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Factors influencing synergistic antimicrobial activity of thymol and nisin against *Shigella* spp. in sugarcane juice

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Abstract: Two food-grade antimicrobial substances, thymol and nisin were tested for their antimicrobial activity against 4 species of *Shigella* including *S. boydii*, *S. dysenteriae*, *S. flexneri* and *S. sonnei* inoculated in sugarcane juice at 4 °C. When used separately, only thymol, but not nisin, exhibited a dose-dependent and species-dependent inhibitory effect on the bacteria. When thymol and nisin were used together, the concentrations of thymol required for complete inhibition were decreased in all cases of the bacteria as the concentrations of nisin were raised, indicating the synergistic antimicrobial activity between both compounds. The effects of bacterial cell number and temperature on the antimicrobial activity of thymol and nisin against *S. sonnei* in sugarcane juice were investigated. The doses of both compounds required for complete inhibition were directly proportional to the number of bacterial cells in sugarcane juice. Furthermore, they inhibited the bacterium at 30 °C more efficiently than at 4 °C. The sensory evaluation showed that thymol- and nisin-treated sugarcane juice was acceptable to consumers. This study suggests that thymol and nisin can be used as food preservatives with the consideration of their dependence on bacteria species, number of contaminated bacterial cells and temperature.

Key words: nisin; *Shigella*; sugarcane juice; thymol.

Abbreviations: CFU, colony-forming unit.

Introduction

Shigella, the causative agent of shigellosis, is a genus of gram negative, facultative anaerobic, non-spore forming, non-motile, rod-shaped bacteria. The pathological importance of different *Shigella* species varies geographically. *Shigella flexneri* and *Shigella sonnei* are the most prevalent species found in developing and industrialized countries, respectively. *Shigella dysenteriae* is seen mostly in South Asia and sub-Saharan Africa, and *Shigella boydii* has been reported worldwide with about 4% of the total shigellosis cases (Kotloff et al. 1999). Shigellosis has been a major global health concern causing more than 100,000 deaths per year worldwide (Kotloff et al. 1999). *Shigella* infection may spread from person to person or via contaminated food or water. The pathogenic bacteria are generally found in the diarrheal stools of infected persons, while they are sick or up to 1 to 2 weeks afterwards. Symptoms of the disease include abdominal cramps, diarrhea that sometimes contains blood, nausea, vomiting and fever (Niyogi 2005; Sjolund Karlsson et al. 2013). Shigellosis not only affects health of people but also has an economic impact on individuals and nations. Substantial economic losses are documented annually due to clinical treatment costs and lost working hours.

Treatment of shigellosis relies mainly on antibiotics, such as norfloxacin, ciprofloxacin, ofloxacin, azithromycin and ceftriaxone (Bhattacharya & Sur 2003). However, this therapeutic approach is currently questionable. It can lead to bacteria developing drug resistance, which can be transferred to environmental and other human pathogenic bacteria (Swartz 1997; Gould 2000; Davidson & Harrison 2002; Soulsby 2005). Increasing prevalence of antimicrobial resistance in *Shigella* (Isenbarger et al. 2002; Niyogi 2007; Kuo et al. 2008) is also of concern because it limits treatment options. Since the treatment of shigellosis with antibiotics has been jeopardized and less effective, prevention of the *Shigella* contamination in food and beverage has gained increasing attention as an alternative approach to control the disease.

Some food preservation systems, such as heat treatments and addition of synthetic preservatives, can be used to prevent *Shigella* infection. However, these systems can have undesired effects and are contrary to food industries' and consumers' demands, who ask for additive-free, fresher, and more natural-tasting food products, while maintaining microbiological safety (Gould 1996). A food preservation system that might fulfill this demand is to use natural compounds generally recognized as safe (GRAS) to prevent *Shigella*

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contamination in food and beverage. Many substances produced by living organisms can be used for such purposes including thymol and nisin.

Thymol (2-isopropyl-5-methylphenol), a natural monoterpen phenol, is a major component of the essential oils of thyme and oregano. It has potent antimicrobial activity against a variety of gram positive and gram negative bacteria including *Escheirchia coli*, *Salmonella typhimurium*, *Staphylococcus areus*, *Listeria monocytogenes* and *Bacillus cereus* (Cosentino et al. 1999; Lambert et al. 2001; Burt 2004). However, the antimicrobial concentration of thymol when used in food and beverage may create undesired changes in their aroma and favor. Therefore, when thymol is used in food and beverage not previously associated with an herby or spicy flavor, its concentration must be kept as low as possible in order to produce the desired antimicrobial effect without such adverse effect.

Nisin is a bacteriocin produced by some strains of *Lactococcus lactis* subsp. *lactis*. It is a 34-amino-acid-long ribosomally synthesized and posttranslationally modified peptide containing five lanthionine rings (Gross & Morell 1971). It is currently a permitted preservative in many countries where it is used in a variety of food (Delves-Broughton 1990). Although nisin is active against a wide range of gram positive bacteria, it is not effective against gram negative bacteria (Delves-Broughton 1990). However, when used in combination with agents destabilizing the outer membrane, it can inhibit gram negative bacteria (Stevens et al. 1992).

