PAPER

DIPPING SOLUTIONS OF NISIN AND BUFFERED SODIUM CITRATE SUPPLEMENTED WITH SODIUM DIACETATE FAIL TO PREVENT GROWTH OF *LISTERIA MONOCYTOGENES* ON VACUUM PACKAGED BEEF FRANKFURTERS STORED AT 4 AND 10°C UNDER MODEL CONDITIONS

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ABSTRACT

This study evaluated commercially available dip solutions of nisin alone, buffered sodium citrate combined with sodium diacetate (BSCSD), and combined solution of the three antimicrobials for potential to control growth of surface-inoculated *Listeria monocytogenes* during vacuum packaged refrigerated storage of model beef frankfurters. None of the treatments prevented growth of *L. monocytogenes* during frankfurters storage. The combined treatments slowed growth of *L. monocytogenes* better than individual treatments. Failure to completely eliminate *L. monocytogenes* on frankfurters or inhibit outgrowth during storage might be attributed to high initial *L. monocytogenes* inoculum levels, insufficient quantities of antimicrobials to interact with all the target cells, low nisin activity at high pH, or presence of resistant subpopulations, such as nisin-resistant strains. The model conditions used in the experimental setup, such as elimination of natural microbiota from frankfurters and nutrient-rich diluent used for Listeria introduction on the surface, could also contributed to enhanced survival and growth of the pathogen.

Keywords: ready-to-eat meats, *Listeria monocytogenes*, nisin, sodium citrate, sodium diacetate, refrigerated storage

1. INTRODUCTION

Recontamination of cooked ready-to-eat (RTE) meat products with *L. monocytogenes* is a major food safety concern. In the U.S.A., *L. monocytogenes* caused an estimated 2,500 cases of foodborne illnesses with 20% mortality annually (MEAD *et al.,* 1999). A study performed over a decade later (SCALLAN *et al.*, 2011) also listed *L. monocytogenes* as the third leading cause of death due to foodborne illnesses at 19% mortality rate, only behind nontyphoidal *Salmonella* spp. and *Toxoplasma gondii*. In addition, a significant annual monetary loss is incurred by the food industry (USDA-ERS, 2014). U.S.-based food industries were forced to recall food products due to possible *L. monocytogenes* contamination on seven different occasions in December, 2018 alone (US FDA, 2018). The national health plan of reducing listeriosis cases to 0.25 person per 100,000 population has not yet been achieved since 2005 (BUCKNER, 2008). *L. monocytogenes* can survive under normally limiting and extreme physicochemical conditions (RYSER and DONNELLY, 2001). Moreover, *L. monocytogenes* is ubiquitous in nature. Though *L. monocytogenes* is susceptible to cooking, i.e. temperatures above 70°C (LUNDEN *et al.,* 2003), post-processing recontamination during cooked frankfurter cooling, case splitting, and packaging is difficult to avoid. At present, RTE meat facilities inspected by the U.S. Food Safety and Inspection Service (FSIS) operate under a 'zero tolerance' policy, which means any food contaminated with detectable levels of *L. monocytogenes* is deemed adulterated (USDA-FSIS, 2014). Therefore, hurdles are required to prevent initial contamination and inhibit growth of this pathogen during storage. One such hurdle is to use post-cooking application of antimicrobials to prevent growth of *L. monocytogenes*, which is accepted as one of three alternatives to control *L. monocytogenes* (U.S. Code of Federal Regulations, Title 9, Part 430, Section 430.4)

There are many potential methods to prevent spoilage and pathogenic bacteria from growth on RTE meats (TOKARSKYY and MARSHALL, 2010). For example, salts of organic acids (lactates, acetates, diacetates, sorbates, and benzoates) have been studied as antilisterial agents on meat products (BARMPALIA *et al.,* 2005; SAMELIS *et al.,* 2005; SIVAROOBAN *et al.,* 2007; STOPFORTH *et al.,* 2005; ZHU *et al.,* 2005). Activity of these compounds depends on agent concentration, product composition (pH, water activity, fat, nitrite, and salt content), storage temperature and packaging atmosphere (CLEVELAND *et al.,* 2001; GEORNARAS *et al.,* 2006a; LUNDEN *et al.,* 2003; MARTINIS *et al.,* 1997; NILSSON *et al.,* 1997). The contamination level of the food product is another factor that influences the activity of these antimicrobials (BEDIE *et al.,* 2001; WEDERQUIST and SOFOS, 1994).

Nisin is a Generally Recognized as Safe (Federal Register, 1988) bacteriocin that is produced by *Lactococcus lactis* subsp. *lactis* (NAIDU, 2000). Nisin has greatest antimicrobial activity in the pH range of 3.0-3.5 (FANG and LIN, 1994) with rapid activity loss at greater pH values (MONTVILLE and CHEN, 1998). Therefore, it is recommended to use nisin at a pH not greater than 5.5, especially in the presence of sodium chloride and nitrite in meat products (MARTINIS *et al.,* 1997; UKUKU and SHELEF, 1997). Studies have shown that nisin is effective in reducing *L. monocytogenes* counts on RTE meat products (DELVES-BROUGHTON, 2005; FANG and LIN, 1994; TOKARSKYY and MARSHALL, 2008; UKUKU and SHELEF, 1997). However, the use of nisin has been limited in food products due to the potential for emergence of nisin-resistant strains of *L. monocytogenes* (CRANDALL and MONTVILLE, 1998; LIU *et al.,* 2002). To overcome this problem, nisin has been combined with other treatments to achieve antilisterial effects (DELVES-

