P-ISSN 2586-9000
E-ISSN 2586-9027
Homepage : https://tci-thaijo.org/index.php/SciTechAsia
Science & Technology Asia
Vol. 23 No.4 October - December 2018
Page: [53-59]
Original research article

Efficacy of a Combination of Nisin and Citric Acid Against *Listeria Monocytogenes* 10403S *in Vitro* and in Model Food Systems

Mathira Wongchai, Wasinee Churklam,
Natthaporn Klabthawee and Ratchaneewan Aunpad*

Faculty of Allied Health Sciences, Thammasat University, Pathum Thani 12120, Thailand

Received 10 July 2018; Received in revised form 28 August 2018
Accepted 14 October 2018; Available online 19 December 2018

ABSTRACT

Nisin has been extensively used as a safe food preservative; therefore, the occurrence of nisin resistance in various bacteria including nisin-exposed *Listeria monocytogenes* has increased in recent years. This problem could be overcome by using nisin in combination with other antimicrobial agents resulting in synergistic effects. Citric acid is a safe food additive granted GRAS status by the Food and Drug Administration, USA. In the present investigation, the antibacterial activity of nisin and citric acid alone or in combinations against *L. monocytogenes* 10403S was determined, and their potential as food preservative in food model systems was evaluated. The nisin and citric acid showed minimum inhibitory concentration (MIC) at 250 and 4,000 µg/ml, respectively. Checkerboard microdilution method using both compounds showed synergistic effect at concentration of 62.5µg/ml and 1,000 µg/ml, respectively, with the fractional inhibitory concentration index (FICI) value of 0.5. The potent anti-listeria effect of nisin in combination with citric acid on the growth of *L. monocytogenes* in pork ham (food model) was observed during six days of storage at 4°C. This might be exploited to inhibit foodborne bacteria and minimize the nisin-resistant problem of *L. monocytogenes* in the food industry.

Keywords: Listeria monocytogenes; synergistic effect; nisin; citric acid; pork ham

1. Introduction

Foodborne infections are still the major public health concerns worldwide. *Listeria* (*L.*) *monocytogenes* is an opportunistic intracellular foodborne pathogen associated with listeriosis caused by the consumption of contaminated foods especially ready-to-eat (RTE) foods such as poultry, beef, dairy products, and vegetables [1].

***Corresponding author:** aratchan@tu.ac.th **doi:** 10.14456/scitechasia.2018.32

Recently, *L. monocytogenes* has been described as one of the top five pathogens contributing to domestically acquired foodborne illnesses resulting in death [2]. Interestingly, consumption of contaminated food appears to be the main route of transmission of listeriosis and has been estimated as the source of *L. monocytogenes* up to 99% of the cases [3]. The interest and use of food preservatives, particularly natural food preservatives, to control *Listeria* contamination in foods has dramatically increased in recent years.

Nisin, a bacteriocin produced by lactic acid bacterium (LAB), *Lactococcus lactis* subspecies *lactis*, has been approved by World Health Organization as a food biopreservative [4]. It is one of most effective and widely use antimicrobial agents in food industry as a safe and natural food preservative. It has been reported to exhibit antimicrobial activity against a broad range of bacteria including strains of *L. monocytogenes*. Due to the extensive use, the occurrence of nisin resistance in various bacteria has increased in recent years [5]. The major mechanisms involved in nisin resistance of *L. monocytogenes* included the modifications of membrane phospholipid composition. The nisin-resistant strains of *L. monocytogenes* represented the decrease of anionic phospholipid which is important for the interaction of nisin with bacterial cell membranes. This resulted in a decreased net negative charge and disruption of nisin binding [5].

The risk of emergence of nisin-resistance among nisin-exposed *L. monocytogenes* could be minimized by the use of nisin in lower concentrations [6], notably with combination with other food preservatives which have synergistic effect. Low-molecular-weight organic acids such as citric, acetic, lactic and malic have a long history of being used as food preservatives. Their antimicrobial action is based on their ability to reduce the pH and on other mechanisms such as inhibition of enzymes [7]. Based on equal molar concentration, citric acid showed the highest effectiveness against *L. monocytogenes* when compared to that of malic, lactic, acetic and HCl [8]. Citric acid is a general purpose food additive and has been granted Generally Recognized as Safe (GRAS) status by the Food and Drug Administration, USA. The combination of nisin and citric acid might be used to reduce the number of *L. monocytogenes* in RTE foods. The objective of the present study was to determine and compare antibacterial activity of nisin, citric acid and their combination against *L. monocytogenes* both *in vitro* and in food model.

