

# Decontamination of Marketed Mullet (*Mugil cephalus*) Infected with *Aeromonas hydrophila* by Organic Acids

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## ABSTRACT

**Background and Objective:** *Aeromonas hydrophila* (*A. hydrophila*) is an emerging enteric pathogen that is causing outbreaks in global fish farms, resulting in significant financial losses. **Materials and Methods:** Using the plate count method, this study investigated the antimicrobial activity of organic acids such as acetic and citric acid on *A. hydrophila* in mullet (*Mugil cephalus*) at various treatment times (0.5, 1.5, 2.25 and 24 hrs) and temperatures (30±2°C and 5±2°C). **Results:** After a long time (24 hrs) of treatment, the findings of organic acids treatment revealed that acetic acid (5%), citric acid (5-6%) and acetic-citric acids mixes were effective against *A. hydrophila* at room temperature with a reduction rate of 98.2%, 38.2% and -45.97%, respectively and refrigerator temperature with reduction rate of 97.01%, 15.22% and -28.95%, respectively. At both refrigeration and room temperatures, the acetic acid (5%) showed rising reduction rates that reached almost their highest value after 24 hrs (97.01 and 98.20%, respectively). In addition, citric acid was more effective at room temperature than at refrigerator temperature (0.5, 1.5 and 2.25 hrs). At both temperatures, however, the decrease rate generated by the acetic-citric acid mixture vanished and was almost similar to that of untreated groups. **Conclusion:** The results of the laboratory investigation suggested that using organic acids (acetic and citric acid) to decontaminate *A. hydrophila* infection in mullet aquaculture farms is a safe and cost-effective option.

## KEYWORDS

*Aeromonas hydrophila*, decontamination, mullet (*Mugil cephalus*), organic acids (acetic acid and citric acid)

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## INTRODUCTION

*Aeromonas hydrophila* as gram-negative non-sporulated bacilli is a facultative anaerobic bacterium commonly existing in aquatic environments where it has emerged as a food-borne pathogen of great significance<sup>1,2</sup>. The infections with *A. hydrophila* are associated with a lot of syndromes including swelling of tissues, dropsy, red sores, necrosis, ulceration and haemorrhagic septicaemia in tilapia fish<sup>3</sup>. *Aeromonas hydrophila* has the ability to tolerate frying, grilling and freezing temperatures<sup>4</sup>. Hence, there are extreme public health risks posed by the ready-to-eat fish involving gastroenteritis, wound infection, septicaemia and skin disease, bacteremia, respiratory tract infections, gastroenteritis, urinary tract infection and traveler's diarrhea<sup>5-7</sup>.



As a result of extensive requirements of consumers to safe, normal and nontoxic preservatives which could be utilized at the domestic level, organic acids Generally Recognized As Safe stations (GRAS) are widely utilized as food preservatives as well as food additives<sup>8</sup>. The use of organic acids is due to their antimicrobial activity and acidic pH. They have two forms, un-dissociated form (uncharged form) and dissociated (charged form). The bacterial lipid membrane could be penetrated only by the un-dissociated form which detaches into anions and protons in the microbial cytoplasm. The bacterial cell must utilize further energy in the form of ATP to retain its impartiality and therefore exhaustion of cell energy and hampering of cellular growth existence<sup>9</sup>.

There are various organic acids such as citric, acetic, ascorbic and formic acids among others. Foundations of citric acid are the juice of citrus and further acidic fruits for instance, lemon, limes, pineapples and gooseberries. Citric acids are extremely water solvable and mainly fat-unsolvable. It has an inhibitory influence on several pathogens such as bacteria, yeast and molds<sup>10</sup>. Furthermore, acetic acid is frequently recognized as vinegar that has antimicrobial abilities owing to its capability to produce lesser pH and affect the stability of the cellular membrane of pathogen<sup>11</sup>.

Therefore, the objective of this research was to evaluate the antimicrobial activity of organic acids (acetic and citric acids) on *Aeromonas hydrophila* in marketed mullet (*Mugil cephalus*) in Egypt.

## MATERIALS AND METHODS

**Study area:** This study was conducted at the Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt from April to August, 2019.

**Ethical statement:** This investigation was performed in accordance with the recommendations approved by the Ethics Committee of the Faculty of Veterinary Medicine, Mansoura University University (Permit numbers 20-17). The duration of the experiment was from April to August, 2019.

**Experimental fish:** Apparently healthy mullets (n = 160) of approximately 400-500 g were obtained and transferred alive to the Laboratory of the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Mansoura University, Mansoura City, Egypt to be applied for experimental challenge. Mulletts were adapted to Lab condition and glass aquaria for 3 weeks and arbitrarily sampled for exclusion of *A. hydrophila* infections. At that time, mullets were fed *ad libitum* with a commercial diet (Uccma feed, Egypt, crude protein, 32%, crude lipid, 6.2%, crude fiber, 5.7%) and evaluated for their general health.