Since the use of thymol in combination with nisin to inhibit gram negative bacteria in food and beverage has not been reported, it is of interest to find out whether they have such ability. Therefore, in this study, the inhibitory effect of thymol and nisin alone and combined against *Shigella* species artificially contaminated in sugarcane juice was investigated. Several factors that may influence the effect including bacterial species, bacterial cell number, and temperature were also evaluated.

Material and methods

Bacterial strain and culture conditions

All *Shigella* species used in this study including *S. boydii* DMST 28180, *S. dysenteriae* DMST 15111, *S. flexneri* DMST 4423 and *S. sonnei* DMST 561 were obtained from the Department of Medical Sciences, The Ministry of Public Health, Nonthaburi, Thailand. The identity of the bacterial strains was confirmed by using an API 20 E test kit (bioMerieux Industry, Hazelwood, MO, USA). All the bacteria used in this study were grown at 37 °C in brain heart infusion broth. Bacterial stock cultures were stored as frozen cultures at –80 °C in brain heart infusion broth containing 20% glycerol (v/v).

For the preparation of bacterial suspension to inoculate food samples, fresh overnight culture of each *Shigella* sp. in brain heart infusion broth was washed three times and re-suspended in sterile phosphate buffered saline. Viable cell count of the washed cell suspension was determined by serial dilution (1 : 10) phosphate buffered saline, spreading 0.1 mL of the suspension (in triplicate) on xylose lysine deoxycholate agar (Merck, Bangkok, Thailand), a selective medium for *Shigella*, and incubating the plates at 37 °C for 24 h. The plates with bacterial colonies in the range of 30 to 300 colonies were used to determine viable cell count.

Chemicals

Thymol and nisin were obtained from Himedia, Mumbai, India. The stock solution of thymol (1 M) was made in 95% ethanol and kept at 4 °C. The stock solution of nisin (1 mM) was made in 0.02 M HCl, sterilized through a 0.22-μm pore size filter membrane (Sartorius, Goettingen, Germany) and kept at –20 °C.

Sugarcane juice

Freshly made sugarcane juice with pH of 6.7 was obtained directly from a manufacturer in Ubon Ratchathani Province, Thailand. Upon arrival at the laboratory, the juice was filtered through a cheesecloth and was sterilized through 0.2 μm polyethersulfone membrane using a vacuum filtration unit (Sartorius, Goettingen, Germany). The sugarcane juice was stored in a refrigerator at 4 °C and used within 2 days of storage. Prior to the experiments at 30 °C, the juice was taken out from the refrigerator, and its temperature was adjusted to 30 °C by placing it in an incubator at 30 °C for 2 h.

Microbiological analysis

One mL of sugarcane juice samples was taken and subjected to serial (1 : 10) dilutions in phosphate buffered saline. Appropriate dilutions of the samples were spread on xylose lysine deoxycholate agar (in triplicate). The numbers of the bacterial cells grown on xylose lysine deoxycholate agar plates (having 30–300 colonies) were used to calculate the concentration of bacteria in the juice. Single samples from each duplicate batch of the juice were removed periodically during incubation for counting viable cells and plating out in triplicate; therefore, mean counts for each time point were calculated from six replicate determinations.

Antimicrobial activity of nisin and thymol againts Shigella sp. in sugarcane juice

Sugarcane juice was prepared in duplicate for each treatment. Ten mL of the juice was inoculated with each of the *Shigella* suspensions (prepared as mentioned above) to give an approximate count of 10^5 colony-forming unit (CFU)/mL. Thymol and nisin were added (alone or in combination) to the inoculated sugarcane juice to give final concentrations as shown in Table 1. The juices with the inoculated bacteria but without thymol and nisin were used as controls. Samples were taken for determination of viable cell counts after storing for 4 h at 4 °C. For each treatment, the possible contamination during the experiment was assessed from the juice having no inoculated bacteria, thymol and nisin.

Influence of bacterial cell number on antimicrobial activity of thymol and nisin

Sugarcane juice (in duplicate) was inoculated with *S. sonnei* DMST 561 to obtain the final concentration of 10^3, 10^5, or 10^7 CFU/mL. To the treated juice, thymol (0.75 mM) and nisin (0.5 μM) were added simultaneously. Controls consisted of the juice inoculated with the bacterial strain without thymol and nisin. Inoculated juice was stored at 4 °C and enumerated for *S. sonnei* DMST 561 at the time of bacterial inoculation and every 30 min for 4 h during the storage period. For each treatment, the possible contamination during the experiment was assessed as mentioned above.

