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Efficacy of natural and consumer-friend applications to control *Aeromonas hydrophila*, growth in Bluefish

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ABSTRACT

Fish is one of the main transmission routes of *Aeromonas (A.) hydrophila*, an emerging pathogen that threatens public health due to its high antibiotic resistance. This study aimed to control the growth of *A. hydrophila* in cold-stored Bluefish (*Pomatomus saltatrix*) using natural, consumer-friendly practices. Samples were inoculated with *A.hydrophila*, dipped or sprayed with acetic acid, citric acid, ascorbic acid, sodium lactate, or sodium chloride solutions (4%), and stored at 4°C. Dipping was very effective since the growth of *A. hydrophila* was inhibited by all dipping treatments and remained below the inoculation dose after 72 hours. During this time, dipping in acetic acid, ascorbic acid, and citric acid reduced the initial load of *A. hydrophila* (7.03 log cfu/g) to 5.27, 5.51, and 5.64 log cfu/g, respectively. Acetic acid, ascorbic acid, and citric acid dipping treatments reduced the *A. hydrophila* number 1 log/cfu more than other treatments ($P<0.05$). Acetic acid and ascorbic also provided the best results for the sprayed samples. Our results showed that dipping in natural acids such as acetic acid, ascorbic acid, and citric acid yielded successful results in inhibiting *A. hydrophila* growth. Using consumer-friendly, natural substances to ensure food safety by controlling the growth of this emerging pathogen will provide significant benefits for the food industry.

Keywords: *Aeromonas hydrophila*, Bluefish, Organic acid, Dipping, Spraying

Introduction

Aeromonas hydrophila is an important opportunistic foodborne pathogen that causes human gastroenteritis or septicemia. It is widely distributed in the environment and found in the intestinal flora of humans and animals. *Aeromonas hydrophila* and other members of *Aeromonas* spp. have been isolated from many fish species, roe, prawns, shellfish, and ready-to-eat seafood products, and seafood has a crucial role in the transfer of this bacteria to humans. (Vivekanandhan et al., 2005; Di Pinto et al., 2011; Stratev et al., 2015; Praveen et al., 2016). Due to the high resistance of *A. hydrophila* to antibiotics, it is difficult to cure diseases caused by this pathogen, and its importance for public health has become prominent. Therefore, it has been regarded as a foodborne pathogen having an emerging importance; and most (> 85%) of human gastroenteritis cases are associated with *Aeromonas* sp., including *A. hydrophila*. The crucial need for further investigation into the control of this pathogen has been reported due to its role in public health and food safety (Praveen et al., 2016; Pal, 2018; Hoel et al., 2019). Various techniques have been developed for the inhibition of pathogenic bacteria. However, in recent years, consumers have been concerned about using artificial additives or preservation methods even more than the pathogen risk. Therefore, the food industry continuously seeks efficient and natural solutions (Mahmoud, 2014).

A growing significance of *A. hydrophila* as an emerging pathogen and fish has been regarded as one of the main transmission routes. Since *A. hydrophila* can grow at refrigerated temperatures, it may significantly impact cold-stored foods and pose a risk during fish transportation, processing, and marketing (Daskalov, 2006; Praveen et al., 2016). The majority (>85%) of gastroenteritis cases caused by Aeromonas are responsible for three *Aeromonas* species. One is *A. hydrophila* (hybridization group HG1) (Daskalov, 2006). Therefore, this study aimed to control the growth of *A. hydrophila* in cold-stored fish using natural, consumer-friendly practices, and the effects of organic acids or salts were studied.

Material and Methods

Sample Preparation

Fresh Bluefish *Pomatomus saltatrix* (Linnaeus, 1766) were purchased from the Istanbul, Türkiye, wholesale market. Bluefish was chosen as raw material, as it is an economically valuable species (Bal et al., 2018) and a treasure for Turkey (Mol & Varlik, 2019).

