Synergistic Antimicrobial Effect of Nisin and $\beta$-Cymene on Salmonella enterica Serovar Typhi in Vitro and on Ready-to-Eat Food

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Foods contaminated with Salmonella enterica serovar Typhi are a major cause of typhoid fever, leading to public health problems and economic losses worldwide. Nisin and $\beta$-cymene were tested in this study for their antimicrobial activity against S. Typhi at 4°C and 37°C. Nisin and $\beta$-cymene, when used separately, did not inhibit the bacterium at either temperature. A synergistic antimicrobial effect between both compounds was observed when they were used simultaneously. This synergism was greater at 37°C than at 4°C. The lowest concentrations of nisin and $\beta$-cymene required for complete inhibition of S. Typhi at 37°C were 0.3 ppm and 1.5 ppm, respectively, and 0.3 ppm and 2.5 ppm at 4°C. The potential of nisin and $\beta$-cymene to control an S. Typhi population on ready-to-eat Thai-style pork sausage was also examined. The compounds were able to eliminate the contaminating bacterium with concentrations depending on the bacterial cell number on the food.

Key words: antimicrobial effect; $\beta$-cymene; nisin; Salmonella Typhi

Salmonella enterica serovar Typhi (hereafter as Salmonella Typhi), originally isolated in 1880 by Karl J. Erberth, is a gram-negative enteric bacillus belonging to the family Enterobacteriaceae. It is a non-spore forming, motile, facultative anaerobe. The bacterium is restricted to humans and does not have a reservoir in animals. Thus, the spread of the infection is from person to person, usually through contaminated food and drinking water. After infection, people can carry the organism for months or years, providing a continuing source from which others may become infected. S. Typhi is a causative agent of typhoid and enteric fever. This disease is characterized by the sudden onset of a sustained and systemic fever, severe headache, nausea, and loss of appetite. Other symptoms include diarrhea, enlargement of the spleen, and possible development of general malaise. In severe cases, there can be perforation of the intestinal wall. The mortality rate of untreated cases ranges from 12 to 30%, while that of treated cases is about 1%. Typhoid was an extremely common disease before the days of proper sewage disposal, water treatment, and food sanitation. Although its incidence in the developed countries has been declining, typhoid fever is still a frequent cause of death in parts of the world with poor sanitation.¹

Treatment of typhoid fever relies mainly on such antibiotics as chloramphenicol, ampicillin, trimethoprim, and ciprofloxacin.² This therapeutic approach is now considered to be unsafe. Prolonged and improper use of antibiotics can lead to bacteria developing drug resistance which can be transferred to the environment and other human pathogenic bacteria.³–⁶ Since 1989, multidrug-resistant strains of S. Typhi have been found to be responsible for numerous outbreaks in many countries in the Indian subcontinent, Southeast Asia, and Africa.²¹ Since the bacterial strains are resistant to many antibiotics, the treatment of typhoid fever based on antibiotics has been jeopardized. In addition, the emergence of multidrug-resistant strains of S. Typhi sends us a message that it is of importance to limit the use of antibiotics. One approach that may be useful for controlling S. Typhi infection is to use natural compounds generally recognized as safe (GRAS) to inhibit the bacterium from contaminating food and drinking water. Many substances produced by living organisms can be used for such purposes, including nisin produced by Lactococcus lactis and plant-originated $\beta$-cymene.

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.³ It is the only bacteriocin to have been accepted by the Food and Agriculture Organization/World Health Organization as a food preservative (accepted in 1969). Nisin is currently a permitted preservative in many countries where it is used in a variety of products.³ It is active against a wide range of gram-positive bacteria, although it is not effective against gram-negative bacteria.⁵ However, when used in combination with agents destabilizing the outer membrane, it can inhibit gram-negative bacteria.⁵

$\beta$-Cymene (4-isopropyltoluene or 1-isopropyl-4-methylbenzene) is a biological compound naturally present in the essential oils of oregano and thyme. It is a precursor of carvacrol, an active compound having antimicrobial activity against a variety of gram-positive and gram-negative bacteria. $\beta$-Cymene by itself does not have a bactericidal effect on bacteria.¹⁰ However, several reports have shown that it enhanced the antimicrobial activity of carvacrol both in vitro and in food.¹⁰–¹²

Since neither nisin nor $\beta$-cymene by itself can inhibit gram-negative bacteria, and synergistic antimicrobial activity between both compounds against gram-negative bacteria has not been reported, it is of interest to find out whether nisin and $\beta$-cymene used together could inhibit gram-negative bacteria. Nisin and $\beta$-cymene were tested

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in vitro in this study for their antimicrobial activity against S. Typhi when used alone and used in combination. The synergistic effect between nisin and ρ-cymene was also investigated on ready-to-eat food that had been experimentally contaminated with S. Typhi.

