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Original Article

Antimicrobial activity optimization of nisin, ascorbic acid and ethylenediamine tetraacetic acid disodium salt (EDTA) against *Salmonella* Enteritidis ATCC 13076 using response surface methodology

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ABSTRACT

Nisin is a commercial bacteriocin produced by *Lactococcus lactis* subsp. *lactis* and widely used as a natural preservative in the food industry. However, while nisin alone cannot inhibit the growth of Gram-negative bacteria, it can in combination with a chelating agent or organic acid. This study combined nisin with some chelating agents, weak organic acids and their salts to inhibit *Salmonella* Enteritidis ATCC 13076. The combinations of nisin (2000 parts per million; ppm) and ascorbic acid (2000 ppm) or ethylenediamine tetraacetic acid disodium salt (EDTA; 7400 ppm) showed significant inhibitory effects on the target strain. Due to regulatory limits, the second part of the study reduced the concentrations of nisin, ascorbic acid and EDTA to 500 ppm, 2000 ppm and 250 ppm, respectively. The mixture of nisin, ascorbic acid and EDTA showed the highest inhibitory effect with a reduction number of 3.41 log colony forming units ($p < 0.05$). To minimize the growth of *S.* Enteritidis ATCC 13076, central composite design and response surface methodology were applied to investigate the combined effect of nisin (0–500 ppm), ascorbic acid (0–2000 ppm), and EDTA (0–250 ppm) on the target strain growth. Among the three factors, nisin had a higher antimicrobial effect than ascorbic acid or EDTA, while an increase in nisin resulted in a decrease in *S.* Enteritidis ATCC 13076 growth. The optimum concentration was 500 ppm nisin with 1515 ppm ascorbic acid and 250 ppm EDTA. Under these conditions, the growth of *S.* Enteritidis ATCC 13076 predicted by the model was 24.99%.

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Introduction

It has been well established that fish and shellfish are nutrient-rich foods and consumption of them provides high quality protein, energy and essential nutrients, including the long-chain n-3 poly-unsaturated fatty acids (LCn3PUFAs; Food and Agriculture Organization, 2010). During 2010–2030, the total global consumption of seafood is expected to increase by 30% (World Bank, 2013). This trend potentially leads to an increase in consumer health risk, because of the greater risk of bacterial contamination. Contamination of fish and shellfish with *Salmonella* is a major public health concern as 99% of human infections are caused by *Salmonella enterica* and *Salmonella* Enteritidis, a non-typhoid *Salmonella* serotype, is the most often implicated in human infections (Amagliani et al., 2012). It causes gastrointestinal disease, which presents as nausea, vomiting, diarrhea, cramps and fever (Iwamoto et al., 2010). The U.S. Food and Drug Administration (USFDA) has demonstrated the presence of salmonellae in a variety of fish and shellfish, including ready-to-eat (RTE) products, products requiring minimal cooking and shellfish eaten raw (Iwamoto et al., 2010). The most common factors contributing to *Salmonella* outbreaks are improper cooking, unsuitable storage, cross-contamination and the use of raw ingredients to prepare food (Amagliani et al., 2012). One approach for the inhibition of *Salmonella* is to use natural antimicrobial agents. Peptide or metabolites from bacteria have been used to control pathogenic bacteria in food for a long time (Bagenda and Yamazaki, 2011). Among these, nisin is the only a kind of bacteriocin which is permitted for use as a food preservative (E234) by the European

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Union since 1983 (Phongphakdee and Nitisinprasert, 2015). Nisin is a short-chained antimicrobial peptide produced by *Lactococcus lactis* subsp. *lactis* and was classified to be Generally Recognized As Safe (GRAS) for human consumption by the USFDA in 1988 (Phongphakdee and Nitisinprasert, 2015). Nisin shows a broad antimicrobial spectrum against vegetable cells and endospores of Gram-positive bacteria but has no antimicrobial effect on Gram-negative bacteria, including *Salmonella* sp., because of their outer membrane (OM) barriers. The lipopolysaccharides (LPSs) in the OM of Gram-negative bacteria are strongly anionic and are stabilized by Ca^{2+} and Mg^{2+} (Facon and Skura, 1996). It has been reported that the antimicrobial activity of nisin could be enhanced by the use of membrane destabilizing agents, such as chelating agents, polycationic acid and weak organic acids as well as their salts (Facon and Skura, 1996; Alakomi et al., 2000; Helander et al., 2001). Most previous studies focused on combinations of nisin and other substances. These combinations can increase the broad spectrum of antimicrobial activity because nisin and combined substances act on different targets within the cell (Dai et al., 2010). However, nisin and/or combined substances must be used at high concentrations to achieve activity against target microorganisms, and these levels may exceed the flavor threshold acceptable to consumers and exceeds the allowed regulatory level (López-Malo et al., 2005). In theory, the use of combinations of three antimicrobial agents should be more effective than combinations of two antimicrobial agents because it is more difficult for the target organism to adapt to and resist a simultaneous attack on several targets in its cell wall and interior (Dai et al., 2010). However, reports on the use of combinations of three antimicrobial agents are scarce. Thus, the objectives of this study were to identify combinations of nisin with some chelating agents against *S.* Enteritidis ATCC 13076 and to determine the optimum concentrations of nisin and potential combined substances using a central composite design with response surface methodology. The information from this study not only can be used as an effective alternative for controlling target, food-borne, pathogenic bacteria in the food industry but also to reduce consumer risk from an overdose of food additives.

Materials and methods

Bacterial strains

S. Enteritidis ATCC 13076 and *Micrococcus luteus* IFO 12708 were used as the target indicator strains. Stock cultures of each strain were kept in tryptic soy broth (TSB; Difco; Sparks, MD, USA) containing 20% (v/v) glycerol (Merck, Darmstadt, Germany) and 0.6% (w/v) yeast extract (Difco; Sparks, MD, US) at −18 °C. Before use, both target strains were cultured in TSB and incubated at 35 °C for 18 h. After incubation, each strain was serially diluted with TSB to a final population of approximately 7.0 log colony forming units (CFU)/mL.

Chemicals and reagents

Commercial nisin (Nisaplin®) containing 10^6 international units (IU) of pure nisin per gram was purchased from Danisco (Grindsted, Denmark). Stock solution of nisin and all chelating agents including ascorbic acid (Riedel-Graham; Barrington, IL, USA), citric acid (Carlo Erba; Rodano, MI, Italy), ethylenediamine tetraacetic acid disodium salt (EDTA; Ajax; Taren Point, NSW, Australia), sodium acetate (Carlo Erba; Rodano, MI, Italy), sodium citrate (M&B; Dagenham, UK), sodium lactate (Wako; Osaka, Japan) and potassium sorbate (Fluka; St. Louis, MO, USA) were prepared by dissolving each chemical in distilled water to the designed final concentration before sterilization using filtration through a syringe filter membrane pore size 0.22 μm (Minisart; Göttingen, Germany).

