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Assaying the formation of beneficial biofilms by lactic acid bacteria and the effect of ayurvedic plant extracts on their enhancement

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Summary

Antibiotic resistance is increasingly becoming more dangerous and harmful. This study aimed to obtain the optimal non-antibiotic, natural method to suppress the growth of pathogenic bacteria within the body. The two-fold purpose of this project was to determine which combination of bacteria would result in the most biofilm formation and then to assess the lowest, median, and highest biofilm formation combinations to see the effect of ayurvedic plant extracts on the biofilm. *S. thermophilus, L. lactis,* and *L. mesenteroides subsp. mesenteroides* alone and *L. lactis, L. mesenteroides subsp. mesenteroides* plus gooseberry extract resulted in the most biofilm formation. The results show that the addition of a plant extract can affect the biofilm growth of a bacteria combination. The applications of this study can be used to design probiotic supplements with added beneficial plant extracts.

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Introduction

Bacteria are a large domain of prokaryotic microorganisms found in the air, soil, water, and food and vary in shape, size, and means of transportation (1). Some bacteria cause bacterial infections that are often the cause of death or disease, and now, with antibiotic resistant–infection numbers rising, new treatments for bacterial infections have become necessary (2) .

Bacteria are often referred to as harmful microorganisms; however, the human body, despite having some amounts of detrimental bacteria, is replete with beneficial bacteria that thrive and decrease the number of harmful bacteria. Many treatments for bacterial infections have been focused on getting rid of the pathogenic bacteria, but this project focuses on multiplying the nonpathogenic bacteria in the body (increasing biofilm formation) under the most favorable conditions, so these beneficial bacteria can thrive in the body and reduce the number of detrimental bacteria. This project studied probiotic bacteria, a form of beneficial bacteria that are found in the body and in natural substances that humans consume. The four bacteria used in this study, *Lactobacillus acidophilus*, *Streptococcus thermophilus, Lactococcus lactis, and Leuconostoc mesenteroides subsp. mesenteroides*, are known as lactic acid bacteria, are gram-positive, are beneficial for the body, and are found in food products (3). *L. acidophilus* is found in the intestines, helps digestion, and can be consumed through dairy products like yogurt (4). *S. thermophilus* helps relieve many gastrointestinal symptoms associated with lactose intolerance and is used as the starter culture in many fermented dairy products as well, like mozzarella cheese (5). A promising application of *L. lactis* is its use as a protein delivery vehicle for the development of live mucosal vaccines in the gastrointestinal mucosal immune system, and almost all forms of cheese and buttermilk are manufactured with strains of *L. lactis* (6). *L. mesenteroides subsp. mesenteroides* is probably the most versatile probiotic bacteria, found in fruits, vegetables, bread dough, and fermented dairy products (7). All possible combinations of these bacteria will be used to see which results in the most biofilm formation.

Studying biofilm formation of different bacteria can be useful in discovering a new treatment for bacterial infections. A biofilm is defined as a community of either one type or multiple types of microorganisms that adheres to a surface. Biofilms are found everywhere, ranging from the environment to the human body. There are different types of bacteria concerning biofilm formation; some are planktonic, or free-living and suspended, while others are biofilm microorganisms because they are surface-associated (8). Planktonic cells form a mature biofilm after being initiated by environmental cues and initial interactions and then being matured by developmental signals. Afterward, some cells from the mature biofilm return to a planktonic lifestyle, completing the cycle of biofilm development (9). Biofilm formation can be enhanced through synergistic interactions among multispecies biofilms, along with some other nutritional and environmental conditions (10). Biofilms can form with both good and bad bacteria, and in the case of beneficial bacteria, biofilm formation needs to be assessed to see what causes the “good” bacteria to transition from free-living to adherent in order to form a biofilm and have the most of it in the body. Biofilms formed by detrimental bacteria are responsible for several diseases that humans contract, and because these structures are developing resistance to antibiotics, diseases and infections caused by biofilms are difficult

to treat (11). Studying the biofilm formation of beneficial bacteria can be useful in curing bacterial infections (8)