BROUGHTON, 2005; GEORNARAS et al., 2006a; SAMELIS *et al.*, 2005; TOKARSKYY and MARSHALL, 2008; ZHU *et al.*, 2005).
Buffered sodium citrate combined with sodium diacetate (BSCSD) is a mixture of citric acid, sodium citrate, and sodium diacetate (HULL, 2007). Buffered sodium citrate (BSC) is approved in the US for cured (9 CFR 318.7 [c] (4)) and uncured (9 CFR 381.7 [f] (4)) processed whole-muscle meat and poultry products (USDA-FSIS, 1996). BSC is used to enhance flavor in meat and poultry products and the recommended usage level is 1.0 to 1.3% by weight of total formulation (USDA-FSIS, 1996). In muscle-food products, BSC increases ionic strength, which in turn increases water holding capacity, lowers water activity, and causes less purge loss (CEYLAN *et al.*, 2003; HULL, 2007). Sodium diacetate is primarily used as flavor enhancer in meat products at no more than 0.25% of product formulation (USDA-FSIS, 2000). Sodium diacetate has been reported to have antilisterial properties in combination with lactates or nisin in meat and poultry products (SAMELIS *et al.*, 2002; SAMELIS *et al.*, 2005). BSCSD has been shown to be effective in controlling germination and outgrowth of *Clostridium perfringens* during cooling of cooked meat and poultry products (THIPPAREDDI *et al.*, 2003) and a single study showed possibility for inhibiting *L. monocytogenes* on beef frankfurters (CEYLAN *et al.*, 2003).
These antimicrobials have been used as individual solutions (PATEL *et al.*, 2006; SAMELIS *et al.*, 2005) or as separate solutions applied sequentially to food surfaces (GEORNARAS *et al.*, 2006a; GEORNARAS *et al.*, 2006b). Most previous studies have used salts of organic acids and nisin as additives in the meat formulation, while relatively fewer studies have evaluated their efficacy as dipping solutions post-processing. Little is known about the potential of these agents as processing aids applied as surface treatments rather than as ingredients. Therefore, the present study was designed to investigate dip solutions of nisin alone, BSCSD alone or the two in various combined treatment sequences on ability to control *L. monocytogenes* attached on RTE beef frankfurters during storage at 4 or 10°C.

2. MATERIALS AND METHODS

2.1. Preparation of inoculum

A cocktail for inoculation was prepared using five strains of *L. monocytogenes*. Strains were acquired from American Type Culture Collection (ATCC, Manassas, VA, USA) and included ATCC 15313 (rabbit isolate, England), ATCC 51414 (raw milk associated with listeriosis outbreak, Massachusetts, USA), ATCC 43256 (Mexican-style cheese isolate, California, USA) ATCC 19115 (human isolate), and ATCC 7644 (human isolate). Strains were maintained on trypticase soy agar slants with 0.6 % yeast extract (TSAYE; BD Diagnostic Systems, Sparks, MD) at 4°C with monthly transfers to fresh TSAYE slants. To prepare study inocula, each strain was streaked separately onto TSAYE plates and incubated 35°C for 24 h. A single colony of each strain from a TSAYE plate was transferred into separate 30 ml portions of trypticase soy broth with 0.6% yeast extract (TSBYE) and incubated at 35°C for 24 h to obtain stationary phase cultures (approximately 9 $\log_{10}$ CFU/ml). A multistrain cocktail was prepared by combining 2-ml portions of each turbid broth in a sterile test tube and vortexed for 10 sec. A 2-ml portion of this mixed-strain cocktail was then dispersed into 2 L of 0.1% peptone water (BD Diagnostic Systems) to achieve a final *L. monocytogenes* inoculum solution containing approximately 6 $\log_{10}$ CFU/ml.

