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Role of Water Hardness in Rinsing Bacteria from the Skin of Processed Broiler Chickens

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Abstract: The effect of water hardness on the ability of water to rinse bacteria from broiler skin was examined. Very hard water (total hardness = 200 ppm) was prepared by dissolving calcium chloride and magnesium chloride in distilled (soft) water and moderately hard water (total hardness = 100 ppm) was prepared by diluting 1 part very hard with 1 part soft water. After five consecutive rinses of skin in soft, moderately hard, or very hard water, samples were stomached in 0.01 M potassium phosphate buffer with 0.025% ethylenediaminetetraacetic acid to recover bacteria remaining on the skin. Bacteria in stomached rinsates were enumerated on Plate Count (PC), Levine Eosine Methylene Blue (EMB), Campylobacter (CA), Pseudomonas (PS) and Staphylococci (ST) Agars. Results indicated that significantly ($p \leq 0.05$) fewer bacteria were recovered on CA and PS Agars from skin rinsed in soft water than from skin rinsed in moderately or very hard water, and fewer bacteria were recovered on EMB Agar from skin rinsed in soft water than from skin rinsed in very hard water. Skin was also rinsed in very hard water that had been softened by adding 0, 1.0, 2.5, or 5.0% potassium citrate. Results indicated that fewer bacteria were recovered on EMB and CA Agars from skin rinsed in water softened with 5.0% citrate than from skin rinsed in water with 0, 1.0, or 2.5% citrate. Chemically softened water was not bactericidal. Findings indicate that reducing water hardness may increase the ability of water to remove bacteria from broiler skin.

Key words: water hardness, water softeners, broiler skin, bacteria

INTRODUCTION

Processing water used in commercial poultry processing facilities can play a major role in the qualities of poultry meat produced at the facility. The pH, ammonia concentration, level of microbial contamination and hardness of water used in scald-tanks, washers and chiller tanks are some factors that may influence the ability of sanitizing procedures to remove microorganisms from carcasses during processing (Russell, 2002). Water hardness is related to the concentration of minerals, such as calcium and magnesium, dissolved in the water (Kovach, 2007). These minerals are naturally found in soil and rocks in locations with high concentrations of limestone, dolomite, or gypsum in the ground. Hard water is produced as minerals from these ground deposits become dissolved in water flowing through the earth. High concentrations of calcium and magnesium contained in hard water can bind to cleansers and sanitizers used in processing operations and decrease the effectiveness of these processing aids (Russell, 2002).

The process of softening hard water may be achieved by mechanically or chemically removing calcium, magnesium, and other minerals from water (Dickerson *et al.,* 1992). Mechanical water softeners pass hard water over ion exchangers that remove calcium and magnesium ions from the water and replace them with sodium ions. Chemical water softeners may be added to hard water to reduce hardness by binding or chelating minerals and keeping them in solution, by precipitating minerals to form insoluble particles that may be filtered from the water, or by exchanging calcium or magnesium ions in the water with sodium or potassium ions. Citric acid is a well known chelator that is used as a “builder” in soaps and detergents to increase the cleansing activity of these products by reducing water hardness (Schweiker, 1981). The purpose of the present study was to determine if water hardness can play a role in the ability of water to rinse away bacteria from skin of processed broiler chickens and to determine if the process of chemically softening hard water improves the ability to water to remove bacteria from broiler skin.

MATERIALS AND METHODS

Broiler skin samples: Broiler carcasses exiting the defeathering machine in the processing line of a local, commercial poultry processing facility were removed from the line, immediately placed in separate plastic bags and placed on ice. Carcasses were transported to the laboratory where breast skin was removed and cut into 1 g samples. Skin samples were pooled, placed in a sterile plastic bag and stored at 4^{o}C until ready for use. Unused skin samples were discarded after 5 days of storage.

Artificial hard water samples: Artificial hard water with a total hardness of approximately 200 ppm (very hard water) was prepared by dissolving 0.35 g of $CaCl_2$ (Spectrum Chemical Manufacturing Corp., Gardena, CA, USA) and 0.175 g of $MgCl_2 \cdot 6H_2O$ (Acros Organics, Fair Lawn, NJ, USA) per 1 liter of distilled (soft) water. Very hard, artificial hard water was diluted 1:1 with soft water to produce hard water with approximately 100 ppm total hardness (moderately hard water). Artificial hard waters and soft water samples were sterilized by autoclaving at 121°C at 15 psi for 15 min. Total hardness of sterilized water samples was measured with a Mini Analyst Water Analysis System (Orbeco-Hellige, Inc., Farmingdale, NY, USA).

