INDONESIA INTERNATIONAL INSTITUTE FOR LIFE SCIENCES

Improving The Quality of Life

PROTOCOL

Clinical Trial Protocol for Antimicrobial Evaluation of Roll-On Deodorant

Laboratory : Indonesia International Institute for Life Sciences
Authors : apt. Pietradewi Hartrianti, M.Farm., Ph.D.
Putu Virgina Partha Devanthi, Ph.D.
Ihsan Tria Pramanda, M.Sc.
Brian Christopher Budiono - 18010041 (Biomedicine)
Erica - 18010068 (Biomedicine)
Evelyn Jeanette Wijaya - 18010072 (Pharmacy)
Gabrielle Audrey Gandasasmita - 18010090 (Biomedicine)

INDONESIA INTERNATIONAL INSTITUTE FOR LIFE SCIENCES
2022

FOREWORD

This protocol is based on the clinical trial conducted at Indonesia International Institute for Life Sciences (i3L), Jakarta, Indonesia. The clinical trial is done to test out a deodorant sample and aim to observe its antibacterial activity. It is conducted through a three-day testing period, where subjects undergo specific conditioning. The study consists of bacterial sampling from axillary areas treated with the tested deodorant and axillary areas not treated with any deodorant, serial dilution, and bacterial culture on agar media, observation and counting of colony forming unit (cfu), and identification of bacteria using selective agar media and gram-staining.

This protocol covers all steps except for the identification of bacteria using selective agar media and gram-staining and DNA sequencing, which will be covered in a separate protocol with title “Isolation and Identification of Bad Axillary Odors Producing Microorganism from Human Axillary Region“. This protocol can be an example for those conducting similar studies testing antimicrobial activity in cosmetic products through clinical trials, as adjustments can be made according to each one of the study.

