Effectiveness of microbial culture supernatants against multi-drug resistant microbes

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of B.Sc. in Biotechnology

Department of Mathematics and Natural Sciences

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Declaration

It is hereby declared that

1. *The thesis submitted is my own original work while completing degree at Brac University.*
2. *The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.*
3. *The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.*
4. *I have acknowledged all main sources of help.*

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Approval

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Abstract:

The experiment started with collecting microorganisms from different sources like soil samples from 10 different places and storing them on CMCA, & SA, and MRS agar for yogurt samples from 7 different places. Among the soil samples, 20 cellulolytic and 15 amylolytic bacteria were found and 12 cellulolytic and 6 amylolytic bacteria showed clear zones when treated with gram's iodine and indicated that they were able to produce enzymes that can degrade cellulose and starch and these probiotics could be a possible source for treating multidrug-resistant bacteria. Then, the same types of broth: CMC broth, starch broth, and MRS broth, Luria Broth & lactose broth media according to selectivity were used. After 48 hours of incubation, the bacterial culture broths were centrifuged and the culture supernatants were collected and the supernatants were used to check the effectiveness of the supernatants against several multidrug-resistant bacteria in different compositions of the cell-free culture supernatants. The main goal of the experiment was to perform an antibiogram within the Mueller-Hinton agar medium and observe the zone of inhibition after an 18-22 hours incubation against several MDR bacteria with the presence of different supernatant compositions. Almost against 11 MDR bacteria, the culture supernatants from specific yogurt samples could inhibit the growth of superbugs as indicated by the zone of inhibition in Muller Hinton Agar, but neither amylolytic nor cellulolytic bacterial culture supernatants have shown any desired effect against the superbugs, it also decreased the effect of LAB supernatant's zone of inhibition if they were mixed in different compositions. After biochemical tests of the LAB, it can be called *Lactobacillus bulgaricus* (presumptive) the culture supernatants of which had shown positive output against most of the MDR bacteria.

Keywords: Carboxymethylcellulose agar, Starch agar, de Man, Rogosa and Sharpe (MRS) agar, Luria Broth (LB), Lactose broth, Superbug, Cellulolytic, Amylolytic, LAB

Dedication

Dedicated to

My parents, without whose love and encouragement I could not have envisioned beginning my adventure for knowledge. My sister for encouraging me mentally, as well as my family and friends for consistently motivating me to keep going on my research journey.

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List of Acronyms

CMCA – Carboxymethylcellulose agar

SA – Starch agar

CMCB – Carboxymethylcellulose broth

SB – Starch broth

DNS – Dinitro salicylic Acid

MRS– de Man, Rogosa and Sharpe

LB – Luria Broth

MHA– Mueller Hinton Agar

LAB- Lactic acid bacteria

Chapter 1

Introduction

1.1 Background

Since prehistoric times, antibiotics have been used in different forms. An antibiotic is a particular class of antimicrobial agent that works against bacteria. The use of antibiotic drugs is common in the treatment and prevention of bacterial infections because they represent the most effective type of antibacterial agent. Ancient Egypt, Nubia, China, Serbia, Greece, and Rome were among the civilizations that applied moldy bread topically and made numerous references to its therapeutic properties. John Parkinson (1567–1650) was the first to formally record the utilization of molds for the treatment of infections. The 20th century saw a revolution in medicine owing to antibiotics. The widespread use of contemporary penicillin, which was developed in 1928 by Alexander Fleming (1881–1955), was extremely helpful throughout the war. The effectiveness and availability of antibiotics have also resulted in their abuse, and certain bacteria have developed antibiotic resistance as a consequence[1]. (Gould, 2016)

A variety of insensitivity that microorganisms can develop to lethal antibiotic dosages is known as multidrug resistance (MDR). Multidrug-resistant bacteria can no longer be controlled or killed by certain antibiotics because they have developed a resistance to the following antibiotics.

Drug resistance is a serious issue. Through horizontal gene transfer, the number of drug-resistant bacteria has significantly expanded worldwide. Multidrug-resistant (MDR) bacteria have risen frighteningly in the last few decades, creating a severe threat to both human and animal health. In order to maintain the health and productivity of animals, antibiotics are utilized. The spread of infections that are resistant to treatment in both humans and livestock is facilitated by these practices, which pose a serious risk to the public's health[2] (Boeckel et al., 2015). The development of antimicrobial drug resistance has significant impacts on both

[1] Kate Gould, Antibiotics: from prehistory to the present day, *Journal of Antimicrobial Chemotherapy*, Volume 71, Issue 3, March 2016, Pages 572–575, https://doi.org/10.1093/jac/dkv484

[2] Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., Teillant, A., & Laxminarayan, R. (2015). Global trends in antimicrobial use in food animals. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(18), 5649–5654. https://doi.org/10.1073/pnas.1503141112

personal and societal health as the world is on the approach to returning to the "pre-antibiotic period" [3] (Hoque et al., 2020). Today, more than 70% of bacteria that cause infectious diseases are resistant to at least one antibiotic used in traditional antimicrobial therapy[4] (Samanipour et al., 2016).

Combating bacterial resistance has become an urgent and demanding challenge that must be effectively handled as the prevalence of diseases caused by MDR bacteria has been accompanied by sickness and death. Even though antibiotics are still frequently used to cure diseases, they have unintended consequences that include the development of resistant strains and microbial substitution. To avoid negative side effects, several natural compounds are being employed[5] (Khalil et al., 2016).

To treat the MDR bacteria, different techniques can be used, such as- nanoparticles therapy, phase therapy, immunoglobulin therapy, Fecal microbiota transplants, Antisense RNA-based treatments, CRISPR-Cas9-based treatments, Natural product-based antibiotic, etc.

Among Natural product-based antibiotic treatment, culture supernatants of different bacteria can be effective against several MDR bacteria. The media in which the cells were growing is called a cell culture supernatant. Here, cell-free culture supernatants were used to experiment.

In this experiment, 3 types of bacterial culture supernatants were used. To find cellulolytic and amylolytic bacteria, soil samples were taken from several areas in Dhaka. Another type of bacteria collected from Yogurt samples from renowned brands as probiotics has effective characteristics against different microbes [6] (Barzegari et al., 2019).

After testing the enzyme activity of the following soil bacteria with Gram's iodine, the selective bacteria who showed zone were kept for 36-72 hours incubation within the selective

[3] Hoque, R., Ahmed, S. M., Naher, N., Islam, M. A., Rousham, E. K., Islam, B. Z., & Hassan, S. (2020). Tackling antimicrobial resistance in Bangladesh: A scoping review of policy and practice in human, animal and environment sectors. *PloS one*, *15*(1), e0227947. https://doi.org/10.1371/journal.pone.0227947

[4] Samanipour, A., Dashti-Khavidaki, S., Abbasi, M. R., & Abdollahi, A. (2016). Antibiotic resistance patterns of microorganisms isolated from nephrology and kidney transplant wards of a referral academic hospital. *Journal of research in pharmacy practice*, *5*(1), 43–51. https://doi.org/10.4103/2279-042X.176559

[5] Khalil, D., Hultin, M., Rashid, M. U., & Lund, B. (2016). Oral microflora and selection of resistance after a single dose of amoxicillin. *Clinical Microbiology and Infection*, *22*(11), 949-e1. https://doi.org/10.1016/j.cmi.2016.08.008

[6] Barzegari, A., Kheyrolahzadeh, K., Hosseiniyan Khatibi, S. M., Sharifi, S., Memar, M. Y., & Zununi Vahed, S. (2020). The Battle of Probiotics and Their Derivatives Against Biofilms. *Infection and drug resistance*, *13*, 659–672. https://doi.org/10.2147/IDR.S232982

broth media. The yogurts' bacteria were also kept for 48 hours of incubation. After suitable incubation, the culture supernatants were collected by centrifugation [7](Shofiyah et al., 2020).

The culture supernatants of those bacteria were used to know whether they can show any effectivity against MDR or not. Here, the culture supernatants were applied individually along with mixed among them in different proportions within the MHA plates and observed their effectivity at 37°C within 18-22 hours[8] (Maslennikova et al., 2017).

Here, the supernatants were loaded within the Petridis by using antibiogram techniques. The supernatants which could show a zone of inhibition, might be considered as effective against the MDR bacteria.

The purpose of the study is to treat MDR bacteria with different type of cell free culture supernatants.

1.2 Cellulolytic bacteria

Photosynthesis is the process in which plants produce biomass with cellulose as its main constituent, which is essential for maintaining life on Earth. Because of the activity of cellulose-consuming bacteria found in soil and animal guts, the carbon cycle is primarily closed. The carbon flux at both local and global scales is relevant about microbial cellulose consumption, which is responsible for one of the greatest material flows in the biosphere. Ruminants' role as a significant dietary protein source only serves to emphasize the significance of microbial cellulose consumption in natural habitats[9] (Lynd et al., 2002).

