Project Title: Formation, Survival, and Virulence of Stress-Induced Filamentous *Listeria monocytogenes* (SIFL) – Phase I
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Research Institution: University of Wisconsin-Madison
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Objectives:

Listeria monocytogenes is well known as a hardy organism that can survive many environmental stresses. One response to stress is the formation of filaments (elongated cells without septation) that retain the ability to divide into multiple normal-sized cells. This study was based on our hypothesis that certain stresses encountered by *L. monocytogenes* in the ready-to-eat (RTE) meat processing environment induce filamentation, which contributes to the pathogen's ability to survive in the food processing environment and enhances its virulence potential. Under favorable conditions, the filaments divide into multiple normal-sized cells, leading to a sudden increase in the number of *L. monocytogenes*, which can impact safety and risk assessment of food products.

Our objective was to determine the conditions that affect the formation and persistence of *L. monocytogenes* filaments, and growth and survival properties of these filaments.

Conclusions:

L. monocytogenes developed filaments when exposed to osmotic stress induced by low water activity. When the stress was removed, the filaments septated and divided into normal-sized cells at 30°C and 4°C, leading to an increase in bacterial numbers more quickly than control (non-filamented) cells. The relative ability of filaments and control cells to survive stresses commonly encountered in the RTE meat environment varied depending on the stress and exposure time. Filaments were less sensitive than control cells to acid (pH 2) and drying on a stainless steel surface, but survival was similar at high temperature (55°C). Three sanitizers tested were all effective against both filaments and control cells. *L. monocytogenes* did not grow or develop filaments on three types of luncheon meat tested; however, there was a significant decrease in viability on pepperoni, which had the lowest water activity. Control cells and filaments decreased in viability to a similar extent when exposed to either nitrite or a nisin/rosemary extract blend. Both control cells and filaments increased in viable numbers in diacetate/lactate, with greater increases in the filament culture, while in sodium hexametaphosphate, the viable control cell number generally decreased but that of filaments increased. Overall, exposure to luncheon meats or an antimicrobial appeared to prolong the persistence of the filamentous phenotype.

Deliverable:

The presence of *L. monocytogenes* filaments in meat and processing ingredients could impact the safety and risk assessment of meat products. If filaments form septa when favorable conditions are encountered, there will be a sudden increase in the number of *L. monocytogenes* that will significantly affect estimations of the total number of viable cells of *L. monocytogenes* in the food. This could affect retrospective assessments of the infectious dose. Likewise, occurrence of filamentous *L. monocytogenes* in the processing environment can influence detection, enumeration, effective processing parameters, and risk assessments.

Formation, Survival, and Virulence of Stress-Induced Filamentous *Listeria monocytogenes* (SIFL) – Phase I

Final Report submitted to the American Meat Institute Foundation

August 5, 2011

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Abstract

Listeria monocytogenes is well known as a hardy organism that can survive many environmental stresses. One response to stress is the formation of filaments (elongated cells without septation) that retain the ability to divide into multiple normal-sized cells. This study was based on our hypothesis that certain stresses encountered by *L. monocytogenes* in the ready-to-eat (RTE) meat processing environment induce filamentation, which contributes to the pathogen's ability to survive in the food processing environment and enhances its virulence potential. Our objective was to determine the formation, survival, and growth of stress-induced filamentous *L. monocytogenes* (SIFL). Five strains of *L. monocytogenes* of three serotypes were able to develop filaments when subjected to osmotic stress on tryptic soy agar (TSA) with 6% NaCl (TSA-6, a_w 0.96) or in tryptic soy broth (TSB) with 8% NaCl (a_w 0.95). Control (non-filamented) cells developed on TSA without added NaCl and filaments of strain Scott A developed on TSA-6 were tested for growth in fresh TSB and on TSA, and survival in TSB with sodium nitrite (156 ppm), sodium diacetate/lactate (0.1%/1.5% and 0.25%/3.75%), or sodium hexametaphosphate (0.1, 0.3, and 0.5%), and on TSA with a nisin/rosemary extract blend (0.02, 0.05, and 0.1%). Survival was also determined at 55°C and pH 2, on three types of luncheon meats, chicken, beef, and pepperoni, when desiccated on stainless steel, and in a quaternary ammonium, acid, or chlorine-based sanitizer. Scott A filaments septated and increased in colony-forming units (CFU) more rapidly than control cells when grown in TSB or on TSA at both 4°C and 30°C. The relative ability of Scott A filaments and control cells to survive stresses commonly encountered in the RTE meat environment varied depending on the stress and exposure time. At pH 2, filaments were inactivated more rapidly than control cells initially, but with longer exposure, there was a greater decrease in viability for control cells. At 55°C survival of control cells were similar although control cells inactivated more rapidly initially. More Scott A control cells were inactivated than filaments during desiccation on stainless steel surfaces. Both control cells and filaments were inactivated by the three sanitizers. Scott A did not grow or develop filaments on the three types of luncheon meat tested. There was a significant decrease in viability on pepperoni, which had the lowest a_w (0.86). Control cells and filaments decreased in viability to a similar extent when exposed to either nitrite or a nisin/rosemary extract blend. Both control cells and filaments increased in CFU in diacetate/lactate, with greater increases in the filament culture, while in sodium hexametaphosphate, the CFU of control cells generally decreased but that of filaments increased. Overall, exposure to luncheon meats or an antimicrobial appeared to prolong the persistence of the filamentous phenotype. The presence of *L. monocytogenes* filaments in food and the food processing environment could impact the safety and risk assessment of food products.

Objectives

1. Determine conditions that affect formation and persistence of SIFL
2. Determine growth and survival properties of SIFL

Material and Methods

Our overall approach was to test the ability of *L. monocytogenes* to form filaments and compare the ability of control (non-filamented) cells and filaments to survive under stress conditions that are relevant to RTE meats and their processing environments. These include low water activity (a_w), low and high temperatures, acid pH, surface desiccation, sanitizers, and exposure to RTE meats and antimicrobials used in these products.