Ayurvedic medicines have a beneficial impact on our body's immune system. Many herbs and plants used in ayurveda, the study of natural medicine, may also have an impact on probiotic biofilm formation. The extracts that are used in this study have some known impact on the immune system, but their impact on biofilm formation is not that researched. The six plant extracts used in this study come from bitter gourd, fenugreek, gooseberry, holy basil, and pomegranate seeds and peel extracts. The extracts of bitter gourd and fenugreek were chosen because these vegetables are used in the treatment for diabetic patients who have a weak immune system and are susceptible to bacterial infections (12, 13). Since bitter gourd and fenugreek help treat diabetes, they might also have a connection to improving a weak immune system by enhancing probiotic biofilm formation. The extracts of gooseberry, holy basil, and pomegranate seeds and peel were chosen because they are consumed to boost the immune system, so these plants could also possibly have an effect on probiotic biofilm formation (14-16).

It was hypothesized that if the probiotic bacteria *L. acidophilus, S. thermophilus, L. lactis,* and *L. mesenteroides subsp. mesenteroides* were assessed for biofilm formation, the combination of bacteria that would have the most biofilm formation would contain all four bacteria. This hypothesis was formulated due to past studies asserting the synergistic benefits of multi-species biofilms (10). It was further hypothesized that if the combinations of bacteria with the lowest, median, and highest biofilm formation were tested in the presence of the ayurvedic plant extracts from gooseberry, bitter gourd, holy basil, fenugreek, and pomegranate seeds and peel, the plant extract that would boost the biofilm formation the most would be the extract from gooseberry. This hypothesis was developed due to the numerous reported beneficial implications of gooseberry on human health (17).

When fifteen different combinations of the four bacteria were tested, the bacterial combination that resulted in the most biofilm formation contained *S. thermophilus, L. lactis,* and *L. mesenteroides subsp. mesenteroides*, disproving the hypothesis. When the bacterial combinations were tested with the six plant extracts, the combination containing *L. lactis, L. mesenteroides subsp. mesenteroides*, and gooseberry extract resulted in the most biofilm formation, supporting the second hypothesis. The results show that biofilm formation of a bacteria combination with a plant extract is different than the biofilm growth of just the bacteria combination.

Results

Assessing Biofilm Formation with Different Combinations of Lactic Acid Bacteria

The abilities of probiotic bacteria to form biofilms both alone and in combination were assessed using a crystal violet–staining assay. Measured absorbance for each of the experimental conditions are presented in **Figure 1**. Assessment of probiotic biofilm formation across fifteen different combinations of lactic acid bacteria showed that the combination resulting in the most biofilm formation was N14, consisting of *S. thermophilus, L. lactis,* and *L. mesenteroides subsp. mesenteroides*. Overall, it was evident that biofilms with *L. acidophilus* did not have great biofilm growth. A wide range and high degree of variability in biofilm formation was observed. Through statistical analysis of these results and their averages, standard deviations, and standard errors, it can be said that the combination N14 (*S. thermophilus, L. lactis, L. mesenteroides subsp. mesenteroides*) had the highest level of biofilm formation, with a value of 1.59 Au at a 99.9% confidence level and a value of 2.045 Au (absorbance units) at a 95% confidence level (**Table 2**). Au, the unit of measurement for absorbance of crystal violet dye in this experiment, is proportional to the amount of biofilm formation that occurs. For the next part of the project, the combination with the least biofilm growth, B2 (*S. thermophilus*); the combination with median biofilm growth, J10 (*L. lactis, L. mesenteroides subsp. mesenteroides*); and the combination with the highest biofilm growth, N14 (*S. thermophilus, L. lactis, L. mesenteroides subsp. mesenteroides*) were chosen.

