AND METHODS

Study area

Two marine stations, Broom Station and Mouth of Khor Al-Mukalla Station, located along the coast of Al-Mukalla City, the capital of Hadhramout Governorate, Yemen, were selected for this study (Figure 1).

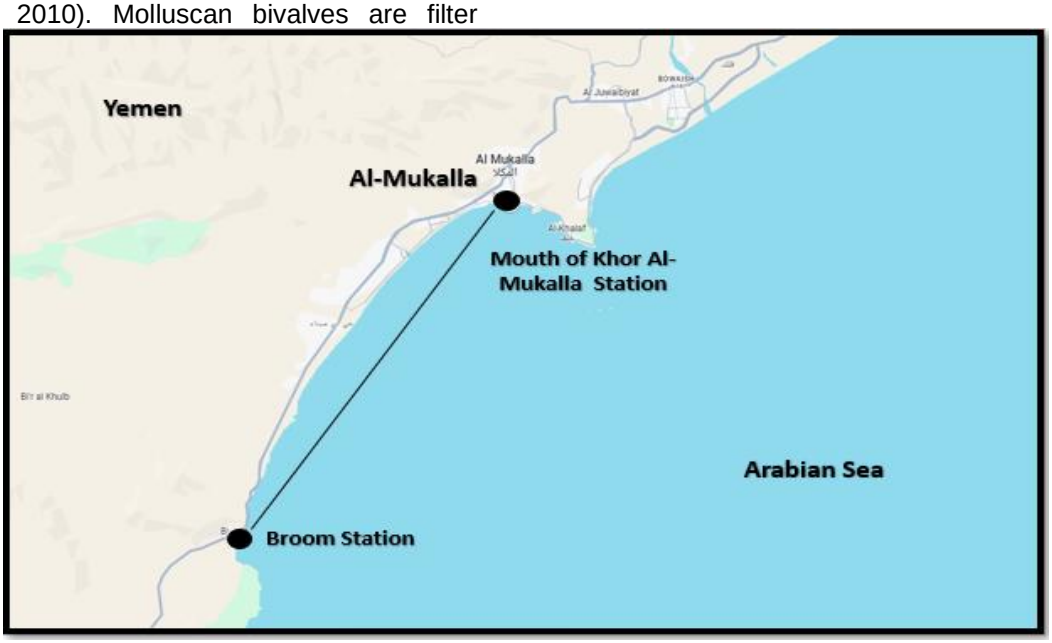

Figure 1: **A map showing the study area on the Al-Mukalla coast (Google Maps**). 14.4464676, 49.0710433

Khor Al-Mukalla, known as “Al aaeqah,” is a water channel that extends along more than 1,870 m with an average width of 70 m and 3 to 5 m and 30 cm depth (Al-Amary, 2006) (Figure 2). Khor Al Mukalla receives the disposed sewage. Brown mussel colonies are densely distributed at the entrance of the channel in the direction

of the sea in the south and less distributed near the Chinese bridge in the north.

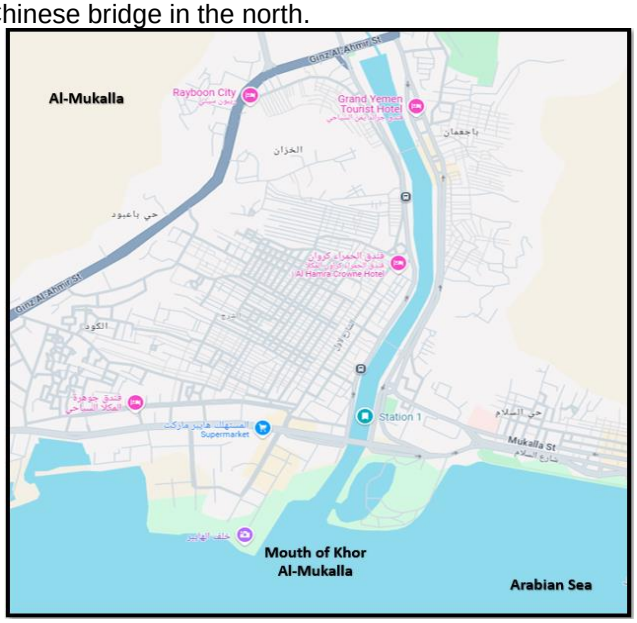

Figure 2: A map showing the location of Khor Al-Mukalla. 14.535648107427845, 49.126826074810616 (Google Maps)

Sampling.

