International Baccalaureate Biology

Extended Essay

The effect of different types of mouthwashes on bacterial growth

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ABSTRACT

The oral cavity is a favourable environment for the growth of bacteria. The use of machinal techniques such as brushing or flossing are lacking the potential for reaching deep gingival surfaces, therefore the use of mouthwashes is high in demand. This research aims to compare five different types of mouthwashes which contain different chemicals inside based on their antibacterial effect on the growth *of E. coli* measured through ZOI.

The research question of the investigation is: "Which alternative is the best between five different types of mouthwashes (Colgate, Parodontax, Listerine Advanced White, Listerine Total Care, and Listerine Cool Mint) as an antibacterial agent, in terms of preventing the growth of bacteria Escherichia coli measured through the zone of inhibition by the programme ImageJ?"

The method used to isolate bacteria in the investigation is the strake-plating method. 0.50 ± 0.03 McFarland *E. coli* was prepared with LB Broth and diluted 10^3 times. Petri dishes 6.00 ± 0.50 cm in diameter are seeded with 40.00 ± 0.01 μL LB Agar with bacteria solution and spread via a cell spreader. Later on, 10.00 ± 0.01 μL of mouthwash solution is added to every dish's centre. After incubation at 36.60 ± 0.01℃ for 24.00 ± 0.50 hours, the IZD created are measured through the computer programme "ImageJ".

The mean result of diameters is as follows: Colgate (1537.06 ± 0.50 mm), Parodontax (1132.233 ± 0.50 mm), Listerine Advanced White (748.241 ± 0.50 mm), Listerine Total Care (166.372 ± 0.50 mm) and Listerine Cool Mint (125.801 ± 0.50 mm). The conclusion is that Colgate is proven to be the most effective antibacterial mouthwash in contrast with Listerine Cool Mint, which is the least effective type.

LIST OF ABBREVIATIONS & TERMS

1. *E. coli*: *Escherichia coli*
2. BSL-1: Biosafety level 1
3. K-12: Name of the non-pathogenic strain of *E. coli*
4. ZOI: Zone of inhibition
5. LB: Lysogenia Broth/ Luria Broth
6. ATTC: American Type Culture Collection
7. IZD: Inhibition zone diameter
8. McFarland: A standard used in microbiology for adjusting the turbidity of a bacterial solution. ($1\ \text{McFarland} = 1.00 \times 10.00^{8} \text{cells/mL}$)
9. SD: Standard deviation
10. ANOVA: Analysis of Variance
11. NaF: Sodium fluoride
12. $ZnCl_2$: Zinc chloride
13. C_6H_5COOH: Benzoic Acid
14. $C_{10}H_{14}O$: Thymol
15. $C_{21}H_{38}ClN$: Cetylpyridinium chloride
16. F^-: Fluoride ion
17. Zn: Zinc
18. LHS: Left-hand side

1. INTRODUCTION

1.1 Research Question

Which alternative is the best between five different types of mouthwashes (Colgate, Parodontax, Listerine Advanced White, Listerine Total Care, and Listerine Cool Mint) as an antibacterial agent, by preventing the growth of bacteria *Escherichia coli* measured through the zone of inhibition by the programme ImageJ?

1.2 Background Information

The oral cavity is favourable for the growth of distinct types of microorganisms, such as bacteria.[1] Bacterial plaque can be described as a thin film of bacteria that forms on teeth.[2] It can be considered the most common issue in the destruction of teeth and periodontal tissues.[3] One of the most effective ways to minimize these oral microorganisms is to control the amount of plaque accumulation on the teeth and adjacent surfaces. Therefore, maintaining good oral hygiene should be considered through mechanical methods such as brushing and flossing. However, due to a lack of proper plaque control, there is a high occurrence of gingival inflammation with the use of mechanical methods, so the use of chemical methods such as toothpaste and mouthwash are highly preferred.

The antibacterial activity of a molecule is associated with the presence of compounds that kill bacteria or slow down their rate of growth, without being extremely toxic to the tissues nearby.[4] Studies have proven the effect of mouthwashes as an anti-plaque and anti-inflammatory agent which contributes to oral hygiene. Although it is not a replacement for daily brushing and

[1] Yousefimanesh, H., Amin, M., Robati, M., Goodarzi, H., & Otoufi, M. (2015). Comparison of the Antibacterial Properties of Three Mouthwashes Containing Chlorhexidine Against Oral Microbial Plaques: An in vitro Study. Jundishapur journal of microbiology, 8(2), e17341. https://doi.org/10.5812/jjm.17341

[2] (2022, November 6). Mouthwash - Mouthrinse | MouthHealthy - Oral Health Information from the ADA. Mouthhealthy. https://www.mouthhealthy.org/all-topics-a-z/mouthwash

[3] Yousefimanesh, H., Amin, M., Robati, M., Goodarzi, H., & Otoufi, M. (2015). Comparison of the Antibacterial Properties of Three Mouthwashes Containing Chlorhexidine Against Oral Microbial Plaques: An in vitro Study. Jundishapur journal of microbiology, 8(2), e17341. https://doi.org/10.5812/jjm.17341

[4] Singh, K., Mishra, A., Sharma, D. & Singh, K. (2019). 13 - Antiviral and Antimicrobial Potentiality of Nano Drugs. Micro and Nano Technologies, 343-356. https://doi.org/10.1016/B978-0-12-814029-1.00013-2

flossing, frequent use of mouthwash may be beneficial for daily dental hygiene.[5] The usefulness of mouthwash is that it can get in between teeth and reach areas that toothbrushes cannot. Also, it reduces the speed that tartar (hardened plaque) forms on the teeth, provide a fresh breath and decline the possibility of tooth decay.

As a person who cares a lot about dental hygiene, I realized that although I brush my teeth three times a day, I still get tooth decay. Mentioning the problem to the dentist, she recommended me to use mouthwash. However, while I was searching for mouthwashes, I realized that there were many varieties. To decide the best antibacterial type, I decided to investigate which brand is better at reducing the bacteria present in the oral microbiome.

Two types of mouthwashes are therapeutic and cosmetic.[6] Therapeutic ones contain active ingredients to eradicate dental problems in contrast with cosmetic ones which are usually used to freshen breath. All mouthwashes are effective to some extent however, due to the presence of some indicator materials, their antibacterial effect could be enhanced or limited.

There are many ingredients present in mouthwashes. All types usually contain aqua, sorbitol, aroma, or glycerol for convenience in use. The main ingredients in the ones I used differed by their content through the presence of alcohol, non-alcohol, $ZnCl_2$, NaF, C_6H_5COOH, and $C_{21}H_{38}ClN$ *(Table 1)*. I wanted to compare which main ingredient is the most effective antibacterial agent.

