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Full Length Research Paper

Effects of ascorbic acid, citric acid, lactic acid, NaCl, potassium sorbate and *Thymus vulgaris* extract on *Staphylococcus aureus* and *Escherichia coli*

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Staphylococcus aureus **and some strains of** ***Escherichia coli*** **are frequently implicated in foodborne diseases. This study examined the effects of some compounds (ascorbic acid, citric acid, lactic acid, sodium chloride, potassium sorbate and** ***Thymus vulgaris*** **extract) on growth of** ***S. aureus*** **and** ***E. coli*****. Lactic acid (0.03% or 0.1%) alone nearly completely inhibited growth of** ***S. aureus*** **or** ***E. coli*****, respectively. Citric acid (0.03%) reduced growth and ascorbic acid (0.1%) nearly completely inhibited the growth of** ***S. aureus*****; the percentages of inhibition after 24 h incubation in nutrient broth were 33 and 91%, respectively. Citric acid (0.03%) and ascorbic acid (0.1%) did not inhibit growth of** ***E. coli*****, but a lag occurred before increase in number could be observed. NaCl (5%) significantly reduced growth of both strains; the percentages of inhibition of** ***S. aureus*** **and** ***E. coli*** **after 24 h incubation were 55 and 64%, respectively.** ***Thymus vulgaris*** **extract (0.3%) alone or potassium sorbate (0.09%) alone reduced growth of both strains. A combination of citric acid (0.03%) and potassium sorbate (0.05%) or citric acid (0.03%) and NaCl (5%) nearly completely or completely inhibited, respectively, the growth of** ***S. aureus*****. For** ***E. coli*****, combination of citric acid (0.03%) and potassium sorbate (0.05%) together completely inhibited the growth. A combination of citric acid (0.03%) and NaCl (3%) or** ***T. vulgaris*** **extract (0.3%) and NaCl (3%) greatly reduced the growth of** ***E. coli*** **strains and the percentages of inhibition after 24 h incubation were 65 and 70%, respectively.**

Key words: *Staphylococcus aureus*, *Escherichia coli,* inhibition, chemicals.

INTRODUCTION

Staphylococcus spp., especially *S. aureus* is one of the most common causes of food related diseases throughout the world (Hennekinne et al., 2012). For example, in Canada, 10.5% of foods submitted for outbreak investigation between 2007 and 2010 contained *S. aureus* (Crago et al., 2012). The symptoms of staphylococcal food poisoning appear within short time (1- 6 h) after ingestion of the contaminated food. The symptoms include vomiting, diarrhea and abdominal cramps; about 10% of the affected persons may be admitted to hospital but the mortality rate is very low or nil (Jay, 2000). Staphylococcal food poisoning results from consumption of one or more preformed enterotoxins that are produced in food contaminated with *Staphylococcus* (Dinges et al., 2000). The amount of enterotoxin needed to cause food poisoning is very low; it has been reported that 20 ng to < 1 ug can cause symptoms in humans (Berdgoll, 1989). Most of these enterotoxins are highly resistant to heat. Enterotoxin A of *S. aureus*, for example, remains active in breaded chicken cutlets that are prefried at 180°C for 55 s and baked at 180°C for 13 min and stored at 4°C (Pepe et al., 2006).

Many strains of *E. coli* are nowadays considered as foodborne pathogens. Strains belonging to the subgroups enteropathogenic and enterotoxigenic *E. coli* produce gastroenteritis and mild or severe diarrhea when contaminated food or water is ingested (Ray, 2001). Also, shigatoxigenic *E. coli*, including *E. coli* O157: H7, produce heat resistant toxins which are not inactivated by pasteurization temperatures (63°C for 30 min, 72°C for 15 s, or 89°C for 1 s) (Rasooly and Do, 2010).

