Practical activity (9)

Antimicrobial Study of Fruit Juice and Herbal Tea

(Student version)

I. Scenario

Our laboratory technician Nancy likes consuming healthy drinks. Apple juice, pomegranate juice, and honeysuckle flower tea are her favourites. Recently, she learnt from a news report that fruit juices and Chinese herbal tea are found to have some antimicrobial properties. She wants us to test for her whether these three drinks provide any antimicrobial properties. To perform an antimicrobial test, we will first collect some microbes from our environment. Then, we will grow them in nutrient broth for use in our experiments, we will compare the effectiveness of these three drinks on the inhibition of colony formation on agar plates.

II. Guiding questions about the design of the experiment

1. What equipment is suitable for the collection of microbiological samples from the environment?
2. How can we estimate the microbial density of a broth culture without a sophisticated instrument?
3. If a test drink is claimed to be antimicrobial, what do we expect to observe on the agar plates after the microbial incubation period?

III. Objectives of the experiment

1. To collect and culture microbial samples from the daily environment.
2. To estimate the microbial density of a broth culture by comparing the cloudiness of broth culture with the 0.5 McFarland turbidity standard.
3. To investigate the antimicrobial effect of the selected fruit juices and herbal tea.

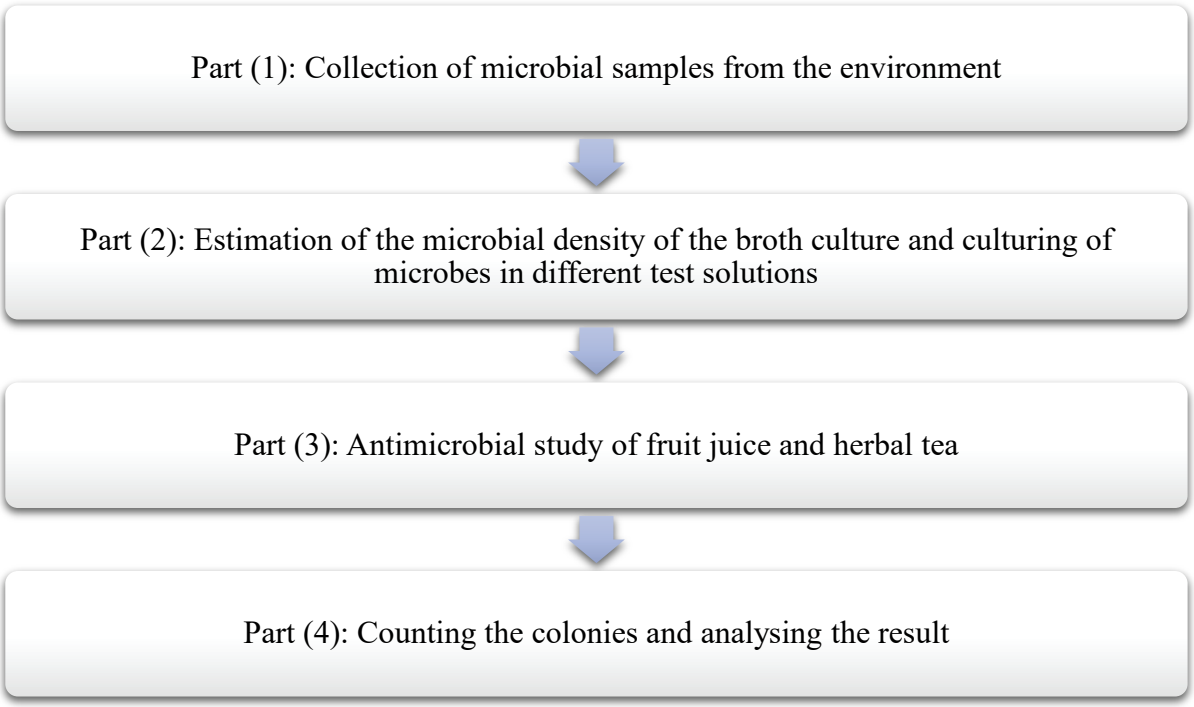
IV. Expected Learning Outcomes

Upon completion of the learning activities, students should be able to:

1. culture microorganisms collected from the environment;
2. estimate the microbial density of broth culture by comparing it to turbidity standards; and
3. evaluate the antimicrobial effect of fruit juices and herbal tea according to inhibition of colony formation.

