Characterization of Bacteria Isolated From Fermented Food and Evaluation of Their Potential to be Probiotic

By

Shucheta Nazia Archi
ID: 19136016

Nafisa Tabassum
ID: 18236018

Juairiah Bakth Juhana
ID: 19136025

A Thesis report submitted to the Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
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Declaration

It is hereby declared that

1. The thesis submitted is our original work while completing a 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 that has been accepted, or submitted, for any other degree or diploma at a university or other institution.

4. We have acknowledged all main sources of help.

Students' Full Names & Signatures:

Shucheta Nazia Archi	Nafisa Tabassum	Juairiah Bakth Juhana
Student ID: 19136016	Student ID: 18236018	Student ID: 19136025

Supervisor's Full Name & Signature:

Dr. M. Mahboob Hossain
Professor, Microbiology Program
Department of Mathematics and Natural Sciences
School of Data and Sciences
BRAC University
66, Mohakhali, Dhaka-1212

Approval

The thesis/project titled “Characterization of Bacteria Isolated From Fermented Food and Evaluation of Their Potential to be Probiotic” submitted by

1. Shucheta Nazia Archi	2. Nafisa Tabassum	3. Juairiah Bakth Juhana
ID: 19136016	ID: 18236018	ID: 19136025

of Summer, 2023 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of B.Sc. in Biotechnology on 14th August 2023.

Examining Committee:

Supervisor
(Member)

_____

Dr. M. Mahboob Hossain
Professor, Microbiology Program
Department of Mathematics and Natural Sciences
School of Data and Sciences
BRAC University

Program Director:

_____

Dr. Munima Haque
Associate Professor
Department of Mathematics and Natural Sciences
BRAC University

Department Head:
(Chair)

_____

A F M Yusuf Haider
Professor and Chairperson
Department of Mathematics and
Natural Sciences
BRAC University

Ethics Statement:

We, Shucheta Nazia Archi, Nafisa Tabassum, and Juairiah Bakth Juhana hereby solemnly declare that the research work necessary for this thesis was completed by the three of us under the supervision of Dr. M. Mahboob Hossain, Professor of Microbiology Program, BRAC University at the thesis laboratory of the Department of Mathematics and Natural Sciences, BRAC University. We again proclaim that the research presented here is original and suitable for the submission for the partial fulfillment of the degree of Bachelor of Science in Biotechnology, BRAC University, Dhaka.

The samples and materials used in this research were in no way ethically compromised and the purpose of this study is the betterment of humans.

Abstract

This study aimed to isolate and biochemically characterize bacteria that are not exclusively LABs from popular fermented food products and evaluate their potential to be probiotics. As primary screening, isolated bacterial colonies were tested for antagonism against seven laboratory stains (LS) of enteric or infectious bacteria using three methods: spot-on-lawn with direct bacterial colonies, disk diffusion and well diffusion with cell-free supernatant (CFS). Biochemical tests, morphological observation and growth on selective media were used to characterize the potential antagonistic bacteria and their survivability in GI-tract conditions was determined by culturing in different media modified with bile salts and acid. Antibiotic susceptibility tests in the presence of ten different antibiotics were performed to study their resistance or susceptibility. Their potential for pathogenicity was tested via hemolysis test on blood agar and DNase test on DNase agar. Although a total of eight isolates showed signs of antagonism via a competitive advantage and/or inhibition of some of the LSs, most of them tested positive for hemolytic activity deeming them potentially harmful. Two of the bacteria that were isolated from yogurt showed no apparent sign of pathogenicity and survived well in high concentrations of bile salts as well as acidic pH. They were presumed to be *Pediococcus* and *Paenibacillus* type of bacteria which had shown antagonism against *Shigella flexneri* and *Salmonella typhi* respectively. Overall, further molecular-level research is necessary to understand the true identity and probiotic potential of the isolated bacteria.

Keywords:

Fermented food, Probiotics, Antagonism, Pathogenicity, Hemolysis, Enteric bacteria.

Dedicated to

Our beloved **mothers** who dedicated their lives to our future**.**

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List of Acronyms

CA	Cetrimide Agar
CFS	Cell Free Supernatant
ETEC	Enterotoxin Escherichia coli
FAO	Food and Agriculture Organization
GI-tract	Gastrointestinal tract
GRAS	Generally Recognized as Safe
LAB	Lactic Acid Bacteria
MAC	MacConkey Agar
MIU	Motility Indole Urease
mL	Milliliter
MR	Methyl Red
MRSA	De Man, Rogosa and Sharpe agar
MSA	Mannitol Salt Agar
NA	Nutrient Agar
ºC	Degree Celsius
PCR	Polymerase Chain Reaction
Spp.	Species
TLR	Toll-like receptors
TSI	Triple Sugar Iron
VP	Voges-proskauer
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate agar
ZOI	Zone Of Inhibition
µL	Micro Letter

Chapter 1

Introduction

1.1. Background

A rich and diverse microbiota in the human gastrointestinal tract has always been a major contributing factor to good health. Thus the widespread search for probiotic bacteria originating mainly from food and gastrointestinal sources began. An easy and cost-effective vehicle for the delivery of probiotics to achieve a diversified gut microbiota is fermented foods. Fermented dairy products like yogurt, cheese, and kefir as well as fermented vegetables like kimchi can be great sources of good bacteria or probiotics. The mechanism of action of probiotics may be one or more of Enzymatic activities, Production of volatile Fatty acids, forming a Protective layer on the host intestinal lining, Competitive Exclusion of or advantage over Pathogens, Bacteriocin production, Modulation of the Host Immune System, etc. (Plaza-Diaz at el., 2019; Kosgey at el., 2019).

1.2. Literature Review

1.2.1. Importance of Gut Microbiota

In the recent decade, westernized food habits have massively affected the composition of human gut microbiota. According to Statovci at el. (2017), diets containing high amounts of refined sugar, and red meat and are low in fiber, fruits, and vegetables may contribute to metabolic diseases including diabetes and obesity. Moreover, the risk of chronic mucosal inflammatory conditions such as Inflammatory bowel diseases (IBD) and asthma also increases with such dietary habits. To maintain a well-functioning metabolism, immunology, and host protection, the gut microbiota must be symbiotic with the gut mucosa. To protect a gut from enteric infections a microbiota should have up to 1×10^{14} commensal bacteria which essentially bestows colonization resistance against pathogens. Westernized diets which invite inflammatory diseases as well as the use of antibiotics disturb the commensal microbiota thus increasing the chance for

colonization by pathogens. Only a healthy commensal community consisting of a variety of good gut bacteria can provide the necessary defense against such events. Therefore, a diversified microbiota is a very important factor in good health (Statovci et al., 2017).

1.2.2. Food Products that Contain Good Bacteria

When it comes to probiotics, the most popular worldwide are the Lactobacilli strains which are explored in various fields including biotechnology, food preservation, and even therapeutics (Halder et al., 2017). The popularity of Lactobacilli is for valid reasons. It was mentioned in a paper by Halder et al. that *L. acidophilus*, *L. plantarum*, *L. fermentum*, *L. casei* and *L. rhamnosus* are some pathogens that have shown effectiveness against the strains *P. mirabilis* and *P. vulgaris* which are known to cause urinary infection. Moreover, *Lactobacillus plantarum* isolates interfere with the growth of pathogenic *Salmonella typhi* while many lactobacilli isolated from fermented cereal and dairy-based foods have shown antibacterial activity against *Escherichia coli* ATCC 700728 standard strain (Halder et al., 2017)

1.2.3. About probiotics

The 2002 definition of probiotics by FAO and WHO states that probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Caggia et al., 2015; Kosgey et al., 2019). Some welfare activities that have an impact on metabolism such as reducing cholesterol absorption, lactase activity, assistance in producing certain vitamins, and antagonism against pathogenic bacteria, etc. may be conferred to a host who ingests such probiotics (Caggia et al., 2015).

The terms Biogenics and synbiotics are also related to probiotics. The former means the involvement of probiotic-based bioactive compounds the activity of which is not related to the viability of the source bacteria and the latter term signifies the utilization of prebiotics and probiotics (Kosgey et al., 2019).

A study by Skowron et al. (2022) mentions that fermented foods containing functional microorganisms which either arise naturally (e.g. *Lactobacillus, Lacticaseibacillus, Levi Lactobacillus*) or are generally recognized as safe (GRAS) (e.g. *Bifidobacterium)* tend to have positive effects on health. It is also mentioned that many ongoing types of research focus on the

effects of such functional foods on improving intestinal function, treatment of gut dysfunction, and dysbiosis-related diseases. Probiotics from these foods can antagonize pathogens and reduce the chance of colonization by them, and have a role in bile acid salt metabolism, production of short-chain fatty acids, and homogenization of gastrointestinal microbiota. Thus, fermented foods can be used for temporary improvement of the gut microbiome via probiotics, prebiotics, and synbiotics (Skowron et al., 2022).

1.2.4. Dairy products

According to Caggia et al. (2015), bacteria of the *Lactobacillus* and *Bifidobacterium* genera are the most studied probiotics as they have shown inhibitory effects on intestinal pathogens and themselves are non-pathogenic. Human or animal intestine, oral cavity, and vagina are some common habitats of many lactobacillus species. Apart from the intestinal sources, potential probiotics are mainly isolated from dairy products which are also a medium for the delivery of probiotics to the human body. For example, diverse strains of Lactobacillus with functional probiotic properties have been found in traditionally prepared dairy products such as cheese. Among cheeses made in Sicily that are Protected Denomination of Origin (PDO) including Ragusano and Pecorino, are made from raw milk without using commercial starter cultures. During their aging process, they develop a diverse culture of food-safe bacteria with probiotic potential (Caggia at el., 2015).

1.2.5. About plant-based fermented food

A study by Choi et al. (2018) states that probiotic bacteria do not necessarily originate from the intestine or dairy products despite these being the common sources of probiotics isolation. In fact, dairy products are losing popularity due to associated issues such as milk allergy and lactose intolerance (Choi et al., 2018). Veganism may also be included among the reasons for the lost popularity of milk-based foods. This is why the demand for plant-based fermented products with probiotic enrichment has risen among consumers. To fulfill such demands much focus has been cast on the research of screening functional probiotics from unconventional sources such as fermented vegetables, fruits, cereals, etc. However, the rate of successful employment of probiotics from these sources is still limited (Choi et al.,2018).

1.2.6. Mechanism of Probiotic Actions

There is a wide range of mechanisms a probiotic bacteria may follow to confer protection to the host. Most probiotic bacteria may display one or more of the following most common mechanisms:

- **Enzymatic activities:** Probiotics may function by either producing beneficial enzymes or disrupting the activities of harmful enzymes. The enzyme bile salt hydrolase produced by most probiotic bacteria has been shown to influence cholesterol absorption by means of modifying bile acid metabolism in the gut lumen. Whereas, a functional bacteria, *B. longum* showed modifications in the gut microbiota which in turn lowered the activity of β-glucuronidase, an enzyme associated with the toxification of glucuronidated metabolites in the intestine which causes damage there (Plaza-Diaz et al., 2019).
- **Production of volatile Fatty acids:** Probiotic bacteria such as *L. gasseri* CECT5714 and *L. coryniformis* CECT5711 have been found to improve the production of short-chain fatty acids (SCFA) (Plaza-Diaz et al., 2019). SCFAs such as acetic acid, propionic acid, and butyric acid have important metabolites that have a role in increasing insulin sensitivity in tissues thus benefiting patients that suffer from Type-2 diabetes (Al-Lahham at al., 2010). This attribute of probiotics also improves the metabolism of carbohydrates and antioxidants status, reduces biomarkers of cardiovascular diseases, etc. which in turn helps patients of obesity and non-alcoholic fatty liver syndrome (Plaza-Diaz et al., 2019).
- **Protective layer:** Some probiotics can protect the host intestine by adhering to the epithelial cells so that pathogens cannot attack. For example, *L. rhamnosus* has been shown to maintain an epithelial barrier and protect against infection by promoting the activation of the intestinal epithelial cells (Plaza-Diaz et al., 2019).
- **Competitive Exclusion of or Advantage over pathogens:** One of the most important mechanisms of probiotic activity is to win in competition against the pathogens by occupying binding sites or receptor sites as well as exhausting nutrients and growth factors (Plaza-Diaz at el., 2019; Kosgey et al., 2019).
- **Bacteriocin production:** Bacteriocins are cationic peptides that have antimicrobial properties. Some lactobacillus and bifidobacterium can produce bacteriocins that contain about 30-60 amino acids and disrupt the cytoplasmic membrane of enteric pathogens,

thus preventing colonization and infection. There are some cases of inhibition of *Helicobacter*, *C. difficile*, rotaviruses, multidrug-resistant *Shigella spp*., and *E. coli,* etc. with bacteriocins produced by *L. plantarum* and *L. acidophilus* under specific conditions (Plaza-Diaz et al., 2019).

- **Modulation of Host Immune System:** Some probiotics, especially *Bifidobacterium*, can produce molecules that can stimulate the immune cells with their immunomodulatory and anti-inflammatory functions. Some specifics include stimulation of sIgA production, Cytokine production, lowering intestinal inflammation via downregulation of Toll-like receptors (TLR) expression and cell cascade signaling, interaction with the brain-gut axis, etc. (Plaza-Diaz et al., 2019).

1.2.7. Antagonism Test

One of the most promising methods of screening bacteria with probiotic properties is to test their antagonism against pathogenic bacteria. In a study by Karimi et al. (2018), the antagonistic effects of certain probiotic isolates were tested against pathogenic *E. coli* strains. In this study, the probiotics were isolated from different natural sources first and then identified via molecular assessment. Then the isolates were tested for antagonistic properties against pathogens by using disk diffusion agar and well diffusion agar methods. Cell-free supernatant (CFS) of the isolates was used in wells as well as for impregnation of disks placed in Muller-Hinton agar plates lawned with the target pathogens. After the incubation period, the zones of inhibitions (ZOI) were measured with a ruler and according to the size of the zones, antagonism was evaluated (Karimi et al., 2018).

1.2.8. Side Effects and Risks of Probiotics Sourced from Fermented Food

Although generally considered safe, theoretically, there may be some risks associated with probiotics. After all, they are bacterial species that may have a possibility of conferring drug or antibiotic resistance to surrounding pathogens. Moreover, susceptible individuals such as critically ill patients or premature infants may be subject to sepsis by them. As examples, there are records of fungemia caused by *Saccharomyces boulardii* and bacteremia in certain susceptible individuals by *Bacillus subtilis* and *Lactobacillus rhamnosus*. This is why, any

potential probiotic must be screened thoroughly for characteristics like drug resistance plasmids, and antibiotic susceptibility (Kosgey et al., 2019).

