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Analysis of Antimicrobial Activity Using Hand Sanitizers

Leong Xin Yi[1], Regina Leong Zhi Ling[1], Lim Lai Huat[1], Su Shao Feng*[2], and Teo Swee Sen*[1]

[1]Faculty of Applied Sciences, UCSI University, No.1 Jalan Menara Gading, UCSI Heights, 56000 Cheras, Kuala Lumpur, W. P. Kuala Lumpur, Malaysia

[2]Biotechnology Research Centre, Inner Mongolia Academy and Animal Husbandry Sciences, Hohhot, China

Corresponding Author: teoss@ucsiuniversity.edu.my; & sushaofeng2020@163.com

Abstract

Alcohol-based hand sanitiser is introduced to replace hand washing when water is unavailable to decrease the rate of infectious diseases. Proper hand hygiene is essential during pandemics to transmit pathogens through contaminated surfaces. The efficacy of hand sanitisers is reviewed based on a quantitative suspension test according to European Standards against microorganisms Staphylococcus aureus, Saccharomyces cerevisiae and Escherichia coli. Five selected products, 96% (v/v) ethanol, 65% (v/v) ethanol, World Health Organization (WHO) formulated hand rub, Commercial oil-based and non-oil-based (Brand A and Brand B, respectively), are used as treatments against the microbes. The post-treatment results of hand sanitisers as colony-forming units on tryptone soya agar plates are counted. The inoculation of microbes using the spread plate technique can identify CFU units where the logarithmic reduction factor is determined. The minimum requirement of the log reduction factor is 2 logs to provide sufficient bactericidal activity. Based on the results, Brand A and B, 96% (v/v) and WHO-formulated products achieve the minimum standards with high efficacy against the test organisms. However, 65% ethanol (v/v) is less effective. The minimum amount of hand sanitiser doses is 3 mL of complete coverage on hand to ensure high inhibition percentages of microbes. Moreover, the WHO recommended that the application time requirement is 60 seconds.

Keywords: Hand sanitiser, ethanol, WHO, antimicrobial activity, log reduction

Introduction

The last documented outbreak of lethal disease, the Black Death, occurred in the Middle age from 1347 to 1351. It is the uttermost disastrous epidemic of humanity as ten million people, which is estimated to be half of the Europe population, have been erased because the mortality rate caused by the Bubonic plague with the causative agent's bacterium Yersinia pestisis exceptionally high (1). Infection droplets and contact with contagious body fluids transmit plague transmission. The recent outbreak of influenza A H1N1 emerged when the initial case was discovered in California, United States, in April of 2009. The influenza H1N1 spread swiftly across 70 countries in a short period of 2 months. The H1N1 strain is a new variation of the influenza virus, which mutates from RNA human flu strains and avian and swine strains. Based on the Centers for Disease Control and Prevention (CDC) data, the H1N1 affected cases range from 43 to 89 million people, with 12469 deaths in the United States, whereas approximately 151,700- 575,400 deaths worldwide (2). The transmission of H1N1 flu is from infected animals to humans, and the virus also spreads between humans as airborne droplets as infected patients cough or sneeze in the air. Influenza A H1N1 is highly contagious when someone touches the surface with a virus and contacts the mucous membrane, including the mouth, nose, and eyes will be infected (3).

In December 2019, a new virus outbreakwas detected in wet markets in Wuhan, China, and the Chinese government announced an epidemic alert in January 2020 (4). However, the infected patients spread the disease to other

provinces in China. They later spread globally as the patients travel despite having noticeable symptoms such as fever, dry cough and tiredness. By 6 February, 28276 confirmed cases had been reported, with 565 deaths globally, according to the data from WHO. The virus has been identified as a novel beta-coronavirus derived from the same family of viruses with severe acute respiratory syndrome (SARS) that occurred in 2003 as a viral respiratory disease (5).

It is necessary to follow health measures tightly to prevent exposure to coronavirus, which is highly contagious. The protective measures to decrease the risk of transmission include physical distancing because the respiratory droplets of a patient with a virus can infect a healthy individual when deposited on the mucous membrane. Besides, wearing a surgical mask reducesthe risk of airborne transmission and washing hands regularly with soap. Because the virus may survive on various surfaces for a specific time ranging from a few hours to days, known as hidden transmission. For instance, disinfectants with different mechanisms to destroy microorganisms act as antimicrobial agents (6).

