

Bactericidal effects of triclosan in soap both *in vitro* and *in vivo*

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Objectives: On December 2013, the US FDA proposed a rule stating that manufacturers must provide data to demonstrate that antibacterial soap is more effective than plain soap or water. The objective of the present study was to examine the *in vitro* and *in vivo* bactericidal effect of triclosan (the most widely used antiseptic agent in soap) in soap.

Methods: Twenty bacterial strains (proposed by the FDA) were exposed to plain and antibacterial soaps (the same formulation as plain soap, but containing 0.3% triclosan) for 20 s at 22°C (room temperature) and 40°C (warm temperature). The temperature and time were selected to simulate the hand washing conditions and procedures used by consumers. The triclosan concentration of 0.3% is the maximum allowed by law. The decontamination efficacy of plain soap and antibacterial soap was also examined *in vivo*: the hands of volunteers were artificially inoculated with *Serratia marcescens*.

Results: There was no significant difference ($P > 0.05$) in bactericidal activity between plain soap and antibacterial soap at either test temperature. However, antibacterial soap showed significantly greater bactericidal effects after 9 h. These results suggest that although triclosan-containing soap does have antibacterial activity, the effects are not apparent during the short time required for hand washing.

Conclusions: Antibacterial soap containing triclosan (0.3%) was no more effective than plain soap at reducing bacterial contamination when used under 'real-life' conditions. The present study provides practical information that may prove useful for both industry and governments.

Introduction

Hand washing with water and/or soap is an important and inexpensive method for preventing the transmission of infection because it is effective at removing contaminants, including pathogenic bacteria or viruses, from hands.^{1–3} Nowadays, industry produces a variety of commercial soaps described as 'antibacterial' or 'antimicrobial'. Antibacterial soap refers to soap containing ingredients with active antimicrobial activity; plain soap on the other hand contains no such ingredients.³ Millions of consumers in the USA use antibacterial hand soap and body wash products,⁴ spending nearly \$1 billion annually: the expectation is that these products will provide more protection from pathogens than plain soap.⁵

In December 2013, the Center for Drug Evaluation and Research (CDER) of the US FDA proposed an amendment to the 1994 tentative final monograph (TFM) regarding over-the-counter antiseptic products.⁶ This stated that manufacturers of antibacterial hand soaps intended for use with water must demonstrate that they are safer and more effective than plain soap and water when it comes to preventing illness and/or the spread of infection.

If the manufacturer cannot supply scientific evidence to support the claims, then these products will have to be reformulated or relabelled to remain on the market.

Triclosan ($C_{12}H_{17}Cl_3O_2$; 2,4,4'-trichloro-2'-hydroxydiphenyl ether) is the most common active antiseptic ingredient used in soap. Triclosan is a phenoxyphenol antimicrobial agent first developed in the early 1960s and has been widely used as an antibacterial or antifungal agent since the 1970s.³ It is added to various personal care products and cosmetics, including soap, toothpaste, lotions and shampoos, as well as to other products, such as clothing, kitchenware, furniture and toys, with the aim of reducing or preventing bacterial contamination and growth.^{7,8} The majority of liquid hand soaps marketed as 'antibacterial' contain triclosan as the active ingredient.⁹ A study conducted in 2001 found that ~76% of liquid hand soaps and 29% of bar soaps contained triclosan.¹⁰

Triclosan has antibacterial activity against bacteria, fungi and viruses; indeed, there is little doubt that the compound has antimicrobial activity.^{11,12} However, the use of triclosan remains controversial because various adverse effects have been reported, including allergies, antibiotic resistance, endocrine disruption,

acute/chronic toxicity and bioaccumulation; one study even identified carcinogenic impurities.^{9,11,13–19} Also, the effectiveness of antibacterial soap has been inconclusive and questions remain about whether antibacterial soap is more effective than plain soap. There have been two literature reviews on the effectiveness of antibacterial soap.^{9,20} Aiello et al.⁹ concluded that antibacterial soaps containing triclosan were no more effective than plain soap. On the other hand, Montville and Schaffner²⁰ concluded that antibacterial soaps resulted in significantly greater reductions in bacterial counts than plain soaps, although the differences were small (around 0.5 log reduction difference). These concerns about safety and effectiveness have provided the background for the proposed ruling by the FDA.⁶

