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Identification of yeasts and bacteria from fermented masau juice.

By

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A research project submitted in partial fulfilment of the requirements for the Bachelor of Science Honours Degree in Biological Sciences.

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Approval form

The undersigned certify that they have read the dissertation titled ‘Identification of yeasts and bacteria from fermented masau juice.’ and confirm that it is suitable for submission to the Biological Sciences Department, Faculty of Science and Engineering, for assessment.

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Declaration

I, Previledge Christine Chinembe (B202785B) declare that this research herein is my own work and has not been plagiarized from another source(s) without acknowledgement of the concerned author(s) either electronically or otherwise

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Supervisor

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Dedication

I dedicate this project to my parents who have been by my side and supported me every step of the way during my academic pursuits.

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List of abbreviations

SDA- Sabouraud Dextrose Agar

PDA- Potato Dextrose Agar

List of figures

List of tables

List of appendices

Abstract

The fermentation of masau juice is a natural process that involves the activities of various microorganisms, primarily yeasts and bacteria, which play a crucial role in transforming the sugars into alcohol and other metabolites. This study aimed to identify the yeast and bacterial species present in fermented masau juice. Four samples of fermented masau juice were collected from the BUSE Innovation HUB. The samples were plated onto Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) to encourage the growth of yeasts, and Nutrient Agar to allow the growth of bacteria. The plates were incubated for 48 hours at 37°C. Isolated yeast colonies were further subcultured and plated on yeast extract. After isolation, the yeast colonies were examined and identified based on their morphological characteristics, and gram staining was performed to identify the bacterial species. The study found that the bacterial species in the fermented masau juice included *Leuconostoc minor, Leuconostoc agilis, Lactobacillus fermentum, Lactobacillus fructosus,* and *Lactobacillus divergens.* The yeast species identified were *Saccharomyces cerevisiae, Aureobasidium pullulans, Hanseniaspora opuntiae, Saccharomycopsis fibuligera* and *Pichia kudriavzevii*. The findings from this research have highlighted the need for more controlled and consistent fermentation practices to enhance the quality and safety of traditional masau beverages, while still preserving their unique sensory attributes. The uncontrolled nature of the traditional fermentation process can lead to inconsistencies in product quality and potential food safety concerns.

Contents

Chapter 1

Introduction

1.1. Background of study

Fermentation is one of the oldest methods of food processing used to produce food and drinks. It is defined as bioprocessing that employs microorganisms and their enzymes to obtain desired quality features (Nout 2003). African traditional fermented foods and drinks are mostly produced by spontaneous fermentation, which is an unregulated process. Traditional fermented food include drinks made from local fruits, such as masau. The fruit known as masau (*Ziziphus mauritiana)* has a rich cultural history and unique qualities. The fruit is sometimes referred to as "desert apple," "ber," "Indian jujube," and "Indian plum" (Kaaria, 1998). Although it is widespread in Zimbabwe, only the lower lying regions bear fruit. In particular, the lower Zambezi valley is linked to it. As the fruit ripens, it starts out green before turning yellow and then brown. The ripe fruit quenches thirst and tastes sweet to sour. The fruits are a great source of vitamins, minerals, calcium, potassium, phosphorus, iron, zinc, copper, and carbs. They are also a strong source of protein. It has significant levels of antioxidants, vitamin C, and vitamin A (Morton, 1987). The fruits sweet and lightly acidic taste lends itself well to a range of dishes and beverages for the local market. Masau juice has gained popularity for its unique taste, nutritional benefits and refreshing qualities. Usually, the ripe masau fruits are squeezed or pressed gently to obtain the juice. Traditionally, masau fruit is fermented into drinks using random, spontaneous processes. In Zimbabwe, the traditional beverage known as kachasu is produced by fermenting masau fruits for 6-7 days, and then distilling the fermented liquid into a potent and highly

intoxicating spirit (Gadaga *et al.*, 1999). Kachasu is commonly consumed by the local population, but its alcohol content can vary significantly depending on the individual producer.

The fermentation process is spontaneous and uncontrolled, leading to inconsistent product microbiota and variable quality in the final fermented beverage (Gadaga, 2000). The fermentation relies primarily on the microbial flora present on the surface of the masau fruits, as well as to some extent from the utensils used during the fermentation process. This diverse microbial community on the fruit surfaces plays an important role in the spontaneous fermentation. The types of microorganisms involved in the fermentation process include coliforms, lactic acid bacteria, yeasts, and molds (Mbugua, 1985). The spontaneous fermentation of grape juice into wine, as observed by Louis Pasteur approximately 150 years ago, established the widely accepted understanding that yeasts have a natural association with fruits and fruit products (Fleet, 2003). Similarly, *Ziziphus mauritiana*, an indigenous Zimbabwean fruit, has been reported to harbor high numbers of microbes that contribute to the natural fermentation process following ripening (Chivero et al., 2001). However, limited research has been conducted on the specific microbial species present in masau juice and their impact on its quality.

1.2. Problem Statement

The microbial composition of fermented masau juice has not been extensively studied. This knowledge gap hinders understanding of the fermentation process, the impact of different microbial strains on product quality and the potential risks associated with microbial contamination. The most serious concern is the spoilage of masau juice by microorganisms even after a preservative has been added. Insufficient sanitation practices or inadequate control of fermentation conditions can allow harmful microorganisms to proliferate leading to the growth of molds, yeasts, and bacteria. The presence of these uncontrolled microbial contaminants not

only degrades the overall quality of the masau juice, but also potentially poses health risks to consumers if the fermented masau juice is consumed. Thus, if not properly controlled fermentation can cause the masau juice to be spoiled. Additionally, masau juice contains natural sugars that when exposed to yeast and bacteria ferment converting them into alcohol. Inadequate fermentation control can result in excessive alcohol concentration making the juice unsuitable for some customers, such as children or individuals with alcohol-related health conditions. However, the lack of comprehensive information regarding the microbial composition of fermented masau juice poses challenges for its commercial production, quality control and safety. Hence by knowing which bacteria strains and yeast cause spoilage, this can enable producers take appropriate measures to ensure consistent product quality. Therefore, there is a need to investigate and identify the yeasts and bacteria present in fermented masau juice.

1.3. Aim of the study

To identify yeasts and bacteria present in fermented masau juice.

1.4. Objectives

1. To isolate and identify the yeast species in fermented masau juice.

2. To isolate and identify bacterial strains responsible for the fermentation of masau juice.

3. To develop strategies to prevent spoilage and ensure food safety.

1.5. Research questions

1. What are the yeast and bacteria strains present in fermented masau juice?

2. What strategies can be used to avoid growth of yeast and bacteria in masau juice?

1.6. Hypothesis

$H_{o.}$ There are no yeasts and bacteria in masau juice as the juice has been pasteurized.

$H_{1.}$ There are yeasts and bacteria found in masau juice.

1.7. Significance of the study

This research is of essence as it contributes to the scientific understanding of the microbial composition of fermented masau juice which will enable producers to develop appropriate quality control measures for masau juice production, ensuring consistent flavor profiles and product safety. Secondly, identifying the microorganisms responsible for fermentation will contribute to the development of preservation techniques that extend the shelf life of masau juice while maintaining its desirable characteristic.

1.8. Limitations of the study

Molecular methods based on DNA sequencing were supposed to be implemented to identify the yeasts and bacteria in fermented masau juice but due to shortage of PCR reagents in the laboratory the research was limited to identification based on morphological characteristics.

1.9. Delimitations of the study

This study specifically targeted the identification of yeasts and bacteria present in fermented masau juice and not any other microorganisms. Also, the study was limited to a specific production area at Bindura Innovation Hub, limiting the generalizability of the findings.

1.10. Definition of terms

Fermentation is a chemical process where bacteria, yeast or a combination of these microorganisms break down food into new components, often lowering pH and altering the flavor of the resulting product (Bamforth, 2019).

Yeasts are unicellular fungi that reproduce vegetatively through budding or fission and have sexual states that are not contained in a fruiting body (Boekhout et al., 2003).

Bacteria are ubiquitous, mostly free-living organisms often consisting of one biological cell (Winiecka-Krusnell, 2001).

