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Virulence and Antimicrobial Resistance Profiling of *Aeromonas hydrophila* Recovered from Retail fish in Sharkia Province, Egypt

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Abstract

Fish is one of the most important foods because of its high nutritional value, high palatability, and easy digestion. At the same time, it acts as a vehicle for many types of pathogenic microorganisms especially *Aeromonas* species, which results in public health hazards. Therefore, the present study was conducted to evaluate the prevalence of *Aeromonas* species in fresh fish (catfish, mullet, lizardfish, and coralfish) marketed in Zagazig city, Sharkia Governorate, Egypt. In addition, multiplex PCR was performed to detect some virulence-associated genes in *A. hydrophila* isolates. Furthermore, antimicrobial susceptibility testing was carried out on *A. hydrophila* isolates using commonly used antimicrobials in Egypt through the disc diffusion method. The achieved results indicated contamination of fish with different species of *Aeromonas* such as *A. veronii*, *A. sobria*, *A. caviae* and *A. hydrophila*. The results revealed that *Aeromonas* species isolated with an overall percentage of 55% of all examined fish. Bacteriological examinations revealed 20% *A. hydrophila*, 20% *A. sobria*, 10% *A. caviae* and 5% *A. veronii*. Antibiotic sensitivity declared high resistance of the isolates to different antimicrobial agents used in Egypt, including penicillin (100%), Ampicillin (90.0%), Streptomycin (90.9%), Cephalothin (72.7%), Tetracycline (72.7%), Cefotaxime (63.6%), and Sulfamethoxazole (54.5%). Therefore, hygienic measures should be adopted to control microbial contamination either in the aquatic environment or in fish markets.

KEYWORDS
Aeromonas hydrophila, Antibiotic sensitivity, Fish, Virulence genes.

INTRODUCTION

Fish is considered one of the most foods consumed in Egypt as it provides consumers with high biological value animal protein and solving the problem of red meat shortage (Mohamed *et al*., 2023). Fish are considered one of the vital sources of nutrition. It provides humans with essential fatty acids, especially Omega 3 fatty acids, amino acids, high quality animal protein, vitamins and minerals that are required for health and growth (Morshdy *et al*., 2022a). Although seafood is a nutritious element, it is incriminated as a possible cause of many foodborne pathogens (Hussein *et al*., 2019).

Aeromonas species are emerging foodborne pathogens that are linked to conditions such as septicemia, gastroenteritis, enterocolitis, and wound infections in humans (Morshdy *et al*., 2022b). *Aeromonas* infections are more commonly seen in young, elderly, or immunocompromised individuals (Gluskin *et al*., 1992) and outbreaks seem to be more prevalent in summer than other seasons (Nishikawa and Kishi, 1988). *Aeromonas* is an opportunistic microorganism, constituting a part of the normal microbial flora of many aquatic animals and being widely distributed in the aquatic environments, including fresh, marine, and ground water. It is considered a primary pathogen of fish and can be isolated from healthy or diseased ones (Figueras and Beaz-Hidalgo, 2014). In addition, *Aeromonas* species have been isolated from various food products such as meats, seafood, dairy products, and vegetables (Krovacek *et al*., 1992). According to Bergey's manual, *A. hydrophila*, *A. sobria*, and *A. caviae* are motile members of the genus *Aeromonas* in the family Vibrionaceae. *Aeromonas* species are commonly associated with many environmental sources and food (Das *et al*., 2013).

In developing countries, the shortage and high price of animal proteins is the principal cause of the high demand for fish; so many trials have been developed by National Authorities during the last years in order to improve the fish quality. There is little information about the prevalence of *Aeromonas* species in fish marketed in Egypt. Therefore, this study was conducted to determine the prevalence of *Aeromonas* species in different types of fresh fish sold in fish markets in Zagazig city, Sharkia Governorate, Egypt. Due to the significant roles of *A. hydrophila* as foodborne pathogen, characterization of its virulence attributes and antibiotic resistance profile were also studied.

MATERIALS AND METHODS

Collection of fish samples

A total of one hundred fish samples of mullet, catfish, lizard and coralfish (25 of each) were randomly collected from different fish markets at Zagazig city, Sharkia Governorate, Egypt. The collected samples were identified and packaged separately in a sterile plastic bag; then transported in cooled aseptic conditions without any delay to the Laboratory of Meat Hygiene, Faculty of Veterinary Medicine, Zagazig University, Egypt for bacterial isola-

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tion and identification.