Bacterial strains

Five *L. monocytogenes* strains of three different serotypes were used: Scott A (serotype 4b; clinical isolate), LM101 (serotype 4b; hard salami), LM310 (serotype 4b; goat milk cheese), V7 (serotype 1/2a; raw milk), and LM108 (serotype 1/2b; hard salami). Strains were stored in 40% glycerol at -70°C. Working stock cultures were maintained on tryptic soy agar (TSA) (Becton Dickinson, Sparks, MD) plates. Inoculum was prepared by transferring a colony from a TSA plate into 3 ml TSB, followed by overnight (20 to 24 hours) incubation at 37°C.

Filament formation at reduced a_w

Listeria monocytogenes survival and filament formation at reduced a_w was determined with tryptic soy broth (TSB; a_w 0.99) supplemented with 4, 8, and 12% NaCl to achieve a_w levels of 0.98, 0.95, and 0.92, respectively. The a_w levels were measured using an Aqualab model 4TE a_w meter (Decagon Devices, Pullman, WA) according to the manufacturer's instructions. An overnight *L. monocytogenes* culture was adjusted to an optical density at 600 nm (OD_{600nm}) of 1 and 40 µl portions were added to wells containing 2 ml of the appropriate medium in polystyrene 24-well plates (Falcon, Becton Dickinson, Franklin Lakes, NJ), followed by incubation at 4 or 30°C for 6 days. Samples were removed periodically and colony-forming units per milliliter (CFU/ml) were determined on plate count agar (PCA). Cell morphology was assessed microscopically after the bacteria were heat-fixed on a glass slide and stained with crystal violet. The stained cells were viewed using an Olympus BH2 microscope equipped with an Olympus model DP70 digital camera. Images were captured and processed using DP Controller version 1.2.1.108 software. The size and percentage of elongated cells were estimated from ten fields of view. *Listeria monocytogenes* cells were normally 1 to 2 µm in length. Cells at least three times longer (>7 µm) than the maximal normal length were considered filaments.

Filament formation was also determined on TSA agar (a_w 0.99) supplemented with 6 and 7% NaCl to achieve a_w levels of 0.96 and 0.95, respectively. One hundred microliters of an overnight *L. monocytogenes* culture were spread on the surface of a plate. The plate was sealed with parafilm to prevent evaporation and incubated at 25, 30, or 37°C for 3 days.

L. monocytogenes cells were harvested by pipetting 2 ml of 0.01M phosphate-buffered saline (PBS, pH 7.4) onto each plate and scraping cells off the agar surface with a sterile plastic rod. Viable counts and cell morphology were determined as described above.

Survival and growth of *L. monocytogenes* filaments and control cells

Strain Scott A was grown on TSA and TSA supplemented with 6% NaCl (TSA-6) at 30°C for 3 days to generate control (non-filamented) and filamented cells, respectively, for use in survival experiments. Cells harvested from TSA and TSA-6 were adjusted to a concentration of ~ 7 log CFU/ml in sterile tap water unless noted otherwise. The cells were exposed to the

following stress conditions and assayed for their ability to survive, the potential for control cells to form filaments, and the ability of filamented cells to septate and divide into normal-sized cells.

Low pH: TSB was adjusted with 6N HCl to pH 2 or 4 and filter-sterilized using a 0.22 μm filter unit (Millex®GV). Tubes were filled with 9 ml of medium and tempered at 37°C. One milliliter of control cells or filaments containing ~7 $\log_{10}$ CFU/ml in sterile distilled water was added to each tube. Samples were removed immediately after inoculation and after 2, 5, 10, 20, and 30 minutes to determine CFU/ml and cell morphology.

Heat: Tubes containing 900 μl of TSB tempered at 55°C were inoculated with 100 μl of control and filamentous cells containing ~7 $\log_{10}$ CFU/ml in sterile distilled water. Tubes were removed immediately after inoculation and after 2, 5, 10, 20, and 30 minutes to determine CFU/ml and cell morphology.

Anti-microbials: Sodium diacetate and sodium lactate (Optiform SD4; 4% sodium diacetate, 60% sodium lactate, and 36% water; PURAC, Lincolnshire. IL), sodium nitrite (Sigma), sodium hexametaphosphate (Hexaphos; Astaris, St. Louis, MO), and a blend of nisin and natural rosemary extract (NovaGARD® (Guardian) NR 100; Danisco, Grindstad, Denmark) were used. Except for the nisin/rosemary extract, all antimicrobials were tested in TSB. They were dissolved in TSB to achieve the final test concentrations and filter-sterilized using a 0.22 μm filter unit (Millex®GV). Forty microliters of the inoculum culture were added to wells containing 2 ml of TSB with the appropriate antimicrobial in 24-well plates, followed by incubation at 4°C. The nisin and rosemary extract was added to TSA at the desired concentrations and autoclaved. One hundred microliters of the inoculum were spread on the surface of TSA plates, which were incubated at 4°C. Cell morphology and CFU/ml were determined at 0, 24, 74, and 144 hours.

RTE meats: Beef (a_w 0.96) and chicken (a_w 0.96) luncheon meats and pepperoni (a_w 0.88) were purchased from a local grocery store. One gram meat samples were cut and weighed aseptically and surface-inoculated with 10 μl of a culture adjusted to a concentration of ~10 $\log_{10}$ CFU/ml. The inoculated samples were placed in sterile petri dishes, wrapped with parafilm, and incubated at 4°C. After 72 and 144 hours the meat samples were placed in 5 ml of PBS + 0.5% Tween 80 and vortexed for 1 minute to recover bacterial cells. Cell morphology and CFU/g were determined.

Desiccation: Stainless steel type 304 with a no. 4 finish (Temperature Systems, Madison, WI) was cut into 1-cm^2 chips, washed with detergent (Micro, International Products Corp., Trenton, NJ), and sterilized by autoclaving. Each chip was inoculated with 40 μl of an overnight Scott A culture adjusted to an OD_{600nm} of 1 in sterile tap water. Inoculated chips were placed in petri dishes. The latter were put in desiccators containing saturated $MgCl_2 \cdot H_2O$ or NaCl solutions to maintain 32.5 or 75.5% relative humidity (RH), respectively, and incubated at 25°C. Chips were removed at 24, 72, and 144 hours and placed in 2 ml PBS in a tube with several glass beads. *Salmonella* cells were dislodged from the chips by vortexing for 15 seconds. The number of CFU/chip and cell morphology were determined.