[5] (2022, November 6). Mouthwash - Mouthrinse | MouthHealthy - Oral Health Information from the ADA. Mouthhealthy. https://www.mouthhealthy.org/all-topics-a-z/mouthwash

[6] (2020, November 5). General Dentist FAQs: What Active Ingredients Should I Look for in a Mouthwash? - Dental Care of Madison Mississippi. Dentalcareofmadison. https://www.dentalcareofmadison.com/blog/general-dentist-faqs-what-active-ingredients-should-i-look-for-in-a-mouthwash/

Listerine Cool Mint	Aqua, **Alcohol**, Sorbitol, Poloxamer 407, **Benzoic Acid**, Sodium Saccharin, Eucalyptol, Aroma, Methyl Salicylate, **Thymol**, Menthol, Sodium Benzoate, CI 42053.
Listerine Total Care	Aqua, **Alcohol**, Sorbitol, Aroma, Poloxamer 407, **Benzoic Acid**, **Zinc Chloride**, Eucalyptol, Methyl Salicylate, Sodium Saccharin, **Thymol**, Menthol, Sodium Benzoate, **Sodium Fluoride**, Sucralose, Benzy| Alcohol, CI 16035, Cl 42090.
Listerine Advanced White	Aqua, Sorbitol, Propylene Glycol, Tetrapotassium Pyrophosphate, Pentasodium Triphosphate, **Citric Acid**, Poloxamer 407, Aroma, Sodium Methyl Cocoyl Taurate, Caprylyl Glycol, Eucalyptol, **Thymol**, Sodium Saccharin, Menthol, **Sodium Fluoride**, Sucralose.
Parodontax	Aqua, Glycerine, PEG-60 Hydrogenated Castor Oil, Sodium Citrate, Sodium Lauryl Sulfate, Aroma, Methylparaben, Propylparaben, **Zinc Chloride**, Gellan Gum, o-cymen-5-ol, **Sodium Fluoride**, Sodium Saccharin, CI 17200
Colgate Plax	**Cetylpyridinium chloride**, glycerine, sorbitol, propylene glycol, polysorbate 20, sodium benzoate, phosphoric acid, **sodium fluoride**, sodium saccharin.

Table 1: Ingredients in five types of mouthwash tested with bold-written active ingredients.

$C_{21}H_{38}ClN$ and F^- are commonly found effective substances in mouthwashes showing anti-bacterial properties.[7] F^-, when in complex with a divalent metal ion and ADP, forms a non-functional copy of ATP that can inhibit many metabolic enzymes.[8] Due to the enzyme inhibition, F^- is toxic to all organisms including bacteria. Another active agent, $C_{21}H_{38}ClN$, has antiseptic properties.[9] By increasing the permeability of the bacterial cell wall, and promoting its lysis, it contributes to the reduction in metabolism and interruption of the microorganism's ability to adhere to the tooth surface. Additionally, Zn has proven to be an antibacterial by

[7] Alawamleh, H. (2021). Antibacterial Effect of Mouthwashes against Selected Bacteria. Systematic Reviews in Pharmacy, 12(03), 795-799. doi:10.31838/srp.2021.2.84

[8] Nelson, James W.., Plummer, Mark S.., Blount, Kenneth F.., Ames, Tyler D.. & Breaker, Ronald R.. (2015). Small Molecule Fluoride Toxicity Agonists. Science Direct, 22(4), 527-534. https://doi.org/10.1016/j.chembiol.2015.03.016

[9] RAUJO, Danilo Barral de et al. Mouthrinses: active ingredients, pharmacological properties and indications. RGO, Rev. gaúch. odontol. (Online) [online]. 2012, vol.60, n.3, pp. 349-357. ISSN 1981-8637.

altering bacteria's membranes, through oxidizing which results in disruption and inhibited growth.[10] A study has shown that C_6H_5COOH and its derivatives have antibacterial properties on *E. coli* as well.[11] However, these substances may react with each other and hence their effect might be limited.

The use of essential oils (thymol, peppermint, and eucalyptus oils) has also been proven to increase the antiseptic capacity of mouthwashes. Although these cannot be compared to active materials, their effect can be enhanced by methyl salicylate. Alcohol is not a prominent factor in mouthwashes since it is just used to diffuse substances and has a slight antibacterial capacity compared to others.[12] Moreover, flavours present in cosmetic mouthwashes do not contribute to their performance.

1.3 Use of *E. coli* as a Model Organism

Escherichia coli is a gram-negative bacterium in the family of Enterobacteriaceae.[13] It is usually found in the lower intestine of warm-blooded organisms. By its fast-growing rate, cheap culture media, and well-adaptation to the laboratory environment; it is frequently used in biological research. Additionally, most *E. coli* are considered harmless although some can cause diarrhoea or food poisoning.

Furthermore, *E. coli* spreads through ingestion of undercooked, unpasteurized, or contaminated raw foods and oral contact.[14] Improvement in sanitation is the fundamental prevention of diseases spread by *E. coli*. Therefore, choose of *E. coli* as a model organism is related to the aim of the study since it might be found in an oral environment.

[10] Chevalier, J., Pelletier, J. & Bolzinger, C. (2014). The contribution of zinc ions to the antimicrobial activity of zinc oxide. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 457, 263-274. https://doi.org/10.1016/j.colsurfa.2014.05.057

[11] Synowiec, A., Żyła, K., Gniewosz, M., & Kieliszek, M. (2021). An effect of positional isomerism of benzoic acid derivatives on antibacterial activity against Escherichia coli. Open life sciences, 16(1), 594–601. https://doi.org/10.1515/biol-2021-0060

[12] Lid, S. (2021, October 19). Which Mouthwash Is Best for You?. Verywellhealth. https://www.verywellhealth.com/which-type-of-mouthwash-works-best-4126424

[13] (2022, November 1). Escherichia coli - Wikipedia. En. https://en.wikipedia.org/wiki/Escherichia_coli

[14] (2019, April 3). 4 Bacteria Hiding in Your Teeth | Cambrian Dental. Cambriandental. https://cambriandental.ca/blog/4-bacteria-hiding-in-your-teeth/#:~:text=Escherichia%20coli%20spreads%20through%20ingestion,end%20up%20in%20your%20mouth.

2. HYPOTHESIS

The difference between the diameters of the active material containing and cosmetically produced mouthwashes will be observable on the petri dish. The more natural and less metal ion-containing mouthwash will have the least effect on the bacteria since it will not go under reaction as an oxidizing agent and break down the cell wall of the bacteria. Despite this, the chemical molecule-rich type will have reactive oxygen atoms and attract the cell wall's electrons.[15] With fewer electrons, bacteria cells' walls will be deteriorated and break apart or their enzymatic activity will be inhibited, suppressing bacteria's growth.

Considering the substances in five types of mouthwash, I hypothesized that: Even though the mouthwash containing NaF and $C_{21}H_{38}ClN$ will be effective and have a broad area on the petri dish indicating the efficiency of the antibacterial agent (Colgate); Zn containing type will be the most prominent antibacterial agent through its disruption of cellular membranes (Parodontax). The alcoholic type (Listerine Total Care) will be slightly more efficient than the non-alcoholic (Listerine Advanced White) but will still be lower than Colgate and Parodontax. The remaining cosmetic mouthwash, Listerine Cool Mint, will demonstrate the least antibacterial effect by the smallest area on the petri dish by lacking an active ingredient altering with the bacterium.

3. METHOD DEVELOPMENT & PLANNING

3.1 Rationale

Deciding on the research question, I started researching the method and the bacteria I will be using. I referred to biosafety levels for deciding the bacteria. The non-pathogenic strain of *E. coli* (K-12) considered BSL-1 suggests the strain is not known to consistently cause disease

[15] Park, J., Lee, G., Yun, B.G., Kim, C., & Ko, Y. (2014). Comparative effects of chlorhexidine and essential oils containing mouth rinse on stem cells cultured on a titanium surface. Molecular Medicine Reports, 9, 1249-1253. https://doi.org/10.3892/mmr.2014.1971

and presents a minimal potential hazard.[16] Therefore, I preferred *E. coli* due to its availability and possible presence in the mouth correlating with the study. Also, it was suitable for the strake plating method which will be discussed later.