The samples were packed in polystyrene boxes with ice and then transferred to the laboratory. The samples' average lengths (cm) were 32 ±1.32, and the average weights (g) were 408.68 ±10.64. The samples were cut into portions approximately 2x5x1 cm (10g), and each sample was decontaminated under a U.V. cabinet (CRYSTE, Korea) at 366 nm for 2 hours. Before inoculation, decontaminated fish samples were analyzed for the presence/absence of *A. hydrophila* (Xanthopoulos et al., 2010).

Preparation of Bacterial Inoculum

Aeromonas hydrophila was obtained from the culture collection of the Department of Aquaculture and Fish Diseases Faculty of Aquatic Sciences, İstanbul University. The bacterial stock culture was kept in Tryptone Soy Broth (TSB) (Merck, 1.05499) with 20% glycerol (v/v) at –80°C before use. *Aeromonas hydrophila* was activated in TSB at 30°C for 24h. After incubation, the culture was centrifuged (EBA 20 Hettich, Germany) at 4000 RPM for 10 min. After centrifugation, the pellet was washed two times in 10 mL TSB. After this process, the supernatant was removed, and the pellet was resuspended in TSB (10mL). Then, serial dilutions were prepared, and the bacteria cell count was determined (Doğruyol et al., 2020).

Sample Inoculation and Treatments

Both sides of the fish samples were inoculated with 0.2 mL of *A. hydrophila* inoculum (10 log cfu/mL) and spread with a sterile Drigalski spatula. The inoculated fish samples were left to stand in a sterile cabinet for 15 min for inoculum attachment. For the natural acid /natural salt treatments, food grade acetic acid (Sigma Aldrich, 4% v/v), citric acid (Sigma Aldrich, 4% w/v), ascorbic acid (Sigma Aldrich, 4% w/v), sodium lactate (Fluka, 4% v/v) and sodium chloride (Merck, 4% w/v) solutions were prepared in sterile distilled water. The inoculated fish samples were divided into thirteen groups. Six batches of these groups were treated by dipping for two minutes in one of 4% acetic acid, 4% citric acid, 4% ascorbic acid, 4% sodium lactate, 4% sodium chloride solution, or sterile distilled water. The ratio of Bluefish samples to treatment solution volume was 1:2 (w/v). After dipping, the fish samples were kept for 15 min at room temperature (20°C) to allow draining. The other six batches were treated by spraying (0.50 mL) one of the formerly mentioned solutions to one side of each fish sample. The fish samples were left to stand for 15 min for the attachment. The same procedure was repeated for the other sides of each sample. The thirteenth group was untreated (UNT). All samples were treated at the same time. Then, samples from different treatment groups were placed in sterile plastic bags, stored at 4 ±1°C for 72 hours, and analyzed every 24 hours.

Enumeration of A. hydrophila

Microbiological analyses were performed in duplicate. Aseptically, 10 g of Bluefish sample was homogenized with 90mL TSB in a stomacher bag using a stomacher (IUL Instruments, Barcelona, Spain) for 60 seconds. Serially diluted samples were spread out (0.1 mL) onto Aeromonas Isolation Medium Base (Himedia, M884), supplemented with Aeromonas selective supplement (HIMEDIA, FD039), for quantitative enumeration. Three agar plates per dilution were incubated at 28°C for 24 hours (Xanthopoulos et al., 2010).

pH Measurement

All pH measurements were performed in triplicate. Fish were homogenized (fish/distilled water; 1:10 w/v), and Hanna pH 211 Micro-processor pH meter was used (Vyncke, 1981).

Statistical Analysis

The experimental study was repeated twice using 12 individuals of Bluefish per treatment (12 fish x 13 treatments x 2 replications). *Aeromonas hydrophila* counts were presented as $\log_{10}$ cfu/g values. The reduction in *A. hydrophila* counts was calculated by subtracting the $\log_{10}$ cfu/g in the treated samples (dipped and sprayed) from the $\log_{10}$ cfu/g in the untreated samples. Analysis of variance (ANOVA) was used to compare the results of mean bacterial levels. Significance was determined at the $P < 0.05$. Data were analyzed using the IBM SPSS 21 software program.