Preparation of fish samples

After removal of the dorsal, pectoral, and ventral fins by sterile scissors and forceps, the scales were removed from the body surface everywhere by a sterile scalpel. The surface was sterilized by a hot spatula and then removed. About 25 grams of the back muscles were aseptically homogenized with 225 ml of sterile 0.1% alkaline peptone water for 2.5 min and then were allowed to stand for 5 min (ICMSF, 1978).

Bacterial isolation

From the prepared homogenate, 1 ml was transferred into a sterile test tube containing 0.1% alkaline peptone water as an enrichment broth and incubated at 37°C for 18 h. After incubation, a loopful of the alkaline peptone water was streaked on the surface of *Aeromonas* agar medium (Himedia, Mumbai, India) and incubated at 37°C for 18-24 h (Palumbo *et al*., 1985). Typical colonies (Pale green with dark centers, their size is typically between 0.5 and 1.5-mm) were sub-cultured on a non-selective medium (Nutrient agar, Himedia, Mumbai, India), and incubated for 24 h.

Primary characterization and identification of the isolates

The pure cultures of the isolates were morphologically, biochemically, and serologically identified (Garrity, 2001). Morphological characters including shape, size, Gram staining, and motility of the isolated *Aeromonas* were confirmed (Koneman *et al*., 1994; Garrity, 2001). Biochemical identification was done using the following tests: oxidase, esculin hydrolysis, arginine hydrolysis, indole, urease, hydrogen sulphide production, nitrate reduction, gelatin liquefaction, pigment formation, sugars fermentation and detection of ornithine decarboxylase, L- lysine decarboxylase, arginine decarboxylase and β- galactosidase (MacFaddin, 2000). Biochemical reagents used were from Himedia, Mumbai, India. Physiological properties were investigated by observing the growth of each isolate at various temperatures (4, 24, 37 and 40°C) and different NaCl concentrations (0, 1, 2, 3 and 4%) (Garrity 2001; Nahar *et al*., 2016).

Detection of virulence genes of Aeromonas hydrophila by multiplex PCR

The extraction of the bacterial DNA was carried using QIA amp kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Application of PCR for identification of 16SrRNA, aerolysin (*aerA*) and haemolysin (*ahh1*) virulence genes of *A. hydrophila* was performed by using primers purchased from Pharmacia Biotech, Sweden. The 16SrRNA primers were sense 5'GGG AGT GCC TTC GGG AAT CAG A'3 and antisense 5'TCA CCG CAA CAT TCT GAT TTG'3 with a product size of 356 bp. while the primers used for detection of *aerA* were sense 5' CAA GAA CAA GTT CAA GTG GCC A '3 and antisense 5'ACG AAG GTG TGG TTC CAG T'3 with a product size of 309 bp. The primers used to detect *ahh1* were sense 5'GCC GAG CGC CCA GAA GGT GAG TT'3 and antisense 5'GAG CGG CTG GAT GCG GTT GT'3 with a product size of 130 bp (Stratev *et al*., 2016).

The amplification reaction was performed on a thermal cycler (Master cycler, Eppendorf, Hamburg, Germany). The PCR reaction (10 µl) consisted of bacterial DNA (2µl), 10X EX Taq buffer (1µl), forward primer (1µl), reverse primer (1µl), 2.5 mM dntp (0.8 µl), EX Taq polymerase (TaKaRa, Japan) (0.05µl) and nuclease free water (4.15µl) (Shah *et al*., 2009). Amplification consisted of an initial denaturation at 95°C for 5 min, 50 cycles at 95°C for 30 sec., 59°C for 30 sec., 72°C for 30 sec., followed by final elongation at 72°C for 7 min. Amplified DNA fragments were analyzed by 2% of agarose gel electrophoresis (Applichem, Germany, GmbH). Finally, the gel was stained with ethidium bromide and captured as well as visualized on UV transilluminator. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes (Wang *et al*., 2003).