According to the Centre for Disease Control and Prevention (CDC), when there is no access to water, washing steps can be replaced with rubbing alcohol-based hand sanitiser that has microbicidal activity with convenience because maintaining hand hygiene is essential to decrease infectious diseases. Hand sanitisers are widely used in healthcare systems and public areas as hygiene facilities to reduce the spread of diseases. However, the efficiency of hand sanitiser in the market against certain viruses is in doubt because different contents are formulated as there are several types of hand sanitisers such as gel, water and foam forms. Choosing the approved hand sanitisers is essential to ensure the safety and ability to kill harmful microorganisms. Besides that, the main active ingredients of hand sanitisers, like alcohol and benzalkonium chloride, contribute respective functions. The Food and Drug Administration (FDA) must approve formulation usage in terms of composition. The recommended percentage of alcohol ranges from 60% to 95% to denature the microorganism protein while avoiding skin irritation because of high alcohol content (7). The purpose of the research is to investigate the effectiveness of hand sanitiser actions in terms of the broadness of the spectrum by applying European Standard EN 1040 guidelines.

Materials and Methods

Test Organisms Working Culture Preparation: An in-vitro study was used to evaluate the effectiveness of several products,Hand sanitiser, oil-based on non-oil-based, WHO formula (Ethanol 80% (v/v), glycerol 1.45% (v/v), hydrogen peroxide 0.125% (v/v)), ethanol (96% and 65%) against three selected isolates (Escherichia coli, Staphylococcus aureus and Saccharomyces cerevisiae). The selected isolates used in this study were provided in Tryptic Soy Broth (TSB) and maintained at 37°C, 150rpm for 24 hours. After overnight culture, the densities of the 50mL culture suspension were compared with the McFarland standard and diluted with Phosphate-buffered Saline (PBS), if necessary, to obtain a final concentration equivalent to 0.5 McFarland standard (1.5 x 10^8CFU/mL). A 50mL culture suspension was precipitated through centrifugation at 800 x g for 10 minutes, and the pellet was washed with PBS, pH7,three times. The bacterial pellet was resuspended in 5 mL of PBS. After undergoing 15-sec mixing, cell density will be determined next. Enumeration was carried out using TSA at 37°C for 24 hours to verify the inoculum size further.

Suspension Validation

The working culture was diluted from 3.0 x 10^2 CFU/mL to 1.6 x 10^3 CFU/mL by using a diluent through serial dilution. The Tryptic Soy Agar (TSA) plates were incubated for 20 to 24 hours at 37°C, and the number of colonies that grew on the plates was counted. The TSA agar plates were further incubated for 20 to 24 hours

(40-48 hours), and the number of colonies was recounted. The plate with the higher number of colonies was used.

Suspension Test for Bactericidal Activity of Hand Sanitizer

5ml of validation suspension of the test organism was pipetted into a sterile beaker, and 5mL of treatment solution (Hand sanitiser, oil-based on non-oil-based, WHO formula, ethanol (96% and 65%) was added. The time was counted immediately, and the solutions were mixed by vortex briefly for 1 min (contact time) at room temperature. Then, 1mL of the solution was transferred onto the surface TSA agar plate after 1 minute in triplicate using the spread plate method. After 5 min contact time, 1mL of the solution was spread into a TSA agar plate in triplicate. All the TSA agar plates were incubated and observed after 20-24 hours.

The count limit for the viable microbial count in the test mixtures is 15 to 300, with a 10% deviation. Thus, the range is between 14 and 330. However, the ideal content may differ according to microbes' morphological characteristics (8). In this experiment, the recommended range is between 0 and 330 because the hand sanitizers successfully inhibit the growth of tested microbes, as there is no bacterial colony growth after incubation. To conclude, the colony-forming unit result from statistical analyses using a common logarithm. The log reduction factor (rf) represents the percentage of bacterial reduction by hand sanitizers where the formula is applied $\log_{10}$ (A) - $\log_{10}$ (B), and (A-B)/A% determines the percentage of reduction. A represents the log reduction pre-treatment of hand sanitiser, and B represents the number of microbial cells post-treatment. A higher log reduction value shows the higher bactericidal activity of the products to decrease the pathogenic microbes on the hands. The minimum requirement for hygienic products must induce a log reduction for each hand sanitiser product more than the mean $\log_{10}$ reductions of > 2.00 through the inactivation of microbes (9).