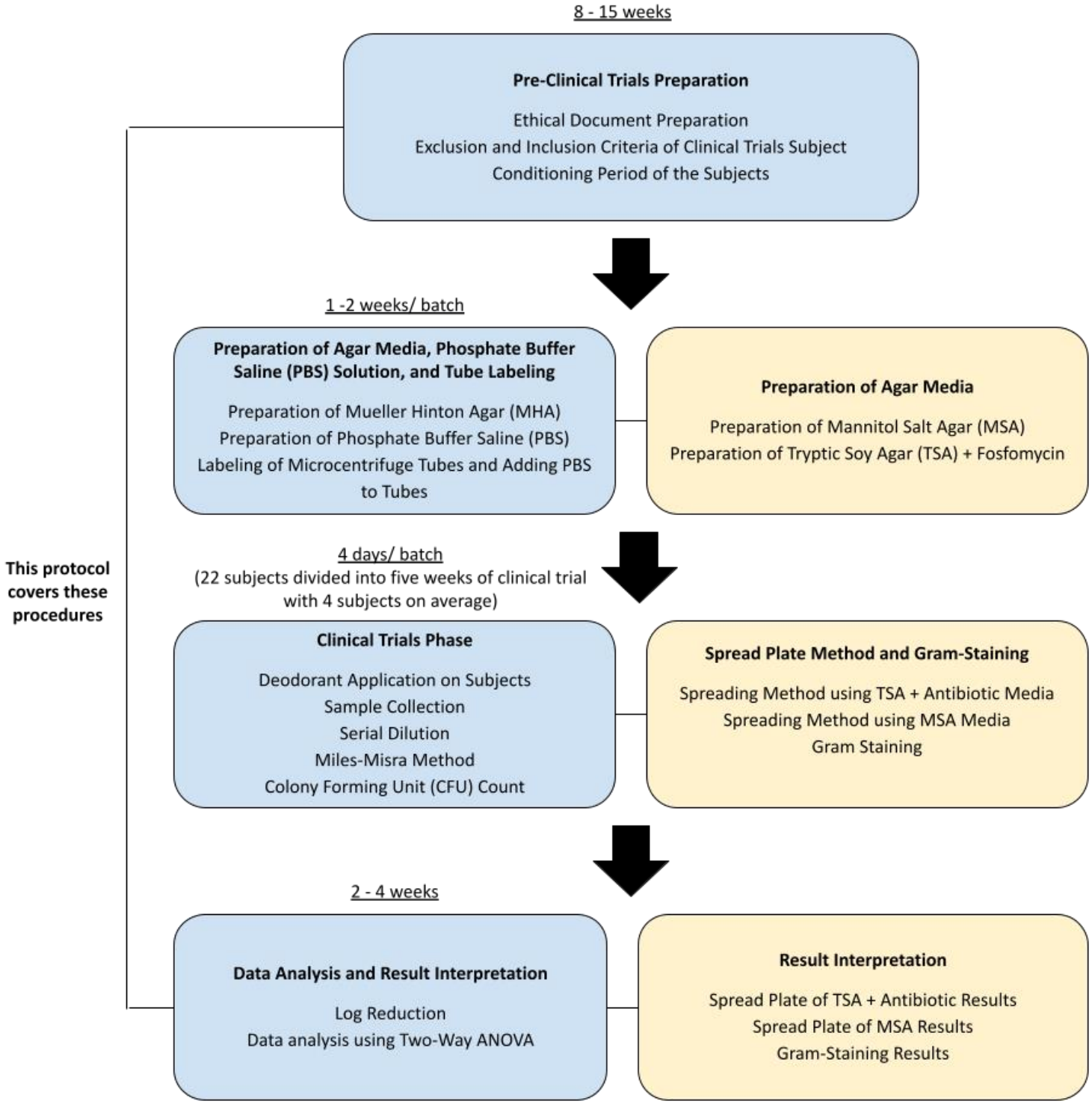
INTRODUCTION

Bad body odor or bromhidrosis is a common skin problem that many people experience. In a chronic stage, patients can experience psychological problems due to social stigma given by people in their society. There are multiple factors that influence the appearance of this malodor including gender, bad body odor-causing bacteria, age, sweating intensity, climate condition, and psychological stress (Sorokowska, Sorokowski & Szmajke, 2012). Among them, the secretion of sweat by the sweat glands and the presence of bacteria on the surface of the skin are the main factors that cause body odor. Sweat glands will produce an odorless secretion that contains fatty acids, steroids, sodium chloride, and many more. However, bad body odor-causing bacteria on the skin will transform these compounds into Volatile Odor Compounds (VOC) which trigger the appearance of bad body odor (Rudden et al., 2020). There are 3 dominant genera of bacteria that live on the axillary skin and contribute to body odor: *Staphylococcus spp*, *Corynebacterium spp*, and *Micrococcus spp*. The *axilla* area provides a humid environment which promotes the growth of these bacteria (Mogilnicka, Bogucki, & Ufnal, 2020).

Deodorant is a cosmetic product used to counteract bad body odor, especially in the *axilla* region. There are 3 main components of deodorant to show its action: antimicrobial agents, antiperspirant agents, and perfume (Bretagne, 2017). A common antimicrobial used in deodorant is triclosan which is able to disturb the fatty acid pathway and colony formation of bacteria (Lee et al., 2019). For antiperspirant agents, aluminum-based substances such as Aluminum Chlorohydrate and Aluminum Zirconium tetrachloro hydrate are the priority options for the deodorant product (Sakhawoth et al., 2021). These agents will block sweat gland secretion which thereby reduces the amount of substrate in the biotransformation process of VOC. Lastly, the addition of perfume agents, such as amyl cinnamal and anisyl alcohol, are used to mask the malodor scent (Minamino & Kanda, 2017).

Mueller-Hinton Agar (MHA) is a common, non-selective, and non-differential agar media used to grow bacteria colonies. One of the methods that can be done to grow bacteria is the Miles-Misra Method, where small drops of bacterial samples are placed on the agar before being incubated to let the bacteria grow. The results of the Miles-Misra method are similar to the spread plate method and only differ in the number of countable range of colonies. This is due to the volume of bacterial samples being plated on the agar initially. While the spread plate method is often done by spreading 100 µL of the bacterial sample, the Miles-Misra method it is done by adding three drops of 10 µL bacterial sample. Some other agar media might be used to specifically grow or identify particular bacteria, and these agar media are called selective media and differential media.