Rinsing carcass skin in artificial hard water: One g skin samples were placed in separate 50 ml sterile, plastic Pro Cent Tubes with Screw Cap (Tyco Healthcare Group, Mansfield, MA, USA) and 20 ml of sterile soft, moderately hard, or very hard water were added. The filled plastic tubes were placed on a Burrell, Model 75 wrist action mechanical shaker (Burrell Scientific, Inc., Pittsburg, PA, USA) and shaken at high speed for 1 min. Liquid in each tube was decanted, fresh water was added, and the rinsing procedure was repeated for a total of five, consecutive 1 min rinses. After the final rinse, skin was placed in a stomacher bag, and 9 ml of 0.01 potassium phosphate buffer with 0.025% Ethylenediaminetetraacetic acid, dipotassium salt dehydrate (EDTA) (Sigma Chemical Co., St. Louis, MO, USA) was added. Rinsed skin was blended in the buffer solution by stomaching on high speed in a Stomacher 80 (Seward Medical Limited, London SE1 IPP, UK) for 2 min. Skin was discarded and aliquots of the skin rinsates were removed for microbial analyses. An Autoplate 4000 Automated Spiral Plater (Spiral Biotech, Bethesda, MD, USA) was used to plate rinsates on Plate Count (PC) (Becton Dickinson and Co., Sparks, MD, USA); Staphylococci, Medium 110 (ST) (Remel Inc., Lenexa, KS, USA); Levine Eosin Methylene Blue (EMB) (Oxoid Limited, Basingstoke, Hampshire RG24 8PW, England); Oxoid Blood Agar Base (Oxoid Limited) supplemented with 7.0% lysed horse blood (Lampire Biological Laboratories, Pipersville, PA, USA) and Oxoid *Campylobacter* Selective Supplement (Blaser-Wang) (CA) (Oxoid Limited) and Pseudomonas (PS) (Oxoid Limited) Agars. Inoculated PC, ST, EMB and PS agars were incubated aerobically at 35-37°C for 24-48 h and inoculated CA plates were incubated at 42°C for 48 h in a BBL GasPak Jar System (Becton Dickinson and Co.) with an activated BBL CampyPak Plus gas generator envelope. After incubation, colonies on agar plates were counted using the QCount™ Colony Counting System (Spiral Biotech).

Rinsing carcass skin in chemically softened hard water: Very hard water was chemically softened by dissolving 0.0, 1.0, 2.5, or 5.0% (w/vol) potassium citrate, tribasic monohydrate (Sigma Chemical Co.) in the water, then autoclaving water samples at 121°C at 15 psi for 15 min. Total hardness of the very hard water and chemically softened waters was measured with a Mini Analyst Water Analysis System. Skin samples were rinsed in the water samples, and bacteria were recovered from the skin as described above.

Determination of the bactericidal activity of potassium citrate: Cultures of bacterial isolates recovered from skin of poultry carcasses were mixed in solutions of very hard water and chemically softened water and size of the bacterial populations recovered from the solutions was determined. *Staphylococcus simulans* and *Escherichia coli* that were previously isolated from poultry carcasses were grown aerobically at 35-37°C for 18-24 h in Tryptic Soy Broth (Becton Dickinson and Co.). *Campylobacter jejuni* PPMQ2B that was previously isolated from poultry carcasses was grown microaerobically at 42°C on blood agar (Becton Dickinson and Co.) and then harvested from the agar by adding 9 ml of 0.1% peptone water to the plate and using a sterile bacterial spreader stick to remove bacterial growth from the plate. All bacterial suspensions were centrifuged at 5500 g in a Hermle Z300 centrifuge (National Labnet Co., Woodbridge, NJ, USA) for 5 min, supernatant was discarded, cell pellet was suspended in 10 ml of fresh 0.1% peptone and centrifugation was repeated. A suspension of the final pellet was added to an aliquot of 0.1% peptone to produce an absorbance of 1.00 at 625 nm in a Spectronic® 20D+ spectrophotometer (Spectronic Instruments, Inc. Rochester, NY, USA). Serial dilutions of the bacterial suspensions were prepared to produce cultures containing approximately 106 cfu/ml and 0.2 ml of the bacterial suspension was added to test tubes containing 19.8 ml of very hard water to which 0.0, 1.0, 2.5, or 5.0% (w/vol) potassium citrate had been added. Tubes were shaken for 5 min on a wrist action mechanical shaker, 1 ml of the suspensions were transferred to 9 ml of 0.1% peptone. The Autoplate 4000 Automated Spiral Plater was used to plate the diluted *E. coli* onto EMB Agar, *S. simulans* on ST Agar and *C. jejuni* onto CA Agar. Agar media were incubated as described above and cfu's/ml were enumerated.