2.2. Frankfurter inoculation

The experimental setup was rather a model than real-life in order to simulate the worst possible conditions for *Listeria* survival and growth, such as destruction of frankfurters natural microbiota to avoid competition, use of stationary culture of pathogen in nutrient-rich medium, modifying of frankfurters texture by autoclaving, overnight adaptation and attachment of *Listeria* to the meat surface before application of antimicrobials. Briefly, low-fat frankfurters without added antimicrobial agents were purchased from a commercial distributor and transported in a cooler on ice packs directly to the laboratory. The proximate composition of the frankfurters remained unknown. The frankfurters were immediately frozen and stored at -20°C until use but no longer than for a month.
For each replicate, 44 frankfurters (11 treatments x 4 sampling days) for 4°C storage and 66 frankfurters (11 treatments x 6 sampling days) for 10°C storage were thawed at 4°C overnight. After thawing, the frankfurters were aseptically cut in half perpendicular to the longitudinal axis (calculated: 2.5 cm diameter; 6 cm length; 57 cm^2 surface area; 22 ± 2 g). Each replicate portion of halved frankfurters were vacuum packaged (Multivac A300/16; Kansas City, MO) in vacuum bags (Vacuum Pouches; Prime Source, Kansas City, MO) at 999 mbar vacuum for 1 sec with a 2.5 sec seal. The vacuum-packaged frankfurters were autoclaved for 15 min at 121°C to destroy indigenous microorganisms (DORSA *et al.* 1993), and cooled for approximately 2 h at 25 ± 2°C in a laminar flow hood before inoculation with *L. monocytogenes*.
A solution of sterile 0.1 % peptone water was used as a negative control. Up to 20 halved frankfurters were placed into 4 L sterile steamer strainers (Progressive International, Kent, WA). Each batch of 20 was dipped in containers with either 2 L of negative control buffer or 2 L of the five-strain inoculum of *L. monocytogenes* for 5 min with constant agitation. After dipping, the strainers were removed and drained for 10 min into empty sterile containers. The inoculated frankfurters were aseptically transferred to plastic storage bags with a zip closure (Ziploc, S. C. Johnson and Son Inc., Racine, WI) and stored over night at 4°C to allow for low temperature adaptation and maximum adherence of *L. monocytogenes* to product surfaces. This practice is similar to commercial frankfurter manufacturing where cooked links can be hydrocooled after cooking or rack cooled overnight in a low temperature cooler.

2.3. Antimicrobial treatments

Commercially-available antimicrobials were secured from the U.S. food ingredients suppliers and prepared according to manufacturer's instructions but using sterilized distilled water for antimicrobial dissolution "as is". Nisin (1,000 IU/mg, ProFood International, Inc., Chicago, IL, USA) and buffered sodium citrate combined with sodium diacetate (BSCSD, World Technology Ingredients, Inc., Jefferson, GA, USA) were used to prepare eleven treatment dipping solutions. The exact composition of BSCSD was not declared by the supplier, but according to Hull (2007), it may have contained 65 to 95% sodium citrate and 5 to 35% of sodium diacetate. The inoculated frankfurter samples were dipped into 2-L portions of the following individual agent treatment solutions: 1) 0.1% sterile peptone water (no antimicrobials, *L. monocytogenes* positive control); 2) 2000 IU/ml nisin; 3) 4000 IU/ml nisin; 4) 6000 IU/ml nisin; 5) 2.5% (w/v) BSCSD; 6) 3.0% (w/v) BSCSD; 7) 3.5% (w/v) BSCSD. In addition, two sequential treatment solution treatments were conducted; 8) 6000 IU/ml nisin followed by 3.5% (w/v) BSCSD (nisin-BSCSD) and 9) 3.5 % (w/v) BSCSD followed by 6000 IU/ml nisin (BSCSD-nisin). Finally, a combined

solution treatment was used: 10) 6000 IU/ml nisin and 3.5% (w/v) BSCSD; along with a negative control: 11) non-inoculated samples dipped in 0.1% peptone water.
Dipping was conducted by using sterile steamer strainers as used earlier for frankfurter inoculation. For a single treatment, not more than 20 frankfurter samples were dipped into 2.0 L of treatment solution for 5 min and drained for 10 min before vacuum packaging. For sequential treatments, samples were dipped into the first solution for 5 min, drained for 10 min, and then dipped into another bucket containing the second solution for 5 min followed by draining for 10 min.
Treated half-frankfurter samples were aseptically individually placed into separate vacuum bags (Prime Source, 15.24 cm x 21.59 cm) and vacuum packaged (Multivac A300 /16). The packages from each treatment were randomly selected into two batches, which were stored at 4 or 10°C for 42 and 20 days, respectively.

2.4. Bacterial enumeration

Frankfurters stored at 4°C were analyzed on days 0, 14, 28 and 42, and those stored at 10°C were analyzed on days 0, 4, 8, 12, 16 and 20. Twelve hours post-treatment was designated as day 0. On each sampling day, two packages from each treatment were randomly selected and analyzed for microbial counts. Twenty-three milliliters of 0.1% peptone water was added to each frankfurter sample (1:1 ratio) in a stomacher filter bag (Thermo-Fisher Scientific, Fairlawn, NJ). The samples were homogenized for 2 min using a stomacher (Stomacher 400 Lab Blender, Seward Medical, London, UK) and the homogenate was serially diluted with 0.1% peptone water. An aliquot of 0.1 ml was taken from each dilution, and spread plated on duplicate modified Oxford agar (BD Diagnostic Systems) plates followed by incubation at 35°C for 48 h. Colonies that appeared 2 to 3 mm in diameter and grayish black with a halo were enumerated as *L. **monocytogenes*.

2.5. pH measurements

The surface pH of treated frankfurters was determined using Corning Pinnacle 530 pH meter (Corning, NY). Half-cut frankfurter samples were dipped into treatment solutions for 5 min and then drained for 10 min before pH measurement. The pH values of the antimicrobial solutions were also measured prior to application.

