MICROBIAL ANALYSIS OF LOCAL ALCOHOLIC BEVERAGES USING PALM WINE AS A CASE STUDY

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OCTOBER 2025.

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A RESEARCH PROJECT SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY, IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF DEGREE OF B.Sc. (HONS) IN MICROBIOLOGY, UNIVERSITY OF BENIN, BENIN CITY.

OCTOBER 2025.

CERTIFICATION

This is to certify that this project work was carried out by **TREASURE OSAKAMWENYOR EGHE** with matriculation number **LSC2103930**, of the Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City, Edo State, Nigeria, under my supervision.

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DEDICATION

This project is dedicated to the Almighty God for His ever-guiding presence, strength and grace during the course of this study and my academic journey in general.

ACKNOWLEDGEMENTS

First and foremost, my profound gratitude goes to God Almighty for His great show of love, mercy, kindness and peace in my life.

My gratitude goes to the Holy spirit, whom with His aid and guidance, I have come thus far.

My sincere appreciation goes to my supervisor, Dr. (Mrs.) C.G. DIMOWO, for her commitment, encouragement and insights towards the success of this work. I also express my gratitude to the Head of Department, Microbiology, PROF. E.O. IGBINOSA, for his support towards the Department and students in general.

I acknowledge my course advisers, Mr. G.O. Oribhabor and Dr. (Mrs.) O.B. Agbonwaneten for their unwavering support throughout my years of study.

I am also grateful to all lecturers who have in one way or another impacted me throughout this journey.

My deepest gratitude goes to my immediate family, my parents most especially. For every ounce of support, care and love, I am extremely grateful.

I am thankful to my friends, Sarah, Faith and Esohe for their presence in my social and academic life. The journey was memorable because of you girls.

Finally, thanks to all MCB 21 graduating class for this wondrous journey together.

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ABSTRACT

Microbial analysis in this study was conducted on palm wine samples gotten from five locations in Benin City. The samples were collected from different palm wine bars in the different locations. The microorganisms isolated were identified based on their various morphological, cultural and biochemical characteristics. The microbial analysis of palm wine in this study revealed the presence of diverse microorganisms including *Lactobacillus*, *Acetobacter, Enterobacter, Bacillus*, and *Staphylococcus* specie as well as *Saccharomyces cerevisiae, Candida* sp., and *Aspergillus* sp. The highest heterotrophic bacterial count was recorded for Sample A, in which the values were too numerous to count for the first dilution and $12\pm1.00\times10^{4}$cfu/ml for the fourth dilution plated. This was followed by Sample E with values of $1.4\pm1.00\times10^{2}$cfu/ml and $8.0\pm1.73\times10^{4}$cfu/ml for both dilutions plated. The total fungal counts were recorded as $2.06\pm0.58\times10^{2}$cfu/ml and $1.6\pm1.00\times10^{4}$cfu/ml for Sample A**,** $2.54\pm1.53\times10^{2}$cfu/ml and $8.6\pm2.08\times10^{4}$cfu/ml for Sample B and $4.0\pm0.81\times10^{1}$cfu/ml and $2.6\pm0.58\times10^{4}$cfu/ml for Sample C. Sample D was recorded as $2.26\pm1.53\times10^{2}$cfu/ml and $2.24\pm0.58\times10^{5}$cfu/ml while Sample E was recorded as $2.24\pm5.69\times10^{2}$cfu/ml and $5.4\pm1.53\times10^{4}$cfu/ml. The lowest count was found in Sample B in which the value was $2.6\pm0.58\times10^{1}$cfu/ml for the first dilution plated and no growth was recorded for the 10^{4} dilution The presence of *Saccharomyces cerevisiae* and *Lactobacillus spp.* with a 100% occurrence in the samples showed their usefulness in the fermentation of the beverage. Some of the organisms isolated such as the coliforms, indicated unhygienic handling of the beverage. It is therefore pertinent that hygiene practices are taken into great considerations in the production and distribution of the beverage.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

The role of microorganisms in various aspects of the ecosystem cannot be overemphasized. Their diversity and ubiquitousness are evident in our everyday life ranging from putrefaction to maintaining a sustainable biosphere. They are known for their positive and negative roles in the environment. In the health sector, they are known to cause various diseases in humans. Plants and livestock are not excluded as lots of disease-causing microbes have been reported in the agricultural sectors. In a positive light, vaccines and antimicrobials have been generated by the aid of microbes. They are also used for bioremediation purposes whereby microorganisms are used to clean up our environments in cases such as oil spills and refuse degradation. They have also been known to form mutual associations with higher organisms creating a more suitable environment for these organisms. These associations include, *Rhizobium* sp. and the root nodule of leguminous plants, microorganisms and the human gut etc. (Khorsheed, 2024).

Microorganisms play very crucial roles in food production and processing, serving as both beneficial agents in fermentation and potential hazards that cause spoilage and foodborne illnesses. Microorganisms can be used to produce food additives such as citric acids, flavour enhancers and pH adjusters. Some microorganisms such as *Lactobacillus plantarum* and *Saccharomyces cerevisiae* can increase the nutritional value of foods by producing vitamins, enzymes, or other beneficial compounds. Certain microorganisms produce antimicrobial compounds that can inhibit the growth of spoilage causing bacteria, thereby, extending the shelf life of food (Kuley *et al*., 2020). Microorganisms, particularly bacteria and yeast, are used in fermentation processes to

produce various food products such as cheese, bread, yoghurt, fermented milk as well as alcoholic beverages (Bowden, 2023).

These fermentation processes and conditions contribute to the unique flavours and aromas of many foods, improving their sensory qualities. Microbial contamination can occur at any point in the food production process, from harvesting to processing, to packaging thereby impacting food safety. Microbial food contamination can lead to spoilage and might cause food borne illnesses. Spoilage microorganisms can cause food to become unedible, unappealing and undesirable developing off-odours, off-flavours and visible changes such as changes in colour and texture. Pathogenic microorganisms, i.e. disease causing organisms can contaminate food and cause food borne illnesses, ranging from mild symptoms to severe illnesses. Examples of spoilage organisms include, *Pseudomonas* sp., *Bacillus* sp., *Clostridium* sp., *Saccharomyces* sp., and *Aspergillus* sp. Examples of pathogenic microorganisms include *Salmonella* sp., *Escherichia coli, Campylobacter* sp., *Staphylococcus aureus* etc. While spoilage organisms primarily affect food quality, some can produce compounds that may be toxic or allergic to humans. For example, Fungi like *Aspergillus* sp., *Penicillin* sp., and *Fusarium* can produce mycotoxins which can be toxic to the human health. In all, as biotechnology and microbiology advance, the exploitation of microbial processes offers sustainable and innovative solutions to global food challenges, highlighting the need for continued research, responsible use, and improved understanding of microbial interactions in food systems (Iwegbue *et al*., 2014).

Local alcoholic beverages also referred to as traditional alcoholic beverages are alcoholic drinks which are produced and consumed within a specified region, community or cultural context. These drinks often reflect local traditions, ingredients, and brewing practices. Ingredients utilized may include locally grown fruits, grains, or other agricultural products. They include things like home-brewed beers, locally

fermented fruit wines, or distilled spirits made from local plants or grains (Eze and Iroha, 2020). These local beverages are often made using traditional brewing or fermentation techniques which have been passed down from generation to generation. They are often consumed within the community where they are produced, fostering social connections and celebrations. Examples of local alcoholic beverages in Nigeria include palm wine (fermented from the sap of palm trees), sorghum beer also called burukutu (fermented from sorghum grains), and cassava spirits (produced from fermented cassava roots) (Amoa-Awua *et al*., 2007).

Palm wine is a local alcoholic beverage produced by fermenting the sap of palm trees such as oil palm, coconut palm, and date palm. The palm tree is tapped by making a small incision in the trunk allowing the sap to flow out. The sap is collected in a gourd or container and left to ferment. The fermentation process which occurs by action of naturally occurring yeasts, converts sugars present in the sap to alcohols. Freshly tapped palm sap has a sweet taste and is non-alcoholic, but it starts fermenting quickly, developing an alcoholic content of up to 4% within a couple of hours. Longer fermentation can increase the alcohol content and alter the taste (usually a balance of sweet and sour taste). The natural fermentation process of palm wine produces probiotics and enzymes that aid digestion, promote gut health and improve overall digestive function. It also possesses antioxidant properties as well as anti-inflammatory properties. Excessive consumption though, can lead to negative health effects, such as liver damage and increased risk of accidents and injuries (Adakaren *et al*., 2017).

Burukutu is brewed from the grains of guinea corn (*Sorghum bicolor*) and millet. The alcoholic beverage is often produced in tropical African countries such as Togo, Nigeria and Kenya as one of the major traditional and local alcoholic drinks. It has various production processes depending on where it is been produced but five major processes have been known to be consistent all through. The five basic steps include:

steeping, malting, mashing, fermentation and maturation. The production process begins by malting which involves the conversion of the grain used into malt, usually carried out on a malting floor. This stage is followed by steeping. Here, the grains are soaked in water for about three days in order to allow it absorb moisture and sprout. Afterwards, it is air-dried and then followed by mashing in. In this procedure, the milled grain known as the grain bill is mixed with water and the mixture is heated. This process allows the enzymes in the grain bill to decompose the starch in the grain into sugar to form a wort. The product is allowed to ferment using the sugar fungi form of yeast and allow maturation for 48 hrs. Burukutu is generally considered to be healthy and nutritious as it contains vitamins, minerals, and carbohydrates, providing essential nutrients for the body (Kolawole *et al*., 2007).

Palm spirit popularly called ogogoro is a distilled spirit derived from fermented palm wine. The fermentation process is followed by distillation, which concentrates the alcohol content, resulting in a high-proof beverage (around 30-50%, and sometimes higher). It is a popular gin in Nigeria, particularly in the southern region. Generally, local alcoholic beverages play a vital role in the cultural, social, and economic fabric of many communities across Nigeria and other parts of West Africa. They are greatly embedded in our cultural ceremonies, social interactions and economic livelihoods (Okafor, 2007).

The fermentation process of palm wine is a complex biological process driven by the metabolic activities of diverse microbial communities which are either naturally present in the sap or introduced during tapping, handling, storage or packaging. These organisms include yeasts, lactic acid bacteria, and acetic acid bacteria (Karamoko *et al*., 2012). Yeasts ferment the simple sugars in palm sap (mainly glucose and sucrose) to produce ethanol and carbon dioxide (Hoffman *et al*., 2015). LAB contribute to acidification by producing lactic acid, which can inhibit the growth of spoilage and

pathogenic organisms as well as contribute to the sour taste of palm wine. Species such as *Acetobacter aceti* oxidize ethanol into acetic acid, particularly when the beverage is exposed to air, leading to a sour taste as the wine ages.

