RESEARCHREPORT
PORK SAFETY
pork checkoff

Title: Efficacy of novel food antimicrobial combinations for control of *Listeria monocytogenes* for preservation of ready-to-eat (RTE) products. **(07-186)**

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Industry Summary

Objectives: The primary objective of this study was to determine the minimum inhibitory concentrations (MIC) of USDA-FSIS approved food antimicrobials necessary for the inhibition of the foodborne pathogen *Listeria monocytogenes*. The second major purpose of this work was to determine the interactions of food antimicrobials with one another for *L. monocytogenes* inhibition.

Study Methods: These objectives were completed by the use of a broth dilution microassay. Antimicrobials were serially diluted in sterile water immediately prior to sample inoculation. A single strain of the pathogen was incubated at 35°C for 24 hours in a non-selective microbiological medium to which the test antimicrobial was added. Following incubation, experimental samples were removed and the change in sample optical density (turbidity) at 630 nm (OD630) was determined. If experimental samples exhibited no change in OD630 (<0.05 ΔOD630) following incubation in the presence of the antimicrobial, the pathogen was pronounced inhibited. With respect to testing of combined antimicrobials, single strains of *L. monocytogenes* were again incubated at 35°C in a nutritious medium containing two antimicrobials. The fractional inhibitory concentrations (FICs) were calculated following determination of MICs from combinations that contained the lowest concentrations of antimicrobials A and B that resulted in a <0.05 ΔOD630. Calculated FIC values were then used to prepare FIC index values (FIC_I), used to classify the type of interaction observed between antimicrobials in sample wells. The FIC_Is were used to classify the interaction of antimicrobials as synergistic, additive, or antagonistic.

Study Results: With respect to antimicrobial interactions, the combination of Nisin and Acidic Calcium Sulfate as well as Octanoic Acid and Acidic Calcium Sulfate produced synergistic-type inhibition of *L. monocytogenes* strains within experimental parameters. Additive-type inhibition of *L. monocytogenes* was observed with the application of Nisin and Lauramide Arginine Ethyl Ester. Conversely, pairing of Nisin with Octanoic Acid resulted in an increase in the minimum concentrations of Nisin required for inhibition as compared to the MIC of Nisin required for pathogen inhibition when applied singly. Similar trends were observed for other antimicrobial pairings, whereby the minimum concentration of one or both antimicrobials capable of inhibiting the pathogen in combination was more than what was needed for *L. monocytogenes* inhibition when one or both antimicrobials were applied alone. Furthermore, the resulting antagonistic-type interactions of many antimicrobial pairings indicates that use of dual antimicrobials does not necessarily guarantee the user significantly enhanced inhibition of foodborne bacterial pathogens over the use of a single compound.

These research results were submitted in fulfillment of checkoff-funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer-reviewed.

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Industry Significance: Results of this study indicate that some experimental combinations of food antimicrobials produced synergistic-type inhibition of *L. monocytogenes* when incubated at 35°C at pH 7.3 or 5.0, whereas others produced additive-type and antagonistic type behavior. However, it is recommended that before processors use a food antimicrobial or a combination of antimicrobials they must first experimentally validate the actions and interactions of antimicrobials *in vitro* and on surfaces of the food product and their ability to produce enhanced inhibitory effects against the target microorganism.

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Scientific Abstract

The primary purposes of this study were to: (1) define the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of various food antimicrobials approved for use with ready-to-eat (RTE) meat products for the inhibition of the Gram-positive foodborne pathogen *L. monocytogenes*, (2) to define the MIC and fractional inhibitory concentrations (FIC) of combined antimicrobials for the inhibition of *L. monocytogenes*, and (3) to define the interactions of antimicrobials as antagonistic, additive, or synergistic. It was hypothesized that use of dual antimicrobials would result in inhibition of the pathogen at lower levels than those observed for single antimicrobial use as a result of pathogen inability to adapt to simultaneous multi-antimicrobial attack.

For the determination of single antimicrobial MICs and MBCs against experimental strains, a broth dilution microassay was employed. A checkerboard broth dilution microassay was used to detect the inhibition of individual strains of *L. monocytogenes* following exposure to food antimicrobials in combination. Following incubation of pathogenic strains in a non-selective microbiological medium and antimicrobial at 35°C for 24 hr, growth or inhibition was detected via observation of change in sample optical density (turbidity) at 630 nm (OD630). Samples for which the change in OD630 was <0.05 were pronounced as exhibiting inhibition; MICs for each antimicrobial were defined as the lowest concentration for which <0.05 ΔOD630 was observed across duplicate replications. Following incubation, samples demonstrating <0.05 ΔOD630 were subjected to MBC testing via the spread plating of 0.1 ml of sample solution on the surface of a Petri dish containing a non-selective agar-solidified microbiological medium. Minimum bactericidal concentrations were defined as the lowest concentration of antimicrobial for which a 3-log cycle reduction was observed between the sample inoculum and the resulting plate following incubation across duplicate replications.

Results indicate that combinations of Acidic Calcium Sulfate with Nisin or Octanoic Acid resulted in synergistic-type inhibition of the pathogen, while other combinations displayed either additive or antagonistic-type interactions. The use of single compounds was generally found to be as effective as the use of combined antimicrobials for the purpose of pathogen inhibition. The use of combined antimicrobials must be experimentally validated prior to adoption.

Introduction

The U.S. Department of Agriculture (USDA) has estimated that the annual financial burden of foodborne Listeriosis approximates $2.3 billion.[1] Recognition of the potential for post-process contamination of ready-to-eat (RTE) meat products, including frankfurters and some deli meats, has spurred the development and use of various food antimicrobials as interventions for the control of *L. monocytogenes* growth on products.[2]

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[1]E.R.S./U.S.D.A. 2007. Economics of foodborne disease: *Listeria monocytogenes*. Available at: http://www.ers.usda.gov/Briefing/FoodborneDisease/listeria.htm. Accessed June 26, 2007.
[2]F.S.I.S./U.S.D.A. 2003. Control of *Listeria monocytogenes* in post-lethality exposed ready-to-eat products. 9CFR430.4.

Antimicrobials have been developed that function to inhibit bacterial pathogens via different mechanisms of action. Antimicrobial polypeptides (e.g. nisin, lysozyme) destroy the integrity of the cellular peptidoglycan, allowing leakage of cellular solutes and ions. Surfactants that bury in the membrane, destabilizing the bacterial membrane, can result in uncontrolled loss of essential nutrients. Organic acids such as lactic and citric acid may acidify the cytoplasm, resulting in decreased ability to synthesize macromolecules or irreversible over-utilization of energy stores. However, the advent of novel antimicrobials with modes of action that may not yet be fully elucidated requires investigation into their inhibitory potential in model systems and on foods prior to their adoption into processing operations.