Most studies examined the antibacterial activity of triclosan by determining its MIC.^{12,21–26} However, the FDA maintains that MIC testing is not relevant when evaluating the effectiveness of antiseptic ingredients, since the MIC test requires a long exposure time (at least 1 day); clearly, this does not replicate the typical exposure time of a consumer to antimicrobial-containing products.⁶ The FDA recently recommended that the ‘modified time–kill assay’ be used to provide evidence for the efficacy of active antiseptic ingredients and that the concentration of the compound and the contact time should reflect real-life situations.⁶ Also, they recommended that *in vitro* studies should be performed using reference strains and representative clinical isolates [20 strains (nine genera); 10 Gram-positive and 10 Gram-negative].⁶ The nine genera are as follows: *Campylobacter*, *Enterococcus*, *Escherichia*, *Listeria*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Shigella* and *Pseudomonas* (Table 1).

Table 1. The 20 bacterial strains proposed by the US FDA that were tested in the present study

Bacterial strain	ATCC number
Gram-positive	
<i>Enterococcus faecalis</i>	19433
<i>Enterococcus faecalis</i>	29212
<i>Staphylococcus aureus</i>	6538
<i>Staphylococcus aureus</i>	29213
MRSA	33591
MRSA	33592
<i>Streptococcus pyogenes</i>	14289
<i>Streptococcus pyogenes</i>	19615
<i>Listeria monocytogenes</i>	7644
<i>Listeria monocytogenes</i>	19115
Gram-negative	
<i>Campylobacter jejuni</i>	33291
<i>Campylobacter jejuni</i>	49943
<i>Escherichia coli</i>	11775
<i>Escherichia coli</i>	25922
<i>Pseudomonas aeruginosa</i>	15442
<i>Pseudomonas aeruginosa</i>	27853
<i>Salmonella</i> Enteritidis	13076
<i>Salmonella</i> Typhimurium	14028
<i>Shigella sonnei</i>	9290
<i>Shigella sonnei</i>	25931

To the best of our knowledge, no published study has examined the antibacterial efficacy of triclosan in soap against all 20 listed strains. Therefore, the objectives of the present study were: (i) to examine the bactericidal effects of triclosan in soaps against all 20 strains at room temperature (22°C) and in warm water (40°C) after a short exposure time (20 s) (*in vitro* study); and (ii) to compare the ability of plain soap and antibacterial soap to remove/inactivate *Serratia marcescens* artificially inoculated onto human hands (*in vivo* study).

Materials and methods

Experiment 1 (in vitro antimicrobial testing)

The treatment temperatures (22 and 40°C), time (20 s) and triclosan concentration (0.3%) were in line with those recommended for hand washing, consumer habits and the maximum level of triclosan allowed in products.

The Healthcare Infection Control Practices Advisory Committee, the WHO, the CDC and the Ministry of Food and Drug Safety of Korea (MFDS) recommend that consumers wash their hands for 20 s (the time taken to sing ‘happy birthday’ twice).^{27–29}

They also suggest washing hands with warm water, but did not specify the water temperature. However, the American Society for Testing and Materials (ASTM) standard (ASTM E1174: Standard test method for evaluation of the effectiveness of healthcare personnel or consumer hand wash formulations) suggested the water temperature for hand washing should be 40°C. Also, in previous research examining the decontamination effect of hand washing, the volunteers washed their hands with water heated to 40°C.^{30,31} Also, most consumers wash their hands with water at the temperature at which it comes out of the tap.

Triclosan is governed by different regulations in different countries, although the generally accepted maximum concentration is 0.3% in Europe [Regulation (EC) no. 1223/2009], Australia (Poisons Standard 2012), Canada (Canadian Environmental Protection Act 1999) and China (Hygiene Standard for Cosmetics 2007).

Therefore, we performed experiments using antibacterial soap containing 0.3% triclosan, a water temperature of 22°C (ambient) or 40°C (warm) and a time of 20 s.

The antibacterial activity of plain and triclosan-containing soap was assessed using an official time–kill method (M26-A; a method for determining the bactericidal activity of antimicrobial agents) as suggested by the CLSI (Wayne, PA, USA).

