# COMPARATIVE TEST OF EFFECTIVENESS OF ALCOHOL MIXTURE-BASED AND CHLORHEXIDINE MIXTURE-BASED HAND-SANITIZERS ON *ESCHERICHIA COLI* AND *SHIGELLA SONNEI*

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## ABSTRACT

Hand sanitizer is a hand cleanser which is easy and practical in use. Using hand sanitizer can be an alternative for washing hands if in condition without water and soap. Hand sanitizer is used to clean pathogens on hands. Hands are the source of various diseases, therefore it's important to maintain hand hygiene. The ability to kill pathogens in hand sanitizers is due to the active substance content in them. In this research, the effectiveness of 2 hand sanitizers on the market was tested. Hand sanitizer 1 has a composition consisting of 70% ethyl alcohol and 0.5% chlorhexidine. Hand sanitizer 2 has a composition consisting of 55% ethyl alcohol and 18% isopropyl alcohol with a total of 73% alcohol. This study aims to see the effect of the active substances in each preparation on the effectiveness of killing pathogens. There are two bacteria observed in this study, i.e. *Escherichia coli* and *Shigella sonnei*. The method used was Kirby Bauer with the aim of looking at the bacterial barrier zone on the disk. Based on observations, it was found that hand sanitizer 1 was more effective than hand sanitizer 2. The chlorhexidine content in hand sanitizer 1 increased its effectiveness in killing pathogens.

**Keywords:** Effectiveness; Hand sanitizer; Kirby bauer.

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## INTRODUCTION

An antimicrobial agent is defined as a natural or synthetic substance that kills or inhibits the growth of microorganisms such as bacteria, fungi and algae to prevent infection. (S.C. Burnett, 2011). Antiseptics can be distinguished from disinfectants, while antiseptics are used on living organisms, disinfectants are used on inanimate objects. One example of an antiseptic is hand sanitizer.

Hand sanitizers are popularly used to reduce pathogens on the hands as they are practical, easy to carry, and easy to use in various situations. Therefore, hand sanitizers are often used in people's lives and science.

The active ingredients of hand sanitizers may vary and can be combined, in this study two branded hand sanitizers were tested with ingredients containing isopropyl alcohol, ethyl alcohol, and chlorhexidine.

Alcohol-based hand sanitizers usually contain the active ingredients ethanol, isopropanol, n-propanol or a mixture thereof. Alcohols can denature and agglomerate proteins in the lipid membrane layer of microbes. In addition, ethanol kills bacteria by denaturing proteins and membranes resulting in disruptions in metabolism and cell lysis. The OH-group in ethanol binds to microbial proteins and damages protein structure and function, resulting in enzyme inhibition and protein deposition (Setiawan, 2022).

Chlorhexidine is one type of antiseptic. Chlorhexidine is a bactericidal that works by damaging the cell wall and outer membrane of the cell, resulting in intracellular leakage, and ultimately cytosolic coagulation (Komang, 2019).

## MATERIALS AND METHODS

### a. Material

Tools used in this test include sterile petri dish, screw tube, flask, measuring pipette, drip pipette, measuring cup, micropipette, disc paper (Oxoid), caliper/ruler, ose needle, tweezers, markers, label paper, vortex mixer, spreader, incubator, autoclave, oven, refrigerator, petri dish, colony counting device, and analytical balance.

The materials used in this test are samples of two kinds of samples of hand sanitizer 1 and 2, *Escherichia coli* ATCC 25922 and *Shigella sonnei* in 24-hour NB media (HiMedia), Mueller Hinton Agar (MHA) media (HiMedia),and sterile sterile water (Merck) as test compound solvents.