Antibiotic resistance of the isolated A. hydrophila

Antimicrobial susceptibility was tested by the single diffusion method according to Stratev *et al*. (2015) using the antibiotic discs (Oxoid Limited, Basingstoke, Hampshire, UK) with variable concentrations (µg) including cephalothin (CN, 30µg), ampicillin (AM, 10µg), chloramphenicol (C, 30µg), Sulfamethoxazole (SXT, 25µg), Ofloxacin (O, 5µg), Meropenem (M, 10µg), Cefozolin (CZ, 30µg) , Azithromycin (AZ, 15µg), Tetracycline (T, 30µg), gentamicin (G, 10µg), amikacin (AK, 30µg), ciprofloxacin (CIP, 5µg), cefotaxime (CF, 30µg), streptomycin (S, 10 µg) and Imipenem (IPM, 10µg). Interpretation of the results was carried out according to the guidelines stipulated by National Committee for Clinical Laboratory Standards (NCCLS, 2001). The tested isolates were evaluated as susceptible, intermediate, and resistant and multiple antibiotic resistances (MAR) index for each strain was determined; MAR index = No. of resistance (isolates classified as intermediate were considered sensitive for MAR index) / Total No. of tested antibiotics (Vivekanandhan *et al*., 2002).

RESULTS

Prevalence of *Aeromonas* spp. in fish samples, including mullet, catfish, lizard, and coralfish, is recorded in Table 1. Out of 100 fish samples analyzed by bacteriological examination revealed the presence of *Aeromonas* species in 55% of all examined samples. Bacteriological and chemical examinations revealed 20% *A. hydrophila*, 20% *A. sobria*, 10% *A. caviae* and 5% *A. veronii*. The isolation percentage was the highest in catfish (60%), mullet (60%), lizard fish (60%) and the lowest percentage was observed in coralfish (40%). Table 2 shows 100% resistance of the tested *Aeromonas* isolates for penicillin, 92.9%, 92.9% and 72.7 resistance to Ampicillin, streptomycin and cephalothin, respectively. Meanwhile, *Aeromonas* species were more sensitive to Imipenem (36.4%), Gentamicin (18.2%), amikacin (18.2%). The resistance profile of each *Aeromonas* isolates is presented in Table 3. All isolates were categorized as multi-drug resistant (MDR) *Aeromonas*, and their multiple antibiotic resistance (MAR) index ranged from 0.062 to 1.00, with an average of 0.59. Additionally, It was discovered that the 16S rRNA gene was found in all tested *A. hydrophila* isolates. However the *ahhl* virulence gene was found in 75 % of the examined samples, while, only 25% were positive for *aerA* (Figure 1).

DISCUSSION

Fish flesh is an excellent substrate for growth of large number of bacteria with compositional attributes, which affect the bacterial growth and the related biochemical activities (Darwina *et al*., 2012). Various sources are responsible for microbial contamination of fish such as water, soil, and fish handlers. Bad handling of fish during fishing, transporting, and shipping plays an important role in cross contamination of fish from the surrounding environment and can act as a stress factor which results in bacterial

migration from the gut to fish muscles (Mahmoud *et al*., 2022). Some of these bacteria are associated with many diseases in humans, making aquaculture products a potential risk factor for customer's health. *Aeromonas* bacterium is considered as one of the emerging food-borne pathogens (Massa *et al*., 2001).

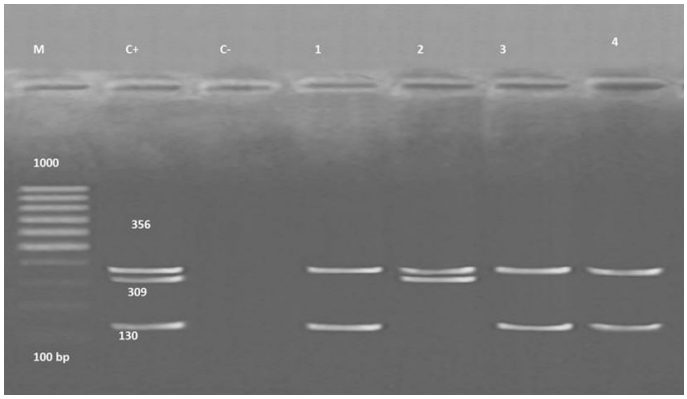

Fig. 1. Agarose gel electrophoresis of multiplex PCR of 16S rRNA (356 bp), *aerA* (309 bp) and *ahhl* (130 bp) genes for characterization of *Aeromonas hydrophila*. Lane M: 100 bp ladder as molecular size DNA marker. C+: Control positive *A. hydrophila* for 16S rRNA, *aerA* and *ahhl* gene, C-: Control negative, 1, 3 & 4: Positive *A. hydrophila* for 16S rRNA and *ahhl* genes, 2: Positive *A. hydrophila* strain for 16S rRNA and *aerA* genes.

