Effects of Caproic and Caprylic Acids on Microbial Growth and Cytotoxicity

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Abstract: In order to assess the potential use of fatty acids and their derivatives as alternatives to the regionally banned antibiotics, the bacteriocidal and cytotoxic effects of caproic (hexanoic) and caprylic (octanoic) acids were investigated. Fatty acids at various concentrations were added to media containing *Staphylococcus aureus*, *Escherichia coli* and murine fibroblast cells. At 0.5% (w/v) concentration of hexanoic acid, both *S. aureus* and *E. coli* growth occurred, however, octanoic acid was effective on inhibition of growth of both bacteria above 1% level. At the same time octanoic acid above 0.5%, hexanoic acid above 0.25% levels inhibited mammalian cell growth. Both acids decreased the medium pH linearly with increasing concentration. Further investigation is needed to prove that they may be usable for *in vivo* applications.

Key words: Hexanoic acid, octanoic acid, *S. aureus*, *E. coli*, cytotoxicity

INTRODUCTION

Post-antibiotic era in animal nutrition has led the researchers to search for new and safe substitutes. Medium chain fatty acids seem to have promising future for antibacterial activity *in vitro* and since they are incorporated in the structure of the diet naturally, common side effects of other compounds may well be eliminated.

Mode of action of fatty acids on the inhibition of human pathogens such as viruses and bacteria has mostly been attributed to their detrimental effects on cell membrane (Bergsson *et al.*, 2001), however they might additionally inactivate cellular enzymes and denaturate cellular proteins. Lauric (dodecanoic), capric (decanoic), caprylic (octanoic), caproic (hexanoic) acids and their monoglycerides have effects on wide range of pathogens starting from enveloped viruses of infants to meat spoilage microorganisms suggesting that hydrophobic group in saturated fatty acids interact with the lipids and protein on the cell surfaces although increased chain length reduces the solubility resulting in low interaction with cell components (Ouattara *et al.*, 1997; Nair *et al.*, 2005). Intestinal colonization of some common pathogens such as *Vibrio cholera* and *Escherichia coli* was decreased by monoacylglycerides and free fatty acids when they were applied at the time of onset of the infection (Petschow *et al.*, 1998). Additionally, their

bactericidal effects with some emulsifying agents have been used to eliminate Helicobacter pylori colonization when the bacteria were still in the lumen and the partial digestion of milk fat took place in the stomach (Sun *et al.*, 2007). Some poultry processing pathogens were able to be removed from the broiler skins when the lauric acid was combined with KOH more than KOH was used alone (Hinton and Ingram, 2006).

Few studies investigated the cytotoxic effects of those fatty acids and their monoamines especially on microorganisms that their monoamines may be more cytotoxic to the target microorganisms than free fatty acids, although their effect on endothelial cells were found to be dose dependent and weak attributing that they might be more selective to the peptidoglycans of bacteria than those of host organism cells (Kitahara *et al.*, 2004, 2006). Additionally most of the researches focused on either short chain (less than C4) or medium chain (C10 or above) fatty acids. Therefore, the aim the present study is to investigate antimicrobial and cytotoxic effects of caproic and caprylic acids *in vitro*.

MATERIALS AND METHODS

The following materials were cased in the research:

- Ethyl Caprate 99% (Ethyl Decanoate), Code AL148970, Aldrich.

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- Octanoic Acid 99%, Code SIC2875, Sigma.
- Caproic Acid, 99%, Code ALH12137, Aldrich.

Antibacterial performance of the fatty acids: The antibacterial performance of the fatty acids was examined by pursuing the following assessment technique: The hexanoic and octanoic acid concentrations were adjusted in Brain Heart Broth as 4, 2.1, 0.5, 0.25, 0.125, 0.0625, 0.0312, 0.0156 % for each fatty acid in tubes. A calibrated bacterial suspension (10^7 CFU mL^{-1}) 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 fatty 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 fatty 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 fatty acids in the tubes for 48 h. After 48 h of cell culturing in the presence of each fatty 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 (Perkin Elmer, 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 AND DISCUSSION

The bacteriocide effects of hexanoic and octanoic fatty acids at certain concentrations on *S. aureus* and *E. coli* were summarized in Table 1.