Results and Discussion

Effect of Treatments on Growth of A. hydrophila

Overall results indicated that pre-processing organic acids, sodium lactate, and NaCI salt treatments as dipping solutions might provide more effective inhibition compared to treatments with sprayed solutions and untreated samples. Some similar studies demonstrated that dipping treatment considerably reduced bacterial load (Anderson et al., 1988; Okolocha & Ellerbroek, 2005; Leceta et al., 2015). Organic acids have been reported to be more effective in decreasing microbial load (Xiong et al., 1998; Phillips, 1999; Samelis et al., 2001; Geornaras et al., 2006; Rio et al., 2007; Neetoo et al., 2008; Schelegueda et al., 2012; Gonzales-Fandos & Herrera, 2014; Zaki et al., 2015; Mohan & Pohlman, 2016). Before the treatment, the uninoculated fish samples were found to be free of *A. hydrophila*. After the inoculation, *A. hydrophila* load was found to be 7.03 ±0.06 log cfu/g, then reached 9.51 ±0.19 log cfu/g after 72 hours at 4°C in UNT samples. The populations of *A. hydrophila* in treated Bluefish samples, either by dipping or spraying, are presented in Table 1. Dipping treatment resulted in significantly ($P<0.05$) lower *A. hydrophila* counts. The growth of *A. hydrophila* was suppressed by all dipping treatments, and its amount was reduced below the inoculation dose after 72 hours (Table 1). Spraying also reduced the initial load, but *A. hydrophila* counts were significantly higher ($P<0.05$) than dipped samples and exceeded the initial load after 24 hours of storage. Sterile water treatment also succeeded in reducing the initial load.

Table 1. *A. hydrophila* counts on bluefish samples treated with different organic acids under refrigerated storage

			Storage Hours			
Applications method	**Groups**	**Chemical solutions**	**0 hour**	**24th hour**	**48th hour**	**72th hour**
Spraying	S1	Sterile Water	6.97 ±0.12 ^{aeA}	7.86 ±0.04 ^{adB}	9.23 ±0.05 ^{aC}	9.64 ±0.11 ^{ajD}
	S2	NaCl %4	6.93 ±0.22 ^{aeA}	7.85 ±0.05 ^{adB}	8.86 ±0.06 ^{bC}	9.76 ±0.15 ^{aD}
	S3	Acetic Acid %4	6.88 ±0.07 ^{aAB}	6.96 ±0.31 ^{bAB}	6.82 ±0.06 ^{cA}	7.34 ±0.32 ^{bB}
	S4	Ascorbic Acid %4	6.97 ±0.03 ^{aeA}	7.12 ±0.10 ^{cA}	8.66 ±0.10 ^{dB}	8.15 ±0.15 ^{cC}
	S5	Sodium lactate %4	6.89 ±0.08 ^{aA}	7.89 ±0.05 ^{agB}	9.23 ±0.12 ^{aC}	9.14 ±0.06 ^{dC}
	S6	Citric Acid %4	7.01 ±0.06 ^{aeA}	7.73 ±0.05 ^{dgB}	8.57 ±0.13 ^{eC}	9.38 ±0.27 ^{ejD}
Dipping	D1	Sterile Water	5.70 ±0.03 ^{bA}	6.09 ±0.03 ^{eB}	6.12 ±0.05 ^{fB}	6.76 ±0.07 ^{fC}
	D2	NaCl %4	6.26 ±0.04 ^{cA}	6.04 ±0.05 ^{eB}	6.06 ±0.04 ^{fhB}	6.40 ±0.08 ^{gC}
	D3	Acetic Acid %4	5.71 ±0.04 ^{bA}	5.40 ±0.11 ^{fB}	5.79 ±0.07 ^{gA}	5.27 ±0.16 ^{hB}
	D4	Ascorbic Acid %4	5.74 ±0.08 ^{bAC}	5.41 ±0.04 ^{fB}	5.83 ±0.02 ^{gA}	5.51 ±0.15 ^{iCB}
	D5	Sodium lactate %4	6.07 ±0.07 ^{dA}	5.94 ±0.06 ^{eB}	6.01 ±0.07 ^{hB}	6.35 ±0.03 ^{gC}
	D6	Citric Acid %4	5.67 ±0.08 ^{bA}	5.44 ±0.08 ^{fB}	5.56 ±0.10 ^{iAB}	5.64 ±0.06 i^{A}
Untreated	UNT		7.03 ±0.06 ^{eA}	8.02 ±0.28 ^{gB}	9.15 ±0.05 ^{aC}	9.51 0.19 ^{jD}