Also, common systems like anaerobic digestion and composting also depend on the consumption of microbial cellulose. Cellulolytic bacteria release free enzymes that rely on the breakdown of lignocellulose into useable sugars by enzymes with distinct substrate specificities. Cellulases and hemicellulases are examples of Glycoside hydrolases (GHs)

[7] Shofiyah, S. S., Yuliani, D., Widya, N., Sarian, F. D., Puspasari, F., Radjasa, O. K., Ihsanawati, & Natalia, D. (2020). Isolation, expression, and characterization of raw starch degrading α-amylase from a marine lake *Bacillus megaterium* NL3. *Heliyon*, *6*(12), e05796. https://doi.org/10.1016/j.heliyon.2020.e05796

[8] Maslennikova, I. L., Kuznetsova, M. V., Nekrasova, I. V., & Shirshev, S. V. (2017). Effect of bacterial components of mixed culture supernatants of planktonic and biofilm Pseudomonas aeruginosa with commensal Escherichia coli on the neutrophil response in vitro. *Pathogens and disease*, *75*(8), 10.1093/femspd/ftx105. https://doi.org/10.1093/femspd/ftx105

[9] Lynd, L. R., Weimer, P. J., van Zyl, W. H., & Pretorius, I. S. (2002). Microbial cellulose utilization: fundamentals and biotechnology. *Microbiology and molecular biology reviews: MMBR*, *66*(3), 506–577. https://doi.org/10.1128/MMBR.66.3.506-577.2002

which are enzymes that break down the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate element. Cellulolytic bacteria can be used as probiotics[10] (Ren et al., 2021).

So, its cell free culture supernatants might be effective against MDR bacteria, as they have strong enzymatic backgrounds.

1.3 Amylolytic bacteria

A polymeric complex carbohydrate called starch also referred to as amylum, is composed of a number of glucose units linked together by glycosidic linkages. All green plants naturally generate starch, a well-known polymer, which serves as a food source for people and a source of energy. It is essential for a healthy, balanced diet since it gives the body glucose, which serves as each cell's primary source of energy. With that, it supplies a variety of vitamins, minerals, fiber, and other nutrients. Starch typically has an amylose content of 20–25% and an amylopectin content of 75–80%, based on the source[11] (Habibi et al., 2012).

The bacteria that can degrade starch is called as amylolytic bacteria. Amylolytic bacteria can be performed as probiotics. So, its cell free culture supernatants might be effective against MDR bacteria, as they have strong enzymatic backgrounds.

[10] Ren, W., Xu, X., Long, H., Zhang, X., Cai, X., Huang, A., & Xie, Z. (2021). Tropical Cellulolytic Bacteria: Potential Utilization of Sugarcane Bagasse as Low-Cost Carbon Source in Aquaculture. *Frontiers in microbiology*, *12*, 745853. https://doi.org/10.3389/fmicb.2021.745853

[11] Visakh, P. M., Mathew, A. P., Oksman, K., & Thomas, S. (2012). Starch-based bionanocomposites: Processing and properties. *Polysaccharide building blocks: A sustainable approach to the development of renewable biomaterials*, 287-306. DOI:10.1002/9781118229484

1.4 Yogurts' bacteria

Since it originated in Western Asia and the Middle East, yogurt has become a common meal in so many cultures. When heated milk is mixed with bacteria, particularly *Lactobacillus bulgaricus* and Streptococcus thermophilus, and left to ferment for several hours at a warm temperature (110-115°F), yogurt is formed. Bifidobacteria and lactobacilli of several varieties could be present. The bacteria convert milk sugar lactose into lactic acid, which solidifies the milk and gives it a distinctly acidic flavor [12] (Fisberg et al., 2015).

Lactic acid is produced by a range of fermenting microorganisms found in fermented products that come from different genera and species. Milk and yogurt both contain nearly equivalent amounts of vitamins and minerals. Folic acid is produced and vitamins B-12 and C are absorbed during fermentation. Depending on the type of bacteria used for fermentation, there are minor variations in other vitamins between milk and yogurt. Although milk and yogurt both contain equivalent amounts of minerals, some elements, such as calcium, are more readily available in yogurt than in milk. In contrast to milk, yogurt typically contains less lactose and more lactic acid, galactose, peptides, free amino acids, and free fatty acids[13] [14]. (Tamime et al., 2006) , (Shahani et al., 1979).

Due to yogurt's decreased lactose content, people who cannot tolerate milk products due to lactose intolerance may be able to eat some of it. These bacteria can inhibit the growth of harmful bacteria, which results in fewer infections and stronger anticancer benefits. It is well known that consuming fermented milk that contains lactic acid bacteria is beneficial to the body since it prevents intestinal infections and acts as an anticarcinogen[15] (Meydani et al., 2000).

[12] Fisberg, M., & Machado, R. (2015). History of yogurt and current patterns of consumption. Nutrition reviews, 73(suppl_1), 4-7.

[13] Kurmann, J. A. (1978). Fermented fresh milk products and their cultures. Technical Dairy Publishing House (diff.).

[14] Shahani, K. M., & Chandan, R. C. (1979). Nutritional and healthful aspects of cultured and culture-containing dairy foods. Journal of Dairy science, 62(10), 1685-1694.

[15] Meydani, S. N., & Ha, W. K. (2000). Immunologic effects of yogurt. *The American journal of clinical nutrition*, *71*(4), 861-872.

1.5 Culture Supernatants

The medium in which the cells are growing is known as the cell culture. Prokaryotic and eukaryotic organisms can be cultivated within controlled environments using the cell culture technique. The clear liquid that floats above the solid residue from cell culture following centrifugation, precipitation, and relaxation is referred to simply as the supernatant. The liquid usually has a lower density and is devoid of precipitate.

The goal of the cell culture supernatant is to be free of any floating cells collected that may have broken off from the cell cultures, as well as of any cellular debris (vesicles or particles) that may potentially be present in the cell culture.

Here, cell-free bacterial culture supernatant was used, in which bacteria create a wide range of secondary metabolites, such as antibiotics, enzymes, siderophores, and toxins within the culture supernatant[16] (Blumer et al., 2000).

Three types of derived bacterial cell-free culture supernatants were mixed in different proportions and observed their efficiency against MDR bacteria. Also, they were applied individually to know their effectiveness. The supernatants were tested by using agar diffusion and disk diffusion method. Here, within the agar diffusion method, the supernatants were loaded directly and disk diffusion method were performed by soaking the antibiotic disk within the cell free culture supernatants in different proportions.

[16] Blumer, C., & Haas, D. (2000). Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. Archives of microbiology, 173(3), 170–177. https://doi.org/10.1007/s002039900127

Chapter 2

Materials and Methods

The present study was carried out at the Biotechnology and Microbiology laboratories of the Department of Mathematics and Natural Sciences, BRAC University.

2.1 Apparatus and Reagents

Apparatus

- Sample collection box
- Petri dish
- Test Tubes
- Erlenmeyer Flasks
- Falcon tubes
- Eppendorf Tubes
- Bunsen burner
- Spirit lamp
- Glass Rods
- Pipette
- Laminar Air Flow
- Shaker incubator
- Incubator
- Multipurpose Centrifuge Machine
- Cork borer
- Blank antibiotic disk
- Loop
- Needle
- Cotton swab
- pH indicator
- Autoclave
- Balance
- Spatula
- Foil Paper

Reagents

- Nutrient agar
- Agar
- Carboxymethylcellulose
- Starch
- MRS
- Gram's Iodine
- Lactose broth
- LB broth
- Mueller Hinton Agar
- Dextrose
- Sucrose
- Sodium Chloride
- Sodium hydroxide
- Peptone
- 40% Urease Solution
- 70% ethanol
- 0.1M NaOH

2.2 Methodology

2.2.1 Collection of Samples and Identification of Cellulolytic, Amylolytic and Yogurts' Bacteria

Soil Samples were collected from different places inside and outside Dhaka. From those soil samples, Cellulolytic and Amylolytic bacteria were isolated.

Yogurts' bacteria were isolated from different companies in Bangladesh. Yogurts' bacteria usually consist of Bifidobacteria and lactobacilli which can be commonly said as Lactic acid bacteria.

The obtained samples were diluted up to 10^{-4} times to get fewer bacterial colonies. The diluted samples were spread on CMCA, SA, and MRSA to get the following Cellulolytic, Amylolytic, and Lactic acid bacteria. After 24-48 hours of incubation at 37°C temperature, the selective media showed the zone of the desired bacteria. Based on their size, color, and attitudes of the colony, they were differentiated.

After that, the differentiated bacterial colony was inoculated on CMCA and SA plates. After incubation, they were swamped with Gram's Iodine. After 4-5 minutes of applying the Gram's Iodine, some of the bacterial colonies showed a ring within their area. The bacterial colonies were again taken from the replica plates and streaked to obtain pure cultures.

Yogurts' bacteria were isolated according to their size, color, and attitudes of the colony they were streaked and differentiated.

The pure cultures were stored as stock cultures along with 30% glycerol at -20°C temperature for long-time preservations. The following bacteria were inoculated within the selective broth, such as- cellulolytic bacteria at CMC broth[17] (Otajevwo & Aluyi, 2011), amylolytic bacteria at starch broth[18] (Sjofjan & Ardyati, 2011).

[17] Otajevwo, F. D., & Aluyi, H. S. A. (2011). Cultural conditions necessary for optimal cellulase yield by cellulolytic bacterial organisms as they relate to residual sugars released in broth medium. Modern Applied Science, 5(3), 141.

[18] Sjofjan, O., & Ardyati, T. (2011). Extracellular amylase activity of amylolytic bacteria isolated from quail's (Coturnix japonica) intestinal tract in corn flour medium. International Journal of Poultry Science, 10(5), 411-415.

For Lactic acid bacteria, the bacteria were inoculated in 3 different broths, such as- MRS broth, Lactose broth, and LB broth[19] (Carr et al., 2002).

