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Investigation of the Antibacterial Effect of Astaxanthin and the Prevalence of Virulence and Antimicrobial Resistance Genes of *Aeromonas hydrophila* and *Aeromonas sobria* strains

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Abstract: In the study, in addition to the antibacterial effect of astaxanthin on *Aeromonas hydrophila* and *A. sobria* strains, the presence of virulence genes (*Aero*, *act*, *ast*, and *hylA*) and antibiotic resistance genes (*tetC* and *sulI*) in the strains was investigated. Antibiotic profiles of the strains were also investigated as part of the study. Strains were identified by conventional biochemical tests and PCR assay using a 16S rDNA primer pair specific for *A. hydrophila*. According to the results of bacteriological and molecular studies, two of the six *Aeromonas* strains were identified as *A. hydrophila* and four of them as *A. sobria.* The *Aero* virulence gene and the *act* virulence gene were found in all strains, while the *ast* and *hylA* virulence genes were detected only in *A. hydrophila* strains. All strains were resistant to chloramphenicol, tetracycline, nalidixic acid, and ampicillin in the standard disk diffusion test. Although all strains showed resistance to tetracycline and moderate resistance to oxytetracycline in the antibiogram tests, *tetC* antibiotic resistance gene was not detected in the strains and *sulI* antibiotic resistance gene was not detected in the strains. In the study, acetone solutions containing 0.1 g and 0.5 g of astaxanthin were found to have an antibacterial effect on *A. hydrophila* strains. Acetone solutions containing 0.1 g, 0.5 g, and 1.0 g of astaxanthin showed antibacterial effects on *A. sobria* strains. It was found that 0.1 g, 0.5 g, and 1.0 g astaxanthin solutions prepared with methanol and distilled water had no antibacterial effects on the strains.

Keywords: *Aeromonas hydrophila*, *Aeromonas sobria*, astaxanthin, virulence genes, antibiotic genes, antibiotics

1. Introduction

The motile *Aeromonas* species, including *Aeromonas hydrophila*, *A. caviae*, *A. sobria*, *A. dhakensis*, *A. jandaei*, and *A. veronii*, also known as mesophilic bacterial species (Ebied et al. 2022), cause motile Aeromonas septicemia (MAS), which can lead to findings such as soft tissue and haemorrhage (Joseph et al. 2013; Hossain and Heo 2020). MAS is observed in farmed and wild fish as well as terrestrial animals and causes up to 80% mortality under farmed conditions (Saharia et al. 2021). The motile *Aeromonas* species *A. hydrophila* and *A. sobria* have been reported to infect freshwater fish species such as tilapia, catfish, carp, and rainbow trout, as well as many tropical or ornamental fish species including goldfish (Elsheshtawy et al. 2019; Yardımcı and Turgay 2021). Also, they have recently been identified as causative agents of intestinal and other infections in humans, e.g., infections associated with natural disasters such as hurricanes and tsunamis, and hospital infections. Therefore, these bacteria are of interest as opportunistic and primary pathogens (Robertson et al. 2014; Hoel et al. 2017).

Members of the genus *Aeromonas* are Gram-negative, rod-shaped, cytochrome oxidase- and catalase-positive, capable of reducing nitrates to nitrites, fermenting glucose, and resistant to the vibriostat agent (2,4-diamino-6, 7-di-iso-propylpteridine phosphate) (Fernández-Bravo and Figueras 2020). *Aeromonas* species are phenotypically divided into two groups: the motile and non-motile groups (Hossain and Heo 2020). The non-motile group consists of psychrophilic *Aeromonas* species that exhibit optimal growth at 22-28 °C. These bacterial species are considered to cause furunculosis, especially in salmonids. The other group is the species that can develop at 35-37 °C and cause motile *Aeromonas* septicemia (MAS) in fish (Hossain and Heo 2020).

So far, a number of potential virulence factors such as pore-forming hemolytic toxins, cytotonic heat-labile (*alt*), cytotonic heat-labile (*ast*), cytotoxic heat-labile enterotoxin (*act*), aerolysin (*Aero*), flagellin (*fla*), elastase (*ela*), serine protease (*ser*), lipase (*lip*), collagenase (*acg*), Dnase (*exu*), and cholesterol acyltransferase (*gcat*) have been identified (Robertson et al. 2014; Guz et al. 2021); however, *ast*, *act*, *alt*, and aerolysin toxin (*Aero*) of mesophilic *Aeromonas* species have been reported as virulence factors that are commonly reviewed in the context of infections (Robertson et al. 2014; Hoel et al. 2017). Robertson et al. (2014) noted

that the aforementioned toxins may be a potential clue for distinguishing pathogenic *Aeromonas* species from non-pathogenic *Aeromonas* species. In addition to virulence factors associated with infection in *Aeromonas* species, another important issue is the detection of multi-antibiotic resistance in these bacteria (Sreedharan et al. 2012; Guz et al. 2021).