Results and Discussion

The antimicrobial efficacy of the selected hand sanitiser was evaluated against gram-positive, gram-negative and fungus. Two commercial hand sanitizers were included in this study, name Brand A (oil-based with ingredients: Denatured Alcohol, aqua, propylene glycol, glycerin, fragrance, Aloe barbadensis leaf juice, niacinamide, calcium pantothenate, sodium ascorbyl phosphate, pyridoxine HCl, tocopheryl acetate, acrylates/C10-30Alkyl acrylate crosspolymer, methyl gluceth-20, aminomethyl propanol) and Brand B (non-oil based with ingredient: Ethanol, aqua, glycerin, C12-15 pareth-12, fragrance, chlorhexidine digluconate). Oil or non-oil-based labels depend on their thickening and drying status. Oil-based hand sanitisers in this experiment are slightly greasy compared to non-oil-based hand sanitisers, which are greasiness.

Due to a shortage of hand sanitiser, the WHO recommended a formulation for healthcare products in a sterile manufacturing environment. WHO claimed that the provided alcohol-based formulation is a broad spectrum of pathogenic microbes with a low risk of developing antimicrobial resistance. The active ingredients contained in WHO formulations are ethanol or isopropyl alcohol. A range of alcohol concentrations from 60% to 95% is needed to inhibit the microbes through bactericidal activity. Based on the formulation given in (Table 1), ethanol 96% diluted from pure ethanol is evaluated to ensure the alcohol concentration using an alcoholmeter. The final concentration of ethanol in WHO hand sanitiser is 80% (v/v), recommended by WHO to inhibit pathogens.

A high concentration of alcohol enables broad-spectrum efficacy against vegetative cells such as bacteria, fungi and selectively viruses. The mechanism of alcohol as an active ingredient with a broad spectrum of antimicrobial functions against vegetative bacteria through cell membrane disruption or damage in metabolic pathways of the microbes, thus leading to cell death (10). Adding water to the WHO formulations enhances the protein denaturation

Table 1: The alcohol-based formulations provided by WHO (WHO 2009)

Component	Formulation (mL for 1L)	
	Ethanol	Isopropyl Alcohol
Ethanol 96%	833.3	-
Isopropyl Alcohol 91%	-	824.2
Hydrogen Peroxide 3%	41.7	41.7
Glycerol 98%	14.5	7.5

of the microbe. Therefore, the increased effectiveness of bactericidal activity compared to pure alcohol without any percentage of water (7).

However, alcohol with potent antimicrobial towards vegetative bacteria but not effective towards spore-form bacteria usually causes foodborne diseases. Spore-forming bacteria such as Bacillus cereus and Clostridium botulinum have high adaptability towards disinfectants as bacterial spores can withstand high temperatures and resist environmental pH changes (11). Hydrogen peroxide can decrease the formation of the bacterial spore by removing the protein that the structure spore coats; therefore, the dormant bacterial spore is vulnerable to environmental factors. However, a high concentration of hydrogen peroxide is corrosive to the skin and highly irritates when in contact with the eyes (12). Therefore, the final concentration of hydrogen peroxide is determined by titrimetric as a quantitative analysis where an oxidation-reduction reaction takes place.

This experiment used two types of hand sanitiser: an oil-based and non-oil-based variety of alcohol-based hand sanitisers (ABHS). The portable design of both hand sanitisers increases the frequency of usage among the community and healthcare workers. The texture of oil-based hand rub is thicker, requiring more time to dry than the non-oil-based type. A higher volume is needed to increase antimicrobial activity to ensure complete coverage on the hands when applying the hand sanitizer as complete disinfection. The runny consistency of the liquid type affects healthcare workers during rushed shifts because it requires a longer application time. Therefore, workers tend to apply less volume to speed up the drying process. The drawback of a non-oil-based hand rub includes the excess liquid solution that causes dripping to the ground during sanitization practice. The disadvantages of ABHS tend to leave skin in dry conditions with adverse effects. Therefore commercial products will add humectant to retain moisture on the skin (13).

