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# Combined Antimicrobial Effect of Nisin, Carvacrol and EDTA against *Salmonella* Typhimurium in TSBYE at 4°C and 37°C

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## Abstract

*In this study, the antimicrobial effect of low concentrations of nisin, carvacrol and EDTA alone and their combinations against S. Typhimurium SL1344 in TSBYE at 4°C and 37°C for 24 h were investigated. Carvacrol was found the most effective chemical among the single antimicrobial treatments. Carvacrol at 0.5 or 1 mM plus EDTA at 2 mM showed a synergistic effect at 37°C. This effect was increased when they were combined with nisin both at 4°C and 37°C. The most effective application was determined the combinations of nisin at 400 IU/mL, carvacrol at 1 mM and EDTA at 2 mM. This combination reduced S. Typhimurium counts to undetectable level after 12 and 6 h of incubation at 4°C and 37°C, respectively. The results of this study indicated that combinations of low concentrations of nisin, carvacrol and EDTA could potentially be used as food preservative against S. Typhimurium, to provide better quality foods.*

**Keywords**: *Salmonella Typhimurium, nisin, carvacrol, EDTA, antimicrobial activity*

## 1. Introduction

*Salmonella* is Gram-negative, non-spore forming, facultative anaerobe and rod-shaped bacteria that is a member of the family of Enterobacteriaceae (1). The genus *Salmonella* is divided into two species, *S. enterica* and *S. bongori*, based on the high sequence similarity of the genome. There are six main subspecies under *S. enterica* species namely *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*, whereas no subspecies has been assigned to *S. bongori* species (2). These subspecies are further subdivided into serovars that are over 2500 recognized serovars characterized by their flagella, carbohydrate and lipopolysaccharide (LPS) structures (3, 4). *Salmonella* is an important pathogen for both humans and animals. This bacterium is one of the leading causes of gastrointestinal disease (non-typhoidal) in both developing and developed countries (3). *Salmonella* is not only responsible of a large number of illnesses but also there is a cost associated with these outbreaks. The cost for salmonellosis in the United States was estimated to range from $600 million to $3.5 billion each year (5). *S. enterica* subsp. *enterica* serovar Typhimurium (called *S.* Typhimurium) is broad-host range serovar that causes gastrointestinal disease in humans for this reason it is important to public health (1, 4, 6). Nowadays, consumers demand natural and chemical additives-free food products, but that are free from pathogens, therefore the food industry look for novel and alternative strategies to food biopreservation (7). For this reason, there has been an increasing interest in the search for natural antimicrobials such as organic acids, plant essential oils, enzymes obtained from animal sources, naturally-occurring polymers and bacteriocins from lactic acid bacteria (8). Nisin is a class I bacteriocin, lantibiotic, produced by certain strains of *Lactococcus lactis*. Nisin is a hydrophobic and cationic polypeptide that exhibits a broad-spectrum of inhibitory activity against Gram-positive bacteria and their spores, but shows little or no activity against Gram-negative bacteria, yeasts and fungi. Nisin is generally