At the initial stage of production, i.e. 0-6 hrs of tapping, the sap is sweet, non-alcoholic, and rich in sugars. Yeasts and lactic acid bacteria (LAB) begin to multiply rapidly as fermentation sets in. The alcohol content rises steadily due to the action of yeast. LAB contribute to a drop in pH, enhancing microbial stability. The beverage becomes sour and less palatable as ethanol is significantly oxidized to acetic acid by the acetic acid bacteria. Several biochemical transformations occur during the process due to the various metabolic activities carried out by these microbes and volatile compounds like esters and organic acids contribute to the characteristic flavour and aroma of the beverage. Various factors influence the microbial fermentation of palm wine. These include: temperature, oxygen availability, sanitation, sap quality. Ambient temperatures between 25-35ºc favour rapid fermentation. Anaerobic conditions promote alcohol production while exposure to air enhances acetic acid formation. The sugar content and microbial composition of the fresh sap also influence fermentation rate and microbial succession (Santiago-Urbina and Ruiz-Teran, 2014). Fermentation enhances the nutritional value of palm wine by enriching it with B-vitamins and amino acids. Some yeasts and LAB may also exhibit probiotic properties. While fermentation enhances the nutritional value and shelf-life of the product, poor handling and storage practices can lead to contamination by spoilage and pathogenic organisms. Understanding the microbiology of the beverage is therefore essential for ensuring a safe and quality product for commercial development.

Despite the widespread consumption and socio-cultural relevance of palm wine, there is still the lack of routine microbiological assessment of the beverage. This exposes consumers to health risks especially when it is produced under unhygienic conditions.

The lack of quality control measures during production, handling and storage of the product poses a significant risk to microbial contamination. The preparation of palm wine is often done in informal settings, without strict adherence to hygiene or standardization. Though produced naturally via fermentation, it is often tapped, stored and sold using containers and methods that promote microbial contamination. During tapping, unclean knives or collection vessels may be used. Contamination might occur during transportation due to exposure to dust or flies. Sometimes, the containers used for storage are not properly washed, some lack good covering mechanism thereby exposing its contents to contaminants. Sometimes, even the vendors themselves lack appropriate personal hygiene and proper etiquette and could contaminate the product directly, through touching, sweat and saliva droplets, sneezing, coughing etc. Also, most times, palm wine is sold in open markets, roadside stalls and exposed shops making it feasible for vector carrying pathogens to have access to the product (Ezeagu *et al*., 2003).

The presence of spoilage organisms or pathogens in palm wine and other local alcoholic beverages has been reported in several studies, yet most vendors and consumers remain unaware of the associated health implications. Common contaminants identified in palm wine include *Escherichia coli, Staphylococcus aureus, Klebsiella* spp*., Bacillus* spp*.*, and moulds, many of which are indicators of faecal contamination or poor sanitary practices. These organisms may compete with the natural fermenting flora, affect the quality of fermentation and pose serious health risks to consumers. Health risks include illnesses such as diarrhoea, vomiting, or even systemic infections, particularly in immunocompromised individuals. Negative microbial interference may also result in variable alcohol content and poor product shelf-life of the product. Also, changes in taste, smell and appearance can also be noticeable (Santiago-Urbina and Ruiz-Teran, 2014). Improved hygiene practices, such as using clean and sterilized containers for

storage, making sure to properly cover collection vessels, and observing proper personal hygiene by handlers, are essential steps to reduce microbial hazards. Public health awareness should be prioritized as palm wine tappers and vendors should be properly trained and educated on food handling and safe practices. In all, the quality and safety of palm wine are closely linked to hygiene practices, making microbial contamination a key area of concern in the production and handling processes (Ezeagu *et al*., 2003).

1.2 Aim/Objectives of the Study

The aim of this study was to conduct a comprehensive microbial analysis of local alcoholic beverages using palm wine as a case study.

The specific objectives were:

i. to isolate and enumerate microbial species from palm wine

ii. to purify microbial isolates gotten from the palm wine samples

iii. to characterize and identify the microorganisms involved in the fermentation of palm wine

iv. to determine the pH of the palm wine samples

v. to provide insights into improving the safety, shelf life, and potential industrial applications of palm wine.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction to Palm Wine and Traditional Alcoholic Beverages

Palm wine is a traditional beverage which is naturally fermented from the sap of various palm trees such as the oil palm (*Elaeis guineensis*), raffia palm (*Raphia hookeri*), coconut palm (*Cocos nucifera*), and Palmyra palm (*Borassus flabellifer*). It is an alcoholic beverage popular in sub-Saharan Africa that is made from palm sap (Amadi, 2022, Ojo and Agboola, 2019). It is widely consumed in Western Africa, Central Africa, South Asia, and parts of South America (Okafor, 2007). Palm wine plays important and significant roles in many cultures especially in sub-Saharan Africa. It is useful in various ceremonial events and social gatherings, such as, marriages, burial ceremonies, naming ceremonies, religious rituals, as well as other social celebrations. Its cultural significance cannot be overemphasized as both men and women enjoy its consumption thereby fostering harmony.

Local alcoholic beverages such as palm wine differ from industrially produced alcoholic beverages in terms of their chemical composition, means of production, microbial composition as well as their shelf life. These drinks are basically fermented naturally by action of microbes present naturally in the sap, the environment and even the collection containers. The fermentation process is rather uncontrolled unlike in industrial alcoholic production where the fermentation process is controlled using

starter cultures. Hence, local alcoholic beverages tend to show a higher degree of microbial diversity, which consequently affects their flavour profile, nutritional composition, alcoholic value, and consumption safety (Eze and Iroha, 2020). Microbial diversity of palm wine ranges from fermentation organisms (such as yeast, lactic acid bacteria and acetic acid bacteria) to spoilage and pathogenic organisms among which are the popular *E. coli*, *Staphylococcus aureus*, and *Bacillus* sp., and moulds (Amoa-Awua *et al*., 2007).

Palm wine is a cloudy, whitish beverage with a sweet alcoholic taste and very short shelf life of only one day (Joshi and Panesar, 2017). Palm wine when stored traditionally, typically has a shelf life of just 1-2 days before it turns acidic due to fermentation. However, methods like pasteurization and bottling can significantly extend its shelf life to over six months, potentially longer (Okafor, 2008). Fresh palm wine, usually sold in gourds and kegs, is best consumed within a day or two to avoid the sour taste caused by the preceding fermentation process. Refrigerating palm wine is known to extend its shelf life for an additional 1-2 days. Other methods of preservation have been explored to preserve the beverage such as the use of sodium bisulphite, pasteurization and bottling. Some organizations are working on bottling palm wine using biotech methods to halt fermentation and consequently extend its shelf life for up to twelve months (Rahul, 2025).

2.2 Relevance and Usefulness of Palm Wine

Palm wine serves vital roles and holds practical and social relevance particularly in sub-Saharan Africa. It serves as an alcoholic beverage, a source of income to those who are in the business of producing or selling it, and has also been known to provide various health benefits in terms of its nutritional value and treatment of ailments. The following are some of the relevance of palm wine:

1. Palm wine could be used as an extractant for local medicinal herbs in traditional medicine in order to increase the effect of these herbs against the organisms on which they are specifically acting on (Adedayo and Ajiboye, 2011).
2. It is an important economic resource in West Africa especially in Nigeria where it is estimated that per capita income from palm wine tapping can equal or exceed Nigeria's per capita income with a production estimate of two million metric tons of palm wine (Onyeukwu *et al*., 2024).
3. Palm wine is rich in various nutrients such as amino acids, minerals, vitamins (such as B1 and B2), and antioxidants which benefit overall health (Eze *et al*., 2019).
4. Some studies suggest that it may help lower blood glucose levels (Odafe and Oluwayemisi, 2023).
5. Palm wine has various use in ceremonial occasions such as weddings and other social gatherings.

2.3 Biochemical Composition of Palm Wine

Palm wine is a complex, naturally fermented beverage with its composition changing rapidly due to the action of microbe shortly after its extraction from the palm trees. Its constituents include carbohydrates (mainly sugar), amino acids, organic acids, alcohol, vitamins, as well as minerals each present in varying amount and contributing to the uniqueness, taste, nutritional value, and microbial dynamics of the beverage (Ezeagu *et al*., 2003). Its microbial community include yeasts, acetic acid bacteria and lactic acid bacteria which play crucial role in the fermentation process.

2.3.1 Carbohydrate constituent of palm wine

Carbohydrates are the major constituents of freshly tapped palm wine consisting of various sugars in varying proportions. These sugars include sucrose, glucose, fructose

and maltose. Palm wine primarily contains the carbohydrate sucrose as its main fermentable sugar (Ezeagu *et al.*, 2003). The unfermented sap is a sweet, colourless, clear syrup containing about 10%-12% sugar, which is mainly sucrose. Upon fermentation, the level of this sugar decreases as it is converted to alcohol and other products and the sap turns milky white due to the growth and action of the microbes present (Karamoko *et al*., 2016). Glucose and fructose are monosaccharides produced when sucrose is broken down by invertase during the fermentation process. The disaccharide maltose is also present, though in trace amounts. Maltose can also be fermented by yeast. These fermentable sugars serve as substrate for yeasts and lactic acid bacteria.

2.3.2 Protein constituent of palm wine

According to Ndom (2003), there is relatively little protein in the sap of the Raffia palm. The protein content of palm wine is generally low typically around 39.03 mg per 100 ml, but microbial growth is supported by trace amounts of amino acids and peptides. Free amino acids are about 59.63 mg per 100 ml. the amino acid profile can also change during fermentation as a result of proteolytic activity by certain bacteria strains. The protein component not only support the fermentative microbes in their growth but also play vital roles in flavour development and foam formation. (Amoa-Awua *et al*., 2007).

2.3.3 Minerals and vitamin content of palm wine

Palm wine contains a variety of minerals and vitamins. It is known to be a good source of vitamin B such as thiamine (B1), riboflavin (B2), and niacin (B3). It also contains vitamin A and vitamin C (Faparusi, 2019, Edema and Atayese, 2021). Minerals commonly found in palm wine include zinc, calcium, iron, potassium, magnesium and phosphorus. These are gotten from the tree sap and contribute generally to its health

benefits as they are essential electrolytes and trace nutrients beneficial to human health. The mineral content is relatively stable during fermentation, although some leaching or complex formation with organic acids may occur. (Amoa-Awua *et al*., 2007).

Palm wine is also rich in organic acids such as lactic acid, acetic acid, butyric acid and traces of tartaric acid and citric acid, which affects the beverage by lowering its pH value, thereby giving it that sour taste and influencing the microbial ecology. These acids lower the pH content from about 6.5 (fresh sap) to 3.5 or lower within 24-48hrs. Lactic acid bacteria play a crucial role in acid production, which can inhibit spoilage and pathogenic organisms (Obire, 2005). The progressive souring due to acid accumulation is one reason palm wine is best consumed fresh.

2.3.4 Alcohol content of palm wine

The alcohol (ethanol) content of palm wine typically starts low (about 0.5%-1.5% v/v in fresh sap) but increases progressively as fermentation proceeds, reaching levels of 4-6% within 24 hours (Eze and Iroha, 2020). Ethanol contributes to the beverage's intoxicating effect and helps in preserving it temporarily by inhibiting the growth of certain spoilage microbes. However, in cases of prolonged preservation, improper handling or bad hygiene practices, palm wine can become overly sour or even hazardous due to the accumulation of certain by-products that are undesirable such as methanol, fusel alcohols, and acetic acid (Uzochukwu *et al*., 2022).

