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DEPARTMENT OF BIOENGINEERING

ANTAGONISTIC EFFECTS OF PROBIOTIC MICROORGANISMS ISOLATED FROM DIFFERENT NATURAL SOURCES AGAINST SELECTED HUMAN PATHOGENS

MASTER'S THESIS

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DECLARATION

I declare that the rules of scientific ethics are followed in the writing of this thesis, which is prepared in accordance with the thesis writing rules, that in case of benefiting from the works of others, they are referred to in accordance with scientific norms, that the innovations and results contained in the thesis are not taken from anywhere else, that no falsification is made in the data used, and that any part of the thesis is not presented as another thesis work in this university or another university.

JOSEPH OLASUBOMI BISHI

August , 2023

PREFACE

First, I would like to express my deepest gratitude to my supervisor, Prof Dr. Isa Karaman, for his unwavering support and guidance throughout the process of completing this thesis. I thank him for providing some of the materials used in the duration of this study, and particularly his ideas and guidance being a more experienced scholar.

The motivation behind this research lies primarily in my personal interest to understand the regulation of diseases and the control of pathogenic microorganisms in humans via natural resources. I was intrigued by the potential capacity of nature to moderate diseases, circumventing the prolonged use of artificial and synthetic drugs which could eventually pose harm to the human body.

I would also like to express my heartfelt gratitude to my parents, who have stood behind me in all my decisions in life. They have made the greatest efforts for me to attain peak heights in life and I hope to pay them back for all that they have done in my life.

I would also like to appreciate my friends who cheered me up and gave me the push to reach the finish line when things got tough.

Last but not least, I would like to thank God almighty for keeping me and bringing me to this stage of my life, and may He continue to open doors for us all.

JOSEPH OLASUBOMI BISHI

July, **2023**

ABSTRACT

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In this research, we gathered samples from red and white cabbages and carrots from a secluded Tokat village. Remarkably, these vegetables were grown without the use of fertilizers. From these samples, we successfully isolated microorganisms.

We further identified pure colonies from those microorganisms, which potentially possess probiotic properties. The selected colonies underwent testing against pathogens harmful to humans to observe potential antagonism.

Finally, we analyzed the potentially probiotic microorganisms for any indicators of antibacterial and antifungal characteristics.

KEYWORDS: Probiotics, Carrots, Cabbages, Human Pathogens

ÖZET

FARKLI DOĞAL KAYNAKLARDAN İZOLE EDİLEN PROBİYOTİK MİKROORGANİZMALARIN SEÇİLMİŞ İNSAN PATOJENLERİNE ANTAGONİSTİK ETKİLERİ

Bishi, Joseph Olasubomi

TOKAT GAZİOSMANPAŞA ÜNİVERSİTESİ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

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Bu araştırmada tenha bir Tokat köyünden kırmızı ve beyaz lahana ve havuç örnekleri topladık. Dikkat çekici bir şekilde, bu sebzeler gübre kullanılmadan yetiştirildi. Bu örneklerden mikroorganizmaları başarıyla izole ettik.

Ayrıca potansiyel olarak probiyotik özelliklere sahip olan mikroorganizmalardan saf koloniler belirledik. Seçilen koloniler, potansiyel düşmanlığı gözlemlemek için insanlara zararlı patojenlere karşı test edildi.

Son olarak, potansiyel olarak probiyotik mikroorganizmaları antibakteriyel ve antifungal özelliklerin herhangi bir göstergesi için analiz ettik.

ANAHTAR KELİMELER: Probiyotikler, Havuç, Lahana, İnsan Patojenleri

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
MAMP-PRR	Microbe-associated molecular pattern-pattern recognition receptors
FAO	Food and Agriculture Organization
WHO	World Health Organiztaion
LAB	Lactic Acid Bacteria
AMR	Antimicrobial Resistance
GRAS	Generally Regarded as Safe
UN	United Nations
ROS	Reactive Oxygen Species
VOC	Volatile Organic Compound
DNA	Deoxyribonucleic Acid
℃	Degree Centigrade
mL	Millilitre
NA	Nutrient Agar
$CaCO_2$	Calcium Carbonate

AHL	N-acyl homoserine lactone
kDa	Kilo Daltons
Mw	Molecular weight
SCFA	Short Chain Fatty Acids
EPS	Exo Polysaccharides
L	Litre
LB	Luria Bertani Broth
PDA	Potato Dextrose Agar
%	Percentage
g	grams
cm	Centimetre

CHAPTER ONE

INTRODUCTION

Over the years, the relationship between foods and the health benefits arising from it has continued to be examined. Probiotic microorganisms have attracted widespread recognition due to the growing commercial interest. Studies have characterized specific probiotic organisms and some of their health benefits (Prabhurajeshwar and Chandrakanth, 2018). Generally, probiotics are used in the production of fermented food and are considered safe. Also, they have numerous applications in medical and veterinary activities. In the food industry, they are used as starter cultures. The main sources of probiotics are cheese, yogurt, and fermented milk products (Prabhurajeshwar and Chandrakanth, 2018). The genera *Lactobacillus* and *Bifidobacterium* are commonly studied and widely used probiotic bacteria (Edalati et al., 2018). Lactic acid bacteria (LAB) can be found in a variety of environments, such as fermented foods, breast milk, oral tracts, GI tracts of humans and animals, human vagina, and dairy and dairy-based goods (Zakaria et al., 2021). Because of their ability to manage infections, have immunomodulatory effects, and have anti-inflammatory properties, lactic acid bacteria are generally recognized as safe (GRAS) and have been the subject of intensive research to create probiotic microorganisms for human and animal health (Jung et al., 2021).

An effective immune system, the absorption of nutrients, and defense against infectious diseases are all influenced by healthy gut flora (Ubeda et al., 2017). Probiotics, bacteriophages, and antimicrobial peptides (AMPs) are some of the antibiotic substitutes that have been the focus of research (Mulani et al., 2019; Rasmussen et al., 2020). One of the key solutions to restoring the gut microbiota is the consumption of probiotics (Jung et al., 2021). Probiotics are live microorganisms that provide health benefits when ingested in adequate amounts (FAO/WHO, 2001; Silva et al., 2020). Probiotic organisms can establish themselves in the intestinal tract due to their ability to withstand the acidic conditions of gastric juices and bactericidal properties of bile salts, and the production of lactic acid that inhibits the growth of other microorganisms (Diguta et al., 2020). Probiotic organisms produce a variety of antimicrobial metabolites such as organic acids, acetoin, hydrogen peroxide, diacetyl, and bacteriocins. These compounds control the growth

of other microorganisms and inhibit pathogenic bacteria (Prabhurajeshwar and Chandrakanth, 2018; Petrova et al., 2022).

Microorganisms cause different types of disease in humans (Silva et al., 2020). Some of the reported pathogens are *Staphylococcus aureeus*, *Escherichia coli*, *Campylobacter spp*., *Klebsiella*, Shigella, and Salmonella. They induce human ailments such inflammation, diarrhea, fever, cramping in the abdomen, and vomiting (Jung et al., 2021). The Council for Agricultural Science and Technology, reported an estimate of about 6.5 - 33 million cases of human illness caused by microbial pathogens in food and up to 9000 deaths annually (Prabhurajeshwar and Chandrakanth, 2018). Intestinal pathogenic bacteria are cause food poisoning and diarrhoea, particularly in developing nations (Edalati et al., 2018).

The resistance of pathogens to antibiotics is due to some intrinsic mechanisms such as the production of antibiotics modifying enzymes to degrade antibiotics, efflux pumps (altering the outer membrane permeability), and modification of the active sites for the antibiotics. Also, bacteria survive in the presence of antibiotics by acquiring antibiotic resistant genes via horizontal gene transfer mechanisms or mutations that evolved as part of natural selection (Nataraj and Mallappa, 2021). Due to the widespread of antibiotic-resistant pathogens as a result antibiotics misuse, these properties are desired in a potential probiotic strain, studies have focused on the use of probiotic antimicrobial metabolites alone and in combination with antibiotics for the treatment and prevention of microbial infections (Zakaria et al., 2021). Probiotics are also viewed as an alternative to antibiotics due to the growth in bacteria that are multi-drug resistant. This has prompted increased study into their therapeutic and preventative applications (Ahmadnejad and Dolatabadi, 2020).

A probiotic strain should possess specific desirable properties such as bile tolerance and gastric acidity, adhesion to epithelial and mucosal surfaces, antimicrobial activity against various pathogens, among others (Sornsenee et al., 2021). Most probiotic bacteria are Gram-positive, and they help to regulate and maintain the health of the intestinal system (Marco et al., 2006). Evidence suggest that probiotics help in the treatment and prevention of infectious diseases. Antibiotics are currently utilized to treat infectious infections, however their indiscriminate use has negative effects on patients' health as well as the health of the general public (Yang et al., 2019). This makes finding new methods and alternatives for antimicrobial therapy a top concern, especially for natural products (Silva et al., 2020). Also, in spite of the proven bioactive potentials of probiotics,

it still needs further elucidation. Thus, this study focuses on the antagonistic effects of probiotic organisms against human pathogens.

1.1 Statement of Problem

Approximately 200 diseases are brought on by eating contaminated food. Globally, close to 600 million people get sick from consuming contaminated foods, consequently leading to 420,000 deaths annually (Petrova et al., 2022). Also, the alarming and persistent increase in antimicrobial resistance (AMR) by pathogens pose a great challenge to public health. Antibiotics are readily available at low cost and also there are few laws governing the use of antibiotic. Globally, the emergence of AMR is a direct consequence of the irrational use of antibiotics in human medications, agriculture etc (Nataraj and Mallappa, 2021). In the past few decades, AMR has been a global menace facing modern medicine.

1.2 Justification

Antimicrobial resistance (AMR) is a great public health concern because the therapeutic effects of antimicrobials on pathogens are becoming less effective. The magnitude of the challenge posed by AMR led to the exploration for alternatives therapies, such as consumption of probiotics. For many years, research focused on understanding pathogens and finding ways to prevent and treat human diseases. However, the symbiotic relationship of probiotics microorganisms may mitigate the challenges posed by AMR. Hence, the need for this study.

1.3 Aims and Objectives of this research work

This research was carried out to investigate the potential antagonistic effects of probiotics gotten from carrots and cabbages (that fertilizers had not been used on in any way) on pathogenic microorganisms, and hopefully propose new modes of treatments of infections caused by these pathogens.