Sanitizers: Sodium dichloro-s-triazinetrione dehydrate (Trichlor-O-Cide® XP-160), peroxyoctanoic acid (Octave™), and dimethyl benzyl ammonium chloride (Ster-Bac®) (Ecolab, St. Paul, MN) were diluted with sterile tap water to twice the concentrations recommended by the manufacturer. Control cells and filaments suspended in sterile tap water were added to equal volumes of the double-strength sanitizer solutions and incubated for 0.5, 2, 5, and 10 minutes at room temperature. At each sampling time, sanitizers were neutralized with 0.01M sodium

thiosulfate (for Trichlor-O-Cide® XP-160), 0.003N NaOH (for Octave™), or Tween 80-lecithin (for Ster-Bac®) prior to cell morphology and CFU/ml determination.

Data analysis

Data presented are the average of two independent trials with two replications per trial. Statistical analysis was performed using the *t*-test with Microsoft Excel.

Results

Filament formation under reduced a_w conditions

We tested the ability of each of five strains of three different serotypes, Scott A, LM101, LM310, V7, and LM108 to form filaments under reduced a_w conditions. When grown in TSB (a_w 0.99) or on TSA (a_w 0.99), *L. monocytogenes* cells are generally 1 to 2 μm in length. All strains elongated into filaments (defined as at least 7 μm, or greater than three times the normal length) when grown at 30°C in TSB supplemented with 8% NaCl (TSB-8; a_w 0.95) or on TSA supplemented with 6% NaCl (TSA-6; a_w 0.96). Filaments of varying lengths started to develop after 1 day, and increased in number and size with time, with some >120 μm after 3 days of incubation (Fig. 1). Four of the five strains developed filaments to similar degrees, while V7 was least able to form filaments.

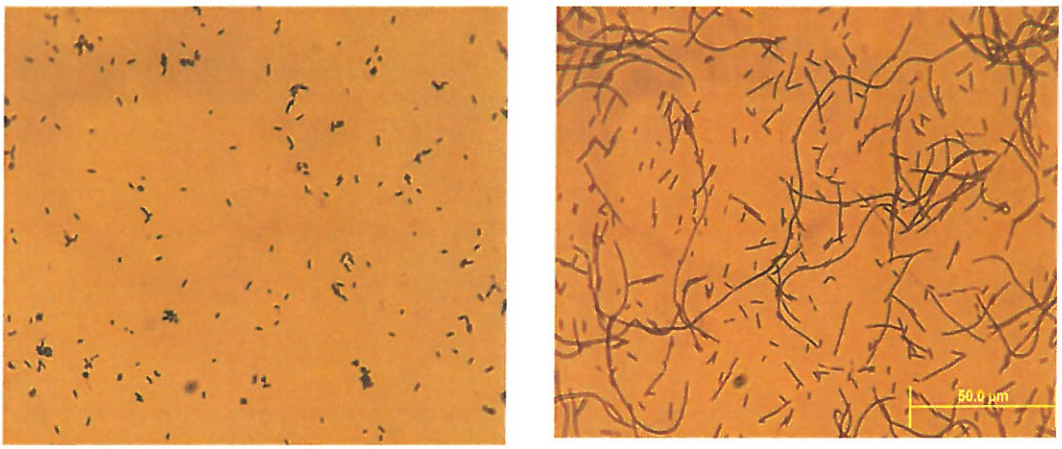

Fig. 1. *L. monocytogenes* Scott A control cells (left) and filaments (right).

Scott A was further tested for its ability to form filaments at different a_w levels and temperatures. TSB-8 was used initially because in a previous study we observed that it caused *Salmonella* to form filaments. *Listeria monocytogenes* Scott A cells started to elongate in this medium after 1 day at 30°C, with >50% of the cells longer than 7 μm, and the maximum cell length observed being ~60 μm. After 3 or 6 days, the % filamentation remained constant; however, there was a greater percentage of long filaments, with the longest >120 μm. When the NaCl concentration was decreased to 4% in TSB (a_w 0.98), no filamentation was observed; with 12% NaCl (a_w 0.92), < 1% of the cells were filamented after 6 days at 30°C. When incubated at 4°C, Scott A did not form filaments in TSB or TSB supplemented with 4, 8, or 12% NaCl.

Scott A developed filaments at 30°C to similar degrees on TSA-6 (a_w 0.96) or TSA supplemented with 7% NaCl (a_w 0.95). After 3 days, >50% of the cells were longer than 7 μm, with the longest >120 μm. When incubated at 37°C, filamentation was slightly greater; however, there was less total growth. A total of about 2×10^{10} CFU and 1×10^{10} CFU were recovered from

TSA and TSA-6 plates, respectively, when incubated at 30°C, while 8 x 10^9 CFU and 7 x 10^9 CFU were recovered when incubated at 37°C. At 25°C, < 1% of the cells were >7μm.

As strain V7 was the least able to form filaments of the five strains tested on TSA-6, we examined whether increasing the NaCl concentration or incubation temperature would have an effect. On TSA-6 at 30°C, 15 to 30% of V7 cells were filamented, with the longest ~ 40 μm. When incubated at 37°C, < 2% of the cells were filamented, with the longest ~25 μm. Increasing the NaCl to 7% resulted in 2 to 4% filamentation.

When filaments of Scott A were incubated in fresh TSB at 4 or 30°C, they septated to form individual regular sized cells. As a result, viable numbers increased more quickly than for non-filamented cells. Septation initiated within 2 h of incubation at 30°C, and no filaments were observed by 12 hours. At 4°C, septation also occurred although more gradually. After 6 days, < 10% of the cells remained filamentous.

Growth and survival of Scott A filaments

Because the greatest concentration of filaments was recovered from TSA-6 plates incubated at 30°C for 3 days, this method was used to develop *L. monocytogenes* Scott A filaments for subsequent survival experiments. Control cells were developed on TSA at 30°C for 3 days. Bacteria exposed to one type of stress may develop cross protection toward other stresses. We compared the ability of Scott A filaments and control cells to survive under the following stress conditions that could be encountered in RTE meats and their processing environments. Additionally the potential for Scott A to form filaments was determined.