Material and Methods

Bacterial strain and culture conditions. The S. enterica serovar Typhi used in this study was S. enterica serovar Typhi ATCC 19430 (referred to as S. Typhi from now on) obtained from the American Type Culture Collection (ATCC). The identity of the bacterial strain was confirmed by using an API 20 E test kit (bioMérieux Industry, Hazelwood, MO, USA). A brain heart infusion (BHI) medium (Oxoid, Wesel, Germany) was used to culture the bacterial strain at 37°C.

The bacterial stock culture was stored as a frozen culture at −80°C in BHI broth containing 20% glycerol (vol/vol). Throughout the experiments, the bacterial strain was subcultured every 2 weeks on BHI agar and kept at 4°C. A liquid culture prepared from a single colony was transferred twice to BHI broth and incubated at 37°C before being used.

Chemicals. Nisin, in the form of Nisinap (containing 2.5% nisin), was obtained from Aplin & Barrett (Trowbridge, UK). Its stock solution was made in 95% ethanol, sterilized through a filter membrane with a 0.22-μm pore size (Sartorius, Göttingen, Germany) and kept at −20°C. ρ-Cymene was obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Its stock solution was held in 95% ethanol at 4°C.

Determination of the antimicrobial activity. The antimicrobial activity against S. Typhi of nisin, ρ-cymene, and nisin plus ρ-cymene was examined. Bacterial cells in an overnight culture of S. Typhi were washed twice with physiological saline (0.9% w/v of NaCl) and then resuspended in fresh BHI broth to obtain a final concentration of 10^7 cfu/ml. The bacterial cell suspension was exposed to different concentrations of nisin and/or ρ-cymene, and kept at a constant temperature (4°C or 37°C). The concentrations of nisin used ranged from 0 to 0.4 ppm, while those of ρ-cymene ranged from 0 to 2.5 ppm. During exposure, samples taken every 20 min for 4 h were subjected to serial dilution with BHI broth. One hundred microliters of appropriate dilutions were plated on BD CHROMagar Salmonella (Becton, Dickson and Company, Sparks, MD, USA), a selective medium for Salmonella sp., and incubated at 37°C for 24 h. Tests were carried out in three replicates.

Since the lower limit of detection of S. Typhi was found to be 0.5 log cfu/ml, a concentration of S. Typhi lower than the detection limit is defined as undetectable. “Complete inhibition” was used only when no growth of S. Typhi was observed after the S. Typhi culture with an undetectable concentration has been transferred to the fresh BHI broth (5% inoculum size) and incubated at 37°C for 24 h.

Preparation of the food sample. The food used in this study was Si KroK Moo, a ready-to-eat Thai-style pork sausage. It was sold in a bacteria-free form (by steaming for 20 min) at a local supermarket and confirmed to be free from bacteria before use.

Examination of the antimicrobial activity of nisin and ρ-cymene in food. The bacterial cell suspension used to inoculate the food was prepared from an overnight culture of S. Typhi by washing the bacterial cells twice with physiological saline and then resuspending them in BHI broth. The food samples (1 g each) were inoculated with 0.1 ml of the S. Typhi suspension so that the final concentration on the samples was 10^5 cfu/g or 10^6 cfu/g. The bacterial suspension was spread thoroughly over the surface of each food sample with a sterile bent glass rod. To the treated sample, 0.01 ml of nisin and ρ-cymene at respective final concentrations of 0.5 and 5.0 ppm or 0.7 and 10.0 ppm were deposited on the surface by using a pipette and then spread with a sterile bent glass rod. The food inoculated with the bacterium with no nisin or ρ-cymene was used as a control. The treated samples were left undisturbed for 30 min to allow residual moisture to be absorbed, and were then stored in plastic bags (International PBI, Milan, Italy) at 4°C, a temperature normally used to store the sausage. Three replicates of each treatment were performed.