Statistical analysis of data: All experiments were replicated 5 times. The number of cfu recovered/ml of rinsate was transformed to log10 cfu/ml before conducting statistical analysis using GraphPad InStat® version 4.00 for Windows 95 GraphPad Software. San Diego, CA, USA). One-way Analysis of Variance (ANOVA) with Tukey-Kramer Multiple Comparison tests was performed to determine significant differences in group means.

RESULTS AND DISCUSSION

Bacteria recovered from skin rinsed in distilled water or artificial hard waters: Water hardness played a significant role in determining the number of bacteria recovered from the skin of processed chickens that was

Table 1: Log cfu/ml bacteria recovered on Plate Count, Levine Eosin Methylene Blue, Campylobacter, Staphylococcus, and Pseudomonas Agars from rinsates of broiler skin washed 5 times in soft, moderately hard, or very hard water[1]

		Agar Medium				
Wash	Water Hardness[2] (ppm)	Plate Count	Levine Eosin Methylene Blue	Campylobacter	Staphylococcus	Pseudomonas
Soft water	0.00 ± 0.00	3.54^{a}±0.96	3.00^{a}±0.46	1.44^{a}±0.34	3.52a+0.57	2.67^{a}±0.70
Moderately hard water	106.80 ±10.9	4.25^{a}±0.48	3.71^{ab}±0.35	2.33^{b}±0.28	3.91^{a}±0.51	3.46b±0.57
Very hard water	200.80 ±16.33	4.17^{a}±0.47	3.97^{b}±0.27	2.29^{b}±0.26	3.93^{a}±0.21	3.59b±0.55

[1]Values are averages ± standard deviation. n = 5. [2]Total water hardness measured as ppm calcium carbonate ($CaCO_3$).

[a,b]Within columns, different letters indicate significant ($p \leq 0.05$) differences in the number of bacteria from recovered from skin rinsates

rinsed in the water (Table 1). Significantly fewer bacteria were recovered on EMB Agar from skin samples rinsed in soft water than from skin rinsed in very hard water. Bacteria recovered on EMB Agar are Gram negative bacteria, such as *Escherichia coli*, that are associated with the intestinal tract, but these bacteria may contaminate other parts of the carcass as intestinal contents are forced out of the digestive tract during processing. Additionally, significantly fewer bacteria were recovered on CA and PS Agar from skin rinsed in distilled water than from skin rinsed in moderately hard or very hard water. The *Campylobacter* recovered on CA Agar are also intestinal bacteria, while *Pseudomonas* bacteria recovered on PS Agar are spoilage bacteria found in processing water (Thomas and McMeekin, 1980) and on processing equipment (Hinton *et al*., 2004). Intestinal bacteria and spoilage bacteria are transient members of the bacterial flora of the skin of healthy chickens (Thomas and McMeekin, 1980) that are primarily found floating in thin water layer on the surface of the skin of processed broiler carcasses (Thomas and McMeekin, 1980; Kim *et al*., 1996). Since hard water is a less effective cleanser than soft water (Kovach, 2007), rinsing skin in hard water may not have been as effective as rinsing in soft water in removing these bacteria from the water layer on the surface of the skin. Differences in the degree of hardness of water used to rinse the skin may have played a role in the significant differences in number of bacteria recovered from skin rinsed in soft or hard water. Furthermore, high concentrations of calcium found in hard water can cause aggregations of protein molecules (Molina and Wagner, 1999) that could entrap bacteria found on the surface of the skin, thereby reducing the number of bacteria that could be rinsed from the surface.

There was no significant difference in the number of bacteria recovered on ST or PC Agar from skin rinsed in soft, moderately hard, or very hard water. Staphylococci bacteria that are isolated on ST Agar are members of the native flora of the skin of live chickens and other animals. These bacteria colonize the skin and become imbedded in fats and proteins secreted by the skin (Thomas and McMeekin, 1980). These fats and proteins may firmly attach these bacteria to the skin and protect the bacteria from physical and chemical treatments used to sanitize broiler carcasses (Hinton and Cason, 2008). Since staphylococci can also grow on PC Agar, these bacteria may have been partially responsible for the reason that there was no significant difference in the number of bacteria recovered PC Agar from skin rinsed in soft or hard water.