In the present study, *Aeromonas* species were identified in 55% of the examined fish samples (catfish, mullet, lizard fish and coral fish) consumed in Egypt. This result show that the prevalence of *Aeromonas* species in catfish, mullet, lizard fish and coral fish were 15(60%), 15(60%), 15(60%), and 10(40%), respectively indicated that the examined fishes were subjected to contamination during transportation and marketing. Leaving fish at room temperature in fish markets is a favorable condition for growth of *Aeromonas* spp.

These findings were in agreement with *Aeromonas*-related studies conducted in other countries. For instances, in Malaysia, *Aeromonas* spp. were isolated from 69% of fish samples (Radu *et al*., 2003) while in Turkey, it was isolated from 82.8% of the examined fish (Yucel *et al*., 2005). Generally, the studies on prevalence of *Aeromonas* spp. in fish focused on 3 species, *A. hydrophila*, *A. sobria* and *A. caviae* (Karabasil *et al*., 2002). In the current study, identification of the isolated *Aeromonas* spp. from the examined fish samples revealed four different species: namely *A. hydrophila*, *A. sobria*, *A. caviae* and *A. veronii*. The most predominant species was *A. sobria* (20%) and *A. hydrophila* (20%) followed by *A. caviae* (10 %) then *A. veronii* (5%). This result indicated the wide spreading of motile *Aeromonas* spp. in the aquatic environment; consequently, fish can be easily contaminated by these microorganisms after exposure to stress as a result of rough handling during fishing and overcrowding in fish boxes. In addition, cross contamination during transportation to fish markets can increase the contamination by pathogenic motile *Aeromonas* spp.

In agreement with the current report, a study conducted in Serbia found that *A. hydrophila* and *A. sobria* were isolated from 66.7% and 33.3% of the examined fish, respectively (Karabasil *et al*., 2002), while in Malaysia, *A. caviae* was the predominant species isolated from fish samples (47.1%) followed by *A. hydrophila*, *A. sobria* and *A. veronii* (Radu *et al*., 2003). In India, *A. sobria* was the most common species isolated from apparently healthy fish (Rathore *et al*., 2005). In China, *A. veronii* was the dominant *Aeromonas* spp. (Cai *et al*., 2012). Variation in the prevalence of *Aeromonas* spp. in catfish, mullet, lizard and coral fish could be attributed to the differences in the contamination levels during handling and transportation.

Aeromonas is recognized as an emerging pathogen that has the ability to cause various diseases in humans including food poisoning, gastroenteritis, septicemia, skin affections, soft-tis-

Table 1. Prevalence of *Aeromonas* species isolated from fish samples (n = 25, each).

Fish type	No. of positive (%)	*A. hydrophila*	*A. sobria*	*A. caviae*	*A. veronii*
Catfish	15 (60%)	-	10 (40%)	5 (20%)	-
Mullet	15 (60%)	10 (40%)	-	5 (20%)	-
Lizard fish	15 (60%)	10 (40%)	5 (20%)	-	-
Coral fish	10 (60%)	-	5 (20%)	-	5 (20%)
Total	55 (55%)	20 (20%)	20 (20%)	10 (10%)	5 (5%)

Table 2. Antimicrobial susceptibility of *Aeromonas* species (n = 55).