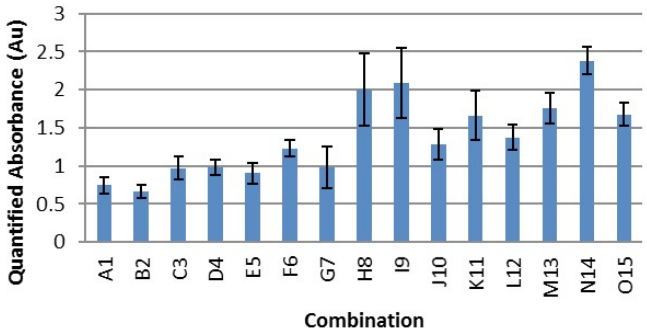

Figure 1: Mean value of quantified absorbance of each probiotic biofilm formation combination. Absorbance (Au) of crystal violet dye indicates the amount of biofilm formation. Ten replicates were done for each combination. Error bars show standard error.

Combination	Average (Au)	Standard Deviation	Standard Error	95% & 99.9% Confidence that Combination will have Au >=	
				95%	99.9%
A1	0.745	0.332	0.105	**0.552**	**0.294**
B2	0.661	0.265	0.084	**0.507**	**0.301**
C3	0.971	0.490	0.155	**0.688**	**0.306**
D4	0.978	0.307	0.097	**0.800**	**0.560**
E5	0.904	0.443	0.140	**0.647**	**0.302**
F6	1.232	0.362	0.114	**1.022**	**0.740**
G7	0.986	0.868	0.274	**0.482**	**-0.194**
H8	2.004	1.510	0.478	**1.129**	**-0.048**
I9	2.088	1.453	0.459	**1.246**	**0.114**
J10	1.287	0.636	0.201	**0.918**	**0.422**
K11	1.664	1.045	0.330	**1.058**	**0.244**
L12	1.372	0.531	0.168	**1.065**	**0.651**
M13	1.763	0.634	0.200	**1.396**	**0.902**
N14	2.383	0.584	0.185	**2.045**	**1.590**
O15	1.676	0.480	0.152	**1.398**	**1.024**

Table 2: Statistical figures for assessing biofilm formation with different combinations of lactic acid bacteria.

Assessing Biofilm Formation with Different Plant Extracts

Probiotic biofilm formation with the plant extracts was also assessed using a crystal violet–staining assay. Measured absorbance for each of the experimental conditions are presented in **Figure 3**. The results from the second part of this project concerning the biofilm formation of the lowest, median, and highest bacteria combinations along with the six plant extracts of gooseberry, bitter gourd, holy basil, fenugreek, pomegranate seeds, and pomegranate peel showed that the combination and plant extract that yielded the most biofilm formation was J10, containing *L. lactis* and *L. mesenteroides subsp. mesenteroides*, with the gooseberry extract. Overall, the extract of gooseberry, bitter gourd, and holy basil enhanced biofilm formation the most. Fenugreek and pomegranate peel enhanced biofilm formation slightly, but sometimes even lowered it. Pomegranate seeds showed the least enhancement of biofilm formation, lowering the biofilm formation for each combination. Through statistical analysis of these results and their averages, standard deviations, and standard errors, it can be said that the combination J10 gooseberry (*L. lactis, L. mesenteroides subsp. mesenteroides*, gooseberry extract) has the highest level of biofilm formation with a value of 3.809 Au at a 99.9% confidence level and a value of 3.854 Au at a 95% confidence level (**Table 4**).

Combination	Average (Au)	Standard Deviation	Standard Error	95% & 99.9% Confidence that Combination will have Au >=	
				95%	99.9%
B2 Gooseberry	3.305	0.848	0.268	**2.813**	**2.152**
B2 BitterGourd	3.122	0.730	0.231	**2.698**	**2.129**
B2 HolyBasil	3.409	0.966	0.306	**2.849**	**2.096**
B2 Fenugreek	1.206	1.240	0.392	**0.487**	**-0.479**
B2 P. Seeds	0.332	0.236	0.074	**0.196**	**0.012**
B2 P. Peel	1.245	0.325	0.103	**1.057**	**0.804**
J10 Gooseberry	3.887	0.057	0.018	**3.854**	**3.809**
J10 BitterGourd	2.008	0.655	0.207	**1.628**	**1.117**
J10 HolyBasil	2.889	1.305	0.413	**2.133**	**1.115**
J10 Fenugreek	1.226	0.634	0.200	**0.859**	**0.364**
J10 P. Seeds	0.629	0.221	0.070	**0.500**	**0.328**
J10 P. Peel	1.716	1.245	0.394	**0.994**	**0.023**
N14 Gooseberry	3.657	0.391	0.124	**3.430**	**3.126**
N14 BitterGourd	3.479	0.627	0.198	**3.115**	**2.627**
N14 HolyBasil	2.458	0.980	0.310	**1.890**	**1.126**
N14 Fenugreek	2.259	0.831	0.263	**1.777**	**1.130**
N14 P. Seeds	1.483	0.891	0.282	**0.967**	**0.273**
N14 P. Peel	1.832	0.900	0.285	**1.310**	**0.609**

