EFFECT OF LACTIC ACID AND BACTERIOCIN ON IMPROVIVING MICROBIOLOGICAL SAFETY OF E-SAN SAUSAGE, A THAI FERMENTED MEAT PRODUCT

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Abstract –This study was to evaluate the application of lactic acid solution, bacteriocin and their combination to improve the microbial safety of E-san sausage, a Thai fermented meat product. There were 6 treatments in the experiment as follows: 2 and 4 % of lactic acid, 2 and 4% of bacteriocin, the combination of 2% lactic acid solution and 2% bacteriocin and control (no adding solution). This study composed of 2 experiments. The first was application of above solutions to E-san sausages before casing. Then, lactic acid bacteria and yeast/mold were determined. The second was inoculation of *Staphylococcus aureus* to E-san sausage before adding solutions and casing. The survival of *S. aureus* was counted. These sausages underwent fermentation process by hanging under room temperature for 4 days. The results indicated that all treatments which adding solutions had an effect on number of microorganisms. The number of lactic acid bacteria in E-san sausage was lower in control and the combination treatments. However, the number of yeast/mold and *S. aureus* added with the mixture of lactic acid and bacteriocin was the lowest to other groups ($p<0.05$). Therefore, this research was beneficial for E-san sausage production to improve the microbial safety.

Key Words – bacteriocin, Lactic acid, Thai E-san sausage

I. INTRODUCTION

Thai fermented sausage or E-san sausage or Sai krok E-san is a kind of well-known traditional and popular meat product in Thailand. E-san sausage are made from minced pork, fat, salt, garlic, cooked rice, pepper and soy sauce that are mixed well and stuffed into pork casing or other edible casings and then fermented until becoming sour and cooked before eating [8,13]. E-san sausage fermentation by indigenous microorganism generally takes 2-3 days at room temperature. During this natural fermentation, lactic acid bacteria such as pediococci and lactobacilli have been shown to become the dominant microorganisms [6,13]. Mostly they are produced by home industry or small factories and sold in both cooked and uncooked pieces. Therefore the quality is not stainable. In addition, spoilage and pathogenic microorganisms contamination were found. Lactic acid have long been use as an antimicrobial substance and widely employed in food, meat and meat products. It has a generally recognize as safe (GRAS) status for direct addition to food [4]. Bacteriocins are small proteins produced by many bacteria, including lactic acid bacteria. Due to various reports in many countries informed the advantage of using bacteriocin to control the microbiological quality and safety during meat products fermentation [12]. Bateriocin produced by *Lactobacillus plantarum* KL-1 showed inhibitory activity against *L. sakei*, *Leuconostoc mesenteroides* and *Enterococcus faecalis* [9]. Therefore the objective of this study was to evaluate the application of bacteriocin, lactic acid solution and their mixture to improve product quality to meet the safety standard of the E-san sausage.

II. MATERIALS AND METHODS

Preparation of fermented sausage

The sausage composition was as follows: ground pork (50.60%), back fat (33.73%), cooked rice (6.73%), garlic (6.73%), prague powder (salt : sodium nitrite ratio 94% : 6%) (1.52%), phosphate (P_2O_5 : salt ratio 57.9% : 42%) (0.34%) and sugar

(0.34%). The ingredients were thoroughly mixed and divided into six batches; (1) control (no adding solution or bacteriocin) (2) added 2% lactic acid solution (3) added 4% lactic acid solution (4) added 2% bacteriocin (5) added 4% bacterocin and (6) added 2% lactic acid solution and 2% bacteriocin. For experiment I, the mixture was then stuffed into 2.3 cm diameter collagen casings and tied with tread. Each piece of sausage was around 2.5 cm in length. After that, the sausages were kept by hanging under room temperature. Lactic acid bacteria and yeast/mold analysis was carried out for 0, 1, 2, 3 and 4 days. For experiment II, *S. aureus* (approximately 10^3 cfu/g) was inoculated in E-san sausage before stuffing. The number of *S. aureus* was count at the same period as above.