Many types of food (milk, dairy products, chicken, meat) are associated with *S. aureus* or *E. coli* related diseases. It has been reported that 3.1% (12/ 384) of

cooked food products were contaminated with *S. aureus* in China; and *S. aureus* strains were detected in 11% (40/ 365) of raw meats and 16.3% (34/ 209) of raw milk (Chao et al., 2007). In Italy, Normanno et al. (2007) reported that 17% of dairy product samples and 10% of meat product samples were contaminated with *S. aureus*; and ~ 60% of these strains produced enterotoxins. In Portugal, viable counts (log CFU/ ml or g) of Enterobacteriaceae (including *E. coli*, *Klebsiella*, *Enterobacter sakazakii*) ranged between 5.9 and 7.0 in raw milk and between 0.0 and 1.3 in 4 months old cheeses (Kongo et al., 2008). The prevalence of shigatoxigenic *E. coli* (STEC) - positive samples in raw milk was 21% in France (Perelle et al., 2007), and ~ 7% of hard raw ewe's milk cheese harbored shigatoxigenic *E. coli* in Spain (Caro and Garcia- Armesto, 2007). Also, more than 90% of raw food samples (beef, chicken, shellfish) contained *E. coli* in Vietnam (Van et al., 2007). Introduction of the organisms to food may occur in their original environment (farms) and / or during slaughtering and processing. Therefore, application of compounds to eliminate these organisms before storage may improve the quality of food and extend their shelf life. There is little information on the effect of food preservatives on *S. aureus* and *E. coli*. Therefore, this study was undertaken to investigate the effects of potassium sorbate, lactic acid, citric acid, ascorbic acid, sodium chloride, *Thymus vulgaris* extract, and combinations of these compounds on these bacteria.

MATERIALS AND METHODS

Bacterial strains

Clinical strains of *E. coli* and *S. aureus* were used in this study. The strains were obtained from a central laboratory in 2010, and were identified to the genus and species level using the API-20E and API-Staph systems, respectively (Biomerieux, Marcy l'Etoile, France). Bacterial strains were maintained on nutrient agar slants at 4°C. To prepare the inoculum, nutrient broth (20 ml) was inoculated with *E. coli or S. aureus* strains and incubated at 30°C for 24 h.

Media and chemicals

Lactic acid, citric acid, ascorbic acid, sodium chloride and potassium sorbate were obtained from Gainland Chemical Co. (Hampshire, UK). Crude extract of *T. vulgaris* (extracted by hydrodistillation) was obtained from Systema Co. Ltd. (Amman, Jordan). Stock solutions of potassium sorbate, citric acid and ascorbic acid were freshly prepared before each use and sterilized by filtration through membrane filters (0.45 um, Micron Separation Inc., Philadelphia, Pa., USA). Nutrient broth was obtained from Scharlau Chemie (Barcelona, Spain).

Growth conditions

Flasks containing 30 ml of nutrient broth (pH 7.2), nutrient broth plus lactic acid (0.03 or 0.1% w/v), nutrient broth plus citric acid (0.03% w/v), nutrient broth plus ascorbic acid (0.05 or 0.1 % w/v), nutrient broth plus NaCl (3, 4 or 5% 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 0.5 ml of overnight grown bacterial culture. For experiments containing potassium sorbate (0.05% or 0.09%), the pH of nutrient broth was adjusted to 5.9 (using HCl or NaOH) before addition of the tested compounds. Then, the tested compounds were added to flasks containing 30 ml of nutrient broth (pH 5.9) and treated as previously described. After inoculation, the flasks were incubated static at 30°C for 24 h. At suitable intervals, samples were withdrawn from each flask and growth was monitored by measuring optical density at 560 nm spectrophotometrically (Spectronic, Cheshire, UK). During incubation, at least five readings were obtained from each flask.

All experiments in this study were performed five times, and the optical density readings presented are the mean values. Student's t- test was used to determine the significant differences ($p < 0.05$) among the different compounds tested.

RESULTS

Effect of citric acid, lactic acid, ascorbic acid and sodium chloride

The OD_{560} readings of *E. coli* and *S. aureus* subjected to citric acid alone are shown in Tables 1 and 3. The presence of citric acid (0.03%) in the growth medium did not inhibit the growth of *E. coli* but significantly inhibited growth of *S. aureus* ($p < 0.05$). However, both strains grew in this medium after a long lag (~ 3 h). The presence of ascorbic acid (0.1%) alone in the growth medium of *E. coli* did not cause substantial inhibition of growth, but there was a lag of more than 3 h before growth could be detected (Table 1). However, ascorbic acid (0.1%) nearly completely inhibited ($p < 0.05$) growth of *S. aureus* (the percentage of inhibition after 24 h incubation was 91%) (Table 3).