Screening of the potential chelating agents for enhancing nisin antimicrobial activity

A stock solution of nisin, ascorbic acid, citric acid, sodium acetate, sodium citrate, sodium lactate, potassium sorbate and a mixture of nisin with each chelating agent were separately added into 10 mL of Mueller Hinton broth (MHB; Difco; Sparks, MD, USA) to get a final concentration of 2000 parts per million (ppm) except for the EDTA which was added to a final concentration of 7400 ppm. Thereafter, 100 μL of each target strain was separately inoculated into each tube of treated MHB to a final population of approximately 5.0 log colony forming units (CFU)/mL and then incubated at 35 °C for 24 h. After incubation, the growth of the target strain under different treatments was determined using optical density measurement at 600 nm (OD_{600}; Suksathit and Tangwatcharin, 2013) with a microplate reader (Power wave; Biotek; Winooski, VT, USA).

Antimicrobial activity of nisin in combination with ascorbic acid and ethylenediamine tetraacetic acid disodium salt at legal limit concentration

Stock solutions of nisin in combination with ascorbic acid or EDTA or both were added into 10 mL of MHB to a final concentration of 500 ppm; 2000 ppm or 250 ppm. Then, 100 μL of each target strain was separately inoculated into treated MHB to a final population of approximately 5.0 logCFU/mL and then incubated at 35 °C. After 24 h, the viable cell number in each treatment were enumerated using the spread plate technique with tryptic soy agar (TSA; Difco; Sparks, MD, USA) and incubated at 35 °C for 24 h (Suksathit and Tangwatcharin, 2013).

Determination of the optimal mixing concentration of nisin, ascorbic acid and ethylenediamine tetraacetic acid disodium salt

In this study, central composite design (CCD) and response surface methodology (RSM) were applied to determine the optimal nisin, ascorbic acid and EDTA concentrations in simulated mixed solutions that exhibited the highest antimicrobial activity against *S.* Enteritidis ATCC 13076 (Bezerra et al., 2008). Then nisin, ascorbic acid and EDTA concentrations were assigned as independent variables, whereas the percentage of target strain growth was assigned as the response variable in the proposed regression equation (Table 1). Moreover, the upper-limit concentrations for nisin, ascorbic acid and EDTA were designed to comply with the legal limit concentrations of European Food Safety Authority, 2006 and Food and Agriculture Organization, 2015. Using this approach, 20 experiments consisting of 2^3 factorial central composite experimental designs with six axial points and six replicates at the central point were conducted. MHB without any antimicrobial agent was used as a control experiment.

A stock solution of nisin, ascorbic acid, and EDTA was added into 5 mL of MHB to the designed final concentrations. *S.* Enteritidis ATCC 13076 was inoculated into prepared MHB to a final population

Table 1
Levels of independent variables—nisin, ascorbic acid and ethylenediamine tetraacetic acid disodium salt (EDTA)—in a 2^3 full factorial experimental design.

Independent variable	Unit[a]	Level				
		−1.682 (-α)	−1	0	+1	1.682 (+α)
nisin	ppm	0.00	101.37	250.00	398.63	500.00
ascorbic acid	ppm	0.00	405.47	1000.00	1594.53	2000.00
EDTA	Ppm	0.00	50.68	125.00	199.32	250.00

[a] ppm = parts per million.

of approximately 5 logCFU/mL and then incubated at 35 °C for 24 h. After incubation, the viable cells in each experiment were enumerated using the pour plate technique with TSA and incubated at 35 °C for 48 h. The viable cell numbers were determined as logCFU/mL and expressed as growth percentages.

All experiments were carried out in three replications and the average growth in percentage of *S.* Enteritidis ATCC 13076 in each experiment was used to generate the regression Equation (1):

$$Y = b_0 + b_1x_i + b_2x_{ii} + b_3x_{iii} + b_4x_i^2 + b_5x_{ii}^2 + b_6x_{iii}^2 + b_7x_ix_{ii} + b_8x_ix_{iii} + b_9x_{ii}x_{iii} \quad (1)$$

where Y is the response variable, b is the regression coefficient and x is the coded or un-coded level of the independent variables. This equation evaluates the effects of the linear (x_i), quadratic (x_i^2) and interactive terms (x_ix_j) of the independent variables on the response variable (Nwabueze, 2010). Surface response plots and contour plots were generated as a function of two variables, while the other variable was fixed at a coded level of 0.

Statistical analysis

Each reported value was the mean of three replications. Analysis of variance was performed and means were compared using Duncan's multiple range tests at the 95% confidence interval (significance level at $p < 0.05$).

Results and discussion

Screening of the potential chelating agents for enhancing nisin antimicrobial activity

The antimicrobial activity of nisin and some chelating agents on *S.* Enteritidis ATCC 13076 and *M. luteus* IFO 12708 is represented by OD_{600} values (Fig. 1). When the nisin and chelating agents were individually used, nisin (2000 ppm) showed significant inhibitory effects on *M. luteus* IFO 12708. *M. luteus* is the most nisin-sensitive microorganism and is used as an indicator strain to study nisin activity (Wolf and Gibbons, 1996). However, nisin had no effect on the growth of *S.* Enteritidis ATCC 13076 ($p > 0.05$) when compared with the control (growth of each target strain in MHB without any agent). The results in this study showed that growth of *M. luteus* IFO 12708 was also significantly inhibited under all antimicrobial agent treatments. Various researchers have used nisin at different concentrations (25–1000 ppm) to inhibit *Salmonella* and have reported that nisin had no effect on the growth of *Salmonella* even at 1000 ppm (Elliason and Tatini, 1999; Yuste and Fung, 2004; Govaris et al., 2010; Lee and Kaletunç, 2010). However, the MIC value of nisin was reported as 8000 ppm against *Salmonella indica* by Selim et al. (2012).

