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Original Article

Inhibition of *Aeromonas caviae* and *A. sobria* by Sodium Chloride, Citric Acid, Ascorbic Acid, Potassium Sorbate and Extracts of *Thymus vulgaris*

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SUMMARY: The respective and combined effects of sodium chloride, ascorbic acid, citric acid, potassium sorbate, and *Thymus vulgaris* extract on the growth of *Aeromonas caviae* and *Aeromonas sobria* were investigated. Sodium chloride (3%) significantly reduced the growth and 4% NaCl inhibited growth of the tested strains. Ascorbic acid (0.1%), potassium sorbate (0.05%), and citric acid (0.03%) slightly inhibited growth. *T. vulgaris* extract (0.3%) greatly reduced the growth. Various combinations of these compounds prevented growth of the tested strains. A combination of NaCl (3%) and ascorbic acid (0.1%), citric acid (0.03%) and potassium sorbate (0.05%), or citric acid (0.03%) and ascorbic acid (0.1%) inhibited growth of *A. caviae* and *A. sobria*. In fish homogenates, the addition of ascorbic acid (0.1%) and citric acid (0.03%) was the most effective combination tested.

INTRODUCTION

Aeromonas spp. have recently come to be considered an important cause of food-borne diseases in humans, and are implicated as a cause of gastroenteritis in both developed and developing countries. In studies of bacterial pathogens associated with diarrhea, *A. hydrophila* and *A. caviae* were isolated from both adult and pediatric patients (1,2). In another study, *Aeromonas* spp. were recovered from 52% of children with acute diarrhea (3). Many types of food, particularly contaminated seafood, were implicated as the cause of food poisoning (4). *Aeromonas* spp. were isolated from 34% of finfish taken from retail outlets in New Zealand (5) and in the United Kingdom, 15 out of 47 (approximately 32%) fish purchased from retail outlets were found to be contaminated with *Aeromonas* (6). In Jordan, 13% of sampled retail fish (locally grown and imported) were contaminated with *Aeromonas* spp., a large proportion of which were *A. caviae* or *A. sobria* (unpublished data).

The initial bacterial content in fresh fish is affected by the method of harvesting. Trawled fish carry bacterial loads that are 10-100 times greater than those of line-caught fish. This is due to the fact that the trawling net drags along the sea bottom, stirring up the mud and contaminating the fish (7). Also, during periods of heavy fishing, trawling nets become full, and fish are bruised from compression, which results in poor quality and decreased shelf life. After harvesting, fish should be cleaned and cooled down to 0°C as quickly as possible (8). However, small boats, which leave the docks in the morning and return after several hours to store fish, often store them without ice. This increases the number of spoilage bacteria, and necessitates the application of compounds to eliminate spoilage bacteria and extend the shelf life of fish. Chemicals may be added in a variety of ways, including spraying onto fish; dipping fish into a solution containing chemicals; direct incorporation into fish; or application of chemicals to the wrapping material (9). To my knowledge, there are no published data on the effect of preservatives on *A. caviae* and *A. sobria*. Therefore, this study was conducted to investigate the respective effect of citric acid, ascorbic acid, potassium sorbate, sodium chloride, *Thymus vulgaris* extract, and combinations of these compounds on the two *Aeromonas* spp.

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MATERIALS AND METHODS

Bacterial strains: Two *A. sobria* strains and one *A. caviae* strain were used in this study. *A. sobria* 172 was isolated from local fish, and *A. sobria* 74 and *A. caviae* 166 were isolated from imported fish. All strains had been previously isolated by the author, and were identified at the genus and species levels using the method described by Carnahan et al. (10) and Kirov (11). In brief, samples were enriched in 0.1% alkaline peptone water, and incubated at 34°C for 18-24 h. A loopful of the above culture was streaked on MacConkey agar and incubated overnight at 34°C. Lactose fermenter and lactose non-fermenter colonies appearing on the MacConkey agar plates were plated on non-selective medium (nutrient agar) and an oxidase test was performed. Strains that were oxidase positive were subjected to the following tests: Gram stain, glucose fermentation using a triple sugar iron agar (TSI) slant, and resistance to O/129 vibriostatic agent (150 μg). The strains that were Gram-negative rods, glucose-fermenting, and resistant to O/129 vibriostatic agent were further characterized by using the API-20E system (Biomerieux, Marcy l'Etoile, France) and other complementary tests (e.g., production of gas from glucose fermentation and growth in 6% NaCl).

