Received: 02 October 2023, Accepted: 16 November 2023

Original Research

Journal of Advanced Veterinary Research
(2023) Volume 13, Issue 10, 2058-2062

Prevalence of Food Poisoning Microorganisms in Bluespot Mullet Meal

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Abstract

Fish have number of bacteria naturally that can cause foodborne infections. The present work was conducted to determine the prevalence of *Staphylococcus aureus*, *E. coli*, *Pseudomonas* spp. and *Aeromonas* spp. in Bluespot mullet and the antibacterial effect of citric acid against the isolated pathogens. *S. aureus* was detected in 11 and 5 samples representing 44% and 20% of the total examined raw and cooked samples, respectively. Also, *E. coli* was detected in 6 and 2 samples representing 24% and 8% of the total examined raw and cooked samples, respectively. *Pseudomonas* spp. and *Aeromonas* spp. were detected in 10 and 5 samples, representing 40% and 20% of the total examined raw bluespot mullet samples, respectively. While they weren't detected in cooked bluespot mullet samples. The enterotoxigenic strains of *S. aureus* were detected in 27.27% and 20% of raw and cooked samples, respectively. Enterotoxins A, A & C and D were 9.09% while enterotoxin A was identified in 20% cooked bluespot mullets. The serological identification of *E. coli* showed the presence of O156: H7, O127: H6, O125:H21, O15: H12 and O91: H21. *P. aeruginosa* strains were divided into 2 serotypes *P. aeruginosa* serotype (O11 group E and O2 group B). *pslA*, *oprL* and *toxA* virulence genes of *P. aeruginosa* were detected in all examined isolates. Citric acid has a great antimicrobial effect against *S. aureus*, *E. coli*, *P. aeruginosa* and *Aeromonas* spp as it causes complete inhibition for them. The findings suggest the application of citric acid during preparation of bluespot mullet is beneficial.

KEYWORDS
Bluespot mullet, citric acid, *E. coli*, *S. aureus*, *P. aeruginosa*, *Aeromonas* spp.

INTRODUCTION

Fish is a vital source of food for people globally. Around 60 per cent of the developing countries derive 30 percent of their annual protein from fish (Abisoye *et al*., 2011). Salted fish is a traditional food product that is produced by salting and drying fish, and it is widely consumed in many parts of the world (Belton *et al*., 2022). Bluespot mullet, *Valamugil seheli*, is a fish belonging to the *Mugilidae* family, and found worldwide in coastal temperate and tropical water. The mullets are of considerable importance in the capture and culture fisheries in many parts of the world and demand has increased during the last decade (Al-Asous and Al-Harbi, 2017).

It is the most important source of high quality protein, providing approximately 16 percent of the animal protein consumed by the world's population. Fish has become an increasingly important source of protein and other elements necessary for the maintenance of healthy body and constitute an important food component for a large section of the world population. Fish has high consumer preference due to its inherent nutritive value, taste and easy digestibility other essential nutrients and omega 3-fatty acids and its low fat content as compared to other meats (Pal and Das, 2010).

The microbial association with fish compromises safety and the quality for human consumption; particularly critical is when the micro-organisms are opportunistic or pathogenic in nature (Mhango *et al*., 2010). However, fish are susceptible to a wide variety of bacterial pathogens, most of which are capable of causing disease and are considered by some to be saprophytic in nature (Petronillah *et al*., 2013). The prevalence of food poisoning microorganisms in blue spot mullet meat may vary depending on a number of factors, such as the conditions under which the fish were harvested, stored, and transported, as well as the methods used to process and prepare the meat (Edris *et al*., 2020).The pathogenic strains of *E. coli* may cause diarrhoea by producing and releasing toxins (enterotoxigenic *E. coli*) and cause of food borne illness in fish (Austin *et al*., 1995).