Evaluation of five products against *Staphylococcus aureus*, *Saccharomyces cerevisiae* and *Escherichia coli*

There are five products, including commercial hand sanitizers (Brand A and Brand B), WHO-formulated hand sanitizer, ethanol(96% and 65%, v/v) and control that wereenumerated against Staphylococcus aureus by using the spread plate method to test the efficacy of hand sanitizer samples which are alcohol-based hand sanitizers. The results obtained and recorded after 48 hours of incubation for 1 min and 5 min, respectively, are used to evaluate the efficacy effects. It is because the growth of gram-negative microorganisms is maximized from 24 hours to 48 hours. The viable count on the TSA plates is counted in total colony-forming units where the TSA plates are incubated under conditions at 37 °C. According to the Association Advancement of Medical Instrumentation, the cell culture yields better assay at 37 °C compared to lower incubation temperature and more than 48 hours to prevent other microbes' recovery (14). According to the European Standard EN 1040, the results of viable count after 48 hours of incubation are applied instead of 24 hours because longer incubation time maximises the cell growth of test bacteria. However, the TSA plates are more condensed and less countable after 48 hours because they are too much to count (15). The results show 24 hoursof incubation of TSA agar plates with lower viable cell count compared to a further 48 hoursof incubation time at 37 °C (Table 2 and Table 3).

Table 2: Logarithm Reduction: TSA plates enumerated with test organisms treated with various tested products

Products	Contact times (minute)	Number of Colony (CFU/mL)		
		S. aureus	*S. cerevisiae*	*E. coli*
Control	1	3.3×10^5	3.3×10^5	3.3×10^5
	5	3.3×10^5	3.3×10^5	3.3×10^5
WHO formulation	1	1.5×10^5	2.7×10^4	<30
	5	2.3×10^4	1.6×10^3	<1
Ethanol 65%	1	2.4×10^5	1.7×10^5	1.0×10^2
	5	0.7×10^5	3.1×10^4	<1
Ethanol 96%	1	1.9×10^5	2.9×10^4	<1
	5	3.1×10^4	1.8×10^3	<1
Brand A (oil-based)	1	3.2×10^4	4.0×10^2	<1
	5	1.3×10^2	<1	<1
Brand B (none-oil-based)	1	<1	2.7×10^2	<1
	5	<1	<1	<1

Table 3: Percentage Reduction: TSA plates enumerated with test organisms treated with various tested products

Products	Contact times (minute)	Percentage Reduction (%)		
		S. aureus	*S. cerevisiae*	*E. coli*
Control	1	NA	NA	NA
	5	NA	NA	NA
WHO formulation	1	54.54	91.81	99.99
	5	93.03	99.51	99.99
Ethanol 65%	1	27.27	48.48	99.96
	5	78.78	90.60	99.99
Ethanol 96%	1	42.43	91.21	99.99
	5	90.61	99.45	99.99
Brand A (oil-based)	1	99.20	99.87	99.99
	5	99.96	99.99	99.99
Brand B (none-oil-based)	1	99.99	99.91	99.99
	5	99.99	99.99	99.99

Based on the result of TSA agar after being treated with WHO-formulation hand sanitizer and ethanol (96% and 65%, v/v) against S. aureus does not show potential to inhibit bacterial growth. After 48 hours of incubation, the viable count result with 1 minute contact time for these three products shows a high microbial count. The control count is 3.3 x 10^5 CFU/mL, where the 96% and 65% ethanol is 1.9 x 10^5 CFU/mL and 2.4 x 10^5 CFU/mL, respectively, and the WHO formulation is 1.5 x 10^5 CFU/mL (Table 2). To improve the efficacy of both hand sanitizers, the contact time should be increased from 1 to 5 minutes or through formulation modification. For instance, the ethanol concentration of the WHO formulation rises by 5% to increase the antimicrobial activity of the hand rub (16). By decreasing the glycerol concentration in WHO formulation from 1.45% (v/v) to 0.50%− 0.73% (v/v), able to improve bactericidal effects as well as speed up the drying process as stated that glycerol reduces the efficacy of ABHS and by replacing natural humectant such as Aloe vera extract and ethylhexylglycerin which able to retain the antimicrobial function (17). In 5 minutes of contact time, both WHO-formulation hand sanitizer and ethanol (96% v/v) show the abilityto inhibit the growth of *S. aureus*. According to CDC, hand sanitizer with a higher alcohol concentration of 80% to 85% (v/v) can decrease the contact time with the same efficacy (17). The post-treatment log reduction factor of WHO formulation and ethanol (96% v/v) compared to pre-treatment control is $\log_{10}$ 1 reduction, indicating approximately 90% reduction of *S. aureus* (Table 3).

