Journal of Animal and Veterinary Advances 7 (6): 681-684, 2008
ISSN: 1680-5593

Investigation of Antibacterial and Cytotoxic Effects of Organic Acids Including Ascorbic Acid, Lactic Acid and Acetic Acids on Mammalian Cells

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Abstract: Organic acids are becoming more accepted as substitutes for antibiotics. The aim of this study was to determine the antibacterial effects of ascorbic acid, lactic acid and acetic acid and at the same time to analyze the cytotoxicity of these organic acids to mammalian cells. The antibacterial effects of ascorbic acid, lactic acid and acetic acid in Brain Heart Infusion (BHI), at 4, 1, 0.5, 0.25 and 0.1% concentrations, on the bacteria suspensions of *Staphylococcus aureus* ATCC29740 and *Escherichia coli* DH5á strains, were determined by dilution methods. The cytotoxic effects of organic acids in Dulbecco's Modified Eagle's Medium (DMEM) at 4, 1, 0.5, 0.25 and 0.1% concentrations on cell cultures of murine fibroblast NIH 3T3 cells, were analyzed by the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) test. Antibacterial study showed that acetic acid was the most effective acid on both bacterial strains followed by lactic acid and ascorbic acid. Interestingly, although lactic acid was highly antibacterial, it produced the least cytotoxic effect on murine fibroblast cells. Acetic acid produced the strongest cytotoxic effect. At the same concentrations, the lowest pH values were measured in lactic acid containing media. It was followed by acetic acid. This study showed that the antibacterial and cytotoxic effects of organic acids may follow different mechanisms, on the other hand, pH changes caused by organic acids are not the only determining factor. Therefore, testing the cytotoxicity of organic acids on mammalian cells is usefull in preventing the detrimental effects of organic acids to the mammalian cells before using them as antibacterial agents.

Key words: Antibacterial, cytotoxicity, ascorbic acid, lactic acid, acetic acid

INTRODUCTION

Organic acids are becoming more accepted as substitutes for antibiotic growth promoters. Organic acids have been used for a long time as preservative in animal feed and feed raw material in order to protect them from bacteria and fungal deterioration as well as to prolong shelf life of animal feed. Antibiotic growth promoters and their prophylactic usage against enteric diseases in pigs within the EU have been banned. Thus, organic acids become more popular for their antimicrobial effects (Van Dam, 2006).

Organic acids reduce pH and the buffering capacity of the feed while their antibacterial effect inhibits the growth of bacteria, yeasts and moulds. Some parts of organic acid molecules are in the undissociated form both in feed and in the gastrointestinal (GI) tract of the animals (Cherrington *et al*., 1991; Russell, 1992). In the undissociated form, acid molecules enter specifically Gram negative bacterial cell wall, dissociate within the cell and hence reduce intracellular pH of bacteria. Cells metabolic pathways will be redirected towards the efflux of excess protons (H^+) produced by the acids. This energy-intensive process exhaust the cell metabolism and result in cell death. Furthermore, remaining anions can also inhibit certain important processes in the cytoplasm or the nucleus of bacterial cell (Eklund, 1983; Baronofsky *et al*., 1984; Salmond *et al*., 1984; Russell and Diez-Gonzalez, 1998; Kroll and Patchett, 1991).

Organic acids not only prevent animal feed from microbial deterioration but they most probably also have an effect on the pathogenic microorganisms in the

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gastrointestinal tract. Therefore, they can be used as profilactic agents against enteric diseases. Acidic anion binds with Ca, P, Mg and Zn which results in improved absorption of these minerals in gastro-intestinal tract. Moreover, organic acids can be used as substrates in the intermediary metabolism (Kichgessner and Roth, 1988). The major obstacle to using organic acids as antibacterial agents is the fact that they may be detrimental to the mammalian cells *in vivo*, therefore it is of utmost importance to understand the effect of organic acids on mammalian cells. Hence in this study, cytotoxicity of organic acids including ascorbic acid, lactic acid and acetic acid was investigated by analyzing their antibacterial effects on *Staphylococcus aureus ATCC* 29740 and *Escherichia coli* DH5α cells and cytotoxic effects on Murine fibroblast NIH 3T3 cell line.