2.2.2 Preparation of culture supernatants and extraction of cell-free culture supernatants

To incubate for 48-72 hours, bacteria inoculated in CMC broths, Starch broths, MRS broth, Lactose broth, and LB broth were kept within a shaker incubator at 37°C and 160 rpm. After incubation, 5ml of each cell culture was taken to falcon tubes and centrifuged at a multipurpose centrifuge machine. For cellulolytic and amylolytic bacteria, they were centrifuged at 11000 rpm at 4°C temperature for 30 minutes[20] (Ibarra et al., 2004) and yogurts' bacteria were centrifuged at 3000 rpm at 4°C temperature for 20 minutes[21] (An et al., 2010). The clear liquid that floats above the solid residue from cell culture following centrifugation, precipitation, and relaxation is referred to simply as the supernatant. The liquid usually has a lower density and is devoid of precipitate. Cell culture supernatants need to be free of any floating cells that may have broken off from the cell cultures, as well as any cellular debris (vesicles or particles) that may potentially be present in the cell culture.

Cell-free bacterial culture supernatants were used, in which bacteria create a wide range of secondary metabolites, such as antibiotics, enzymes, siderophores, and toxins within the culture supernatant[22] (Blumer & Hass, 2000).

The supernatants were preserved at -20°C for further use[23] (Hamad et al., 2018).

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[19] Carr, F. J., Chill, D., & Maida, N. (2002). The lactic acid bacteria: a literature survey. Critical reviews in microbiology, 28(4), 281-370. https://doi.org/10.1080/1040-840291046759

[20] Ibarra, D., del Rıo, J. C., Gutiérrez, A., Rodrıguez, I. M., Romero, J., Martınez, M. J., & Martınez, Á. T. (2004). Isolation of high-purity residual lignins from eucalypt paper pulps by cellulase and proteinase treatments followed by solvent extraction. Enzyme and Microbial Technology, 35(2-3), 173-181. https://doi.org/10.1016/j.enzmictec.2004.04.002

[21] An, H. M., Baek, E. H., Jang, S., Lee, D. K., Kim, M. J., Kim, J. R., ... & Ha, N. J. (2010). Efficacy of Lactic Acid Bacteria (LAB) supplement in management of constipation among nursing home residents. Nutrition Journal, 9(1), 1-7.

[22] Blumer, C., & Haas, D. (2000). Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. Archives of microbiology, 173(3), 170–177. https://doi.org/10.1007/s002039900127

[23] Hamad, G. M., Abu-Serie, M. M., Ali, S. H., & Hafez, E. E. (2018). Combination probiotic supernatants reduce growth and aflatoxin production by Aspergillus spp in food contamination. American Journal of Food Science and Technology, 6(2), 57-67. DOI:10.12691/ajfst-6-2-1

2.2.3 Supernatants combination for antibiogram

The supernatants collected from **cellulolytic** bacteria were named as CL1, CL2, CK1, CK2, CI, CJ2, 3CS, CA1, CA2, CA3, CB, and Cx.

The supernatants collected from **amylolytic** bacteria were named as SA2, SA3, SA4, SA12, SA13, and SA16.

The supernatants collected from **Lactic acid** bacteria were named Ar, Mo, Po & Sh and the 4 samples were inoculated within MRS broth, Lactose broth, and LB broth separately.

Ar bacteria inoculated within MRS broth's was marked as ArB, Lactose broth was marked as ArA, LB broth was marked as ArC.

Mo bacteria inoculated within MRS broth's was marked as MoB, Lactose broth was marked as MoA, LB broth was marked as MoC.

Po bacteria inoculated within MRS broth's was marked as PoB, Lactose broth was marked as PoA, LB broth was marked as PoC.
Sh bacteria inoculated within MRS broth's was marked as ShB, Lactose broth was marked as ShA, LB broth was marked as ShC.

The following synbiotics culture supernatants were applied-

Table 1: Different combinations of synbiotics culture supernatants

CL1	CL2	CK1	CK2	CI	CJ2	3CS	CA1
CA2	CA3	CB	Cx	SA2	SA3	SA4	SA12
SA13	SA16	ArB	ArA	ArC	MoB	MoA	MoC
PoB	PoA	PoC	ShB	ShA	ShC	CA1 CA2	CA1 CA3
CA1 CB	CA1 CL1	CA1 CL2	CA1 CK1	CA1 CK2	CA1 CI	CA1 CJ2	CA1 3CS
CA1 CA2 CL1	CA1 CA2 CL2	CA1 CA2 CK1	CA1 CA2 CK2	CA1 CA2 CI	CA1 CA2 CJ2	CA1 CA2 3CS	CL1 SA2
CL2 SA2	CL2 SA3	CL2 SA4	CL2 SA12	CL2 SA13	CL2 SA16	ArB ArA ArC	ArB ArA
ArA ArC	MoB MoA MoC	MoB MoA	MoB MoC	MoB	MoA	MoC	ArB
ArA	ArC	PoB	PoA	PoC	PoB PoA PoC	PoB PoA	PoB PoC
PoA PoC	ArB ArA ArC CL1	ArB CA1 CA2 CK2	ArA CA1 CA2 CJ2	MoB CA1 CA2 CK2	MoA CA1 CA2 3CS	MoC CA1 CA2 CK2	PoB SA2 CA1 CA2 3CS
PoA SA3 CA1 CA2 3CS	ShB ShA ShC	ShB SA3 CA1 CA2 3CS	ShC SA3 SA4 CA1 CA2 CI	ShB ShA ShC SA1 SA3 CA1 CA2 3CS	ArB SA2 SA12 CA1 CA2 3CS CI	ArB MoB SA3 SA16 CA1 CA2 3CS	PoA PoB SA3 SA12 CA1 CA2 3CS

C= Cellulolytic culture supernatant, **SA**= Amylolytic culture supernatant

Yogurt samples, **Ar**= Lactic acid bacterial culture supernatant 1, **Mo**= Lactic acid bacterial culture supernatant 2, **Po**= Lactic acid bacterial culture supernatant 3. **Sh**= Lactic acid bacterial culture supernatant 4, A= Growth on Lactose broth, B= Growth on MRS broth, C= Growth on LB broth

2.2.4 Antibiogram

Antibiogram was done on MHA plates. Fresh 24 hours MDR bacterial cultures on NA were taken by a loop and collected in a single colony and taken to the test tube of saline solution. Then, using vortex to shake the sample of MDR bacteria within the saline water to maintain 0.5 McFarland Standard. With a sterile cotton swab, the MDR bacteria were taken and the MHA plates were inoculated in a such manner that the bacteria could spread in every space of the MHA plates.

Two methods were followed to do an antibiogram, such as the agar diffusion method and Kirby-Bauer disk diffusion susceptibility test method. For the agar diffusion method, the inoculated MHA plates were introduced with small holes with the help of a sterile Cork borer and filled with the holes with the upper mentioned supernatants' combination with the help of a pipette.

Kirby-Bauer disk diffusion susceptibility test method was done by soaking the sterile filter paper within the supernatants and keeping it sometimes to absorb the supernatants. After absorbing the disk were placed on the MHA places and marked as their symbolic name.

After that, it was kept for 16-20 hours in the incubator at 37°C temperature.

2.2.5 Streak the effective bacterial culture

The sample of Lactic acid bacteria was shown effective against MDR bacteria, the effective bacterial colony was streaked and isolated to gain pure culture and test which bacterial culture

supernatant is effective against MDR bacteria. After getting 2 types of a bacterial colonies, they were inoculated within MRS broth and kept under a shaker incubator for 48 hours at 37°C temperature.

2.2.6 pH and supernatants effectiveness

The effective culture supernatants were found from Lactic acid bacteria from a certain sample. The effective culture supernatants were used to know whether they are effective against MDR bacteria or not. For doing that, 0.1M NaOH was used to neutralize the pH which is 7, and more than 7 which is basic. To add, the initial pH of the culture supernatants was acidic found in Lactic acid bacteria.

2.2.7 Biochemical tests

A few biochemical assays (refer to Appendix B for further information) were conducted in order to identify the effective bacteria strain against that was acquired. The identified microbial strains were determined by comparing the observed characteristics to a chart[24] .

[24] Microbiology Lab: MOLB 2210. (n.d.). Retrieved from https://www.uwyo.edu/molb2210_lab/info/biochemical_tests.htm?fbclid=IwAR1rf9GTh1gXq4-823iTQy_3XrB5frc2NzWKoq_YN1hBzFlB4iUWqiYLyrE#sulfur

Results

3.1 Soil sample collections on selective media

The 10^{-4} diluted soil samples were spread on selective media, such as – CMC agar and starch agar. After incubation for 48 hours, both types of selective media showed bacterial colonies on the plates. The bacterial colonies were streaked according to their shape, color, and attitude and streaked in other plates of the same selective media. After incubation of 72 hours, the plates were shown enough growth.

Figure 1:Cellulolytic and amylolytic Bacterial growth on selective media (Cellulolytic bacteria on CMCA and amylolytic bacteria on starch agar media)

3.2 Streaked and cultured cellulolytic and amylolytic bacteria on Nutrient Agar media

The isolated colonies were streaked on NA plates to observe its difference from each other that were isolated from CMCA and Starch agar plates.

Figure 2: Cellulolytic and amylolytic bacteria streaked on NA plates

3.3 Cellulolytic and amylolytic bacterial colony cultured on selective media

Differently, isolated colonies were again cultured on selective media like CMCA and SA.

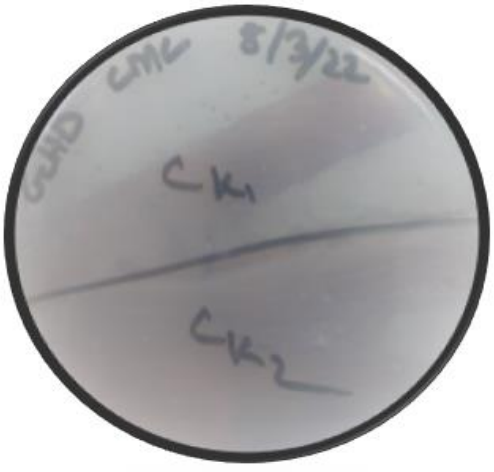

Figure 3: Cultured on CMCA again and observed growth

3.4 Isolation and identification of cellulolytic and amylolytic bacteria

The cellulolytic and amylolytic bacteria were identified initially by using Gram's iodine. The bacteria which showed the zone were isolated and collected and the bacteria which were failed to show the zone were discarded.

