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CHARACTERIZATION AND ANTAGONISTIC ACTIVITY OF YEAST AND BACTERIA ISOLATED FROM FRUITS

MASTER'S THESIS (MSC)

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Abbreviations and acronyms

BCA	Biological Control Agents
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
HCCA	α-Cyano-4-hydroxycinnamic acid
KOH	Potassium Hydroxide
MALDI	Matrix-Assisted Laser Desorption/Ionization
NaCl	Sodium Chloride
PCR	Polymerase Chain Reaction
rDNA	ribosomal DNA
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
VAS-PAR	Vaseline-Paraffin
YEPD	Yeast Extract Peptone Dextrose
TE	Tris-EDTA.

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1. Introduction

Fruits and vegetables are widely acknowledged as vital components of a healthy diet, with recommendations from the Dietary Guidelines for Americans 2010 emphasizing their importance by suggesting that half of one's plate should consist of them. These plant-based foods offer a diverse range of nutrients and energy levels. They are rich in dietary fiber, which has been associated with reduced risks of cardiovascular disease and obesity. Moreover, fruits and vegetables provide essential vitamins, minerals, and phytochemicals, which act as antioxidants, phytoestrogens, and anti-inflammatory agents, offering further protective benefits(*International Journal of Food Science and Agriculture-Hill Publishing Group*, n.d.).

Fresh fruits have tough skin that prevents the majority of plant pathogenic bacteria from entering, as well as an exterior layer that may be waterproof, wax-coated, or both. However, because the skin is home to a wide range of microorganisms, fruits typically have a diverse microbiota that includes both bacteria and fungi. The skin and exterior surface of fruits are colonized or infected with a range of Gram-negative bacteria (including *Pseudomonas, Erwinia)* through various sources, including windblown, composted soil, insects, like *Drosophila melanogaster*, and other fruit flies. Similarly, *Staphylococcus aureus* (a Gram-positive bacterium) is contaminated the fruit surfaces during the hand-picking of fresh fruits. Furthermore, interaction with soil, particularly partially processed manure or compost, introduces a variety of human pathogenic microorganisms, mostly of the fecal-oral type, including the member of *Enterobacteriacea, Shigella*, and even *E. coli 0157:H7*, as well as certain bacteria. Molds such as *Rhizopus, Aspergillus, Penicillum, Eurotium*, and *Wallemia* are typical members of the fungal microbiota found in fruits, whereas *Saccharomyces, Zygosaccharomyces, Hanseniaspora, Candida, Debaryomyces*, and *Pichia sp*. are the most common yeasts. As long as the fruit's skins are intact, these microorganisms remain on surface. Any cuts or bruises that develop during the postharvest processing procedures give them access to the inside soft tissue, which is less protected, which can cause damage and product loss. (Hui, 2006).

Fungal diseases result in significant losses of fruits and vegetables during handling, transportation and storage. At present, post-production fungal spoilage is predominantly controlled by using synthetic fungicides Under the global climate change scenario and with the need for sustainable

agriculture, biological control methods of fungal diseases, using antagonistic microorganisms, are emerging as ecofriendly alternatives to the use of fungicides. (Dukare et al., 2018).

The potential of microbial antagonists, isolated from a diversity of natural habitats, for postharvest pathogen suppression has been investigated in this study.

1.2 Statement of the Problem:

Preserving fruits and their products is crucial for food safety, especially with concerns about microbial spoilage and reducing reliance on synthetic preservatives. Yeasts and bacteria found on fruits show promise as natural preservatives and biocontrol agents against plant pathogens. However, there are knowledge gaps:

1. Lack of Comprehensive Characterization: Research lacks in-depth studies on the diverse yeasts and bacteria across various fruits. Understanding their genetic diversity, traits, and applications is limited. (Zhang et al., 2020)
2. Limited Understanding of Antagonistic Activity: While some microorganisms show antagonistic activity against foodborne pathogens, we don't fully understand these interactions. Factors, like fruit type and environment, affect their effectiveness. (Pandit et al., 2022)
3. Implications for Food Safety: The lack of knowledge about fruit-derived microorganisms affects food safety and security. By better understanding these microorganisms, we can reduce reliance on synthetic chemicals while ensuring the safety of fruit-based products. However, bridging this knowledge gap is crucial to unlock their full potential. (Palumbo et al., 2022)

1.2 Significance of study

This research is significant because it aims to identify and characterize yeast and bacterial strains found in various fruits, such as Grapefruit (*Vitis vinifera),* Dog Rose (*Rosa canina*), Apple (*Malus domestica*), and Sloe fruit *(Prunus spinosa*). By employing multiple techniques like morphological observations, biochemical assays, MALDI-TOF identification, PCR, and sequencing, the study seeks to understand the diversity and characteristics of these microorganisms. Additionally, evaluating the antagonistic activity of selected strains against foodborne pathogens and spoilage

organisms can provide insights into potential natural methods for food preservation and safety enhancement. Overall, this study contributes to our knowledge of the microbial ecology of fruits and its implications for food quality and safety.

Lastly, using antagonistic yeast strains as natural preservatives offers a viable strategy for decreasing the food industry's dependency on artificial chemical preservatives, matching with consumer inclinations toward clean-label goods and encouraging environmentally friendly methods of food preservation (Ribes et al., 2017).

1.3 Objectives

1. To carefully select microbial strains with antagonistic activity from both yeast and bacteria isolated from a variety of fruits. This selection process aims to identify strains demonstrating inhibitory effects against other microorganisms, potentially valuable for applications in food preservation or biocontrol strategies.
2. To characterize the diverse yeast and bacterial species present in different fruits. This characterization involves employing traditional microbiological methods, along with assessments of morphological and biochemical properties.
3. To identify the selected strains using Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) technics, an advanced analytical technique enabling rapid and accurate microbial identification based on protein profiles.
4. To identify the selected strains through ribosomal DNA (rDNA) sequencing, a molecular method offering precise microbial identification.
5. By comparing sequencing results with those obtained from MALDI testing and traditional methods, we aim to evaluate the reliability and efficiency of these modern approaches in the identification processes.

2. Literature review

2.1 Importance of fruits in human health

Fruits are rich sources of essential nutrients crucial for maintaining good health. Water- and fat-soluble vitamins, such as vitamins A, B, C, D, E, and K, play vital roles in ensuring the balanced functioning of the body. Vitamin C and B, among the water-soluble vitamins, and vitamins A, D, E, and K, among the fat-soluble ones, are particularly noteworthy for their roles in preventing infections and bolstering the body's defense mechanisms. Consuming vitamins through food is highly recommended, as it facilitates their absorption in the body. Fruits and vegetables serve as excellent sources of vitamins, alongside other beneficial nutrients like minerals, water, and fiber. They are integral components of daily nutrition and can be regarded as functional foods due to their abundance of biologically active compounds. In addition to supporting immune function, vitamins and other nutrients found in fruits and vegetables also contribute to the proper functioning of various organ systems, helping to maintain overall equilibrium in the body. (Balali et al., 2020)

Moreover, incorporating fruits into one's diet can have a multitude of positive effects, including hydration, prevention and treatment of gastrointestinal, cardiovascular, nervous, and metabolic diseases, as well as enhancing overall energy balance. Therefore, emphasizing the consumption of fruits and vegetables can significantly contribute to overall health and well-being. (www.hillpublisher.com, n.d.).

2.2 Microbiota of fruits

Microorganisms find unique ecological niches in fresh fruits and vegetables, including plant components like leaves, roots, bulbs, and tubers, due to their distinct morphology and metabolic functions. Depending on the type of crop, agronomic techniques, production region, and preharvest weather, different microorganisms may be present in different quantities. Produce handling, harvesting, and subsequent processing can all have a significant impact on the microbiome pattern (Ramos et al., 2013). Fruits and vegetables can become contaminated with harmful microorganisms from the farm to the table at any point because they come into constant contact with soil, insects, animals, and people. Contamination can also occur from handling human waste, harvesting equipment, processing, distribution, and transportation. On the unharmed outside surface of fresh fruits or vegetables, the protective qualities of the plant's natural barriers—the cell

walls and natural waxy layer—may inhibit the growth of microorganisms. Because of the lack of nutrients and is exposed to the moisture, temperature and humidity changes, furthermore to the UV light, the physical environment on the fruit surface in the field is thought to be unfavorable for microbial growth and survival. On the other hand, bacterial and yeast populations can be greatly impacted by environmental factors (Qadri et al., 2015).

Recent studies have focused on understanding the diversity of bacteria living inside grapevines, revealing that species like *Pseudomonas* and *Bacillus* can act as natural agents for disease control, promoting plant growth and overall health. However, research on bacteria residing on the surface of grapevines has been limited. Most studies have centered on bacteria relevant to winemaking, such as lactic acid bacteria found on grape berries. Lactic acid bacteria such as *Leuconostoc mesenteroides* and various species of *Lactobacillus* are commonly present. (Zhang et al., 2017) There's a lack of information regarding the effects of surface bacteria on other parts of the plant, like leaves and bark. Grapevine bacteria not only contribute to plant health but also impact crop quality and yield. The microbial population on grape berries serves as a natural reservoir that affects the cleanliness of grapes and can influence the winemaking process, thus affecting wine quality. Despite their significance, the diversity of surface bacteria on grape berries remains poorly understood, as does their colonization of other plant parts and vineyard soil. The microbial composition of vegetables and fruits primarily consists of *Pseudomonas spp., Erwinia herbicola*, *Flavobacterium spp., Xanthomonas spp.*, and *Enterobacter agglomerans*, alongside various molds like species belonging to genera *Alternaria, Penicillium, Fusarium*, and *Aspergillus genera*. Yeasts such as *Torulopsis,* and *Candida* are also prevalent, especially on fruits due to their high sugar content. (Ramos et al., 2013)

2.3 Plant Pathogens associated with fruits

Plant pathogens, which encompass fungi, bacteria, viruses, and nematodes, cause significant losses and damage to crops worldwide, leading to a notable decline in both the quality and quantity of agricultural produce. These losses pose a substantial threat to global food production on an annual basis. Furthermore, pathogenic infections occurring either in the field or during post-harvest storage can have adverse effects on human and livestock health, particularly when the pathogens produce toxins in or on consumable products. (Jones et al., 2014) .The high-water content present in orchards due to the moisture content of plant products renders vegetables and fruits vulnerable

to pathogen attacks. Additionally, the presence of wounds on plant organs, which often occur during harvesting and transportation, provides an ideal entry point for pathogenic fungi, especially necrotrophic ones. Many bacteria and fungi typically exploit these wounds or natural openings, such as lenticels or stomata, to invade the plants. For instance, *Erwinia amylovora* causes fire blight in apples and pears through hydathodes, while *Puccinia graminis* causes stem rust in wheat through natural openings. *Streptomyces scabies* leads to potato common scab, and *Penicillium expansum* causes blue mold rot. However, certain fungal species can secrete specific enzymes that allow them to penetrate intact cuticles, stems, and fruits by exerting mechanical pressure. (Mostafidi et al., 2020)

Various fungal genera, including *Alternaria, Botrytis*, *Botryosphaeria, Colletotrichum, Lasiodiplodia, Monilinia*, and *Phomopsis*, can remain inactive and unnoticed by visual inspection while unripe fruits are stored until they ripen. Fungal pathogens proliferate rapidly as the fruit ripens. Some harmful fungi may reside on fruit tissue until ripening. Consequently, as the fruit's disease resistance decreases, they become more susceptible to fungal infections. Hence, it is crucial to implement disease prevention measures both before and after harvest to mitigate crop damage in terms of both quantity and overall quality. (Fenta et al., 2023) Many moulds and yeast are involved in the spoilage of fruits. These include *Aspergillus spp*, *Fusarium spp, Geotrichum spp, Penicillium spp, Rhizopus spp, Saccharomyces spp, Candida spp* and *Trichosporon spp*. These fungi render the fresh fruits unfit for human consumption by causing their deterioration, leading to the reduction in quality and texture, off-flavour development and loss of nutrients. (Samuel et al., 2015).