2.6. Statistical analysis

Three replicate experiments were performed for each storage temperature. At each sampling point, duplicate samples from each treatment were removed from storage. Plate counts were converted to $\log_{10}$ CFU/g. Least-square means of bacterial counts for each treatment were estimated and significance of differences were determined using analysis of variance using general linear model of Statistical Analysis System 9.1 (SAS 2002). All differences were reported at a significance level of $P \leq 0.05$.
In addition, the logarithm of the *L. monocytogenes* counts from each of the two storage temperatures were modeled as a function of time using the Baranyi model (BARANYI AND ROBERTS, 1994). For curve fitting, the program DMFit (provided by Dr. J. Baranyi, IFR (Institute of Food Research, Reading, UK) was used (BARANYI, 2005). Four Baranyi model parameters were measured: 1) lag phase; 2) μ_{max}, which expresses the maximum specific growth rate (per day); 3) Y_0, which represents the lower asymptote, corresponding to the initial bacterial counts ($\log_{10}$ CFU/g); and 4) Y_{end}, represents the upper asymptote,

corresponding to the maximum bacterial counts ($\log_{10}$ CFU/g) when the growth curve forms an upper plateau at the stationary phase of growth. The lag phase is formally separated from the exponential and the stationary phase, which can be regarded as part of the potential growth curve defined by the model. The main difference between this model and other sigmoid curves is the mid-phase curve is similar to a linear curve, unlike other classical sigmoid curves that have a pronounced curvature.

3. RESULTS AND DISCUSSION

3.1. pH of frankfurters dipped in antimicrobial solutions

Table 1 shows treatment solution and frankfurter surface pH values. BSCSD solutions had pH values above 5.6, which is not considered inhibitory to growth of *L. monocytogenes* (USDA-ARS, 2019, Pathogen Modeling Program Online). Nisin solutions had growth inhibitory pH values below 4.0 (USDA-ARS, Pathogen Modeling Program Online). Despite the low pH of the nisin solutions, no significant (P>0.05) change in the surface pH of frankfurters was observed after dipping in any of the treatment solutions (Table 1).

Table 1. pH values of antimicrobial solutions and treated frankfurters.

	pH mean ±SD [a]	
Treatment	**Treatment solution**	**Surface pH of treated frankfurters**
Positive control	6.3±0.07	6.0±0.12 ab
Nisin (2000 IU/ml)	3.9±0.02	5.8±0.03 b
Nisin (4000 IU/ml)	3.5±0.42	5.8±0.11 b
Nisin (6000 IU/ml)	3.7±0.07	5.8±0.03 b
BSCSD (2.5%)	5.8±0.03	5.8±0.05 b
BSCSD (3.0%)	5.8±0.01	5.8±0.10 b
BSCSD (3.5%)	5.6±0.19	6.1±0.06 a
Nisin-BSCSD	3.9±0.10 - 5.6±0.03	5.8±0.02 b
BSCSD-Nisin	5.8± 0.02 - 3.7±0.11	5.7±0.12 b
Combined	5.6±0.11	5.8±0.08 b

[a]All means are duplicate measurements from three different experiments. Means within a column followed by the same letter(s) are not significantly different (P>0.05).

Similar pH results were seen by PATEL *et al.* (2006) after dipping turkey frankfurters in solutions of nisin, sodium lactate, or sodium diacetate, either alone or in combination. BEDIE *et al.* (2001) also did not observe significant changes in the pH of frankfurters when sodium acetate, sodium diacetate, or sodium lactate were added as ingredients. These and other studies (SAMELIS *et al.*, 2005; SCHLYTER *et al.*, 1993; SHELEF and ADDALA, 1994) reveal that pH reduction is not a major contributor to the antilisterial property of these antimicrobials. Instead, activity within the meat system accounts for antilisterial activity (DOORES *et al.*, 2005; TOKARSKYY and MARSHALL, 2008).

3.2. Effects of nisin, BSCSD, and combined solutions

Tables 2 and 3 show effects of 2000, 4000, or 6000 IU nisin/ml, 2.5, 3.0, or 3.5% BSCSD, sequential nisin-BSCSD treatment, sequential BSCSD-nisin treatment, and a combined nisin-BSCSD treatment solution on surface inoculated frankfurters stored at 4 and 10°C.

Table 2. Effect of nisin, BSCSD, and combined solutions on growth of *L. monocytogenes* on beef frankfurters stored at 4°C*.

Treatment	*L. monocytogenes* population (mean $\log_{10}$ CFU/g ± SD) Time (day)			
	0	14	28	42
Positive control	4.4±0.4 a	8.0±0.1 a	8.5±0.2 a	9.0±0.2 a
Nisin (2000 IU)	2.2±1.1 b	7.0±0.1 bc	8.2±0.3 a	9.0±0.1 a
Nisin (4000 IU)	2.1±0.8 b	7.2±0.3 bc	8.1±0.1ab	9.0±0.2 a
Nisin (6000 IU)	2.3±0.2 b	7.0±0.1 bc	8.2±0.2 a	9.0±0.2 a
BSCSD (2.5%)	4.0±0.5 ab	7.3±0.1 bc	8.2±0.2 a	8.9±0.3ab
BSCSD (3.0%)	3.2±1.1 ab	7.3±0.0 b	8.4±0.3 a	8.8±0.2ab
BSCSD (3.5%)	3.3±0.8 ab	7.1±0.1 bc	8.3±0.3 a	8.7±0.0 b
Nisin - BSCSD	3.1±0.8 ab	6.9±0.3 c	7.5±0.4 bc	8.7±0.1 b
BSCSD - Nisin	2.7±1.2 ab	7.3±0.2 bc	7.5±0.4 bc	9.0±0.1 a
Combined	2.7±1.3 ab	6.3±0.2 d	7.4±0.3 c	8.9±0.3 ab

*Means within a column followed by the same letter(s) are not significantly different (P>0.05). Minimum detection limit was 2.0 $\log_{10}$ CFU/g.

