

## Fate of Gram-Positive Bacteria in Reconditioned, Pork-Processing Plant Water†

### ABSTRACT

This study investigated the responses of *Enterococcus faecium* (ATCC 19433), *Staphylococcus aureus* (196E), and *Listeria monocytogenes* Scott A in water from a local meat-processing plant. Each bacterium was added to a starting count of 3 log<sub>10</sub> CFU/ml and held from 5 to 28°C. At intervals (0, 2, 7, 14, and 21 days), aliquots were plated on appropriate selective agars. In contrast to the gram-negative bacteria studied previously and which grew, the three gram-positive bacteria survived with some slight increase in number in only nonchlorinated, reconditioned water, either filtered (0.22 μm pore size) or nonfiltered. The presence of chlorine in either potable or reconditioned water contributed to the rapid decline in viable counts for all three bacteria. These results further emphasize the importance of residual chlorine in preventing the growth of these gram-positive bacteria in potable and reconditioned waters.

Water use and conservation in food-processing plants is of increasing concern because of increased initial cost of water and sewage fees that are based on the volume and biological oxygen demand of the wastewater produced and released from the plant. To reduce usage and costs, various food-processing plants are reconditioning waste water and then using this reconditioned water for different food-processing operations (6). Recent work from this laboratory investigated the safety of using reconditioned water for certain pork slaughter operations (4) in a pork slaughter and processing plant. Specifically, this meat plant operates a water treatment facility on their premises (see Miller et al. (4) for a detailed description) and purifies the water by pH adjustment, dissolved air flotation, denitrification, nitrification, clarification, sand filtration, and chlorination. This reconditioned water is used primarily for carcass scalding and washing. We determined that the bacteriological quality (pathogen level and total aerobic plate count) of carcasses was similar whether they were washed with potable or reconditioned water (4).

Recent studies from this laboratory have indicated that reconditioned water from this plant (either nonchlorinated or with the residual chlorine neutralized by the addition of thiosulfate) would support the survival and growth of various gram-negative bacteria including *Aeromonas hydrophila*, *Salmonella*, and *Vibrio cholerae* (5, 8). This survival

and growth was temperature dependent. Unpublished data (7) indicate that *Pseudomonas aeruginosa* and *Escherichia coli* also exhibit this survival and growth response.

We previously demonstrated that reconditioned water from this water treatment facility was free of *Staphylococcus aureus*, *Listeria monocytogenes*, and fecal streptococci (*Enterococcus faecalis/faecium*) (4). In this study, we simulate recontamination of this reconditioned (nonchlorinated filtered and nonfiltered) water by inoculating samples of these waters with cultures of the gram-positive pathogens *L. monocytogenes* and *S. aureus* and the gram-positive indicator bacterium *E. faecium* and followed the changes in their numbers using selective and nonselective media; we also determined the influence of temperature and residual chlorine levels and compared their response to that in potable water used in this plant.

### MATERIALS AND METHODS

**Water.** The nonchlorinated and chlorinated reconditioned water samples used in this study were obtained on four separate occasions from a local meat-processing plant that operates its own wastewater treatment facility; see Miller et al. (4) for details of the process used to recondition the water. Potable water used in the plant, supplied by the local township, was the control. Some water samples were filtered (0.22 μm filter, Nalgene, Rochester, N.Y.) to remove the background microflora (cells).

**Bacteria.** The following cultures, from our research unit's culture collection, were used as representative gram-positive bacteria of interest to food microbiologists: *S. aureus* 196E (*Sa*), *L. monocytogenes* Scott A (*Lm*), and *E. faecium* ATCC 19433 (*Ef*). *Sa* and *Ef* were grown in brain heart infusion broth (Difco, Detroit, Mich.) and *Lm* was grown in tryptic soy broth (Difco) overnight

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† Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