Table 1. Viable count at 4 °C (mean ± SD) (log CFU/mL) of *Shigella* species in sugarcane juice after addition of different combinations of thymol and nisin for 4 h.[a]

Shigella spp.	Concentration of thymol (mM)	Concentration of nisin (μM)			
		0	0.1	0.25	0.5
S. boydii	0	5.09 ± 0.06	5.11 ± 0.05	5.09 ± 0.03	5.08 ± 0.09
	0.25	4.02 ± 0.02	3.22 ± 0.02	2.67 ± 0.08	2.01 ± 0.05
	0.5	3.54 ± 0.04	2.70 ± 0.06	1.42 ± 0.02	0.89 ± 0.04
	0.75	2.78 ± 0.07	1.36 ± 0.03	nd	nd
S. dysenteriae	0	5.10 ± 0.03	5.09 ± 0.03	5.08 ± 0.06	5.10 ± 0.07
	0.25	3.52 ± 0.02	2.88 ± 0.05	2.01 ± 0.01	1.41 ± 0.03
	0.5	2.54 ± 0.04	1.41 ± 0.04	nd	nd
	0.75	2.11 ± 0.06	0.97 ± 0.03	nd	nd
S. flexneri	0	5.10 ± 0.02	5.09 ± 0.04	5.09 ± 0.06	5.09 ± 0.07
	0.25	4.13 ± 0.05	3.31 ± 0.07	2.98 ± 0.05	1.99 ± 0.06
	0.5	3.01 ± 0.01	2.77 ± 0.04	1.80 ± 0.03	1.16 ± 0.07
	0.75	2.53 ± 0.04	1.11 ± 0.03	nd	nd
S. sonnei	0	5.09 ± 0.09	5.11 ± 0.04	5.10 ± 0.07	5.10 ± 0.06
	0.25	4.54 ± 0.07	4.13 ± 0.08	3.66 ± 0.03	2.90 ± 0.04
	0.5	4.02 ± 0.07	3.22 ± 0.08	2.40 ± 0.02	1.26 ± 0.02
	0.75	3.51 ± 0.03	2.78 ± 0.09	1.34 ± 0.05	nd

[a] nd: no detectable viable cell count.

Table 2. Viable count (mean ± SD) (log CFU/mL) of *S. sonnei* DMST 561 in sugarcane juice containing 10^7 CFU/mL of the bacterium after treatment with different combinations of thymol and nisin for 4 h at 4 °C.[a]

Concentration of thymol (mM)	Concentration of nisin (μM)		
	0.5	0.75	1.0
0.75	2.52 ± 0.03	1.68 ± 0.07	1.03 ± 0.03
1.0	2.04 ± 0.04	1.25 ± 0.08	nd
1.25	1.47 ± 0.02	0.84 ± 0.05	nd

[a] nd: no detectable viable cell count.

To determine the concentrations of thymol and nisin required for complete inhinbition of 10^7 CFU/mL of *S. sonnei* DMST 561 in sugarcane juice at 4 °C, different combinations of both compounds were added to the *S. sonnei* DMST 561 inoculated sugarcane juice (in duplicate) to give final concentrations as shown in Table 2. Viable counts were determined in samples taken after 4 h of storage.

Influence of temperature on antimicrobail activity of thymol and nisin

Sugarcane juice was prepared to contain 0.75 mM of thymol, 0.5 μM of nisin and 10^5 CFU/mL of *S. sonnei* DMST 561. The juice inoculated with the same amount of the bacterial strain, but not with thymol and nisin, was used as a control. Two sets of the treated and control juices were prepared for storage at 2 different temperatures, 4 °C and 30 °C. Viability of the bacterial strain was examined in the samples taken from each group at the time of bacterial inoculation and every 30 min for 4 h post-inoculation. This experiment was performed in duplicate. For each treatment, the possible contamination during the experiment was assessed as mentioned above.

To determine the lowest concentrations of thymol and nisin resulting in complete inhibition of 10^5 CFU/mL of *S. sonnei* DMST 561 after 2 h of treatment at 30 °C, thymol and nisin were added to the *S. sonnei* DMST 561 inoculated sugarcane juice (in duplicate) to give final concentrations as shown in Table 3. Samples were taken for determination of viable cell counts after storing for 2 h.

Sensory evaluation

Sugarcane juice was treated with thymol and nisin at respective concentrations of 1.0 μM and 1.0 mM. For control, no thymol and nisin were added in the sugarcane juice. The treated and control juices were stored for 4 h at 4 °C prior to evaluation. To determine whether the addition of thymol and nisin to sugarcane juice influences the consumer liking of the juice, 20 panelists evaluated the juice on 9-point hedonic scale (ranging from 1 = dislike extremely to 9 = like extremely) for overall liking of the food and liking of the appearance, aroma and flavor. The FIZZ sensory analysis software (Biosystems, Couternon, France) was used for data analysis.

Statistical analysis

The data expressed as mean ± SD were analyzed by ANOVA with the SPSS Win program, version 9.0 (SPSS, Chicago, IL, USA). The significance of differences between means was assessed by Tukey's test based on a 5% significance level ($p < 0.05$), using the same program.

Table 3. Viable count (mean ± SD) (log CFU/mL) of *S. sonnei* DMST 561 in sugarcane juice containing 10^5 CFU/mL of the bacterium after treatment with different combinations of thymol and nisin for 2 h at 30 ℃.[a]

Concentration of thymol (mM)	Concentration of nisin (μM)			
	0	0.1	0.25	0.5
0	5.05 ± 0.10	5.05 ± 0.06	5.04 ± 0.07	5.05 ± 0.03
0.25	3.92 ± 0.04	3.25 ± 0.10	2.51 ± 0.04	1.83 ± 0.06
0.5	3.16 ± 0.05	2.63 ± 0.03	1.97 ± 0.05	nd
0.75	2.65 ± 0.09	2.05 ± 0.07	1.03 ± 0.03	nd

[a] nd: no detectable viable cell count.

