*Journal of Food Protection, Vol. 80, No. 10, 2017, Pages 1670–1675*
doi:10.4315/0362-028X.JFP-17-088


**Research Paper**

# Removal of *Escherichia coli* and *Enterococcus faecalis* after Hand Washing with Antimicrobial and Nonantimicrobial Soap and Persistence of These Bacteria in Rinsates

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MS 17-088: Received 1 March 2017/Accepted 31 May 2017/Published Online 6 September 2017

## ABSTRACT

Food handlers are important sources of contamination in the agricultural environment. This study was conducted (i) to evaluate the activity of antimicrobial soaps against *Escherichia coli* and *Enterococcus faecalis* using a hand washing model with soiled hands and (ii) to determine the survival and persistence of these bacteria in rinsates. Sterilized agricultural soil from tomato and pepper farms was inoculated with *E. coli* or *E. faecalis* at $10^3$ or $10^6$ CFU/g. Decontaminated hands were placed in contact with contaminated soil for 2 min and were then washed with soaps with or without antimicrobial compounds (citric extracts, chloroxylenol, triclosan, or chlorhexidine gluconate). As the control, hands were washed with sterile distilled water. The levels of bacteria remaining on the hands and recovered from the rinsates were determined using a membrane filtration method and selective media. Antimicrobial soaps removed levels of *E. coli* similar to those removed by distilled water and nonantimicrobial soap on hands contaminated with *E. coli* at $10^3$ CFU/g. However, when hands were contaminated with *E. coli* at $10^6$ CFU/g, more *E. coli* was removed with the chlorhexidine gluconate soap. When hands were contaminated with *E. faecalis* at $10^3$ CFU/g, bacteria were removed more effectively with soaps containing chloroxylenol or chlorhexidine gluconate. When hands were contaminated with *E. faecalis* at $10^6$ CFU/g, all of the antimicrobial soaps were more effective for removing the bacteria than were distilled water and nonantimicrobial soap. *E. coli* grew in all of the hand washing rinsates except that containing triclosan, whereas *E. faecalis* from the $10^6$ CFU/g treatments grew in rinsates containing chlorhexidine gluconate and in the distilled water rinsates. Washing with antimicrobial soap was more effective for reducing bacteria on soiled hands than was washing with water or nonantimicrobial soap. However, persistence or growth of bacteria in these rinsates poses health risks.

Key words: Antimicrobial agent; Decontamination; *Enterococcus faecalis*; *Escherichia coli*; Soap formulations

Because human skin is a reservoir for many microorganisms that are associated with foodborne illnesses *(25)*, hand hygiene is important for reducing the transmission of infectious agents in clinical and community settings *(20)*. In the agricultural environment, the hands of farmworkers are key vectors in the spread of pathogenic microorganisms, leading to contamination of produce and subsequent consumer health risks *(2, 9)*.

Usually, microbial quality certifications are required for farms that export their produce and that want to obtain better prices and reduce the risks of outbreaks due to consumption of their produce *(38)*. Good agricultural practices are used on these farms, including hand hygiene, which is a simple and effective method for reducing the cross-contamination of produce *(9, 26)*.

To reduce the microbial load on hands, the Public Health Service of the U.S. Food and Drug Administration (FDA) recommends rubbing hands with a cleaning compound for at least 10 to 15 s and then rinsing them with warm water *(35)*. Although the number of people receiving hand hygiene training has increased, numerous foodborne illness outbreaks still occur, indicating that hand hygiene issues must still be addressed *(3, 6)*.

The efficacy of soap for reducing bacteria on hands depends on the types and physiological characteristics of the bacteria, the presence of organic matter, the volume of hand soap used, and the time of contact with hand soap *(18, 31, 32)*. Because of the mechanical and cleaning effects of hand washing with water and soap, a certain level of microbes can be removed. The addition of antiseptic agents could also help to eliminate or reduce most of the bacteria found on hands *(13, 32)*; however, various studies have shown that hand washing without soap may reduce the bacterial load more effectively than hand washing with soap. This difference is primarily due to the friction that occurs during hand washing *(23, 32)*.