**Bacterial inoculum:** *Aeromonas hydrophila* originated from mullet were kindly obtained from Zoonosis Laboratory, Faculty of Veterinary Medicine, Mansoura University, Egypt<sup>12</sup>. Inoculation of tryptic soya broth (TSB) (Oxoid, Hampshire, UK) with *A. hydrophila* colonies was made, followed by incubation at 35°C for 24 hrs. Ten-fold serial dilutions from the inoculated TSB were prepared and then 100 µL of every dilution was sub-cultured consistently onto the MacConkey-ampicillin agar plates (Oxoid, Basingstoke, UK), followed by incubation at 35°C for 24 hrs. *Aeromonas hydrophila* ( $3.35 \times 10^6$  CFU mL<sup>-1</sup>) was prepared as mentioned by Brenner *et al.*<sup>13</sup>, Janda and Abbott<sup>14</sup>.

**Sources of organic acids solutions:** One liter of commercial vinegar (5%) and freshly squeezed lemon fruit juice were utilized to examine the antimicrobial effect of both acetic acid and citric acid on *A. hydrophila*, respectively. One lemon produces around 45 mL of juice. The lemon juice had approximately 5-6% citric acid, with acidic pH (of 2.2)<sup>8</sup>.

**Design description:** The freshly purchased mullet samples (n = 160) were arbitrarily separated into 8 groups with 20 fish each. Four groups were separated into the same division but at room temperature (RT) (30±2°C) as follows: Untreated group (G1R) and treated groups with acetic acid (G2R), citric acid (G3R),

Table 1: Experimental description of the organic acid treated mullet experimentally infected with *A. hydrophila*

Temperature	Treated groups			Untreated group
	Acetic acid	Citric acid	Acetic-citric acids mixture	
Refrigeration T (5±2°C)	20	20	20	20
Room T (30±2°C)	20	20	20	20

acetic-citric acids mixture (G4R). Also, the other four groups were divided into an untreated group (G1F) and treated groups with acetic acid (G2F), citric acid (G3F) and acetic-citric acids mixture (G4F) at refrigerator temperature (5±2°C) (Table 1).

**Experimental examination and parameters studied:** All mullets were bathed using sterilized phosphate-buffered saline solution (Difco, Detroit, MI) and then dribble on the aseptic metal mesh. After that, disinfection of mullet was performed by sinking in ethanol (70%) for 5 min, followed by dribbling on sterilized metal mesh in a laminar flow hood. Once thorough occurred, mullets were immersed for 45 min in 1 L of bacteria suspension (with 10 times clockwise and anticlockwise trembling each five min for the full time)<sup>8,15</sup>. Then, the mullet groups (G2R, G3R, G4R) were dipped in baths containing acetic acid, citric acid and acetic-citric acids mixture and kept at RT (30±2°C), while other groups (G2F, G3F, G4F) were dipped in baths containing acetic acid, citric acid, acetic-citric acids mixture and kept at refrigerator temperature (5±2°C). The untreated groups (G1F and G1R) were dipped in a sterile saline solution without any organic acids.

After each specified time (0.5, 1.5, 2.25 and 24 hrs), treated mullets were collected by sterilized forceps and permitted to dry on aseptic material. Ten grams of the musculature of treated mullet were weighed up into 90 mL of sterilized physiological saline (0.85% NaCl) and then homogenization was made in a stomacher under aseptic condition. A serial ten-fold dilution in sterile phosphate buffered saline solution was made from the original homogenate up to 10<sup>6</sup>. Then, 0.1 mL from every dilution was inoculated into MacConkey-ampicillin agar plates (Oxoid, Basingstoke, UK) and the plates were kept in the incubator at 35°C for 24 hrs. On the plate surface, the colonies were counted and the log<sub>10</sub> CFU g<sup>-1</sup> was determined<sup>16</sup>.

**Statistical analysis:** The Kruskal-Wallis test (non-parametric ANOVA) was used for the differentiation of the treatment and control means. The p-value was established at ≤0.05 (SPSS version 20).

Log<sub>10</sub> reduction and reduction percentages were designed by Excel software version 2010:

$$\log_{10} \text{ reduction} = A - B$$

Where,

A = log<sub>10</sub> number of viable microbes before treatment

B = log<sub>10</sub> number of viable microbes after treatment

$$\log \text{ reduction percent (\%)} = \frac{A - B}{A} \times 100$$

If the number is negative, this indicates a log<sub>10</sub> increase in number and percentage.