To isolate *E. coli*, I chose the strake plating method which is creating areas of colony growth on a single plate by making zig-zag patterns with a sterile inoculum.[17] After cultivating the isolated *E. coli* with LB Broth, the antibacterial activity could be tested by seeding 40.00 ± 0.01 μL *E. coli* solution in every Petri dish via a cell spreader and pipetting 10.00 ± 0.01 μL of mouthwash into the centre. Measuring the diameter of the experimentation area (ZOI) after 24.00 ± 0.50 hours, data can be collected.[18]

In the middle of the Petri dish, where the mouthwash is added, bacteria will not be able to grow due to the antibacterial substance. However, as the distance from the centre increase, the effect of the antibacterial substance will decrease; thus, bacteria will be forming colonies. After the incubation, a presence of a circular zone which is the inhibited growth area (ZOI), will correlate with the antimicrobial capacity of the substance**.** Hence, the effectiveness of the mouthwash could be determined.

Before the experimentation, preliminary testing was made. Viscous substances were hard to use with a micropipette so, all the independent variables are taken as liquid form as possible to reduce random errors.

After the preliminary testing, I started searching for the most appropriate five types of mouthwashes. I went to three local supermarkets and came with 5 types of mouthwash: Colgate, Parodontax, Listerine Cool Mint, Listerine Advanced White and Listerine Total Care

[16] (2022, November 9). Biosafety Level | ATCC. Atcc. https://www.atcc.org/support/order-support/biosafety-level
[17] Sanders E. R. (2012). Aseptic laboratory techniques: plating methods. Journal of visualized experiments: JoVE, (63), e3064. https://doi.org/10.3791/3064
[18] Borthagaray, G., Mondelli, M., Facchin, G. & Torre, María H.. (2018). Silver-containing nanoparticles in the research of new antimicrobial agents against ESKAPE pathogens - ScienceDirect. Science Direct, 317-386. https://doi.org/10.1016/B978-0-12-813661-4.00008-0.

(Appendix 4- Figure 10). Due lack of availability of another brand, three of them belong to the same brand however, they differed in their use and ingredients. This contributes to the experimentation since it will illustrate to what extent different types of products of the same brand differ in antibacterial properties.

To conduct my experiment, I started searching for a laboratory due unsterile environment in a school lab which is not appropriate for a microbiological investigation. After searching, I got permission for conducting my experiment at a scientific lab at TOBB University of Economics and Technology.

3.2 Justification

I chose the bacteria *Escherichia coli* due to its safe strain (K-12), fast-growing rate, cheap culture, and high availability of it. Additionally, it is related to the aim of the study as well since it might be found in an oral environment.

To increase accuracy, I will be evaluating the antimicrobial effect on five independent variables and five trials for each of them with one control set for error detection. The measurements will be taken from each trial by considering the largest IZD on the Petri dish for data stabilization. From the quantitative data, the mean, SD, and variance of IZD will be calculated for further comparison.

Except for the five types of mouthwashes (independent variables), everything will be stable throughout the experiment. Samples will be kept for incubation at $36.60 \pm 0.01℃$ for 24.00 ± 0.50 hours at the same environmental conditions. ZOI for all samples will be measured through a computer program (ImageJ) to avoid random errors. Measurements will be taken immediately after $24.00 \pm 0.50\ hours$ of incubation to compare the IZDs. Timing of measurement is significant for ZOI creation since zones may be enclosed beyond a given time due to the temporary antibacterial effect of mouthwashes and the gain of resistance of bacteria.

That is also why ZOI will be measured through a computer program, which considers the photo captures at the selected time so, IZDs will not be affected by the time difference in measuring.

Furthermore, LB Broth Agar will be used since it is the optimal environment for *E. coli* growth.[19] The amount of LB agar solution with bacteria $(40.00 \pm 0.01\ \mu L)$ and the amount of mouthwash $(10.00 \pm 0.01\ \mu L)$ will be added in a ratio of $\frac{1}{4}$ and the resulting solution will be 0.50 ± 0.03 McFarland (turbidity) which will be diluted 10^3 times. The turbidity, dilution and amount of other substances added are preferred for an observable *E. coli* growth on the petri dish since a higher amount of mouthwash eliminates all bacteria or a less concentrated solution will not be able to display the bacteria growth whereas a high concentration will lack in ZOI. The optimal temperature and minimum time requirement for the growth of *E. coli* is $36.60 \pm 0.01℃$ and 24.00 ± 0.50 hours which is chosen to see the results faster.

Although further actions are taken, unpreventable factors may affect the investigation results making IZD appear different, such as the proportion of added substances, cross-contamination, temperature $(36.60 \pm 0.01℃)$, incubation time $(24.00 \pm 0.50$ hours$)$ or pH & humidity of the environment due to imprecision of lab apparatus. Therefore, experimentation will be conducted in a microbiologic lab cleaned with ethyl alcohol, with a Bunsen burner and use of scientific equipment. The random and systematic errors will be minimized in these conditions thus, results will be more accurate.

[19] MacWilliams, Maria P.. & Liao, M. (2006). Luria Broth (LB) and Luria Agar (LA) Media and Their Uses Protocol. American Society for Microbiology, 1-4.

3.3 Variables

	Name of Variable	Method of Control/Measurement
Independent Variable	Five different types of mouthwashes (10.00 ± 0.01 μL)	Colgate, Parodontax, Listerine Advanced White, Listerine Total Care and Listerine Cool Mint (10.00 ± 0.01 μL) are used and measured with a micropipette.
Dependent Variable	Diameter (millimetres) of inhibition zone (IZD)	The same type of bacteria of the brand ATCC, *E. coli*, is used and the IZD is measured by the programme "ImageJ".
Controlled Variables	Temperature of incubation	All samples are incubated at 36.60 ± 0.01℃.
	Time of incubation	All samples are incubated for 24.00 ± 0.50 hours using a digital clock (± 0.01 minutes).
	Timing and method of measurements	The measurements are taken after 24.00 ± 0.50 hours with the programme "ImageJ" through photos taken with the same camera in the same quality.
	Petri dish	6.0 ± 0.50 cm diameter polystyrene Petri dishes were used for each trial.
	Amount of mouthwash added	A 10.00 ± 0.01 μL mouthwash sample was added in each trial using a micropipette.
	Bacteria nutrient source	LB Broth Agar (1000.00 ± 0.01 μL) is used in each trial as a bacteria nutrient source.
	Amount of bacteria solution seeded	40.00 ± 0.01 μL solution with *E. coli* is seeded in each trial through a micropipette.
	Sterilization	The experiment is conducted in a lab environment cleaned with ethyl alcohol sanitizer and with a Bunsen burner.
	Lab equipment	The same type of equipment; clock, micropipette, incubator, inoculum, Bunsen burner and petri dish is used for all experiments.

Table 2: Table showing dependent, independent, and controlled variables with methods for controlling or measuring.

