INTERNATIONAL BACCALAUREATE DIPLOMA PROGRAMME

BIOLOGY EXTENDED ESSAY

How does the diameter of the inhibition zones formed on agar plates containing bacteria (*S. aureus*, *P. aeruginosa*, *E. coli*) exposed to different strains of *Lactococcus* (1.2, 6.2, 10.2) change observed by well diffusion method and how may *Lactococcus* be used for food coating observed by disc diffusion method?

Word Count: 3902

TABLE OF CONTENTS

INTRODUCTION

Bacteria have been usual sample organisms in biological research for a long time. But they are not merely experimentation subjects. They are everywhere around us. From the handle of the cup we use every day to drink water to the food we eat. While some of these bacteria are harmless, some of them are. The negative effects of bacteria include sicknesses, disruption of the biological balance of an organism and food decaying. For example, if raw meat is left out for too long, it decays. As it makes contact with air, oxidation reactions occur, encouraging bacterial growth on the meat. In time, as more bacteria forms, the meat decays more and becomes unhealthy. When consumed, these bacteria release toxins that disrupt the order of the intestines[12]. What if there was a way to prevent this? Maybe not fully but to some extent. This research aims to identify three species of bacteria produced on decaying food and inhibit them.

Lactic acid bacteria, with *Lactococcus* being a type, is microorganism known for its antibacterial traits[7]. Microorganisms like this are known as antibacterial agents. Being effective in eliminating bacterial formations, antibacterial agents have a broad field of usage. When added on bacteria, they release many substances that has antibacterial effects. Hydrogen peroxide and organic acids (lactic acid and acetic acid) being the most well-known ones, ethanol, diacetyl, acetaldehyde, acetoin, carbon dioxide, reuterin, reutericyclin and bacteriocins are amongst the antibacterial products of *Lactococcus*[11]. The effects of *Lactococcus* can usually be seen on both Gram-positive and Gram-negative types of bacteria. By the observation of inhibition zones formed on the bacteria, the effectiveness of *Lactococcus* can be measured. Larger inhibition zones indicate more effective antibacterial activity. So, *Lactococcus* causing the formation of wide inhibition zones would be a sign that it is a strong antibacterial agent.

There are many types of bacteria found on decay food. Three of those types are *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Two of these – *E. coli and P. aeruginosa* – are Gram-negative bacteria, whereas, *S. aureus* is Gram-positive. The difference between Gram-positive and Gram-negative bacteria is the thickness of the peptidoglycan layer outside of them[17]. Gram-negative bacteria have a thin peptidoglycan layer at around 2-3 nanometers with an outer lipopolysaccharide layer[6]. However, Gram-positive bacteria do not have an outer layer. Instead, they have a thicker peptidoglycan layer reaching up to 80 nanometers[15]. When put through Gram staining procedure, a test where different colored dyes are applied to determine the presence and type of bacteria in a sample, Gram-positive bacteria appear violet and Gram-negative appear red[15]. The antibacterial agent used in this research, *Lactococcus*, acts on both types of bacteria. Additionally, it has previously been found to be effective against all bacteria used in this research[2,10,20]. The well diffusion method will be used to apply the *Lactococcus* to the bacteria. In the well diffusion method, small wells are opened on a solution of bacteria and an antibacterial agent is applied in those wells. The expected result would be the formation of inhibition zones around the wells.

Carboxy-methyl cellulose (CMC) is a natural polymer that is commonly used as a thickening agent for obtaining a desired body in solutions[1]. After *Lactococcus* is added in the agar plates, CMC will be added on discs to ensure they stick on the agar plates while performing the disc diffusion method. This method will be used to determine whether the antibacterial effects of *Lactococcus* strains continue when in solid form.

I was inspired to do this research after I suffered food poisoning from meat. The meat was left out enough time for foreign microorganisms to produce. Even after cooking, the bacteria continued to live and eventually got into my digestive system after I ate the meat. So, I wondered if there was a way to make sure this would not happen again and came up with

this research idea. In conclusion to this research, it will be determined whether *Lactococcus* is eligible for use in the food industry.

Observing the Experiment

This research is based on the investigation of the antibacterial effects of *Lactococcus*. The antibacterial effects will be tested on three bacteria: *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli*. Although the strains of bacteria used in this research do not contain any hazardous effects that may cause disease, other strains of the same bacteria do. Those hazardous strains may be found on rotten or spoiled food. Therefore, food is usually covered while it is put on sale at the supermarkets. This coating prevents microorganisms from attaching on the surface of the food. The antibacterial agents found in the coverage also eliminate any previous bacterial formations. It usually also eliminates foreign bacterial formations on the food. So, food coating is crucial for human health.

The expected result of this research is that all strains of *Lactococcus* show antibacterial activity and cause inhibition zone formation on the bacteria. This antibacterial activity is expected to begin with the well diffusion method and continue after the disc diffusion method.

If the expected results are proven to be true, this would mean that *Lactococcus* would make an effective antibacterial agent to use in food coating. This would prevent the bacteria from reproducing on the food, keeping the food healthy as long as possible. In a case where the results do not come out as expected, a repetition of the experiment could be necessary. On the contrary, if the results do turn out to be true, this would indicate that the research was an overall success.

