ADDIS ABABA UNIVERSITY
ADDIS ABABA INSTITUTE OF TECHNOLOGY
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Development of Inoculated Fermentation System for the Production of Ethiopian Honey Wine, *Tej*, with Suspended Mixed Starter Culture

PhD Dissertation

by

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Submitted to the School of Chemical and Bio Engineering in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY (PROCESS ENGINEERING STREAM)

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Declaration

I hereby declare that this PhD Dissertation is my original work, submitted to Addis Ababa Institute of Technology's School of Chemical and Bio Engineering in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemical Engineering (Process Engineering Stream). Furthermore, this dissertation has never been submitted for any other degree or examination at this or any other institution before. Moreover, proper citation and acknowledgement have been made.

Eskindir Getachew Fentie _____ _____

Name Date Signature

Approval

Addis Ababa Institute of Technology

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As PhD research advisor, I hereby certify that I have read and evaluated this dissertation entitled "Development of Inoculated Fermentation System for the Production of Ethiopian Honey Wine, *Tej*, with Suspended Mixed Starter Culture" prepared under my guidance by Eskindir Getachew Fentie. Thus, I recommend that it can be submitted as fulfilling the requirements of the degree of Doctor of Philosophy (PhD).

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As member of the, *Board of Examiners of the Dissertation*, we certify that we have read, evaluated the dissertation prepared by Eskindir Getachew Fentie. We recommend this dissertation to be accepted as fulfilling the requirements for the Degree of Doctor of Philosophy (Ph.D.) in Chemical Engineering (Process Engineering) with respect to its originality and quality.

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Abstract

Traditional alcoholic beverages are widely produced and consumed in Ethiopia. *Tej*, Ethiopian honey wine, is one of Ethiopia's most popular spontaneously fermented traditional alcoholic beverages, usually made from honey and gesho (*Rhamnus prinoides*). However, this spontaneous way of fermentation increases the likelihood occurrence of suck and/or slow fermentation, making the process difficult to predict, control, and correct. Furthermore, it may also cause the product to have off flavor and inconsistent quality. As a result, the overall goal of this research is to create a direct fermentation system for *Tej* fermentation system, which is the assumed solution to the aforementioned problems. To achieve this goal, a physico-chemical and microbial ecological investigation, microbial isolation and strain selection, and kinetic study were carried out. Based on the honey production potential, *Tej* consumption culture, accessibility, and suitability for sample collection, study area for this research was selected to be Addis Ababa (AA), Debre Markos (DM), and Bahir Dar (BD), Ethiopia. The physicochemical properties of *Tej* samples collected from the aforementioned areas varied significantly from household to household and location to location. The alcohol and sugar contents ranged from 6.36 to 11.34 g/100 mL and 0.37 to 31.6 g/L, respectively. However, the microbial profiles were dominated by a few fermentative microorganisms. The bacterial community structure of *Tej* samples was dominated by the genera *Lactobacillus* (53.15%) and *Zymomonas* (38.41%). Similarly, the fungal community was exclusively dominated by the genus *Saccharomyces* (99.66%). Moreover, the microbial communities shifted to *Lactobacillus* and *Saccharomyces* dominance as *Tej* fermentation period progressed to the end. Furthermore, throughout these spontaneous fermentation period, the number of bacterial gene copies was half that of fungal gene copy numbers. After the intensive purpose-oriented screening and genotypic identification the isolated strains were *Saccharomyces cerevisiae*, *Pichia fermentans*, *Wickerhamomyces anomalus*, *Lactobacillus hilgardii*, *Lactobacillus paracasei*, and *Lactobacillus parabuchneri*. These isolated microbial strains were then used to ferment honey-must in various combinations. The more mixed the microbial strains are, the more the fermented product resembles the control sample in terms of physicochemical properties and volatile compound profile. Moreover, to reduce the possibility of sluggish and stuck fermentation, the medium was supplemented by diammonium phosphate (DAP). Higher fermentation rate was observed for the medium supplemented with the nitrogen concentration greater than 140 mg/L. A predictive kinetic model for microbial growth, substrate utilization, and product formation rate was developed using two substrate limitation (Monod and Teissier) and product inhibition (Ghose and Tyagi) models.