Nowadays, the number of studies aimed at determining the antimicrobial profiles of motile *Aeromonas* species, particularly *Aeromonas hydrophila, A. caviae, A. sobria, A. dhakensis, A. jandaei*, and *A. veronii*, has increased (Fernández-Bravo and Figueras 2020; Hossain and Heo 2020). The use of antibiotics to treat infections has also been shown to be effective in this situation. Due to the zoonotic properties of motile *Aeromonas* species, the development of antibiotic resistance in these species is of concern not only for fish under farmed conditions, but also for general public health, including fish farmers (Fernández-Bravo and Figueras 2020).

Alternative products of plant origin to antibiotics have been proposed due to the multiantibiotic resistance found in bacteria (Pandey 2018). The ketocarotenoid astaxanthin (AST) (3, 3'-dihydroxy-ß, ß'-carotene-4,4'-dio) is a fat-soluble xanthophyll (Dhankar et al. 2012; Lotfi et al. 2021). It can be naturally synthesized by microorganisms such as the bacterium *Agrobacterium aurantiacum*, the fungus *Xanthophyllomyces dendrorchous* and the green alga *Haematococcus pluvialis* (Olaizola 2007; Dhankhar et al. 2012). In addition, it can be produced synthetically from petrochemicals (Marinho et al. 2021). Today, its human health benefits such as its antioxidant properties, are of interest as it is used as a feed additive for poultry and salmonids. It is also used commercially to color ornamental fish such as goldfish (*Carassius auratus*) and *Pseudochromis fridmani*, and shellfish such as crabs and shrimp (Olaizola 2007; Dhankar et al. 2012; Marinho et al. 2021; Montaya et al. 2021). Although the effects of astaxanthin on reproductive performance, egg production, and egg quality of aquatic animals are well known, increased resistance to bacterial and viral pathogens has also been observed in fish fed astaxanthin-supplemented diets (Lim et al. 2018).

In this study, the antibacterial effect of astaxanthin on *Aeromonas hydrophila* and *A. sobria* strains previously isolated from sick goldfish (*Carassius auratus)*,, antibiotic resistance, and multiple resistance (MAR) of the strains were investigated. The virulence genes (*Aero*, *act*, *ast*, and *hylA*) and antibiotic resistance genes (*tetC* and *sulI*) of the strains were also studied.

2. Materials and Method

2.1. Bacterial strains and phenotypic characterization of the strains

For the study, 6 strains of *Aeromonas* spp. previously isolated from freshly dead goldfish (*Carassius auratus*) showing signs of MAS were used. After an incubation period of 24-28 h at 24 ± 2 °C, bacterial colonies grown on the plates were examined for morphology and color. To determine the morphological and biochemical characteristics of the strains, all strains were tested using conventional identification methods, including hanging drops for motility, Gram stain, cytochrome oxidase (tetramethyl-p-phenylenediamine dihydrochloride), catalase (3% H_2O_2), fermentation test in O/F glucose broth, Voges-Proskauer (VP) and methyl red (MR), citrate utilization in Simmon's Citrate agar, onpg (o-nitrophenyl-β-D-galactopyranoside), vibriostat assay (10 µg and 150 µg, respectively). NaCl tolerance was determined using nutrient broth (NB) spiked with different NaCl concentrations. To determine temperature tolerance, strains were cultured in NB at 4, 25, and 37 °C. H_2S production on Triple Sugar Iron (TSI) agar, gas formation from glucose, hemolysis on blood agar (BA), dihydrolase and decarboxylase assays, acid production from sugars such as glucose, lactose, sorbitol, inositol, fructose, mannose, xylose, galactose, mannitol and sucrose in peptone water, nitrate production, amylase and gelatinase production, and growth on MacConkey agar were studied (Austin and Austin 2007).

2.2. Molecular studies

2.2.1. DNA extractions and PCR studies

For PCR amplification of 16S rDNA, DNA from the strains was extracted using a commercially available kit for purification of bacterial and yeast genomic DNA (Hibrigen, Türkiye). After DNA isolation, samples were stored at -20 °C in the freezer until used for PCR studies (Temizkan and Arda 2004). Prior to testing, the DNA samples were thawed at room temperature. Mytaq HS DNA polymerase kit was used to obtain the PCR products. For this purpose, a standard reaction of 50 µl was prepared. PCR components and amounts are listed in Table 1. For identification of the 16S rDNA, virulence genes (*aero*, *act*, *ast* and *hylA*), and antibiotic resistance genes (*tetC* and *sulI*), the primer pairs used in the study were listed in Table 2 and Table 3.