V. The experiment

A. Overview



B. Part (1) of the experiment: Collection of microbial samples from the environment

a) Equipment and materials (per group)

Equipment

No equipment needed

Materials

- Culture tube with sterile LB broth (10 ml) × 1 (per student)
- Sterile swab (breakable stick with a cotton bud) × 1 (per student)
- Culture tube rack × 1
- Masking tape × 1
- Ruler × 1

- Permanent marker × 1
- 70% ethanol in spray bottle × 1
- Paper towel × 1 box
- Biohazard bag × 1
- Disposal container with 10% chlorine bleach × 1

b) Safety precautions

- Wear a laboratory gown.
- Tie up long hair.
- Wear gloves during the experiment.
- Wash hands to remove all possible contamination before and after the experiment.
- Keep ethanol away from fire.
- Make sure ethanol is fully vaporised before approaching any flame.
- Dispose of or disinfect all materials properly after each experiment.

c) Procedure

1. Label the LB broth-containing culture tube with the date of the experiment, your group number, and choice of environmental surface*.

***Caution: Collection of samples from potentially dangerous sources such as sewage is prohibited.**
Collection of samples from the environment during a pandemic is prohibited.

2. Choose an appropriate size of the area (e.g. 5 × 5 cm) for surface sampling collection (e.g. door handle, benchtop, lift button, mobile phone screen).