Bacteriocin preparation

L. plantarum KL-1, isolated from traditional Vietnamese fermented pork called "Nem chua", was cultured in MRS broth (de Man Rogasa and Shape; Merck, Germany) at 30 °C overnight. The culture was stored at -80 °C in glycerol at final concentration of 15% to use as stock culture. Stock culture was transferred into MRS broth with 1% (w/v) NaCl and incubated 14-18 h at 37 °C for refresh culture. Then, transferred 2% (v/v) culture in MRS broth again and incubated for 14-18 h at 37 °C. Subsequently, culture broth was centrifuged at 1,500xg at 4 °C (Jouan CR3i, France). Cell-free supernatant (CFS) known as bacteriocin was adjusted to pH 6.9-7.0 and sterilized by filter (0.2 µm, Sartorius stedim biotech). Therefore, inhibition due to acid of CFS was excluded.

Microbiological analysis

Lactic acid bacteria, Yeast/mold and *S. aureus* count were examined according to AOAC (2006) [2], AOAC (2005) [1] and Bam (2001) [3], respectively. For microbiological analysis, 25 g fermenting sausage sample was aseptically transferred to a sterile plastic bag and mixed for 1 min with 225 mL of sterile 0.1% peptone (w/v) solution. Appropriate decimal dilutions of the sample solutions were prepared using sterile peptone solution and 0.1 mL of each dilution was spreaded on selective agar plates in duplicate. The lactic acid bacteria was determined on MRS agar (Merck, Germany) added 0.5% $CaCO_3$ incubated at 37°C for 48h. Yeast/mold was growth on malt agar (Merck, Germany) was adjusted pH to 4.5, then incubated at 26 °C for 7 days. *S. aureus* was propagated by using Baird parker agar (Merck, Germany). Bacterial counts were expressed in log colony forming units per gram of sample (log cfu/g).

Statistical analysis

All the experiments were carried out using Completely Randomized Design (CRD). Significant differences between means were separated using Duncan's New Multiple Rang test (DMRT) with SPSS (SPSS 16.0 for windows, SPSS Inc., Chicago, IL, USA).

III. RESULTS AND DISCUSSION

Effect of lactic acid solution and bacteriocin on microbial safety of E-san sausage

Number of lactic acid bacteria, yeast/mold and *S. aureus* in E-san sausage were determined at 0, 1, 2, 3 and 4 days during fermentation process. The results displayed that number of lactic acid bacteria was low in control and the combination solutions of 2% lactic acid and 2% bacteriocin groups. The lowest number of lactic acid bacteria was observed in the combination solutions groups. However, number of lactic acid bacteria in E-san sausage of all groups increased during longer period of fermentation ($P<0.05$) when compared to the first day of fermentation (Table 1). The lowest number of yeast/mold in E-san sausage were observed in the combination of 2% lactic acid and 2% bacteriocin group ($P<0.05$). There was no different between E-san sausage using different concentration (2 and 4%) of single solution. The lowest number of *S. aureus* was found in 4% lactic acid group and the combination of 2% lactic acid and 2% bacteriocin group. However, it was noticed that adding either lactic acid or bacteriocin could decrease number of yeast/mold and *S. aureus* when compare to control group. In addition, the highest number of yeast/mold and *S. aureus* exhibited in control group (Table 2 and 3). Fermentation of E-san sausage was dominated by lactic acid bacteria. [11]. Similarly, our result displayed the increasing of lactic acid bacteria during longer period of fermentation. During this period, the growth of yeast and mold increased in every treatment because yeast and mold can growth in the presence of acidity. The results showed that lactic acid, bactericoin and the combination of lactic acid and bacteriocin could inhibit the growth of yeast/mold and *S. aureus*. This explained that lactic acid could disturb pH homeostasis of bacteria resulting in stress cell. It is also enable diffuse across the bacterial cell membrane. Once inside, the acid may dissociate and release protons that acidity to the cytoplasm, hence the growth is reduced and inhibited [10]. Moreover, bacteriocins have an effect on bacterial act by forming membrane chanels or pores that destroy the energy potential of sensitive cells, leads to cell death [7]. Many bacteriocins are safe and effective natural inhibitors against pathogenic and food spoilage bacteria in various foods [5].