2.4 Human pathogens associated with fruits

Salmonella enterica (for example, *S. enterica* serovar Typhimurium) and *Escherichia coli* (such as *E. coli* O157:H7) are the most commonly identified causative agents of foodborne infections linked to the consumption of fresh produce. Interestingly, these human pathogens are not typically known to infect plants. Instead, they colonize and firmly attach to the surface of plants or may internalize into plant tissues, persisting within the mesophyll without causing disease in the plant itself. Numerous studies utilizing microscopic techniques have demonstrated the presence of these human microbial pathogens, particularly *E. coli* O157:H7 and *Salmonella spp*., associated with

plant stomata, wounds, and lesions present on plant leaves. Notably, these pathogens are resistant to removal or decontamination using standard disinfection methods. (Esmael et al., 2023).

2.5 Interaction of microbes

Two microorganisms may interact in an antagonistic (helpful for one partner but detrimental to the other) or synergistic (useful for both) manner. There are numerous ways in which yeasts and bacteria might interact antagonistically with other microbes. The most frequent ones are shown in figure 1 below including physical (contact) inhibition, competition for nutrients (yeast and bacterial cells utilise, consume a particular nutrient faster than the other microorganism), inhibition by the secretion of cell-wall lytic enzymes, killer factors or agents that immobilize certain nutrients (e.g., pulcherriminic acid), by releasing volatile organic compounds, etc. (Zhang et al., 2020).

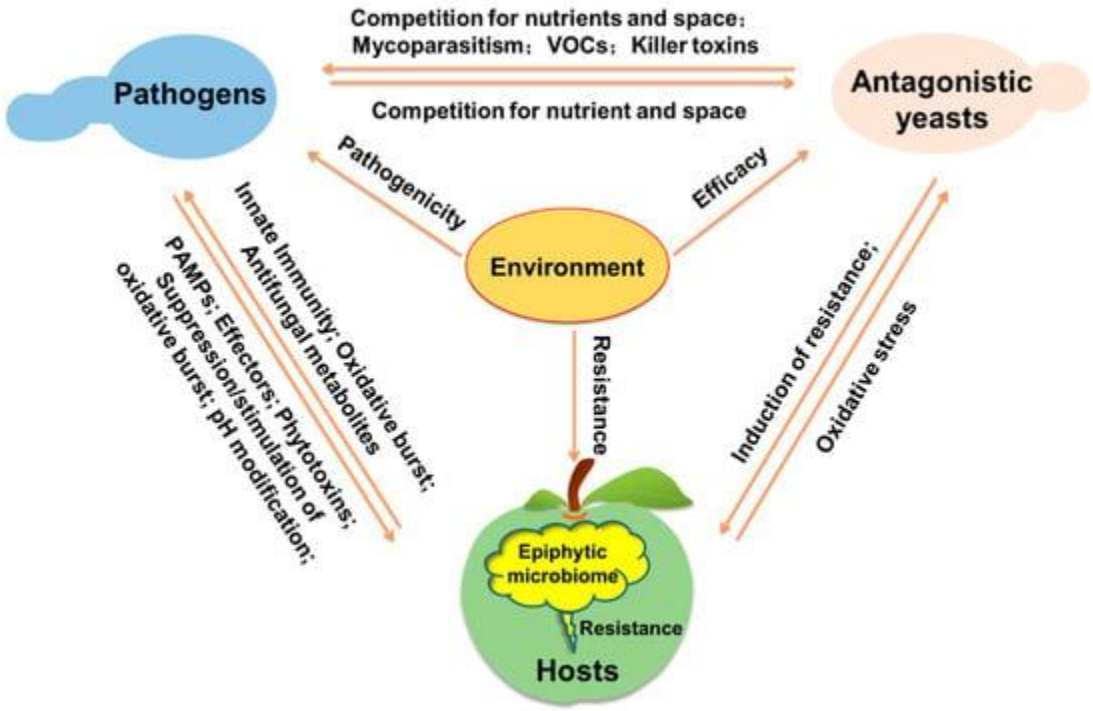


Figure 1: Diagram of possible interactions between host, pathogen and antagonist and their environment *(Zhang et al., 2020)*

2.6 Isolation and identification of yeast antagonists

Yeasts or yeast-like fungi that have the ability to impede or disrupt the growth, development, reproduction, or activity of phytopathogens are referred to as antagonistic yeasts, or biocontrol yeasts. With the intention of isolating and testing these species *(Candida saitoana, C. oleophila, C. sake, C. guillermondi, Debaryomyces hansenii, Metschnikowia fruticola, M. pulcherrima, Pichia anomala, Rhodotorula glutinis,* etc.) for their antimicrobial properties, there has been a surge in interest in yeast antagonists over the last ten years (ref). The mechanisms of action and methods for increasing the effectiveness of the different *M. pulcherrima* strains' biocontrol abilities against different fungal strains, such as *Botrytis cinerea, Alternaria spp., Penicillium spp., Aspergillus spp.,* and *Rhizopus spp*., were investigated, either in isolation or in conjunction with different fungicides. (Csutak et al., 2013).

The screening parameters for antagonistic yeasts have steadily improved as a result of the substantial study on antagonistic yeasts. An ideal antagonistic yeast should be low in concentration, efficient against several fungal infections on a variety of fruits, genetically stable, have simple nutrient requirements, and be effective under harsh environmental circumstances. Additionally, an antagonistic yeast must to have promising business prospects: It should be compatible with various physical and chemical treatments (such as controlled environment, low/high temperature, chemical fungicides/pesticides, and phytohormones) and be able to grow on a low-cost growing medium. It should also be simple to store and administer. Regarding biosafety, an ideal antagonistic yeast would not harm humans or the environment, create no metabolites that are hazardous to humans, and not be able to infect humans. It would also not be pathogenic toward the host fruits (Zhang et al., 2020).

Wisniewski et al. (2007) have provided a wealth of information regarding the commercial scale production of yeast, encompassing aspects such as fermentation, formulation, storage, and handling (Liu et al., 2013).

Developing a biocontrol agent starts with the isolation and screening procedure. Although the majority of hostile yeasts are isolated straight from fruit surfaces, they are found in a wider range of natural environments, including on leaves, roots, in soil.

Gray mold (*Botrytis cinerea*) on tomato leaves and fruits has been shown to be suppressed by the phyllosphere yeast *Rhodotorula glutinis* (strain Y-44), which was isolated from tomato leaves (Kalogiannis et al., 2006). According to reports, *Kloeckera apiculata* (strain 34-9) that was isolated from citrus roots is useful in suppressing *Penicillium italicum* on citrus and *B. cinerea* on grapes (Long et al., 2005). *Leucosporidium scottii* (strain At17), a psychrotrophic yeast that was isolated from Antarctic soil, was found to be an effective biocontrol agent against apple gray and blue mold, which are caused by *B. cinerea* and *P. expansum*, respectively (Vero et al., 2013). Marine yeasts usually have higher osmotolerance than yeasts isolated from fruit surfaces. Following isolation in pure culture, morphological and physiological characterisation and/or DNA sequencing of conserved sections of ribosomal DNA are used to identify a suitable yeast antagonist. (Liu et al., 2013).

Numerous antagonistic yeasts have been identified and evaluated thus far as shown in figure 2 below, including *Candida oleophila, Candida sake, Metschnikowia fructicola, A. pullulans, Saccharomyces cerevisiae*, and *Cryptococcus albidus*, have been developed as commercial products. Some of them, like *Candida spp., Cryptococcus spp., Metschnikowia spp., Pichia spp., Rhodotorula spp*., and yeast-like fungus *A. pullulans,* have been the subject of extensive research. (Zhang et al., 2020).

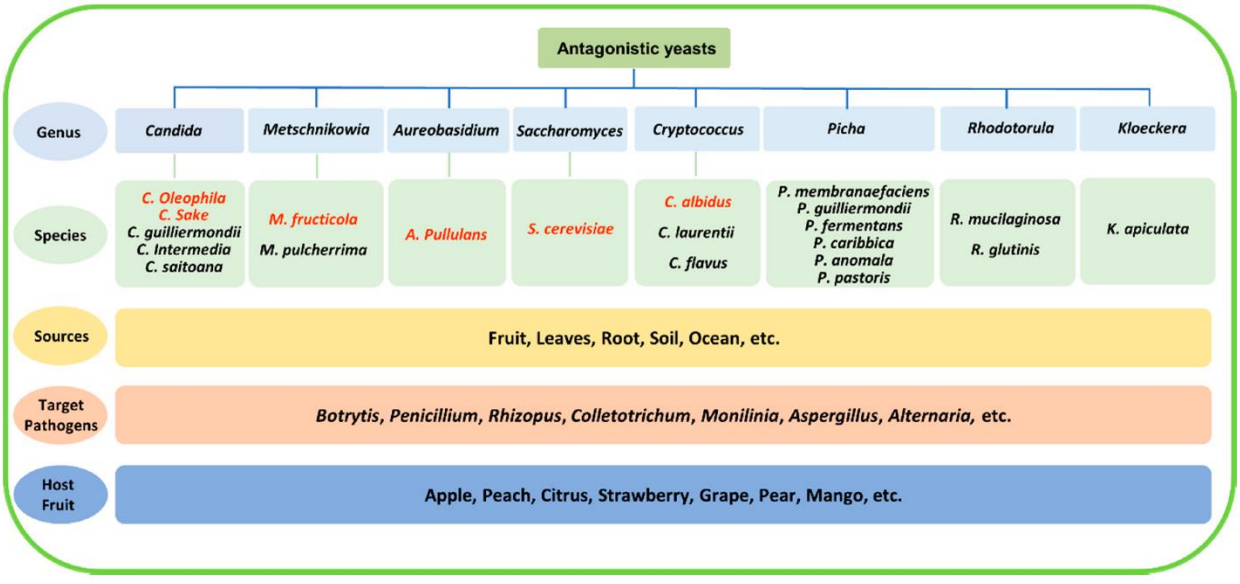


Figure 2: Types and sources of Antagonistic Yeast *(Zhang et al., 2020)*

2.7 Isolation and identification of bacteria antagonists

All bacterial isolates are characterized and identified based on their cultural, morphological, and biochemical characteristics. Staining and biochemical tests include the following: Gram stain, catalase test, oxidase test, urease test, and sugar fermentation test. Epiphytic microorganisms, predominantly bacteria, have emerged as promising agents for biocontrol against plant pathogens. These bacteria, naturally inhabiting the surfaces of plant organs, offer significant potential in managing various plant diseases. For instance, research has identified strains of *Pantoea, Pseudomonas, Enterobacter*, and *Serratia*, sourced from pear blossoms, which exhibit efficacy in mitigating flower rot caused by *Erwinia amylovora.* Similarly, *Bacillus* strains isolated from maize leaves have demonstrated notable antifungal activity against *Exserohilum turcicum* (Bolivar-Anillo et al., 2021) . In the realm of fruit protection, investigations on avocados and grapes have unveiled *Bacillus* species with the ability to impede the growth of phytopathogenic fungi, thereby curbing diseases such as anthracnose and grape rot (Khan et al., 2021). The prevalence of bacterial communities as the dominant constituents of the epiphytic microbiota on plants could be attributed to their adaptability to nutrient-poor and adverse environmental conditions (Methe et al., 2020). While current knowledge on antagonistic bacteria associated with apple fruit remains somewhat limited, *Bacillus amyloliquefaciens, Bacillus subtilis, Gluconobacter oxydans*, and *Rahnella aquatilis* have demonstrated efficacy against major apple fungi. These findings underscore the importance of native bacterial populations residing on fruit surfaces as valuable resources for combatting post-harvest fungal decay. However, a significant gap exists in understanding the dynamics of antagonistic bacterial strains throughout the developmental stages of apple fruit, which hampers the full exploitation of their biocontrol potential (Juhnevič a et al., 2011).

2.8 Mechanisms of Action of Biocontrol Agents

Biological control (BC) methods offer effective plant protection against fungal diseases, presenting a promising alternative for safeguarding fruits from phytopathogens during the postharvest stage. The adoption of BC within cultivation practices brings forth various advantages, including the reduction of disease-causing agents, enhanced cultivation protection, cost-effectiveness, minimal contamination of soil, water, and plants, as well as the absence of waste management issues. However, despite its potential, BC approaches have notable limitations such as sensitivity to factors like temperature and humidity, along with a narrow spectrum of activity, which can lead to

significant variability in field applications, thereby hindering their practical implementation. Despite these challenges, after over three decades of research, BC has progressed to a point where it can be more seamlessly integrated into agricultural production systems. (Carmona-Hernandez et al., 2019).