2.3.5 Sensory characteristics of palm wine

As a result of microbial activities and metabolism, a variety of alcohols and organic acids are produced and these contribute distinctively to the beverages aroma and effervescence. For example, in *Elaeis guineesis* palm wine, 73 compounds (comprising 23 esters, 14 alcohols and phenols, 11 carbonyls, 10 acids, 5 sulphur compounds, 3

terpenes, 2 acetals, 2 hydrocarbons, 2 nitrogen compounds and 1 lactone) have been identified. The main compounds which give palm wine its aroma are esters, higher alcohols, as well as acids, aldehydes and alkanones (Santiago-Urbina and Ruiz. Teran, 2014). The most potent odorants in palm wine include 3-isobutyl-2 metoxipyrazine, fruity ethylhexanoate, 3methylbutylacetate and 2-acetyl-1-pyrroline (Lasekan and Abbas, 2010). These compounds contribute to the characteristic fruity aroma and effervescence of fresh palm wine, making it palatable when consumed immediately after tapping (Okafor, 2007).

2.3.6 Microbiome of palm wine

Local drinks such as palm wine contain various nutrients such as vitamins, minerals, sugar, proteins and amino acids (Ogu *et al,* 2022). These provides a suitable substrate for the growth of various microorganisms. The nutrient-rich palm sap supports a diverse microbiota which includes both resident flora and the invading flora brought by tappers, collection containers and even insects (Djeni *et al*., 2020). The regions geography and the palm tree species also have big effects on the local flora (Bi *et al*., 2019). The sugars in the palm sap are fermented by a broad array of yeast and bacterial species (Stringini *et al*., 2009, Karamoko *et al*., 2012).

In the study carried out by Nwachukwu *et al*., (2016) on the microbiological quality of different palm wine, it was revealed that palm wine harbours several species of microbial genera which include *Staphylococcus, Lactobacillus, Micrococcus, Serratia, Bacillus, Streptococcus, Saccharomyces cerevisae, and Candida tropiclis.* Yeast populations have been reported in palm wine in concentrations of about 10^4 to 10^7 CFU/mL, while, LAB ranging between 10^7 and 10^9 CFU/mL, AAB from concentrations of 10^5 to about 10^8 CFU/mL, total aerobic mesophiles ranging between

10^6 and 10^9 CFU/mL, and total coliforms have been reported in a range of 10^3 to 10^7 CFU/mL (Ouoba *et al*., 2012).

2.4 Factors affecting palm wine fermentation

There are various factors which influence the fermentation of palm wine. These factors affect and impact the final composition and quality of the wine.

1. Microbial communities: The microorganisms present in palm sap are its natural fermenters. Their activity and biochemical composition determine the types and amounts of products formed, such as alcohol, acids, esters.
2. Temperature: Temperature affects the speed and types of fermentation as well as the final products taste and quality. Optimal temperatures for fermentation typically range from 22 to 25ºC. Fermentation is a process which generates heat, hence it is important to regulate the temperature during the fermentation process to prevent excessive build-up of heat and to ensure a desired fermentation process is achieved. While temperature primarily affects fermentation rate, it can also influence the final alcohol content. It can also affect the production of various esters, acids, and other compounds that contribute to the taste and aroma of palm wine (Ghosh *et al*., 2012).
3. pH: The pH of the palm sap influences the growth of various microorganisms. Freshly tapped palm wine has a pH of about 6.92, which is close to neutral (Onourah *et al*., 2016). Fermentation occurs shortly after tapping, primarily due to yeast and lactic acid bacteria. The production and accumulation of the acids produced leads to a decrease in the pH of palm wine. The lower pH contributes to the sour taste and characteristic aroma of the beverage. The pH continues to decrease as fermentation progresses, with the most significant changes occurring in the initial hours and days after tapping. pH decrease can be affected by the type of

microorganism present, the fermentation temperature and the sugar content of the beverage (Ukwuru and Awah, 2013).

4. Nutrient availability: The availability of nutrients and essential elements such as sugars, proteins, nitrogen, can affect microbial growth and fermentation rates. Sufficient sugars, particularly sucrose, acts as a substrate for yeast to convert into ethanol and other byproducts. However, competition for these nutrients from other microorganisms, like the lactic acid bacteria and acetic acid bacteria, can limit ethanol production and introduce unwanted acidic flavours (Chandrasekhar *et al*., 2012).
5. Oxygen: The presence or absence of oxygen can influence the type of fermentation that occurs i.e. either aerobic fermentation or anaerobic fermentation. While initial fermentation in palm sap is typically anaerobic, the introduction of oxygen can alter the fermentation process and the quality of the final product. Excessive oxygen exposure can lead to oxidation, causing the wine to darken in colour, lose its sweetness, and develop off-flavours. Controlled oxygen additions can help yeast synthesize fatty acids and sterols, which are crucial for cell wall maintenance and population growth, leading to an improved fermentation process. In essence, while oxygen is not essential for the initial stages of palm wine fermentation, its presence can significantly impact the overall quality and shelf life of the wine (Santiago-Urbina and Ruiz-Teran, 2014).
6. Palm tree variety: Different palm tree variants may have varying sugar and acidity levels, which can influence the fermentation process (Santiago-Urbina and Ruiz-Teran, 2014). For example, the Tenera variety of oil palm may have a lower sugar concentration, leading to a weaker fermentation. Varieties with higher sugar content will lead to more vigorous and rapid fermentation. Palm sap usually contain various varieties of microorganisms, including yeasts, lactic acid bacteria, and acetic acid

bacteria. The type and abundance of these microorganisms are influenced by the palm species, tapping methods, and environmental factors. The presence of specific microorganisms can affect the flavour profile of the beverage (Owhoudue and Efejene, 2024). Examples of these palm tree varieties include, *Elaeis guineesis* (oil palm), *Cocos nucifera* (coconut palm), *Borassus flabellifer* (Palmyra palm) etc.

7. Tapping methods: The methods applied during tapping can affect the contamination and microbial diversity in the collected sap (Santiago-Urbina *et al*., 2014). Destructive methods, like felling of palm tree, can expose a larger volume of sugars in the sap to the surrounding environment, increasing the concentration of sugars available for fermentation. With more sugars available, fermentation can proceed more rapidly and completely, potentially resulting in a higher alcohol content in the final palm wine. Nondestructive methods which is basically slower and with less fermentation, can offer a more sustainable approach to palm wine production and potentially result in a more complex and nuanced flavour profile.
8. Storage means and conditions: Storage methods significantly impact palm wine fermentation by controlling the progression of natural fermentation and the growth of spoilage microorganisms. Heat treatment e.g. pasteurization, can kill many microorganisms, thereby stopping or slowing fermentation. Temperatures below 0ºC can also halt or slow down microbial activity and fermentation. In many cultures, palm wine is stored in calabashes or other containers, which can still lead to fermentation if not properly cleaned or sealed. Improper storage, like leaving palm wine at room temperature for extended periods without proper preservation can lead to over-fermentation (in which case its alcohol content becomes higher leading to a sour or vinegar-like flavour), and spoilage which further spoils the wine causing cloudiness or sediment formation (Karamoko *et al*., 2012).

Overall, palm wine's rich biochemical profile reflects in its traditional use not only as a refreshing beverage, but also as a source of nutrients and health uses. Understanding its compositional changes and microbial dynamics is thus crucial for both public health and product development. Furthermore, improper handling and unhygienic collection methods contribute to microbial contamination, including the presence of coliforms, spoilage fungi, and opportunistic pathogen. Studies have isolated several microorganisms which pose serious health risks such as *Escherichia coli*, *Staphylococcus aureus* and *Bacillus* spp. (Ezeagu *et al*., 2003). This therefore points out the need for improved postharvest preservation strategies and sanitary practices during production and distribution. Efforts should be made at enhancing the shelf-life and marketability of this beverage to enhance consumer safety and the beverage quality in general.

2.5 Microorganisms Involved in Palm Wine Fermentation

Due to the availability of nutrient substrate in palm wine, there is non-arguably, the growth of microorganisms in the beverage. At the start of fermentation, microbes serve as catalyst that aids the process. Palm wine fermentation is a complex, natural and spontaneous process that is primarily driven by indigenous microorganisms which colonize the sap immediately after tapping. Palm sap is a naturally sweet, nutrient-rich liquid which is fermented into a mildly alcoholic beverage characterized by its distinct flavour, aroma, and probiotic potential. The fermentation process involves a mixed microbial community.

The predominant groups involved in this process include yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB), each contributing unique metabolic and sensory outcome. Lactic acid bacteria ferment sugars into lactic acid, contributing to the acidity

and thereby affecting the flavour of the final product. Yeasts are the main drivers of alcohol production, converting sugars into ethanol. Acetic acid bacteria can oxidize ethanol into acetic acid, which contributes to the vinegar-like flavour and potential spoilage of the wine (Santiago-Urbina and Ruiz-Teran, 2014). *Saccharomyces cerevisae* is the main microorganism that has been identified to be responsible for the alcoholic fermentation and odorant production in the beverage.

On the other hand, *Lactobacillus plantarum* and *Leuconostoc mesenteroides* have been reported as the predominant lactic acid bacteria. *Gluconobacter* and *acetobacter* are the predominant acetic acid bacteria genera (Santiago-Urbina and Ruiz-Teran, 2014). Also prominent in the latter part of the fermentation process are the spoilage and pathogenic microorganism, which affect the quality of the beverage, causing spoilage thereby making it toxic for consumption. These include *Staphylococcus aureus, Escherichia coli, Bacillus cereus, Candida* spp*., Clostridium perfringens* etc.

2.5.1 Yeasts

Yeasts are eukaryotic, single-celled microorganisms classified as members of the fungus kingdom (Hoffman *et al*., 2015). They come from the domain Eukaryota and the kingdom Fungi. Yeasts do not form a single taxonomic or phylogenetic grouping. The term "yeast" is often taken as a synonym for *Saccharomyces cerevisiae*, but the phylogenetic diversity of yeasts is shown by their placement in two separate phyla: the Ascomycota and the Basidiomycota. The budding yeasts or true yeast are classified in the order Saccharomycetales, within the phylum Ascomycota (Kurtzman, 1994).

Yeasts are known to be chemoorganothrophs, as they use organic compounds as their source of energy and do not require sunlight for their growth. Carbon is mostly obtained from hexose sugars such as glucose and fructose, or from dissacharides such

as sucrose and maltose. Some species can metabolize pentose sugars such as ribose (Bernett, 1975).

They also metabolize alcohols and organic acids. Unlike bacteria, no known yeast species grow only anaerobically as they have the ability to grow in a no-oxygen environment, but also have aerobic methods of energy production.

Most yeast grow best in slightly neutral or acidic pH environment with a range of about 4.5-6.5. They also vary in regards to the temperature range in which they grow best. For example, *Saccharomyces telluris* grows at 5-35ºC, *Leucosporidium frigidum* grows at -2 to 20ºC, and *Candida slooffi* at 28 to 45ºC. Though, with viability decreasing with time, the cells can survive freezing under certain conditions (Authur and Watson, 1976).