### b. Method

1. Aseptic techniques are performed on hands and workbenches in a one-way way to the right or left using 70% alcohol and tissue.
2. The spirit fire was ignited and then placed in the center.
3. Mueller Hinton Agar media prepared and marked with a label.
4. The surface of the media is zoned using a permanent marker, petri dish 1 with 3 zones (blank, hand sanitizer 1, and hand sanitizer 2), petri dish2 and petri dish 3 with 2 zones (hand sanitizer 1 and hand sanitizer 2).
5. A threaded tube containing a pure culture of bacteria was taken using the left hand.
6. A little finger used to open the lid of the threaded tube (the cap of the threaded tube remains held as it was originally).
7. Burned the mouth of the screw tube, then took the bacteria with sterile cotton swab media using thumb and index finger.
8. Re-burned the mouth of the threaded tube and close the screw tube back.
9. Inoculated the *Escherichia coli* and *Shigella sonnei* on MHAdishes by streak plate.
10. The sterile disc paper that has been dipped in the hand sanitizer solution is removed. After removal, the remaining drops of excessive solution on the disc paper are applied to the walls of the container because it is feared that the solution will expand on the surface if the solution is too much.
11. Paper discs laid on the surface of the agar with tweezers. Press with tweezers so that the disc paper actually sticks to the agar. Three times replication is performed.
12. Incubation was carried out for 48 hours at a temperature of 37ºC.
13. The inhibitory zone formed is measured in diameter, compared to the working power of various hand sanitizers.

## RESULTS

**Table 1.** Observations

| Petri dish 1 | Petri dish 2 | Petri dish 3 | Mean ± SD |
|---|---|---|---|
| ***Escherichia coli*** | | | |
| <br><br>H1: 6,85 mm<br>H2: 1,45 mm | <br><br>H1: 6,87 mm<br>H2: 2,57 mm | <br><br>H1: 5,90 mm<br>H2: 1,43 mm | 2 mm ±2,41 |
| ***Shigella*** | | | |
| <br><br>H1: 9,40 mm<br>H2: 1,01 mm | <br><br>H1: 7,00 mm<br>H2: 1,64 mm | <br><br>H1: 4,56 mm<br>H2: 1,13 mm | 3 mm ±3,19 |

Descriptions:
H1 = Hand sanitizer 1 (ethyl alcohol 70%and chlorhexidine 0,5%)
H2 = Hand sanitizer 2 (Ethyl alcohol 55%and isopropyl alcohol 18%)

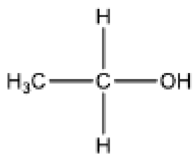
## DISCUSSION

Hand sanitizers that contain alcohol levels between 60 and 95% are antiseptic substances. According to the Food and Drug Supervisory Agency (FDA) (Radji, 2007), hand sanitizer has the ability to remove germs in less than 30 seconds. The alcohol contained in hand sanitizers has bactericidal properties which are effective against gram-positive and gram-negative bacteria. Apart from alcohol, hand sanitizers also contain antibacterial ingredients such as triclosan or other antimicrobial agents which can inhibit the growth of bacteria such as *Escherichia coli* and *Staphylococcus aureus* (Radji, 2007). The advantage of hand sanitizers lies in their ability to kill germs quickly, thanks to the content of alcohol compounds such as ethanol, propanol and isopropanol with concentrations of around 60% to 80%, as well as phenol group substances such as chlorhexidine and triclosan (Ambari, et al. 2020).

In these studies, tests were carried out on 2 hand sanitizers with different brands, namely hand sanitizer 1 and hand sanitizer 2). Hand sanitizer 1 has a composition consisting of 70% ethyl alcohol and 0.5% chlorhexidine. Meanwhile, hand sanitizer 2 has a composition consisting of 55% Ethyl alcohol and 18% isopropyl alcohol with a total of 73% alcohol.

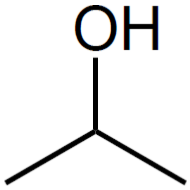

Source : Cattaneo, et al., 2016

**Figure 1**. Chlorhexidine chemical structure.




Source : Antonius, et al., 2021

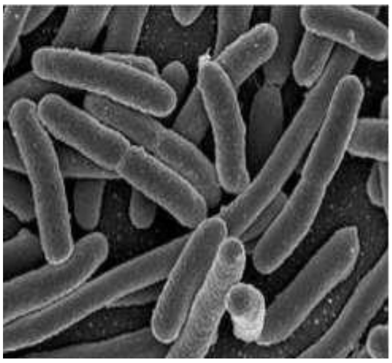
**Figure 2**. Ethyl alcohol chemical structure.