Table 3. Effects of nisin, BSCSD, and combined solutions on the growth of *L. monocytogenes* on beef frankfurters stored at 10°C*.

Treatment	*L. monocytogenes* population (mean $\log_{10}$ CFU/g ± SD) Time (day)					
	0	4	8	12	16	20
Positive control	4.7±0.5 a	7.7±0.5 a	8.6±0.1 abc	8.7±0.1 ab	8.8±0.1 a	9.0±0.1 abc
Nisin (2000 IU/ml)	2.9±0.3 bc	5.6±0.1 d	7.9±0.5 abcd	8.3±0.2 bc	8.4±0.2 b	8.6±0.1 d
Nisin (4000 IU/ml)	2.8±0.2 c	6.5±0.2 bcd	7.9±0.4 abcd	8.3±0.3 bc	8.5±0.3 ab	8.7±0.1 cd
Nisin (6000 IU/ml)	2.2±1.0 c	6.1±0.7 d	8.0±0.1 abcd	8.0±0.1 cd	7.9±0.1 c	8.5±0.3 d
BSCSD (2.5%)	4.2±0.9 ab	7.5±0.7 ab	8.9±0.2 a	9.0±0.2 a	8.6±0.1 ab	9.1±0.2 ab
BSCSD (3.0%)	4.5±0.5 a	7.4±0.6 abc	8.5±0.8 abc	9.0±0.2 a	8.6±0.1 ab	9.3±0.2 a
BSCSD (3.5%)	4.5±0.4 a	7.4±0.5 abc	8.8±0.6 ab	8.8±0.3 a	8.6±0.1 ab	9.0±0.2 abc
Nisin - BSCSD	3.0±0.5 bc	6.3±0.4 cd	7.7±0.3 cd	7.9±0.3 cd	8.4±0.1 b	8.8±0.1 bcd
BSCSD -Nisin	2.7±0.5 c	6.3±0.2 cd	7.9±0.3 bcd	8.0±0.1 cd	8.6±0.2 ab	8.9±0.1 bc
Combined	2.7±0.6 c	6.1±0.3 d	7.5±0.1 d	7.7±0.1 d	8.3±0.2 b	8.5±0.1 d

*Means within a column followed by the same letter(s) are not significantly different (P>0.05). Minimum detection limit was 2.0 $\log_{10}$ CFU/g.

Treatment and sampling of negative control beef frankfurters followed the same procedures as inoculated samples and showed no *L. monocytogenes* contamination (results not shown). Positive-control untreated samples supported *L. monocytogenes* growth to populations at or exceeding 8 $\log_{10}$ CFU/g by 14 days of storage at 4°C (Table 2) and 8 days at 10°C (Table 3). Treatment with nisin alone initially reduced ($P<0.05$) *L. monocytogenes* counts by approximately 2 logs at both storage temperatures; however, there was no observed ($P>0.05$) dose-response relationship. Although treatment with BSCSD alone appeared to reduce counts initially at both temperatures, these reductions were not significant ($P>0.05$). Sequential nisin-BSCSD and BSCSD-nisin treatments and combined nisin-BSCSD treatment initially reduced ($P<0.05$) counts by approximately 2 logs at 10°C (Table 3) but not ($P>0.05$) at 4°C (Table 2). This is suggestive that warmer temperature application may increase antimicrobial activity of the combined treatments.

Despite some initial count reductions, none of the treatments were able to prevent growth of *L. monocytogenes* during frankfurter storage at 4 or 10°C under proposed model conditions (Tables 2 and 3). By 14 days at 4°C, all treatments were able to keep *L. monocytogenes* counts significantly lower ($P<0.05$) than the untreated control, but the count reduction was small (around 1 log) (Table 2). The combined nisin-BSCSD treatment achieved a larger 1.7 log reduction ($P<0.05$) at 14 days of storage at 4°C. Sequential and combined nisin-BSCSD treatments were able to keep counts around 1 log lower ($P<0.05$) than the untreated control for up to 28 days at 4°C. No meaningful count differences were observed among the treatments on the last day of testing, 42 days at 4°C. Similar trends were observed when frankfurters were stored at 10°C, with no meaningful treatment differences observed by 16 days of storage.

Based on previous reports we expected that sequential or combined nisin-BSCSD treatments would be more effective than treatment with either antimicrobial preparation alone (CEYLAN *et al.,* 2003; CLEVELAND *et al.,* 2001; DELVES-BROUGHTON, 2005; JUNEJA AND THIPPAREDDI, 2004; SAMELIS *et al.,* 2001; SAMELIS *et al.,* 2005; THIPPAREDDI *et al.,* 2003; ZHU *et al.,* 2005). The combined application of antimicrobials (either sequential or as a single solution) did not influence *L. monocytogenes* counts, except day 14 at 4°C, where nisin-BSCSD yielded significantly lower counts. Perhaps application of low-pH nisin solution before BSCSD application influenced activity at this time point.