recognized as safe (GRAS status) by the US Food and Drug Administration and World Health Organization since 1969 and is permitted currently for use as a food preservative in over 50 countries (9). The primary target of nisin is the cytoplasmic membrane of vegetative cells. Nisin forms pores that collapse the proton motive force and the pH equilibrium, causing leakage of ions and hydrolysis of ATP, resulting in the death of the cell (10). Normally, Gram-negative bacteria are resistant to nisin because of their LPS layer. On the other hand, use of nisin in conjunction with EDTA (9) or plant essential oils (11, 12) may increase the effectivness. Carvacrol is a monoterpenoid phenol that presents in the essential oil fraction of oregano, thyme, pepperwort and Alaska yellow cedar (13). Carvacrol exhibits inhibitory activity against Gram-negative bacteria that increasing the cell membrane permeability to ATP and ions (14, 15). Carvacrol is a GRAS flavoring that is typically used in the minimum concentration required to produce the intended effect (16). Carvacrol exhibits a strong antibacterial effect, but its application in foods has been limited by its strong flavor (17). Ethylenediamine tetraacetic acid (EDTA) is a chelator that is used in a wide variety of food products either as a preservative or as an inhibitor of product discoloration. EDTA can increase the inhibitory effects of some antimicrobial substances such as nisin, lysozyme and monolaurin against Gram-negative bacteria by disrupting the outer membrane protein layer of these organisms (18). Although the synergistic antimicrobial effect of nisin with EDTA or carvacrol on the viability of different pathogenic bacteria has been observed before, there are not enough data on synergistic antimicrobial activity of these three antimicrobials. This survey is the first report of the synergistic activity of these three antimicrobials against *Salmonella* Typhimurium at 4°C and 37°C. The purpose of the present investigation was to determine the antimicrobial effect of low concentrations of nisin, carvacrol, EDTA alone or in combination against *Salmonella enterica* serovar Typhimurium SL1344 in TSBYE at 4°C and 37°C.

## 2. Material and Methods

**2.1. Bacterial strain** *Salmonella enterica* serovar Typhimurium SL1344 strain used in this study was obtained from the Laboratory of Microbial Gene Technology, NLH, Ås, Norway. The SL1344 was routinely grown in tryptone soy broth (TSB, LAB M Ltd, Lancashire, United Kingdom) supplemented with 0.5% (w/v) yeast extract (Merck, Darmstadt, Germany) (TSBYE) for 24 h at 37°C. Stock culture of SL1344 was stored at -20°C in TSBYE with added 20% sterile glycerol. For cell suspension preparation, the stock culture of SL1344 was grown in TSBYE for 24 h at 37°C, with two consecutive transfers to obtain a population of approximately $10^9$ CFU/mL. The bacterial cells were centrifuged at 12.000 rpm for 5 min and then washed twice in 1 mL of 0.1 M phosphate-buffered saline (PBS, pH 7.0). Initial cell counts were confirmed by tenfold serial dilution and then enumerated on tryptone soy agar supplemented with 0.5% (w/v) yeast extract (TSAYE). The cell suspension of *Salmonella* Typhimurium SL1344 was inoculated into TSBYE approximately $10^4$ log CFU/mL (12).

**2.2 Preparation of nisin, carvacrol and EDTA stock solutions** Nisin and carvacrol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). EDTA was obtained from Riedel-de Haën AG (Seelze, Germany). Nisin and EDTA stock solutions were prepared according to ALVAREZ & al. (16). Stock solutions of nisin (50.000 IU/mL) and EDTA (200 mM) were prepared in deionized water. Nisin and EDTA stock solutions were sterilized by passing them through a 0.45 μm membrane filter (Whatman Schleicher & Schuell FD 30/0.45 CA-S, Dassel, Germany). Nisin and EDTA stock solutions were stored at

-20°C and 4°C, respectively. Carvacrol stock solution (1 M) was made in 95% ethanol and stored at 4°C until used (19).

**2.3. Antibacterial activity in TSBYE** For determination of antimicrobial activity of nisin, carvacrol and EDTA against *S.* Typhimurium SL1344 were used in alone or 22 combinations. The flow chart and treatment diagram of antimicrobial system application protocols were given in Figure 1. Nisin, carvacrol and EDTA were added to TSBYE from stock solutions to obtain the desired concentrations. Following inoculation with the *S.* Typhimurium SL1344 cells, broth samples of all examined treatments were incubated at 4°C or 37°C for 24h. Sampling for microbiological analysis of inoculated broth was performed after 0, 6, 12, 24 h of incubation. The counts of bacterial survivors in TSBYE were detected by direct plating method. Serial dilutions of TSBYE samples were prepared with sterile saline (0.85%, w/v). Duplicate of 0.1 mL of appropriate dilution were inoculated onto TSAYE. Survivor counts were enumerated after incubation at 37°C for 24 h.