2. Materials and Methods

2.1 Reagents and microorganisms

Nisin (N5764, containing 2.5% pure nisin, activity of $1x10^6$ IU/g, according to the manufacturer's certificate of analysis) and citric acid (251275, 99.5% purity) were obtained from Sigma-Aldrich. Nisin was resuspended in sterile 0.02 N HCl and citric acid was resuspended in sterile distilled water. *L. monocytogenes* strain 10403S was maintained at -80°C and grown in Tryptic Soy Agar supplemented with 0.6% Yeast Extract (TSAYE). The culture was incubated at 37°C for 24 h.

2.2 Antibacterial assay *in vitro*

The minimum inhibitory concentration (MIC) values of nisin and citric acid against the *L. monocytogenes* 10403S were determined by standard broth micro-dilution assay (CLSI2010). In brief, the cells were grown in TSBYE at 37°C until the exponential phase (OD_{600} ~ 0.5–0.6). Then, they were centrifuged (10 min at 6,000xg) and resuspended in TSBYE at the concentration of 10^5 colony forming units/ml or CFUs/ml (0.5 McFarland). Bacteria were distributed in triplicate into

96 well microplates (50 µl/well), mixed with two-fold serial dilutions of nisin or citric acid (50 µl/well) and incubated at 37°C for 24 h. A TSBYE medium containing 0.02 N HCl or sterile distilled water was used as a control. The MIC values were defined as the lowest concentration of antimicrobial that prevented the growth as determined by measuring the absorbance at 600 nm (Varioskan™ Flash Multimode Reader). All tests were performed in triplicate.

2.3 Checkerboard synergy testing

Mechanistic interactions of drug combinations are usually measured by an established technique known as checkerboard method [9]. The synergy between nisin and citric acid was performed in 96-well microtiter plates. In this study, the serial two-fold dilutions ranging from 1/256 - 2 times the MIC for nisin and from 1/256 - 2 times for citric acid were tested (Table 1). The antibacterial effects of each combination was evaluated in terms of the fractional inhibitory concentration index (FICI), which was calculated as the ratio of the MIC of agents A and B in combination to the MIC of agent A (or B) alone as follows:

$$FICI = FIC_A + FIC_B = (C_A^{COMB} / MIC_A^{alone}) + (C_B^{COMB} / MIC_B^{alone})$$

Where MIC_A^{alone} and MIC_B^{alone} are the MIC values of agent A and B, respectively, acting alone and C_A^{COMB} and C_B^{COMB} are the MIC of agents A and B, respectively, when in combination. The interpretation of the FICI was as follows: FIC $\leq$0.5 were classified as synergistic; those resulting in FIC indices of >4 were designated as antagonistic [10]. All tests were performed in triplicate.

2.4 Agar diffusion test

The agar diffusion synergy testing was used to determine the antimicrobial activities of nisin and citric acid along or in combination against *L. monocytogenes* 10403S. In brief, 50 µl of inoculum of an approximately $1x10^6$ CFU/ml bacterial suspension was added in 100 ml of sterile TSBYE with 1% agar and pour into the plate. Wells of 8 mm diameter were punched using sterile cork borer and filled with nisin and citric acid alone or in combination. The tests were performed in triplicate. The antimicrobial activity can be classified into three levels (Rota): weak activity (inhibition zone <12 mm); moderate activity (inhibition zone = 12-20 mm) and strong activity (inhibition zone $\geq$ 20 mm). The assays were measured after incubation at 37°C for 24 h. 0.02N HCl and sterile distilled water were used as negative controls.