After simulation and parameter estimation, the Teissier model adequately described yeast assimilated nitrogen (YAN) utilization and ethanol production. Whereas Monod-Cheirsilp and Monod kinetic modes effectively described lactic acid bacteria (LAB) growth and sugar consumption rates. In general, the microbiological ecology analysis, isolation of dominant strains, and design of fermenting medium performed in this study yielded promising results for the modernization of Ethiopian honey wine, *Tej*. However, much more input from future research findings is required for the complete process upgrade, particularly in the areas of fermentation parameter optimization.

Keywords: *Tej*, *Fermentation*, *Lactobacillus*, *Saccharomyces*, antioxidant, physicochemical, Kinetic modeling

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List of Abbreviations

ABTS – 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt

DPPH – 2,2-diphenyl-1-picrylhydrazyl

ANOVA – Analysis of Variance

FAO – Food and Agricultural Organization

FAOSTAT – Food and Agricultural Organization Statistical Database

HPLC – High Performance Liquid Chromatography

SPME- GC-MS – Solid Phase Micro Extractor Gas Chromatography Mass Spectroscopy

SD – Standard Deviation

EFFCA – European Food and Feed Cultures Association

IDF – International Dairy Federation

SSF – Solid State Fermentation

TP – Total Phenol

YAN – Yeast Assimilable Nitrogen

PCR – Polymerase Chain Reaction

NGS – Next Generation Sequencing

DNA – Deoxyribonucleic acid

RNA – Ribonucleic acid

ANN – Artificial Neural Network

OIV – International Organization of Vine and Wine

TA – Titratable Acidity

LefSe – Linear Discriminant Analysis Effect Size

PCoA – Principal Co-ordinate Analysis

ITS – Internal transcribed spacer

qPCR – Quantitative Polymerase Chain Reaction

RAPD-PCR – Randomly Amplified Polymorphic DNA Polymerase Chain Reaction

YPD – De Man, Rogosa and Sharpe

MRS – Yeast Extract Peptone Dextrose

CCA – Canonical Correspondence Analysis

OD – Optical Density

DAP – Diammonium Phosphate

LAB – Lactic Acid Bacteria

List of Mathematical Nomenclatures

X_Y – yeast cell concentration

X_L – lactic acid bacteria concentration

N – nitrogen concentration

S – substrate (sugar) concentration

P – product concentration

t – time

P_{max} – maximum product concentration

μ – specific growth rate

ε – mixed culture effect factor

q – specific product formation rate

q_{max} – maximum product formation rate

$Y_{Y/N}$ – yeast cell mass yield coefficient

$Y_{L/N}$ – lactic acid bacteria cell mass yield coefficient

$Y_{e/S}$ – ethanol yield coefficient

$Y_{l/S}$ – lactate yield coefficient

$\mu_{max,Y}$ – yeast cell maximum specific growth rate

$\mu_{max,L}$ – lactic acid bacteria maximum specific growth rate

$K_{SX,Y}$ – yeast cell half saturation coefficient

$K_{SX,L}$ – lactic acid bacteria half saturation coefficient

$K_{SP,E}$ – ethanol half saturation coefficient

$K_{SP,L}$ – lactate half saturation coefficient

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CHAPTER 1

Introduction

1.1 Background

Fermentation is a biochemical process in which energy source substrates (carbohydrates and related compounds) are partially oxidized and energy is released in the absence of external electron acceptors (Buglass, 2010; Voidarou *et al.*, 2020). Organic compounds produced directly from the breakdown of carbohydrates serve as final electron acceptors (Mannaa *et al.*, 2021; Tamang & Kailasapathy, 2010). It is among the most ancient and cost-effective methods of food preservation (Lemi, 2020; Steinkraus, 2004). In addition to preservation, it also serves as a natural way to improve the nutritional quality of food through synthesizing essential amino acids and vitamins (Dimidi *et al.*, 2019; Kabak & Dobson, 2011). Basically, fermented foods and beverages are made by the action of microorganisms and their respective enzymes.