Bacteria recovered from skin rinsed in chemically softened hard water: Reducing water hardness by the addition of potassium citrate significantly increased the ability of water to rinse some bacteria from carcass skin (Table 2). Adding potassium citrate to very hard water decreased water hardness to from approximately 200 ppm in water containing no citrate to 0 ppm in very hard water with 5.0% potassium citrate added. Significantly fewer bacteria were recovered on EMB and CA Agar from skin rinsed in very hard water softened with 5.0% potassium citrate than from skin rinsed in very hard water with 0.0, 1.0, or 2.5% potassium citrate added. By binding to calcium and magnesium in very hard water, citrate can keep these ions in solution and prevent them from reducing the cleansing activity associated with soft water. Citrate can also reverse the ability of calcium to cause aggregation of protein molecules (Molina and Wagner, 1999). Since it is possible that aggregated protein molecules may entrap bacteria on the surface of the skin and reduce the number of bacteria that are recovered from skin rinsed in hard water, another chelator, EDTA, was included in buffer rinse. Preliminary results indicated that stomaching recovered more bacteria from skin rinsed in very hard water when EDTA was added to the stomaching rinsates (data not shown). Softening water with citrate did not improve that ability of the water to remove staphylococci recovered on ST Agar and bacteria recovered on PC Agar, however.

Potassium citrate possesses no bactericidal activity towards bacterial isolates recovered from broiler skin (Table 3). There was no significant difference in the number of *E. coli*, *S. simulans*, or *C. jejuni* recovered from cultures mixed in very hard water or chemically softened hard water; therefore, changes in the number of bacteria recovered from skin rinsed in chemically softened hard water were probably due to the physical removal of bacteria instead of bactericidal activity.

Water hardness varies in different geographical locations, but most treated water in the United States

Table 2: Log cfu/ml bacteria recovered[1] on Plate Count, Levine Eosin Methylene Blue, Campylobacter, and Staphylococcus Agars from rinsates of broiler skin washed 5 times in very hard water softened with 0.0, 1.0, 2.5, or 5.0% potassium citrate[1]

		Agar Medium			
% Citrate	Water Hardness[2] (ppm)	Plate Count	Levine Eosin Methylene Blue	Campylobacter	Staphylococcus
0.0	211.20±13.48	4.08[a]±0.55	3.50[a]± 0.47	1.89[a]±0.74	4.02[a]±0.48
1.0	54.00±4.64	4.00[a]±0.39	3.49[a]± 0.38	1.53[a]±0.93	3.86[a]±0.55
2.5	13.60±4.89	3.81[a]±0.56	3.34[a]± 0.45	1.53[a]±0.95	4.03[a]±0.57
5.0	0.00±0.00	3.37[a]±0.57	2.85[b]± 0.27	0.52[b]±0.72	3.39[a]±0.56

[1]Values are averages ± standard deviation. n = 5. [2]Total water hardness approximately 200 ppm measured as calcium carbonate ($CaCO_3$). [a,b]Within columns, different letters indicate significant ($p \leq 0.05$) differences in the number of bacteria from recovered from skin rinsates.

Table 3: Log cfu/ml *Escherichia coli*, *Staphylococcus simulans,* and *Campylobacter jejuni* recovered[1] after mixing pure cultures of isolates for 5 min in artificial hard water[2] softened with 0.0, 1.0, 2.5 and 5.0% potassium citrate

% Citrate	*E. coli*	*S. Simulans*	*C. jejuni*
0.0	4.48[a]±0.07	3.98[a]±0.10	3.62[a]±0.29
1.0	4.45[a]±0.04	4.07[a]±0.06	3.59[a]±0.45
2.5	4.42[a]±0.06	4.07[a]±0.04	3.14[a]±0.19
5.0	4.40[a]±0.06	4.01[a]±0.09	3.21[a]±0.15

[1]Values are averages ± standard deviation. n = 5. [2]Total water hardness = 200 ppm measured as $CaCO_3$. [a]Within columns, no significant differences in cfu/ml recovered from water samples

has some degree of hardness (Water Quality Association, 2008). Therefore, water hardness may be one of the characteristics of processing water that should be monitored by poultry processors. By controlling water hardness, poultry processors may be able to improve the ability of processing water to remove bacteria from the skin of processed poultry.

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