Yeasts are very common in the environment, and are often isolated from sugar-rich materials, e.g. skins of fruits and extracts from plants such as plant saps. Yeasts are able to grow in foods with a low pH, mostly in foods with sugar contents, organic acids, and other easily metabolized sources. They are able to metabolize these food components ad produce metabolic end products of which might cause a change in the physical, and chemical structure of the food. This can be seen as a change in colour, texture, taste, or smell of the food (as seen in beverage fermentation).

The yeast species *Saccharomyces cerevisiae*, converts carbohydrates to carbon dioxide and alcohols via the process of fermentation. The end products of the reaction have been used in the baking process as well as the production of alcoholic beverage. *Saccharomyces cerevisiae* is one of the most studied microorganisms in modern cell biology research. Researchers have cultured it in order to understand the biology of the eukaryotic cell and ultimately human biology in general (Ostergaard *et al*, 2000). Other species of yeasts, such as *Candida albicans*, are opportunistic pathogens and can cause infections in humans.

Yeasts have recently been used to generate electricity in microbial fuel cells and to produce ethanol for the biofuel industry (Chen *et al*, 2010). In all, yeasts are very useful organisms especially in the food industry where they are used in bread making, beer brewing, wine production, cheese production, food flavouring, nutritional supplements, biofortification, and food waste reduction, though wild yeast strains can contaminate food products, cause over-fermentation and food spoilage.

2.5.1.1 *Saccharomyces cerevisiae*

This is a species of yeast believed to have been originally isolated from the skin of grapes and is one of the most intensively studied eukaryotic model organisms in molecular and cell biology. It is commonly called the baker's yeast or brewer's yeast as it is instrumental in winemaking, baking and brewing since ancient times. It is known to cause common types of fermentation.

Saccharomyces cerevisiae, the predominant yeast employed in fermented beverage production, is generally round or oval in shape with a diameter of about 5-10µm. They are unicellular fungi with ultrastructure features similar to that of higher eukaryotic cells (Walker and Stewart, 2016). High water activity is required for *S.cerevisiae* cells which typically possess a minimum a_w of around 0.65. Water is absolutely essential for fermentation, as high sugar media can impose osmotic stress on the cells, adversely affecting cell physiology.

Regarding temperature and pH requirements for alcoholic fermentations, yeasts thrive in warm and acidic environments with most *S.cerevisiae* species growing well between 20 and 30ºC and pH range of 4.5-6.5. Exceptions to these characteristics are larger brewing yeast strains of the species *Saccharomyces pastorianus* which are adapted to ferment in lower temperatures (typically 8-15ºC). Fermenting yeasts acidify their growth environment through a combination of proton secretion during nutrient

transport, direct secretion of organic acids, removal of buffering agents and carbon dioxide evolution and dissolution.

For the production of alcoholic beverages by *Saccharomyces cerevisiae*, the nutrient composition of the fermentation medium is crucially important for yeast growth and metabolism, and consequently, the quality of the final beverage. Most *S.cerevisiae* strains can grow if supplied with glucose, ammonium salts, inorganic ions and a few growth factors. Macronutrients need to be supplied at millimolar concentrations, and these includes sources of carbon (sugars), free amino nitrogen (amino acids, small peptides, and ammonium salts), oxygen, sulphur, phosphorus, magnesium and potassium. Micronutrients are only needed by yeast at micromolar concentrations, and they comprise micro elements such as copper, calcium, copper, iron, manganese and zinc (Walker and Stewart, 2016).

Saccharomyces cerevisiae is regarded as an ethanologenic yeast which can readily ferment glucose, fructose, sucrose, maltose, mannose and galactose into ethanol and CO_2. The sequence of enzyme-catalyzed reactions that convert glucose to pyruvic acid is known as glycolysis and can be summarizes as:

$$Glucose + 2ADP + 2Pi + 2NAD^{+} \rightarrow 2Pyruvate + 2ATP + 2NADH^{+} + 2H^{+}$$

Fermentative yeasts are able to use sugars anaerobically as electron donors, electron acceptors, and carbon sources. This pathway provides yeast cells with energy and reducing power for growth. In the latter stages of glycolysis, four molecules of ATP are formed and this results in the formation of two molecules of pyruvic acid, with 2 molecules net of ATP produced. This is the only source of energy obtained by *S.cerevisiae* during fermentative metabolism. Secondary fermentation metabolites of *S.cerevisiae* include higher alcohols, polyols, esters, organic acids, vicinal diketones, and aldehydes. These metabolites, although produced by yeast in much lower

concentrations compared with ethanol and CO_2, are very important flavour congeners in fermented beverages (Walker and Stewart, 2016).

Palm wine fermentation involves a diverse range of yeast species, many of which are classified as non-*Saccharomyces* yeasts. They typically dominate the early phase of spontaneous fermentation and contribute significantly to the complexity of palm wine's flavour, aroma, and biochemical properties (Amoa-Awua *et al*., 2007). Common non *Saccharomyces* yeasts present in palm wine include, *Hanseniaspora* spp*., Pichia* spp*., Candida* spp., and *Rhodoturula* spp*.* (Oguntoyinbo, 2011). These yeasts are known for their ability to metabolize a wide range of sugars and produce secondary metabolites such as organic acids, esters, and alcohols, which influences the physio-chemical properties of the beverage (Ezeronye, 2004).

For example, *Hanseniaspora uvarum* is often associated with fruity esters and volatile compounds, while *Pichia anomala* has been reported to exhibit killer toxin activity that suppresses spoilage organisms (Amoikon *et al*., 2014). Although their ethanol-producing capacity is generally lower than that of *Saccharomyces cerevisiae,* their metabolic activities contribute to the microbial succession and environmental conditions that eventually favour *Saccharomyces cerevisiae* dominance.

These yeasts can influence fermentation kinetics, affecting the rate of sugar consumption and ethanol production. Research suggests that non-*Saccharomyces* yeasts can be used in combination with *Saccharomyces cerevisiae* to increase polysaccharide, glycerol, and volatile compound production, reduce volatile acidity, and potentially improve the overall quality of palm wine. In palm wine fermentation, yeast populations dynamically shift throughout the process with *Saccharomyces cerevisiae* initially dominating and other yeasts like *Hanseniaspora* becoming more prominent later on (Santiago-urbina et al., 2015).

Factors like palm tree species, and tapping duration play a role in shaping these population dynamics. Environmental conditions and factors such as temperature, pH, and oxygen availability also play a role in the growth and survival of these different yeast species (Stringini *et al.,* 2009). Probiotic yeasts, like *Saccharomyces boulardii*, offer significant health benefits, primarily by improving gut health and bolstering the immune system. They can enhance the gut barrier, compete with harmful bacteria, and even aid in nutrient absorption and anti-inflammatory compound production. Some yeast strains like *Saccharomyces cerevisiae*, can stimulate the immune system and potentially help the body recognize and eliminate harmful microbes (Moslehi-Jenabian *et al.*, 2010).

Palm sap is naturally exposed to various microorganisms, including bacteria, which can interfere with yeast activity and lead to undesirable by products like acetic acids. Proper hygiene during sap collection and storage is essential to minimize contamination and ensure that yeast predominates. Yeast activity is highly temperature-dependent. Extreme temperatures can inhibit yeast growth and the fermentation process in general leading to incomplete or even stalled fermentation. The fermentation process itself can be difficult to control. Factors like oxygen availability or lack, nutrient availability, and pH can all affect yeast activity and the final flavour and quality of the palm wine. Hence, understanding the yeast community and its impact on fermentation is very essential for enhancing product quality. Also, ensuring adequate nutrients are available for the yeast to thrive is important for efficient fermentation and desired flavour development (Stringini *et al.,* 2009).

2.5.2 Lactic Acid Bacteria

Lactobacillales are an order of Gram-positive, acid-tolerant, either rod-shaped (bacilli) or spherical (cocci) bacteria that share common metabolic and physiological

characteristics. They are mostly found in decomposing plants and milk products and they produce lactic acid as their major metabolic end product in the process of carbohydrate fermentation, giving them the common name lactic acid bacteria (LAB). The genera that comprise the LAB are *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus*, and *Streptococcus*, as well as the more peripheral *Aerococcus, Enterococcus, Oenococcus, Sporalactobacillus, Tetragenococcus, Vagococcus* and *Weissella.* All but *Sporactobacillus* are members of the Lactobacillales order, and are all members of the Bacillota phylum.

Production of lactic acid has linked lactic acid bacteria with the fermentation of foods, as acidification inhibits the growth of spoilage agents. Proteinaceous bacteriotoxins are produced by several lactic acid bacteria strains and this provides an additional hurdle for spoilage and pathogenic microorganisms. Furthermore, lactic acid and other metabolic products contribute to the organoleptic and physical profile of a food item. Lactic acid bacteria are used in the food industry for a variety of reasons such as the production of cheese and yoghurt products. The beer and wine making process utilizes certain lactic acid bacteria, mostly *Lactobacillus* in their fermentation processes (Ezeronye and Legras, 2009).

Lactic acid has complex nutritional requirements for amino acids, peptides, nucleotic bases, vitamins, minerals, fatty acids, and carbohydrates. These are components which are present in the natural niches they inhabit. Their ability to inhabit different niches is as a result of their metabolic diversity and adaptability. Depending on the genus and species, lactic acid bacteria ferment sugars solely to lactic acid (Homofermentative), or to lactic acid, ethanol, and carbon dioxide (Heterofermentative) (Fossi *et al*., 2016).

Lactic acid bacteria represent a type of microorganism of considerable importance, given their usability in the food industry, ranging from nutritional contribution to the

optimization of food preservation processes. This group of bacteria can induce alterations in food taste and texture and has been utilized for the production of a wide variety of commercial products, such as fermented meats, cereals, and vegetables. LAB derive their energy through the phosphorylation of sugar molecules. Bacteria classified as homofermentative (e.g. *Lactobacillus plantarum* and *Pediococcus pentosaceus*) have the ability to exclusively degrade hexoxes, mainly relying on the process of glycolysis. These bacteria are characterized by the production of two molecules of lactate from one molecule of glucose.

On the other hand, bacteria classified as heterofermentative (e.g. *Limosilactobacillus fermentum, Lentilactobacillus buchneri* etc.) are notable for their ability to degrade both hexoses and pentoses. In the case of pentoses, they employ the pentose phosphate pathway, involving the action of specific enzymes such as ribose-5P epimerase and phosphoketolase. This process leads to the generation of lactate, carbon dioxide, and either ethanol or acetate as fermentation products (Chandrasekhar *et al*., 2012).

The presence of palm wine is closely linked to the spontaneous fermentation that occurs immediately after tapping. As the sap's sugars begin to ferment, lactic acid bacteria begin to multiply rapidly, especially under the anaerobic, sugar-rich conditions of freshly tapped palm wine. *Lactobacillus plantarum* and *Leuconostoc mesenteroides* are commonly isolated species, and they play key roles in reducing the pH of the wine, which in turn restricts the growth of spoilage or pathogenic organisms (Amoa-Awua *et al*., 2007). This acidification is not only beneficial for microbial control but also enhances the unique tangy taste of fresh palm wine.