3. Use masking tape to mark the borders of the selected area as shown in Fig. 1.

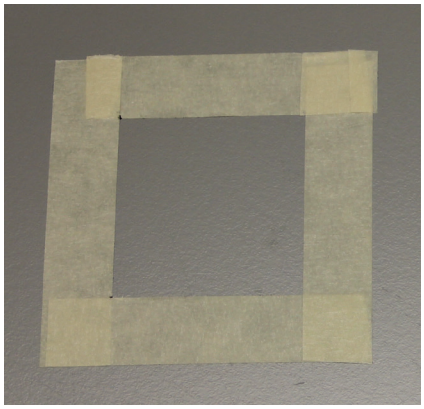


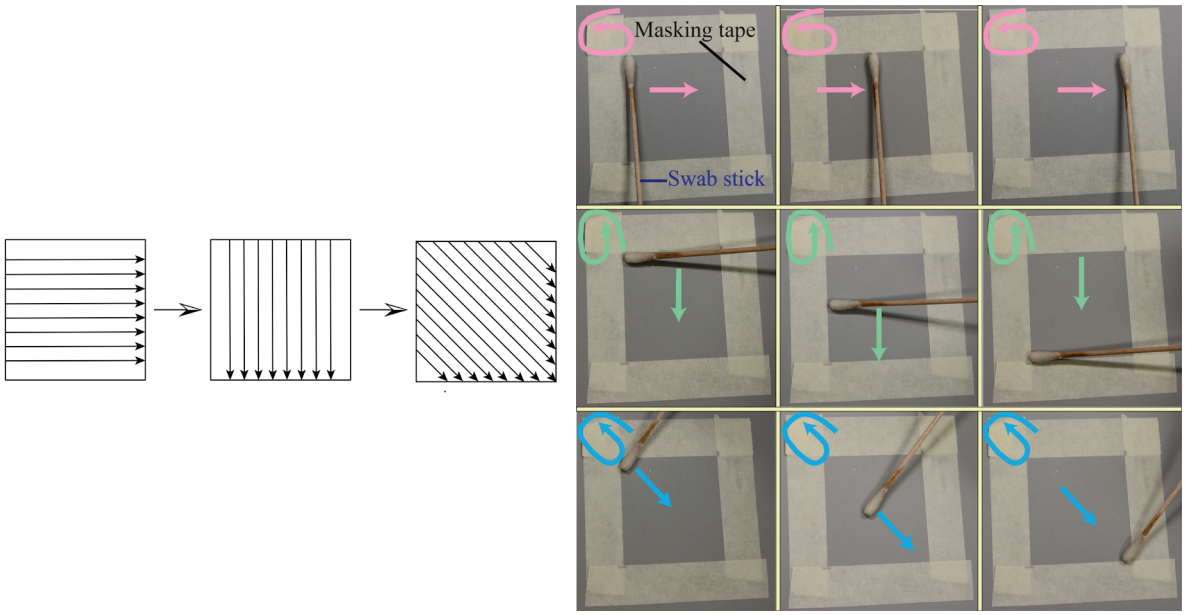
Fig. 1

4. Remove a swab stick from the sterile wrapping. Moisten the head by immersing it in the LB broth of the culture tube.

5. Press the swab head against the tube wall to remove excess liquid (Fig. 2).

Fig. 2

6. Swab across the selected surface* according to Fig. 3a in three different swab directions, while rotating the swab continuously, to collect as many microbes as possible from the selected area (Fig. 3b).



(a) (b)

Fig. 3

7. Aseptically break off the head of the swab into the sterile LB broth. Cap the tube. (Fig. 4).

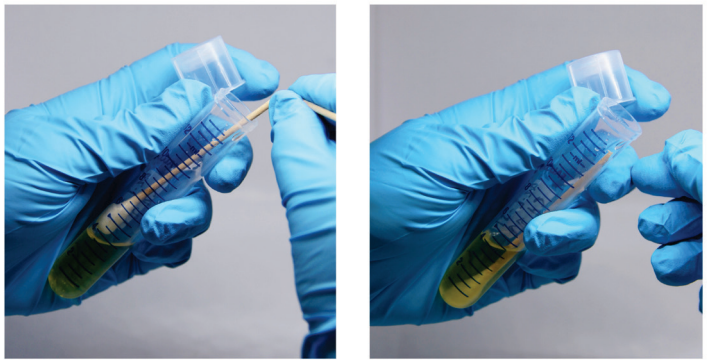


Fig. 4

8. Incubate the culture tube at room temperature# or in an incubator set at 25−30 °C for 2 days.

> #Note: Room temperature may vary, depending on the ambient conditions of the laboratory environment. Hence, the incubation time may also vary.

9. Discard the swab in the designated disposal container with 10% chlorine bleach (no autoclave processing required) or in a biohazard bag for autoclave processing afterwards.

C. Part (2) of the experiment: Estimation of the microbial density of the broth culture and culturing of microbes in different test solutions

a) Equipment and materials (per group)

Equipment

- Bunsen burner × 1
- Spark lighter × 1
- Micropipettes (P5000 and P1000) and sterile tips

Materials

- LB broth culture from Part (1) × 1 (per student)
- Sterile LB broth (30 ml) × 1
- Apple juice (1.5 ml) × 1
- Pomegranate juice (1.5 ml) × 1
- Honeysuckle flower tea (1.5 ml) × 1
- Ampicillin (25 mg/ml, 1.5 ml) × 1
- Sterile distilled water (1.5 ml) × 1
- 0.5 McFarland turbidity standard × 1
- Cardboard with black and white strips × 1
- Culture tube rack × 1
- Micro-centrifuge tube rack × 1

- Permanent marker × 1
- 70% ethanol in spray bottle × 1
- Paper towel × 1 box
- Biohazard bag × 1
- Disposal container with 10% chlorine bleach × 1

b) Safety precautions

- Wear a laboratory gown.
- Tie up long hair.
- Wear gloves during the experiment.
- Wash hands to remove all possible contamination before and after the experiment.
- Keep ethanol away from fire.
- Make sure ethanol is fully vaporised before approaching any flame.
- Dispose of or disinfect all materials properly after each experiment.

c) Procedure

I. Estimation of microbial density of the broth culture by comparing it with the 0.5 McFarland standard

1. Finger-flick or wrist-flick your microbial culture tube from Part (1) and the 0.5 McFarland turbidity standard* tube thoroughly.