Results

Antimicrobial activity of nisin and thymol against Shigella in sugarcane juice

Thymol and nisin were tested for their antimicrobial activity against four *Shigella* species in sugarcane juice at 4 ℃ by determining the viable cell counts 4 h after treatment. When thymol and nisin were not applied in the sugarcane juice (control group), total viable cell counts of all tested *Shigella* species increased from 5.0 log CFU/mL to 5.1 log CFU/mL (Table 1) indicating the concordance in survival and growth ability of the bacteria in the juice at 4 ℃. Application of thymol alone showed a dose-dependent reduction of the viable counts of all four species of *Shigella*, though with different degree (Table 1). On the other hand, the results obtained from the application of nisin independently were similar to those of control group (Table 1) indicating the inhibitory incompetence of nisin towards all *Shigella* species. Although nisin lacked antimicrobial activity against *Shigella* species, it was found to be able to enhance the inhibitory effect of thymol. Regardless of *Shigella* species, the antimicrobial activity of thymol increased as the concentration of nisin increased. Of all studied *Shigella* species, *S. dysenteriae* DMST 15111 was the most sensitive strain to thymol and nisin, whereas *S. sonnei* DMST 561 was the least sensitive one. The lowest concentrations of thymol and nisin that resulted in complete inhibition (no detectable viable cell counts) of *S. dysenteriae* DMST 15111 were 0.5 mM and 0.25 μM, respectively, whereas those of *S. sonnei* DMST 561 were 0.75 mM and 0.5 μM, respectively (Table 1). Since *S. sonnei* DMST 561 was the most tolerant species in this study, it was selected for further experiments to analyze the effects of bacterial cell number and temperature on antimicrobial activity of thymol and nisin.

Influence of bacterial cell number on antimicrobial activity of thymol and nisin

To study the effect of bacterial cell number on the antimicrobial activity of thymol and nisin against *S. sonnei* DMST 561 in sugarcane juice at 4 ℃, experiments were conducted in the juice, containing different bacterial concentrations including 10^3, 10^5, and 10^7 CFU/mL. When 0.75 mM of thymol and 0.5 μM of nisin were used simultaneously, the complete inhibition was observed in the juice containing 10^3 and 10^5 CFU/mL of *S. sonnei* DMST 561 at different time, at 1 h (for 10^3 CFU/mL) and at 3 h (for 10^5 CFU/mL) (Fig. 1). On the other hand, 2.52 log CFU/mL of *S. sonnei* DMST 561 was detected in the juice having the initial bacterial concentration of 10^7 CFU/mL after 4 h observation period. However, in this circumstance the complete inhibition of *S. sonnei* DMST 561 could be obtained by increasing the concentrations of thymol and nisin. The lowest treatment doses of thymol and nisin causing the result were 1.0 mM and 1.0 μM, respectively (Table 2).

Influence of temperature on antimicrobail activity of thymol and nisin

Effect of temperature on the ability of thymol and nisin to inhibit the growth of *S. sonnei* DMST 561 in sugarcane juice was examined at 4 ℃ and 30 ℃. In the control groups (without thymol and nisin), *S. sonnei* DMST 561 survived in the juice throughout the 4 h period of observation at both temperatures (Fig. 2). In the treatment groups (with thymol and nisin), *S. sonnei* DMST 561 reacted to thymol (0.75 mM) and nisin (0.5 μM) differently depending on the temperature. The reduction of the bacterial cells to the undetectable level was observed at 4 ℃ and 30 ℃ at different time, at 3 h (for 4 ℃) and 2 h (for 30 ℃) after treatment (Fig. 2). Furthermore, by varying the concentrations of thymol and nisin in the sugarcane juice at 30 ℃, it was found that to obtain the undetectable viable count within 2 h after treatment the concentration of thymol could be lowered from 0.75 mM to 0.5 mM (Table 3).

Sensory evaluation

Effect of the addition of thymol (1.0 mM) and nisin (1.0 μM) in sugarcane juice on overall liking, appearance, aroma and flavor was investigated. Results of analysis of variance showed that there were no significant differences ($p < 0.05$) among the treatment group (with thymol and nisin) and control group (without thymol and nisin) for hedonic scores for overall liking, appearance, aroma and flavor. The means of all attributes fell between like moderately (score = 7) and like very much (score = 8) on the hedonic scale (Table 4). These results suggest that the addition of thymol and nisin to sugarcane juice would be acceptable to consumers.