Table 4: Statistical figures for assessing biofilm formation with different plant extracts.

Discussion

The first hypothesis, concerning biofilm formation with the fifteen different combinations of the lactic acid bacteria, *L. acidophilus, S. thermophilus, L. lactis*, and *L. mesenteroides subsp. mesenteroides*, was not supported by the results. The combination that resulted in the most biofilm formation contained only *S. thermophilus,*

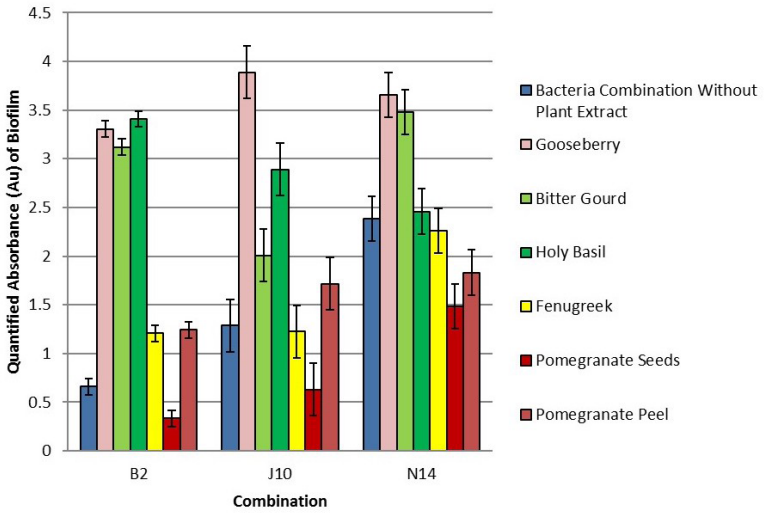

Figure 3: Mean value of quantified absorbance of the lowest, median, and highest probiotic biofilm formation combination with plant extracts. Absorbance (Au) of crystal violet dye indicates the amount of biofilm formation. Ten replicates were done for each combination. Error bars show standard error.

L. lactis, and *L. mesenteroides subsp. mesenteroides*. As for general analysis of the results, *L. acidophilus* seemed to grow the best on its own compared to other combinations containing it (but not the most compared to the other single-species biofilms) (A1); when grown with other bacteria, it tended to lower the overall biofilm formation (e.g., E5, F6, G7). *S. thermophilus*, although it had the lowest growth when grown alone, in a single-species biofilm (B2), it tended to support and increase biofilm formation when grown with *L. lactis* and *L. mesenteroides subsp. mesenteroides* (as seen in H8 and I9). *L. lactis* was overall a very conducive bacteria, bringing about an increase in biofilm formation when it was in a multispecies biofilm with *S. thermophilus* and *L. mesenteroides subsp. mesenteroides* (e.g. H8, M13, N14) as well as when it is in a single-species biofilm by itself (C3). *L. mesenteroides subsp. mesenteroides* is also a lactic acid bacteria that brought about an increase in biofilm formation both when by itself (D4) and when in a multispecies biofilm with *S. thermophilus* and *L. lactis* (e.g., I9, N14). Overall, *L. acidophilus* lowered biofilm formation when grown with other beneficial bacteria. It was speculated that this was due to *L. acidophilus's* tendency to maximize biofilm growth when grown alone in a single-species biofilm. It is possible that due to certain, possibly morphological, characteristics of *L. acidophilus*, it did not enhance biofilm growth when grown with the other bacteria. All of these analyses of the behavior of each bacterium when in the biofilm are important to understand when researching and applying the results of probiotic biofilm formation in the real world. Probiotic biofilm formation can potentially be used as a model for limiting pathogen growth.