* Uppercase letters on the same line show significant differences ($p < 0.05$) and lower case letters in the same column show significant differences ($p < 0.05$)

Considering that the inoculation level is 7.03 log cfu/g, sterile water treatment reduced the *A. hydrophila* load by spraying (6.97 log cfu/g) or dipping (5.70 log cfu/g), and dipped samples remained below the initial load even after 72 hours. Our study also showed that the dipping process provides mechanical reduction of the bacterial load by washing, enhancing the decontaminant effect. The inoculated *A. hydrophila* count (7.03 log cfu / g) decreased significantly ($P < 0.05$) after 72 hours in samples (D1) dipped in sterile water (Table 1). Treatment-related changes in the inoculated *A. hydrophila* count (7.03 log cfu/g) after 72 hours of cold storage are presented in Figure 1. Dipping treatment was found effective since the growth of *A. hydrophila* was inhibited by all dipping treatments and remained below the inoculation dose after 72 hours. Dipping in acetic acid (5.27 log cfu/g), ascorbic acid (5.51 log cfu/g), and citric acid (5.64 log cfu/g) were the most successful treatments, reducing the load of this pathogen. A study on oysters found that the use of five percent citric acid significantly reduced the bacterial population (Mahmoud, 2014). The dipping method is quite effective because all the food is covered with the dipping solution (Meredith et al., 2013).

Contrary to the results we obtained in our study, Smyth et al. (2018) reported that the total viable count did not change significantly in cod fillets dipped in 5% citric acid. In sprayed samples, acetic acid, and ascorbic acid were also more effective than other treatments (Figure 1). In the study by Dorsa et al. (1997), refrigerated beef carcasses were washed with a 3% acetic acid solution, and a significant reduction in total bacterial load was determined. Delmore et al. (2000) reported that different treatments, including acetic acid, effectively reduced the bacterial count on beef samples. Another study demonstrated that acetic acid reduced Listeria populations in fresh meat (Samelis et al., 2001). Carpenter et al. (2011) reported that the application of 2% acetic acid reduced the count of Salmonella and prevented residual growth of *E. coli* and *L. monocytogenes* in chickens. Gonzales-Fandos and Herrera (2014) observed a significant reduction in microbial counts after dipping chicken legs in acetic acid (1-2%) compared to control groups. Studies showing the effect of acetic acid on other pathogens in different foods support our results.

