Efficacy Enhancement of Trisodium Phosphate against Spoilage and Pathogenic Bacteria in Model Biofilms and on Adipose Tissue

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MS 01-311: Received 28 August 2001/Accepted 30 November 2001

ABSTRACT

A two-step approach for enhancing the efficacy of trisodium phosphate (TSP) was evaluated using meat spoilage and pathogenic bacteria in flow cell biofilms and adipose tissue model systems. The process was based on the plasmoslysis of attached bacteria (biofilms) with a hyperosmotic solution (1.5 M NaCl) and the subsequent deplasmolysis of cells with a low-osmotic-strength solution containing different concentrations of TSP (0.1, 0.25, 0.5, 0.625, and 1.0 % [wt/vol]). Escherichia coli, Salmonella Enteritidis, Pseudomonas sp., Listeria monocytogenes, and Brochothrix thermosphacta strains were cultivated for 24 h as pure culture biofilms in glass flow cells with complex media and were then treated with either 0.1, 0.25, 0.5, 0.625, and 1.0 % TSP, or the same TSP concentrations delivered in conjunction with plasmoslysis-deplasmolysis (PDP). Confocal scanning laser microscopy, a commercial fluorescent viability probe, and image analysis were then used to quantify the relative abundances of living and dead cells remaining after the different treatment regimes. With the exception of L. monocytogenes (which was resistant to TSP concentrations of up to 5%), the PDP process increased the sensitivity of the test strains to TSP. However, when similar experiments were conducted with pork adipose tissue, it became evident that higher TSP concentrations were necessary to produce significant decreases in the number of viable cells and that the PDP process generally failed to enhance TSP efficacy. An exception was L. monocytogenes, which exhibited an increase in sensitivity to TSP when inoculated tissue was pretreated with 1.5 M NaCl. It is thought that factors contributing to the failure of the PDP process to enhance the activity of TSP in meat systems involves the mode of TSP antimicrobial activity, alkaline pH stress, and the chemically complex, buffered nature of meats. It remains to be determined whether the PDP process is suitable for use with other food grade antimicrobial agents or can be used in nonfood biofilm control applications.

During the slaughter, dressing, and processing of meat animals, spoilage bacteria and pathogens can contaminate equipment and exposed meat surfaces (20). Subsequent bacterial attachment (15) and biofilm formation (48) are of concern to the industry. Thus, biofilms have been quantified in meat-processing plants (23) and visualized on meat tissue surfaces (12).

From a meat-processing perspective, the most critical biofilm-related issues include the increased resistance of biofilm bacteria to sanitizers compared with the resistance of planktonic cells (13, 38, 40, 43) and the difficulty in removing biofilm bacteria from food contact surfaces (19). Because of their ubiquity in processing plants, biofilms have the potential to constitute a long-term recontamination threat for meat and meat products (2, 34, 35, 47). An understanding of bacterial attachment as well as biofilm formation and persistence is therefore fundamental for the development of antibacterial intervention strategies suitable for the meat industry (7).

Regulatory authorities (44) have advocated the use of certain antimicrobial agents, including trisodium phosphate (TSP), to assist the meat-processing sector in meeting pathogen reduction performance standards for red meats. TSP has been approved for use in hog scalding and poultry processing (36) and as a spray to be applied to beef carcasses prior to chilling (17). Research has shown that TSP can facilitate significant reductions in meatborne spoilage bacteria (18) and pathogens (10, 16). It is also important that TSP was found to be effective in removing attached Escherichia coli O157:H7 and Salmonella spp. from beef (24).

Problems associated with the use of TSP include the necessity for relatively high concentrations (8 to 12%) to obtain measurable reductions in bacterial populations on meat, as well as the associated potential for the production of undesirable organoleptic changes (9). Approaches that reduce the concentration of TSP required to effect significant reductions in viable cell numbers would have the potential for commercial application. While the mode of TSP action at the bacterial-membrane level is not completely clear, it appears that high pH values play a large role in the antimicrobial activity of TSP (9, 41). Other possible consequences of TSP application include metal ion sequestration and cell detachment caused by surfactant effects. Since TSP’s primary active site appears to be the cell membrane,
a number of researchers have used approaches whereby other membrane-active agents are applied in concert with TSP. Thus, TSP has been used in combination with nisin, sodium chlorite–based oxyhalogen disinfectants, lysozyme, and chelators in an effort to enhance antimicrobial efficacy (9, 11, 37).