Flowchart


8 - 15 weeks
Pre-Clinical Trials Preparation
Ethical Document Preparation
Exclusion and Inclusion Criteria of Clinical Trials Subject
Conditioning Period of the Subjects
1 -2 weeks/ batch
Preparation of Agar Media, Phosphate Buffer Saline (PBS) Solution, and Tube Labeling
Preparation of Mueller Hinton Agar (MHA)
Preparation of Phosphate Buffer Saline (PBS)
Labeling of Microcentrifuge Tubes and Adding PBS to Tubes
Preparation of Agar Media
Preparation of Mannitol Salt Agar (MSA)
Preparation of Tryptic Soy Agar (TSA) + Fosfomycin
This protocol covers these procedures
4 days/ batch
(22 subjects divided into five weeks of clinical trial with 4 subjects on average)
Clinical Trials Phase
Deodorant Application on Subjects
Sample Collection
Serial Dilution
Miles-Misra Method
Colony Forming Unit (CFU) Count
Spread Plate Method and Gram-Staining
Spreading Method using TSA + Antibiotic Media
Spreading Method using MSA Media
Gram Staining
2 - 4 weeks
Data Analysis and Result Interpretation
Log Reduction
Data analysis using Two-Way ANOVA
Result Interpretation
Spread Plate of TSA + Antibiotic Results
Spread Plate of MSA Results
Gram-Staining Results

Methodology

Pre-Clinical Trials Preparation

Background

Ethical documents are necessary when conducting a study that involves human or animal subjects. Ethical documents ensure that the study being conducted is ethical and has minimum risks of harming the subjects. A particular team reviews and decides on whether or not the study is ethical enough to conduct. In this case, human subjects participated by testing out a deodorant on their axillary areas. Several inclusion and exclusion criteria are made to ensure that unwanted factors interfering the study such as the possibility of irritation due to the presence of wound or allergic reaction do not occur. Age group is also decided as sweat production is also affected by age. Other factors such as daily habit are controlled during the conditioning period, ensuring that all subjects undergo similar conditions to avoid variations due to external factors.

Goal

To obtain ethical clearance for the clinical trial, subjects based on the criteria, and to ensure that all subjects are under similar conditions throughout study which allow comparison to be made (excluding external factors that might affect the results).

Ethical Document Preparation

1. Prepare several documents such as clinical trials protocol; ethical form; explanatory form for clinical trials participant; cover letter for revision of ethics review application; list of researcher members and main researcher biodata; approval letter of the head of the institution; transfer receipt of payment for ethical review; and Good Clinical Practice (GCP)
2. Send all documents mentioned above to the University of Indonesia ethics committee that has a medical major.
3. The ethical form will be issued by the ethics committee once all documents are complete. This form is required for legal human clinical trials activity.

Exclusion and Inclusion Criteria of Clinical Trials Subject

1. Screen all subjects involved in this study with the designated inclusion and exclusion criteria.
2. All subjects have to fulfill the inclusion criteria and are excluded from this study if they meet one of the exclusion criteria.
3. List of inclusion and exclusion criteria of the subjects.

Inclusion	Exclusion
Healthy Male with age from 19-23 years old	Subject with skin problem at *axilla* area
The subject with a healthy skin condition in the *axilla* area (no acne, psoriasis, erythema,	The subject has an allergy to the product

edema, allergy)	
The subjects already received the COVID-19 vaccine	Subject tested positive for COVID-19 via antigen test
The subjects agree to sign an informed consent form	
The subjects tested negative for the COVID-19 antigen test	

Conditioning Period of the Subjects

1. All clinical trial participants need to have a conditioning period one week before day one of sample collection begins.
2. During the conditioning period, participants need to:
    a. Shave their *axilla* hair on the first day of the conditioning period.
    b. Not using other cosmetic/dermatological products and dermatological interference at their armpit area during the conditioning period until the last day of sample collection.
    c. Use a designated soap (Oilum soap bar) given by the researcher for showers.
    d. Not doing a heavy outdoor activity that results in massive sweat production such as exercise, vacation, sunbathing.
    e. Not eating spicy food that may result in massive sweat production.