Table 1 PCR components used in the study

Components	Volume
5xMytaq reaction buffer	10 µl
DNA	5 µl
Primer Fd	1 µl
Primer Rs	1 µl
Mytaq HS DNA polymerase	1 µl
Water (ddH_2O)	32 µl

Table 2 16S rDNA primer sequence for *A. hydrophila* (Gardenia et al. 2010)

Primer	
16S rDNA Fd	
16S rDNA Rs	
Primer sequence	
5′-GAAAGGTTGATGCCTAATACGTA-3′	
5′-CGTGTGGCAACAAAGGACAG-3′	
Annealing	
56 °C	
bp	
685	

Table 3 Primer sequence for DNA amplification of virulence and antibiotic resistance genes

Primer	
Aero Fd*	
Aero Rs	
Primer sequence	
5′-CCAAGGGGTCTGTGGCGAAC-3′	
5′-TTTCACCGGTAACAGGATTG-3′	
bp	
209	
Primer	
act Fd**	
act Rs	
Primer sequence	
5′-GAGAAGGTGACCACCAAGAACA-3′	
5′-AACTGACATCGGCCTTGAACTC-3′	
bp	
232	
Primer	
ast Fd**	
ast Rs	
Primer sequence	
5′-TCTCCATGCTTCCTTCCACT-3′	
5′-GTGTAGCGATTGAAGCCG-3′	
bp	
331	
Primer	
hylA Fd**	
hylA Rs	
Primer sequence	
5′-GGCCGGTGGCCCGAAGATACGGG-3′	
5′-GGCGGCGCCGGACGAGACGGGG-3′	
bp	
592	
Primer	
tetC Fd***	
tetC Rs	
Primer sequence	
5′-AACAATGCGCTCATCGT-3′	
5′-GGAGGCAGACAAGGTAT-3′	
bp	
1138	
Primer	
sulI Fd***	
sulI Rs	
Primer sequence	
5′-CGGCGTGGGCTACCTGAACG-3′	
5′-GCCGATCGCGTGAAGTTCCG-3′	
bp	
433	

*Gardenia et al. (2010), **El-Bahar et al. (2019), ***Duman (2017)

The different thermocyclers were programmed for amplifications of 16S rDNA primer pairs, virulence genes, and antibiotic resistance genes; however, each cycle consisted of an initial denaturation, annealing, extension, and final phase (El-Bahar *et al*., 2019; Duman, 2017; Gardenia *et al*., 2010). The thermocycler programme for each target gene except 16S rDNA is given in Table 4. The PCR cycle for 16S rDNA primer pairs was set to 30 cycles, with initial denaturation for 5 min at 95 °C, 1 min at 94 °C, annealing for 1 min at 56 °C, extension for 1 min at 72 °C. The final stage was incubated for 10 min at 72 °C (Gardenia *et al*., 2010). The thermocycler programme for each target gene except 16S rDNA is given in Table 4.

Table 4 The thermocycler programme for each target gene except 16S rDNA

Thermocycler programme	Target gene	
	*Aero**	*act***
Initial denaturation	95°C/4 min	95°C/4 min
Cycles	30	30
Denaturation	95°C/30 sec	94°C/30sec
Annealing	54°C/45 sec	42°C/30sec
Extension	72°C/30 sec	72°C/1 min
Final stage	72°C/10 min	72°C/10 min
Thermocycler programme	Target gene	
	*Ast***	*hylA***
Initial denaturation	95°C/5 min	94°C/2 min
Cycles	30	35
Denaturation	95°C/1 min	94°C/30 sec
Annealing	55°C/1 min	94°C/30 sec
Extension	72°C/1 min	72°C/1 min
Final stage	72°C/5 min	72°C/5 min
Thermocycler programme	Target gene	
	*tetC****	*sulI****
Initial denaturation	94°C/4min	94°C/4 min
Cycles	35	30
Denaturation	94°C/1 min	94°C/30 sec
Annealing	62°C/2 min	60°C/30 sec
Extension	72°C/3 min	72°C/1 min
Final stage	72°C/7 min	72°C/7min

*Gardenia et al. (2010), **El-Bahar et al. (2019), ***Duman (2017)

2.2.2. Gel electrophoresis

To prepare a 2 % agarose gel, 5 x TBE buffer was diluted 80:20 ml (distilled water: buffer) to 100 ml 1 x TBE buffer. 2 g agarose was added to 1 x TBE buffer and cooled to 50-60 °C at room temperature. Then 2 µl of ethidium bromide solution was added to the cooled agarose. After placing the combs of the electrophoresis apparatus, the prepared gel was poured onto the dish, and the gel was allowed to drain at room temperature. A 100 bp marker was used as a DNA marker. The marker was added to the first well, which contained 5 µl, and 5 µl of the PCR amplification products (4 µl of sample + 1 µl of 6 x dye) were added to the other wells. The test samples were run at 80 V for 60 min. After running the test samples, the bands on the agarose gel were visualised in a U.V. transilluminator.

2.3.1. Preparation of astaxanthin solutions

The commercial form of astaxanthin (Roche, Switzerland) was used for the study. Distilled water, methanol (Merck, Germany) and acetone (Merck, Germany) were used as solvents for the experiments.

2.3.2. Antibacterial effect of astaxanthin by disc diffusion method

To determine the antibacterial activity of astaxanthin, sterile discs were placed on Petri plates containing Mueller-Hinton agar (MHA). 100 µg distilled water, acetone, and methanol solutions containing 0.1g, 0.5g, and 1.0gastaxanthin were added to the empty discs, and zone diameters around the discs were measured at the end of the 16-18 h incubation period at 24 ± 2 °C. Oxytetracycline (OT30, 30 µg) was used as a control antibiotic. The tests were performed in duplicate and the average values were recorded (CLSI M49 2006).