The treated food samples were taken for determining the bacterial population after 0, 2, 4, 6, 8, 10, 12, and 14 days of storage. On each sampling day, two samples from each inoculation level (10^5 cfu/g or 10^6 cfu/g) of every concentration of nisin and ρ-cymene were assayed. To each sampling bag, 9 ml of a 0.1 M phosphate buffer (pH 7.0) was added, and the content was thoroughly homogenized for 1 min in a Stomacher 400 laboratory blender (Seward, London, UK). Only the liquid part of the homogenate was collected and serially diluted with the phosphate buffer. One hundred microliter aliquots of appropriate serial dilutions were spotted on BD CHROMagar Salmonella and then incubated at 37°C for 24 h to determine the population of S. Typhi.

Sensory evaluation. A preliminary sensory evaluation of the sausage treated by surface application of nisin and ρ-cymene at respective concentrations of 0.5 and 5.0 ppm or 0.7 and 10.0 ppm was carried out. The samples were evaluated for aroma, taste and overall acceptance by a three-member untrained taste panel. A five-point hedonic scale was used to assess the treated food, where 1 = “extremely disliked” and 5 = “extremely liked.”

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.

Results

The antimicrobial activity of nisin and ρ-cymene against S. Typhi was studied at 4°C and 37°C. At 37°C, with no added nisin or ρ-cymene, the viable cell concentration of the S. Typhi culture increased from 7 log cfu/ml to 9 log cfu/ml over a 4-h incubation period (Table 1). The same result was obtained in the cultures containing nisin alone or ρ-cymene alone at all concentrations used. These results indicated that nisin or ρ-cymene by itself did not inhibit S. Typhi. When nisin and ρ-cymene were applied simultaneously, however, dose-related inhibition of S. Typhi was observed. The bacterial cell counts reduced as the concentrations of nisin and ρ-cymene increased. The minimum concentrations of nisin and ρ-cymene resulting in undetectable cell count (complete inhibition) were 0.3 and 1.5 ppm. The time required to kill all of the bacterial cells was dependent on the concentration of nisin but not on that of ρ-cymene. Regardless of the concentration of ρ-cymene, nisin at concentrations of 0.4 and 0.3 ppm killed all of the cells within 80 min and 120 min, respectively (Fig. 1).
Effect of Nisin (N) and \( \rho \)-Cymene (C) on the Viable Count of S. Typhi at 37 °C.

With both concentrations of nisin, the viable count of S. Typhi dropped very rapidly in the first 20 min after treatment, after which the bacterial cells continued to decrease to the undetectable level (Fig. 1). When aliquots of all the treated cultures with an undetectable concentration of S. Typhi were transferred to the fresh BHI broth (5% inoculum size) and incubated at 37 °C for 24 h, no bacterial growth was apparent in all cases.

At 4 °C, the S. Typhi cell number in the control (without nisin and \( \rho \)-cymene) remained stable throughout the experiment at around 7 log cfu/ml (Table 2). At all concentrations of nisin and \( \rho \)-cymene used separately, no change of the viable cell count of S. Typhi occurred. A decrease of the viable count in a dose-dependent pattern was noted when nisin and \( \rho \)-cymene were used simultaneously. Of all the combinations of nisin and \( \rho \)-cymene tested, only two combinations (0.3 ppm of nisin, 2.5 ppm of \( \rho \)-cymene, and 0.4 ppm of nisin, and 2.5 ppm of \( \rho \)-cymene) were found to cause complete inhibition (Table 2). With both combinations of nisin and \( \rho \)-cymene, all of the bacterial cells were killed after 180 min of exposure to the test compounds. As observed at 37 °C, the viable count of S. Typhi reduced sharply in the first 20 min after treatment, and then continuously dropped to the undetectable level (Fig. 2). In all of the treated cultures with an undetectable concentration of S. Typhi, no bacterial growth was evident when they were transferred to the fresh BHI broth (5% inoculum size) and incubated at 37 °C for 24 h.