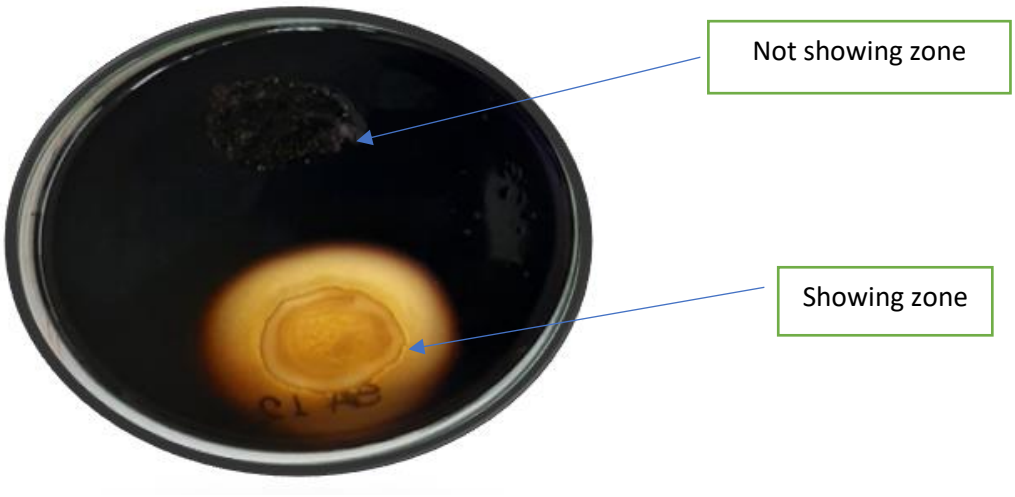

Figure 4: One colony showed positive results (showed zone) and another colony showed a negative result for Gram's iodine test

Figure 5: Colonies showed negative result for Gram's iodine test

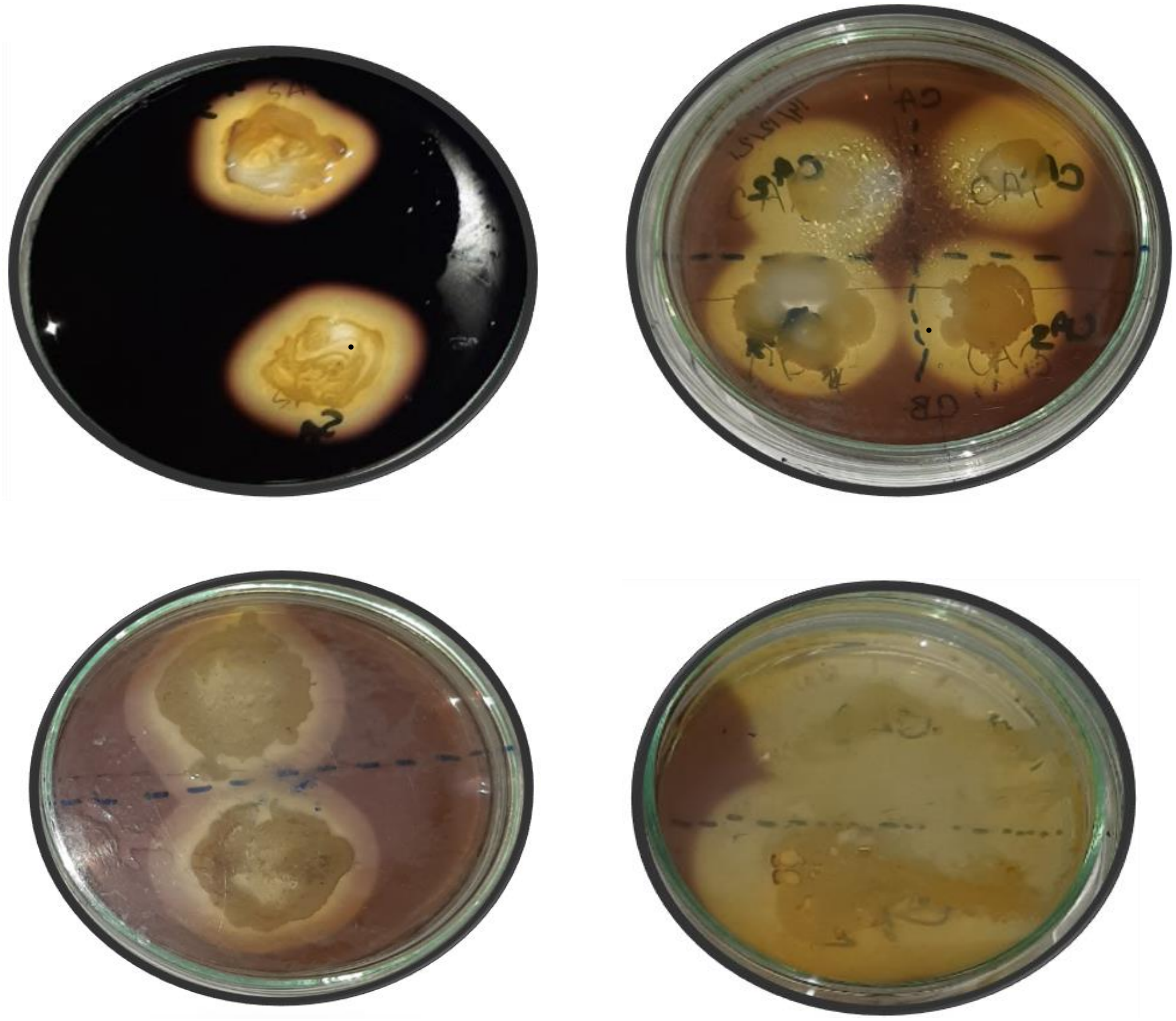

Figure 6: Colonies showed a positive result (showed zone) for Gram's iodine test

3.5 Isolated amylolytic bacteria can also grow on CMCA media:

The isolated amylolytic bacteria were shown to grow on CMCA media after 48 hours of incubation. So, the isolated bacteria showed both cellulolytic and amylolytic characteristics. To confirm this, the gram's iodine test was performed again and it showed positive results.

Figure 7: Amylolytic bacteria's growth on CMCA media

3.6 Antibiogram result:

After loading the culture supernatants by using the agar well diffusion method or Kirby-Bauer disk diffusion susceptibility test method, some supernatants showed a zone of inhibition after 16-24 hours against MDR bacteria and some failed to show a zone of inhibition.

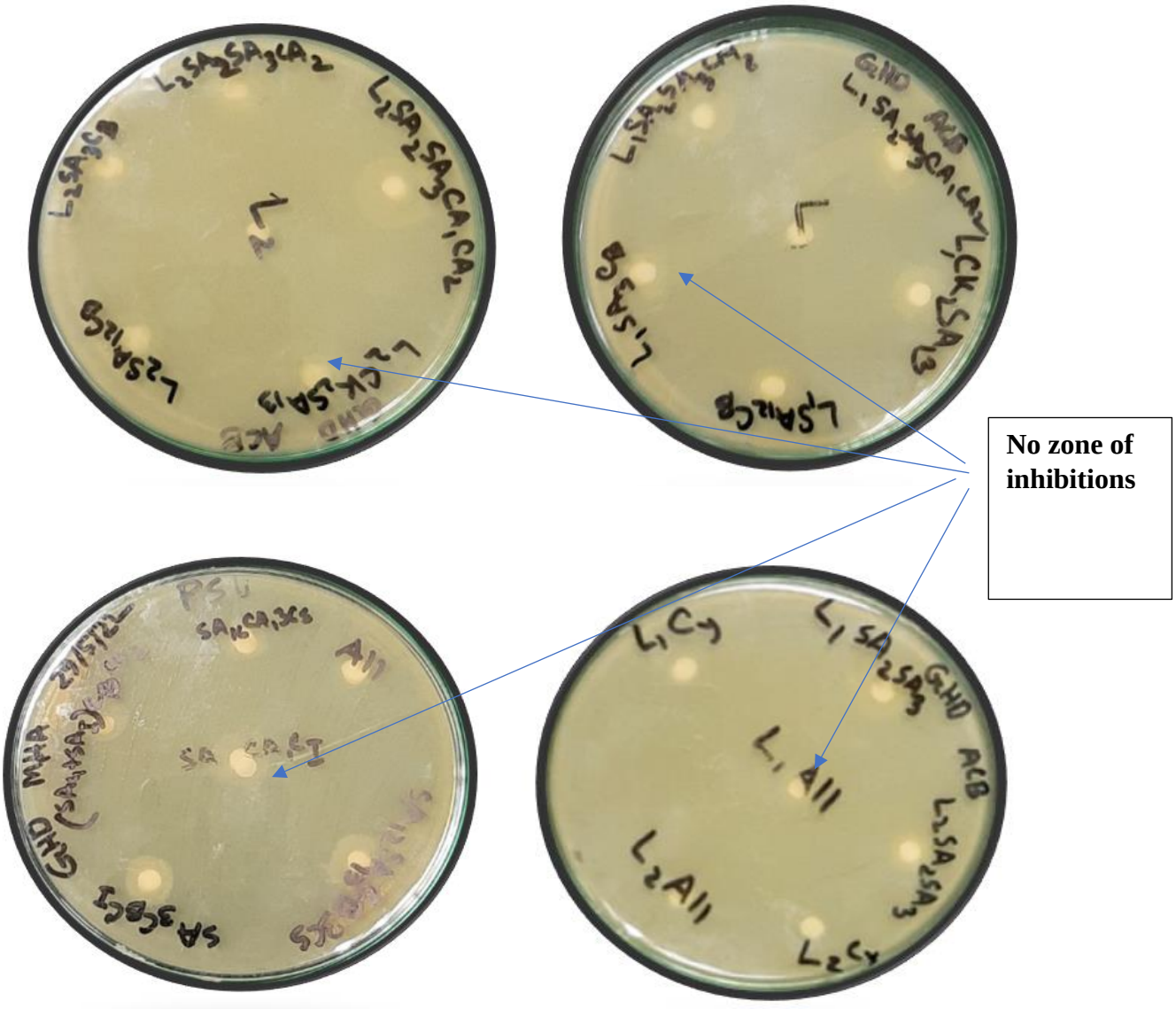

Figure 8: No zone of inhibition was observed against different supernatants combination (cellulolytic, amylolytic and LAB supernatants)