HYPOTHESIS

All strains of *Lactococcus* (1.2, 6.2, 10.2) will have an antibacterial effect on all types of bacteria (*S. aureus*, *P. aeruginosa*, *E. coli*) and the effects will carry on after combining *Lactococcus* with carboxy-methyl cellulose which will prove the solutions to be appropriate for use as food coating material.

RESEARCH QUESTION

How does the diameter of the inhibition zones formed on agar plates containing bacteria (*S. aureus*, *P. aeruginosa*, *E. coli*) exposed to different strains of *Lactococcus* (1.2, 6.2, 10.2) change observed by well diffusion method and how may *Lactococcus* be used for food coating observed by disc diffusion method?

VARIABLES

Independent Variable: Strains of *Lactococcus* (1.2, 6.2 and 10.2) added on *S. aureus*, *P. aeruginosa* and *E. coli*

Dependent Variable: Diameter of inhibition zones (mm) after exposure to *Lactococcus*

Controlled Variables	Why is it controlled?	How is it controlled?
Amount of *Lactococcus* (300 µL of each strain)	A change in the amount of *Lactococcus* will cause a change in the size of inhibition zones.	The amount of *Lactococcus* will be measured before use.
Concentration of *Lactococcus* (1.00×10^9 Colony Forming Unit(CFU)/mL)	The concentration should be high enough to create an observable inhibition zone but low should not be too high to completely inhibit the bacteria.	The same concentration of *Lactococcus* will be taken for every trial.
Concentration of bacteria (1.00×10^9 CFU/mL)	The concentration of bacteria will affect the size of inhibition zones.	The same concentration of bacteria will be taken for every trial.
Temperature (30°C)	The temperature should be at a level in which the *Lactococcus* can operate.	The temperature will be set to 30°C in the incubator for optimum bacterial growth.
Size of agar plates (25 cm diameter)	The size of the agar plate should be sufficient for observation of inhibition zones.	Same type of agar plates will be used in each trial.

Table 1: Controlled Variables

MATERIALS

Materials	Uncertainty	Quantity
Petri dishes	-	60
Mueller-Hinton agar (Appendix 1)	-	1500 mL
S. aureus (1×10^9CFU/mL)	-	2 mL = 2000 μL
P. aeruginosa (1×10^9CFU/mL)	-	2 mL = 2000 μL
E. coli (1×10^9CFU/mL)	-	2 mL = 2000 μL
Lactococcus (Strains 1.2, 6.2 and 10.2)	-	300 μL of each strain
Micropipettes	±2.50 μL	1 (with 1-10,000 μL range)
Spectrophotometer	-	1
Centrifuge	-	1
Magnetic stirrer	-	1
Autoclave machine	-	1
Laminar flow	-	1
Precision scale	±0.0005 grams	1
Incubator	-	1
Vernier caliper	±0.01mm	1
Cork borer	-	1
Medical gloves	-	1 pair
Medical mask	-	1 (Keep at least 5 spare masks)

Table 2: Material List[9]

PROCEDURE

1. Take two tubes of *S. aureus* suspension and put them in the centrifuge.

2. Centrifuge the *S. aureus* suspensions at 4500 rounds per minute for 15 minutes to precipitate the bacteria.

3. Adjust the *S. aureus*–physiological saline (PS) (Appendix 2) ratio by putting the solution in the spectrophotometer until it reaches the appropriate optical density (OD) (Appendix 3).

4. Take the *S. aureus* and dilute it to 1×10^6 CFU/mL using the appropriate amount of PS but do not change the ratio found in step 3.

5. Prepare four agar plates by spreading the Mueller-Hinton agar evenly on the petri dishes and letting it solidify.

6. Calibrate the micropipette to be 100 μL.

7. Take the *S. aureus* from the tube and transfer it on the agar plates using the micropipette so that 100 μL of it is present on the agar.

8. Spread the *S. aureus* evenly on the agar plates by a Drigalski spatula.

9. Take the cork borer and pass its tip through the Bunsen burner to sterilize it.

10. Use the cork borer to open a well in each agar plate.

11. Label the agar plates as "1.2" , "6.2" , "10.2" and "Control".

12. Take the electronic pipette controller and attach a pipette to it.

13. Take 5 μL of the 1.2 strain of *Lactococcus* and transfer it into one of the wells. Then, do the same for strains 6.2 and 10.2 on separate agar plates.

14. Create a control group by not adding *Lactococcus*, but instead PS in the fourth agar.

15. Measure 100 mL of pure water by a beaker glass.

16. Weigh 1.50 grams of CMC by a precision scale.

17. Mix the CMC and the pure water inside the beaker glass.

18. Put a magnet inside the beaker glass.

19. Put a cap on the beaker glass and leave it on a magnetic stirrer for 2 hours.

20. Pour half of the CMC-water solution into another beaker glass.

21. Take separate empty beaker glass and leave its cap open.

22. Take a syringe without attaching its needle and pull 10 mL from the 50 mL CMC-water solution.

23. Attach the syringe to a 22-micron filter while the 10 mL of CMC-water solution is inside the syringe.

24. Pour the solution inside the empty beaker glass.

25. Repeat steps 23-25 until all 50 mL of the CMC-water solution is transferred into the empty beaker glass.

26. Prepare CMC films[8].

27. Cut four disks to be 7 millimeters wide approximately each.

28. Place the disks on the agar plates, then close the petri dishes and leave them for incubation for 24 hours at 30°C.

29. After the incubation process is done, measure the diameters of the inhibition zones using a Vernier caliper.

30. "Measured diameter of inhibition zone − diameter of cork borer = Final diameter of inhibition zone" do this calculation for each of the agar plates to find the inhibition zone formed on them.