Not all fermented products have standard starter cultures or follow controlled conditions and rely on spontaneous fermentation. This may give rise to some health risks in case ingredients are of poor quality or manufacturing hygiene and quality control are compromised (Skowron et al., 2022). Improper storage or processing, contaminated raw materials, and water, lack of Good Manufacturing Practices (GMPs), etc. may also be included in the risk factors. Due to these factors, pathogenic bacteria, fungi, or their toxins could remain in consumable products and cause illnesses (Skowron et al., 2022). In many regions of Africa and Asia, enterotoxigenic and enterohemorrhagic *Escherichia coli*, *Shigella* spp., *Salmonella* spp., enterotoxigenic *Staphylococcus aureus*, *Listeria monocytogenes, and Bacillus cereus*, etc. Laboratory stains have been found in fermented foods (Skowron et al., 2022). Fermented products made by small-scale producers and households in low to middle-income countries are more prone to such safety risks. Since countries of low income tend to reserve good quality ingredients for export purposes and keep the secondary crops for domestic usage which is one of the reasons for the lack of standards. However, only a small percentage of foodborne illness cases reported in the healthcare system were caused by the consumption of traditional fermented foods (Skowron et al., 2022).

Even if the bacteria is potentially beneficial for the gut there always lies the risk of it not surviving the harsh conditions of the human digestive tract. Because the varying pH values of the digestive tract range between 1.5 to 7.5 with acidic conditions in the stomach and sometimes colon, and basic conditions in the mouth, intestine, and colon (Allegany Nutrition, n.d.). Theoretically, an in-vivo test would be more accurate to understand the survival capacity of a prospective probiotic in the GI tract. However, for practicality and efficiency purposes in-vitro conditions are used to test an organism's ability to tolerate gastric juice and bile salts. This first level of screening would narrow down the selection to move on to an in vivo approach for confirmation (Caggia at el., 2015).

1.3. Aims and objectives

- The initial objective of the study is to find bacteria that are not exclusively LABs but can potentially be explored as a probiotic as they show a pattern of antagonism against some selected Laboratory grown enteric or infectious bacteria;
- To evaluate the most popular fermented foods through their bacterial composition;
- To compare antagonism essays such as disk diffusion and well diffusion with cell-free supernatant (CFS) as well as the spot-on-lawn method which allows direct interaction among cells of opposing bacteria;
- Finding which is the more common pattern of antagonism, competitive exclusion/advantage or inhibition through producing antimicrobial metabolites;
- To compare between the selected Laboratory strains and Isolates in terms of antibiotic resistance and potential pathogenicity (e.g. Hemolytic ability, DNase production, etc.);
- To evaluate the tolerance of the antagonists towards GI-tract conditions (e.g. acidity and bile salts), etc.

1.4. Research Gap:

Since most research related to probiotics focuses on LABs this study aims to explore unknown territories by screening for bacteria that are not exclusively LABs. This however comes with the risk of dealing with many research gaps. Firstly, the primary growth media not being selective to LABs but being a general nutrient broth (NA) may not support the growth of all potentially probiotic bacteria. A study by Wade (2002) points out that the number of bacteria culturable in laboratories is lower than 2% of all bacteria that are known to man. The percentage of culturable bacteria found in different parts of the human or animal body may be close to 50% which still leaves a wide gap in research. Moreover, the survivability of the bacteria in the GI tract conditions cannot be guaranteed unless extensive in-vivo studies are involved.

Lastly, the growth of many probiotic bacteria requires specific conditions and even specific co-cultures including many other factors that may affect their probiotic activity. For example, a bacteria from yogurt may have specific interactions in its source environment depending on carbohydrate availability, hydrolysis of milk proteins, the degree of milk lipid hydrolysis, etc. to display probiotic qualities. Even the interaction between the probiotic bacteria and the starter culture of a fermented product may play a big role in the degree of probiotic activity (Heller, 2001). According to Heller (2001), the growth phase or physiologic state of the probiotic during addition, harvest, and termination of fermentation are also some important factors. Most of these factors have not been explored for particular locally produced fermented foods. Therefore, this study can only look for bacteria that grow vigorously in nutrient media and show antagonism against the selected Laboratory strains.

Chapter 2

Materials and Methods

2.1 Experimental Design

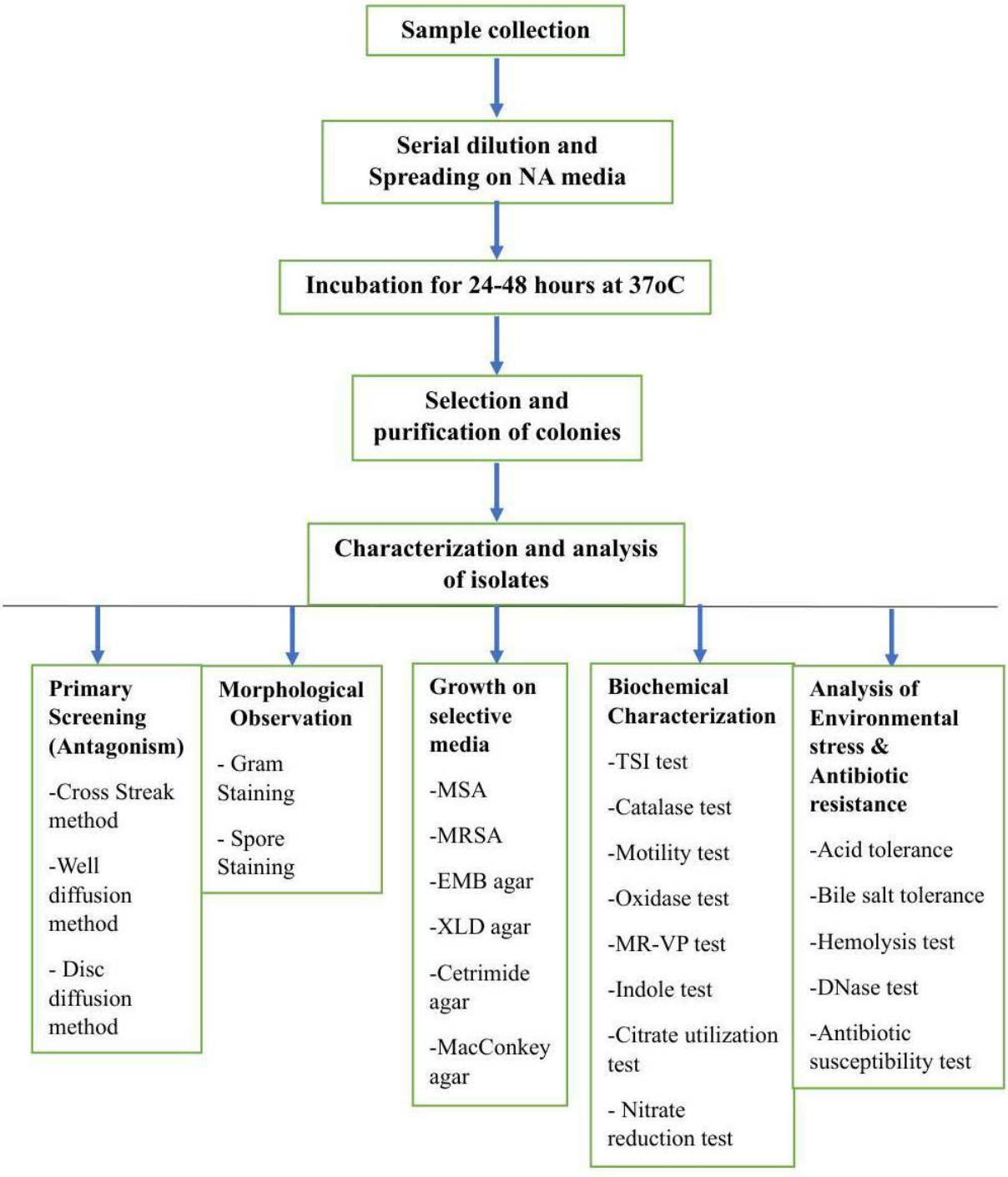


Figure 1: Experimental Design.

Table 1: Sources, food types, number of isolated organisms, and their given names

No.	Food type	Source type	No. of isolated organisms	Labeled as
01	Honey	Natural	5	HM1-HM5
02	Yogurt	Packed	2	UL1-UL2
03	Yogurt	Packed	3	AS1-AS3
04	Yogurt	Local	7	BK1-BK7
05	Yogurt	Local	6	TT1-TT6
06	Yogurt	Local	5	PS1-PS5
07	Yogurt	Local	4	RS1-RS4
08	Yogurt	Local	4	VK1-VK4
09	Sauerkraut/Kimchi	Homemade	2	SK1-SK2
10	Yogurt	Packed	5	MV1-MV5
11	Yogurt	Packed	8	SwR1-SwR8
12	Yogurt	Packed	5	PR1-PR5

***Total number of isolates = 56.**

Table 2: Type of Laboratory Strains (LS) used against isolates for Antagonism

LS	Description of Bacteria	Type of LS	Labeled as
Pseudomonas aeruginosa	Commonly found in soil and water. But if pathogenic, it causes an infections called pneumonia in the lungs.	Not Enteric	A
Klebsiella pneumoniae	Normally lives in human intestines where it doesn't cause any disease. It is a common gut bacteria, causing infection only when it moves from the gut to other body parts.	Enteric	B
Salmonella typhi	A gram-negative gut microbiota. Normally it is present in human and animal intestines but it can cause Typhoid fever if it enters the body through contaminated food or drink.	Enteric	C
Staphylococcus aureus	The most common human pathogen. It is a normal intestinal flora but it can be pathogenic and cause high fever, nausea, vomiting, diarrhea, and stomach pain.	Enteric	D
Enterotoxin E.coli	ETEC is present in the gut of humans and animals however it is a major enteric pathogen that causes bacterial diarrheal illness.	Enteric	E
Shigella flexneri	An enteric bacteria that is responsible for diarrhea in humans. Also, shigellosis or shigella infection affects the digestive system.	Enteric	F
Vibrio cholerae	Normally it is found in the environment but when it enters the human body it colonizes the small intestine and causes Cholera.	Not Enteric	G

2.2 Location of research

This study was carried out in the Biotechnology and Microbiology Laboratory, Department of Mathematics and Natural Sciences, University Building 2 (10th floor), BRAC University, Dhaka 1212, Bangladesh.

2.3 Sample collection and processing

Samples from different fermented food sources were collected. Then, 1gm of each sample was taken using a sterile spatula, mixed with 9 ml of 0.9% saline solution. Next, the prepared sample was used for further analysis.

2.4 Isolation, purification, and storage of the sample

The samples were serially diluted from 10^{-1} to 10^{-5} in 0.9% saline solution. Then 100 µL of each dilution was spread on each nutrient agar (NA) media plate. Liquid samples were directly spread as 10^{0} on agar plates. Also, for each dilution, 2 NA plates were prepared for replication purposes. Next, all the plates were incubated for 18-24 hours at 37ºC. After the incubation, single colonies were selected and isolated based on colony size, color, and texture.

Long-term preservation: To store the isolates, T_1N_1 stock media was prepared in sterile vials. Bacteria were taken from the fresh culture plates with a sterile inoculating needle and stabbed into the set T_1N_1 agar media. Then, the vials were incubated for 18-24 hours at 37ºC. Once enough growth of bacteria was observed in vials, 100 µl of sterile glycerol was added to flood the surface of the media. Lastly, the vials were aseptically stored at room temperature.

2.5 Characterization and analysis of isolates

Screening the isolates was done based on the following category:

- Primary screening tests
- Morphological characterization
- Growth in selective media
- Biochemical identification
- Analysis of environmental stress and antibiotic resistance

2.5.1 Primary screening of the isolates for antimicrobial activity

a. Cross-streak method (Spot-on-Lawn)

This method was utilized to co-culture the isolates with each of the Laboratory Strains to observe their interaction such as competitive advantage or exclusion, over one another. The surface of an NA plate was lawned with a laboratory strain and each isolate was inoculated as a spot on that lawn. This process was replicated for all of the laboratory strains and isolates. After 18-24 hours of incubation at 37ºC, the plates were observed for zones of inhibition or overpowering growth patterns of the isolates which might indicate antagonism-positive results.

b. Well Diffusion Technique

The well diffusion technique was done to test the antimicrobial activity of the secondary metabolites produced by the isolates in in-vitro conditions. In this test, a 0.9% saline suspension (concentration = Mcfarland 0.5 standard) of each Laboratory strain was swabbed on the MHA medium. Then, adequately dispersed wells were created in the medium where 100 µL cell-free culture supernatants of the isolates were poured. After 18-24 hours of incubation at 37ºC, the zones of inhibition around the wells were observed and measured using a ruler.

c. Disk diffusion technique

In the disk diffusion method, filter-paper disks of 6 mm diameter were impregnated with 20-30 µL culture supernatants of the isolates. Then an MHA medium plate was swabbed with a specific Laboratory strain (suspension in 0.9% saline; concentration = Mcfarland 0.5 standard). Next, the impregnated disks were placed on the MHA plates using sterile forceps. Antimicrobial activity of the isolates was observed within 18-24 hours of incubation at 37°C. The diameters of the zones Of Inhibition around the disks were measured with a ruler.

2.5.2 Morphological observation

a. Gram staining: The gram staining method was conducted to understand the structure of the cell membranes of the isolates. On a glass slide, a heat fixed smear of each bacteria was primary stained with crystal violet following the addition of a mordant (Gram's iodine), then decolorized with acetone and finally counterstained with safranin. After staining, color, cell shape and colony morphology were observed under a compound microscope using the 100x lens.

b. Spore staining: For spore staining the Malachite green staining or Schaeffer-Fulton method was used. First, a smear of bacteria was dried and heat fixed on a glass slide. Next, the stain was flooded with malachite green and placed on top of a beaker of boiling water for 2-3 minutes. Then, the slides were cooled, washed under running tap water, and counterstained with safranin for 30 seconds. After a final wash with tap water, they were dried and observed under a microscope with the 100x lens. Under the microscope, appearance green spores and red vegetative cells were noted down.

