Pathogenic Bacteria and Their Effect on Organoleptic Characters in Gilthead Sea bream, *Sparus aurata*

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I affirm that, the manuscript has been prepared in accordance with Instructions. The contribution during the whole process of the articles as the following:
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Abstract

In this study, 60 naturally infected seabreams (*Sparus aurata*) were randomly collected from several fish farms in Port-Said Governorate. Fish were subjected to clinical, postmortem, bacteriological, and molecular examinations. Furthermore, the organoleptic changes of fish fillet, including odor, color, general appearance, slime, and flesh consistency, were investigated. Grossly, the infected fish showed exophthalmia, cloudiness in the eyes, and abdominal ascites with a prolapsed vent. The visceral organs also appeared enlarged and varied in color from pale to congest following dissection. Bacteriologically, *Vibro parahaemolyticus* (44.8%), *Aeromans hydrophila* (34.5%), and *Pseudomonas aeruginosa* (20.7%) were recovered from the diseased fish. Molecular studies of the retrieved isolates detected various virulence genes, such as *trh* for *V. parahaemolyticus*, aerolysin for *A. hydrophila*, *toxA*, and *psl*A for *P. aeruginosa*. Following 7 days of ice storage, the control samples were in acceptable condition, whereas the *P. aeruginosa*-inoculated samples became inedible and only usable after 4 days of storage.

The above-mentioned findings showed the importance of implementation a strict control measures to combat the problems posed by resistant pathogens in the mariculture industry that cause food spoilage.

Keywords:
Sea bream, Bacterial pathogens, Virulence genes, Organoleptic Changes.

Introduction

Fish and fish products are the most significant source of protein, making aquaculture one of the most significant industries **(*Beveridge et al.*, 2013).** Fish has therefore become a key resource in Egypt to meet the needs of a rapidly expanding human population in terms of food and nutrition security. Additionally, Egypt has the largest aquaculture industry in Africa with a market value of over 2.18 billion$ *(CAPMAS 2014). **Khalil and Abd El-Latif, (2013)*** declared that mariculture is a significant investment for fishermen in Egypt; nevertheless, the main challenges to the sustainability and profitability of this industry are diseases and high feeding costs. For instance, bacterial diseases, particularly those caused by resistance pathogens, pose a serious threat to aquaculture systems and cause severe damage and mortality in Egyptian fish farms ***(Aly et al., 2019, and Aly et al., 2020).*** Additionally, according to ***Austin and Austin (2016a),*** among the economically relevant bacterial fish diseases infecting marine fishes are those caused by the *Aeromonas, Vibrio*, and *Pseudomonas* bacterial species. The bacterial pathogens not only have an economic impact on the aquaculture industry, but they also have an impact on the quality and durability of fish meat, threatening human consumer health ***(Umaraw et al., 2020).***

The greatest threats to public health are thought to be consumption of contaminated seafood, the emergence and spread of bacteria resistant to antibiotics and their resistance genes, as well as the dissemination of resistance genes through horizontal gene transfer, that poses several risks to public health ***(Amarasiri et al., 2020).*** Additionally, fish shelf-life is influenced by a variety of factors, including the amount of ice used, the period of time the fish is stored, the temperature, the species, and the degree of stress the fish experienced during catch ***(Duarte et al., 2020).***

For instance, one of the main fish-borne spoilers is *Pseudomonas* species, which has a lipolytic activity that causes fish products to lose more weight, hold less water, change in texture, and smell ***(Tomaś and Myszka, 2022).***

Thus, the current study aims to isolate and identify the pathogenic bacteria from Gilthead seabream (*Sparus aurata*), besides

investigating how they may affect fish organoleptic alterations and shelf life.

Materials and methods

1. Sampling

In the current study, 60 clinically affected Gilthead seabream (*Sparus aurata*) were collected randomly from different fish farms in Port-Said Governorate. Moribund and freshly dead fish were kept in tightly closed plastic bags and then packed in an ice box surrounded with crushed ice prior to being transferred immediately to the bacteriological unit at Animal Health Research Institute, Port-said lab. for clinical, postmortem, and bacteriological examinations.