Traditional food products are those in which (1) the recipe (mix of ingredients), origin of raw material, and/or production process are authentic, (2) the key production steps are carried out in a specific area at the national, regional, or local level, (3) the product has been commercially available for at least 50 years, and (4) the product is part of the gastronomic heritage (Galanakis, 2019). Particularly, traditional fermented beverages, are products produced by the naturally available microorganisms through spontaneous fermentation system (Chaves-López *et al.*, 2014).

Traditional alcoholic beverages are one of the most common fermented products, in which ethanol is the primary end product of its spontaneous fermentation process (Egea, 2016; Lee *et al.*, 2015b). These traditional alcoholic beverages are commonly produced and consumed products in Asia and Africa (Tamang *et al.*, 2016). Nigeria's oti-oka (Temitope & Taiyese, 2012), Rwanda's ikigage (Lyumugabe *et al.*, 2010), Kenya's Busaa (Mary *et al.*, 2014), Uganda's kwete (Muyanja & Namugumya, 2009), Mexico's pulque (Valadez-Blanco *et al.*, 2012), and Korea's makgeolli (Kim *et al.*, 2013) are among the most common traditional alcoholic beverage consumed and produced in each respective country. Likewise, traditional alcoholic consumption and production in Ethiopia is a very common practice (Lemi, 2020). The country's per capital pure alcohol consumption is about 2.8 liters per annum. From this figure, traditional alcoholic beverages takes half of the market share (WHO, 2019). *Tella*, *Tej*, *Borde*, *Areki*, *Keribo*, and *Korefe* are among the popular traditional

fermented alcoholic beverages in Ethiopia (Fentie *et al.*, 2020). These traditional beverages are produced at the house hold level and consumed locally at the vicinity of production area. Producing and selling a traditional fermented beverages in Ethiopia also serves as a source of income for the large portion of the community (Nemo & Bacha, 2020b).

Fermented beverages produced from cereals usually referred to as beers while those produced from fruits are classified as wines (Peppler & Perlman, 1979). On the bases of this definition, beverages made form sugar, palm and/or honey are under the category of wine. Thus, *Tej*, which is made from honey, water, and gesho *(Rhamnus Prenoides)*, is definitely under the category of wine. *Tej* fermentation is based on naturally occurring fermentative microorganisms found in substrates and/or fermenting equipment. However, *Saccharomyces* and *Lactobacillus* genera have been identified as the dominating microbes for the *Tej* samples collected from Addis Ababa, Ethiopia. These two microbial species from two different domain acted harmonically in this acid-alcoholic fermentation system of *Tej* (Bahiru *et al.*, 2006a).

Only a few reports on Ethiopian traditional beverages in general, and *Tej* in particular, have been published so far. Even the literature that is available is outdated and lacking in depth (Fentie *et al.*, 2020; Lemi, 2020). The first ever full-length article exclusively focused on *Tej* were reported by Bahiru *et al.*, (2001b). They studied the physicochemical properties of *Tej* samples collected from different house hold venders in Addis Ababa, Ethiopia. Their findings revealed that the pH, titratable acidity, alcohol content, fuse oil content, and proximate composition of the collected samples from different households varied significantly. The authors also claimed that the major source of variability is spontaneous and uncontrolled fermentation process during *Tej* production. The same authors had also reported the microbiological profile of *Tej* samples collected from the same site (Bahiru *et al.*, 2006b). Yeast and Lactic acid bacteria were the dominate microbes for the encountered samples. Furthermore, the microbial and physicochemical properties of *Tej* samples collected from Jimma (Ethiopia) district alongside another traditional fermented beverage, were recently studied (Nemo & Bacha, 2020b). Generally, almost all of these reports recommended further research into designing appropriate fermentation medium, selecting desirable fermenting cultures, and optimizing processing conditions for large-scale production. Thus, the overall aim of this PhD research work is to fill this gap by developing a direct fermentation system to modernize this Ethiopian honey wine *Tej*.