MATERIALS AND METHODS

Antibacterial performance of the organic acids: The antibacterial performance of the organics acids was examined by pursuing the following assessment technique: The organic acids concentrations were adjusted in Brain Heart Broth as 4, 1, 0.5, 0.25 and 0.1% for each organic acid in tubes. A calibrated bacterial suspension (10^7 CFU mL) of *S. aureus ATCC* 29740 and *E. coli* DH5α was added into each tube including control. Samples were incubated at 35-37°C under rotational agitation for 24 h. At the end of the incubation period, cultures were diluted serially using sterile PBS and viable counts were carried out in triplicate on nutrient agar media (BD., U.S.A.). Growth medium with bacteria and without organic acids were used as positive control. All experiments were carried out in duplicate on at least 2 separate occasions and the graphs represent mean values.

Cytotoxicity test: Cytotoxic effects of organic acids were tested in Murine fibroblast NIH 3T3 cell line. The cells (seeding density 1.5×10^4 per well) were precultured for 18 h in Dulbecco's Modified Essential Medium (DMEM) supplemented with bovine serum (10%) in 96-well plates and exposed to the organic acids in the tubes for 48 h. After 48 h of cell culturing in the presence of each organic acid, the medium was removed and washed with PBS three times and subsequently, 100 μL of growth medium with MTT (5 mg mL^{-1} in PBS) was added to the cultures. Cells were incubated at 37°C in humidified atmosphere for 3 h. Then the growth medium was removed, 100 μL of lysis solution (99.4% DMSO, 0.6% acetic acid, 10% SDS) was added to each well to dissolve purple crystals of formazan. The absorbance was measured in a spectrophotometer (PerkinElmer, Lambda 35, U.S.A) at a wavelength of 570 nm. Reported values are the means of three replicates and are expressed as percentages of the control values. Acidic pH measurements were taken using a pH meter (Mettler Toledo, GmbH, Germany).

Statistical methods: The differences between the average numbers of growing bacteria concerning the indicated concentration were examined by one-way variance analysis (ANOVA with SPSS Software 1997). Values of $p<0.05$ were considered significant.

RESULTS

Viable count results of experiments performed in order to compare the anti-bacterial performance of the organic acids are summarized in Table 1. Ascorbic acid is found to have the least antibacterial effect on both bacterial strains. At 1% concentration of lactic and acetic acid, no bacterial colonies could be observed on agar plates. At 0.5% lactic acid concentration, 4.99±0.10 CFU mL^{-1} was detected for *S. aureus*, however, no colonial growth was observed for *E. coli*. At 0.25% lactic acid concentration, a total of 6.99±0.09 CFU mL^{-1} for *S. aureus* and 3.98±0.10 CFU mL^{-1} for *E. coli* were counted. At 0.1% lactic acid concentration, 7.98±0.11 CFU mL^{-1} and 7.99±0,009 CFU mL^{-1} were counted for *S. aureus* and *E.coli*, respectively (Table 1).

At 0.5% acetic acid concentration, 2.99±0.10 CFU mL^{-1} was detected for *S. aureus*, on the other hand, there were not any *E. coli* colony on plates. At 0.25% acetic acid concentration, 5.00±0.09 CFU mL^{-1} for *S. aureus* and 2.98±0.12 CFU mL^{-1} for *E. coli* were detected. At 0.1% acetic acid concentration, 7.99±0.08 CFU mL^{-1} for *S. aureus* and 8.98±0.11 CFU mL^{-1} for *E. coli* were detected. For both bacterial strains, 10.58±0.14 CFU mL^{-1} were detected on solid media with no organic acids (Table 1). Results are means±SEM (Standard Error of the Mean) of 5 experiments. Prior to the statistical analysis of antibacterial performance of the organic acids, their logarithmic conversions were done, due to the wide range of their values (0-10^{10} CFU mL^{-1}).