In addition to the production of lactic acid, some LAB produces antimicrobial compounds such as hydrogen peroxide, diacetyl, and bacteriocins. These substances aid in the suppression of growth of undesirable microbes, hence, enhancing the safety of the beverage (Jespersen *et al*., 2005). Several lactic acid bacteria species, have been

suggested to possess probiotic properties. For instance, *Lactobacillus fermentum* has been studied for its ability to withstand acidic and bile conditions, suggesting potential benefits for gut health in consumers of fresh palm wine (Sanni *et al*., 2002).

Lactic acid bacteria also interact with yeasts and other microorganisms during fermentation. These interactions can be synergistic (whereby there is co-metabolism of sugars) or antagonistic (in which the acid and antimicrobial production by lactic acid bacteria suppresses the growth of ethanol-sensitive microbes). The succession from non-*Saccharomyces* yeasts to *Saccharomyces cerevisiae* is partially influenced by changes in environmental pH and nutrient availability due to LAB mediated activities (Oguntoyinbo and Sanni., 2005).

In commercial production, controlling lactic acid bacteria populations may be necessary to ensure consistent product quality as over-fermentation and acid accumulation, particularly in stored palm wine can result in over-souring, spoilage and other undesired qualities. This sourness is one of the primary reasons why palm wine is best consumed fresh, or refrigerated almost immediately after tapping.

In conclusion, lactic acid bacteria are important functional microorganisms in palm wine fermentation. Their ability to inhibit spoilage organisms, acidify the medium and contribute to overall flavour developments makes them exceptional. Further research into strain-specific roles and interactions may open avenues for controlled fermentation techniques and potential probiotic applications in palm wine based beverages.

2.5.3 Acetic acid bacteria

These are group of Gram-negative bacteria which oxidize sugars or ethanol and produce acetic acid during fermentation. These group of bacteria consist of ten genera in the family Acetobacteraceae. They are rod-shaped, obligate aerobes which are actively present in environments where ethanol is being formed as a product of the

fermentation of sugars. Fruit flies or vinegar eels are considered common vectors in the propagation of acetic acid bacteria as they can be isolated from the nectars of flowers and from damaged fruits. Other good sources are fresh apple cider and unpasteurized beer that has not been filter sterilized. In this liquid, they grow as a surface film due to their aerobic nature and active motility. Their growth can be suppressed through effective sanitation, by complete exclusion of air from wine in storage, and by the use of moderate amounts of SO_2 in the wine as a preservative (Raspor and Goranovic, 2008).

When acetic acid bacteria act on alcoholic beverages such as wine, vinegar is produced. Specific oxidation reactions occur through oxidative fermentation, which creates vinegar as a by-product. In the biotechnological industry, the oxidation mechanism of these bacteria is exploited to produce a number of compounds such as l-ascorbic acid, dihydroacetone, gluconic acid, and cellulose (Mamlouk and Gullo, 2013). Besides its use in the food industry, some acetic acid bacteria are used as biocatalysts for the industrial production of compounds (e.g. they are used as important biocatalysts for the development of eco-friendly fermentation processes as an alternative to chemical synthesis (Mamlouk and Gullo, 2013). Some genera, such as *Acetobacter*, can oxidize ethanol to carbon dioxide and water using Krebs cycle enzymes. Other genera, such as *Gluconobacter*, do not oxidize ethanol, as they do not have a full set of Krebs cycle enzymes. These bacteria are acid tolerant, growing well below pH 5.0, although the optimum pH for growth is 5.4-6.3 (Kaushal and Walker, 1951).

2.5.3.1 *Acetobacter aceti*

Acetobacter aceti is a Gram-negative bacterium recognized as a species within the genus *Acetobacter*, belonging to the family Acetobacteraceae in the class Alphaproteobacteria. It is widely distributed in various environmental niches. It thrives

in habitats abundant in fermentable sugars, such as flowers, fruits, honey, water, soil, and wherever sugar fermentation occurs. *Acetobacter aceti* grows best within temperatures ranging from 2530ºC, with an upper limit of 35ºC. It has been classified as an acidiophile, able to survive in acidic environments with pH between 5.5 and 6.3. *Acetobacter aceti* has long been in the fermentation industry to produce acetic acid from alcohol. The microorganism's ability to thrive in environments rich in fermentable sugars shows its potential as an organism for studying microbial metabolism and adaptation (Raspor and Goranovic, 2008).

Acetobacter aceti holds a significant economic value, particularly in vinegar production, where it catalyses the conversion of ethanol in wine or cider into acetic acid. Its unique metabolic capabilities have gained attention on biotech research. The acetic acid it generates is used in the manufacturing of acetate rayon, plastics production, rubber production, and photographic chemicals. Studies have found that it has the potential to be key player in the production of bio-based chemicals and renewable materials, using its enzymatic machinery for sustainable manufacturing processes (Raspor and Goranovic, 2008).

This microbe utilizes a two-step oxidation of ethanol to acetate. Ethanol is oxidized by membrane-bound proteins called pyrroloquinoline quinone-dependent alcohol dehydrogenase (PQQ-dependent ADH) to produce acetyl aldehyde. PQQ-dependent ADH proteins reside within the periplasm (Nakayo and Fukaya., 2008). Acetyl aldehyde is then oxidized by the enzyme aldehyde dehydrogenase to produce acetate resulting in the incomplete oxidation of ethanol. Subsequently, acetate can be used in the TCA cycle after ethanol is depleted. Acetate is then converted into acetyl-CoA synthetase or mediated by phosphotransacetylase and acetate kinase. *Acetobacter aceti* strains can tolerate extracellular acetic acid concentration of 5-20% (Gomes *et al.,* 2018).

Acetic acid bacteria (specifically those in the genera *Acetobacter* and *Gluconobacter*), like other microorganisms, utilize the sugars in palm sap as a source of energy. They convert the alcohol produced by yeasts into acetic acid, a process known as acetification. The amount of acetic acid produced depends on factors like the duration of the tapping period, the presence of oxygen, and the type of acetic acid bacteria present. Unlike lactic acid bacteria that thrive in low oxygen environments, acetic acid bacteria require oxygen to grow. Their presence and activity are thus strongly influenced by aeration and handling methods during palm wine collection and storage. The traditional practice of tapping palm wine into open containers or calabashes exposes the sap to air, thereby favouring the growth of acetic acid bacteria.

This becomes one of the reasons why palm wine deteriorates rapidly within hours after tapping if not refrigerated or pasteurized. As the fermentation process progresses, more acetic acid is produced and the palm wine begins to develop a sour taste (more like that of vinegar). This is particularly noticeable after the initial 24 hours of fermentation. The production of acetic acid can also affect the overall fermentation process and the composition of the palm wine. While it is desirable for flavour and preservation, excessive acetic acid can negatively impact the quality and taste of palm wine (Amoa-Awua *et al.,* 2007).

2.5.4 Other Bacteria and Fungi in Palm Wine Fermentation

While yeast, lactic acid bacteria, and acetic bacteria are the dominant microorganisms in palm wine fermentation, other microbial groups, particularly certain bacteria and filamentous fungi- have also been isolated during various stages of production, especially under poor hygienic conditions or in situations of improper storage and preservation techniques. These organisms may not necessarily partake in the

fermentation processes, but will often contribute to spoilage, contamination, and off-flavours.

2.5.4.1 *Zymomonas mobilis* and Enterobacteriaceae

Zymomonas mobilis is one of the notable bacteria present in palm wine. It is a Gram negative, facultative anaerobic bacterium that produces ethanol via the Entner-Doudoroff pathway. While its ethanol yield is comparable to *Saccharomyces cerevisiae,* it is generally less dominant in natural fermentations. Its presence is more commonly reported in controlled or semi-controlled fermentations (Okafor, 1975).

Members of the Enterobacteriaceae family including *Klebsiella, Enterobacter* and *Citrobacter* species have also been reported, especially in freshly tapped sap. Their presence is mostly attributed to environmental contamination or unhygienic tapping methods. They are not desirable in the fermentation process as they may introduce pathogenic traits or produce unwanted by-products such as biogenic amines, CO_2, and compounds that might cause off-flavours. Their growth is often suppressed as fermentation progresses due to increase in acidity of the beverage (Chandrasekhar, 2012).

2.5.4.2 Filamentous Fungi

Fungal genera like *Aspergillus* and *Penicillium* are not typically associated with fresh palm wine but may emerge in poorly stored samples or during advanced spoilage. These fungi are aerobic and thrive on residual sugars and organic acids in the wine. Their presence is significant due to the potential to produce mycotoxins and spoilage enzymes, which can affect both safety and quality.

In traditional palm wine production, there is little or no use of starter cultures. As a result, wild microbiota from the environment, tapping tools, and the palm tree itself

play a major role in shaping the microbial ecology of fermentation. These include a wide range of organisms which may appear briefly, compete with beneficial microbes, or modify the fermentation profile in unpredictable ways. While this contributes to the unique regional flavours of palm wine, it also creates variability in quality and safety (Sanni *et al*., 2001).

The presence of unwanted bacteria and fungi highlights the importance the good hygienic practices in palm wine production. These microorganisms are usually indicators of contamination or poor handling and may be linked to gastrointestinal disturbances in consumers. Some spoilage microbes introduce toxins, enzymes or gaseous substances that compromise the beverage's shelf life and sensory characteristics.

2.6 Microbial Succession Dynamics during Palm Wine Fermentation

Palm wine fermentation is a spontaneous process which is usually dynamic and non-sterile. It is characterized by a sequential and colonization and interaction of different microbial groups. This succession is shaped by various factors including substrate composition, time of fermentation, temperature, availability of oxygen, and hygienic conditions.

2.6.1 Initial phase: Dominance of wild Microbiota

Fermentation begins immediately the sap is tapped at 0-6 hrs. This freshly tapped sap is rich in sugars (sucrose, glucose, fructose) and slightly acidic, providing an ideal environment for a variety of yeasts, bacteria, and mould that originate from the palm tree, collecting vessels, and tapping tools. At this stage, non-Saccharomyces yeasts such as *Candida tropicalis, Pichia anomala*, and *Hanseniaspora* spp. dominate (Amoa-Awua *et al*., 2007). In cases of environmental conditions, Enterobacteriaceae and other

Gram-negative bacteria may also be abundant at the start of fermentation. These organisms initiate fermentation by converting sugars into ethanol and organic acids, while also producing aroma-active esters. However, at this phase, the microbial population is still diverse and unstable.

