Control of Pathogenic Bacteria in Cooked Duck Blood Curd using Sodium Diacetate and Sodium Chloride

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Abstract

The effects of sodium diacetate (SD) and sodium chloride (NaCl) on growth inhibition of four bacterial strains: *Escherichia coli*, *Salmonella* Typhimurium, *Pseudomonas fluorescens* and *Staphylococcus aureus* were investigated by determining the minimum inhibitory concentration and bactericidal concentrations (MIC and MBC), fractional inhibitory and bactericidal concentration index (FICI and FBCI) and survival of these pathogenic bacteria in cooked duck blood curd. SD provided greater overall inhibition to all tested bacterial strains in comparison with NaCl. The effect of SD + NaCl in combination was partially synergistic or synergistic against these pathogenic bacterial, exhibiting FICI and FBCI of 0.75 and 0.25 respectively for *E. coli*, 0.62 and 0.62 respectively for *S.* Typhimurium, 1.00 and 0.62 respectively for *P. fluorescens* and 0.50 and 0.37 respectively for *S. aureus*. For cooked duck blood curd, 0.15% (w/v) SD + 1.25% (w/v) NaCl in combination reduced *E. coli*, *Salmonella* spp., *Pseudomonas* spp. and *S. aureus* ($p < 0.05$) in the range of 0.63 - 1.10 log cycles and controlled the growth of all four bacteria more than the control sample ($P < 0.05$) did. Furthermore, the 0.15% (w/v) SD + 1.25%(w/v) NaCl combination controlled the growth of these bacteria in cooked duck blood curd for 6 days of storage, except in the case of *E. coli*, for which growth was controlled for 4 days of storage. All experimental results were compared to the control sample before storage ($p > 0.05$). The results indicate that SD in combination with NaCl can be incorporated into cooked duck blood curd to effectively reduce and control growth of pathogenic bacteria at 10°C of storage.

Keywords: organic acid salt, pathogenic bacteria, cooked duck blood curd, cold storage
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1. Introduction

Cooked duck blood curd is one of the favorite blood foods in Thailand. This traditional product is produced from a combination of raw duck blood and water and is clotted by heat. It is distributed as a freshly cooked product soaked in low concentration of salt with a shelf life of approximate two days. In recent times, preservation by chilling has been used to extend the product's shelf life and thus expand existing markets [1]. However, safety issues to do with the presence and growth of pathogenic bacteria in this product are of ongoing concern.

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Problems associated with the safe preservation of this by-product of slaughter houses have grown to be more complex as today's products require more safety and greater assurance of protection from pathogenic microorganisms. Many chemical antimicrobials have been used to inhibit pathogen growth on meat and meat product surfaces. Sodium diacetate (SD) was shown to be effective in limiting the growth of *Escherichia coli*, *Salmonella* Typhimurium, *Pseudomonas fluorescens* and *S. aureus* [2, 3]. However, in developed countries, the maximum level of sodium diacetate allowed for use in meat products is 0.25% of final product [4, 5]. Sodium chloride (NaCl) was reported to affect the morphology of *E. coli* and *S. aureus*. It was also reported to have a milder effect on cell damage, particularly on *S. aureus* [6]. At low salinity, it causes an immediate influx of small solutes thus relieving physical stress. On the other hand, at high salinity, it causes water efflux which is balanced by an augmentation of compatible solutes, for example glutamate, proline, glycine betaine, trehalose and ectoine [7]. Therefore, the objective of the present study was to compare the antibacterial activity of SD, NaCl and SD+NaCl against *E. coli*, *Salmonella* Typhimurium, *P. fluorescens* and *S. aureus* in cooked duck blood curd.

2. Materials and Methods

2.1 Strains of bacteria

E. coli DMST 4212, *S.* Typhimurium DMST 22842, *P. fluorescens* DMST 20076 and *S. aureus* DMST 4745 were obtained from the culture collection at the Department of Medical Sciences, Ministry of Public Health, Thailand. These bacteria were cultivated on Mueller Hinton agar (MHA, Merck, Germany). Inocula were prepared by transferring 2-3 colonies from MHA to 10 ml of 0.85%(w/v) NaCl (Sigma-Aldrich Pte. Ltd., Singapore) and diluted in 0.85%(w/v) NaCl to 10^8 cfu/ml (McFarland standard of 0.5). These suspensions were further diluted with 0.85%(w/v) NaCl as required. The initial concentration of approximately 5×10^5 cfu/ml was adopted for the minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and synergy methods and approximately 1×10^6 cfu/ml for cooked duck blood curd model.