The inhibitory effects of nisin and \( \rho \)-cymene against S. typhi experimentally inoculated on the ready-to-eat Thai-style pork sausage were examined for 14 d at a storage temperature of 4 °C. Two inoculum sizes of S. Typhi were used, 10⁵ cfu/g (low inoculum) and 10⁶ cfu/g (high inoculum), and two combinations were tested of nisin and \( \rho \)-cymene (0.5 ppm nisin and 5.0 ppm \( \rho \)-cymene, and 0.7 ppm nisin and 10.0 ppm \( \rho \)-cymene).

No alteration in the viable count of the control (no nisin and \( \rho \)-cymene) was observed throughout the

### Table 1. S. Typhi Viable Count (mean ± SD; log cfu/ml) after 4 h of Incubation at 37 °C in the Presence of Different Combinations of Nisin and \( \rho \)-Cymene

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<thead>
<tr>
<th>Concentration of ( \rho )-cymene (ppm)</th>
<th>Concentration of nisin (ppm)</th>
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<tr>
<td>0</td>
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<tr>
<td>1.0</td>
<td>0.00 ± 0.01</td>
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<td>1.5</td>
<td>0.00 ± 0.04</td>
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### Table 2. S. Typhi Viable Count (mean ± SD; log cfu/ml) after 4 h of Incubation at 4 °C in the Presence of Different Combinations of Nisin and \( \rho \)-Cymene

<table>
<thead>
<tr>
<th>Concentration of ( \rho )-cymene (ppm)</th>
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![Fig. 1](image1.png)  
![Fig. 2](image2.png)
experiment with the low S. Typhi population treatment (Fig. 3). This result indicates that the bacterium could survive on the food at 4 °C during 14 d of storage. Both combinations of nisin and \(\rho\)-cymene killed all of the bacterial cells, although to a different degree. Complete inhibition was observed on day 2 of storage when the combination with more nisin (0.7 ppm) and \(\rho\)-cymene (10.0 ppm) was used and on day 4 of storage when the other combination was used. After no bacterial cells had initially been detected on the food samples, the viable count remained undetectable throughout the experiments (Fig. 3). Complete inhibition of S. Typhi was confirmed in all of the food samples with an undetectable concentration of S. Typhi.

The viable count of S. Typhi of the control (no nisin and \(\rho\)-cymene) with the high S. Typhi population treatment was stable throughout the experiment, indicating the survival ability of the bacterium on the food at 4 °C over a 14-d storage period (Fig. 4). The two different combinations of nisin and \(\rho\)-cymene affected the population of S. Typhi on the sausage differently. When the combination with more concentrations of nisin (0.7 ppm) and \(\rho\)-cymene (10.0 ppm) was applied, complete inhibition was observed on day 6 of storage. The undetectable cell count was maintained throughout the storage period. Complete inhibition of S. Typhi was confirmed in all of the food samples with an undetectable concentration of S. Typhi. Although nisin with a concentration of 0.5 ppm together with 5.0 ppm of \(\rho\)-cymene did not kill all of the bacterial cells on the sausage, it substantially lowered the bacterial load on the sample (from 6 log cfu/g to 2.5 log cfu/g). This bacterial cell reduction was observed during 6 days of storage, and after that, no increase in the cell population was detected (Fig. 4).

A statistical analysis of the sensory panel evaluations for aroma, taste and overall acceptability revealed no significant difference (\(p < 0.05\)) between the food samples untreated (no nisin and \(\rho\)-cymene) and treated (with 0.5 ppm nisin and 5.0 ppm \(\rho\)-cymene, and 0.7 ppm nisin and 10.0 ppm \(\rho\)-cymene).

