

## BACTERIOCIN-LIKE ACTIVITY FROM *WEISSELLA CONFUSA* AND *PEDIOCOCCUS ACIDILACTICI* ISOLATED FROM TRADITIONAL THAI FERMENTED SAUSAGES

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### Summary

*Bacteriocin-like activity (BLA) was screened in 133 strains of lactic acid bacteria (LAB) isolated from traditional Thai fermented sausages. The inhibition assay against the test organisms showed that eight out of 133 isolates (CP1-15, CP2-11, CP3-1, CP7-3, CP10-3, CP11-6, CP14-1 and CP14-4) suppressed the growth of Bacillus cereus and four (CP1-15, CP7-3, CP14-2 and CP14-3) suppressed the growth of Staphylococcus aureus but none could suppress the growth of Escherichia coli and Salmonella sp. The isolates with the highest activity against the two Gram positive test strains (CP3-1 for B. cereus and CP7-3 for S. aureus) were further investigated for the effect of heat treatment at different pH values and for combined effects of dual mixtures. The increase in heating temperature (between 65 and 100 °C) and time (between 5 and 60 min) significantly decreased BLA, while the changes in pH (between 4 and 6) had little effect. When the filtrates of the two isolates were mixed, BLA seemed to be synergistic against each test strain. Regression equations were obtained by fitting the experimental percent inhibition data with second-order polynomial equations. The simulated data agreed well with the experimental data within 10 % when the filtrates were incubated at 65 °C for 5, 15, 30 and 60 min and at 80 °C for 5, 15 and 30 min. The isolates CP3-1 and CP7-3, which had an antagonistic effect against the two strains of Gram positive foodborne bacteria, were formerly identified as Weissella confusa and Pediococcus acidilactici, respectively. It is the researcher's belief that this is the first report on BLA from W. confusa against B. cereus.*

**Key words:** *lactic acid bacteria, bacteriocin, antibacterial activity, inhibitory activity, Weissella confusa, Pediococcus acidilactici*

### Introduction

"Sai-krork-prieo" is a traditional Thai fermented sausage, consumed all around the country and produced from minced pork, sliced pork/beef liver, garlic, pepper, spices, salt and sugar. "Mum" is a product similar to "Sai-krork-prieo" but can be made from beef as well

as pork, and is popular only in the Northeast of Thailand. In a previous study [9], lactic acid bacteria (LAB) from "Sai-krork-prieo" and "Mum" were isolated and characterized. It was found that *Lactobacillus plantarum* and *Pediococcus pentosaceus* were the species most frequent-

ly identified. On the other hand, *Weissella* sp., *P. acidilactici*, *L. fermentum*, *L. brevis*, *L. farciminis* and *L. sakei* were isolated from fewer samples. The study first reported the isolation of *W. cibaria/kimchii*, *W. confusa*, *L. brevis*, and *L. farciminis*, from "Sai-krork-prieo", and *P. pentosaceus*, *P. acidilactici*, *L. fermentum*, *L. brevis*, and *L. sakei* from "Mum".

Information on bacteriocin-producing LAB isolated from traditional Thai fermented sausages was rather limited. Some LAB isolated from tropical fermented sausages may produce new bacteriocins with properties suitable for adaptation to a tropical niche. This study

screened for bacteriocin-like activity (BLA) of 133 LAB strains previously isolated from "Sai-krork-prieo" and "Mum". Representative test organisms included *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* sp. These organisms are foodborne bacteria which are known to cause food poisoning worldwide [4]. In particular, the tropical climate is an optimum environment for cell multiplication leading to an awareness of food safety in the Southeast Asian region. The effect of heat treatment at different pH values and the combined effect of selected bacteriocin-like activities were also described.

## Materials and methods

### Bacterial strains and growth conditions.

One hundred and thirty-three LAB isolated from "Sai-krork-prieo" and "Mum" were grown on tryptic soy agar (without glucose) with 2.0 % yeast extract (TSAYE) slant at 37 °C for two days before use. The test organisms were *B. cereus* TISTR 037, *S. aureus* TISTR 029, *E. coli* TISTR 073 and *Salmonella* sp. isolated from chicken intestine. The test strains were cultured on nutrient agar (NA) slant at 37 °C for one day. All strains were maintained as frozen stocks at minus 20 °C in their medium supplement with 30 % glycerol.

**Cell suspension preparation.** Each one-day old test strain on NA slant was suspended in normal saline solution (NSS) to an OD<sub>600</sub> of 0.5 in order to obtain a viable cell number of 10<sup>8</sup> – 10<sup>9</sup> CFU/ml. Subsequently, the cell suspension was diluted to an initial cell number of 10<sup>5</sup> – 10<sup>6</sup> CFU/ml.