2.8.1 Antibiosis

Antibiosis is a fundamental mechanism employed by biocontrol agents, involving the production of substances that inhibit or kill potential pathogens in close proximity. *Bacillus spp*. produce various antibiotic compounds such as iturin, pyrrolnitrin, and syringomycin, along with other metabolites like bacillomycin, surfactin, and fengycin, which exhibit antibacterial and antifungal properties. These compounds, particularly cyclic lipopeptides like iturin, surfactin, and fengycin, not only inhibit pathogen growth but also enhance the resistance response in fruit. However, the involvement of antibiosis in postharvest disease control remains debated. While some studies have shown the importance of antimicrobial compounds, others suggest additional mechanisms at play (Manzar et al., 2022).

2.8.2 Competition for nutrients and space

BCAs, particularly yeasts, compete for nutrients and space with pathogens, rapidly colonizing wounds and inhibiting fungal spore germination. However, competition effects may only be effective under certain nutrient conditions, and other mechanisms may become more prominent as nutrient availability changes. This mode of action has been widely observed in various antagonists, and its significance in disease control has been demonstrated in several studies (Di Francesco et al., 2017).

2.8.3 Induced resistance

Induced resistance is a mechanism by which BCAs activate plant defense responses, such as the production of pathogenesis-related proteins and defense-related enzymes, in harvested fruits. This induction of resistance can contribute to disease control by enhancing the fruit's ability to resist pathogen invasion. Several studies have shown how BCAs induce disease resistance by activating defense-related enzymes and proteins, although the precise mechanisms involved require further elucidation. (de Lamo & Takken, 2020).

2.8.4 Parasitism

Parasitism involves direct interaction between the antagonist and the pathogen, including the secretion of lytic enzymes and active development of the antagonist within the fungal pathogen.

Although limited information is available on this mechanism in postharvest systems, lytic enzyme production by BCAs, such as β-1,3-glucanase and chitinases, may play a crucial role in breaking down fungal cell walls and inhibiting pathogen growth. Some studies have observed direct parasitic interactions between BCAs and fungal pathogens, suggesting its potential significance in disease control. (Dancau et al., 2016).

2.8.5 Biofilm formation

Biofilm formation by BCAs creates a mechanical barrier between the wound surface and the pathogen, serving as a site exclusion system. Certain antagonist yeasts have been shown to form biofilms, which may contribute to their biocontrol activity. Biofilm formation is considered a promising mechanism for disease control, although further research is needed to understand its full potential and optimize its application in agricultural practices (Zeriouh et al., 2013).

Figure 3 below shows various mechanisms of action of biocontrol agents

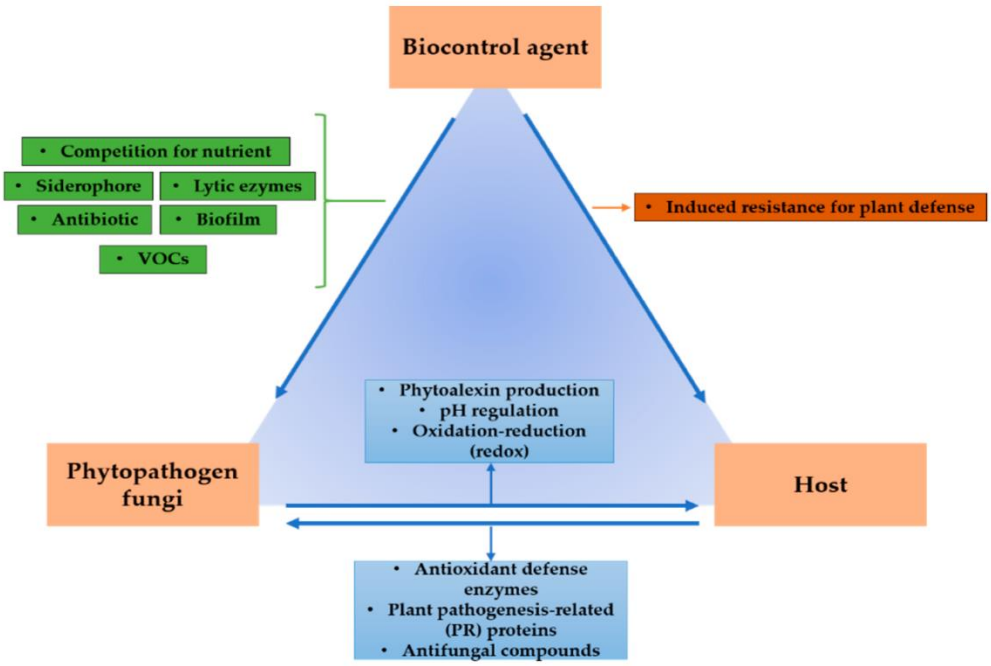


Figure 3: Mechanisms of Action of Biocontrol agents *(Carmona-Hernandez et al., 2019)*

3. Methodologies

3.1 Media Preparation; *YEPD medium*

YEPD medium was prepared by dissolving 5 grams of yeast extract, 5 grams of peptone, 10 grams of dextrose (glucose) and 15 grams of agar in 1 liter of distilled water. The components were thoroughly mixed and heated for complete dissolution, and the pH of the solution was adjusted to approximately 7.2 ± 0.2 at 25 °C. After pH adjustment, the medium was sterilized by autoclaving at 121°C for 15. Once autoclaved, the medium was allowed to cool to approximately 50°C before being poured into sterile Petri dishes. After solidification, the Petri dishes were labeled with the date of preparation and stored in a cool, dark place until needed for yeast culture experiments.

3.2 Strain Isolation and Preparation

Fruit samples were collected from gardens and viticulturers in the period of September/2021 to until May/2022 in Hungary. The strains studied were isolated from the specific fruits Grapes (*Vitis vinifera*), Dog Rose (*Rosa canina*), Medlar (*Mespilus germanica*), Crab Apple *(Malus sylvestris)*, and Sloe fruit (*Prunus spinosa*) and maintained in the culture collection of the Department. The strike technique was applied on the selected microorganisms in order to obtain a pure culture on YEPD medium. Colonies were selected from these plates and maintained using YEPD medium at 4°C. List of sample strains, their origin common names and species names as illustrated in table 1(Department of Food Microbiology, Hygiene and Safety).

Table 1: List of Sample strains, origin and Species

No.	Strain	origin	species
1.	Bc65.2.2	Grape	*Vitis vinifera*
2.	Bc65.1.1	Grape	*Vitis vinifera*
3.	Bc65.2.1	Grape	*Vitis vinifera*
4.	2Mg.1.2	Medlar	*Mespilus germanica*
5.	VC.1.2	Dog-Rose	*Rosa canina*
6.	2Ha.1.2.1RE	Grape	*Vitis vinifera*
7.	VC.1.1	Dog-Rose	*Rosa canina*
8.	FHa.1.90.2	Grape	*Vitis vinifera*

9.	Mg.1.1	Medlar	*Mespilus germanica*
10.	Bc64.2.1	Grape	*Vitis vinifera*
11.	3svc.3.1rc&p	Dog-Rose	*Rosa canina*
12.	La.1.2	Crab Apple	*Malus Sylvestris*
13.	Bc60.2.1	Grape	Vitis vinifera
14.	BB.3.1p	Sloe Fruit	*Prunus spinosa*
15.	G2.F2.1	Grape	*Vitis vinifera*
16.	2.Ha.1.1.w	Grape	*Vitis vinifera*
17.	BB.2.1pg	Sloe Fruit	*Prunus spinosa*
18.	FU.1.2.1	Grape	*Vitis vinifera*
19.	VC.1.2.3	Dog-Rose	*Rosa canina*
20.	FHa.1.2.40	Grape	*Vitis vinifera*
21.	2FU.1.2	Grape	*Vitis vinifera*
22.	Bc64.2.2	Grape	*Vitis vinifera*
23.	2svc.1.1rc	Dog-Rose	*Rosa canina*

3.3 Contact Method used for selection of antagonistic Yeasts and Bacteria

Summary of the yeast microbial strains used in antagonistic test as listed in table 2-3

Table 2: Yeast test strains

No.	Yeast strain
Y1	*Zygosaccharomyces rouxii 19*
Y2	*Galactomyces geotrichum*
Y3	*Zygosaccharomyces lentus Y. 1200*
Y4	*Zygosaccharomyces bailii PM 167*
Y5	*Pichia anomala T121*
Y6	*Candida parapsilosis Y1011*

Y7	*Saccharomyces cerevisiae CBS 1171*

Table 3: Bacterial test strains

No.	Bacterial strain
B1	*Bacillus cereus B 2078*
B2	*Pantoea agglomerans 3493T*
B3	*Staphylococcus aureus 1755*
B4	*Pseudomonas aeruginosa ATC 9027*
B5	*Escherichia coli ATCC 8739*
B6	*Listeria innocua CCM 4030*

Antagonistic Activity of isolated Bacterial and Yeast strains against the above test strains were studied by the contact method (The contact method for detection of inhibition on solid nutrient medium involves assessing the ability of microorganisms to inhibit the growth of neighboring colonies through physical contact) on solid nutrient medium. The test organisms were inoculated in lawn, then the antagonistic strains were inoculated onto in a line. The plates were incubated for 24 h at 25°C. The antagonistic activity was detected as a clear zone of growth inhibition around the strains examined.

These species (table 2 and 3) were selected as a test strain because they play a role in food spoilage, and they can cause significant product loss or can cause illness through food poisoning (*S. aureus*) or infection (certain strains of *E. coli*), or are indicator species of a food born pathogen, as *Listeria innocua indicates L. monocytogenes*. *Zygosaccharomyces rouxii* is a primary factor in the spoilage of concentrated apple juice, often resulting in product waste or recalls. While essential oil components from plants have shown antimicrobial properties against various microorganisms, there's limited research on their effectiveness against *Z. rouxii* specifically in concentrated apple juice (Wang and Sun, 2020). *Galactomyces geotrichum*, referred to as "machinery mold" in common terms and as *Geotrichum candidum* in its anamorphic form within the food industry, is a frequent spoilage agent in processed fruit and vegetable items. This fungus is responsible for soft-

rot spoilage in both fresh-cut and processed fruit and vegetable products, including canned tomato paste, blanched and frozen green beans, citrus fruits and juices, as well as cut carrots. (Cai and Snyder, 2019). Yeasts causing spoilage were extracted from ready-to-drink calamansi juice left at room temperature. After conducting biochemical and morphological analyses, it was determined that both isolates belonged to the *Candida parapsilosis* species. (Gabriel et al., 2018). Freshly cut jackfruit obtained from urban markets in Bangkok were investigated for bacterial presence. The predominant contaminant bacterium identified in 90% of the 30 samples examined was *Pantoea agglomerans*, with a significant number of samples exhibiting high levels of contamination. (Ruttipron Pothimon et al., 2021).

3.4 Macroscopic and microscopic examination of isolated yeast and bacteria

The fungal and bacterial morphology was studied macroscopically by observing the colony features including shape, margin, elevation, texture and pigment production, and microscopically by a light microscope using a slide mounted with a small portion of the inoculum prepared by mixing loop of cells with distilled water and maintaining an optical density of 0.5. The morphological characteristics of the yeast antagonists were examined by cell and colony patterns observation using a light microscope. Colony morphology of each yeast isolate was examined in cultures grown in YEPD Medium at 25 °C. The features of the yeast cultures on the plates, i.e., colony shape, margin, elevation, texture and pigment production, were recorded after 1-2 days of incubation.

3.5 Gram staining properties of bacteria:

The procedure began with the preparation of a slide smear, where a drop of suspended culture was transferred onto a microscope slide using an inoculation loop. Culture was then spread into a thin film over a 15 mm diameter circle on the slide using the inoculation loop. The slide was gently dried over a flame to facilitate cell adhesion. Next, Gram staining was performed by adding crystal violet stain to the fixed culture, followed by rinsing off the excess stain with water. Iodine solution was applied to fix the dye, then ethanol was added and rinsed off after a few seconds. Counterstaining with safranin solution followed. Finally, the slide underwent microscopic examination under oil immersion, initially at X100 magnification to assess smear distribution and then at X1000 magnification to examine individual cells. All areas of the slide were examined,

with particular attention given to areas that were only one cell thick layer to avoid variable results. Gram-positive bacteria retain the purple crystal violet-iodine complex and appear purple under the microscope, while Gram-negative bacteria lose the stain and are stained pink or red by the safranin counterstain (PMC, 2020)

3.6 Biochemical and physiological characterization of yeast

Yeasts physiological characteristics were determined according to their ability to ferment glucose anaerobically and to assimilate various carbon compounds (lactose, sucrose, glucose, raffinose) as the major source of carbon under aerobic condition. All yeasts were preliminarily grouped based on urease production and physiological characteristics, determined by assimilation tests of carbon sources and the fermentation ability of the stains.