Source : Monument Chemical, 2021

**Figure 3**. Isopropyl alcohol chemical structure.

***1. Escherichia coli***



Source : Chandana, 2015

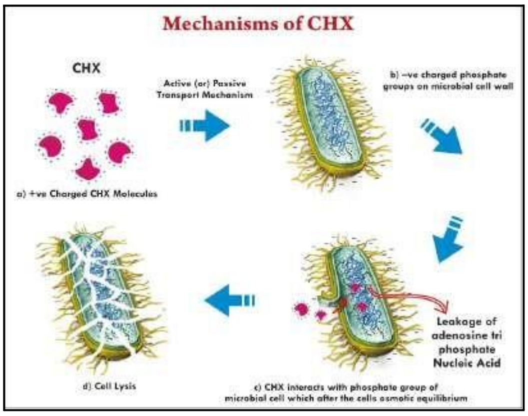
**Figure 4**. *Escherichia coli*.

*Escherichia coli* is a gram-negative bacterium that is facultative anaerobic. This bacteria belongs to the Coliform group or can ferment lactose by producing acid and gas within 48 hours at a temperature of 35℃ (Badjoeri, 2007). *E. coli* bacteria belong to the Enterobacter family which have a short rod-like shape (Jawetz, et al., 2007). This bacterium has flagella and pili extending from the surface of its body (Budiyanto, 2002). Under normal circumstances, *E. coli* bacteria are included in the normal flora that live in the human intestine (Karsinah, et al., 2011). However, in certain conditions such as digestive disorders and immunosuppression, these bacteria can be pathogenic (Kartikasari, et al., 2019). *E. coli* bacteria can cause several diseases such as diarrhea, stomach cramps, vomiting and fever (Sinaga, 2017; Elfidasari, 2011).

In these studies, a hand sanitizer containing the active ingredients Chlorhexidine and Alcohol is used. Chlorhexidine is a bactericide that works by damaging the cell wall and outer cell membrane, resulting in intracellular leakage, and ultimately coagulation of the cytosol. (Al-adham et al, 2013) Alcohol is another ingredient in the antiseptic in the hand sanitizer used. Alcohol acts as a bactericidal, by damaging the cell membrane of bacteria, so that intracellular components come out. Alcohol also works by denaturing proteins in cells, so that the performance of bacterial enzymes will be hampered, resulting in metabolic processes being disrupted (Komang, 2019).

Alcohol acts as a bactericidal by denaturation and coagulation proteins. Protein denaturation and coagulation will damage enzymes so that microbes cannot fulfill their living needs and ultimately their activity stops (Purwatiningsih S, 2015). The action of alcohol as a bactericidal can be achieved if the alcohol content used is 60% -95%. If the alcohol content is less than 60%, it is not effective in killing bacteria (Srikartika, Suharti and Anas, 2016). If you use an alcohol concentration that is too high, namely more than 95%, it is also not good because your hands will become dry, thereby reducing the ability of the hand sanitizer to denature proteins because the protein denaturation process requires water (Situmeang and Sembiring, 2019). Our samples use 70% and 73% alcohol so the role of alcohol in both samples is bactericidal.

The chlorhexidine content in hand sanitizer influences the effectiveness of the hand sanitizer's performance. The mechanism by which chlorhexidine inhibits microbial growth is the interaction between the positive charge of chlorhexidine and the negatively charged microbial cell walls. This interaction can increase the permeability of the microbial cell wall which can cause the cell membrane to tear, leak the cytoplasm, and ultimately cause death (Nuryani, 2017). The addition of chlorhexidine will affect the quality of the inhibition zone.




Source : Ilango et al., 2013

**Figure 5**. Mechanism of action of chlorhexidine

The inhibition zone is the area around the disc where no growth of *Escherichia coli* bacteria is found or it can also be called the clear zone on the media. The zone of inhibition can be measured using a caliper. Inhibition zones can form because they contain antibacterial or antimicrobial test materials. According to research by Oktaviani et al., (2019), inhibition zones can form because they have antimicrobial activity caused by materials containing secondary metabolites such as materials containing flavonoids, phenolics and terpenoids. According to Putri et al., (2016) the differences between the antimicrobial inhibitory power of bacteria can be influenced by the antimicrobial mechanism of action, concentration, bacterial cell walls and the peptidoglycan layer that makes up the bacteria. *Escherichia coli* bacteria are included in the group of gram-negative bacteria which have a cell wall structure consisting of 3 layers, namely, the first layer is lipoprotein, the second layer is lipopolysaccharide and phospholipid, the outer layer is thin peptidoglycan (Amalia et al., 2014). The average diameter of the inhibition zone can be grouped based on the resistance category. Categories that have antimicrobial inhibition zone activity can be divided into four, namely: weak activity has an inhibition zone < 5 mm, medium activity has an inhibition zone measuring 5-10 mm, and very strong activity has an inhibition zone > 20-30 mm (Davis and Stout, 1971).