2.5 Evaluation of antibacterial efficacy of nisin in combination with citric acid in food system

Before each trial, *L. monocytogenes* from overnight culture was adjusted to 0.5 McFarland turbidity with normal saline (0.85% NaCl). Freshly sliced pork hams were bought from a department store supplied by a local distributor on the same day with the experiment. A total of 12 pork hams, weighing approximately 45 g, were randomly allocated to three groups: (i) uninoculated controls (control), (ii) inoculated with *L. monocytogenes* at a level of $2x10^3$ CFU/g (pork ham) by randomly distributing dropwise to the surface of slices and spread throughout the surface using glass rod and allowed to dry, (iii) inoculated with *L. monocytogenes* at a level of $2x10^3$ CFU/g (sliced bologna sausage) together with nisin and citric acid (at synergism concentration) by randomly distributed dropwise to the surface of slices and spread throughout the surface using glass rod and allowed to dry. After assuring good contact, the samples were kept in a clean plastic box

and stored at 4ºC. The samples weighing 0.5 g from each group were aseptically taken after 0, 1, 2, 3, 4, 5, and 6 days of storage. They were vigorously mixed in 4.5 ml normal saline for 5 min. The numbers of *L. monocytogenes* CFUs from each group were determined following decimal dilution and plating the appropriate dilutions on *Listeria* Selective Agar (Oxoid, UK) for 48 h at 37ºC. Three independent experiments were performed. A Student's t-test was computed to determine the statistical significance of the results. Differences were judged to be statistically significant when $p<0.05$.

2.6 Statistical analysis

All measurements were made in triplicate and each experiment was performed on three separate occasions. For MIC determination and synergy testing, the results were expressed as mean value of the three parallel assays. A Student's t-test was used to evaluate the antibacterial efficacy of nisin in combination with citric acid in food system.

3. Results and Discussion

3.1 Determination of alone and in combination effects

The *in vitro* activities of citric acid or nisin alone or in combination against *L. monocytogenes* strain 10403S was determined by standard broth micro-dilution assay. The results showed that citric acid or nisin alone were able to reduce the bacterial population and the MIC values of citric acid and nisin against *L. monocytogenes* 10403S was 4,000 and 250 μg/ml, respectively. The calculated MIC of the nisin against various foodborne *L. monocytogenes* isolates including ATCC 19115 strain using microbroth dilution method was 32-64 μg/ml [11]. For citric acid, the average MIC value against six isolates of *L. monocytogenes* was 3.8 mM [12], which were similar to our study.

In the checkerboard assay, the FICI values were calculated to analyze the interaction of the combinations [13]. Different combinations of nisin and citric acid ranging from several dilutions below the MIC to twice of MIC were tested. As shown in Table 1, nisin at concentration of 62.5 μg/ml showed synergistic effect with citric acid at concentration of 1,000 μg/ml against *L. monocytogenes* 10403S with the fractional inhibitory concentration index (FICI) values of 0.5. Nisin not only decreased the MIC of citric acid (from 4,000 to 1,000 μg/ml), but also the MIC of nisin itself was reduced (from 250 to 62.5 μg/ml) by citric acid. Furthermore, it is worth noting that all the FICI values were smaller than 4.0, which indicates that there was no antagonism between nisin and citric acid. The synergistic interactions of nisin combined with citric acid against nine food isolates and one ATCC 191115 strain were also observed with FICI values ranging from 0.19 to 0.375 [11]. In conclusion, a substantial interaction occurs when using nisin and citric acid in combination whereas nisin and citric acid alone showed weak antibacterial activity against *L. monocytogenes* 10403S.

Table 1. Synergistic effects of nisin with citric acid against *L. monocytogenes* 10403S

	MIC (μg/ml)									
Nisin	500	250	125	62.5	31.25	15.63	7.82	3.91	1.95	0.98
Citric acid	15.63	250	500	1000	1000	2000	2000	2000	2000	2000
FICI value	2.004	1.063	0.625	0.5	0.625	0.563	0.531	0.516	0.508	0.504
Interpretation	IND	IND	IND	SYN	IND	IND	IND	IND	IND	IND

SYN, synergism; ANT, antagonism; IND, indifference. For the FICI model, synergy was defined as an FICI of ≤0.5, antagonism was defined as an FICI of >4.0, and indifference was defined as an FICI of >0.5–4 (i.e., no interaction)

3.2 Agar diffusion test

The results obtained from agar diffusion tests helped to visualize the synergy between nisin and citric acid (Fig. 1). The bigger clear zone indicated higher activity. The agar diffusion method is simple, reliable, inexpensive, and currently used for susceptibility testing of bacteria and fungi in clinical laboratories. This method has been widely used to assay the interaction between antimicrobial agents as it can provide more visually convincing results [14]. Nisin or citric acid at 62.5 and 1,000 μg, respectively, showed very weak antimicrobial activities. In contrast, the combination of nisin and citric acid showed moderate activity with a clearer and larger zone (16 mm diameter), which were much larger than for a single antibacterial on the agar plate. This result confirmed the results of the checkerboard synergy testing.