The ability of microorganisms to survive in the presence of some food antimicrobials has led researchers to hypothesize that applying multiple antimicrobials to simultaneously attack differing cellular targets will enhance the overall inhibition of a foodborne pathogen.[3] The synergistic interaction of antimicrobials that inhibit a foodborne pathogen by a multi-pronged attack would represent a significant advancement in the industry's ability to suppress the growth of *L. monocytogenes* for protection of RTE pork product safety.

Objectives

1) Investigate *L. monocytogenes* strain-specific inhibition by USDA-FSIS approved generally recognized as safe (GRAS) food preservatives and antimicrobials, applied singly *in vitro*. Determine the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of Sodium-L-Lactate (SL), Octanoic Acid (OCT), and Acidic Calcium Sulfate (ACS). Investigate and determine MICs for the naturally occurring food antimicrobials Nisin (NIS), Lauramide Arginine Ethyl Ester (LAE), and ε-Poly-L-Lysine (EPL).

2) Determine efficacy of selected antimicrobial combinations for the inhibition of target pathogen strains *in vitro*. Determine the MICs and MBCs of antimicrobials applied in combination against *L. monocytogenes* strains *in vitro*.

3) Characterize antimicrobial interactions for the inhibition of *Listeria monocytogenes*. Determine differences in MICs for antimicrobials against target strains when combined as compared to single antimicrobial application. Characterize antimicrobial interactions (antagonistic, additive, synergistic) against target strains.

Materials and Methods

<u>Test Antimicrobials:</u> Powdered Nisin fermentate (NIS; Sigma-Aldrich, St. Louis, MO, 2.5% w/w Nisin) was dissolved in sterile 0.02 M hydrochloric acid (HCl) (Thermo-Fisher Scientific, Waltham, MA) and boiled for 4 minutes to obtain a stock solution of 25,000.0 ppm w/w active Nisin.[4] Save-ory® PL-25 (EPL; Chisso America, Inc., Rye, NY; 50% w/w ε-Poly-L-Lysine) was dissolved in sterile distilled water (DI H_2O) to obtain a working stock of 5,000 ppm w/w active EPL. Octa-Gone® (OCT; Ecolab, Inc., St. Paul, MN; 3.6% w/w Octanoic Acid/Sodium Octanoate) was diluted in sterile DI H_2O to obtain a working stock of approximately 9,000 ppm w/w active Octanoic Acid/Sodium Octanoate. Safe$_2$O® RTE:01 (ACS; Mionix Corp., Round Rock, TX; saturated Acidic Calcium Sulfate solution), CytoGuard LA (LAE; A&B Ingredients, Fairfield, NJ; 10% w/w Lauramide Arginine Ethyl Ester), and Purasal®S (SL; Purac America, Inc., Lincolnshire, IL; 60% w/w Sodium-L-Lactate) were all dissolved in sterile DI H_2O to obtain working solutions. After preparation, 100 µl of stock solutions were spread-plated on the non-selective microbiological medium Tryptose Phosphate Agar (TPA; Becton Dickinson and Co., Sparks, MD) and incubated at 35°C for 48 h to ensure that solutions were free of microbial contamination. Antimicrobials, active agent, concentration of stock solution, and manufacturers are presented in Table 1.

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[3] de Gonzalez, M.T.N., J.T. Keeton, G.R. Acuff, L.J. Ringer, and L.M. Lucia. 2004. *J. Food Prot.* 67:915-921.
[4] Rogers, A.M. and T.J. Montville. 1991. *Food Biotechnol.* 5(2): 161-168.

Table 1: Experimental antimicrobials, active agents, and manufacturer.

Antimicrobial	Active Agent	Stock Concentration	Study Concentration	Manufacturer	Headquarters
Nisin	Nisin	25,000.0 ppm	6.25 ppm	Sigma-Aldrich, Inc.	St. Louis, MO
Save-ory®	ε-Poly-L-Lysine	5,000.0 ppm	50.0 ppm	Chisso America, Inc.	Rye, NY
Octa-Gone®	Octanoic Acid	3.60% (w/w)	100.0 ppm	Ecolab, Inc.	St. Paul, MN
$Safe_2O$® RTE:01	Acidic Calcium Sulfate	330,000.0 ppm Lactic Acid	5.0% v/v	Mionix Corp.	Round Rock, TX
CytoGuard	Lauramide Arginine Ethyl Ester	10.0% (w/w)	50.0 ppm	A&B Ingredients, Inc.	Fairfield, NJ
Purasal®S	Sodium L-Lactate	60.0% (w/w)	5.0% (w/w)	Purac America	Lincolnshire, IL

Bacterial Culture Preparation and Maintenance: *Listeria monocytogenes* Scott A, 310, National Animal Disease Center (NADC) 2783 and 2045 were obtained from the Department of Animal Science Center for Food Safety culture collection at Texas A&M University, College Station, TX. All strains were identified as *Listeria monocytogenes* using the API® *Listeria* identification system (bioMérieux, Inc., Hazelwood, MO) according to manufacturer instructions. Thereafter, cultures were maintained on Tryptic Soy Agar (TSA; Becton Dickinson and Co.) slants at 5°C. In order to prevent potential development of antimicrobial resistance via repeated sub-culturing, working cultures were obtained by transferring a loopfull of culture from TSA slants to 10 ml of Fraser Broth (Becton Dickinson and Co., supplemented with 0.5 g/L Ferric Ammonium Citrate) and incubating for 24 h without agitation at 35°C. After confirming a *Listeria*-species correct phenotype (esculin hydrolysis) via medium blackening, a loopfull of culture was transferred to 10 ml Tryptose Phosphate Broth (TPB; Becton Dickinson and Co.), which was incubated for an additional 24 h at 35°C without agitation. Cultures from Fraser Broth were streaked for isolation on TSA plates at the time of TPB inoculation and incubated at 35°C for 48 h to confirm culture purity.

Inoculum Preparation: Overnight (24 h) cultures of each *L. monocytogenes* strain in TPB were serially diluted in 9.9 ml volumes of double-strength TPB (2xTPB) to achieve a final concentration of approximately 5.0 log CFU/ml. Double-strength TPB was adjusted to pH 5.0 using 6 M HCl for analysis of certain antimicrobials according to manufacturer recommendations, but was otherwise used at pH 7.3 to provide optimal conditions for pathogen survival and growth. Enumeration of cultures was completed by diluting to approximately 3.0 log CFU/ml in sterile 0.1% Peptone (Becton Dickinson and Co.), and then spread-plating 100 µl on both TPA and PALCAM Agar (Becton Dickinson and Co., supplemented with 8 mg/L Ceftazidime). Plates were aerobically incubated at 35°C for 48 h prior to enumeration. Use of PALCAM Agar served to ensure that cells from the inoculum displayed a correct phenotype for *Listeria* species (medium blackening via esculin hydrolysis), while simultaneous enumeration on TSA plates confirmed inoculum purity.