Addition of lactic acid (0.1%) alone to the growth medium of *E. coli* and lactic acid (0.03%) alone to the growth medium of *S. aureus* nearly completely inhibited growth of the tested strains ($p < 0.05$) (Tables 1 and 3). Only a slight increase in optical density was observed after 24 h incubation in nutrient broth with lactic acid.

Optical density readings of *E. coli* and *S. aureus* subjected to NaCl alone are presented in Tables 1 and 3. The presence of 3% NaCl in the growth medium of *E. coli* slightly reduced the growth rate ($p < 0.05$); the percentage of inhibition after 24 h incubation was 29%. Exposure of *E. coli* to 4% NaCl caused enhanced inhibition of growth, where the percentage of inhibition was 44%. NaCl (5%) greatly inhibited ($p < 0.05$) growth of *E. coli* or *S. aureus*; the percentages of inhibition after 24 h incubation were 63.5 and 55%, respectively (Tables 1 and 3).

Interaction between the tested compounds

Addition of NaCl (3%) and citric acid (0.03%) together to the growth medium greatly reduced ($p < 0.05$) the growth

Table 1. Inhibition of growth of *E. coli* in nutrient broth by single or combination of compounds

Citric acid (%)	NaCl (%)	Lactic acid (%)	Thyme extract (%)	Ascorbic acid (%)	Growth (OD_{560}) after			Reduction in growth (%)[a]
					2 h	3 h	24 h	
0	0	0	0	0	0.04	0.1	0.63	-
0.03	0	0	0	0	0	0.02	0.68	-
0	3	0	0	0	0.027	0.03	0.45*	29
0	4	0	0	0	0	0	0.35*	44
0	5	0	0	0	0	0	0.23*	63.5
0.03	3	0	0	0	0	0	0.22*	65
0	0	0.1	0	0	0	0	0.01*	98
0	0	0	0.3	0	0	0	0.41*	35
0	3	0	0.3	0	0	0	0.19*	70
0	0	0	0	0.1	0	0.01	0.61	3

[a]Reduction in growth (%) = 100 × Growth in broth without chemicals – growth in broth with chemical (s) / Growth in broth without chemicals. *Means are significantly different ($p< 0.05$) from control (compound(s) was not added to growth medium) .

Table 2. Effect of potassium sorbate and other compounds on *E. coli* .

Potassium sorbate (%)	Citric acid (%)	NaCl (%)	Thyme extract (%)	Growth (OD_{560}) after			Reduction in growth (%)[a]
				2 h	3 h	24 h	
0	0	0	0	0.03	0.08	0.62	-
0.05	0	0	0	0.01	0.03	0.55*	11.3
0.09	0	0	0	0	0.01	0.51*	18
0.05	0.03	0	0	0	0	0*	100
0.05	0	3	0	0	0.01	0.26*	58.1
0.05	0	0	0.3	0	0	0.41*	34

[a]Reduction in growth (%) = 100 × Growth in broth without chemicals – growth in broth with chemical (s) / Growth in broth without chemicals.* Means are significantly different ($p< 0.05$) from control (compound(s) is not added to growth medium).

rate of *E. coli*, where the percentage of inhibition after 24 h incubation was 65% (Table 1). Exposure of *S. aureus* to NaCl (5%) and citric acid (0.03%) together completely inhibited growth. Increase in optical density was not observed after 24 h incubation in nutrient broth containing these compounds (Table 3). Addition of NaCl (5%) and ascorbic acid (0.05%) together to the growth medium of *S. aureus* caused significant reduction of the growth rate ($p< 0.05$), and the percentage of inhibition after 24h incubation was 52% (Table 3).

Effect of potassium sorbate

Addition of potassium sorbate (0.05%) alone to the growth medium of both strains only slightly inhibited growth. However, 0.09% potassium sorbate significantly reduced ($p< 0.05$) the growth rate of both strains and the percentages of inhibition of *E. coli* and *S. aureus* after 24 h incubation were 18 and 34%, respectively. Also, a lag(~ 3 h) was observed before increase in number of either strain could be detected (Tables 2 and 4).

