Reduction of *Listeria* on Ready-to-Eat Sausages after Exposure to a Combination of Pulsed Light and Nisin†

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ABSTRACT

The risk of listeriosis associated with ready-to-eat foods is a major concern in the United States. Pulsed light (PL) treatment has been effective for killing *Listeria*. The possibility of enhancing the antilisterial capability of PL treatment by combining PL with an additional hurdle, the natural antimicrobial nisin, was explored in this study. First, the ability of *Listeria innocua* to mimic the response of *Listeria monocytogenes* to PL treatment was demonstrated. Subsequently, a series of inoculation studies was performed in which canned sausages were surface inoculated with *L. innocua* as a surrogate for *L. monocytogenes* and then treated with a commercial preparation of nisin (Nisaplin), PL, or a combination of the two treatments. The application of a Nisaplin dip alone resulted in an immediate reduction of *L. innocua* by 2.35 ± 0.09 log CFU. PL reduced *L. innocua* by 1.37 ± 0.30 log CFU after exposure to 9.4 J/cm². A total reduction of 4.03 ± 0.15 log CFU was recorded after the combined treatment of Nisaplin and PL for 48 h at 4°C. The long-term survival of *L. innocua* was evaluated on sausages stored at 4°C. Treatment with Nisaplin and PL resulted in a 4- to 5-log reduction for two replicate studies. The combination treatment resulted in no significant microbial growth during 28 and 48 days of refrigerated storage in the first and second replicates, respectively. These results suggest that this combination treatment can be used as an effective antilisterial step in the production of ready-to-eat foods.

*Listeria monocytogenes* is a gram-positive, non–spore-forming foodborne bacterial pathogen that targets both high-risk people such as immunocompromised individuals, pregnant women, neonates, and the elderly and healthy people. Numerous major foodborne listeriosis outbreaks have occurred since the first documented outbreak associated with coleslaw in 1981 (33). The Centers for Disease Control and Prevention estimated that in the United States there are approximately 2,500 cases of listeriosis yearly, leading to about 500 deaths per year (32). Most recent cases of listeriosis have been linked to ready-to-eat (RTE) foods such as frankfurters (6, 20), butter (28), and deli turkey meat (7–9). Many cases of listeriosis seem to be the result of postprocessing contamination, which is a serious concern with RTE foods. The ability of *L. monocytogenes* to survive and grow at refrigeration temperatures (3), in foods with high salt concentrations, and in acidified foods of pH ≥5.2 (24, 26, 44) makes this pathogen a serious problem for many refrigerated RTE products with a long shelf life. Tompkin (46) reported that *Listeria* cannot be eliminated from the processing environment of RTE meat and poultry products but that steps could be implemented to control *Listeria* in those environments.

The choices of postprocess bactericidal interventions for RTE products are very limited. The recent development of pulsed light (PL) technology, which can reduce the microbial load on foods and food contact surfaces (29–31, 47, 48, 50), may provide a viable solution for controlling post-process contamination by *Listeria* in RTE products. PL treatment consists of very short high-intensity pulses of broad spectrum light that is used to inactivate vegetative bacteria, spores, yeasts, and molds. The PL treatment dose, called fluence, is expressed in joules per square centimeter. PL treatment has been approved by the U.S. Food and Drug Administration for decontamination of food and food surfaces with the restriction that a xenon flash lamp be used as a light source, with pulse durations of <2 ms and the cumulative treatment not exceeding 12 J/cm² (48). Although the exact mechanisms responsible for cell death in PL treatment are not yet fully elucidated, a majority of the PL studies indicate a significant contribution of the UV portion of the spectrum, and the lower wave length UV radiation has a more pronounced role in inactivation (38, 49, 51). UV radiation, especially within 250 to 260 nm, is damaging to most microorganisms because it alters the microbial DNA through the formation of pyrimidine dimers, pyrimidine adducts, and DNA-protein cross-links (5).