2.5.3 Growth on selective media

Bacteria from a fresh culture were taken with a sterile loop and inoculated on the surface of each of the selective agar media listed bellow. They were observed for growth after incubation at 37ºC for 24 - 48 hours.

a. Mac Conkey agar (MAC) is used for the isolation of gram-negative enteric bacteria. It is also used to differentiate lactose fermenting from lactose non-fermenting gram-negative bacteria. Also, this media is useful in isolating pathogens present in food and water samples e.g. *E. coli* would form pink colonies with dark centers on MAC agar (Cappuccino & Sherman, 2005).

b. Eosin Methylene Blue Agar (EMB) is a selective and differential medium used for the isolation of different types of gram-negative rods. The selective and inhibitory agents against gram-positive bacteria are the dyes eosin and methylene blue. For example, *E.coli*, a gram-negative bacteria grows with green metallic sheen colonies (Cappuccino & Sherman, 2005).

c. Xylose Lysine Deoxycholate agar (XLD) XLD agar selectively promotes the growth of *Salmonella* and *Shigella* by inhibiting other enteric Laboratory strains. *Salmonella* gives black colonies and Shigella produces red colonies on XLD agar. It also differentiates gram-negative enteric bacteria based on xylose fermentation, lysine decarboxylation, and the production of hydrogen sulfide from sodium thiosulphate (Cappuccino & Sherman, 2005).

d. Mannitol salt agar (MSA) is a selective medium used to identify Halophilic organisms. Incorporating 7.5% sodium chloride in the medium helps select only those bacteria that can tolerate high salt concentrations. Halophilic species can form yellow colonies on the MSA plates when they can ferment mannitol sugar and forms pink colonies when they can not.

e. Cetrimide agar is a type of agar used for the selective identification and isolation of the gram-negative bacterium, *Pseudomonas aeruginosa* which appear as blue-green or yellow-green colonies. The media contains cetrimide, which is the selective agent against alternate microbial flora. Glycerol is added to the media as a source of carbon (Cappuccino & Sherman, 2005).

f. Man, Rogosa, and Sharpe agar (MRS Agar)

This test is used to determine whether an isolate can form gas during glucose fermentation or not. MRS media is selective for *lactobacilli* and also helpful in the growth of some *pediococci.* After the incubation period if the inoculated organism can grow and form gas, it is considered as a positive result. In the case of the growth of some organisms, no bubble can form at all, which is considered a positive result also. Whereas no growth with no gas production is considered a negative result (Cappuccino & Sherman, 2005).

g. Facultative Anaerobic Condition: Some bacteria show better growth in the presence of oxygen but they can also grow without oxygen. These are called facultative anaerobes. Isolates were inoculated in NA media and incubated for 18-24 hours in the absence of oxygen. After the incubation period, growth on the media would mean that the bacteria can proliferate in anaerobic conditions. If no growth is formed then it indicates negative results.

2.5.4 Biochemical characterization

The tests described bellow were used for biochemical identification of the isolates. The ABIS online software was used to generate a probable genus and species-level identity of the isolates based on their morphology and chemical test results. ABIS stands for Advanced Bacterial Identification Software which uses microbial databases and is a powerful tool connected with an encyclopedia.

a. Catalase test: In this test, whether a bacteria can produce the enzyme catalase or not is identified. Catalase acts as a catalyst in breaking down hydrogen peroxide into oxygen and water. 18-24 hours fresh culture of the organisms to be tested were spotted on glass slides. A few drops of 3% hydrogen peroxide solution on top of each spot. The release of bubbles immediately would indicate a positive test while no bubble formation would mean a negative result.

b. Oxidase test: In an oxidase test, a Whatman filter paper (1mm) was soaked with the oxidase reagent. One loop which contains a colony from a pure culture of bacteria was placed on it. Within 30 seconds to 1 minute, if the purple color appeared over the bacteria then it would mean an oxidase-positive, in case of no color it would be considered a negative result.

c. Methyl Red test: Methyl red test was done to determine the ability of a bacteria to utilize glucose and produce acidic end products. For the methyl red test, a test tube containing 5 ml of sterile glucose phosphate broth was inoculated with a fresh culture of experimental bacteria. The tubes were then incubated for 40-48 hours at 37ºC. After incubation, 5 drops of methyl red were added to each tube and the color of the tubes was observed. The development of red and yellow color would indicate positive and negative results respectively (Cappuccino & Sherman, 2005).

d. Voges-Proskauer (VP) test: The VP test was done to determine if an organism can produce acetyl methyl carbinol from glucose fermentation or not. For this test, 1 ml of sterile glucose phosphate broth was placed in a test tube and inoculated with a fresh culture of isolates by using a sterile loop. After 40-48 hours of incubation at 37ºC, 10 drops of Barritt's A (40% Potassium Hydroxide) and 15 drops of Barrit's B (alpha-naphthanol) were added sequentially. The color was observed after 15-30 minutes. The development of a dark red color would indicate a positive result and no color would indicate a negative result. (Cappuccino & Sherman, 2005)

e. Motility test: For this test, a fresh culture of test bacteria was stabbed into a test tube of set MIU agar media. The tubes were then incubated for 18-24 hours at 37ºC. The growth of the organism would spread around the stabline or the whole media would become cloudy if the bacteria were motile.

f. Indole test

An indole production test was done to determine the ability of microorganisms to degrade the amino acid tryptophan by the enzyme tryptophanase. To detect the indole production, 10 drops of Kovacs reagent were added into an 18-24 hours-old culture in tryptone broth. For indol-positive bacteria, a red ring of rosindol would form on the surface of the media. (Cappuccino & Sherman, 2005)

g. Citrate utilization test

A citrate utilization test was done to differentiate among enteric organisms based on their ability to ferment citrate by the production of citrase enzyme. Using an aseptic technique, a small amount of the test bacteria from 18-24 hours old fresh culture was stabbed into a slant of Simon's citrate agar. After incubation at 37ºC for 18-24 hours, the development of blue color in the media and/or any growth would indicate the citrate positive result which means the organism is capable of utilizing citrate as the sole source of carbon.

h. Nitrate reduction test

In 18-24 hours-old bacterial culture in nitrate broth, reagents alpha-naphthylamine and sulphanilic acid were added. The two reagents in the presence of nitrite, and the reduced form of nitrate produce a red pigment. Thus, the observation of a deep red color would mean that nitrate reductase enzyme was present in an test bacteria. In case of no color, a bit of zinc was added. Red color after zinc addition would demote a negative result as nitrate in this case is reduced by the zinc and not the enzyme produced by the bacteria. No color after zinc addition would also mean a positive result.

i. Triple sugar-iron (TSI) agar test

Triple sugar iron agar test was done to differentiate between Gram-negative enteric bacilli based on their ability to ferment monosaccharides and/or disaccharides and to produce gas and/or hydrogen sulfide. The test bacteria were inoculated into slants of TSI agar by stabbing them with a sterile inoculating needle. After incubation at 37˚C for 18-24 hours, the color of the butt and slant was observed for any acid production due to fermentation of the sugars, cracks or bubbles due to gas, and black precipitate due to hydrogen sulfide production.

2.5.5 Analysis of Environmental Stress Tolerance, Antibiotic Resistance, and Pathogenic Potential

a. Acid Tolerance Test: Through modification of the pH of the nutrient broth, acid tolerance of the isolates was observed. By using 1N HCl, the pH of the broth was controlled and adjusted to pH 3, 4, and 5 in respective batches. By using sterile technique a small amount of 18-24 hours fresh culture of positive isolates was inoculated in the different pH media. They were then incubated for 18-24 hours at 37°C to observe the results. Growth was noted through the turbidity of the media. In case of subtle or no turbidity, the culture was observed under a microscope to find viable cells.

b. Bile salt tolerance: Nutrient broth (NB) modified with a commercial bile salt mixture (pH 8) was used to observe the bile salt tolerance of the test bacteria. Fresh cultures of positive isolates were inoculated in NB that contained bile salts of concentrations 0.5%, 1.0%, and 1.5% in respective batches. Uninoculated NB was used as the control and all were incubated at 37°C for 18-24 hours. They were observed for turbidity due to growth after the incubation.

c. AST: Antibiotic Susceptibility Test was used to determine a isolates' and LSs' growth pattern in the presence of antibiotics. For this experiment, the Kirby-Bauer disk diffusion susceptibility method was utilized using MHA as media. Test bacteria was lawned on MHA media plates in a concentration equivalent to Macfarland 0.5 standard and different antibiotic disks were placed on them keeping enough space between two disks. After incubation at 37°C for 18-24 hours they were observed for Zones of inhibition. The diameter of the zone of inhibition was measured and compared to the antibiotic susceptibility chart of the selected antibiotics to determine resistance or susceptibility.

d. DNase Test: This test was used to determine an isolate's ability to produce DNase enzymes. The isolates were streaked on DNase medium containing nutrients and polymerized DNA. If DNase-producing organisms were to grow on the media they would break down DNA into oligo-nucleotides or mono-nucleotides. When the media surface was flooded with 1N HCl after 18-24h incubation at 37°C, the HCl would depolymerize the remaining DNA in the media making it cloudy with precipitation of free nucleic acids. In DNase-positive bacteria, a clear zone around the growing colonies would appear as the DNA would already have been already broken down by the bacteria.

e. Hemolysis test on Blood agar: Blood agar was used to determine the type of hemolysis a bacteria can perform. It tested the bacteria's ability to lyse red blood cells catalyzed by hemolysin enzymes. Different bacteria contain different hemolysins such as alpha, beta, or gamma. In this experiment, commercial blood agar base media was prepared and autoclaved at 121° C for 15 minutes. After cooling down the media to 45-50°C, 5% sterile defibrinated sheep blood was added. Once the agar was completely mixed with blood it was poured into sterile petri dishes. Then by using a sterile inoculating loop a small number of isolates were inoculated on the set agar media. The plates were then incubated at 37°C for 18-24 hours. After incubation, the growing bacterial colonies were observed for clear or green zones which would respectively mean complete or partial hemolysis.

Chapter 3

Results

3.1 Primary screening result

Primary screening results (the cross streak, well diffusion, and disk diffusion methods): The isolates were evaluated as possible antagonists based on either Zone of inhibition or growth that overpowers/minimizes the growth of Laboratory strains observed on a cross streak, well diffusion, and disk diffusion method.

a. SK (Homemade Sauerkraut):

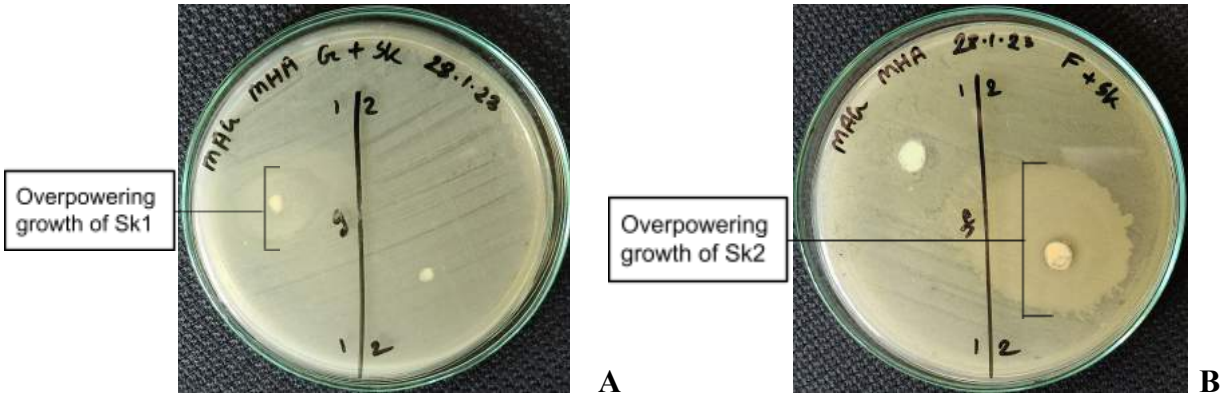
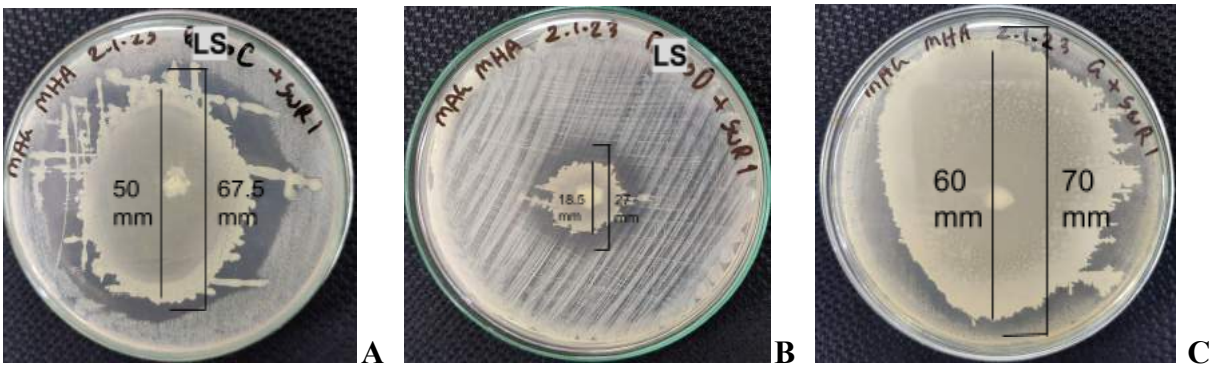


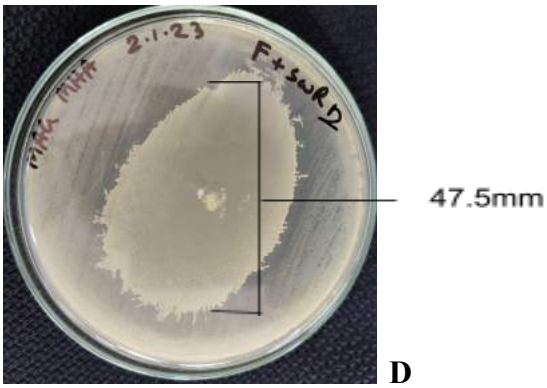
Figure 2: After the incubation period, (A) Cross streak of SK1 on the lawn of LS-G showing an overgrowth of 19 mm average diameter and no zone of inhibition. (B) Cross streak of SK1 on the lawn of LS-F showing an overgrowth of 37.5 mm average diameter and no zone of inhibition.

b. SwR (Packed Yogurt):



A B C

Figure 3: SwR1 showing overpowering growth and zones of inhibition against LS - C, D, and G respectively in figures A, B, and C after incubation. (A) A cross streak of SwR1 on the lawn of LS-C shows an overgrowth of 50 mm average diameter and a zone of inhibition of 67.5 mm average diameter. (B) A cross streak of SwR1 on the lawn of LS-D shows an overgrowth of 18.5mm average diameter and a zone of inhibition of an average diameter of 27 mm. (C) Cross streak of SwR1 on the lawn of LS G showing an overgrowth of 65 mm average diameter and a zone of inhibition of an average diameter of 70 mm.