CHAPTER TWO

2.0 Literature review

2.1 Probiotics

Probiotic is derived from the combination of Greek words "pro" and "bios" which means "for life." It is the opposite of “antibiotics” which means “against life” (Khaneghah et al., 2019; Nataraj and Mallappa, 2021). Beneficial bacteria have long been ingested routinely as fermented foods. These products not only confer nutritional but also therapeutic benefits, however there was no scientific evidence (Basavaprabhu et al., 2020). Eli Metchnikoff, a Russian Nobel winner, proposed probiotics and the idea that it was feasible to use host-friendly bacteria to alter the intestinal microbiome for better health (Mackowiak, 2013). Lilly and Stillwell introduced and describe it as the consumption of a living microorganism with a positive effect on the resident microflora (Gibson, 2004). Fujiya and Kohgo in 2010, added other positive effects on human health to the definition of probiotics. Probiotics maintain a balance between pathogens and beneficial bacteria thereby preventing GIT infections and disorders (Petrova et al., 2022). Different factors such as age, the kind of food we eat, diseases, drugs used, among others directly alters the physical and/or chemical composition of the gut microbiota and this is commonly known as “dysbiosis”. Thus, preserving the balance between the microbiota is a must in order to maintain the wellness of the intestine. Evidence suggest that the gut microbiota is crucial for the well ness of the gut and changes to the composition of the microbiota leads to disorders inside in the guy and intestine (Wan et al. 2018). A lot of microorganisms show probiotic capabilities, and some are lactic acid bacteria (LAB) (Khaneghah and Fakhri, 2019). The most important genera of LAB are *Lactobacillus, Weisella, Carnobacterium, Lactococcus, Bifidobacterium, Enterococcus, , Streptococcus, Pediococcus, Leuconostoc, Weisella*, *Carnobacterium* and *Tetragenococcus,* Also, the leading probiotic species are *Bifidobacterium. animalis, L. salivarius, Lactobacillus acidophilus, L. casei, L. lactis, L. johnsoniin, L. bulgaricus, L. plantarum* and *L. reuteri* (Yadav and Srikanth 2018), even though probiotic properties are strain specific. The next generation probiotics include bacteria strains like *Escherichia coli* Nissle 1917, *Bacillus* spp., *Wisella* spp.,

Enterococcus faecium SF68, *Bacteroides ovatus* D6, *Clostridium butyricum* MIYAIRI 588, among others (O'Toole et al., 2017).

Probiotics are living bacteria or yeast that colonize the host and offer health advantages, thereby showing symbiotic properties. several characteristics allow them withstand adverse conditions (enzymatic properties and acidity) in the host organism. They multiply within the host, managing the microbiome and carrying out biological processes to promote the host's health (Silva et al., 2020). The UN Food and Agriculture Organization and World Health Organization released the "Guidelines for the Evaluation of Probiotics in Food" in 2002 (FAO/WHO, 2002). The several factors that should be considered when choosing probiotics were given in these guidelines. It contains the antibacterial activity, capacity to adhere to epithelial tissues, resilience to unfavorable bodily conditions, and usability. Pharmaceutical probiotics contain *L. amylovorus, Lactiplantibacillus plantarum, Lactobacillus johnsonii, Lacticaseibacillus paracasei, Lactiplantibacillus rhamnosus, and Lactiplantibacillus pentosus*, whereas those frequently used in the production of functional foods are *L. acidophilus, Limosilactobacillus, Lactobacillus gasseri* (Markowiak and Slizÿewska, 2017).

2.2 Probiotic Bacteria

Both dairy-based and non-dairy-based sources can be used to obtain probiotic microorganisms. Milk and milk products are sources that are dairy-based, whereas cereals, vegetables, and fruits are sources that are non-dairy-based. Dairy-based sources have been investigated for their potential health benefits, however persons with excessive cholesterol, lactose intolerance, or allergies to milk proteins should avoid them (Shori, 2016). In the non-dairy sources, the nutritional content such as minerals, vitamins, antioxidants and other properties of fruits, cereals, and vegetables promotes the survival of probiotic strains (Homayouni et al., 2012). Food sources containing fermentable sugars can potentially promote the growth of probiotics. Novel probiotic LABs have been isolated from fermented foods (Angellin and Kavitha, 2020).

Lactobacillus is a genus with Gram-positive, micoaerophilic, non-spore forming, catalase-negative, rod-shaped bacteria and comprise about 183 known species commonly applied in different industrial food processes (Edalati et al., 2018). The mechanisms of action of probiotics include production of antimicrobial substances, increased adhesion to the intestinal mucosa, increased epithelial barrier, competitive exclusion of pathogenic microorganisms, and immune system modulation (Silva et al., 2020). LAB are capable of the production of lactic acid from a lot

of carbon sources, and also, produce secondary metabolites (such as exopolysaccharides, bacteriocins) that stop the growth of most other microorganisms. These factors contribute to the antimicrobial activity of **LAB** as probiotics (de Melo Pereira et al., 2018). The need for new strains of LAB that convey probiotic qualities and that have an impact on human well-being and health continue to increase. LAB can be acquired from different natural ecologic niches that have remained unexploited. Also, there are many research focused on LAB because of their role in most fermented food and their capacity to produce different antimicrobial compounds that advances probiotic properties (Khedid et al., 2009). The protease-sensitive bacteriocins produced by LAB appeared to have inhibitory activities, mostly towards Gram-positive pathogens and closely related bacteria (Edalati et al., 2018).

A significant part of human intestinal microflora is the *Lactobacilli* and their role in human health has continued to be investigated (Prabhurajeshwar and Chandrakanth, 2018). The genus *Lactobacillus* is used in food fermentation and is of great economic importance.

In recent years, the taxonomy had changed. Studies have been conducted to identify and classify LAB including conventional biochemical tests, such as carbohydrate fermentation kits, physiological tests (Prabhurajeshwar and Chandrakanth, 2018) as well as molecular-biology methods. Generally, *Lactobacilli* are regarded as nonpathogenic members of urogenital and intestinal flora because they have not been associated with any disease. Due to their antagonistic interactions with pathogens, LAB maintain the gastrointestinal ecosystem in a healthy state. Regulatory processes are carried out by LAB that produce lactic and other organic acids, bacteriocins, and hydrogen peroxide (H_2O_2). Bacteriocins are biologically active, low-molecular weight peptides capable of inhibit the growth of various pathogenic bacteria (Prabhurajeshwar and Chandrakanth, 2018).

The probiotic Bifidobacterium can be used to prevent and treat intestinal flora disorders. Bifidobacterium is a member of the human gut microbiome. Some species of Bifidobacteria are used as probiotics in medicine, food, and feed (Li et al., 2022). During metabolism, Bifidobacteria may produce lactic acid and/or acetic acid. The lactic acid reduces intestinal pH, thus, inhibiting the proliferation of pathogenic microorganisms (Fukuda et al., 2011). *Bifidobacterium longum* BB536 isolated from the feces of healthy infants was regarded as safe and has been used commercially in various food applications (Li et al., 2022).

2.3 Probiotic Yeast

Compared to bacterial probiotics, only a few research has focused on the use of yeasts as probiotics (Shruthi et al., 2022). The most common yeast probiotics is *Saccharomyces cerevesiae var boulardii*. Yeasts make minerals available through the hydrolysis of phytate and detoxify fungi toxins. The rapid growth rate, limited space for cultivation, and diverse biochemical properties made microbes-based systems essential in the production of various chemicals (Afolabi et al., 2018). Probiotic yeast with the potentials to produce important compounds are used in industrial applications. Phagocytic cells use high concentrations of ROS (reactive oxygen species) like hydrogen peroxide during phagocytosis to attack invading pathogens. The ROS generated can cause mutations in DNA and damage other biomolecules such as proteins and lipids. In this context, superoxide dismutase and catalase convert ROS into less detrimental oxygen species in the host (Angulo et al., 2019). Volatile organic compounds (VOCs) are metabolites with a low molecular weight, low polarity, and high vapor pressure. They include molecules like thioesters, alcohols, thioalcohols, aldehydes, phenols, among others (Morath et al., 2012). Some yeast genera produce mycocin, regarded as killer toxins. They include *Saccharomyces, Cryptococcus, Candida, Kluyveromyces, Debaryomyces,* among others. The killer toxins block the DNA synthesis, interrupt cell division, and cell wall synthesis (Shruthi et al., 2022).

The antibacterial activity of probiotic bacteria has been well documented many authors (Diguță et al., 2020; Ahmadnejad and Dolatabadi, 2021); however, probiotic yeast exerts its antibacterial activity through indirect mode of action such as co aggregation, auto aggregation, adhesion ability, and immunomodulation (Shruthi et al., 2022). The β-Glucans in the cell wall of some yeast either in particulate or soluble forms stimulates innate immune cells such as macrophages, natural killer (NK) cells, among others, on antibacterial activity and the production of cytokines (Jang et al., 2009). The antimicrobial property of probiotic organisms is essential because they inhibit the growth pathogens. Hence, they can be used in treating gastro-intestinal tract infections. The probiotic yeast produces an array of molecules such as organic acids, proteases, polyamines, and mycocins which acts as antimicrobial compounds that kill pathogenic bacteria (Simoes et al., 2021). In a study by Czerucka and Rampal, (2019), it was observed that *Saccharomyces boulardii* CNCMI-745 exhibited significant antimicrobic effect against pathogens like *E. coli, Salmonella, Vibrio cholerae, C. difficle,* among others, using both cellular and animal models. In another study by Hasan et al. (2019), antimicrobial peptides produced by *Saccharomyces cerevesiae* inhibits the

survival of *S. aureus E. coli, B. subtilis, K. aeruginosa* when compared to the control. Furthermore, Lara-Hidalgo et al. (2019) reported that a 7.5-fold supernatant of *Hanseniaspora opuntiae* IPNFG2 isolated from *Guajillo pepper* exerted antimicrobial activity on *S. typhimurium* and *Candida albicans*. Also, they observed that there was a reduction in the adhesion of selected pathogenic bacteria (*E. coli, L. monocytogens,* and *S. aureus*) to $CaCO_2$ cells. Similar observation was recorded for the commercial probiotic, *Saccharomyces boulardii*. In sum, their experiment demonstrated that the incubation of yeast and pathogenic bacteria together reduces the adhesion capacity of pathogens to Caco-2 cells.

Probiotic yeast and their metabolites could be used as therapeutic agents in treating gastrointestinal and urinary tract diseases (Shruthi et al., 2022). Yeasts isolated from fruits are valuable in food and feed industry because they have demonstrated antifungal activity against molds (Shruthi et al., 2022). Furthermore, Yeasts and *Lactobacillus* species are regarded as safe biocontrols against mycotoxins are in demand in the food industry (Shruthi et al., 2022).