The second hypothesis, concerning biofilm formation with the six plant extracts from gooseberry, bitter gourd, holy basil, fenugreek, and pomegranate seeds and peel was supported by the results. The combination with the plant extract that had the most biofilm formation contained *L. lactis, L. mesenteroides subsp. mesenteroides*, and gooseberry extract. In a general analysis of the effects of the presence of the six plant extracts on the probiotic biofilm, the extract of gooseberry enhanced the probiotic biofilm formation the most, especially in the J10 and N14 combinations. "Runner-ups" to the gooseberry extract were bitter gourd and holy basil. Bitter gourd enhanced the biofilm formation of N14 while holy basil enhanced the biofilm formation of B2. Next in effect of enhancement were fenugreek and pomegranate peel, which had lower benefits than gooseberry, bitter gourd, and holy basil, but still enhanced the biofilm formation somewhat (only in B2 and J10, but not N14). Pomegranate seeds, on the other hand, were detrimental to the probiotic biofilm; their presence decreased the amount of biofilm formation. Looking individually at each of the bacteria for the effects of plant extracts on biofilm formation, gooseberry extract was a universal enhancer for all three bacteria; bitter gourd and holy basil extracts had some strong, enhancing effects on *S. thermophilus*; fenugreek extract enhanced the biofilm formation of *L. mesenteroides subsp. mesenteroides*; and pomegranate seeds and peel were not effective in enhancing the biofilm formation of all three bacteria. As in the results, the combination with a median level of biofilm formation (J10 with gooseberry extract) actually had the highest biofilm formation, even exceeding the combination that had the highest biofilm formation (N14). The most important point drawn from the results of this part of the project was that biofilm formation of a bacteria combination with a plant extract could have different amounts of biofilm formation than the biofilm growth of just the bacteria combination.

Applications

Antibiotic resistance is a natural phenomenon that occurs when the bacteria targeted by the antibiotic grows resistant to the antibiotic and continues to multiply. Antibiotic resistance is on the rise and a new way to prevent and treat bacterial infections is needed. This study provides a basis of a new solution to antibiotic resistance. Instead of focusing on killing the harmful bacteria that cause an infection, this study focuses on ways to maximize the growth of good or helpful bacteria that will thrive and destroy the detrimental bacteria naturally.

The main goal of this project was to determine the best combination of lactic acid bacteria and natural plant extracts that would maximize probiotic biofilm formation. This can be applied to the real world in many ways, one just explained above. Pharmaceutical companies can use this study to design probiotic pills, tablets, and supplements. These companies can use the combination of bacteria that results in the most probiotic biofilm formation in their tablets or even add the plant extracts into the probiotic pills. With a probiotic supplement that has the potential to maximize probiotic biofilm formation within the body in the market, people can intake these supplements either to increase and strengthen their immunity or to fight off a bacterial infection that is not affected by antibiotics. Based on statistical analyses, even if the process of making these probiotics has some variability, it can be said with 99.9% confidence that combination N14 (the combination containing *S. thermophilus, L. lactis*, and *L. mesenteroides subsp. mesenteroides* that had the most biofilm formation) or J10 with gooseberry extract (the combination with *L. lactis, L. mesenteroides subsp. mesenteroides*, and gooseberry plant extract that had the most biofilm formation) will always have a value of biofilm formation greater than or equal to 1.59 Au and 3.809 Au respectively, and this can be advertised on product bottles.

The four bacteria used in this study were specifically chosen because they are lactic acid bacteria and can be found naturally in fermented and other natural products and be consumed by the average person daily in a natural form. If the combination of bacteria that proved to have the most biofilm formation is consumed through natural products daily, the average person can experience the benefits without taking supplements or pills. The plant extracts that enhanced probiotic biofilm formation the most, gooseberry, bitter gourd, and holy basil, can also be found naturally and consumed along

with the bacterial combination to enhance and support probiotic biofilm formation within the body.