D

Figure 4: : After the incubation period, a Cross streak of SwR2 on the lawn of LS-F showed an overgrowth of 47.5 mm average diameter and a slight zone of inhibition irregularly surrounding the growth area.

c. MV (Packed Yogurt):

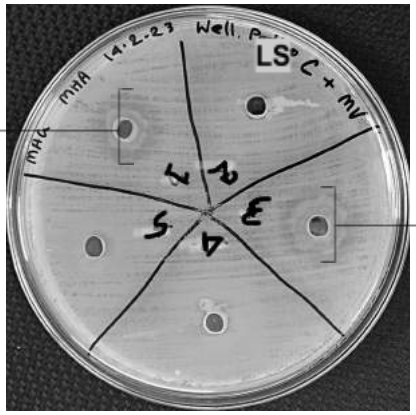
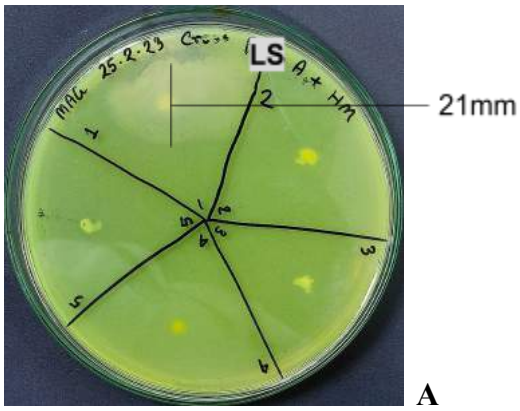
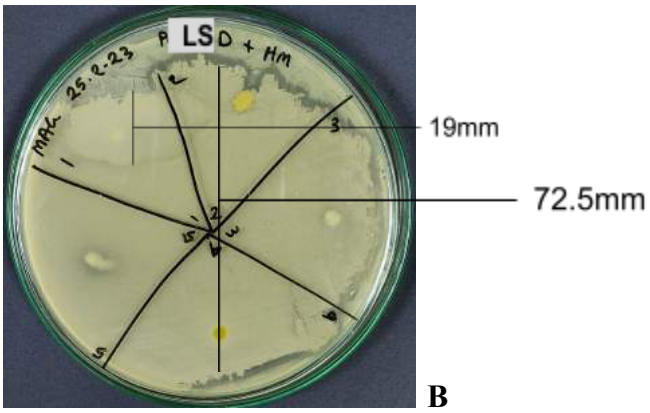
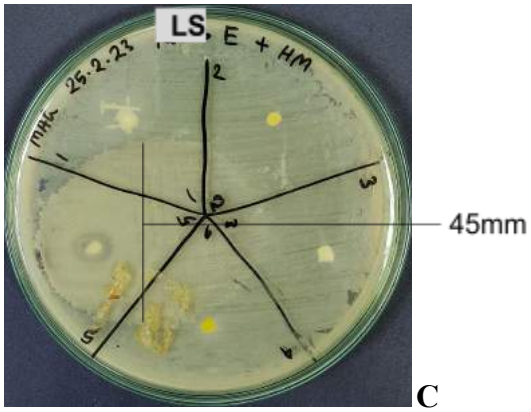


Figure 5: After the incubation period, well diffusion of MV1 (15mm ZOI) and MV3 (16.5mm ZOI) on the lawn of LS-C.

d. HM (Natural Honey):







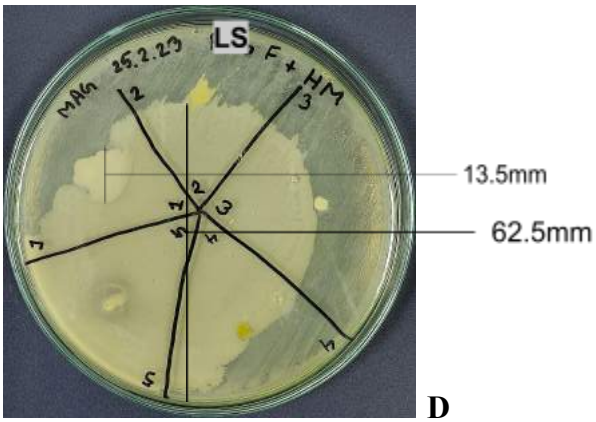


Figure 6: Results of the cross streak of the isolated HM after the incubation period. (A) Colony of HM1 on the lawn of LS-A showing an overgrowth of 21 mm average diameter. (B) The colony of HM1 on the lawn of LS-D showed an overgrowth of 19 mm average diameter and the Colony of HM5 showed an overgrowth of 72.5 mm average diameter with a slight, irregular zone of inhibition. (C) Colony of HM1 on the lawn of LS-E showing an overgrowth of 45 mm average diameter. (D) The colony of HM1 on the lawn of LS-F showed an overgrowth of 13.5 mm average diameter and the Colony of HM5 showed an overgrowth of 62.5 mm average diameter.

Table 3. Primary Screening Based on Antagonism Against Selected LSs

Primary screening test				
No.	**Isolated Bacteria**	**Antagonism in Cross streak (Spot-on-lawn)**	**Antagonism in Well diffusion**	**Antagonism in Disk diffusion**
1	BK1	x	x	x
2	BK2	x	x	x
3	BK3	x	x	x
4	BK4	x	x	x
5	BK5	x	x	x
6	BK6	x	x	x
7	BK7	x	x	x
8	TT1	x	x	x
9	TT2	x	x	x
10	TT3	x	x	x
11	TT4	x	x	x
12	TT5	x	x	x
13	TT6	x	x	x
14	UL1	x	x	x
15	UL2	x	x	x
16	PS1	x	x	x
17	PS2	x	x	x
18	PS3	x	x	x
19	PS4	x	x	x
20	PS5	x	x	x
21	RS1	x	x	x
22	RS2	x	x	x

23	RS3	x	x	x
24	RS4	x	x	x
25	VK1	x	x	x
26	VK2	x	x	x
27	VK3	x	x	x
28	VK4	x	x	x
29	SK1	√	x	x
30	SK2	√	x	x
31	MV1	x	√	x
32	MV2	x	x	x
33	MV3	x	√	x
34	MV4	x	x	x
35	MV5	x	x	x
36	PR1	x	x	x
37	PR2	x	x	x
38	PR3	x	x	x
39	PR4	x	x	x
40	PR5	x	x	x
41	As1	x	x	x
42	As2	x	x	x
43	As3	x	x	x
44	HM1	√	x	x
45	HM2	x	x	x
46	HM3	x	x	x
47	HM4	x	x	x
48	HM5	√	√	x
49	SwR1	√	x	x
50	SwR2	√	x	x
51	SwR3	x	x	x
52	SwR4	x	x	x
53	SwR5	x	x	x
54	SwR6	x	x	x
55	SwR7	x	x	x
56	SwR8	x	x	x

x = Antagonism Negative
√ = Possible antagonism Positive

Table 4: Antagonism positive result of isolates against different LSs

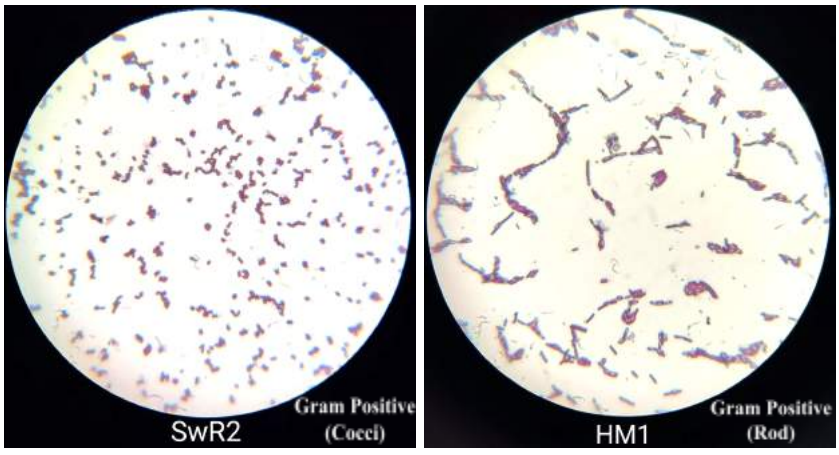
No	Isolated Bacteria	Description of cross streak results	Description of well diffusion results	Interpretation
1	Sk1	19 mm of overgrowth & No ZOI against LS-G	x	Competitive advantage over LS-G
2	Sk2	37.5 mm overgrowth & No ZOI on LS-F	x	Competitive advantage over LS-F
3	SwR1	50mm overgrowth & 67.5 mm ZOI against LS-C; 18.5 mm of overgrowth & 27 mm ZOI against LS-D; 65 mm overgrowth & 70 mm ZOI on LS-G	x	Competitive advantage over and inhibition of LS-C, LS-D & LS-G
4	SwR2	47.5 mm overgrowth & slight, irregular ZOI surrounding the growth on LS-F	x	Competitive advantage over & slight inhibition of LS-F
5	MV1	x	15 mm ZOI against LS-C	Possible inhibition of LS-C
6	MV3	x	16.5 mm ZOI against LS-C	Possible inhibition of LS-C
7	HM1	Overgrowth 21 mm against LS-A; 19 mm against LS-D; 13.5 mm against-F	x	Competitive advantage over LS-A, LS-D & LS-F
8	HM5	Overgrowth 72.5 mm against LS-D; 45 mm against LS-E with slight ZOI; 62.5 mm against-F	25 mm ZOI against LS-C; 15 mm ZOI against LS-D	Competitive advantage over LS-D, LS-E & LS-F; inhibition of LS-C & LS-D

***Zone of inhibition (ZOI) and area of overgrowth measured in average diameter (mm).**
***Due to the irregularity in shape, the measurements are approximate.**

Major Findings of Tables 3 & 4: Primary screening was done with 56 organisms and only 8 (SK1, SK2, SWR1, SWR2, MV1, MV3, HM1, HM5) displayed signs of antagonism. Among these isolates, one showed signs of competitive advantage against *Pseudomonas aeruginosa*; one was possibly competitively advantageous and four were inhibitory against Salmonella typhi; competitive advantage was also indicated against *Staphylococcus aureus*, *Enterotoxic E. coli*, *Shigella flexneri* and *Vibrio cholerae* by three, one, four, and two of the isolates respectively. Moreover, two of the isolates were inhibitory towards *Staphylococcus aureus* while *Shigella flexneri* and *Vibrio cholerae* each had one isolate acting on inhibiting their growth.

3.2 Bacterial Characterization

a. **Gram staining:** After primary screening, Gram staining of antagonism-positive isolates was observed for morphological identification. Some representitives of the photos taken while observing under a microscope using the 100x lens are displayed in Figure 7.



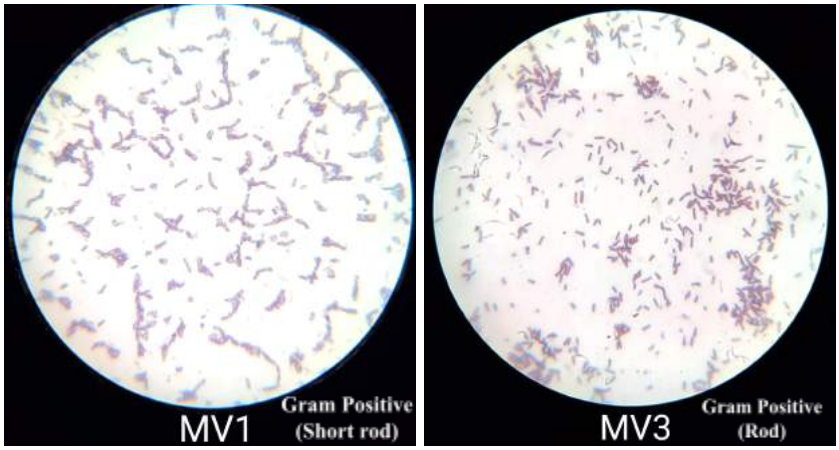


Figure 7: Representative images of Gram Staining.

Table 5: Gram staining result of Selected Antagonism Positive Isolates

No	Given name of Isolates	Gram stain result	Shape
1	Sk1	Negative	Rod
2	SK2	Positive	Rod
3	SwR1	Positive	Rod
4	SwR2	Positive	Cocci
5	MV1	Positive	Rod
6	MV3	Positive	Rod
7	HM1	Positive	Rod
8	HM5	Negative	Rod

Major Findings of Table 5: Five of the isolates were gram positive rods, two were gram negative rods and only one was gram positive cocci.

b. Spore staining: Under the microscope, spores showed light green color and vegetative cells form brownish-red or pink color.

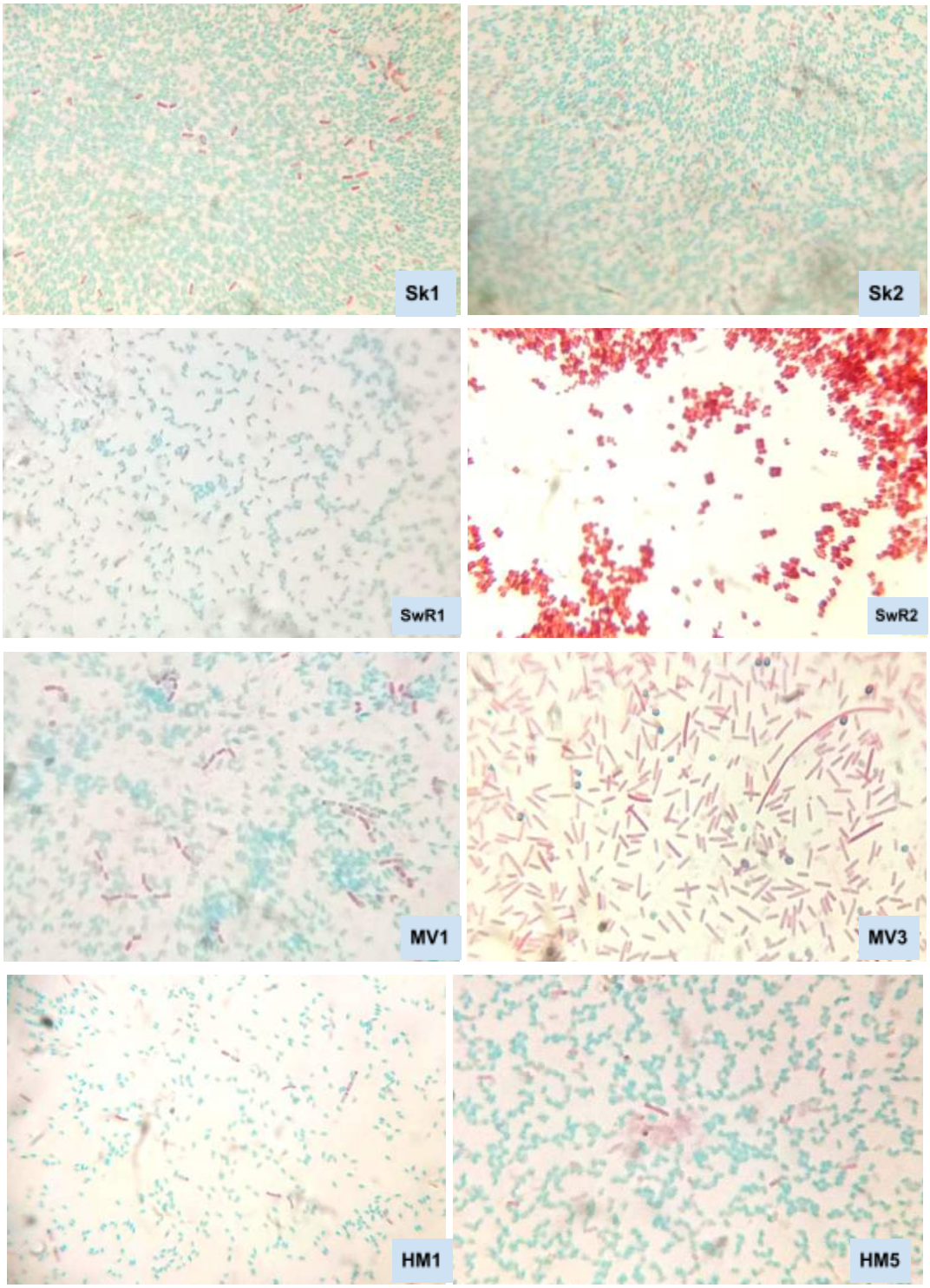


Figure 8: Spore staining results observation.