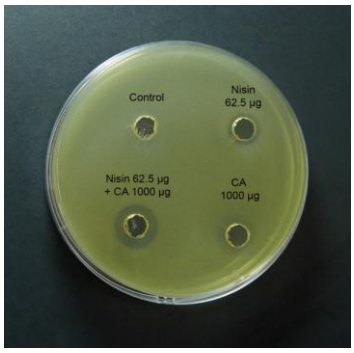

Fig. 1. Agar diffusion assay for nisin combined with citric acid against *L. monocytogenes* 10403S.

3.3 Evaluation of antibacterial efficacy of nisin in combination with citric acid in food system

The *in situ* anti-listeria activity of nisin in combination with citric acid against *L. monocytogenes* 10403S was studied using pork ham as a model. Figure 2 showed the viable counts of *L. monocytogenes* in the batches inoculated with $2x10^3$ CFU/g *L. monocytogenes* with nisin and citric acid (treated group) at 62.5 and 1,000 μg/ml, respectively or without (untreated group) and the control group without nisin and citric acid and *L. monocytogenes*. After six days of storage at 4°C, the viable cells count of treated group was reduced significantly (p<0.05) when compared to that of untreated group. It was observed that the addition of nisin and citric acid significantly reduced the viable count (p<0.05) upto four days. Indeed, the initial *L. monocytogenes* population ($2x10^3$ CFU/g) increased to a level of $3.2x10^4$ CFU/g for the untreated group (inoculated with only *L. monocytogenes*). By contrast, the addition of nisin and citric acid led to an inhibition of the growth for more than 10^4 CFU. In control sample without *L. monocytogenes* and nisin and citiric acid, the viable cells count of *L. monocytogenes* was not detected.

Due to the high prevalence of *L. monocytogenes* contamination in RTE foods and spontaneous nisin-resistant variants of this strain are reported to occur at a relatively high frequency [15]. The combination of nisin with non-nisin anti-listeria compound might be used to minimize the resistant problem of nisin [11]. In this study, nisin in combination with citric acid was able to inhibit the growth of *L. monocytogenes* 10403S throughout the storage period (6 days). *L. monocytogenes* populations in untreated group grew quickly while application of nisin and citric acid was able to inhibit or slow down the growth of *L. monocytogenes* by more than seven folds. The inactivation of *L. monocytogenes* in raw ground pork stored aerobically was attained mainly with the combination of nisin and lactic acid [16]. The nisin–sodium citrate (5%) combination was significantly effective against *L. monocytogenes* inoculated on fresh-cut tomato stored at

4 °C for three days [17]. This study showed the synergistic effect of nisin and citric acid against *L. monocytogenes* 10403S in food model.

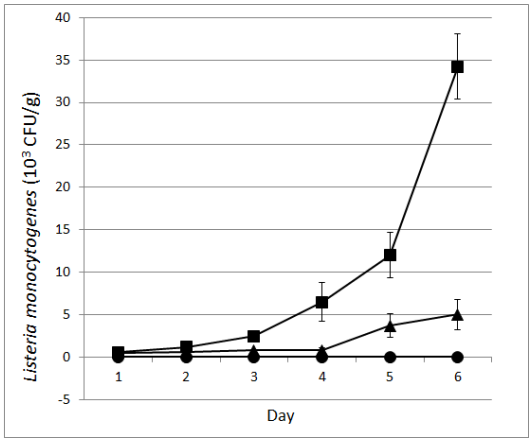

Fig. 2. Growth of *L. monocytogenes* in pork ham storage at 4°C. (■), inoculated only *L. monocytogenes* 10403S; (▲), inoculated with *L. monocytogenes* and nisin with citric acid; (●), control group without *L. monocytogenes* and nisin and citric acid. Values are mean and standard deviation of three independent experiments.

4. Conclusion

The use of natural antimicrobials such as nisin in combination with salts of organic acids such as citric acid is a promising and safe food biopreservatives. Nisin and citric acid at 62.5 and 1,000 μg/ml, respectively, showed synergistic effect toward *L. monocytogenes* 10403S both *in vitro* and in food model. This might be exploited to inhibit foodborne bacteria and minimize the nisin-resistant problem of *L. monocytogenes* in the food industry. Further investigations would be required to evaluate this synergy using a wider range number of fresh products and challenging pathogens.