31. Repeat steps 1-30 for a total of 5 times.

32. Repeat the whole procedure for *P. aeruginosa* and *E. coli* separately.

    (5 trials should be conducted for each bacteria.)

RISK ASSESSMENT

Environmental Problems	The microorganisms used in this research might gain resistance against the antibacterial agent applied. If these resistant bacteria are dumped in the nature and this treat mutates, a new generation of resistant bacteria would form. Therefore, all solutions containing any microorganism is autoclaved.
Safety Issues	Exposure to the bacteria used in this research by respiration or ingestion may cause the bacteria to spread through human activities. A medical mask, medical gloves and a *Lactococcus* coat must be worn. All equipment must be sterilized before and after it is used. Steps 4-13 are advised to be conducted inside a laminar flow.
Ethical Considerations	*E. coli* is a Biosafety Level 1 (BSL-1) bacteria, whereas, *S. aureus* and *P. aeruginosa* are BSL-2 microorganisms. Although BSL-2 is not a health-threatening level, there still is a risk factor involved. So, everything that might be contaminated must be autoclaved.

Table 3: Risk Assessment Table[5,13,14,18,19]

DATA

Raw Data Table

Type of Bacteria	Trials	Diameter of Inhibition Zone (±0.01 mm)			
		Lactococcus Strain 1.2	*Lactococcus* Strain 6.2	*Lactococcus* Strain 10.2	Control Group (No *Lactococcus* Strain)
S. aureus	1	8.59	13.10	9.65	0.00
	2	8.52	14.03	12.05	0.00
	3	8.14	13.41	11.52	0.00
	4	8.48	13.57	11.28	0.00
	5	8.76	13.88	11.17	0.00
P. aeruginosa	1	10.89	15.85	16.27	0.00
	2	12.20	16.67	15.95	0.00
	3	12.03	16.16	16.04	0.00
	4	11.14	16.30	15.99	0.00
	5	11.59	15.98	16.12	0.00
E. coli	1	2.27	5.86	6.15	0.00
	2	2.93	6.52	7.02	0.00
	3	3.06	6.22	6.56	0.00
	4	2.11	6.10	6.23	0.00
	5	2.51	5.97	6.75	0.00

Table 4: Inhibition Zones by Bacteria and Lactococcus Strains

Processed Data Table

Type of Bacteria	Mean Diameter of Inhibition Zone for *Lactococcus* Strain 1.2(mm)	Standard Deviation (1.2)	Mean Diameter of Inhibition Zone for *Lactococcus* Strain 6.2(mm)	Standard Deviation (6.2)	Mean Diameter of Inhibition Zone for *Lactococcus* Strain 10.2(mm)	Standard Deviation (10.2)
S. aureus	8.50	0.227	13.60	0.371	11.13	0.896
P. aeruginosa	11.57	0.560	16.19	0.318	16.07	0.126
E. coli	2.58	0.411	6.13	0.255	6.54	0.362

Table 5: Processed Data Table

Visual Representations of the Inhibition Zones

Figure 1: Petri dish containing *S. aureus* and *Lactococcus* Strain 1.2

Figure 2: Petri dish containing *S. aureus* and *Lactococcus* Strain 6.2

Figure 3: Petri dish containing *S. aureus* and *Lactococcus* Strain 10.2

Figure 4: Petri dish containing *S. aureus* but no *Lactococcus*

Figure 5: Petri dish containing *P. aeruginosa* and *Lactococcus* Strain 1.2
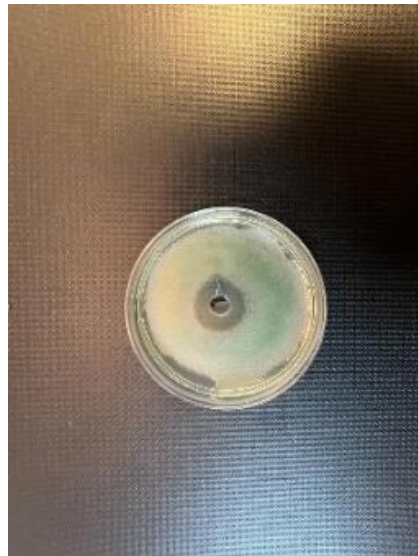