2. Bacteriological analysis

Moribund and freshly dead fish samples were examined bacteriologically under strict aseptic conditions. Loopful from the spleen, liver, kidney, and skin lesions of the infected Gilthead Sea bream were inoculated into 10% tryptic soya agar broth, supplemented with 2% NaCl, incubated at 25° c for 24-48h., then streaked on different lab media, including Pseudomonas selective agar base (LABM) for *pseudomonas* spp., Aeromonas selective agar base supplemented with ampicillin (LABM) for *Aeromonas* spp., and thiosulfate citrate bile salt sucrose agar TCBS agar (Oxoid) for *Vibrio* spp., and incubated at 28 ºC for 24 hrs. The retrieved suspicious colonies (*Pseudomonas*, *Aeromonas* and *Vibrio*) were then plated onto tryptic soy agar, supplemented with 2% NaCl, and purification was carried out overnight at 28 °C. Pure bacterial isolates were stored in glycerol (20%) media for further identification. The bacterial isolates were identified phenotypically through Gram's stain, oxidase, and catalase tests, **(Austin and Austin, 1999)** as well as confirmed biochemically by the Vitek2 system, (bioMerieux, France).

3. Molecular Detection of virulent genes from the isolated bacteria

Three oligonucleotide primers were used for each bacterial isolate for the detection of virulence genes. These genes were *tlh*, *tdh*, and *trh*, for *Vibrio parahaemolyticus*, Hly, Aerlysin and lipase for *Aeromonas hydrophila* and *tox*A, *las*I, and *psl*A for *Psudomonas aeruginosa* **(Table 1).** For amplification of *V. parahaemolyticus tlh*, *tdh* and *trh* gene mixture, include the following: 0.5µM each primer, 200 µM each deoxynucleoside triphosphate (dNTP), 1X PCR buffer, 1.25 units of Taq, and yielded single bands of 450 bp (*tlh*), 245 bp (*tdh*) or 410 bp (*trh*). The thermal-cycling programme for each gene was as follows: denaturation at 95°C for 5 min, followed by 40 cycles involving 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min, and a final expansion at 72°C for 2 min **(Bej *et al.*, 1999).**The amplification of *A. hydrophila hly*, Aerolysin and

lipase genes, according to ***(Rozi et al., 2018, Singh et al., 2008, Sen and Rodgers, 2004)*** and *P. aeruginosa tox*A, *las*I and *psl*A genes, according to ***(Ghadaksaz et al., 2015, Bratu et al., 2006, Matar et al., 2002)*** was performed for each primer; respectively.

4. Detection of the organoleptic alterations of experimentally inoculated *Sparus aurata*.

To judge the fish quality, the grading of fish using scores on its characteristics has been done following the European Communities Commission (ECC) freshness grade for fishery products **(Howgate *et al*., 1992).** The organoleptic characteristics of quality changes during the storage period was divided into three phases, which were of 0-2, 2-4, and 4-7 days in ice box, containing bags of crushed which was replaced daily during the storage period (7 days). Ten marine fish, Gilthead seabream (average weight of 150 gm), were collected and transferred immediately in sterile plastic bags on ice to the laboratory. *Pseudomonas aeroginosa* was selected to experimentally studying the organoleptic changes, because of its' lipolytic activity that causes fish products to lose more weight, hold less water, change in texture, and smell (**Tomaś and Myszka, 2022**). After that, fish were divided into two groups; the first is the control group (not inoculated), and the second is the *P. aeruginosa* inoculated group with 10^5 cfu of bacterial suspension. The organoleptic changes were assessed in the fish fillets of fish groups after their storage on the ice for zero, 96, and 168 h. (10 fish fillet samples/each storage period).

5. Ethical statement

Strict guidelines for the use of laboratory animals were followed in the conduct of this study. All experimental procedures were approved by the local Administrative Panel on the Laboratory Animal Care Committee and conformed by Suez Canal University's Animal Care and Use Guidelines.