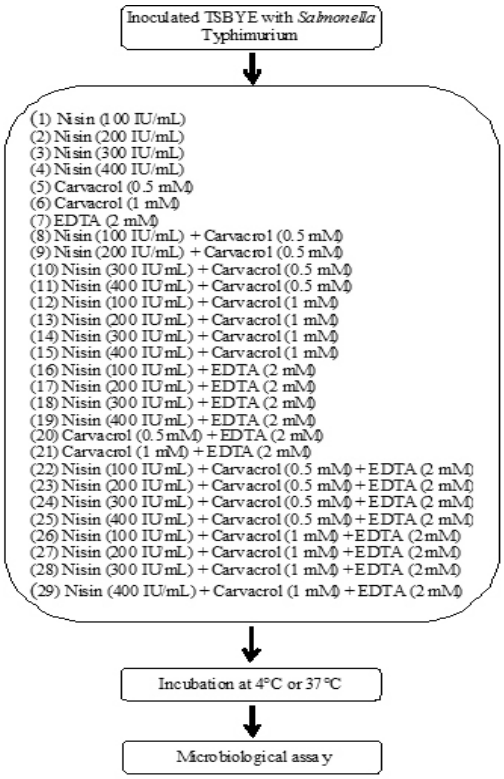



**Figure 1.** The flow chart and treatment diagram of antimicrobial system application protocols

**2.4. Statistical analysis** Microbiological analyses were performed in triplicate. Colony counts were transformed to log values before analyses of repeated measurements ANOVA and Tukey's test were used. Mean levels of colony counts at 0. hour were included in the analysis as a covariant. Significance was expressed at $P \leq 0.05$.

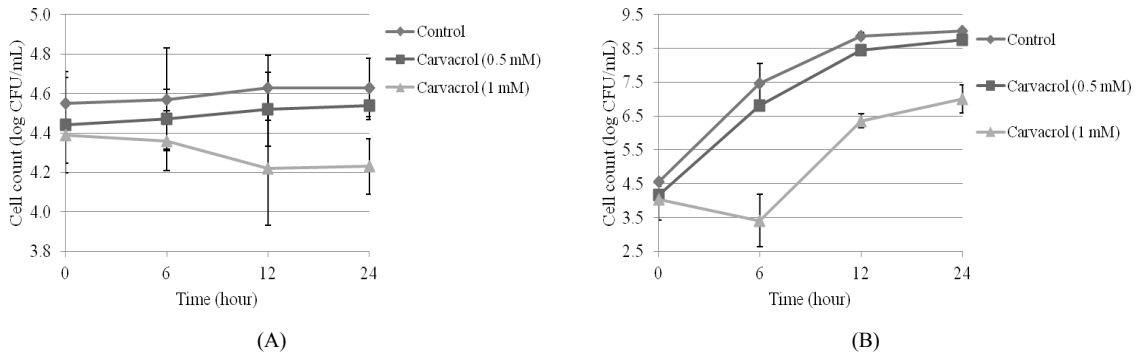
## 3. Results and Discussion

The initial populations of *Salmonella* Typhimurium SL1344 in all control samples (without addition of any antimicrobial) used in this study were not significantly changed during the incubation time at 4°C. In contrast at 37°C, the initial populations of the *S.*

Typhimurium SL1344 in all control samples were significantly increased ($P$>0.05) at the end of the incubation period.