**Screening for BLA.** The screening method was modified according to the lawn method from Ohmomo et al. [8]. The LAB cultures from TSAYE slant were streaked on TSAYE plates (without glucose) and incubated under anaerobic conditions at 37 °C for one day. One ml of the suspended test bacteria was mixed with 15 ml of melted TSA at 50 °C and was overlaid on overnight-grown LAB plates. The plates were further incubated anaerobically at 37 °C for

1-2 days and were observed for inhibition zones in parallel with the overlay plate without LAB serving as a control.

**Broth dilution method.** The broth dilution method modified from Waterworth [14] was employed to investigate BLA in broth medium. Each LAB strain was grown anaerobically in tryptic soy broth (glucose excluded) supplemented with 2.0 % yeast extract (TSBYE) at 37 °C for 24 hours. The broth was centrifuged at 5000 rpm for 15 min and the supernatant was collected and filtered through a 0.45 µm of cellulose acetate membrane (no. 41334, Gelman). The filtrate was diluted with Mueller Hinton broth (MHB) to a concentration of 1/2-, 1/4-, 1/8-, 1/16- and 1/32 fold of an undiluted filtrate. One volume of each test bacterial suspension, prepared as described above, was added to an equal volume of each undiluted and diluted filtrate and the mixtures were incubated at 37 °C for 24 hours. Optical density at 600 nm of each mixture was measured and compared with a control (the test bacterial suspension diluted by half with MHB) and the differences were reported as a percent inhibition according to equation (1) [1, 6]. One bacteriocin unit (B.U.) was defined as the amount of bacteriocin that inhibited growth of the test microorganism by 50 %, when compared with a control culture without bacteriocin [2].

$$\text{Percent Inhibition} = 100 \times (\text{OD}_{600, \text{control}} - \text{OD}_{600, \text{test}}) / \text{OD}_{600, \text{control}} \quad (1)$$

**Heat treatment at different pH values and BLA of dual mixtures.** Selected LAB strains were grown on TSBYE at 37 °C for two days. Cell-free supernatants were harvested and filtered through 0.45 µm cellulose acetate membranes. The filtrate was adjusted to pH 4, 5 and 6 and heated at 65, 80 and 100 °C for 5, 15, 30 and

60 min according to Yin et al. [15]. One volume of each test bacterial suspension was added to one volume of each filtrate and the mixtures were incubated at 37 °C for 24 hours. Optical density at 600 nm of each mixture was measured and percent inhibition was calculated. To investigate the combined effect of BLA against the test orga-

nisms, one volume of dual filtrate mixture (1:1 ratio) was added to one volume of each test bacterial suspension and the final mixtures were incubated at 37 °C for 24 hours. Optical density at 600 nm of each mixture was measured and the percent inhibition was calculated.

**Statistical analysis and regression model.** Each treatment was repeated twice and the measurement of optical density of each sam-

ple was performed in triplicate. Average values were reported along with standard deviations. Tests of significant difference based on t-statistics were performed using MINITAB software (Minitab Inc., USA): non-significant difference if (probability;  $P > 0.05$ ) and a significant difference if ( $P < 0.05$ ). Data were computed with MINITAB software for correlation coefficients and regression models.

## Results and Discussion

### BLA of the isolates

The screening method modified according to the lawn method showed that 8 out of 133 (CP1-15, CP2-11, CP3-1, CP7-3, CP10-3, CP11-6, CP14-1 and CP14-4) suppressed the growth of *B. cereus* and 4 out of 133 isolates (CP1-15, CP7-3, CP14-2 and CP14-3) suppressed the growth of *S. aureus*. However, there was no antagonistic activity against *E. coli* and *Salmonella* sp. The isolate CP3-1 was previously identified as *Weissella confusa*, whereas CP7-3 was *Pediococcus acidilactici*, and CP1-15, CP2-11 and CP10-3 were *Lactobacillus plantarum* [9]. The use of tryptic soy media without glucose limited the effect of organic acids produced from glucose catabolism. The exclusion of the effect of organic acids produced from glucose catabolism was also ensured by the pH at the time of filtrate collection which was approximately 6.5.

The inhibition activities against *B. cereus* and *S. aureus* from the broth dilution test at various dilutions are shown in Fig. 1, A and B, respectively. There were 8 isolates with BLA

against *B. cereus*. The degree of inhibition was reduced more than twice when the filtrate was two-fold diluted. The isolate CP3-1 showed the highest BLA against *B. cereus* (94 %) at an undiluted concentration. Four isolates showed inhibitory activities against *S. aureus* and the isolate CP7-3 showed the highest inhibition (48 %). Although the activity against *S. aureus* at undiluted concentrations was less than 50 % inhibition, the inhibition activities could still be detected at the lowest concentration used in this study (diluted to 1/32 fold). Bacteriocin units at the highest concentration of the filtrate (1 ml filtrate/ml total volume) of each isolate are reported in Table 1. It was also noted that the filtrates from two isolates, CP1-15 and CP7-3, showed antibacterial activity against both *B. cereus* and *S. aureus* with different percentages.