Brown mussels of *P. perna* (250 samples) were monthly collected from October 2018 until October 2019 from the exposed coast rocks, especially from the intertidal zone, using knives and sharp devices. Samples were placed in sterile plastic containers with ice cubes and taken directly to the laboratory. Diving was sometimes necessary to collect samples, especially in Broom Station due to the attachment of brown mussels to the concrete wall of the creek channel about 1 m deep in the station of Khor al-Mukalla mouth.

Mussel processing.

Samples of *P. perna* were washed from byssal threads and from sediments adhering on the shell surface using clean water and knife. The collected samples were tested monthly to isolate the most common associated pathogenic bacteria from their soft tissue brown mussel by inoculation on special media and incubation under the suitable conditions. The most important pathogenic bacteria isolated from the brown mussels were subjected to microscopic examination using Gram stain and some biochemical tests.

Media used.

All media used in this study are products of HiMedia, Mumbai, India. MacConkey Broth Purple medium was used for enumeration and isolation of fecal coliforms (37°C) and *E. coli* (44°C). MacConkey Agar was used to distinguish Gram-negative bacteria that can ferment the sugar lactose, such as *Escherichia coli, Enterobacter,* and *Klebsiella,* from those that cannot ferment lactose, such as *Salmonella, Proteus* species*, Yersinia, Pseudomonas aeruginosa,* and *Shigella*. Alkaline peptone water medium was used for the enrichment of Vibrio species. Peptone water was used as a growth medium and as a base for carbohydrate fermentation media. Baird-Parker agar medium was used for selective isolation of Gram-positive *Staphylococci species.* Thiosulfate-Citrate-Bile Salts-Sucrose Agar (TCBS) was used for the isolation of *Vibrio cholerae* and other enteropathogenic *Vibrio*s (in particular *Vibrio parahaemolyticus*). Selenite broth was used as an enriched medium for the isolation of *Salmonella* species. Xylose Lysine Deoxycholate (XLD) agar was used as a selective differential medium for the isolation of *Salmonella* species

Microbiological experiments.

Fifteen grams of fleshy body parts of brown mussels were placed into sterile stomacher bags, then diluted (1:10) with buffered peptone water medium and mixed well until homogenized. Samples were used for further experimentation.

Enumeration, isolation and identification of Coliform and *Escherichia coli.*

Afterward, decimal dilutions with sterile buffered diluents were prepared. Using at least 3 consecutive dilutions, each one contains 10 ml of macconkey broth (himedia, Mumbai, India) (Adams & M. O. Moss., 2008). Two ml were taken from the mixture, 1 ml was added to a tube of dilution 10-1, and the second ml was added to a tube containing macconkey broth. Then, two ml were taken from dilution 10-1 using a sterile pipette, 1 ml was added to a tube of dilution 10-2, and the second ml was added to a tube containing macconkey broth. Finally, 1 ml was taken from dilution 10-2 and added to a tube containing macconkey broth using a sterile pipette and incubated at 37°C ± 0.5°C for 24 ± 2 h. The tubes in which the organism produced gas due to lactose fermentation were recorded as a positive result. Tubes lacking gas were re-incubated for another 24 h. A confirmation test was made for the positive tubes, and for enumeration, the most probable number (MPN g^{-1}) was calculated using the data of Anderson & Baird Parker and Spencer & de Spencer (anderson & baird-parker, 1975; Spencer & Spencer, 2001). For *E. Coli* detection, from each positive tube, a loopful of suspension was transferred to a tube of peptone water for the indole test using Kovacs reagent and another loopful to a tube of macconkey broth for the detection of gas production and lactose fermentation; both tubes were incubated at 44°C ± 2 at 24 ± 2 hours (Anderson & Baird Parker, Spencer & de Spencer, ANDERSON & BAIRD-PARKER, 1975; Spencer & Spencer, 2001). If both tubes are positive, the test of *E. coli* presence will be recorded. If only one tube is positive, the test is recorded as fecal coliform positive. MacConkey