Regarding the antimicrobial activity of individual chelating agents, EDTA (7400 ppm) showed complete inhibition of growth of *S.* Enteritidis ATCC 13076 when compared to the control ($p < 0.05$). When compared with previous reports, the concentration of EDTA used in this study was higher than the MIC value in Branen and Davidson (2004), who reported that the MIC value of EDTA against *S.* Enteritidis was only 2500 ppm, whereas citric acid (2000 ppm) and potassium sorbate (2000 ppm) had slight inhibitory effects on the growth of *S.* Enteritidis ATCC 13076 when compared with the control ($p < 0.05$). Similar results were observed for citric acid by Nazer et al. (2005), who found that 2882 ppm of citric acid showed 50% growth reduction of *Salmonella* Typhimurium and its MIC value was 5764 ppm. Norhana et al. (2012) used potassium sorbate at 30,000 ppm to control the growth of *Salmonella* in shrimps stored at 4 °C in vacuum packaging. The results showed that after 3 d storage, potassium sorbate reduced the number of *Salmonella* from 4.64 to 4.12 logCFU/g, whereas the MIC value of potassium sorbate against *S.* Typhimurium was 64,080 ppm (Nanasombat and Chooprang, 2009), which was higher than the concentration of potassium sorbate used in the current study.

Individual ascorbic acid, sodium acetate, sodium citrate and sodium had no effects on the growth of *S.* Enteritidis ATCC 13076 when compared with the control ($p > 0.05$). According to previous reports, the MIC values of ascorbic acid against *Salmonella typhi*, *S. indica* and *Salmonella suis* were 5000 ppm; 16,000 ppm and 32,000 ppm, respectively (Lacroix et al., 2004; Gao et al., 2012; Selim et al., 2012), which were higher than the concentration of ascorbic acid used in the current study. Milillo et al. (2011) and Pundir and Jain (2011) found that sodium acetate at 25,000 ppm had no inhibitory effect on the growth of *S.* Typhimurium. Additionally, Nanasombat and Chooprang, 2009 reported that the MIC values of sodium acetate against *Salmonella* Rissen and *S.* Typhimurium were 210,000 ppm and 105,000 ppm, respectively. Milillo et al. (2011) found that 25,000 ppm sodium citrate had no effect on the growth of *S.* Typhimurium.

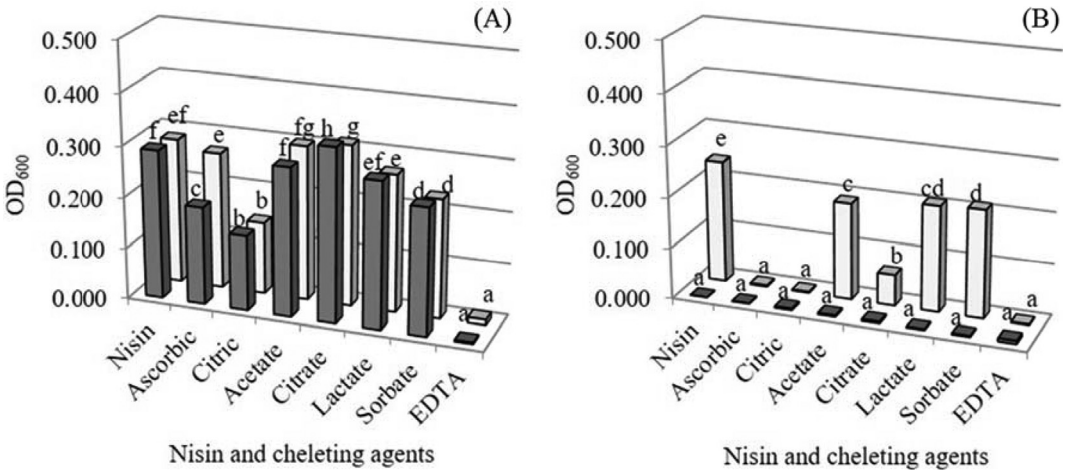


Fig. 1. Effect of target chelating agents as optical density measurement at 600 nm (OD_{600}) on enhancing antimicrobial activity of nisin in Mueller Hinton broth at 35 °C for 24 h against (A) *S.* Enteritidis ATCC 13076; (B) *M. luteus* IFO 12708; (EDTA = ethylenediamine tetraacetic acid disodium salt; mean values, $n = 3$, with different letters indicating a significant difference at $p < 0.05$; ■ + nisin; □-nisin).

When nisin was combined with each chelating agent, the treatment of nisin + ascorbic acid showed a significant reduction in the OD_{600} values of *S.* Enteritidis ATCC 13076 when compared to individual use. The synergistic effects of nisin with various chelating agents have been studied. Cutter and Siragusa (1995) compared the synergistic effect of nisin (50 ppm) in combination with sodium lactate (56,000 ppm), sodium citrate (29,000 ppm), and EDTA (18,000 ppm) to inhibit the growth of *S.* Typhimurium. All the combinations had higher inhibitory effects than those of the individual treatments of each agent. In particular, the nisin + EDTA showed the highest inhibitory effect on the target strain. These findings differed from other research. Ukuku and Fett (2004) compared the synergistic effect of nisin (50 ppm) in combination with EDTA (7400 ppm), sodium lactate (20,000 ppm), and potassium sorbate (200 ppm) against *Salmonella* in culture medium incubated at 36 °C every 3 h for 24 h. The treatment of nisin + EDTA showed a population increase of *Salmonella*, whereas the treatments of nisin + sodium lactate and nisin + potassium sorbate gradually reduced the population of *Salmonella*. Moreover, Cabo et al. (2005) compared the synergistic effect of nisin (1000 ppm) in combination with EDTA (3000 ppm) and ascorbic acid (500 ppm) against *Escherichia coli* in fish extracts stored at 20 °C for 24 h. The results showed that the nisin + EDTA had an inhibitory effect against *E. coli*, whereas the nisin + ascorbic acid had no inhibitory effect against *E. coli.*

Like other Gram-negative bacteria, the OM of *Salmonella* spp. prevents nisin from penetrating to the cytoplasmic membrane and so it is possible to increase the susceptibility to nisin by use of membrane-destabilizing agents, such as chelators (Vaara, 1992). The classical example of a chelator is EDTA, which is a sequestered metal ion from the LPS layer and increases cell sensitivity and permeability to nisin (Vaara, 1992). Besides EDTA, a number of organic acids and their salts are chelating agents as reported elsewhere (Cabo et al., 2005; Suksathit and Tangwatcharin, 2013). Davidson and Branen (2005) discussed antimicrobial efficacy and noted that the efficacy of an organic acid on microorganism inhibition depends on its pKa value, which is the pH at which 50% of the acid is dissociated. Only the undissociated form of an organic acid can pass through the cell membrane of a microorganism and at a lower pH, a more undissociated form of the acid is present. Therefore, an organic acid with high pKa values has more acid in the undissociated form and has a stronger antimicrobial efficacy.