Methodology

Preparation of Agar Media, Phosphate Buffer Saline (PBS) Solution, and Tube Labeling

Background

Mueller-Hinton Agar (MHA) is one of the common agar media used for bacterial growth. This agar media is non-differential and non-selective, allowing any bacteria present in the samples obtained able to grow. Phosphate Buffer Solution (PBS) is used to store the bacterial samples obtained prior to culturing them on MHA. PBS is chosen as it maintains bacterial viability without inducing growth as it has no essential nutrients for bacterial growth. Tubes are used as containers for the PBS and bacterial samples. The tubes are labeled accordingly to avoid confusion.

Goal

To prepare agar media, tubes, and PBS needed for the bacterial samples obtained in the clinical trial.

Materials

Mueller-Hinton Agar (MHA), Phosphate Buffer Solution (PBS) tablet, Sterile Petri dishes, 1.5 ml microcentrifuge tubes, micropipette tips, sterile cotton swabs.

Equipment

Marker, Ruler, Parafilm, Plastic wrapper, Scissors, Labels, Paraflims, Magnetic stirrer, Aluminum foil, Microcentrifuge racks, Biosafety Cabinet (BSC), Mason jar.

Procedure

A. Preparation of Mueller Hinton Agar (MHA)

1. In total, 451.44 ≈ 456 grams of MHA powder are needed for 22 subjects of this clinical trial.
2. Weight 19 grams of MHA powder and place it in 500 mL of Erlenmeyer Flask. Therefore, 24 Erlenmeyer Flasks are needed to make all agar for the experiment.
3. Measure 500 mL of Distilled Water Type III (DW Type III) using a graduated cylinder.
4. Add the measured DW Type III into the Erlenmeyer Flask.
5. Place the Erlenmeyer on a magnetic stirrer hotplate, then put a magnetic stirrer to dissolve the agar solution.
6. Take out the magnetic stirrer from the Erlenmeyer. Cover the opening part of the Erlenmeyer with aluminum foil.
7. Autoclave at 121°C for 15 minutes.
8. After the autoclave process, pour 15 mL of agar solution into the sterile Petri dish. Perform this step in the Biosafety Cabinet (BSC).
9. Let the agar solidify inside the BSC.

10. Close the petri dish with the cover. At the bottom part of the dish, using a marker divide the dish into 6 quadrants.
    a. **Note**: 5 quadrants are for dilution sample and 1 quadrant is dedicated for PBS solution as the negative control
11. Give the label for each plate according to
    a. Deodorant product: I/II
    b. Subject number: 1-22
    c. Day of sampling: 1/2/3
    d. Sampling time: 9/13/17
    e. Treatment/ Control : T/C
12. Group the Petri dish according to its sampling time and wrap it with plastic wrap. Store the agar in the 4°C fridge until further usage.

B. Preparation of Phosphate Buffer Saline (PBS)
    1. Take PBS tablets and dissolve them in type III water (1 tablet for 100ml), and calculate how much is needed in each preparation (1 mL for stock and 800ul for dilution).
    2. Try not to make too much at once to avoid contamination.
    3. Dissolve the tablet inside a Schott bottle.
    4. Close cap but not too tight.
    5. Autoclave the solution at 115℃ for 10 minutes.
    6. Secure cap after autoclave is done and parafilm the bottle to prevent contamination, and store inside the 4°C fridge until further usage.

C. Labeling of Microcentrifuge Tubes and Adding PBS to Tubes
    1. Tubes Labeling and filling PBS into Tubes
    2. Do inside the BSC
    3. Tubes for dilution have the numbers -1, -2, -3, -4, and -5 on its caps while stocks tubes do not
    4. Ignore “I” or “II” and “A” or “B” on the tube labels (example: I-D1-S3-9-CB, I and B can be ignored)
    5. Fill in tubes with C on its cap with 1,000 ul of PBS (tubes are for control, PBS only)
    6. Ensure tubes are closed properly
    7. Arrange them by the purpose of tubes, batch, day, and time (example: S1-S4 on D1 for 9 a.m. stock tubes are grouped into one and the dilution tubes are grouped into one)
    8. Wrap and secure them nicely in cling wrap
    9. Store in the clean fridge at 4°C temperature until further usage.