Single Antimicrobial Inhibition Assay: A broth dilution microassay was used to determine the strain-specific minimum inhibitory concentration (MIC) for each antimicrobial. Strains were exposed to NIS at 6.250, 3.125, 1.563, 0.781, and 0.391 ppm active at pH 7.3 and at 1.563, 0.781, 0.391, 0.195, 0.098, and 0.049 ppm active at pH 5.0. Acidic Calcium Sulfate was tested at 5.000, 2.500, 1.250, 0.625, and 0.313 % v/v at pH 7.3 and at 0.625, 0.313, 0.156, 0.078, and 0.039 % v/v at pH 5.0. Epsilon Poly-L-Lysine was delivered at 50.000, 25.000, 12.500, 6.250, and 3.125 ppm active at pH 7.3; SL at 5.000, 2.500, 1.250, 0.625, and 0.313 % w/w active at pH

7.3; and LAE at 50.000, 25.000, 12.500, 6.250, and 3.125 ppm w/w at pH 7.3. Finally, OCT was tested at 100.000, 50.000, 25.000, 12.500, and 6.250 ppm at pH 5.0. All stock solutions of antimicrobials were serially diluted in 5 ml vol. flasks with sterile distilled water to obtain working solutions. Concentrations of working solutions were devised so as to deliver the correct concentration of test antimicrobial to the test wells once all additions were made. Sterile barrier tips and pipettes were always used in making dilutions so as to minimize potential for cross-contamination.

Microtiter plates (96-wells; 300 µl capacity) (Microtest™, Becton Dickinson and Co.) were used for all broth dilution assays. Equivalent volumes of test antimicrobial (125 µl) and serially diluted *L. monocytogenes* inoculum in 2xTPB (125 µl) were aseptically loaded into test wells. Cells were diluted and inoculated in 2xTPB to allow the correct content of nutrients in the final sample well upon delivery. Sterile barrier tips were used to load solutions into the wells so as to minimize potential for cross-contamination. Appropriate negative controls containing only antimicrobial-containing solutions and sterile 2xTPB were built in for baseline adjustment of experimental wells. Positive controls containing sterile water and inoculum in 2xTPB were also completed. Immediately following plate preparation, optical density of the test wells at 630 nm (OD630) was measured at 0 h (630 nm) using an EL800 absorbance microplate reader (BioTek® Instruments, Inc., Winooski, VT). Microplates were incubated for 24 h at 35°C before being retrieved for a 24 h OD630 reading.

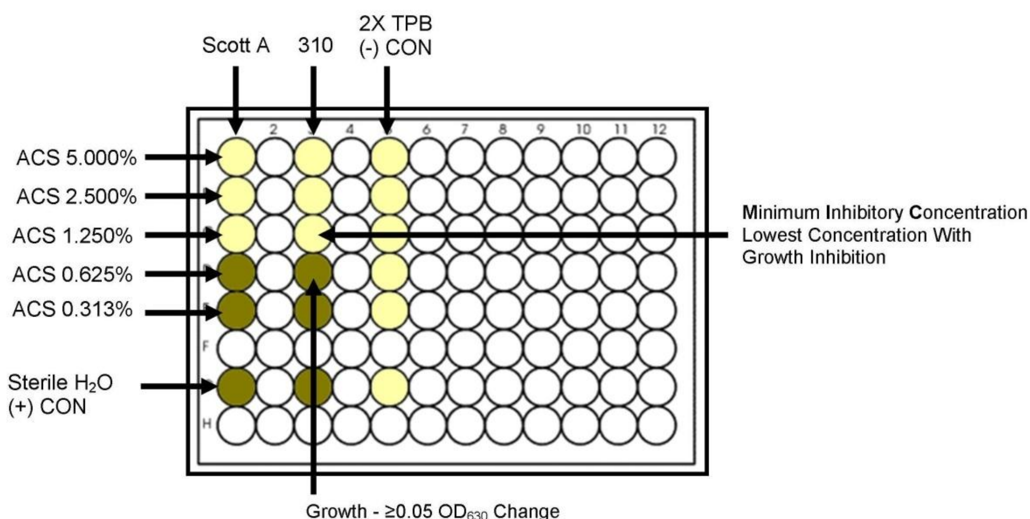

Figure 1: Broth dilution microplate setup and interpretation.

Antimicrobial inhibition testing of each antimicrobial at all concentrations was replicated at least twice for each strain. Concentrations of antimicrobial that produced a <0.05 change (Δ) in OD630 from 0 h to 24 h following appropriate baseline adjustment over replication were classified as inhibitory. The MIC for each antimicrobial was defined as the lowest concentration of antimicrobial that produced <0.05 ΔOD630 after 24 h incubation following baseline adjustment (Figure 1).

<u>Antimicrobial Combinations Susceptibility Testing:</u> The response of *L. monocytogenes* to six combinations of antimicrobials: EPL+ACS at pH 7.3, NIS+ACS at pH 7.3, SL+ACS at pH 7.3, NIS+LAE at pH 7.3, OCT+ACS at pH 5.0, and OCT+NIS at pH 5.0 was evaluated using a checkerboard broth dilution microassay. Minimum inhibitory concentrations previously determined from single antimicrobial susceptibility tests were used as starting concentrations for the combination assays. Serial (1:2) dilutions of antimicrobials were made from these starting concentrations so as to produce a 5X5 checkerboard of combinations between the two antimicrobial compounds. All stock solutions of antimicrobials were serially diluted in 5 ml vol. flasks with sterile distilled water to obtain working solutions. Concentrations of working solutions were again devised so as to provide the correct concentration of test antimicrobial when finally delivered to the test wells. Sterile barrier tips and sterile pipettes were used in making dilutions and in plate loading so as to minimize potential for cross-contamination.

For the EPL+ACS combination, all strains were exposed to 12.500, 6.250, 3.125, 1.563, and 0.781 ppm w/w active EPL in combination with 1.250, 0.625, 0.313, 0.156, and 0.078 % v/v ACS. For the NIS+ACS combination*,* *L. monocytogenes* strains were exposed to 6.250, 3.125, 1.563, 0.781, and 0.391 ppm w/w active NIS in combination with 1.250, 0.625, 0.313, 0.156, and 0.078 % v/v ACS. For the SL+ACS combination, strains were exposed to 5.000, 2.500, 1.250, 0.625, and 0.313 % w/w of active SL in combination with 1.250, 0.625, 0.313, 0.156, and 0.078 % v/v ACS. For the NIS+LAE combination, all strains were exposed to 6.250, 3.125, 1.563, 0.781, and 0.391 ppm w/w active NIS in combination with 12.500, 6.250, 3.125, 1.563, and 0.781 ppm w/w active LAE. For the OCT+ACS combination at pH 5.00, all strains were exposed to 25.000, 12.500, 6.250, 3.125, and 1.563 ppm w/w active OCT in combination with 0.156, 0.078, 0.039, 0.020, and 0.010 % v/v ACS. For the OCT+NIS combination, Scott A and 310 were exposed to 25.000, 12.500, 6.250, 3.125, and 1.563 ppm w/w active OCT in combination with 0.195, 0.098, 0.049, 0.024, and 0.012 ppm active NIS, while NADC 2783 and NADC 2045 were exposed to 5.000, 12.500, 6.250, 3.125, and 1.563 ppm w/w active OCT in combination with 1.562, 0.781, 0.391, 0.195, and 0.098 ppm active NIS. This alteration for the OCT+NIS combination was performed in response to differences observed in strain-dependent inhibition of *L. monocytogenes* Scott A and 310 versus NADC 2783 and 2045 by single application of Nisin at pH 5.0.