The pKa values of the target chelating agents used in this study could be comparatively graded as sodium lactate (3.86) < ascorbic acid (4.10) < sodium acetate (4.73) < potassium sorbate (4.76) < citric acid/sodium citrate (5.41) < EDTA (10.7). These values corresponded well with the results of the current experiments, where EDTA had the highest antimicrobial efficacy against *S.* Enteritidis ATCC 13076 and the other chelating agents had slight or negligible antimicrobial effects. Previous researchers compared the antimicrobial activity of EDTA with some organic acids. Their results showed that EDTA caused significant leakage of UV-absorbing material from *S.* Typhimurium, whereas acetic acid, citric acid and lactic acid were inactive (Helander and Mattila-Sandholm, 2000). Moreover, when Norhana et al. (2012) compared the efficacy of EDTA and potassium sorbate on inactivating *Salmonella* recovered from inoculated shrimps, their results showed that *Salmonella* treated with EDTA had a lower amount of the target strain than did potassium sorbate. Lacroix et al. (2004) found that EDTA showed a 20% increase in irradiation sensitivity of *S.* Typhi, while ascorbic acid showed only a 1% increase in irradiation sensitivity of *S.* Typhi.

The concentration of nisin used in this work (2000 ppm) was higher than the permissible level for nisin. The maximum legal limit for nisin in RTE food in the USA is 10,000 ppm of Nisaplin® (Rulis, 2001), the maximum limits allowed by the European Commission and Codex Alimentarium Commission for use of nisin in ripened cheese and processed cheese is 500 ppm of Nisaplin® (European Food Safety Authority, 2006; Food and Agriculture Organization, 2015). The legal limit concentrations allowed by Codex Alimentarius Commission for EDTA and potassium sorbate in fish and fish products are 50–250 ppm and 2000 ppm, respectively. While no upper limits have been imposed for ascorbic acid, citric acid, sodium acetate, sodium citrate and sodium lactate, they are usually added in concentrations of not more than 2000 ppm. It was found that only citric acid (2000 ppm) showed inhibitory effect against *S.* Enteritidis ATCC 13076, but did not show any additive effect when combined with nisin (2000 ppm). In addition, the results from this study showed that sodium acetate, sodium citrate and sodium lactate all used individually and combined with nisin were not effective at nisin's maximum legally allowed concentration. Therefore, citric acid, sodium acetate, sodium citrate, sodium lactate and potassium sorbate were removed from the remaining parts of the study. Ascorbic acid (which showed an increased inhibitory effect when used in combination) and EDTA were used in the remaining study. Due to regulatory limits, the remaining study lowered the concentration of nisin, ascorbic acid and EDTA to 500, 2000 and 250 ppm, respectively.

Antimicrobial activity of nisin in combination with ascorbic acid and ethylenediamine tetraacetic acid disodium salt at legal limit concentrations.

In this study, nisin, ascorbic acid and EDTA singly and in combination were prepared in MHB to the final legal limit of concentration of the Food and Agriculture Organization and the European Food Safety Authority (500; 2000 and 250 ppm, respectively). The inhibitory effects of all treatments against *S.* Enteritidis ATCC 13076 and *M. luteus* IFO 12708 were represented by the viable cell number (Fig. 2). For *S.* Enteritidis ATCC 13076 inhibition, the mixture of nisin + ascorbic acid + EDTA showed the highest inhibitory effect with a reduction number of 3.41 logCFU ($p < 0.05$) when compared to the control (growth of target strain in MHB without any agent). The mixture of nisin + EDTA, which achieved a significant reduction in lowering OD_{600} values in the previous experiment, showed a significant inhibitory effect on *S.* Enteritidis ATCC 13076 with a reduction number of 1.45 logCFU ($p < 0.05$). In addition, the mixture of ascorbic acid + EDTA had a slight inhibitory effect on the growth of the target strain with a reduction number of 0.63 logCFU ($p < 0.05$), whereas the mixture of nisin + ascorbic acid (which resulted in a significant lowering OD_{600} values in our previous experiment) as well as the treatments of individual nisin, ascorbic acid and EDTA did not reduce the numbers of the target strain ($p > 0.05$). In contrast, the growth of *M. luteus* IFO 12708 was completely inhibited under all treatments.

Helander and Mattila-Sandholm (2000) suggested that nisin could not access the OM of Gram-negative bacteria. The surface of Gram-negative OM is normally anionic, whereas such cationic nisin molecules can bind the anionic OM and remain on its surface without access to the target at the OM. Therefore, nisin only attaches on the OM of Gram-negative bacteria. This phenomenon was supported by the unchanging uptake of the fluorescent dye-1-*N*-phenylnaphtlylamine (NPN) in OM of bacteria pretreated with nisin. The NPN uptake levels in nisin-treated strains were not different from those seen in untreated bacteria. Generally, the combination of nisin with EDTA allowed simultaneous attack on different targets that increased the sensitivity of the bacterial membrane. EDTA releases LPS from the OM of Gram-negative bacteria by chelating Ca and Mg salts that interact with the LPS layer. This allows nisin to reach the cytoplasmic membrane which is its target site. Although many previous researchers have confirmed