Methodology

Clinical Trials Phase

Background

This clinical trial may take up to three days of testing a deodorant on human subjects. The deodorant is applied only on one of the *axilla* as the comparison to the one with no deodorant applied. Three sampling times are done, at 9 a.m., 1 p.m., and 5 p.m. on each of the three days of testing. This represents the number of bacteria not long after deodorant application, at peak perspiration, and the end of the day which might show the longevity of the deodorant effectiveness. The bacterial samples are taken using sterile cotton swabs dipped in PBS for 45 seconds on a 10 by 10 centimeters template. This is done to avoid variations in the collection, which might affect the results. 10 x 10 centimeters area is also found to cover the average areas of a human axillary. Serial dilution is done to the bacterial samples obtained, to allow various bacterial concentrations to be cultured and when observed, a countable range can be achieved. The Miles-Misra method is a way to culture bacteria, similar to spread plate but using less materials. While the countable colonies range of the spread plate method is 30-300 colonies, while the Miles-Misra method ranges from 3-30 colonies per drop. The number of colonies obtained from three dots are averaged and converted to its original concentration, which allows the estimation of the initial bacterial sample concentration in CFU/mL unit.

Goal

To collect bacterial samples from both treated and untreated *axilla*, culture the bacteria on prepared MHA media using Miles-Misra Method, and observe the colony forming unit (cfu) after 24 hours of incubation.

Application of Deodorant Product on Clinical Trials Subjects

Materials

Sponge, Oilum liquid soap, Tested deodorant, Silicon Brush

Equipment

Marker, label, centrifuge rack,

Procedure

1. Approximately at 7.30-7.45 AM for each sampling day during 3 days of the clinical trial each participant comes to the dedicated sampling room
2. Wash both armpits of the participants with the wet sponge
3. Add liquid Oilum Soap to the wet sponge and apply it to both *axilla* of the participants
4. Wash the sponge with running water until no residual soap remains. Once again wash both *axilla* of the participants to clean the soap
5. Dry the axilla area using clean tissue paper
6. Put the tested deodorant on a silicon brush
7. Apply the deodorant using a silicon brush at the left *axilla* of each participant. Apply the deodorant in a zig-zag motion to allow equal distribution of the deodorant.
8. Let the deodorant dry for a few minutes.
9. Allow the participants to wait in the waiting room until sampling time comes.

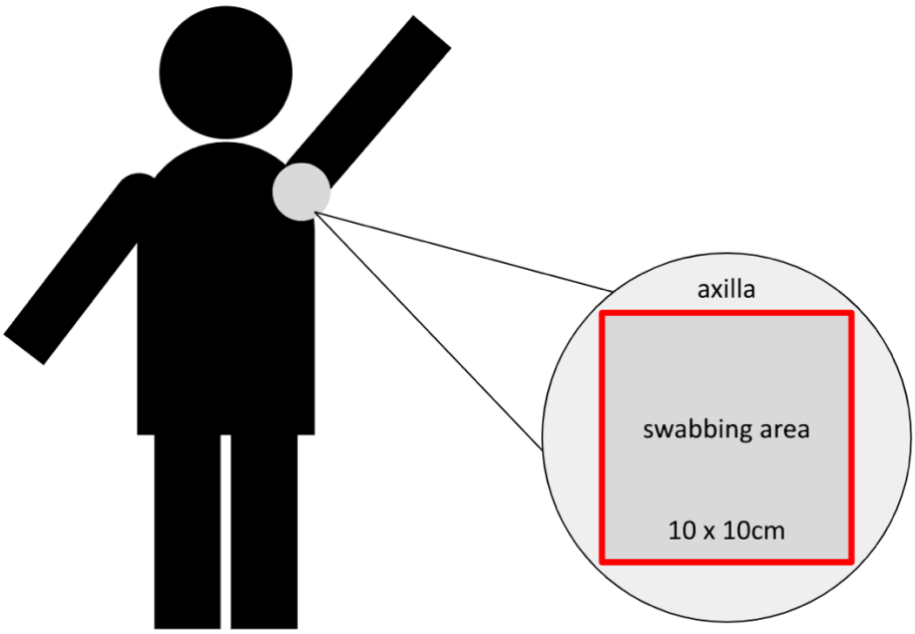


Figure 1. Representative illustration of 10x10 cm *axilla* area to take the *axillary* bacterial sample at both treated and untreated *axilla*