Figure 6: Petri dish containing *P. aeruginosa* and *Lactococcus* Strain 6.2

Figure 7: Petri dish containing
P. aeruginosa and *Lactococcus* Strain 10.2

Figure 8: Petri dish containing
P. aeruginosa but no *Lactococcus*

Figure 9: Petri dish containing *E. coli*

and *Lactococcus* Strain 1.2

Figure 10: Petri dish containing *E. coli*
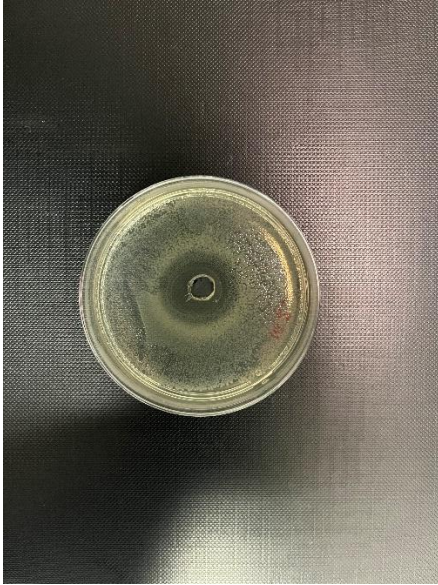
and *Lactococcus* Strain 6.2

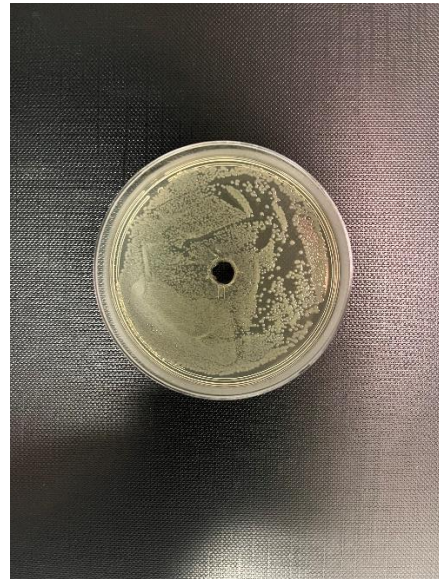Figure 11: Petri dish containing *E. coli* and *Lactococcus* Strain 10.2

Figure 12: Petri dish containing *E. coli* but no *Lactococcus* Strain

ANOVA Single-Factor and TUKEY Tests

The difference between the effect of *Lactococcus* on *S. aureus*, *P. aeruginosa* and *E. coli* is already clear when the data on Table 4 is observed. However, in order to test whether the difference of the antibacterial effect on the bacteria between *Lactococcus* strains 1.2, 6.2 and 10.2 is meaningful, single-factor ANOVA tests will be conducted. These tests will be conducted separately for the three bacteria. Two hypotheses must be made before the tests. Those two hypotheses are:

H$_0$: There is not a significant difference between the effects of same concentrations of *Lactococcus* strains 1.2, 6.2 and 10.2 on *S. aureus*, *P. aeruginosa* and *E. coli*.

H$_1$: There is a significant difference between the effects of same concentrations of *Lactococcus* strains 1.2, 6.2 and 10.2 on *S. aureus*, *P. aeruginosa* and *E. coli*.

The data from Table 4 is entered in Excel and the tests are conducted. The following are the results of the test:

- The α-value for these tests is 0.05.

SUMMARY

Groups	Count	Sum	Average	Variance
Strain 1.2	5	42.49	8.498	0.05152
Strain 6.2	5	67.99	13.598	0.13757
Strain 10.2	5	55.67	11.134	0.80323

ANOVA

Source of Variance	Sum of Squares	df	Mean Square	F	P-value	F crit
Between Groups	65.04965	2	32.52483	98.32965	3.62E-08	3.885294
Within Groups	3.96928	12	0.330773			
Total	69.01893	14				

Table 6: ANOVA Test Results for different strains of *Lactococcus* (1.2, 6.2, 10.2) on *S. aureus*

SUMMARY

Groups	Count	Sum	Average	Variance
Strain 1.2	5	57.85	11.57	0.31405
Strain 6.2	5	80.96	16.192	0.10077
Strain 10.2	5	80.37	16.074	0.01603

ANOVA

Source of Variance	Sum of Squares	df	Mean Square	F	P-value	F crit
Between Groups	69.43804	2	34.71902	241.7478	2.02E-10	3.885294
Within Groups	1.7234	12	0.143617			
Total	71.16144	14				

Table 7: ANOVA Test Results for different strains of *Lactococcus* (1.2, 6.2, 10.2) on *P. aeruginosa*

SUMMARY

Groups	Count	Sum	Average	Variance
Strain 1.2	5	12.88	2.576	0.16868
Strain 6.2	5	30.67	6.134	0.06488
Strain 10.2	5	32.71	6.542	0.13077

ANOVA

Source of Variance	Sum of Squares	df	Mean Square	F	P-value	F crit
Between Groups	47.59164	2	23.79582	195.9418	6.88E-10	3.885294
Within Groups	1.45732	12	0.121443			
Total	49.04896	14				

Table 8: ANOVA Test Results for different strains of *Lactococcus* (1.2, 6.2, 10.2) on *E. coli*

The p-values found with this ANOVA tests are 3.62E-08(Table 6), 2.02E-10(Table 7) and 6.88E-10(Table 8). Since all of these values are below 0.05, the H_0 hypothesis is rejected for all bacteria. Therefore, it is confirmed that there is a significant difference between the effects of same concentrations of *Lactococcus* strains 1.2, 6.2 and 10.2 on *S. aureus*, *P. aeruginosa* and *E. coli*. However, the number of groups in observation is three. The ANOVA tests indicate that there is a significant difference between the means of at least two of those groups but they do not tell what those specific groups are. So, I aim to determine which strains of *Lactococcus* have a significant difference between their effects on bacteria. Using the data from the ANOVA Tests, the TUKEY tests are conducted. Given below are the results of these tests:

Lactococcus Strains	Group Pairs	Absolute Difference	Standard Error	q-score
S. aureus	1.2 and 6.2	5.100	0.2572	19.82892691
	6.2 and 10.2	2.464	0.2572	9.580093313
	1.2 and 10.2	2.636	0.2572	10.24883359
P. aeruginosa	1.2 and 6.2	4.622	0.16948	27.27165447
	6.2 and 10.2	0.118	0.16948	0.696247345
	1.2 and 10.2	4.504	0.16948	26.57540713
E. coli	1.2 and 6.2	3.558	0.15585	22.82964389
	6.2 and 10.2	0.408	0.15585	2.617901829
	1.2 and 10.2	3.966	0.15585	25.44754572

*Table 9: Results of TUKEY Test*

Critical Values of Studentized Range Distribution(q) for Familywise ALPHA = .05.

Denominator DF	Number of Groups (a.k.a. Treatments)							
	3	4	5	6	7	8	9	10
1	26.976	32.819	37.081	40.407	43.118	45.397	47.356	49.070
2	8.331	9.798	10.881	11.734	12.434	13.027	13.538	13.987
3	5.910	6.825	7.502	8.037	8.478	8.852	9.177	9.462
4	5.040	5.757	6.287	6.706	7.053	7.347	7.602	7.826
5	4.602	5.218	5.673	6.033	6.330	6.582	6.801	6.995
6	4.339	4.896	5.305	5.629	5.895	6.122	6.319	6.493
7	4.165	4.681	5.060	5.359	5.606	5.815	5.997	6.158
8	4.041	4.529	4.886	5.167	5.399	5.596	5.767	5.918
9	3.948	4.415	4.755	5.024	5.244	5.432	5.595	5.738
10	3.877	4.327	4.654	4.912	5.124	5.304	5.460	5.598
11	3.820	4.256	4.574	4.823	5.028	5.202	5.353	5.486
12	3.773	4.199	4.508	4.748	4.947	5.116	5.262	5.395
13	3.734	4.151	4.453	4.690	4.884	5.049	5.192	5.318
14	3.701	4.111	4.407	4.639	4.829	4.990	5.130	5.253
15	3.673	4.076	4.367	4.595	4.782	4.940	5.077	5.198
16	3.649	4.046	4.333	4.557	4.741	4.896	5.031	5.150
17	3.628	4.020	4.303	4.524	4.705	4.858	4.991	5.108
18	3.609	3.997	4.276	4.494	4.673	4.824	4.955	5.071
19	3.593	3.977	4.253	4.468	4.645	4.794	4.924	5.037
20	3.578	3.958	4.232	4.445	4.620	4.768	4.895	5.008
21	3.565	3.942	4.213	4.424	4.597	4.743	4.870	4.981
22	3.553	3.927	4.196	4.405	4.577	4.722	4.847	4.957
23	3.542	3.914	4.180	4.388	4.558	4.702	4.826	4.935
24	3.532	3.901	4.166	4.373	4.541	4.684	4.807	4.915
25	3.523	3.890	4.153	4.358	4.526	4.667	4.789	4.897
26	3.514	3.880	4.141	4.345	4.511	4.652	4.773	4.880
27	3.506	3.870	4.130	4.333	4.498	4.638	4.758	4.864
28	3.499	3.861	4.120	4.322	4.486	4.625	4.745	4.850
29	3.493	3.853	4.111	4.311	4.475	4.613	4.732	4.837
30	3.487	3.845	4.102	4.301	4.464	4.601	4.720	4.824
31	3.481	3.838	4.094	4.292	4.454	4.591	4.709	4.813
32	3.475	3.832	4.086	4.284	4.445	4.581	4.698	4.802
33	3.470	3.825	4.079	4.276	4.436	4.572	4.689	4.791
34	3.465	3.820	4.072	4.268	4.428	4.563	4.680	4.782
35	3.461	3.814	4.066	4.261	4.421	4.555	4.671	4.773
36	3.457	3.809	4.060	4.255	4.414	4.547	4.663	4.764
37	3.453	3.804	4.054	4.249	4.407	4.540	4.655	4.756
38	3.449	3.799	4.049	4.243	4.400	4.533	4.648	4.749
39	3.445	3.795	4.044	4.237	4.394	4.527	4.641	4.741
40	3.442	3.791	4.039	4.232	4.388	4.521	4.634	4.735
41	3.439	3.787	4.035	4.227	4.383	4.515	4.628	4.728
42	3.436	3.783	4.030	4.222	4.378	4.509	4.622	4.722
43	3.433	3.779	4.026	4.217	4.373	4.504	4.617	4.716
44	3.430	3.776	4.022	4.213	4.368	4.499	4.611	4.710
45	3.428	3.773	4.018	4.209	4.364	4.494	4.606	4.705
46	3.425	3.770	4.015	4.205	4.359	4.489	4.601	4.700
47	3.423	3.767	4.011	4.201	4.355	4.485	4.597	4.695
48	3.420	3.764	4.008	4.197	4.351	4.481	4.592	4.690
49	3.418	3.761	4.005	4.194	4.347	4.477	4.588	4.686
50	3.416	3.758	4.002	4.190	4.344	4.473	4.584	4.681