In 1 minute contact time, Brand A with 3.2 x 10^4 CFU/mLwith 1 log reduction equalsa 90% reduction of S. aureus. Brand B is the most effective way to decrease the S. aureus among the tested sanitizers because there is no bacterial count (<1) on the TSA agar surface after 48 hours of incubation. The microbial decrease shows a 5 log reduction, which indicates a 99.999% reduction against S. aureus. In 5 minutes contact time, the efficacy of Brand A is greater than 1 minute contact time, which is 99.96% compared to 90.30%, respectively. A longer application time of hand sanitizer will ensure complete coverage of the hands. However, shorter application time in real-lifesituationsencourages people to apply frequently, thus increasingthe compliance rate to decrease the risk of disease transmission (18).

Based on the results in 1 minute contact time, the post-treatment of ethanol (96% and 65%, v/v) against S. cerevisiae shows 2.9 x 10^4CFU/mL and 1.7 x 10^5 CFU/mL, respectively (Table 2). These findingsindicateethanol's poor sanitising effects because the reduction percentage is less than 50% (Table 3), and log reduction equals 0. The log reduction factor is used to evaluate the hand sanitizers' efficacy, food safety evaluation and water purity by summarising the final results in the statistical method. However, by applying the analysis method, the log reduction method may miscalculate efficacy by over or underperforming hand sanitisers. It is because analysis based on the arithmetic mean concentration analysis is more accurate than the log reduction factor. The log reduction of ethanol may cause by a false interpretation that underestimated the efficacy of inhibiting S. cerevisiae (19).

However, Brand A and B inhibitedS. cerevisiae within 1 minute of contact time with a log reduction of 3. Thus, it is indicated that 99.9% of effectiveness reached the minimum requirements, similar to Brand A and B'sclaim to kill 99.9% of microbes (Table 3). It is recommended that antiseptic hand wash clean hands with dirt rather than ABHS because hand washing is effective for manymicroorganisms. It may be because microbes would recover as time passed by, as alcohol is not capable of permanently inhibiting some residual microbes.For instance, ABHS is less effective towardspore-forming bacteria such as Clostridium difficile and ABHS. Based on the results in 5 minutes of contact time, the post-treatment ethanol (65% v/v) shows no log reduction. According to WHO Guideline for Hand Sanitizer (2009), research on a few types of hand rubs, such as alcohol alone, a combination of alcohols has higher efficacy than alcohol alone. The concentration of ethanol as treatment in the

experiment is 96% v/v which is not recommended as not in the range of 60% to 95%. Too high a concentration is not applicable as low-level water cannot—the 85% v/v ethanol with 5 log reduction factors after 15 seconds application time.

In addition, in the WHO formulation, ethanol (96% v/v), Brand A and B can decrease S. cerevisiae by at least 2 log reductions in 5 minutes of contact time. In contrast, Brand B is most effective as it shows 3 log reductions in 1 minute contact time (Table 3). The commercial hand rub producers recommended different application volumes (20). The research concludes that no statistically significant difference exists between the application volume and full hand coverage. The result shows that a 1 mL dose can cover 92.9 %, a 2 mL dose covers 98.3%, and a 3 mL dose can cover 99% of the hand surface, thus increasingthe bactericidal effect. Therefore, 3 mL is recommended for complete hand coverage, but 3 ml requiresan extra 30 s for drying. However, more than 3 ml dose is less effective because the excess liquid will cause spillage (20). The ethanol does not show inhibition against fungal S. cerevisiae because it only contains alcohol without other active ingredients. The three alcohol-based hand sanitisers (ABHS) have a high percentage of ethanol with chlorhexidine digluconate, hydrogen peroxide and moisturizer. According to the Australian guideline review, adding 0.5% chlorhexidine and skin moisturizers such as glycerol and Aloe vera extract in ABHS can increase hand sanitiser's efficacy (21).