The organisms were maintained on nutrient agar slants at 4°C. To prepare the inoculum, nutrient broth (20 ml) was inoculated with appropriate cultures and incubated at 34°C for 20 h.

Media and chemicals: Nutrient broth was obtained from ADSA micro (Scharlau Chemie, Barcelona, Spain). Sodium chloride, citric acid, ascorbic acid, and potassium sorbate were obtained from Gainland Chemical Co. (Hampshire, UK). Stock solutions of citric acid, ascorbic acid, and potassium

sorbate were freshly prepared before each use and sterilized by filtration through filter membranes (0.45 μm, Micron Separation Inc., Philadelphia, Pa., USA). Crude extract of *T. vulgaris* (extracted by hydrodistillation) was obtained from Systema Co. Ltd. (Amman, Jordan).

Growth conditions: (1) Growth in nutrient broth: Flasks containing 30 ml of nutrient broth (pH 7.2), nutrient broth plus NaCl (3% or 4% w/v), nutrient broth plus citric acid (0.03% w/v), nutrient broth plus ascorbic acid (0.1% w/v), nutrient broth plus crude extract of *T. vulgaris* (0.3% v/v), or nutrient broth plus various combinations of these compounds were inoculated with 1 ml of overnight-grown bacterial cultures. For experiments containing potassium sorbate (0.05% w/v), the pH of nutrient broth was adjusted to 5.8 before addition of the tested compounds, then the tested compounds were added to flasks containing 30 ml of nutrient broth (pH 5.8) and treated as mentioned above. After inoculation, the flasks were incubated static at 34°C. Samples were withdrawn from each flask at suitable intervals (~1 h) and growth was monitored for 24 h by measuring optical density at 560 nm spectrophotometrically (Spectronic, Cheshire, UK). During incubation, at least five readings were obtained from each flask.

(2) Growth in fish homogenates: Fish muscle, fat tissue, and skin (60 g) were aseptically obtained from fresh fish, and fish homogenates were prepared by blending the above samples with 400 ml distilled water in a sterile blender. The pH of the homogenate was 6.6 as determined by pH meter. The homogenate was sterilized by heating at 70°C for 10 min, then cooled. A specific volume (30 ml) of the homogenate was dispensed into screw cap test tubes, and sodium chloride, citric acid, ascorbic acid, or combinations of these compounds were added per tube, aseptically. One milliliter of overnight-grown bacterial cultures was added to each test tube, and the tubes were incubated static at 34°C. Samples were withdrawn from each tube at suitable intervals and growth was monitored for 24 h spectrophotometrically.

All experiments in this study were performed five times, and the optical density readings presented are the mean values. The standard error was ± 0.01 for all readings.

RESULTS

Effect of sodium chloride, citric acid and ascorbic acid: The OD_{560} readings of *A. caviae* 166 and *A. sobria* 172 subjected to sodium chloride (3%) alone are presented in Tables 1 and 2. The presence of sodium chloride (3%) in the growth medium reduced the growth rate, whereas the percentage of inhibition after 24 h of incubation was 61% for both strains. Also, a lag was observed before increase in number for either strain could be detected, and the lag was longer for *A. sobria* 172. The effect of NaCl (3%) on *A. sobria* 74 is shown in Figure. The percentage of inhibition after 24 h of incubation was 64%, and the lag was shorter than that for *A. sobria* 172 and more similar to that for *A. caviae* 166.