Figure 13: Table showing the critical values of q by DF[3]

The following interpretation of the results of the TUKEY test is done according to the information found online[16]. The DF number is given to be 14 by the ANOVA tests. As the number of strains observed is three, the intersection of 14 and 3 is found on the table. That value is given as 3.701. The q-scores of all groups from *S. aureus* are above 3.701 so, all strains have a significant difference between their effects on these bacteria. However, for *P. aeruginosa* and *E. coli*, all strains have a significant difference between their effects except 6.2 and 10.2. Given below is a graph visualizing this information:
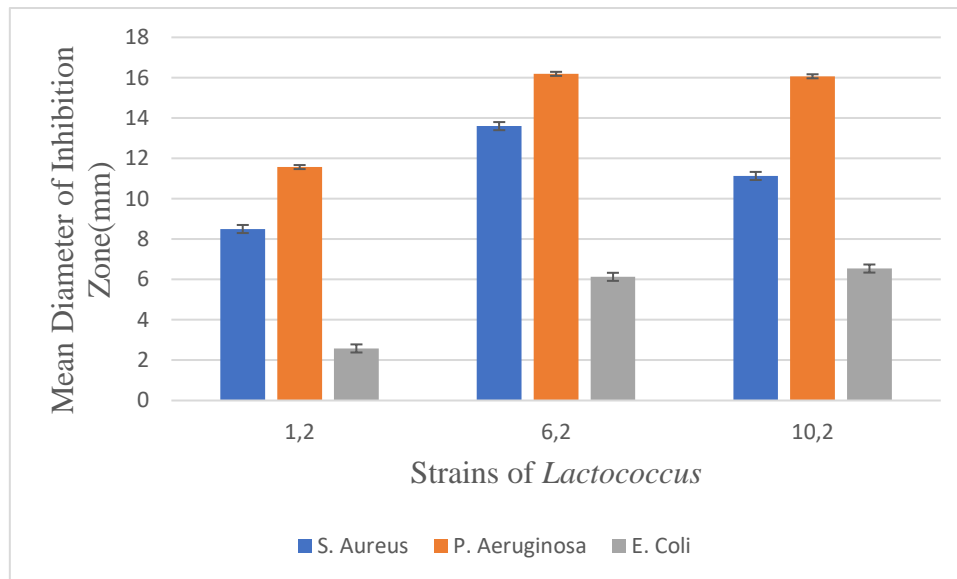
Graph 1: Mean diameters of inhibition zones formed on the bacteria by Lactococcus strains

The error bars are made according to the uncertainties of the equipment used.

CONCLUSION

In conclusion to this research, the results mostly turned out as expected. First of all, every strain of *Lactococcus* was effective against the bacteria as they were successful in forming an inhibition zone. In the control group, where no *Lactococcus* was added, no inhibition zones were formed(Figures 4, 8 and 12). The bacteria that *Lactococcus* was most effective on is *Pseudomonas aeruginosa* since the mean diameters of inhibition zones are highest(Table 5). The bacteria that *Lactococcus* was most effective on after *P. aeruginosa* is *Staphylococcus aureus*. Compared to the other bacteria, every diameter of inhibition zone on *S. aureus* has a mediocre value, standing in between *E. coli* and *P. aeruginosa*. The least affected bacteria is *E. coli* with mean diameters of inhibition zones remaining under 6.54 mm. The diameters of inhibition zones are between 2.11 mm and 7.02 mm. The maximum diameter of inhibition zone on *E. coli*, 7.02 mm, is lower than the minimum value observed on *S. aureus*.

Considering *P. aeruginosa* and *E. coli* are Gram-negative and *S. aureus* is Gram-positive, a relationship between the antibacterial effect and Gram-staining of microorganism may be searched. The bacteria can be ordered from the most susceptible to the antibacterial effects of *Lactococcus* to the least as *E. coli*(Gram-) > *S. aureus*(Gram+) > *P. aeruginosa*(Gram-). According to this order of bacteria, the result would be that there is no correlation between the effectiveness of *Lactococcus* and Gram-staining of microorganism. However, the 6.2 and 10.2 strains don't show much difference between their effects on *S. aureus* which is Gram+, whereas, they do on Gram– bacteria(Table 9).

Another topic that should be observed is the overall effectiveness of the strains of *Lactococcus*. For all bacteria, the least effective strain of *Lactococcus* was 1.2 since the diameters of inhibition zones formed by it are the lowest. The effects of *Lactococcus* strain 1.2 are present in Figures 1, 5 and 9. The 6.2 and 10.2 strains were the most effective. Both strains caused similar values of inhibition zone diameters. The data indicate that the 6.2 strain was slightly more effective than 10.2 but the two strains should be considered the same in effectiveness. The TUKEY test, and Graph 1 also suggest that there is no significant difference between the effectiveness of 6.2 and 10.2 strains. In addition, the inhibition zones formed by 6.2 and 10.2 strains are not very different (Figures 2, 3, 6, 7, 10 and 11). In conclusion, it is certain that the 1.2 strain is the least effective out of the three strains. However, there is little difference between the other two strains. The 6.2 strain can be said to be the most effective strain by only observing the data. However, the TUKEY test must also be taken into consideration when interpreting the results. So, it is unclear whether the 6.2 strain or the 10.2 strain is more effective.