The efficacy of many biocides requires their delivery to either the cell cytoplasm or the cytoplasmic membrane. To enhance this delivery process, we developed an approach that involves an NaCl pretreatment to plasmolyze cells prior to the application of the antimicrobial agent. Plasmolysis has previously been used as a physical indicator of viability (25), since plasmolysis occurs only when cells that possess a functional semipermeable membrane are placed in a hypertonic solution. Under these conditions, cells lose intracellular water while “attempting” to equilibrate with the high-osmotic-strength extracellular solution. When the osmotic pressure is relieved, plasmolyzed cells take up surrounding water, deplasmolyzing, or becoming rehydrated. It is during the deplasmolysis phase that the efficacy of antimicrobial agents may be enhanced (via facilitated uptake) if these antimicrobial compounds are present in the low-osmotic-strength deplasmolysis solution. In the present study, plasmolysis-deplasmolysis (PDP) was used in conjunction with TSP against a number of wild-type meat spoilage and pathogenic bacteria. Efficacy was assessed for model biofilms grown in flow cells and on pork adipose tissue.

MATERIALS AND METHODS

Bacteria and cultural conditions. With the exception of *Salmonella enterica* serovar Enteritidis (ATCC 4931), the bacteria used for these experiments were wild-type strains isolated from commercial meat-processing facilities (*E. coli* 8, *Listeria monocytogenes* 4) or chilled meat cuts (*Brochothrix thermosphacta* S521, *Pseudomonas* C531). Cells to be used in the adipose tissue disk assay were cultivated in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 25°C for 15 h, at which time the inocula were determined to be in the logarithmic phase of growth. To prepare bacterial inocula for biofilm studies, bacteria were first inoculated into 250-ml Erlenmeyer flasks containing the appropriate growth medium and were then incubated on orbital flask shakers (200 rpm) at room temperature (21 ± 2°C) for ~16 to 18 h. *B. thermosphacta* and *Pseudomonas* C531 were cultivated with 50 ml of nutrient broth (Difco), *E. coli* and *Salmonella* Enteritidis cells were cultivated with 10% strength tryptic soy broth, and *L. monocytogenes* was cultivated with brain heart infusion broth (Difco).

Adipose tissue disk assay. The procedures for assessing antibacterial efficacy on pork adipose tissue have previously been described (21, 22). Pork loin muscles (longissimus thoracis) were then incubated on orbital flask shakers (200 rpm) at room temperature (21 ± 2°C) for ~16 to 18 h. *B. thermosphacta* and *Pseudomonas* C531 were cultivated with 50 ml of nutrient broth (Difco), *E. coli* and *Salmonella* Enteritidis cells were cultivated with 10% strength tryptic soy broth, and *L. monocytogenes* was cultivated with brain heart infusion broth (Difco).

Bacterial inocula were diluted in 0.1% peptone water to a concentration of 5 to 6 log CFU of attached bacteria per cm² of disk surface. Adipose tissue disks were suspended from alligator clips and inoculated by immersion in 800 ml of bacterial inocula for 15 s. Inoculated adipose tissue was then held at 20°C for 15 min to permit the attachment of bacteria. Inoculated disks were subjected to (i) no treatment (control); (ii) sterile water for 3 min, immediately followed by sterile water for an additional 3 min; (iii) 1.5 M NaCl for 3 min, immediately followed by sterile water for 3 min; (iv) sterile water for 3 min, immediately followed by 5% TSP for 3 min; or (v) 1.5 M NaCl for 3 min, immediately followed by 5% TSP for 3 min. This final treatment, whereby cells were first plasmolysed with 1.5 M NaCl and then deplasmolysed in the presence of TSP, is hereinafter referred to as the PDP-TSP treatment. All antimicrobial treatments were conducted at 20°C.