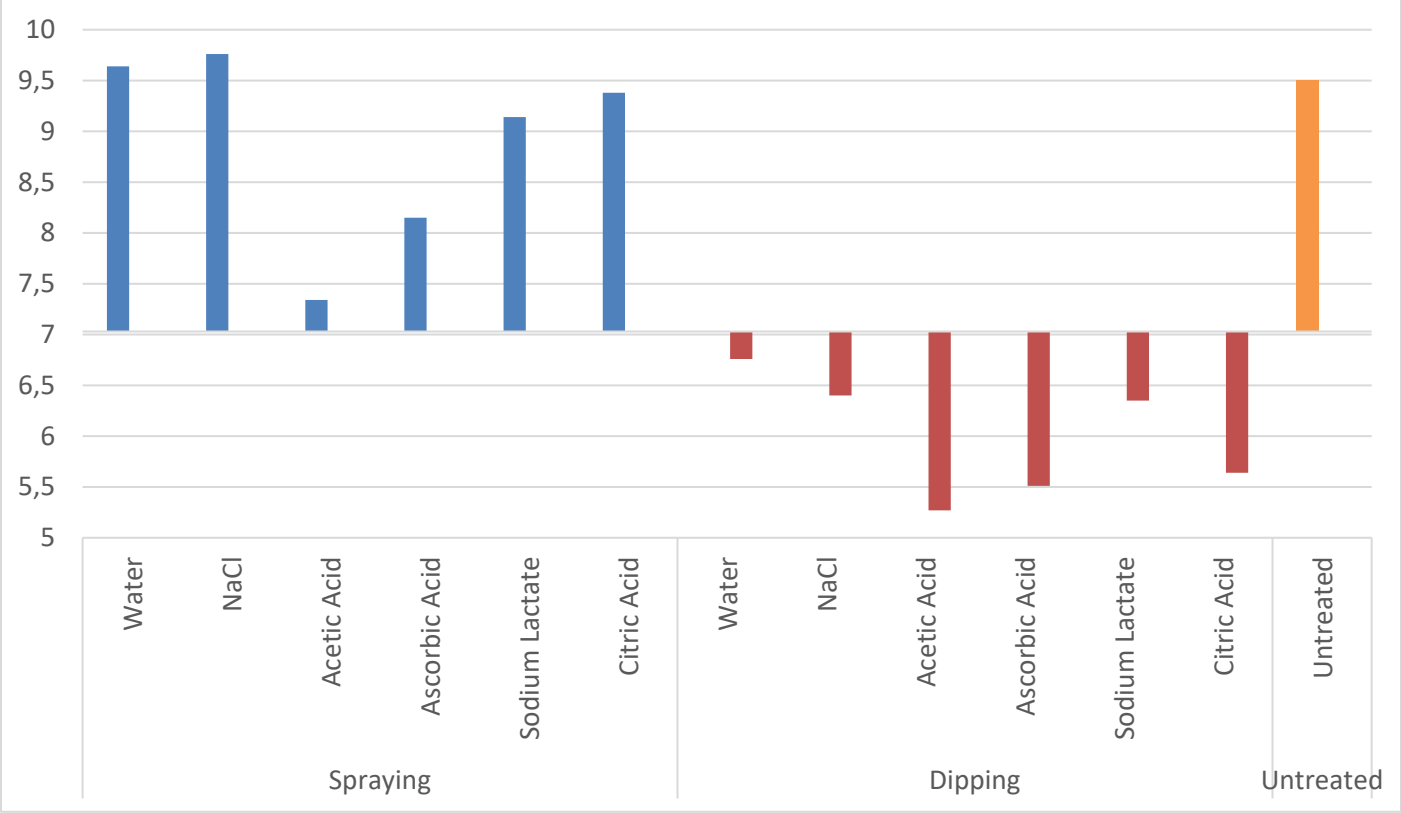

Figure 1. Reduction in *A. hydrophila* counts in bluefish samples after 72 hours.

Ascorbic acid is the other substance found to be effective in our study. Similarly, dipping chicken chunks in 1% ascorbic acid solution slowed microbial growth and increased shelf life (Arafata & Chen, 1978). Ouattara et al. (2002) indicated that ascorbic acid stabilized ground beef's total aerobic plate count. It has also been reported that the addition of ascorbic acid and/or citric acid could reduce bacterial load in food (Tajkarimi & İbrahim, 2011; Mahmoud, 2014). Bolton et al. (2014) reported that citric acid (1-5%) was effective against microbial growth in poultry products. Likewise, Doležalová et al. (2010) reported that 4% citric acid application effectively reduced microbial load on chicken skin. In the present study, the reduced load of *A. hydrophila* after dipping in citric acid is consistent with these studies.

Indigenous microbial flora can be inhibited by sodium chloride. Sodium chloride treatment decreases water activity and thus prevents the development of bacteria. Likewise, chloride ions are toxic for some bacteria that inhibit enzymatic systems (Leroi & Chevalier, 2000). In our study, sodium chloride treatment reduced the initial load of *A. hydrophila* by dipping and spraying treatments. However, dipping treatments resulted in more effectiveness in controlling the growth of *Aeromonas hydrophila* (Figure 1).