(i) Low pH: Both filaments and control cells survived pH 4 for up to 2 hours without any decrease in viable counts (data not shown). At pH 2, viability of filaments and control cells decreased significantly over 30 minutes ($p < 0.05$), resulting in a reduction of about 5 and 6 $\log_{10}$ CFU/ml, respectively (Fig. 2). For the first 10 minutes, the number of CFU in the filament population decreased more rapidly than the control, while at 20 and 30 minutes, a greater decrease in the control population was observed.

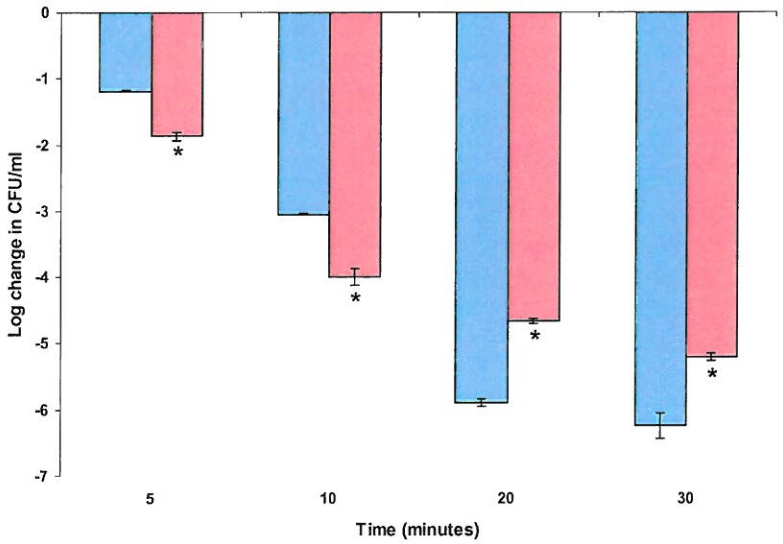

Fig. 2. $\log_{10}$ change in CFU/ml of Scott A control cells (blue) and filaments (pink) after exposure to pH at 37°C. *Significantly different ($p < 0.05$) from control.

(ii) High temperature: Viability of filaments and control cells decreased significantly over 30 minutes of incubation at 55°C (Fig. 3). Control cells decreased in viability more rapidly than filaments during the first 10 minutes ($p < 0.05$) but not thereafter.

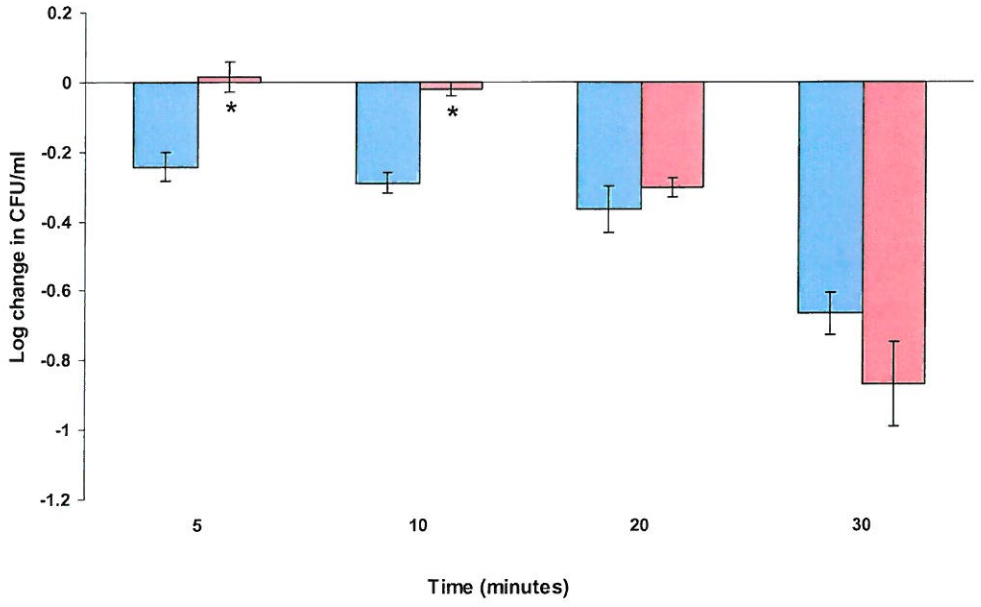

Fig. 3. Log_{10} change in CFU/ml of Scott A control cells (blue) and filaments (pink) after exposure to 55°C. *Significantly different ($p < 0.05$) from control.

(iii) RTE meats: Scott A filaments and control cells were inoculated onto three types of luncheon meats (chicken, beef, and pepperoni), and incubated at 4°C. Cells inoculated onto TSA plates were used as controls. On TSA a significant increase ($p < 0.05$) in CFU/gram was observed in filaments compared to control cells at both 72 and 144 hours (Fig. 4A). A decrease in CFU/gram was observed on all meat types for both filaments and control cells, with the exception of control cells on beef at 72 hours. There were no significant differences ($p > 0.05$) between control cell numbers on beef and chicken compared to that on TSA, but the CFU/gram on pepperoni was significantly less ($p < 0.05$). The CFU/gram of filaments on all meats was significant different ($p < 0.05$) from that of filaments grown on TSA. Survival of filaments and control cells on pepperoni was significantly less (< 0.05) than on beef and chicken. The lower a_w (0.86) of pepperoni might have contributed to the decreased survival. The Scott A filamentous culture initially contained about 60% cells that were longer than 7 μm (Fig. 4B). On TSA, most of the filaments septated (as reflected by the increase in CFU) and 90% of the cells were < 7 μm after 144 hours. In contrast, on RTE meat, septation was not observed and the % filaments remained high, although filaments longer than 60 μm were not observed. Control cells, whether inoculated onto TSA or meat, remained < 7 μm.