It is noteworthy that surviving *L. monocytogenes* populations after antimicrobial treatments were able to initiate growth under model conditions. Baranyi model growth curves at 4 and 10°C are shown in Figures 1 and 2, respectively.

Growth rate kinetics of the bacterium at the two storage temperatures are shown in Tables 4 and 5. There were no lag-phase growth differences observed with any treatment at 4 or 10°C based on the Baranyi model (results not shown). In pork bologna formulated with 1.8% sodium lactate and 0.125% sodium diacetate, BARMPALIA *et al.* (2005) observed a *L. monocytogenes* lag phase of 13.78 days and 5.02 days at 4 and 10°C, respectively, using the same Baranyi model. GEORNARAS *et al.* (2006b) found a 10.2 day lag phase at 10°C for commercial cooked sausages treated with 1.5% potassium lactate plus 0.05% sodium diacetate and no lag phase for untreated sausages. Result differences in lag phase observations may be due to variations in experimental protocol including number of data points taken during the lag phase, different starting inocula populations, type of product, presence or absence of indigenous bacteria that could be inhibitory to *L. monocytogenes*, or differences in absorption rates of antimicrobial solutions due to specific surface characteristics (BARMPALIA *et al.,* 2004; GEORNARAS *et al.,* 2006a; GEORNARAS *et al.,* 2006b; SAMELIS *et al.,* 2001, 2002, 2005).

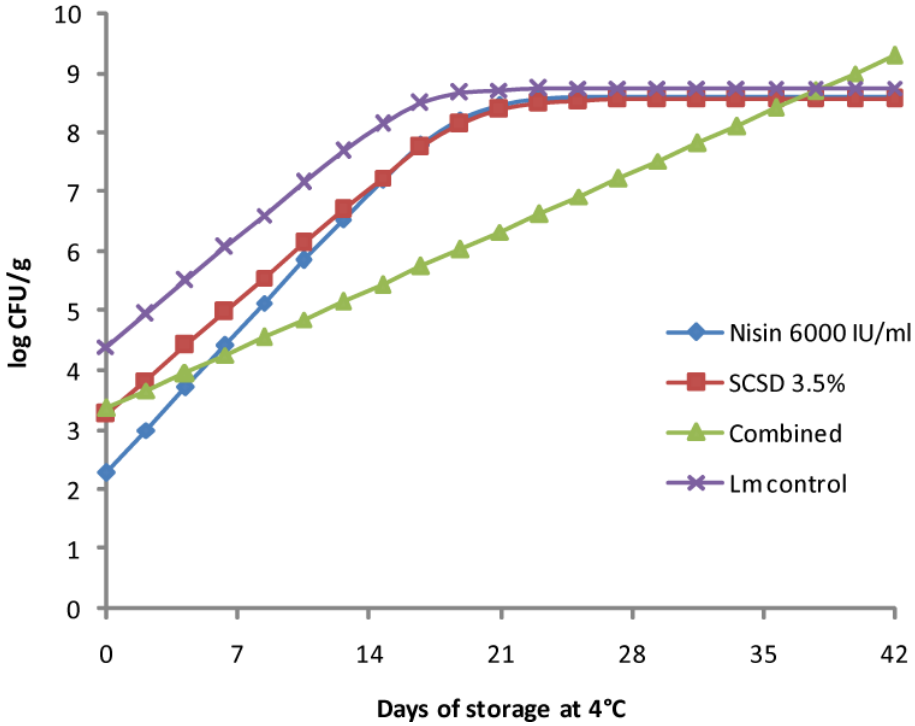

Figure 1. Growth of *L. monocytogenes* on vacuum packaged beef frankfurters dipped in solutions of 6000 IU/ml nisin, 3.5% BSCSD, and combined solution, stored at 4°C. Lm control, *L. monocytogenes* inoculated on beef frankfurters dipped in 0.1% peptone water with no antimicrobials. Data points are not actual experimental values but aids for better visualization of Baranyi graphs.

Table 4. Growth kinetics of *L. monocytogenes* inoculated on the surface of beef frankfurters treated with nisin alone, BSCSD alone, nisin-BSCSD in sequence, BSCSD-nisin in sequence, both combined, then vacuum packaged and stored at 4°C for 42 days.

Treatment	Maximum specific growth rate (μ_{max}; per day ±standard error)	Y_0 [a] (log_{10} CFU/g)	Y_{end} [b] (log_{10} CFU/g)	R^2
Lm control	0.26±0.030	4.4	8.7	0.98
Nisin (2000 IU/ml)	0.35±0.045	2.2	8.6	0.93
Nisin (4000 IU/ml)	0.37±0.046	2.1	8.5	0.93
Nisin (6000 IU/ml)	0.34±0.041	2.2	8.6	0.94
SCSD (2.5%)	0.23±0.025	4.0	8.5	0.96
SCSD (3.0%)	0.30±0.057	3.1	8.6	0.86
SCSD (3.5%)	0.27±0.059	3.2	8.6	0.84
Nisin + SCSD	0.28±0.039	3.1	8.0	0.91
SCSD + Nisin	0.34±0.046	2.7	8.2	0.90
Combined	0.14±0.046	3.3	-c	0.89

[a]Lower asymptote estimated by the Baranyi model.

[b]Upper asymptote estimated by the Baranyi model.