Microbial composition refers to the collective microbial community present in a given sample. It encompasses the diversity, abundance and relative proportions of various microorganisms, including bacteria, archaea, fungi, viruses, and other microbial entities (Riensfeld et al., 2004)

Chapter 2

Literature review

2.1. Background of fermentation

Fermentation is one of the oldest and most fundamental food processing techniques known to humanity. This bioprocessing method utilizes the metabolic activities of various microorganisms, such as bacteria, yeasts, and molds, to transform raw materials into a diverse array of food and beverage products. One of the most well-known applications of fermentation is the production of fermented fruit juices and wines. The fermentation of fruit juices has its roots deeply embedded in human history. The pioneering work of Louis Pasteur in the 19th century further solidified the understanding of the role of yeasts in the fermentation of fruit juices (Fleet, 2003). Pasteur's observations of the spontaneous conversion of grape juice into wine demonstrated the innate association between yeasts and fruit products. He recognized that yeasts were the primary drivers of this transformation, consuming the sugars and producing ethanol and carbon dioxide as byproducts (Carpena *et.,al*, 2020). Building on Pasteur's insights, researchers have since delved deeper into the complex microbial ecology and biochemical changes that occur during the fermentation of fruit juices. It is now known that the fermentation process is not limited to just yeasts, but often involves a diverse consortium of microorganisms, including lactic acid bacteria, acetic acid bacteria, and molds, each playing a unique role in shaping the final flavor, aroma, and stability of the fermented product (Garcia *et.al*., 2019). The fermentation of fruit juices is not only a means of preserving and transforming these perishable raw materials, but also a way to impart desirable sensory characteristics (Anagnostopoulos, 2019). The metabolic activities of the microbes involved can produce a wide range of volatile compounds, organic acids, and other

secondary metabolites that contribute to the complex flavor profiles of fermented fruit juices and wines.

Furthermore, the fermentation process can also enhance the nutritional value of fruit juices by increasing the bioavailability of certain vitamins, minerals, and antioxidants. Some fermented fruit beverages, such as kombucha and kefir, even contain beneficial probiotic microorganisms that can promote gut health (Chong et al, 2023). Fermentation has evolved, so to have the techniques and technologies employed in the production of fermented fruit juices and wines. From traditional, artisanal methods to modern, carefully controlled industrial-scale processes, the fermentation of juices has become an integral part of the global food and beverage industry, offering consumers a diverse array of flavorful and nutritious products.

2.2. Yeasts and lactic acid bacteria

Boekhout et al. (2003) describe yeasts as unicellular fungi with sexual states that are not enclosed in fruiting bodies, and that reproduce vegetatively through budding or fission. Yeast taxonomy and specification are frequently determined by several physiological, biochemical, and DNA characteristics in addition to the morphology of the vegetative and sexual stages. In many different conditions, yeast can consume a broad range of food substrates. According to Holzapfel (1997), yeast species belonging to the genera *Saccharomyces*, *Candida*, *Torula*, and *Hansenula* use plant resources that contain fermentable sugars as their substrates. Yeasts are employed in many different industries, such as dairy, brewing, and medicines, as well as in the production of ethanol and biomass. They have been used for years to produce wines and other alcoholic drinks, and they are a key component in fermented foodstuffs like Indian idli, Nigerian fufu, and Korean nuruk (Nout, 2003).

Lactic acid bacteria (LAB) are a group of microaerophilic, Gram-positive organisms that play a significant role in the global food supply by fermenting hexose carbohydrates to produce lactic acid (Carr, 2002). These bacteria are essential for the production of a wide range of fermented foods and beverages, including dairy products, meats, vegetables, wine, coffee, sourdough, and various indigenous fermentations such as oji and mahewu. LAB are known to produce exopolysaccharides, which can improve the consistency and rheology of fermented milk products (Laws, 2001). Additionally, the interactions between yeast and LAB are frequently observed in the production of beverages and fermented foods, such as cheese, kefir, wine, and sausages (Bhalla, 2017).

2.3. Importance of yeasts and bacteria in the food industry

Fermented foods and beverages have played a pivotal role in human history, dating back to prehistoric times, as a means of preservation and manufacturing (Asghar *et,al.,* 2017). The global food industry relies heavily on the use of yeasts, primarily *Saccharomyces cerevisiae,* and lactic acid bacteria (LAB) to produce a wide variety of fermented products. Certain foods, such as yogurt, fermented vegetables, and sourdough bread, are fundamentally influenced by the presence of LAB during the manufacturing process (Etern, 2014). Yeast, on the other hand, plays a vital role in the production of all alcoholic beverages, contributing to the complexity and sensory quality of these fermented products. During fermentation, yeast cells convert cereal-derived sugars into ethanol and carbon dioxide, which are essential for the production of alcoholic beverages (Akbaria, 2012).

Yeasts, particularly *Saccharomyces cerevisiae,* play a crucial role in enhancing the flavors of fermented beverages, such as wine (Walker, 2016). During the fermentation process, yeasts synthesize a variety of minor metabolites, including volatile sulfur compounds, alcohols, acids,

ketones, polyols, and esters, which collectively define the unique flavor profile of the final product (Lindsay, 2007). The ability of different yeast strains to produce distinct metabolite profiles is the primary reason for the diverse flavors observed in various alcoholic beverages. *S.cerevisiae*, also known as baker's yeast, is widely used in the baking and confectionery industries due to its capacity to rapidly ferment sugars, which is directly related to its ability to leaven dough and promote the development of the gluten network (Parapouli *et.,al.* 2020). Additionally, baker's yeast produces aromatic compounds that contribute to the overall flavor of baked goods. The availability of baker's yeast in various forms, such as frozen, granular, compressed, instant, dry pellet, and cream, further highlights its versatility and importance in the food industry (Romano, 2013).

Yeasts are also found in meat and meat-based processed foods, where they play a beneficial role in the fermentation of these products. Various yeast species, such *as Yarrowia, Debaryomyces, Candida, Pichia, Trichosporon, Cryptococcus,* and *Rhodotorula*, have been isolated from fermented meat products, particularly sausages (Deek, 2003). These yeasts are reported to exhibit lipolytic and proteolytic activity, which contributes to the production of volatile compounds that enhance the flavor profile of the fermented meat products. Among these yeasts, *Debaryomyces hansenii* is particularly noteworthy, as it is frequently used as a commercial starter culture in the production of fermented meat products due to its ability to improve the overall quality of the final product (Toldra, 2007).

Yeasts, including species such as *Debaromyces hansenii, Yarrowia lipolytica, Kluyveromyces marxianus,* and *Saccharomyces* cerevisiae, play a vital role in the maturation and development of unique flavors and textures in cheese (Settani *et al.,* 2011). These yeasts are responsible for transforming lactose through fermentation, as well as facilitating lipolysis and proteolysis, which

are essential processes in cheese ripening. Furthermore, the fermentation of cereal-based foods, such as sourdough, typically involves a complex interplay between yeasts, bacteria, and fungi. In the case of sourdough, the combined activities of lactic acid bacteria and yeasts are crucial for its production. Yeasts play a crucial role in the fermentation of sourdough by breaking down fermentable sugars, which leads to the production of carbon dioxide, enhancing gas production and improving the crumb texture and nutritional value of the final product. Additionally, yeasts contribute to the development of unique flavors and aromas in sourdough (Chavan, 2011).

Additionally, yeasts also play a pivotal role in the production of coffee and cocoa, two widely consumed beverages. In the case of cocoa beans, they undergo fermentation processes that involve a variety of yeast species, including *Saccharomyces, Hanseniaspora, Candida, Issatchenkia,* and *Pichia*. This fermentation process is crucial for the development of the characteristic chocolate flavor in cocoa. Similarly, the processing of coffee beans involves the removal of pulp and other mucilaginous materials surrounding the seeds, and this process is facilitated by the activity of yeasts such as *Candida*, *Saccharomyces, Kluyveromyces*, *Saccharomycopsis*, *Hanseniaspora*, *Pichia,* and *Blastobotrys*. These fermentation processes are essential for the proper preparation of coffee beans before they can be further processed and transformed into the final coffee product (Masoud, 2005).