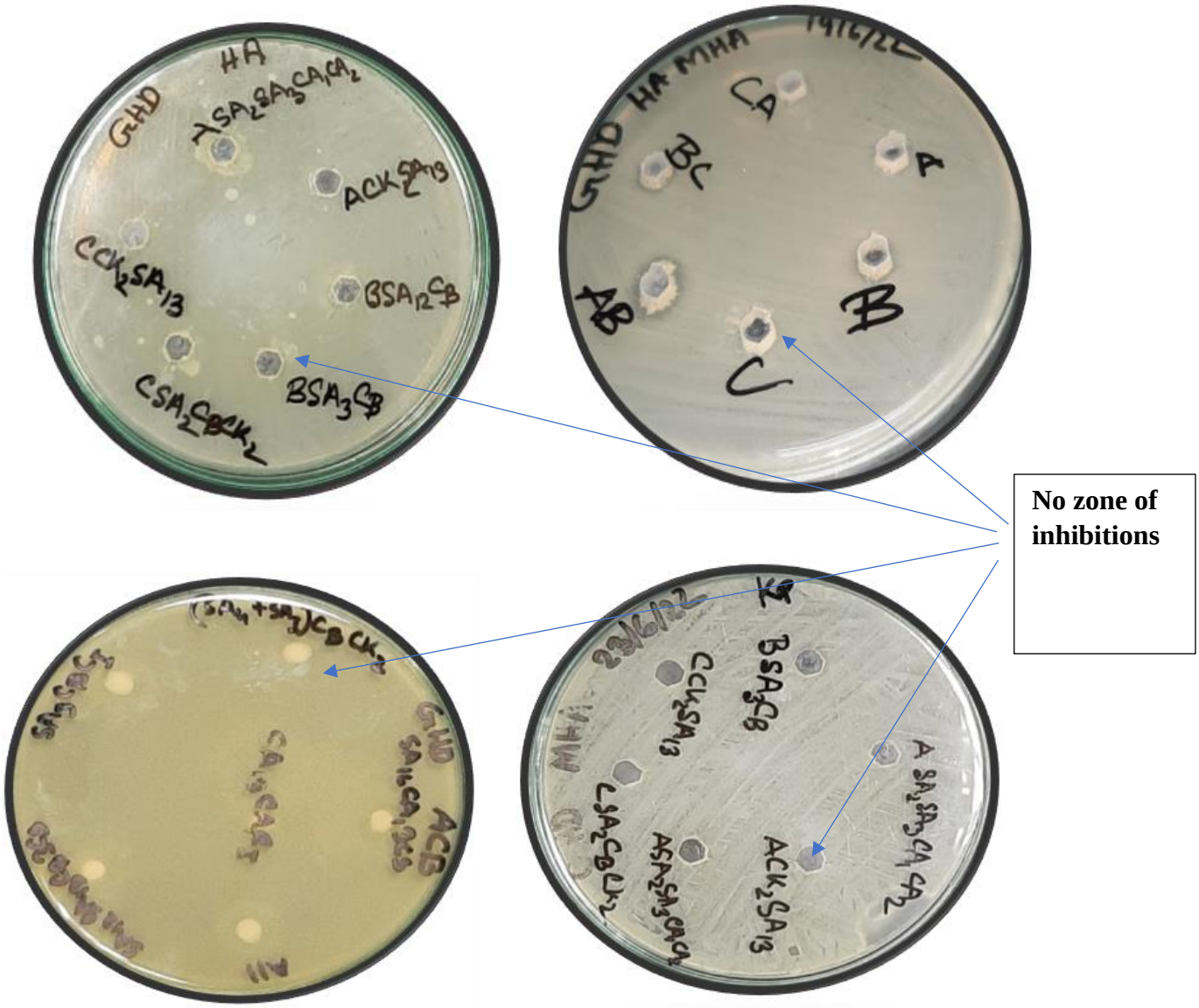

Figure 9: No zone of inhibition was observed against different supernatants combination (cellulolytic, amylolytic and LAB supernatants)

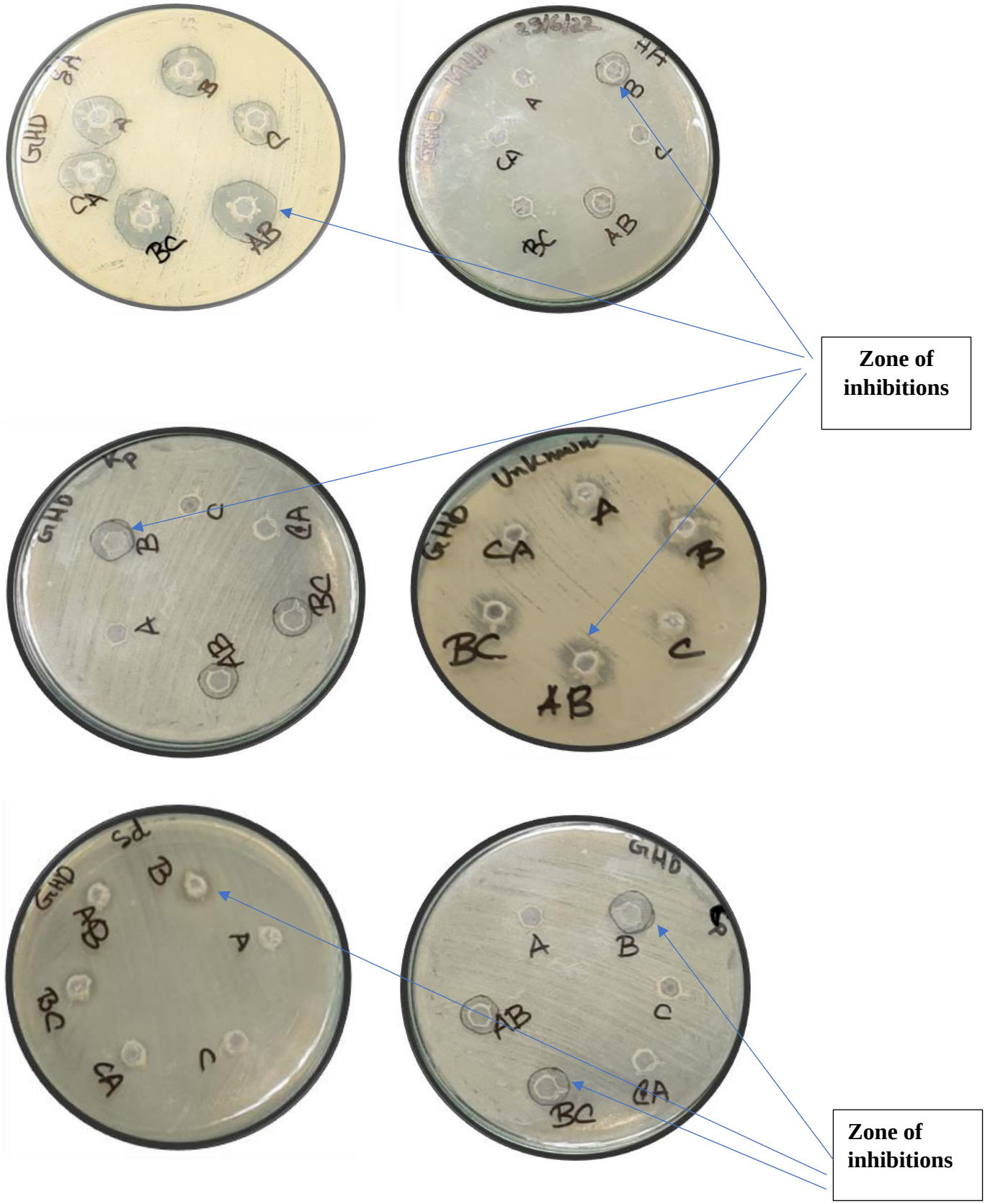

Figure 10: Zone of inhibitions were observed against different supernatants combination (LAB supernatants collected from Ar)

3.6.1 Mixing Cellulolytic and amylolytic bacterial culture supernatants with effective Lactic acid bacterial culture supernatants

Mixing with Cellulolytic and amylolytic bacterial culture supernatants decreased or inhibited the effectiveness of Lactic acid bacterial culture supernatants.