Legislation in various countries allow hand soaps to include antiseptics or disinfectants to further eliminate or reduce bacteria and organic matter *(7, 28, 33)*. Chlorhexidine gluconate, triclosan, and quaternary ammonium compounds are commonly found in hand soaps, and

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chlorhexidine gluconate and triclosan maintain their activity on hands over relatively long periods of time (30 min to 2 h) *(10, 16, 29)*.

Most hand washing procedures are effective when little or no organic matter is present because organic matter helps bacteria attach to hands *(31, 32)*. In the agricultural environment, the hands of farmworkers typically have high levels of organic matter, which in conjunction with the roughness of farmworker hands, makes bacterial removal difficult *(9, 21)*.

Another issue of concern is the presence of persistent bacteria in hand washing rinsates, which might be used for several purposes, including irrigation, when water is scarce *(17)*. Hand washing rinsates contain organic chemicals from hand soaps, organic material, and microorganisms (including pathogens) that were removed from the hands, and these rinsates could pose a risk to the environment and human health *(11, 17)*.

The presence of microbial indicators of fecal contamination suggests the presence of pathogens; thus, detection and enumeration of indicator organisms are widely used to assess the efficacy of sanitation programs *(20)*. The measurements associated with hygiene practices include total viable bacterial counts, total coliform counts, and detection of *Escherichia coli* and *Enterococcus* spp. *(15)*.

The purpose of this study was to analyze the efficacy of hand washing with antimicrobial soaps for reducing indicator bacteria on soiled hands and to evaluate the survival and persistence of indicator bacteria in rinsates.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *E. coli* ATCC 25922 (kindly provided by Dr. Lynne McLandsborough, Department of Food Science, University of Massachusetts, Amherst) and *Enterococcus faecalis* ATCC 19433 (American Type Culture Collection, Manassas, VA) were used as test strains in this study. These reference strains are commonly used for quality control purposes. Strains were maintained as stock cultures in brain heart infusion (BHI) broth (Bioxon, BD, Mexico City, Mexico) with 20% glycerol (Sigma-Aldrich, Toluca, Mexico) at −80°C. Working cultures were made by transferring aliquots of frozen cultures to BHI agar tubes, and after 48 h of incubation, the tubes were stored at 4°C. Fresh cultures were obtained by transferring aliquots from the refrigerated cultures to tubes containing 5 mL of BHI broth and incubating the tubes for 24 h at 37°C.

Growth curves of these strains were generated by inoculating the strains into BHI broth tubes, incubating the tubes at 37°C, measuring the absorbance at 600 nm ($A_{600}$), and determining viable counts by plating on BHI agar every hour. Working cultures were adjusted to an $A_{600}$ of 0.5 to 0.6 ($\sim 10^8$ CFU/mL), and serial dilutions were made with 0.85% sterile saline solution. The dilutions corresponding to $10^4$ and $10^7$ CFU/mL were used for the soil inoculations.

**Soaps.** Four antimicrobial soaps were used in this study: Neutro Germ (containing citric extracts; *s*-citric extracts, Corpo Citrik S.A. de C.V., Mexico City, Mexico), Dial (containing triclosan; *s*-triclosan, Schwarzkopf & Henkel, Mexico City, Mexico), Lysol (containing chloroxylenol; *s*-chloroxylenol, Reckitt Benckiser Centroamérica, S.A., San Jose, Costa Rica), and Hibiclens (containing chlorhexidine gluconate; *s*-chlorhexidine gluconate, Mölnlycke Health Care US, Norcross, GA). The nonantimicrobial soap Lirio (Sanchez y Martin S.A. de C.V., Mexico City, Mexico) was included as a control.

**Soil inoculation.** Agricultural soil was collected from tomato and pepper farms (Cadereyta, Nuevo Leon, Mexico). Large soil particles and stones were removed, and the soil was dispensed into sterile bags and stored at room temperature for no more than 2 weeks until used. Aliquots (10 g) were autoclaved for 30 min at 121°C *(30)*. Soil sterility was evaluated by plating 100 μL of a 1:10 dilution onto BHI plates using the agar overlay method. The plates were incubated at 37°C for 24 to 48 h, and when no colonies were observed after incubation, the soil was determined to be useful for further studies.