3.6.1 Carbohydrate assimilation

The carbohydrate assimilation test determines the ability of a yeast isolate to use a particular carbohydrate substrate as its sole carbon in a medium. If there is an increase in growth then the yeast has a certain enzyme to assimilate that certain carbohydrate and the opposite is true too, if there is no growth then the yeast lacks a certain enzyme. Solid medium containing 6.7g of Bacto Yeast Nitrogen Base and 20g of high-grade agar in 1-liter distilled water and a carbohydrate substrate (e.g., glucose, sucrose, lactose and raffinose) was used.

3.6.2 Carbohydrate fermentation

Fresh Yeast cultures previously cultured on YEPD medium were inoculated using sterile loop onto glucose-containing broth medium. The fermentation basal medium was prepared according to Wickerham (1951). 0.45 g of powdered yeast extract ,0.75 g of peptone and glucose 2% v/v were dissolved in 100 ml of demineralized water. Bromothymol blue was added to give a sufficiently dark green color. Then, 2 ml aliquots of the basal medium were placed into tubes. After inoculation the broth was covered by VAS-PAR plug using small amount of paraffin to create the necessary conditions for the fermentation process to occur efficiently. Gas (CO2) production was observed, indicating fermentation. The indicator changes color from green to yellow showing positive results

3.6.7 Urea hydrolysis

Fresh Yeast cultures previously cultured on YEPD medium were inoculated using sterile loop onto Urea broth prepared by dissolving 5g of urea, 0.025 yeast extract, 0.02375g Na_2HPO_4, 0.02375g KH_2PO_4 and 0.0025g phenol red into 250ml of deionised water and pH was adjusted to 6.8 at 25°C,

then filter sterilized and divided into sterile test tubes. Urea is a product of decarboxylation of certain amino acids. It can be hydrolysed to ammonia and carbon dioxide by microorganisms containing the enzyme urease.

3.7 Biochemical tests for bacteria

3.7.1 Catalase test:

For the catalase test, fresh bacterial colonies previously cultured on YEPD medium were transferred to a glass slide using a sterile loop. A drop of 3% hydrogen peroxide solution was then added to the bacterial sample. Catalase-positive bacteria produced bubbles of oxygen gas due to the breakdown of hydrogen peroxide into water and oxygen catalyzed by the catalase enzyme present in their cells. The presence of bubbles within a few seconds indicated a positive catalase test, suggesting the presence of catalase-producing organisms.

3.7.2 Oxidase test:

To perform the oxidase test, a filter paper strip impregnated with oxidase reagent (e.g., tetramethyl-p-phenylenediamine dihydrochloride) was used. Bacterial colonies were picked from an agar plate using a sterile loop and smeared onto the filter paper. The appearance of a blue-purple color on the filter paper within 10-30 seconds indicated a positive oxidase test, signifying the presence of cytochrome c oxidase in the bacteria. This enzyme plays a role in the electron transport chain and is characteristic of certain bacterial groups.

3.7.3 Potassium hydroxide test

The potassium hydroxide (KOH) test was conducted by emulsifying a small amount of bacterial colony in a drop of 3-10% KOH solution on a clean microscope slide using a sterile inoculating loop. After thorough mixing, the slide was gently tilted to observe the formation of a string-like mucoid consistency between the loop and the slide. Gram-negative bacteria typically exhibited a positive KOH test result, characterized by the formation of a stringy or viscous appearance when the suspension was drawn out with the loop, indicating the presence of Gram-negative bacteria. In contrast, Gram-positive bacteria did not show this stringy appearance and remained relatively unchanged, indicating a negative KOH test result. This test served as a quick and reliable method for presumptive differentiation of Gram-positive and Gram-negative bacteria.

3.8 Molecular identification

3.8.1 DNA extraction and isolation

a. Cell lysis:

Cells were broken open to release the DNA. This was done by suspending a loopful of cells in a 300 µl breaking buffer (10 mM Tris-HCL; 1 mM EDTA; 1% SDS; 2 % Triton X-100; 100 mM NaCl, pH 8), EDTA (Ethylenediaminetetraacetic acid)), and SDS (sodium dodecyl sulfate). SDS disrupted the cell membrane and denatured proteins, while EDTA chelated divalent cations, such as Mg^{2+}, which were required for DNase activity. Approximately 0.3 g of glass beads were added to enhance mechanical breakdown. The mixture was vortexed for 3 minutes then 200 µl TE buffer (10 mM Tris-HCL; 1 mM EDTA, pH 8) and EDTA (Ethylenediaminetetraacetic acid)) was added to each tube.

b. **Phenol-chloroform extraction**:

An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the cell lysate. Phenol denatured proteins and separated them from DNA, while chloroform helped to remove phenol and residual proteins. 250 µl of phenol and 250 µl of chloroform-isoamyl alcohol solution was used and mixed in the tube by turning up and down and centrifuged at 12000 rpm for 5 mins

The aqueous layer (containing DNA) was transferred to a clean eppendorf tube, being careful not to disturb the interphase and 500 µl of chloroform solution was added and mixed by turning up and down. The mixture was centrifuged at 12000 rpm for 5 mins The aqueous layer (containing DNA) was transferred to a clean tube.

c. **Precipitation of DNA**:

Double amount of 96% Ice-cold ethanol or isopropanol (approximately 800 µl) was added to the aqueous layer to precipitate DNA. The solution was mixed gently and incubated at -20°C for at least 10 minutes to allow DNA to precipitate.

d. **DNA pellet formation**:

The solution was centrifuged at high speed (12,000 rpm for 10 mins) to pellet the DNA.

The supernatant was carefully removed without disturbing the DNA pellet.

e. **Drying and resuspension**:

The DNA pellet was air-dried to remove residual ethanol.

The DNA pellet was resuspended in TE buffer (Tris-EDTA pH 8.0).

f. **Quantification and storage**:

The concentration and purity of the extracted DNA were measured using a spectrophotometer.

The extracted DNA was stored at -20°C.

3.8.2 Identification by MALDI-TOF-MS Test

MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight) mass spectrometry is a powerful analytical technique used for the rapid identification of microorganisms, including bacteria, fungi, and viruses. Here is how the process was carried out:

a) *Preparation of Sample Spot:* A small amount of fresh microbial sample was transferred onto a target plate. The target plate typically had several spots or wells for sample application. The spot size had to be small and uniform to ensure consistent ionization and analysis.

b) *Covering of Sample with formic acid and HCCA Matrix*: 1 µl of formic acid and the matrix solution was applied directly onto the microbial sample spot on the target plate.

c) *Drying*: After mixing, the sample spot was allowed to air-dry completely. Drying was essential to remove excess solvent and ensure that the sample was uniformly coated with the matrix.

d) *Analysis:* The prepared sample spots were then loaded into the MALDI-TOF mass spectrometer for analysis. The instrument applied a laser beam to the sample, causing ionization and generating mass spectra that were used for microbial identification.

The score value in MALDI-TOF mass spectrometry represents the similarity between the mass spectrum obtained from the sample and the mass spectra stored in a reference database. It is typically expressed as a numerical value or percentage and indicates the degree of matching between the experimental spectrum and the spectra of known compounds in the database. A higher score value suggests a closer match and higher confidence in the identification of the sample. A

score > 2.3 indicates "highly probable species identification", a score > 2 and < 2.299 indicates "secure genus identification, probable species identification", a score > 1.7 and < 1.999 indicates "probable genus identification", and a score < 1.7 indicates "unreliable identification". The databank used in MALDI-TOF mass spectrometry contains reference mass spectra of known microorganisms, proteins, peptides, or other biomolecules. These spectra are obtained from pure cultures or purified samples of the organisms or molecules of interest. The databank may also include metadata such as taxonomic information, strain details, and spectral characteristics

3.8.3 PCR reaction

The Polymerase Chain Reaction (PCR) is a molecular biology technique used to amplify a specific region of DNA, producing millions of copies of that DNA segment. DNA samples treated with RNase enzyme were used. The process was performed by:

a. The volumes of each component needed for the master mix were calculated based on the number of reactions to be performed and adjusted to account for potential pipetting errors.
b. The components were added in order starting with deionized water followed by buffer, $MgCl_2$, dNTP, Forward and reverse primers and lastly Taq polymerase enzyme respectively and then thoroughly mixed in a microcentrifuge tube and then 29 µl was distributed into PCR tubes and 1 µl of the DNA samples added, ensuring uniform distribution by pipetting up and down or by brief vertexing. For yeast we used NL1 and NL4 as the forward and reverse primers respectively while for bacteria we used 27F and 1492R as the forward and reverse primers respectively. The concentration was 2 µM for bacteria and 10 µM for yeast.
c. The tubes or plates were then securely sealed to prevent evaporation and contamination during thermal cycling.

   The PCR reaction was initiated by placing the tubes or plates in a thermal cycler machine programmed with the desired cycling parameters (Yeast set at 52.5^0C while bacteria at 57 ^{0}C), including denaturation, annealing, extension, and the number of cycles.

3.8.4 Cleaning of PCR product for DNA sequencing

Cleaning PCR products before DNA sequencing is crucial to remove excess primers, nucleotides, enzymes, and salts that can interfere with the sequencing reaction. PCR DNA fragments extraction kit general protocol was used for cleaning PCR products for DNA sequencing as below (Gel/PCR DNA Fragments Kit; Geneaid, Taiwan)

a. Sample preparation: 25 µl of the reaction product was transferred to 1.5 microcentrifuge tube. 125 µl of DF buffer was added and mixed by vortexing
b. Step 1; DNA binding: DF column was placed in a 2ml collection tube and the sample mixture transferred into the DF column. Centrifuged for 30 seconds at 12000rpm. The flow through was discarded and the DF column placed back in the 2ml collection tube.
c. Step 2; Wash: 600 µl of the wash buffer was added into the DF column and it stood for 1 minute at room temperature. It was then centrifuged at 20000rpm for 30 seconds. The flow through was discarded and the DF column placed back in the 2ml collection tube and centrifuged for 3 minutes at 20000rpm to dry the column matrix.
d. Step 3; DNA elution: The dried DF column was transferred to a new 1.5ml microcentrifuge tube. 30 µl of elution buffer was added into the center of the column matrix and stood for at least 2 minutes ensuring the elution buffer is completely absorbed and then centrifuged for 2 minutes at 20000 rpm to elute the purified DNA.

Sequencing was done by Sevice Laborary**: Eurofins BIOMI Kft.**Szent-Györgyi Albert u. 4,2100 Gödöllő and sequence alignment done through NCBI platform by blast program.

4. Results and discussion

4.1 Selection of antagonistic strains through contact method

Results of selection and screening of antagonistic activity of samples strains experimented by contact method as shown in table 4.