Discussion

Nisin is a peptide exhibiting a broad spectrum of antimicrobial activity against many food-borne pathoge-

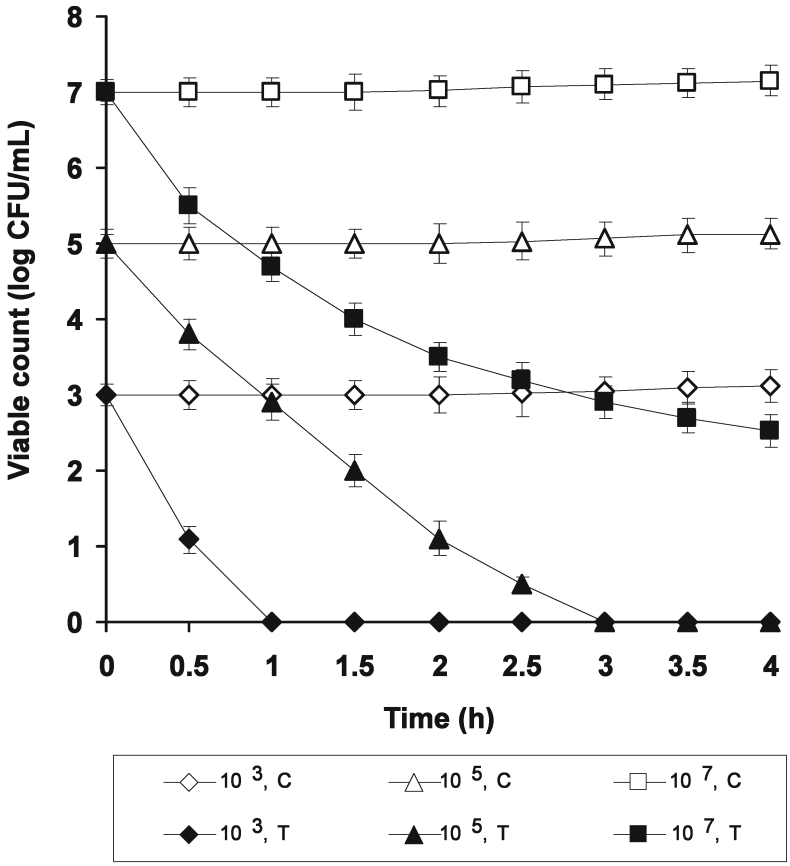

Fig. 1. Effect of bacterial cell number on antimicrobial activity of thymol and nisin against *S. sonnei* DMST 561. In the treatment groups (T), different concentrations (CFU/mL) of *S. sonnei* were inoculated into sugarcane juice with thymol (0.75 mM) and nisin (0.5 μM). In control groups (C), different concentrations (CFU/mL) of *S. sonnei* were inoculated into sugarcane juice with no thymol and nisin.

Table 4. Mean sensory scores for sugarcane juice without thymol and nisin (C) and with thymol and nisin (T).

Attribute	C	T
Overall liking	7.3[a]	7.2[a]
Appearance	7.2[a]	7.3[a]
Aroma	7.2[a]	7.3[a]
Flavor	7.6[a]	7.5[a]

[a] Means with the same row with the same letter are not significantly different ($p < 0.05$).

nic bacteria. The safety and antimicrobial efficacy of nisin have resulted in its widespread use as a bacterial biological control agent in food throughout the world. The bacterial cytoplasmic membrane is the site where nisin activity is believed to occur, and loss of cell viability may involve interaction of the dehydroalanine residues in nisin with membrane sulfhydryl groups (Morris et al. 1984; Klaenhammer 1988). Cellular damage caused by nisin can range from the disruption of proton motive force to loss of membrane integrity (Kordel & Sahl 1986; Liu & Hansen 1990). However, in normal circumstances, gram negative bacteria are resistant to nisin mainly due to the impermeable outer membrane consisting of substantial amounts of proteins, phospholipids and lipopolysaccharides. The outer membrane acts as a barrier to the action of nisin on cytoplasmic membrane (Stevens et al. 1992). Several works have presented that nisin can be effective against gram negative bacteria if used in combination with physical or chemical treatments destabilizing the outer membrane (Stevens et al. 1992; Cotter et al. 2005). In this study, thymol, a food-grade antimicrobial substance, was used as an outer membrane destabilizing agent together with nisin to inhibit *Shigella*, one of the leading bacterial diarrhea causes worldwide. This paper, in our best knowledge, is the first report presenting the effectiveness of using thymol and nisin to inhibit gram negative bacteria in a food model.

Thymol and nisin when used alone as antimicrobial substances in sugarcane juice gave different results. Nisin did not inhibit all *Shigella* species used in this study. This result is in agreement with several previous reports showing the unsuccessful use of nisin in inhibiting gram negative bacteria (Delves-Broughton 1990; Rattanachaikunsopon & Phumkhachorn 2010a). On the other hand, thymol had species-dependent inhibitory effect on *Shigella*. Thymol exerts a broad spectrum of antimicrobial activity against both gram positive and gram negative bacteria. Many reports have described its antimicrobial activity against food-borne pathogens including *Bacillus cereus*, *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Salmonella typhimurium* and