Sodium chloride (4%) nearly completely inhibited growth of *A. caviae* 166 and *A. sobria* 172; only a slight increase in optical density was observed after 24 h incubation in nutrient broth with 4% NaCl (Tables 1 and 2).

The presence of citric acid (0.03%) alone in the growth medium only slightly inhibited growth of *A. caviae* 166 or *A. sobria* 172; OD_{560} readings after 24 h incubation in nutrient broth with and without citric acid were nearly the same (Tables 1 and 2). However, when the broth contained citric acid, a lag occurred before growth of *A. caviae* 166 could be observed.

Addition of ascorbic acid (0.1%) alone to the growth medium slightly inhibited growth of the strains, whereas the percentages of inhibition after 24 h incubation were 11% and 24% for *A. sobria* 172 and *A. caviae* 166, respectively. However, the strains grew in nutrient broth containing ascorbic acid after a lag of about 2 h.

Interaction between sodium chloride or citric acid and ascorbic acid: Exposure of *A. caviae* 166 and *A. sobria* 172 to NaCl (3%) and ascorbic acid (0.1%) together caused enhanced inhibition of both strains; growth of cells was not detected after 24 h incubation (Tables 1 and 2). Addition of citric acid (0.03%) and ascorbic acid (0.1%) together to the growth medium inhibited growth of *A. caviae* 166 and *A. sobria* 172 (Tables 1 and 2).

Effect of potassium sorbate: The presence of potassium sorbate (0.05%) in the growth medium slightly inhibited growth of the tested strains. The percentages of inhibition after 24 h incubation were 12%, 15%, and 34% for *A. caviae* 166, *A. sobria* 74, and *A. sobria* 172, respectively. However,

Table 1. Inhibition of growth of *A. caviae* 166 in nutrient broth by combination of various compounds

NaCl (%)	Potassium sorbate (%)	Citric acid (%)	Ascorbic acid (%)	Growth after 2 h	Growth after 4 h	Growth after 24 h	Reduction in growth (%)[b]
0	0	0	0	0.13	0.25	0.82	–
3	0	0	0	0.01	0.13	0.32	61
4	0	0	0	0	0	0.01	99
0	0	0	0.1	0.05	0.06	0.62	24
3	0	0	0.1	0	0	0	100
0	0	0.03	0	0.06	0.08	0.72	12
0	0	0.03	0.1	0	0	0	100
0	0.05	0	0	0.01	0.06	0.66	12
3	0.05	0	0	0	0.01	0.45	40
0	0.05	0.03	0	0	0	0	100
0	0	0	0	0.04[a]	0.10[a]	0.75[a]	–

[a]: The values represent growth in nutrient broth (pH 5.8).

[b]: Reduction in growth (%)= $\frac{\text{Growth in broth without chemicals} - \text{growth in broth with chemical(s)}}{\text{Growth in broth without chemicals}} \times 100$

Table 2. Inhibition of growth of *A. sobria* 172 in nutrient broth by combination of various compounds

NaCl (%)	Potassium sorbate (%)	Citric acid (%)	Ascorbic acid (%)	Growth after 2 h	Growth after 4 h	Growth after 24 h	Reduction in growth (%)[b]
0	0	0	0	0.1	0.36	1.14	–
3	0	0	0	0	0.06	0.44	61
4	0	0	0	0	0	0.01	99
0	0	0	0.1	0.03	0.1	1.01	11
3	0	0	0.1	0	0	0	100
0	0	0.03	0	0.08	0.13	1.00	12
0	0	0.03	0.1	0	0	0	100
0	0.05	0	0	0.02	0.08	0.73	34
3	0.05	0	0	0	0.01	0.40	63
0	0.05	0.03	0	0	0	0	100
0	0	0	0	0.1[a]	0.3[a]	1.1[a]	–

[a]: The values represent growth in nutrient broth (pH 5.8).