2.2 MIC and MBC determinations

For each bacterium, MIC was estimated by a broth microdilution method. Distilled water was used for dissolving all the antimicrobials. Subsequent two-fold serial dilutions were performed in medium. The final concentrations of the antimicrobial in wells ranged from 0-2.5% (w/v) for SD (Chemipan Corporation Co. Ltd., Bangkok, Thailand) and 0-20% (w/v) for NaCl. The MIC was reported as the lowest concentration that limited the broth turbidity to below 0.05 at absorbance of 600 nm using a UVM 340 Microplate Reader (Biochrom Ltd., Cambridge, UK). MBC was estimated by comparing the survival numbers of bacteria with their initial numbers. The MBC was then reported as the lowest concentration that killed not less than 99.9% of the initial number of bacteria [8].

2.3 Synergistic effects

To determine whether SD acted synergistically with NaCl, a checkerboard titration was used for analysis of the fractional inhibitory concentration index (FICI) and fractional bactericidal concentration index (FBCI) in Mueller Hinton broth (Merck, Germany). The MIC, MBC, FICI and FBCI analyses were repeated three times and their means were calculated. Synergy was demonstrated when FICI and FBCI < 0.5; partial synergy/additive effect was obvious when the

FICI and FBCI ranged from > 0.5 to 1.0; there was no interaction when the FICI and FBCI was > 1 to < 2, and antagonism was exhibited when the FICI and FBCI was > 2 [9].

2.4 Cooked duck blood curd model

Cooked duck blood curd samples (*Cherry valley* crossbred ducks at 47 d) were collected in three batches at different days from a cooked duck blood curd line at an industrial slaughterhouse in Chachoengsao province, Thailand. 500 g samples were taken, kept in polystyrene boxes containing ice, and transported to the Department of Animal Production Technology and Fisheries, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang for analysis within 3 hrs of sampling.

The samples were used to estimate the effects of the antimicrobials on *E. coli*, *S.* Typhimurium, *P. fluorescens* and *S. aureus*. Each sample was inoculated with all 4 bacteria suspensions as follows: the cooked duck blood curd was soaked in 300 ml of the bacterial inoculum (approximately 10^6 cfu/ml) for 20 min and dried in laminar air flow for 20 min before the addition of the antimicrobials. The initial count of each pathogenic bacterium was approximately 10^4 cfu/g. The sample was randomly separated into two treatments and soaked in 300 ml of the antimicrobial as follows: (1) control –water and (2) 0.15% (w/v) SD +1.25%(w/v) NaCl in combination. Each treatment was packed in a polyethylene plastic bag. Air-circulated refrigeration was used for storing samples at 10ºC for 8 days. The stored samples under refrigeration were sampled for microbiological determination at 0, 2, 4, 6 and 8 days.

2.5 Microbiological analysis

The samples were submitted to count for *E. coli* [10], *Samonella* spp. [11], *Pseudomonas* spp. [12] and *S. aureus* [13] according to standard procedures. *E. coli*, *Samonella* spp., *Pseudomonas* spp. and *S. aureus* were enumerated on violet red bile agar (Merck, Germany) , xylose lysine deoxycholate (Merck, Germany), *Pseudomonas* CFC agar (Merck, Germany) and Baird Parker agar (Merck, Germany) contained 5% (v/v) of egg-yolk tellurite emulsion 20% (Merck, Germany), respectively. The plates were incubated at 35 ± 2ºC for 24-48 h, except for *Pseudomonas* spp., which was incubated at 25 ± 2ºC for 24-48 h. Then, colonies were counted. IMViC test for *E. coli*, triplate sugar iron agar test, lysine iron agar test for *Salmonella* spp., oxidase, aerobicity and ability to ferment glucose for *Pseudomonas* spp. and coagulase test for *S. aureus* by standard methods [14] were determined. The results were calculated to log cfu/g.

2.6 Statistical analysis

Data showing bacterial loading in the cooked duck blood curd model were showed as means and standard deviations. The general linear model procedure was used for estimating significant differences ($p < 0.05$) of all statistical computations. Least squares means were computed and separated ($p<0.05$) with the PDIFF option of GLM. All statistical analyses were executed by SAS v. 9.0 [15].