Based on the 1 and 5 minutes of contact time, the post-treatment of ethanol (96% and 65%, v/v), Brand Aand B against E. coli show excellent antimicrobial effects because there are five logarithm reductions, respectively,indicating 99.99% effectiveness towards E. coli (Table 3). The humectant used in oil-based Brand A hand sanitiser is Aloe barbadensis leaf juice as an inactivated ingredient that increases efficacy because aloe vera contains bioactive compounds such as salicylates and β-sitosterol (22). Aloe vera is an effective antimicrobial agent against S. aureus, E. coli, Streptococcus pyogenes and Klebsiella pneumoniae because Aloe vera contains saponin and anthraquinones that activate phagocytic leucocytes to kill microbe by the anti-inflammatory reaction. Gram-negative bacteria have lipopolysaccharide (LPS), whereas gram-positive do not. The bactericidal activity of Aloe vera is more effective against gram-positive bacteria (23). E. coli is susceptible to most hand sanitisers except WHO formulation, where no log reduction is shown in 1 minute of contact time compared to other microbes. Most hand sanitisers have high inhibition and antibacterial effects on E. coli compared to other test hand sanitisers because of strain-to-strain variation in hand sanitiser efficacy against different microbes. Thus, E. coli is rapidly more susceptible to all hand sanitiser in 1 minute, except for WHO-formulated hand sanitiser (24).

Brand B's non-oil-based hand sanitisers performed the most effective antimicrobial among the five samples. The efficacy analysis is determined using 1 minute application time because it indicates the real-world application time. Non-oil-based hand sanitiser is suitable for preventing transmission of infectious diseases in a public area because alcohol will denature most bacteria with at least 99.9% effectiveness, ensuring less residual leave and causing mutation (25). The high efficacy of Brand B is because of formulations that combine ingredients with active ingredients and additives (26). It also shows a higher rate of inhibition kinetics against three test organisms. Ethanol (96% and 65%, v/v) alone without adding other ingredients shows less efficacy in S. aureus and S. cerevisiae. It is because ethanol is tested independently without combining other active ingredients or humectants. WHO-formulation hand sanitiser with a final concentration of 80% lacks antimicrobial activity against three test microbes compared to commercial hand sanitisers. The products with a 70% final ethanol concentration may be more effective than high concentrations because they contain a minimum percentage of water in the hand rub (27). The efficacy increases with the exposure time towards hand sanitisers, as 5 minutes of contact time can inhibit more microbes than 1

minute. However, effective hand sanitisers such as Brand A and B can inhibit shorter exposure time, indicating a more effective daily life. Based on thereduction percentage (Table 2), Brand A and B can inhibit the microbe's growth in 1 minute contact time.

In contrast, othersamplesdo not effectively kill the microbe in 1 minute contact time. Ethanol (96% and 65%, v/v) and WHO formulation areless effective when testing with a 1-minute contact time (Table 2 and Table 3). For instance, post-treatment of WHO formulation against S. aureus shows no logarithm reduction in 1 minute contact time and can onlyyield 2 log reductions in 5 minutes. The results indicate that ethanol and WHO formulation required a longer contact time to kill pathogenic microbes effectively.

WHO recommended 60 seconds of application time to ensure effectiveness while protecting the skin barrier from dehydration. If the application of ethanol (96% and 65%, v/v) and WHO formulation require longer than 60 seconds, the lengthy hand hygienic step could be more practical, thus reducing the usage frequency (28). The manufacturer did not state the actual concentration used in the ingredient list despite the high efficacy of Brand B hand sanitiser with chlorhexidine digluconate as an active ingredient with a combination of ethanol. The concentration ranges from 0.5% to 4%, inhibiting gram-positive, gram-negative bacteria and selective fungi with a broad spectrum of antimicrobial activity. A1% combination of chlorhexidinedigluconate and ethanol can inhibit microbes in less than 45 seconds.

Conclusion

In conclusion, based on these findings, it is recommended to apply hand sanitisers with sufficient doses and rub at least 60 seconds to ensure proper sanitation. According to the evaluation of antiseptics, alcohol-based hand sanitisers with ethanol of at least 75% have reached the minimum requirement. The broad spectrum of alcohol-based hand sanitiser with active ingredients can effectively inhibit gram-positive and gram-negative bacteria. This experiment applies the spread plate technique to the observed colony-forming unit on TSA plates post-treatment with hand sanitisers. The study showedthat non-oil-based Brand B has the highest efficacy against test microorganisms as it can inhibit microorganism growth on TSA plates with the highest average log reduction factor.

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Conflict of Interest

The authors declare that they have no conflict of interest in the publication.

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