While yeasts possess numerous beneficial properties that contribute to the production and enhancement of various food products, they can also lead to food spoilage under certain conditions. Yeasts are particularly adept at thriving in environments with a pH of 5.0 or lower, as well as in the presence of readily available sugars, organic acids, and other carbon sources (Kurtzman, 2006). On a wide range of food matrices, including bread, dairy, fruits, and vegetables, fungi, particularly certain yeast species, are often identified as the primary

microorganisms responsible for food deterioration. Some of the yeast species that have been recognized as spoilage microorganisms include *Penicillium, Aspergillus, Fusarium, Candida, Rhodotorula, Kluyveromyces,* and *Debaryomyces*. These yeasts can thrive in the favorable conditions presented by various food products, leading to undesirable changes in the food's appearance, texture, and flavor, ultimately rendering it unfit for consumption. Therefore, while yeasts play a vital role in the production and maturation of many foods, their potential for causing food spoilage must be carefully managed to ensure food safety and quality (Schnürer & Magnusson, 2005).

2.4. Control of yeasts and bacteria during food processing

The most practical and effective way to destroy microorganisms such as yeasts and bacteria, is through the application of heat. These microorganisms thrive in warm temperatures, making temperature control a critical factor during food processing. One of the primary methods used to control yeasts and bacteria in food processing is pasteurization. Pasteurization involves the destruction of vegetative cells of disease-producing microorganisms, typically achieved by exposing the food to a temperature of 60°C for 30 minutes or 71.6°C for 16 seconds (Jay, 2005). At these pasteurization temperatures, yeasts, molds, and the vegetative cells of spoilage bacteria are effectively killed. The rate of microbial cell reduction increases significantly as the temperature is raised, even just slightly above the maximum growth temperatures. In the case of certain canned foods, the processing temperatures can reach as high as 115.56°C, with the duration of exposure varying depending on the product and can size, to ensure the complete elimination of microorganisms. By carefully controlling the temperature during food processing, the growth and proliferation of yeasts, bacteria, and other spoilage microorganisms can be effectively managed, thereby enhancing the safety and shelf-life of food products.

In addition to temperature control, the pH of the food product is another crucial factor in limiting the growth of yeasts and bacteria. Microorganisms have specific pH requirements for optimal growth, and by adjusting the pH to levels that are hostile to them, their proliferation can be effectively inhibited (Marayanan et al., 2016). To achieve the desired pH levels, the use of acidulants or alkalizing agents during food processing is essential. By carefully manipulating the pH, the food environment can be made less suitable for the growth and proliferation of yeasts and bacteria. Another strategy employed in food processing to prevent microbial contamination is the use of preservatives. Substances such as salt, sugar, and various chemical additives can be incorporated into the food to extend its shelf life and inhibit the growth of yeasts and bacteria. Furthermore, controlled fermentation can be utilized as a natural means of inhibiting the growth of these microorganisms. The fermentation process produces acids and other compounds that create an environment unfavorable for the proliferation of yeasts and bacteria. By ensuring that the fermentation process is properly managed, the growth of harmful microorganisms can be effectively prevented. Therefore, by manipulating the pH, using preservatives, and implementing controlled fermentation processes, food processing methods can successfully limit the growth and contamination of yeasts and bacteria, thereby enhancing the safety and shelf-life of food products.

Yeasts and bacteria require water to grow and multiply. By controlling the water activity of the food, the growth of microorganisms can be inhibited. However, it is crucial to ensure that the food is properly dehydrated or processed to reduce the water activity to levels below what is needed for microbial growth. Proper sanitation practices are essential to control yeasts and bacteria during food processing. Cleaning and sanitization of all equipment, surfaces, and utensils regularly prevents cross-contamination and the spread of harmful microorganisms. Also,

proper packaging can help control yeasts and bacteria during food processing. The use of airtight packaging materials that are impermeable to moisture and oxygen to inhibit the growth of harmful microorganisms prevents contamination from external sources hence, extending the shelf life of the food.

Controlling the water activity of food is a critical factor in limiting the growth and proliferation of yeasts and bacteria (Rojo et al., 2017). These microorganisms require the presence of available water to thrive, so by reducing the water activity to levels below their growth requirements, their ability to multiply can be effectively inhibited. To achieve this, it is vital that the food is properly dehydrated or processed to ensure the water activity is adequately lowered. This may involve techniques such as drying, evaporation, or the addition of solutes to the food matrix to decrease the availability of water for microbial growth. Alongside water activity control, maintaining proper sanitation practices during food processing is essential for managing the growth of yeasts and bacteria. Regular cleaning and sanitization of all equipment, surfaces, and utensils helps prevent cross-contamination and the spread of harmful microorganisms. Additionally, the use of proper packaging can contribute to the control of these microorganisms. The employment of airtight, moisture- and oxygen-impermeable packaging materials can help inhibit the entry of external contaminants and limit the growth of yeasts and bacteria, thereby extending the shelf life of the food product. By combining effective water activity control, precise sanitation practices, and the application of appropriate packaging, food processing methods can successfully limit the growth and contamination of yeasts and bacteria, ensuring the safety and quality of the final food product.

Commercial sterility refers to the process of destroying and inhibiting the growth of microorganisms that are of public health significance, as well as those that could potentially spoil

the food product, even if they are not directly harmful to human health (Barba et al., 2017). To achieve commercial sterility, microbiologists employ high-temperature treatments, such as autoclaving media at 250°F (121°C) for 15 or 20 minutes. These examples demonstrate the importance of both high temperatures and sufficient exposure time to effectively kill a population of bacteria. The combination of elevated temperature and extended duration is crucial for ensuring the complete inactivation of microbial contaminants. This comprehensive approach to sterilization is necessary to eliminate both pathogenic and spoilage-causing microorganisms, thereby maintaining the safety and quality of the final food product. By upholding high standards of commercial sterility through rigorous time and temperature control, food processing methods can effectively eliminate the microbiological risks associated with the presence of harmful and undesirable microorganisms.

2.5. Isolation of yeasts

Yeasts are ubiquitous organisms, having been recovered from a wide variety of aquatic and terrestrial environments, as well as the atmosphere. While some yeasts are widely distributed, others appear to be limited to specific habitats. Yeasts rarely occur in the absence of other microorganisms, such as molds or bacteria. Consequently, selective techniques are often employed to recover yeasts such as utilizing media that promote yeast growth while suppressing the growth of molds and bacteria. The composition of these selective media is influenced by the fact that yeasts are generally capable of developing at pH levels and water activities that inhibit the growth of bacteria. Antibiotics may also be used to selectively suppress bacterial growth, allowing for the isolation of yeasts. Incubation temperatures for yeast cultures are typically in the range of 20-25°C, as most yeasts are mesophilic. However, lower temperatures, between 4 and 15°C, are essential for the recovery of psychrophilic yeast taxa. In contrast, higher temperatures,

ranging from 30 to 37°C, are often required for the cultivation of yeasts that are closely associated with warm-blooded hosts or environments. By employing selective media, temperature control, and targeted techniques, researchers can effectively isolate and cultivate yeasts from diverse environmental sources, while accounting for the unique growth requirements and ecological niches of these ubiquitous microorganisms.

2.5.1. Use of acidic media

When acidifying culture media for the isolation of yeasts, either hydrochloric acid or phosphoric acid is typically the preferred choice over organic acids. The use of organic acids, such as acetic acid, is generally not recommended for general yeast isolation purposes. This is because organic acids, like acetic acid, are only slightly dissociated at pH levels around 3.5-4.0, and the high concentrations of undissociated acid forms can have an inhibitory effect on the growth of most yeast species. However, there are some notable exceptions to this general rule. Certain yeast species, such as *Zygosaccharomyces bailii*, *Z. bisporus*, *Schizosaccharomyces pombe*, and some strains of *Pichia membranifaciens* and similar species, are more tolerant of the higher concentrations of undissociated organic acids. These yeasts may be able to grow and thrive even in the presence of the inhibitory organic acid forms. Therefore, while hydrochloric and phosphoric acid are typically the preferred acidifying agents for general yeast isolation, the use of organic acids may be suitable for the selective recovery of specific yeast species that are known to be more resistant to their effects.