2.6.2 Active Fermentation Phase: Shift to *Saccharomyces* and LAB

As the environment becomes more anaerobic and acidic at 6-18 hrs because of the accumulation of ethanol, there is a progressive decline in microbial diversity. *Saccharomyces cerevisiae* becomes dominant, due to its acid tolerance. This yeast takes over alcoholic fermentation, producing significant levels of ethanol, CO_2, and flavour compounds such as higher alcohols and aldehydes. Simultaneously, lactic acid bacteria such as *Lactobacillus plantarum* and *Leuconostoc mesenteroides* increase in number. They ferment the remaining sugars into lactic acid, further acidifying the medium. Their metabolic activity also enhances microbial safety by suppressing spoilage or pathogenic bacteria (Adekunle *et al*., 2020).

2.6.3 Late Fermentation: Rise of Acetic Acid Bacteria and Spoilage Microbes

As the wine continues to undergo fermentation at 18-36 hrs and is subsequently being exposed to air, acetic acid bacteria, such as *Acetobacter aceti* begin to oxidize ethanol into acetic acid, leading to an increase in sourness and a decline in palatability. Meanwhile, if the wine is not consumed early enough, microorganisms such as *Aspergillus, Penicillium*, and other spoilage bacteria may appear. The sugar content begins to decline as the acid level increases. Sensory appeal becomes lowered and spoilage is prone to increase. There might also be cases of possible health risks due to the action of microbial metabolites and toxins (Falegan and Akoja, 2014).

2.7 Factors Influencing Succession

Various factors influence the microbial succession in palm wine. They include:

Temperature: Warmer temperatures accelerate the growth of microbes and the fermentation rate in general.

Oxygen: Aerobic conditions favour the activity of acetic acid bacteria and spoilage fungi

Hygiene conditions: Poor sanitation methods introduces unwanted microbes such as spoilage bacteria and moulds

Palm species and age of tree: The type of palm species and the age of the palm tree affects the sap's composition and the microbial load of the sap.

Fermentation vessel: The fermentation vessels or containers may harbour native microbiota, influencing the fermentation process (Hebbar *et al*., 2018).

2.8 Fermentation Process and Metabolic Activities

Fermentation is a type of anaerobic metabolism in which organic molecules such as glucose or other sugars, are catabolized and reduced to give adenosine triphosphate (ATP) as its end product alongside other organic end products such as alcohols and organic acids. This is mostly accompanied by effervescence noticed by the release of gases e.g. (CO_2). Fermentation is used by organisms to generate ATP as energy for metabolic activities. As it does not need an exogenous electron acceptor, it is able to occur regardless of the environmental conditions.

However, the primary disadvantage of fermentation is that it is relatively inefficient and produces lesser ATP (**2-4.5 ATP**) as compared to aerobic respiration (**32 ATP** produced). Over 25% of bacteria and archaea carry out fermentation. The fermenting

microbes are most frequently found in host-associated habitats, such as the gastrointestinal tracts, food substrates, and other habitats. The most common fermentation products include lactate, acetate, ethanol, carbon dioxide (CO_2), succinate, hydrogen (H_2), propionate, and butyrate. Both bacteria and archaea share the capacity for fermentation, leading to a wide variety of organic end products.

2.9 Enzymatic activities and substrate utilization

Like many biochemical reactions, fermentation is an enzyme catalysed reaction with the goal of either changing the initial substrate or forming useful end-products. Naturally occurring fermentation carried out by microorganisms is usually to obtain useful metabolic products such as ATP, pyruvate, or lactic acid. The substrates used in this form of fermentation are usually simple sugars such as glucose, fructose, which serve as carbon source (Folch *et al*., 2021). Food as a substrate for fermentation is the most common and oldest use of fermentation as it was used to preserve food such as cereals, honey, dairy products (cheese, milk etc.), bread, and beers.

This type of fermentation which occurs naturally has constantly been harnessed by humans to achieve preservative effects, improve flavour and texture profiles. Advances in fermentation has led to the engineering and industrialization of specific microbes and substrates in order to obtain certain flavour and texture profiles (most observable in beer fermentation) (Mannaa *et al*., 2021).

When an organic compound is fermented, it is broken down to a simpler molecule and releases electron. These electrons are then transferred to a redox cofactor, which then transfers them to an organic compound. ATP is generated via this process. It can be formed via substrate-level phosphorylation or by ATP synthase. When glucose is fermented, it enters glycolysis or the pentose phosphate pathway and is converted to

pyruvate. From this point, the pathways branch out to form a number of end products e.g. lactate. At several points of the pathways, electrons are released and accepted by redox cofactors (NAD^+ and ferredoxin). At later points, these cofactors donate electrons to their final acceptor and become oxidized. ATP is also formed at several points in the pathway (Folch *et al*., 2021).

2.10 Biochemistry of Organic Acid and Alcohol Products

2.10.1 Ethanol

Ethanol fermentation, also referred to as alcoholic fermentation, is a biological process which converts sugars such as glucose, fructose and sucrose into cellular energy. One glucose molecule is converted into two ethanol molecules and two carbon dioxide (CO_2) molecules. Ethanol fermentation is used to make bread dough rise (the carbon dioxide released forms bubbles expanding the dough into a foam). Ethanol is known for its intoxicating effects in alcoholic beverages such as wine, beer, and liquor. Fermentation of feedstock, including sugarcane, maize, and sugar beets, produces ethanol that is added to gasoline.

Before fermentation, a glucose molecule breaks down into two pyruvate molecules (glycolysis). The energy derived from this exothermic reaction is used to bind inorganic phosphates to ADP, which converts it to ATP, and convert NAD^+ to NADH. The pyruvates break down into two acetyldehyde molecules and give off two carbon dioxide molecules as waste products. The acetyldehyde is reduced o ethanol using the energy and hydrogen from NADH, and the NADH is oxidized into NAD^+ so that the cycle may repeat. The reaction is catalysed by the enzymes pyruvate decarboxylase and alcohol dehydrogenase (Logan and Distefano, 1997).

2.10.2 Lactic acid

There are two forms of lactic fermentation which are homolactic fermentation (simplest type of fermentation) and heterolactic fermentation. In homolactic fermentation, pyruvate from glycolysis undergoes a simple redox reaction, forming lactic acid. Overall, one molecule of glucose (or any six-carbon sugar) is converted to two molecules of lactic acid:

$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH$

It occurs in the muscles of animals when they need energy faster than the blood can supply oxygen. It occurs in some kind of bacteria (such as the lactobacillus) and in some fungi. The lactic acid bacteria can carry out either homolactic fermentation, where the end-product is usually lactic acid, or heterolactic fermentation, where some lactate is further metabolized to ethanol and carbon (iv) oxide, acetate, or other metabolic products.

$C_6H_{12}O_6 \rightarrow CH_3CHOHCOOH + C_2H_5OH + CO_2$

Heterolactic fermentation is an intermediate between lactic acid fermentation and alcoholic fermentation. In this case, the high concentration of lactic acid produced drives the equilibrium backwards. This decreases the rate at which the fermentation process occurs. Ethanol, into which lactic acid can be easily converted, is volatile and will readily escape allowing the reaction to proceed easily. CO_2 is also produced, but it is only weakly acidic and even more volatile than ethanol.

Glucose + ADP + P_i → Lactate + Ethanol + CO_2 + ATP

2.10.3 Acetic acid and others

As fermentation progresses beyond 1-2 days, the metabolic activities of spoilage organisms particularly acetic acid bacteria begin to dominate. Under aerobic conditions, ethanol serves as a substrate for acetic acid bacteria, hence, the conversion of ethanol to acetic acid.

$C_2H_5OH + O_2 \rightarrow CH_3COOH + H_2O$

This biochemical reaction increases acidity and ultimately renders the palm wine unpalatable. A sharp sourness and vinegary odour is noticeable which diminishes the beverage's sensory appeal. Higher alcohols such as isoamyl alcohol and isobutanol are produced through the catabolism of amino acids via the Ehrlich pathway. These compounds contribute to the warm, alcoholic scent and flavour. Esters, particularly ethyl acetate and isoamyl acetate, are the major aroma contributors in fermenting beverages contributing to its fruity aroma.

The fermentation of palm wine is a biochemical process primarily governed by the action of yeasts and bacteria through their various metabolic activities. The process not only elevates the alcohol content but also preserves the beverage to some extent. Understanding these biochemical transformations is not only essential for appreciating the beverages value but is also useful for optimizing its stability, quality, and industrial commercialization through controlled fermentation techniques (Lasekan and Abbas., 2010).

2.11 Health Implications of Palm Wine Consumption

Palm wine as a naturally fermented beverage presents both nutritional benefits and potential health risks depending on its production method, microbial quality, and consumption patterns. Palm wine contains a wide range of bioactive compounds,

microorganisms, and metabolites which play various roles in human health. While some studies shows that it is good for reducing the rate of cardiovascular diseases, it can lead to serious health problems, including liver damage and neurological issues.

2.11.1 Nutritional and probiotic benefits of palm wine intake

Nutrient source: Palm is naturally rich in amino acids, potassium, zinc, iron, and vitamins including B1, B2, B3, B6, and vitamin C making it a very nutritious beverage. It also contains various minerals and organic acids which supports physiological functions.

Antioxidant properties: Palm wine contains antioxidants such as riboflavin, which may help protect against cell damage and fight cancer.

Heart health: Some studies suggests that moderate consumption of palm wine may impact heart health by reducing the risk of cardiovascular diseases, lower blood pressure as well as keep a steady heart rate.

Lactation: Palm wine is believed to increase breast milk production in nursing mothers. Fresh palm wine is typically consumed by lactating women who seek to increase their breast milk supply (Amadi, 2022).

Vision: Studies have shown that the yeast content in palm wine may contribute to improved eyesight (Amadi, 2022).

Weight and stress managements: Palm wine may be used as a stress reliever, though this is not scientifically proven. It is also believed that palm wine can aid in weight management.

Treatment of malaria: In times of malaria attacks, fresh palm wine is frequently blended with local herbs and consumed in cases of local treatments (Amadi, 2022).

2.12 Potential health risks of palm wine

Despite its nutritional advantages, palm wine poses health challenges when hygiene is compromised or when fermentation becomes prolonged. Poor production practices can lead to contamination by spoilage organisms. These contaminants may cause foodborne illnesses, especially when the wine is not consumed fresh. Its negative impact include:

Neurological effects: Palm wine can affect body rigidity and cause muscle contraction, potentially leading to neurological symptoms.

Liver damage: Extended fermentation of palm wine leads to an increase in the alcohol content. Excessive intake at this point can affect liver function and lipid metabolism, potentially leading to fatty liver disease.

Increased risk of diabetes and cardiovascular diseases: Sometimes during production, artificial sweeteners and sugars are added to palm wine. This can raise the risk of diabetes, hypertension, and other cardiovascular disease.

Effects on reproductive health: Some research indicates that palm wine can negatively affect reproductive health, potentially decreasing testosterone levels and sperm counts. It is also advised that pregnant women limit intake of the beverage as it could potentially harm the development of the foetus.

Also, an increase in acetic acid concentration during the course of fermentation can make the drink more acidic. This may harm the dental enamel and affect gastric health when consumed excessively. In general, moderation is key in order to minimize these risks and reap any potential benefits.