After treatment, five tissue disks for each storage time were placed in petri plates and overwrapped with an oxygen-permeable film to prevent desiccation during storage. Incubation was carried out at 4°C, and five samples were taken for bacterial enumeration at 0, 2, 4, and 8 days after treatment. Each tissue disk was homogenized in 90 ml of 0.1% peptone water, diluted, and surface plated on tryptic soy broth plus 0.1% yeast extract (Difco). Bacteria were enumerated after 48 h at 25°C and converted to log CFU/cm² values. Preliminary trials were conducted over a wide range of TSP concentrations to select a level that would result in a 1- to 2-log reduction in bacterial counts. With an initial bacterial concentration of about log 5 or 6 CFU/cm², synergistic effects of PDP-TSP could more readily be observed.

The efficacy of the PDP-TSP treatment was evaluated in at least two trials. The sensitivities of two different strains of *Pseudomonas* and *B. thermosphacta* were compared. The susceptibilities of *E. coli* and *Salmonella* Enteritidis were determined at both 4°C (nonpermissive for growth) and 10°C (permissive for growth).

Construction of flow cells and culture apparatus. Six-channel flow cells with internal channel dimensions of 3 (width) by 3 (depth) by 40 mm (length) were milled from a single piece of polycarbonate plastic (lexan; GE Structured Products, Mt. Vernon, Ind.). A number 1 coverslip (45 by 50 mm; Fisher Scientific, Ltd., Nepean, Ontario, Canada) was glued over the top of the device with silicone sealant (Translucent RTV 110, G.E. Silicons, Waterford, N.Y.), resulting in six separate flow cell channels. S/P medical grade silicone tubing (inside diameter 1.5 mm, outside diameter 3.18 mm; Dow Corning Co., Baxter, Ill.) was then fitted to 3-mm holes drilled at the channel ends and sealed with silicone adhesive. Figure 1 presents a schematic illustration of the flow cell as used in this study, along with the approximate site of inoculation. Additional information on the construction and sterilization of flow cells has previously been published (28, 46).

A reservoir containing a supply of sterile growth medium (either brain heart infusion broth, tryptic soy broth, or nutrient broth) was connected to flow cells, providing a once-through delivery of growth substrate to the flow cells. All tubing and fittings were connected and sealed with Teflon connectors (Cole Parmer, Vernon Hills, Ill.) and silicone adhesive. Flow cells were inoculated with test strains by injecting a 1-ml pulse of mid-log-phase cells into flow cell channels with a sterile syringe and 27G hypodermic needle as previously described (28). Following inoculation, flow cells were continuously irrigated with the appropriate medium for 24 h at a bulk flow rate of 5 ml/h with a 201Z multi-channel peristaltic pump (Watson-Marlow, Cornwall, UK). This flow rate corresponded to a laminar flow velocity of 0.015 cm/s. Flow cells were then mounted on the stage of a Nikon FXA microscope for antimicrobial treatment and confocal laser imaging.
Treatment of biofilms cultivated in flow cells with antimicrobial solutions. After biofilms were grown for 24 h, flow was stopped and biofilms were pulse-treated either with TSP (0.1, 0.25, 0.5, 0.625, or 1.0% [wt/vol] in double-distilled H₂O) for 1 min or with 1.5 M NaCl (in double-distilled H₂O) for 3 min (inducing plasmolysis) followed immediately by the TSP solution for 1 min as above (thereby inducing deplasmolysis). In both cases, 0.5 ml of TSP (AV-Guard, Rhône Poulenc, Inc.) or NaCl was injected into the appropriate flow cell channel at a rate of approximately 100 µl/s with a sterile syringe and 27G hypodermic needle. Following the 1-min TSP treatment period, the peristaltic pump was turned on at a rate of 100 ml/h for 3 min to ensure that no residual TSP remained. The 0.1, 0.25, 0.5, 0.625, and 1.0% TSP solutions had pH values of 10.9, 11.45, 11.65, 11.7, and 11.85, respectively.