2.5.1.1. Solid media for direct isolation

When yeasts are present in high numbers, they can be isolated through the technique of plating the material or suspensions of the material on either acidified media or media containing antibiotics. In the case of acidified media, it is important to note that agar can undergo hydrolysis

when autoclaved at low pH levels. To address this, the sterilized molten agar is typically cooled to approximately 45°C before the determined volume of acid is added. The medium and acid are then rapidly mixed and immediately poured into Petri dishes. For example, the addition of approximately 0.7% (v/v) 1N hydrochloric acid (HCl) to YM agar and glucose-peptone-yeast extract agar usually results in the desired pH range of 3.7 to 3.8. This acidic pH can support the growth of many yeast species. However, it is important to note that some yeast species, particularly those within the genus *Schizosaccharomyces*, are inhibited by highly acidic media. These yeasts are better isolated on moderately acidic media with a pH range of 4.5 to 5.0. By carefully controlling the pH of the culture media, either through the addition of specific acids or the use of antibiotic-supplemented media, researchers can selectively isolate and recover a diverse range of yeast species from various sources.

2.5.1.2. Liquid media for enrichment purposes

When the number of yeasts present is low, their isolation may require enrichment techniques that favor the growth of yeasts over other microorganisms. In such cases, the material is inoculated into a liquid medium with a pH of 3.7 to 3.8. This procedure promotes the growth of fermentative yeast strains but may fail to recover aerobic yeast strains. This prevents molds from sporulating and causes them to aggregate into pellets, which are then outgrown by the yeasts. The yeasts can then be separated from the mold pellets by either allowing the pellets to settle and then streaking the yeast-containing suspension onto agar plates. This technique recovers both fermentative and non-fermentative yeast strains. Wickerham (1969), also described a useful medium, referred to as IM, for isolating yeasts from soil and insect frass. This medium contains Yeast Nitrogen Base, glucose, and six other carbon sources. The pH is not adjusted, and it drops after inoculation due to the utilization of the ammonium sulfate nitrogen source by the growing

organisms. By employing these enrichment and isolation techniques, researchers can effectively recover a diverse range of yeast species, including both fermentative and non-fermentative strains, from environments where they are present in low numbers.

2.5.2. Use of membrane filter

Yeasts can be recovered from liquid substrates, or from solid substrates by first washing to suspend the yeast cells, through the use of membrane filtration (Yarrow, 1998). The liquid substrate, or the suspension obtained from washing solid substrates, is passed through a membrane filter. The filter, which now retains the yeast cells, is then incubated on the surface of a selective agar medium. This membrane filtration technique is particularly useful for isolating yeasts when they are present in extremely low numbers within the sample. By concentrating the yeasts on the filter surface and providing them with a suitable growth medium, this method allows for the effective recovery and cultivation of yeast species that may otherwise be difficult to detect using conventional plating or enrichment techniques.

2.5.3. Use of media with high concentration of sugars

Yeasts generally have a higher tolerance for high sugar concentrations compared to many bacteria. This characteristic can be leveraged to facilitate the isolation of yeasts from samples that may contain a mix of microbial populations. By using culture media with relatively high sugar concentrations, the growth of bacteria can be inhibited or suppressed, while allowing yeasts to thrive and proliferate. The elevated sugar levels create an environment that is more favorable for yeast growth and development, effectively outcompeting the less sugar-tolerant bacterial species. This selective growth advantage of yeasts in high-sugar media is a useful tool for researchers and microbiologists when attempting to isolate and recover yeast strains from complex samples.

2.5.3.1. Solid media for direct isolation

To isolate osmophilic and osmotolerant yeasts from food products and juice concentrates with low water activity, a medium containing high concentrations of glucose, typically 30 to 50%, can be employed (Jermini, 1987). Suitable media for this purpose include glucose-peptone-yeast extract agar or YM agar. The selective action of these high-sugar media can be further enhanced by lowering the pH to around 4.5. This combination of elevated sugar levels and acidic pH creates an environment that is particularly favorable for the growth of osmotolerant yeast species, while inhibiting the development of many other microorganisms, including less osmotolerant yeasts and bacteria. This gradual reduction in sugar concentration allows for the adaptation and growth of the isolated osmotolerant yeast strains under less restrictive conditions, facilitating their further study and characterization. By utilizing this approach, researchers and microbiologists can effectively recover and isolate osmophilic and osmotolerant yeast species from food and beverage samples with low water activity, such as fruit juices, concentrates, and other high-sugar products. This selective isolation technique is a valuable tool for understanding the diversity and ecological roles of these specialized yeast populations in various food and industrial contexts.

2.5.3.2. Liquid media for enriching cultures

Yeasts can also be isolated by cultivating them in liquid media that contain high concentrations of glucose, typically ranging from 30 to 50%. Suitable liquid media for this purpose include glucose-peptone-yeast extract broth and YM broth. Another useful medium for isolating yeasts from soil and insect frass (waste) samples is the D-20 medium, as described by (Wickerham, 1969). This medium contains Difco Yeast Nitrogen Base, 20% glucose, 0.1% yeast extract, and 0.1% malt extract. The combination of a moderate sugar concentration, along with the added

nitrogen and growth factors, creates a selective environment that supports the growth and recovery of yeast species from these complex substrates. By employing these liquid media with high sugar levels, either in static or agitated conditions, researchers can effectively isolate and cultivate a diverse range of yeast strains from various environmental and industrial sources, including soils, insect habitats, and food or beverage products with low water activity.

2.5.4. Use of antibiotics and selective compounds

Media containing specific antibiotics can be employed as a last resort for isolating yeasts. These selective media may be formulated to target the recovery of particular yeast genera, species, or those with specific properties. The use of antibiotics, combined with other inhibitory compounds or selective carbon and nitrogen sources, forms the basis of these specialized isolation techniques. For example, Van der Walt and van Kerken (1961) described a medium containing cycloheximide and sorbic acid at pH 4.8 for the isolation of Dekkera species. Harder and Van Dijken (1982) used a medium with methanol as the sole carbon source, along with cycloserine and penicillin G, to inhibit bacterial growth and isolate yeasts capable of utilizing methanol. Similarly, Kwon-Chung et al. (1978) developed a medium with creatinine as the nitrogen source and diphenyl to reduce mold growth, for the isolation of *Filobasidiella neoformans*. These specialized, antibiotic-containing media can be valuable tools when other yeast isolation methods have proven ineffective, particularly for the recovery of specific yeast species or those with unique metabolic capabilities.

2.6. Isolation of bacteria

The ability to isolate and culture individual bacterial species is a fundamental practice in microbiology. Isolating bacteria allows for the study of their physiological, genetic, and biochemical characteristics, which is crucial for understanding their roles in various

environments and applications (Alain, 2009). Several techniques have been developed to selectively recover and cultivate bacterial strains from complex samples, each with its own advantages and limitations.

2.6.1. Dilution plating

The dilution plating method for bacterial isolation involves systematically diluting a sample containing a mixture of bacterial cells. As the sample is diluted, the individual bacterial cells become increasingly separated from one another. Aliquots of these diluted samples are then spread onto solid growth media, such as agar plates. On the agar surface, the isolated bacterial cells are able to proliferate into distinct, visible colonies. This separation and colony formation is crucial, as it allows for the isolation of pure bacterial cultures. Individual colonies, each derived from a single progenitor cell, can be selectively picked and transferred to fresh growth media. This approach of serial dilution and plating facilitates the isolation of pure bacterial isolates from complex, mixed samples. By steadily reducing the cell density through dilution, the technique ensures that individual bacterial cells are spatially separated on the agar medium. This, in turn, enables the recovery of unadulterated cultures of the constituent bacterial species present in the original sample.

2.6.2. Streak plating

Streak plating is another commonly employed technique for isolating bacteria. In this method, a sample containing a mixture of bacterial cells is directly inoculated onto the surface of a solid growth medium, such as an agar plate. Using a sterile loop or swab, the sample is then systematically streaked across the agar surface in a specific pattern. This streaking action creates a gradient of cell density across the plate. Regions with higher cell concentrations near the point of inoculation gradually transition to areas with fewer, more isolated cells towards the end of the

streak. This variation in cell density enables the isolation of individual bacterial colonies. As the cells proliferate on the agar, the regions with fewer initial cells give rise to discrete, well-separated colonies that can be easily picked and subcultured. Streak plating is a straightforward and effective way to obtain pure bacterial isolates from complex samples. The creation of the cell density gradient, through the systematic streaking of the inoculum, facilitates the recovery of unadulterated cultures of the constituent bacterial species present in the original sample.