Table 1.1: Most studied probiotic microrganisms with antimicrobial activity (Silva et al., 2020)

Lactobacillus	*Bifidobacterium*	Other bacteria
L. amylovorus	*B. lactis*	*Propionibacterium freudenreichii*
L. casei	*B. bifidum*	*Saccharomyces bourlardii*
L. acidophilus	*B. infantis*	*Enterococcus faecalis*
L. plantarum	*B. longum R0175*	*Streptococcus thermophilus*
L. crispatus	*B. animalis subsp. lactis*	*Sporolactobacillus inulinus*
L. casei Shirota	*B. adolescentis*	*Pediococcus acidilacticiei*
L. johnssonii	*B. breve*	*Saccharomyces cerevisiae var.*

	Boulardi
L. fermentum	*Leuconstoc mesenteroides*
L. reuteri	*Enterococcus faecium*
L. rhamnosus	*Lactococcus lactis*
L. paracasei	
L. helveticus R0052	

2.4 Antimicrobial activity of probiotics against human pathogens

According to the United Nations Food and Agriculture Organization and the World Health Organization, probiotics are living microorganisms that confer a benefit to the health of the host when administered in adequate amounts (FAO/WHO, 2019).

Most of the probiotic microorganisms used by humans are *Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei, L. acidophilus, L. casei, L. reuteri, L. paracasei, L. johnsonii, L. rhamnosus Bifidobacterium bifidum, B. lactis, B. infantis, Propionibacterium freudenreichii and Saccharomyces bourlardii* (Markowiak and Slizewska, 2017; Silva et al., 2020). Several studies have revealed the different benefits of consuming probiotics, it ranged from improving the host immune system functions to directly inhibiting the pathogenic microorganisms (Markowiak and Slizewska, 2017; Moraes et al., 2019).

The aim of consuming probiotic microorganisms is to support the health of the host. The various mechanisms of action of probiotics include the production of inhibitory substances, such as hydrogen peroxide and bacteriocins; competition for nutrients; blockage of adhesion sites; biosynthesis of biosurfactants and exopolysaccharides to overcome biofilms; inhibition of quorum sensing by MDR pathogens (Markowiak and Slizewska, 2017; Moraes et al., 2019; Satpute et al., 2019). They modulate immune response by increasing nonspecific phagocytic activity through the activation of macrophage and **inducing** the release of pro and anti-inflammatory cytokines (Silva et al., 2020). Other benefits of probiotics are antimutagenic antidiarrheal properties, anticarcinogenic and reduced blood cholesterol, among others (Liu et al., 2017; Nath et al., 2018; Devaraj et al., 2019). Thus, probiotics usage is a promising strategy for the preventing and controlling infectious diseases (Silva et al., 2020).

In a study by Lahtinen et al. (2007), the antibacterial activity of 38 *Bifidobacterium* strains against *Staphylococcus aureus* commonly found in systemic and peri-implant infections, was tested. They observed that only three of the 38 *Bifidobacterium* strains inhibited the growth of the *S. aureus*. In another study by Lee et al. (2019), the antibacterial activity of probiotic *Pediococcus acidilactici* HW01 against *Pseudomonas aeruginosa* (*P. aeruginosa*) was examined. They observed the decrease in *P. aeruginosa* motility and decrease in production of pyocyanin, and decreased biofilm formation on the surface of stainless steel, among other observations.

Furthermore, probiotics are being used in combination with antibiotics to eradicate *Helicobacter pylori*. They are useful in treating several intestinal diseases such as diarrhea. The merits of including probiotic *H. pylori* therapy are decreased microbial load and improved host tolerability. Studies have revealed the favorable effects of using probiotics against *H. pylori*, it strengthens the mucosal barrier and promotes competition for adhesion and immunomodulation (Silva et al., 2020). An opportunistic pathogen that colonizes the human gut, and is multi-drug resistant with a high biofilm forming ability is *Klebsiella pneumoniae*. Lagrafeuille et al. (2018), reported that of the 140 species of *Lactobacillus* (supernatant cultures) examined, the supernatant of 13 strains showed anti-biofilm activity against *K. pneumoniae*. Also, they observed that *L. plantarum* CIRM653 disrupted *K. pneumoniae* preformed biofilms. In an *in vivo* study by Vieira et al. (2016), revealed that *Bifidobacterium longum* 5 reduced *K. pneumoniae* infection in mice and protected it from *K. pnemoniae* lung infection, by inducing the secretion of pro-inflammatory cytokines.

2.5 Mechanism of action of Probiotics

2.5.1 Adhesion and Antiadhesion by Probiotics

Bacterial can adhere to epithelial surfaces and this is criterion for selecting probiotic strains. The adherence of probiotic organisms to the host epithelial cells results in responses such as immunomodulation. This is done through microbe-associated molecular pattern-pattern recognition receptors (MAMP-PRR). Teichoic acid, lipoteichoic acid, mannose-binding protein, fibronectin protein, among other cell-wall associated surface components recognized as MAMP, which recognize the PRR on the gut (Pyclik et al., 2020). The process of adhesion to epithelial cells is complex and it is a function of physicochemical characteristics of the organisms (Rokana et al., 2017). The ability of probiotic microorganisms to adhere to epithelial cells is one of the proposed mechanisms of action used to protect the host from invasion and adherence of pathogens.

It is an efficient function of probiotics (Campaniello et al. 2018). For this reason, microorganisms are classified into three based on their adhesive characteristics, namely non-adhesive strains, adhesive strains, and highly adhesive strain (Candela et al. 2005). The *in-vitro* assays for the bacterial adhesion are used to ascertain their ability to colonize in the gut (Duary et al., 2011). The human epithelial cell models (HT-29 and Caco-2) are commonly used since these cell lines have similar structural and functional features with human enterocytes (Vemuri et al., 2018).

2.5.2 Interaction between probiotics and the immune system

Another benefit of consuming probiotics is the modulation and regulation of immune responses Probiotics modulate and stabilize the microbiota composition leading to the enhancement of the immune system and prevention of inflammatory responses. Probiotics modulate the immune system directly by inducing the secretion of cytokines and expression of co-stimulating molecules (Ren et al., 2019). The immune system of humans recognizes the intestinal microorganisms through the Toll-like-TLR receptors. Several biological interactions occur after probiotics adhere to the cell such as the release of cytokines and chemokines as associated proteins with systemic immunity (Khaneghah et al., 2019). A crucial immune modulation factor is regulating the production of immune-modulating substances like pro- and anti-inflammatory cytokines (Khaneghah et al., 2019). Also, probiotic bacteria can stimulate the production of dendritic cells and activate lymphocytes to produce antibodies (IgA) (Khaneghah et al., 2019).

2.5.3 Competition by exclusion

Pathogens have a favorite location such as intestinal villosities, colon cryptal, among others. The physical blocking and colonization of these sites by probiotic cells is referred to as competition by exclusion. The presence of probiotics creates a physical barrier which limit the proliferation of the pathogenic by colonization of the intestinal surface by probiotics, competition for nutrients and energy, and production of several organic acids and volatile fatty acids. Probiotics cells compete with pathogens such as *B. vulgatus, E. coli, Clostridium histolyticum, Clostridium difficile, Salmonella choleraesuis, Staphylococcus aureus* and *Listeria monocytogenes* for epithelial surfaces thereby preventing their intestinal colonization (Goldenberg et al., 2017; Khaneghah et al., 2019).

2.5.4 Coaggregation

Coaggregation is a process of cell-to-cell aggregation by the assistance of cell surface appendages or the electrostatic forces of attraction or repulsion (Datta et al., 2017). It is a phenomenon widely

observed in all microbial systems, however, the coaggregation ability of a probiotic organism is advantageous since their inter-bacterial flocculation hinders pathogens colonizing ability. Coaggregation by probiotics depends on a number of factors, which include cell surface hydrophobicity, co/aggregation-promoting factor, cell surface molecules, among others (Van Zyl et al., 2021).

2.5.5 Antimicrobial Activity

The antagonistic effect of probiotics on pathogens has promoted the use of probiotics as a preventive medicine to curb various diseases. LAB produce various antimicrobial compounds such as short-chain fatty acids and organic acids, ethanol, hydrogen peroxide, carbon dioxide and diacetyl, acetaldehyde, reuterin, bacteriocin, extracellular polymeric substances etc. (Satpute et al., 2016; Nataraj and Mallappa, 2021). These metabolites defend the host against pathogens invasion (Gaspar et al. 2018). The antibacterial activity of lactic acid (LA) has been well-established. Arena et al. (2018), reported that LA impairs the pH gradient between the acidic external environment and alkaline cytosol using its protonated form. Also, Birt et al. (2013) demonstrated that branched short-chain fatty acids (SCFA) such as isovalerate and isobutyrate has antimicrobial effects. Fazeli et al. (2004) reported the antimicrobial effect of acetate, formate, succinate, butyrate, among others. Carbonyl derivatives such as diacetyl, acetoin, acetaldehyde, and 2,3-butanediol, are antimicrobial compounds that act against toxigenic *E. coli*, *S. aureus*, and *Lis. monocytogenes*, (Lanciotti et al., 2003). The oxidative effect hydrogen peroxide damages cellular structures subsequently disrupting the membrane redox potential. Carbon dioxide inhibits enzymatic decarboxylation and increases the permeability of the membrane (Reid, 2008).

Bacteriocins are produced in the ribosome and stable to heat and acid. Bacteriocins are grouped into four according to its DNA sequence and amino acid composition; heat-stable, lanthionine-containing and molecular weight (Mw) smaller than 5 kDa; heat-stable, non-lanthionine-containing and Mw below 10 kDa; heat-sensitive proteins with Mw higher than 30 kDa; and bacteriolysins (Kumariya **et al., 2019**). The efficiency of pediocin-like bacteriocins (class II) against *Lis. monocytogenes* qualified them as an alternative to chemical preservatives (Kumariya **et al., 2019**). Bacteriocins with antitoxigenic activity are grouped in the third class: helveticin M and J, and enterolysin A. They are produced by *Lactobacillus helveticus, L. crispatus*, and *Ent. Faecalis*. The fourth class has leuconocin S and lactocin 27, comprising of complexes of protein, carbohydrates, lipids. Plantaricin A is an active antibacterial compound produced by many food-

derived LAB: *Furfurilactobacillus rossiae*, *Companibacillus paralimentarius*, *Levilactobacillus brevis*, *Leuc. mesenteroides*, *Leuc. citreum*; *Weissella paramesenteroides*, *Lactiplantibacillus paraplantarum*, among others. Other bacteriocins secreted by food LAB are bavaricin, sakacin, pentocin, lactocillin, and gassericin A (**Petrova et al., 2022**). Bacteriocins produced by different probiotics such as acidocin, enterolysin, enterocin, and lysostaphin have exhibited similar mode of action. However, in order to exert their antimicrobial activity, bacteriocins require a receptor to bind to. Nevertheless, newly-produced enterocin (AS-48) require no receptor on the targeted pathogens exhibit bactericidal activity (Khaneghah et al., 2019).