The antimicrobial effect of nisin on *S.* Typhimurium SL1344 in TSBYE at 4°C and 37°C for 24 h incubation period were tested. At 4°C, nisin treated samples at 100, 200, 300 or 400 IU/mL presented population of *S.* Typhimurium SL1344 strain were not significantly different than those of control samples throughout the incubation period. During incubation at 37°C, samples treated with nisin at 200, 300 or 400 IU/mL were significantly different than those of controls and treated with nisin at 100 IU/mL after 6 h of incubation. Compared with the control, samples treated with nisin at 200, 300 or 400 IU/mL reduced *S.* Typhimurium SL1344 counts by approximately 0.1, 0.14 and 0.29 log CFU/mL after 6 h of incubation, respectively. On the other hand, all nisin treated samples were not significantly different ($P$ 0.05) than control samples after 12 and 24 h of incubation at 37°C. According to our results, there is no significant antibacterial activity of nisin at 100, 200, 300 or 400 IU/mL against *S.* Typhimurium SL1344 in TSBYE at 4°C and 37°C after 24 h of incubation. Our findings are consistent with previous observations that nisin shows little or no activity against Gram-negative bacteria (9, 20-24). The outer membrane of Gram-negative bacteria acts as a barrier to the activity of the nisin on the cytoplasmic membrane (10).

The antimicrobial effect of carvacrol at 0.5 or 1 mM on *S.* Typhimurium SL1344 in TSBYE at 4°C and 37°C are shown in Figure 2. During incubation at 4°C, samples treated with carvacrol at 0.5 or 1 mM were not exhibited significantly different ($P$>0.05) than control samples. Conversely, the initial populations of *S.* Typhimurium SL1344 treated with carvacrol at 1 mM were showed significantly different ($P$>0.05) than control and samples treated with carvacrol at 0.5 mM at 37°C. The populations of *S.* Typhimurium SL1344 in the samples treated with carvacrol at 1 mM showed an initial decrease to 3.41 log CFU/mL after 6 h of incubation and then increased, reaching ca 6.36 log CFU/mL and 7.01 log CFU/mL after 12 and 24 h of incubation, respectively. Compared with the control, 1 mM of carvacrol reduced *S.* Typhimurium SL1344 counts by approximately 4.05, 2.51 and 2.0 log CFU/mL by 6, 12 and 24 h of incubation, respectively. A number of studies have shown the antibacterial effect of different concentration of carvacrol against Gram-negative bacteria (25-27), as confirmed in this study. JONHY & al. (25) reported that 10, 50 and 75 mM of carvacrol showed the antibacterial effect on *Salmonella* Enteritidis inoculated at approximately $10^7$ CFU/mL in chicken cecal contents. Compared with the control, carvacrol at 10 mM reduced *S.* Enteritidis counts by approximately 3.4 log CFU/mL by 8 h of incubation. Carvacrol at 50 and 75 mM decreased *S.* Enteritidis counts to <1.0 log CFU/mL at 15 s after addition of the molecules.




(A) (B)

**Figure 2.** Effect of carvacrol at 0.5 or 1 mM on *S.* Typhimurium SL1344 in TSBYE at 4°C (A) and 37°C (B)

Samples treated with EDTA at 2 mM did not show significantly different ($P$>0.05) than control samples at 4°C. However, the population of *S.* Typhimurium SL1344 in the 2 mM of EDTA added samples were found significantly lower ($P$>0.05) than control samples during the incubation period at 37°C. The results of this study showed that EDTA treated samples were grown slowly than control samples at 37°C. Compared with the control, 2 mM of EDTA reduced *S.* Typhimurium SL1344 counts by approximately 1.41 and 2.56 log CFU/mL after 6 and 12 h of incubation at 37 °C, respectively. On the other hand, the initial populations of *S.* Typhimurium SL1344 ca 4.73 and 4.60 log CFU/mL in control and EDTA treated samples reached ca 8.96 and 8.29 log CFU/mL at the end of the incubation period, respectively. According to present findings, when *S.* Typhimurium was grown in the presence of 2 mM EDTA at 37°C, significant difference was observed regarding control samples, even though this difference was small after 24 h of incubation. EDTA binds divalent cations notably $Mg^{+2}$ that stabilize molecular interactions in the outer membrane of Gram-negative bacteria, which results in a loss of lipopolysaccharides and lipids from the outer membrane (28). Antibacterial activity of EDTA against some Gram-negative bacteria was shown by different researchers (29-31). Furthermore, PRUDÊNCIO & al. (31) reported that *S.* Typhimurium cells treated with low concentration of EDTA (1.6 mM/L) grown slowly than the control sample without addition of EDTA, as confirmed in this study.