Table 4:The result of the antagonistic test against of yeasts

(Source: Own work)

No.	Strain	Activity against Yeast strains							Activity against bacteria strains					
		Y1	Y2	Y3	Y4	Y5	Y6	Y7	B2	B3	B4	B5	B6	B2
1.	Bc65.2.2	-	-	-	-	-	-	-	-	++	-	++	++	-
2.	Bc65.1.1	-	-	-	-	-	-	-	-	-	-	++	-	-
3.	Bc65.2.1	-	-	-	-	-	-	-	-	++	+	-	-	-
4.	2Mg.1.2	+	-	+	-	-	+	++	-	-	-	++	+	-
5.	VC.1.2	+	-	-	-	-	+	++	-	++	-	++	+	-
6.	2Ha.1.2.1RE	-	-	-	-	-	-	-	-	-	-	-	-	-
7.	VC.1.1	+	-	-	-	-	+	++	-	++	-	++	++	-
8.	FHa.1.90.2	-	-	-	-	-	-	-	-	-	-	-	-	-
9.	Mg.1.1	++	-	-	-	-	-	++	-	++	+	++	+	-
10.	Bc64.2.1	++	-	++	-	-	++	++	-	-	+	+	-	-
11.	3svc.3.1rc&p	-	-	-	-	-	-	-	-	-	-	-	-	-
12.	La.1.2	++	-	-	-	-	-	+	-	++	-	-	++	-
13.	Bc.60.2.1	-	-	-	-	-	-	-	-	-	-	-	-	-
14.	BB.3.1p	-	-	-	-	-	-	-	-	-	-	-	-	-
15.	G2.F2.1	-	-	-	-	-	-	-	-	-	-	-	-	-
16.	2.Ha.1.1.w	+		-	-	-	-	-	-	-	+	-	-	-
17.	BB.2.1pg	++	+	-	-	-	+	+	-	++	-	++	+	-
18.	FU.1.2.1	-	-	-	-	-	-	-	-	-	-	-	-	-
19.	VC.1.2.3	-	-	-	-	-	-	-	-	-	-	-	-	-
20.	FHa.1.2.40	+	+	++	+	+	+	-	-	++	-	++	++	-
21.	2FU.1.2	-	-	++	-	-	-	+	+	++	-	++	++	+
22.	Bc64.2.2	-	-	-	-	-	-	-	-	-	-	-		-
23.	2svc.1.1rc	++	-	-	-	-	+	+	+	++	-	++	+	+

++, Clear inhibition; +, Mild/shy inhibition; -, No inhibition

In this study, 23 strains isolated from specific fruits, such as Grapes (*Vitis vinifera*), Dog Rose (*Rosa canina*), Medlar (*Mespilus germanica*), Crab Apple *(Malus sylvestris)*, and Sloe fruit (*Prunus spinosa*) were screened for their antagonistic activity against 7 spoilage yeast strains and 6 spoilage bacterial strains shown in tables 2 and 3 above through contact method. In vitro microbial interactions result in one of the following: production of a clear zone of inhibition, mild/shy zone of inhibition or no inhibition (Ortansa Csutak et al., 2013). During this experiment all the three aspects were recorded for the interactions between the isolated bacterial and yeast strains and the pathogen strains: production of a clear zone of inhibition, mild/shy zone of inhibition or no inhibition.

The results indicate varying levels of inhibition exerted by the isolated strains against the test spoilage yeast strains. Figure 4 below shows an example of isolate 5 showing clear inhibition zone against Y7 and a mild inhibition zone against Y1. Thus, showing the difference in intensity of inhibition. These results suggest that the effectiveness of the isolates in inhibiting spoilage yeast strains varies depending on the specific strain tested. A total of 14 strains were selected from the sample strains tested as they showed either clear zone of inhibition or mild zone of inhibition against the tested spoilage strains, and were recorded by numbers from 1 to 14 for easier use in the proceeding tests. Table 6 below depicts invitro antagonism effect and inhibition percentage of 14 selected strains against test species. Out of the selected antagonistic strains, strain FHa.1.2.40 isolated from Grape (*Vitis vinifera*) had the highest inhibition frequency of 75% against the spoilage microorganisms while strain Bc65.1.1 isolated from Grape (*Vitis vinifera*) had the lowest inhibition rate of 8.3%.

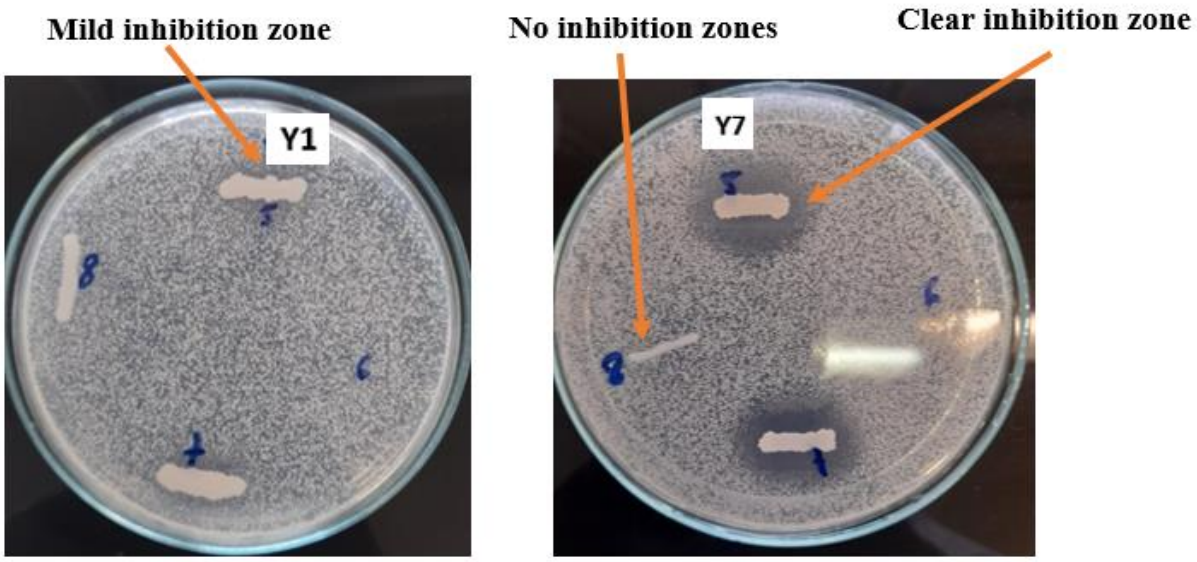


Figure 4: Sample 5 (VC.1.2) showing mild inhibition zone with Y1(Zygosaccharomyces rouxii 19) and clear zone of inhibition with Y7 (Saccharomyces cerevisiae) while sample 8 (FHa.1.90.2) shows no inhibition zone against both Y1 and Y7

After the screening and selection of antagonists, new numbering was introduced to for the antagonistic strains as shown in table 6 below for proceeding tests.

Table 5: Antagonistic strains selected

(Source Own Work)

No.	Antagonistic Strains	Inhibition frequency%	Test strains											
			Y1	Y2	Y3	Y4	Y5	Y6	Y7	B2	B3	B4	B5	B6
1	2MG.1.2	50	+	-	+	-	-	+	++	-	-	-	++	+
2	VC.1.2	50	+	-	-	-	-	+	++	-	++	-	++	+
3	VC.1.1	50	+	-	-	-	-	+	++	-	++	-	++	++
4	2.Ha.1.1.w	16.7	+	-	-	-	-	-	-	-	-	+	-	-
5	Bc64.2.1	50	++	-	++	-	-	++	++	-	-	+	+	-
6	BB.2.1pg	58.3	++	+	-	-	-	+	+	-	++	-	++	+
7	FHa.1.2.40	75	+	+	++	+	+	+	-	-	++	-	++	++
8	2svc.1.1rc	58.3	++	-	-	-	-	+	+	+	++	-	++	+
9	2FU.1.2	50	-	-	++	-	-	-	+	+	++	-	++	++

10	Mg.1.1	50	++	-	-	-	-	-	++	-	++	+	++	+
11	La.1.2	33.3	++	-	-	-	-	-	+	-	++	-	-	++
12	Bc65.2.2	25	-	-	-	-	-	-	-	-	++	-	++	++
13	Bc65.2.1	16.7	-	-	-	-	-	-	-	-	++	+	-	-
14	Bc65.1.1	8.3	-	-	-	-	-	-	-	-	-	-	++	-

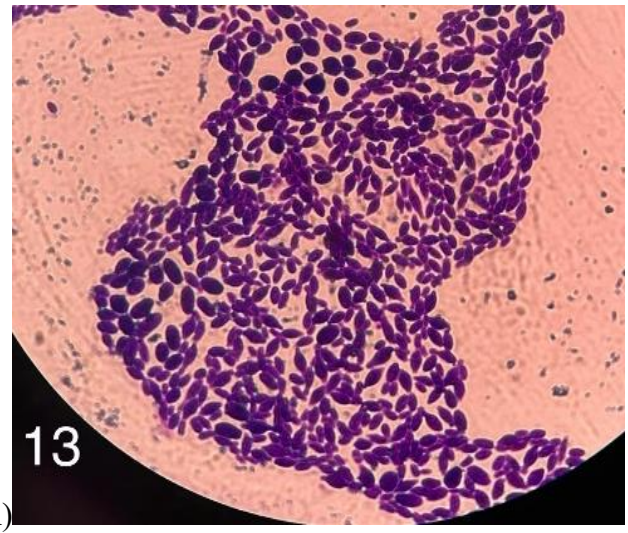
++, Clear inhibition; +, Mild/shy inhibition; -, No inhibition

Test strains B3 (*Staphylococcus aureus),* B5 (*Escherichia coli) and* B6 *(Listeria innocua)* were inhibited by the majority of the isolated antagonists, 10 out of the total 14 antagonistic strains selected while Y4 *(Pichia anomala T121) and* Y5 (*Pantoea agglomerans)* were least inhibited with only one selected strain out the 14 strains.

4.2 Cell and colony morphological results

Based on the cell size and morphology the 14 strains were grouped into two major types: yeast and bacteria. Under a stereo-microscope, yeast colonies were observed as small, smooth, and round structures on solid agar media, exhibiting a creamy or opaque coloration and often displaying a slightly raised or domed shape. Individual yeast cells visualized by light microscope (100x objective) appeared as oval or spherical structures. In contrast, bacterial colonies displayed a diverse range of morphologies including circular, irregular, and rhizoid shapes, with varying in sizes and surface characteristics such as smoothness, roughness, or mucoid appearance. Bacterial colonies exhibited a wide range of coloration, from translucent to opaque, and could present shades of white, cream, or red. Under microscopic examination (100x objective), individual bacterial cells appeared as small, rod-shaped cells, reflecting the diversity of bacterial species present as shown in table 6 below. It was concluded that 8 isolated microbial samples showed bacterial characteristics while 6 samples showed yeast characteristics.

Figure 5. below shows the oval and lemon shaped cells of strain 13 as observed under light microscope.

A) 

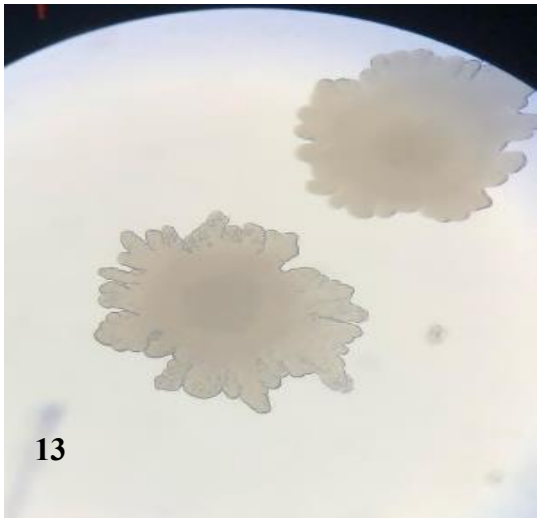
B) 

Figure 5: A) Cell morphology of Strain 13 under light microscope (100x objective) showing oval and lemon shape - and B) Colony morphology observed under stereomicroscope

These differences in morphology properties provided valuable tools for distinguishing between yeast and bacteria for further microbiological analysis. We had 8 bacterial strains including: 2MG.1.2, VC.1.2, VC.1.1, Bc64.2.1, BB.2.1pg, FHa.1.2.40, 2FU.1.2 and La.1.2 and 6 yeast strains including 2.Ha.1.1.w, 2svc.1.1rc, Mg.1.1, Bc65.2.2, Bc65.2.1 and Bc65.1.1

Colony morphological characteristics of the 14 selected antagonistic sample strains were analyzed under light microscope (100x objective) and stereomicroscope presented in table 6 below.