2.4.1. Antibiotic profiles of the strains

Antibiotic resistance of the strains was determined by the standard disc diffusion method (Bauer et al. 1966). Briefly, inoculations from 16-18 hours broth cultures were applied to the surface of Petri plates containing MHA using sterile swabs. Then, the antibiotic-containing discs were placed on the surface of the medium and incubated at 24 ± 2 °C for 16-18 hours. After the incubation period, the diameter of the zone of inhibition around the discs was measured and recorded. The tests were performed in duplicate, and the average of the values was reported. The antibiotics used in the study were ampicillin (AMP10; 10 µg), chloramphenicol (C30; 30 µg), erythromycin (E15; 15 µg), flumequine (UB30; 30 µg), kanamycin (K30; 30 µg), nalidixic acid (NA30; 30 µg), oxytetracycline (OT30; 30 µg), streptomycin (S10; 10 µg), sulfamethoxazole (RL25; 25 µg), tetracycline (TE10; 10 µg), tetracycline (TE30; 30 µg), and trimethoprim (W5; 5 µg). Zone diameter results were interpreted as susceptible ≥ 18 mm, intermediate resistance 13-17 mm and resistance ≤ 13 mm (Odeyemi et al. 2012).

2.4.2. Multi-antibiotic resistance index (MAR)

The multiantibiotic resistance index (MAR) is calculated from the ratio between the number of antibiotics resistant to test organisms and the total number of antibiotics tested. It provides information about the spread of bacterial resistance in populations (Krumperman 1983). The calculated index MAR indicates the presence of environmental strains using multiple antibiotics if it is greater than 0.2 (Ehinmidu 2003).

3. Results

3.1. Phenotypic characterization of the strains

The bacterial colonies were grown between 24 and 48 hours and formed the cream-colored colonies on BHIA. Since the strains were Gram-negative, motile, fermentative, cytochrome oxidase- and catalase-positive, resistant to O/129 vibriostatic agents (10 µg and 150 µg) and reduced nitrate to nitrite, they were classified as putative *Aeromonas* spp. The results of a series of physiological and biochemical tests to further identify putative *Aeromonas* strains are listed below. The isolates produced indole and citrate. They were tolerant to NaCl up to 4% and could grow at 37 °C but not at 4°C. Hydrolysis of urea was negative for all strains. Hydrolysis of gelatin was also negative, but the isolates produced amylase. The strains were able to metabolize lactose, mannitol, mannose, xylose, and galactose. Two strains were able to utilize sucrose, but four strains were unable to utilize sorbitol, inositol, and fructose. The two strains were identified as *Aeromonas hydrophila* (Fig.1)and the 4 strains as *A. sobria*. All phenotypic characteristics of the strains are listed in Table 5.

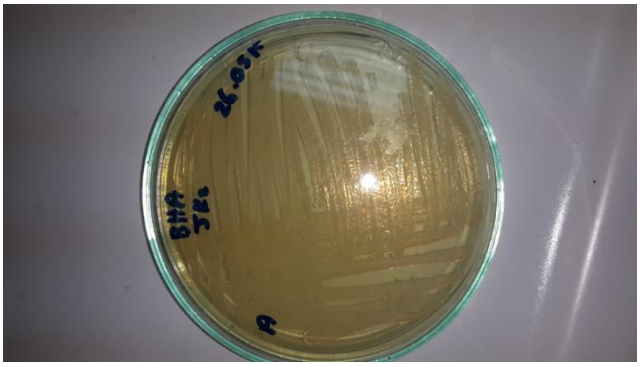

Fig.1. *Aeromonas hydrophila* strain on BHIA

Table 5 Results of morphological, physiological and biochemical tests of the *Aeromonas* strains

Tests	1 (2 strains)	2 (4 strains)	3*	4*
Gram-staining	-	-	-	-
Motility	+	+	+	+
C.oxidase	+	+	+	+
Catalase	+	+	+	+
O/F	F	F	F	F
Indole	+	+	+	+
MR	+	+	-	.
VP	+	+	+	+
H_2S	-	-	-	-
ADH	+	+	+	+
LDC	-	-	V	+
ODC	-	-	-	-
ONPG	+	+	+	+
Citrate	+	+	.	.
Urease	-	-	-	-
Gelatinase	+	+	+	+
Amylase	+	+	+	+
Nitrate red.	+	+	+	+
Growth on MacConkey agar	+	+	.	.
Haemolysis	+, β	+,β	+	+
Growth at:				
37°C	+	+	+	.
4°C	+	+	-	.
Growth in:				
0% NaCl	+	+	+	+
2% NaCl	+	+	+	+
4% NaCl	+	+	+	+
6% NaCl	-	-	-	-
8% NaCl	-	-	-	-
Acid production				
Glucose (acid/gas)	+/+	+/+	+/+	+/+
Lactose	-	-	V	.
Sorbitol	-	-	-	-

Mannitol	+	+	+	+	
Sucrose	+	-	+	+	
Inositol	-	-	-	-	
Fructose	-	-	+	.	
Mannose	+	+	.	.	
Xylose	+	+	-	.	
Galactose	+	+	+	.	
Resistance to Vibriostatic agents					
10 µg		R	R	R	R
150 µg		R	R	R	R

* *A. hydrophila* and *A. sobria* strains from Austin and Austin (2007), +: positive, -: negative, ADH: Arginine dihydrolase, LDC: Lysine decarboxilase, ODC: Ornithine decarboxylase, ONPG: o-nitrophenyl-β-D-galactopyronoside, V: Variable results, .: not stated.