Table 1: Mean bacterial growths (CFU g^{-1}) at various concentrations of hexanoic and octanoic acids

Acid	Concentration (% w/v)	Staphylococcus aureus	Escherichia coli
Hexanoic acid	4	0 (No growth)	0 (No growth)
	2	0 (No growth)	0 (No growth)
	1	0 (No growth)	0 (No growth)
	0.5	8.3×10^7 CFU mL^{-1}	1.5×10^5 CFU mL^{-1}
Octanoic acid	4	0 (No growth)	0 (No growth)
	2	0 (No growth)	0 (No growth)
	1	6.48×10^8 CFU mL^{-1}	2.72×10^8 CFU mL^{-1}

Table 2: pH values of brain heart infusion and Dubecco's Modified Eagle's Medium (DMEM) of hexanoic acid and octanoic acid

	Concentration (% w/v)	pH	DMEM
Hexanoic acid	4	4.33	-
	2	4.70	-
	1	5.46	-
	0.5	6.40	5.10
	0.25		6.40
			(The pH where the cells are viable)
Octanoic acid	4	5.14	-
	2	5.74	-
	1	6.79	-
	0.5		-
	0.25		5.91
	0.125		7.25
			(The pH where the cells are viable)

(Brain Heart Infusion pH = 7.28)

Strong bacteriocide and cytotoxic effects of these fatty acids at 4 and 2% concentrations and hexanoic acid at 1% concentration were observed on Gram positive *S. aureus* and Gram negative *E. coli*. On the other hand, the hexanoic acid at 0.5% concentration, the colonies were seen as 8.3×10^7 and 1.5×10^5 CFU mL^{-1} for *S. aureus* and *E. coli*, respectively. Similarly, the octanoic acid at 1% concentration, the colonies were detected as 6.48×10^8 and 2.72×10^8 CFU mL^{-1} for *S. aureus* and *E. coli*, respectively.

Cytotoxic effects of these fatty acids at certain concentrations on mammals' cells were shown in Fig. 1 and 2. These fatty acids at concentrations of 4, 2, 1% and octanoic acid at 0.5% concentration were determined as cytotoxic. Therefore, eventhough they have strong bacteriocidal effects at the above concentrations, it could be suggested that they are not yet suitable for *in vivo* use.

There was 10% cell growth in the presence of hexanoic acid, at 0.5% concentration. On the other hand, at 0.25, 0.125, 0.625 and 0.0312% concentrations, the rates of cell growth became 37, 74, 92 and 100%, respectively. At this context, this fatty acid at 0.25 % concentration has moderate toxicity.

In octanoic acid, at 0.25% concentration, 16% mammalian cell growth was detected and at concentrations of 0.0125, 0.0625, 0.0312 and 0.0156%, cell growths were measured as 32, 54, 81 and 100%,
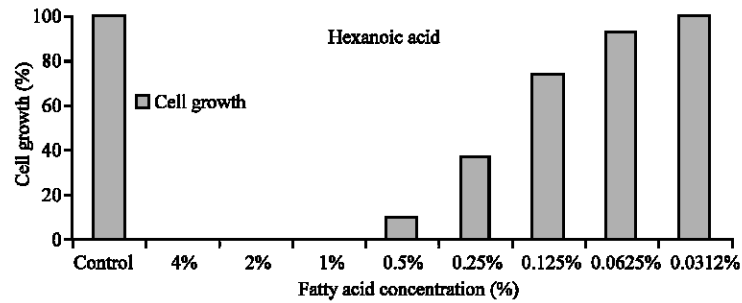
Fig. 1: Mean cell growth (%) at various hexanoic acid concentrations
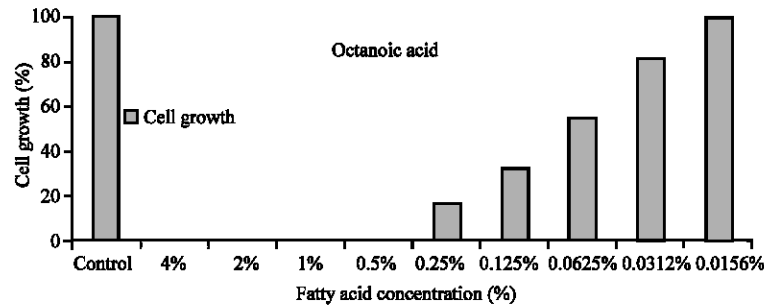

Fig. 2: Mean cell growth (%) at various octanoic acid concentrations

respectively. Thus, the moderate cytotoxic concentration of this fatty acid on mammalian cell was 0.0625%.