The high isolation rate of *S. aureus* indicates poor hygiene and working practices of the meat handlers during the processing stage as well as lack of sterilization of utensils and working surfaces most *Staphylococci* occur as commensally; however, *S. aureus* strains producing various toxins and enzymes are responsible for diseases in animals and humans. The pathogenic properties of *S. aureus* are mainly due to various virulence factor such as heamolysin, and enterotoxins (Choudhary *et al*., 2022). *Pseudomonas aeruginosa* is a particular food safety concern, as it is known to cause poisoning (Visciano *et al*., 2012). Most outbreaks of food poisoning associated with fish and seafood derive from the consumption of raw or insufficiently heat treatment, insufficient cooking and cross contamination during processing

ISSN: 2090-6277/2090-6269/

(Mohammed *et al*., 2017).

Fish sold at open market and exposed to ambient temperature increases likelihood of spoilage. Marketing and handling chain involving fresh fish is important from public health point of view (Kapute *et al*., 2012). So, the present work focused on the studying of microorganisms that are associated with the processing and storage of salted fish and the antibacterial effect of citric acid against the isolated pathogens.

MATERIALS AND METHODS

Preparation of samples

A total of 50 bluespot mullet fish samples, including 25 raw and 25 cooked samples were collected randomly and under aseptic conditions from various retail outlets of Sharqia governorate. The samples were processed within 4-6 hours of collection. Twenty-five grams of the examined samples were aseptically transferred to a sterile stomacher bag and homogenized with 225 ml sterile peptone water (0.1%) for 30-60 seconds to give an initial dilution of 1/10. One ml of the initial dilution was transferred by means of sterile pipette to another sterile tube containing 9 ml of sterile buffered peptone water (0.1%) then mixed thoroughly by using vortex for 5-10 seconds to obtain the next dilution (1:100). This operation was repeated to obtain further decimal serial dilutions up to 10 7 according to ISO (1999).

Isolation of Staphylococcus aureus.

From the previously made serial dilution 0.1 ml dispersed over Baird-Parker agar plates, incubation took place at 35°C for 24-48 h. Suspected colonies were picked up onto nutrient agar slant and incubated at 37°C for 24 hours to help identify them. They were circular, smooth, convex, moist, grey to jet black, with light colored (off-white) margin, surrounded by opaque zone with an outer zone, and had gummy consistency when touched with inoculating needle microscopical examination and biochemical tests performed according to ISO (1999).

Direct extraction of Staphylococcus aureus enterotoxins from salted fishes.

Each sample's ten gram was combined with ten ml of physiological saline (0.85%). The blended sample was homogenized for 30 minutes at a speed of 32.000 x g and a temperature of 4°C in a high-speed cooling centrifuge. 0.2 m low protein binding membrane filter (Mintain plates 4/pk 0.2 m, Millipore Corporation, Bedford) was used to filter the clear supernatant solution. The sample's toxin content was determined using the clear filtrate. According to Oda *et al*. (1979) and Shingaki (1981) the clear culture supernatant fluid was tested serologically by SET-RPLA TD0900 (Oxoid) (A detection kit for the of enterotoxins of staphylococci A, B, C and D) using RPLA technique.

Isolation and identification of E. coli according to ISO (1999)

Serological identification of *E. coli*

Following the procedure outlined by Kaper *et al*. (1979), the detected *E. coli* isolates were serologically typed using the slide agglutination test (standard polyvalent and monovalent *E. coli* antisera).

Isolation of *Pseudomonas* spp. and *Aeromonas* spp.

Twenty five grams of the sample were homogenized with 225 ml of peptone water. After initial enrichment, the bacteria were cultured in Aero Pseudo Selective Agar, Granulated media (HI-MEDIA GM1620) and incubated at 37°C for 24 hours. Colonies were selected based on color and odor (green blue pigment with a specific odor). To confirm *Pseudomonas* and *Aeromonas* species, biochemical tests were done similar to Austin *et al*. (2007) and Shahrokhi *et al*. (2022).