Fluorescent viability probes, confocal laser scanning microscopy (CLSM), and image processing. The Live/Dead BacLight viability assay (Molecular Probes Inc., Eugene, Oreg.), which has previously been described in detail (25, 26), was used to assess the survival of bacteria in untreated, PDP-TSP-treated, and TSP-treated biofilms. A 200-µl aliquot of BacLight probe was aseptically injected into each flow cell channel (with flow off and in darkness) following the application and removal of the antimicrobial agent. After a 15-min reaction period, unbound probe was washed from the flow cell by resuming flow with a fluoro-free medium. Untreated biofilms and NaCl-treated biofilms were used as positive (viable) controls for the staining procedure (data not shown).

After staining with the viability probe, horizontal (in the x-y plane) optical thin sections of TSP-treated and untreated biofilms were acquired at the solid-liquid interface (the biofilm base) by dual-channel CLSM imaging (providing green and red fluorescence emission images from the same microscopic field) with a Bio-Rad MRC-600 Lasersharp fluorescence confocal laser system mounted on a Nikon FXA microscope equipped with a ×60 objective with a numerical aperture of 1.4 (25). The base of the biofilm was chosen as the site of analysis because it was typically the biofilm region with the highest density of bacteria, often with overlying biofilm material. Analysis of this location has been suggested to provide the most rigorous challenge to the antimicrobial procedure, and, more importantly, this region could reliably be located on the z axis. The membrane-permeant nucleic acid stain SYTO 9 was used to obtain fluorescent (green wavelengths) images of all biofilm bacteria for a qualitative comparison of different biofilms.

Experiments (with each organism either treated with PDP-TSP, treated with TSP alone, or not treated) were replicated in separate flow cell channels, from which 10 random images were obtained along a transect near the channel center. Fluorescence emitted in the red and green channels was quantitated with a Macintosh G4 computer and the public domain image analysis program NIH Image (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/NIH-image/) and used to calculate the average area of viable cells following each treatment. Color presentations of the image data were prepared by merging red- and green-channel images from the same field using Adobe Photoshop (Adobe Systems, Inc., Palo Alto, Calif.).

RESULTS

The effect of PDP-TSP on biofilm bacteria cultivated in flow cells. Some organisms used in this study did not adhere well to surfaces; hence, a number of isolates were evaluated for their ability to adhere to surfaces and form biofilms. For example, significant variability in adherence and biofilm formation was observed for the 9 pseudomonad and 10 B. thermosphacta meat and processing-plant isolates screened (data not shown). Qualitative assessments, carried out directly with high-magnification microscopy, were performed after 24 h of growth on flow cell surfaces and used to identify Pseudomonas C531 and B. thermosphacta S521 as the isolates most amenable to this study. The remaining organisms used in these experiments (L. monocytogenes 4, E. coli 8, and Salmonella Enteritidis) readily attached to flow cell surfaces and formed biofilms.

Biofilms of Pseudomonas C531, B. thermosphacta S521, E. coli 8, Salmonella Enteritidis, and L. monocytogenes 4 were cultivated in flow cells for 24 h prior to treatment with either TSP (concentration range, 0.125 to 1.0%) or PDP-TSP (at the same concentrations as TSP). During these trials, it was found that a 1-min treatment with TSP at any of the concentrations tested was insufficient to completely kill E. coli 8 or Salmonella Enteritidis (Fig. 2A and 2C, respectively). The two lowest concentrations of TSP (0.125 and 0.2%) had little impact on the number of living Salmonella Enteritidis or E. coli cells remaining in the flow cells, with ≥95% of the biofilm biomass remaining viable. Following treatment with 0.5 to 1.0% TSP, the relative abundance of viable Salmonella Enteritidis and E. coli cells remaining decreased in a TSP-concentration-dependent manner, although ≥65% of each cell population remained
FIGURE 2. The effect of PDP on the viability of biofilm bacteria grown in flow cells for 24 h following various TSP or PDP-TSP treatments. The viability of biofilm bacteria was determined with the BacLight viability probe, CLSM, and image analysis. Panels A, B, C, and D show the percentages of viable cells remaining for E. coli 8, B. thermosphacta S521, Salmonella Enteritidis, and Pseudomonas C531, respectively.