The change in pH of the Brain Heart Broth liquid media due to the addition of 0.5 and 1% of the fatty acids was also followed and summarized in Table 2. Prior to adding the fatty acids, pH of the medium was measured as 7.28. At 1 % concentration of hexanoic and octanoic acid pH was lowered to 5.46 and 6.79, respectively. As expected, lowering the concentration of hexanoic acid to 0.5% resulted in less acidic media where pH value of 6.40 was measured for hexanoic acid (Table 2).

Fatty acids which are used as antimicrobials in soaps, 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 fatty acids on mammalian cells, whereas there is a vast number of literature on antibacterial activities of fatty acids.

The antibacterial and cytotoxic effects of fatty acids on the bacterial and mammalian cells were at different levels. Hexanoic acid and octanoic acid, at 4, 2 and 1% concentrations, made the strongest cytotoxic effect on mammalian cells. In addition, their antibacterial effect were very strong as well. It is not convenient to use hexanoic and octanoic acids at the stated above mentioned concentrations, for prolonging shelf life, *in vivo* and chemotherapeutic purposes. Other researchers have

claimed that Gram negative bacteria are less sensitive to the antimicrobial effect of fatty acid and monoglycerides than gram-positive bacteria (Kabara, 1978; Monk *et al.*, 1996).

Hexanoic acid at 0.5% and octanoic acid at 0.25% concentrations and their more dilute concentrations have less cytotoxic effects on mammallian cells. However, the antibacterial effects of these fatty acids at lower concentrations were 0.5% for hexanoic and 1% for octanoic acids. Therefore, there is a need for investigating the optimum antibacterial concentration as well as the cytotoxic concentration of these fatty acids *in vivo*.

Hexanoic acid is less cytotoxic than octanoic acid and its bacteriocidal effect is much more stronger than octanoic acid, statistically ($p<0.05$). However, it decreases the PH of the growth media at lower values when compared with octanoic acid. Therefore, it could be concluded that there was no any direct correlations between acidity, bacteriocidal effect and cytotoxicity (Table 1 and 2 and Fig. 1).

Petschow *et al.* (1996) proved that medium-chain fatty acids and their mono glycerol esthers have a bacteriocidal effect on Gram negative *H. pylori*. In our study, the similar effect was done by hexanoic and octanoic fatty acids for Gram negative *E. coli*. Therefore, our study is consistent with Petschow's. Additionally, methicillin-resistant *Staphylococcus aureus* was inhibited by lauric acid and myristilamine and synergistic activity of

lauric acid and gentamycin *in vitro* (Kitahara *et al.* 2006). Furthermore Nair *et al.* (2005) postulated that caprylic acid and monocaprylin have bacteriocidal effect in some microorganisms in milk. Present study indicated that in addition to caprylic acid, caproic acid also has antibacterial activity on *S. aerous* and *E. coli*.

Previous studies postulated that killing effects of free fatty acids and their monoglycerides are atributable to their damaging nature to the microbial membrane outer surfaces resulting in demolishing regular passage through membrane (Galbraith and Miller, 1973a, 1973b). Thompson *et al.* (1994) showed in their results that longer chain polyunsaturated fatty acids also damage *H. pylori* cell membrane by incorporating themselves into the structure resulting in altered conformation and nutrient passage and further lysis. It is possible that medium-chain Free Fatty Acids (FFA) and their Monoglycerides (MG) utilize a similar mechanism to inactivate bacteria. Others have suggested that the bacteriocidal effects of polyunsaturated fatty acids are created by cellulary toxic lipid peroxides generated from an oxidative process involving H_2O_2 and iron (Knapp and Melly, 1986).

Although, there were many studies published to prove the *in vivo* antibacterial effects of FFAs and MGs, concrete improvements has not established yet. Because FFAs and MGs are digested and absorbed in the earlier stages of GI tract (Borgstrom *et al.*, 1957) and therefore using MGs or FFs as feed additives for the interruption of intestinal pathogen microorganisms' life cycles needs further investigations. These questions can be addressed only through careful evaluation of the effectiveness of MGs and FFAs aganist *S. aureus* and *E.coli* infections in either animal studies or human clinical trials.

The pressure to limit antibiotic use in food-producing animals is one of the biggest challenges for the dairy industry, animal health and to prolong the shelf life of animal food, eventhough antibiotics still remain the most effective tool for these objectives. Because fatty acids and their monoglycerides are believed to kill bacteria by multiple mechanisms, the potential for developing bacterial resistance to these molecules is relatively negligible.