Based on observation data, the results of the hand sanitizer effectiveness test on *Escherichia coli* bacteria are:

$$\text{Hand sanitizer 1} = \frac{6{,}85 + 6{,}87 + 5{,}90}{3} = 6{,}54 \text{ mm} \pm 0{,}55$$

$$\text{Hand sanitizer 2} = \frac{1{,}45 + 2{,}57 + 1{,}43}{3} = 1{,}81 \text{ mm} \pm 0{,}65$$

Based on average data, the results of the *Escherichia coli* bacteria inhibition test on hand sanitizers 1 and 2 were 6.54 mm and 1.81 mm respectively. Thus, hand sanitizer 1 is included in the moderate inhibition zone category, while hand sanitizer 2 is included in the weak inhibition zone category. Hand sanitizer 1 is more effective than hand sanitizer 2. The difference in compound content in each hand sanitizer will affect the effectiveness of the inhibition. When compared with the blank, the blank showed no inhibition at all. This is because the blank does not contain compounds that can inhibit bacterial growth.

Hand sanitizer 1 contains 70% ethyl alcohol and 0.5% chlorhexidine. Hand sanitizer 2 contains 55% Ethyl alcohol and 18% isopropyl alcohol with a total of 73% alcohol. Based on the content, there is a difference in the presence/absence of the 0.5% chlorhexidine compound. These differences will affect the performance of each hand sanitizer.

*Escherichia coli* bacteria are included in gram-negative bacteria, where the mechanism of resistance of gram-negative bacteria to antiseptics and disinfectants is the presence of an outer membrane which acts as a barrier that limits the entry of various types of chemically unrelated antibacterial agents (Mcdonnell and Russell, 1999). Disinfection levels are not achieved due to the development of biofilm which is difficult to remove by the sanitation process because it adheres strongly to various surfaces and consists of organic materials such as exopolysaccharides and proteins (Osland et al, 2020). Biofilm is a micro environment in the form of microorganism cells that are attached and do not easily come off on a surface. The purpose of forming a biofilm by a microbe is as a form of defense against environmental factors that are less favorable for bacterial growth. With this biofilm, the time required for a compound to disinfect bacteria becomes longer. This is because the compound carries out the process of influencing bacterial cells in stages, namely from the outermost layer to the inner layer of the aggregate (Retnowati and Dama, 2009).

One of the distinctive characteristics of biofilms is the presence of an extracellular matrix that protects bacteria or plays a protective role as a diffusion barrier and absorbs toxic molecules such as antimicrobials, hydroxyl radicals and superoxide anions. This aspect of the extracellular matrix contributes to the development of phenotypic resistance in *E. coli* biofilms. Some of the exopolysaccharides found in this matrix are cellulose, PGA, and cholanic acid which play an important role in biofilm formation. PGA or b-1,6-N-acetylglucosamine (b-1,6-GlcNAc) functions to stabilize E. coli. Cholanic acid functions to form a protective capsule around bacterial cells under growth conditions. Meanwhile, cellulose functions to spread the formed biofilm. Apart from the extracellular matrix, cell surface polysaccharides also play an important role in the phenotype of the biofilm. Cell surface polysaccharides consist of lipopolysaccharide which is the main component of the outer layer of gram-negative bacteria and the capsule which acts to protect the cell (Beloin et al, 2008).

### 2. *Shigella sonnei*

Source : Judaibi, A., 2014

**Figure 6**. *Shigella sonnei*.

*Shigella* is a bacterium of the Enterobacteriaceae family that is a pathogenic bacterium that causes diseases of the gastrointestinal tract in animals and humans. *Shigella* bacteria are transmitted through contamination of food or water. Classically, *Shigella* arises with symptoms of abdominal pain, fever, and slimy stools (Suyana et al., 2015). *Shigella* is a gram-negative bacteria and rod-shaped, facultative anaerobic but most often grows aerobically, non-spore, grows optimally at 37 ° C and pH 7.4 (Lucchini, et al., 2005).