In several studies, the ability of PL to inactivate various microorganisms on food surfaces has been demonstrated: *L. monocytogenes* and *Escherichia coli* O157:H7 on raw salmon fillets (35), *E. coli* O157:H7 on alfalfa seeds (42), yeast on flour and black pepper (14), and fungi from fresh fruit (25), corn meal (22), and strawberries (30). Woodling and Moraru (50) reported a nearly 4-log reduction of *Listeria innocua* on the surface of stainless steel coupons of different surface roughness after a PL dose less than 12 J/cm². Because of its effectiveness against *Listeria*...
and appropriateness for the inactivation of surface microflora, PL could be applied as a postprocessing safety step to reduce surface contamination in RTE products. Particularly interesting is the potential to enhance the effectiveness of PL treatment using a hurdle approach by combining PL with another antilisterial treatment, such as an antimicrobial chemical compound.

Nisin, a low-molecular-weight antimicrobial protein produced by *Lactococcus lactis* subsp. *lactis*, has a broad antimicrobial spectrum of activity against gram positive bacteria and spore formers. In vegetative cells, nisin complexes with lipid II and inserts itself into the cytoplasmic membrane (11). This insertion leads to the formation of pores within the membrane, resulting in leakage of cellular components and cell death. Nisin is generally recognized as safe and is approved in more than 80 countries, including the European Union, the United States, India, China, and Australia (1). Commercially, nisin has been available since 1953 under the name Nisaplin, which contains milk and milk solids and approximately 2.5% nisin (11). Nisin has been incorporated into various foods, including meat products (1, 11, 12, 27, 37). Applied as a spray, a dip, or incorporated into the product, nisin can control, with variable efficiency, postprocessing microbial growth in various meat products, including smoked rainbow trout (34), raw meat (2), fresh pork sausage (41), bologna (10, 40), hot dogs (16, 43, 45), and frankfurters (18, 19).

The objective of this work was to develop a hurdle treatment for the control of *Listeria* on the surface of RTE foods by combining the bactericidal action of PL treatment with the bactericidal and bacteriostatic effect of nisin. The appropriateness of using *L. innocua* as a surrogate for *L. monocytogenes* was tested, and subsequently *L. innocua* was used as a challenge organism. Commercial canned Vienna sausages were used as the food substrate. The survival of *L. innocua* was examined over prolonged storage at 4°C for four sausage treatments: (i) no treatment (control), (ii) nisin dip, (iii) PL, and (iv) nisin dip plus PL. The results of this work should help the food industry develop efficient hurdle treatments for control of postprocess *Listeria* surface contamination in RTE foods.

**MATERIALS AND METHODS**

**Substrate.** Armour brand Vienna sausages (Pinnacle Foods Corporation, Cherry Hill, NJ) were used as a model for RTE hot dogs and frankfurters. These sausages were made from mechanically separated chicken, water, beef, pork, salt, corn syrup, and less than 2% mustard, natural flavor, dried garlic, and sodium nitrite and were canned in a solution of chicken broth and caramel color. The pH of the sausages was 5.7. The sausages were skinless and had a diameter of 20 mm and an average length of 53 mm.

**Nisin.** The commercial preparation Nisaplin (Danisco, New Century, KS) was used as a nisin source. A 0.5% Nisaplin solution was prepared by dissolving 1 g of Nisaplin into 200 ml of sterile, deionized water. Nisaplin contains a standardized nisin activity of 10⁶ IU/g, resulting in a final nisin solution of about 5,000 IU/ml. This 0.5% Nisaplin solution is referred to as nisin throughout the article.

**PL treatment.** PL treatments were performed with an RS-3000C SteriPulse System (Xenon Corporation, Woburn, MA). The system consists of a controller unit and a treatment chamber that houses a xenon flash lamp. Each sample was centered individually on an adjustable stainless steel shelf in the PL unit 50.8 mm beneath the lamp and was treated with a variable number of pulses at a frequency of three pulses per second and a pulse width of 360 μs.