1.2 Statement of the problem

Base line information on final product characteristics is critical for upgrading any traditional fermentation to a direct inoculated fermentation system. However, limited studies are available on characterization of spontaneously fermented Ethiopian honey wine, *Tej*. Even available literature lacks the depth required to upgrade this beverage fermentation system. As a result, gathering detailed information on raw materials, production techniques, physicochemical properties, and microbiological profiles of the final product from various locations and households is a first must-do task.

Since microbial communities and enabling environmental conditions change constantly during *Tej* fermentation, information generated from the characterization of the final product alone is insufficient for the successful transition from spontaneous to direct fermentation system. The dominant microbes that lasted from start to finish have a significant impact on the spontaneous fermentation system. Furthermore, understanding the physicochemical properties with a given time interval during *Tej* fermentation will provide a significant advantage in the fermentation system's predictive power. However, *Tej* fermentation dynamics, which provide more information about the aforementioned issues, have not yet been revealed.

The most known draw back of the spontaneously fermented products is their lack of consistency from place to place and/or from household to household. This was clearly observed in previous studies, despite the fact that it was not on the required depth (Bahiru *et al.*, 2001b, 2006a). This variation could be due to differences in raw materials, equipment used, processing techniques, and environmental conditions. The most important step in properly addressing these limitations is to develop a starter culture. The process is more likely to have a predictable nature and consistent final product quality with a well-defined starter culture. To the best of our knowledge, no previous reports on the isolation, characterization, and identification of microbial strains that could be used as starter cultures for the fermentation of Ethiopian honey wine, *Tej*, have been published.

The occurrence of sluggish and/or stuck fermentation is another most common problems observed during spontaneous honey-must fermentation. Since it holds extra working capital for frequent *Tej* producers, this prolonged duration of fermentation has become a major constraint for producers' profitability. This type of failure in the fermentation systems affects not only the profitability, but also the quality attributes of the final product. The problem even exacerbated by the absence of a

starter culture and designed fermentation medium. Nonetheless, no report has been submitted so far to address this issue in a scientific manner.

1.3 Research questions

- Are there any differences in *Tej* production techniques between Ethiopian localities?
- How varied are the physicochemical properties and microbiological profile of *Tej* samples collected from various locations and households?
- What will happen to the fermentation dynamics over time during the *Tej* fermentation period?
- Is it possible to upgrade the spontaneous *Tej* fermentation system to a direct inoculation fermentation system by using a well-defined mixed starter culture without compromising the final product major quality attributes?
- Which microbial strain combination will result in a higher final product quality attribute?
- How do these mixed culture microbial strains interact with one another during *Tej* fermentation?
- How will the fermentation kinetics react to the addition of diammonium phosphate to the honey-must fermentation medium?
- Which kinetic model will best describe the mixed culture *Tej* fermentation system?

1.4 Objectives

1.4.1 General objective

The general objective of this research was to develop a direct fermentation system for the production of alcoholic beverage *Tej* with a suspended mixed culture of yeast and lactic acid bacteria.

1.4.2 Specific objectives

The specific objectives of this research were to:

- Assess the indigenous *Tej* making process, microbiome, physicochemical and antioxidant profiles for the samples from different local vendors.
- Study the fermentation dynamics of spontaneously fermented *Tej* samples.

- Isolate, characterize, screen and identify microbial strains that has a potential to be used as *Tej* fermentation starter culture.
- Conduct a comparative assessment for different isolated strains combination for mixed culture selection to produce good quality *Tej*.
- Develop a kinetic model as a function of nitrogen supplement concentration for batch suspended mixed culture *Tej* fermentation system.