*Shigella* is an acid-resistant bacterium that can pass through stomach acid and reach the intestine, then multiply in the cell and push the bacterial body through the cell cytoplasm and invade adjacent cells. When bacteria begin to enter the cell, enterocytes will be phagocytosed by macrophages, but *Shigella* can induce macrophages to occur apoptosis (Jorgensen James H, et al, 2015).

The mechanism of action of hand sanitizers in general is by denaturering and coagulating cell proteins (Asngad &; Bagas, 2018). In addition, compounds contained in hand sanitizers such as triclosan also play a role by inhibiting lipid biosynthesis by binding to the enzyme enoyl-acyl carrier protein reductase (ENR) which prevents the synthesis of fatty acids necessary for lipid production in microbes. Optimum triclosan is used as an antiseptic at a concentration of 0.2-0.3 % (Weatherly, et al., 2017).

No specific studies have been found that explain the mechanism of hand sanitizers against *Shigella* bacteria. However, there are several factors that can be a reference to several mechanisms of action of hand sanitizers against these bacteria, such as denaturing the cell wall and coagulating proteins in the cytoplasm in *Shigella* bacteria.

The microbial inhibition zone is the area around the disc where there is no bacterial growth found on the medium. The inhibitory zone usually measures the diameter of the area around the disc that is not overgrown by microbes. The larger the inhibition zone, the more effective the antiseptic and disinfectant will be.

Antibacterial activity is said to be weak if the diameter of the inhibitory zone is <5 mm, medium 5-10 mm, strong 10-20 mm, and very strong >20 mm (Emelda, et al., 2021).

According to Sudarwati (2016), there are several factors that can affect the diameter of the microbial inhibition zone, namely the type of bacteria used, the chemical properties of antibacterial substances, the sensitivity of each bacterium. In addition, according to some literature there is mention of the turbidity of the bacterial suspension, growing conditions, media thickness, and incubation time.

*Shigella* is a rod-shaped, single gram-negative bacterium, has no flagellum, aerobic or facultative aerobics and does not form spores (Aini, 2018). The growth of *shigella* can be inhibited by several chemical compounds that are usually present in antiseptics and disinfectants, namely alcohol (ethanol, isopropyl alcohol), chlorhexidine, triclosan, etc. In addition, there are also antiseptics that combine alcohol and chlorhexidine. The combination of alcohol and chlorhexidine results in higher reduction factor values compared to chlorhexidine or alcohol alone. The mechanism of chlorhexidine in inhibiting microbial growth, namely the interaction between the positive charge of chlorhexidine and the negatively charged microbial cell wall. This interaction can increase the permeability of microbial cell walls which can cause cell membranes to tear, leak in the cytoplasm, and eventually cause death (Nuryani, 2017). Meanwhile, the mechanism of alcohol in inhibiting microbial growth, namely by denaturation of microbial membrane proteins (Wahyuni, 2017).

In the results of the *Shigella sonnei* bacterial inhibition zone test, our group obtained that the bottom result of hand sanitizer 1 has a wider inhibitory zone compared to blank and hand sanitizer 2 on the three cups as in **Table 1.** Based on observational data, the results of the hand sanitizer effectiveness test on *Shigella sonnei* bacteria are:

$$\text{Hand sanitizer 1} = \frac{9{,}40 + 7{,}00 + 4{,}56}{3} = 6{,}99 \text{ mm} \pm 2{,}42$$

$$\text{Hand sanitizer 2} = \frac{1{,}01 + 1{,}64 + 1{,}13}{3} = 1{,}26 \text{ mm} \pm 0{,}33$$

In the stamp test, of course, it does not have an inhibitory zone because there are no compounds that act as antiseptics to kill bacteria and the blank here is only as a negative control. Furthermore, hand sanitizer 2 obtained less effective results when compared to hand sanitizer 1. This is due to the different compositions contained in the two hand sanitizers. Hand sanitizer 1 contains 70% ethyl alcohol and 0.5% chlorhexidine while hand sanitizer 2 only contains 73% alcohol consisting of 55% ethyl alcohol and 18% isopropyl alcohol. With this difference in content, causing hand sanitizer 1 to be more effective. As mentioned above, the combination of the content in hand sanitizer with alcohol and chlorhexidine will certainly be much more effective than just using alcohol. Chlorhexidine can kill bacteria by increasing the permeability of microbial cell walls which can cause cell membranes to tear (Nuryani, 2017) while alcohol can only inhibit microbial growth by denaturing membrane proteins (Wahyuni, 2017). This is in accordance with the results of the studies we conducted where hand sanitizer 1 containing chlorhexidine is more effective than hand sanitizer 2.