Table 1. Oligonucleotide primers of target genes for *V. parahaemolyticus, A. hydrophila,* and *P. aeruginosa.*

Primer	Primer Sequence 5'-3'	Amplified product	Reference
V. parahaemolyticus			
L-tlh	5´-AAAGCGGATTATGCAGAAGCACTG-3´	450 bp	**(Bej *et al*., 1999)**
R-tlh	3´-GCTACTTTCTAGCATTTTCTCTGC-5´		
L-Tdh	5´-GTAAAGGTCTCTGACTTTTGGAC-3´	245 bp	
R-Tdh	3´- TGGAATAGAACCTTCATCTTCACC-5´		
L-Trh	5´- TTGGCTTCGATATTTTCAGTATCT-3´	410 bp	
R-Trh	3´- CATAACAAACATATGCCCATTTCCG-5´		
A. hydrophila			
Hly-F	5´-GGCCGGTGGCCCGAAGATACGGG-3´	592	***(Rozi et al., 2018)***
Hly-R	3´-GGCGGCGCCGGACGAGACGGGG-5´		
AHA-F	5´-CACAGCCAATATGTCGGTGAAG-3´	326	***(Singh et al., 2008)***
AHA-R	3´-GTCACCTTCTCGCTCAGGC-5´		
Lip-F	5´-ATCTTCTCCGACTGGTTCGG-3´	382	***(Sen and Rodgers, 2004)***
Lip-R	3´-CCGTGCCAGGACTGGGTCTT-5´		
P. aeruginosa			
ETA-1	5´-GGACAACGCCCTCAGCATCACCAGC-3´	396	***(Matar et al., 2002)***
ETA-2	3´-CGCTGGCCCATTCGCTCCAGCGCT-5´		
LasI-F	5´-ATGATCGTACAAATTGGTCGGC-3´	606	***(Bratu et al., 2006)***
LasI-R	3´-GTCATGAAACCGCCAGTCG-5´		
pslA-F	5´-TCCCTACCTCAGCAGCAAGC-3´	656	***(Ghadaksaz et al., 2015)***
pslA-R	3´-TGTTGTAGCCGTAGCGTTTCTG-5´		

Results

Clinical and Postmortem examination

The clinical examination of naturally collected *Sparus aurata* revealed scale detachment, exophthalmia, ocular opacity, darkening of the exterior body surfaces, and some massive, irregular hemorrhages that were dispersed throughout the body, particularly at the base of the fins and in the anal region. Some of the fish had quite obvious abdominal distention **(Fig. 1).** Moreover, postmortem examination showed white to yellow serous fluid with some red tinged fluid in the abdominal cavity. In some cases, the liver was engorged and hemorrhagic, while in others, they were pale, friable, and dotted with hemorrhages. In many cases, the spleen was pallor, enlarged, and swollen; in others, it seemed normal. The kidneys had a little enlargement and were congested **(Fig.2).**

Bacteriological examinations

The isolated Gram-negative bacteria were recovered from the examined *Sparus aurata* fish that were identified biochemically as *A. hydrophila*34.50%, *P. aeruginosa* 20.7% and *V. parahaemolyticus* 44.8%

3.3 Molecular detection of virulence genes among isolated *V. parahaemolyticus, A. hydrophila, and P. aeruginosa*

The *trh* virulence gene was identified among *V. parahaemolyticus* isolates by molecular analysis of the PCR

products of the studied bacterial isolates. Additionally, *A. hydrophila* isolates contained the *aerA* virulence gene. Meanwhile *tox*A and *psl*A virulence genes were detected in isolated *P. aeruginosa* **(Fig. 3).**

Detection of the organoleptic alterations of experimentally inoculated *Gilthead sea bream*

The three phases of the organoleptic features of quality changes that happened throughout the storage period correspond to periods of 0-2, 2-4, and 4-7 days in ice displayed that in phase 1, both groups of fish had their original flavor and odor. At this point all of the samples had the traits of recently captured fish. Except for a minor loss of natural flavor in the control sample during phase 2 and some meat softening with slime coverage as well as a mildly sour odor in the inoculated sample with not much degradation**.** At phase 3, there was some softening of the flesh with slight sour odor of control sample, while the fish fillet begins to soften, sour odor and flesh covered with slime in inoculated sample, **(Table 2).**

Fig.1. Naturally infected *Sparus auratus* wih *V.parahaemolyticus* showing darkening of the back (white arrow) and emaciation.