2.6.3. Enrichment culture and selective media

Enrichment culture techniques leverage the selective growth of target bacteria by providing specific nutrients or growth conditions in the culture medium. For instance, incubating samples under anaerobic conditions can be used to isolate obligate or facultative anaerobic bacteria, as these microbes will be able to proliferate while aerobic species are suppressed. Similarly, the inclusion of selective substrates, such as methanol or urea, can enrich for bacteria capable of utilizing those compounds as their primary carbon or nitrogen sources. This approach of enrichment culturing often precedes the use of selective media to further purify the desired bacterial isolates. Selective media contain specific compounds, such as antibiotics, dyes, or inhibitory agents that allow the growth of certain bacterial groups while inhibiting the proliferation of others. This strategy is particularly useful for isolating fastidious or slow-growing bacteria from complex samples containing diverse microbial populations, as the selective agents can suppress the growth of more competitive species. By leveraging the unique nutritional requirements or growth conditions preferred by target bacteria, enrichment culturing techniques enable the selective isolation of these microorganisms. The subsequent use of selective media further refines the purity of the bacterial isolates, allowing the study the of physiology, genetics, and other characteristics of the desired bacterial species in isolation.

2.6.4. Membrane filtration

Membrane filtration is another technique employed for bacterial isolation. This method involves passing a liquid sample, containing a mixed population of bacterial cells, through a porous membrane (Yarrow, 1998). As the sample is filtered, the bacterial cells become trapped within the membrane's pores. After filtration, the membrane bearing the trapped bacterial cells can then be transferred directly to growth media, such as agar plates. This allows the captured cells to proliferate and form distinct colonies, which can be individually selected and subcultured for further study. The key advantage of membrane filtration is that it physically separates the bacterial cells from the liquid sample, effectively concentrating the cells and removing potential inhibitors or contaminants present in the original sample. By trapping the cells on the membrane surface, this technique facilitates the isolation of bacteria from complex matrices, such as water or soil samples, where the target microorganisms may be present at low abundances.

2.6.5. Micromanipulation

Micromanipulation is a technique that uses a microscope-mounted micromanipulator to physically isolate individual bacterial cells or colonies. This approach involves the precise, manual capture and transfer of targeted microbial cells or small colony fragments using a specialized micromanipulator instrument. The process typically begins with the examination of a sample under a microscope, which allows to visually identify and select individual cells or small groups of cells of interest. The micromanipulator, equipped with a fine-tipped glass or metal probe, is then precisely maneuvered to gently capture the selected microbial target. Once isolated, the captured cells or colony fragments can be transferred to a fresh growth medium, such as an agar plate, for further cultivation and isolation of pure cultures. This direct, microscope-guided manipulation of individual bacterial cells or colonies enables the recovery of

pure isolates from complex samples, even when the target microorganisms are present at very low abundances.

2.6.6. Immunomagnetic

Immunomagnetic separation is a technique that utilizes antibody-coated magnetic beads to capture and isolate target bacteria from complex samples. In this method, the sample containing a mixture of bacterial cells is incubated with the antibody-functionalized magnetic beads. The antibodies on the bead surface bind specifically to target bacterial cells, effectively "capturing" them. By applying a magnetic field, the beads with the bound target cells can then be separated from the rest of the sample. This selective capture and magnetic separation allow for the efficient isolation of the desired bacterial cells from the complex mixture.

2.7. Enumeration of yeasts and bacteria

The enumeration of bacteria and yeasts is a crucial step in microbiology to determine the population size of these microorganisms in a given sample (Harrigan, 1998).There are several methods used for enumerating bacteria and yeasts.

2.7.1. Direct microscopic counts

This method involves counting the cells directly under a microscope using a counting chamber or a hemocytometer. It provides quick results but may not differentiate between live and dead cells.

2.7.2. Viable plate counts

Viable plate counts involve serial dilution of the sample followed by plating on agar plates. After an incubation period, colonies formed are counted to estimate the viable cell count. This method provides information on only viable cells.

2.7.3. Membrane filtration

In this method, the sample is passed through a membrane filter with specific pore sizes that trap bacteria and yeasts. The filter is then placed on a suitable growth medium for colony formation and enumeration.

2.7.4. Most Probable Number (MPN) Method

The MPN method is based on statistical probability calculations from multiple tube dilutions to estimate the microbial population in a sample. It is commonly used for samples with low microbial counts.

2.7.5. Modern molecular techniques

Polymerase chain reaction (PCR), quantitative PCR (qPCR), next-generation sequencing (NGS), and metagenomics are advanced molecular techniques used for enumerating bacteria and yeasts based on their genetic material. These methods provide high sensitivity and specificity in identifying microbial populations.

2.8. Identification of yeasts and bacteria

Identifying bacteria and yeasts to species or strain level is essential for various fields such as clinical diagnostics, food safety, environmental monitoring, and biotechnology (Hameed, 2018). Several traditional and molecular methods are employed for accurate identification.

2.8.1. Phenotypic methods

Phenotypic characteristics like morphology, staining properties, growth requirements, and metabolic activities are used for preliminary identification of bacteria and yeasts.

2.8.2. Biochemical tests

Biochemical tests such as catalase test, oxidase test, sugar fermentation tests, help in differentiating between bacterial species based on their metabolic capabilities.

2.8.3. Molecular methods

Molecular techniques like 16S rRNA gene sequencing for bacteria or internal transcribed spacer (ITS) sequencing for yeasts provide highly accurate identification at the genetic level. These methods are rapid and reliable compared to traditional approaches.

2.9. Characterization of yeasts and bacteria

Characterizing bacteria and yeasts involves understanding their physiological traits, pathogenicity factors, antibiotic resistance profiles, virulence factors, genetic relatedness and ecological roles. Various methods are employed for characterizing these microorganisms.

2.9.1. Antibiotic susceptibility testing

Determines the susceptibility of bacteria or yeasts to specific antibiotics using methods like disk diffusion or broth microdilution assays.

2.9.2. Virulence factor analysis

Identifies genes or proteins associated with virulence in pathogenic strains through molecular techniques like PCR or genome sequencing.

2.9.3. Genomic analysis

Whole-genome sequencing helps in understanding the genetic makeup of bacteria and yeasts, aiding in phylogenetic analysis, evolutionary studies, and functional genomics research.

Chapter 3

Materials and methods

3.1. Sample collection

Four bottles of fermented masau juice (figure 1), were randomly selected from BUSE Innovation Hub. This was done through visual observation on the bottles which had changed shape and reduction in volume of the juice, which shows fermentation. The bottles were transported to the laboratory for tests at Astra Campus Laboratory of Bindura University of Science Education.

Figure 1. Fermented masau juice samples

3.2. Preparation of Media

Agar is a gelatinous substance derived from seaweed that is widely used in microbiology for the cultivation and identification of microorganisms. It provides a solid surface for microbial growth, allowing researchers to isolate and study different types of bacteria and fungi. Agar is

particularly useful in microbial identification because it can be customized with specific nutrients and indicators to encourage the growth of certain organisms while inhibiting others. In this study, Sabouraud Dextrose Agar, Potato Dextrose Agar were used to isolate yeasts and Nutrient agar was used to isolate bacteria in fermented masau juice as showed in figure 2.

Sabouraud Dextrose agar, is a type of agar medium used in microbiology to culture and identify different microbes, particularly yeast and fungi. The characteristic growth patterns, colony morphology, color, texture, and other features observed on the SDA plate can provide valuable information for identifying the specific microbe present in the sample.

Potato Dextrose Agar, is a common medium used to culture and identify different microbes particularly useful for fungi and molds due to its high carbohydrate content from dextrose and potato infusion. Colonies of different shapes, sizes, colors, and textures will appear on the agar surface. These characteristics can provide initial clues about the identity of the microbe.

3.2.1. Preparation of nutrient agar

Measured 9g of Nutrient agar, was dissolved in 200ml of distilled water then boiled until the ingredients were dissolved completely. The mixture was autoclaved at 121˚C for 15 minutes. After sterilization, the agar was left to cool down before poured into petri dishes.