> *Note: The 0.5 McFarland turbidity standard is commonly used as a reference in antimicrobial tests. The standard provides an optical density comparable to that of a bacterial suspension with 1.5×10^8 colony forming unit (CFU) per ml. In this experiment, we would like to obtain a broth culture with microbial density of about 1.5×10^8 colony forming unit (CFU) per ml.

2. Place the two tubes in front of the cardboard with black and white stripes (Fig. 5).

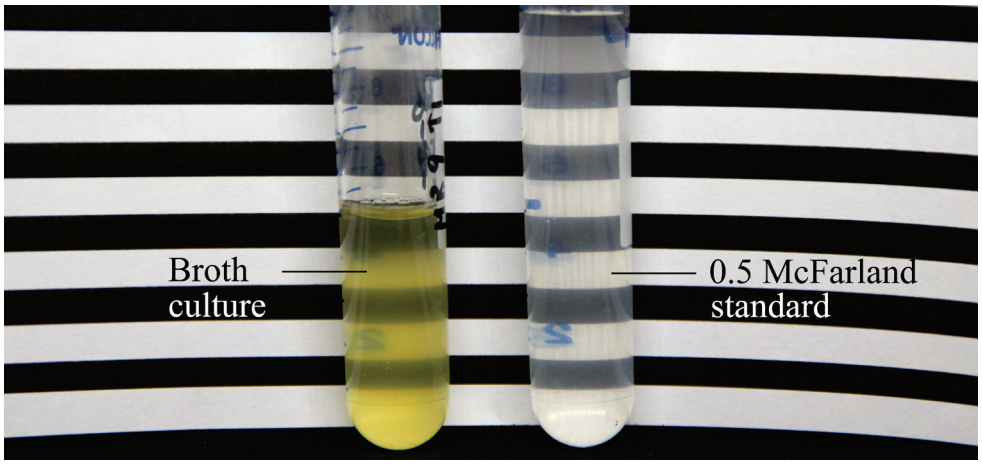


Fig. 5

3. Compare the turbidity of the microbial broth culture with the 0.5 McFarland standard, with the naked eye.

4. Record the turbidity of the microbial broth culture of each member of the group as compared to the 0.5 McFarland standard (i.e. higher than / lower than / similar to the standard).

5. Choose the culture broth among the group whose turbidity is the closest to the 0.5 McFarland standard. This tube of broth culture will be used in Section (II) of this experiment.

II. Microbial culture in test solutions

1. Disinfect the benchtop and gloves with 70% ethanol^.

^Caution: Keep away from fire! DO NOT light the flame until the ethanol has been completely vaporised!

2. Label 5 culture tubes with the different test solutions according to Table 1, the date of the experiment, your class, and group number (Fig. 6).

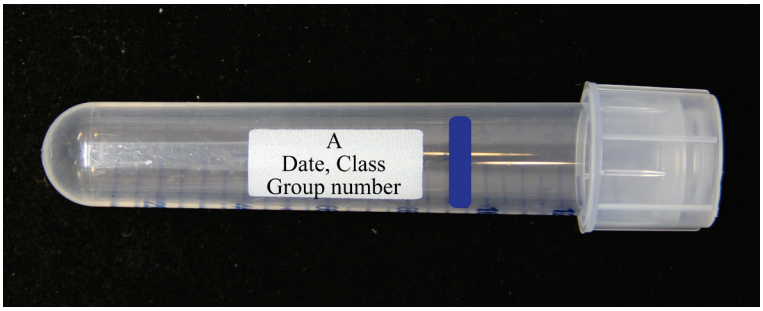


Fig. 6

Table 1

Label	A	P	H	Amp	W
Test solution	Apple juice	Pomegranate juice	Honeysuckle flower tea	Ampicillin	Sterile Distilled water

3. Light a Bunsen burner.

4. Aseptically# use a P5000 micropipette to transfer 3 ml fresh LB broth to each of the 5 culture tubes.

#Note: Please refer to Step 2 of Appendix 1 for details of the aseptic technique.