Overall, the strains of *Lactococcus* can be ordered as 6.2 ≥ 10.2 > 1.2 in antibacterial activity effectiveness. As the bacteria's Gram-staining does not affect the antibacterial activity of *Lactococcus*, inhibition zones were formed on all bacteria.

EVALUATION AND FURTHER INVESTIGATION

Three different strains of *Lactococcus* were used in this research. The results indicate that all strains of *Lactococcus* were effective on the bacteria. Another observation was the clarity of inhibition zones. All inhibition zones formed on *E. coli* and *P. aeruginosa* were clearly observed. However, static inhibition zones were formed on *S. aureus*. Therefore, the inhibition zones were not clear. Bacteria remained alive in the shape of spots on the agar plate. Despite, more faded areas compared to other parts of the agar plate were present. So, the inhibition zones were measured as the diameters of these faded areas. The fade in these areas show that the antibacterial agent was more effective near the well it was added in.

The disc diffusion method was conducted in order to observe whether the antibacterial effect would continue after the solution is solidified. In every trial, the results turned out to be positive. Instead of the control group, in which the effect was expected to never occur, the antibacterial agent kept on the inhibition of the bacteria after it was solidified. This proves both CMC to be a successful solidifying agent and *Lactococcus* to be an effective antibacterial agent which can carry on its effects in different mediums.

This research was conducted in the safest and most detailed way possible with the equipment available. However, no matter how carefully the experimentation was performed, the presence of flaws should be considered. The table below shows a list of things that could be done to improve the accuracy of this research and increase overall quality of the research.

More Trials	This research consisted of 5 trials for each bacteria. More trials could have been performed and more data could have been gathered. This would have decreased the standard deviation, obtaining more accurate results. Although the current number of trials were enough to observe the expected results, an increase in the number of trials would be highly accurate. It may also have shown if exceptional data that lies out of the expected results exist.
Better Materials	The uncertainty of the materials used in this research are already small enough to neglect. However, the use of equipment with even less uncertainties would increase the accuracy of the results obtained.
Tests and Calculations until Desired Values Are Reached	More calculations and tests could have been made to reach the desired values. For example, when setting the optical densities some, values were rounded up to the desired values. The actual value measured by the spectrophotometer was 0.988 for S. aureus, whereas, the value should have been 1.000. Instead of testing for the actual bacteria-SF ratio, the ratio used to obtain this optical density was accepted and 0.988 was rounded up to 1.000.

Table 10: Limitations and Recommendations

In conclusion, this research has its limitations but it can be considered as successful. For further investigation, this research could be expanded by testing the coating material on actual food products. Experiments could be conducted by human consumption of food coated by the material containing *Lactococcus*. The potential errors could be eliminated through different methods to get more accurate results. In order to make an assessment in general, there are many aspects to be improved in this research. However, overall, accurate results were obtained on the antibacterial effects of different strains of *Lactococcus* on different bacterial microorganisms.

BIBLIOGRAPHY

References

1. Ballesteros, L. F., Cerqueira, M. A., Teixeira, J. A., & Mussatto, S. I. (2018). Production and physicochemical properties of carboxymethyl cellulose films enriched with spent coffee grounds polysaccharides. *International journal of biological macromolecules*, *106*, 647-655. Accessed on 13 Dec. 2023.

2. Cho, D.-Y., Skinner, D., Lim, D. J., Mclemore, J. G., Koch, C. G., Zhang, S., Swords, W. E., Hunter, R., Crossman, D. K., Crowley, M. R., Grayson, J. W., Rowe, S. M., & Woodworth, B. A. (2020, April). *The impact of Lactococcus lactis (probiotic nasal rinse) co-culture on growth of patient-derived strains of pseudomonas aeruginosa*. International forum of allergy & rhinology. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8058912/. Accessed on 24 Feb. 2024.

3. Critical values of studentized range distribution(q) for familywise alpha=0.05 (n.d.). Accessed https://www.stat.purdue.edu/~lingsong/teaching/2018fall/q-table.pdf. Accessed on 26 Jan. 2024.

4. Dave, D. and Ghaly, A.E. (2011). *Meat Spoilage Mechanisms and Preservation Techniques: A Critical Review*. American Journal of Agricultural and Biological Sciences 6 (4): 486-510. https://www.thescipub.com/pdf/ajabssp.2011.486.510.pdf. Accessed on 3 Mar. 2024

5. Escherichia coli (Migula) castellani and Chalmers - 25922 | ATCC. (n.d.-b). https://www.atcc.org/products/25922. Accessed on 13 Feb. 2024.

6. Glauert, A. M., & Thornley, M. J. (1969). The topography of the bacterial cell wall. *Annual review of microbiology*, *23*, 159–198. https://doi.org/10.1146/annurev.mi.23.100169.001111. Accessed on 4 Dec. 2023.