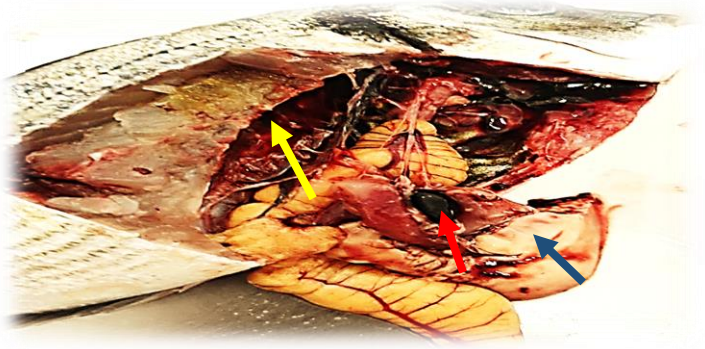

Fig.2. Naturally infected *Sparus auratus* by *A.hydrophila* showing pale liver (blue arrow) , congested kidney(yellow arrow) and spleen (red arrow

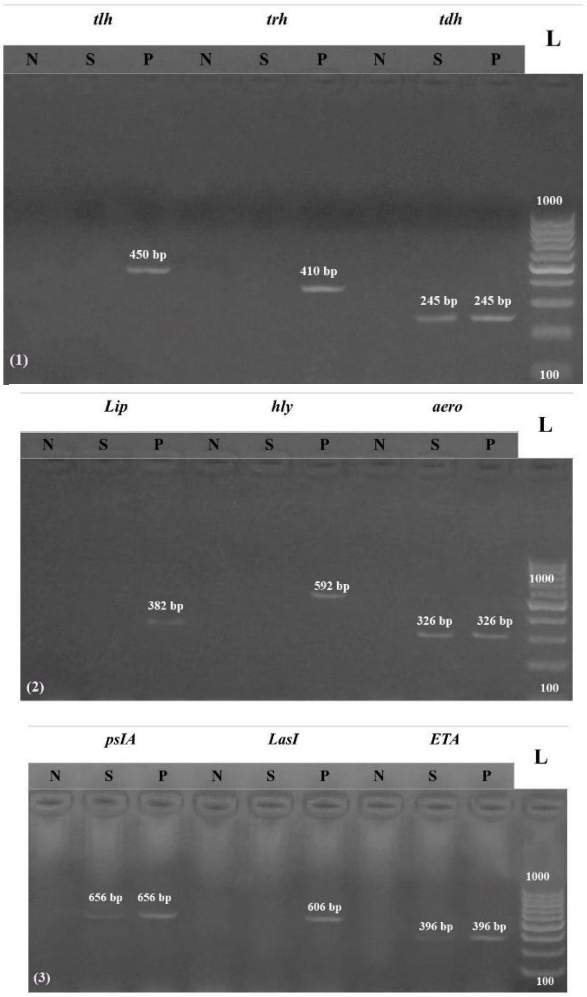

Fig. 3. PCR products of virulence genes among isolated *V. parahaemolyticus, A. hydrophila,* and *P. aeruginosa* from naturally collected *Sparus aurata.* **(1)** Detection of *V. parahaemolyticus* virulence genes (at 450 bp for *tlh* Gene, and 410 bp for *trh* gene, and 245 bp *tdh* gene). **(2)** Detection of *A. hydrophila* virulence genes (at 382 bp for lipase gene, 592 bp for Hly gene and 326 bp for aero gene). **(3)** Detection of *P. aeruginosa* virulence genes (at 656 bp for *pslA* gene, 606 bp for *lasl* gene and 396 bp for *toxA* gene).Lane L: 100-1000 bp. DNA Ladder, N.: Negative control, P.: Positive control *Pseudomonas aeruginosa* toxA gene positive at 396 bp and pslA gene positive at 656 bp. Lane L: 100-1000 bp. DNA Ladder, N.: Negative control, P.: Positive control *A.hydrophila* aero gene positive at 326 bp. Lane L: 100-1000 bp. DNA Ladder, N.: Negative control, P.: Positive control *V. parahaemolyticus* trh gene positive at 250 bp.