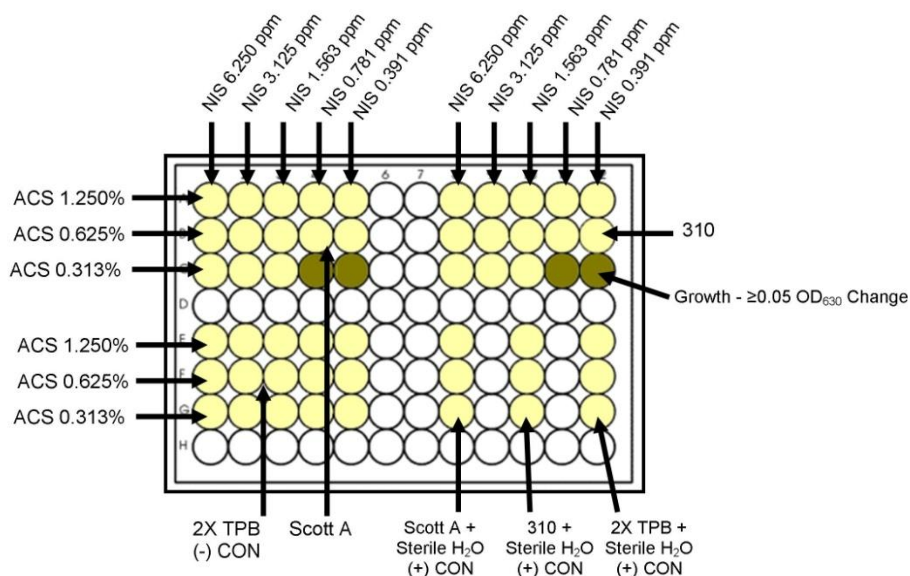

Figure 2: Checkerboard assay microplate setup and interpretation.

Ninety-six well microtiter plates were again used for carrying out the checkerboard assay (Figure 2). 75 µl of antimicrobial A solution (EPL in EPL+ACS, NIS in NIS+ACS and NIS+LAE, SL in SL+ACS, and OCT in OCT+ACS and OCT+NIS) was added to the test wells in columns along with 50 µl of antimicrobial B solution (ACS in EPL+ACS, NIS+ACS, SL+ACS and OCT+ACS, LAE in NIS+LAE, and NIS in OCT+NIS) added in rows. *L. monocytogenes* inoculum in 2x TPB (125 µl) was then added to the 125 µl of combined antimicrobial solutions. Sterile barrier tips were used to load solutions into the wells so as to minimize potential for cross-contamination. Wells containing only antimicrobial-containing solutions and sterile 2x TPB were built in as negative controls for baseline correction adjustment of optical density values. Wells containing only sterile water and inocula in double-strength TPB were again built in as positive controls.

After loading plates, OD630 was measured at 0 h and 24h using the same protocol from the single antimicrobial susceptibility tests (described above). All susceptibility tests for each combination of test antimicrobials were repeated at least twice for each strain. Wells that produced a <0.05 ΔOD630 after 24 h incubation over both replications were again classified as being inhibitory. Inhibitory combinations that utilized the least amounts of one compound in combination with the other were then classified as optimal inhibitory combinations (OIC)

(Figure 2). The respective concentrations of the two compounds that were added together in these OICs were reported as the combination MICs in Tables 4-5.

Assessment of Antimicrobial Interactions: Interaction of antimicrobials was assessed by calculating the fractional inhibitory concentration (FIC) of antimicrobials applied in combination against target pathogen strains (Figure 3). An FIC was defined as the concentration of antimicrobial A in an OIC divided by the MIC of antimicrobial A from single antimicrobial susceptibility testing. To determine and classify the interactions of antimicrobials, FIC indexes (FIC_I) for optimal inhibitory combinations were calculated as the sum of the FIC of antimicrobial A and the FIC of antimicrobial B. Antimicrobial interactions were defined as antagonistic ($FIC_I > 1$), additive ($FIC_I = 1.0$), or synergistic ($FIC_I < 1.0$).[5]

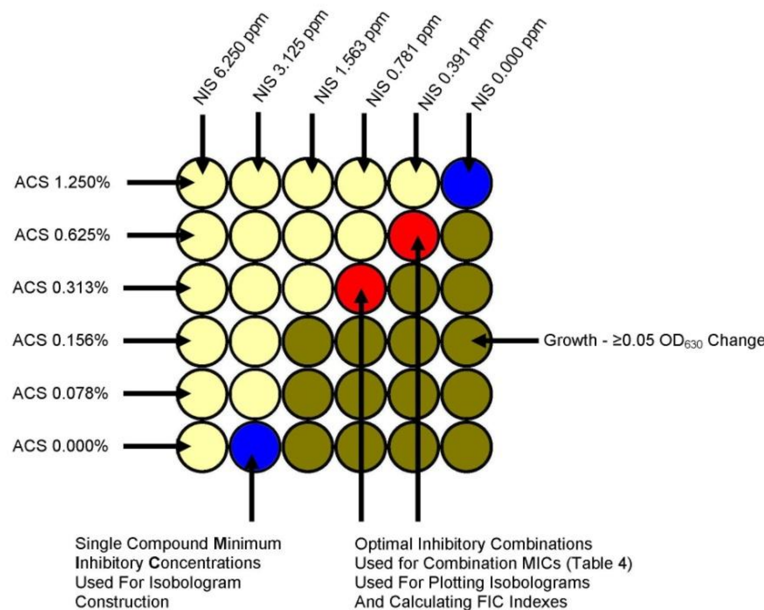

Figure 3: Determination of combined antimicrobials MICs for antimicrobial interaction characterization and isobologram construction.

Antimicrobial Bactericidal Activity: For both single antimicrobial and combined antimicrobials susceptibility testing, single antimicrobials and antimicrobial combinations were evaluated for bactericidal activity. Bactericidal activity was assessed by spreading 100 µl aliquots from wells that were classified as inhibitory onto TPA. Inoculated plates were aerobically incubated at 35°C for 24 h and survivors were enumerated. Concentrations of single antimicrobials and antimicrobial combinations that produced at least a 3 log CFU/ml (99.9%) decrease in viable cells from the starting concentration of the inoculum were classified as bactericidal. The lowest concentration of antimicrobial applied alone or in combination producing bactericidal effects was declared the MBC. Bactericidal combinations that utilized the least amounts of one compound in combination with the other were then classified as optimal bactericidal combinations (OBC). The respective concentrations of the two compounds that were added together in these OBCs were reported as the combination MBCs in Table 4 and Table 5.