The cytotoxic analysis of organic acids were done using murine fibroblast NIH 3T3 cell line and results are shown in Fig. 1. According to the analysis results; at 0.1% lactic acid concentration, there was no decrease in cell proliferation, however, increasing the concentration to 0.25% resulted in 30% reduced cell proliferation rate. On the other hand, at 0.1% acetic acid concentration, cell proliferation was decreased by 8.8%. In the positive control without any organic acids in cell line, the proliferation of the cell was detected as 100.00%±12.74 (Fig. 1).

Table 1: Antibacterial performance of the organic acids

	Ascorbic acid		Lactic acid		Acetic acid	
	S. aureus	*E.coli*	*S. aureus*	*E.coli*	*S. aureus*	*E.coli*
Con%	$\bar{X}$ ±SD	$\bar{X}$ ±SD	$\bar{X}$ ±SD	$\bar{X}$ ±SD	$\bar{X}$ ±SD	$\bar{X}$ ±SD
4%	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a
1%	4,96±0,19b	3,98±0,11b	0,00±0,00a	0,00±0,00a	0,00±0,00a	0,00±0,00a
0,5%	7,98±0,11c	7,98±0,11c	4,99±0,10b	0,00±0,00a	2,99±0,10b	0,00±0,00a
0,25%	9,38±0,64d	9,19±0,50d	6,99±0,09c	3,98±0,10b	5,00±0,09c	2,98±0,12b
0,1%	8,98±0,12d	10,39±0,46e	7,98±0,11d	7,99±0,09c	7,99±0,08d	8,98±0,11c
P	×××	×××	×××	×××	×××	×××

×××: P<0,001; a, b, c, d, e: different letters in the same row are significant. Prior to the statistical analysis of antibacterial performance of the organic acids, their logarithmic conversions were done, due to the wide range of their values (0-10^{10} CFU mL^{-1})

Table 2: pH changes in BHI (pH:7.22) after adding organic acids

Organic acids	%1 conc/pH	%0.5 conc/pH
Lactic acids	3.68	4.28
Acetic acids	4.12	4.47
Ascorbic acids	4.48	5.53

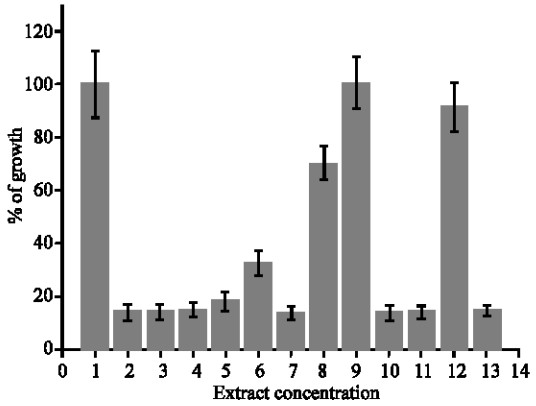

Fig. 1: Cytotoxic effects of organic acids tested in Murine fibroblast NIH 3T3 cell line. (1: Control; 2: 2% Asc A; 3: 1% Asc A; 4: 0.5% Asc A; 5: 0.25% Asc A; 6: 0.1% Asc A; 7: 0.5% Lac A; 8: 0.25% Lac A; 9: 0.1% Lac A; 10: 0.5% A A; 11: 0.25% A A; 12: 0.1% A A; 13: negative control)

The change in pH of the Brain Heart Broth liquid media due to the addition of 0.5 and 1% of organic acids was also followed and summarized in Table 2. Prior to adding the organic acids, pH of the medium was measured as 7.22. At 1% concentrations of lactic, acetic and ascorbic acids pH was lowered to 3.68, 4.12 and 4.48, respectively. As expected, lowering the concentration of the organic acid to 0.5% resulted in less acidic media where pH values of 4.28; 4.47 and 5.53 were measured for lactic, acetic and ascorbic acid, respectively (Table 2).