Serological identification of *Pseudomonas aeruginosa*

Serotyping of isolated *Pseudomonas* spp. was applied by using slide agglutination technique according to Glupczynski *et al*. (2010). The International Antigen Typing System was used to divide *P. aeruginosa* into groups based on *P. aeruginosa* O antisera (IATS) according to Legakis *et al*. (1982).

Genotypic detection of some virulence genes of in Pseudomonas aeruginosa

Three pairs of primers (Biobasic, Canada and metabion, Germany) were used. Their specific sequences are showed in Table 1. Bacterial DNA was extracted following QIAamp DNA Mini Kit (Catalogue no.51304) as it provides silica-membrane-based nucleic acid purification from different types of samples. Master Mix preparation and thermal profile was performed according to the instructions of manufacturer (Emerald Amp GT PCR master mix (Takara) Code No. RR310A, AM 41010A) and 1.5% agarose gel electrophoreses (Sambrook *et al*., 1985).

The susceptibility testing of citric acid on the isolated strains

Preparation of citric acids.

Citric acids (Merck Co., Germany) were purchased as powders of reagent grade from the corresponding manufacturers. The citric acid solution was made by weighing 1 g of citric acid and adding it to 30 ml of previously sterilized distilled water. The mixture was then homogenized for an hour in the magnetic stirrer and kept at 4°C. pH meters were used to calculate the pH of citric acid in water.

Table 1. Genotypic sequences of virulence genes of in *Pseudomonas aeruginosa*

Gene	Sequence	Amplified product (bp)	Reference
pslA	TCCCTACCTCAGCAGCAAGC TGTTGTAGCCGTAGCGTTTCTG	656	Ghadaksaz *et al*. (2015)
toxA	GACAACGCCCTCAGCATCACCAGC CGCTGGCCCATTCGCTCCAGCGCT	396	Matar *et al*. (2002)
oprL	ATG GAA ATG CTG AAA TTC GGC CTT CTT CAG CTC GAC GCG ACG	504	Xu *et al*. (2004)

Testing of antimicrobial susceptibility

Treatment of positive raw samples with 1% citric acid solution for two minutes. Twenty five grams of the examined samples were transferred to a sterile stomacher bag and homogenized with 225 ml sterile peptone water (0.1%) for 30-60 seconds to give an initial dilution of 1/10. One ml from previously prepared solution dispersed on Aero Pseudo Selective Agar, Granulated media (HIMEDIA GM1620) and Eosin methylene blue (EMB), incubated at 37°C for 24 hours and 0.1 ml over Baird-Parker agar plates, incubation took place at 35°C for 24-48 h.

RESULTS

In Figure 1, *S. aureus* was detected in 11 and 5 samples representing 44% and 20% of the total examined raw and cooked samples, respectively. Also, *E. coli* was detected in 6 and 2 samples representing 24% and 8% of the total examined raw and cooked samples, respectively. *Pseudomonas* spp. and *Aeromonas* spp. were detected in 10 and 5 samples, representing 40% and 20% of the total examined raw bluespot mullet samples, respectively. While they weren't detected in cooked bluespot mullet samples.

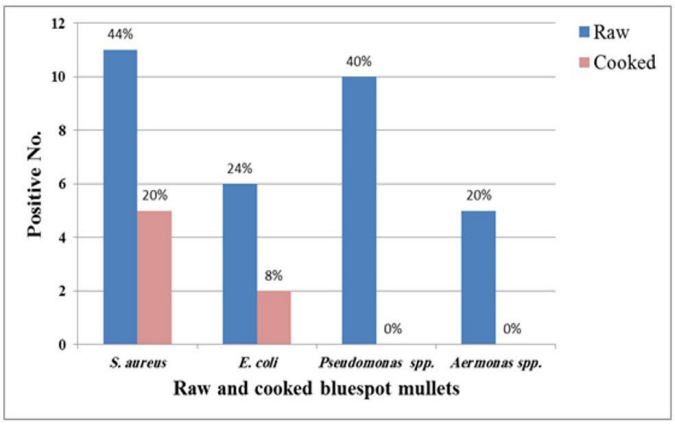

Fig. 1. Prevalence of *S. aureus*, *E. coli*, *Pseudomonas* spp. and *Aeromonas* spp. in the raw and cooked bluespot mullets (n=50).