Bacterial strains

The 20 bacterial strains (Table 1) proposed by the FDA⁶ were obtained from the ATCC (Manassas, VA, USA), the National Culture Collection for Pathogens (NCCP; Osong, Republic of Korea), the Korean Culture Center of Microorganisms (KCCM; Seoul, Republic of Korea) and the Food Microbiology Culture Collection at Korea University (Seoul, Republic of Korea).

Preparation of cell suspensions

Each strain was cultured twice (separately) in 10 mL of Mueller–Hinton broth (MHB; Difco) and incubated under optimal conditions [37°C for 24 h for all strains except *Listeria monocytogenes* (30°C for 24 h) and *Campylobacter jejuni* (42°C for 24 h)]. *C. jejuni* was cultured under micro-aerobic conditions (GasPack Plus; Difco). After incubation, the bacteria were harvested by centrifugation (Centra–CL2; IEC, Needham Heights, MA, USA) for 15 min at 3000 g. The supernatant was decanted and the pellet washed twice with 0.9% sterile saline before being resuspended in the same buffer solution (the maximum time that the bacteria were allowed to remain in the buffer did not exceed 15 min).

Preparation of plain and antibacterial soaps

To examine the sole effect of triclosan in soap, compounds present in soap samples, other than triclosan, should be the same. Plain soap and triclosan-containing antibacterial soap (the same formulation as plain soap except that it contained 0.3% triclosan) were kindly provided by the research centre of SeoKang SPT (Anyang, Republic of Korea), which specializes in the production of antibacterial soap. Plain soap (gel type) comprised various ingredients, including distilled water, sodium laureth sulphate, cocamidopropyl betaine, glycerin, lauramide DEA, sodium chloride, glycol distearate, *Argania spinosa* kernel oil, methylisothiazolinone, methylchlorisothiazolinone, disodium EDTA and citric acid. Antibacterial soap had the same formulation as plain soap except that it contained 0.3% triclosan. All soap samples were used within 2 weeks of the production date.

Bactericidal efficacy assay

Tubes containing soap samples (10 mL) were pre-heated to the designated temperature (22 or 40°C) in a water bath (Vision Scientific Co. Ltd, Daejeon, Republic of Korea). An aliquot (100 µL) of each bacterial suspension was then inoculated into the soap samples. The tubes were then maintained at the designated temperature for 20 s. *Escherichia coli* ATCC 25922 was used to examine the bactericidal effects of the soaps after long-term exposure. Briefly, bacterial suspensions (100 µL) were inoculated into soap samples (10 mL), which were then stored at 22°C for 0, 1, 3, 6, 9, 12, 24, 48 and 72 h. The surviving bacteria were then counted. All treatments were performed in triplicate.

Microbiological analysis

Immediately after the various treatments, 90 mL of Dey/Engley neutralizing buffer (Difco) (Dey/Engley buffer neutralizes a broad spectrum of antimicrobial agents, including phenolics^{32,33}) was added to each soap sample to quench any residual bactericidal activity. The samples were then homogenized using a stomacher (Circulator 400; Seward, Worthing, UK) at 230 rpm for 1 min. One millilitre of sample was serially diluted with 9 mL of 0.9% sterile peptone water. One hundred microlitres of diluent was then spread-plated on two Mueller–Hinton agar (MHA; Difco) plates and incubated under optimal conditions to allow surviving bacteria to form colonies. The number of colonies on the MHA plates was then counted.

Experiment 2 (in vivo hand washing test)

The decontamination efficacy of plain and antibacterial soaps was evaluated using the standard method stipulated by ASTM standard E1174. Briefly, 16 healthy adult (aged >19 years) volunteers with no history of skin problems participated in the test. All volunteers refrained from using any type of antibacterial soap (researchers provided all subjects with plain soap) for at least 1 week before the day of the experiment. All were taught the hand washing procedure recommended by the WHO.²⁹ A paper describing the hand washing procedure of the WHO was distributed to volunteers;²⁹ it contained the following instructions: (i) wet your hands with water; (ii) apply soap to cover all of your hands; (iii) rub hands palm to palm; (iv) place right palm over left dorsum with interlaced fingers and vice versa; (v) place palm against palm with fingers interlaced; (vi) place backs of fingers on opposing palms with fingers interlocked; (vii) perform rotational rubbing of left thumb clasped in right palm and vice versa; (viii) perform rotational rubbing, backwards and forwards, with clasped fingers of right hand in left palm and vice versa; (ix) rinse hands with water; and (x) dry hands thoroughly with a towel. The study was reviewed and approved by the institutional review board of Korea University.