DISCUSSION

Organic acids which are used as preservatives to prolong the shelf life of feed, are considered as the most important substitutes for feed supplements such as antibiotic growth promoters and chemotherapeutics due to their natural structure. Very few studies have been performed on the cytotoxicity of organic acids on mammalian cells, whereas there is a vast number of literature on antibacterial activities of organic acids.

The antibacterial and cytotoxic effects of organic acids on the bacterial and mammalian cells were at different levels. Ascorbic acid, at 2, 1, 0.5 and 0.25% concentrations, made the strongest cytotoxic effect on mammalian cells, on the contrary, its antibacterial effect was weaker than the other acids. Thus, it would not be a suitable option to choose ascorbic acid as preservative in animal feed for prolonging the shelf-life .

Acetic and lactic acids, at 1% concentration, did not lead to any colony formation on solid media for *S. aureus* and *E. coli*. These organic acids had a less cytotoxic effect on murine fibroblast NIH 3T3 cell line, compared with ascorbic acid. Because of these reasons, both of these organic acids could be a tool for serving different aims such as preservative in animal feed, prolongation the shelf-life of feed, even though, the prophylaxy of enteric diseases in gastrointestinal system.

In a *in vivo* study with 0.8 % lactic acid, Cole *et al*. (1968) showed that the organic acid had bactericide effect on some microorganisms Addition of 0.8% lactic acid to a weaner diet was found to reduce *E. coli* effectively in the duodenum and jejunum of 8 weeks old piglets. Furthermore, when compared with the control-fed animals, piglets fed with the acid-added diets had only non-haemolytic *E. coli*.

Malicki and Bruzewick (2003) found that a mixture of 10% lactic and ascorbic acids in equal proportions was more effective than separately added the acids in reducing *Listeria monocytogenes* in raw beef, stored under the refrigeration. They also claimed that the acid mixture reduced the amount of *Enterobacteriaceae* and inhibited the aerobic growth in the meat samples. Therefore, the combination of organic acids may help in preservation of raw meat at 4°C storage condition by protecting from microorganisms.

In another study by Maribo *et al*., (2000), the gastrointestinal parameters showed some alterations with 0.7, 1.4 or 2.8% concentrations of lactic acid. Whereas the pH and amount of *Lactobacilli* were reduced in the small intestine by the presence of 1.4% lactic acid, they were higher in the caecum and colon of pigs fed with diet containing 0.7% lactic acid. Also, lactic acid decreased the amount of coliforms, but increased the counts of yeast in gastrointestinal tract. Unpublished data from Mikkelsen and Jensen showed that liquid feed containing 0.9, 1.8 or 2.7% lactic acid decreased gastric pH, on the other hand increased the number of *Lactobacilli* and yeast in the GI tract (Canibe *et al*., 2001).

The addition of lactic acid in concentration of 1% to drinking water reduced the gastric pH, in comparison to the control animals. Moreover, lactic acid postponed the multiplication of an enterotoxigenic *E. coli strain* and reduced the mortality rate of animals (Thomlinson and Lawrence, 1981). As a result of our study; organic acids at 4% concentration possessed strong bactericidal effect, on the other hand, lactic and acetic acids at 1% concentration showed fairly strong effect. Addition of ascorbic acid resulted in least decrease in medium pH.

CONCLUSION

The lowest pH was measured in the media with lactic acid, in contrast, the strongest antibacterial and cytotoxic effects were not originated by lactic acid. Therefore, it could be related to its chemical feature rather than its acidic property. This general observation also hold for the other two organic acids as well.

The *in vitro* system offers a reliable method to investigate the effect of different concentrations of some organic acids on bacterial and mammalian cells. It provides a useful approach to find out the conditions of organic acids to be subsequently tested *in vivo*.

Finally, we believe that there is a need to investigate the cytotoxic effects of organic acids on mammalian cells which will be used as preservative in animal feed, prolongation the shelf-life of feed and prophylaxy of enteric diseases.

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