Table 6: Observation and interpretation of spore staining test

Isolates	Observation	Interpretation
SK1	Most cells retained malachite green stain. Only a few rod shaped vegetative cells retained the red stain of safranin.	Spore forming bacteria. Most cells in spore form.
SK2	Most cells retained malachite green stain. Only a few rod shaped vegetative cells retained the red stain of safranin.	Spore forming bacteria. Most cells in spore form.
SwR1	Most cells retained malachite green stain. Only a few rod shaped vegetative cells retained the red stain of safranin.	Spore forming bacteria. Most cells in spore form.
SwR2	All cells were cocci vegetative cells and retained the red stain of safranin.	Non spore forming bacteria.
MV1	Most cells retained malachite green stain. Only a few rod shaped vegetative cells retained the red stain of safranin.	Spore forming bacteria. Most cells in spore form.
MV3	A few cells retained malachite green stain. Mostly rod shaped vegetative cells that retained the red stain of safranin.	Spore forming bacteria. Some cells in spore form.
HM1	Most cells retained malachite green stain. Only a few rod shaped vegetative cells retained the red stain of safranin.	Spore forming bacteria. Most cells in spore form.
HM5	Most cells retained malachite green stain. Only a few rod shaped vegetative cells retained the red stain of safranin.	Spore forming bacteria. Most cells in spore form.

Major Findings of Table 6: All of the isolates were spore forming bacteria except one.

3.3 Selective media: The growth of positive isolates observed on 5 different selective media was observed and growth was only observed on one selective media, MSA for 6 out of the 8 isolates. These 6 bacteria were deemed halophilic with 4 of them also being able to ferment mannitol and produce acidic end products. All of the 8 isolates were able to grow under anaerobic conditions.

a. **MAC Agar:** After 18-24 hours of incubation no colony formed on the MacConkey agar plates. The images below show the results of isolates (Figure 9).

Figure 9: MacConkey Agar plates observed.

b. **EMB Agar**: No isolates had shown green metallic shine colonies. There was no growth on the any of the plates. (Figure 10).

Figure 10: EMB agar plates observed.

c. **XLD Agar**: No colonies were seen after the incubation on the plates that indicates no growth of isolates (Figure 11).

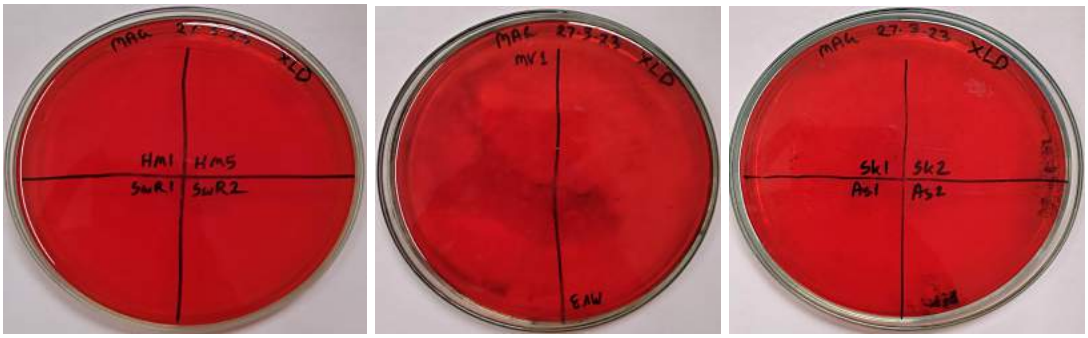


Figure 11: XLD agar plates observed.

d. MSA: Yellow colonies for SK1, SK2, HM5 and SwR1 were observed on the MSA plates. But HM1, MV1 and SwR2 show pink colonies.

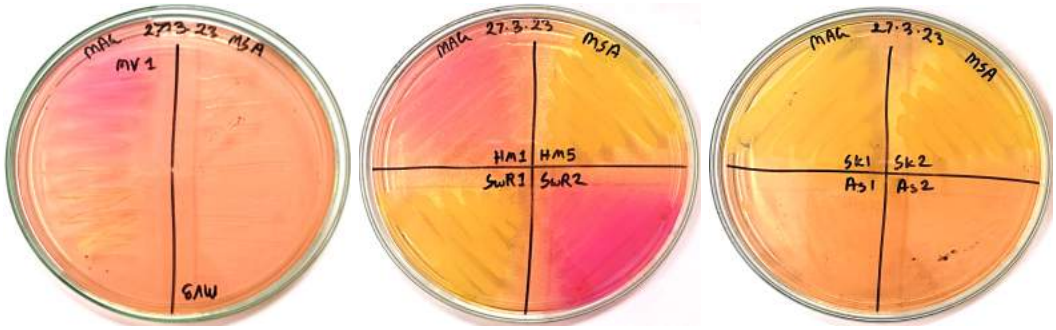


Figure 12: MSA plates observed

e. Cetrimide Agar: No colony formed on cetrimide plates for all the isolates indicating no presence of *Pseudomonas aeruginosa.*

Figure 13: Cetrimide plates observed.

f. **MRSA:** No growth and no gas production indicates negative results for all the isolates.

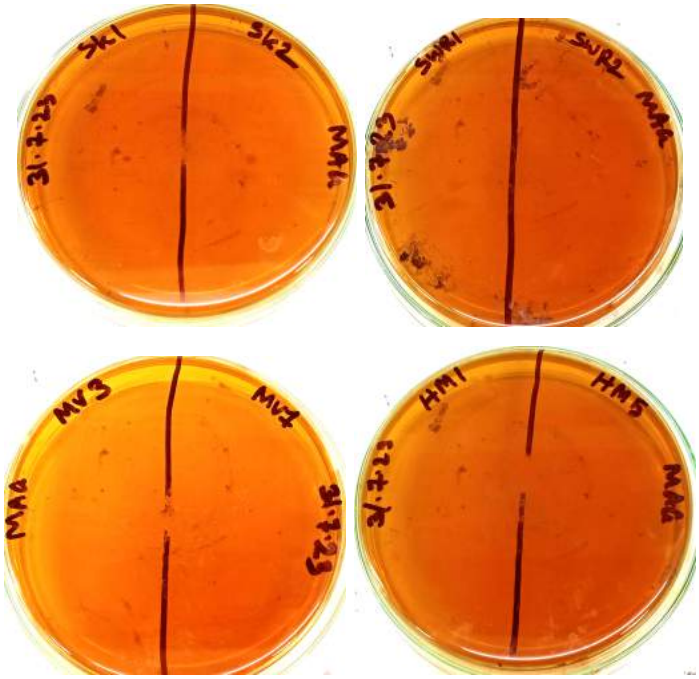


Figure14: MRSA plates observed

g. **Growth in Anaerobic Condition:** Growth of all the isolates observed in the absence of oxygen which indicates positive results.

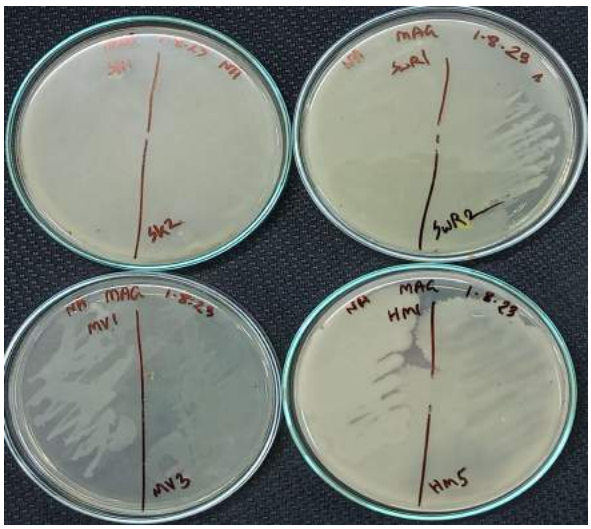


Figure 15: Growth of Facultative anaerobes observed.

Table 7: Observations based on different selective media

Isolates	MacConkey	EMB	XLD	MSA	Cetrimide	MRSA	Interpretation
SK1	x	x	x	Yellow colonies	x	x	Halophilic, (tolerate salt concentration up to 7.5% & can ferment mannitol sugar)
SK2	x	x	x	Yellow colonies	x	x	Halophilic, (tolerate salt concentration up to 7.5% & can ferment mannitol sugar)
SwR1	x	x	x	Yellow colonies	x	x	Halophilic, (tolerate salt concentration up to 7.5% & can ferment mannitol sugar)
SwR2	x	x	x	Pink colonies	x	x	Halophilic, (tolerate salt concentration up to 7.5% & can not ferment mannitol sugar)
MV1	x	x	x	Pink colonies	x	x	Halophilic, (tolerate salt concentration up to 7.5% & can not ferment mannitol sugar)
MV3	x	x	x	x	x	x	Can not tolerate salt concentration up to 7.5% & also can not ferment mannitol sugar
HM1	x	x	x	Pink colonies	x	x	Halophilic, (tolerate salt concentration up to 7.5% & can not ferment mannitol sugar)
HM5	x	x	x	Yellow colonies	x	x	Halophilic, (tolerate salt concentration up to 7.5% & can ferment mannitol sugar)

Major Findings of Table 7: All of the isolates were halophilic except MV3 while four of them could also ferment mannitol to produce acidic end products.

3.4 Biochemical test analysis and observation

a. **Catalase Test:** All the isolates had shown positive results (Figure 16) that confirm these bacteria are capable of producing the catalase enzyme which detoxifies H_2O_2 and showed bubbles.

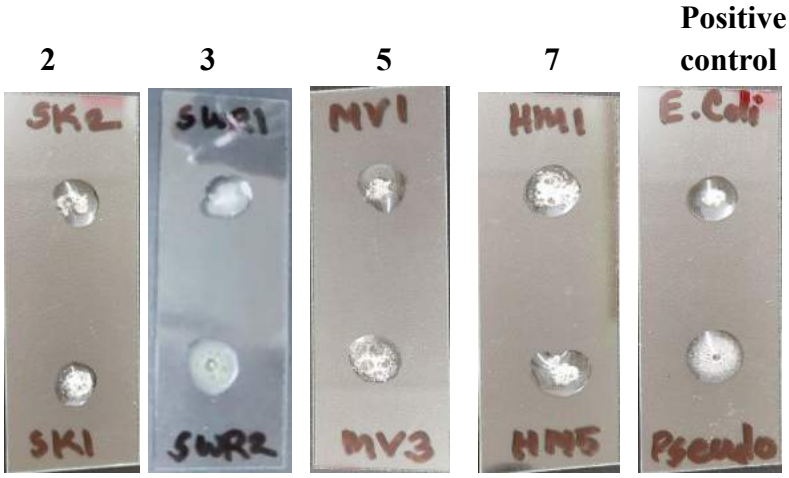


Figure 16: Catalase Test Observation.

b. **Citrate test:** After incubation, color change from green to blue indicates a positive test, which is shown by only SwR1 isolate. It was seen that the rest of the 7 isolates showed no color change or growth indicating negative results (Figure 17).

Figure 17: Simmon Citrate Test Observation.

c. **Motility test:** After the incubation period, cloudiness in the test tubes indicates these bacterias are motile, and non-motile bacteria did not form any cloud in the test tubes. SK1, SK2, SwR1, SwR2, HM1, HM5 showed positive and other isolates showed negative results.

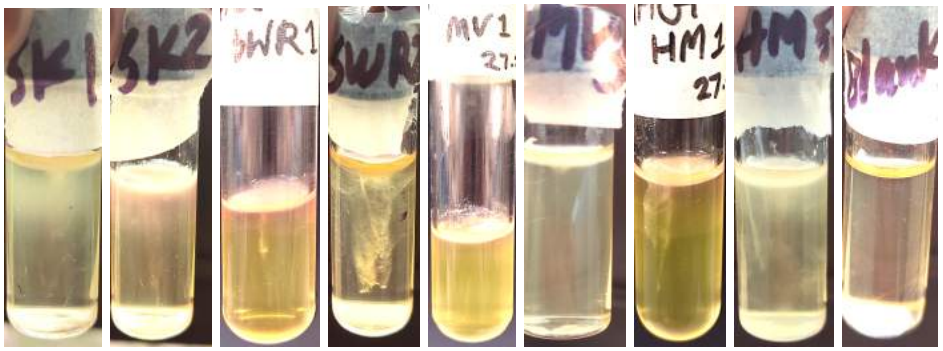


Figure 18: Motility test observation.

d. **Indole test**: For the indole test observation, the formation of no red ring at the top of the media indicates negative results for all the isolates. This means that the bacteria were unable to convert tryptophan into indole.

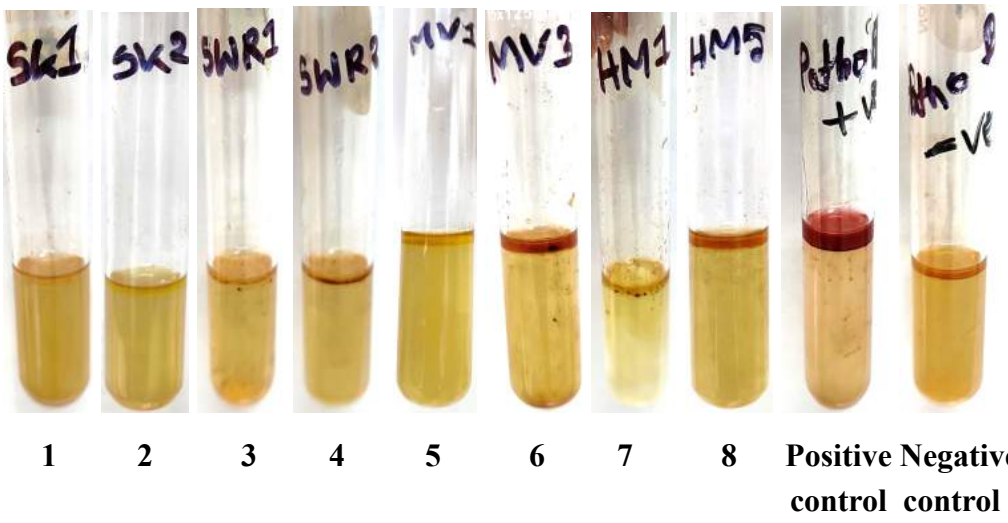


Figure 19: Indole test observation .

e. **MR Test:** Color changing from yellow to red indicate a positive result, while no color change indicates a negative result for MV3 and SwR1 (Figure 19).