From the literature, BLA found in *Lactobacillus* sp. and *Pediococcus* sp. are common [7, 11, 12] but those from *Weissella* sp. are rare. This study showed a clear evidence for the detection of BLA from *Weissella* sp.

Table 1. Bacteriocin units at the highest concentration of filtrate (1 ml filtrate/ml total volume) of each isolate.

Isolate	Bacteriocin Units	
	<i>B. cereus</i>	<i>S. aureus</i>
CP1-15	0.92	0.81
CP2-11	1.56	-
CP3-1	1.84	-
CP7-3	0.72	0.98
CP10-3	1.77	-
CP11-6	0.28	-
CP14-1	0.91	-
CP14-2	-	0.76
CP14-3	-	0.85
CP14-4	0.83	-

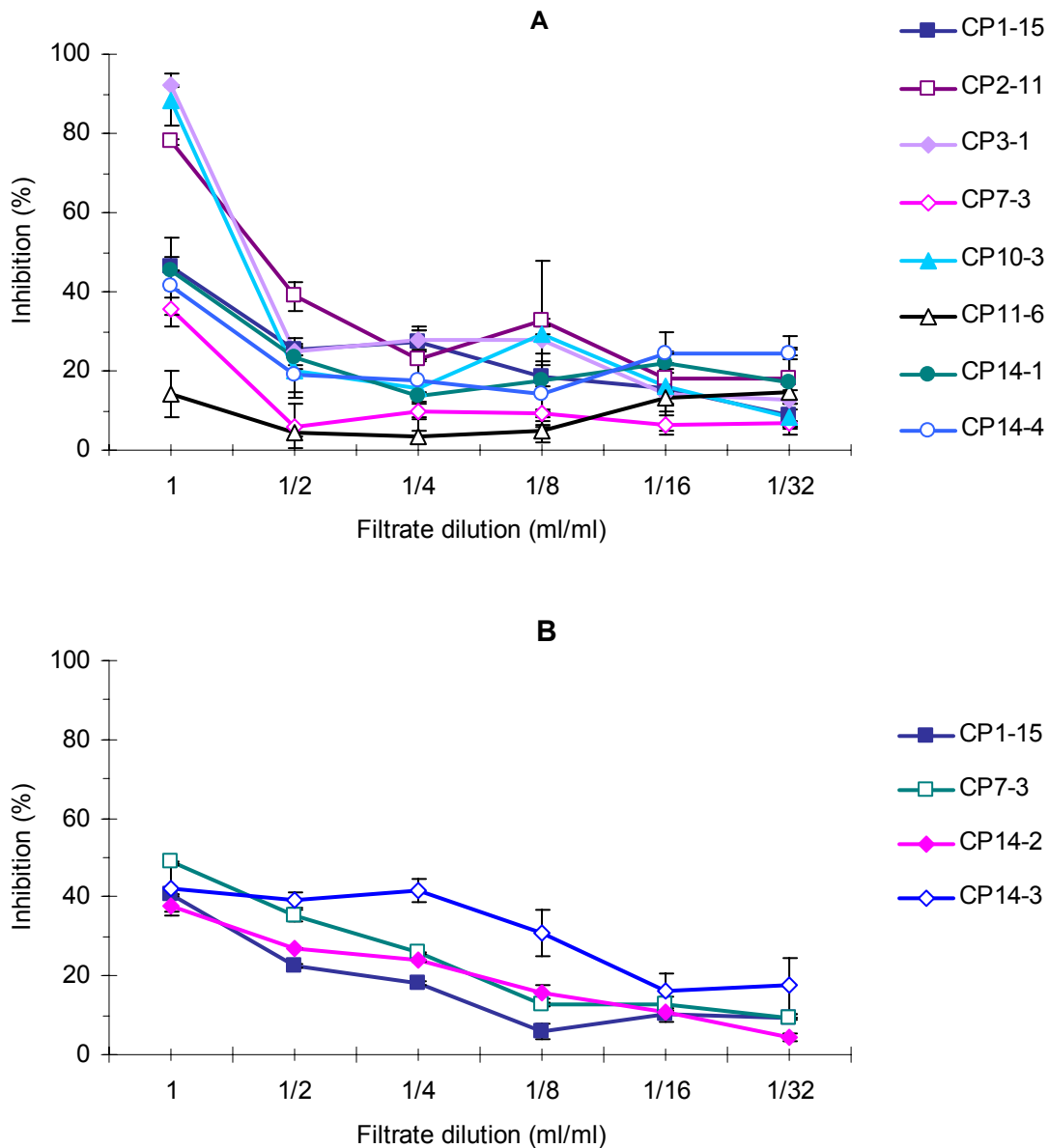


Fig. 1. Inhibitory activities in broth medium at various dilutions against *B. cereus* (A) and *S. aureus* (B).