viable. Application of NaCl in conjunction with TSP (PDP-TSP) enhanced TSP efficacy; PDP with 0.5% TSP resulted in the death of $\approx 95\%$ of bacteria following application to either Salmonella Enteritidis or E. coli 8, with higher TSP concentrations (0.65 and 1.0%) effectively killing all CLSM-detectable bacteria. Treatment of Pseudomonas C531 biofilms with PDP-TSP also led to the enhancement of TSP efficacy (Fig. 2D). The application of 0.2% TSP caused the death of $\approx 95\%$ of the Pseudomonas C531 biofilm cells, while PDP with 0.1% TSP resulted in the death of all cells. B. thermosphacta S521 (Fig. 2B) also showed enhanced TSP susceptibility when applied in conjunction with PDP; however, the difference between the TSP and the PDP-TSP treatments (at the same TSP concentration) was less pronounced than it was for E. coli, Salmonella Enteritidis, or Pseudomonas sp. L. monocytogenes 4 biofilms were not sensitive to TSP with or without the PDP process, even at 10% TSP.

While the combination of time, flow rate, and nutrients was chosen to produce relatively dense biofilms, colonization patterns and 24-h biofilm cell densities varied in a species-dependent manner (Fig. 3A through 3D). The viability probe staining patterns at the biofilm base (0 $\mu$m section depth) following application of sublethal TSP concentrations to E. coli, B. thermosphacta, Salmonella Enteritidis, and Pseudomonas biofilms are shown in Figure 4A through 4D. Notably, E. coli 8 was the only strain for which dense accumulations of cells appeared to impact the survival of embedded bacteria. Regions of surviving E. coli 8 cells were typically observed in areas of high cell density (in the x-y plane) and covered by up to 10 $\mu$m of overlying biofilm biomass.

The effect of PDP-TSP on bacteria on adipose tissue. The data in Figure 5 show the effects of NaCl and TSP on the growth of Pseudomonas C531 and L. monocytogenes 4 on pork adipose tissue disks. On the basis of comparisons with water-treated control tissue, NaCl had only marginal effects on bacterial numbers during the 8-day storage period. In contrast, TSP produced an immediate 1-log reduction in pseudomonads (Fig. 5A) and L. monocytogenes (Fig. 5B). Consequently, levels of bacteria recovered from TSP-treated tissue generally remained about 1 log lower than those for control tissue for 8 days of storage, but rates of bacterial growth were similar for TSP-treated and control tissues.

When Pseudomonas-inoculated tissue discs were pretreated with 1.5 M NaCl immediately prior to treatment with 5% TSP (Fig. 5A), there was only a slight enhancement of antimicrobial activity after 4 days of storage, at which time there was a 2.17-log reduction in pseudomonads, compared with a 1.53-log reduction with TSP alone. Similar trends were observed in an identical trial with another wild-type strain, Pseudomonas D23 (data not shown).

L. monocytogenes 4 appeared to be more susceptible to the synergistic effects of PDP-TSP (Fig. 5B). Two and
4 days after treatment, there were 1.96- and 1.81-log reductions, respectively, in the number of *L. monocytogenes* cells when PDP was used in conjunction with 5% TSP. These reductions were somewhat larger than the 1.19- and 0.64-log reductions observed at the same sampling times for tissue treated with TSP alone. Attempts to enhance this synergistic effect with higher concentrations of TSP (10%) were not successful (data not shown). The growth of *L. monocytogenes* on adipose tissue disks was also compared with that of the three other bacteria (*B. thermosphacta* S521, *Salmonella Enteritidis*, and *E. coli* 8) by using an identical experimental design. Antimicrobial efficacy was greatest at 2 to 4 days posttreatment, and, for convenience, treatment comparisons were arbitrarily summarized after 4 days of storage at 4°C. Table 1 compares the reductions in log bacterial numbers (water control numbers minus antimicrobial treatment numbers) for all five bacteria at 4 days after treatment with NaCl alone, TSP alone, and NaCl and TSP applied in sequential treatments (PDP-TSP).