IV. CONCLUSION

To improve the microbial safety, lactic acid and bacteriocin can be applied in E-san sausage. Those substances could inhibit and reduce yeast/mold and *S. aureus* in Esan sausage. Therefore, this research was beneficial for E-san sausage production for improving the microbial safety.

ACKNOWLEDGEMENTS

The authors would like to thank Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang for support the research financial.

Table 1 Effect of lactic acid solution and bacteriocin on number of lactic acid bacteria in E-san sausage

Time (day)	Number of Lactic acid bacteria (log cfu/g)					
	Con	2% L	4% L	2% B	4% B	2% L + 2% B
0	3.09 ± 1.48 ^{a,C}	3.01 ± 0.01 ^{a,E}	4.04 ± 0.01^{a,E}	4.03 ± 0.02 ^{a,E}	4.03 ± 0.00 ^{a,E}	2.45 ± 0.15 ^{a,B}
1	5.65 ± 0.42 ^{bc,B}	6.11 ± 0.01 ^{b,D}	7.02 ± 0.02 ^{a,D}	6.12 ± 0.01 ^{b,D}	7.05 ± 0.01 ^{a,D}	5.26 ± 0.85 ^{c,A}
2	6.52 ± 0.50 ^{b,AB}	7.11 ± 0.01 ^{ab,C}	8.08 ± 0.01^{a,C}	7.14 ± 0.01 ^{ab,C}	8.12 ± 0.01 ^{a,C}	5.35 ± 1.49 ^{c,A}
3	7.10 ± 0.02 ^{b,A}	8.09 ± 0.01 ^{a,B}	8.32 ± 0.00 ^{a,B}	8.10 ± 0.01 ^{a,B}	8.35 ± 0.01 ^{a,B}	5.99 ± 0.97 ^{c,A}
4	7.21 ± 0.12 ^{ab,A}	8.60 ± 0.42 ^{a,a}	8.36 ± 0.00^{a,A}	8.16 ± 0.12 $^{a.A}$	8.39 ± 0.01^{a,A}	6.13 ± 1.76 ^{b,A}

Note: L means Lactic acid solution B menas Bacteriocin

$^{a-c}$ values with different letters within the same row differ significantly (p<0.05)

$^{A-E}$ values with different letters within the same column differ significantly (p<0.05)

Con means E-san sausage no adding solution 2% B means E-san sausage added 2% bacteriocin

2% L means E-san sausage added 2% lactic acid solution 4% B means E-san sausage added 4% bacteriocin

4% L means E-san sausage added 4% lactic acid solution 2%L + 2% B means E-san sausage added 2% lactic acid combine with 2% bacteriocin

Table 2 Effect of lactic acid solution and bacteriocin on number of yeasts and molds in E-san sausage