4. METHOD

4.1 Materials

- 1 ATCC *Escherichia coli*
- 1 experimental test tube $(2.00 \pm 0.50$ mL) & cap
- Micropipette $(100.00 \pm 0.01$ μL) & 6 pipette tips
- 70.00% Ethyl Alcohol Sanitizer
- One cell spreader
- 1 LB broth agar plate
- 2 Sterile wire inoculums
- Incubation unit $(36.60 \pm 0.01$℃)
- $6 \times (6.0 \pm 0.5$ 0cm) diameter sterilized polystyrene Petri dishes
- A vortex
- A McFarland densitometer $(\pm 0.03$ McFarland)
- 2 Bunsen burners
- One permanent marker
- One lab apron
- 3 pairs of nitrile gloves
- A computer & the programme "ImageJ"
- A high-quality camera
- A digital clock (± 0.01 minutes)
- 4 Masks

4.2 Procedure

"Isolating *E. coli*" and "Preparing LB Broth Agar & E. coli Solution" are preliminary steps. See *Appendix 1-2* for details.

4.2.1 Seeding Bacteria

1) Wear nitrile gloves, a mask, and a lab apron.
2) Clean the environment with 70.00% ethyl alcohol sanitiser and turn on two Bunsen burners to minimize airborne contamination.
3) Label the polystyrene Petri dishes $(6.00 \pm 0.50$ cm) for Listerine Cool Mint as *"1"*, Listerine Total Care as *"2"*, Listerine Advanced White as *"3"*, Parodontax as *"4"*, Colgate as *"5"* and Control Set as *"C"* with a permanent marker.

4) Invert the Petri dishes and open the lid of each. Pipette 40.00 ± 0.01 μL of the previously prepared 0.50 ± 0.03 McFarland (diluted 10^3 times) LB broth agar & *E. coli* solution to each dish using a 100.00 ± 0.01 μL micropipette.
5) Spread the added solution via a cell spreader into every part of the petri dish, carefully rotating it underneath. Close the lid of the Petri dishes immediately after spreading.
6) Using a 100.00 ± 0.01 μL micropipette, pipette 10.00 ± 0.01 μL of the labelled mouthwash type (*1, 2, 3, 4, 5*) on the centre of the Petri dish. Do not add any mouthwash to the control group.
7) Close the lid of the Petri dishes and wait for 1.00 ± 0.01 minutes.
8) Invert the Petri dishes slowly and put them into the incubator at 36.60 ± 0.01℃ for 24.00 ± 0.50 hours.

4.2.2 Collection of Data

1) Wait for 24.00 ± 0.50 hours for samples to incubate at 36.60 ± 0.01℃.
2) After 24.00 ± 0.50 hours, take photos of each sample of the Petri dish in a clear bright light with the same camera making sure the photos are in the same quality and resolution.
3) Measure the diameter of the ZOIs using the programme "ImageJ" *(Appendix 5)* and record the continuous quantitative data in a table similar to *Table 3.*
4) Discard all the Petri dishes and materials to the biohazard bin after you finish.
5) Repeat all the procedures four more times.

5. RISK ASSESSMENT & ETHICAL CONSIDERATIONS

According to IBO ethical guidelines, the safest strain of *E. coli* (K-12) is used, and all the materials are composed into a biohazard bin after the experiment. Sterilization is made before and after the experiment against any cross-contamination, the number of cultures is kept at a minimum, gloves, masks, and lab coats are worn and hands are washed at the end with soap

and water. Eating or drinking is limited during experimentation. Additionally, cruelty to any animal, vertebrate or invertebrate is eliminated.

6. DATA ANALYSIS

6.1 Raw Data

Type of Mouthwash	Trials	Diameter of Inhibition Zone (IZD ± 0.50 mm)	The volume of Mouthwash Added (µL ± 0.01)
Colgate	1	1608.79	10.00
	2	1504.45	
	3	1558.40	
	4	1403.70	
	5	1609.95	
Parodontax	1	1022.33	10.00
	2	1241.04	
	3	1034.04	
	4	1280.94	
	5	1082.81	
Listerine Advanced White	1	593.97	10.00
	2	742.99	
	3	703.78	
	4	895.22	
	5	805.24	
Listerine Total Care	1	227.72	10.00
	2	96.88	
	3	198.04	
	4	167.63	
	5	141.60	
Listerine Cool Mint	1	80.82	10.00
	2	169.72	
	3	157.13	
	4	122.20	
	5	99.13	
Control	1	224.22	10.00
	2	292.06	
	3	262.46	
	4	165.36	
	5	244.88	

Table 3: Table of raw data showing the inhibition zone diameter (IZD) created by E. coli with 5 distinct types of mouthwash for 5 trials for each of them, measured after 24.00 ± 0.50 *incubations at 36.60 ± 0.01℃ with controlled variables.*

6.2 Calculations

6.2.1 Mean

$$Mean\ (\overline{x}) = \frac{1}{n} \times \sum_{i=1}^{n} = \frac{a_1 + a_2 + a_3 + \cdots + a_n}{n}$$

where n is the number of data values in the data set and x_i is the i^{th} data value in the data set.

$$Mean\ IZD = \frac{80.82 + 169.72 + 157.13 + 122.20 + 99.13}{5} = 125.80$$

6.2.1 Standard Deviation

$$SD\ (\sigma) = \sqrt{\frac{\sum(x-\bar{x})^2}{(n-1)}}$$

where n is the number of data values, x is the data value and $\bar{x}$ is the mean of the data set

$$1: (x - \bar{x})^2 = (80.82 - 125.80)^2 = 2023.20$$

$$2: (x - \bar{x})^2 = (169.72 - 125.80)^2 = 1928.97$$

$$3: (x - \bar{x})^2 = (157.13 - 125.80)^2 = 981.57$$

$$4: (x - \bar{x})^2 = (122.20 - 125.80)^2 = 12.96$$

$$5: (x - \bar{x})^2 = (99.13 - 125.80)^2 = 711.29$$

$$\sqrt{\frac{2023.20 + 1928.97 + 981.57 + 12.96 + 711.29}{(5-1)}} = 37.61$$

6.2.2 Variance

$$Variance = \sigma^2 = 37.61^2 = 1414.51$$

where σ variance of the data set.

An example calculation for Listerine Cool Mint is shown above. All the other calculations are repeated for all data sets (*Table 5*). Uncertainties are taken from the electronic measurement apparatus' information sheet and half of the smallest division of digital devices.

6.2.3 ANOVA

The one-way ANOVA formula: F-ratio: $F = \frac{MSB}{MSW}$

Where, F = coefficient of ANOVA, MSB = Mean sum of squares between the groups and

MSW = Mean sum of squares within groups

ANOVA test includes a null hypothesis (all independent variable means are equal) and an alternative hypothesis (at least one independent variable mean will vary from others).[20] If the F-ratio is equal or close to 1, then the two variances are equal, and the null hypothesis is true.

All values are calculated through an online ANOVA calculator[21] and given in *Table 4*.

Source	DF	Sum of Square	Mean Square	F-Value	P-value
Groups (Between groups)	5	8536432.693	1707286.54	250.708	1.11E-16
Error (Within groups)	24	163436.5084	6809.8545	_	_
Total	29	8699869.201	299995.49	_	_

Table 4: Table showing the results obtained from the ANOVA test performed with the inhibition zone diameter (IZD) created by E. coli with 5 distinct types of mouthwash, measured after 24.00 ± 0.50 hours incubation at 36.60 ± 0.01℃.