## Non-chlorinated, Filtered

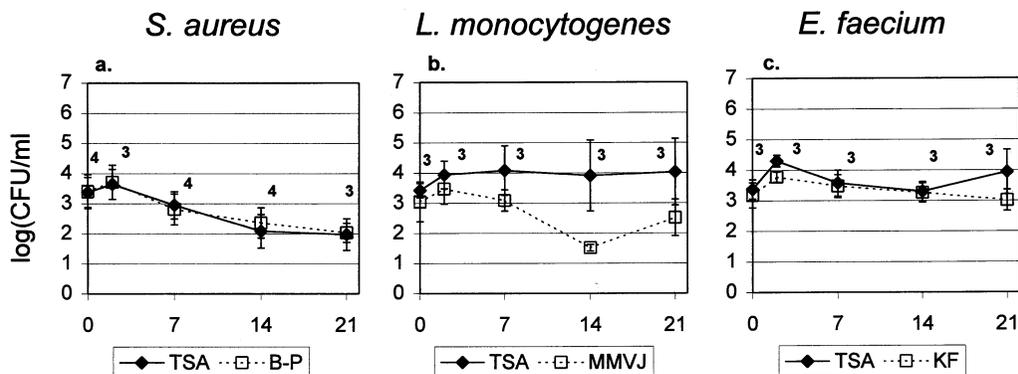


FIGURE 1. Response of gram-positive bacteria in nonchlorinated, filtered (NCF) reconditioned pork-processing plant water at 19°C: total count on TSA, *E. faecium* counted on KF agar (KF), *S. aureus* counted on Baird-Parker agar (BP), and *L. monocytogenes* counted on modified-modified Vogel Johnson agar (MMVJ). Coliform growth response of the water was  $2.51 \pm 0.49$ ; numbers above the data points indicates the number of samples used for the data point on the graph; data points are mean  $\pm$  standard error.

at 37°C with shaking (150 rpm; New Brunswick Scientific Psychrotherm, model G26, New Brunswick, N.J.). The individual cultures were centrifuged (10 min, room temperature, 7,500 rpm, IEC Clinical Centrifuge, Intl. Equipment Co., Needham Hts., Miss.), and the cell pellet was resuspended in an equivalent volume of sterile distilled water (dH<sub>2</sub>O); this was repeated twice. The resuspended cells were diluted in dH<sub>2</sub>O, and the respective water samples were inoculated with the individual cultures to yield a starting count of each bacterium of ca. 3 log<sub>10</sub> CFU/ml of water.

**Counting.** Bacteria were enumerated by surface plating (Spiral Plater, model D, Spiral Biotech, Bethesda, Md.) as follows: *S. aureus* on Baird-Parker agar (Difco; shiny black colonies, 48 h), *E. faecium* on KF agar (Difco; pinpoint pink/red colonies, 48 h), and *L. monocytogenes* on modified-modified Vogel Johnson agar (MMVJ) (11) (small black colonies, 48 h). Incubation was at 37°C, and colonies were counted with a laser colony counting system (Spiral Biotech). Serial dilutions for plate counting were made as necessary in 0.1% peptone (Difco) water. Aerobic plate count was determined by surface plating on tryptic soy agar (TSA; Difco), and colonies were counted after 24 to 48 h at 37°C. The difference in count between TSA and the individual selective media is a reflection of the number of injured cells.

**Temperatures.** Inoculated and uninoculated samples of each water type were incubated in 50-ml Falcon tubes at 5, 12, 19, and 28°C. Aliquots were removed at intervals (0, 2, 7, 14, and 21 days) and plated as above.

**Addition of thiosulfate solution.** Sodium thiosulfate solution (to yield a final level of 10 mg/100 ml) was added to samples of potable and chlorinated reconditioned water to neutralize residual chlorine (2).

**Water analysis.** The nutrient level of various water samples was expressed at the coliform growth response (CGR) as described by Rice et al. (9).

**Data analyses and experimental design.** Complete sets of water samples were collected on four separate occasions, each individual water sample comprising an experiment. The data presented in the figures represent the mean  $\pm$  standard error for from two to four samples assayed at each time point. The CGR of the waters used was  $2.51 \pm 0.49$ . In addition, the data for each individual bacterium at each temperature on either TSA or the se-

lective medium were analyzed by linear regression using the Lotus computer program. While the regression lines are not shown on the figures, they do provide an overall indication (trend) of how each bacterium behaved at each temperature in each water sample.

## RESULTS AND DISCUSSION

Initial experiments on the fate of gram-positive bacteria inoculated into different types of water obtained from the pork-processing plant indicated that: (i) the three gram-positive bacteria investigated survived with only very limited increases in number, in contrast to gram-negative bacteria that readily grew in reconditioned water (either nonchlorinated or with the residual chlorine neutralized by the addition of thiosulfate). For example, *Salmonella* spp. grew at temperatures from 12 to 37°C and *V. cholerae* grew at temperatures from 10.8 to 34.8°C (CGR =  $2.36 \pm 0.52$ ) (8), while *A. hydrophila* grew at temperatures from 5 to 42°C (CGR =  $2.91 \pm 0.61$ ) (5); and (ii) this response occurred only in nonchlorinated, reconditioned water. In the other waters tested, including potable and reconditioned water without the addition of thiosulfate, the three test bacteria declined to undetectable (<21 CFU/ml). Because the bacteria responded similarly in chlorinated, reconditioned water with thiosulfate and nonchlorinated water, we did further studies on the fate of the gram-positive bacteria at different temperatures only in nonchlorinated water, either filtered (NCF) or nonfiltered (NCNF).