[c]No value could be estimated when the upper part of the growth curve ceased without forming an upper asymptote that corresponds to the stationary phase.

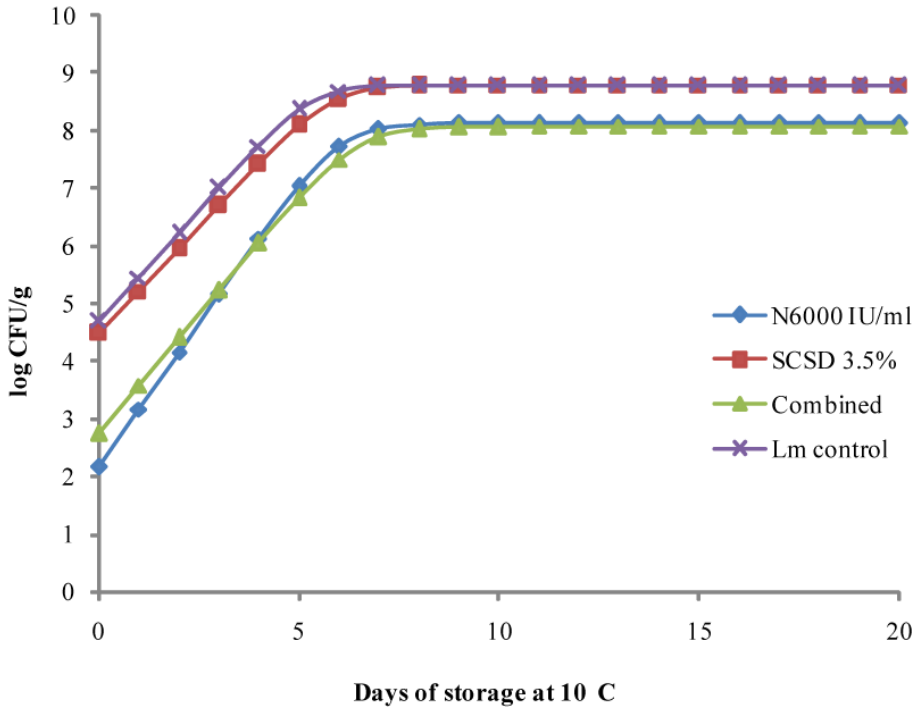

Figure 2. Growth of *L. monocytogenes* on vacuum packaged beef frankfurters dipped in solutions of 6000 IU/ml nisin, 3.5% BSCSD, and combined solution, stored at 4°C. Lm control, *L. monocytogenes* inoculated on beef frankfurters dipped in 0.1% peptone water with no antimicrobials. Data points are not actual experimental values but aids for better visualization of Baranyi graphs.

Table 5. Growth kinetics of *L. monocytogenes* inoculated on the surface of beef frankfurters treated with nisin, BSCSD, alone or in sequence or combination, then vacuum packaged and stored at 10°C for 20 days.

Treatment	Maximum specific growth rate (μ_{max}; per day ±standard error)	Y_0[a] (log_{10} CFU/g)	Y_{end}[b] (log_{10} CFU/g)	R^2
Lm control	0.77±0.020	4.7	8.8	0.95
Nisin (2000 IU/ml)	0.66±0.017	2.9	8.4	0.98
Nisin (4000 IU/ml)	0.95±0.021	2.8	8.4	0.97
Nisin (6000 IU/ml)	1.00±0.04	2.1	8.1	0.92
SCSD (2.5%)	0.84±0.037	4.2	8.9	0.89
SCSD (3.0%)	0.70±0.037	4.6	8.9	0.87
SCSD (3.5%)	0.74±0.027	4.5	8.8	0.92
Nisin + SCSD	0.79±0.031	3.0	8.2	0.93
SCSD + Nisin	0.89±0.027	2.7	8.4	0.96
Combined	0.83±0.29	2.7	8.1	0.94

[a]Lower asymptote estimated by the Baranyi model.
[b]Upper asymptote estimated by the Baranyi model.
[c]No value could be estimated when the upper part of the growth curve ceased without forming an upper asymptote that corresponds to the stationary phase.

The Y_0 values of control samples were similar, 4.7 and 4.4 $\log_{10}$ CFU/g at 4 and 10°C, respectively (Tables 4 and 5). Treatment count reductions were addressed earlier when discussing results shown in Tables 2 and 3.

Maximum specific growth rate (μ_{max}) of nisin-treated frankfurters ranged from 0.339-0.368 day^{-1} while that of BSCSD was 0.230-0.274 day^{-1} and control was 0.263 day^{-1} at 4°C. This implies that surviving nisin-treated *L. monocytogenes* grew faster than non-stressed populations. Others (LUCHANSKY and CALL, 2004; RUTHERFORD, 2004; SAMELIS *et al*, 2005) found similar results of greater μ_{max} values with treated samples than with control samples. Those researchers postulated that nisin could have slowed the growth of other bacteria thus reducing the bacterial competition against *L. monocytogenes*, which allowed it to grow at a faster rate than when in the presence of competitor organisms. In contrast, the present study used sterile frankfurters to avoid possible confounding effects of any indigenous microflora, which implies another potential, as yet unproven, reason for nisin-resistant growth stimulation. For example, it is possible that a fast-growing, nisin-resistant strain was in the inoculum mix. Also, perhaps nisin-exposed cells are primed to multiply quickly as a stress-response survival strategy, especially in case of absence of indigenous microbiota.