2.13 Public health considerations

Traditional palm wine drink has been ascribed as a functional drink due to the fact that it contains a high level of micro and macro nutrients, and most especially beneficial

microbes known to promote certain health benefits. Despite all these, there is still the lack of standardization of its production processes thereby resulting in variations in taste, colour, and aroma due to differences in sugar and ethanol content across different production countries and sites. Research into the biochemical and microbiological properties of the beverage during and after production will be useful in determining the level and methods of production that will be best for obtaining optimum benefits for consumption as well as elongating its shelf life. Additionally, safe practices during the point of extraction, to storage and preservation will eliminate the risk of contamination by extrinsic factors such as insects, dirt and external microbes (Adakaren *et al*., 2017).

It is also noteworthy to emphasize the need to adequately label the packaged drink (especially for commercial purposes) to include the sugar and alcohol content, as this would assist in making the quality of the product consistent. Future research on integrating an eco-friendlier packaging material such as the round bottom calabashes should be investigated. Furthermore, labelling of the product will provide useful information of the product to those that require it for medicinal and health purposes. This will boost the socio-economic development of the producers, thereby promoting the international market for palm wine (Mbuagbaw and Noorduyn., 2012).

CHAPTER THREE

METHODOLOGY

3.1 Study Area and Sample Collection

Palm wine samples were sourced from multiple vendors across selected local communities within Benin City, Edo state. The selection of sampling sites was based on accessibility and reputation for traditional palm wine tapping. Samples were collected aseptically from the vendors' storage kegs using sterile bottles. Each sample was labelled accordingly and stored in an ice-packed container to reduce microbial activity during transit. The samples were analyzed within 2 hrs of collection to preserve their microbial integrity.

3.2 Sterilization of Media Materials

All materials were appropriately sterilized before and after use. Glass wares such as test tubes, conical flasks, pipettes were thoroughly washed with detergents, rinsed properly with water and drained. They were wrapped in aluminium foil and sterilized in hot air oven at 160ºC for 1 hr. The inoculating loops were heated to redness in an open flame before and after use. The laboratory bench was swabbed using 70% alcohol for disinfection before analysis were made.

3.3 Preparation of Media

For total aerobic bacterial counts, Nutrient Agar (NA) was used. MacConkey agar was used to determine coliform presence and for the isolation of fungi, Potato Dextrose Agar (PDA) was used. Lactic acid bacteria were isolated using MRS Agar. All media were prepared according to the manufacturer's instructions. The media were placed in an autoclave to sterilized at 121ºC for 15 mins. After sterilization, the flask was allowed to cool.

3.4 Isolation of Microorganisms by Serial Dilution

Each sample was gently shaken to ensure homogeneity. A volume of 25ml was aseptically transferred into 225ml of sterile distilled water to make the stock solution. Subsequent serial dilutions were prepared up to 10^{-6} to ensure accurate colony enumeration. These dilutions were used for the enumeration of bacteria, yeasts, and moulds using appropriate selective and differential media. The pour plate method was used to isolate microorganisms from the diluted sample (Dimowo and Omonigho,2023). For each dilutions plated, 1ml aliquots were transferred into sterile Petri dishes. Approximately 15-20 mL of molten agar (cooled to about 45ºC) was poured into each Petri dish. The plates were gently swirled to mix and ensure even distribution of the inoculum. This was allowed to solidify. The plates containing Nutrient Agar, MRS Agar and MacConkey agar were incubated at 37 ºC for 48 hrs. The plates containing Potato Dextrose Agar were incubated at 28 ºC for 5-7 days. After the incubation period, distinct colonies were counted using a colony counter (Dimowo and Omonigho,2023).

3.5 Enumeration of Isolates

At the end of the incubation periods, the plates were observed for growth. Colonies that developed both on the surface and within the agar medium were counted using a colony counter. The total viable count for each microbial group was calculated by multiplying the number of colonies by the dilution factor, and results were expressed in CFU/mL of palm wine (Cheesbrough, 2005).

$$\frac{Number\ of\ colonies}{Volume\ plated\ in\ mL} \times \frac{1}{Dilution\ factor} = CFU/ml$$

3.5.1 Purification of Isolates

Distinct colonies obtained from the pour plate cultures were carefully sub-cultured onto freshly prepared Nutrient Agar. This was done using the streak plate method to obtain discrete colonies. The sub-culturing was repeated until pure cultures were obtained. The purified isolates were incubated at optimal conditions and maintained on slants of their respective media at 4 ºC for further characterization and biochemical testing

3.6 Characterization of Isolates

The purified isolates were subjected to both morphological and biochemical characterization to determine their identities.

3.6.1 Morphological Characterization of Isolates

Bacterial colonies were examined for size, shape, elevation, edge, opacity, colour, and texture. Gram staining was performed to determine Gram reaction and cell morphology (cocci, bacilli, etc). Fungi isolates were identified based on spore structure, hyphal arrangements and were microscopically examined using lactophenol cotton blue stain.

3.6.2 Biochemical Characterization of Isolates

Bacterial isolates were subjected to a series of biochemical tests, including Catalase, Oxidase, Indole production, Citrate utilization and Urease tests.

3.7 Identification of isolates

The bacteria isolates were identified using Bergeys's manual (9th Edition, 1994) which is the gold standard reference book for identifying and classifying bacteria. The results obtained from characterization of the bacteria were compared with Bergey's manual to determine the genus and species of the isolates based on the closest affinity in cultural, biochemical, and microscopic features. Fungal isolates were identified based on

morphological and microscopic features. Identification was conducted using standard microbiological identification guides (Cheesbrough, 2005).

3.8 Physiochemical Analysis

The pH of fresh and fermented palm wine was measured using a calibrated pH meter. Measurements were taken immediately after sample collection and repeated after 24 hrs to observe pH variation during fermentation.

3.9 Data Analysis

Data obtained from microbial enumeration were recorded and statistically analyzed using the SPSS package version 21.0. All data are mean of triplicate replicates. Results were expressed in tables as mean ± standard deviation.

CHAPTER FOUR

RESULTS

Table 1 represents the total heterotrophic bacterial counts of microorganisms isolated from the palm wine samples (A-E) collected from different locations in Benin City. Table 2 shows the total coliform counts of isolates cultured on MacConkey agar. All samples had varying coliform counts except samples D which had zero coliform counts. The total fungal counts isolated from the palm wine samples are shown in Table 3. MRS agar was used for lactic acid bacteria isolation and Fig 1 shows the appearance of the Lactic acid bacteria growth on MRS medium after 72 hrs of incubation. The total lactic acid bacteria counts could not be recorded because of flooded growths in the medium which were too numerous to count.

Table 4 shows the various morphological and cultural characteristics of the various bacterial isolates gotten from the samples including the size, shape colour and texture of the different isolates. Table 5 reveals the results gotten from the biochemical tests done to further identify the bacterial isolates as well as the Gram stain reaction of the various isolates. The morphology and microscopic features of the fungal isolates are depicted in Table 6. The percentage occurrences for each isolate on the different samples were recorded in Table 7 while the mean pH values for each samples were recorded in Table 8. These values depicted the acidic nature of the beverage.

Table 1: Total heterotrophic bacterial counts (cfu/ml) isolated from the palm wine samples

S/N	Mean±SD×10^1	Mean±SD×10^4
A	TNC	1.2±1.00×10^5
B	2.6±0.58×10^1	NG
C	1.0±1.00×10^2	6.6±0.58×10^4
D	1.14±0.47×10^2	9.4±0.57×10^4
E	1.4±1.00×10^2	8.0±1.73×10^4

KEYS:

A-Oluku, B-Ekiadolor, C-Ugbogiobo, D-Iwu, E-Ora

Table 2: Total coliform counts (cfu/ml) isolated from the palm wine samples

S/N	Mean±SD×10^1	Mean±SD×10^4
A	1.2±1.00×10^2	NG
B	8.0±1.00×10^1	2.6±0.58×10^4
C	1.0±1.00×10^2	6.0±1.00×10^5
D	NG	NG
E	7.4±0.58×10^1	3.4±0.33×10^4

KEYS:

A-Oluku, B-Ekiadolor, C-Ugbogiobo, D-Iwu, E-Ora

Table 3: Total Fungal counts (cfu/ml) isolated from the palm wine samples

S/N	Mean±SD×10^1	Mean±SD×10^4
A	2.06±0.58×10^2	1.6±1.00×10^5
B	2.54±1.53×10^2	8.6±2.08×10^4
C	4.0±0.81×10^1	2.6±0.58×10^4
D	2.26±1.53×10^2	2.14±0.58×10^5
E	2.14±5.69×10^2	5.4±1.53×10^4

KEYS:

A-Oluku, B-Ekiadolor, C-Ugbogiobo, D-Iwu, E-Ora

Fig 1: Appearance of Lactic Acid Bacteria growth on MRS Medium after 72 hrs of Incubation

Table 4: Morphological description of isolates

S/N	Colonial morphology and cultural characteristics	Isolates
1	short rod in chains, raised irregular white colonies on nutrient agar	*Bacillus* spp.
2	Cocci in chains, translucent grayish-white colonies	*Streptococcus* spp.
3	Small,round,raised, cream-coloured, smooth colonies on MRS agar, rod shaped in small chains when viewed on a microscope	*Lactobacillus* spp.
4	Cocci in clusters, smooth, round and creamy colonies on Nutrient agar	*Staphylococcus* spp.
5	Large, circular, convex, mucoid pink colonies on MacConkey agar, appear rod shaped when viewed on a microscope	*Klebsiella pneumoniae*
6	Short rods in singles, small round pink colonies on MacConkey agar	*Escherichia coli*
7	Short rods, small, circular, smooth and off-white colonies	*Acetobacter* spp.

Table 5: Biochemical description of isolates

S/N	Gram stain Reaction	Catalase	Urease	Citrate	Oxidase	Indole	Isolates
1	+	+	-	+	-	-	*Bacillus* spp.
2	+	-	-	-	-	-	*Streptococcus* spp.
3	+	-	-	-	-	-	*Lactobacillus* spp.
4	+	+	-	-	-	-	*Staphylococcus* spp.
5	-	+	+	+	-	-	*Klebsiella pneumoniae*
6	-	+	-	-	-	+	*Escherichia coli*
7	-	+	-	-	-	-	*Acetobacter* spp.

Keys:

Negative: -

Positive: +

Table 6: Identification of fungal isolates from the palm wine samples

S/N	MORPHOLOGY	MICROSCOPIC FEATURES	ISOLATES
1.	Whitish colonies without well-developed pseudo-mycelium	Single round cells with characteristic absence of spores	*Candida* spp.
2.	Round and creamy colonies on PDA	Single oval cells, some in pairs and elongated. Spores were present and budding was pronounced	*Saccharomyces* spp.
3.	Green colored colonies with powdery like structures	Oval shaped cells with pronounced conidia structures	*Aspergillus* spp.