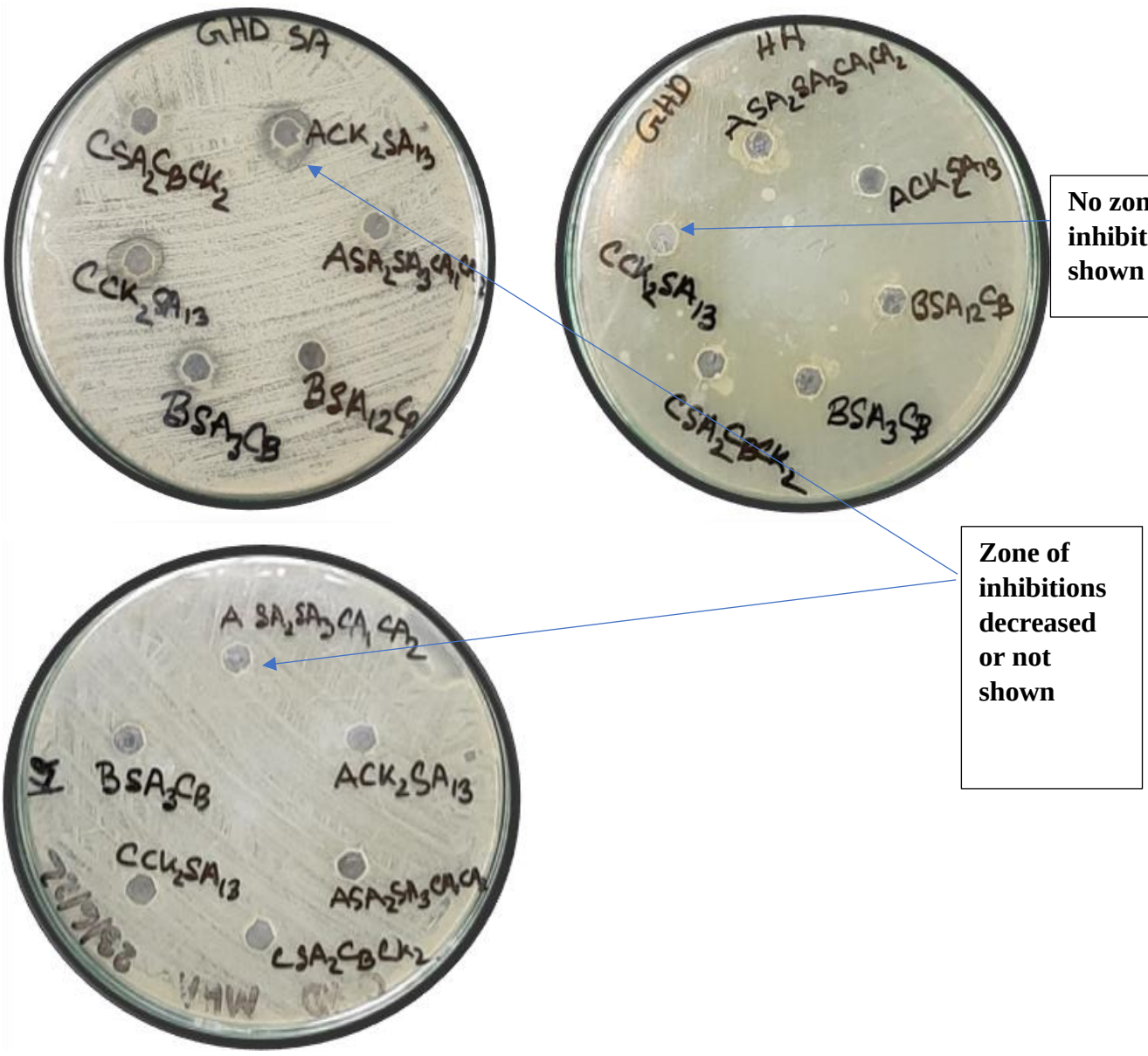

Figure 11: Previously observed zone of inhibitions decreased or not visible against different cellulolytic, amylolytic and LAB supernatants combination

3.6.2 Changing the pH and observing the effectiveness:

The culture supernatants which showed effectiveness against MDR bacteria were treated with different pH and observed the pH sensitivity.

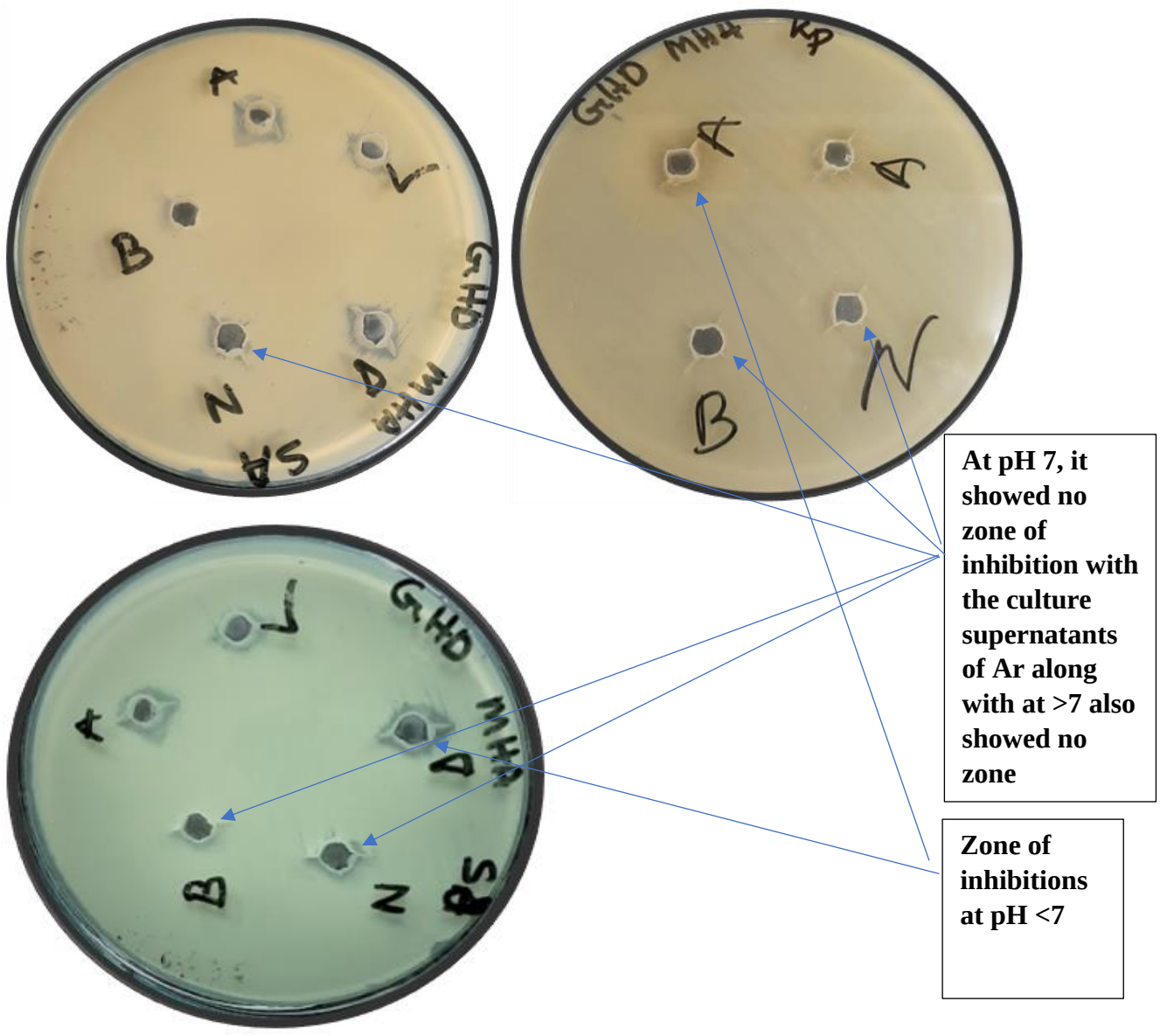

Figure 12: Zone of inhibitions decreased if the pH was maintained as neutral or increased (basic)

3.6.3 Zone measurements (in mm) of effectiveness against MDR bacteria:

Table 2:

Table 2: Zone of inhibition measurement:

	Culture supernatants (zone of inhibition in mm)									
MDR bacteria	**A**	**B**	**C**	**AB**	**BC**	**CA**	**ACK2SA13**	**CCK2SA13**	**BSA3CB**	**MSA3CBCI**
Hafnia alvei	10	13	10	15	13	12	0	0	10	11
Klebsiella pneumoniae	0	15	0	12	11	10	0	0	12	0
Streptococcus aureus	15	18	15	20	20	15	0	10	14	0
Acinetobacter baumannii	0	10	0	11	10	0	0	0	0	10
Pseudomonas aeruginosa	28	15	10	15	18	20	13	9	12	12
Shigella dysenteriae	10	11	0	15	10	0	0	8	11	8
Escherichia coli	11	12	10	12	12	10	8	0	0	0
Neisseria gonorrhoeae	0	0	0	0	0	0	0	0	0	0
Salmonella typhi	13	20	0	17	15	14	9	0	13	0
Helicobacter pylori	15	19	10	17	16	15	0	0	0	0
Unknown	13	20	0	17	15	10	0	0	15	16

In the table, Soil samples,

C= Cellulolytic culture supernatant SA= Amylolytic culture supernatant

Yogurt samples,

A= Growth in Lactose broth, B= Growth in MRS broth, C= Growth in LB broth

3.7 Biochemical tests to identify the Lactic acid bacteria

Effective probiotic culture stain had been isolated and performed biochemical tests. The biochemical test results are below-