agar plates were inoculated from the expected *E. coli* suspended on the positive tubes. Ten colonies were randomly selected and examined for colony morphology, Gram reaction, cell morphology, motility, and biochemical tests: oxidase, gas production, and lactose fermentation. IMVIC reaction was used to confirm their identification.

Enumeration, isolation, and identification of *Staphylococcus aureus*.

One mL of prepared sample was taken spread on a petri dish containing Baird-Parker agar medium (HiMedia, Mumbai, India) and another one mL was used to prepare 10-2 and 10-3dilutions and incubated at 37± 1 oC for 48 hours (Adams & M. O. Moss., 2008). Colonies of expected *Staph. aureus* was enumerated and referred to as colony-forming units (CFU) per one gram of the initial mussel sample. For identification, ten colonies were randomly selected and tested for colony morphology, Gram reaction, cell morphology, catalase and coagulase production, novobiocin sensitivity, and IMVIC reaction was used to confirm their identification.

Enumeration, isolation, and identification of *Vibrio parahaemolyticus.*

Decimal dilutions with sterile alkaline peptone previously prepared were incubated at 37°C for 8 hours to prevent overgrowth of the vibrios by other organisms. The growing colonies were enumerated and referred to as colony-forming units (CFUs) per one gram of the initial mussel sample. After 8 hours, a loopful of the mixture was taken and spread on a petri dish containing thiosulfate citrate bile salt sucrose agar (TCBS agar) and incubated at 37°C for 24 hours (Adams & M. O. Moss., 2008). Ten colonies will be selected randomly and tested for their colony morphology, Gram reaction, cell morphology, motility, catalase and oxidase activity, and IMVIC reactions.

Enumeration, isolation, and identification of *Salmonella typhi*.

Decimal dilutions with sterile buffered peptone water were prepared and mixed well until the sample dissolved, then incubated at 37°C for 16-20 hours. The growing colonies were enumerated and referred to as colony-forming units (CFUs) per one gram of the initial mussel sample. One mL of the broth culture was added to a 10 mL tube of selenite broth and incubated for 24 hours at 37°C. A loop full of the broth culture was planted in a petri dish containing XLD agar and incubated at 37°C for 24 hours (Adams & M. O. Moss., 2008). Ten colonies were selected randomly and tested to confirm the characteristics of *S. typhi.*

Statistical analysis.

The data of the experiments were analyzed using analysis of variance (ANOVA) techniques, t- test and the mean differences between treatments of types and numbers of isolated bacteria, depending on the site of station and on season of growth for significant differences ($P < 0.05$) using a Duncan's Multiple Range Test. Statistical analysis was performed using the version package of the SPSS program.

RESULTS AND DISCUSSION

Enumeration, isolation and identification of bacteria isolated from brown mussels of *P. perna* obtained from the Mouth of Khor Al-Mukalla. Enumeration, isolation, and identification of the most common types of pathogenic bacteria living in the soft tissue brown mussels of P. perna collected from the coasts of Al-Mukalla were the most important purposes of this study.

Enumeration, isolation, and identification of *coliforms.* The total count of coliforms contained in the studied brown mussel *P. perna* was estimated during the whole year; the highest value was achieved during summer (131.33 MPN g^{-1}), while the lowest value was during winter (117.00 MPN g^{-1}) (Table 1 and Figure 3).