**Discussion**

S. Typhi is a major foodborne pathogenic bacterium causing typhoid fever which leads to human health problems and economic loss in many countries worldwide. The bacterium, S. Typhi, was found in all foods (sausage, pork, and minced meat) tested in this experiment. This is consistent with previous reports that S. Typhi is widely distributed in foods.7-9,14) Nisin, a bacteriocin produced by some strains of Lactococcus lactis subsp. lactis, exhibits a broad inhibitory spectrum against gram-positive bacteria, including bacterial endospores.14,15) It has been used for a number of applications, including extending the shelf life of dairy products, preventing the spoilage of canned goods by thermophiles, and preventing spore outgrowth and toxin production by Clostridium botulinum.8,14) The cytoplasmic membrane is the site at which nisin activity is believed to occur, and loss of cell viability may involve interaction of the dehydroalanine residues in nisin with membrane sulphydryl groups.16) Cell inactivation is a result of cellular damage which can range from the disruption of proton motive force to loss of membrane integrity.17,18) However, nisin is not active against gram-negative bacteria. Their outer membrane, consisting of substantial amounts of protein, phospholipid, and lipopolysaccharide, acts as a barrier to the action of nisin on the cytoplasmic membrane.19-22) The combination of nisin and other treatments altering the outer membrane has been found to inhibit gram-negative bacteria. Salmonella species treated with the chelating agent EDTA became sensitive to nisin.23) Cells of Escherichia coli exhibited nisin sensitivity when their outer membrane was subjected to osmotic shock.17,24) Treatment with trisodium phosphate increased the nisin sensitivity of E. coli, Campylobacter jejuni, Pseudomonas fluorescens, and Salmonella enteritidis.25)
As observed with the nisin treatment, \( \rho \)-cymene used alone did not inhibit S. Typhii. This result is in agreement with the findings of Juven et al., Dorman and Deans and Juliano et al. \( \rho \)-Cymene, a hydrophobic substance, has been found to have a preference for liposomal membranes, thereby causing swelling of the cytoplasmic membrane. Since it did not affect the pH gradient across the cytoplasmic membrane, the proton motive force and the ATP pool, it was ineffective in killing cells when used alone.\(^{39}\) All combinations of nisin and \( \rho \)-cymene used in the in vitro study exhibited a synergistic antimicrobial effect between both compounds against S. Typhii. A similar effect against a variety of bacteria, including \( Listeria \) \( monocyctogenes \), \( Bacillus \) \( cereus \), and \( Bacillus \) \( subtilis \), has been found between nisin and such other natural compounds as thymol and carvacrol, the antimicrobial compounds found in oregano and thyme.\(^{20–25}\) Although the actual mechanism for the synergism between nisin and carvacrol and nisin and thymol is not known, several possibilities have been proposed. Pol and Smid have proposed for the synergism between nisin and carvacrol that carvacrol might enhance the action of nisin by increasing the number or size of the pores formed, both leading to an increased reduction of viable cell numbers.\(^{20}\) In respect of the synergism between nisin and thymol, it was believed that thymol destabilized the bacterial membrane, resulting in an increased permeability for nisin.\(^{31}\) How nisin and \( \rho \)-cymene work together to inhibit S. Typhii is still unknown. Due to its lipophilic property, \( \rho \)-cymene may increase the permeability of nisin so that nisin can get to the site of action (the cytoplasmic membrane). Treatment with \( \rho \)-cymene may destabilize the outer membrane of bacteria, helping nisin get to its target site on the cytoplasmic membrane. However, more investigations are needed to explain the actual mechanism for the synergistic antimicrobial effect.

This study found that temperature affected the antimicrobial activity of nisin and \( \rho \)-cymene against S. Typhii. The bacterial strain was less sensitive towards the antimicrobial compounds at 4°C than at 37°C. A similar result has been reported by Periago and Moezelaar. They found that the combined effect of nisin and \( \rho \)-cymene required for S. Typhii ATCC 33459, S. Typhii ATCC 33926, \( Escherichia \) coli O157:H7 ATCC 35150, and \( Shigella dysenteriae \) ATCC 11835, had different degrees of sensitivity to nisin and cymene (data not shown). Many factors which may affect the concentrations of nisin and \( \rho \)-cymene required for complete inhibition of S. Typhii such as the pH, temperature and type of food are being tested in our laboratory.

They were also found to depend on the type of bacteria. Our experiments show that several strains of Gram-negative bacteria, including S. Typhii ATCC 33459, S. Typhii ATCC 33926, \( E. coli \) O157:H7 ATCC 35150, and \( Shigella dysenteriae \) ATCC 11835, had different degrees of sensitivity to nisin and cymene (data not shown). Many factors which may affect the concentrations of nisin and \( \rho \)-cymene required for complete inhibition of S. Typhii such as the pH, temperature and type of food are being tested in our laboratory.

### References