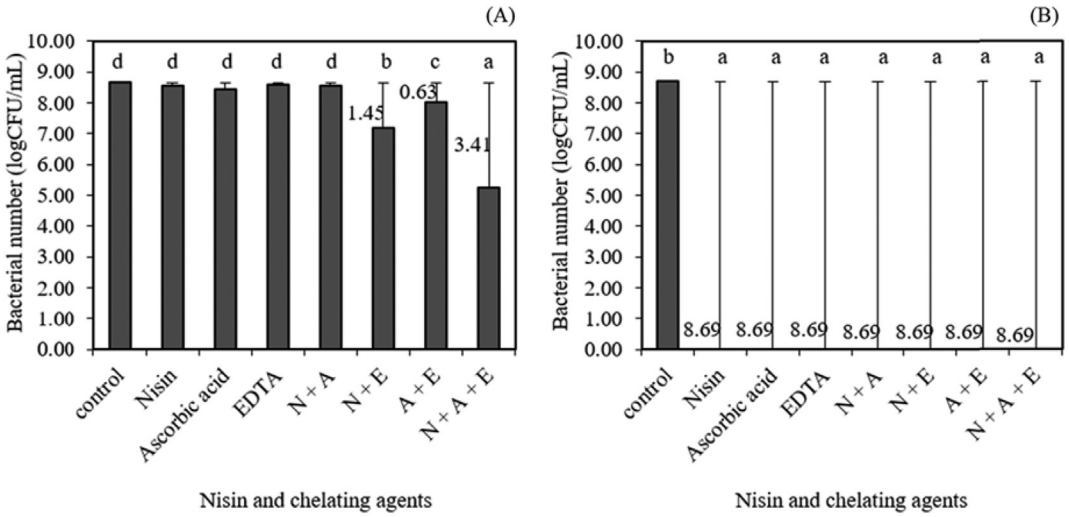


Fig. 2. Synergistic effects in Mueller Hinton broth at 35 °C for 24 h of nisin (N), ascorbic acid (A) and ethylenediamine tetraacetic acid disodium salt (EDTA and E) against: (A) *S.* Enteritidis ATCC 13076; (B) *M. luteus* IFO 12708; (mean values, $n = 3$ with different letters indicating significant difference at $p < 0.05$; numbers show the reduction of viable cells in log colony forming units (CFU) compared to the control).

that EDTA is able to enhance the antimicrobial activity of nisin, the current study found that the combination of nisin and EDTA resulted in only a 1.45 logCFU reduction of *S.* Enteritidis ATCC 13076. In agreement with the current study, Ukuku and Fett (2004) also found that EDTA (7400 ppm) in combination with nisin (50 ppm) had no effect on the growth of a five-strain cocktail of *Salmonella* (*Salmonella* Stanley, *Salmonella* Newport, *Salmonella* Anatum, *Salmonella* Infantis, *Salmonella* Poona). Additionally, Branen and Davidson (2004) and Khan et al. (2015) found that nisin + EDTA was synergistic against *E. coli*, but not *S.* Enteritidis. It is possible that the amounts of LPS released from the OM of each target strain differ.

The antimicrobial activity of ascorbic acid depended on the pH of environment. In general, ascorbic acid is most effective as an antimicrobial when it is in undissociated form which exists in the majority only at a pH of the environment below pKa (4.10) according to Buchanan et al. (1993) and Conner and Kotrola (1995). In the current study, the pH values of MHB with only ascorbic acid and with the combination of nisin, ascorbic acid and EDTA were 5.40 and 5.42, respectively, (data not shown) which was higher than the pKa value of ascorbic acid and caused the ascorbic acid to have no antimicrobial activity against *S.* Enteritidis ATCC 13076. Most researchers used ascorbic acid at concentrations higher than those in the current study (greater than 2000 ppm) with no change in bacterial growth. For example, Selim et al. (2012) reported that a concentration of ascorbic acid less than 32,000 ppm had no antimicrobial effect on *S.* Indica. Moreover, the combination of nisin and ascorbic acid had no antimicrobial activity on the growth of Gram-negative bacteria. Cabo et al. (2005) found that combination of nisin (1000 ppm) with ascorbic acid (500 ppm) had no synergistic effect on *E. coli*. Generally, the antimicrobial activity of ascorbic acid also depends upon the dose of ascorbic acid as previous study reported that ascorbic acid with low concentrations did not show any antimicrobial

Table 2
Results of central composite design and test of significance of model to estimate the effects of nisin, ascorbic acid and ethylenediamine tetraacetic acid disodium salt (EDTA) concentrations on growth of *S.* Enteritidis ATCC 13076.

Nisin	Ascorbic acid	EDTA	*S.* Enteritidis growth (%)		Model	Coefficient	*p*-value
(X_1)	(X_2)	(X_3)	actual	predicted			
−1	−1	−1	98.15	96.88	Constant	70.107	0.000
1	−1	−1	97.01	93.44	N	−12.481	0.000
−1	1	−1	96.59	100.67	A	−3.201	0.034
1	1	−1	79.68	78.35	E	−10.079	0.000
−1	−1	1	90.79	89.55	N^2	1.176	0.377
1	−1	1	68.61	61.96	A^2	6.860	0.000
−1	1	1	90.85	91.84	E^2	4.114	0.009
1	1	1	46.65	45.36	NA	−4.723	0.020
-α	0	0	97.18	94.42	NE	−6.041	0.005
α	0	0	46.05	52.44	AE	0.377	0.830
0	-α	0	88.56	94.89			
0	α	0	86.83	94.13		*F*	*p* > *F*
0	0	-α	98.70	98.70	Model	23.75	0.000
0	0	α	61.16	64.79	Lack of fit	4.50	0.062
0	0	0	69.11	70.11			
0	0	0	70.08	70.11			
0	0	0	65.97	70.11			
0	0	0	72.42	70.11	R^2	= 0.9553	
0	0	0	69.30	70.11	R^2-adjust	= 0.9151	
0	0	0	74.39	70.11			

activity against the tested strains (Abu-Ghazaleh, 2006). A possible explanation for this is that the tested strains utilized ascorbic acid. In contrast, at high concentration, ascorbic acid acts as a reducing agent, so it is able to reduce the bacterial growth by lowering the oxygen availability (Tajkarimi and Ibrahim, 2011). It is assumed that ascorbic acid modifies the outer membrane of bacterial cells, with a resulting loss in selective permeability, allowing other antimicrobial agents to reach their target site (Miller, 1969). Synergy from antimicrobial agent combination is an effect greater than that of the individual antimicrobial agents with an additional one $\log_{10}$ reduction compared to the sum of the effects of each antimicrobial agent (Lambert et al., 2003). Most antimicrobial agent combinations consist of two antimicrobial agents. In theory, the use of combinations of three antimicrobial agents should be more effective than combinations of two antimicrobial agents. The combination of nisin, ascorbic acid and EDTA used in the current study proved to be highly effective against *S.* Enteritidis ATCC 13076. This synergistic antimicrobial activity against *S.* Enteritidis ATCC 13076 can be explained by two key reactions: 1) binding of ascorbic acid on the nisin molecule is able to stabilize the nisin structure and the decrease of the pH in solution can increase the solubility of nisin (Adhikari et al., 2012); and 2) EDTA releases LPS from the OM of *S.* Enteritidis ATCC 13076 by chelating Ca and Mg salts that interact with the LPS layer. This allows nisin to reach the cytoplasmic membrane which is its target site (Helander and Mattila-Sandholm, 2000). The study of antimicrobial activity of a combination of nisin (25 ppm) and lysozyme (100 ppm) plus ascorbic acid (550 ppm), sodium diacetate (1000 ppm) or polyphosphate (5000 ppm) showed a 1–1.5 logCFU/mL reduction of *Listeria monocytogenes* in turkey slurries stored at 4 °C (Glass et al., 2005). Moreover, Norhana et al. (2012) showed a 0.58–0.81 logCFU/mL reduction of *Salmonella* inoculated in shrimps treated with nisin (500 ppm) and EDTA (7400 ppm) plus potassium sorbate (30,000 ppm), sodium benzoate (30,000 ppm) or sodium diacetate (30,000 ppm).