During incubation at 4°C, the effects of combinations of nisin (100, 200, 300 or 400 IU/mL) and EDTA (2 mM) on *S.* Typhimurium SL1344 not showed significantly different ($P$<0.05) than control samples. In contrast at 37°C, the initial populations of the *S.* Typhimurium SL1344 treated with combinations of nisin and EDTA was found significantly different ($P$<0.05) than control samples. The combinations of nisin and EDTA reduced *S.* Typhimurium SL1344 counts by approximately 2.0 log CFU/mL after 6 h of incubation as compared to control ($P$<0.05). In addition, samples treated with combinations of nisin and EDTA reduced *S.* Typhimurium SL1344 counts by approximately range from 0.7 to 0.96 log CFU/mL after 24 h of incubation, as compared to control ($P$<0.05). These reduction rates are similar to the results of EDTA at 2 mM used alone. EDTA can increase the susceptibility of the cells to other antimicrobial agents such as nisin by increasing the permeability of bacterial cellular membranes (32). However, in this study, we did not find any significant effects of nisin combined with EDTA against the growth of *S.* Typhimurium, as confirmed by CUTTER & SIRAGUSA (33) and TU & MUSTAPHA (34).

The antimicrobial effects of the combinations of nisin (100, 200, 300 or 400 IU/mL) and carvacrol at 0.5 mM on *S.* Typhimurium SL1344 at 4°C and 37°C after 24 h of incubation not showed significantly different ($P$ 0.05) than control samples. On the other hand, samples treated with combinations of nisin (100, 200, 300 or 400 IU/mL) and carvacrol at 1 mM were significantly different ($P$<0.05) than those of controls at 4°C and 37°C. When nisin (100, 200, 300 or 400 IU/mL) and carvacrol at 1 mM were combined, synergistic effects were observed at 4°C. During incubation at 4°C, samples treated with combinations of nisin (100, 200, 300 or 400 IU/mL) and carvacrol at 1 mM reduced *S.* Typhimurium SL1344 counts by approximately range from 0.39 to 0.89 log CFU/mL after 24 h of incubation, as compared to control ($P$<0.05). On the other hand, the combinations of maximum nisin level (400 IU/mL) and carvacrol at 1 mM reduced *S.* Typhimurium SL1344 counts by approximately 3.73, 2.51 and 1.19 log CFU/mL by 6, 12 and 24 h of incubation at 37°C, respectively (Figure 3). These reduction rates are similar to the results of carvacrol at 1 mM used alone. In this study, when nisin (100, 200, 300 or 400 IU/mL) and carvacrol at 1 mM were combined, synergistic effects were not observed at 37°C. On the other hand, the synergistic antimicrobial effect of nisin and

carvacrol on the viability of Gram-positive bacteria such as *Listeria monocytogenes* and *Bacillus cereus* has been observed before by different researchers (35, 36).

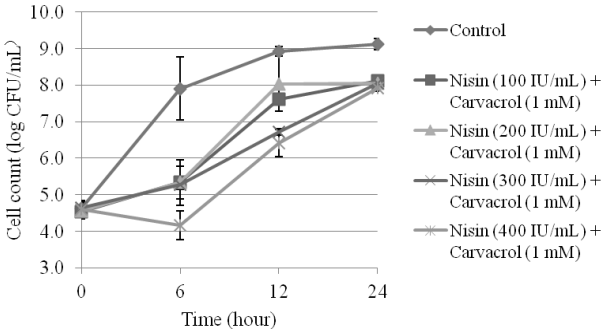



**Figure 3.** Effect of different concentration of nisin at 100, 200, 300 or 400 IU/mL and carvacrol at 1 mM on *S.* Typhimurium SL1344 in TSBYE at 37°C