Enumeration, isolation, and identification of *Escherichia coli*. The *Escherichia coli* isolated from the soft tissue of *P. perna* were enumerated during the seasons using the MPN method, which gives an important indication of fecal pollution of the environment. Their total number was 26.30 MPN g^{-1} during the summer season; it was the highest value away from the lowest value (11.13 MPN g^{-1}), which was achieved during winter (Table 1 and Figure 3). The confirmation study of characteristics of the selected bacteria showed pink colonies, and the cells were motile Gram-negative bacilli, lacked oxidase activity, were indole positive, gas producers, lactose fermenters, methyl red positive, and unable to mediate citrate fermentation or Voges-Proskauer reactions.

Enumeration, isolation, and identification of *Staphylococcus aureus*. Detection of *Staph aureus* in the brown mussel *P. perna* differed in abundance depending on the season. The highest number (90.37 CFU g^{-1}) was recorded in summer, while the lowest value (46.30 CFU g^{-1}) was achieved in winter (Table 1). The colonies randomly selected from the surface of Baird-Parker agar appeared shiny black with clear zones, and the cells were Gram-positive cocci with grape-like arrangements. Culturally, they were novobiocin sensitive. Biochemically, they were negative for the indole test and positive for catalase, coagulase, methyl red, Voges proskauer, citrate, and mannitol fermentation.

Enumeration, isolation, and identification of *Vibrio parahaemolyticus* and *Salmonella typhi*. All brown mussel samples collected from the mouth of Khor Al-Mukalla during the study period were free from the growth of *Vibrio parahaemolyticus* and *Salmonella typhi*.

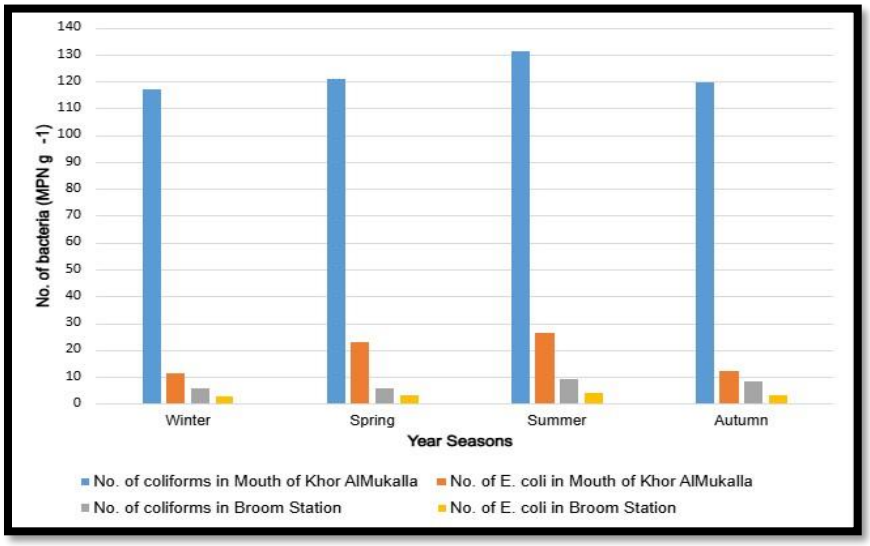

Figure 3: A comparative variation between the counts of coliform and *E. coli* bacteria in the tested samples of mussels obtained from both stations, Mouth of Khor Al-Mukalla and Broom, through the year's seasons.

Table 1: Average count of coliforms, *E. coli, Staph. aureus, V. parahaemolyticus,* and *S. typhi* in *P. perna* mussels obtained from the Mouth of Khor Al-Mukalla during the year's seasons and the Standardization Organization for Gulf Cooperation Council (GSO) standard

Season	No. of coliforms (MPN g^{-1}) Mean ± SD.	No. of *E. coli* (MPN g^{-1}) Mean ± SD.	No. of *Staph. aureus* (CFU g^{-1}) Mean ± SD.	No. of *V. parahaemolyticus* (CFU g^{-1}) Mean ± SD.	No. of *S. typhi* (CFU g^{-1}) Mean ± SD.
Winter	117.00^{a}±75.68	11.13^{a}±4.41	46.30^{a}±17.13	0.0	0.0
Spring	120.78^{a}±61.17	22.72^{b}±8.15	85.93^{b}±55.63	0.0	0.0
Summer	131.33^{a}±54.20	26.30^{b}±5.88	90.37^{b}±51.33	0.0	0.0
Autumn	119.44^{a}±64.46	12.33^{a}±7.6	52.59^{b}±24.59	0.0	0.0

* Results are presented as the mean value of duplicate trials ± standard deviation (SD). 0.0: no growth. No.: number. MPN: Most Probable Number. SD: Standard Deviation. CFU g^{-1}: Colony Forming Unit per gram of sample.