S. marcescens culture

Laboratory stock cultures (100 µL) of *S. marcescens* ATCC 14756 (the test organism suggested by the ASTM) were enriched by growing the bacteria overnight at 25°C in screw-cap tubes containing 10 mL of tryptic soy broth (Difco). Enrichment cultures developed a red pigment. Bacterial cells were harvested by centrifugation at 3000 g for 15 min. After the supernatant was discarded, the bacterial pellets were washed twice with sterile 0.9% saline. The final pellets were resuspended in the same buffer.

Inoculation of *S. marcescens* onto the hands of the volunteers

Subjects washed their hands for 30 s with a non-antibacterial soap. Wet hands were patted dry with a clean paper towel. The hands were then dipped in 70% ethanol for 5 s to remove dirt, oil and natural flora. After air drying, the hands were contaminated as follows. A 1.5 mL aliquot of *S. marcescens* suspension was dispensed into the cupped hands. The hands were then rubbed together for 20 s to spread the bacteria over the entire surface. The hands were then allowed to air dry for 30 s. Next, a second aliquot of bacterial suspension (1.5 mL) was dispensed into the subjects' cupped hands and the procedure repeated. Finally, a third 1.5 mL of suspension was poured onto the hands and distributed for 20 s. The hands were then allowed to dry for 90 s (total volume of bacterial per subject, 4.5 mL; total inoculation time, 210 s). The subject's hands were then immediately tested to measure the baseline bacterial population as described below.

Hand washing procedure

Before the experiment, the water to be used for hand washing was pre-heated to 40°C in a water bath. Then, 5 mL of test material (plain soap or antibacterial soap) was dispensed into the cupped hands of each subject using a sterile syringe, followed by 3 mL of tepid water (40°C). The subjects were asked to lather the soap vigorously for 30 s and spread it over the entire surface of the hands and the lower third of the forearms. The hands (from the fingertips to the elbows) were then thoroughly rinsed with water (40°C; ~2 L of water per subject from a spigot in a water bath with the same flow rate) for 30 s before being dried by lightly patting with dry paper towels.

Recovery of bacteria from a sample solution and counting

Prior to bacterial recovery, a sampling solution containing a neutralizer was prepared as described in a previous study (0.075 M phosphate buffer with 0.1% Triton X-100).³⁰ After washing their hands, the subjects put on a sterile glove on each hand (loose-fitting, unlined and powder-free) into which 75 mL of sampling solution was poured. The glove was then secured above the wrist and massaged uniformly for 1 min. A pipette was inserted into the glove and a 5 mL sample of fluid was aseptically withdrawn and placed in a sterile conical tube. Samples (1 mL) were then diluted with 9 mL of sterile Butterfield's buffer (10-fold dilution). The samples or diluents (0.1 mL) were spread-plated in duplicate on tryptic soy agar (Difco) and incubated at 25°C for 48 h. The number of typical red-pigmented colonies was counted.

Statistical analysis

The average cfu count for duplicate plates from three independent trials was converted into log cfu/mL prior to examination by analysis of variance (ANOVA). The log bacterial population was calculated using the general linear model (GLM) within the SAS package (version 9.13; SAS Institute Inc., Cary, NC, USA). When ANOVA indicated a significant result ($P < 0.05$), the significantly different means were separated using Tukey's multiple range test.

Results and discussion

Experiment 1 (in vitro study)

Figures 1 and 2 show the bacterial counts for each of the 20 strains after exposure to plain or antibacterial soap for 20 s at 22 or 40°C. There was no significant differences in bactericidal activity between plain soap and antibacterial soap against

any of the tested bacteria ($P>0.05$) at either temperature and at an exposure time of 20 s. Neither was there a difference after exposure for 10 or 30 s (data not shown). Triclosan is known to have antibacterial and antifungal properties;^{11,12} therefore, it is difficult to understand why no difference was observed in this experiment. Although there may be many reasons, we propose two major ones.