Ten grams of decontaminated soil was placed into a 92-oz Whirl-Pak sterile bag (Nasco, Ft. Atkinson, WI), and 1 mL of bacterial suspension (adjusted to $10^4$ or $10^7$ CFU/mL) was added. The soil and bacteria mixture was homogenized in the bag to obtain nonmuddy dried contaminated soil, which was used for hand contamination assays. Time between inoculation and hand assays was less than 3 min.

**Efficacy of antimicrobial soaps for removing bacteria: hand contamination.** Hands were predecontaminated by washing with nonantimicrobial soap, rinsing with sterile room temperature water, drying with single-use paper towels, sprinkling with 5 mL of 70% ethanol, and rubbing until dry. Because ethanol could have an impact on adherence or removal of bacteria, only one experiment per day was conducted.

The efficacy of the decontamination protocol was tested by placing hands into a bag containing 750 mL of sterile 0.1% buffered peptone water (BPW) and agitating them for 20 s. The rinsates were then assayed for *E. coli* and *E. faecalis*. Rinsates were discarded when *E. coli* or *E. faecalis* were detected.

Decontaminated hands were placed into a bag containing soil inoculated with *E. coli* or *E. faecalis* at $10^3$ or $10^6$ CFU/g (final level), and the soil was smeared onto the hands for 2 min. The soiled hands were then immediately subjected to hand washing. The initial level of attached bacteria was determined immediately after soiling using the procedure described for enumeration of bacteria in rinsates.

**Efficacy of antimicrobial soaps for removing bacteria: hand washing assays.** Soiled hands were moistened with 1 mL of sterile distilled water, and one pump (~1.7 mL) of antimicrobial soap, nonantimicrobial soap, or distilled water was dispensed into the palm of one hand. After rubbing vigorously for 20 s, hands were rinsed with 100 mL of sterile distilled water at room temperature (25°C). Hand rinsates were collected for further assays, and the bacteria remaining on the hands were immediately analyzed by placing one hand in a bag containing 750 mL of sterile 0.1% BPW, agitating for 20 s, and then massaging the hand in the bag for an additional 20 s with particular attention to the fingers. That hand was removed, the second hand was placed in the same bag, and the process was repeated. The bacteria in the BPW samples were then enumerated.

**Effects of antimicrobial soaps on bacteria in rinsates.** Hand rinsates were analyzed to determine whether the antimicrobial soaps affected the viability of the bacteria removed from hands. Bags containing the hand rinsates were incubated at room temperature, and *E. coli* and *E. faecalis* in the rinsates were quantified at 0, 1, 3, and 20 h by the filtration method described by Heredia et al. *(15)*.

TABLE 1. *Reduction of* E. coli *and* E. faecalis *on hands after washing with different hand soap formulations*[a]

| Treatment | Mean ± SD reduction (log CFU/hand) | | | |
|---|---|---|---|---|
| | *E. coli* | | *E. faecalis* | |
| | $10^3$ CFU/g | $10^6$ CFU/g | $10^3$ CFU/g | $10^6$ CFU/g |
| Control | 3.4 ± 0.2 | 6.0 ± 0.1 | 3.6 ± 0.1 | 6.7 ± 0.4 |
| Distilled water | 2.23 ± 0.1 A | 3.01 ± 0.1 AB | 1.84 ± 0.1 AB | 1.97 ± 0.1 A |
| Nonantimicrobial soap | 3.02 ± 0.2 B | 2.74 ± 0.1 A | 1.84 ± 0.1 AB | 2.05 ± 0.1 A |
| Soap with citric extracts | 2.58 ± 0.4 AB | 3.21 ± 0.1 BC | 1.53 ± 0.1 A | 3.00 ± 0.02 B |
| Soap with chloroxylenol | 2.16 ± 0.1 A | 3.52 ± 0.2 C | 2.61 ± 0.3 C | 4.43 ± 0.3 D |
| Soap with triclosan | 3.17 ± 0.0 BC | 2.88 ± 0.1 AB | 2.09 ± 0.1 B | 3.83 ± 0.2 C |
| Soap with chlorhexidine gluconate | 3.17 ± 0.0 BC | 4.17 ± 0.2 D | 3.15 ± 0.3 D | 3.47 ± 0.02 C |

[a] Bacteria were inoculated on hands at $10^3$ or $10^6$ CFU/g. Within a column, means followed by the same letter are not significantly different (MANOVA, $\alpha = 0.05$). Analyses were performed comparing the treatments for each bacterial species.