Research Results

Pathogen Inhibition via Single Antimicrobials: The MICs and MBCs of antimicrobials applied to strains of *L. monocytogenes* grown in pH 7.3 TPB at 35°C are provided in Table 2. The MIC of NIS tested at pH 7.3 for Scott A was 3.125 ppm and was 6.25 ppm for all others; 6.25 ppm exhibited bactericidal activity against all

[5] Branen, J.K., P.M. Davidson. 2004. *Int. J. Food Microbiol.* 90:63-74.

strains except NADC 2045. The MIC of LAE for *L. monocytogenes* strains was 12.50 ppm; LAE was bactericidal to all strains at 12.50 ppm also. *L. monocytogenes* Scott A and 310 growth was inhibited by EPL at a concentration of 6.25 ppm; for NADC 2783 and NADC 2045, the EPL MIC was 12.50 ppm. The MBC for EPL against *L. monocytogenes* 310 and NADC 2045 was 12.50 ppm, but for Scott A and NADC 2783, the MBC was 25.00 ppm. Acidic calcium sulfate was consistently inhibitory and bactericidal at 1.25% v/v at pH 7.0 against *L. monocytogenes*. Sodium-L-Lactate at pH 7.0 was not inhibitory at any experimental concentration and no MBC was detected. Octa-Gone was not tested at pH 7.0 according to manufacturer recommendations.

Table 2: Experimentally determined MIC and MBC levels of antimicrobials against strains of *L. monocytogenes* at pH 7.3.[c]

Antimicrobial[a] (Units)	*L. monocytogenes* Scott A		310		NADC 2783		NADC 2045	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
NIS (ppm)	3.125	6.25	6.25	6.25	6.25	6.25	6.25	None[b]
EPL (ppm)	6.25	25.00	6.25	12.50	12.50	25.00	12.50	12.50
ACS (% v/v)	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
LAE (ppm)	12.50	12.50	12.50	12.50	12.50	12.50	12.50	12.50

a. NIS = Nisin; EPL = ε-Poly-L-lysine; ACS = Acidic Calcium Sulfate; LAE = Lauramide Arginine Ester.
b. None = No MBC obtained.
c. MIC values are determined as the lowest concentration of antimicrobial for which ΔOD630 is <0.05 after 24 hr incubation at 35°C over two replications. MBC values are determined as the lowest concentration of antimicrobial for which a 3 log-cycle reduction is observed.

Table 3 provides minimum inhibitory and bactericidal concentrations of experimental antimicrobials tested at pH 5.0 in accordance with manufacturer recommendations. Significant strain-dependent sensitivity was observed with NIS-driven inhibition of experimental strains. *L. monocytogenes* Scott A and 310 were inhibited at a concentration of 0.195 ppm NIS with bactericidal effects being observed at 0.391 ppm NIS at pH 5.0. Conversely, *L. monocytogenes* NADC 2783 and NADC 2045 were inhibited at 1.563 ppm NIS and 0.781 ppm NIS, respectively; the observed MBC was 1.563 ppm NIS at pH 5.0 for both NADC 2783 and NADC 2045(Table 3). In the case of ACS, MIC and MBC values of the antimicrobial for all *L. monocytogenes* strains were 0.156% and 0.625%, respectively. The MIC for Octanoic acid (Octa-Gone®, Ecolab, Inc.) against all strains of *L. monocytogenes* was 25.00 ppm; no experimental concentration of the antimicrobial exhibited bactericidal capacity against target pathogen strains.

Table 3: Experimentally determined MIC and MBC levels of antimicrobials against strains of *L. monocytogenes* at pH 5.0.[c]

Antimicrobial[a] (Units)	*L. monocytogenes* Scott A		310		NADC 2783		NADC 2045	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
NIS (ppm)	0.195	0.391	0.195	0.391	1.563	1.563	0.781	1.563
ACS (% v/v)	0.156	0.625	0.156	0.625	0.156	0.625	0.156	0.625
OCT (ppm)	25.00	None[b]	25.00	None	25.00	None	25.00	None

a. NIS = Nisin; ACS = Acidic Calcium Sulfate; OCT = Octanoic Acid.
b. No experimental level tested was bactericidal.
c. MIC values are determined as the lowest concentration of antimicrobial for which ΔOD630 is <0.05 after 24 hr incubation at 35°C over two replications. MBC values are determined as the lowest concentration of antimicrobial for which a 3 log-cycle reduction is observed. Growth medium was pH adjusted with 6.0 N HCl (Thermo-Fisher Scientific).

Pathogen Inhibition by Combined Antimicrobials: The combination MICs determined with SL and ACS in combination against all *L. monocytogenes* strains at pH 7.3 were 0.625% (v/v) ACS + 0.313% SL (w/w) (Table 4). No combinations of ACS and SL produced OBCs, and thus no combination MBCs were determined. At pH 7.3, NIS and ACS in combination was inhibitory at minimum levels of 1.563 ppm NIS + 0.313 % ACS for Scott A; 3.125 ppm NIS + 0.078 % ACS for 310, NADC 2783, and NADC 2045; and 0.391 ppm NIS + 0.625 % ACS for all strains (Table 4). Bactericidal effects were observed when 3.125 ppm NIS was combined with 0.078% ACS for Scott A and 310; 6.25 ppm NIS + 0.078 % ACS for NADC 2045; 3.125 ppm NIS + 0.156 % ACS for NADC 2783 and NADC 2045; 1.563 ppm NIS + 0.625% ACS for NADC 2783; 0.781 ppm NIS + 0.625 % ACS for 310 and NADC 2045; and 0.391 ppm NIS + 0.625 % ACS for Scott A (Table 4). Combining EPL with ACS resulted in MICs of 0.781 ppm EPL + 0.625% ACS (w/w) for all strains but *L. monocytogenes* NADC 2045. Additional EPL+ACS MICs were 3.125 ppm EPL + 0.313 % ACS for Scott A; 6.250 ppm EPL + 0.156 % ACS for 310; and 1.563 ppm EPL + 0.625 % ACS for NADC 2045 (Table 4). No OBCs were able to be determined the EPL+ACS combination and so no combination MBCs were denoted (Table 4). Optimal inhibition via LAE+NIS was observed at 1.563 ppm NIS + 6.25 ppm LAE for 310 and NADC 2045; 0.781 ppm NIS + 6.25 ppm LAE for Scott A; 3.125 ppm NIS + 6.25 ppm LAE for NADC 2783; 3.125 ppm NIS + 3.125 ppm LAE for NADC 2045; and 3.125 ppm NIS + 1.563 ppm LAE for 310 (Table 4). LAE + NIS combination MBCs were observed at 3.125 ppm NIS + 6.25 ppm LAE for 310 and NADC 2045; 3.125 ppm NIS + 3.125 ppm LAE for Scott A; 6.25 ppm NIS + 0.781 ppm LAE for NADC 2045; and 1.563 ppm NIS + 6.25 ppm LAE for Scott A. No combinations of NIS+LAE produced OBCs for NADC 2783 (Table 4).