3.2. Molecular studies

According to the results of PCR assays with 16S rDNA, 685 bp amplicons was detected in two of the six *Aeromonas* strains. No amplicons were detected in the four strains. Two of the six strains were found to be *A. hydrophila* strains when the specific-specific 16S rDNA primer pair was used in the PCR studies (Fig.2).

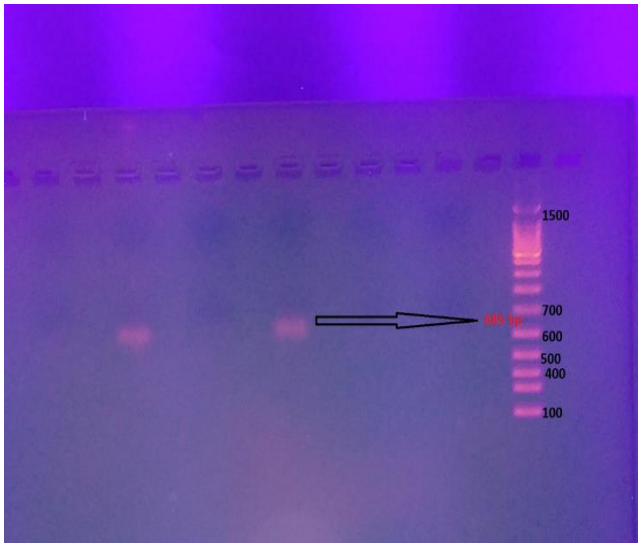

Fig. 2 Result of PCR assay using 16S rDNA pb. M: Marker.

The *aero* virulence gene was detected in 2 strains of *A. hydrophila* and 4 strains of *A. sobria* with a specific band of 209 bp (Fig.3).

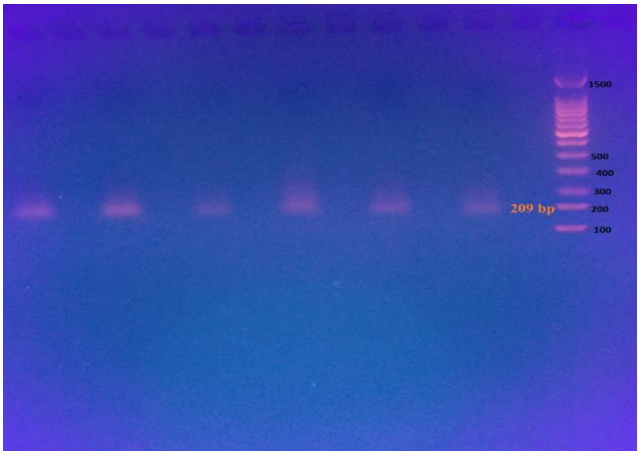

Fig. 3 Result of PCR assay using *Aero* virulence gene. Result of PCR assay using 16S rDNA pb. M: Marker.

In 2 *A. hydrophila* strains, 331 bp amplicons containing the *ast* gene were detected in the PCR assay. However, no amplicons were detected in four *A. sobria* strains (Fig.4). 232 bp amplicons were detected in the 6 strains with the *act* virulence gene (Fig. 5). 592 bp amplicons were observed in 2 *A. hydrophila* strains; however, the 4 *A. sobria* strains had no amplicons for the *hylA* virulence gene (Fig. 6). The antibiotic resistance genes (*tetC* and *sulI*) were not detected in all strains.

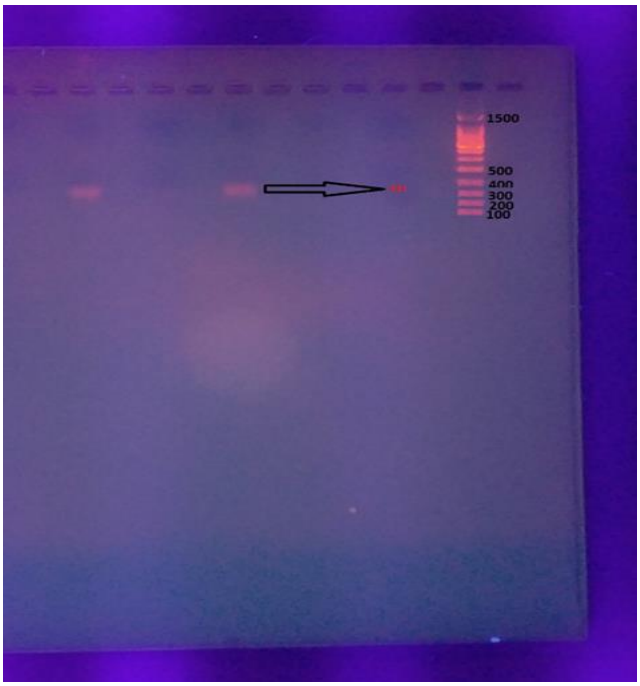

Fig. 4 The amplicons for *ast* gene were detected in two strains of *A. hydrophila*.