In Table 2, the enterotoxigenic strains of *S. aureus* were detected in 27.27% of raw samples, while they were 20% in cooked samples. Enterotoxins A, A &C and D were 9.09% while 20% enterotoxin A was identified in cooked bluespot mullets.

In Table 3, the serological identification of *E. coli* showed the presence of O156: H7 (EPEC), O127: H6 (ETEC), O125:H21 (EHEC), O15: H12 (EPEC) and O91: H21 (EHEC).

P. aeruginosa strains according to (IATS) were divided into 2 serotypes *P. aeruginosa* serotype (O11 group E and O2 group B).

Referring to the obtained results of molecular detection of some virulent genes of *P. aeruginosa* in the examined isolates that present in Figs. 2, 3 and 4; *pslA*, *oprL* and *toxA* genes of *P. aeruginosa* were detected in all examined isolates.

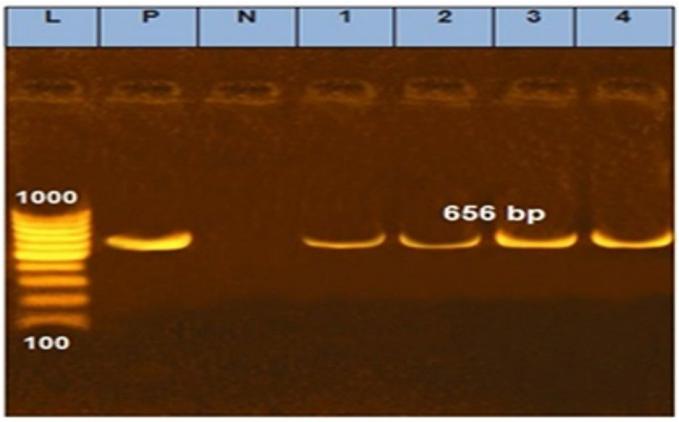

Fig. 2. Amplification profile of *pslA* gene of *P. aeruginosa* isolates at 656bp.

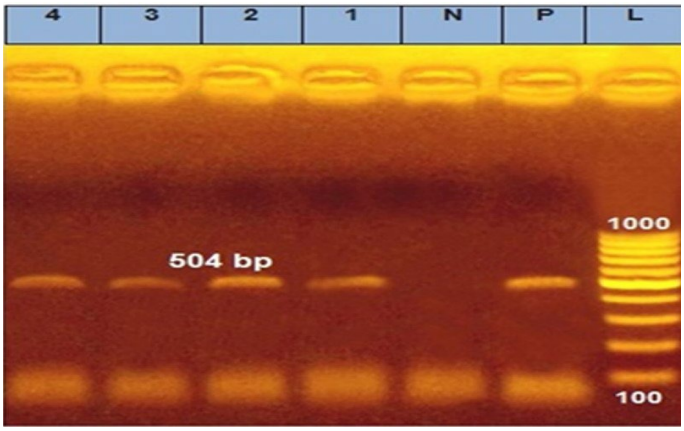

Fig. 3. Amplification profile of *oprL* gene of *P. aeruginosa* isolates at 504bp.

Data in Table 4 showed that citric acid have great antimicrobial effect against *S. aureus*, *E. coli*, *P. aeruginosa* and *Aeromonas* spp. as it causes complete inhibition for them.

Table 2. Occurrence of enterotoxin of *S. aureus* strains isolated from the examined samples of bluespot mullets.