5. Use a P1000 micropipette and a new tip to add 1 ml of each test solution into the corresponding culture tube according to Table 1. Mix them by gently shaking.

6. Use a P1000 micropipette and a new tip to transfer 1 ml microbial culture of the selected tube from Section (I) of this experiment to each of the 5 culture tubes containing the different test solutions. Mix them by gently shaking.

7. Turn off the Bunsen burner.

8. Incubate all culture tubes at room temperature* or in an incubator set at 25–30 °C for 2 days.

*Note: Room temperature may vary, depending on the ambient conditions of the laboratory environment. Hence, the incubation time may also vary.

9. Discard all unwanted microbial culture tubes and used tips in the designated disposal container with 10% chlorine bleach (no autoclave processing required) or in a biohazard bag for autoclave processing afterwards.

D. Part (3) of the experiment: Antimicrobial study of fruit juice and herbal tea

a) Equipment and materials (per group)

Equipment

- Bunsen burner × 1
- Spark lighter × 1
- Micropipettes (P5000, P1000 and P200) and sterile tips

Materials

- Microbial broth culture from part (2) × 5
- Culture tube rack × 1
- LB agar plates × 5
- 15-ml centrifuge tube × 15
- Centrifuge tube rack × 1
- Sterile LB broth (200 ml) × 1
- Inoculating loop × 1

- Permanent marker × 1
- 70% ethanol in spray bottle × 1
- Paper towel × 1 box
- Biohazard bag × 1
- Disposal container with 10% chlorine bleach × 1

b) Safety precautions

- Wear a laboratory gown.
- Tie up long hair.
- Wear gloves during the experiment.
- Wash hands to remove all possible contamination before and after the experiment.
- Keep ethanol away from fire.
- Make sure ethanol is fully vaporised before approaching any flame.
- Dispose of or disinfect all materials properly after each experiment.

c) Procedure

1. Disinfect the benchtop and gloved hands with 70% ethanol*.

***Caution: Keep away from fire! DO NOT light the flame until the ethanol has been completely vaporised!**

2. Collect the complete set of 5 microbial broth culture tubes "A", "P", "H", "Amp" and "W" prepared in Part (2).

3. Label 10 centrifuge tubes A1, A2, P1, P2, H1, H2, Amp1, Amp2, W1, and W2 and with your group number.

4. Light a Bunsen burner.

5. To each of the above centrifuge tubes, aseptically* use a P5000 micropipette and sterile tips to add 9.9 ml sterile LB broth (Fig. 7).

*Note: Please refer to Step 2 of Appendix 1 for details of the aseptic technique.

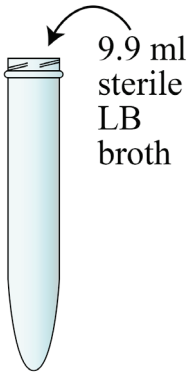


Fig. 7

6. Label 5 centrifuge tubes A3, P3, H3, Amp3, and W3 and with your group number.

7. To each of the above centrifuge tubes, aseptically# use a P5000 micropipette and sterile tips to add 9 ml sterile LB broth (Fig. 8).

#Note: Please refer to Step 2 of Appendix 1 for details of the aseptic technique.