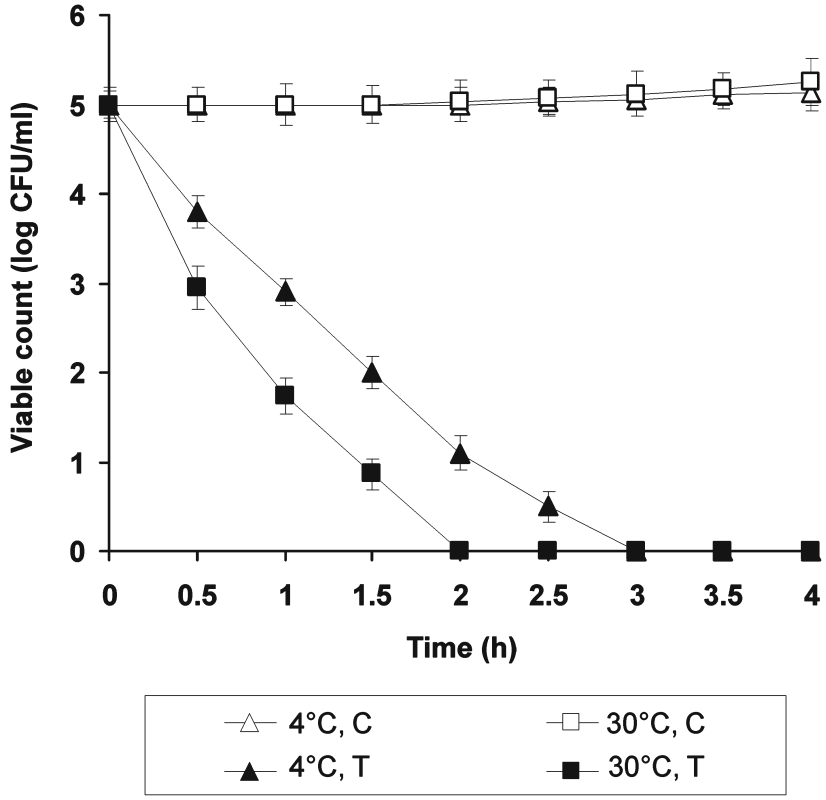

Fig. 2. Effect of temperature on antimicrobial activity of thymol and nisin against *S. sonnei* DMST 561. In the treatment groups (T), thymol (0.75 mM) and nisin (0.5 μM) were added into sugarcane juice with 10^5 CFU/mL of *S. sonnei* at different temperatures (4 °C and 30 °C). In control groups (C), no thymol and nisin were added into sugarcane juice with 10^5 CFU/mL of *S. sonnei* at different temperatures (4 °C and 30 °C).

Listeria monocytogenes (Burt 2004; Cowan 1999; Ettayebi et al. 2000; Xu et al. 2008; Hyldgaard et al. 2012). Its potential to inhibit bacteria was also reported in many foods, such as alfalfa seeds (Weissinger et al. 2001), sprouts (Weissinger et al. 2001) and carp fillets (Mahmoud et al. 2006). The action of thymol on the outer membrane of gram negative bacteria results in the release of the lipopolysaccharides with the consequent cell membrane permeability increase and ATP loss. Thymol can also cause cell death by damaging the cytoplasmic membrane, which leads to the collapse of the proton motive force (Hyldgaard et al. 2012).

This paper describes a synergistic antimicrobial effect between thymol and nisin on *Shigella* species in sugarcane juice. The effect is illustrated in Table 1, which demonstrates that thymol enabled nisin to exhibit antimicrobial activity against *Shigella*, and nisin in turn enhanced the antimicrobial activity of thymol against *Shigella* indicated by the decrease of thymol concentration required to obtain complete inhibition of *Shigella* in sugarcane juice when used in combination with nisin. Although the actual mechanism for the synergism between thymol and nisin is still unknown, it could be explained on the basis of thymol-induced destabilization of bacterial outer membrane structure resulting in an increased permeability for nisin (Ettayebi et al. 2000). In combination treatment, therefore, intracellular nisin concentration would be higher leading to increased cell death. The synergistic effect of thymol and nisin would also mean that effective concentration of thymol could be lowered considerably to achieve the desired antimicrobial activity, thus avoiding irritation, allergenicity and organoleptic impact on food possibly caused by high concentration of thymol (Burt 2004).

This study found that bacterial species influenced the synergistic antimicrobial effect between thymol and nisin on *Shigella* inoculated in sugarcane juice. The difference in sensitivity between bacterial species to the antimicrobials tested is in agreement with previous findings. For example, *Bacillus cereus* and *Bacillus subtilis* had a different degree of sensitivity to *Echinophora platyloba* DC oil and nisin (Saei-Dehkordi et al. 2012); *Salmonella enterica* and *Salmonella typhimurium* exhibited a different degree of sensitivity to *Lippia multiflora* oil and *Mentha* × *piperita* oil (Bassole et al. 2010); and *Staphylococcus aureus* and *Staphylococcus albus* possessed a different degree of sensitivity to oregano oil (Kivanc & Akglul 1986). Our findings suggest that species variation is an important factor to take into account when thymol and nisin are applied together in food.