Table 2: **Average* of the total count of coliforms, *E. coli, Staph. aureus, V. parahaemolyticus,* and *S. typhi* in *P. perna* mussels obtained from Broom Station during the year's seasons and GSO standard**

Season	No. of coliforms (MPN g^{-1}) Mean ± SD.	No. of *E. coli* (MPN g^{-1}) Mean ± SD.	No. of *Staph. aureus* (CFU g^{-1}) Mean ± SD.	No. of *V. parahaemolyticus* (CFU g^{-1}) Mean ± SD.	No. of *S. typhi* (CFU g^{-1}) Mean ± SD.
Winter	5.60^{a}±2.73	3.15^{a}±2.29	0.0	0.0	0.0
Spring	5.75^{a}±2.86	3.69^{a}±2.07	0.0	0.0	0.0
Summer	9.19^{b}±5.07	4.52^{a}±2.64	0.0	0.0	0.0
Autumn	8.14^{ab}±6.17	3.76^{a}±2.62	0.0	0.0	0.0

* Results are presented as the mean value of duplicate trials ± standard deviation (SD). 0.0: no growth. No.: number. MPN: Most Probable Number. SD: Standard Deviation. CFU g^{-1}: Colony Forming Unit per gram of sample

Enumeration, isolation, and identification of bacteria isolated from brown mussels of *P. perna* obtained from Broom Station

Enumeration, isolation, and identification of coliforms. The brown mussel of the studied *P. perna* samples was high in the number of contained coliforms, whereas the highest number was achieved during the summer season (9.19 MPN g^{-1}), and the lowest number was achieved during winter (5.60 MPN g^{-1}). A great difference was detected between the count of coliforms isolated from mussels of Khor Al-Mukalla Station and that of Broom Station through all seasons, mussels of Mouth of Khor Al-Mukalla Station were containing the highest counts (Table 2 and Figure 3).

Enumeration, isolation, and identification of *E. coli*. Determination of the total number of *E. coli* contained in

the soft tissue of *P. perna* during the year's seasons was most important. We noticed that the highest number of the contained *E. coli* was 4.52 MPN g^{-1} during summer, while the lowest number (3.15 MPN g^{-1}) was achieved during winter. The studied brown mussels of Broom Station were containing lower counts of *E. coli* if compared with that of Khor Al-Mukalla Station. (Table 2 and Figure 3).

The characteristics of the selected bacteria were morphologically motile Gram-negative bacilli, biochemically indole and methyl red positive, gas production and lactose fermentation positive, unable to mediate citrate or Voges-Proskauer reactions, and oxidase activity was negative.

The types and numbers of isolated bacteria in the edible mollusk bivalve varied depending on the site of isolation and growth season. The statistical analysis between the numbers of tested bacteria in the two stations (Mouth of Khor Al-Mukalla and Broom) showed significant differences at $P<0.05$, and the statistical analysis showed that there were statistically significant differences ($P<0.05$) among the counts of coliforms in the brown mussel *P. perna* collected from the mouth of Khor Al-Mukalla during year seasons, and no significant differences among the counts of *E. coli* ($P=0.319$). For Broom station, statistically significant differences were detected also among the *E. coli* numbers during year seasons ($P<0.05$), while the differences among the counts of coliforms were not significant ($P=0.107$). The higher total number of coliforms contained in *P. perna* mussels during the warm summer months (Table 1) referred to adequate water temperature and salinity conditions enabling bivalves to increase their filtration capacity and thus can retain many more microorganisms (CEFAS, 2014). The increase of coliform number during summer also occurred in brown mussel *P. perna* obtained from Broom Station, and significant differences ($P<0.05$) were found with that of winter months. The high temperature of the summer season led to the elevation of coliform numbers isolated from mussels obtained from both stations because the summer temperature was optimum to accelerate filtration rates. This result agrees with the results of Jozić and Šolić (Jozić et al. 2012; Šolić et al. 2007). *P. perna* mussels living in Broom station were tested and found somewhat free from sewage pollution due to lack of sewage discharging. This refers to the safe environmental quality of the bivalve health because there is a significant relationship between the level of fecal contamination in aquatic environments and the immune response of mussels, as mentioned by Silva dos Santos et al. (2018).