Antimicrobial agent	S		I		R	
	NO	%	NO	%	NO	%
Penicillin (P)	-	-	-	-	11	100
Ampicillin (AM)	-	-	1	9.1	10	90.9
Streptomycin (S)	-	-	1	9.1	10	90.9
Cephalothin (CN)	1	9.1	2	18.2	8	72.7
Tetracycline (T)	3	27.3	-	-	8	72.7
Cefotaxime (CF)	3	27.3	1	9.1	7	63.6
Sulfamethoxazole (SXT)	2	18.2	3	27.3	6	54.6
Ofloxacin (O)	4	36.4	2	18.2	5	45.5
Ciprofloxacin (CP)	5	45.5	1	9.1	5	45.5
Imipenem (IPM)	4	36.4	3	27.3	4	36.4
Meropenem (M)	6	54.6	1	9.1	4	36.4
Chloramphenicol (C)	7	63.6	-	-	4	36.4
Cefazolin (CZ)	7	63.6	1	9.1	3	27.3
Gentamicin (G)	7	63.6	1	9.1	2	18.2
Amikacin (AK)	9	81.8	-	-	2	18.2
Azithromycin (AZ)	10	90.9	-	-	1	9.1

S: Sensitive; I: Intermediate; R: Resistant.

Table 3. Antimicrobial resistance profile of *Aeromonas* species (n = 55)

NO	*Aeromonas* species	Antimicrobial resistance profile	MAR index
1	*A. hydrophila*	P, AM, S, CN, T, CF, SXT, O, CP, IPM, M, C, CZ, AK, G, AZ	1
2	*A. hydrophila*	P, AM, S, CN, T, CF, SXT, O, CP, IPM, M, C	0.75
3	*A. hydrophila*	P, AM, S, CN, T, CF, SXT	0.44
4	*A. hydrophila*	P, AM, S, CN, T, CF	0.38
5	*A. caviae*	P, AM, S, CN, T, CF, SXT, O, CP, IPM, M, C, CZ, AK, G	0.94
6	*A. caviae*	P, AM, S, CN, T, CF, SXT, O, CP	0.56
7	*A. caviae*	P, AM, S	0.19
8	*A. sobria*	P, AM, S, CN, T, CF, SXT, O, CP, IPM, M, C, CZ	0.81
9	*A. sobria*	P, AM, S, CN, T	0.31
10	*A. sobria*	P, AM, S	0.19
11	*A. veronii*	P	0.06

Average = 0.512

P: Penicillin; S: Streptomycin; CZ: Cefazolin; AM: Ampicillin; CN: Cephalothin; AK: Amikacin; CP: Ciprofloxacin; CF: Cefotaxime; T: tetracycline; SXT: Sulfamethoxazole; G: Gentamicin; AZ: Azithromycin; O: Ofloxacin; M: Meropenem; CZ: Ceftazidime; IMP: Imipenem.

sues and muscles infection. *A. hydrophila*, *A. sobria*, and *A. caviae* are the main causes of *Aeromonas* associated human diseases (Janda and Abbott, 2010). These microorganisms had been linked to an outbreak of *Aeromonas* infection due to ingestion of raw fermented fish (Rasmussen *et al*., 2016). Identification of *A. hydrophila* using conventional methods remains difficult. Therefore, the multiplex PCR was used in the current study. In *A. hydrophila*, as with all pathogenic microorganisms, disease occurs because of complex molecular interactions between bacteria, host and environment (Janda and Abbott, 2010). Virulence in *A. hydrophila* is a multifactorial due to the production of several virulence factors, such as cytotoxins, adhesins, hemolysins, proteases and lipases, as well as the ability to form biofilms by using a specific metabolic pathway and a mediate virulence factor expression (Beaz-Hidalgo, 2014). Enterotoxins, cytotoxins and hemolysins are more frequently detected in isolates obtained from patients with gastrointestinal symptoms; a relationship between hemolysin production and human illness caused by motile *Aeromonas* species has been observed (Soler *et al*., 2002).

In the present study, the selected four isolates of *A. hydrophila* were specific for 16S rRNA gene; while 1 isolate was producer to hemolytic toxins aerolysin (*aerA*) and 3 isolates were producers to haemolysin (*ahhl*). These results agreed with published reports in Canada, Turkey and India, where *ahh1* and aerolysin were detected (Wang *et al*., 2003). Hemolytic toxins were also detected in 82% of *A. hydrophila* (Radu *et al*., 2003) and hemolysin gene was detected in 78% of *A. hydrophila* isolates (Thayumanavan *et al*., 2003). Additionally, *ahh1* and *aerA* were expressed in 60.52% and 13.15%, respectively of *Aeromonas* spp. (Sharma *et al*., 2010). The 16S rRNA gene serves as an excellent and rapid means to determine the identity of *A. hydrophila*, despite its complexity due to the presence of up to 15 copies of this ribosomal operon. It has been used for molecular identification of species by restriction fragment length polymorphism (Ghatak *et al*., 2007) or direct gene sequencing (Kupfer *et al*., 2006). Hemolysin is a group of multifunctional enzymes, which plays important roles in the pathogenicity of *A. hydrophila*. Hemolysins include *aerA*, *ahh1*, *ahyA*, and asa1; *ahh1* is the most widely distributed extracellular heat-labile hemolysin, the synergistic combination of *aerA* and *ahh1* is the most cytotoxic genotype (Wang *et al*., 2003).