The data show that NaCl by itself produced a limited reduction in bacterial numbers (of 0.05 to 0.78 log cycles). The gram-positive bacteria seemed more resistant to TSP, with reductions of 0.84 and 0.64 log cycles for *B. thermosphacta* and *L. monocytogenes*, respectively. When the gram-negative species were similarly treated with 5% TSP, reductions of 1.53, 1.59, and 2.88 log cycles were observed for pseudomonads, *E. coli*, and *Salmonella Enteritidis*, respectively.

When inoculated tissues were pretreated with 1.5 M NaCl immediately prior to the 5% TSP treatment, there was only limited evidence of enhanced antibacterial activity over and above that recorded with TSP alone. With *B. thermosphacta* and *Salmonella Enteritidis, there was no enhancement of susceptibility to TSP. When *Pseudomonas* C531 and *E. coli* 8 were the test strains, the combined PDP-TSP treatment produced only a marginal increase in bacterial susceptibility (~0.5 log cycles) compared with the antibacterial effects of TSP alone. As described in Figure 5B, the only pronounced improvement of TSP efficacy was observed for *L. monocytogenes* 4, for which sequential treatment with NaCl and TSP produced a >1-log decrease in bacterial populations over and above that found for TSP alone.

Similar results were observed for *E. coli*– and *Salmonella Enteritidis–inoculated adipose tissue stored at 10°C,
FIGURE 4. Representative dual-channel CLSM optical thin sections (0 μm section depth) of 24-h biofilms of E. coli 8 (A), B. thermosphacta S521 (B), Salmonella Enteritidis (C), and Pseudomonas C531 (D) following sublethal treatment with TSP and staining with the BacLight viability probe. The red-green pseudocolor presentation shows the result of the merging of fluorescent signals from the green and red wavelengths (corresponding to living and dead bacteria, respectively). Bar = 25 μm.

for which bacterial proliferation was significant (data not shown). Results for B. thermosphacta S521 were also confirmed in an identical trial with a different meat isolate, B. thermosphacta B2 (data not shown).

DISCUSSION

The objective of this study was to determine whether PDP could be used to enhance the efficacy of TSP in killing attached bacteria that are of concern to the meat-processing industry. The first stage of the PDP process plasmolizes the biofilm bacteria with the addition of a high-osmotic-strength (1.5 M NaCl) solution. After 1 to 3 min, the osmotic stress is relieved by pumping a low-osmotic-strength solution supplemented with trisodium phosphate. It was hypothesized that PDP could be used to facilitate the delivery of the antimicrobial agent to the cell and across the plasma membrane. This premise was first examined in an in situ study of the PDP process against biofilms cultivated in a flow cell model system by using CLSM and a fluorescent viability probe (26). The PDP process was then tested against the same organisms on adipose meat disks treated in a similar fashion.

While there has been an increase in the number of in vitro studies applying fluorescent indicators of cellular viability to bacteria following various treatments, relatively few of these studies have used direct fluorescent methods for quantifying the efficacy of antimicrobial agents against biofilm bacteria (3, 26, 27). In the present study, flow cells and digital imaging were used to provide initial evidence that the efficacy of TSP against biofilm bacteria could be enhanced by manipulating the osmotic strength of the system prior to TSP application.

TSP applied alone exhibited antimicrobial activity against all biofilm bacteria examined in this study with the exception of L. monocytogenes 4. The gram-negative isolate, Pseudomonas C531, was most susceptible to the action of TSP when examined in flow cells, with 0.125% TSP causing a 45% reduction of cell viability. When PDP was applied in conjunction with 0.125% TSP against this organism, <0.5% of the biofilm bacteria remained viable. The two other gram-negative organisms tested (E. coli 8 and Salmonella Enteritidis) were also sensitive to TSP; however, even the highest TSP concentration tested (1.0%) failed to produce more than a 35% reduction in living cells.
TABLE 1. Effects of 1.5 M NaCl and 5% TSP on the recovery of bacteria from inoculated pork adipose tissuea