[b]: Reduction in growth (%)= $\frac{\text{Growth in broth without chemicals} - \text{growth in broth with chemical(s)}}{\text{Growth in broth without chemicals}} \times 100$

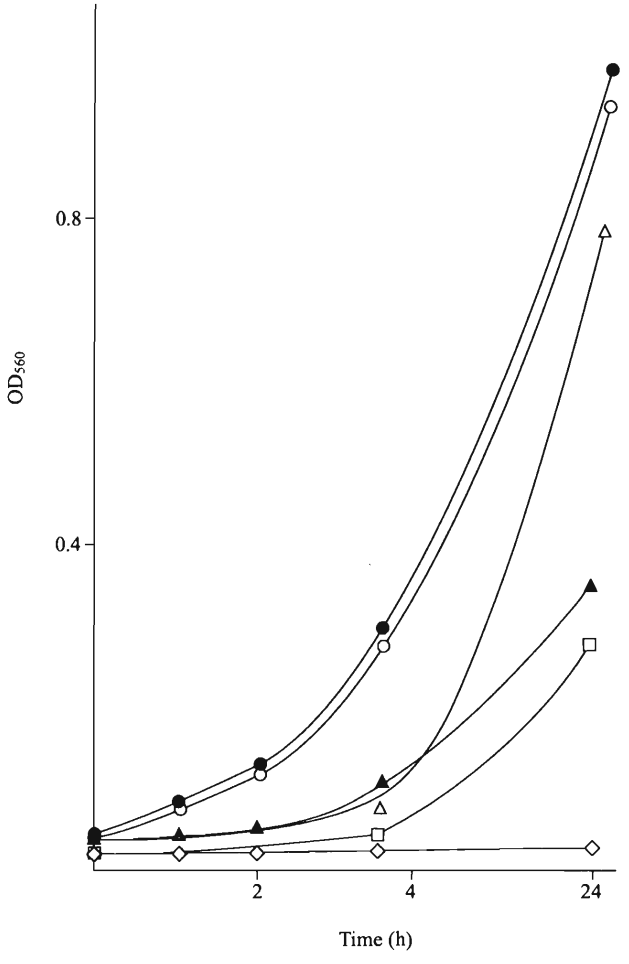

Figure. Influence of NaCl, potassium sorbate, and combination of various chemicals on the growth of *A. sobria* 74. The strain was inoculated into NB medium (pH 7.2) containing no chemicals (●) or 3% NaCl (▲); or into NB medium (pH 5.8) containing no chemicals (○), 0.05% potassium sorbate (△), 0.05% potassium sorbate plus 3% NaCl (□), or 0.05% pottassium sorbate plus 0.03% citric acid (◇).

a lag was observed before increase in number could be detected (Tables 1 and 2, Figure). This inhibitory activity was due to the presence of potassium sorbate and not due to the low pH of the growth medium. The OD_{560} readings of the strains grown in nutrient broth at pH 5.8 for 24 h were nearly similar to OD_{560} readings of strains grown in nutrient broth at pH 7.2 (Tables 1 and 2, Figure).

Interaction between sodium chloride or citric acid and potassium sorbate: The results of exposure of *A. caviae* 166, *A. sobria* 172, and *A. sobria* 74 to NaCl (3%) and potassium sorbate (0.05%) together are presented in Tables 1 and 2 and Figure. Addition of NaCl (3%) and potassium sorbate (0.05%) together caused enhanced inhibition of the tested strains, where the percentages of inhibition after 24 h incubation were 40%, 63%, and 70% for *A. caviae* 166, *A. sobria* 172, and *A. sobria* 74, respectively. There was a lag of about 4 h before growth of the tested strains could be detected.

Exposure of *A. caviae* 166, *A. sobria* 172, and *A. sobria* 74 to citric acid (0.03%) and potassium sorbate (0.05%) together completely inhibited growth of these strains. Increase in optical density was not observed after 24 h incubation in nutrient broth containing these compounds (Tables 1 and 2, Figure).