### Effect of heat treatment and pH of medium

*W. confusa* CP3-1 with highest B.U. against *B. cereus* and *P. acidilactici* CP7-3 with highest B.U. against *S. aureus* were selected for further studies. Fig. 2 and Fig. 3 show the effect of heat treatment on the inhibition activity of CP3-1 filtrate against *B. cereus* and that of CP7-3 filtrate against *S. aureus* at various pH, respectively. The data shows that the alteration of pH of the medium had little effect on the inhibitory activity and the paired t-test analysis yielded ( $P > 0.05$ ). Although bacteriocins have been known for decades, only two are available commercially and only one, nisin, has been allowed for use in the food industry [3]. However, the function of nisin was reported to be depleted in a neutral pH environment [10, 13]. The BLA of CP7-3 and CP3-1 reported in this work has the potential for appli-

cation in a broader pH range since the inhibitory activities in acid and near neutral conditions were not significantly different.

In contrast to pH, heating temperature and time had significant effects on the antibacterial activity ( $P < 0.05$ ). The effect of heat treatment was more distinctive in the case of CP3-1 filtrate against *B. cereus*, i.e., the percent inhibition of CP3-1 filtrate dropped from 92 % to 65 % when the filtrate was incubated at 65 °C for five minutes, while that of CP7-3 filtrate dropped from 49 % to 46 %. For both cases of CP3-1 filtrate against *B. cereus* and CP7-3 filtrate against *S. aureus*, the effect of time became more apparent with increasing temperature, i.e., when heated for the same period, the percent inhibition dropped faster at 80 °C than at

65 °C and heat treatment at 100 °C for longer than five minutes completely destroyed BLA. The heating temperature of 65 °C and 80 °C reflect the temperature in the pasteurization

process applied in the food industry. This study suggests the tendency of where these bacteriocins could be exploited, provided that they must be used in conjunction with the other hurdles.

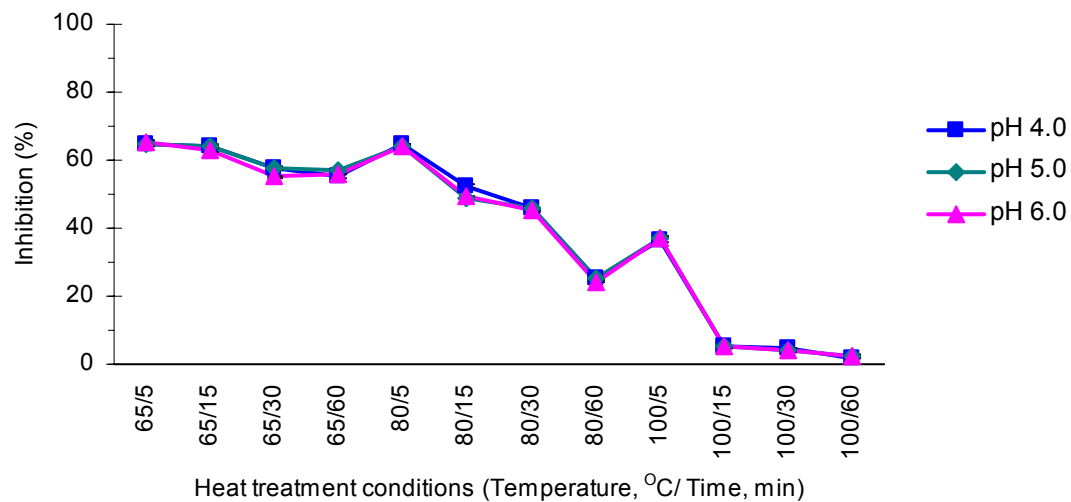


Fig. 2. Effect of the heat treatment on the inhibition activity of CP3-1 filtrate against *B. cereus* at pH 4, 5 and 6.