## RESULTS

**Decontamination of *A. hydrophila* using organic acids at room temperature:** The efficacy of organic acids on *A. hydrophila* at room temperature (RT) was evaluated by the reduction in colony counts after different treatment times (0.5, 1.5, 2.25 and 24 hrs) using the plate count method of viable bacterial cells (Table 2). The acetic acid (98.2%), followed by citric acid (38.2%) and acetic-citric acid mixtures (-45.97%)

Table 2: Effect of organic acids treatments on *A. hydrophila* count at room temperature

Time (hrs)	Treated groups											
	Acetic acid			Citric acid			Acetic-citric acids mixture			Untreated group		
	Log <sub>10</sub> means	Log <sub>10</sub> R	R%	Log <sub>10</sub> means	Log <sub>10</sub> R	R%	Log <sub>10</sub> means	Log <sub>10</sub> R	R%	Log <sub>10</sub> means	Log <sub>10</sub> R	R%
0.5	2.99	0.36	10.74	2.86	0.49	14.62	3.31	0.04	01.19	3.45	-0.10	-02.98
1.5	2.91	0.44	13.13	3.01	0.34	10.14	4.87	-1.52	-45.37	4.99	-1.64	-48.95
2.25	2.09	1.26	37.61	2.96	0.39	11.64	4.09	-0.74	-22.08	4.28	-0.93	-27.76
24	0.00	3.29	98.20	2.07	1.28	38.20	4.89	-1.54	-45.97	4.95	-1.60	-47.76

The difference between the reduction effects of the two acids on *A. hydrophila* was significant in which and  $p < 0.0001$

Table 3: Effect of organic acids treatments on *A. hydrophila* count at refrigerator temperature

Time (hrs)	Treated groups											
	Acetic acid			Citric acid			Acetic-citric acids mixture			Untreated group		
	Log <sub>10</sub> means	Log <sub>10</sub> R	R%	Log <sub>10</sub> means	Log <sub>10</sub> R	R%	Log <sub>10</sub> means	Log <sub>10</sub> R	R%	Log <sub>10</sub> means	Log <sub>10</sub> R	R%
0.5	3.04	0.31	9.25	2.82	0.53	15.82	3.13	0.22	6.56	3.22	0.13	3.88
1.5	2.92	0.43	12.83	2.98	0.37	11.04	3.15	0.20	5.97	3.19	0.16	4.77
2.25	2.15	1.20	35.82	2.89	0.46	13.73	3.18	0.17	5.07	3.31	0.04	1.19
24	0.00	3.25	97.01	2.84	0.51	15.22	4.32	-0.97	-28.95	4.45	-1.10	-32.83

The difference between the reduction effects of the two acids on *A. hydrophila* was significant in which and  $p < 0.0001$

were effective against *A. hydrophila* for a long period of treatment (24 hrs) at RT. The citric acid showed the highest reduction rate (14.62%) of *A. hydrophila* after 0.5 hrs of treatment, while acetic acid revealed the highest reduction rate (13.13, 37.61 and 98.2%) after 1.5, 2.25 and 24 hrs of treatment, respectively. The acetic-citric acid mixture led to a reduction of *A. hydrophila* to 1.19% after a short period of treatment only (0.5 hrs) at RT.

**Decontamination of *A. hydrophila* using organic acids at refrigeration temperature:** The efficiency of organic acids on *A. hydrophila* at refrigerator temperature was assessed by the reduction in colony counts after different treatment times (0.5, 1.5, 2.25 and 24 hrs) using the plate count method of viable bacterial cells (Table 3). The acetic acid (97.01%), followed by citric acid (15.22%) and mixtures (-28.95%) were effective against *A. hydrophila* at refrigerator temperature for a long period of treatment (24 hrs). The citric acid showed the highest reduction rate (15.82%) of *A. hydrophila* after 0.5 hrs of treatment, while acetic acid revealed the highest reduction rate (12.83, 35.82 and 97.01%) after 1.5, 2.25 and 24 hrs of treatment, respectively. The acetic-citric acid mixture led to a reduction of *A. hydrophila* to 6.56% after a short duration of treatment only (0.5 hrs) at refrigerator temperature.

**Comparison of decontamination at both room and refrigeration temperatures:** The results of organic acids treatment demonstrated that acetic acid (5%) had to descend reducing rates which extended approximately its peak value afterward 24 hrs (97.01 and 98.20%) at both refrigerator and RT, respectively. Furthermore, the effectiveness of acetic acid was highly observed at RT than at refrigerator one. Regarding the influence of citric acid, it was higher in effect than acetic acid only at a short duration (0.5 hrs) at both temperatures. Also, citric acid was more effective at refrigerator temperature than at RT for (0.5, 1.5 and 2.25 hrs) (Table 2 and 3). The variance between the reduction influence of the two acids on *A. hydrophila* was statistically significant ( $p < 0.0001$ ).