LAB metabolites with efficient antifungal activity are cyclic dipeptides often produced by sourdough lactobacilli species such as *L. harbinensis*, *L. amylovorus*, *Limosilactobacillus reuteri*, *Levilactobacillus spicheri*, among others (**Axel et al., 2017**). Different *Lp. plantarum* strains generate the fungistatic peptides (**Petrova et al., 2022**).

The extracellular polymeric substances (EPS) from probiotic lactic acid bacteria (LAB) have received immense attention because of their generally regarded as safe (GRAS) status and are utilized for various applications including *in-vitro* and *in-vivo* biological activities (Angellin and Kavitha, 2020). The EPS from probiotic bacteria have gained much attention in therapeutic applications such as antimicrobial, antiinflammatory, immunomodulatory, anti-tumor, antioxidant, among others (Hussain et al., 2017).

2.5.6 Antibiofilm Activity

The diverse biosurfactants produced by probiotics are responsible for the antibiofilm activity of probiotics. These biosurfactants reduces surface tension, prevents bacterial adhesion and dislodges preformed biofilms (73, 74; Nataraj and Mallappa, 2021). Also, the hydrophobic interaction between the biosurfactants and bacterial membrane lipid affects membrane integrity (75; Nataraj and Mallappa, 2021). The multiple antagonistic action of biosurfactants may inhibit antibiotic resistant organisms with a low probability of developing cross-resistance. Furthermore, the exopolysaccharides (EPS) produced by probiotic bacteria inhibit pathogen adhesion by the formation of non-pathogenic biofilms in the gut (Nataraj and Mallappa, 2021).

2.5.7 Interference in the Signaling System to express virulence

Bacteria can communicate with each other and their surrounding environment by signaling chemical molecules which regulates the virulence expression, this is regarded as quorum sensing (Kiymaci et al. 2018). Pathogens such as *Salmonella typhimurium* and *Escherichia coli* O157: H7

have pathogenicity islands and virulence plasmids. They have a type-III secretion system responsible for the transfer and injection of effector proteins into host cells to aid adherence and cellular invasion (Khaneghah et al., 2019). These effectors rearrange the cytoskeleton resulting in a disturbance of the plasma membrane. Enzymes produced by probiotics degrade these self-inducing molecules. Probiotics such as *Lactobacillus, Bifidobacterium* and *B. cereus* prevent the emergence of virulence in pathogenic bacteria (Khaneghah et al., 2019). The bioactive molecules produced by probiotic microorganisms have been reported to repress virulence genes in *E. coli* O157: H7 and *Salmonella* (Khaneghah et al., 2019). N-acyl homoserine lactone (AHL) is a quorum sensing signal molecule that regulates virulence factors in pathogens. The disruption of quorum sensing system been introduced as an essential anti-infective approach. This disruption eliminates or decreases virulence factor production in bacteria without inhibiting their growth (Papenfort and Bassler 2016). In a study by Piewngam and Otto, (2019), they demonstrated that *Bacillus* species probiotics produce a kind of lipopeptides (fengycins) that strongly inhibit the *S. aureus* **accessory gene regulator (*agr*)** quorum-sensing system.

2.6 Effect of Probiotic LAB on selected pathogens

Toxigenic *E. coli* strains, the most clinically significant pathogens in Europe, cause diarrhea, neonatal meningitis, urinary tract infections, enteritis, septicemia, among others (Allocati et al., 2013). The shiga toxin-producing *E. coli* (STEC) cause hemolytic uremic syndrome (HUS) or enteric disease. The enterohemorrhagic *E. coli* (EHEC) colonizes the large intestine causing attaching-and-effacing (AE) lesions. The Shiga toxins 1 and 2 (Stx1, Stx2) aids the adherence to epithelial cells (Petrova et al., 2022). Studies have shown the inhibitory activity of probiotic strains *L. acidophilus* YIT 0070 clinical isolates of *E. coli* O157:H7 and *Lc. casei* Shirota. Bacteriocidal activity on EHEC by both probiotic lactobacilli was observed during batch co-fermentation (Ogawa et al., 2001b). In another study, Ogawa et al. (2001a), investigated the protective ability of orally administered probiotic *Lc. casei* Shirota against EHEC infection using a newborn rabbit. They observed that daily consumption of the probiotic, from birth, prevented the GIT colonization and reduced the concentrations of the shiga toxins 1 and 2.

Listeria monocytogenes is an opportunistic pathogen found in soil, water, and plant material. It is an intracellular parasite and a non-spore-former (Maury et al., 2016). This pathogen causes a disease characterized by ailments of the central nervous system such as meningitis and listeriosis (Petrova et al., 2022). Immunocompromised individuals, newborns, pregnant women are

susceptible to listeriosis (Buchanan et al., 2004). Listeriolysin O (LLO) is responsible for this severe infection, Actin (ActA), the surface proteins internalins InlA and InlB, and transcriptional activator PrfA are present in conjuction to it. These genes are proof that there is a Listeria infection in food (Yap et al., 2021). LAB that produces bacteriocins with anti-listerial activity, are in the genera *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, *Leuconostoc*, and *Carnobacterium* (Sullivan et al., 2002).

C. botulinum, an obligate anaerobe and endospore forming bacteria, was first isolated from human liver. The most powerful natural toxin is botulinum neurotoxins (BoNTs) (Dhaked et al., 2010), They induce the lethal paralytic sickness known as botulism, which can harm both humans and animals.. Based on the difference in the amino acid sequence of botulinum neurotoxins, they are grouped into seven types (A–G) and many subtypes. Initially, BoNTs, which have a low level of toxicity, are formed and synthesized as single-chain polypeptides. Lund and Peck (Lund and Peck, 2013), reported that proteases cleave the single-chain protein, subsequently forming a double-chained highly toxic neurotoxin in proteolytic *C. botulinum* (A, B, and F neurotoxins of Group I). But in non-proteolytic *C. botulinum*, unidentified proteases in host cells activate a single chain pre-toxin. (B, E, and F type, Group II). The genes coding for aforementioned groups of neurotoxins are located either in the chromosomes or plasmid, whereas groups III (C and D) and IV (G) are always plasmid-localized (Petrova et al., 2022). There various antimicrobials produced by LAB are involved in defense against C. botulinum (Roces et al., 2012).

Fungal infection affecting humans globally. Candida is a life-threatening pathogen which accounts for about 80% of fungal infections. According to the most recent reports, C. albicans causes approximately 400,000 cases of blood ailments each year, with a mortality rate of 42% (Li et al., 2022). *C. albicans* is a part of healthy flora, it is found in the mouth, gut, and other parts of the body system. Pathogenic *C. albicans* is pleomorphic, however, it does not cause disease when growing as a unicellular yeast cell. When the body's immune function and defenses decline, *C. albicans* proliferates, grows mycelium, then invade cells to cause disease. It is one of the major cause morbidity and mortality in immunocompromised individuals (Vila et al., 2020). Additionally, the disease-causing capability of *C. albicans* is enhanced by biofilm synthesis (Nobile and Johnson, 2015). Therefore, effective strategy against *C. albicans* infection are the inhibition of hyphal development and biofilm formation (Li et al., 2022). Recent studies have

reported that *L. johnsonii* inhibited the growth of Candida. *L. johnsonii* MT4 gotten from the oral cavity and crevices of healthy mice affected the growth of *C. albicans* individually and in biofilms. The genome study of the strain revealed that it produced metabolites that exhibited antifungal effect on *C. albicans*; however, no active products with actions against fungi have been reported (Li et al., 2022).

2.7 Methods for determining the antimicrobial activity of probiotics against other microorganisms

The antimicrobial activity of a probiotic organism can best be determined using a direct antagonism between a probiotic culture and a pathogenic strain on solid media (Silva et al., 2020). It involves detecting growth inhibition of a pathogenic strain caused by the test culture. The various *in vitro* methods used to evaluate of the antimicrobial activity of probiotics include;

Direct and delayed antagonistic behavior are frequently used in the Agar Spot Test, as described by many authors (Choi & Chang, 2015; Tagg et al., 1976; Macaluso et al., 2016) and with various modifications. The release of a diffusible inhibitor at the start of the test organism growth is what causes antagonism in the direct assay, in which the test and indicator cultures are grown concurrently (Tagg et al., 1976). The probiotic bacterium is grown on agar media for a specific amount of time, then it is inactivated before an overlap of the indicator strain is added to the surface of the molten agar. This procedure is known as the postponed antagonism test. According to Tagg et al. (1976), this technique is thought to be sensitive and can test changes in the incubation times and conditions of the test and indicator cultures. Either the inhibition zone (mm) or the arbitrary units (AU/mL) are used to express the antibacterial activity.

The Agar Well Diffusion assay is used to determine the antagonistic effects of cell-free supernatants. After inoculation with the indicator microorganism, subsequently, the supernatant of the probiotic microorganism is diluted in aliquots at different concentrations and placed in the wells. After incubation, the antimicrobial activity is expressed as an inhibition zone or as arbitrary units (AU/mL) (Tagg et al., 1976).

CHAPTER THREE

3.0 Materials and Methods

3.1 Materials

The probiotic microorganisms used in this study were isolated from carrots and red and white cabbages gotten from different farms in a village in Tokat. Various media, chemicals, spectrophotometers, centrifuges, refrigerators, incubators and some other equipment were used.

3.1.1 Sources from which the microorganisms used in the study will be isolated

The carrots and cabbages were gotten from different farms in a village in Tokat district of Turkey. It was ascertained that there were no fertilizers used in the growing of the carrots and cabbages. The plants were harvested myself, and they were brought to the Microbiology Laboratory in the Bioengineering department of the Faculty of Engineering and Architecture of Tokat Gaziosmanpaşa University for insulation work under appropriate conditions.

3.1.2 Media used in this study

The following media were used in this study:

3.1.2.1 Nutrient Agar

Constituents	Amount (g/L)
Peptone	5.0
Yeast extract	1.5
Beef extract	1.5
Sodium chloride	5.0
Agar	15.0

To prepare 1 litre of Nutrient agar, 28g of nutrient agar is measured using a weighing balance and is then poured into a Conical/Erlenmeyer flask containing 1000ml of distilled water. This mixture is shaken and mixed properly, and a magnetic stirrer is used to stir it in the presence of heat till it is completely boiled and homogenized. After homogenization, the magnetic stirrer is taken out and this newly boiled solution is then autoclaved at 15lbs pressure (121 ^{o}C) for 15 minutes. After it has been autoclaved, the solution is brought out and allowed to cool till it gets to about 40-45℃. Then this media gets poured into sterile petri dishes and are left to solidify in the sterile cabin.