The synergistic antimicrobial activity of thymol and nisin against *S. sonnei* DMST 561 in sugarcane juice was found to be dependent on the number of bacterial cells present in the juice. The concentrations of thymol and nisin required for complete inhibition of the bacterium was directly proportional to the number

of the bacterial cells in sugarcane juice. Similar results were also observed in our previous works using nisin and ρ-cymene to inhibit 10^3 and 10^6 CFU/g of *S. typhi* on pork sausage (Rattanachaikunsopon & Phumkhachorn 2010a) and carvacrol and cymene to inhibit 10^3, 10^5 and 10^7 CFU/mL of *Vibrio cholerae* in carrot juice (Rattanachaikunsopon & Phumkhachorn 2010b).

Temperature was shown to be another factor influencing antimicrobial activity of thymol and nisin against *S. sonnei* DMST 561. The sensitivity of the bacterium to both compounds decreased as the temperature lowered from 30 ℃ to 4 ℃. A similar result was also reported in our previous papers demonstrating that *S. typhi* was less sensitive to nisin and ρ-cymene at 4 ℃ than at 37 ℃ (Rattanachaikunsopon & Phumkhachorn 2010a) and that *V. cholerae* was less sensitive to carvacrol and cymene at 4 ℃ than at 25 ℃ (Rattanachaikunsopon & Phumkhachorn 2010b). Although an actual cause of our observation is not revealed, it is possible that low temperature may change the properties and fluidity of the bacterial cytoplasmic membrane so that the sensitivity of *S. sonnei* DMST 561 to thymol and nisin is decreased. In addition, low temperature may retard the synthesis of target sites of the compounds, which would lower the number of target sites in the cytoplasmic membrane and the sensitivity of the bacterium to the compounds (Periago & Moezelaar 2001). Further investigation is required to elucidate the relationship between temperature and the antimicrobial activity of thymol and nisin.

The concentrations of thymol and nisin added into sugarcane juice used for the sensory evaluation were the concentrations required for complete inhibition at 4 ℃ of the most resistant *Shigella* species (*S. sonnei* DMST 561) with concentration of 10^7 CFU/mL in the juice. The result showed that the treated juice was acceptable to consumers based on appearance, aroma and flavor of the juice. No irritation and allergic reaction was noticed in all of the consumers (data not shown). Furthermore, the concentration of thymol used in this test was below 3% (or about 0.2 M) which is the concentration proposed for use in cosmetics, drug absorption by animal tissues, food preservation and antifungal treatments in plants (Manou et al. 1998; Ettayebi et al. 2000).

Based on its antimicrobial activity against *Shigella* species in sugarcane juice and acceptance by consumers when applied in the juice, thymol and nisin are potential candidates for food preservatives. Since their antimicrobial activity was influenced by several factors including species of bacteria, contaminated bacterial cell number and temperature, these factors need to be taken into account when thymol and nisin are used as food preservatives.

References

Bassole I.H., Lamien-Meda A., Bayala B., Tirogo S., Franz C., Novak J., Nebie R.C. & Dicko M.H. 2010. Composition and antimicrobial activities of *Lippia multiflora* Moldenke, *Mentha* × *piperita* L. and *Ocimum basilicum* L. essential oils and their major monoterpene alcohols alone and in combination. Molecules **15:** 7825–7839.

Bhattacharya S.K. & Sur D. 2003. An evaluation of current shigellosis treatment. Expert Opin. Pharmacother. **4:** 1315–1320.

Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods – a review. Int. J. Food Microbiol. **94:** 223–253.

Cosentino S., Tuberoso C.I.G., Pisano B., Satta M., Mascia V., Arzedi E. & Palmas F. 1999. *In vitro* antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils. Lett. Appl. Microbiol.**29:** 130–135.

Cotter P.D., Hill C. & Ross R.P. 2005. Bacteriocins: developing innate immunity for food. Nat. Rev. Microbiol. **3:** 777–788.

Cowan M.M. 1999. Plant products as antimicrobial agents. Clin. Microbiol. Rev.**12:** 564–582.

Davidson P.M. & Harrison M.A. 2002. Resistance and adaptation to food antimicrobials and other process controls. Food Technol. **56:** 69–78.

Delves-Broughton J. 1990. Nisin and its uses as food preservative. Int. J. Dairy Technol. **43:** 73–76.

Ettayebi K., El Yamani J. & Rossi-Hassani B. 2000. Synergistic effects of nisin and thymol on antimicrobial activities in *Listeria monocytogenes* and *Bacillus subtilis*. FEMS Microbiol. Lett. **183:** 191–195.

Gould G.W. 1996. Industry perspectives on the use of natural antimicrobials and inhibitors for food applications. J. Food Prot. **59 (Supplement):** 82–86.

Gould I.M. 2000. A review of the role of antibiotic policies in the control of antibiotic resistance. J. Antimicrob. Chemother. **43:** 459–465.

Gross E. & Morell J.L. 1971. The structure of nisin. J. Am. Chem. Soc. **93:** 4634–4635.

Hyldgaard M., Mygind T. & Meyer R.L. 2012. Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. Front. Microbiol. **3:** 12.