Other virulence factors encoded by *A. hydrophila* include adherence proteins, catalysts, nucleases, and toxins that may be expressed differently. Adherence proteins are responsible for mucosal adherence, biofilms formation, cell division and motility (Huang *et al*., 2015). These virulence-associated factors are very important in distinguishing between pathogenic and non-pathogenic strains. *A. hydrophila* enterotoxin, which is cytotoxic in nature, is the main cause of gastroenteritis, while aerolysin is the principal virulence-associated factor implicated in various intestinal disorders (Thayumanavan *et al*., 2003). In the current study, the virulence genes (*aerA* and *ahh1*) of *A. hydrophila* were discovered in 25% and 75% of the samples, respectively. These results were nearly similar to those of Ahmed *et al*. (2018), who found that only three isolates (42.86%) from Nile tilapia fish and five isolates (55.56%) from Mugil cephalus were *aerA* positive. Higher findings were made by Morshdy *et al*. (2022b), who discovered that the *ahh1* and *aerA* genes were present in 100% and 75%, respectively of the studied *A. hydrophila* isolates. Hoel *et al*. (2017) observed that all isolates exhibited high prevalence of hemolysin producing genes (99% and 98%) for hlyA and *aerA*, respectively.

Antibiotics are widely used in fish farms for prevention and control of bacterial diseases. Using a wide variety of antimicrobial agents, aquaculture had been implicated in the development of resistant bacteria and a source of transmission of these resistant pathogens to other animals and humans (Srinivasan and Saranraj, 2017). In addition, application of the same antibiotics in different fields including veterinary and human medicine fosters the emergence of the microbial drug resistance phenomenon (Abdallah *et al*., 2022; Morshdy *et al*., 2022c).

In the present investigation, *A. hydrophila* showed variable degrees of resistance to the fourteen tested antimicrobial agents. The isolates were 100% resistant to penicillin 90.9% resistant to ampicillin and streptomycin 72.7% of the isolates were resistant to cephalothin and tetracycline; 63% of isolates were resistant to cefotaxime; 54.5% of the isolates were resistant to Sulfamethoxazole and olfoxacin; 36.4% were resistant to imepinem; 27.3% were resistant to cefozolin, 18.2% were resistant to gentamicin. In agreement with the current study similar resistance profiles were reported in India and Egypt (Thayumanavan *et al*., 2003; Furmanek-Blaszk, 2014). However, higher resistance percentages were recorded in Turkey to gentamicin, and cephalothin; while, low resistance rates were recorded to streptomycin (18.1%), sulfamethoxazole and cefotaxime (63%) (Radu *et al*., 2003). In another report, resistance to sulfamethoxazole and cefotaxime was 67% (Vivekanandhan *et al*., 2002). These differences in the antimicrobial resistance profiles may be related to the differences in fish species and the type of the used antimicrobials.

CONCLUSION

The present study indicated that most of examined fish samples are contaminated with different types of *Aeromonas* species particularly *A. hydrophila*. A higher percentage of isolated *A. hydrophila* had hemolytic genes that pose a potential threat to human health. Furthermore, *A. hydrophila* has variable degrees of resistance to the fourteen tested antimicrobial agents. In order to prevent the prevalence of pathogens, it is advised that hygienic practices be adopted during handling, preparation, processing,

and storage of fish to improve the quality and lower its microbial contamination with *A. hydrophila*. Moreover, it is advised to reduce the overuse of antibiotics in aquaculture. Additionally, strengthening the quality assurance procedures for keeping an eye on the fish's quality.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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