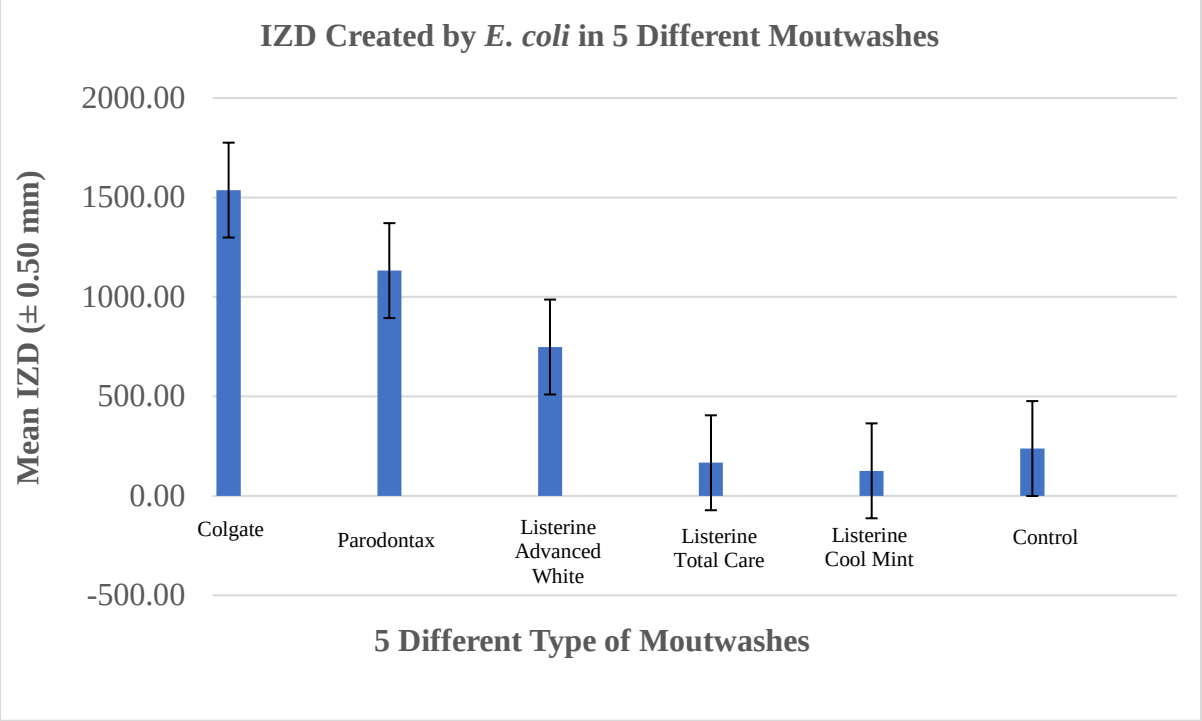
[20] (2022, August 2). One-Way ANOVA - Definition, Formula, Examples, When to Use?. Wallstreetmojo. https://www.wallstreetmojo.com/one-way-anova/#h-anova-formula

[21] (2023, January 22). ANOVA Calculator - One Way ANOVA and Tukey HSD test. Statskingdom. https://www.statskingdom.com/180Anova1way.html

6.3 Processed Data

Type of Mouthwash	Trials	IZD (± 0.50 mm)	Mean IZD (± 0.50 mm)	SD	Variance
Colgate	1	1608.79	1537.06	77.15	5952.59
	2	1504.45			
	3	1558.40			
	4	1403.70			
	5	1609.95			
Parodontax	1	1022.33	1132.23	120.53	14528.45
	2	1241.04			
	3	1034.04			
	4	1280.94			
	5	1082.81			
Listerine Advanced White	1	593.97	748.24	112.53	12663.68
	2	742.99			
	3	703.78			
	4	895.22			
	5	805.24			
Listerine Total Care	1	227.72	166.37	50.53	2552.88
	2	96.88			
	3	198.04			
	4	167.63			
	5	141.60			
Listerine Cool Mint	1	80.82	125.80	37.61	1414.51
	2	169.72			
	3	157.13			
	4	122.20			
	5	99.13			
Control	1	224.22	237.80	47.53	2258.63
	2	292.06			
	3	262.46			
	4	165.36			
	5	244.88			

Table 5: Table of processed data showing the mean, SD, and variance of the inhibition zone diameter (IZD) created by E. coli with 5 distinct types of mouthwash, measured after 24.00 ± 0.50 hours incubation at 36.60 ± 0.0 ℃.



Graph 1: Graph showing the mean inhibition zone diameter (IZD) created by E. coli with 5 distinct types of mouthwash for 5 trials for each of them, measured after 24.00 ± 0.50 hours incubation at 36.60 ± 0.01℃.

7. EVALUATION

This research aimed to investigate the best alternative between five different types of mouthwashes showing the highest antibacterial effect measured through ZOI. The experiment was conducted in a laboratory environment with five different mouthwash types on *Escherichia coli* samples in LB broth agar. It was hypothesized that Parodontax would have the greatest antibacterial effect due to the antibacterial properties of Zn and NaF disrupting the cellular membranes and Colgate, containing NaF which is also an antibacterial substance, will be the second-highest one.

From the quantitative data mean IZD is obtained for each mouthwash. Colgate (1537.06 ± 0.50 mm), Parodontax (1132.23 ± 0.50 mm) Listerine Advanced White (748.24 ± 0.50 mm), Listerine Total Care (166.37 ± 0.50 mm), Listerine Cool Mint

$(125.80 \pm 0.50\ mm)$ has shown antibacterial effects respectively, due to decreasing order of IZD.

Unexpectedly Colgate was observed to be the most effective mouthwash rather than Parodontax, rejecting the hypothesis. However, it was also stated that some chemicals' capacity may be limited because of reacting with other substances present. Therefore, it can be said that Colgate did not have any reverse mechanism to decline its efficiency by reacting with NaF as it could have been in Parodontax. This result verifies a study before, proving that Colgate is an effective antimicrobial agent.[22]

Although the hypothesis for Parodontax was rejected by having the second-highest mean IZD $(1132.23 \pm 0.50\ \text{mm})$, the least effective mouthwash type was hypothesized truly as Listerine Cool Mint by $125.80 \pm 0.50\ \text{mm}$ *(Graph 1)*. Not having any active ingredients, it was cosmetically produced therefore it showed a negligible antibacterial effect. Besides, Listerine Advanced White $(748.24 \pm 0.50\ \text{mm})$ and Listerine Total Care $(166.37 \pm 0.50\ \text{mm})$ have been the tertiary and quaternary effective ones respectively, which also rejects the hypothesis. Since Total Care has too many ingredients, Zn or NaF, reactions that occurred may have decreased its efficiency.

During the experiment, there were no visible mistakes made since it is conducted in a professional laboratory with sterile equipment. However, the high SD, variance, and unexpected results indicate the limitations that may present. Particularly in Parodontax ($\text{SD} = 120.53$, $\text{Variance} = 14528.45$) and Listerine Advanced White ($\text{SD} = 112.53$, $\text{Variance} = 12663.68$); high SD signifies that the distribution of IZD is spread, decreasing the precision while the high variance suggests the IZDs are far from the mean, decreasing the accuracy.[23]

[22] Szymanska, J., Olljenik, E., Biernasiuk, A. & Malm, A. (2020). (PDF) Antimicrobial efficacy of Colgate Plax Cool Mint® mouthwash – in vivo studies. Current Issues in Pharmacy and Medical Sciences, 33, 211-218. 10.2478/cipms-2020-0040

[23] (2022, October 10). Variance and Standard Deviation-Definition, Formula, Relation and Example. Byjus. https://byjus.com/maths/variance-and-standard deviation/#:~:text=Variance%20and%20Standard%20Deviation%20are,the%20distribution%20of%20statistical%20data.