Test	**Result**
Bacterial Shape	Rod-shaped
Gram's stain	Positive
Glucose utilization	Positive
Sucrose utilization	Positive
Lactose utilization	Positive
Mannitol utilization	Negative
Indole	Negative
Methyl Red	Negative
Vogues Proskauer	Negative
Citrate	Negative
Starch hydrolysis	Positive
Urease	Negative
H_2S production	Negative
Gas production	Positive
Nitrate reduction	Negative
Oxidase	Negative

Table 3: Biochemical tests result

The test results suggest that the bacteria is *Lactobacillus bulgaricus.*

Biochemical test results

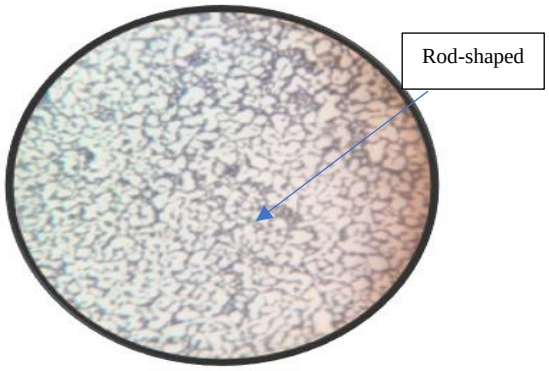

Figure 13: Gram staining: Gram positive

Figure 14: Starch hydrolysis Test: Positive

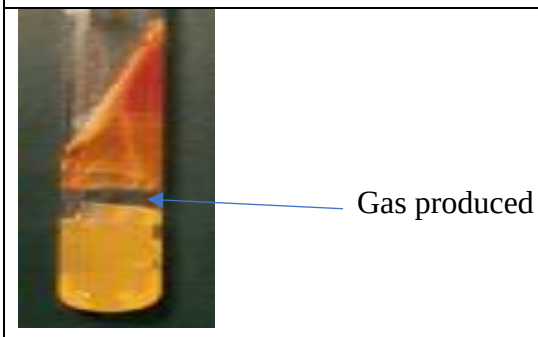

Figure 15: TSI test: Positive

Figure 16: Indole test: Negative

Figure 17: Methyl red Test: Negative

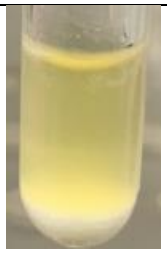

Figure 18: Urease Test: Negative

Figure 19: Vogues- Proskauer Test: Negative

Figure 20: Citrate Test: Negative

Chapter 4

Discussion

Nowadays Multidrug resistant bacteria are a burning issue for the world. According to WHO, treating MDR bacteria are one of the most concerning issues. Scientists are trying to eradicate them by using different techniques[25] (Nikaido, 2009).

Probiotics are live bacteria or fungi that are directly eaten and provide the host with health benefits. Probiotics work by causing metabolic precursors to produce SCFAs, which further modulate the immune system and improve mucosal barrier function. Along with physically inhabiting the epithelia, probiotics may also produce antimicrobial chemicals. Synbiotics are products that include both prebiotics and probiotics in one container [26](Newman et al., 2020).

Probiotics' supernatant treatment is one of them where different probiotic bacterial cell-free culture supernatants are used to treat the MDR bacteria. Probiotics' culture supernatants were aimed to treat the MDR bacteria and it might be the possible solution of treatment from our food habits [27] (Jung et al., 2021). Along with it, synbiotic treatment can also be very effective against MDR bacteria[28] (Davison et al., 2019).

From my experiments, different probiotic culture supernatants of cellulolytic and amylolytic bacteria had no effects on MDR bacteria. They could effectively degrade cellulose and starch, but are unable to combat MDR bacteria. But the probiotic culture supernatants prepared by using the Ar sample showed wonderful results against different MDR bacteria. Other Yogurts' samples had no effect on the MDR bacteria.

My experiments, showed that only certain Lactic acid bacteria's cell-free culture supernatant that was collected from the Ar sample can fight against most of the MDR bacteria effectively. If it was mixed with other probiotic cell-free culture supernatants like cellulolytic or amylolytic bacterial supernatants, it gradually reduced its zone of inhibition or it cannot

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[25] Nikaido H. (2009). Multidrug resistance in bacteria. *Annual review of biochemistry*, *78*, 119–146. https://doi.org/10.1146/annurev.biochem.78.082907.145923

[26] Newman, A. M., & Arshad, M. (2020). The role of probiotics, prebiotics and synbiotics in combating multidrug-resistant organisms. *Clinical therapeutics*, *42*(9), 1637-1648.

[27] Jung, J. I., Baek, S. M., Nguyen, T. H., Kim, J. W., Kang, C. H., Kim, S., & Imm, J. Y. (2021). Effects of probiotic culture supernatant on cariogenic biofilm formation and RANKL-induced osteoclastogenesis in RAW 264.7 macrophages. *Molecules*, *26*(3), 733.

[28] Davison, J. M., & Wischmeyer, P. E. (2019). Probiotic and synbiotic therapy in the critically ill: State of the art. *Nutrition*, *59*, 29-36.

combat the MDR bacteria like as earlier. It might be happened due to the non-reciprocal activity of the culture supernatants. Individually, the cellulolytic and amylolytic bacterial culture supernatants could not fight against the MDR too. Both cellulolytic and amylolytic bacteria were collected from soil samples and they could hydrolysis the selective media in a large manner and showed their high effectiveness during Gram's iodine test. To clarify, Gram's iodine indicated that soil bacteria could degrade cellulose and starch by showing a zone. Gram's iodine reacts with starch or cellulose and forms a blue to black complex with them, but they are unable to react with glucose. To clarify, if the soil bacteria could break down cellulose or starch and as a by-product, it could produce glucose, it shows the zone whereas other areas remain blue to black by reacting with Gram's iodine.

The Lactic acid bacterial culture supernatant which showed effective results against different MDR was cultured in 3 different broths media, such as MRS broth, Lactose broth, and LB broth. Among them, MRS broth's Lactic acid broth showed the highest performance compared to others against the MDR bacteria by showing a clear zone of inhibition. Lactose broth's Lactic acid bacterial culture supernatants showed less effectivity in antibiogram and LB broth's inoculated bacteria showed almost no powerful effectivity against the MDR bacteria. It may happen due to the presence of glucose in the MRS broth which turns the media acidic where pH stays <5. It indicates that by the presence of enough glucose certain Lactic acid bacterial strains can produce enough acid to lower the pH, such as Lactic acid [29](Sikder et al., 2021). The culture supernatant which could effectively be performed against MDR bacteria was treated at different pH during antibiogram and it showed that only it was effective while its pH was less than 5, and in treating with 0.1M NaOH converted the pH of the culture supernatant at 7 and more than 7 which had no effectivity against the MDR bacteria. Most probably, the cell-free culture supernatant of the selected Lactic acid bacteria worked with the help of acidic conditions against the MDR bacteria or the concentration of the cell-free culture supernatants may be decreased due to the presence of 0.1M NaOH solution.

[29] Sikder, A., Chaudhuri, A., Mondal, S., & Singh, N. P. (2021). Recent Advances on Stimuli-Responsive Combination Therapy against Multidrug-Resistant Bacteria and Biofilm. *ACS Applied Bio Materials*, *4*(6), 4667-4683.

The gram-positive Lactic acid bacteria can effectively fight against MDR bacteria in culture supernatants form, the bacteria could ferment glucose, sucrose, lactose, and mannitol and could hydrolysis starch. The bacteria could not give positive results in Indole, Methyl Red, Vogues Proskauer, Citrate, and Urease test which means the bacteria cannot utilize them. After biochemical tests of the LAB, it can be called *Lactobacillus bulgaricus* (presumptive) which culture supernatants had shown positive output against most of the MDR bacteria.

The *Lactobacillus bulgaricus* bacterial culture supernatant can be an effective treatment against MDR bacteria and more probiotics collection can give more expected results.

Conclusion

The experiment began with the collection of microorganisms from various sources, which were then preserved in CMCA and SA for soil samples and MRS agar for yogurt. When exposed to gram's iodine, soil bacteria were shown to be able to develop enzymes that can break down cellulose and starch and may be used as a treatment for pathogens that are resistant to multiple drugs. After that, the following broths were used to culture the bacteria: MRS broth, Luria broth, and lactose broth media for yogurt bacteria; CMC broth for cellulolytic bacteria; and starch broth media for amylolytic bacteria. Following a 48-hour incubation period, the bacterial culture broths were centrifuged to separate the culture supernatants, which were used to examine the effectiveness of the supernatants against a variety of multidrug-resistant bacteria in supernatants with various chemical compositions. The main objective of the experiment was to execute an antibiogram on Mueller-Hinton agar medium and observe the zone of inhibition following an incubation of many multi-drug resistant bacteria for 18 to 22 hours in the presence of various supernatant compositions in different pH. By demonstrating the zone of inhibitions, the culture supernatants from specific yogurt's sample have showed highly effective discoveries in treating the superbugs, but neither amylolytic nor cellulolytic bacterial culture supernatants have showed any desirable effect on the superbugs which had degraded or halted the effective Lactic acid bacterial capacity.

Some new probiotic bacterial culture supernatants' combination may show effectivity against the MDR bacteria and it may be a great finding of MDR bacteria treatment. If it is found, people sufferings from MDR bacteria may need not to be thinking so much.

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Appendix A.

1) **Preparation of Carboxymethylcellulose Agar Medium**[30] [31]

(Ebtesam, 2019)

Reagents required:

- **Ammonium Dihydrogen Phosphate** **0.1%**
- **Magnesium Sulphate** **0.1%**
- **Potassium Chloride** **0.02%**
- **Yeast Extract** **0.1%**
- **Carboxymethylcellulose** **1.2%**
- **Agar** **1%**
- **Distilled Water**

Procedure:

- The following salts were measured and distilled water was added according to need.
- The mixture was heated until the salts were mixed methodically.
- Carboxymethylcellulose and agar were added into the mixture and heated while stirring occurs.