7. Jagoda, S., Kos, B., Beganovic, J., Pavunc, A. L., Habjanic, K., & Matosic, S. (2010). Antimicrobial activity of lactic acid bacteria. *Food Technol. Biotechnol*, *48*(3), 296-307. Accessed on 11 Jan. 2024.

8. Łopusiewicz, Ł., Kwiatkowski, P., Drozłowska, E., Trocer, P., Kostek, M., Śliwiński, M., Polak-Śliwińska, M., Kowalczyk, E., & Sienkiewicz, M. (2021). Preparation and Characterization of Carboxymethyl Cellulose-Based Bioactive Composite Films Modified with Fungal Melanin and Carvacrol. *Polymers*, *13*(4), 499. https://doi.org/10.3390/polym13040499. Accessed on 1 Jan. 2024

9. Making agar plates. (n.d.). https://teach.genetics.utah.edu/content/microbiology/plates/. Accessed on 27 Nov. 2023.

10. Nouaille, S., Rault, L., Jeanson, S., Loubière, P., Le Loir, Y., & Even, S. (2014, November). *Contribution of Lactococcus lactis reducing properties to the downregulation of a major virulence regulator in Staphylococcus aureus, the AGR system*. Applied and environmental microbiology. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4248995/. Accessed on 24 Feb. 2024.

11. Ponce, A. G., Moreira, M. R., Del Valle, C. E., & Roura, S. I. (2008). Preliminary characterization of bacteriocin-like substances from lactic acid bacteria isolated from organic leafy vegetables. *LWT-Food Science and Technology*, *41*(3), 432-441. Accessed on 27 Nov. 2024.

12. professional, C. C. medical. (n.d.). *Food poisoning (foodborne illness): Symptoms, signs, treatment*. Cleveland Clinic. https://my.clevelandclinic.org/health/diseases/21167-food-poisoning. Accessed on 24 Feb. 2024.

13. *Pseudomonas aeruginosa biological agent reference sheet (bars)*. Pseudomonas aeruginosa Biological Agent Reference Sheet (BARS) | Environment, Health and Safety. (n.d.). https://ehs.cornell.edu/research-safety/biosafety-biosecurity/biological-safety-manuals-and-other-documents/bars-other/pseudomonas-aeruginosa. Accessed on 1 Dec. 2023.

14. Pseudomonas aeruginosa (Schroeter) migula - 27853 | ATCC. (n.d.-b). https://www.atcc.org/products/27853. Accessed on 13 Feb. 2024.

15. Rajagopal, M., & Walker, S. (2017). Envelope structures of gram-positive bacteria. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5002265/. Accessed on 1 Dec. 2023.

16. Rynearson, S., & Tsitlidze, L. (Eds.). (2023, October 30). Tukey Test – Excel and Google Sheets. Automate Excel. https://www.automateexcel.com/stats/tukey-test/.

17. Silhavy, T. J., Kahne, D., & Walker, S. (2010). The bacterial cell envelope. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2857177/. Accessed on 1 Dec. 2023.

18. *Staphylococcus aureus biological agent reference sheet (bars)*. Staphylococcus aureus Biological Agent Reference Sheet (BARS) | Environment, Health and Safety. (n.d.). https://ehs.cornell.edu/research-safety/biosafety-biosecurity/biological-safety-manuals-and-other-documents/bars-other/staphylococcus-aureus. Accessed on 5 Dec. 2023.

19. Staphylococcus aureus subsp. aureus Rosenbach - 6538 | ATCC. (n.d.-b). https://www.atcc.org/products/6538. Accessed on 13 Feb. 2024.

20. Yu, D., Xia, Y., Ge, L., Tan, B., & Chen, S. (2021, July 19). *Effects of lactococcus lactis on the intestinal functions in weaning piglets*. Frontiers.

https://www.frontiersin.org/articles/10.3389/fnut.2021.713256/full. Accessed on 24 Feb. 2024.

Appendix

Appendix 1

Why was the Mueller-Hinton agar chosen?

The Mueller-Hinton agar is a suitable medium for fast and efficient growth of all three bacteria used in this research. Used normally as a Gram-negative bacteria (e.g. *E. coli*, *P. aeruginosa*) growth medium, it has been observed that a few types of Gram-positive bacteria including *S. aureus* also grows on this type of agar. Therefore, the Mueller-Hinton agar was the ideal choice for this research.

Appendix 2

What is PS?

Physiological saline, also known as PS, is a form of salty water. It is usually used to reduce the concentration of a substance.

Appendix 3

What are the optimum OD values for each type of bacteria?

The optimum OD value is different for different types of bacteria. The types of bacteria used in this experiment are *S. aureus, P. aeruginosa* and *E. coli*. The OD values for these bacteria should be 1.0, 0.1 and 0.7 respectively. The spectrophotometer should be set to 600, 625 and 600 nanometers respectively for the tests.

Appendix 4

What are the ATCC numbers for the bacteria used in the research?

ATCC stands for American Type Culture Collection. An ATCC number given to a microorganism is used in the database to find detailed information on a specific strain. The ATCC numbers for S. aureus, P. aeruginosa and E. coli bacteria are ATCC 6538, ATCC 27853 and ATCC 25922 respectively[5,14,19].

Appendix 5