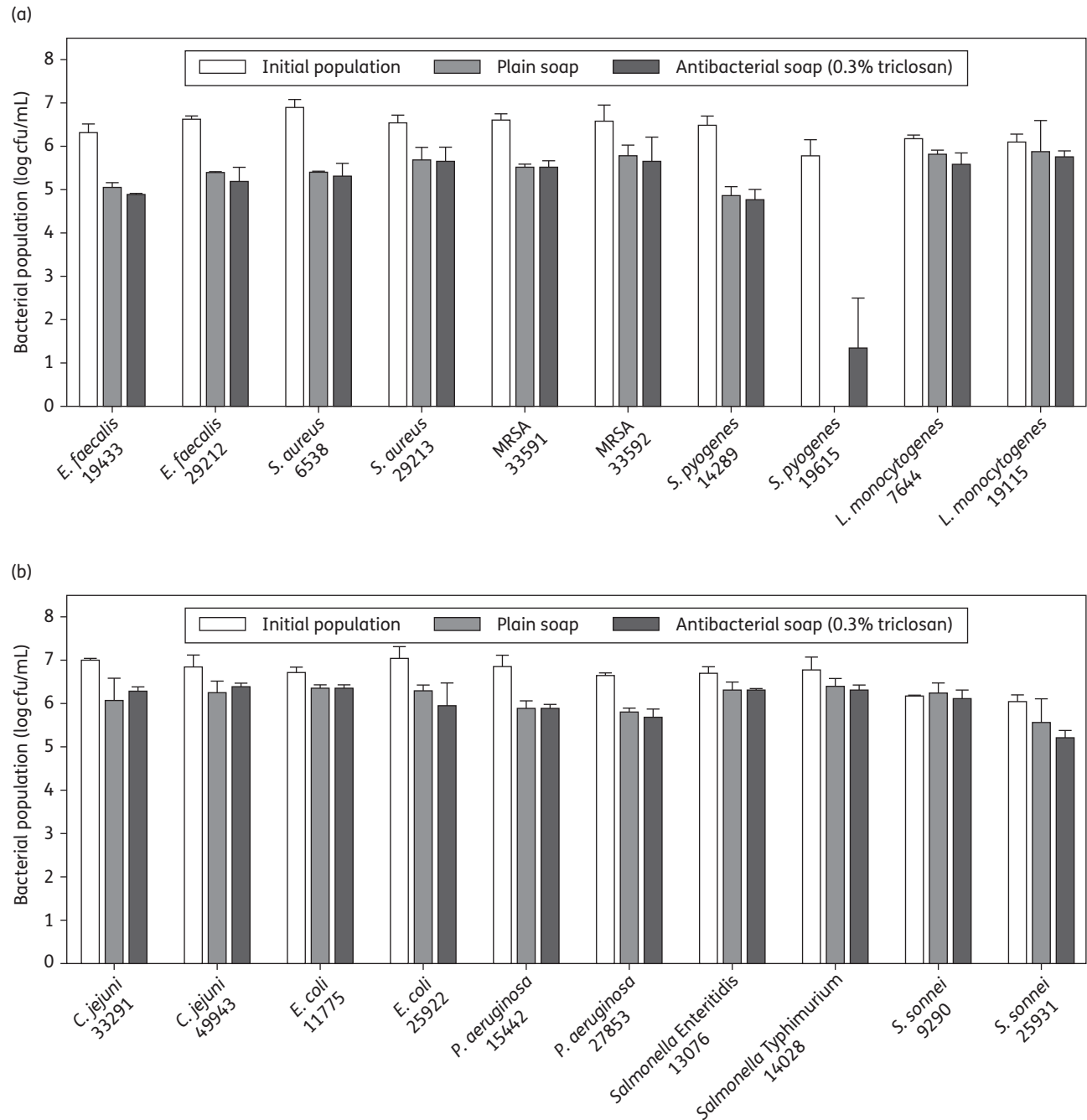


Figure 1. Bactericidal effects of plain and antibacterial (0.3% triclosan) soaps against (a) 10 strains of Gram-positive bacteria and (b) 10 strains of Gram-negative bacteria when used at room temperature (22°C) for 20 s. The bars represent the standard deviation.