Table 6: Colony and cell morphology for antagonistic strains

(Source Own Work)

No.	Strain	Type	Cell morphology		Colony morphology			
			Size	**Shape**	**Margin**	**Elevation**	**Texture**	**Pigment production**
1	2MG.1.2	Bacteria	small	Rod	Entire	Flat	smooth	Red
2	VC.1.2	Bacteria	Small	Rod	Entire	convex	Shiny	creamy
3	VC.1.1	Bacteria	Small	Rod	entire	Raised	wrinkle	Shinny
4	2.Ha.1.1.w	Yeast	Large	spherical	entire	flat	smooth	cream

5	Bc64.2.1	Bacteria	Small	Rod	undulate	Flat	Smooth	Translucent
6	BB.2.1pg	Bacteria	Small	Rod	Entire	Flat	Glistering	Dull
7	FHa.1.2.40	Bacteria	Small	Rod	Entire	Flat	Smooth	Dull
8	2svc.1.1rc	Yeast	Large	oval	filamentous	convex	powdery	cream
9	2FU.1.2	Bacteria	Small	Rod	undulate	flat	smooth	Translucent
10	Mg.1.1	Yeast	Large	oval	lobate	convex	smooth	cream
11	La.1.2	Bacteria	Small	Rod	Entire	raised	wrinkle	shinny
12	Bc65.2.2	Yeast	Large	spherical	entire	flat	smooth	cream
13	Bc65.2.1	Yeast	Large	oval, lemon shaped	undulate	flat	wrinkle	cream
14	Bc65.1.1	Yeast	large	spherical	entire	flat	smooth	cream

The evaluation of morphological characteristics played a crucial role in distinguishing between yeast and bacteria in microbiological analyses. By assessing cell morphology, colony morphology, we were able to classify the selected antagonistic microorganisms. Yeast cells, characterized by their larger size, eukaryotic nature, and typically round to oval shape, contrasted with bacterial cells, which were smaller, prokaryotic, and exhibited various shapes such as bacilli or rod shape. Additionally, the appearance of yeast colonies on solid media differed from bacterial colonies in terms of texture, color, and overall morphology. Utilizing these evaluation techniques ensured accurate differentiation of yeast and bacteria, thereby facilitating various proceeding microbiological test and analysis for accurate differentiation and analysis.

4.3 Biochemical test results for selected antagonistic yeasts strains

4.3.1 Urease and fermentation test

Results of the Urease test and fermentation test for Yeast strains after 72 hours of incubation as shown in table 7

Table 7: Results of the Fermentation and Urease test for Yeast strains

(Source Own Work)

Strain no.	Strain	Fermentation results	Urease test
4	2.Ha.1.1.w	++	-
8	2svc.1.1rc	+	-
10	Mg.1.1	+	-
12	Bc65.2.2	++	-
13	Bc65.2.1	++	-
14	Bc65.1.1	++	-

++, Fast fermenting; +, slow fermenting;−, negative reaction

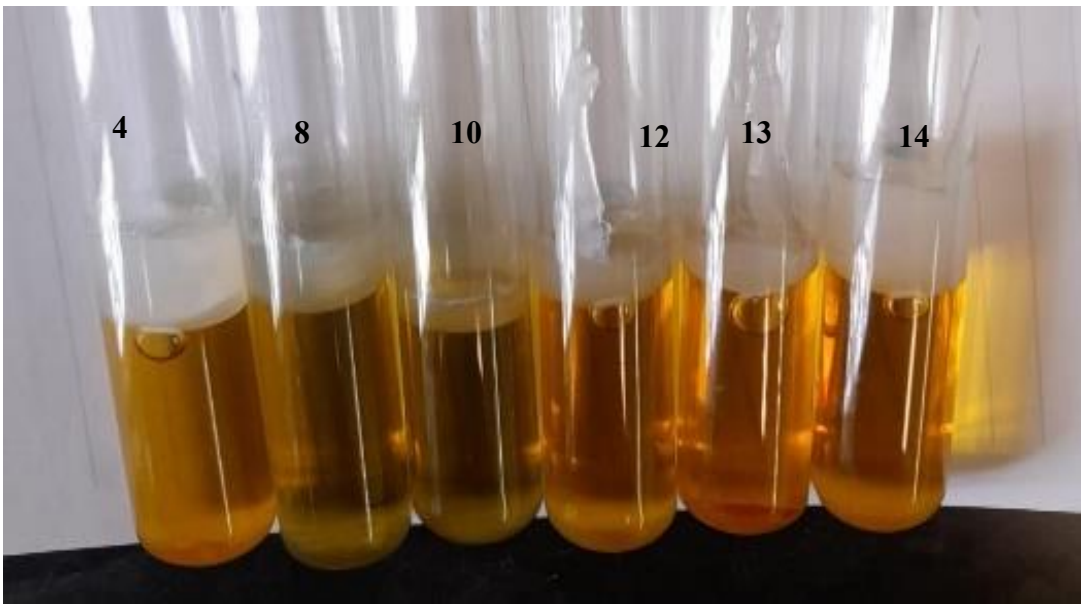


Figure 6: Fermentation test results for antagonistic yeast strains

All the six yeast strains showed no significant color change for the urease test thus showing negative results. Yeasts in *Ascomycota* phylum typically does not give positive results for the urease test. The urease test is primarily used to detect the presence of the enzyme urease, which

hydrolyzes urea into ammonia and carbon dioxide. This reaction causes an increase in pH, leading to a color change to pink if the pH indicator presents in the test medium. Most ascomycetous Yeasts lacks the urease enzyme and therefore do not exhibit a positive reaction in this test

For fermentation results, strains 4 (2.Ha.1.1.w), 12 (Bc65.2.2), 13 (Bc65.2.1) and 14 (Bc65.1.1) were fast fermenting and showed positive results as the color changed from green to distinct yellow color after 72 hours of incubation and there was formation of bubbles indicating gas release while strains 8 and 10 were slow fermenting as the color slightly changed from the green color and there were no bubbles observed after 72 hours of incubation. The fermentation results of antagonistic yeast strains demonstrated their ability to efficiently convert sugars into ethanol and carbon dioxide

4.3.2 Carbon assimilation test for yeast strains

Carbon assimilation test results for yeast isolates as observed after 72 hours of incubation are illustrated in table 8 below

Table 8:Carbon assimilation test for yeast strains

(Source Own Work)

Strain no.	Strain	Carbon Source			
		Glucose	Sucrose	Lactose	Raffinose
4	2.Ha.1.1.w	+	+	-	-
8	2svc.1.1rc	+	-	-	-
10	Mg.1.1	+	-	-	-
12	Bc65.2.2	+	+	-	-
13	Bc65.2.1	+	-	-	-
14	Bc65.1.1	+	-	-	-

+, positive reaction; −, negative reaction

The carbon assimilation test was valuable for characterizing yeast strains and understanding their metabolic capabilities. It provided information on the range of carbon sources that a yeast strain could utilize, which was essential in yeast strain traditional identification and classification. Positive growth or turbidity indicated that the yeast strain could assimilate and utilize the specific carbon source provided in the medium. Lack of growth or turbidity suggested that the yeast strain could not utilize the carbon source provided. All the six strains showed positive reaction with glucose while only strain 4(2.Ha.1.1.w) and strain 12(Bc65.2.2) showed positive reaction with

sucrose. All the strain showed negative reaction with lactose and raffinose meaning they could not utilize these types of sugars.

I could conclude that the six yeast isolates probably represent four different yeast species, as the isolates 4 and 12 have the same characteristics, while 8 and 10 differ only in colony morphology.

4.4 Characterization of selected antagonistic bacterial strains

Gram staining and biochemical characterization results of the selected antagonistic bacterial strains are illustrated in table 9 below

Table 9: Characterization of selected antagonistic bacterial strains

(Source Own Work)

Strain no.	Strain name	Gram staining	KOH test	Catalase test	Oxidase test	Urease test
1.	2MG.1.2	-	+	+	+	-
2.	VC.1.2	+	-	+	+	-
3.	VC.1.1	+	-	+	-	+
5.	Bc64.2.1	+	-	+	-	-
6.	BB.2.1pg	+	-	+	-	-
7.	Fha.1.2.40	+	-	+	-	-
9.	2FU.1.2	+	-	+	-	-
11.	La.1.2	+	-	+	-	-

+, positive reaction; −, negative reaction

Figure 7: Strain 3 (VC.1.1) showing positive results with distinct color change to pink for Urease test

4.4.1 Gram Staining:

Gram staining differentiated bacterial strains into Gram-positive and Gram-negative based on cell wall characteristics. Gram-positive bacteria retained the crystal violet stain and appeared purple or blue, whereas Gram-negative bacteria did not retain the stain and appeared pink or red after counterstaining with safranin. This differential staining helped in initial bacterial classification. Strain 1 (2MG.1.2) was gram negative on the basis of staining results while strains 2(VC.1.2), 3 (VC.1.1), 5(Bc64.2.1), 6(BB.2.1pg),7 (FHa.1.2.40) , 9(2FU.1.2) and 11(La.1.2) were positive.

4.4.2 Biochemical test results for Bacterial strains

a. KOH Test: The KOH test was used to confirm the Gram staining results, because the behavior of the bacteria depends on bacterial cell wall structure. When bacterial cells were treated with potassium hydroxide (KOH), Gram-negative bacteria lysed due to the disruption of their cell walls, resulting in the release of DNA and forming a viscous solution. In contrast, Gram-positive bacteria maintained their cell wall integrity, and no significant change was observed. Only strain 1(2MG.1.2) showed positive results while all other six strains showed negative results. The test helped in confirming the Gram staining results and classifying the bacteria as shown in table 9 above

b. **Catalase Test**: The catalase test assessed the presence of the catalase enzyme, which catalyzes the decomposition of hydrogen peroxide into water and oxygen. Bacterial strains producing catalase exhibited the formation of bubbles when hydrogen peroxide was added to the bacterial

culture. Positive catalase reactions were indicative of the presence of aerobic or facultative anaerobic bacteria capable of producing catalase. All the strains were catalase positive as shown in table 9 above.

c. Oxidase Test: The oxidase test determined the presence of cytochrome c oxidase enzyme in bacterial strains. Bacterial cells were exposed to an oxidase reagent containing a chromogenic substrate, and the development of a purple or blue color within a specified time indicated a positive reaction. Positive oxidase reactions were characteristic of bacteria possessing the oxidase enzyme, only strains 1 (2MG.1.2) and 2 (VC.1.2) showed positive reaction .

d. Urease Test: The urease test assessed the ability of bacterial strains to hydrolyze urea into ammonia and carbon dioxide using the enzyme urease. Positive urease reactions were observed as a color change in the medium from yellow to pink due to the increase in pH caused by ammonia production. This test aided in identifying urease-positive bacteria, only strain 3 (VC.1.1) showed positive results with distinct color change to pink.as shown in figure 7 above

4.5 Bruker MALDI Biotyper Identification Results

Results from the MALDI-TOF mass spectrometer were obtained by comparing the mass spectrum of the sample with spectra in a reference database, with a higher similarity score indicating a closer match and aiding in the identification of the sample as shown in figure 8 below. In this study identifications of 14 selected antagonistic strains using MALDI-TOF mass spectrometer procedure were performed. After the acquisition, we obtained one secure genus identification of strain 4 identified as *Metschnikowia pulcherrima* with a score value of 2.28, 7 out of 14 strains that is 50% showed probable genus identification with a score of > 1.7 and < 1.999 and 6 ‘no identification’ (42.9%) as shown in figure 8 below; This study shows the performance of the MALDI-TOF-MS strategy extended to various identifications: bacteria, and yeast cultures. It is to date the fastest method, providing excellent results without prior extraction. Overall, during the study period, after a single run of MALDI-TOF MS without extraction, over 50% of microorganisms (bacteria and yeasts) from solid cultures, were successfully identified. The low number of non-identified microorganisms suggests that the database contains the majority of bacteria and yeast encountered in fruit microbiota.

As a limitation, for bacteria and yeast belonging to very closely related species or the same, MALDI-TOF MS can only provide a result between two possible identifications. This limitation of MALDI-TOF MS was reported by other authors (Bille et al., 2012)

Figure 8 below relates the identification results and the strain numbers and names

Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
2	no peaks found	0.00	no peaks found	0.00
1	Pseudomonas japonica	1.98	No Organism Identification Possible	1.60
3	Bacillus subtilis	1.85	Bacillus subtilis	1.81
5	no peaks found	0.00	no peaks found	0.00
6	Bacillus mojavensis	1.99	Bacillus subtilis	1.96
7	no peaks found	0.00	no peaks found	0.00
9	no peaks found	0.00	no peaks found	0.00
11	Bacillus mojavensis	1.72	Bacillus mojavensis	1.71
4	Metschnikowia pulcherrima	2.28	No Organism Identification Possible	1.46
8	Geotrichum candidum	1.93	Geotrichum candidum	1.93
10	no peaks found	0.00	no peaks found	0.00
12	Metschnikowia pulcherrima	1.91	No Organism Identification Possible	1.39
13	Hanseniaspora uvarum	1.98	Hanseniaspora uvarum	1.91
14	no peaks found	0.00	no peaks found	0.00

Figure 8: MALDI test identification results

Strains 4 and 12 were similar thus only 12 was forwarded for DNA sequencing since it had a lower score than 4. Strains 8 and 10 also showed similar morphological and biochemical results and 8 was identified as *Geotrichum candidum* thus only 10 was forwarded for DNA sequencing. Strain 5 and 9 also showed similar morphological and biochemical properties thus only 9 was sent for DNA sequencing.