3.1.2.2 Luria-Bertani Agar (LB Agar)

To prepare 1 liter of LB agar, 25g of LB broth medium and 15g of agar are measured separately and poured into a Erlenmeyer flask containing 1000ml of distilled water. This mixture is shaken and mixed properly, and a magnetic stirrer is used to stir it in the presence of heat till it is completely boiled and homogenized. After homogenization, the magnetic stirrer is taken out and this newly boiled solution is then autoclaved at 15lbs pressure (121 ^{o}C) for 15 minutes. After it has

been autoclaved, the solution is brought out and allowed to cool till it gets to about 40-45℃. Then this media gets poured into sterile petri dishes and are left to solidify in the sterile cabin.

3.1.2.3 Nutrient Broth

Constituents	Amount (g/L)
Peptone	15.0
Yeast extract	3.0
Sodium chloride	6.0
D(+) Glucose	1.0

Following the manufacturer's directions, to prepare 1L of Nutrient broth, 15g of nutrient broth is measured and added to 1000ml of distilled water in an Erlenmeyer's flask. This solution is mixed thoroughly using a hotplate magnetic stirrer and allowed to homogenize. After homogenization, the solution is autoclaved at 121℃ for 15 minutes.

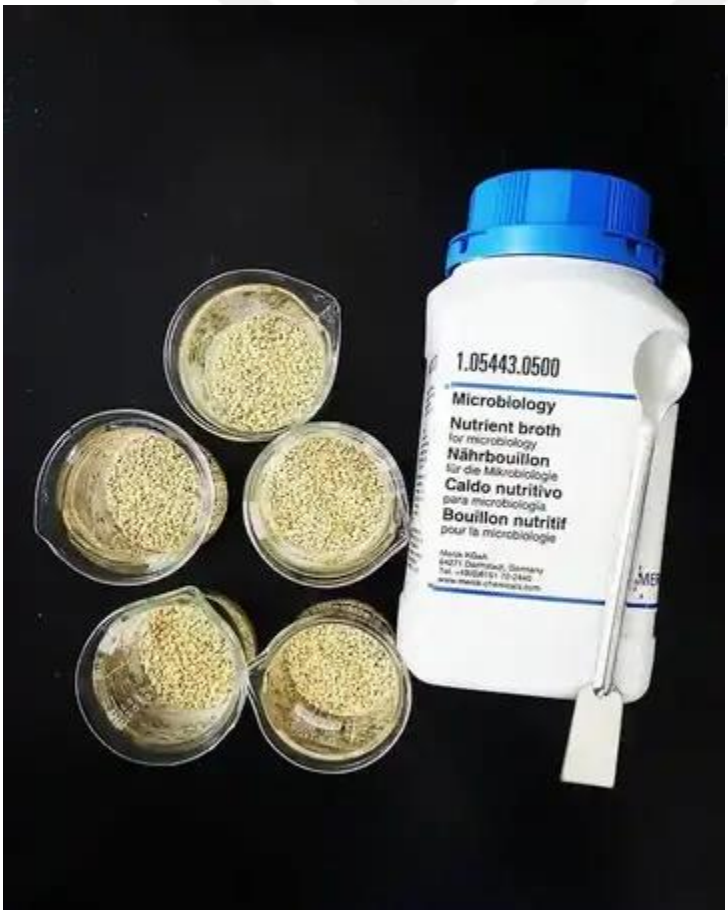

Fig 3.1.2.3 Nutrient broth

3.1.2.4 LB Broth

The constituents of LB Luria Broth are 1% Bacto-tryptone, 0.5% Bacto-yeast extract and 0.05% Sodium chloride. Following the manufacturer's directives, 25g of the LB broth powder is measured and poured into an Erlenmeyer's flask containing 1000ml of distilled water. This mixture is then put on a magnetic stirrer for it to be completely homogenized. After homogenization, the solution is autoclaved at 121℃ for 15 minutes. Luria-Bertani Broth is a fast and rich fattening medium which is normally used for the growth of many bacterial species.

Fig 3.1.2.4 LB Broth

3.1.2.5 Potato Dextrose Agar (PDA)

To prepare Potato Dextrose Agar, according to the manufacturer's directives, 39 grams was measured using the precision balance and poured into an Erlenmeyer's flask containing 1000ml of distilled water. This mixture is mixed thoroughly using a magnetic hot plate stirrer and allowed to

boil and completely homogenize. After homogenization, the solution is autoclaved at 121℃ for 15 minutes. It is allowed to cool till around 40-45℃ before it is poured into agar plates. PDA is a selective media that is used mainly for the cultivation of yeasts and fungi.

3.1.3 List of Fungi used in this study

- *Aspergillus niger*
- *Aspergillus flavus*
- *Didymella sp.*
- *Athraknoz*
- *Fusarium oxisporium*
- *Phoma sp.*
- *Kawun*
- *Alternata Alternaria*
- *Venturia inaequalis*

3.1.4 List of bacteria used in this study

- *Staphylococcus aureus*
- *Streptococcus mutans*
- *Salmonella enteritidis*
- *Pseudomonas aeruginosa*
- *Listeria monocytogenes*
- *Clostridium perfringens*
- *Escherichia coli*
- *Klebsiella pneumoniae*
- *Bacillus cereus*
- *Klebsiella aerogenes*

3.1.5 Devices and Consumables used

The devices used in this study include:

- Class 2 sterile cabin
- Autoclave (Hiroyama)
- Magnetic stirrer
- UV-VIS spectrophotometer (shimadzu uvmini-1240)
- Spectrophotometer curvettes
- Vortex machine
- Centrifuge
- Aerobic incubator (Memmert)
- Refrigerator
- Freezer

- Digital precision balance (presica)
- Plastic and glass petri dishes
- Glass tubes
- Erlenmeyer flasks
- Beaker
- Blender
- Scissors
- Acetate pen
- Aluminium foil
- Automatic pipette
- Pipette tips
- Knife

3.2 Methods

3.2.1 Collection of samples

The samples (a red cabbage, a white cabbage and 3 carrots) were gotten from a remote village in Tokat. It was ensured that there was absolutely no use of fertilizers on the land and they were completely natural. The samples were harvested personally and put into sample bags and brought back to the laboratory.

3.2.2 Purification of samples and isolation of microorganisms

The samples were taken to the sterile cabin so the isolation could be done under sterile and aseptic conditions.

3.2.2.1 Isolation of samples from the cabbages

The sample was cut till the third layer so as to avoid outside contamination and samples were gotten from inside the cabbages' leaf, the center of the cabbage, and when it was cut and blended using a sterilized blender. 10ml of sterile broth was put into 5 sterile test tubes and labelled 10^{-1} to 10^{-5}. Isolation was carried out by swabbing the insides of the leaf using a sterile swab and dipped inside the test tube labelled 10^{-1}. Serial dilution was then carried out till 10^{-4}. The same process was carried out for the center of the cabbage and serial dilution was also carried out. After this process, 0.1ml was inoculated on the pre-prepared Nutrient and PDA agar plates labelled "Lahana Gobegi 10^{-1} to 10^{-5}" for the samples gotten from the center of the cabbage, and "Lahana Yaprak Yuzeyi 10^{-1} to 10^{-5}" for the samples gotten from the cabbages' leaf surface.

After this was done, the cabbage was cut into small parts using a sterilized knife and blended using a blender and distilled water. 10ml of this blended mixture was then serially diluted up to 10^{-5}. 0.1ml was taken from each of the 5 test tubes and inoculated on the pre-prepared nutrient and PDA agar plates using Drigalski's spatula. The plates were labelled "Lahana Gobegi Blender".

All the plates were then incubated for 24-48 hours to allow for optimum growth. After incubation, the resulting colonies were visually inspected using the microscope and selected for further analysis.

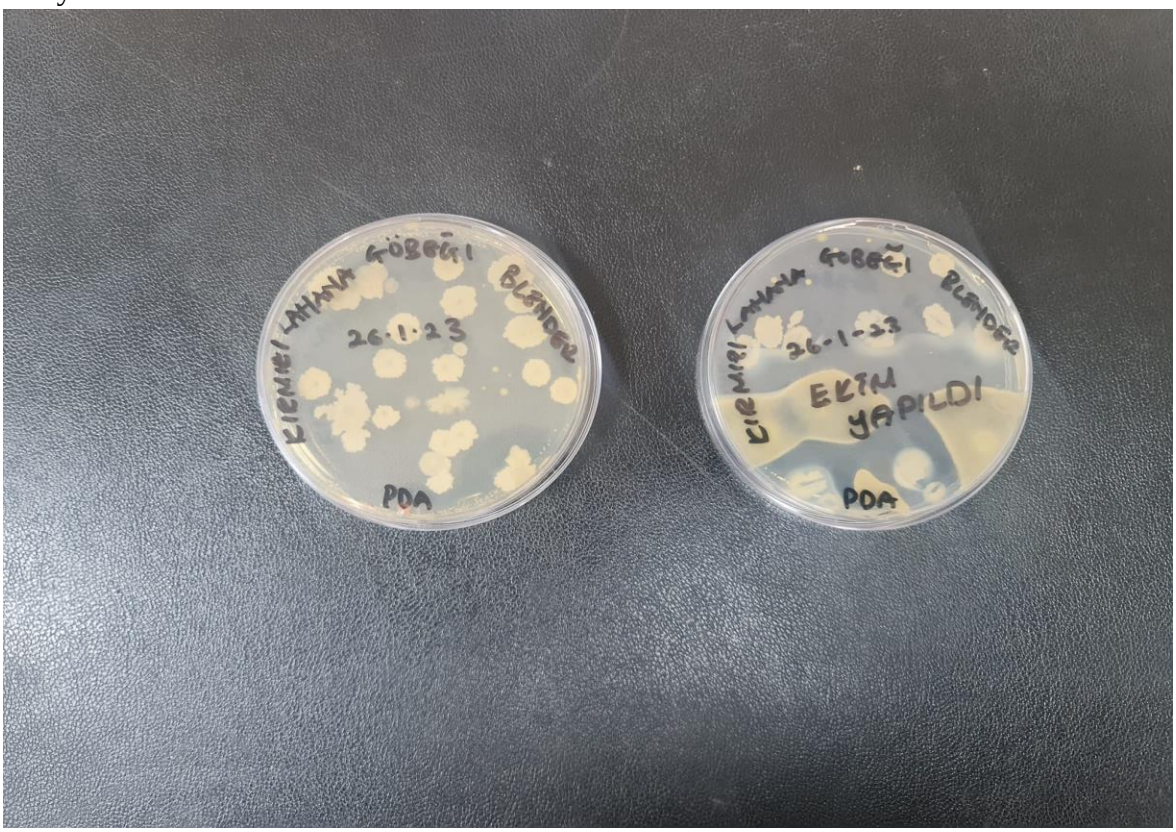

Fig 3.2.2.1 Red Cabbage Probiotic isolation

3.2.2.2 Isolation of samples from the carrots

The process of isolating potential probiotic microorganisms from carrots involved several steps. First, samples were collected from the surface of carrots using sterile swabs and dipped into sterile broth. This was then serially diluted up to 10^{-5} 0.1ml was pipetted and plated onto nutrient and PDA agar plates labelled "Havuc Yuzeyi". The carrots were then cut and blended as previously described with the cabbages above. 10ml was taken from this mixture and out in a test tube containing 10ml broth, and serial dilution was also carried out till 10^{-5}. 0.1ml was taken from each of the test tubes and inoculated onto agar plates using Drigalski's spatula. The agar plates were labelled "Havuc mix 10^{-1} to 10^{-5}".