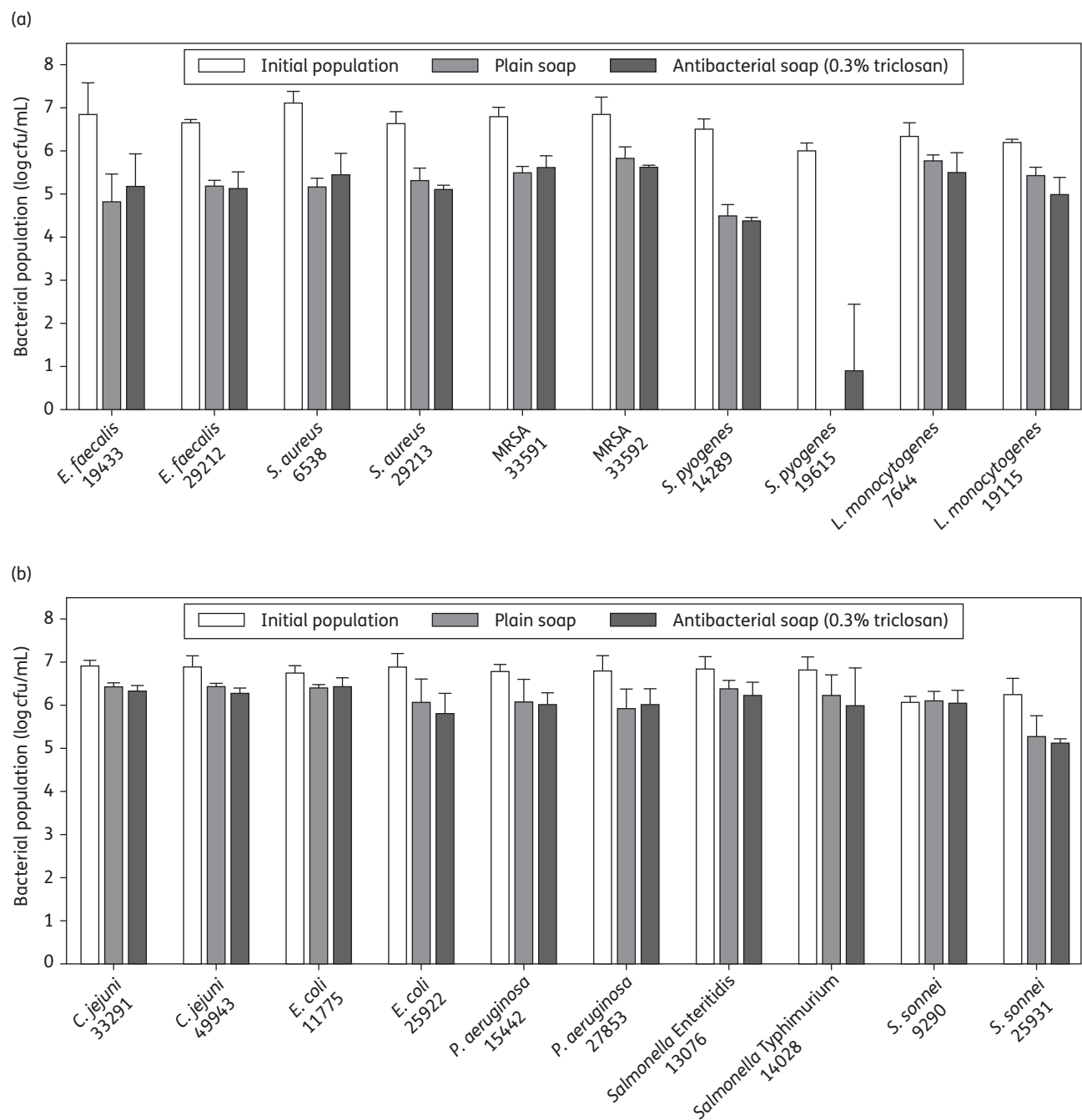


Figure 2. Bactericidal effects of plain and antibacterial (0.3% triclosan) soaps against (a) 10 strains of Gram-positive bacteria and (b) 10 strains of Gram-negative bacteria when used at 40°C for 20 s. The bars represent the standard deviation.

Firstly, the exposure time was too short. Most studies examining the antibacterial activity of triclosan used the MIC method, which requires continuous exposure to soap for at least 24 h.^{12,21–26} When we used longer exposure times, the antibacterial soap did show higher bactericidal efficacy than plain soap (Figure 3). When *E. coli* ATCC 25922 was exposed to antibacterial soap containing 0.3% triclosan, the cells survived for up to 24 h. By contrast, the bacterium survived for up to 72 h after exposure to plain soap. At

exposure times <6 h, there was little difference between the two ($P>0.05$), although antibacterial soap performed significantly better after 9 h of exposure ($P<0.05$). Thus, although the addition of triclosan to soap does provide antibacterial protection, the effect is not seen due to the short exposure time associated with hand washing.