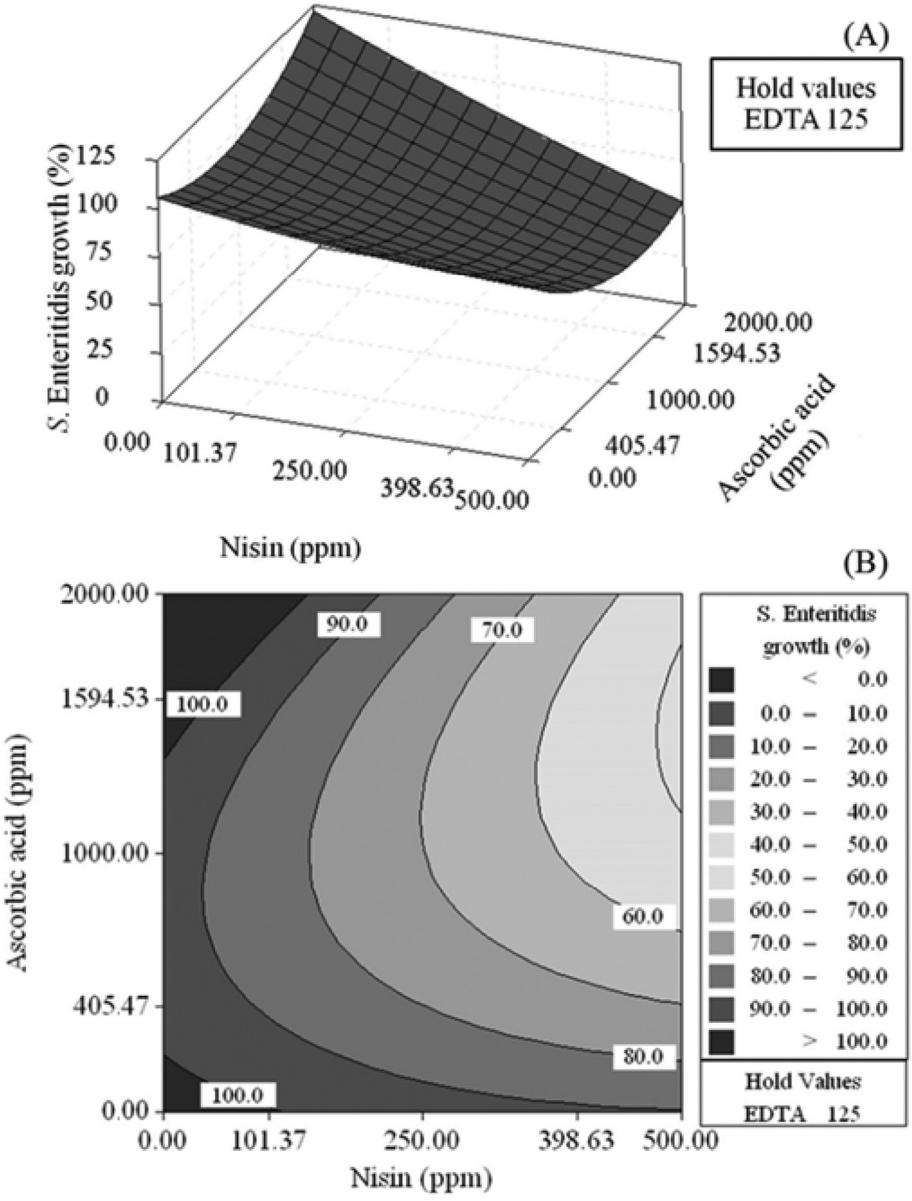


Fig. 3. Response surface and contour plot showing percentage growth of *S.* Enteritidis ATCC 13076 in Mueller Hinton broth as affected by nisin and ascorbic while keeping the concentration of ethylenediamine tetraacetic acid disodium salt at 125 parts per million (ppm).

Determination of the optimal mixing concentration of nisin and each target chelating agent by response surface methodology using central composite design

Previous studies developed an appropriate model by including nisin, other antimicrobial agents and environmental factors in the model. An example of one such model is that of Khan et al. (2015), which can be used to estimate the inhibition of *S.* Typhimurium and *L. monocytogenes* as a function of nisin, EDTA and the pH. Another model was proposed by Aouadhi et al. (2014), which can be used to predict the log cycle reduction of *Bacillus sporothermodurans* spores as a function of nisin, temperature and the temperature-holding time. Another example is the model of Cabo et al. (2005), which can be used to evaluate the total viable counts in refrigerated blue whiting fillets as a function of nisin, CO_2 and sodium metaphosphate. In contrast to previous studies that focused on combinations of two antimicrobial agents and environmental factors, the current study developed a model to estimate the growth of *S.* Enteritidis ATCC 13076 as a function of three antimicrobial agents—nisin, ascorbic acid, and EDTA.

A total of 20 experiments with different combination of nisin, ascorbic acid and EDTA were performed. The growth in the percentage of *S.* Enteritidis ATCC 13076 indicated the antimicrobial effect of three independent variables. The observed responses (growth in percentage of *S.* Enteritidis ATCC 13076) along with the predicted responses are summarized in Table 2. The results obtained were then analyzed using analysis of variance to determine the fit of the model, termed R^2 (the determination coefficient) which was 0.9553 (Table 2), indicating that the model equation could explain 95.53% of the variations and only 4.47% of the total variation could not be explained by the model. The model fit was further confirmed by the significant regression p-value ($p_{model} > F = 0.000$) and no significant ($p = 0.062$) lack of fit at the 95% confidence interval. When the model regression is significant, the terms which are