Sodium lactate has also been studied to prevent bacterial growth, and various products have reported different results. Although sodium lactate has been reported to be effective against bacterial growth in some foods (Sallam & Samejima, 2004; Seydim et al., 2006; Kilinc et al., 2009), it failed in preventing the growth of *L. monocytogenes* in minced beef during cold storage (Serdengeçti et al., 2006). Likewise, sodium lactate did not significantly suppress the growth of *A. hydrophila* in Bluefish in the present study.

Effect of Treatments on pH

It has been known that adding natural acids to food causes a decrease in pH (Bou et al., 2017). Since an undissociated portion of the acid molecule inhibits bacterial growth by creating an unfavorable environment, organic acids have antimicrobial effects (Hardin et al., 1994). Likewise, the treatments showing the highest antimicrobial effect on *A. hydrophila* have to lead to more pH reduction (Table 2). Doležalová et al. (2010) similarly reported a correlation between the decrease in pH and the antimicrobial effect of citric acid on chilled chicken skin. The growth and survival of pathogenic bacteria depend on a diversity of external factors, such as background flora and temperature, as well as internal factors, such as acidity and pH (Shekarforoush et al., 2007). A publication by Daskalov (2006) affirmed that a combination of low temperature and low pH decreases the growth of *A. hydrophila*.

Table 2. pH changes of bluefish samples treated with different organic acids under refrigerated storage

			Storage Hours			
Applications method	**Groups**	**Chemical solutions**	**0 hour**	**24th hour**	**48th hour**	**72th hour**
Spraying	S1	Sterile Water	6.55 ±0.00	6.88 ±0.03	6.32 ±0.03	6.79 ±0.02
	S2	NaCl %4	6.22 ±0.00	6.54 ±0.02	6.33 ±0.04	6.39 ±0.05
	S3	Acetic Acid %4	5.97 ±0.01	6.13 ±0.01	5.81 ±0.01	5.87 ±0.02
	S4	Ascorbic Acid %4	6.01 ±0.01	6.36 ±0.01	6.16 ±0.09	6.27 ±0.04
	S5	Sodium lactate %4	6.41 ±0.01	6.63 ±0.02	6.20 ±0.01	6.45 ±0.02
	S6	Citric Acid %4	5.64 ±0.01	5.85 ±0.01	5.84 ±0.10	6.15 ±0.02
Dipping	D1	Sterile Water	6.35 ±0.02	6.45 ±0.02	6.42 ±0.03	6.85 ±0.01
	D2	NaCl %4	6.22 ±0.00	6.43 ±0.03	6.41 ±0.02	6.30 ±0.10
	D3	Acetic Acid %4	4.56 ±0.01	4.81 ±0.06	5.81 ±0.01	5.13 ±0.04
	D4	Ascorbic Acid %4	5.39 ±0.01	5.54 ±0.02	5.43 ±0.06	5.94 ±0.01
	D5	Sodium lactate %4	6.34 ±0.01	6.53 ±0.01	6.43 ±0.01	6.56 ±0.02
	D6	Citric Acid %4	5.01 ±0.01	5.65 ±0.05	5.12 ±0.10	5.28 ±0.05
Untreated	UNT		6.45 ±0.01	7.28 ±0.12	7.18 ±0.19	6.68 ±0.07

Conclusion

The growth of *A. hydrophila* was inhibited by all dipping treatments and remained below the inoculation dose after 72 hours. Dipping in acetic acid, ascorbic acid, and citric acid (4%) was the most effective treatment to inhibit this emerging pathogen. Considering that the consumer is more worried about using artificial additives or treatments than the risk of pathogens, investigating consumer-friendly, natural substances to ensure food safety will provide significant benefits for the food industry.

Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no actual, potential, or perceived conflict of interest for this article.

Ethics committee approval: Ethics committee approval is not required for this study.

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