This study also provides insight into the workings of biofilm formation and other bacterial processes and furthers research on antibiotic resistance to explore the bacterial interactions that cause the promotion of probiotic biofilm formation within the body. This study can potentially also be used as a basis for further research regarding quorum sensing, a system of stimulus and response to coordinate gene expression according to local population density in a bacterial colony. Quorum sensing explains the "why" of biofilm formation, and it is necessary to understand why and how these helpful lactic acid bacteria are forming a probiotic biofilm in order to maximize the growth of these probiotic biofilms. This project can be used as a basis to explore the relationship between quorum sensing and biofilm formation in order to maximize the beneficial bacteria within the body to overcome antibiotic resistance through natural means and develop a model for limiting pathogen growth.

Limitations

One of the limitations of this project was that one (*L. lactis*) of the four bacteria was in a different starter culture medium than the three others. Another limitation of this study was that not all fifteen combinations were tested with the six plant extracts in the second part of the project. Consistent forms of all plants were not used to create the extracts as the six plants have different characteristics and have to be handled differently in order to make the extracts. Also, testing different sources of the same extracts would help lend support to these conclusions. Other general limitations arise when dealing with bacteria, but best precautions and care were taken to minimize these limitations.

Future Research

In order to expand the spectrum of this project, more variables can be added. Also, in the future, to fix the most important limitation of this project, the same starter medium, whether they all be in the form of Culti-Loops or a MicroKwik culture, for every bacterium in the study should be used in order to achieve the most consistent and reliable results. The next step in this project would be to test this new "formula" for maximizing biofilm formation found in this study by growing the bacteria and plant combination that had the most growth with harmful bacteria that also live in the same environment. By growing the probiotic biofilm with detrimental bacteria that would attack the body otherwise, it can be seen if the probiotic biofilm is actually able to fight off the bad bacteria to prevent bacterial infections and diseases as it would in our body. This can be tested by growing the probiotic biofilm with the detrimental bacteria and then quantifying the DNA within the biofilm using primers and PCR (polymerase chain reaction) to see if the probiotic bacteria enhanced with the plant extract is actually prevalent (more present than the harmful bacteria) in the biofilm.

Methods

Safety Precautions

All necessary care was taken when handling the bacteria specimens. Safety goggles, gloves, and an apron were always worn when handling the bacteria. Any processes requiring the use of bacteria were carried out on a diaper. All disposable equipment used with the bacteria was safely discarded in the biohazard bin. When heating with the hot plate, oven mitts were always worn to avoid burns and other injuries. When using crystal violet dye, an apron, safety goggles, and gloves were worn as it readily stains skin and clothing.

Preparing and Growing Bacteria

MRS (de Man, Rogosa, Sharpe) agar plates were prepared by boiling 62 grams of the MRS agar powder in one liter of distilled water on a hot plate with a stir bar. After the powder had fully dissolved, the solution had come to a boil, and it had cooled, 20 mL of agar was poured into 50 labeled plates with a 25-mL pipette. The plates were flipped over and placed in the refrigerator until use. The MicroKwik dehydrated culture of L. lactis (MicroKwik Culture, Vial) was first hydrated in the broth provided in the kit, as per kit instructions, and incubated at 37°C for 48 hours. After 48 hours, 0.5 mL of the liquid medium with bacteria in it was spotted onto MRS agar plates. The spot was streaked with an inoculating loop sterilized with the Bunsen burner. These labeled plates were then incubated, upside down at 37°C for 48 hours before proceeding with the experiment. The three Culti-Loops bacteria (Thermo Scientific Culti-Loops *Lactobacillus acidophilus* ATCC 314; Thermo Scientific Culti-Loops *Streptococcus thermophilus* ATCC 19258; Thermo Scientific Culti-Loops *Leuconostoc mesenteroides subsp. mesenteroides* ATCC 8293) were streaked with the prepared culture loops, as instructed on the package, onto prepared and labeled MRS agar plates and incubated, upside down, at 37°C for 48 hours before proceeding with the experiment. In order to prepare the 96-well plates, the bacteria must be inoculated into the appropriate liquid culture, anaerobe broth (Thermo Scientific Oxoid Schaedler Anaerobe Broth). This broth is advertised for growing lactic acid bacteria in liquid medium. 2.65 grams of the anaerobe broth powder was added to 100 mL of distilled water in a 250-mL bottle and brought to a boil until fully dissolved, with a stir bar. The broth was labeled and placed in the refrigerator until use. After incubating all bacteria for 48 hours (in the case of *L. lactis*, 96 hours), an inoculating loop that had been sterilized with the Bunsen burner was used to pick up a few colonies and transfer them into microcentrifuge tubes containing 1 mL of the room-temperature anaerobe broth. These tubes were placed in a microcentrifuge rack and incubated at 37°C for 48 hours, and we confirmed that they contained bacteria with a microscope (with glass slide, cover slip, and crystal violet dye).