1.5 Significance of the research

The absence of detailed baseline information on final product quality attributes and on physicochemical and microbiological profile changes during the fermentation period is the first and most significant problem for *Tej* producers as well as scholars interested in studying how to improve *Tej* fermentation systems. As a result, the findings from this study on final product characterization and *Tej* fermentation dynamics will have a significant impact on supplementing existing literatures in order to develop better baseline information that can be used as a reference point for future research.

Tej producers continue to struggle with inconsistent final product quality as well as failure to complete the fermentation process successfully. Particularly, honey wine fermentation has a higher risk of becoming stuck and/or sluggish by nature, which has a greater impact, especially from an economic standpoint. The aforementioned problem will be greatly alleviated through the development of starter culture and the design of the proper fermentation medium. Contamination is the other most important issue in any biochemical process in general, and *Tej* in particular. This issue always results in a failed fermentation. Development of starter culture, will indirectly reduce the risk of contamination during *Tej* fermentation.

According to FAO report in 2018 “The annual honey production in Ethiopia was increased and reached quite higher than 58 thousand tons in 2018”. This honey production has the potential to attain up to 500,000 tons of honey and 50,000 tons of beeswax per year (MoA & ILRI, 2013). From the produced honey; about 80% are used for local consumption with a greater part for *Tej* making process at house hold level (Gobessa *et al.*, 2012). As a result, in addition to focusing on achieving the country's honey production potential, a due attention is also needed to the research on *Tej* production process and marketing.

1.6 Organization of the dissertation

This dissertation is organized in 8 chapters:

Chapter 1 of the dissertations gives a brief introduction of this study. This chapter contains background information, a statement of problems, research questions, objectives of the study, the significance of the study, and the dissertation's organization.

Chapter 2 presents the prior literatures which are related to this study. This chapter in short provides readers with background information on traditional fermented beverages, Ethiopian honey wine, *Tej*, and biological process modeling.

Chapter 3 deals about the general materials and methods used in this study. Study area description, sample collection, and transportation mechanisms, and common analytical techniques are described in this chapter.

Chapter 4 presents the assessment report on the raw materials and traditional *Tej* making practices, quality attributes, and shelf stability of spontaneously fermented *Tej*. Furthermore, it also provides a background information on the characteristics of spontaneously fermented *Tej* samples. This chapter presents detailed physicochemical properties, antioxidant activity, and microbiological profiles of *Tej* samples.

Chapter 5 deals about the microbial and physicochemical dynamics during *Tej* fermentation. The detailed microbiome, substrate, product and antioxidant dynamics over time during the fermentation of *Tej* are covered in this chapter.

Chapter 6 presents about the presumptive starter culture isolation, characterization, screening and identification for direct inoculated *Tej* fermentation system. Besides, it gives information about the microbial strains' interactions, physicochemical properties and volatile compound profile of the *Tej* samples produced from the isolated starter culture strains.

Chapter 7 deals about the effect of nitrogen supplement on the honey-must fermentation system. Furthermore, it compares various first principle kinetic models that best fit the mixed culture *Tej* fermentation system.

Chapter 8, the final chapter of this dissertation, presents the conclusions and recommendations of the entire works covered by this dissertation.

CHAPTER 2

Literature Review

2.1 Food Fermentation

Fermentation is most likely one of the oldest food processing technologies that utilize active microorganisms to transform and extend the shelf life of food materials (Peppler & Perlman, 1979). From a biochemical standpoint, fermentation is a metabolic process for obtaining energy from organic substrates without the use of an exogenous oxidizing agent (Ray & Montet, 2015). According to Steinkraus, (2002) description, fermentation plays five major roles in food processing: (1) preservation of agricultural products; (2) enrichment of nutritional value of foods; (3) detoxification of food materials; (4) increasing of bioactive compounds; (5) reduction of cooking time. Many different fermented foods exist around the world, in which a variety of different substrates are metabolized using variety of microorganisms to produce products with appealing characteristics (Tamang, 2014). Meat and fish, vegetables, dairy, soybeans, other legumes, fruits, and cereals are just a few examples of foods that have been fermented for years (Dimidi *et al.*, 2019). Microorganisms, substrate to be fermented, fermenting vats and vessels, and various tools that can be used to develop and monitor the fermentation are the main components of the fermentation ecosystem (Scott & Sullivan, 2008).