Type of tested samples	No. (%) of *S. aureus* isolates	No. (%) Enterotoxigenic *S. aureus* strains	Type of detected *S. aureus* enterotoxins					
		No. (%)	A		A and C		D	
			No.	(%)	No.	(%)	No.	(%)
Row bluespot mullets	11 (44%)	3 (27.27%)	1	9.09%	1	9.09%	1	9.09%
Cooked bluespot mullets	5 (20%)	1 (20%)	1	20%	-	-	-	-

% in relation to positive samples

Table 3. Serological identification of isolated *E. coli* from bluespot mullets samples.

Product	Raw bluespot mullets	Cooked bluespot mullets	Strain characterization
	NO.	NO.	
O156:H7	1	-	EPEC
O127:H6	2	1	ETEC
O125:H21	1	-	EHEC
O15:H12	1	1	EPEC
O91:H21	1	-	EHEC

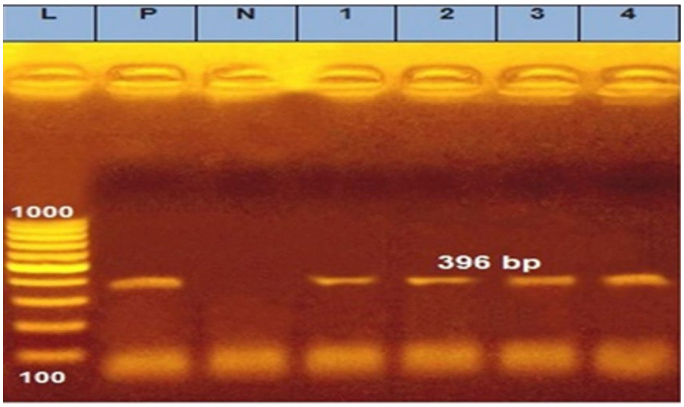

Fig. 4. Amplification profile of *toxA* gene of *P. aeruginosa* isolates at 396bp.

Table 4. The effect of citric acid against *S. aureus*, *E. coli*, *P. aeruginosa* and *Aeromonas* spp.

Strains	Before treatment	After treatment
S. aureus		
6	+ve	-ve
7	+ve	-ve
14	+ve	-ve
16	+ve	+ve
25	+ve	-ve
E. coli		
14	+ve O127:H6 (EPEC)	-ve
19	+ve O15:H2 (EPEC)	-ve
P. aeruginosa		
2	+ve	-ve
6	+ve	-ve
7	+ve	-ve
13	+ve	-ve
14	+ve	-ve
15	+ve	-ve
16	+ve	-ve
21	+ve	-ve
Aeromonas spp.		
7	+ve	-ve
14	+ve	-ve
15	+ve	-ve
21	+ve	-ve
25	+ve	-ve

DISCUSSION

Salted fish products are popular in many countries around the globe, as these have been proven to be safe for millions, even in the developed countries. Numerous kinds of microorganisms such as bacteria can be present in salted fish due to the spontaneous fermentation (Edris *et al*., 2020). The microorganisms in salted fish can be originated from the fish itself and the salt also used in the manufacture. Raw fish naturally have several bacteria, and this can be opportunistic and causing foodborne infections rapidly (Choudhary *et al*., 2022).

The percentage of *S. aureus* that was isolated from raw samples was similar to that isolated by Mohammed *et al.* (2017) who isolated *S. aureus* from 44% of the examined mullet samples which is not accepted with ES (1042/2005), as it should be free. Poor personal hygiene of the food handler, no pre-employment and routine medical examination for *S. aureus* and improper storage of cooked food were identified as the contributing factors of *S. aureus* illness. Moreover, Al-Harbi and Al-Asous (2019) isolated Psychrotrophic bacteria from mullet at (32.26%), *Pseudomonas aeruginosa* (*P. aeruginosa*) is a pathogenic bacterium and one of the seafood's most common spoilage microorganisms (Shahrokhi *et al*., 2022).