4.6 rDNA amplification

The target rDNA sequence was successfully amplified in case of all selected microbial strains as shown in Figure 9 below. The observed bands matched the expected size of the PCR products (600bp for yeast and 1300bp for bacteria), confirming the specificity of the amplification, however the isolate 6 showed less amount of amplified DNA while sample 13 had extra bands. The PCR products were then purified and after measuring of the DNA contentration and purity sent for sequencing for further analysis.In the figure 9 below M is the marker, size 10kb ; B was the bacterial PCR control and Y was the yeast PCR control

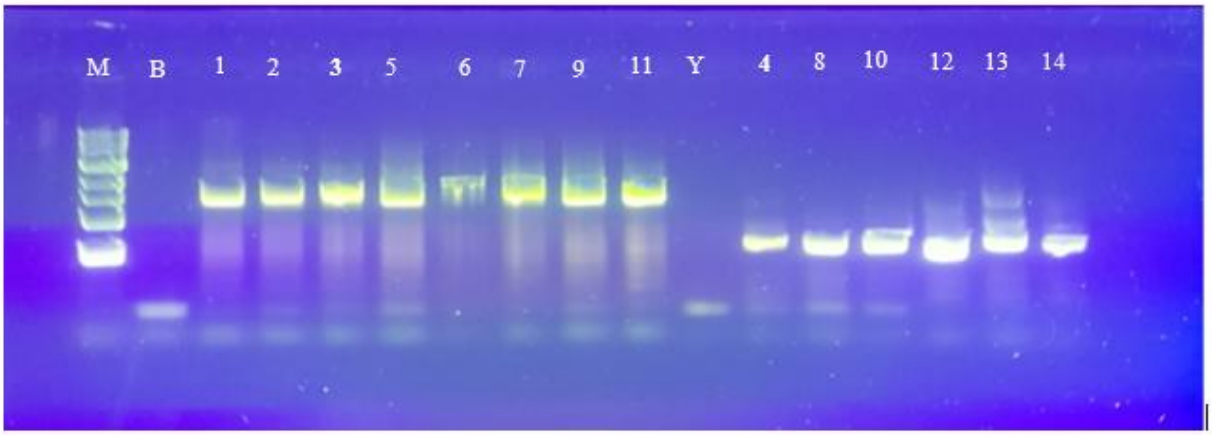


Figure 9: PCR Results

4.7 DNA Sequencing Results

The analysis of DNA sequencing results involved several steps, including the examination of chromatograms using MEGA 11 software and the sequence alignment through the NCBI database using blast program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Chromatogram analysis in MEGA software allowed for the inspection of sequencing peaks and base calls, ensuring the accuracy and

quality of the obtained sequences. Strains 12 and 13 had ambiguous base calls and more background noise. Once sequencing data were validated, the comparison of the obtained sequences to those in the NCBI database provided. We could determine the similarity between their sequences and known rDNA sequences of the type material aiding in species identification as indicated in table 10 below.

Table 10: DNA sequencing results

Sample number	Strain	Definition	Type	Source	Strain number	Accession number	Percent Identity %
1	2MG.1.2	*Pseudomonas japonica*	Bacteria	*Pseudomonas japonica*	NBRC 103040 = DSM 22348	NR_114192	99.47
					NBRC 103040 = DSM 22348	NR_040992	99.21
2	VC.1.2	*Bacillus mojavensis*	Bacteria	*Bacillus mojavensis*	RO-H-1	OQ876687	99.89
					NBRC 15718	OQ876687	99.89
					IFO 15718	NR_024693	99.89
3	VC.1.1	*Bacillus halotolerans*	Bacteria	*Bacillus halotolerans*	ATCC 25096	MN840041	98.89
		Bacillus spizizenii ATCC 6633 = JCM 2499	Bacteria	*Bacillus spizizenii*	ATCC 6633	CP034943	98.89
6	BB.2.1pg	*Paenibacillus ottowii*	Bacteria	*Paenibacillus ottowii*		MH842737	99.15
		Paenibacillus ottowii	Bacteria	*Paenibacillus ottowii*	MS2379	NR_180200.1	99.15

		Paenibacillus polymyxa	Bacteria	*Paenibacillus polymyxa*	NBRC 15309	NR_112641	99.15
7	Fha.1.2.40	*Paenibacillus ottowii*	Bacteria	*Paenibacillus ottowii*		MH842737	99.28
		Paenibacillus ottowii		*Paenibacillus ottowii*	MS2379	NR_180200	99.28
		Paenibacillus polymyxa	Bacteria	*Paenibacillus polymyxa*	NBRC 15309	NR_112641	99.17
9	2FU.1.2	*Bacillus halotolerans*	Bacteria	*Bacillus halotolerans*	ATCC 25096	MN840041	99.27
		Bacillus mojavensis	Bacteria	*Bacillus mojavensis*	NBRC 15718	MH281746	99.27
		Bacillus mojavensis	Bacteria	*Bacillus mojavensis*	RO-H-1	OQ876687	99.27
		Bacillus mojavensis	Bacteria	*Bacillus mojavensis*	NBRC 15718	NR_112725	99.27
		Bacillus mojavensis	Bacteria	*Bacillus mojavensis*	IFO 15718	NR_024693	99.27
10	Mg.1.1	*Geotrichum pandrosion BRIP 74954a*	Yeast	*Geotrichum pandrosion*	BRIP 74954a	NG_242136	99.62
		Geotrichum silvicola UFMG 354-2	Yeast	*Geotrichum silvicola*	UFMG 354-2	NG_060622	99.43
11	La.1.2	*Bacillus mojavensis*	Bacteria	*Bacillus mojavensis*	RO-H-1	OQ876687	99.52
		Bacillus mojavensis	Bacteria	*Bacillus mojavensis*	NBRC 15718	NR_112725	99.52
		Bacillus mojavensis	Bacteria	*Bacillus mojavensis*	IFO 15718	NR_024693	99.52
12	Bc65.2.2	*Metschnikowia pulcherrima*	Yeast	*Metschnikowia pulcherrima*		KY108497	96.60

		Metschnikowia pulcherrima	Yeast	*Metschnikowia pulcherrima*	NRRL Y-7111	JX188179	96.60
13	Bc65.2.1	*Hanseniaspora uvarum*	Yeast	*Hanseniaspora uvarum*		KY107844	95.99
		Hanseniaspora uvarum	Yeast	*Hanseniaspora uvarum*	NRRL Y-1614	NG_055419	95.96
14	Bc65.1.1	*Metschnikowia rubicola*	Yeast	*Metschnikowia rubicola*	NRRL Y-6064	NG_073612	99.27
		Metschnikowia pulcherrima	Yeast	*Metschnikowia pulcherrima*	NRRL Y-7111	JX188179	99.03

Comparison of the MALDI identification results and rDNA sequencing results is shown in table 11 below

Table 11: Comparison between MALDI Identification and DNA sequencing results

Strain no.	Strain	Score value	MALDI Identification	DNA sequencing
1	2MG.1.2	1.98	*Pseudomonas japonica*	*Pseudomonas japonica*
2	VC.1.2	0.0	*No identification*	*Bacillus mojavensis*
3	VC.1.1	1.85	*Bacillus subtilis*	*Bacillus halotolerans; Bacillus spizizenii*
4	2.Ha.1.1.w	2.28	*Metschnikowia pulcherrima*	*Not identified*
5	Bc64.2.1	0.0	*No identification*	*Not identified*
6	BB.2.1pg	1.99	*Bacillus mojavensis*	*Paenibacillus ottowii: Paenibacillus polymyxa*
7	Fha.1.2.40	0.0	*No identification*	*Paenibacillus ottowii*
8	2svc.1.1rc	1.93	*Geotrichum candidum*	*Not identified*

9	2FU.1.2	0.0	*No identification*	*Bacillus halotolerans*
10	Mg.1.1	0.0	*No identification*	*Geotrichum pandrosion: Geotrichum silvicola*
11	La.1.2	1.72	*Bacillus mojavensis*	*Bacillus mojavensis*
12	Bc65.2.2	1.91	*Metschnikowia pulcherrima*	*Metschnikowia pulcherrima*
13	Bc65.2.1	1.98	*Hanseniaspora uvarum*	*Hanseniaspora uvarum*
14	Bc65.1.1	0.0	*No identification*	*Metschnikowia rubicola; Metschnikowia pulcherrima*

DNA sequencing results were comparable to the MALDI identification results, both methods showed same identity for strain 1 (2MG.1.2) as *Pseudomonas japonica* with a high percentage identity of above 99% for DNA sequencing. Strain 2(VC.1.2) was not identified by the Maldi test but was identified as *Bacillus mojavensis* by rDNA sequencing. Strain 3 (VC.1.1) was identified as *Bacillus subtilis* by MALDI test but rDNA sequencing gave two possible identifications of *Bacillus halotolerans and Bacillus spizizenii (syn B. subtilis ssp. spizizenii).* Strain 4(2.Ha.1.1.w) was identified as *Metschnikowia pulcherrima*: but was not sent for sequencing since it had a high score of 2.28 with MALDI test. Strain 5 (Bc64.2.1) was not identified by MALDI test and was not sent for sequencing since it showed similar morphological characteristics with strain 9. Strain 6 (BB.2.1pg) was identified as *Bacillus mojavensis* by MALDI test while rDNA sequencing gave two possible identifications of *Paenibacillus ottowii and Paenibacillus polymyxa.* Strain 7 was not identified by MALDI test but was identified as *Paenibacillus ottowii* by rDNA sequencing. Strain 8 (2svc.1.1rc) was identified as *Geotrichum candidum* by Maldi identification but was not sent for sequencing as it showed similar morphological characteristics with strain 10. Strain 9 was identified as *Bacillus halotolerans* by rDNA sequencing but was not identified by MALDI test. Strain 10 gave two possible identifications of *Geotrichum pandrosion and Geotrichum silvicola* with rDNA sequencing but was not identified by MALDI test.Strain 11 (La.1.2) as *Bacillus mojavensis* by both methods. Strain 12 (Bc65.2.2) was identified as *Metschnikowia pulcherrima*

and strain 13 (Bc65.2.1) as *Hanseniaspora uvarum* by both methods however rDNA sequencing results showed low percentage identity of 96.60% and 95.96% respectively. Strain 8 (2svc.1.1rc) was identified as *Geotrichum candidum* by MALDI identification and showed similar characteristics to strain 10 (Mg.1.1) identified as *Geotrichum pandrosion* by rDNA sequencing. Strains 2 (VC.1.2) and 11 (La.1.2) were also similar and were identified as *Bacillus mojavensis* however they showed different properties in cell and colony morphology and in biochemical properties. Strain difference could have led to this discrepancy. Strain 1(2MG.1.2) was identified as *Pseudomonas japonica:* which is gram-negative and rod-shaped bacterial species, and showed the expected positive results for catalase and oxidase tests, and negative results for urease tests. These results were in harmony with the morphological and biochemical characterization initially done. Strain 3 (VC.1.1) was identified as *Bacillus subtilis* which typically is Gram-positive with rod-shaped cells arranged in chains and has expected positive result for the catalase test. Negative results for the KOH, oxidase, and urease tests. The result conformed to the morphological and biochemical characterization of the strain. Strain 4(2.Ha.1.1.w) was identified as *Metschnikowia pulcherrima*: which typically has creamy or pink-colored colonies with a wrinkled appearance and expected positive result for the fermentation test. Negative results for urease test. Strain 5 (Bc64.2.1) was not identified. Strain 6 (BB.2.1pg) was identified as *Bacillus mojavensis* which is typically Gram-positive, rod-shaped cells and expected positive result for the catalase test. Negative results for the KOH, oxidase, and urease tests. Strain 7 (Fha.1.2.40) was not identified. Strain 8(2svc.1.1rc) was identified as *Geotrichum candidum:* exhibiting typically creamy or white-colored colonies with a powdery texture and expected negative results for urease test. Strain 9 ,10 and 14 were not identified. Strain 11 (La.1.2) was identified as *Bacillus mojavensis:* Gram-positive, rod-shaped cells and expected positive result for catalase test. Negative results for the KOH, oxidase, and urease tests. Strain 12 (Bc65.2.2) was identified as *Metschnikowia pulcherrima* with creamy or pink-colored colonies with a wrinkled appearance and expected positive result for fermentation test. Negative results for urease test. Strain 13 (Bc65.2.1) was identified as *Hanseniaspora uvarum:* Strains 4 and 12 were similar thus only 12 was forwarded for DNA sequencing. Strains 8 and 10 also showed similar morphological and biochemical results thus only 10 was forwarded for DNA sequencing. Strain 5 and 9 also showed similar morphological and biochemical properties thus only 9 was sent for DNA sequencing.