Isenbarger D.W., Hoge C.W., Srijan A., Pitarangsi C., Vithayasai N., Bodhidatta L., Hickey K.W. & Cam P.D. 2002. Comparative antibiotic resistance of diarrheal pathogens from Vietnam and Thailand, 1996-1999. Emerg. Infect. Dis. **8:** 175–180.

Kivanc M. & Akgul A. 2006. Antibacterial activities of essential oils from Turkish spices and citrus. Flavour Frag. J. **1:** 175–179.

Klaenhammer T.R. 1988. Bacteriocins of lactic acid bacteria. Biochimie **70:** 337–349.

Kordel M. & Sahl H.G. 1986. Susceptibility of bacterial, eukaryotic and artificial membranes to the disruptive action of the cationic peptides Pep 5 and nisin. FEMS Microbiol. Lett. **34:** 139–144.

Kotloff K.L., Winickoff J.P., Ivanoff B., Clemens J.D., Swerdlow D.L., Sansonetti P.J., Adak G.K. & Levine M.M. 1999. Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. Bull. World Health Organ. **77:** 651–666.

Kuo C.Y., Su L.H., Perera J., Carlos C., Tan B.H. & Kumarasinghe G. 2008. Antimicrobial susceptibility of *Shigella* isolates in eight Asian countries, 2001-2004. J. Microbiol. Immunol. Infect. **41:** 107–111.

Lambert R.J.W., Skandamis P.N., Coote P. & Nychas G.J.E. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J. Appl. Microbiol. **91:** 453–462.

Liu W. & Hansen J.N. 1990. Some chemical and physical properties of nisin, a small-protein antibiotic produced by *Lactococcus lactis*. Appl. Environ. Microbiol. **56:** 2551–2558.

Mahmoud B.S., Yamazaki K., Miyashita K., Kawai Y., Shin I.S. & Suzuki T. 2006. Preservative effect of combined treatment with electrolyzed NaCl solutions and essential oil compounds on carp fillets during convectional air-drying. Int. J. Food Microbiol. **106:** 331–337.

Manou I., Bouillard L., Devleeschouwer M.J. & Barel A.O. 1998. Evaluation of the preservative properties of *Thymus vulgaris* essential oil in topically applied formulations under a challenge test. J. Appl. Microbiol. **84:** 368–376.

Morris S.L., Walsh R.C. & Hansen J.N. 1984. Identification and characterization of some bacterial membrane sulfhydryl groups which are targets of bacteriostatic and antibiotic action. J. Biol. Chem. **259:** 13590–13594.

Niyogi S.K. 2005. Shigellosis. J. Microbiol. **43:** 133–143.

Niyogi S.K. 2007. Increasing antimicrobial resistance – an emerging problem in the treatment of shigellosis. Clin. Microbiol. Infect. **13:** 1141–1143.

Periago P.M. & Moezelaar R. 2001. Combined effect of nisin and carvacrol at different pH and temperature levels on the viability of different strains of *Bacillus cereus*. Int. J. Food Microbiol. **68:** 141–148.

Rattanachaikunsopon P. & Phumkhachorn P. 2010a. Synergistic antimicrobial effect of nisin and p-cymene on *Salmonella enterica* serovar Typhi *in vitro* and on ready-to-eat food. Biosci. Biotechnol. Biochem. **74:** 520–524.

Rattanachaikunsopon P. & Phumkhachorn P. 2010b. Assessment of factors influencing antimicrobial activity of carvacrol and cymene against *Vibrio cholerae* in food. J. Biosci. Bioeng. **110:** 614–619.

Saei-Dehkordi S.S., Fallah A.A., Saei-Dehkordi S.S. & Kousha S. 2012. Chemical composition and antioxidative activity of *Echinophora platyloba* DC. essential oil, and its interaction with natural antimicrobials against food-borne pathogens and spoilage organisms. J. Food Sci. **77:** M631–M637.

Sjolund Karlsson M., Bowen A., Reporter R., Folster J.P., Grass J.E., Howie R.L., Taylor J. & Whichard J.M. 2013. Outbreak of infections caused by *Shigella sonnei* with reduced susceptibility to azithromycin in the United States. Antimicrob. Agents Chemother. **57:** 1559–1560.

Stevens K.A., Klapes N.A., Sheldon B.W. & Klaenhammer T.R. 1992. Antimicrobial action of nisin against *Salmonella typhimurium* lipopolysaccharide mutants. Appl. Environ. Microbiol. **58:** 1786–1788.

Soulsby E.J. 2005. Resistance to antimicrobials in humans and animals. Br. Med. J. **331:** 1219–1220.

Swartz M.N. 1997. Use of antimicrobial agents and drug resistance. N. Engl. J. Med. **337:** 491–492.

Weissinger W.R., McWatters K.H. & Beuchat L.R. 2001. Evaluation of volatile chemical treatments for lethality to *Salmonella* on alfalfa seeds and sprouts. J. Food Prot. **64:** 442–450.

Xu J., Zhou F., Ji B.P., Pei R.S. & Xu, N. 2008. The antibacterial mechanism of carvacrol and thymol against *Escherichia coli*. Lett. Appl. Microbiol. **47:** 174–179.

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