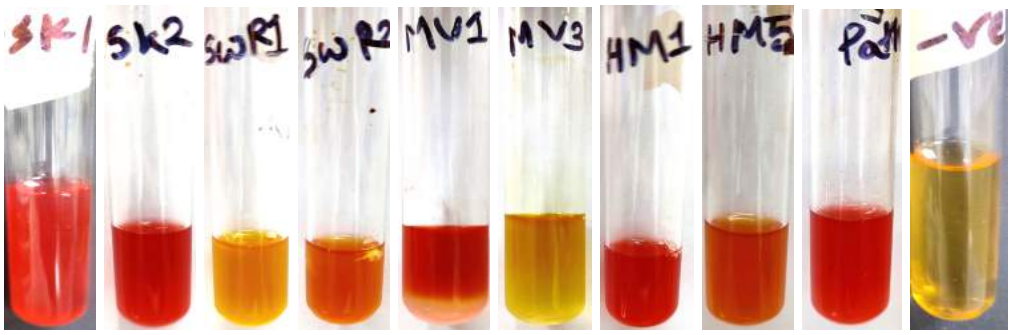


Figure 20: MR Test Observation.

f. **VP Test:** After incubation and addition of reagents, red color indicated a positive and no color change indicated a negative result. (Figure 20). It was observed that 4 isolates had shown positive results, and the other 4 isolates showed negative results.

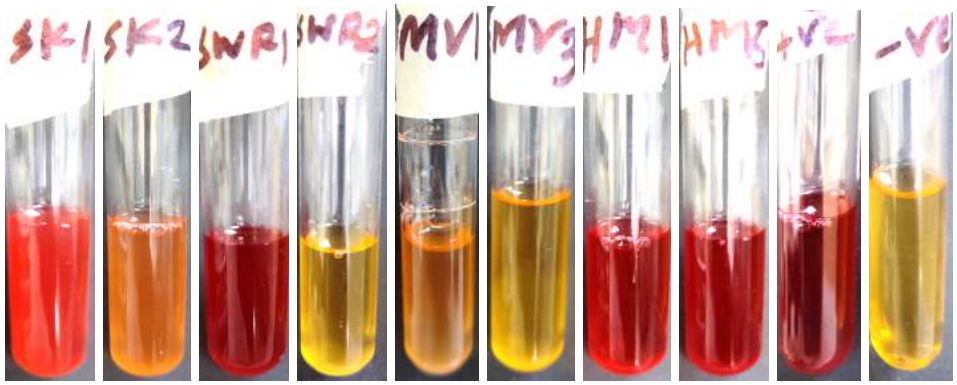


Figure 21: VP Test Observation.

g. **Nitrate reduction test:** After the incubation period, reagents A and B were added. The color of the media changed from a light pink to a deep red quickly which indicated that nitrate reductase enzyme was present. One isolate showed positive result and the other 7 isolates showed negative results in the nitrate reduction test.

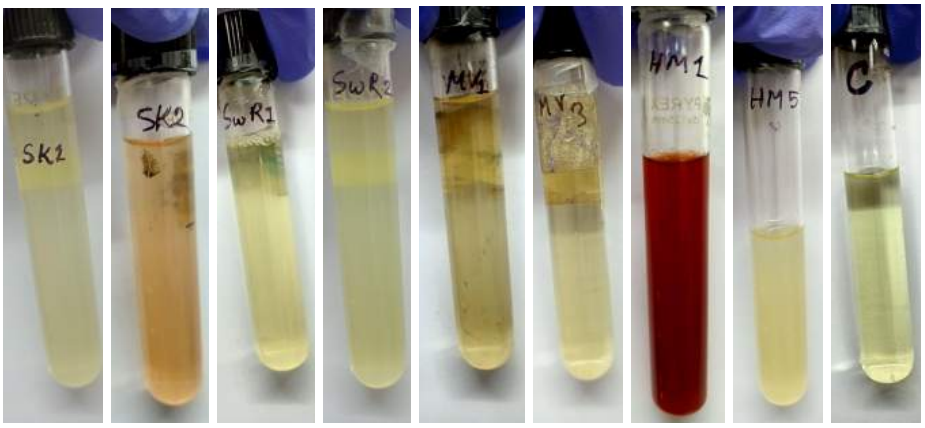


Figure 22: Nitrate reduction test observation.

h. **Oxidase Test:** A blue color indicated a positive result, meaning that the bacteria are able to produce cytochrome oxidase. On the contrary, no color change indicated a negative result. (Figure 23). SK2 and SwR2 showed negative results, and the other 6 isolates showed positive results.

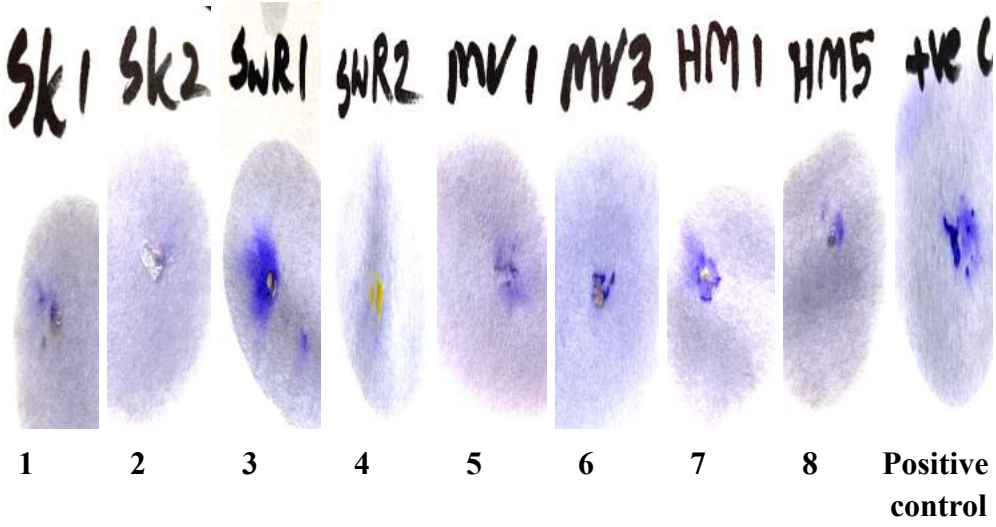


Figure 23: Oxidase test observation

i. **TSI Test:** After incubation, the result was observed in the test tubes (Figure 23). No gas formed in the test tubes of all the isolates. For, both the slant and the butt turning yellow indicated that the pH of the agar had become acidic. Whereas, for both slant and butt turning red indicated the alkaline condition of the media.

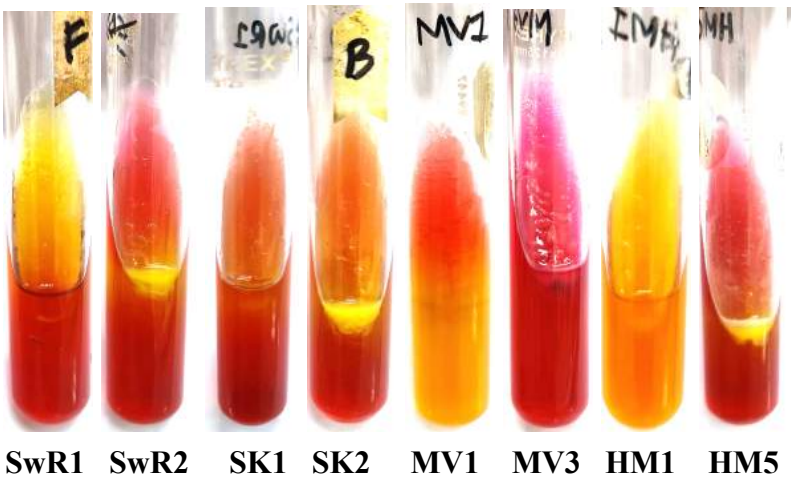


Figure 24: TSI test observation.

Table 8: Biochemical test results

Isolates	Gram Staining	Catalase	Citrate	Indole	Motility	MR	VP	Nitrate reduction	Oxidase	TSI	Presumed Organism
SK1	-, rod	+	-	-	+	+	+	-	+	K/K	Pasteurellaceae
SK2	+, rod	+	-	-	+	+	-	-	-	A/K	Bacillus
SwR1	+, rod	+	+	-	+	-	+	-	+	K/K	Bacillus
SwR2	+, cocci	+	-	-	+	+	-	-	-	A/K	Pediococcus
MV1	+, rod	+	-	-	-	+	-	-	+	A/A	Bacillus
MV3	+, rod	+	-	-	-	-	-	-	+	K/K	Bacillus
HM1	+, rod	+	-	-	+	+	+	+	+	A/A	Bacillus
HM5	-, rod	+	-	-	+	+	+	-	+	K/K	Bacillus

Table 9: Possible genus, species and probability of selected isolates

Serial	Isotale Code	Possible Genus	Possible Species	Probability
1	SK1	*Pasteurellaceae*	Unknown	–
2	SK2	*Bacillus*	*Paenibacillus illinoisensis* *Bacillus simplex*	71.7% 18.3%
3	SwR1	*Bacillus*	*Bacillus pumilus* (possibility of *B. safensis*)	28.4%
4	SwR2	*Pediococcus*	Unknown	–
5	MV1	*Bacillus*	*Paenibacillus mendelii*	39%
6	MV3	*Bacillus*	*Paenibacillus mendelii*	97.5%
7	HM1	*Bacillus*	*Paenibacillus cookii*	39.9%
8	HM5	*Bacillus*	*Bacillus smithii*	84.6%

Major Findings of Table 8 & 9: Four of the isolates were predicted to be *paenibacillus* Spp., one *Bacillus*, one *Pediococcus*, and one *Pasteurellaceae* based on their morphology, growth on selective media and biochemical test results.

3.5 Analysis of Environmental Stress Tolerance and Antibiotic Resistance

a. Acid Tolerance Test

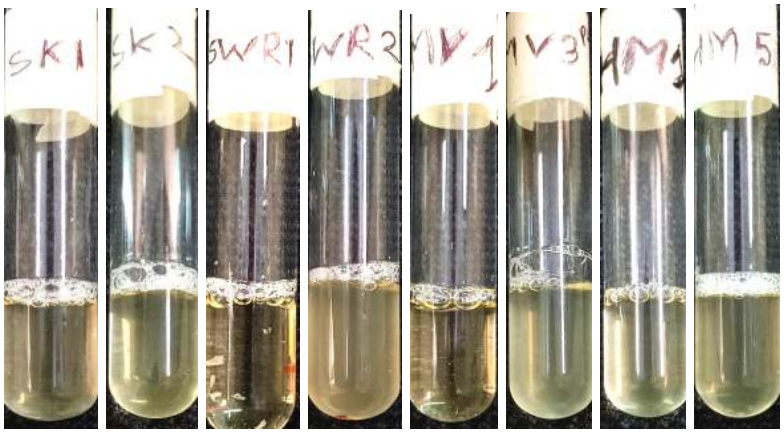
After the incubation period, the acid tolerance of isolates in 3 different pH solutions were observed.

pH3:

SK1 SK2 SwR1 SwR2 MV1 MV3 HM1 HM5

Figure 25: Acid tolerance test in pH 3 solution.

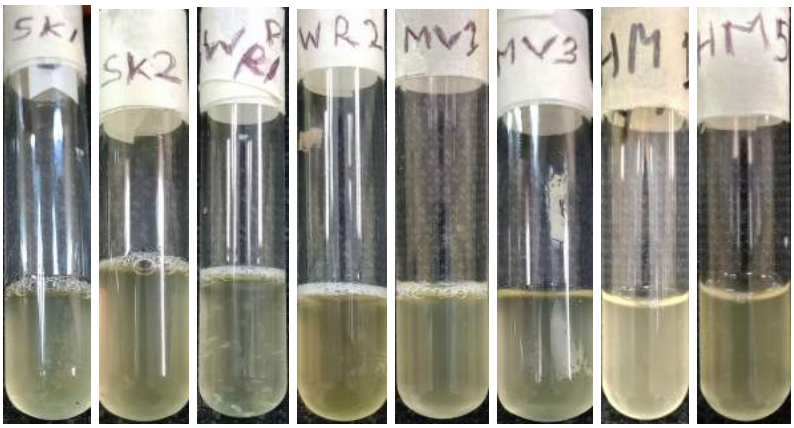
pH4:



1 2 3 4 5 6 7 8

Figure 26: Acid tolerance test in pH 4 solution.

pH5:



1 2 3 4 5 6 7 8

Figure 27: Acid tolerance test in pH 5 solution.

Table 10: Acid tolerance test

Isolates	pH 3	pH 4	pH 5
SK1	-	-	++
SK2	-	+	++
HM1	-	-	++
HM5	-	+	++
MV1	-	-	++
MV3	+	+	++
SwR1	++	+	++
SwR2	++	++	++

(-) = No growth; (+) = Minimal growth; (++) = Heavy growth.

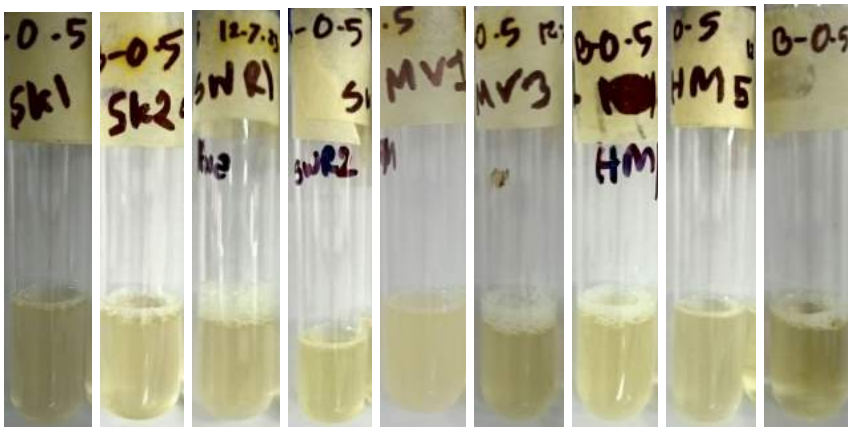
Major findings of Table 10: Three of the isolates survived and proliferated in pH3, five in pH4 and all of them survived very well in pH5.

b. Bile salt tolerance

After the incubation period, the bile salt tolerance of isolates in 3 different bile salt concentrations were observed.

Bile salt concentration (0.5%):

Only SK1 showed negative results and others showed positive results in bile salt concentration (0.5).



1 2 3 4 5 6 7 8 Control

Figure 28: Result of bile salt concentration (0.5%) observed after incubation.

Bile salt concentration (1%):

Only SK1, SK2, SwR1 showed negative results and others showed positive results in bile salt concentration (1).