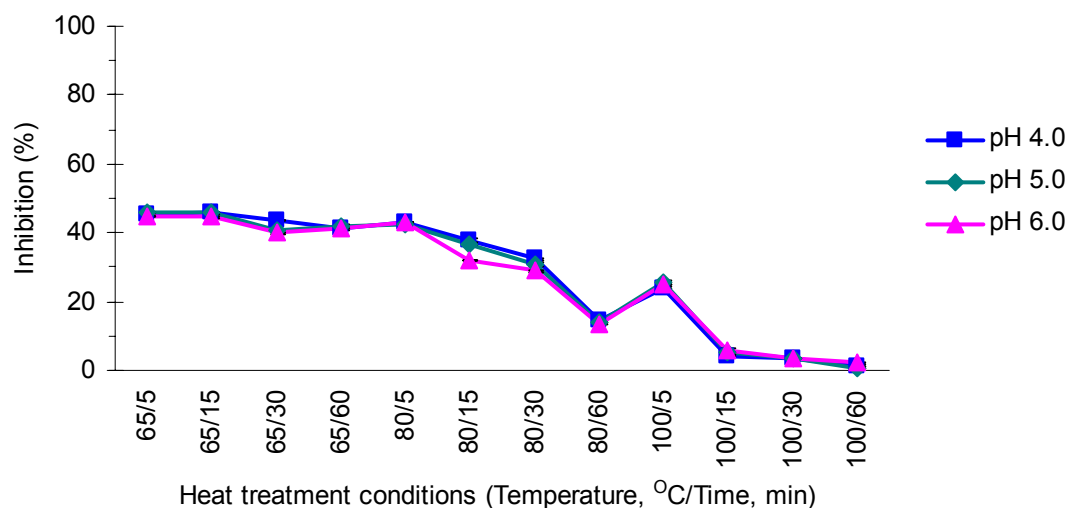


Fig. 3. Effect of the heat treatment on the inhibition activity of CP7-3 filtrate against *S. aureus* at pH 4, 5 and 6.

### Combined effects of dual filtrate mixtures

By adding one volume of dual filtrate mixture (1:1 ratio) to an equal volume of the test bacteria, the working concentration of each filtrate was reduced by half. However, the activity only slightly decreased from that of the undiluted filtrate of each individual strain (Fig. 4, A and B). The results in Fig. 1, A and B show that when the individual filtrate was diluted to half strength, the percent inhibition dropped from 94 % to 28 % in the case of CP3-1 filtrate against *B. cereus* and from 48 % to 36 % in the case of CP7-3 filtrate against *S. aureus*. This

suggested that the combined effect of the BLA was synergistic. In the literature, the combination of Pediocin AcH and nisin was reported to have a greater bactericidal effect than their individual use [5]. The food ecosystem in reality may have more than one food borne disease involved, thus bacteriocins that possess synergistic activity or one bacteriocin that have functional duality are desirable for bio-preservation in the food industry. These synergistic effects and the BLA of *W. confusa* prompt attention for further investigation.