Table 2. Organoleptic changes in experimentally inoculated Gilthead sea bream, *Sparus aurata.*

Examined samples	Days in ice	Organoleptic changes	Grade	Overall quality
Control sample	**0**	Firm, elastic flesh and natural fishy odor.	A	Excellent
	4	Some loss of elasticity of flesh and natural odor	B	Good
	7	Some softening of flesh and slightly sour odor.	B	Good
Inoculated sample	**0**	Firm, elastic flesh and natural fishy odor.	A	Excellent
	4	Softening of flesh and moderately sour odor.	B	Acceptable
	7	Soft flesh , sour odor and flesh covered with slime	C	Rejected

A= Excellent B= Good / Acceptable C= Rejected

Discussion

The most significant cause of disease outbreaks in all types of fish farming is bacterial infections. Epizootics are brought on by bacterial infections in almost all marine and freshwater fish, both cultivated and wild ***(Defoirdt et al., 2011).***

Marine fish are susceptible to a wide variety of bacterial pathogens. In the current study, clinically naturally infected marine fish (*S. aurata*) exhibited various clinical lesions ranging from darkness of the external body surface, opacity of the eye, exophthalmia, and scale detachment to large irregular hemorrhagic areas in many parts of the body. Previously, vibriosis-infected Gilthead sea bream displayed similar symptoms ***(Aly et al., 2019, Aly et al., 2020).*** Additionally, the postmortem examination of the examined fish pointed out that the liver appeared to be pale, anemic, and friable, with some hemorrhagic patches on its surface. Kidney and spleen were congested and slightly enlarged. Our results agreed with those detected in by ***(Austin et al., 2007)***.

The present study showed that, *V. parahaemolyticus* was the most prevalent (44.8%), followed by *A. hydrophila* (34.5%), and *Ps. aeruginosa* (20.7%). These results could be attributed to the fact that *vibrio* pathogen is a halophilic bacterium, making it one of the main bacterial pathogen threats to the mariculture system, followed by the *Aeromonas* and *Pseudomonas* species. Similar studies support these findings ***(Thune et al., 1993, Wellington et al., 2013, Elsayed et al., 2018).*** According to ***Meyer (1991),*** Gram-negative bacteria are the most common cause of bacterial disease infection in aquaculture. Additionally, ***Elsayed* et al. *(2018)*** reported that *Aeromona*s, *Vibrio*

and *Pseudomonas* species were the most common isolated bacterial pathogens from naturally examined marine fishes. In the same context, ***Zorrilla et al. (2003)*** reported that, *Vibrio* species were the most common species isolated from seabream fish, accounting for 69.90% of the total. These findings could be attributed to the difference between the geographical sampling location, fish species and health condition, immune system of the fish, and water, as well as water quality.

The present study elucidated that the virulence gene, the thermostable direct hemolysin-related hemolysin, *trh*, was detected in *V. parahaemolyticus* isolates from diseased fish, which is in agreement with an earlier study ***(Bej* et al., *1999).*** Likewise, this finding is consistent with that recorded by ***Serracca* et al. *(2011)*** who identified the virulence genes (*tox*R, *trh*, and *vvh*) of *V. parahaemolyticus* and *V. vulnificus* and detected *trh* and *tdh* in 16% of samples. Also, ***Casandra* et al. *(2013)*** confirmed that the virulence genes encoding the thermostable direct hemolysin (*tdh*) and *trh* are strongly correlated with the virulence of the emergent human pathogen *V. parahaemolyticus*.