Acknowledgements

The authors gratefully acknowledge the financial support provided by Thammasat University Research Fund under the TU Research Scholar, Contract No. 11256. Miss Wasinee Churklam was supported by Thailand Research Fund under the Royal Golden Jubilee Ph.D. programme (PHD/0159/2556,5.L.TU/56/ I.1).

References

[1] Neetoo H, Ye M, Chen H. Potential antimicrobials to control *Listeria monocytogenes* in vacuum-packaged cold-smoked salmon pâté and fillets. Int J Food Microbiol 2008;123:220-227.

[2] Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, et al. Foodborne illness acquired in the United States—major pathogens. Emerg Infect Dis 2011;17:7-15.

[3] Sauders BD, Sanchez MD, Rice DH, Corby J, Stich S, Fortes ED, et al. Prevalence and molecular diversity of *Listeria monocytogenes* in retail establishments. J Food Prot 2009;72:2337-2349.

[4] Gahan GC, Collins JK. Listeriosis: biology and implications for the food industry. Trends Food Sci Tech 1991;2:89-93.

[5] Zhou H, Fang J, Tian Y, Lu XY. Mechanisms of nisin resistance in Gram-positive bacteria. Annals Microbiol 2014; 64:413–420.

[6] Chen X, Zhang X, Meng R, Zhao Z, Liu Z, Zhao Z, et al. Efficacy of a combination of nisin and p-Anisaldehyde against *Listeria monocytogenes*. Food Cont 2016:66:100-106.

[7] Campos CA, Castro MP, Gliemmo MF, Schelegueda LI. Use of natural antimicrobials for the control of *Listeria monocytogenes* in foods. Science against microbial pathogens: Communicating current research and technological advances. Formatex Badajoz 2011;1112-1123.

[8] Samelis J, Sofos JN. Natural antimicrobials for the minimal processing of foods. Cambridge: Woodhead Publishing Limited and CRC Press LLC; 2003.

[9] Mukherjee PK, Sheehan DJ, Hitchcock CA, Ghannoum MA. Combination treatment of invasive fungal infections. Clin Microbiol Rev 2005;18:163-194.

[10] Pillai SK, Moellering RC, Eliopoulos GM. Antimicrobial combinations. In: Lorian V, editor. Antibiotics in laboratory medicine. 5th ed. New York: Lippincott Williams & Wilkins; 2005. p. 365–440.

[11] Zhao X, Zhen Z, Wang X, Guo N. Synergy of a combination of nisin and citric acid against *Staphylococcus aureus* and *Listeria monocytogenes*. Food Addit Contam 2017;34:2058-2068.

[12] Wemmenhove E, van Valenberg HJ, Zwietering MH, van Hooijdonk TC, Wells-Bennik MH. Minimal inhibitory concentrations of undissociated lactic, acetic, citric and propionic acid for *Listeria monocytogenes* under conditions relevant to cheese. Food Microbiol 2016;58:63-67.

[13] Belkum A, Halimi D, Bonetti EJ, Renzi G, Cherkaoui A, Sauvonnet V, et al. Meropenem/colistin synergy testing for multidrug-resistant *Acinetobacter baumannii* strains by a two-dimensional gradient technique applicable in routine microbiology. J Antimicrob Chemoth 2014;70:167-172.

[14] Zhao X, Liu Z, Li W, Li X, Shi C, Meng R, et al. In Vitro synergy of nisin and coenzyme Q10 against *Staphylococcus aureus*. Food Cont 2014;46:368-373.

[15] Cartwright E, Jackson K, Johnson S, Graves L, Silk B. Listeriosis outbreaks and associated food vehicles United States, 1998–2008. Emerg Infect Dis 2013;19:1–10.

[16] López-Mendoza MC, Ruiz P, Mata CM. Combined effects of nisin, lactic acid and modified atmosphere packaging on the survival of *Listeria monocytogenes* in raw ground pork. Int J Food Sci & Technol 2007;42:562-566.

[17] Oladunjoye AO, Singh S, Ijabadeniyi OA. Inactivation of *Listeria monocytogenes* ATCC 7644 on fresh-cut tomato using nisin in combinations with organic salts. Braz J Microbiol 2016;47:757-76.