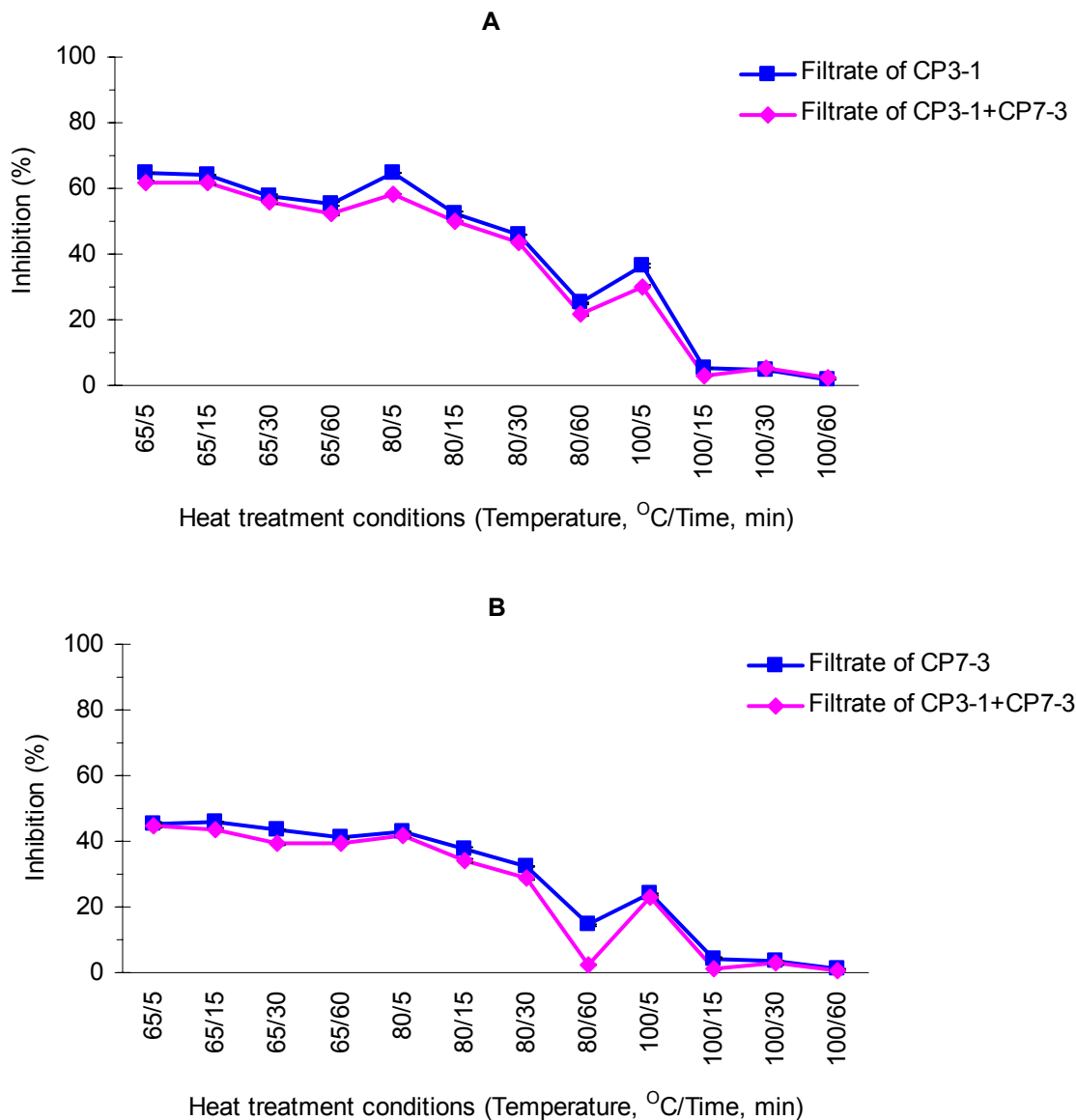


Fig. 4. Effect of the heat treatment on the inhibitory activities of: dual filtrate mixture and CP3-1 filtrate against *B. cereus* (A); dual filtrate mixture and CP7-3 filtrate against *S. aureus* (B).

### Regression equations predicting the effect of heat treatment

Using regression analysis, model equations for the prediction of the effect of heat treatment were given by Equations (2), (3), (4) and (5) for CP3-1 filtrate against *B. cereus*, CP7-3 filtrate

against *S. aureus*, the mixture of CP3-1 and CP7-3 filtrates against *B. cereus* and the mixture of CP3-1 and CP7-3 filtrates against *S. aureus*, respectively.

$$Y = -7.0 + 2.89T - 5.89 \times 10^{-1}t - 2.47 \times 10^{-2}T^2 + 1.07 \times 10^{-2}t^2 - 7.09 \times 10^{-3}Tt \quad (2)$$

$$Y = 40.0 + 8.90 \times 10^{-1}T - 1.50 \times 10^{-1}t - 1.07 \times 10^{-2}T^2 + 4.54 \times 10^{-2}t^2 - 7.09 \times 10^{-3}Tt \quad (3)$$

$$Y = 30.0 + 1.89T - 7.41 \times 10^{-1}t - 1.92 \times 10^{-2}T^2 + 6.93 \times 10^{-3}t^2 - 1.80 \times 10^{-5}Tt \quad (4)$$

$$Y = 120 - 1.09T - 5.79 \times 10^{-1}t + 1.10 \times 10^{-3}T^2 + 5.01 \times 10^{-3}t^2 - 1.80 \times 10^{-5}Tt \quad (5)$$

where Y is the output response (i.e. percent inhibition), T the temperature in °C and t the time in minutes.

Regression equations were obtained by fitting the experimental data with second-order polynomial equations. Fig. 5, A and B show the model prediction of heat treatment effect on antibacterial activity of CP3-1 filtrate against

*B. cereus* and of CP7-3 filtrate against *S. aureus*, respectively. Fig. 6, A and B show the model prediction of heat treatment effect on antibacterial activity of the dual filtrate mixture against *B. cereus* and *S. aureus*, respectively.

The simulated data agreed well with the experimental data within 10 % when the filtrates were incubated at 65 °C for 5, 15, 30 and 60 min and at 80 °C for 5, 15 and 30 min. The effect

of heat treatment at 80 °C for 60 min and at 100 °C for longer than five minutes was more severe than that predicted by the second-order polynomial equations.