The other processed data can be summarized as Colgate ($SD = 77.15$, $Variance = 5952.59$), Listerine Total Care ($SD = 50.53$, $Variance = 2552.88$) and Listerine Cool Mint ($SD = 37.61$, $Variance = 1414.51$) which are calculated for detection of errors.

The most surprising result was Listerine Cool Mint showing a minor antibacterial effect *(Graph 1)*. Although it is cosmetically produced, it still contains some antibacterial ingredients like menthol or eucalyptus. This might be due to a random error or a limitation of the experimental technique since it rejected the studies before stating Listerine Cool Mint is effective for cleansing the oral cavity.[24] Since the IZD is measured after 24.00 ± 0.50 hours, the minimal antibacterial effect might have disappeared by the gain of resistance of bacteria to the substance, resulting in the disappearance of the ZOI. This can also be true for other mouthwashes, therefore ZOIs might not fully correlate with the antibacterial results due timing of measurement.

As a weakness of the experiment, all mouthwashes had different content which may have affected the results. Listerine Total Care and Cool Mint had an alcohol-based content whereas Advanced White did not. Alcohol, having a lower boiling point might have evaporated during the addition to the Petri dishes and may lead to lower results in ZOI. Thus, the hypothesis might appear to be rejected by Advanced White being more effective than Total Care although it did not contain alcohol. The viscosity of the mouthwashes was not standard as well although the most fluid ones are selected. Consequently, the addition amount of mouthwashes (10.00 ± 0.01 µL) may have varied, resulting in a lower ZOI. This may be a reason Parodontax has been the second most effective mouthwash with a slight difference from Colgate. The experiment could be improved by determining the density of the mouthwashes and using fewer volatile ones.

[24] Kato, T., Iijima, H., Ishihara, K., Kaneko, T., Hirai, K., Naito, Y., & Okuda, K. (1990). Antibacterial effects of Listerine on oral bacteria. The Bulletin of Tokyo Dental College, 31(4), 301–307.

Another weakness is that the LB broth agar environment is not a full representation of the oral cavity. The pH of the mouth is maintained near (6.70- 7.30)[25], whereas LB broth agar is near 9.00[26] for the optimal growth of *E. coli*. This may result in smaller ZOI in the agar environment than in an oral cavity. Additionally, the only bacteria used in the experiment was *Escherichia coli* so there was only one factor of resistance. Other types of bacteria may have different resistance towards antibacterial mouthwashes. Indeed, *E. coli* is found in the intestine of people and animals, so it is not always present in the oral cavity ignoring exposure to contaminated water or food.[27] Therefore, these weaknesses could be improved by testing on different organisms or epithetical cells.

A strength of the experiment is that the safest model organism is selected and, not any organism is harmed. The use of most liquid mouthwashes minimized the experimental errors since they did not stick inside the micropipette while adding them. The use of *E. coli* enabled a cheaper and faster observation that can be made in one day without high costs. Experimenting in a scientific lab was another strength since the sterile conditions and professional equipment enabled working with bacteria without any visible cross-contamination or systematic error. Additionality, the replicability of the experiment allowed for increasing the precision by conducting 25 trials which are indicated by low uncertainties of IZDs (± 0.50 mm).

ANOVA test used has provided that the mean IZD created is highly different for each mouthwash. F-ratio being higher than 1 *(Table 4)*, rejected the null hypothesis thus, it is proven that using the most antibacterial mouthwash has a significant improvement in oral sanitation.

[25] Baliga, S., Muglikar, S., & Kale, R. (2013). Salivary pH: A diagnostic biomarker. Journal of Indian Society of Periodontology, 17(4), 461–465. https://doi.org/10.4103/0972-124X.118317

[26] Sezonov, G., Joseleau-Petit, D., & D'Ari, R. (2007). Escherichia coli physiology in Luria-Bertani broth. Journal of bacteriology, 189(23), 8746–8749. https://doi.org/10.1128/JB.01368-07

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8. CONCLUSION

My research question “What is the best alternative between five types of mouthwashes by preventing the growth of *Escherichia coli* in the oral cavity?” was answered with Colgate. By containing NaF and lacking a significant ingredient reacting with it, it has been the most effective mouthwash with an IZD of 1537.06 ± 0.50 mm. It was unanticipated that it will be the most antibacterial type by containing the least amount of ingredients.

My inspiration while choosing this topic was having dental issues for years. Now founding the most effective alternative, I understood the uselessness of cosmetically produced mouthwashes for the prevention of dental problems and hygiene. Although, many ingredients can be antibacterial agents; chemical ones (Zn, NaF) are more useful than natural ones (menthol, eucalyptus).

Having many antibacterial alternatives, users are not acknowledged of the type of mouthwash they use. Thus, many people including me, suffer from dental problems because of the brand’s lack of labelling whether their mouthwash is therapeutically or cosmetically produced. After this investigation, I learned the essentialism of checking mouthwash brand ingredients and decided that the brands should improve the labelling of their products.

A further investigation could be conducted in a saliva medium taken by samples from the human mouth. This could enhance the experiment since bacteria will be observed in its potential habitat. Also, the varying concentration of mouthwash can be tested on bacteria to determine how much mouthwash shows an antibacterial effect.

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APPENDIX 1- Isolating *E. coli*

1) Wear nitrile gloves, a mask, and a lab apron.
2) Obtain the LB broth agar plate and label it on the centre with the name of the bacteria (*E. coli)*, the number of the subculture and the date (day/month/year) with a permanent marker.
3) Clean the environment and apparatus with 70.00% ethyl alcohol, then turn on two Bunsen burners to minimize airborne cross-contamination.
4) Sterilize the wire inoculation loop by passing it through the flame until it is red hot and allow it to cool down before touching it to the bacteria.
5) Pass the *E. coli* containing specimen tube through the flames and take a loop of *E. coli* with the sterilized wire inoculum.
6) Pass the *E. coli* containing specimen tube through the flames again before recapping.
7) Hold the sterilized wire inoculum like a pencil and streak the plate by dragging the inoculum through one-third of the LB broth agar plate back and forth in a zig-zag motion, creating a synodical function *(Appendix 4- Figure 6)*. Close the lid of the LB broth agar plate.
8) Resterilise the loop by passing it through the flames and allow it to cool down before the second streak.
9) Open the plate's lid. Drag the loop again back and forth in a zig-zag motion, creating the second streak in the other one-third and close the lid afterwards.
10) Flame the inoculum again, allow it to cool down and perform the third streak in the remaining one-third. Close the lid of the agar plate afterwards.
11) Incubate the agar plate in an inverted position at 36.6 ± 0.01℃ for 24.00 ± 0.50 hours.