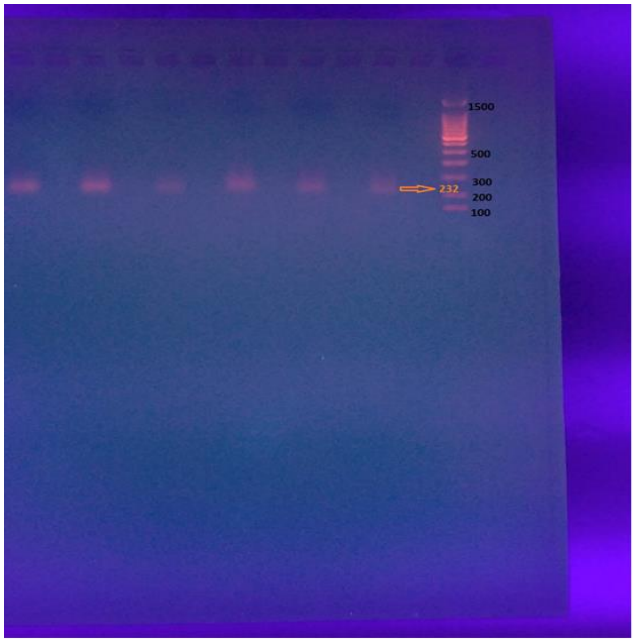

Fig. 5 Result of the PCR assay using the *act* virulence gene.

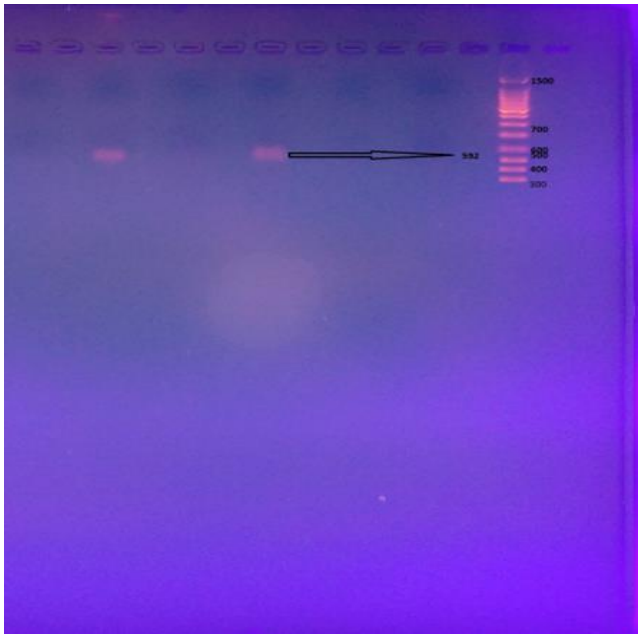

Fig. 6 The amplicons for *hyl A* were detected in 2 *A. hydrophila* strain.

3.3. Results of the antibacterial activity of astaxanthin on *A. hydrophila* and *A. sobria strains*

According to the disc diffusion results, astaxanthin solutions prepared with water and methanol were not effective in *A. hydrophila* and *A. sobria* strains, whereas 0.1g and 0.5g were effective in the strains (Figure 7, Tables 6 and 7); however, 1.0g astaxanthin solutions prepared with acetone were found to be effective in A. sobria strains but not in *A. hydrophila*. *A. hydrophila* strains showed resistance to oxytetracycline, while *A. sobria* strains showed intermediate resistance to OT30, which was used as a control.

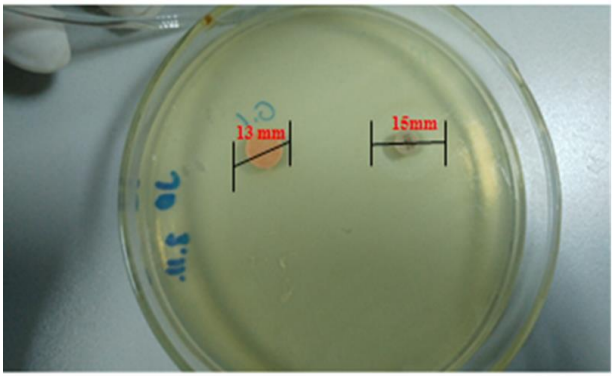

Fig. 7 Effect of acetone solution of astaxanthin on *A. sobria* a: acetone solution containing 0.1g astaxanthin, b: control (OXT 30)