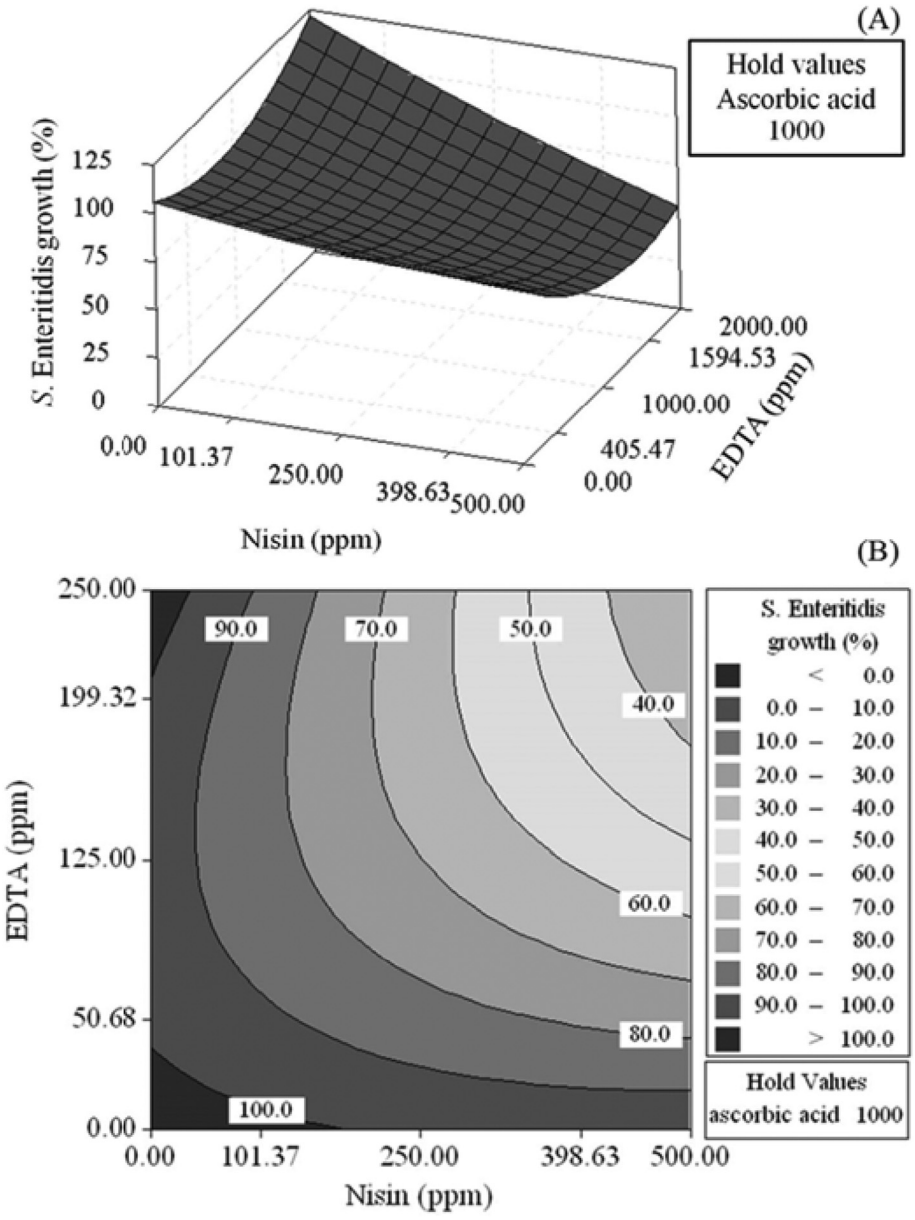


Fig. 4. Response surface and contour plot showing percentage growth of *S.* Enteritidis ATCC 13076 in Mueller Hinton broth as affected by nisin and ethylenediamine tetraacetic acid disodium salt while keeping the concentration of ascorbic acid at 1000 parts per million (ppm).

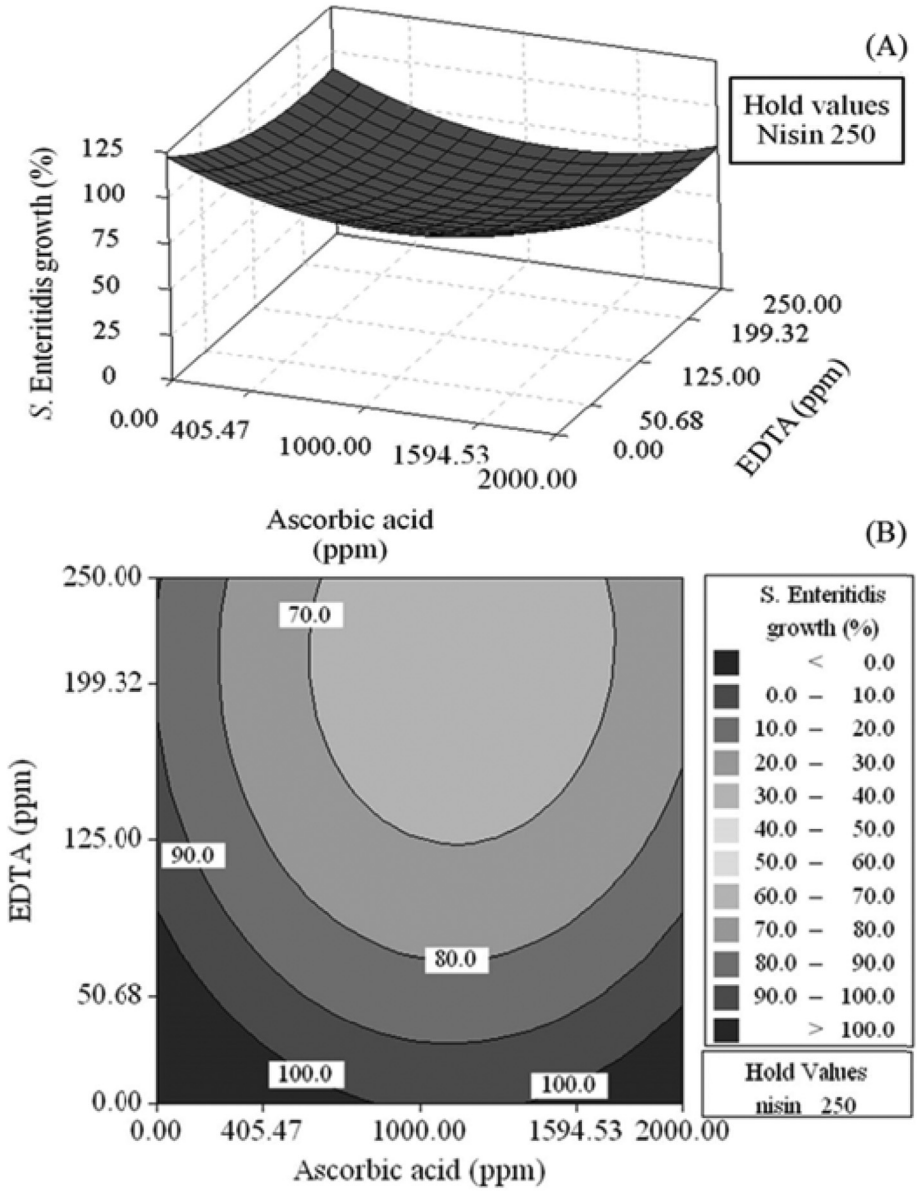


Fig. 5. Response surface and contour plot showing percentage growth of *S.* Enteritidis ATCC 13076 in Mueller Hinton broth as affected by ascorbic acid and ethylenediamine tetraacetic acid disodium salt while keeping the concentration of nisin at 250 parts per million (ppm).