APPENDIX 2- Preparing LB Broth Agar & *E. coli* Solution

1) Wear nitrile gloves, a mask, and a lab apron.
2) Clean the environment with 70.00% ethyl alcohol sanitiser and turn on two Bunsen burners to reduce airborne cross-contamination.
3) Obtain a 2.00 ± 0.50 mL test tube and add 1000 ± 0.01 µL LB Broth into it using a 100.00 ± 0.01 µL micropipette.
4) Sterilise the inoculum by passing it through the flames until it is red hot, allow it to cool down and take a piece of *E. coli* from the previously *E. coli* isolated LB broth agar plate.
5) Add the piece taken into the test tube $(2.00 \pm 0.50$ mL), close the cap of the test tube, and vortex them for 3.00 ± 0.50 seconds altogether.
6) By McFarland densitometer, measure the turbidity of the solution of LB broth with *E. coli* and make sure it is 0.50 ± 0.03 McFarland.
7) If it is too dense dilute it by adding LB broth (repeat step 3) if it is less dense add more *E. coli* (repeat step 4). If it is 0.50 ± 0.03 McFarland, follow the remaining step.
8) After adjusting the solution to 0.50 ± 0.03 McFarland, dilute it 10^3 times by adding 3.00 ± 0.01 mL LB Broth.

APPENDIX 3- Qualitative Data for ZOI (Trial 1) Created by Each Mouthwash Sample

Figure 1: Listerine Cool Mint & its IZD.

Figure 2: Listerine Total Care & its IZD.

Figure 3: Listerine A. White & its IZD.

Figure 4: Parodontax & its IZD.

Figure 5: Colgate Plax & its IZD.

APPENDIX 4- Photos from The Experiment

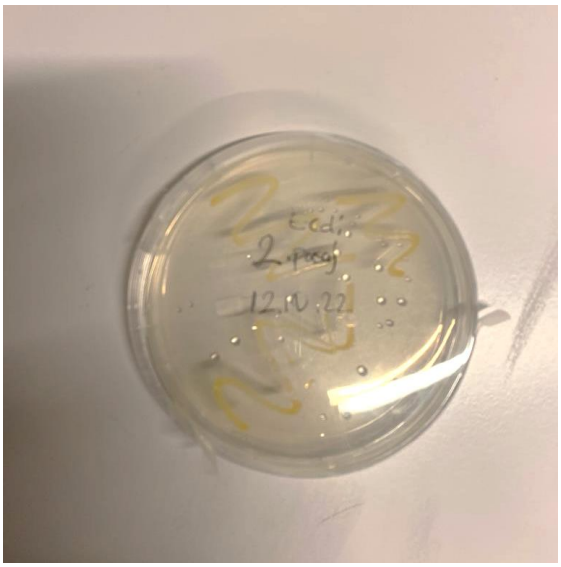


Figure 6: Isolated E. coli by a streak plating method.

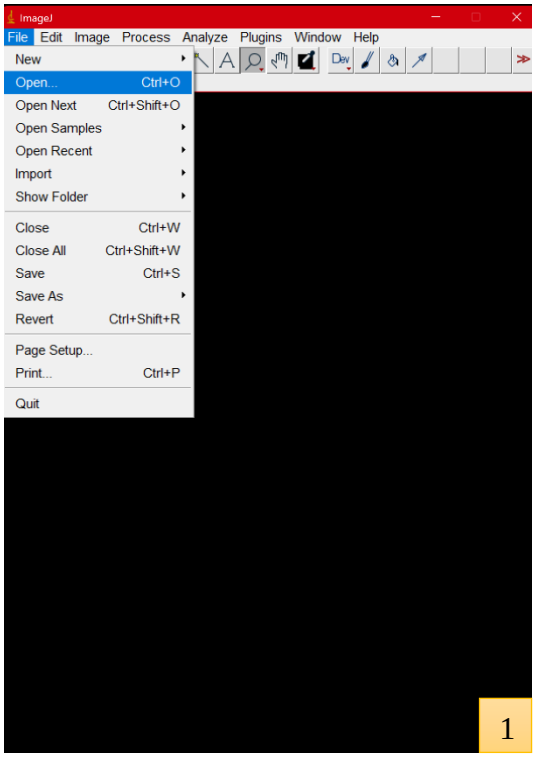
Figure 7: Experimental set-up.

Figure 8: Incubator at 36.60 ± 0.01℃.

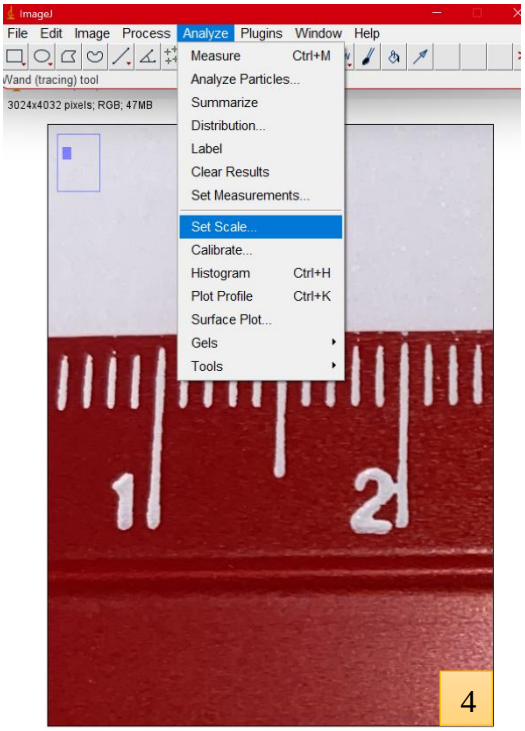
Figure 9: Incubating samples.

Figure 10: 5 different types of mouthwash tested.

APPENDIX 5- Procedure for ImageJ

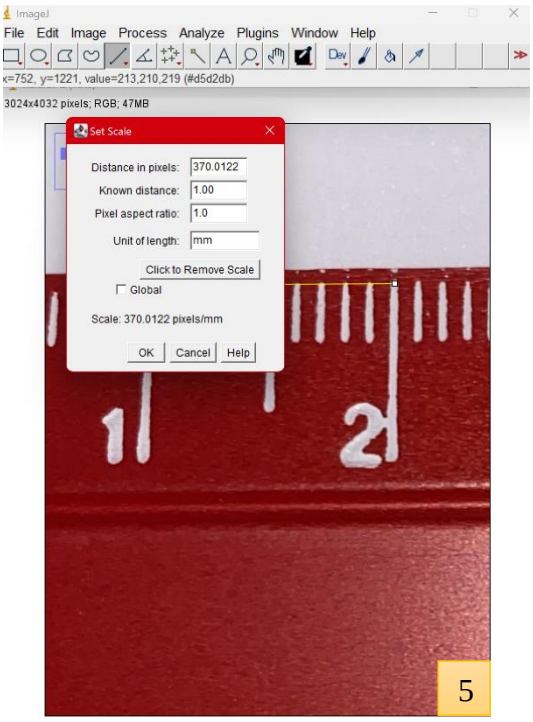


1. Open ImageJ and transfer the photos of all samples and the measurement tool (mm).
2. Zoom to the measurement tool by using the 10th tool from LHS and adjust the area of measurement to the centre with the 11th tool from the LHS.