Table 6 Result of disc diffusion test of water, methanol and acetone solutions of astaxanthin on *A. hydrophila* strains

Solvent	Astaxanthin (0.1 g)	Astaxanthin (0.5 g)	Astaxanthin (1.0 g)
Water	R	R	R
Methanol	R	R	R
Acetone	13 mm	18 mm	R
Control (OT30)	R		

R: Resistant

Table 7 Result of disc diffusion test of water, methanol and acetone solutions of astaxanthin on *A. sobria* strains

Solvent	Astaxanthin (0.1 g)	Astaxanthin (0.5 g)	Astaxanthin (1.0 g)
Water	R	R	R
Methanol	R	R	R
Acetone	13 mm	13 mm	16 mm
Control (OT30)		R	

R: Resistance, I. R. : Intermediate Resistnace

3.4. Antibiotic susceptibility profiles of strains

According to the standard disc diffusion technique, the *A. hydrophila* strains were resistant to ampicillin, chloramphenicol, tetracycline, streptomycin, and nalidixic acid. The strains were sensitive to sulfamethoxazole, flumequine, and trimethoprim, while showing intermediate resistance to erythromycin, kanamycin, and oxytetracycline. Strains of *A. sobria* showed resistant to ampicillin, chloramphenicol, sulfamethoxazole, erythromycin, and nalidixic acid, while the strains showed intermediate resistance to flumequine, trimethoprim, kanamycin, and oxytetracycline; strains were not sensitive to any of the antibiotics used in the study. The antibiogram test results of the strains are shown in Table 8.

Tablo 8 Antibiogram profiles of *A. hydrophila and A. sobria* strains against twelve antibiotics used in the study

Species	Antibiotics			
	C30*	RL25	E15	UB30
A.hydrophila (2 strains)	R	18 mm (S)	17 mm (I.R)	22 mm (S)
	Antibiotics			
A.hydrophila (2 strains)	W5 24 mm	TE10 R	S10 R	NA30 R
	Antibiotics			
	AMP10	K30	TE30	OT30
A.hydrophila (2 strains)	R	16 mm (I.R)	R	15 mm (I. R)
	Antibiotics			
	C30	RL25	E15	UB30
A.sobria (4 strains)	R	R	R (I.R)	17 mm
	Antibiotics			
	W5	TE10	S10	NA30
A.sobria (4 strains)	14 mm (I.R)	R 12 mm (I.R)	R	
	Antibiotics			
	AMP10	K30	TE30	OT30
A.sobria R (4 strains)	15 mm (I.R)	R	15 mm (I.R)	

C30: Chloramphenicol, RL25: Sulfamethaxazole, E15: Erythromycin, UB30: Flumequine, W5: Trimethoprim, TE10: Tetracycline, S10: Streptomycin, NA30: Nalidixic acid, AMP10: Ampicillin, K30: Kanamycin, TE30: Tetracycline, OT30: Oxytetracycline, R: Resistance, S: Susceptibe, I. R: Intermediate Resistance

3.5. Results of MAR Index

The *A. hydrophila* strains proved resistant to 7 of 12 antibiotics used in the study. The strains were found to be intermediate resistance to two antibiotics and sensitive to three antibiotics. *A. sobria* strains were resistant to 7 of the 12 antibiotics. The MAR index value for *A. hydrophila* was 0.5 and the MAR index value for *A. sobria* was 0.6. The results are shown in Table 9.

Table 9 The results of the MAR index for *A. hydrophila* and *A. sobria*

	A.hydrophila	*A.sobria*
Number of the resistant antibiotic disc (a)	6	7
Total number of antibiotics Used in the study (b)	12	12
The MAR index value (a/b)	0.5	0.6

4. Discussion

This study was carried out to determine the antibacterial effect of astaxanthin on *A. hydrophila* and *A. sobria* strains as an alternative to antibiotics. 2 of the 6 strains isolated from goldfish were phenotypically identified as *A. hydrophila* and four as *A. sobria*. PCR study using 16S rDNA-specific primers specific for *A. hydrophila* detected 685 bp amplicons in two of the six strains. The complex pathogenicity mechanism of *A. hydrophila* has been reported to be effective in causing such widespread infections (Ahangarzadeh et al. 2022). Proteinaceous toxins such as hemolysin (*hylA*) and aerolysin (*aerA*) involved in this pathogenicity mechanism, make the *A. hydrophila* strain virulent (Ahangarzadeh et al. 2022). PCR studies using specific primers for the *aerA* and *hylA* virulence genes of *A. hydrophila* strains detected amplicons of 209 bp and 592 bp, respectively, for both strains. Amplicons specific for the *hylA* virulence gene were not detected in four strains defined as *A. sobria*, but amplicons of 209 bp in the PCR study using specific primers for the *aerA* virulence gene were detected in all strains. Robertson *et al*. (2014) reported that the virulence genes hemolysin (*hylA*) and aerolysin (*aerA*) can be useful clues for distinguishing pathogenic *Aeromonas* species from nonpathogenic *Aeromonas* species. A number of conventional microbiological tests are used to determine the phenotypic characteristics of *Aeromonas* spp. Reading the results of these tests can be both time consuming and cause difficulties in accurately identifying bacterial species (Yadav et al. 2014). In the present study, the *hylA* gene was not detected in *A. sobria*, but was detected in *A. hydrophila* strains. The O/129 vibriostat test is important for distinguishing *Aeromonas* and *Vibrio* species. Like this test, the *hylA* virulence gene can also be used to distinguish *A. hydrophila* from other *Aeromonas* species.