**Fluence measurements.** The PL fluence was measured with a pyroelectric head (PE25BBH) and a Nova II display (Ophir Optronics Inc., Wilmington, MA) and was expressed in joules per square centimeter. To perform the fluence measurement, a stainless steel aperture cover was placed over the power meter head. The aperture covered the top surface of the detector head except for a 1-cm² circular opening that exposed the detector’s surface. The pyroelectric head was placed 50.8 mm from the quartz face of the lamp. The settings on the Nova II display were a pulse width of 1.0 ms and a wavelength of <0.3 μm. Pauses of at least 30 s between measurements were allowed to prevent possible overheating of the pyroelectric head. All fluence measurements were performed in triplicate.

**Optical penetration depth measurement.** To determine the optical penetration of PL into the sausage, slices of sausage of various thicknesses (0.58, 1.19, 2.46, and 3.76 mm) were placed over the 1-cm² opening of the pyrodetector head, which was located 50.8 mm from the lamp face. The detector was centered in the PL chamber, and the fluence from three pulses was measured for each thickness. The resulting fluence values were plotted versus distance (thickness of the sausage slice), and the exponential decay of fluence with distance was used to calculate the optical penetration depth of the sausage. All fluence measurements were performed in triplicate.

**Light absorption analysis.** The absorption spectrum of the sausage was measured with a HR2000+CG-UV-NIR spectrometer (Ocean Optics Inc., Dunedin, FL). A 0.58-mm-thick slice of sausage was placed into a cuvette, which was then inserted into the spectrometer. The absorbance measurements were performed for the entire spectral range of the PL treatment (180 to 1,100 nm). The absorption spectrum of the 0.5% Nisaplin in sterile deionized water also was determined.

**Culture and inoculum preparation.** *L. innocua* FSL C2-008 (environmental isolate from a smoked fish plant) and a five-strain *L. monocytogenes* human disease cocktail recommended by Fugett et al. (17) were obtained from the Food Microbiology and Safety Laboratory (Cornell University). The five-strain cocktail comprised the following *L. monocytogenes* isolates: FSL C1-056 (human isolate, sporadic infection), FSL J1-177 (human isolate, sporadic infection), FSL N1-227 (food-associated outbreak isolate, RTE meat product), FSL N3-013 (food-associated outbreak isolate, pâté), and FSL R2-499 (human isolate, outbreak associated with sliced turkey).

Before the experiment, the culture was streaked onto tryptic soy agar (TSA; Becton Dickinson, Sparks, MD) and incubated for 24 ± 2 h at 35 ± 2°C. A single isolated colony was transferred into tryptic soy broth (TSB; Becton Dickinson) and incubated for 24 ± 2 h at 35 ± 2°C. A loopful of this culture was transferred into TSB and incubated for 24 ± 2 h at 35 ± 2°C to produce an initial inoculum of 10⁶ to 10⁷ CFU/ml.

**Building of growth curves for *L. innocua* and *L. monocytogenes.** A single colony of each isolate was selected from a TSA plate and inoculated into 5 ml of TSB and incubated for 12
to 18 h at 37 ± 2°C with shaking (225 rpm). After incubation, 50 μl of the culture was transferred to 5 ml of fresh TSB and incubated at 37 ± 2°C with shaking (225 rpm). When the optical density at 600 nm \( (OD_{600}) \) reached 0.4, 100 μl was transferred to 10 ml of TSB and incubated at 37 ± 2°C with shaking (225 rpm) for 24 h. For the \( L. monocytogenes \) cocktail, 2 ml of each strain culture at an \( OD_{600} \) of 0.4 were combined to create 10 ml of cocktail, which was incubated at 37 ± 2°C with shaking (225 rpm) for 24 h. At 0, 3, 4, 5, 6, 7, 9, 12, 18, and 24 h, appropriate dilutions were made into Butterfield’s phosphate buffer (BPB), and 100 μl of \( L. innocua \) or \( L. monocytogenes \) cocktail was spread plated in duplicate onto TSA and incubated for 24 ± 2 h at 37 ± 2°C.