For combinations in which Octa-Gone® (octanoic acid) was utilized, samples were tested at pH 5.0 following manufacturer recommendations for product testing. No experimental combination of OCT and ACS at pH 5.0 exhibited bactericidal activity, resulting in no MBC identification (Table 5). However, optimal OCT + ACS inhibition was seen at 12.5 ppm OCT + 0.039 % ACS for 310 and NADC 2045; 12.5 ppm OCT + 0.020 % ACS for Scott A; 12.5 ppm OCT + 0.010 % ACS for NADC 2783; 6.25 ppm OCT + 0.020 % ACS for NADC 2783; 3.125 ppm OCT + 0.078 % ACS for 310; and 1.563 ppm OCT + 0.078 % ACS for all strains except 310 (Table 5). With respect to OCT and NIS, No combinations of OCT and NIS produced optimal inhibition (lower than that expected for the compounds by themselves) against strains Scott A and 310, but increased amounts were required for inhibition in some cases. Strain NADC 2783 was consistently inhibited at 12.5 ppm OCT + 0.195 % ACS and NADC 2045 was consistently inhibited at 3.125 ppm OCT + 0.391 % ACS; no combinations of OCT+NIS were bactericidal and thus no combination MBCs could be calculated (Table 5).

Table 4. Minimum inhibitory and bactericidal concentrations of antimicrobials against *L. monocytogenes* tested at pH 7.3.[b, c]

	Minimum Inhibitory Concentrations				Minimum Bactericidal Concentrations			
L. monocytogenes	SL (%) + ACS (%)	EPL (ppm) + ACS (%)	NIS (ppm) + ACS (%)	NIS (ppm) + LAE (ppm)	SL (%) + ACS (%)	EPL (ppm) + ACS (%)	NIS (ppm) + ACS (%)	NIS (ppm) + LAE (ppm)
Scott A	0.313+0.625	3.125+0.313 0.781+0.625	1.563+0.313 0.391+0.625	0.781+6.25	None[a]	None[a]	3.125+0.078 0.391+0.625	3.125+3.125 1.563+6.25
310	0.313+0.625	6.250+0.156 0.781+0.625	3.125+0.078 0.391+0.625	3.125+1.563 1.563+6.25	None[a]	None[a]	3.125+0.078 0.781+0.625	3.125+6.25
NADC 2783	0.313+0.625	0.781+0.625	3.125+0.078 0.391+0.625	3.125+6.25	None[a]	None[a]	3.125+0.156 1.563+0.625	None[a]
NADC 2045	0.313+0.625	1.563+0.625	3.125+0.078 0.391+0.625	3.125+3.125 1.563+6.25	None[a]	None[a]	6.25+0.078 3.125+0.156 0.781+0.625	6.25+0.781 3.125+6.25

a. No combinations produced bactericidal activity at lower levels in combination than the bactericidal activity of the antimicrobials used singly.

b. MIC values are determined as the lowest concentration of antimicrobial for which ΔOD630 = <0.05 after 24 hr incubation at 35°C. MBC values are determined as the lowest concentration of antimicrobial for which a 3 log-cycle reduction is observed.

c. ACS: Acidic Calcium Sulfate; SL: Sodium L-Lactate; NIS: Nisin; EPL: ε-Poly-L-lysine

Table 5. Minimum inhibitory and bactericidal concentrations of octanoic acid in combination with antimicrobials against *L. monocytogenes* tested at pH 5.0.[b, c]

	Minimum Inhibitory Concentrations		Minimum Bactericidal Concentrations	
L. monocytogenes	OCT (ppm) + ACS (%)	OCT (ppm) + NIS (ppm)	OCT (ppm) + ACS (%)	OCT (ppm) + NIS (ppm)
Scott A	12.5+0.020 1.563+0.078	None[a]	None[b]	None[b]
310	12.5+0.039 3.125+0.078	None[a]	None[b]	None[b]
NADC 2783	12.5+0.010 6.25+0.020 1.563+0.078	12.5+0.195	None[b]	None[b]
NADC 2045	12.5+0.039 1.563+0.078	3.125+0.391	None[b]	None[b]

a. No combinations produced inhibition at lower levels in combination than the inhibitory activity of the antimicrobials used singly.

b. No combinations produced bactericidal activity at lower levels in combination than the bactericidal activity of the antimicrobials used singly.

c. MIC values are determined as the lowest concentration of antimicrobial for which ΔOD630 is <0.05 after 24 hr incubation at 35°C. MBC values are determined as the lowest concentration of antimicrobial for which a 3 log-cycle reduction is observed. Growth medium was pH adjusted with 1.0 N HCl (Thermo-Fisher Scientific).

d. OCT: Octanoic Acid; ACS: Acidic Calcium Sulfate; NIS: Nisin

Characterization of Antimicrobial Interactions: Figures 4-6 contains the isobolograms depicting interactions for compound combinations tested at pH 7.3. MIC values in Table 4 were transformed into FIC values and were plotted on the isobolograms. FIC_I values for each point on the graph can be obtained by adding the values of the two individual compound FIC values together and determinations of synergism, additivism, and antagonism can be deduced using these values and the scale that was previously described (<1, 1, >1). With the exception of *L. monocytogenes* 310, which showed slight antagonism, the interaction of EPL with ACS was mostly additive in nature (Figure 4). In combination with ACS, NIS functioned synergistically against all strains of *L. monocytogenes* (Figure 5). Combination of Nisin with LAE resulted in additive-type inhibition between antimicrobials (Figure 6). When octanoic acid was combined with acidic calcium sulfate at pH 5.0, experimental strains of *L. monocytogenes* excepting NADC 2783 were inhibited synergistically (Figure 7). Conversely, the combination of NIS and OCT resulted in evidence of significant antagonism between antimicrobials; all FIC_I values were >1.0. While ACS and SL were tested in combination (Data not shown), no MIC was determined for SL at pH 7.3 either alone or in combination, disallowing the conclusive determination of the antimicrobial interaction type.