In both stations, the count of *E. coli* varied from one season to another and was high in summer and low in winter because the warm temperature is optimum to accelerate filtration rates and suitable for proliferation. Using one-way ANOVA, data showed significant differences ($P<0.05$) between the values of the number of *E. coli* isolated from the samples of both stations. During winter, the reduced presence of *E. coli* in *P. perna* obtained from both stations indicates that the high temperature has a strong influence on the germination of bacteria and plays a major role in the physiological status of bivalves because such organisms are ectothermic; the same results were reported by Bayne (Bayne, 1976). In the same season, the count of *E. coli* found in the brown mussel collected from the mouth of Khor Al-Mukalla Station exceeded that found in the mussel collected from Broom Station. Kueh (Kueh, 1987) and Diego & Júlia (Martinez & de Oliveira, 2010) reported that the accumulation of *E. coli* and other enteric organisms in bivalves resulted from a dynamic process due to filtration by gills as mentioned above.

Regarding the pollution of the studied mussels with *Staph. aureus*, it was noticed positively in the mussels of the mouth of Khor Al-Mukalla station, while in Broom station, mussels lack their presence due to absence of discharging of wastewater to Broom coast. Like coliforms and *E. coli*, *Staph. aureus* germinates well during the suitable summer temperature. Consumption of mussels containing a high number of foodborne pathogenic *Staph. aureus* is regarded as a potential risk leading to the development of foodborne diseases (Lanciotti et al. 2001; Salán et al. 2008). Pathogenic *Staph. aureus* is a causative microorganism for the most common enteric infections in humans (Kumar et al. 2009; Lei et al. 2008). (Mus et al. 2014) reported that isolates of *Staph. aureus* was isolated from mussels.

In this study, it was confirmed that *E. coli* and *Staphylococcus aureus* isolated from brown mussels were well identified depending on their cultural properties, colony and cell morphology, and biochemical characteristics. *V. parahaemolyticus* and *S. typhi* were absent in the studied brown mussel obtained from both stations, the same result was reported by many researchers such as (Mus et al. 2014) and (Helms et al. 2005). The study indicated that the aquatic coasts of Al-Mukalla city are free from *V. parahaemolyticus* and *S. typhi*.

CONCLUSIONS

Finally, the study confirmed that the numbers of total coliform, *E. coli*, *Staph. aureus*, *S. typhi,* and *V. parahaemolyticus* isolated from the brown mussel *P. perna* obtained from the mouth of Khor Al-Mukalla and from Broom stations were still located in the allowed range of (GSO) that states to avoid eating mussels if the count of bacteria, *E. coli*, *Staph. aureus*, *V. parahaemolyticus,* and *Salmonella typhi* exceeds 7×102, 103, 103, and 0.0, respectively (Global Standardization Organization, 2015).

Supplementary materials

Not applicable

Author contributions

Conceptualization, M.S.B. and M.A.B.; methodology, M.S.B. and M.A.B.; formal analysis, M.S.B. and M.A.B.; Data Collection, M.S.B. and M.A.B.; writing original draft, M.S.B. and M.A.B.; writing-review and editing, F.S.B. and A.H.A.; supervision, M.S.B. All authors read and approved the final version.

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Informed Consent Statement

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Data Availability Statement

All the data included in this manuscript will be available on demand.

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Conflict of interest

The authors declare no conflict of interest.

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