The plates were then incubated at 37 degrees Celsius to promote the growth of potential probiotics. After incubation, the resulting colonies were visually inspected using the microscope and selected for further analysis.

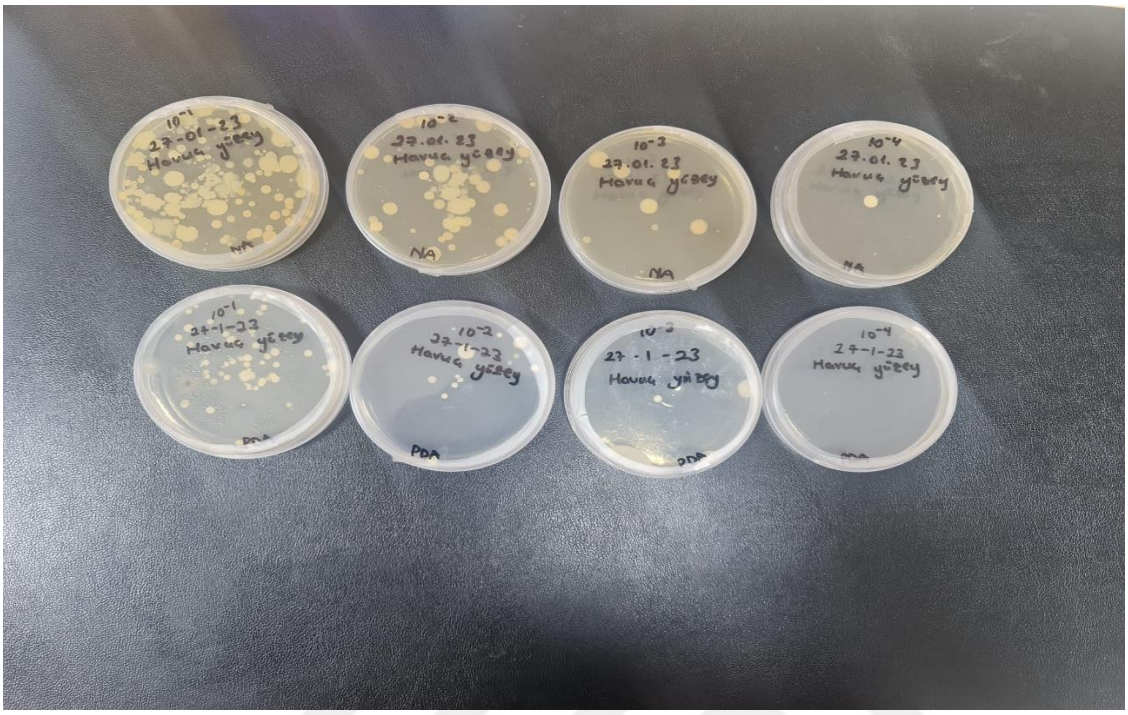

Fig 3.2.2.2a Selected Probiotic isolates from carrot's surface

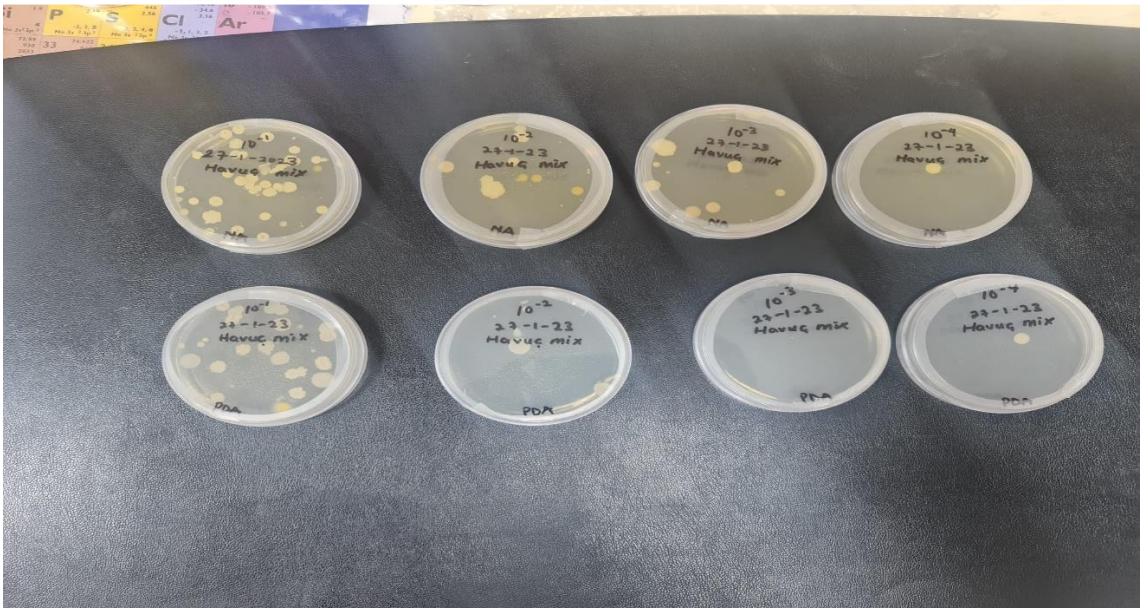

Fig 3.2.2.2b Selected Probiotic isolates from blended carrot

3.3 Antagonistic test

An antagonistic test is a laboratory procedure used to determine the ability of one bacterial strain to inhibit the growth of another bacterial strain. In the context of human health, antagonistic tests can be used to identify bacteria that have the potential to be used as probiotics, or to develop new antibiotics.

To perform an antagonistic test against human pathogenic bacteria, the following steps are typically taken:

1. Select the bacterial strains to be tested: One strain will be the "target" pathogenic bacteria, while the other will be the "antagonist" bacteria.
2. Culture the strains: Grow the target and antagonist bacterial strains separately in appropriate growth media.
3. Inoculate the plates: Use a sterile swab to spread the antagonist bacterial strain onto a petri dish containing the appropriate growth media. Allow the bacteria to grow for a few hours.
4. Create a "zone of inhibition": Place a small amount of the target bacteria onto the agar plate and observe the growth. If the antagonist bacteria are effective, there will be a clear zone around the area of the inoculation where the growth of the target bacteria has been inhibited.
5. Measure the zone of inhibition: Use a ruler or caliper to measure the diameter of the zone of inhibition.

By measuring the size of the zone of inhibition, researchers can determine the effectiveness of the antagonist bacteria in inhibiting the growth of the target pathogenic bacteria. This information can be used to develop new antibiotics or probiotics that may be effective in treating or preventing bacterial infections.

In this study, a total of 74 isolates was isolated from the three sources after careful and visual inspection and variations of antagonistic tests were carried out to determine their potential antimicrobial and antifungal properties. These isolates were labelled JB1 to JB74.

CHAPTER 4

4.1 Results

4.1.1 Antibacterial antagonistic tests

The diagram below gives a brief description of how the antagonistic test used in this study was carried out. The vertical line straight down the middle is the potential probiotic isolate, while the horizontal lines that are slightly touching the isolate are the bacteria that were tested against the potential probiotic isolates.

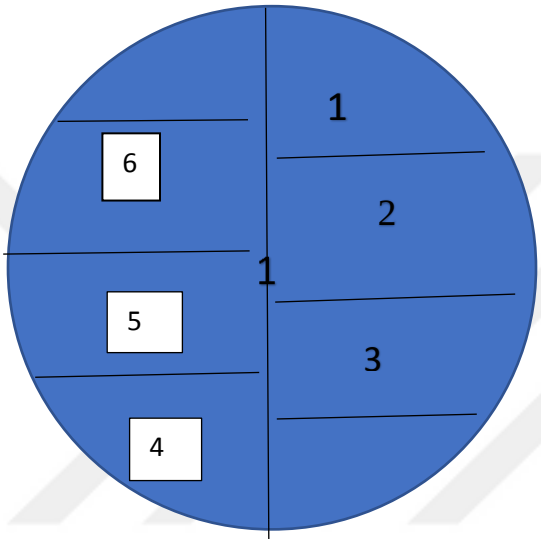

Fig 4.1.1 Antibacterial antagonistic test schemework

The bacteria used in this study were numbered serially from 1 to 10 for easy identification as follows;

1. *S. aureus* ATCC 6438
2. *S. mutans* ATCC 35668
3. *S. enteritidis*
4. *P. aeruginosa* ATCC 15442
5. *L. monocytogenes* ATCC 51774
6. *C. perfringes* ATCC
7. *E. coli* ATCC 35218
8. *K. pneumonia* ATCC 700603
9. *B. cereus* ATCC
10. *K. aerogenes* ATCC 13048

After 24 hours incubation there was little to no clear zone formation in almost all the isolates but after the end of 48 hours, there were some discoveries. The following isolates showed considerable antibacterial antagonism against only *K. pneumonia*:

1. JB13- There was a considerable a clear zone formation against *K. pneumonia* (Up to 10mm)
2. JB10- There was a considerable clear zone formation against *K. pneumonia* (Up to 6mm)

3. JB51- There was a considerable clear zone formation in *E. coli* and *K. pneumonia*. After 48 hours, the inhibition range with E. coli closed up but *with K. pneumonia*, it was observed that the clear zone was more visible up to a range of 18mm.

4. JB58- There was a considerable clear zone formation against *K. pneumonia* (Up to 10mm)

5. JB59- There was a considerable clear zone formation against *K. pneumonia* (Up to 14mm)

The only other pathogenic bacteria that was inhibited by any probiotic isolate was *E. coli* and it was inhibited by only JB50. It had a clear zone measurement of about 10mm.

4.1.2 Antifungal antagonistic tests.

In another variation of the antagonistic test, the PDA agar plates were inoculated with the fungi to be tested against the potential probiotic isolates. The following was the series by which the fungi was lettered for easy identification;

A. *Aspergillus niger*
B. *Didymella sp.*
C. *Anthraknoz*
D. *Fusarium oxisporium*
E. *Aspergillus flavus*
F. *Phoma sp.*
G. *Kawun*
H. *V. inea*
İ. *Alternata Alternaria*

After 24-48 hours, clear zone formation was visible in almost all the fungi tested against the potential probiotic isolates except fungus I. However, the fungus with the highest number of clear zones was found to be fungus C whie the fungus with the least number of clear zones was found to be fungus I.