The presence of potassium sorbate (0.05%) and NaCl (3%) together in the growth medium caused enhanced inhibition of *E. coli* and the percentage of inhibition after 24 h incubation was 58.1% (Table 2). Exposure of *E. coli* or *S. aureus* to potassium sorbate (0.05%) and thyme extract (0.3%) together significantly decreased the growth rate of E. coli ($p< 0.05$) but did not affect growth of *S. aureus* ($p> 0.05$) (Tables 2 and 4). Addition of potassium sorbate (0.05%) and citric acid (0.03%) together to the growth medium completely inhibited growth of *E. coli* and greatly reduced growth of *S. aureus* (Tables 2 and 4).

Effect of *Thymus vulgaris* extract

The presence of thyme extract (0.3%) alone in the growth medium slightly but significantly inhibited growth ($p< 0.05$) of the tested strains; where the percentages of inhibition after 24 h incubation were 35 and 15% for *E. coli* and *S. aureus*, respectively (Tables 1 and 3). Also,

Table 3. Inhibition of growth of *S. aureus* in nutrient broth by single or combination of compounds

Citric acid (%)	NaCl (%)	Lactic acid (%)	Thyme extract (%)	Ascorbic acid (%)	Growth (OD_{560}) after			Reduction in growth (%)[a]
					2 h	3 h	24 h	
0	0	0	0	0	0.006	0.05	0.33	-
0.03	0	0	0	0	0	0	0.22*	33
0	5	0	0	0	0	0	0.15*	55
0.03	5	0	0	0	0	0	0*	100
0	0	0.03	0	0	0	0	0.009*	97
0	0	0	0.3	0	0	0	0.28*	15
0	0	0	0	0.05	0	0	0.27*	18
0	0	0	0	0.1	0	0	0.03*	91
0	5	0	0	0.05	0	0	0.16*	52
0	0	0	0.3	0.05	0	0	0.19*	42

[a]Reduction in growth (%) = 100 ×Growth in broth without chemicals – growth in broth with chemical (s) / Growth in broth without chemicals. *Means are significantly different ($p< 0.05$) from control (compound(s) is not added to growth medium).

Table 4. Effect of potassium sorbate and other compounds on *S. aureus.*

Potassium sorbate (%)	Citric acid (%)	Thyme extract (%)	Growth (OD_{560}) after			Reduction in growth (%)[a]
			2 h	3 h	24 h	
0	0	0	0	0	0.32	-
0.05	0	0	0	0	0.29	9
0.09	0	0	0	0	0.21*	34
0.05	0.03	0	0	0	0.06*	81
0.05	0	0.3	0	0	0.31	3

[a]Reduction in growth (%) = 100 ×Growth in broth without chemicals – growth in broth with chemical (s) / Growth in broth without chemicals. *Means are significantly different ($p< 0.05$) from control (compound(s) is not added to growth medium).

both strains grew in this medium after a long lag (about 3 h). Addition of thyme extract (0.3%) and NaCl (3%) together to the growth medium greatly reduced growth of *E. coli*, and the percentage of inhibition after 24 h incubation was 70% (Table 1). Also, presence of thyme extract (0.3%) and ascorbic acid (0.05%) together in the growth medium of *S. aureus* caused enhanced inhibition of its growth (the percentage of inhibition after 24 h incubation was 42%) (Table 3).

DISCUSSION

S. aureus and some *E. coli* strains are among the most common causes of foodborne diseases, where they produce enterotoxins that are resistant to heat. Therefore, it is important to control growth of these bacteria before processing or storage of food to extend its shelf life and prevent production of enterotoxins. In this study, the effects of citric acid, ascorbic acid, lactic acid, sodium chloride, potassium sorbate, *T. vulgaris* extract, and combinations of these compounds on the growth of *S. aureus* and *E. coli* strains were investigated. Ascorbic acid (0.1%) did not show an inhibitory effect on *E. coli*, but nearly completely inhibited *S. aureus*. This is consistent with results obtained by other investigators. Richter et al. (1988) reported that addition of ascorbate to aerobically growing cultures of *E. coli* B caused only a short pause in growth, and no subsequent change in the rate of growth. Tabak et al. (2003) reported that 10- 20 mg/ ml ascorbic acid inhibited *Helicobacter pylori* growth under microaerophilic conditions. Also, it is possible that *E. coli* strains tested utilized this compound. Citric acid (0.03%) significantly reduced growth of *S. aureus* but did not inhibit the growth of *E. coli* strains tested. It is possible that *E. coli* strains utilized this compound. Other studies reported that citric acid (2%) was more effective against gram- positive bacteria (*Bacillus cereus*, and *S. aureus*) than gram- negative bacteria (*Salmonella enteritidis*, and *E. coli*) (del Rio et al., 2007). Also, increasing concentration of citric acid may reduce the growth of *E. coli* (Sagong et al., 2011).