On the other hand, when acetic and citric acids were mixed, they resulted in a buffering effect between them (weak and strong acids). The acetic-citric acid mixture led to a reduction of *A. hydrophila* to an undetectable level after 0.5 hrs at both temperatures. The reduction was continued for 1.5 and 2.25 hrs

at refrigerator temperature and then the reduction level disappeared at both temperatures after 24 hrs (Table 1 and 2). So, the reduction rate in the mixture of treated samples was nearly equal to that of treated ones.

## DISCUSSION

The present results demonstrated that the acetic acid had a reduction rate that reached its peak value after 24 hrs from treatment at either room or refrigerator temperatures. On the other hand, citric acid was more efficient than acetic acid at a short time of 0.5 hrs of treatment at both room and refrigerator temperatures. Regarding temperature, acetic acid and citric acid were more effective at room temperature than refrigerator one after 24 hrs of treatment. The *A. hydrophila* is not only associated with frequent fish diseases and heavy economic losses, but it also has public health hazards in Egypt<sup>5,12,17</sup>. Thus, for consumers, protection against foodborne pathogens such as *A. hydrophila* is a global public health concern. Thus, the current investigation studied the decontamination of mullet from *A. hydrophila* using organic acids. Although the antimicrobial activities of acetic and citric acids have previously been approved<sup>18</sup>, the antimicrobial activity of acetic acid is higher than citric acid<sup>19</sup>. Acetic acid interferes with cytoplasmic membrane structure and membrane proteins for instance the electron transportation is detached and a consequent reduction of ATP production has occurred<sup>20</sup>. Our results were consistent with a previous investigation which demonstrated the inhibitory effect of acetic acid against bacteria and molds<sup>15</sup>. Also, organic acids (citric, lactic and acetic acids alone or in a mixture) had been revealed to be efficient in the reduction of either pathogenic or spoiled organisms<sup>21</sup>. The previous study applied citrus lemon to protect freshwater fish from *Aeromonas* infection and established efficient protection against *Aeromonas* species infection in juvenile labeo victorious fingerlings fish through the improvement of immunological response, oxidative condition, or growth performance as feed additives<sup>22</sup>. Recently, the effect of organic acids (citric and sorbic acid) on the gut microbiota of European sea bass juveniles was discovered<sup>23</sup>. The *Aeromonas* species are mostly affected by lactic and acetic acid in bovine carcasses<sup>24</sup>. In fact, the variation in antibacterial activity of organic acids might be regarded as the physiological state of the pathogens and the physicochemical characteristics of the external environment<sup>25</sup>.

To date, no studies have tested the effect of acetic acid or citric acid on *A. hydrophila* in mullet fish species in Egypt. Some studies have investigated the effects of certain organic acids in limiting bacterial contamination and propagation of foodborne germs in pre-harvest and post-harvest production of food and processing<sup>26</sup>. Because of the antimicrobial activity of organic acids, they are utilized in animal and human nutrients. Foods cured with organic acids have been thought to make limitations of microbial colonization via the transformation of organic acids into certain antimicrobial phases, just once organic acids are reached the gastrointestinal tract of individuals, animals and fish ingesting the cured foods or feed<sup>9,27,28</sup>.

**Limitations:** The present study recommended improving the quality and safety in mullet aquaculture farms through the application of organic acids particularly acetic acid for the decontamination of *A. hydrophila* strains. It should be noted that there are some limitations to the present study. Therefore, additional studies are warranted to explore the improvement of growth performance and immune responses of mullets against fish pathogens, especially *A. hydrophila* infection.

## CONCLUSION

In light of the results obtained in the current research, it could be assumed that application of organic acids particularly acetic acid could effectively decontaminate *A. hydrophila* strains in mullet. Further studies should be applied to the improvement of growth performance and immune responses of mullet against *A. hydrophila* infection.

## SIGNIFICANCE STATEMENT

The evaluation of the antimicrobial activity of organic acids (acetic and citric acids) on *Aeromonas hydrophila* that is commonly distributed in aquaculture, particularly marketed mullet (*Mugil cephalus*) in Egypt. The implication of the results was that the highest reduction rate of acetic acid occurred at 24 hrs of treatment, while citric acid was more efficient at 0.5 hrs of treatment at either room or refrigerator temperatures. Also, acetic acid and citric acid were more effective at room temperature than at refrigerator one after 24 hrs of treatment.

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