*E. coli* and *E. faecalis* levels were quantified according to the protocol described by Fabiszewski de Aceituno et al. *(8)*. Various volumes of rinsates (0.1 to 100 mL) were filtered through a 0.45-μm-pore-size cellulose filter (EMD Millipore Corp., Billerica, MA) using a vacuum-manifold filtration system (Pall Corp., Port Washington, NY). When volumes to be filtered were less than 1 mL, the funnel (with the vacuum closed) was prefilled with 20 mL of sterile 0.1% BPW before the sample was added to allow even sample dispersion across the membrane before the vacuum was opened. When the colonies were too numerous to be counted, decimal dilutions of the sample were made with 0.1% BPW. After filtration, duplicate membranes for each serial volume of rinsate were placed on petri dishes containing solidified chromogenic RAPID'*E. coli* 2 agar for *E. coli* (Bio-Rad, Hercules, CA) or Kenner fecal *Streptococcus* agar for *E. faecalis* (Difco, BD, Sparks, MD). The plates were incubated at 37°C for 24 to 48 h, and then typical colonies (pink and purple colonies for *E. coli* and colonies with red centers for *E. faecalis*) were enumerated. The limit of detection was 37 CFU per hand *(8)*.

**Statistical analysis.** All of the experiments were performed at least twice independently, and each condition was tested in duplicate. Results from bacterial removal treatments were analyzed with a multivariate analysis of variance (MANOVA; $\alpha = 0.05$) using the Number Cruncher Statistical System, version 6.0 software (NCSS, Kaysville, UT). Graphs were generated using SigmaPlot, version 10.0 (Systat Software, San Jose, CA).

## RESULTS

**Efficacy of antimicrobial soaps for removal of hand bacteria.** When hands were in contact with contaminated soil at $10^3$ CFU/g, hand washing with distilled water or with chloroxylenol removed similar amounts of *E. coli* (2.23 ± 0.1 log CFU per hand with distilled water and 2.16 ± 0.1 log CFU per hand with of chloroxylenol) (Table 1). Hand washing with chlorhexidine gluconate led to greater reductions ($P \leq 0.05$) of *E. faecalis* (3.15 ± 0.3 log CFU per hand) than hand washing with distilled water or with nonantimicrobial soap (both 1.84 ± 0.1 log CFU per hand). Hand washing with citric extracts or with triclosan removed *E. coli* and *E. faecalis* amounts similar to those removed by hand washing with distilled water or nonantimicrobial soap (Table 1).

When hands were in contact with contaminated soil at $10^6$ CFU/g, hand washing with chlorhexidine gluconate or with chloroxylenol resulted in greater log reductions ($P \leq 0.05$) of *E. coli* (4.17 ± 0.2 log CFU per hand with chlorhexidine gluconate and 3.52 ± 0.2 log CFU per hand with chloroxylenol) than hand washing with distilled water or nonantimicrobial soap (3.01 ± 0.1 CFU per hand with distilled water and 2.74 ± 0.1 log CFU per hand with nonantimicrobial soap). However, hand washing with citric extracts or triclosan removed *E. coli* amounts similar to those removed by hand washing with distilled water or nonantimicrobial soap. All of the antimicrobial soaps were more effective ($P \leq 0.05$) than distilled water and nonantimicrobial soap for removing *E. faecalis* (Table 1).

**Effects of antimicrobial soaps on bacteria in rinsates.** *E. coli* grew in almost all of the rinsates from the hands contaminated with $10^3$ and $10^6$ CFU/g in soil (Fig. 1A). However, *E. coli* was not detected in the rinsates containing triclosan in the $10^3$ CFU/g experiment. The *E. coli* levels in the rinsates containing triclosan decreased over time in the $10^6$ CFU/g experiment (Fig. 1).