Preparing 96-Well Plates with Different Combinations

Table 5 and **Figures 6a-b** were used to prepare

the 96-well plates for biofilm formation analysis with the fifteen different combinations of the lactic acid bacteria. The indicated amount of each bacteria listed for each combination was added. 10 replicates for each combination were carried out. The following abbreviations were used: *Lactobacillus acidophilus* = LA; *Streptococcus thermophilus* = ST; *Lactococcus lactis* = LL; *Leuconostoc mesenteroides subsp. mesenteroides* = LM. After appropriately filling the designated wells, the two 96-well plates were labeled and placed in the incubator for 48 hours at 37°C until analysis (explained in "Analyzing Biofilm Formation").

A_1	(120 µl) LA
B_2	(120 µl) ST
C_3	(120 µl) LL
D_4	(120 µl) LM
E_5	(60 µl) LA, ST
F_6	(60 µl) LA, LL
G_7	(60 µl) LA, LM
H_8	(60 µl) ST, LL
I_9	(60 µl) ST, LM
J_{10}	(60 µl) LL, LM
K_{11}	(40 µl) LA, ST, LL
L_{12}	(40 µl) LA, ST, LM
M_{13}	(40 µl) LA, LL, LM
N_{14}	(40 µl) ST, LL, LM
O_{15}	(30 µl) LA, ST, LL, LM

Table 5: Chart with combination names. This chart details the bacteria and their amount in each combination.

Preparing Plant Extracts

The bitter gourd was sliced and dried in the oven at 75°C for 24 hours and then powdered in the coffee grinder and stored in a labeled polystyrene container until use. The same process was repeated with the holy basil (the leaves were just placed in the oven), the pomegranate seeds, and the pomegranate peel (which was diced and then placed in the oven). The fenugreek seeds did not need oven-drying so they were just powdered and stored. The gooseberry powder did not need any preparation. Two grams of each powder was mixed with 25 mL of methanol in an Erlenmeyer flask secured with Parafilm and placed in the shaker for 24 hours at 37°C. The use of methanol ensured a certain degree of the various plant extracts. After shaking, the solutions were filtered and 1 mL of each filtered extract was placed in microcentrifuge tubes and left open for methanol evaporation. After 24 hours, the tubes were closed and ready for use.

Preparing 96-Well Plates with Plant Extracts

After analysis of the plates (flat-bottomed, UV transparent 96-well plates) with the fifteen different combinations was done and the three combinations with the lowest, median, and highest biofilm formation were identified, analysis of biofilm formation with the six different plant extracts needed to be done. Three 96-well plates were prepared (one for each bacteria combination) following **Figure 7**. For each plant extract, 50 µL of the extract was added to the wells. Once the plant extract had been added, the appropriate bacteria combinations, following **Table 5** were added into the wells with the extracts. After appropriately filling the designated wells, the three 96-well plates were labeled and placed in the incubator for 48 hours at 37°C until analysis (explained in "Analyzing Biofilm Formation).