Act gene, which belongs to virulence factors, is the most important enterotoxin with hemolytic, cytotoxic, and enterotoxic activities (Sreedharan et al. 2012). Sreedharan et al. (2012) reported that all isolates amplified at least one virulent gene related to the virulent genes of *Aeromonas* species they isolated from ornamental fish culture systems, and 58.3% of *Aeromonas* strains amplified the *act* gene. In the present study, the strains of *A. hydrophila* and *A. sobria* all amplified the *act* gene. The ast gene was detected only in *A. hydrophila* strains.

Antibiotics and other chemicals are used in aquaculture to prevent and treat disease outbreaks. However, the use of antibiotics for therapeutic purposes is not recommended. The development of antimicrobial resistance in pathogenic bacterial species that cause disease affects this condition (Mohd-Aris et al. 2019). *Aeromonas* species play an important role as a source of antimicrobial resistance genes and they can be considered as indicator bacteria for antibiotic resistance detection (Conte et al. 2020). In this study, the antimicrobial resistance genes *tetC* and *sulI* of 6 strains were investigated. No amplification was detected in the PCR study using primers specific for the sulfonamide resistance gene (*sulI*). Similar results were obtained in the PCR study with primers specific for the *tetC* resistance

gene. However, in the antibiogram study using the standard diffusion method, strains were found to be resistant to both 10 µg and 30 µg tetracycline discs. This could be due to the presence of other organisms that cause tetracycline resistance, such as flow pumps, where strains are phenotypically resistant to tetracycline, as noted by Natarajan et al. (2018).

In the study, 6 *Aeromonas* strains were resistant to chloramphenicol. El-Gohary et al. (2020) informed that isolates were highly resistant (80%) to chloramphenicol in their study of *Aeromonas* spp. Hossain et al. (2020) reported that the resistance rate of *Aeromonas* isolates from ornamental guppies (*Poecilia reticulata*) to chloramphenicol was 5.8%. Although chloramphenicol is a broad-spectrum antibiotic, resistance to this antibiotic has been frequently reported (Dinos et al. 2016). Resistance to ampicillin is observed in *Aeromonas* species, with the exception of *Aeromonas trota* and a few strains (Fernández-Bravo and Figueras 2020). In this study, resistance to ampicillin was observed in *A. hydrophila* and *A. sobria*. While *A. hydrophila* strains were moderately resistant to erythromycin in the study, *A. sobria* strains were resistant. Jagoda et al. (2014) investigated the susceptibility of 53 *Aeromonas* isolates from freshwater ornamental fish to 8 antimicrobial agents. In addition to amoxicillin in the beta-lactam antibiotic group, the highest resistance was found to tetracycline at 58.5% and erythromycin at 54.7%. Eid et al. (2022), in their study investigating the resistance of *Aeromonas* isolates isolated from fish and water samples to antibiotics from seven different classes, reported that the isolates showed extremely high resistance (90%) to tetracycline and significant resistance (63.33%) to streptomycin. The isolates showed low resistance to nalidixic acid. In the study, all *Aeromonas* strains showed resistance to 10 µg and 30 µg tetracycline discs. While 2 *A. hydrophila* strains showed intermediate resistance to oxytetracycline, 4 *A. sobria* strains proved resistant. All strains showed resistance to streptomycin and nalidixic acid. In the study, both *A. hydrophila* and *A. sobria* strains showed intermediate resistance to kanamycin.

5. Conclusion

The widespread use of antibiotics in agriculture and aquaculture has led to a global increase in antibiotic resistance. However, because antibiotic resistance arose millions of years before the era of modern antibiotics, it has been shown that the development of antibiotic resistance cannot be completely eliminated (Dinos et al. 2016). In the study, the MAR index value of *A. hydrophila* strains was 0.5; the MAR index value of *A. sobria* strains was 0.6. All strains showed resistance to more than one antibiotic. Strains with multiple resistance to antibiotics, the presence of the *aero* virulence gene and the *act* virulence gene in all strains; this indicates that the treatment of infections that may arise from these pathogenic bacteria will be difficult. Therefore, in this study investigating the antibacterial activity of astaxanthin, it was found that 0.5 g and 0.1 g astaxanthin solutions prepared with acetone effectively showed antibacterial properties in both *A. hydrophila* and *A. sobria* strains. According to the results of the study, it can be assumed that the use of astaxanthin as a feed additive in fish farming has a prophylactic significance in relation to bacterial fish diseases, but there is also a need for more experimental studies that can show the effect of astaxanthin in relation to fish health.

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