The antibacterial effects of the combinations of carvacrol at 0.5 or 1 mM and EDTA at 2 mM on *S.* Typhimurium SL1344 in TSBYE at 4°C and 37°C are shown in Figure 4. During incubation at 4°C, the effects of the combinations of carvacrol at 0.5 mM and EDTA on *S.* Typhimurium SL1344 not showed significantly different ($P$ 0.05) than control samples. In contrast, the initial population of the *S.* Typhimurium SL1344 treated with the combinations of carvacrol at 1 mM and EDTA was found significantly different ($P$ 0.05) than control samples. Compared with the control, the combinations of carvacrol at 1 mM and EDTA reduced *S.* Typhimurium SL1344 counts by approximately 1.48 log CFU/mL by 24 h of incubation. During incubation at 37°C, the effects of the combinations of carvacrol at 0.5 or 1 mM and EDTA on *S.* Typhimurium SL1344 showed significantly different ($P$ 0.05) than control samples. When both antimicrobials were combined, synergistic effects were observed at 37°C. The effect of the combinations of carvacrol at 1 mM and EDTA was found more effective than combinations of carvacrol at 0.5 mM and EDTA. During incubation at 37 °C, the initial populations of the *S.* Typhimurium SL1344 (ca 4.96 log CFU/mL) in control strains were significantly increased ($P$ 0.05) and reached ca 9.26 log CFU/mL after 24 h of incubation. Conversely, the combinations of carvacrol at 1 mM and EDTA decreased *S.* Typhimurium SL1344 counts to 2.35, 1.41 and 0.67 log CFU/mL after 6, 12 and 24 h of incubation. Compared with the control, this combination reduced the initial populations of *S.* Typhimurium SL1344 by approximately 8.59 log CFU/mL after 24 h of incubation. Similar to our results, ZHOU & al. [17] reported that combinations of carvacrol and EDTA significantly reduced populations of *Salmonella* Typhimurium.

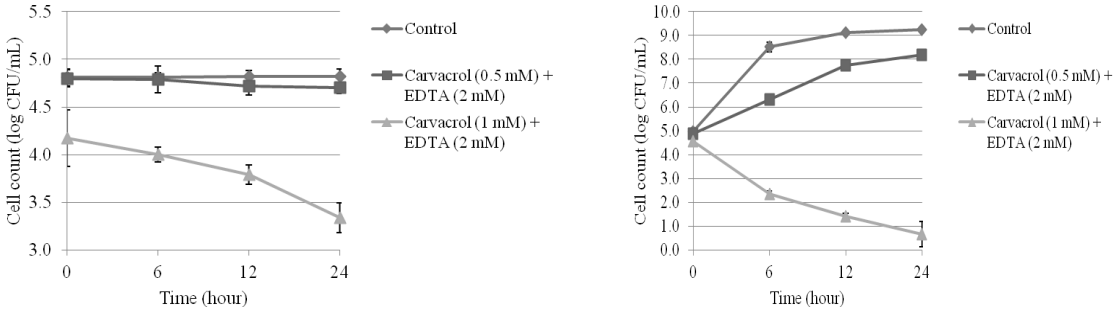



**Figure 4.** Effect of different concentration of carvacrol at 0.5 or 1 mM and EDTA at 2 mM on *S.* Typhimurium SL1344 in TSBYE at 4°C (A) and 37°C (B)

The antimicrobial effects of the combinations of nisin at 100, 200, 300 or 400 IU/mL, carvacrol at 0.5 mM and EDTA at 2 mM on *S.* Typhimurium SL1344 at 4°C after 24 h of incubation not showed significantly different ($P$ 0.05) than control samples. In contrast at 37°C, samples treated with the combinations of nisin, carvacrol and EDTA were significantly different than those of controls ($P$ 0.05). When nisin (100, 200, 300 or 400 IU/mL), carvacrol (0.5 mM) and EDTA were combined, synergistic effects were observed at 37°C but the inhibitory effects of these combinations against *S.* Typhimurium were low. Compared with the control, the maximum level (400 IU/mL) nisin added combination reduced *S.* Typhimurium SL1344 counts by approximately 2.76, 2.36 and 1.59 log CFU/mL by 6, 12 and 24 h of incubation at 37°C, respectively (data not shown).