The detection of the *aerolysin* (*aer*A) gene from isolated *A. hydrophila* isolates in this study was based on an earlier study ***(Rozi* et al., *2018).*** Similarly, ***Algammal* et al. *(2020)*** stated that the *aer*A gene was detected in 100% of *A. hydrophila* isolates. ***Kingombe et al. (1999)*** confirmed these findings, stating that the virulence genes of Aeromonas species were classified as aerolysins-hemolysins, cytolytic enterotoxins, or cytotoxic enterotoxins. Additionally, ***Singh* et al. *(2009)*** investigated that the highly pathogenic strains of *A. hydrophila* always carry two or more virulence genes, such as the *aer*A, alt, *hly*A, and *ahp* genes. Furthermore, the virulence ability of *P. aeruginosa* was confirmed via detection of the *toxA* gene at 396 bp and *psl*A at 656 bp; the same results were obtained by previous research ***(Ghadaksaz* et al., *2015; Al-Shwaikh* et al., *2017*).**

Organoleptic analyses of the inoculated fish samples with spoilage bacteria demonstrated meat softening with slime production as well as a mildly sour odor when the time of preservation was prolonged compared to the non-spoiled sample. These results are consistent with some previous investigations ***(Gram and Huss, 1996; Koutsoumanis and Nychas, 2000; Parlapani* et al., *2014; Parlapani and Boziaris, 2016).*** Thus, because the samples were inoculated with *P. aureginosa*, the most major spoiler of fresh fish at low temperatures, the organoleptic changes in the fish flesh samples could be attributable to bacterial enzymatic activities during long-term ice storage.

Conclusion

The current study revealed that *Vibrio parahaemolyticus, Aeromonas hydrophila, Pseudomonas aeruginosa* were the most bacterial cause of diseases in marine fish.

Organoleptic quality assessment sensory methods were used to assess the degree of freshness based on organoleptic characteristics such as odor, color, general appearance, slime and consistency of flesh. Besides, *P. aeruginosa* - as one of spoilage bacteria – can make the fish flesh inedible in 7 days of storage.

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البكتيريا المسببة للأمراض وتأثيرها على الصفات الحسية في الدنيس البحري

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الملخص العربي

اشتملت هذه الدراسة على تم جمع ستون سمكه من أسماك الدنيس المصابة طبيعياً (*Sparus aurata*) بشكل عشوائي من عدة مزارع سمكية في محافظة بورسعيد. خضعت الأسماك للفحوصات السريرية ، وفحوصات ما بعد النفوق ، والبكتريولوجية ، والجزيئية الجينية. علاوة على ذلك ، تم فحص التغيرات الحسية لشرائح الأسماك ، بما في ذلك الرائحة واللون والمظهر العام وملمس اللحم. بشكل عام ، أظهرت الأسماك المصابة عده تغيرات مرضيه كجحوظً العين ، وغيما في العينين ، واستسقاءًا في البطن مع تدلى الفتحة الاخراجية. ظهرت الأعضاء الحشوية أيضًا متضخمة ومتنوعة في اللون من شاحب إلى احتقان بعد التسلخ. من الناحية البكتريولوجية ، تم العزل البكتيرى لكل من ميكروب الفيبريو باراهيموليتكس 44.74) ٪ (و ميكروب الايروموناس هيدروفيلا 31.58) ٪ (وميكروب السودوموناس اريجينوزا 23.68)٪ (من الأسماك المريضة. كشفت الدراسات الجزيئية الجيبة للعزلات البكتيرية عن جينات ضراوة مختلفة ، مثل ت ار دى من ميكروب الفيبريو باراهيموليتكس؛ الايرولايسين من ميكروب الايروموناس هيدروفيلا ، و توكس ا ، و بي اس ا ال من ميكروب السودوموناس اريجينوزا . و بعد سبعه أيام من تخزين فى الثلج ، كانت عينات المجموعه الضابطه في حالة مقبولة ، في حين أن العينات المحقونة بميكروب السودوموناس اريجينوزا أصبحت غير صالحة للأكل وبذلك لا يمكن استخدامها إلا بعد 4 أيام فقط من التخزين.

توصي نتائج الدراسة بتنفيذ تدابير رقابة صارمة لمكافحة المشاكل التي تطرحها مسببات الأمراض المقاومة في صناعة تربية الأحياء البحرية والتي تسبب فساد الأغذية.