2.1.1 Classification of food fermentation

Based on the type involved microorganisms and type of final product, food fermentation can be broadly classified into alcoholic fermentation, lactic acid fermentation, and alkaline fermentation. Alcoholic fermentation is the anaerobic conversion of sugars, primarily fructose and glucose, to ethanol and carbon dioxide using yeast and bacteria like *Zymomonas mobilis* (Zamora, 2009). Lactic acid fermentation, also known as lacto-fermentation, is the process of converting sugar into cellular energy and lactate under anaerobic condition, which is primarily accomplished through the action of Lactic acid bacteria (Malo & Urquhart, 2016). Alkaline-fermentation is aerobic fermentation process at which the pH of the fermentation medium increases to alkaline values by the action of *Bacillus spp* specifically by *B. subtilis* (Parkouda *et al.*, 2009; Wang & Fung, 1996).

Based on the technique of processing food fermentation could be categorized into two. Natural or spontaneous fermentation is one of the types of fermentation described above, in which the

microorganisms are derived from the raw materials and equipment used (Chaves-López *et al.*, 2014). Whereas, direct inoculated fermentation, commonly referred to culture-dependent fermentation, is the other type of fermentation process which a microbial strain of a well-defined starter culture is applied to the fermentation medium (Cuvas-Limon *et al.*, 2021). Natural (back-sloping) or commercial starters can be used to initiate fermentation and standardize the final product's organoleptic characteristics. Back-sloping fermentation, one of the culture dependent methods, is performed by inoculating a small amount of a previously fermented product is introduced to this fermentation (Mannaa *et al.*, 2021). Frequent use of back-slopping increases the probability of selecting the best-adapted strains, some of which may have characteristics that make them suitable for use as starter cultures. European Food and Feed Cultures Association (EFFCA) defines starter culture: “Microbial food cultures are live bacteria, yeasts or moulds used in food production”. However, the application of this microbial culture has strict regulatory issue. Several regulatory frameworks exist for the use of microorganisms in food fermentations, with an emphasis on “history of use”, "traditional food", or “general recognition of safety”. The updated version of “2002 International Dairy Federation (IDF)”, which recommends 62 genera and 264 species as food starter, is the most common reference for food culture (IDF, 2018).

Furthermore, depending on the moisture content of the fermentation medium, fermentation can be divided into submerged and solid states (Steinkraus, 2004). In the first case, microbial activity occurs in the liquid phase at a relatively low biomass concentration, whereas microbial growth and product generation occur on the surfaces, or within, solid substrates in the latter case (Capozzi *et al.*, 2017). Submerged culture fermentation has progressed to the point where large-scale industrial processes using single-microorganism cultures are now available for a variety of products (Ray & Montet, 2015). on the other hand, SSF processes involves three-phase interactions (gas–liquid–solid) and in many cases mixed microbial cultures (Table 2.1).

2.1.2 Advancements in food fermentations

Traditionally, fermented foods and beverages were made by relying on the microbiota that naturally existed on the food substrate (Giraffa, 2004). Throughout history, spontaneous fermentation based on native microbes was the primary method for producing fermented foods, and it remains the main method in domestic, small-scale, and household productions (Voidarou *et al.*, 2020). Even without knowing the types of active microbes, the fermentation process may begin by transferring a small amount of a previously fermented batch into fresh medium as an inoculum to facilitate the

initial stage of fermentation; this process is known as back-slopping (Hansen, 2002). Back-slopping decrease the chance of failure and improves the competitiveness of fermentation microbes; the repeating process may result in a more refined selection of useful microbes which are best suited to the food substrate and fermentation conditions (Voidarou *et al.*, 2020).