Further, Jawa *et al*. (2021) isolated *E. coli* at higher level (48.33%) than the present study. Also cooking of mullets resulted in decrease the incidence of *S. aureus*, *E. coli*, *Pseudomonas* spp. and *Aeromonas* spp. similar results revealed by Ulusoy *et al*. (2019), while Yılmaz *et al*. (2005) reported a complete elimination of *S. aureus*, after cooking.

E. coli is classified into six pathotypes: enteroaggregative, enterohemorrhagic Shiga toxin-producing *E. coli* (STEC), enteroinvasive, enteropathogenic, enterotoxigenic, and diffuse adherent (Jafari *et al*., 2012). lethal STEC named EHEC were also detected (Beutin *et al*., 2007). Previous studies indicated that STEC represents one of the most significant pathotypes which lead to foodborne illnesses compared with other types *E. coli* (Brett *et al*., 2003).

The pathogenic properties of *S. aureus* are mainly due to various virulence factor such as protein A, clumping factor, coagulase, fiberonectin, heamolysin, nucleases, exfoliative toxin and enterotoxins (Fournier, 2008). Most outbreaks of food poisoning associated with fish and seafood derive from the consumption of raw or insufficiently heat treatment, insufficient cooking and cross-contamination during processing (Mohammed *et al*., 2017). Fish sold at open market and exposed to ambient temperature increases likelihood of spoilage. Marketing and handling chain involving fresh fish is important from public health point of view (Kapute *et al*., 2012).

P. aeruginosa has got an enormous numbers of extracellular virulence factors and cellular components which implicated in pathogenesis (Kebede, 2010). For those reasons, this study was designed for the detection of these virulence genes (*pslA*, *oprL* and *toxA*,) in *P. aeruginosa* isolates by using PCR. Concerning the results of virulence factors, it was found that the detection of *pslA*, *oprL* and *toxA* gene in all our isolates (100%) confirmed the existence of *P. aeruginosa* DNA because considers a specific marker for molecular detection of *P. aeruginosa* and encodes a protein in the inner and outer membranes, which is essential for the invasion of epithelial cells (De Vos *et al*., 1997), the same result obtained by Xu *et al*. (2004) and Abdullahi *et al*. (2013).

The incidence rate of *toxA* gene was 71.42% (Shahat *et al*., 2019). Also, *toxA* were reported by Lavenir *et al*. (2007) and Qin *et al*. (2003). Furthermore, the *exoS* and *lasB* genes were detected by (Shahat *et al*., 2019), in five isolates of *P. aeruginosa* (71.42% for each). Tripathi *et al*. (2011) found that most *P. aeruginosa* isolates had *exoS* gene (78.6%).

The mentioned virulence genes in this work such as, *toxA*, *exoS* and *lasB* were coordinated by a critical global regulatory system consisted of transcriptional activator protein (LasR) and *Pseudomonas* auto- inducer, (PAI), the central gene responsible for activation of this system was putative auto-inducer synthase (lasI) (Saleh *et al*., 2012).

To prevent growth of spoilage and pathogenic microorganisms in fish and fishery products, various preservation techniques using acids such as citric acids have been used universally to extend shelf life. Organic acids such as citric acid have been found to have antimicrobial properties which are the precursor of the shelf life extension of fish (In *et al*., 2013).

Organic acids kill microorganisms via a mechanism wherein un dissociated molecules flow through the cell membranes of microorganisms and are ionized inside; thus, in order to maintain intracellular pH, hydrogen ions are released, and the acidic pH inside the cell causes deformation and damage to enzymatic activities, proteins and DNA structure, thereby damaging the extracellular membrane (Mani-López *et al*., 2012).

CONCLUSION

Bluespot mullets are contaminated with different types of pathogens as *S. aureus*, *E. coli*, *Pseudomonas* and *Aeromonas* spp. Also, citric acid has shown great effect against these microor-

ganisms.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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