Nisin was more effective than BSCSD in both initial population size reduction and growth inhibition during storage (Tables 1 and 2). Combining nisin with BSCSD did not improve nisin activity. Similar results were reported when nisin was used with sodium diacetate (FANG and LIN, 1994; SAMELIS *et al.*, 2005; STOPFORTH *et al.*, 2005), sodium acetate (SAMELIS *et al.*, 2002), potassium benzoate (GEORNARAS *et al.*, 2006b; SAMELIS *et al.*, 2005), potassium sorbate (SAMELIS *et al.*, 2005), sodium lactate (FANG and LIN, 1994), lactic acid (STOPFORTH *et al.*, 2005), acetic acid (STOPFORTH *et al.*, 2005) or grape seed extract (SIVAROOBAN *et al.*, 2007). Consistent with our findings in most combinations, nisin appeared to be the main contributing antimicrobial factor and lacked significant combined activity with other antimicrobials.

The mode of action of nisin involves pore formation in the cytoplasmic membrane, which leads to rapid removal of free amino acids, adenosine triphosphate, and cations from the cell (ABEE *et al.*, 1994). The antilisterial effect of nisin occurs immediately after cells are exposed to the bacteriocin resulting in cell death (EL-KHATEIB *et al.*, 1993). Present results are in agreement with previous studies (GEORNARAS *et al.*, 2005; NILSSON *et al.*, 1997; SAMELIS *et al.*, 2002; SAMELIS *et al.*, 2005) showing initial reductions of *L. monocytogenes* populations following treatment with nisin and subsequent cell recovery and growth during storage. The inability of nisin to maintain its antilisterial activity has previously been observed in RTE meat products (LUCHANSKY and CALL 2004; SAMELIS *et al.*, 2005). Some apparent reasons for loss of nisin activity include insufficient quantities of nisin to interact with all the target cells, nisin activity dependent on low pH, increased resistance of some strains of *L. monocytogenes*, or uneven distribution of nisin within the food (BOUTTEFROY *et al.*, 2000; HENNING *et al.*, 1986; MURIANA, 1996; SAMELIS *et al.*, 2005, MARTINIS *et al.*, 1997). It is known that *L. monocytogenes* is prone to spontaneous development of resistance to nisin due to mutations (CRANDALL and MONTVILLE, 1998; MARTINIS *et al.*, 1997; LIU *et al.*, 2002); however, the strains used in the present study are not believed to be nisin resistant. Addressing these limitations with nisin, TOKARSKYY and MARSHALL (2008) reported synergistic activity between nisin, lactic acid and monolaurin against *L. monocytogenes* growth when lactic acid was able to increase membrane fluidity and hence increase nisin activity. Additionally, model conditions used in this study were favorable for *Listeria* growth and different from real-life situations. Model conditions included destruction of frankfurters natural microbiota to avoid

competition, use of stationary culture of pathogen in nutrient-rich medium for inoculation, modifying of frankfurters texture by autoclaving, overnight adaptation and attachment of *Listeria* to the meat surface before application of antimicrobials.
Dipping in 2.5, 3.0 and 3.5% BSCSD had little impact on *L. monocytogenes* growth at 4 or 10°C (Tables 4 and 5). THIPPAREDDI *et al.* (2003) reported that BSCSD of ≥ 1.0 % was effective in reducing *C. perfringens* populations in roast beef or injected pork. The antimicrobial mechanism of BSCSD may be similar to organic acid esters, which lower the intracellular pH within the microbial cells, alters cell membrane permeability that affect substrate transport, and inhibits the electron transport system required for energy regeneration (THIPPAREDDI *et al.*, 2003). BSCSD activity increases as product pH decreases (CEYLAN *et al.*, 2003; HULL, 2007).
Previous studies with BSCSD against *L. monocytogenes* in meat products are limited. One study observed inhibition of *L. monocytogenes* by 0.2% sodium diacetate, and stimulated growth with 1% buffered sodium citrate (15 parts sodium citrate, 1 part citric acid w/w) in cooked cured ham products (STEKELENBURG and KANT-MUERMANS, 2001). In contrast to present results, CEYLAN *et al.* (2003) found significant inhibition of *L. monocytogenes* using 1% buffered sodium citrate in combination with 0.1% sodium diacetate in beef frankfurters stored at 3.9°C. The most likely explanation for disparate results is whether agents are added to product formulation (growth inhibition) compared to use as dipping agents (not inhibitory). The present protocol also allowed for *L. monocytogenes* to attach and adapt during overnight storage at 4°C, a process not unlike commercial practice where frankfurter links potentially exposed to *L. monocytogenes* environmental contamination are often cooled overnight prior to packaging the next day.

4. CONCLUSION

The results of this study demonstrate that dip antimicrobial treatments with nisin and/or BSCSD were not effective in preventing low-temperature growth of *L. monocytogenes* on the surface of frankfurters under proposed model conditions. Traditional use of such antimicrobials either as direct product ingredients or as in-package solutions remain as more appropriate application methods. Effort to increase activity by combining the different antimicrobial agents did not increase activity.

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