3.2.2. Preparation of Sabouraud Dextrose agar (SDA)

Measured 13g of Sabouraud Dextrose Agar and was dissolved in 200ml of distilled water then heated on a flame to completely dissolve the ingredients. The mixture was sterilized by autoclaving at 121°C for 15 minutes. After sterilization, the agar was left to cool down around 45-50°C.

3.2.3. Preparation of Yeast extract

Measured 10g of yeast extract and was dissolved in 200ml of distilled water. The mixture was heated on a flame to dissolve the ingredients completely and sterilized by autoclaving at 121°C for 15 minutes. After sterilization, the agar was allowed to cool around 45-50°.

3.2.4. Preparation of Potato Dextrose agar (PDA)

Measured 7.8g of Potato Dextrose Agar and was dissolved in 200ml of distilled water. The mixture was mixed to dissolve the ingredients completely and sterilized by autoclaving at 121°C for 15 minutes. After sterilization, the agar was allowed to cool around 45-50°.

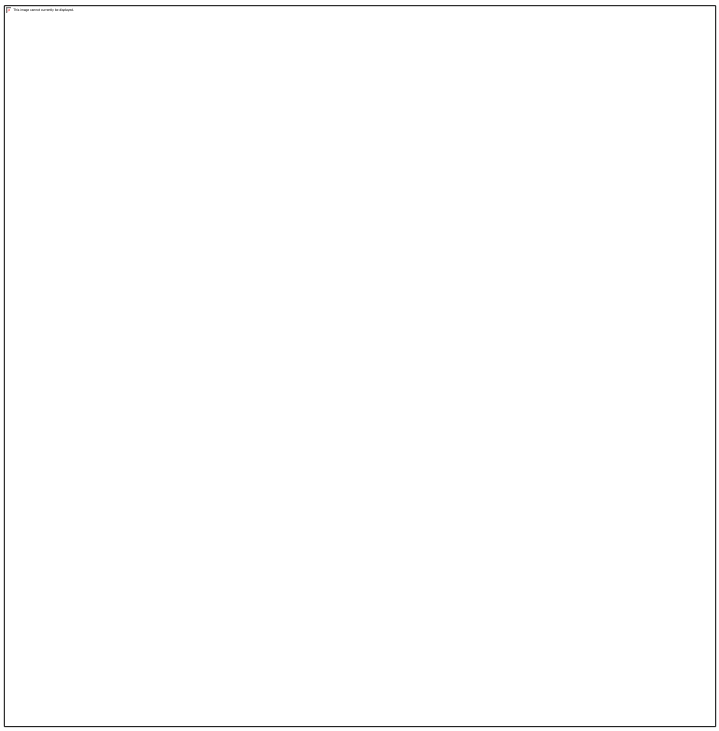

Figure 2. Preparation of agar

3.3. Serial dilution

To perfom the serial dilutions of the masau juice samples, 9ml of distilled water was dispensed into 40 test tubes, with 10 test tubes replicating each sample and autoclaved for 15 minutes. 1ml of each juice sample was added into 9ml of distilled in the first test tube to make a dilution factor of 1×10^{-1} and serial dilutions was carried out up to dilution factor 1×10^{-9}. 1ml of juice sample with serial dilutions of 1×10^{-9} and 1×10^{-7} were transferred using a pipette into petri dishes. Adopting the pour plate method, SDA and PDA was poured into petri dishes and after solidification the plates were incubated for the growth of yeasts at 37˚C for 48 hours. Nutrient agar was also poured into petri dishes with 1ml of inoculum with serial dilutions of 1×10^{-9} and 1×10^{-7} and after solidification the plates were incubated for the growth of bacteria at 37˚C for 48 hours.

3.4. Colony count

The colonies present were counted using a colony counter and number of colonies was recorded for each media and dilution factor as shown in figure 3.

Figure 3. Colony count of bacteria and yeast isolates

3.5. Morphological characteristics

Colonies obtained from PDA, SDA and Nutrient agar were observed for color, opacity, margin, form, elevation, size and surface.

3.6. Isolation of yeasts

Yeasts colonies were isolated from agar plates and subcultured in yeast extract. Plates were incubated at 37C for 48hours and identified.

3.7. Isolation of bacteria

Isolation and identification of bacteria was done by adopting the methods outlined by Ruangpan and Tendencia (2004) and Muhammad, Iqra and Muhammad (2020).

Gram's staining technique

Measured 20 grams of crystal violet dye and was added to 100 ml of ethanol to create a concentrated stock solution. 1 gram of ammonium oxalate was then dissolved in 100 ml water to make a separate stock solution. The working solution was obtained by mixing 1 ml of the crystal violet stock solution with 10 ml of water and 40 ml of the oxalate stock solution. The mixture was stored in a dropper.

Gram Iodine Solution:

Measured 1 gram of iodine, 2 gram of potassium iodide and 3 gram of sodium bicarbonate and added in 300 ml of water.

Gram Decolorizer Solution:

A mixture of equal volumes of 95% ethanol and acetone was made.

Gram Safranin Solution:

Measured 2.5 grams of Safranin O and was dissolved in 100 ml of 95% ethanol to make a stock solution. The working solution was obtained by diluting one part of the stock solution with five parts of water.

Method

An inoculating loop was heated on the Bunsen burner and a drop of water was transferred onto a slide. The inoculum was transferred by an inoculating loop onto the glass slide. The culture was spread creating a thin film in a circular manner. The glass slide was gently flamed and air dried. 5 drops of Crystal Violet were added on the glass slide for 60 seconds and rinsed off with water. Iodine solution was poured off to cover the fixed culture for 30 seconds and the excess was rinsed off with water. The decolorizer, Acetone was added to let the solution trickle down the slide and after 5 seconds, it was rinsed off with water. 5 drops of Safranin solution were added as

a counterstain for 20 seconds. The solution was poured off and the excess rinsed with water. A blotting paper was used to remove excess water. The slide was examined on the light microscope at X4, X10, X40 and X100 magnification, where an oil immersion was used. Gram positive and Gram-negative bacteria were differentiated.

Chapter 4

Results

4.1. Colony morphology

The yeast species isolated from fermented masau juice were *Saccharomyces cerevisae, Aureobasidium pullulans, Hanseniaspora opuntiae, Saccharomycopsis fibuligera* and *Pichia kudriavevi.* Table 1 shows the morphological characteristics of the yeast colonies.

Table 1. Morphologial characteristics of yeast colonies

colony morphology	**colony 1**	**colony 2**	**colony 3**	**colony 4**	**colony 5**
color	cream	white	yellow	cream	Greenish
opacity	opaque	translucent	opaque	opaque	Translucent
margin	smooth	irregular	lobate	entire	Undulate
form	round	oval	circular	ellipsodial	Irregular
elevation	flat	raised	convex	flat	Raised
size	punctiform	puntiform	punctiform	punctiform	Punctiform
surface	smooth	wrinkled	rough	smooth	Granular

The dominant bacterial species isolated from fermented masau juice *were Lactobacillus minor, L .agillis, L. fermentum, L. fructosus and L. divergens*. Table 2 shows the morphological chracteristics of the bacterial colonies.