The antibacterial effects of the combinations of nisin at 100, 200, 300 or 400 IU/mL, carvacrol at 1 mM and EDTA at 2 mM on *S.* Typhimurium SL1344 in TSBYE at 4°C and 37°C are shown in Figure 5. When three antimicrobials were combined, synergistic effects were observed at 4°C and 37°C. During incubation at 4°C, all combinations decreased *S.* Typhimurium counts to undetectable level after 24 h of incubation. In these treatments, inhibition of *Salmonella* cells was increased depending on increasing nisin concentrations. The 400 IU/mL nisin added combination was found more effective than other combinations at 4 °C. The 400 IU/mL nisin, 1 mM carvacrol and 2 mM EDTA concentration reduced *S.* Typhimurium SL1344 counts to 1.05 log CFU/mL and undetectable level after 6 and 12 h of incubation at 4°C, respectively. During incubation at 37°C, 300 and 400 IU/mL nisin added combinations were found more effective than 100 and 200 IU/mL nisin added combinations. The 300 and 400 IU/mL nisin added concentrations reduced *S.* Typhimurium SL1344 counts to 0.33 log CFU/mL and undetectable level at 6 h after addition of the molecules at 37°C, respectively.

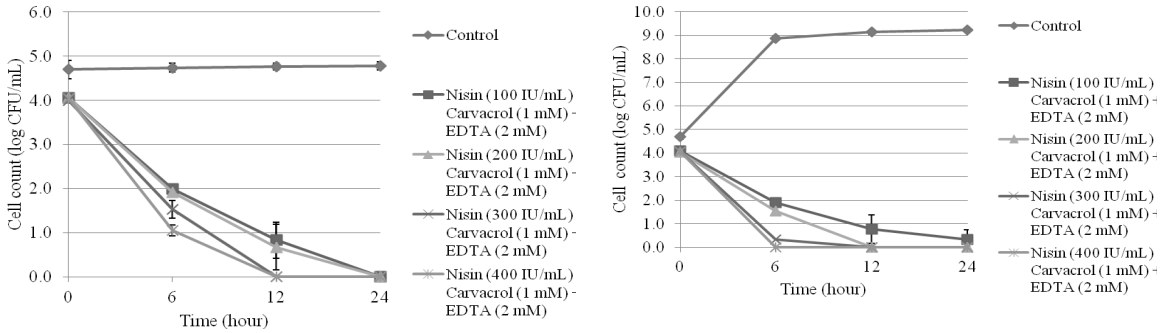



**Figure 5.** Effect of different concentration of nisin at 100, 200, 300 or 400 IU/mL, carvacrol at 1 mM and EDTA at 2 mM on *S.* Typhimurium SL1344 in TSBYE at 4°C (A) and 37°C (B)

## 4. Conclusions

In conclusion, nisin treatments alone not showed the significant antimicrobial effect against *S.* Typhimurium. Carvacrol was found the most effective chemical among the single antimicrobial treatments. Carvacrol and EDTA exhibited the synergistic effect against *S.* Typhimurium. When nisin, carvacrol and EDTA were combined, the growth of *S.* Typhimurium was inhibited at 4 °C and 37 °C, and a synergistic effect was observed. The most effective application against *S.* Typhimurium was determined the combinations of nisin at 400 IU/mL, carvacrol at 1 mM and EDTA at 2 mM both at 4 °C and 37 °C. The results of present investigation showed that low concentration of nisin, carvacrol and EDTA

combinations could potentially be used as food preservative against *S.* Typhimurium, intended to provide better quality foods.

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