Effect of *T. vulgaris* extract: Addition of thyme extract

Table 3. Effect of *Thymus vulgaris* extract on (A) *A. caviae* 166 and (B) *A. sobria* 172

(A)

Thyme extract (%)	NaCl (%)	Growth after 2 h	Growth after 4 h	Growth after 24 h	Reduction in growth[a] (%)
0	0	0.12	0.25	0.81	–
0.3	0	0	0	0.06	93
0.3	3	0	0	0.02	98

(B)

Thyme extract (%)	NaCl (%)	Growth after 2 h	Growth after 4 h	Growth after 24 h	Reduction in growth[a] (%)
0	0	0.1	0.35	1.1	–
0.3	0	0	0	0.14	87
0.3	3	0	0	0.09	92

[a]: Reduction in growth (%)=

$$\frac{\text{Growth in broth without chemicals} - \text{growth in broth with chemical(s)}}{\text{Growth in broth without chemicals}} \times 100$$

Table 4. Inhibition of growth of *A. caviae* 166 in fish homogenates by combination of various compounds

NaCl (%)	Citric acid (%)	Ascorbic acid (%)	Growth after 2.5 h	Growth after 24 h	Reduction in growth[a] (%)
0	0	0	0.06	0.48	–
0	0	0.1	0.02	0.46	4
3	0	0.1	0	0.33	31
0	0.03	0.1	0	0	100

[a]: Reduction in growth (%)=

$$\frac{\text{Growth in broth without chemicals} - \text{growth in broth with chemical(s)}}{\text{Growth in broth without chemicals}} \times 100$$

(0.3%) to the growth medium significantly reduced the growth rate. The percentages of inhibition of *A. caviae* 166 and *A. sobria* 172 after 24 h of incubation were 93% and 87%, respectively (Table 3). Addition of NaCl (3%) and thyme extract (0.3%) together to the growth medium caused enhanced inhibition of both strains (Table 3).

Effects of ascorbic acid and combination of ascorbic acid with sodium chloride and citric acid in fish homogenates: The growth of *A. caviae* 166 in fish homogenates containing the tested compounds is presented in Table 4. The presence of ascorbic acid (0.1%) alone in fish homogenates did not inhibit growth of *A. caviae* 166; OD_{560} readings after 24 h incubation in fish homogenates with and without ascorbic acid were nearly the same. Addition of ascorbic acid (0.1%) and NaCl (3%) together to fish homogenates caused reduction of the growth rate of the tested strain; the percentage of growth inhibition after 24 h incubation was 31%, and the strain showed growth after a lag of more than 2.5 h. On the other hand, addition of ascorbic acid (0.1%) and citric acid (0.03%) together to fish homogenates inhibited growth of *A. caviae* 166; increase in optical density was not observed after 24 h incubation. Similar results were obtained for *A. sobria* (data not shown).

DISCUSSION

Aeromonas spp. are able to grow and produce exotoxins at both low and moderate temperatures (e.g., 5°C and 37°C, respectively) (12), which capacity suggests that, in food stored

at ambient or refrigeration temperature, they could be of public health significance. Therefore, addition of chemicals before storage to control the growth of *Aeromonas* spp. is intended to extend the shelf life of food products. In this study, the respective effects of NaCl, citric acid, ascorbic acid, potassium sorbate, extracts of *T. vulgaris*, and combinations of these compounds on the growth of *A. caviae* and *A. sobria* were investigated. The tested strains showed marginally similar responses after exposure to NaCl, citric acid, or ascorbic acid. Variations in lag time were generally observed. Upon exposure to 3% NaCl, *A. sobria* 172 showed a lag time longer than that of *A. caviae* 166 or *A. sobria* 74. *A. sobria* 172 was isolated from freshwater fish, while the latter strains were isolated from salt water fish and were therefore adapted to growth in an environment of relatively high osmotic pressure. Similar findings were reported by Abu-Ghazaleh et al. (13), who reported that genetic and environmental factors influence the tolerance of food poisoning bacteria to heat. Also, Cheroutre-Vialette et al. (14) emphasized the need to take into account the previous history of a product, and therefore the previous history of a strain, which could impact the lag time of the microorganism.