[30] "CMCA (Carboxymethylcellulose agar) - Bugwoodwiki,". Retrieved from: https://wiki.bugwood.org/CMCA_(Carboxymethylcellulose_agar)

[31] Ebtesam, (2019). ISOLATION, SCREENING AND PRODUCTION OF CELLULASE FROM BACTERIA. Brac University, Dhaka

- Heated the mixture of the media until the components were completely and consistently dissolved.
- The media was autoclaved and poured into sterile petri dishes for further uses.

2) Preparation of Carboxymethylcellulose Broth

Reagents required:

- **Ammonium Dihydrogen Phosphate** **0.1%**
- **Magnesium Sulphate** **0.1%**
- **Potassium Chloride** **0.02%**

- **Carboxymethylcellulose** **1.6%**
- **Distilled Water**

Procedure:

- The following salts were measured and distilled water was added according to need.
- The mixture was heated until the salts were mixed methodically.
- Carboxymethylcellulose was added into the mixture and heated while stirring occurs.

- Heated the mixture of the media until the components were completely and consistently dissolved.
- The media was then poured into Erlenmeyer flasks for autoclaving.

3) Preparation of Starch Agar Medium[32]

Reagents required:

- **Ammonium Dihydrogen Phosphate** **0.1%**
- **Magnesium Sulphate** **0.1%**
- **Potassium Chloride** **0.01%**
- **Sodium Chloride** **0.1%**
- **Starch** **1%**
- **Agar** **1.3%**
- **Distilled Water**

Procedure:

- The following salts were measured and distilled water was added according to need.
- The mixture was heated until the salts were mixed methodically.

[32] Starch Casein Agar (SCA) – Composition, Principle, Uses, Preparation and Result Interpretation retrieved from https://microbiologyinfo.com/starch-casein-agar-sca-composition-principle-uses-preparation-and-result-interpretation/

- Starch and agar were added into the mixture and heated while stirring occurs.
- Heated the mixture of the media until the components were completely and consistently dissolved.
- The media was autoclaved and poured into sterile petri dishes for further uses.

4) Preparation of Starch broth Medium[33]

Reagents required:

- **Ammonium Dihydrogen Phosphate** **0.1%**
- **Magnesium Sulphate** **0.1%**
- **Potassium Chloride** **0.01%**
- **Sodium Chloride** **0.1%**
- **Starch** **1%**
- **Distilled Water**

[33] Starch Casein Agar (SCA) – Composition, Principle, Uses, Preparation and Result Interpretation retrieved from https://microbiologyinfo.com/starch-casein-agar-sca-composition-principle-uses-preparation-and-result-interpretation/

Procedure:

- The following salts were measured and distilled water was added according to need.
- The mixture was heated until the salts were mixed methodically.
- Starch was added into the mixture and heated while stirring occurs.
- Heated the mixture of the media until the components were completely and consistently dissolved.
- The media was autoclaved and poured into sterile petri dishes for further uses.

5)Preparation of MRS Agar Medium[34]

Reagents required:

- **Peptone** **1%**
- **Yeast extract** **0.5%**
- **Meat extract** **1%**
- **Glucose** **2%**
- **Polysorbate 80** **0.1%**
- **Sodium acetate** **0.5%**
- **Magnesium sulfate** **0.01%**
- **Manganese sulfate** **0.005%**
- **Disodium phosphate** **0.2%**
- **Agar** **1.5%**

[34] MRS agar (deMan, Rogosa, Sharpe) | Principle | Preparation | Interpretation retrieved from https://microbiologie-clinique.com/mrs-agar.html

- **Distilled water**

Procedure:

- The following components were measured and distilled water was added according to need.
- The mixture was heated until the mixtures were mixed methodically.
- Heated the mixture of the media until the components were completely and consistently dissolved.
- The media was autoclaved and poured into sterile petri dishes for further uses.

6) Preparation of MRS broth Medium

Reagents required:

- **Peptone** **1%**
- **Yeast extract** **0.5%**
- **Meat extract** **1%**
- **Glucose** **2%**
- **Polysorbate 80** **0.1%**
- **Sodium acetate** **0.5%**
- **Magnesium sulfate** **0.01%**
- **Manganese sulfate** **0.005%**
- **Distilled water**
- **Disodium phosphate** **0.2%**

Procedure:

- The following components were measured and distilled water was added according to need.
- The media was then poured into Erlenmeyer flasks for autoclaving.

7) Preparation of Lactose broth Medium[35]

Reagents required:

- **Peptone** **0.05%**
- **HM Peptone B#** **0.05%**
- **Lactose** **0.05%**
- **Distilled Water**

Procedure:

- The following components were measured and distilled water was added according to need.
- The media was then poured into Erlenmeyer flasks for autoclaving.

[35] Lactose broth retrieved from https://himedialabs.com/TD/M1003.pdf

8) Preparation of LB broth Medium

Reagents required:

- **Tryptone** **1%**
- **Sodium Chloride** **1%**
- **Yeast extract** **0.5%**
- **Distilled Water**

Procedure:

- The following components were measured and distilled water was added according to need.
- The media was then poured into Erlenmeyer flasks for autoclaving.

Appendix B.

Biochemical tests[36]

Gram staining

Gram staining was done to observe cell shape, size, and morphology as well as to determine the gram sensitivity of the Lactic acid bacterial strain. Fresh culture of the bacteria was smeared with the help of loop and made a smear on clean glass slide with the help of distilled water and heat fix them. The slides were poured with crystal violet solution and was left on them for 45-60 seconds. Gram's Iodine solution was flooded onto the slide after it had been cleaned with distilled water and allowed to sit for 60 seconds. In order to prevent the bacteria from being washed off, the slides were washed with 90% ethanol or acetone. Following that, the slides were submerged in the Safranin solution for 45-60 seconds. The slide was washed off and keep it for air dried. After drying, slide was kept under microscope for observation.

Gram positive organisms are stained a blue or purple color, while Gram negative organisms are stained a pink color or red color. Although gram-positive bacteria lack an outer membrane, they are encased in layers of peptidoglycan that are much thicker than those found in gram-negative bacteria.

TSI test

The Triple Sugar Iron (TSI) test is a microbiological procedure that evaluates a microorganism's capacity to produce hydrogen sulfide and ferment sugars. The test is performed using an agar slant containing a specific medium that contains a pH-sensitive dye (phenol red), 1% lactose, 1% sucrose, 0.1 % glucose, sodium thiosulfate, and ferrous sulfate or ferrous ammonium sulfate. When all of these components are combined and allowed to solidify at an angle, an agar test tube is produced that is angled. This medium's slanted form offers a variety of surfaces that are either exposed

[36] Microbiology Lab: MOLB 2210. (n.d.). Retrieved from https://www.uwyo.edu/molb2210_lab/info/biochemical_tests.htm?fbclid=IwAR1rf9GTh1gXq4-823iTQy_3XrB5frc2NzWKoq_YN1hBzFlB4iUWqiYLyrE#sulfur

to air containing oxygen to variable degrees (an aerobic environment) or are not exposed to air at all (an anaerobic environment), under which fermentation patterns of organisms are identified. With the help of needle the bacterial colony was picked and inoculated by touching the butt and marking the slant. If fermentation happen, the pH decreases while acid accumulates. While acids are existing, the color of the carbohydrate medium changes from orange to yellow, indicating the existence of carbohydrates that are fermenting that's happen when the acid base indicator Phenol red comes in. Alkaline compounds are created and the pH increases when peptone is decarboxylated oxidatively. The medium contains sodium thiosulfate and ferrous ammonium sulfate, which detect hydrogen sulfide generation and cause the tube's bottom to become black.

Mannitol utilization

Using this test, it can be determined if indeed the organism is able to use mannitol as a carbon source. The media works as both selective and differential media. The media becomes yellow when bacteria that can use mannitol as a carbon source and grow on it. If bacteria are unable to ferment mannitol, the medium remains red.

Indole test

The test is conducted to find the microorganisms that can generate the tryptophanase enzyme. The bacteria which have the enzyme can easily use the amino acid tryptophan. During the reaction, indole is released which can be detected by the help of Kovac's reagent forms a red dye called as rosindole. Positive indole test results display the distinctive red color due to this dye.

Methyl Red test

Some bacteria are capable of using glucose and producing lactic, acetic, or formic acid as the final product. The bacteria convert glucose to pyruvic acid and then metabolized them into different stable mixed acids. Depending on the particular metabolic pathways of the bacteria different types of acids can be created depending

on the species. A switch in the color of methyl red from yellow to red indicates that the acid so formed reduces the pH to 4.5 or below. The pH won't be significantly decreased if the mixed acid pathway hasn't been used. The pH in the media often stays over 6. In that circumstance, Methyl Red emits a yellow color, suggesting that insufficient acids are present to penetrate the phosphate buffer wall.

Citrate test

The capacity of an organism to utilize citrate as a source of energy is examined using citrate agar test. Citrate serves as the only source of carbon in the medium, while inorganic ammonium salts ($NH_4H_2PO_4$) serve as the only supply of nitrogen. Citrate-permease, an enzyme that can convert citrate into pyruvate, is produced by bacteria that can grow upon the medium. The organism's metabolic cycle can then incorporate pyruvate to produce energy. Growth is a sign that citrate, a Krebs cycle intermediate metabolite, is being used.