*Shigella* has the ability to form biofilms. A biofilm is a structure consisting of colonies of bacteria bound in the extracellular matrix. Bacteria residing in biofilms are less sensitive to antimicrobial agents and more resistant to environmental stresses such as dehydration and oxidation. Biofilm formation processes involving multifactors, such as cellular entanglement, exopolysaccharide secretion, as well as gene regulation, allow *Shigella* to produce a biofilm layer that protects itself from the unfavorable external environment. (Ellafi, et al., 2011) investigated the formation of biofilms by *Shigella* strains cultured in various concentrations of NaCl, and they showed that all isolates produced biofilms.

According to their study, biofilm formation is a protective system under different environmental stress conditions.

Recent studies have shown that bile salts increase the capacity of *S. flexneri* strains to attach to and penetrate epithelial cells. Indeed, extended exposure to *Shigella* to bile salts occurs in the case of increased biofilm formation, and thus is an important resistance mechanism for *Shigella sp*. Similar biofilm phenotypes have been observed for Campylobacter, Listeria, and Vibrio, suggesting that bile salt-induced biofilm production is conserved among members of the Enterobacteriaceae family. In addition, biofilm formation has been shown to require the presence of glucose, while diffusion of biofilms requires elimination of bile salts from the medium. Bacteria in biofilm form can be 100,000 times more resistant to antimicrobial agents than planktonic forms of bacteria in the same species. During biofilm formation, effects of shf, mdoH, VpsT, and LuxR-like genes as well as OpgH protein expression have been confirmed among enteric bacteria, including *Shigella*.

Another study described the potential for biofilm formation and pathological behavior of various mutant strains of S. flexneri with nuclei in an incomplete LPS containing only Kdo fragments. Interestingly, mutant 1 rfaC (also called waaC), with an incomplete core in LPS due to deficiencies in Hep biosynthesis, showed strong biofilm formation capabilities as well as high enough aggressiveness and adhesion in human epithelial cells compared to mutant strains of LPS. However, this strategy succeeded in providing high levels of resistance only in bacterial species with deficiencies in Hep synthesis from LPS. The relationship between biofilm formation and pathogenicity, as well as virulence factors and antimicrobial properties, has not been thoroughly studied in *Shigella sp.* and further studies are needed. (Ranjbar, R & Farahani, A, 2019).

### 3. The effectiveness of hand sanitizer 1 compared to hand sanitizer 2 against both bacteria

As discussed in the previous paragraph, the effectiveness between hand sanitizer 1 and 2 can be compared to looking at the content in the hand sanitizer. In addition, the effectiveness can be compared against both bacteria. If the bacteria are gram-negative which has a lipopolysaccharide coating, it will cause the effectiveness of hand sanitizer 1 to decrease.

This is because the lipopolysaccharide layer can limit the cationic chlorhexidine molecule so that it will reduce its effectiveness. In addition, the outer membrane of gram-negative bacteria serves protection against cationic antibacterial agents such as chlorhexidine. It is possible that the negatively charged phosphorylated heptose and glucosamine groups of the gram-negative bacterial wall of LPS can lock on to stronger cationic chlorhexidine molecules, making them less effective at working (Sinaredi et al, 2014). Gram-negative bacteria have more peptidoglycan layers. Peptidoglycan compounds are polar and easily soluble in ethanol. This property makes it an antibacterial compound that is effective against gram-positive bacteria. This is because the thick peptidoglycan layer on the cell wall of gram-positive bacteria is polar and easily soluble in ethanol (Rini et al, 2018).

Based on the results of the lab that we got, it can be seen that the results of the inhibition zone on the two hand sanitizers in the two bacteria are not too much different. This is because the bacteria that our group got both included gram-negative bacteria. Therefore, it can be seen that the results are not too much different in the two hand sanitizers when compared to their effectiveness.

## CONCLUSION

In these studies, two hand sanitizers with different composition have been tested. Based on the results of bacterial inhibition tests, *Escherichia coli* and *Shigella sonnei* hand sanitizer 1 is more effective than hand sanitizer 2 because of the combination of alcohol and chlorhexidine content. Furthermore, hand sanitizer 1 is more effective against *Shigella sonnei* bacteria than *Escherichia coli*.

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