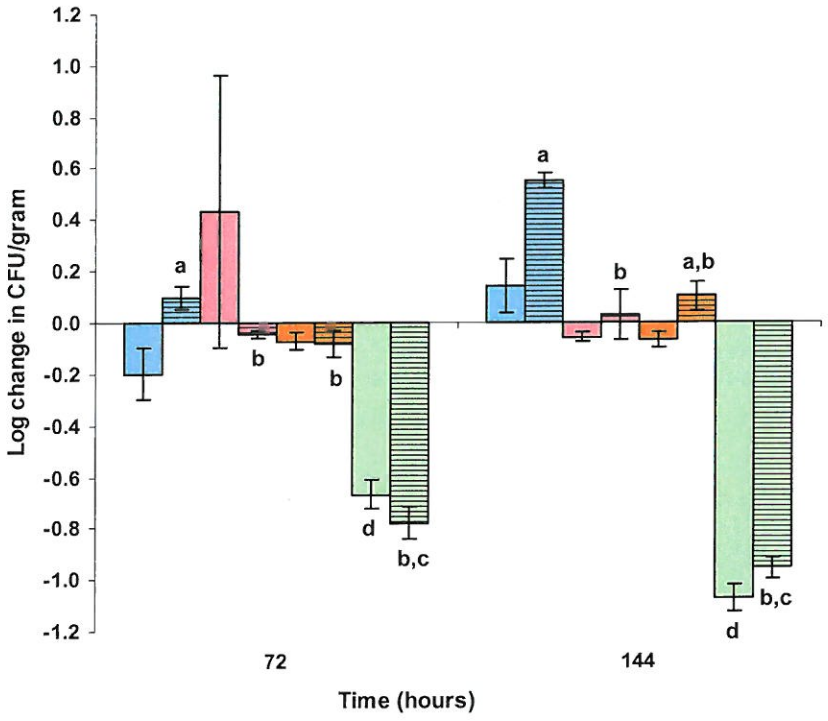

Fig. 4A. Log_{10} change in CFU/gram (ml for TSA) of Scott A control cells (solid) and filaments (striped) after incubation on TSA (blue), beef (pink), chicken (orange), and pepperoni (green) at 4°C. Compared with samples harvested from the same time point, [a]significantly different from respective control; [b]signficantly different from filaments on TSA; [c]significantly different from filaments on beef and chicken; and [d]significantly different from all control cells on TSA, beef, and chicken. Statistical significance for all comparisons was set at $p < 0.05$.

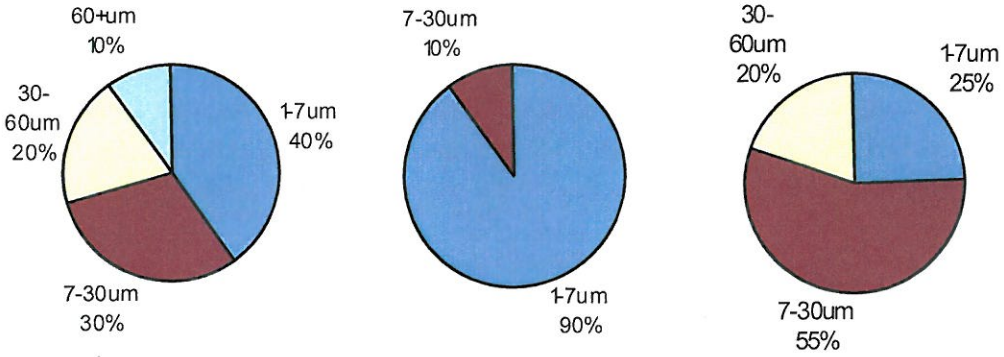

Fig. 4B. Percent cell lengths of Scott A filaments (left) and after incubation on TSA (center) and RTE meats (right) at 4°C for 144 hours.

(iv) Sodium diacetate/sodium lactate: This antimicrobial combination was tested at two concentrations, 0.1%/1.5% (diacetate/lactate) and 0.25%/3.75%. Scott A control cells and filaments increased in CFU/ml when incubated in both levels of diacetate/lactate at 4°C (Fig. 5). The levels of increase were generally lower than those in TSB except for filaments and control cells in 0.1%/1.5% diacetate/lactate at 24 and 72 hours, respectively. The greatest increases were observed with filaments in TSB at 72 and 144 hours. The majority of the filaments septated in TSB at 144 hours when > 90% of the cells were < 7 μm. Sodium diacetate/sodium lactate appeared to affect the persistence of filaments. There was some septation of the filaments which was accompanied by a significantly ($p < 0.05$) greater increase in CFU/ml compared to control cells; however, 20% and 45% of the cells recovered from the 0.1%/1.5% and 0.25%/3.75% concentrations of antimicrobial, respectively, remained filamented. Filamentation by the control cells was not observed when incubated in diacetate/lactate.

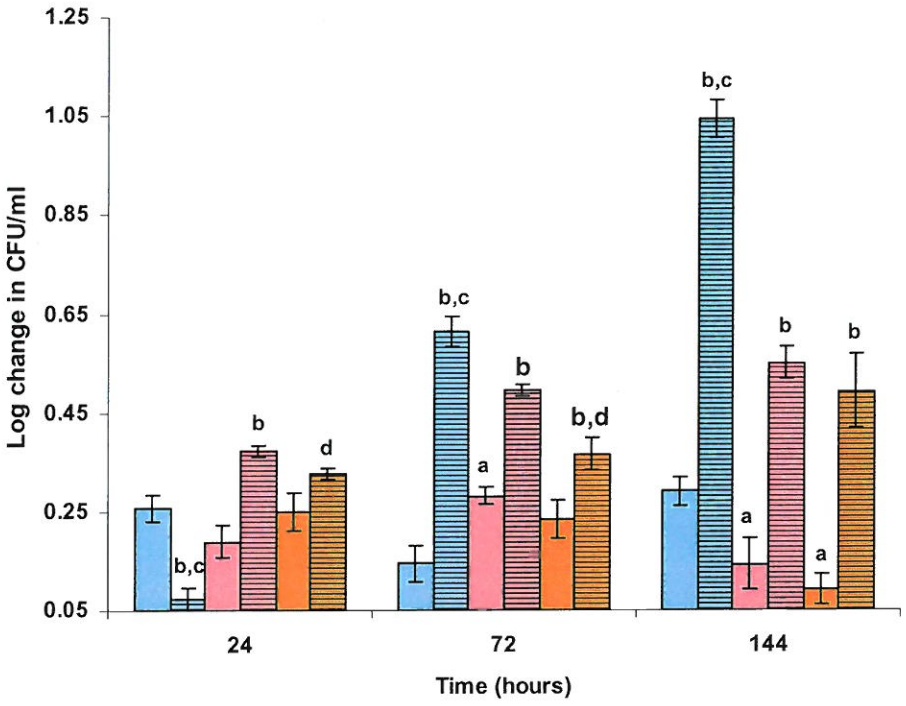

Fig. 5. Log_{10} change in CFU/ml of Scott A control cells (solid) and filaments (striped) after incubation in TSB (blue) and TSB with 0.1%/1.5% (pink) or 0.25%/3.75% (orange) sodium diacetate/sodium lactate 4°C. Compared with samples harvested at the same time point, [a]significantly different from control cells in TSB, [b]significantly different between control cells and filaments, [c]significantly different between filaments in TSB and either concentration of diacetate/lactate, and [d]significantly different from filaments in 0.1%/1.5% diacetate/lactate. Statistical significance for all comparisons was set at $p < 0.05$.