Sample Collection

Materials
PBS solutions, 1.5 mL Microcentrifuge tube, Sterile Cotton Swab, 10x 10 cm plastic mold

Equipment
Microcentrifuge rack, Scissor, Stopwatch

Procedure
1. The sample collection is done three times at different times which are at 9 AM, 1 PM, and 5 PM
2. Open the seal of the sterile cotton swab and dip the sterile cotton swab into a PBS solution for 5 seconds
3. Place the 10 cm x 10 cm plastic mold at the *axillary* area
4. Swab the axillary area using an up and down motion for 45 seconds (this will be done for both *axillary* areas) by following the mold area
    a. **NOTE**: Start at the top left corner of the axillary area and go downwards, following an up and down motion
5. Dip the swabbed cotton swab into a PBS solution in a 1.5 mL microcentrifuge tube and cut the cotton swab to fit it into the microcentrifuge tube
6. Put the centrifuge tube that had the cotton swab into the centrifuge rack

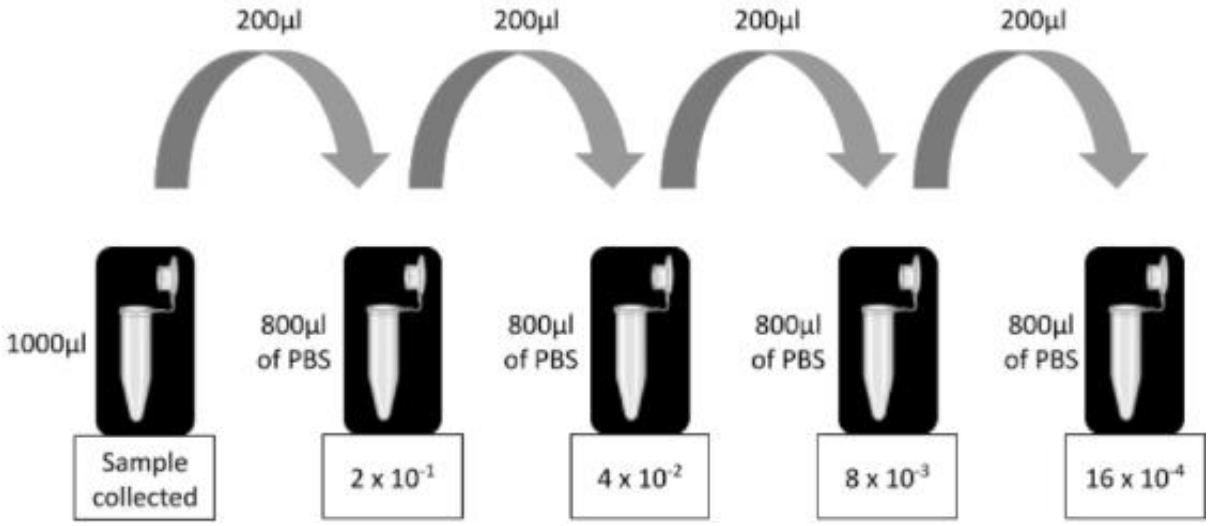
Serial Dilution

Materials
1.5 ml Microcentrifuge tube, Phosphate buffer solution (PBS), Micropipette tips 1000, Stock solution, 10% Bleach solution

Equipment
Beaker, Biosafety Cabinet (BSC), Micropipette 1000 µL, 1.5 mL Microcentrifuge rack

Procedure
1. Transfer 200 µL of PBS solution from the stock sample of both treated and control to the next centrifuge tube containing 800 µL of sterile PBS solution.
2. Resuspend several times to mix the solution and create $2x10^{-1}$ dilution. Discard the tip to 10% bleach solution
3. Using a different 1000 µL tip, transfer 200 µL of solution from the tube containing $2x10^{-1}$ solution into the second tube containing 800 µL PBS to form a $4x10^{-2}$ dilution and resuspend. Discard the tube to bleach solution
4. Take a different 1000 µL tip, and transfer 200 µL of solution from the tube containing $4x10^{-2}$ solution into the second tube containing 800 µL PBS to form an $8x10^{-2}$ solution. Discard the tip to bleach solution
5. Transfer 200 µL of solution from $8x10^{-3}$ to another tube containing 800 µL of PBS to form $16x10^{-4}$ dilution and resuspend it. Discard the tip to the bleach solution.