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>NaCl</th>
<th>TSP</th>
<th>NaCl + TSP</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas C531</td>
<td>0.78</td>
<td>1.53</td>
<td>2.17</td>
<td>0.09</td>
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<tr>
<td>B. thermosphacta S521</td>
<td>0.05</td>
<td>0.84</td>
<td>0.30</td>
<td>0.19</td>
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<tr>
<td>L. monocytogenes 4</td>
<td>0.30</td>
<td>0.64</td>
<td>1.81</td>
<td>0.25</td>
</tr>
<tr>
<td>E. coli 8</td>
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<td>1.59</td>
<td>2.11</td>
<td>0.24</td>
</tr>
<tr>
<td>S. Enteritidis ATCC 4931</td>
<td>0.46</td>
<td>2.88</td>
<td>2.78</td>
<td>0.18</td>
</tr>
</tbody>
</table>

a Each value is the difference between the bacterial number for the control treatment (water only) and that for the biocide treatment after 4 days at 4°C. Data are LS (Least Squares) means of bacterial counts for five adipose tissue disks. The initial level of inoculated bacteria was 5 to 6 log CFU/cm².

In an earlier study, Korber et al. (26) demonstrated that 15 s of exposure to 10% TSP was sufficient to completely kill all Salmonella Enteritidis biofilm bacteria; however, not all of the bacteria were killed when the same biofilms were located between artificial crevices (1,500 μm wide by 500 μm high). In the present study, only when PDP was applied in conjunction with ≥0.5% TSP was the efficacy of TSP antimicrobial activity increased to ≥95%. For the gram-negative organisms, the maximum efficacy enhancements (reduction in viability) for each strain were 95, 69, and 55% for Salmonella Enteritidis, E. coli, and Pseudomonas, respectively. It was hypothesized that the PDP approach would result in a similar, or greater, enhancement of TSP efficacy when applied in a pork tissue assay system employing a fivefold-higher TSP concentration (5%).

To determine whether enhancement of the efficacy of TSP by PDP in flow cells would carry over to the meat environment, pork adipose tissue disks were artificially inoculated with meat spoilage bacteria (Pseudomonas sp. and B. thermosphacta) and pathogens (L. monocytogenes, E. coli, and Salmonella Enteritidis). In accord with the findings of other researchers, relatively high concentrations of TSP used alone could be expected to produce a 1- to 2-log reduction in bacteria attached to beef tissue (17, 24). In contrast, the more recent results of Cutter and Rivera-Betancourt (10) indicated that pathogens associated with beef surfaces could be substantially reduced by >3 log cycles with 10% TSP spray treatments.

In the current study, TSP was used at a concentration of 5% to produce a measurable reduction, but not the maximum reduction (determined during preliminary studies; data not shown), in bacterial numbers. A range of TSP concentrations (4 to 12%) were previously evaluated by others (36) to investigate possible approaches for treating commercial pork carcasses. It was concluded that a concentration of ≥8% TSP would achieve a ~2-log reduction in Salmonella spp. on pork skin for contact times of 5 to 15 s. Under the same conditions, the total aerobic plate count was unaffected.

Osmotic stress caused by NaCl has been found to improve the efficacy of acetic acid in reducing L. monocytogenes and Salmonella Typhimurium numbers on beef tissues (14). There is also evidence that a combination of NaCl and polyphosphates can act synergistically to limit the growth of Aeromonas hydrophila in ground pork (39) and that of L. monocytogenes in milk (32). However, these
treatments involved the simultaneous application of NaCl and an antimicrobial agent, whereas the PDP process is sequential, with NaCl pretreatment being followed by exposure to TSP.