(v) Sodium nitrite: Sodium nitrite (156 ppm) significantly inhibited growth of both Scott A control cells and filaments ($p < 0.05$) (Fig. 6). At 72 hours, a decrease of about 1 log CFU/ml was observed. However, at 144 hours, growth occurred and the number of CFU/ml returned to the original levels. Septation was not observed with the filamentous cultures, nor did the control cells form filaments when exposed to nitrite.

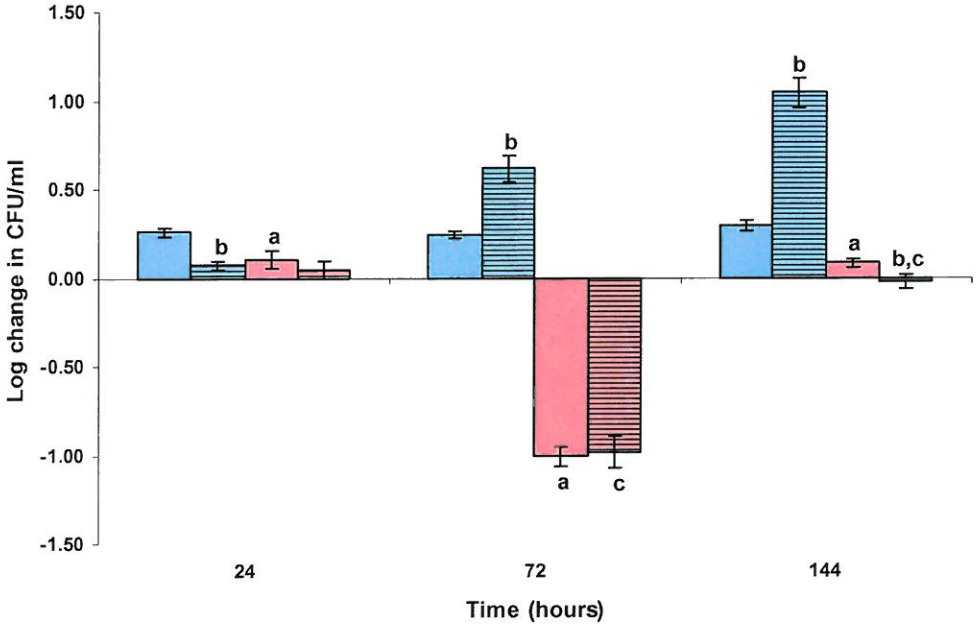

Fig. 6. Log_{10} change in CFU/ml of Scott A control cells (solid) and filaments (striped) after incubation in TSB (blue) and TSB with 156 ppm sodium nitrite (pink) 4°C. Compared with samples harvested at the same time point, [a]significantly different from control cells in TSB, [b]significantly different between control cells and filaments, and [c]significantly different from filaments in TSB. Statistical significance for all comparisons was set at $p < 0.05$.

(vi) Nisin/natural rosemary extract: A commercially available blend of nisin and rosemary extract (NovaGARD NR 100; contains 1.25% nisin and 4% phenolic diterpenes [present in rosemary extract]) was used at 0.02% and 0.05% (0.02% to 0.05% was recommended by the manufacturer) as well as 0.1%. The antimicrobial was incorporated into TSA as it did not disperse well in TSB. Overall there were significant ($p < 0.05$) decreases in CFU/ml in both control cells and filaments after 24, 72, and 144 hours on TSA with nisin/rosemary extract compared to without the antimicrobial (Fig. 7). On TSA, most of the filaments had septated after 144 hours, and < 10% of the population was > 7 μm, while the presence of nisin/rosemary extract appeared to help maintain the filamentous phenotype, especially at the 0.02% level, where > 80% of the cells were > 7 μm after 144 hours. At 0.05% and 0.1%, about 20% of the cells were > 7 μm.

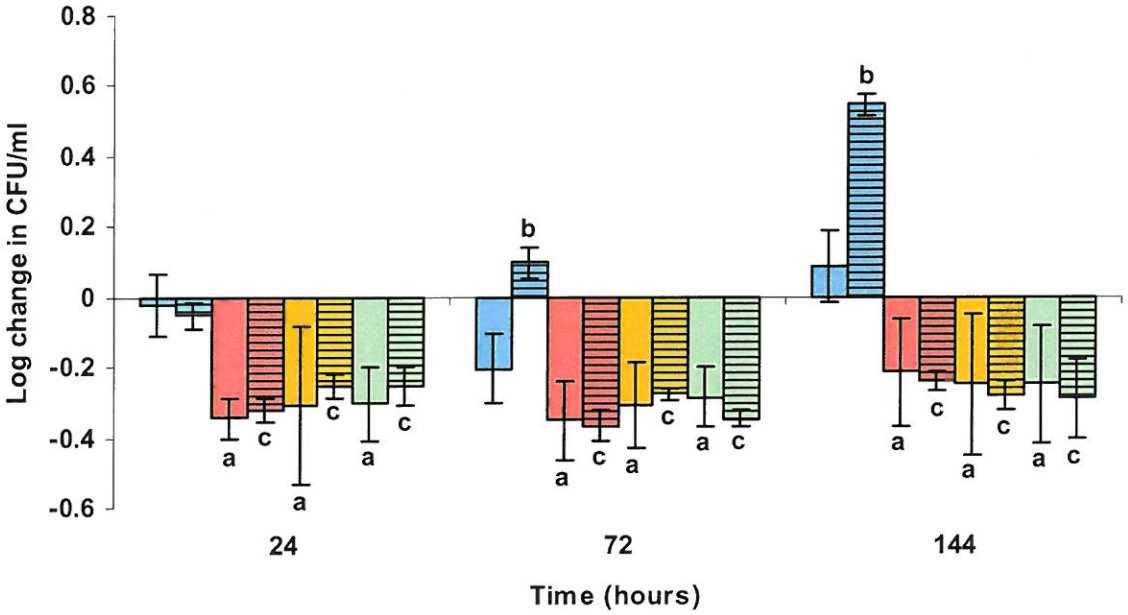

Fig. 7. Log_{10} change in CFU/ml of Scott A control cells (solid) and filaments (striped) after incubation on TSA (blue) and TSA with 0.02% (pink), 0.05% (orange), and 0.1% (green) nisin/rosemary blend incubated at 4°C. Compared with samples harvested at the same time point, [a]significantly different from control cells on TSA, [b]significantly different between control cells and filaments, and [c]significantly different from filaments on TSA. Statistical significance for all comparisons was set at $p < 0.05$.