6. Take 200 µL of solution from $16x10^{-4}$ to another tube containing 800 µL of PBS to form a $32x10^{-5}$ dilution and resuspend it. Discard the tip.
7. Perform all steps for making serial dilution inside BSC.



Miles-Misra Method

Materials

1.5 mL Microcentrifuge Tube, Mueller-Hinton Agar (MHA), Micropipette tips 10 µL, Dilution sample, Phosphate Buffer Saline (Negative control), 10% Bleach solution, Parafilm

Equipment

Beaker, 1.5 mL Microcentrifuge rack, Micropipette 10 µL, Biosafety Cabinet (BSC), Incubator

Procedure

1. Take 10 µL of each dilution sample ($2x10^{-1}$ - $32x10^{-5}$) and place it at the dedicated quadrant on the MHA plates.
2. Each dilution was created as a triplicate. Therefore, each quadrant contains 3 drops of the corresponding dilution sample.
3. Take 10 µL of sterile PBS solution three times to make triplicate and place it on the dedicated quadrant for negative control on the MHA plates.
4. Make a duplicate for each sample for every participant. Therefore, there are 2 MHA plates for the control armpit sample (Control A and Control B) and 2 MHA plates for the treated armpit sample (Treatment A and Treatment B).
5. Let the sample on the agar dry for a while inside BSC.
6. Parafilm the plates 2 times.
7. Flip the plates upside down and incubate at 37°C for 24 hours.

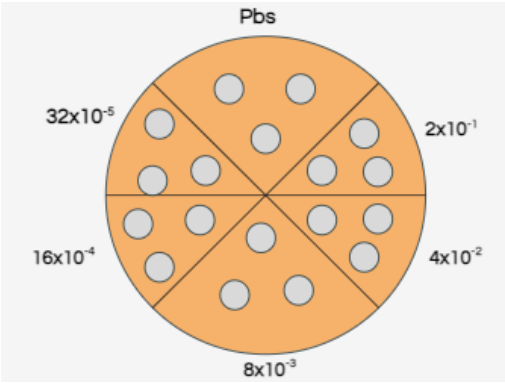


Figure 2. Illustration model of six quadrants division on Mueller Hinton Agar plate to culture dilution $2x10^{-1}$ until $32x10^{-5}$ and PBS culture using Miles-Misra Method

Colony Forming Unit (CFU) Count

Materials

24 hours incubated sample

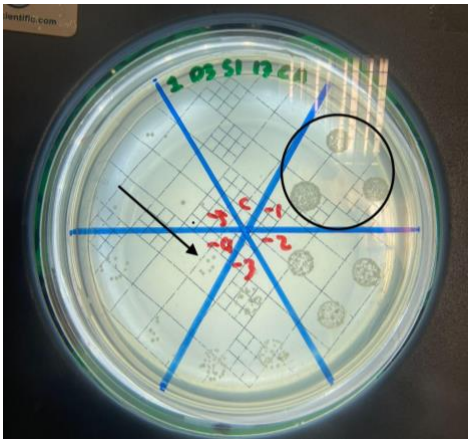
Equipment

CFU counter

Procedure

1. Take the sample out from the incubator following one-night incubation
2. Count the number of colonies present at each drop of the sample in every quadrant
    a. Range of countable colonies for Miles-Misra is between 3-30 colonies
    b. If a colony in each drop is found to be below 3 colonies, it will be considered as Too View Too Count (TVTC).
    c. If colonies in each drop are more than 30 colonies it will be considered Too Many Too Count (TMTC)
3. Average the number of colonies of each triplicate in every quadrant that represents the dilution of the sample.
4. Calculate the number of colonies in the initial sample (CFU/mL) according to the formula:

$$\text{No. of countable colony} \times \frac{1}{10^{-x}\ (Lowest\ dilution\ with\ the\ countable\ colonies\ in\ miles\ misra)} \times 0.01$$



Methodology

Data Analysis and Result Interpretation

Background

Log reduction indicates the reduction in bacterial concentration in samples collected from axillary areas with no deodorant compared to samples collected from axillary areas with deodorant. Two-Way ANOVA is done to analyze the data sets and its significance

Goal

To calculate the reduction of bacterial concentration between treated (axillary with deodorant) and non-treated (axillary without deodorant) group, and to see its significance.