Secondly, the soap used in the present study contained surfactants such as sodium laureth sulphate, which may also play a role

in reducing the bactericidal effects of triclosan. Triclosan inhibits bacterial growth and/or kills cells by diffusing into the cell wall; it disrupts the cell membrane and the synthesis of essential cellular components such as lipids and proteins.^{25,34,35} The bactericidal effects of triclosan may also be affected by the detergent base, emollients and humectants, pH and the ionic nature of the formulation.^{1,11} Surfactant molecules form micelles in water, which sequester triclosan,^{1,11} thereby reducing its bactericidal activity. Thus, the bactericidal activity of triclosan may be significantly lower in a surfactant-based solution than in a comparable water-based solution.

Both soaps were more effective against Gram-positive bacteria (*Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *L. monocytogenes*) than Gram-negative bacteria (*C. jejuni*, *E. coli*, *Pseudomonas aeruginosa*, *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Shigella sonnei*). Exposing Gram-positive bacteria to plain soap yielded 1.47 and 1.83 log reductions (average value for the 10 strains of Gram-positive bacteria) in the number of cfu at 22 and 40°C, respectively. Smaller log reductions (0.56 and 0.58 log cfu/mL at 22 and 40°C, respectively) were observed for Gram-negative bacteria. Similar results were observed for the antibacterial soap (reductions of 1.44 and 1.80 log cfu/mL for Gram-positive bacteria and 0.63 and 0.67 log cfu/mL for Gram-negative bacteria, at 22 and 40°C, respectively). These results are consistent with previous observations that Gram-positive bacteria are more susceptible to soap than Gram-negative bacteria.^{36–38} Among the tested bacteria, *S. pyogenes* ATCC 19615 was the most susceptible to soap. Exposure to either plain soap or antibacterial soap at 22°C reduced the population of *S. pyogenes* ATCC 19615 to undetectable or negligible levels, respectively (initial population, 5.77 log cfu/mL; detection limit, 1.70 log cfu/mL) (Figure 1a). Exposure to plain or antibacterial soap at 40°C resulted in similar reductions (undetectable levels after exposure to plain soap and 0.9 log cfu/mL after exposure to antibacterial soap; initial population, 6.01 log cfu/mL) (Figure 2a). Some soap manufacturers provide data regarding the antibacterial activity of their products against *S. pyogenes* ATCC 19615. However, because this bacterium is extremely susceptible to plain soap, it may not be the

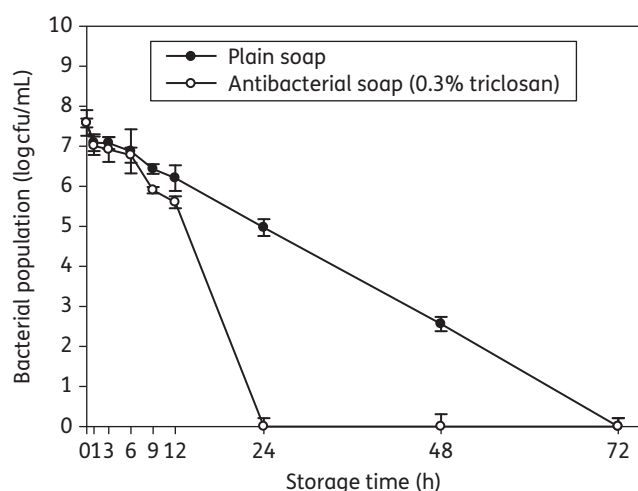


Figure 3. Survival of *E. coli* ATCC 25922 after exposure to plain and antibacterial (0.3% triclosan) soap at 22°C was monitored for up to 72 h.

most suitable organism upon which to base claims of bactericidal activity.