Time (day)	Number of Yeasts and molds (log cfu/g)					
	Con	2% L	4% L	2% B	4% B	2% L + 2% B
0	2.61 ± 0.26 ^{ab,C}	2.63 ± 0.02 ^{ab,E}	2.89 ± 0.01^{c,E}	2.52 ± 0.04 ^{b,E}	2.54 ± 0.01 ^{b,D}	2.62 ± 0.25 ^{ab,B}
1	3.00 ± 0.18 ^{a,C}	2.88 ± 0.01 ^{ab,D}	3.07 ± 0.02 ^{a,D}	2.78 ± 0.06 ^{ab,D}	3.07 ± 0.01 ^{a,C}	2.46 ± 0.54 ^{b,B}
2	3.80 ± 0.30 ^{b,B}	4.01 ± 0.00 ^{b,C}	4.09 ± 0.01^{ab,C}	3.82 ± 0.28 ^{b,C}	4.08 ± 0.02 ^{ab,B}	4.40 ± 0.28 ^{a,A}
3	4.73 ± 0.06 ^{a,A}	4.08 ± 0.01 ^{bc,B}	4.14 ± 0.01 ^{b,B}	4.06 ± 0.01 ^{c,B}	4.12 ± 0.02 ^{bc,B}	4.09 ± 0.05 ^{bc,A}
4	4.78 ± 0.07 ^{a,A}	4.55 ± 0.03 ^{ab,A}	4.46 ± 0.03^{b,A}	4.62 ± 0.01 $^{ab.A}$	4.57 ± 0.06^{ab,A}	4.06 ± 0.32 ^{c,A}

Note: L means Lactic acid solution B menas Bacteriocin

$^{a-c}$ values with different letters within the same row differ significantly (p<0.05)

$^{A-E}$ values with different letters within the same column differ significantly (p<0.05)

Con means E-san sausage no adding solution 2% B means E-san sausage added 2% bacteriocin

2% L means E-san sausage added 2% lactic acid solution 4% B means E-san sausage added 4% bacteriocin

4% L means E-san sausage added 4% lactic acid solution 2%L + 2% B means E-san sausage added 2% lactic acid combine with 2% bacteriocin

Table 3 Effect of lactic acid solution and bacteriocin on number of *S. aureus* in E-san sausage

Time (day)	Number of *S. aureus* (log cfu/g)					
	Con	2% L	4% L	2% B	4% B	2% L + 2% B
0	$3.98 \pm 0.07^{a,A}$	$3.84 \pm 0.10^{a,A}$	$3.69 \pm 0.07^{a,A}$	$3.69 \pm 0.17^{a,A}$	$3.36 \pm 0.25^{b,A}$	$3.37 \pm 0.53^{b,A}$
1	$3.69 \pm 0.32^{a,A}$	$2.75 \pm 0.18^{a,B}$	$2.15 \pm 0.91^{b,B}$	$2.70 \pm 0.70^{ab,A}$	$2.48 \pm 0.65^{ab,A}$	$3.20 \pm 0.76^{ab,A}$
2	$2.79 \pm 0.52^{a,A}$	$1.95 \pm 0.67^{a,BC}$	$1.56 \pm 0.12^{a,B}$	$2.91 \pm 0.86^{a,A}$	$2.17 \pm 0.98^{a,A}$	$1.98 \pm 0.00^{a,AB}$
3	$3.17 \pm 1.83^{a,A}$	$1.00 \pm 0.00^{a,C}$	$1.55 \pm 0.44^{a,B}$	$2.60 \pm 1.20^{c,A}$	$2.71 \pm 1.53^{a,.A}$	$1.87 \pm 0.81^{a,B}$
4	$3.45 \pm 1.42^{a,A}$	$2.00 \pm 0.53^{a,BC}$	$1.37 \pm 0.18^{a,B}$	$2.95 \pm 2.55^{a,A}$	$3.08 \pm 1.14^{a,.A}$	$1.55 \pm 0.07^{a,B}$

Note: L means Lactic acid solution B menas Bacteriocin
$^{a-c}$ values with different letters within the same row differ significantly (p<0.05)
$^{A-E}$ values with different letters within the same column differ significantly (p<0.05)
Con means E-san sausage no adding solution
2% L means E-san sausage added 2% lactic acid solution
4% L means E-san sausage added 4% lactic acid solution
2% B means E-san sausage added 2% bacteriocin
4% B means E-san sausage added 4% bacteriocin
2%L + 2% B means E-san sausage added 2% lactic acid combine with 2% bacteriocin

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