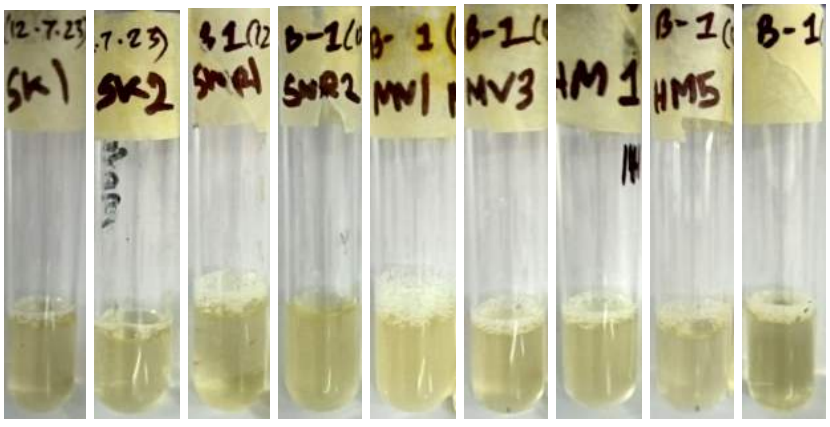


Figure 29: Result of bile salt concentration (1%) is observed.

Bile salt concentration (1.5%):

HM1 and MV1 showed positive results and others showed negative results in bile salt concentration (1.5).

Figure 30: Result of bile salt concentration (1.5%) is observed.

Table 11: Bile salt tolerance test

Isolates	0.5%	1%	1.5%
SK1	-	-	-
SK2	+	-	-
MV1	+	+	+
MV3	+	+	-
HM1	+	+	+
HM5	+	+	-
SwR1	+	-	-
SwR2	+	+	-

(-) = No growth; (+) = Minimal growth; (++) = Heavy growth.

Major findings of Table 11: Out of 8 isolates, 7, 5, and 2 exhibit survival at 0.5% , 1%, and 1.5% bile salt concentrations respectively.

c. Antibiotic Susceptibility Test

Zone of inhibition Observed after 18-24 hours of incubation on MHA media. The antibiotic susceptibility of both the isolates and Laboratory Strains was observed. The resistance and susceptibility of the known bacteria were determined by comparing them to the Zone of Inhibition interpretation chart that came with the antibiotic disks (HIMEDIA ®). In the antibiotic susceptibility test, some of the isolates did display large ZOIs, especially around one or more disks of the antibiotics Imipenem(10 Units), Ciprofloxacin 5 Units) and Chloramphenicol (30 units). This may be an indication of possible susceptibility as most of the LSs were also susceptible to the same three antibiotics. However, all of the bacteria (both sample isolates and LSs showed resistance towards Penicillin(10 Units) and Cefixime (5 Units).

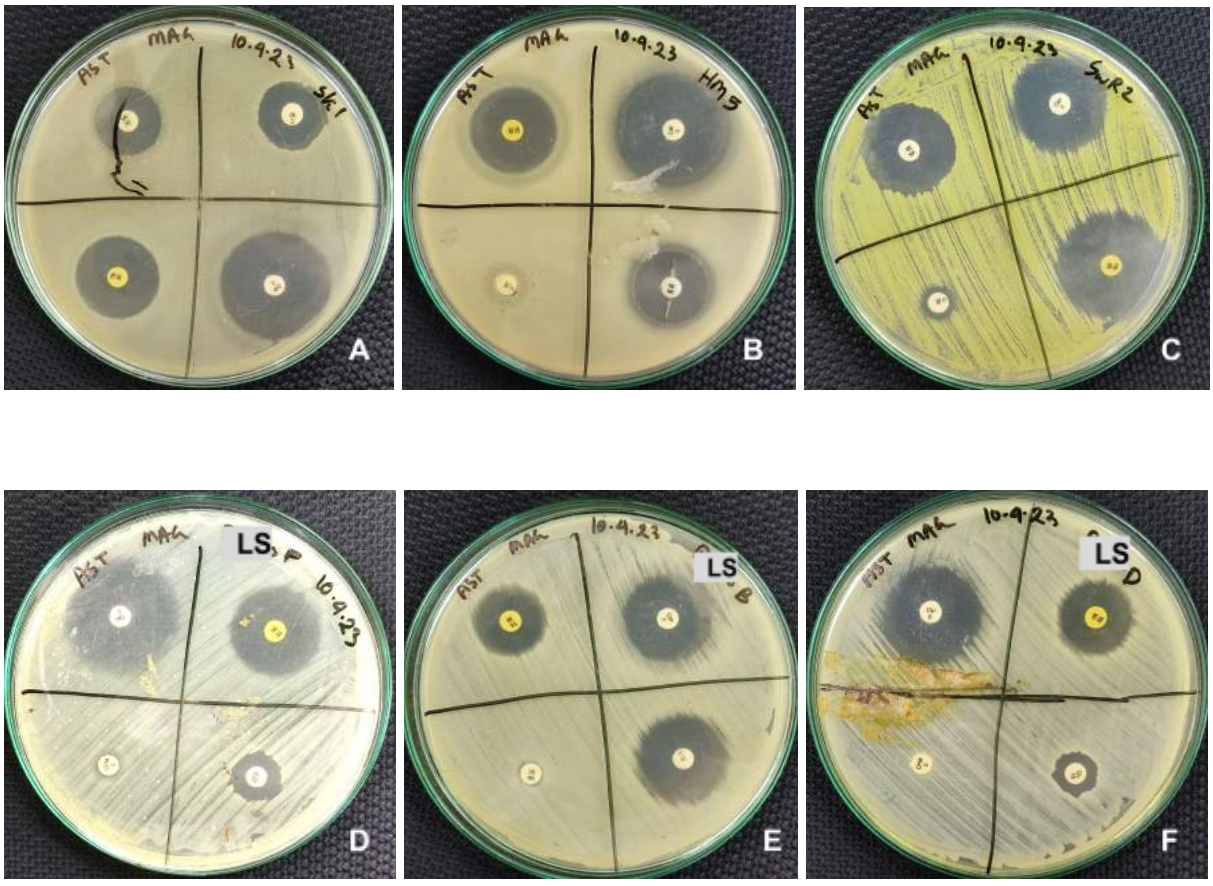


Figure 31: Representative images of AST results. In A, B & C respectively SK1, HM5 & SwR2 against Chloramphenicol, Ciprofloxacin, Tetracycline, and Vancomycin. (D) LS-F against Chloramphenicol, Ciprofloxacin, Tetracycline, and Cefixime. (E) LS-B against

Chloramphenicol, Ciprofloxacin, Tetracycline, and Vancomycin. (F) LS-D against Chloramphenicol, Ciprofloxacin, Tetracycline, and Cefixime.

Table 12: AST results of isolates and LS

Isolates and LSs	Erythro-mycin (mm)	Imipenem (mm)	Cefixime (mm)	Colistin (mm)	Penicillin (mm)	Amikacin (mm)	Tetracycline (mm)	Ciproflox acin (mm)	Vancomycin (mm)	Chloramphenicol (mm)
Code	**E-15**	**IPM-10**	**CFM-5**	**CL-10**	**P-10**	**AK-30**	**TE-30**	**CIP-5**	**VA-30**	**C-30**
SK1	20	38.5	NZ (R)	9.5	NZ (R)	25	26	33.5	20	30.5
SK2	22.5	41.5	NZ (R)	1	NZ (R)	21.5	27.5	35	22	21
MV1	24	33.5	NZ (R)	NZ (R)	NZ (R)	25.5	25	34	22.5	NZ (R)
MV3	16	29	NZ (R)	11.5	NZ (R)	27	24.5	28	23	22.5
HM1	16.5	3	NZ (R)	NZ (R)	NZ (R)	24.5	21	23.5	17.5	20
HM5	2	38	NZ (R)	NZ (R)	NZ (R)	25.5	25	34	22.5	NZ (R)
SWR1	23	30	NZ (R)	NZ (R)	NZ (R)	25.5	16.5	31	NZ (R)	24
SWR2	11	41	NZ (R)	10.5	NZ (R)	30	30	27.5	27	8.5
LS-A	8.5	23 **(S)**	NZ (R)	14	NZ (R)	**20.5 (S)**	13.5	34 **(S)**	13.5	13.5 (R)
LS-B	9.5	28 **(S)**	NZ (R)	12.5	NZ (R)	19.5 **(S)**	21.5 **(S)**	27.5 **(S)**	NZ (R)	27 **(S)**
LS-C	8 (R)	20 (I)	NZ (R)	20	NZ (R)	34 **(S)**	28 **(S)**	37 **(S)**	25	23 **(S)**
LS-D	NZ (R)	27.5 **(S)**	NZ (R)	13	NZ (R)	25 **(S)**	22.5 **(S)**	30	NZ (R)	28 **(S)**
LS-E	18	34 **(S)**	NZ (R)	20.5	NZ (R)	25.5 **(S)**	24.5 **(S)**	34 **(S)**	10	35 **(S)**
LS-F	20	35 **(S)**	NZ (R)	16	NZ (R)	22 **(S)**	27 **(S)**	37 **(S)**	NZ (R)	37 **(S)**
LS-G	29	22.5	10 (R)	NZ (R)	NZ (R)	19.5 **(S)**	19	23.5	16.5	32.5 **(S)**

NZ = No Zone
(S) = Susceptible
(I) = Intermediate
(R) = Resistant

Table 13: Antibiotic susceptibility test

Isolate Code	Most likely Susceptible to Antibiotics	Most likely Resistant to Antibiotics
SK1	Imipenem(10 Units), Ciprofloxacin 5 Units) & Chloramphenicol (30 units)	Penicillin(10 Units), Cefixime (5 Units)
SK2	Imipenem(10 Units) & Ciprofloxacin 5 Units)	Penicillin(10 Units), Cefixime (5 Units)
MV1	Imipenem(10 Units), Ciprofloxacin 5 Units)	Penicillin(10 Units), Cefixime (5 Units)
MV3	—	Penicillin(10 Units), Cefixime (5 Units)
HM1	—	Penicillin(10 Units), Cefixime (5 Units)
HM5	Imipenem(10 Units), Ciprofloxacin 5 Units)	Penicillin(10 Units), Cefixime (5 Units)
SWR1	Imipenem(10 Units), Ciprofloxacin 5 Units)	Penicillin(10 Units), Cefixime (5 Units)
SWR2	Imipenem(10 Units), Tetracycline(30 Units & Amikacin (30 Units)	Penicillin(10 Units), Cefixime (5 Units)

Major Findings of Table 12 & 13: Almost all of the LSs and isolates were resistance to Penicillin(10 Units) and Cefixime (5 Units) showing no ZOI. Most LSs showed susceptibility to Imipenem(10 Units), Amikacin (30 Units), Ciprofloxacin 5 Units), and Chloramphenicol (30 units) and most of the isolates also showed large ZOIs around the disks of Imipenem(10 Units) and Ciprofloxacin (5 Units) which could mean potential susceptibility.

3.6. Evaluation of Potential for Pathogenicity

a. **Hemolysis test on Blood agar:** SK1, SK2, MV1 and HM5 showed clear zones of hemolysis around the growth of the bacterial colonies. SwR1 and HM1 showed partial hemolysis while SwR2, MV3 and all the Laboratory strains showed no hemolysis.

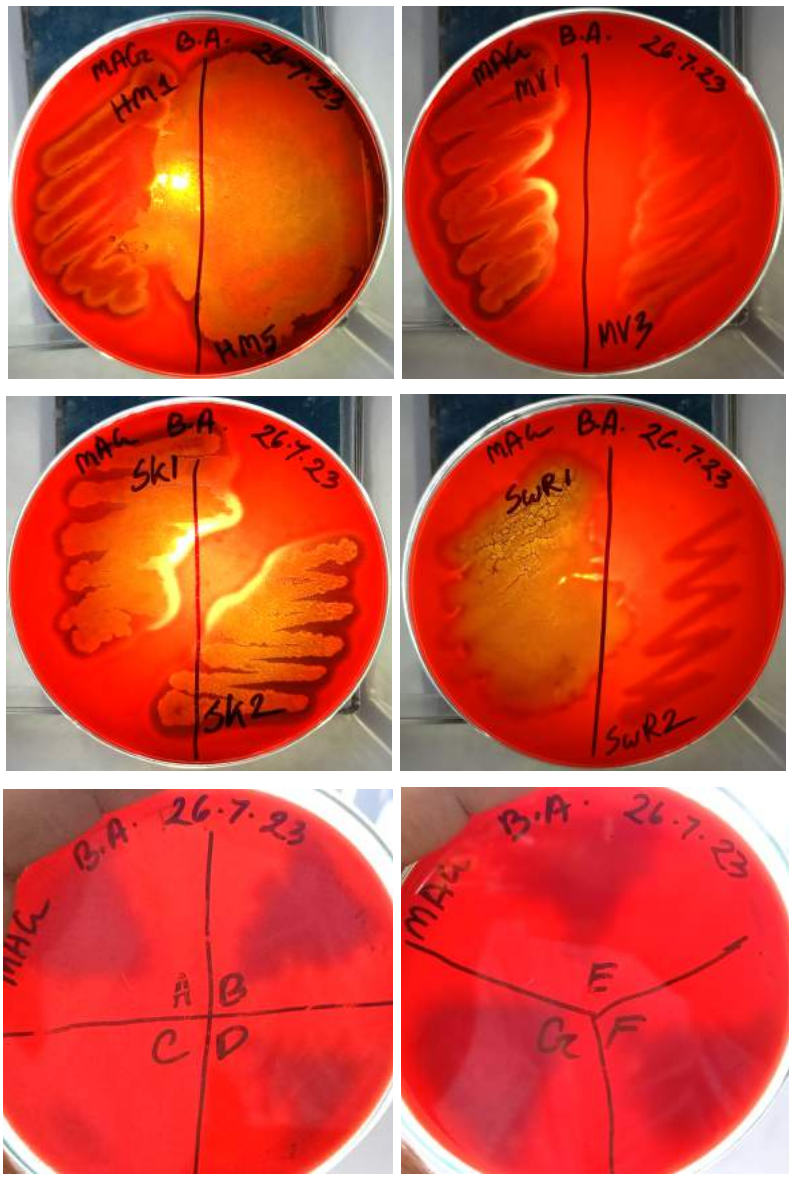


Figure 32: Results of Hemolysis test.

b. DNase test: There was no precipitation observed around the growth after flooding the surface with 1N HCl. All the isolates and Laboratory Strains showed negative results in the DNase test.