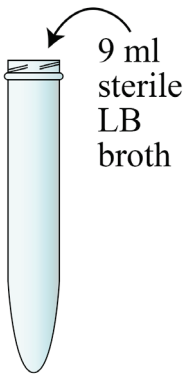


Fig. 8

8. Label 5 LB agar plates A3 (Fig. 9), P3, H3, Amp3, and W3 and with the date of the experiment and your group number.

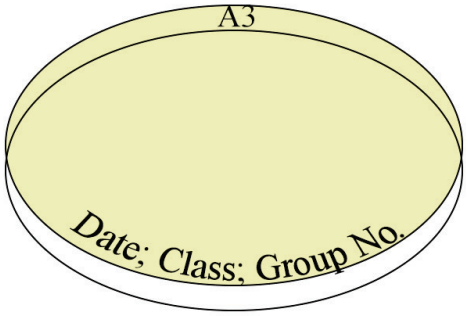


Fig. 9

9. Aseptically^ use a P200 micropipette and a new tip to transfer 100 µl microbial broth culture from tube "A" to tube "A1" (Fig. 10). Hence, the mixture in tube "A1" is a 100-fold dilution of the microbial broth culture in tube "A". Mix tube "A1" by turning it upside down three times.

^Note: Please refer to Step 2 of Appendix 1 for details of the aseptic technique.

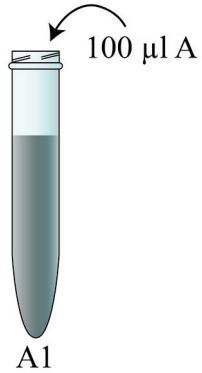


Fig. 10

Please complete the table below.

Table 2

	Tube A	Tube A1	Tube A2	Tube A3
Dilution fold	0	100-fold dilution of tube A	100-fold dilution of tube A1	10-fold dilution of tube A2
Dilution fold of the microbial broth culture				

10. Next, use a P200 micropipette and a new tip to transfer 100 µl mixture from tube "A1" to tube "A2" (Fig. 11). Hence, the mixture in tube "A2" is a 100-fold dilution of the "A1" mixture. Mix tube "A2" by turning it upside down three times.

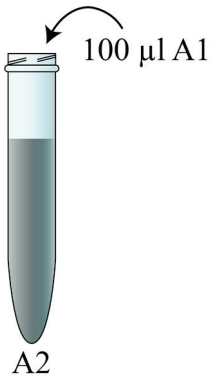


Fig. 11

11. Use a P1000 micropipette and a new tip to transfer 1 ml mixture from tube “A2” to tube “A3” (Fig. 12). Hence, the mixture in tube A3 is a 10-fold dilution of the “A2” mixture. Mix tube “A3” by turning it upside down three times.

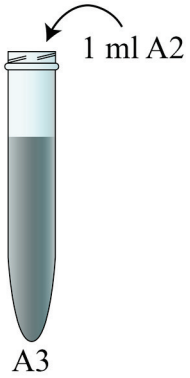


Fig. 12

12. Repeat Steps 9–11 to make three sets of dilutions for the broth culture of tubes “P”, “H”, “Amp” and “W”.

13. Flame the inoculating loop to red hot (Fig. 13) and allow it to cool*.

> *It is essential to flame the metal inoculating loop to red hot to eliminate any previous bacterial residue, to prevent contamination of the current sample.
>
> It is also important to cool the loop, as insufficient cooling kills the inoculated bacteria.
>
> If a sterile plastic disposable inoculating loop is provided, flaming is not required.
>
> Use a new inoculating loop when handling different microbial samples.