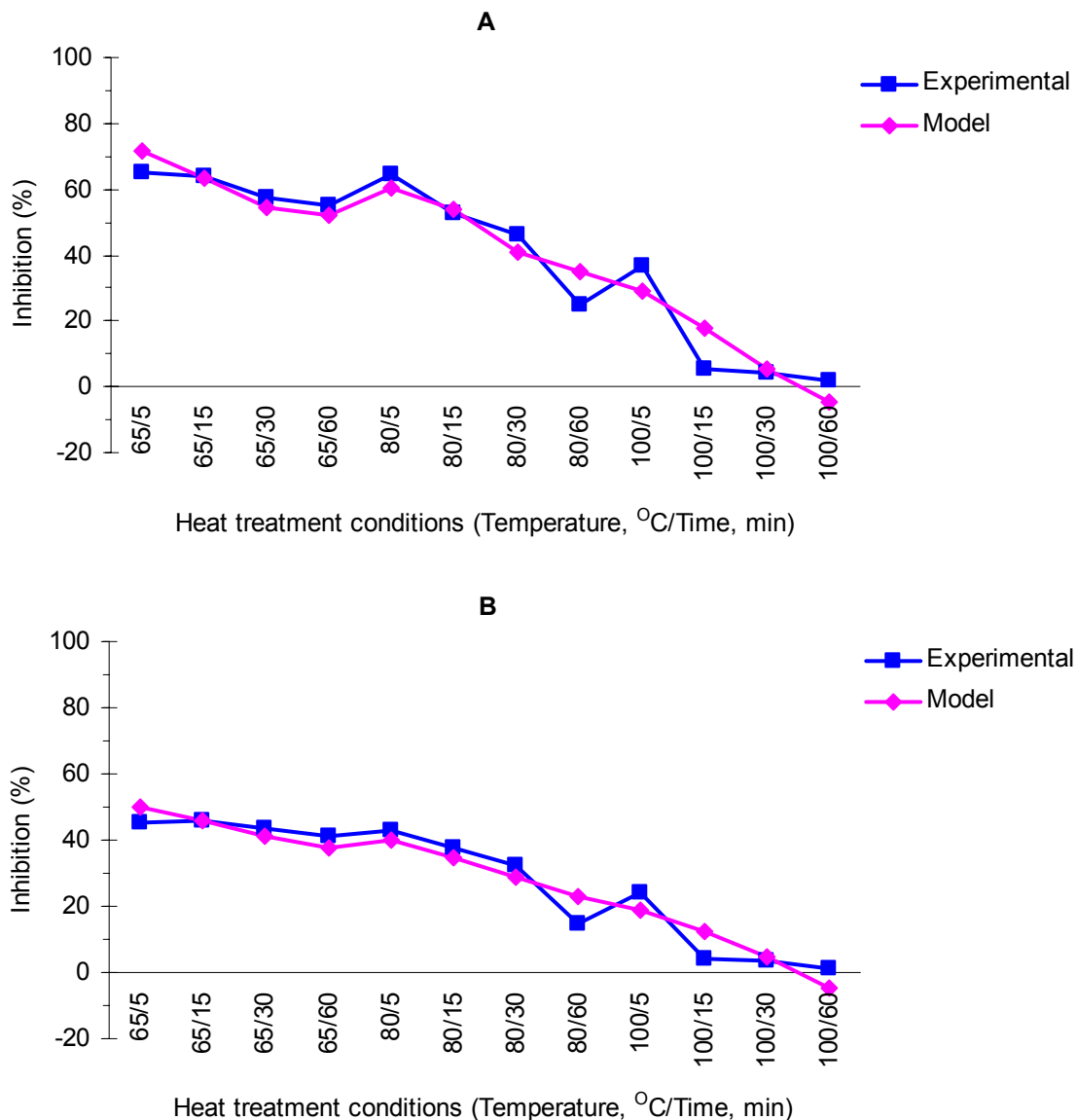


Fig. 5. Model prediction of the heat treatment effect on the antibacterial activity of CP3-1 filtrate against *B. cereus* (A) and CP7-3 filtrate against *S. aureus* (B).

## Conclusion

Bacteriocin-like activity was found in *Weissella confusa* CP3-1 providing a restriction to the growth of *B. cereus*. *Pediococcus acidilactici* CP7-3 also showed antagonistic effects against *S. aureus*. Bacteriocins produced from both strains can be applied in a wide pH range (4–6). Though their stability decreased by increasing the temperature, the activity was found to withstand a pasteurization processing temperature. As the food industry does not always employ purified bacteriocins due to the need of regulation approval, fermented ingre-

dients or bacteriocin-producing cultures become more appealing. Also, highlighted was the potential of exploiting the mixture of crude bacteriocins to gain greater effectiveness against two Gram positive food borne disease.

**Acknowledgement.** The study was supported by a Research Grant for New Scholars (RGNS) from the Commission on Higher Education and the Thailand Research Fund (TRF). Suggestions from mentor Assoc. Prof. Somboon Tanasupawat are gratefully acknowledged.