Ascorbic acid (0.1%) showed a slight inhibitory effect on the tested *Aeromonas* spp. Similar results were obtained by Fletcher et al. (15), who reported that ascorbic acid showed some inhibitory properties toward *Campylobacter jejuni*. Citric acid (0.03%) did not demonstrate an antimicrobial effect on the *Aeromonas* spp. tested. Other studies have reported that citric acid is not as effective as other acids or preservatives. The growth of *Escherichia coli* and *Listeria monocytogenes* was reduced by 0.2% sodium citrate at 7°C and 18°C (16). However, at higher concentrations, citric acid significantly lowered the microbial load of fish and the level of salmonellae on poultry carcasses (9).

In this study, the antimicrobial effect of potassium sorbate (0.05%) on growth of *Aeromonas* spp. was slight. There are few comparative studies extant on the effect of potassium sorbate on bacterial growth. Zhao et al. (17) reported that 0.1% potassium sorbate had a minimal effect on enterohemorrhagic *E. coli* O157:H7. On the other hand, studies that examined higher concentrations of potassium sorbate showed that the shelf life of poultry and seafood was increased by this treatment (18, 19).

In this study, interactions between the tested compounds were investigated for possible additive effects against *Aeromonas* spp. The antimicrobial activity of ascorbic acid (0.1%) was greatly increased in the presence of NaCl (3%) or citric acid (0.03%). This is consistent with results obtained by other studies. Stecchini et al. (20) demonstrated that the death of *A. hydrophila* upon exposure to mild heat (46°C) was not influenced by the presence of ascorbic acid alone. However, increased mortality was observed in the presence of both NaCl and ascorbic acid in the heating menstruum. Addition of *T. vulgaris* extract (0.3%) and NaCl (3%) together prevented growth of *Aeromonas* sp. Potassium sorbate activity was increased in the presence of NaCl (3%), but its activity was more pronounced in the presence of, rather, citric acid (0.03%) in the growth medium. Addition of NaCl intensifies the action of sorbate primarily by reducing a_w. It has been reported that a combination of NaCl and sorbate resulted in synergistic inhibition of *Staphylococcus aureus* (21). Also, Thomas et al. (22) reported that a combination of potassium sorbate (0.2%) and NaCl effectively inhibited the growth of Vero cytotoxigenic *E. coli*, *Bacillus cereus*, and *S. aureus*. Zhao et al. (17) showed that enterohemorrhagic *E. coli* O157:H7 was inactivated in apple cider at higher rates in the presence of 0.1% potassium sorbate and 0.1% sodium benzoate together. Other studies have reported that citric and lactic acids also potentiate the antimicrobial action of sorbate (23).

The effects of chemicals on *A. caviae* 166 and *A. sobria* 172 in fish homogenates were examined in this study. Both strains were sensitive to a combination of ascorbic acid and citric acid when treated in fish homogenates and in broth. On the other hand, the tested strains were more resistant to the effect of ascorbic acid (0.1%) alone or to the combination of ascorbic acid (0.1%) and NaCl (3%) when treated in fish homogenates than in broth. Fish homogenates are proteinaceous products (16% proteins), and, therefore, bacteria may be able to survive by assuming a protective coating of these proteinaceous materials (24). Further, proteins and other organic molecules in fish homogenates may react with the chemicals added, causing a decrease in their antimicrobial activity. Banwart (9) reported that the composition of food is one of the factors affecting the activity of chemicals against microorganisms in it.

In conclusion, this study showed that various combinations of the tested compounds inhibited growth of *Aeromonas* sp. This study also showed that some compounds [e.g., NaCl (3%), ascorbic acid, citric acid, potassium sorbate] increased the lag time of *Aeromonas* sp. Application of these compounds may increase the shelf life of stored foods.

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