3. Results and Discussion

3.1 MIC and MBC determinations

The MICs of SD and NaCl for antibacterial action against *E. coli* DMST 4212, *S.* Typhimurium DMST 22842, *P. fluorescens* DMST 20076 and *S. aureus* DMST 4745 were determined to be 0.31, 0.15, 0.15 and 0.31% (w/v) respectively for SD, and 5.00, 10.00, 5.00 and 20.00% (w/v) respectively for NaCl. However, the MBCs of SD were two-fold for all bacteria, except for *E. coli* (eight-fold), which were higher than the analogous MIC. The MBCs of NaCl against all bacteria were 10.00% (w/v), except in the case of *S. aureus* (20%(w/v) (Table 1). An earlier study found that the MIC and MBC of SD against *S. aureus* were 0.78 and 6.25% (w/v) [9]. *Salmonella* Typhimurium was able to grow in the presence of up to 7-8% NaCl at 37ºC [16]. Normally, *Salmonella* and *E. coli* (bacteria belonging to the Enterobacteriaceae family) do not tolerate high salt levels [17]. However, it has been reported that *S. aureus* can tolerate high concentrations of NaCl in liquid medium, with no damage or shrinkage of cellular structure being observed. Under similar conditions, cell injury of *E. coli* appeared [6].

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of sodium diacetate (SD) and sodium chloride (NaCl) to inhibit growth of some pathogenic bacteria.

Strains	Antimicrobials	MIC % (w/v)	MBC % (w/v)
Escherichia coli	SD	0.31	1.25
	NaCl	5.00	10.00
Salmonella Typhimurium	SD	0.15	0.31
	NaCl	10.00	10.00
Pseudomonas fluorescens	SD	0.15	0.31
	NaCl	5.00	10.00
Staphylococcus aureus	SD	0.31	0.62
	NaCl	20.00	20.00

3.2 Synergistic effects

The FICIs for the combined action of SD with NaCl on *E. coli* DMST 4212, *S.* Typhimurium DMST 22842, *P. fluorescens* DMST 20076 and *S. aureus* DMST 4745 are shown in Table 2. FICI and FBCI indicated that utilization of SD in combination with NaCl resulted in improved inhibition of all pathogenic bacteria.The enhancing effect of the combination was also evidenced by the bactericidal responses produced at sub-MBC levels for each bacterium. FICI of the combined action of SD +NaCl was 0.75 and 1.00 (0.07%(w/v) + 2.50%(w/v)) for *E. coli* and *P. fluorescens* respectively and 0.62 (0.07% (w/v) +1.25% (w/v)) for *S.* Typhimurium, suggesting partial synergy of the analyzed antimicrobials and 0.50 (0.07%(w/v) +5.00%(w/v)) for *S. aureus*, suggesting synergy of the analyzed antimicrobials. Similarly, FBCI of the combined action of SD + NaCl was 0.25 (0.15%(w/v) +1.25%(w/v)) for *E. coli* and 0.37 (0.15%(w/v) +2.50%(w/v)) for *S. aureus*, again suggesting synergy and 0.62 (0.15%(w/v) +1.25%(w/v)) for *S.* Typhimurium and *P. fluorescens*. The calculation and analysis of FICI and FBCI indicated that the utilization of SD with NaCl resulted in the synergistic inhibition of pathogenic bacteria, potentially resulting from SD. SD is a weak organic acid salt and effectively inhibits most tested bacteria.

Table 2. Fractional inhibitory concentration index (FICI) and fractional bactericidal concentration index (FBCI) of the combined action of sodium diacetate (SD) with sodium chloride (NaCl) to some pathogenic bacteria.

Strains	Indices	Concentrations of SD + NaCl in combination %(w/v)	Values	Interpretation
Escherichia coli	FICI	0.07+2.50	0.75	partial synergy
	FBCI	0.15+1.25	0.25	synergy
Salmonella Typhimurium	FICI	0.07+1.25	0.62	partial synergy
	FBCI	0.15+1.25	0.62	partial synergy
Pseudomonas fluorescens	FICI	0.07+2.50	1.00	partial synergy
	FBCI	0.15+1.25	0.62	partial synergy
Staphylococcus aureus	FICI	0.07+5.00	0.50	synergy
	FBCI	0.15+2.50	0.37	synergy