individually non-significant are also considered in the equation (Singh et al., 2012). The effects of nisin (N), ascorbic acid (A) and EDTA (E) in terms of coded values on the growth of *S.* Enteritidis ATCC 13076 (SE growth) can be described by Equation (2):

$$\text{SE growth (\%)} = 70.107 - 12.481N - 3.201A - 10.079E + 1.176N^2 + 6.860A^2 + 4.114E^2 - 4.723NA - 6.041NE + 0.377AE \quad (2)$$

The significance of each coefficient was determined by evaluating the *F*-value and *p*-value. As shown in Table 2, N, E and A^2 were significant at the 99% probability level ($p < 0.01$), while E, E^2, NA and NE were significant at the 95% probability level ($p < 0.05$). N^2 and AE had no significant effect on the growth of *S.* Enteritidis ATCC 13076. The effects of the nisin, ascorbic acid and EDTA concentrations on the growth of *S.* Enteritidis ATCC 13076 were also illustrated in the three dimensional response surfaces and two dimensional contour plots. The effect of the nisin and ascorbic acid concentrations by keeping the EDTA concentration at 125 ppm is shown in Fig. 3. The growth of *S.* Enteritidis ATCC 13076 decreased with the increase in the nisin concentration. In contrast, both low and high concentrations of ascorbic acid (0 and 2000 ppm) resulted in increases in *S.* Enteritidis ATCC 13076. The lowest growth of *S.* Enteritidis ATCC 13076 was 47.91% at 500 ppm of nisin and 1499.41 ppm of ascorbic acid. The interaction of nisin and the EDTA concentration at a constant concentration of ascorbic acid (1000 ppm) is shown in Fig. 4. *S.* Enteritidis ATCC 13076 growth decreased with the increase in the nisin concentration. The growth of *S.* Enteritidis ATCC 13076 also decreased with the increase in the EDTA concentration at 500 ppm of nisin. In the absence of nisin (0 ppm), *S.* Enteritidis ATCC 13076 decreased with an increase in the EDTA concentration up to 127.23 ppm and increased thereafter. The lowest growth of *S.* Enteritidis ATCC 13076 was 30.04% at 500 ppm

of nisin and 250 ppm of EDTA. The growth of *S*. Enteritidis ATCC 13076 as a function of the ascorbic acid and EDTA concentrations at a fixed nisin concentration at 250 ppm is shown in Fig. 5. Both low and high concentrations of ascorbic acid (0 and 2000 ppm) resulted in an increase in the *S*. Enteritidis ATCC 13076 growth. By contrast, *S*. Enteritidis ATCC 13076 decreased with an increase in the EDTA concentration. The lowest growth of *S*. Enteritidis ATCC 13076 was 79.68% at 2000 ppm of ascorbic acid and 250 ppm of EDTA. In this study the linear coefficient of nisin was found to be higher than that of EDTA and ascorbic acid. As a result, nisin could be considered to be the most influential factor affecting the antimicrobial activity of a mixture solution against *S*. Enteritidis ATCC 13076. Bhatia and Bharti (2014) also found that nisin had the highest linear coefficient compared to EDTA and lysozyme when used to inhibit *L. monocytogenes* in a model system. Similarly, Branen and Davidson (2004) and Selim et al. (2012) concluded that an increase in the nisin concentration tended to slow the growth of *Salmonella*. The interaction coefficient of nisin + ascorbic acid and nisin + EDTA had a significant effect on the growth of the target strain, indicating that the antimicrobial effect of nisin increased with an increase in the ascorbic acid or EDTA concentrations. Bhatia and Bharti (2014) also found that EDTA seemed to stimulate the antimicrobial activity of nisin because the interaction coefficient of nisin-EDTA had a significant effect in the studied model. The antimicrobial effect as indicated by the quadratic coefficient of ascorbic acid and EDTA, was that both an increase and a decrease of their concentration resulted in an increase in *S*. Enteritidis ATCC 13076. The response surface also indicated that both low and high concentrations of ascorbic acid or EDTA resulted in an increase in *S*. Enteritidis ATCC 13076 inhibition. An increase in the ascorbic acid or EDTA concentration caused a decrease in the pH of the mixture (data not shown). The ionization of a metal chelator complex (such as ascorbic acid and EDTA) depends on the pH of mixture, so lowering the pH decreases the proportion of its ionized form, so the stability of ascorbic acid or EDTA complex decreases and cannot bind and replace the metal ions from the OM of Gram-negative bacteria, whereas the decrease of inhibition at an alkaline pH can be due to the low antimicrobial activity of nisin at this pH (Oyetade et al., 2012; Khan et al., 2015). The RSM results indicated that the maximum antimicrobial activity was obtained with concentrations of nisin, ascorbic acid and EDTA in the mixture of 500, 1515 and 250 ppm, respectively. Under these conditions, the growth of *S*. Enteritidis ATCC 13076 predicted by the model was 24.99%.

Although nisin has been shown to be effective at inhibiting the growth of Gram-negative bacteria when combined with EDTA, the reduction of the target strain was low. In theory, the combination of three antimicrobial agents could have higher antimicrobial activity than for a combination of two antimicrobial agents. The current study proved that a combination of nisin, ascorbic acid and EDTA had high antimicrobial activity against *S*. Enteritidis ATCC 13076 with the reduction number of 3.41 logCFU. Further optimization is needed to achieve the minimum growth of the target strain with the lowest concentration of each compound. RSM based on CCD was used to determine the effects of the concentration of nisin, ascorbic acid and EDTA on the growth of *S*. Enteritidis ATCC 13076. This study indicated that nisin + ascorbic acid and nisin + EDTA were very effective at inhibiting *S*. Enteritidis ATCC 13076. The optimum concentration was at concentrations of nisin, ascorbic acid and EDTA in the mixture at 500 ppm, 1515 ppm and 250 ppm, respectively. Under the above conditions, the growth of *S*. Enteritidis ATCC 13076 predicted by the model was 24.99%.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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