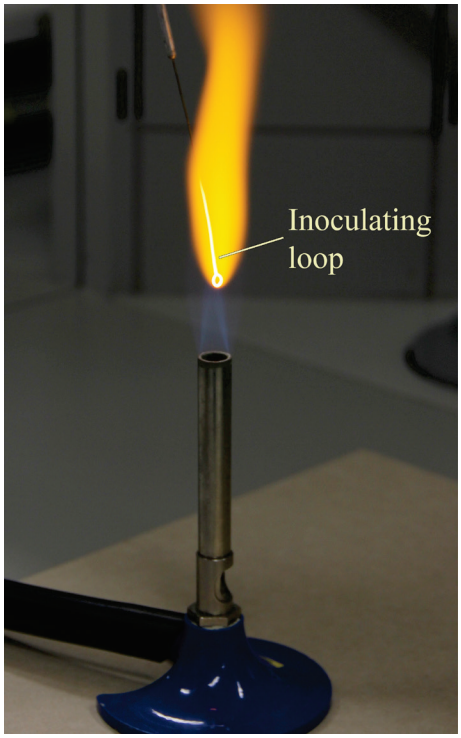


Fig. 13

14. Aseptically* obtain a loopful of solution from tube "A3" (Fig. 14).

*Note: Please refer to Steps 3–4 of Appendix 1 for details of the aseptic technique.

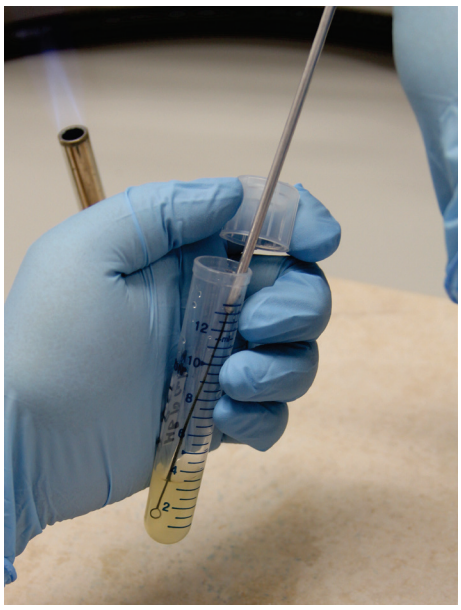


Fig. 14

15. Raise the lid of agar plate "A3" at about 45° (Fig. 15)# . Put the inoculating loop onto the agar.

#Note: This is to minimise the exposure of agar to the atmosphere.

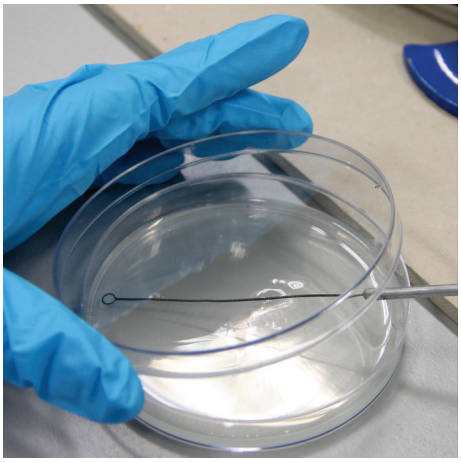


Fig. 15

16. Streak the whole agar plate "A3" in a zigzag pattern (Fig. 16).

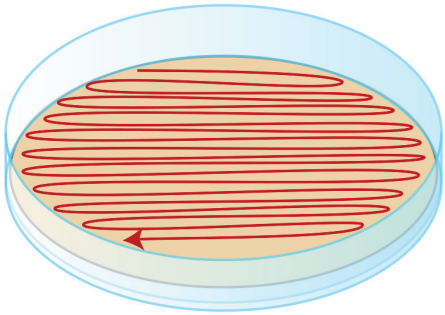


Fig. 16

17. Replace the lid.

18. Repeat Steps 13–17 for the 4 remaining solutions (P3, H3, Amp3, and W3) and their corresponding plates.

19. Flame the loop and allow it to cool.

20. Turn off the Bunsen burner.

21. Stick short strips of adhesive tape at opposite edges of the agar plates to hold the lid and base together as shown in Fig 17. Invert all the plates and incubate them at room temperature* or in an incubator set at 25−30°C for 2 days.