Table 2. Morphological characteristics of bacterial colonies

colony morphology	colony 1	colony 2	colony 3	colony 4	colony 5
color	white	yellow	cream	pink	Brown
opacity	opaque	translucent	opaque	opaque	Translucent
margin	smooth	irregular	entire	undulate	Lobed
form	circular	irregular	circular	oval	Filamentous
elevation	flat	raised	convex	umbonate	Raised
size	small	medium	large	small	Large
surface	smooth	rough	wrinkled	glistening	Rough

4.2. Evaluation of yeast colonies

The data provided (table 3), compares the results of colony counts on samples (A, B, C, and D) under Dilution A 10^7 and Dilution B 10^9 from SDA media ($f=1.787$ $p=0.3131$)

Table 3. Assessment of yeast colonies form SDA

Masau juice sample	Dilution A 10^7	Dilution B 10^9
A	330	267
B	560	540
C	497	215
D	230	198
Mean	404.25	305

Standard deviation	151.368	159.393
Standard error	75.684	79.696

The data provided (table 4), compares the results of colony counts on samples (A, B, C and D) under Dilution 10^7 and 10^9 on PDA (f=0.0007245 p=0.9399)

Table 4. Assessment of yeast colonies isolated from PDA

Masau juice sample	Dilution 10^7	Dilution10^9
A	600	461
B	803	455
C	570	506
D	703	673
Mean	669	523.75
Standard deviation	105.947	102.070
Standard error	52.973	51.035

4.3. Identified yeast species in fermented masau juice

Figure 4 shows the identified yeast species from fermented masau juice during the study

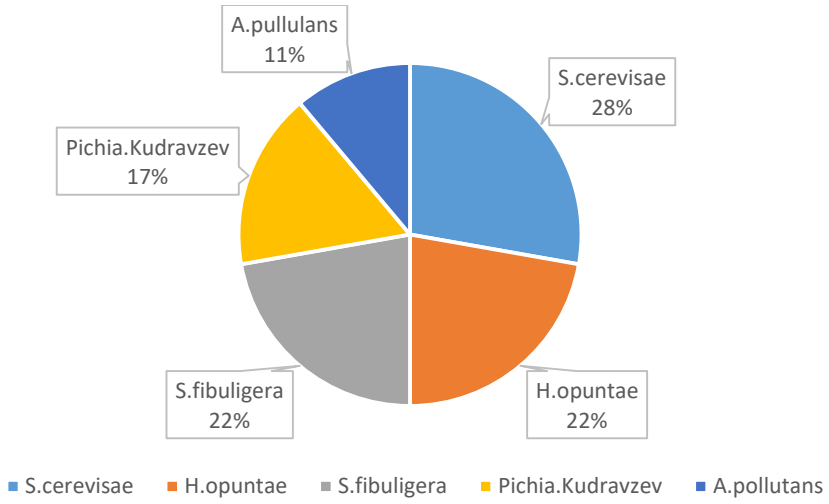

Figure 4. Identified yeast species

4.4. Evaluation of bacterial colonies

The data provided (table 5), compares the bacterial colonies isolated in nutrient agar under Dilution 10^7 and 10^9 (f=0.3691 p=0.6053)

Table 5. Bacteria colonies isolated in nutrient agar

Masau juice sample	Dilution 10^7	Dilution10^9
A	560	267
B	1020	538
C	738	715
D	730	680
Mean	762	550
Standard deviation	190.585	190.585

Standard error	95.293	101.800

4.4.1. Identification of bacteria species

Table 6, shows the physiological identification of bacterial species in fermented masau juice using gram staining.

Table 6. Physiological identification of bacterial species

Bacterial species (Lactobacillus)	A	B	C	D	Total
L.minor	+	+	+	+	4
L.agilis	+	+	+	+	4
L.fermentum	+	+	+	+	4
L.fructosus	+	+	-	_	2
L.divergens	+	-	-	+	2
Totals bacterial strains	5/5(100%)	4/5(80%)	3/5(60%)	4/5(80%)	

4.4.2. Identified bacteria species in fermented masau juice

Figure 5, shows the bacteria species that were identified during the study.

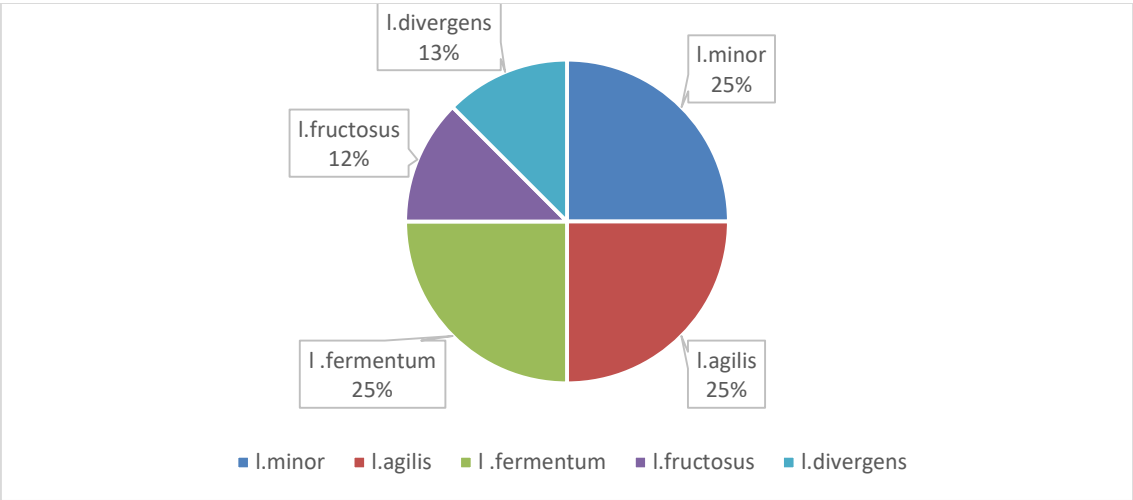

Figure 5. Identified bacteria species

Chapter 5

Discussion

5.1. Discussion

Indigenous fruit trees play a crucial role in the diets and livelihoods of communities across Africa. These fruits provide essential energy and nutrients, including valuable vitamins and minerals. In many regions, these indigenous fruits are traditionally fermented into various beverages. This study specifically focused on one of the most widely utilized wild fruits in Zimbabwe, *Ziziphus mauritiana*, locally known as "masau.". It aimed to document the biodiversity of yeast species and dominant Lactobacillus species involved in the traditional fermentation of masau fruit juice. To achieve this, the current study employed isolation and identification methods to characterize the strains of yeasts and Lactobacillus bacteria that are prevalent in the fermented masau juice.

5.1.2. Assessment of yeast species in fermented masau juice

Yeasts have found widespread applications in various industries, such as the pharmaceutical, brewing, and dairy sectors, as well as in the production of biomass and ethanol. Yeasts have been utilized for thousands of years in the creation of wines and other alcoholic beverages, and they have been found to be the predominant microorganisms in traditional fermented foods like Korean nuruk, Nigerian fufu, and Indian idli (Nout, 2003). During the study on quantitative analysis of yeast colonies among samples A,B,C and D using serial dilution of 10^7 and 10^9 on SDA media, it was noted that the number of yeast colonies for all samples on dilution 10^7 had a mean colony count of 404.25 compared to dilution of 10^9 (mean 159.393), the variance and standard deviation of the second dilution counts was higher compared to the initial serial dilution

of 10^7 , this is because as the dilution becomes weak the yeast cells become less concentrated in terms of limited amount of yeast cell to replicate compared to yeast cell in high concentrated solutions. Compared to SDA media, the appearance of yeast colonies on PDA was highly intergrated symbolizing that the PDA media is better in terms of yeast growing properties compared to SDA. The higher standard deviations in both dilutions indicate that there was substantial variability in the microbial counts across the different masau juice samples. This could be due to factors such as variations in the ripeness, maturity, or handling of the fruits, or differences in the microbial compositions of the individual masau fruits.

5.1.3. Biodiversity evaluation of yeast species in fermented masau juice

Methods used for identification of yeasts at species and strain levels can be classified roughly into two groups based on either phenotypic- or genotypic methodologies. Phenotypic methods include standard taxonomical tests, most recently described in the current taxonomic key edited by Kurtzman et al. (2011) as well as several older publications such as the simplified identification method (SIM) described by (Deak, 2007). All these methods are based on morphological and physiological characteristics (colony and cell morphology, growth conditions, assimilation and fermentation of carbohydrates as well as nitrogen compounds, osmotolerance). In the current study, all samples of fermented masau juice contained diverse yeast population, with 5 different yeast species identified, suggesting a complex microbial community involved in the traditional fermentation process. *Saccharomyces cerevisiae* is the most dominant yeast species, accounting for 28% of the total yeast population. *Saccharomyces cerevisiae* is a well-known and widely used yeast in many fermentation processes, including wine and beer production. *Hanseniaspora opuntiae* and *Saccharomycopsis fibuligera* each account for 22% of the total yeast population. These yeast species are also commonly found in traditional fermented

foods and beverages and can contribute to the development of unique flavor profiles. *Aureobasidium pullulans* and *Pichia kudriavzevii* have lower proportions, at 11% and 17% respectively. These yeast species may play supporting roles in the fermentation process or contribute to specific metabolic activities. The co-existence of these diverse yeast species suggests potential synergistic or antagonistic interactions within the microbial community. Each yeast species may contribute unique metabolic capabilities, enzyme activities, and other functional traits that collectively shape the fermentation dynamics and the final product characteristics. The observed yeast diversity in the fermented masau juice is an important aspect of the traditional fermentation process. Maintaining this diverse yeast population can help preserve the unique sensory properties, nutritional value, and cultural significance of the traditional masau juice fermentation. Expectedly, *S. cerevisiae* is the most frequently occurring yeast in alcoholic beverages and has been reported to dominate the fermentation in 93% of the indigenous sub-Saharan African alcoholic beverages. *S. cerevisiae* has additionally been reported to dominate the fermentation of the fermented dairy products. The biodiversity seen in isolates of yeasts has also been observed in other studies on African indigenous fermented foods and beverages (Jespersen, 2003).