Log Reduction

1. Calculate Log reduction with the following formula:

$$Log\ reduction\ =\ log10\frac{N0}{N}$$

   note: *N0* represents the CFU of untreated while *N* represents CFU of treated
2. Collect data and analyze using Two-Way ANOVA, there should be a significant difference between the control and the treated group, where the amount of bacterial colony present in the treated group should be less than the control group ($p<0.05$)

REFERENCES

Abrutyn, E. (2010). Antiperspirants and Deodorants. Cosmetic Dermatology, 150-155. https://doi.org/10.1002/9781444317657.ch20

Baker, L. (2019). Physiology of sweat gland function: The roles of sweating and sweat composition in human health. Temperature, 6(3), 211-259. https://doi.org/10.1080/23328940.2019.1632145

Bensen, D., Rodriguez, S., Nix, J., Cunningham, M., & Tari, L. (2012). Structure of MurA (UDP-N-acetylglucosamine enolpyruvyl transferase) fromVibrio fischeriin complex with substrate UDP-N-acetylglucosamine and the drug fosfomycin. Acta Crystallographica Section F Structural Biology And Crystallization Communications, 68(4), 382-385. https://doi.org/10.1107/s1744309112006720

Butcher, B., & Helmann, J. (2006). Identification of Bacillus subtilis sigmaW-dependent genes that provide intrinsic resistance to antimicrobial compounds produced by Bacilli. Molecular Microbiology, 60(3), 765-782. https://doi.org/10.1111/j.1365-2958.2006.05131.x

Falagas, M., Athanasaki, F., Voulgaris, G., Triarides, N., & Vardakas, K. (2019). Resistance to fosfomycin: Mechanisms, Frequency and Clinical Consequences. International Journal Of Antimicrobial Agents, 53(1), 22-28. https://doi.org/10.1016/j.ijantimicag.2018.09.013

Menberu, M., Hayes, A., Liu, S., Psaltis, A., Wormald, P., & Vreugde, S. (2020). Tween 80 and its derivative oleic acid promote the growth of Corynebacterium accolens and inhibit Staphylococcus aureus clinical isolates. International Forum Of Allergy &Amp; Rhinology, 11(4), 810-813. https://doi.org/10.1002/alr.22730

Manayi, A., & Saeidnia, S. (2014). Cosmetics and Personal Care Products. Encyclopedia Of Toxicology, 1043-1049. doi: 10.1016/b978-0-12-386454-3.00979-9

Minamino, M., & Kanda, F. (2017). Global Cosmetic R&D Trends Unveiled From Past IFSCC Award-Winning Papers. Cosmetic Science And Technology, 15-38. https://doi.org/10.1016/b978-0-12-802005-0.00002-1

Mogilnicka, I., Bogucki, P., & Ufnal, M. (2020). Microbiota and Malodor—Etiology and Management. International Journal Of Molecular Sciences, 21(8), 2886. https://doi.org/10.3390/ijms21082886

Rudden, M., Herman, R., Rose, M., Bawdon, D., Cox, D., & Dodson, E. et al. (2020). The molecular basis of thioalcohol production in human body odour. Scientific Reports, 10(1). doi: 10.1038/s41598-020-68860-z

Shrestha, P., Zhang, Y., Chen, W., & Wong, T. (2020). Triclosan: antimicrobial mechanisms, antibiotics interactions, clinical applications, and human health. Journal Of Environmental Science And Health, Part C, 38(3), 245-268. https://doi.org/10.1080/26896583.2020.1809286

Scybert, S., Pechous, R., Sitthisak, S., Nadakavukaren, M., Wilkinson, B., & Jayaswal, R. (2003). NaCl-sensitive mutant ofStaphylococcus aureushas a Tn917-lacZinsertion in itsarsoperon. FEMS Microbiology Letters, 222(2), 171-176. https://doi.org/10.1016/s0378-1097(03)00312-4