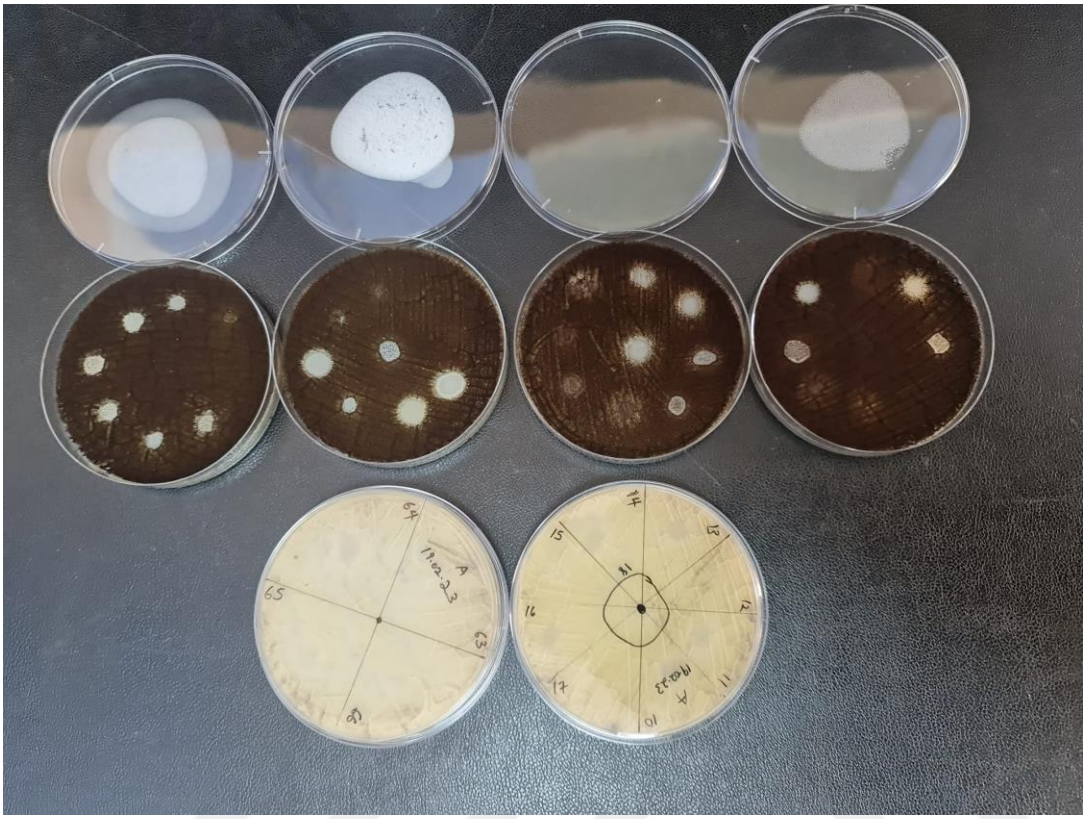

Fig 4.1.2.1 Result of antagonism test against *Aspergillus niger*

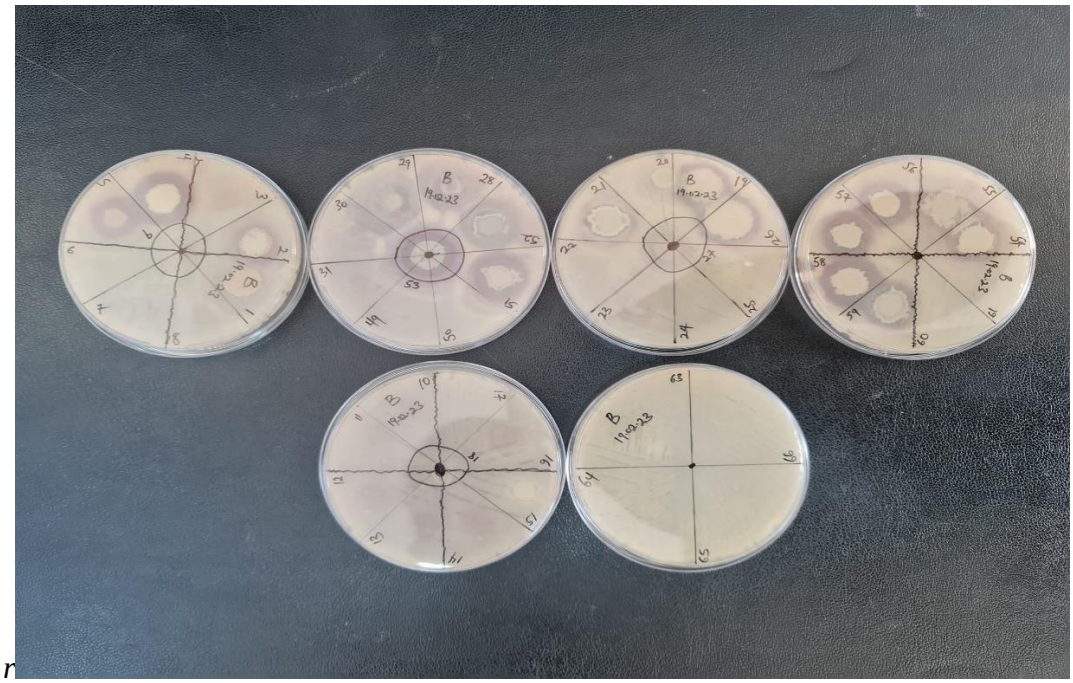

Fig 4.1.2.2 Result of antagonism test against *Didymella sp.*

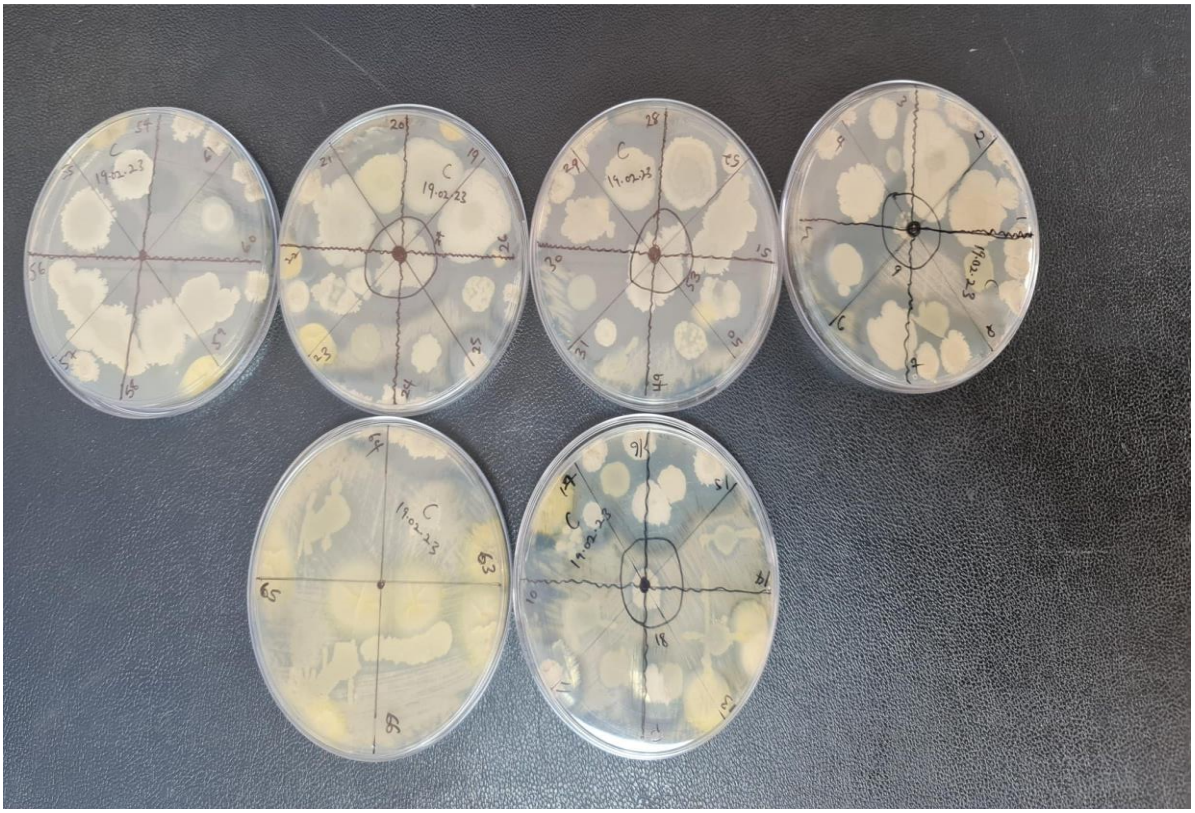

Fig 4.1.2.3 Result of antagonism test against *Anthraknoz*

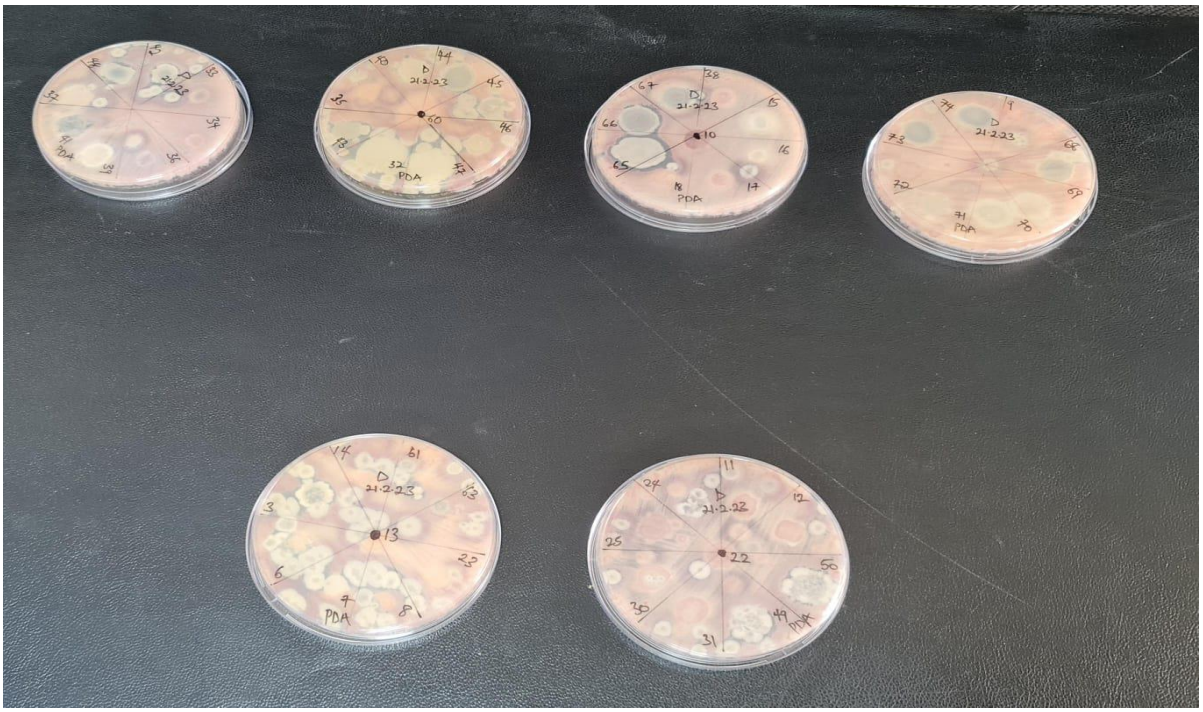

Fig 4.1.2.4 Result of antagonism test against *Fusarium oxiporium*

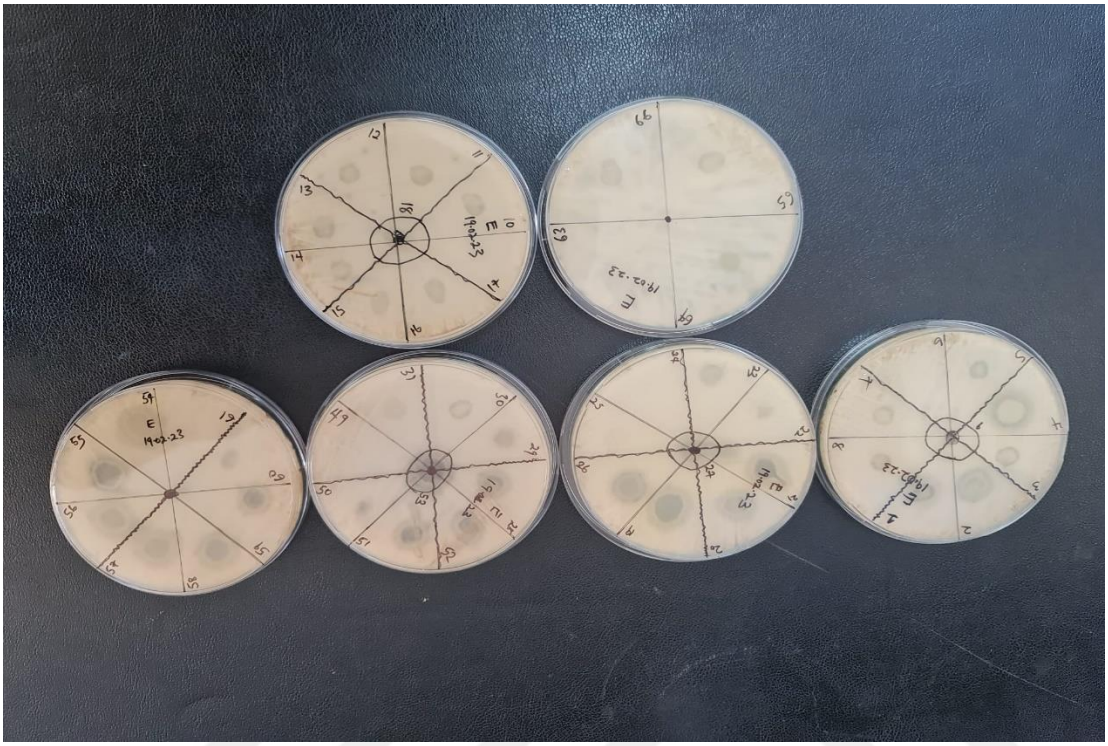

Fig 4.1.2.5 Result of antagonism test against *Aspergillus flavus*

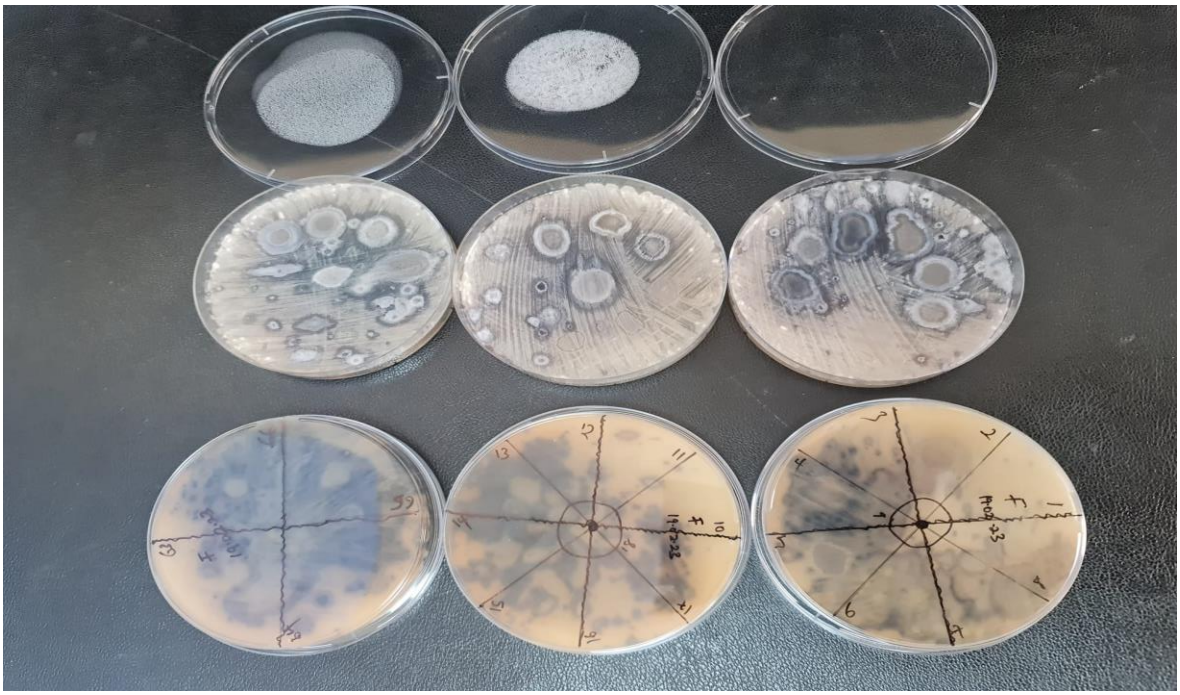

Fig 4.1.2.6 Result of antagonism test against *Phoma sp.*

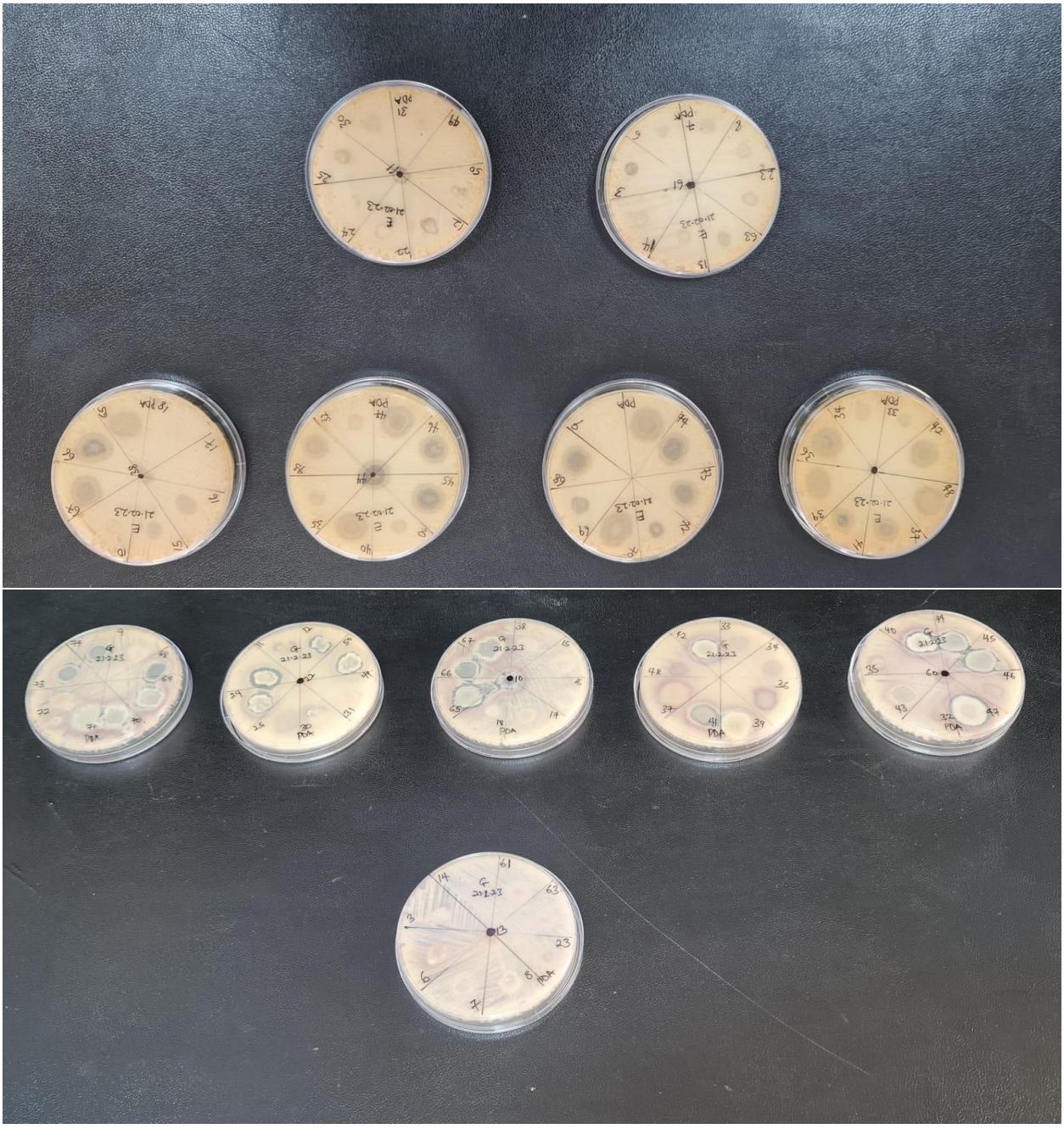

Fig 4.1.2.7 Result of antagonism test against *Kawun*

CHAPTER 5

In conclusion to the investigations carried out in this study, it was garnered that there could be potentially useful bacterial strains present in both carrots and cabbages. It was seen that some isolates had antibacterial properties particularly against *K. pneumonia*, and just once against *E. coli*. This could go a long way in treating diseases and infections caused by these pathogenic bacteria.

It was also discovered that most of these isolates actually have antifungal properties that could be useful in a variety of ways from drugs to skincare, and various other ways of improving the value of life for humans. The isolates labelled JB66, JB67, JB 68 and JB69 particularly had high antifungal activities against all the fungi they were tested against apart from *Alternata Alternaria*.

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