In the *E. faecalis* $10^3$ CFU/g experiments, levels in the rinsates containing antimicrobial agents remained stable over time, levels in the nonantimicrobial soap rinsates decreased over time ($P \leq 0.05$), and levels in the distilled water rinsates increased over time. In the *E. faecalis* $10^6$ CFU/g experiments, levels in the nonantimicrobial soap rinsates and the rinsates containing chloroxylenol decreased over time; however, levels in the distilled water rinsates and the rinsates containing chlorhexidine gluconate increased over time (Fig. 2).

## DISCUSSION

The results of this study are consistent with those of previous studies; hand washing with water was less effective than washing with antimicrobial soaps, but washing with water did reduce contamination *(4)*. In the present study, we tested various antimicrobial compounds to determine their ability to decontaminate soiled hands: (i) chlorhexidine gluconate, which is recommended for health care environments *(19)*, (ii) citric extracts, which are environmentally

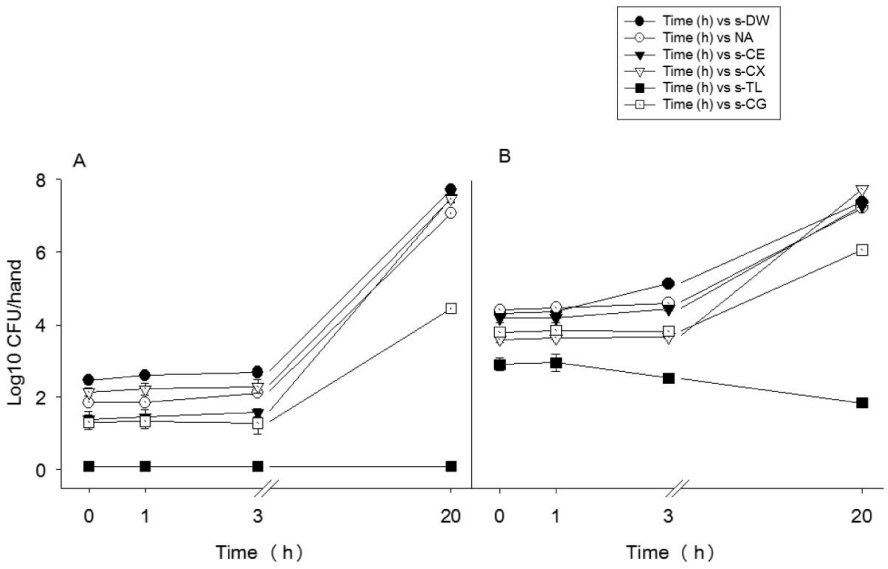


FIGURE 1. *Persistence of* E. coli *in hand washing rinsates. Hands were soiled with* $10^3$ *CFU/g (A) and* $10^6$ *CFU/g (B). DW, distilled water; NA, nonantimicrobial soap; TL, triclosan; CG, chlorhexidine gluconate; CE, citric extracts; CX, chloroxylenol.*

friendly natural products, and (iii) chloroxylenol and triclosan, which are commonly found in antimicrobial soaps. Although triclosan is not generally recognized as safe and effective by the FDA *(34)* for use in soaps in the home, this agent is still used in many countries. The FDA has not published any actions on chloroxylenol and chlorhexidine gluconate *(34)*.

For numerous industrial and health procedures, hand washing is a reliable method to prevent the transmission of harmful pathogens *(15, 32)*. Although many agree that hand washing with soap and water (or even with water alone) is effective for reducing the spread of disease-causing bacteria *(12, 25, 32)*, doubts remain concerning the benefits of adding antimicrobial compounds to soaps. Studies comparing the efficacy of antimicrobial soaps and nonantimicrobial soaps for reducing pathogenic bacteria continue to produce conflicting results *(13)*. Although there are various soap formulations to remove dirt and soil in industrial and health care facilities, little information is available regarding the efficacy of these formulations in the agricultural environment, in which workers' hands my have a different topography and degree of roughness *(9, 37)*.