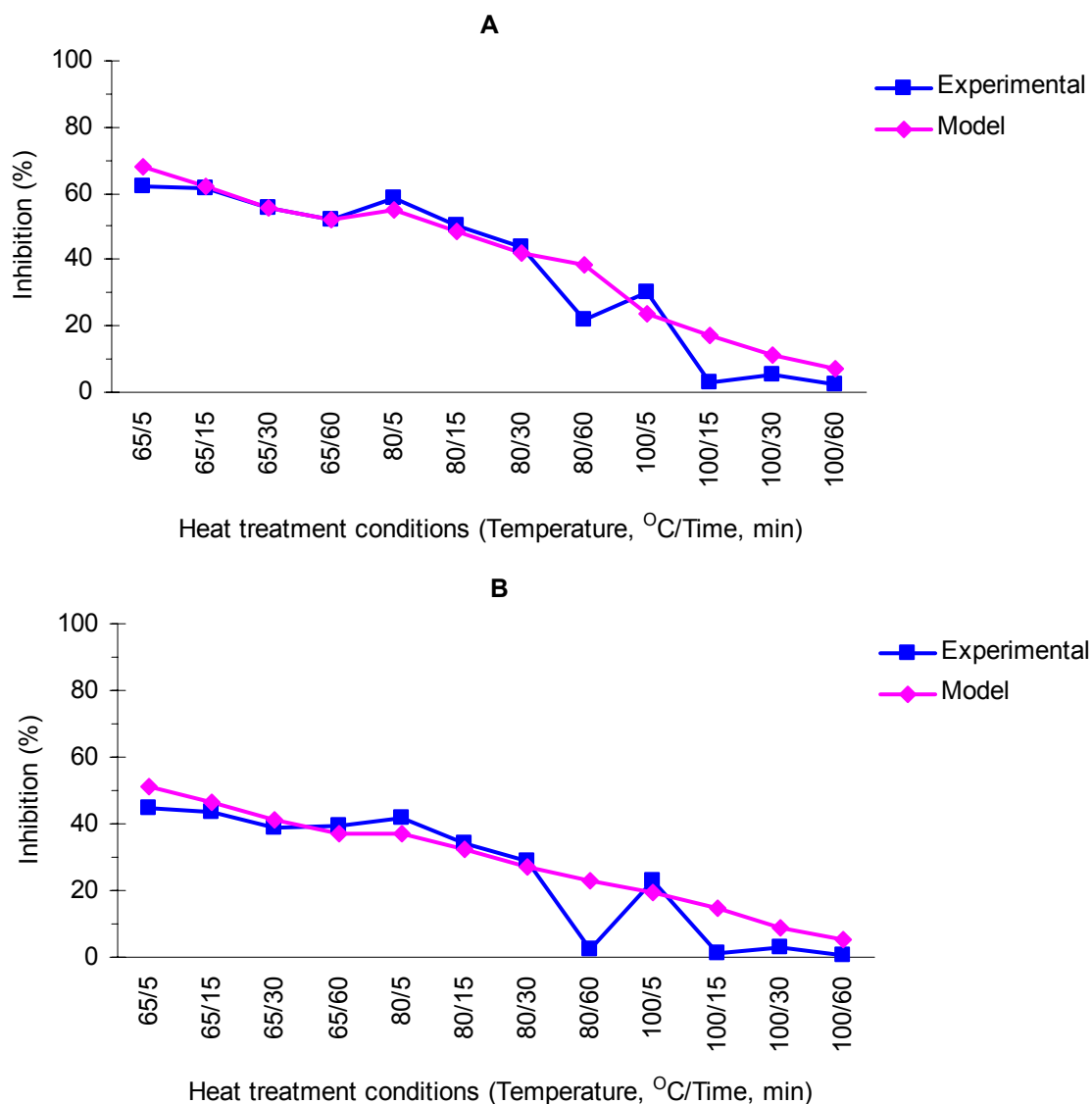


Fig. 6. Model prediction of the heat treatment effect on the antibacterial activity of the dual filtrate mixture against *B. cereus* (A) and *S. aureus* (B).

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# БАКТЕРИОЦИНОПОДОБНА АКТИВНОСТ ПРИ *WEISSELLA CONFUSA* И *PEDIOCOCCUS ACIDILACTICI*, ИЗОЛИРАНИ ОТ ТРАДИЦИОННИ ФЕРМЕНТИРАЛИ НАДЕНИЦИ ТНАІ

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## Резюме

133 щамa млечнокисели бактерии, изолирани от традиционни ферментирани наденици Thai, са скринирани за бактериоциноподобна активност (БПА). Изследването на инхибиращото действие срещу тест-организми показва, че осем от изолатите (CP1-15, CP2-11, CP3-1, CP7-3, CP10-3, CP11-6, CP14-1 и CP14-4) подтискат растежа на *Bacillus cereus*, а четири (CP1-15, CP7-3, CP14-2 и CP14-3) на *Staphylococcus aureus*, но нито един не подтиска *Escherichia coli* и *Salmonella* sp. Изолатите с най-висока активност срещу двата Грам-положителни тест-щамa (CP3-1 за *B. cereus* и CP7-3 за *S. aureus*) са изследвани за ефекта на топлинната обработка при различно рН и за комбинирано действие на двойните смеси. Повишаването на температурата на нагряване (между 65 и 100 °C) и удължаването на времето (между 5 и 60 мин) значително намаляват БПА, докато промените в рН (между 4 и 6) имат слаб ефект. При смесване на филтратите на двата изолата БПА показва синергизъм срещу всеки тест-щам. Получени са уравнения на регресия чрез екстраполиране на експерименталните данни за процента на инхибиране с полиномни уравнения от втори порядък. Прогнозираните данни съответстват добре на експерименталните в рамките на 10 % при инкубиране на филтратите при 65 °C за 5, 15, 30 и 60 мин, и при 80 °C за 5, 15 и 30 мин. Изолатите CP3-1 и CP7-3, които имат антагонистичен ефект срещу двата щамa Грам-положителни бактерии, са идентифицирани по-рано съответно като *Weissella confusa* и *Pediococcus acidilactici*. Авторите смятат, че това е първото съобщение за БПА от *W. confusa* срещу *B. cereus*.