**PL treatment of \( L. innocua \) and \( L. monocytogenes \) in clear liquid suspensions.** A 10-fold dilution of an inoculated 24-h TSB culture was made with BPB for each \( Listeria \) isolate. For the \( L. monocytogenes \) cocktail, equal volumes of each strain were combined. Sterile transparent one-well glass Lab-Tek II Chamber Slides (Nagle Nunc International, Naperville, IL) with chamber dimensions of 25.4 by 50.8 mm were used to hold 1 ml of the liquid cell suspensions. The height of the liquid inoculum in the glass chamber was 1.16 mm. The chamber containing the suspension was centered individually and parallel to the lamp on an adjustable stainless steel shelf in the PL unit approximately 50.8 mm beneath the xenon lamp and was treated with up to 12 pulses at a frequency of 3 pulses per s. The treated inoculum (1 ml) was transferred to 7 ml of TSB, and the chamber was rinsed twice with 1 ml of TSB, adding the rinse TSB to the treated inoculum. The resulting 10 ml was serially diluted, plated, and incubated for 48 ± 2 h at 37 ± 2°C, and the survivors were enumerated. When plate counts fell below the limit of detection, the number of survivors was estimated with the most-probable-number technique. The recovery broth was diluted, 1 ml of each dilution was transferred into 10 ml of TSB (three tubes for each dilution), and the tubes were incubated at 37 ± 2°C for 48 ± 2 h. Turbidity was used to presumptively identify positive samples. Presumptive-positive samples were streaked onto modified Oxford medium (Becton Dickinson) and incubated at 37 ± 2°C for 24 ± 2 h. Turbidity was used to presumptively identify positive samples. Presumptive positive samples were streaked onto modified Oxford medium and incubated at 37 ± 2°C for 24 ± 2 h. Tubes were considered positive when typical black esculin-positive colonies were observed.

**Statistical analysis.** An analysis of variance and Tukey’s honestly significant differences test were used to determine whether differences between treatments were significant at \( P < 0.05 \) using the statistical package JMP 6.0.0 (SAS Institute, Cary, NC).

**RESULTS AND DISCUSSION**

**Optical characteristics of the substrates.** Light absorption by the sausage and nisin, particularly in the UV region, is very important for the outcome of the PL treatment because absorption can affect the amount of fluence delivered to the microbial cells on the sausage surface.

The absorbance of the Vienna sausage was high across a broad spectrum, from 200 to 1,100 nm, but no particular spectral absorbance preference was observed, which indicates that the sausage itself will attenuate the intensity of the treatment without modifying the spectral distribution of the incident light. The attenuation of light inside the sausage followed an exponential decay with sausage thickness (Fig. 1). Based on this decay, the optical penetration depth of the sausage (defined as the depth at which the fluence decreases to \( 1/e \) of its value at the sausage surface) was calculated to be 2.3 mm. Thus, light treatments such as PL will be effective only as a surface treatment for this type of substrate. Bacterial cells located beneath the sausage surface will not be effectively killed by the treatment because the sausage absorbs a significant amount of the incident light.

Nisin had a lower absorbance value than the sausage, with near zero absorbance between 300 and 1,100 nm. Ni-
sin had preferential absorbance in the UV region, between 200 and 300 nm, with a sharp peak at approximately 225 nm. Consequently, nisin should diminish somewhat the amount of lethal UV delivered to the microbial cells, which might slightly reduce the effectiveness of PL treatment when nisin is present on the sausage surface.