Increasing the temperature to 40°C yielded results similar to, or slightly better than, those obtained at 22°C. The average difference between 22 and 40°C was only 0.20 log cfu/mL; indeed, the maximum incremental increase was 0.87 log cfu/mL (for *L. monocytogenes* ATCC 19115), achieved with antibacterial soap at 40°C. These results refute the common belief that washing hands in warm water is more effective than washing in water at ambient temperature. This result is consistent with the previous observation that water temperature was not a significant parameter in hand decontamination.^{39,40}

Experiment 2 (in vivo study)

The average counts of *S. marcescens* ATCC 14756 on the hands of 16 volunteers after washing with plain or antibacterial soap are shown in Figure 4. The viable cell counts before hand washing ranged from 6.31 to 8.87 log cfu/hand (mean, 7.96 log cfu/hand). Washing with either plain or antibacterial soap led to a significant reduction in bacterial populations. Plain soap and antibacterial soap yielded log reductions of 1.96 log cfu/hand (range, 1.00–3.22) and 2.05 log cfu/hand (range, 0.94–3.30), respectively. The difference between plain soap and antibacterial soap was non-significant ($P > 0.05$). This result supports the data showing that antibacterial soap is no more effective than plain soap when used for hand washing.

Many studies have compared the ability of antibacterial soap containing various concentrations of triclosan (ranging from 0.2% to 2.0%, although the most common concentration is 0.3%) and plain soap to kill natural flora or artificially inoculated bacteria, including *S. marcescens*, *E. coli* and *Shigella flexneri*, present on the hands.^{30,41–47} It is difficult to compare the results of these studies with those of the present study due to differences in

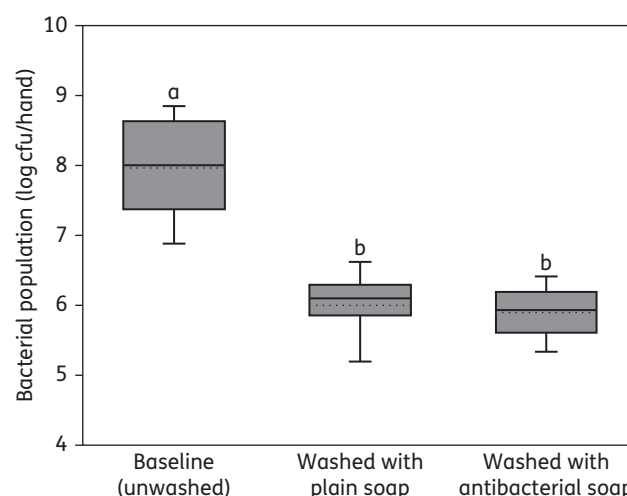


Figure 4. Population of *S. marcescens* ATCC 14756 present on the hands of 16 volunteers after washing with plain or antibacterial (0.3% triclosan) soap. The bars (whiskers) above and below the boxes represent the 90th and 10th percentiles, respectively. The box itself represents the IQR (25th–75th percentiles). The black horizontal line within the box represents the median value and the dotted horizontal line within the box represents the mean value. Data designated by different superscript letters are significantly different ($P < 0.05$).

the experimental procedures, soap formulations and target bacteria. However, in general, soaps containing $\leq 0.3\%$ triclosan were no more effective than plain soap.^{44,45,47} However, soaps containing $>0.3\%$ triclosan (i.e. 0.45%, 1.0%, 1.5% and 2.0%) were significantly more effective than plain soap in reducing bacterial counts on the hands.^{30,41–43} The results of the present study are in agreement with those of previous studies showing that soap containing $\leq 0.3\%$ triclosan is no more effective than plain soap. However, because most of these studies performed experiments using commercial soaps, the formulation of the plain and antibacterial soaps may have been different; this may have affected the results. The soaps used in the present study were identical; the only difference was the inclusion of triclosan in the antimicrobial version. Thus, the data presented herein may be a more accurate reflection of the effectiveness of these soaps.

Conclusions

The major finding of the present study was that there is no significant difference between the bactericidal effects of plain soap and antibacterial soap when used under real-life conditions. Our own survey of soap manufacturers (performed from July to August 2014) revealed that many have removed triclosan from their products due to continuing controversy about the health risks and lack of effectiveness (only 13 of 53 antibacterial soaps contained triclosan). This reflects companies' reactions to the proposed FDA rule. Thus, both advertisement and consumer belief regarding the effectiveness of antibacterial soaps need to be addressed. To the best of our knowledge, this is the first study to examine the bactericidal effects of triclosan in soap against the 20 bacterial strains proposed by the FDA and it provides empirical data regarding the bactericidal activity of antibacterial/plain soap that could form the basis of international regulation.

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Transparency declarations

None to declare.

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