Lactic acid (0.03% or 0.1%) nearly completely inhibited the growth of *S. aureus* or *E. coli* strains, respectively. Similar results were obtained by other investigators. Kolsarici and Candogan (1995) reported that number of total psychrotrophic aerobic bacteria, staphylococci and coliform bacteria on chicken was decreased after

treatment with lactic acid and storage at 4±1°C. Sagong et al. (2011) observed that immersion of lettuce leaves in 0.5% lactic acid significantly reduced growth of *E. coli* O157: H7. Also, lactic acid (0.03 and 0.1%) lowers the pH of the medium to 3.88 and 3.32, respectively; and *S. aureus* and *E. coli* are not able to grow at this pH.

Thyme extract (0.3%) lowered the growth rate of both strains and the effect was more on *E. coli* strains; however, increasing the concentration of thyme extract may enhance its effect on both strains. This is consistent with results obtained by other investigators. Selim (2011) reported that addition of thyme oil at concentrations of 0.5 and 1% caused significant reduction in the growth rate of vancomycin- resistant enterococci and *E. coli* O157: H7 in cheese and meat stored at 7°C. Also, Gupta and Ravishankar (2005) reported that commercial ginger paste completely inactivated *E. coli* O157: H7 in the paste after 3 days of incubation at 4°C and 8°C, and commercial garlic paste reduced (1 log CFU/ g) number of *E. coli* O157: H7 at 4 and 8°C.

Potassium sorbate (0.09%) slightly reduced ($p < 0.05$) growth of the tested strains. Similar results were obtained by Lim and Mustapha (2004), who reported that surface spraying of beef cubes containing *E. coli* O157: H7, *Listeria monocytogenes*, or *S. aureus* with potassium sorbate (0.1%) was not as effective as other preservatives (0.12% acidified sodium chlorite or 0.5% cetylpyridinium chloride) in reducing microbial numbers during storage at 4°C for 2 weeks. Also, Zhao et al. (1993) reported that potassium sorbate (0.1%) had a minimal effect on enterohemorrhagic *E. coli* O157 :H7.

In this study, interactions between the tested compounds were investigated for possible synergism against *E. coli* and *S. aureus* strains. The antimicrobial activity of citric acid (0.03%) against both strains was greatly increased in the presence of NaCl. Thyme extract (0.3%) activity against *E. coli* or *S. aureus* was enhanced in the presence of NaCl (3%) or ascorbic acid (0.05%), respectively. Addition of potassium sorbate (0.05%) and citric acid (0.03%) prevented growth of *E. coli* and greatly inhibited growth of *S. aureus*. Also, antimicrobial activity of potassium sorbate (0.05%) against *E. coli* strains was greatly increased in the presence of NaCl (3%) or thyme extract (0.3%). This is consistent with results obtained by other studies. Betoni et al. (2006) observed that there was synergism between antimicrobial drugs and extracts of clove (*Syzygium aromaticum*), lemongrass (*Cymbopogon citratus*) or guava (*Psidium guajava*). Glass et al. (2007) reported that addition of 0.05% sodium benzoate and 0.05% potassium sorbate together or 0.05% sodium benzoate and 0.05% sodium propionate together to cured Pork- Beef Bologna prevented growth of *L. monocytogenes* during storage for 13 weeks at 4°C. Oksuztepe and Inanli (2007) reported that there was synergistic association between NaCl (10 and 15%) and potassium sorbate (1, 5 and 10%) against microorganisms in vacuum packaged rainbow trout fillets.

In conclusion, this study shows that lactic acid or sodium chloride alone nearly completely inhibited or greatly reduced the growth of *S. aureus* and *E. coli* strains tested. Citric acid or ascorbic acid alone greatly reduced the growth of *S. aureus*, and increased the lag time of *E. coli*. Various combinations of the tested compounds greatly reduced the growth of both strains. These compounds can be used as additives to the preservation in food industries.

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