CONCLUSION

Hexanoic and octanoic acids at 4, 2, 1% and octanoic acid at 0.5% concentrations were tested as bacteriocides against *S. aureus* and *E.coli*. These results provide justifications for evaluating the hexanoic and octanoic acids as alternatives to antibiotics for the dairy industry, animal health and to prolong the shelf life of animal food. Finally, we believe that there is a need to investigate the cytotoxic effects of fatty acids on mammalian cells which will be used as preservative in animal feed, prolongation of the shelf-life of feed and prophylaxy of enteric diseases.

REFERENCES

Bergsson, G., J. Arnfinnsson, O. Steingrimsson and H. Thormar, 2001. *In vitro* killing of *Candida albicans* by fatty acids and monoglycerides. Antimicrobial Agents and Chemotherapy, 45 (11): 3209-3212.

Borgstrom, B., A. Dahlqvist, G. Lundh and J. Sjovall, 1957. Studies of intestinal digestion and absorption in the human. J. Clin. Investigations, 36: 1521-1536.

Galbraith, H. and T.B. Miller, 1973a. Physicochemical effects of long chain fatty acids on bacterial cells and their protoplasts. J. Applied Bacteriol., 36: 647-658.

Galbraith, H. and T.B. Miller, 1973b. Effects of long chain fatty acids on bacterial respiration and amino acid uptake. J. Applied Bacteriol., 36: 659-675.

Hinton, A.J. and H.D. Ingram, 2006. Antimicrobial activity of potassium hydroxide and lauric acid against microorganisms associated with poultry processing. J. Food Prot., 69 (7): 1611-1615.

Kabara, J.J., 1978. Fatty acids and derivatives as antimicrobial agents: A review. In: Kabara, J.J. (Ed.). Symposium on the pharmacological effect of lipid. The American Oil Chemists' Society Champaign, IL, U.S.A., pp: 1-14.

Kitahara, T., Y. Aoyama, Y. Hirakata, S. Kamihira, S. Kohno, N. Ichikawa, M. Nakashima, H. Sasaki and S. Higuchi, 2006. *In vitro* activity of lauric acid or myristillamine in combination with 6 antimicrobial agents against methicillin-resistant *Staphylococcus aureus* (MRSA). Int. J. Antimicrob. Agents, 27: 51-57.

Kitahara, T., N. Koyama, J. Matsuda, Y. Aoyama, Y. Hirakata, S. Kamihira, S. Kohno, M. Nakashima and H. Sasaki, 2004. Antimicrobial activity of saturated fatty acids and fatty amines against methicillin-resistant *Staphylococcus aureus*. Biol. Pharm. Bull., 27 (9): 1321-1326.

Knapp, H.R. and M.A. Melly, 1986. Bactericidal effectts of polyunsaturated fatty acids. J. Infec. Dis., 154: 84-94.

Monk, J.D., L.R. Beuchat and A.K. Hathcox, 1996. Inhibitory effects of sucrose monolaurate, alone and in combination with organic acids, on *Listeria monocytogenes* and *Staphylococcus aureus*. J. Appled Bacteriol., 81 (1): 7-18.

Nair, M.K.M., J. Joy, P. Vasudevan, L. Hinckley, T.A. Hoagland and K.S. Venkitanarayanan, 2005. Antibacterial effect of caprylic acid and monocaprylin on major bacterial mastitis pathogens. J. Dairy Sci., 88: 3488-3495.

Petschow, B.W., R.P. Batema, R.D. Talbott and L.L. Ford, 1998. Impact of medium-chain monoglycerides on intestinal colonisation by *Vibrio cholerae* or enterotoxigenic *Escherichia coli*. J. Med. Microbiol., 47 (5): 383-389.

Petschow, B.W., R.P. Batema and L.L. Ford, 1996. Susceptibility of Helicobacter pylori to bactericidal properties of medium-chain monoglycerides and free fatty acids. Antimicrob. Agents Chemother., 40 (2): 302-306.

Outtara, B., R.E. Simard, R.A. Holley, G.J.P. Piette and A. Begin, 1997. Antibacterial activity of selected fatty acids and essential oils against 6 meat spoilage organisms. Int. J. Food Microbiol., 37: 155-162.

Sun, C.Q., C.J. O'Connor, A.K.H. MacGibbon and A.M. Roberton, 2007. The products from lipase catalysed hydrolysis of bovine milkfat kill *Helicobacter pylori in vitro*. FEMS Immunol. Med. Microbiol., 49: 235-242.

Thompson, L.C., A. Cockayne and R.C. Spiller, 1994. Inhibitory effect of polyunsaturated fatty acids on the growth of *Helicobacter pylori*: A possible explanation of the effect of diet on peptic ulceration. Gut, 35: 1557-1561.