The results shown in Figures 1 and 2 represent the typical response of gram-positive bacteria inoculated into NCF and NCNF water, respectively. Data for 19°C are presented; similar responses were observed at 5, 12, and 28°C (data not shown). As anticipated, the response of the bacteria was both bacterium and water sample (NCF and NCNF) dependent. There was a marked difference in the response of the three bacteria in NCF compared to NCNF water. In general, more injury was detected in NCNF samples, with greater amounts of injury seen at the higher temperatures, and with *L. monocytogenes* and *E. faecium* (data not shown). Whether portions of the flora of the recondi-

# Non-chlorinated, Non-filtered

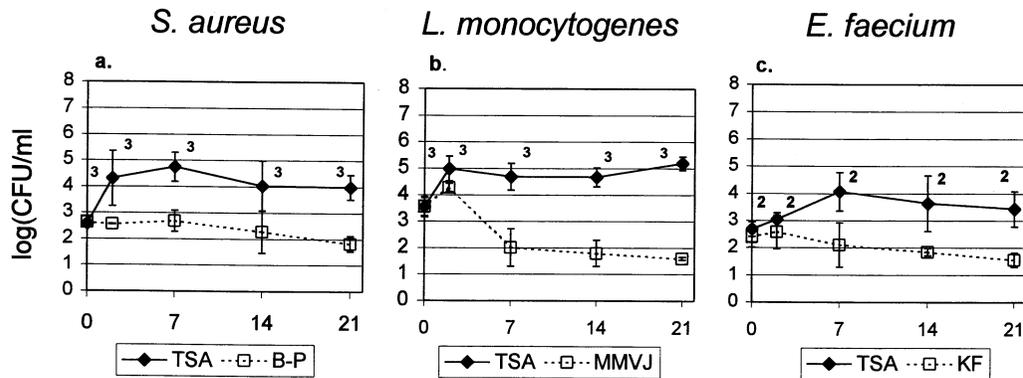


FIGURE 2. Response of gram-positive bacteria in nonchlorinated, nonfiltered (NCNF) reconditioned pork-processing plant water at 19°C: symbols as in Figure 1. Coliform growth response of the water was  $2.51 \pm 0.49$ ; numbers above the data points indicates the number of samples used for the data point on the graph; data points are mean  $\pm$  standard error.

tioned water were contributing to the increase in counts on TSA, and thus the greater difference between TSA and the selective media (more injury), is not known. However, the general responses of each bacterium were constant. *L. monocytogenes* and *E. faecium* appeared to be the hardest, surviving with no changes in viable count, especially at 5 and 28°C. This long-term survival was not expected in that none of the three bacteria have water as their natural habitat.

As indicated, the three gram-positive bacteria survived for long periods in water, especially in filtered samples in which the counts on TSA remained essentially constant (Fig. 1). Though not shown on the figures, the linear regression lines (slopes) for the data in Figure 1 and 2 were very small, particularly for TSA, indicating little change in counts over time. This provides further support for the long-term survival of gram-positive bacteria in NCF reconditioned water. The ability of enterococci (fecal streptococci) to survive in water has prompted the suggestion that they are better indicators of risk than fecal coliforms (10). Our data appear to provide further support for long-term survival of fecal streptococci, at least in nonchlorinated water. While not an aquatic bacterium, *L. monocytogenes* has been isolated from both sewage and river water (12). Watkins and Sleath also determined that *L. monocytogenes* survived better than *Salmonella* on land sprayed with sewage sludge (12). Byrd et al. reported that the non-spore-forming *E. faecalis* and *Micrococcus flavus* rapidly decreased to undetectable in filtered sterilized drinking water (tap water) (chlorine level,  $<0.1$  mg/liter) held at room temperature (1). Gurijala and Alexander (3) reported the rapid decline of *M. flavus* in filtered lake water. In addition, Gurijala and Alexander (3) were not able to detect any injury during the decline of both gram-negative and gram-positive bacteria in filtered lake water.

Uninoculated water samples (NCF and NCNF) were plated periodically during the holding times. While there were some colonies on TSA, there were no colonies on the three selective media used, indicating that there was not a resident background flora capable of growth on the media.

Thus, what was counted on the selective media was the specific bacterium inoculated into the water. Our previous study (4) demonstrated that none of these three bacteria were detected in the reconditioned water from this plant.

In conclusion, the gram-positive bacteria studied survived in NCF and NCNF reconditioned water. This is in contrast to the gram-negative bacteria studied (5, 7) that grew in amounts proportional to the CGR of the reconditioned water. While not shown specifically, it was also observed this study that the residual chlorine levels of chlorinated, reconditioned (2 to 6 ppm free chlorine) and potable waters (1 to 2 ppm residual chlorine) inactivated gram-positive bacteria, which further supports the importance of maintaining the residual chlorine level in reconditioned water to prevent survival of any gram-positive bacteria that might recontaminate it.

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