Comparing the strains that showed similar identity with both MALDI test and DNA sequencing against the inhibition results we found out that the strain 1 (2MG.1.2) identified as *Pseudomonas japonica* had a great biocontrol potential as could inhibited the growth of both the bacterial and yeast strains, such as Y1 (*Zygosaccharomyces rouxii* 19), Y6 (*Candida parapsilosis* Y1011), Y7 (*Saccharomyces cerevisiae* CBS 1171), B5 (*Escherichia coli* ATCC 8739) and B6 (*Listeria innocua* CCM 4030). *Bacillus mojavensis* strain 11 (La.1.2) inhibited efficiently the growth of Y1 (*Zygosaccharomyces rouxii* 19), Y7 (*Saccharomyces cerevisiae* CBS 1171), B3 (*Staphylococcus aureus* 1755) and B6 (*Listeria innocua* CCM 4030). Two yeast strains were also effective against the tester strains. Strain 12 (Bc65.2.2) was identified as *Metschnikowia pulcherrima* inhibited the growth of B3 (*Staphylococcus aureus* 1755), B5 (*Escherichia coli* ATCC 8739) and B6 (*Listeria innocua* CCM 4030), while strain 13 (Bc65.2.1) as *Hanseniaspora uvarum* showed only inhibition against bacteria such as B3 (*Staphylococcus aureus* 1755) and B4 (*Pseudomonas aeruginosa* ATC 9027)

These findings are supported by researches done before as identified species have been studied for their antagonistic activity in literature. *Bacillus mojavensis* strain YL-RY0310, isolated from soil samples, demonstrated the ability to suppress the growth of *Penicillium expansum* and degrade patulin (PAT) primarily through the synthesis of metabolites. Analysis via PCR amplification of genes associated with lipopeptide production and the Oxford cup assay using crude extracts indicated that YL-RY0310 primarily inhibited *P. expansum* growth by secreting lipopeptides such as surfactin, iturins, and fengycin. The findings also suggested that PAT degradation was primarily mediated by intracellular enzymes, followed by extracellular enzymes. These findings imply that *B. mojavensis YL-RY0310* holds promise as a potential biocontrol agent for mitigating *P. expansum* growth and PAT accumulation in apples. (Ding et al., 2023).The utilization of *B. halotolerans* KLBC XJ-5 presents an effective strategy for controlling grey mold decay caused by *B. cinerea* during fruit storage, thereby preserving postharvest quality. The bacterial genome of *B. halotolerans* KLBC XJ-5 contains six gene clusters that are likely responsible for producing a diverse range of antimicrobial compounds, along with at least four genes associated with chitin degradation. These genetic traits confer natural resistance, enhancing the organism's ability to combat fungal pathogens through defense-related enzymatic activities and the production of antifungal compounds. Consequently, we propose that *B. halotolerans* KLBC XJ-5 holds significant potential as a bio-control agent against *B. cinerea*, offering a promising solution for

managing postharvest strawberry grey mold. (Wang et al., 2021) *Paenibacillus sp*. strain UY79, isolated from wild peanut nodules, exhibited antagonistic activity against various fungi and oomycetes, including *Botrytis cinerea* and *Fusarium species*. The antagonism was attributed to both agar-diffusible and volatile compounds, excluding hydrogen cyanide. Volatile compounds like acetoin, 2,3-butanediol, and 2-methyl-1-butanol were identified as potential contributors. UY79 did not affect the symbiotic association or growth promotion of alfalfa plants when co-inoculated with rhizobia. Whole-genome sequencing revealed UY79 as a new species within the *Paenibacillus polymyxa* complex, possessing genes linked to biocontrol activity. Additionally, genome analysis suggested UY79's ability to modulate the growth of bacteria commonly found in soil and plant communities. (Costa et al., 2022) Another study examined *P. polymyxa* APEC128 as a biocontrol agent for anthracnose caused by different fungi in harvested apples. APEC128 was most effective out of 30 bacterial isolates tested, inhibited fungal growth by increasing protease and amylase production. Higher APEC128 concentrations led to greater suppression of anthracnose. The authors concluded that the findings suggest APEC128's potential to extend the shelf life of apples by controlling anthracnose (Young Ho Kim et al.,2016). *Metschnikowia pulcherrima* strains from apples showed promise as biocontrol agents as they not only reduced *P. expansum* incidence but also could degrade patulin. Strain Y29 exhibited the best results, inhibiting blue mold decay significantly when applied to stored apples. (Settier-Ramírez et al., 2021)The *H. uvarum* strains were also used successfully as biocontrol agents. Combining the yeast *H. uvarum* with pre-treatment of kiwifruit using BABA (β-Aminobutyric acid) proved more successful in controlling postharvest diseases compared to either treatment alone. The researchers proposed this integrated approach, combining a biocontrol agent with an elicitor, as it holds great promise for managing postharvest diseases in fruit crops. (Cheng et al., 2019).

5. Conclusion and recommendations

This study revealed the presence of indigenous yeasts and bacteria from specific fruits Grapefruit (*Vitis vinifera*), Dog Rose (*Rosa canina*), Medlar (*Mespilus germanica*), Crab Apple *(Malus sylvestris)*, and Sloe fruit (*Prunus spinosa*), which demonstrated the ability to inhibit the growth of different fruit contaminants and bacteria, such as yeasts: *Zygosaccharomyces rouxii*, *Z. lentus, Z. bailii, Pichia anomala*, *Candida parapsilosis*, *Galactomyces geotrichum, Saccharomyces cerevisiae*, and bacteria: *Bacillus cereus*, *Pantoea agglomerans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Listeria innocua* .

The study findings showed that fruits can harbor a variety of microorganisms with potential inhibitory activity against spoilage microorganisms and can be isolated as antagonistic microorganisms. Morphological and biochemical characterization is necessary for preliminary grouping of microorganisms. MALDI-TOF-MS is a good strategy for various identifications: bacteria, and yeast cultures. It is to date the fastest method, providing excellent results without prior extraction however as a limitation can only provide a result between two possible identifications. DNA sequencing was more effective as it gave elaborate identity of the sample strains giving all the matches within the same genera but different species. Thus, although DNA sequencing is an expensive approach, it's a more robust, accurate and elaborate method of species identification.

These isolates show potential as biocontrol agents for preserving fruits during post-harvest storage. However, the effectiveness of biocontrol agents can vary and depends on factors such as the population of the pathogen and environmental conditions. Initial laboratory screening by contact method was valuable methods for selection of antagonists, particularly when microorganisms are sourced from the natural microbiome.

This study marks an initial step toward potential applications of yeasts and bacteria for fruit protection against spoilage microorganisms. Future efforts should focus on detailed characterization of these isolated strains, especially their biocontrol ability between various conditions. However, successful outcomes in laboratory experiments may not directly translate to high antagonistic efficacy in field conditions, where various factors, including climate and nutrient availability, influence the survival of biocontrol agents. Therefore, further research is needed to understand their mode of action and to assess the most promising selected isolates in field trials.

6. Summary

The research specifically aimed to select, characterize and identify antagonistic yeast and bacteria obtained from specific fruits by examining their morphological and biochemical traits, as well as their biocontrol efficacy against pathogens through contact method and finally identification by MALDI test and DNA sequencing. In this study, 23 strains isolated from specific fruits were screened and selected for their antagonistic activity against 7 spoilage yeast strains and 6 and 14 strains out of 23 isolated strains(60.8%) showed positive antagonistic activity against the tested spoilage microorganisms and were selected for further characterization and identification.

Initial cell and colony morphology characterization was done and the selected antagonists grouped into two types: yeast and bacteria, from this grouping we had 8 bacteria strains and 6 yeast strains. Yeast cells, characterized by their larger size, eukaryotic nature, and typically round to oval shape, contrasted with bacterial cells, which were smaller, prokaryotic, and exhibited various shapes such as bacilli or rod shape. Additionally, the appearance of yeast colonies on solid media differed from bacterial colonies in terms of texture, color, and overall morphology. Utilizing these evaluation techniques ensured accurate differentiation of yeast and bacteria, thereby facilitating various proceeding microbiological test and analysis for accurate differentiation and analysis.

Biochemical characterization of the antagonistic strains was carried out. For yeast strains, fermentation test, urease test and carbon assimilation test were carried out. All the six yeast strains showed no significant color change for the urease test thus showing negative results. Yeast typically do not test positive for the urease test. For fermentation 4 strains were fast fermenting while 2 strains slow fermenting. For carbon assimilation test, all the six strains showed positive reaction with glucose while only strain 4(2.Ha.1.1.w) and strain 12(Bc65.2.2) showed positive reaction with sucrose. All the strain showed negative reaction with lactose and raffinose. For the bacterial antagonistic strains, Gram staining, KOH test, Catalase test, Oxidase test and Urease test were carried out only strain 1(2MG.1.2) was gram. All the strains were catalase positive. Only strains 1(2MG.1.2) and 2(VC.1.2) showed positive reaction for oxidase test. Only strain 3 (VC.1.1) showed positive results for urease test.

Both MALDI test and DNA sequencing methods showed same identity for strain 1 (2MG.1.2) as *Pseudomonas japonica* , strain 11 (La.1.2) as *Bacillus mojavensis* with a high percentage identity of above 99%. Strain 12(Bc65.2.2) was identified as *Metschnikowia pulcherrima* and strain

13(Bc65.2.1) as *Hanseniaspora uvarum* by both methods however rDNA sequencing results showed low percentage identity of 96.60% and 95.96% respectively. Comparing and relating the identification results and the inhibition results, the study findings showed that strain 1 (2MG.1.2) identified as *Pseudomonas japonica* inhibited the growth of Y1 (*Zygosaccharomyces rouxii* 19), Y6 (*Candida parapsilosis* Y1011), Y7 (*Saccharomyces cerevisiae* CBS 1171), B5(*Escherichia coli* ATCC 8739) and B6 (*Listeria innocua* CCM 4030). Strain 11(La.1.2) as *Bacillus mojavensis* inhibited the growth of Y1 (*Zygosaccharomyces rouxii* 19), Y7 (*Saccharomyces cerevisiae* CBS 1171), B3 (*Staphylococcus aureus* 1755) and B6 (*Listeria innocua* CCM 4030). Strain 12 (Bc65.2.2) was identified as *Metschnikowia pulcherrima* inhibited the growth of B3 (*Staphylococcus aureus* 1755), B5(*Escherichia coli* ATCC 8739) and B6(*Listeria innocua* CCM 4030). Strain 13 (Bc65.2.1) as *Hanseniaspora uvarum* showed inhibition against B3 (*Staphylococcus aureus* 1755) and B4(*Pseudomonas aeruginosa* ATC 9027).

MALDI-TOF-MS is a good strategy for various identifications: bacteria, and yeast cultures. It is to date the fastest method, providing excellent results without prior extraction however as a limitation can only provide a result between two possible identifications. DNA sequencing was more effective as it gave elaborate identity of the sample strains giving all the matches within the same genera but different species. Thus, although DNA sequencing is an expensive approach, it's a more robust, accurate and elaborate method of species identification.

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8.0 appendix

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