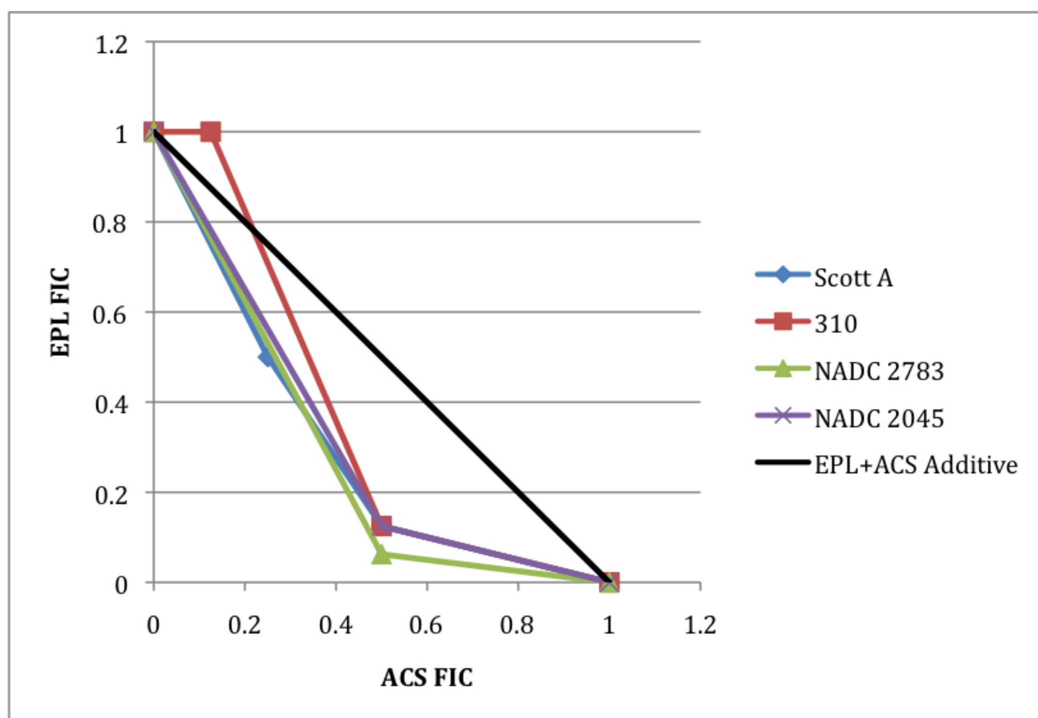

Figure 4: Isobologram of antimicrobial fractional inhibitory concentrations (FIC) for ε-Poly-L-lysine in combination with Acidic Calcium Sulfate at pH 7.3. An FIC was defined as the concentration of antimicrobial A/MIC of antimicrobial A from single antimicrobial susceptibility testing. Symbols represent the mean of duplicate replications.

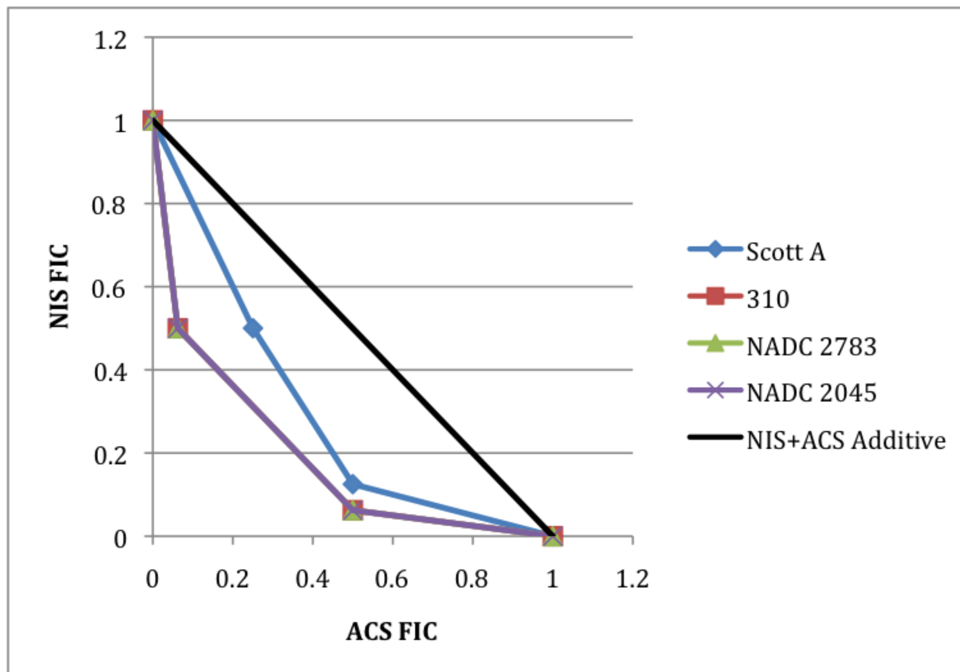

Figure 5: Isobologram of antimicrobial FIC values for Nisin in combination with Acidic Calcium Sulfate at pH 7.3. An FIC was defined as the concentration of antimicrobial A/MIC of antimicrobial A from single antimicrobial susceptibility testing. Symbols represent the mean of duplicate replications.

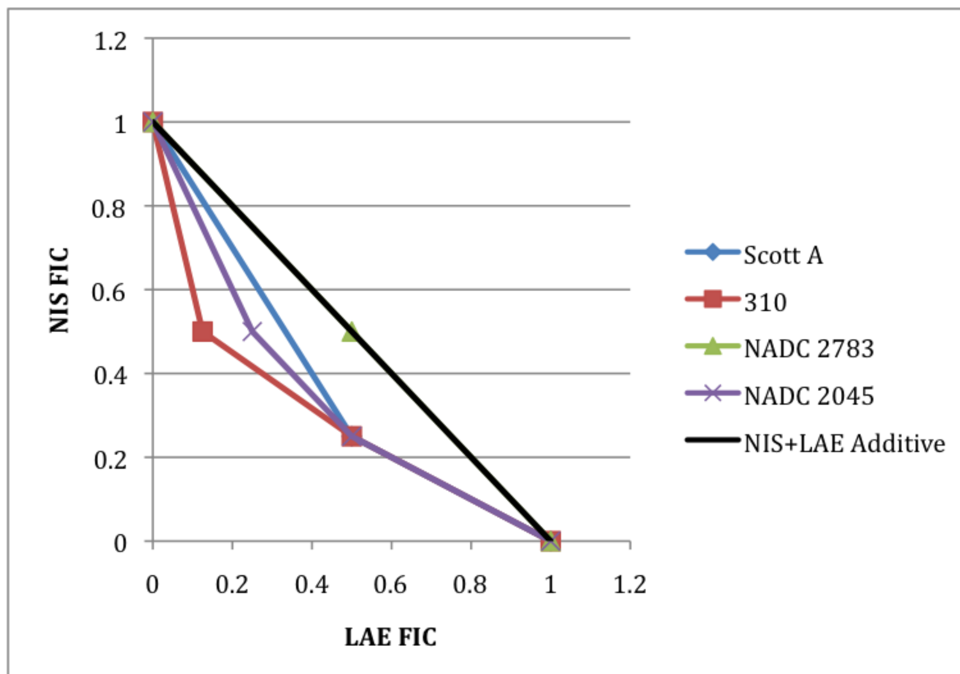

Figure 6: Isobologram of antimicrobial FIC values for Nisin in combination with Lauramide Arginine Ethyl Ester at pH 7.3. An FIC was defined as the concentration of antimicrobial A/MIC of antimicrobial A from single antimicrobial susceptibility testing. Symbols represent the mean of duplicate replications.