Of the five bacteria evaluated in the present study, *L. monocytogenes* was the only organism demonstrating a marked increase in sensitivity to TSP following pretreatment of inoculated pork adipose tissue with NaCl. This finding was of interest because *L. monocytogenes* was most resistant to PDP-TSP treatment in the model biofilms. Other researchers have similarly found that biofilm cells of *L. monocytogenes* were the most resistant of a number of foodborne bacteria to TSP (22). Somers et al. (42) reported that *L. monocytogenes* was highly resistant to TSP treatment; a 12% TSP treatment for 5 min or an 8% TSP treatment for 10 min was required to obtain a 1-log reduction in *Listeria* cell numbers. In the present study, TSP concentrations in the range of 0.1 to 1.0% failed to inhibit *L. monocytogenes* biofilms cultivated in flow cells whether applied alone or in conjunction with PDP (data not shown). TSP concentrations of 2.0, 5.0, and 10% were subsequently tested with and without the PDP process and were found to have no significant effect. A recently published study concluded that one must be cautious in selecting a concentration of TSP for use in foods because low concentrations may actually enhance the growth of *L. monocytogenes* (8). It has been well documented for various pathogenic organisms that the application of sublethal concentrations of various stress factors (e.g., heat, acid, osmotic shock) results in an acquired resistance to otherwise lethal stress factors (5, 31); hence, possible collateral effects stemming from TSP application require further investigation.

There are additional reports indicating that antimicrobial agents that are highly efficacious in broth systems often lose activity in the more complex environments of foods (22). It was recently shown that TSP was far less active in a cooked ground meat medium than it was in commercial broth systems (45). The presence of proteins (i.e., bovine serum albumin) and complex organic molecules has been shown to impair the efficacy of antimicrobial agents (37). Moreover, fresh red meat is a highly buffered system, and since the mode of TSP action is related to high pH values, TSP’s long-term effectiveness may be impaired in this environment.

Concerns about undesirable organoleptic changes associated with the use of high-pH TSP may prohibit commercial application at the concentrations (8 to 12%) required to reduce bacterial numbers on red meat (9). However, there are no data to support this contention (36). It is unfortunate that the PDP approach appears to be ineffective in meat systems, since the PDP-TSP synergy was very pronounced in the biofilm model in flow cells and hence might have provided an acceptable means of enhancing TSP efficacy and reducing the amount of the antimicrobial agent needed for meat.

There is considerable value in the use of in vitro model systems such as flow cells for testing the efficacy of antimicrobial compounds against foodborne organisms of concern. The natural habitat of organisms associated with meats and meat processing are solid-liquid interfaces (e.g., meats, plastics, stainless steel (4, 29, 34)); hence, this approach is significant in assessing reports of increased antimicrobial resistance of biofilm bacteria compared with their planktonic counterparts (1, 6, 13, 29, 38, 40, 43). Moreover, the composition of the overflowing medium can rapidly be changed so as to permit the pulse application of various test and wash solutions (25, 26), precluding procedural difficulties that might exist in testing these treatments on meat surfaces. Finally, the growth, viability, and abundance of biofilm bacteria can be examined in situ with fluorescent probes (3, 30, 33), which often provide indications of other antimicrobial effects or other correlative relationships that might not be observed with growth-based methods of monitoring antimicrobial efficacy. For example, the tendency for bacteria positioned at the base of an *E. coli* biofilm to survive TSP treatment suggests either that these cells are intrinsically more resistant to TSP than are other biofilm bacteria or that the overlying bacteria and their exopolymeric substances somehow prevent TSP from producing antimicrobial effects. It has commonly been contended that overlying biofilm material (cells and exopolysaccharides) provides a protective mechanism. This protective mechanism may, in part, contribute to the observed differences in antimicrobial resistance between biofilm bacteria and their planktonic counterparts (6).

In conclusion, food and food-processing equipment, together with the highly buffered system typical of meats, offer microorganisms a complex environment that suppresses the efficacy of antimicrobial agents. Additional study is required to determine whether the potential exists for integration of the PDP strategy with a different antimicrobial agent or whether PDP can more effectively be integrated in other industrial sanitization situations.

**ACKNOWLEDGMENTS**

Saskatchewan Agriculture Development and Food, the Alberta Agriculture Research Institute, and NSERC Canada are acknowledged for financial support, Bryan Dilts and Brij Verma are acknowledged for technical assistance, and Loree Verquin is acknowledged for clerical support.

**REFERENCES**