Table 7: The occurrence of each isolates in the different samples

Isolates	Samples					
	A	B	C	D	E	Percentage
Bacillus spp.	-	-	+	-	+	40%
Streptococcus spp.	+	-	-	+	-	40%
Lactobacillus spp.	+	+	+	+	+	100%
Staphylococcus spp.	+	-	-	+	+	60%
Klebsiella pneumoniae	+	+	+	-	+	80%
Escherichia coli	+	-	+	-	+	60%
Acetobacter spp.	-	+	+	+	-	60%
Saccharomyces spp.	+	+	+	+	+	100%
Aspergillus spp.	-	-	+	-	-	20%
Candida spp.	+	+	-	+	-	60%

KEYS:

A-Oluku, B-Ekiadolor, C-Ugbogiobo, D-Iwu, E-Ora

Table 8: pH Mean±S.D value

S/N	Mean±S.D
A	3.46±0.21
B	3.54±0.11
C	3.59±0.01
D	3.52±0.05
E	3.49±0.01

KEYS:

A-Oluku, B-Ekiadolor, C-Ugbogiobo, D-Iwu, E-Ora

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

The palm tree sap is a natural rich medium capable of supporting the metabolic activities of several types of microorganisms such as a high number of aerobic mesophilic bacteria, coliforms, lactic acid bacteria, acetic acid bacteria and fungi species (Amoa-Awua *et al,* 2007). Insects are major factors in the contamination of the palm saps as well as the activities of humans either during the process of tapping, storage or distribution. The insect *Drosophilia melanogaster* (the fruit fly) is a major insect contaminant of this product. It is attracted to the fresh product due to the natural aroma that emanates from the tapped sap. Contamination occurs when microbes are transferred from the insect to the palm sap which is a natural supporting medium for the growth of various microorganisms (Karamoko *et al.,* 2012). Usage of contaminated tools during tapping, unsterilized bottles for storage and poor handling of the beverage in general are human activities that results in the microbial contamination of the product.

The microbial analysis of palm wine in this study revealed the presence of diverse microorganisms including *Lactobacillus*, *Acetobacter, Enterobacter, Bacillus*, and *Staphylococcus* specie as well as *Saccharomyces cerevisiae, Candida* sp., and *Aspergillus* sp. The total heterotrophic bacterial counts of isolates obtained from this study indicated varying levels of bacterial populations across the different palm wine samples. The highest heterotrophic bacterial count was recorded for Sample A, in which the values were too numerous to count for the first dilution and $12\pm1.00\times10^{4}$cfu/ml for the fourth dilution plated. This was followed by Sample E with values of $1.4\pm1.00\times10^{2}$cfu/ml and $8.0\pm1.73\times10^{4}$cfu/ml for both dilutions plated. The

lowest count was found in Sample B in which the value was $2.6\pm0.58\times10^{1}$cfu/ml for the first dilution plated and no growth was recorded for the 10^{4} dilution. These results suggest that Sample A might have contained more bacteria load than the other samples. Lower microbial count especially from Sample B could have resulted from insufficient homogenization of the sample or other sampling errors.

The total coliform count varied among the various samples. Sample A recorded $1.2\pm1.00\times10^{2}$cfu/ml for the first dilution plated while the other dilution plated recorded no growth. Sample B gave values of $8\pm1.00\times10^{1}$cfu/ml and $2.6\pm0.58\times10^{4}$cfu/ml. Values for Sample C was recorded as $1.0\pm2.00\times10^{2}$cfu/ml and $6.0\pm1.00\times10^{4}$cfu/ml for both dilutions. Sample D recorded no coliform growth while values for Sample E were recorded as $7.4\pm0.58\times10^{1}$cfu/ml and $1.7\pm0.33\times10^{4}$cfu/ml. The presence of coliforms in Samples A, B, C, and E was an indicator of poor hygienic handling of the beverage gotten from those areas.

The total fungal counts were recorded as $2.06\pm0.58\times10^{2}$cfu/ml and $1.6\pm1.00\times10^{4}$cfu/ml for Sample A, $2.54\pm1.53\times10^{2}$cfu/ml and $8.6\pm2.08\times10^{4}$cfu/ml for Sample B and $4.0\pm0.81\times10^{1}$cfu/ml and $2.6\pm0.58\times10^{4}$cfu/ml for Sample C. Sample D was recorded as $2.26\pm1.53\times10^{2}$cfu/ml and $2.24\pm0.58\times10^{5}$cfu/ml while Sample E was recorded as $2.24\pm5.69\times10^{2}$cfu/ml and $5.4\pm1.53\times10^{4}$cfu/ml. The total lactic acid bacteria count from this study could not be collated as the plates were too flooded to count.

The microbial analysis of palm wine in this study revealed the presence of diverse microorganisms including *Lactobacillus*, *Acetobacter, Enterobacter, Bacillus*, and *Staphylococcus* species as well as *Saccharomyces cerevisiae, Candida* sp., and *Aspergillus* sp. From the results gotten in this study, *Lactobacillus* spp. and *Saccharomyces* spp. were observed as most frequently occurring isolates with percentage occurrence of 100% each. This was followed by *Klebsiella pneumoniae* with 80% occurrence. *Acetobacter* spp., *Candida* spp., *Escherichia coli* and

Staphylococcus spp. had 60% occurrences each. 40% occurrence was seen in *Bacillus* spp. and *Streptococcus* spp., while a 20% occurrence was recorded for *Klebsiella pneumoniae* and *Aspergillus* spp. According to the microbiological safety of the palm wines observed in this work, the palm wine samples were not up to the standard required due to the presence of faecal coliforms and moulds in the samples making the beverage unfit for consumption.

In comparison to other studies, Obi *et al*., (2015) accessed the presence, growth and survival of microbes in fresh palm wine samples which gave rise to the isolation of eight bacterial species: *Staphylococcus aureus, Escherichia coli, Lactobacillus* spp., *Micrococcus luteus, Serratia marcessens, Acetobacter* spp., *Bacillus* spp. and *Schizosaccharomyces pombe.* The presence of yeast and other bacteria isolates was also reported by Karamoko *et al*., (2012). Similar results are also reported by Ouoba *et al*., (2012) which showed *Lactobacillus plantarum* as the dominant species representing 46.7% of the total isolates from the study. These microorganisms particularly *Saccharomyces* spp., *Lactobacillus* spp., and *Acetobacter* spp., are mainly responsible for the fermentation of palm wine which gives rise to its characteristic sour taste. This is due to the production of organic acids and alcohol fermentation causing the pH of the beverage to drop to an acidic position (pH below 4.0) (Amoa-Awua *et al,* 2007). The survival of microorganisms in the palm wine is dependent on their ability and capability to withstand the alcohol and acid content of the beverage. Alcohol has the ability to destroy microbes and limit their growth hence, the metabolic products from the fermentation processes helps in preserving and extending the shelf life of the beverage by inhibiting the growth of spoilage and pathogenic microbes (Chandrasekhar *et al*., 2015).

Traditional palm drink has been ascribed a functional drink due to the fact that it contains a high level of micro and macro nutrients which are beneficial to consumer's

health. Despite these, the lack of standardization of its production processes results in variations in taste, colour and aroma due to differences in sugar and alcohol content across various production sites. Research into the microbial and biochemical characteristics of the product during and after production will be paramount in determining the best level and methods of production to achieve desirable outcomes in both the nutrient quality of the beverage and its shelf life. Most importantly, it is necessary to observe safe hygiene practices during the point of tapping, storage, preservation, sales, and even consumption of the beverage in order to limit if not eliminate permanently the risk of contamination by external factors such as dirt, unwanted microbes and even insects. These measures should be taken by the producers, food safety authorities, sales personnel and even the consumers. This will play a part in boosting the socio-economic development of the product both in the local and international markets, ensuring safe and quality beverages are distributed for consumption.

5.2 Conclusion

Palm wine as a naturally fermented beverage presents both nutritional benefits and potential health risks depending on its production method, microbial quality and consumption patterns. Its wide range of bioactive compounds, microorganisms, and metabolites play various roles even in human health. This study revealed that most of the microorganism isolated are indigenous with palm wine and play a part in the fermentation of the beverage and could also cause spoilage of the beverage. It is therefore important to observe safe hygiene practices during the point of tapping, storage, preservation, sales, and even consumption of the beverage in order to reduce the risk of contamination by external factors such as dirt, unwanted microbes and insects.

5.3 Recommendation

It is recommended that proper investigations by the necessary authorities should be carried out to check the quality of production of these local alcoholic beverages. Proper education should be given to producers and sellers of the beverages. This should include education on personal and environmental hygiene as well as other food safety practices. Routine checks should be carried out by food safety authorities to ensure the beverage does not fall below the various microbiological standards. Also, emphasis should be made on the need to adequately package and label the product especially for commercial purposes. Labelling should include useful information e.g the sugar and alcohol content for those that require it for medicinal and health purposes. This will help in improving the market for the product.

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APPENDIX 1

PROCEDURES FOR THE BIOCHEMICAL TESTS CARRIED OUT

1. Gram staining: This test helps differentiate between Gram positive bacteria from Gram negative bacteria. An inoculum of the organism was emulsified in a drop of saline on a sterile clean slide to obtain a thin film. It was then heat fixed over a gentle flame. Crystal violet was added to the fixed smear and allowed to stand for about 1min, then it was rinsed with distilled water. Iodine was added, allowed to stay for a minute and then rinsed with distilled water. It was then decolourized by adding alcohol and then rinsed off after 30 seconds with distilled water. Safranin was used as a counter stain, left to stand for 1 minute and then rinsed off. Gram positive bacteria retained the crystal violet stain after alcohol decolourization while the Gram negatives did not.
2. Catalase test: A smear of the organism was made on a sterile slide. 2-3 drops of hydrogen peroxide solution were applied on the smear. Immediate bubbles indicated a positive result for the enzyme catalase and no bubbles indicated a negative result.
3. Indole test: This test is used to determine the ability of the organism to convert tryptophan into indole. The test organisms were isolated into bijou bottles containing 3ml of sterile peptone water and incubated at 35^0C for 48 hours. 5-7 drops of Kovac's reagent were added to test for indole production and observation was done for a ring of red colour in the surface layer within 5 minutes. Absence of a ring of red layer indicates a negative result.
4. Citrate test: Slopes of Simmons citrate agar were prepared in bijou bottles as recommended by the manufacturer. The slope was first streaked using a sterile

wire loop and then the butt stabbed. They were then incubated at 35^{O}C for 48 hours. A bright blue colour indicates a positive colour.

5. Oxidase: This is used to identify bacteria that produce the cytochrome oxidase enzyme. Two drops of freshly prepared oxidase reagent were placed in a filter paper on a petri dish. The test organism was picked and smeared on the filter paper with a sterile glass rod. A deep purple colour indicated a positive result.
6. Urease test: The urea agar was prepared according to manufacturer instructions and was then inoculated with the test organism. It was then incubated at 35^{O}C for 48 hours. A pink or red coloured change indicated the organism ability to hydrolyse urea to ammonia. No colour change or a yellow colour indicated no urease activity.
7. Yeast viability staining: A smear of the yeast was prepared, heat fixed and covered with methylene blue stain for 1 minute and then washed off with tap water. It was allowed to drain and air dry after which it was viewed on a microscope.

APPENDIX 2

Subcultured growth on Nutrient agar

Appearance of Lactic acid bacteria on MRS agar