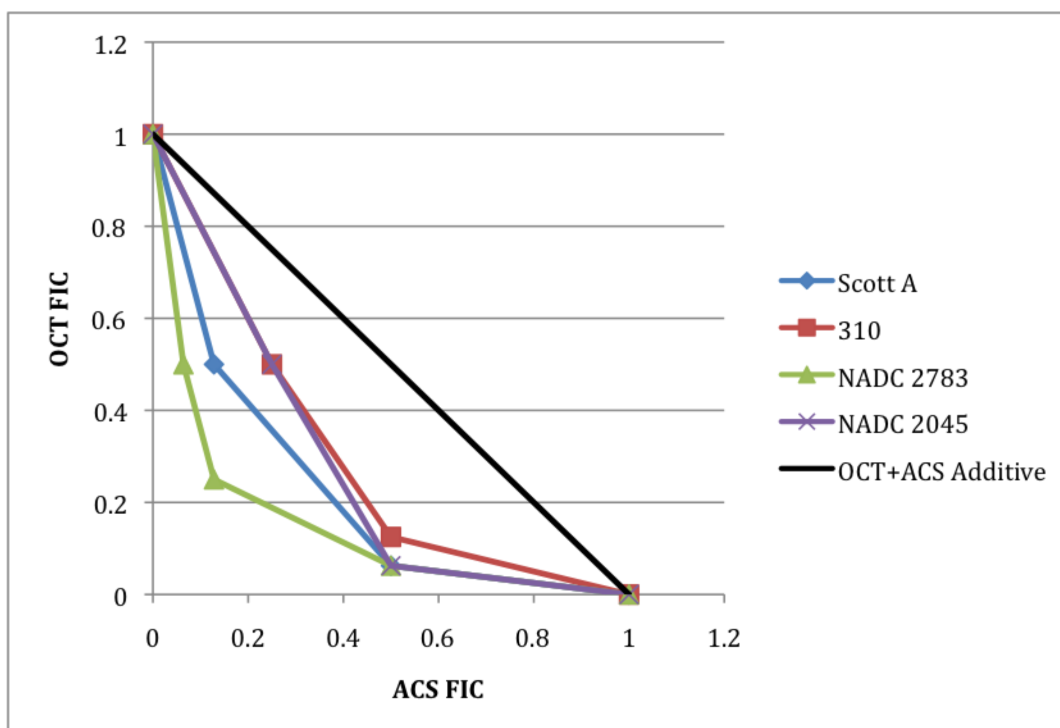

Figure 7: Isobologram of antimicrobial FIC values for Octanoic Acid in combination with Acidic Calcium Sulfate at pH 5.0. An FIC was defined as the concentration of antimicrobial A/MIC of antimicrobial A from single antimicrobial susceptibility testing. Symbols represent the mean of duplicate replications.

Discussion

Experimental results indicate that *in vitro* inhibition and inactivation of *L. monocytogenes* strains occurred with intermittent strain-dependency. While some antimicrobials were capable of exerting bactericidal effects at levels equivalent to the MIC, this phenomenon was not observed for all antimicrobials (EPL, SL). Indeed, the observation of MBCs for all strains of *Listeria monocytogenes* exposed to the bacteriocin Nisin at pH 7.3 except NADC 2045 indicates that this strain may possess some physiological feature that enhances its ability to survive and grow in the presence of the antimicrobial. The acidification of the growth medium to pH 5.0 resulted in significant enhancement of Nisin or ACS-driven inhibition of pathogen strains, likely due to increased bacteriocin solubilization or protonation of the active agents, respectively. In the case of Nisin, its solubility has been reported to increase at acidic pH. Acidic Calcium Sulfate contains large amounts of lactic acid; acidification of the medium to pH 5.0 would result in greater protonation of the molecule, likely enhancing its ability to penetrate the bacterial cell. This might also explain the lack of an MBC for NADC 2045 at pH 7.3 with the acquisition of bactericidal effects at pH 5.0 in that protonation of the antimicrobial would therefore enhance its effect, inactivating the pathogen more effectively.

With regards to combination of antimicrobials against *L. monocytogenes* strains, experimental findings demonstrate that pairing of antimicrobials may result in enhancement of the activity of antimicrobials as indicated by a decrease in antimicrobial MICs. The combination of Nisin and

ACS did result in synergistic inhibition of the pathogen, potentially a result of the acidulant enhancing bacteriocin solubility and likely differing cellular targets. Despite consistent inhibition of *L. monocytogenes* strains at 25.00 ppm (MIC), Octanoic Acid activity was enhanced by addition of Acidic Calcium Sulfate, again resulting in synergistic inhibition of *L. monocytogenes*. The surfactant LAE interacted additively with NIS, possibly a result of surfactant-induced membrane destabilization aiding the bacteriocin. The pairing of EPL with ACS also resulted in additive inhibition of *L. monocytogenes*. Conversely, the interaction of NIS and OCT was strongly antagonistic, indicating that the combination of the fatty acid with bacteriocin resulted in deleterious effects upon the activity of each against the pathogen. While the fatty acid antimicrobial also contained other ingredients that might have aided the inhibition of the pathogen by Nisin, it is unlikely that any such interactions took place considering the FIC_I values observed. Finally, while ACS did function to potentiate the inhibition potential of SL, likely via protonation, synergistic interaction of the two compounds was not expected due to similarities in chemical makeup and likely targets for cellular attack. While it may be assumed that the interaction of ACS and SL might be antagonistic, as no MIC was determined for SL at pH 7.3 applied singly, no interaction classification can be conclusively ascribed here.

The interpretation of antimicrobial interactions is difficult and subject to a number of factors, including antimicrobial chemistry, test conditions, antimicrobial concentrations, target pathogen inherent immunity, etc.[6] For those who might wish to combine antimicrobials with differing modes of inhibition, the solubility of each antimicrobial must be carefully considered in addition to the MIC of each compound alone, the food matrix, the potential for development of antimicrobial resistance, and the potential for negative effects on the organoleptic qualities of the product. In general, the use of a previously validated single antimicrobial that performs in a consistent manner is preferable to the use of novel antimicrobials whose modes of action have not been fully elucidated or for which there exists little data verifying its activity.

In conclusion, with the exceptions of NIS+ACS and OCT+ACS, no combination of antimicrobials produced synergistic inhibition of *L. monocytogenes*. Further, NIS+ACS inhibition was consistently observed across all test strains. These data can provide assistance in the determination of antimicrobial usage conditions for the inhibition of *L. monocytogenes* on RTE meats. Future studies should focus on determining the interactions of NIS+ACS and OCT+ACS on the surfaces of RTE meats.

[6]Davidson, P.M. and M.E. Parish. 1989. *Food Technol*. 43:148-155.