**L. innocua as a surrogate for L. monocytogenes.** Because of the pathogenic nature of *L. monocytogenes*, its use in the laboratory and particularly in pilot plant validation studies can pose significant risks. Thus, an indicator organism is needed in its place, in much the same way as *Clostridium sporogenes* is used as an indicator for the pathogenic *Clostridium botulinum* when evaluating process efficiency (23). *L. innocua* is often regarded as the nonpathogenic variant of *L. monocytogenes* (21) because it possesses many traits that are similar to those of its pathogenic relative; the greatest difference is the lack of hemolysin production (23). Kamat and Nair (23) concluded that *L. innocua* is a reasonable indicator organism for *L. monocytogenes* in a variety of treatments, including radiation, heat, lactic acid, NaCl, and nitrates. Because no data were available regarding the comparative response of *L. innocua* and *L. monocytogenes* to PL treatment, a comparison between the growth characteristics and response to PL for these two organisms was performed.

Numerous studies have been conducted to compare the growth of *L. innocua* and *L. monocytogenes* over a range of temperatures (0 to 36°C) and on a variety of substrates, including minced beef (13), lettuce (15), and crab meat (36). In these studies, no significant differences were noted between *Listeria* species when grown at the same temperature. In this study, the growth kinetics of *L. innocua* and *L. monocytogenes* were compared at 37°C, which was the incubation temperature used for evaluating the survivors of the treatment. When comparing the growth curves at 37°C, no difference between the nonpathogenic *L. innocua* strain and the pathogenic *L. monocytogenes* cocktail were observed (Fig. 2). Additionally, when comparing the survival of *L. innocua* and *L. monocytogenes* cocktail after treatment with PL (Fig. 3), similar reductions were observed at low fluence levels. As PL dose increased, *L. innocua* appeared to have a slightly higher resistance to PL than did *L. monocytogenes*. This finding suggests that PL reductions that occur when utilizing *L. innocua* as the test organism may underestimate the PL reduction of *L. monocytogenes* under similar conditions. Thus, reduction of *L. innocua* by PL will give a conservative estimate of reduction of *L. monocytogenes* by PL, which is a favorable result.

Overall, the physiological and metabolic similarity of these two organisms and the results in Figure 3 indicate that it is reasonable to use *L. innocua* as an indicator for *L. monocytogenes* in PL treatment situations where the pathogen cannot be used. *L. innocua* also can be used as a general model for a food-associated microorganism for evaluating the influence of substrate-related or treatment-related factors on the effectiveness of PL treatments. Because the present study involved prolonged refrigerated storage of the inoculated and treated sausage samples in facilities that did not allow the use of pathogens, *L. innocua* was used as an indicator for *L. monocytogenes*.

**Reduction of *L. innocua* on sausages by exposure to PL and nisin.** As a first step, the necessary level of fluence and nisin exposure for each type of treatment was established. When subjecting the *L. innocua*-inoculated sausages to PL alone, the inactivation curve had a clear plateau (Fig. 4), with the highest reduction (1.37 log CFU per sausage)
achieved after exposure to 9.4 J/cm² (nine pulses). The application of nisin alone resulted in an immediate reduction of 2.35 log CFU per sausage. This reduction was similar to that of L. monocytogenes reported by Geornaras et al. (18, 19) in both frankfurter and smoked sausages dipped in nisin, which had 2.4- and 2.1-log reductions, respectively. The combined PL plus nisin treatment resulted in a significantly greater reduction compared with that achieved with the individual treatments, suggesting an additive effect of PL and nisin (Fig. 4). Additional reduction seemed to occur during refrigerated storage, although the reductions were not significantly different for the sausage samples held at 4°C for 0, 24, or 48 h. A total reduction of 4.03 log CFU per sausage was recorded for the samples subjected to nisin and PL at 9.4 J/cm² after 48 h of storage at 4°C (Fig. 4). The combined treatment was able to repeatedly achieve a greater than 4-log reduction of L. innocua, with four replicates yielding very similar results. Although combination treatments involving PL have not been reported previously, the ability to enhance the effectiveness of nisin by combining it with another antimicrobial treatment has been demonstrated. Combinations of nisin and organic acids (18, 19) or nisin and grape seed extracts in soy protein films (45) have also yielded better control of Listeria in frankfurters or sausages than did the individual treatments.