Recent studies in the agricultural environment have revealed that hand washing removes dirt but does not always effectively eliminate fecal indicators *(8, 9)*, thus; the addition of antimicrobial compounds to soap combined with mechanical friction could increase removal of bacteria. However, a variety of conditions in the agricultural environment could exist that could affect the removal of microbes.

Although controversial results about the effectiveness of antimicrobial versus nonantimicrobial soaps for reducing bacteria and consequently providing health benefits have been reported *(1, 27)*, in the present study substantial reductions in bacterial levels were found after washing hands with antimicrobial soaps. An important finding of this study is that the efficacy of soaps for removing bacteria differs by bacterial species. At low levels of contamination ($10^3$ CFU/g), the amount of *E. coli* removed from hands with water or nonantimicrobial soap was similar to that removed with antimicrobial soaps. At higher contamination

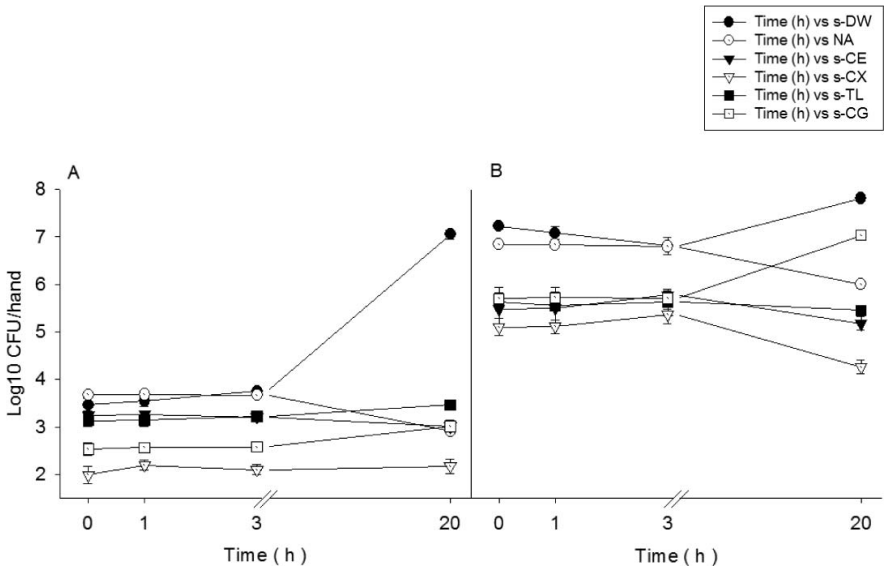


FIGURE 2. *Persistence of* E. faecalis *in hand washing rinsates. Hands were soiled with* $10^3$ *CFU/g (A) and* $10^6$ *CFU/g (B). DW, distilled water; NA, nonantimicrobial soap; TL, triclosan; CG, chlorhexidine gluconate; CE, citric extracts; CX, chloroxylenol.*

levels ($10^6$ CFU/g), the amount of *E. coli* removed from hands with chlorhexidine gluconate or chloroxylenol was higher than that removed with water or nonantimicrobial soap ($P \leq 0.05$).

In most treatments, *E. faecalis* was more difficult to remove than *E. coli* ($P \leq 0.05$), and hand washing with antimicrobial soap was necessary to reduce *E. faecalis* on hands by more than 2 log CFU when the inoculum was $10^3$ CFU/g or by more than 3 log CFU when the inoculum was $10^6$ CFU/g. This difficulty could be due to the ability of gram-positive bacteria such as *Enterococcus* spp. to better persist in the environment than gram-negative bacteria such as *E. coli (5, 14, 22)*.

*E. coli* can persist in terrestrial and aquatic habitats for various periods of time *(36)*. Persistence or growth of bacteria in rinsates collected after hand washing with antimicrobial or nonantimicrobial soap poses health risks, especially on farms without proper sewage systems *(24)*.

The results of this study suggest that antimicrobial compounds in liquid hand soaps improve the reduction of bacteria on agriculturally soiled hands when compared with distilled water and nonantimicrobial soap, but their efficacy depends on the type and level of the contaminating bacteria. Persistence of bacteria in hand washing rinsates necessitates implementation of appropriate disposal systems, and these rinsates should not be used for irrigation.

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