(vii) Sodium hexametaphosphate: Three concentrations of sodium hexametaphosphate were used (0.1%, 0.3%, and 0.5%), with the latter being the greatest concentration allowable in meat products. All three concentrations significantly ($p < 0.05$) inhibited growth of control cells at all time points and of filaments after 72 and 144 hours (Fig. 8). Interestingly, the CFU/ml for the filamentous cultures in 0.1 and 0.3% hexametaphosphate were significantly ($p < 0.05$) greater than in TSB at 24 hr. The CFU/ml for controls and filaments were significantly ($p < 0.05$) different at all hexametaphosphate concentrations tested. As with the other antimicrobials tested, sodium hexametaphosphate appeared to help maintain the filamentous phenotype. About 30% of the cells were > 7 μm, while < 10% of the cells were > 7 μm in TSB after 144 hours.

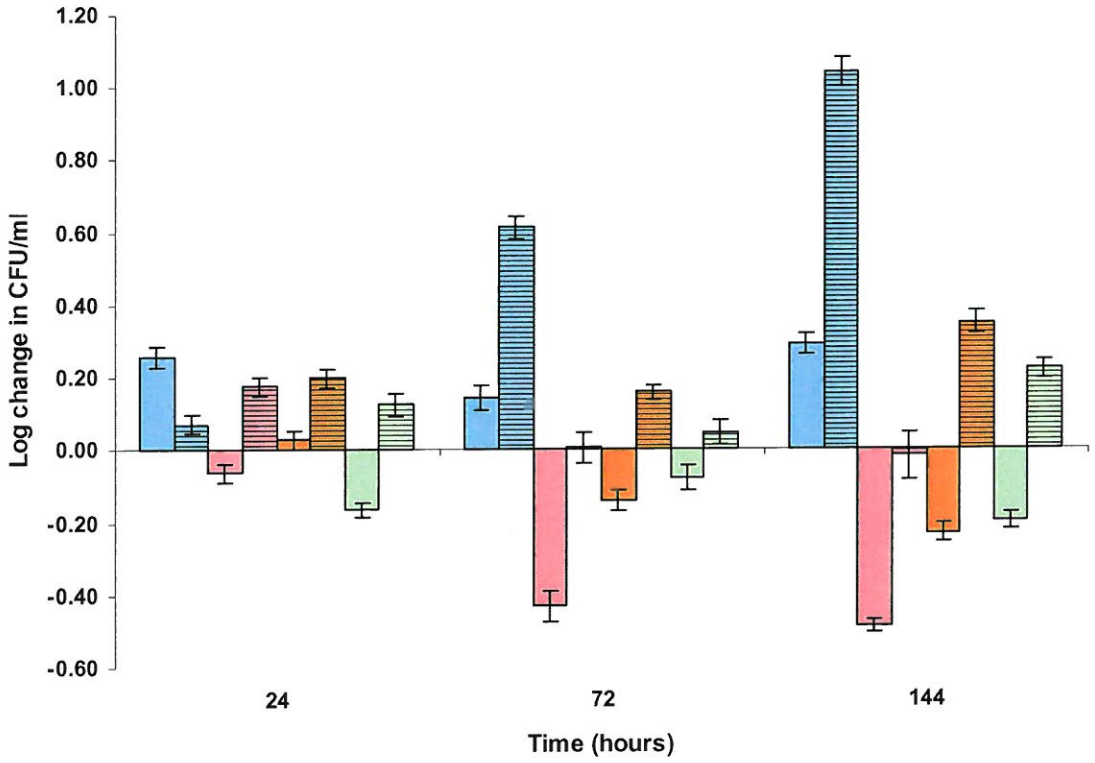

Fig. 8. Log_{10} change in CFU/ml of Scott A control cells (solid) and filaments (striped) after incubation in TSB (blue) and TSB with 0.1% (pink), 0.3% (orange), or 0.5% (green) sodium hexametaphosphate at 4°C. All filament and control cell counts in the presence of sodium hexametaphosphate were significantly different ($p < 0.05$) from their respective filament and control cell counts in TSB without hexametaphosphate. All filament CFU/ml were significantly different ($p < 0.05$) from their respective control counts.

(viii) Desiccation: Scott A control cells and filaments were desiccated on stainless steel surfaces at 32.5% and 75% RH and 25°C. A significant decrease ($p < 0.05$) in CFU/stainless steel chip was observed after 24 hours, with a greater decrease in the control population compared to filaments (Fig. 9). Decrease was more gradual at 75.5% RH for the first 72 hours. No filament formation by control cells or septation by filaments was observed.