3. Select the fourth tool from LHS and draw a line between 1.00 mm and 2.00 mm.

4. Click Analyze > Set Scale.

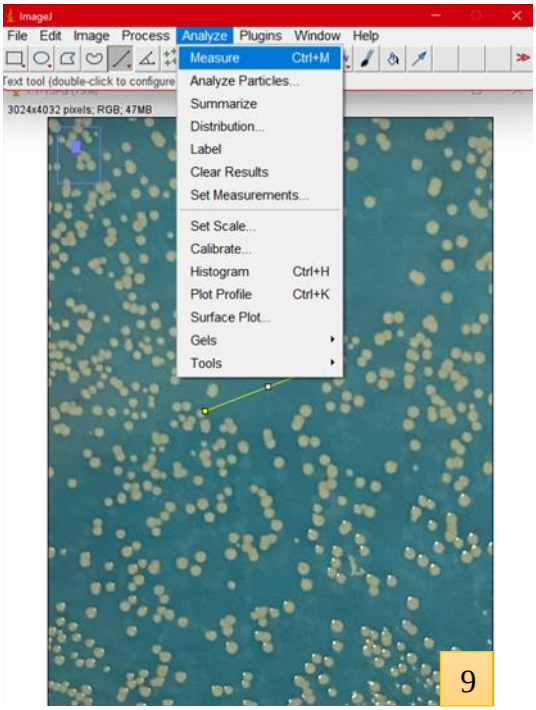


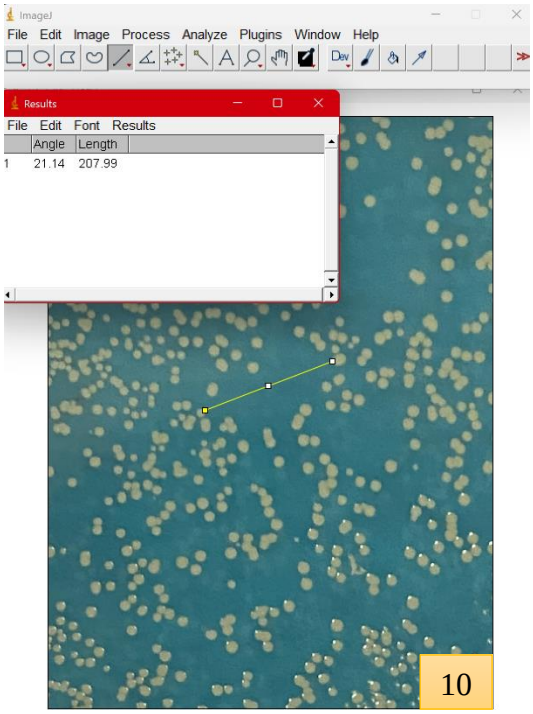
5. Set the known distance to “1.00” and the unit of length to “mm”.

6. Return to the sample you want to measure which you have opened in step 1.

7. Zoom to the sample by using the 10th tool from LHS and adjust the ZOI to the centre with the 11th tool from the LHS.

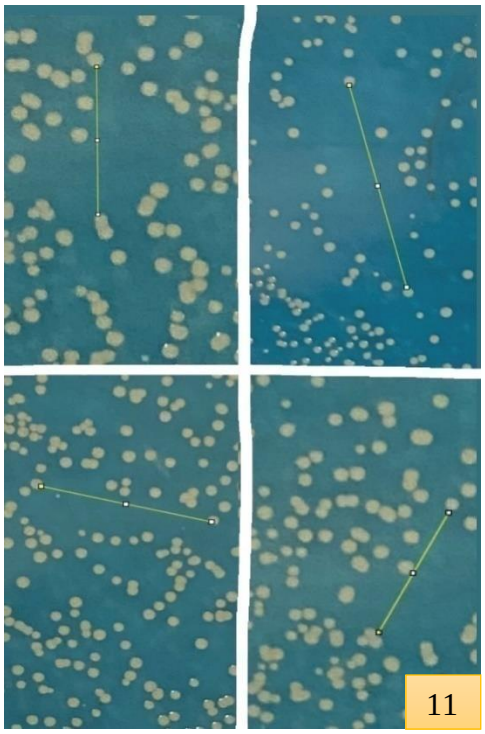
8. Select the fourth tool from LHS and draw a line between the bacteria colonies to measure the IZD, considering the largest possible distance.

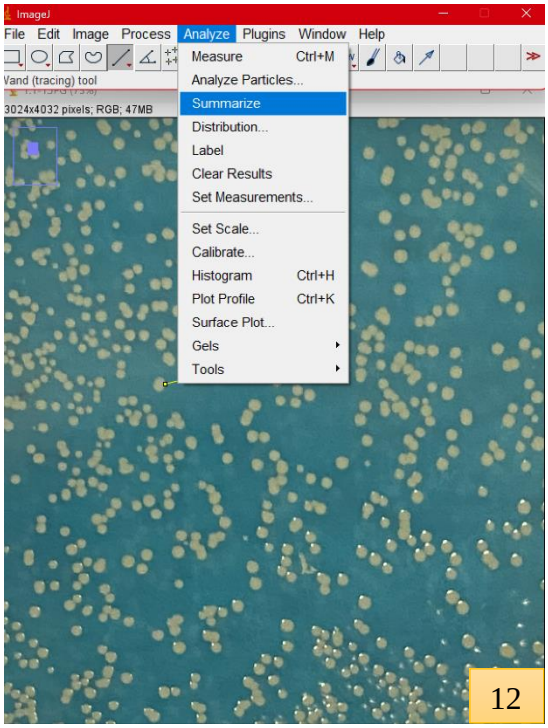




9. Click Analyze > Measure to save the measured distance.

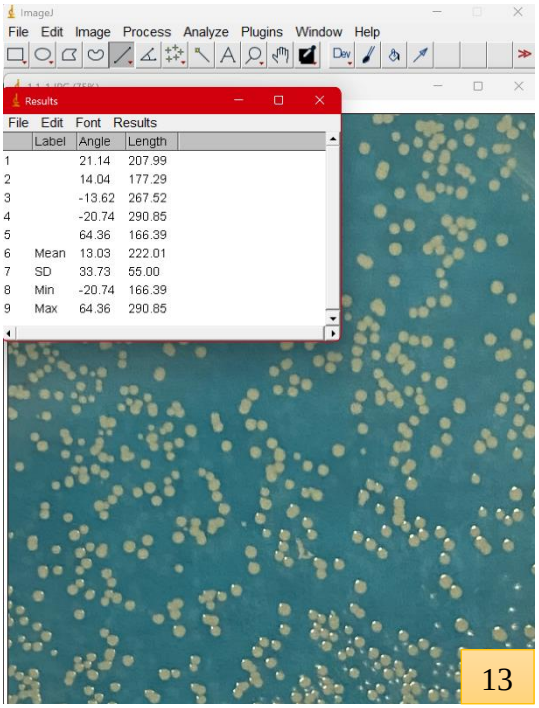
10. After the results table is shown, minimize the window tab to the back.





11. Repeat the same procedure from steps 6 to 10, measuring all five trials for one type of mouthwash. Do not forget to click Analyze > Measure each time after taking the largest diameter.

12. After finishing step 11 click Analyze > Summarize.



13. Record the data and repeat the same process from 6 to 13 for five trials of another type of mouthwash.

APPENDIX 6- Research Consent

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The student has performed their experiments at TOBB University of Economics and Technology Biomedical Engineering Laboratory themselves with supervision from our research assistant.

Associate Professor Dr. Birsen Can Demirdöğen

TOBB University of Economics and Technology
Department of Biomedical Engineering