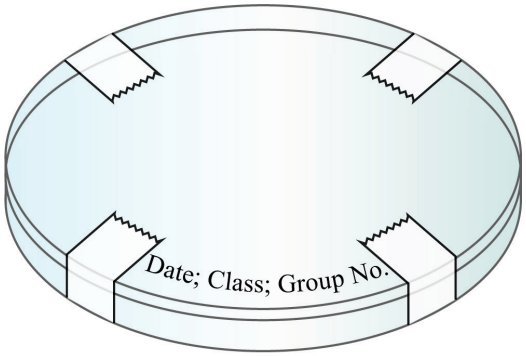


Fig. 17

*Note: Room temperature may vary, depending on the ambient conditions of the laboratory environment. Hence, the incubation time may also vary.

22. Discard all unwanted microbial culture tubes and used tips in the designated disposal container with 10% chlorine bleach (no autoclave processing required) or in a biohazard bag for autoclave processing afterwards.

E. Part (4) of the experiment: Counting the colonies and analysing the result

a) Equipment and materials (per group)

Equipment

- Mobile device × 1

Materials

- LB agar plate from Part (3) × 5
- Permanent marker × 1
- 70% ethanol in spray bottle × 1
- Paper towel × 1 box
- Biohazard bag × 1
- Disposal container with 10% chlorine bleach × 1

b) Safety precautions

- Wear a laboratory gown.
- Tie up long hair.
- Wear gloves during the experiment.
- Wash hands to remove all possible contamination before and after the experiment.
- Keep ethanol away from fire.
- Make sure ethanol is fully vaporised before approaching any flame.
- Dispose of or disinfect all materials properly after each experiment.

c) Procedure

1. Collect all the LB agar plates of your group. Using a permanent marker, count the colonies by marking dots on the back of the plates.

2. Record the number of (CFU)[#] on all the plates.

> [#]Note: Only plates with colony-forming units (CFU) between 25 and 250 can be used for counting.
>
> Using the term "CFU" instead of "colony" is to reflect the fact that a single colony observed on an agar plate may not be formed by a single bacterium.In fact, such a colony can be formed by several bacteria (more than one bacterium) growing in proximity.
>
> If we present our data using the term "colony", then the actual number of bacteria in the test sample may be underestimated.

3. Take pictures of the plates, using a mobile device.

4. After observation and counting, discard all unwanted microbial culture plates in the designated disposal container with 10% chlorine bleach (no autoclave processing required) or in a biohazard bag for autoclave processing afterwards.

VI. Results

1. The choice of your sampling environmental surface is

   _____, and the sampling area is _____ cm^2.

2. Record of the microbial samples collected and the turbidity of the broth culture of the group.

Name of member	Sampling environmental surface	Area of the sampling surface (cm^2)	Turbidity of the broth culture as compared with the 0.5 McFarland turbidity (higher / similar to / lower)	Whose broth culture is selected for further culturing in different test solutions? (Put a "✓".)

3. Paste the photo or draw a picture of the LB agar plates after incubation in the respective boxes below.

“A” Apple juice	“P” Pomegranate juice
“H” Honeysuckle flower tea	“Amp” Ampicillin solution
“W” Sterile distilled water	

4. Record the number of CFU on the LB agar plates in the following table.

Test solution	**Number of CFU**
Apple juice	
Pomegranate juice	
Honeysuckle flower tea	
Ampicillin solution	
Sterile distilled water	

VII. Discussion

1. Suggest a reason why we have to make dilutions of the microbial broth culture before plating.

2. The number of colony-forming units (CFU) shows a positive correlation with the growth of the microbes. Thus, which test solution allows the most growth of microbes? Which test solution provides the best antimicrobial effect?

3. Based on the rationale of question 2 and the experimental data, arrange the antimicrobial property of the test fruit juices and herbal tea in descending order.

4. Ampicillin solution and sterile distilled water served as controls in this experiment with different functions. State the rationale for setting these two different controls.