5.1.4. Assessment of bacteria species in fermented masau juice

The total bacteria counts across the 4 masau juice samples showed considerable variation. At dilution 10^7, the counts range from 560 to 1020, with a mean of 762 and at dilution 10^9 the counts range from 267 to 715, with a mean of 550. The standard deviation of 190.585 indicates a significant level of variability in the yeast counts across the samples. This suggests that the masau juice fermentation process may have some inconsistencies or heterogeneity between different samples. The variability in bacteria counts could be influenced by factors such as

differences in the starting masau juice composition, fermentation conditions, or microbial interactions during the fermentation process.

5.1.5. Evaluation of the biodiversity of bacterial colonies in fermented masau juice

Yeasts are not the only organisms of importance in the microbiology of fruit and fruit products. Bacteria, especially lactic acid bacteria (LAB) and acetic acid bacteria, also play a prominent role in the spoilage of some fruits and fruit products (Fleet, 2003). Furthermore, certain species of LAB can make positive contributions to the production of wines. In most indigenous fermentation processes, yeasts are found coexisting with LAB. This co-occurrence of yeasts and LAB was also observed in the fermented masau juice. In the current study, the data showed the presence of 5 different Lactobacillus species across the 4 fermented masau juice samples (A, B, C, and D). The most prevalent Lactobacillus species found in the juice samples are *L. minor*, *L. agilis*, and *L. fermentum*, as they are present in all 4 samples. *L. fructosus* and *L. divergens* are less prevalent, being present in only 2 out of the 4 samples. This indicates a moderately diverse bacterial population in the fermented masau juice, with multiple Lactobacillus species contributing to the overall microbial community. Sample A contains all 5 identified Lactobacillus species, representing the highest bacterial diversity among the samples. Samples B and D contain 4 out of the 5 Lactobacillus species, indicating slightly lower bacterial diversity compared to Sample A. Sample C contains the lowest number of Lactobacillus species, with only 3 out of the 5 species present. Lactobacillus species are commonly found in traditional fermented foods and beverages, as they play a crucial role in the fermentation process. These lactic acid bacteria can contribute to the development of desirable flavor profiles, enhance the nutritional value, and inhibit the growth of potentially harmful microorganisms in the fermented masau juice. The presence of diverse Lactobacillus species in the fermented masau juice suggests a

well-established and stable bacterial community that can contribute to the overall quality and safety of the final product. The variation in bacterial diversity across the samples may indicate differences in fermentation conditions, microbial inoculum, or other factors that influence the bacterial community composition.

Chapter 6

Summary, recommendations and conclusion

6.1. Summary

Fermentation of masau juice is a natural process that involves the activity of various organisms primarily yeast and bacteria which play a crucial role in the transformation of sugars into alcohol and other metabolites. This study aimed to identify the yeast and bacteria in fermented masau juice. 4 samples of fermented masau juice were collected from the BUSE Innovation HUB. The samples were plated onto SDA and PDA to encourage the growth of yeasts and Nutrient agar to allow growth of bacteria. The plates were incubated for 48hours at 37^{o}C. Once isolated, yeast colonies were examined based on their morphological characteristics and gram staining was performed to identify bacteria species. In this study the dorminat biodiversity of bacterial species in fermented included *L. minor, L. agilis, L. fermentum, L. fructosus* and *L. divergens* and the dominant yeast species from the study were *S. cerevisae, Aureobasidium pullulans, Hanseniaspora opuntiae, Saccharomycopsis fibuligera* and *Pichia kudriavzevi*. The findings from this research have highlighted the need for more controlled and consistent fermentation practices to enhance the quality and safety of traditional masau beverages, while still preserving their unique sensory attributes. The uncontrolled nature of the traditional fermentation process can lead to inconsistencies in product quality and potential food safety concerns.

6.2. Recommendations

Further research must be conducted through molecular identification methods since the laboratory results are preliminary and being based on phenotypic properties. Conduct in-depth characterization of the isolated yeast and bacterial strains, including their physiological,

biochemical, and genetic properties. This information can provide insights into the specific roles and functionalities of the microorganisms in the traditional fermentation process.

To ensure the safety and quality of freshly extracted masau juice, a pasteurization step should be implemented to inactivate naturally occurring yeast and bacterial populations, identifying the optimal time-temperature combination that effectively eliminates spoilage organisms while preserving sensory and nutritional properties. Strict hygiene practices must be followed during harvesting, transportation, and processing to minimize initial microbial load, using clean, sanitized equipment for juice extraction and aseptic handling techniques to prevent post-processing contamination. The pasteurized juice should be packaged in sterilized, airtight containers to protect against microbial re-entry. Additionally, evaluating food-grade preservatives like benzoates, sorbates, or sulfites can help inhibit microbial growth, with careful optimization of type and concentration to maintain sensory qualities and consumer acceptance. Finally, the packaged juice should be stored at low temperatures (4-8°C) to slow down any remaining microbial activity, ensuring that the cold chain is maintained throughout distribution and storage to preserve product integrity and extend shelf life.

6.3. Conclusion

The identification of the yeast and bacterial species involved in the traditional fermentation of *Ziziphus mauritiana* (masau) fruit in Zimbabwe have provided valuable insights into the microbial diversity and dynamics underlying this culturally significant process. The findings have highlighted the need for more controlled and consistent fermentation practices to enhance the quality and safety of traditional masau beverages, while preserving their unique sensory attributes. By developing a comprehensive understanding of the key microbial players and their roles in the fermentation, this research has laid the foundation for the development of improved,

tailor-made starter cultures and fermentation protocols. This can contribute to the valorization and sustainable commercialization of traditional masau beverages, ultimately benefiting local communities and promoting the preservation of indigenous food processing knowledge in Zimbabwe and beyond.

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Appendices

Appendix 1 Summary statistics table for analysis of differences in yeast colonies in each dilution of samples in SDA

Coefficients:

Estimate Std. Error t value Pr (>|t|)

(Intercept) 12.5900 229.9610 0.055 0.961

Dilution A 0.7233 0.5411 1.337 0.313

Residual standard error: 141.9 on 2 degrees of freedom

Multiple R-squared: 0.4719, Adjusted R-squared: 0.2078

F-statistic: 1.787 on 1 and 2 DF, p-value: 0.3131

Appendix 2 Summary statistics table for analysis of difference of yeast colonies in each dilution of samples in PDA

Coefficients:

Estimate Std. Error t value Pr (>|t|)

(Intercept) 485.02930 459.18022 1.056 0.402

Dilution A 0.05788 0.68000 0.085 0.940

Residual standard error: 124.8 on 2 degrees of freedom

Multiple R-squared: 0.003609, Adjusted R-squared: -0.4946

F-statistic: 0.007245 on 1 and 2 DF, p-value: 0.9399

Appendix 3 Summary statistics table for analysis of differences of bacterial colonies in each dilution of samples in nutrient agar

Coefficients:

Estimate Std. Error t value Pr (>|t|)

(Intercept) 228.6772 541.1321 0.423 0.714

Dilution A 0.4217 0.6941 0.608 0.605

Residual standard error: 229.1 on 2 degrees of freedom

Multiple R-squared: 0.1558, Adjusted R-squared: -0.2663

F-statistic: 0.3691 on 1 and 2 DF, p-value: 0.6053

Appendix 4 Comparative analysis of colonies using paired t test evaluation

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Dilution107Totalyeastcounts	12.629	3	.001	669.000	500.42	837.58
@109Totalyeastcounts	10.263	3	.002	523.750	361.33	686.17