3.3 Cooked duck blood curd model

The results revealed that the use of 0.15% (w/v) SD + 1.25% (w/v)) NaCl in combination reduced the numbers of *E. coli*, *Salmonella* spp., *Pseudomonas* spp. and *S. aureus* on cooked duck blood curd stored at 10ºC (Figure 1). The four bacteria of the sample soaked in SD in combination with NaCl decreased by 0.63 log cfu/g for *E. coli*, 0.75 log cfu/g for *Salmonella* spp., 1.10 log cfu/g for *Pseudomonas* spp. and 0.82 log cfu/g for *S. aureus*, compared to control sample before storage. Then, growth of the four bacteria was retarded throughout the 6 days of storage at 10ºC, except for *E. coli*, which was retarded for 4 days of storage when compared to the control sample before storage ($p > 0.05$). At the end of the 8-day storage time, the four bacteria in the samples soaked in water (control) were 2.61 log cfu/g for *E. coli*, 2.26 log cfu/g for *Salmonella* spp, 1.59 log cfu/g for *Pseudomonas* spp. and 2.17 log cfu/g for *S. aureus* which were higher than the numbers of the four bacteria in samples soaked in SD in combinations with NaCl ($p < 0.05$). This result could probably explain the role of SD in combination with NaCl as antimicrobial agents. Salts of organic acid have been shown to have antimicrobial effects by causing hyper-acidification via proton donation at the plasma membrane interface of the microorganism and intracellular cytosolic acidification, an excess of which can disrupt the H+ -ATPase enzyme, which is required for ATP synthesis [18]. Lag phase extension and growth rate reduction for *L. mononcytogenes* were also observed in ground ham to which had been added 0.25% SD [19]. *S.* Typhimurium was able to grow in the presence of up to 4%NaCl at 12ºC [16].

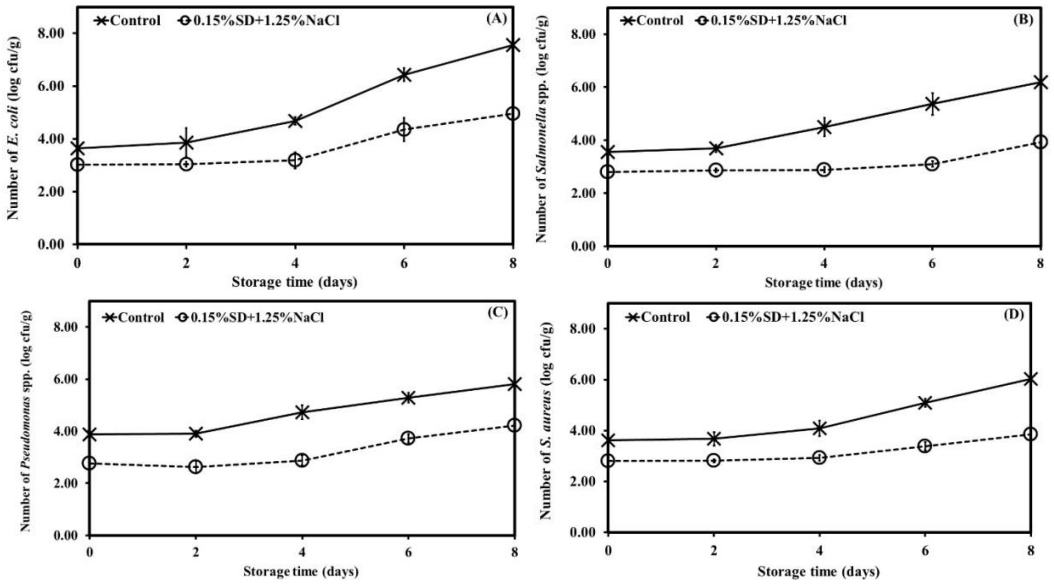

Figure 1. Effect of sodium diacetate (SD) in combination with sodium chloride (NaCl) on *Escherichia coli* (A), *Salmonella* spp. (B), *Pseudomonas* spp. (C) and *Staphylococcus aureus* (D) on cooked duck blood curd stored at 10°C for 8 days.

4. Conclusions

Using SD in combination with NaCl at concentrations lower than the MIC values of each antimicrobial agent is capable of decreasing the number of pathogenic bacteria in cooked duck blood curd, thereby enhancing microbiological safety of cooked duck blood curd. Furthermore, the addition of SD in combination with NaCl is recommended in order to control the growth of pathogenic bacteria effectively.

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