Analyzing Biofilm Formation

The contents of the 96-well plate incubated for 48 hours were dumped out into a plastic container. The plate was then gently submerged in a plastic container filled with water and washed twice, blotting the plate on a stack of paper towels in between washes to lower background staining. 150 µL of crystal violet dye (handled with care as it readily stains) was added to each well containing a biofilm with a micropipette, and the plate was incubated at room temperature for 15 minutes. The plate was washed as described above four times and dried upside down for three hours, and after drying, photographs were taken. To be added into the wells after the plate dried, 30% acetic acid was made by making a 1 M solution from a 6 M solution by adding 5 mL of acetic acid to 25 mL of distilled water, and then making the 1 M solution into a 30% acetic acid solution by adding 30 mL of the 1 M solution to 70 mL of distilled water. 150 µL of the 30% acetic acid solution was added into each well with a biofilm and incubated at room temperature

A.

1	1	2	3	4	5	6	7	8	9	10	11	12
A	A_1	A_1	A_1	A_1	A_1	A_1	A_1	A_1	A_1	A_1	B_2	B_2
B	B_2	B_2	B_2	B_2	B_2	B_2	B_2	B_2	C_3	C_3	C_3	C_3
C	C_3	C_3	C_3	C_3	C_3	C_3	D_4	D_4	D_4	D_4	D_4	D_4
D	D_4	D_4	D_4	D_4	E_5	E_5	E_5	E_5	E_5	E_5	E_5	E_5
E	E_5	E_5	F_6	F_6	F_6	F_6	F_6	F_6	F_6	F_6	F_6	F_6
F	G_7	G_7	G_7	G_7	G_7	G_7	G_7	G_7	G_7	G_7	H_8	H_8
G	H_8	H_8	H_8	H_8	H_8	H_8	H_8	H_8	I_9	I_9	I_9	I_9
H	I_9	I_9	I_9	I_9	I_9	I_9						C

B.

2	1	2	3	4	5	6	7	8	9	10	11	12
A	J_{10}	J_{10}	J_{10}	J_{10}	J_{10}	J_{10}	J_{10}	J_{10}	J_{10}	J_{10}	K_{11}	K_{11}
B	K_{11}	K_{11}	K_{11}	K_{11}	K_{11}	K_{11}	K_{11}	K_{11}	L_{12}	L_{12}	L_{12}	L_{12}
C	L_{12}	L_{12}	L_{12}	L_{12}	L_{12}	L_{12}	M_{13}	M_{13}	M_{13}	M_{13}	M_{13}	M_{13}
D	M_{13}	M_{13}	M_{13}	M_{13}	N_{14}	N_{14}	N_{14}	N_{14}	N_{14}	N_{14}	N_{14}	N_{14}
E	N_{14}	N_{14}	O_{15}	O_{15}	O_{15}	O_{15}	O_{15}	O_{15}	O_{15}	O_{15}	O_{15}	O_{15}
F												
G												
H												C

Figure 6: 96-well plates set-up used in assessing biofilm formation with different combinations of lactic acid bateria. A: This diagram illustrates the 96-well plate set-up for combinations A1 through I9, along with a control. B: This diagram illustrates the 96-well plate set-up for combinations J10 through O15, along with a control.

Lowest/Median/Highest	1	2	3	4	5	6	7	8	9	10	11	12
A				Gooseberry								
B				Bitter Gourd								
C				Holy Basil								
D				Fenugreek								
E				Pomegranate Seeds								
F				Pomegranate Peel								
G												
H												C

Figure 7: 96-well plate template for assessing biofilm formation with plant extracts. This diagram illustrates the 96-well plate set-up for the three bacterial combinations (lowest, median, highest) with the five plant extracts.

for 15 minutes again. The absorbance (which correlates to the amount of biofilm formation) was quantified in the spectrophotometer (SpectraMax Plus 384 Microplate Reader from Molecular Devices) at 550 nanometers. Since the limit of detection on the spectrophotometer is 4.0 Au, high readings are not artifacts of maxing out the signal, but instead are real and significant differences. Results were statistically analyzed after the first part and then all together at the end.

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