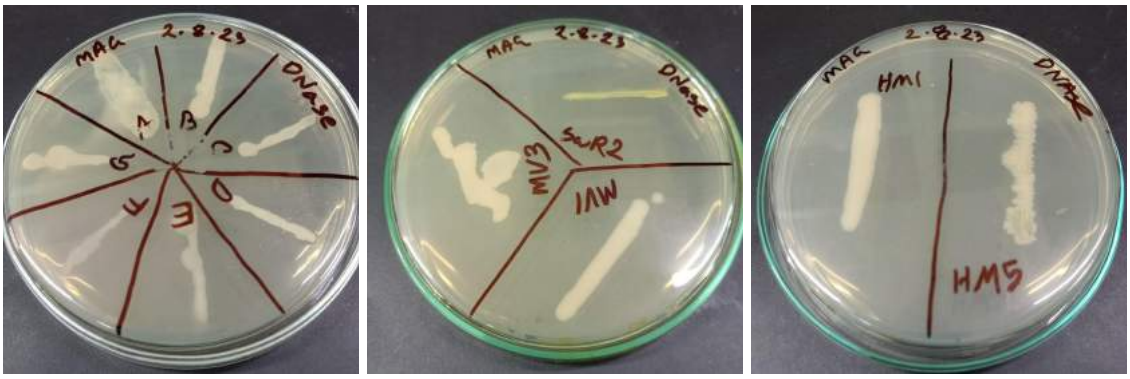


Figure 33: Results of DNase test.

Table 14: Hemolysis and DNase test of Selected Isolates and LSs

Isolate/LS code	Hemolysis	DNase
SK1	Complete hemolysis with transparency: β	Negative
SK2	Complete hemolysis with transparency: β	Negative
SwR1	Partial hemolysis without transparency: α	Negative
SwR2	No hemolysis: γ	Negative
MV1	Complete hemolysis with transparency: β	Negative
MV3	No hemolysis: γ	Negative
HM1	Partial hemolysis without transparency: α	Negative
HM5	Complete hemolysis with transparency: β	Negative
LS-A	No hemolysis: γ	Negative
LS-B	No hemolysis: γ	Negative
LS-C	No hemolysis: γ	Negative
LS-D	No hemolysis: γ	Negative
LS-E	No hemolysis: γ	Negative
LS-F	No hemolysis: γ	Negative
LS-G	No hemolysis: γ	Negative

Major Findings of Table 14: Four out of the eight isolates showed signs of complete hemolysis of blood cells, two showed incomplete hemolysis and two were non hemolytic. None of the isolates could produce DNase to break down DNA. None of the LSs were hemolytic or DNase positive.

Chapter 4

Discussion

In this study, three different tests were used to detect antagonism of bacteria isolated from fermented food against 7 selected Laboratory strains of bacteria that are commonly found in human GI-tract. The spot-on-lawn cross streak method is to observe antagonism in cell-to-cell direct contact conditions and determine the competitive advantage of an unknown isolate against the known bacteria (Denkova et al., 2017). On the other hand, Cell-free supernatants (CFS) of the isolates are used in well-diffusion and disk diffusion methods to observe any inhibitory activity by the metabolites or secretions of the isolates (Denkova et al., 2017). Most of the antagonism-positive isolates were found by the spot-on-lawn method and a few were from the well diffusion method. No significant outcome was shown in the disk diffusion tests as the disks only held 20-30 µL of CFS while the wells held up to 100 µL. According to Balouiri et al. (2016), antimicrobial agents diffuse through the agar to inhibit the germination and growth of test organisms. For that to happen the concentration of the inhibitory compounds in the CFS-impregnated disks had to be high enough which was hard to achieve with such a small volume. Therefore, it can be reasoned that well diffusion is a more efficient technique than disk diffusion for screening inhibitory activity.

According to Denkova et al. (2017), nutrients are limited in the intestine for which pathogens and probiotics compete. Probiotics with competitive advantage usually win in this situation and deprive the pathogens of nutrients. Some probiotics even display competitive exclusion where they colonize the gut so that pathogens cannot (Denkova et al., 2017). A total of 56 isolates were isolated from various dietary sources, and eight (SK1, SK2, SWR1, SWR2, MV1, MV3, HM1, HM5) showed some signs of antagonism against the selected Laboratory strains. Most of the isolates showed signs of either a competitive advantage or inhibition by antibacterial metabolites (cell-free supernatant) against one or more of the lab strains. Some, however, had both competitive advantage and inhibitory action against the selected lab strains. Therefore, the eight isolates were attempted to be identified based on morphology, physiology, and biochemical characterization.

A probiotic must survive in the highly acidic pH of the stomach and reproduce in the intestine withstanding the effects of bile salts (Denkova et al., 2017). The capacity to survive in the digestive tract of the selected isolates was tested under artificially mimicked conditions using nutrient media modified with bile salts and acid (Succi et al., 2005). This resulted in a specific tolerance of 8 isolates to acid at three different pH levels, with three isolates surviving at pH3, 70% survival at pH4, and 100% survival at pH5. Out of 8 isolates, 7, 5, and 2 exhibit survival at 0.5% , 1%, and 1.5% bile salt concentrations respectively. With the increase of concentration of bile salt the survival rate of the isolates decreased. However, the strains HM1 and MV3 survived well even with a higher concentration of bile salt (1.5%) than any other strain.

A potentially probiotic bacteria should not have any pathogenic, toxigenic, or invasive traits (Denkova et al., 2017). In this experiment, the DNase test and Hemolysis test were used to initially verify if the isolates possessed any potential for Pathogenicity. Firstly, none of the isolates or LSs produced DNase enzymes which means they are not capable of breaking down DNA and showing no pathogenicity. On the other hand, when cultivated in blood agar, two of the eight examined isolates showed α-hemolytic activity, four showed β -hemolytic activity, and the rest showed γ or no hemolytic activity. Lack of hemolytic activity is significant during probiotic strain selection since such strains are non-virulent and lack of hemolysin ensures that virulence will not arise among the bacterial strains (Mangia et al., 2019). Therefore, the hemolytic bacteria may not be considered promising as probiotics.

Since some of the isolates may have the potential for pathogenicity, their susceptibility to antibiotics becomes a significant factor. In case the isolates show pathogenicity, the antibiotics, Imipenem, Ciprofloxacin, and Chloramphenicol can be further studied with different doses against these bacteria. However, the data generated in this study is not enough to assign susceptibility or resistance yet.

Only two of the isolates, SwR2 and MV3 are worth exploring further as they are non-hemolytic, do not produce DNase, are moderately tolerant of bile salts and very tolerant of acidic environment. This means, besides antagonizing against potentially harmful gastrointestinal bacteria, they have the potential to survive in the harsh conditions of the GI tract and do not yet display any signs of pathogenicity.

From ABIS online tool, the probable identity of SwR2 and MV3 were assumed to be Pediococcus and *Paenibacillus mendelii* respectively. Pediococcus is a type of LAB (Raccach, 2014) which is highly likely since the source of this isolate was yogurt. Whereas, *Paenibacillus* are found in soil, fresh and saltwater, food, plants, insect larvae, etc. (Sáez-Nieto, 2017). This again is not impossible as it was sourced from yogurt as well and could have been introduced from one of the mentioned sources during production. Because, according to a study by Skowron et al. (2022), during the production of fermented food, the conditions of storage or processing, quality of raw materials, water, etc. may result in the introduction of unexpected microorganisms in the food. But there is no surety of the identity of the found isolates until 16S ribosomal RNA sequencing is performed. For example, in a study on the antagonistic activity of a novel bacteria by Khusro, Preetam, and Panicker (2014), after isolation, screening, and morphological as well as biochemical testing, the isolated bacteria were identified at the molecular level. The chemical testing indicated the bacteria would be of the *Bacillus* spp. After using Taq DNA polymerase and primers 27F (5` AGAGTTTGATCCTGGCTCAG 3`) and 1492R (5`ACG GCT ACC TTG TTA CGA CTT 3`) for 16S rRNA sequencing, the bacteria was confirmed to be *Bacillus subtilis* with 99% similarity profile.

Conclusion

The findings of this experiment were mostly unexpected having most of the isolated bacteria as hemolytic. All in all, there were hardly any significant findings in terms of the isolation of probiotic bacteria. Most of the isolates being hemolytic may pose a threat to public health as they were indeed isolated from food items. However, two isolates from yogurt sources showed promising results and may be further explored to understand them at the molecular level as well as in more complex culture conditions. Further extension of the experiment with a modified methodology such as the addition of PCR with pathogenicity specific primers, antagonism testing against clinical pathogens, etc. may result in better discoveries.

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Appendix A

Media compositions:

Nutrient agar

Component	**Amount(g/l)**
Peptone	5.0
Sodium chloride	5.0
Beef extract	3.0
Agar	15.0
Final pH	7 at 25°C

Saline

Component	**Amount(g/l)**
Sodium chloride	9.0

Mannitol salt agar

Component	**Amount(g/l)**
Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	75.0
D-mannitol	10.0
Phenol red	0.025
Agar	15.0
pH	7.4 ± 0.2 at 25°C

Eosin methylene Blue (EMB) Agar

Component	**Amount(g/l)**
Peptone	10.0
Dipotassium phosphate	2.0
Lactose	5.0
Sucrose	5.0
Eosin yellow	0.14
Methylene blue	0.065
Agar	13.50
pH	7.1 ± 0.2 at 25°C

Nutrient Broth

Component	**Amount(g/l)**
Nutrient Broth	13.02

Cetrimide

Component	**Amount(g/l)**
Agar	15.0
pH	7.0 ± 0.2 **at 25°C**

Methyl Red - Voges Proskauer (MRVP) media

Component	**Amount(g/l)**
Peptone	7.0
Dextrose	5.0
Dipotassium hydrogen phosphate	5.0
pH	7.0 at 25°C

Triple Sugar Iron Agar

Component	**Amount(g/l)**
Bio-polytone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous ammonium sulfate	0.2
Phenol red	0.0125
Agar	13.0
pH	7.3 at 25°C

Nitrate Reduction Broth

Component	**Amount(g/l)**
Beef extract	3.0
Gelatin peptone	5.0
Potassium nitrate	1.0

T1N1

Component	**Amount(g/l)**
Tryptone	1.0
Sodium chloride	1.0
Agar	0.75

Blood Agar

Component	**Amount(g/l)**
Beef heart infusion from (beef extract)	500.0
Tryptose	10.0
Sodium chloride	5.0
Agar	15.0
pH	6.8 ± 0.2 at 25°C

Simmon's Citrate agar

Component	**Amount(g/l)**
Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bacto bromothymol blue	0.08

MRS Agar (oxoid)

Component	**Amount(g/l)**
Peptone	10.0
Lab-Lemco Powder	8.0
Yeast Extract	4.0
Glucose	20.0
Di-potassium hydrogen phosphate	2.0

Sodium acetate 3H2O	5.0
Tri-ammonium citrate	2.0
Magnesium sulfate 7H2O	0.2
Agar	10.0

Appendix B

Reagents and buffers:

Gram's iodine (300ml)
To 300 ml distilled water, 1 gram iodine and 2 gram potassium iodide was added. The solution was mixed on a magnetic stirrer overnight and transferred to a reagent bottle and stored at room temperature.

Crystal Violet (100ml)
To 29ml 95% ethyl alcohol, 2gm crystal violet was dissolved. To 80 ml distilled water, 0.8gm ammonium oxalate was dissolved. The two solutions were mixed to the stain and stored in a reagent bottle at room temperature.

Safranin (100ml)
To 10 ml 95% ethanol, 2.5gm safranin was dissolved. Distilled water was added to the solution to make a final volume of 100ml. The final solution was stored in a reagent bottle and stored at room temperature.

Kovac's reagent (150ml)
To 150ml (reagent grade) isoamyl alcohol, 10 gm of p-dimethylaminobenzaldehyde (DMAB) and 50 ml of HCL (concentrated) were added and mixed. Next, the prepared reagent was kept in an aluminum foiled reagent bottle to prevent light exposure and stored at 40 C.

Methyl Red (200ml)
To 1 gm of methyl red powder, 300ml of 95% ethanol was completely dissolved. Next, 200ml distilled water was added to make 500ml of 0.05 %(wt/vol) solution in 60 %(vol/vol) ethanol and stored at 40 C.

Barrit's Reagent A (100ml)
5% (wt/vol) α-naphthol was added to 100ml absolute ethanol and stored at 4 0 C.

Barrit's Reagent B (100ml)
40% (wt/vol) KOH was added to 100ml absolute ethanol and stored at 4 0 C.

Catalase reagent (20ml 3% hydrogen peroxide)
From a stock solution of 35% hydrogen peroxide, 583µl solution was added to 19.417ml distilled water and stored at 40 C.

Malachite green (100 ml)
To 20 ml distilled water, 5 g malachite green was dissolved in a beaker. The solution was transferred to a reagent bottle. The beaker was washed two times with 10 ml distilled water separately and a third time with 50 ml distilled water and the solution was transferred to the reagent bottle. The remaining malachite green in the beaker was washed a final time with 10 ml distilled water and added to the reagent bottle. The stain was stored at room temperature.

Oxidase Reagent (100 ml)
To 100 ml distilled water, 1% tetra-methyl-p-phenylenediamine dihydrochloride was added and stored in a reagent bottle covered with aluminum foil at 4°C to prevent exposure to light.

Nitrate Reagent A (100 ml)
5N acetic acid was prepared by adding 287 ml of glacial acetic acid (17.4N) to 713 ml of deionized water. In a reagent bottle, 0.6 g of N,N-Dimethyl-α-naphthylamine was added along with 100 ml of acetic acid (5N) and mixed until the color of the solution turned light yellow. The reagent was stored at 4°C.

Nitrate Reagent B (100 ml)
In a reagent bottle, 0.8 g of sulfanilic acid was added along with 100 ml acetic acid (5N)to form a colorless solution and stored at 4°C.

Ethyl Alcohol (95%)
95 ml of ethyl alcohol (100%) was added to 5 ml of distilled water. This solution was stored at room temperature.

Appendix C

Instruments:

Autoclave Model: WIS 20R Daihan Scientific Co.ltd, Korea

Laminar airflow cabinet: Model-SLF-V, vertical, SAARC group Bangladesh

Incubator: Model-OSI-500D, Digi system Laboratory Instruments Inc. Taiwan

Vortex mixer: Digi system Taiwan, VM- 2000

Electronic balance: Model: WTB 200 RADWAG Wagi Electronics

Refrigerator (4°C): Model: 0636 Samsung

Sterilizer: Labtech, Singapore

Shaking Incubator: Model: WIS-20R Daihan Scientific Companies, Korea

Water Bath: Daihan Scientific Companies, Korea

Table Top Centrifuge: Digisystem, Taiwan

Microscope: A. Krüssoptronic, Germany

-20°C Freezer: Siemens, Germany

Magnetic Stirrer: Model: JSHS-180 JSR, Korea

pH Meter: pHep Tester Hanna Instruments, Romania

Micropipette: Eppendorf, Germany

Disposable Micropipette tips: Eppendorf, Ireland

Microcentrifuge tubes: Tarsons Products, Pvt Ltd, Kolkata

Online Tool: ABIS online (https://www.tgw1916.net/bacteria_logare_desktop.html)