Under the conditions of this study, the sausages treated with PL and/or nisin did not appear to undergo any noticeable changes in color or appearance compared with the untreated sausages. To accurately evaluate such aspects, a systematic sensory study is needed.

**Long-term survival of Listeria on refrigerated sausages.** The long-term survival of L. innocua was evaluated under refrigeration conditions on sausages subjected to the individual and combination treatments. A single level of PL treatment was used at a fluence of 9.4 J/cm² (nine pulses). The results of two replicates are shown in Figure 5. Replicates are displayed separately because of quantitative differences observed between the two experiments.

In addition to the higher level of microbial inactivation achieved by the combination treatment compared with the individual treatments, the PL plus nisin treatment also inhibited the growth of surviving L. innocua cells for a much longer period as compared with all other treatments.

In the first replicate, the starting inoculum was 7.0 to 7.3 log CFU per sausage. After an initial drop of 4.61 log CFU per sausage immediately after the treatment (at day 0), the number of survivors for the combined treatment did not change over 28 days of refrigerated storage (Fig. 5A). The greatest reduction, 5.10 log CFU per sausage, was observed for the combination treatment at day 4. For the treatments with PL only and nisin only, significant cell growth started to occur at the same time as for the untreated samples (after day 8) after an initial reduction of 2.74 and 1.24 log CFU per sausage, respectively. After 28 days of storage, the L. innocua counts increased to more than 3 log CFU above the initial inoculation level for the untreated samples. The number of survivors reached the initial inoculum level after 28 days in both the nisin- and PL-treated samples and after approximately 40 days in samples from the combined treatment.

For the second replicate (Fig. 5B), the starting inoculum was 6.6 log CFU per sausage. In the combined treatment, there was an initial reduction of 3.53 log CFU per sausage, with a reduction of 4 to 5 log CFU per sausage during the 28 days of storage. By day 60, the combined treatment counts had reached the initial inoculum level. Similar to the combined treatment, the number of survivors for the nisin treatment did not change over 28 days. The PL treatment produced an initial reduction of 1.58 log CFU per sausage, but microbial counts became similar to those of the untreated samples after 12 days. After 60 days, the untreated (control) and PL treatment samples had L. innocua counts about 3 log CFU per sausage higher than the initial inoculum level.

These results demonstrate that the combination treatment of nisin and PL can significantly reduce L. innocua on RTE food surfaces and is more effective for delaying cell growth than is either treatment alone. Similar results were reported by Samelis (39, 40), who found that combinations of nisin and different organic acids used against L. monocytogenes extended the shelf life of frankfurters much longer than did the individual antimicrobials.

In this study, PL is believed to have caused damage to microbial DNA, whereas the bactericidal and bacteriostatic activity of nisin was probably the result of both membrane pore formation and the disturbance of cell wall biosynthesis. When used together, the two treatments may cause ma-
jor damage to bacterial cells, preventing the recovery of sublethally injured cells and significantly impeding the growth of the surviving cells. Like the use of organic acid or salt dips to control postprocessing Listeria contamination (4, 18, 19, 39, 40), the use of a nisin dip and PL together provide another option for improving the safety and shelf life of RTE meat products.

PL and nisin had additive antimicrobial effects when applied to the surface of Vienna sausages inoculated with L. innocua. The combination treatment also significantly delayed the growth of survivors as compared with the individual treatments. Because the results of this study indicated that L. innocua can be used as an indicator organism for the pathogenic L. monocytogenes in PL treatment studies, the combination treatment should efficiently control L. monocytogenes on the surface of RTE meat products. The advantage of using PL to reduce postprocess contamination is that this treatment would reduce the amount of chemical compounds need for such applications. Preliminary data also indicate that a unique attribute of PL treatment is the possibility of applying it through UV-transparent packaging material. This could enable the development of an in-package terminal antimicrobial treatment, which would represent a quality step in ensuring the safety of RTE food products.

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