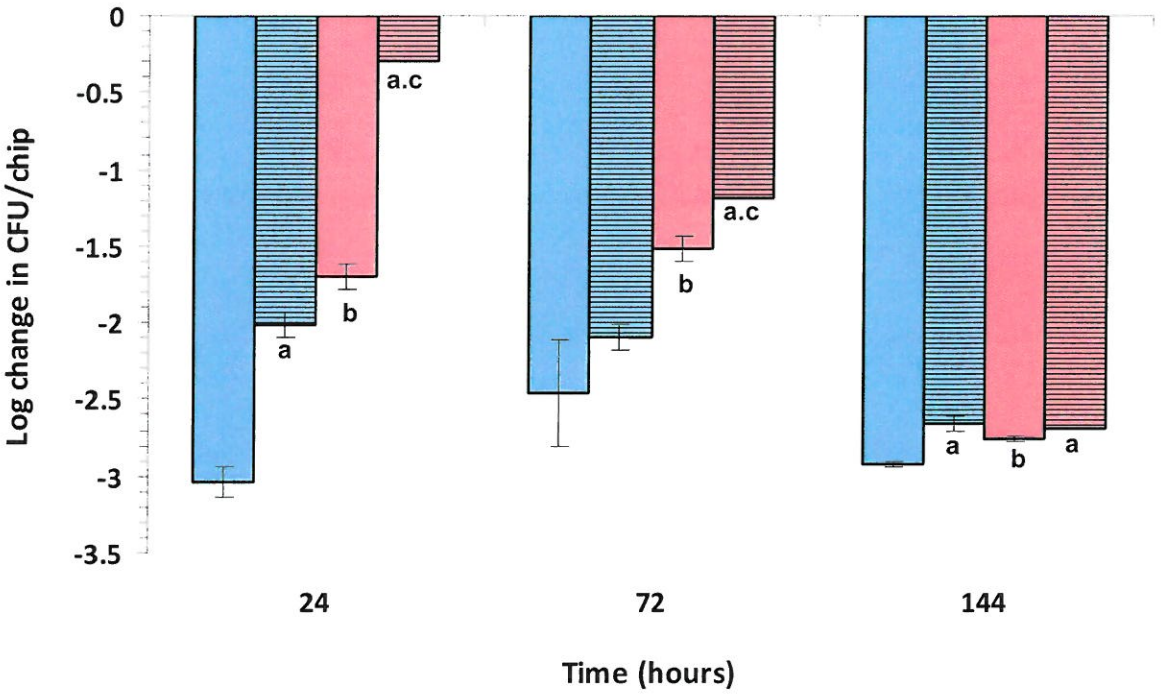

Fig. 9. Log_{10} change in CFU/chip of Scott A control cells (solid) and filaments (striped) after desiccation on stainless steel surfaces at 32.5% (blue) or 75.5% RH (pink) and 25°C. Compared with samples harvested at the same time point, [a]significantly different from control cells at the same %RH, [b]significantly different from control cells at 32.5% RH, and [c]significantly different from filaments at 32.5% RH. Statistical significance for all comparisons was set at $p < 0.05$.

(ix) Sanitizers: Both Trichlor-O-Cide® XP-160 and Ster-Bac® inactivated a significantly greater ($p < 0.05$) percentage of filaments than control cells after a 30-second exposure. Survival of control cells and filaments was 0.007% and < 0.0002%, respectively after Trichlor-O-Cide® XP-160 treatment, and 0.33% and 0.26%, respectively after Ster-Bac® treatment. Survival of control cells and filaments was the same at 0.007% after exposure to Octave™ for 30 seconds. After 2 minutes, all treated cultures were below the detection limit of 5 CFU/ml.

Conclusions

The ability of *L. monocytogenes* to develop filaments and survive different stress conditions was evaluated. All five strains tested formed filaments when grown in TSB or TSA with a_w levels reduced to 0.95 or 0.96 by the addition of NaCl. Filamentation occurred at 30 and 37°C, rarely at 25°C, and none was observed at 4°C. When filaments were transferred to fresh medium, they started to septate and divide into normal-sized cells within 2 hours at 30°C, and more slowly but steadily at 4°C, causing the number of CFU to increase more rapidly than a comparable population of non-filamented cells.

The relative ability of Scott A filaments and control cells to survive stresses commonly encountered in the RTE meat environment varied depending on the stress and exposure time. At pH 2, filaments were inactivated more rapidly than control cells initially, but with longer exposure, there was a greater decrease in viability for control cells. At 55°C survival of control cells were similar although control cells inactivated more rapidly initially. More Scott A control cells were inactivated than filaments during desiccation on stainless steel surfaces. The three sanitizers tested were effective in inactivating both control cells and filaments. Scott A did not grow or develop filaments on the three types of luncheon meat tested. There was a significant decrease in viability on pepperoni, which had the lowest a_w (0.86). Control cells and filaments decreased in viability to a similar extent when exposed to either nitrite or a nisin/rosemary extract blend. Both control cells and filaments increased in CFU in diacetate/lactate, with greater increases in the filament culture, while in sodium hexametaphosphate, the CFU of control cells generally decreased but that of filaments increased. Overall, exposure to luncheon meats or an antimicrobial appeared to prolong the persistence of the filamentous phenotype.

Recommendations for Future Research

It is unknown whether filamentation by *L. monocytogenes* occurs in food or in the processing environment, and whether filamentation is a survival strategy or a consequence of stress. It is crucial to address these and other questions related to stress response and the physiology of the filamentation phenotype. It is also important to determine the survival properties of the filaments relative to the normal form. If filaments form septa when encountering favorable conditions, there will be a sudden increase in the number of *L. monocytogenes* that will significantly affect estimations of the total number of viable cells of *L. monocytogenes* in the food. This could impact retrospective assessments of the infectious dose. Likewise, occurrence of filamentous *L. monocytogenes* in the processing environment can influence detection, enumeration, effective processing parameters, and risk assessments.

This study was a first attempt to address some of these questions. Research is needed to determine the potential for filament formation in food products and the food processing environment and to develop methods for detection and estimation of filament numbers. The mechanism(s) of filament formation is unknown. Research toward understanding what causes filaments to form under stress would be helpful in designing control strategies. We have shown in a separate study that stress-induced *Salmonella* filaments can invade and grow in human intestinal Caco-2 cells as well as colonize the mouse gastrointestinal tract and disseminate to other organs. Similar studies need to be conducted with *L. monocytogenes* filaments to determine their virulence potential.

Presentations and Publications

1. Wong, A. C. L. Physiological response of *L. monocytogenes* and *Salmonella* to low moisture environments. Food Research Institute Spring Meeting, May 17, 2011, Madison, WI.

2. Stackhouse, R.R., K. Yang, C. W. Kaspar, C. J. Czuprynski, and A. C. L. Wong. 2011. Formation and survival of stress-induced filaments by *Listeria monocytogenes*. 98th Ann. Meet. International Association for Food Protection, Milwaukee, WI.

3. A manuscript describing the results presented in this report is in preparation.