Table 2. 1 Comparison between submerged and solid-state fermentation process

Submerged state fermentation
Lower pure and mixed microbial culture concentration
Relatively fast microbial growth and product formation rate
Higher level of aeration and agitation
Easy for scale up, and process control
Easy for product recovery (e.g., cell separation, distillation)
Solid state fermentation
Slower microbial growth and product formation rate
Higher biomass and enzyme concentration is need
Lower level of mixing, due to the shear sensitivity of filamentous fungi
Scale up problem, due to difficulty in heat and mass transfer problems
Difficult process control, due to three phase (solid-liquid-gas) process
Higher probability of happening contamination

Source:(Steinkraus, 2004)

Later, the spontaneous fermentation system was improved by inoculating a well-defined starter culture isolated, identified, and characterized from fermented foods (Malo & Urquhart, 2016; Steinkraus, 2004). These cultures are now widely used, particularly on an industrial scale, to ensure that the fermentation process is controlled and the outcome is consistent in terms of quality and properties (Mannaa *et al.*, 2021). The primary function of starter cultures is to speed up the fermentation process by converting carbohydrates into alcohols and organic acids, this in turn limits the growth of harmful microbes and imparts distinct organoleptic properties to the product (Grumezescu & Holban, 2019). The most commonly used microbial strains in food fermentation are yeast and lactic acid bacteria (Tamang & Kailasapathy, 2010).

Starter cultures are not always composed of a single strain; in several cases, a consortium of various organisms and strains is involved (Smid & Lacroix, 2013). Most food fermentation relies on microbe mixtures that work together to produce the desired product characteristics (Tamang & Kailasapathy,

2010). In general, diverse microbial consortia perform more complex activities and tolerate more environmental variation than pure cultures (Champagne *et al.*, 2010). The robustness and versatility can be described by two features (Brenner *et al.*, 2008). First, members of the microbial strains in the consortium communicate with to each other by exchanging metabolites or molecular signals. The consortium's second distinguishing feature is its division of labor, which results in an overall output which can only be explained by combining the performance of constituent individuals or sub-populations (Mannaa *et al.*, 2021).

The advancement in molecular biology had assisted the fermentation process to improve further in terms of using starter culture. Previously, starter cultures were chosen after screening a large number of isolates and selecting those that performed well and produced end products with desirable organoleptic characteristics (Vinicius De Melo Pereira *et al.*, 2020). Recently, the advanced tools of high-throughput screening for specific target genes and metabolic pathways enables to select the better performing and well-adapted starter cultures (Andeta *et al.*, 2019). For decades, recombinant DNA technology has been used to create starter cultures, which may improve fermentation processes and provide better-quality products with desired properties However, most of the developed strains is not widely used in the industry due to strict government regulations and consumers rejection for genetically modified food ingredients (Johansen, 2017). As a result, in the food industry, non-recombinant DNA strain improvement methods like directed or adaptive evolution, random mutagenesis, and dominant selection, as well as natural mechanisms like natural competence, bacteriophage transduction, and conjugation are widely used (Derkx *et al.*, 2014).

2.1.3 Alcoholic fermentation

In general, alcoholic beverages are produced by yeasts, primarily strains of the species *S. cerevisiae*, by fermenting sugars (Grumezescu & Holban, 2019). Indeed, yeasts play an important role in the production of alcoholic beverages, and selecting suitable yeast strains is critical not only for maximizing alcohol yield, but also for maintaining beverage sensory quality (Tamang & Kailasapathy, 2010). *S. cerevisiae* is the most common yeast species used in worldwide alcoholic beverages production, and the strains used in fermentation have a significant impact on the flavor and aroma characteristics of various beverages (Vinicius De Melo Pereira *et al.*, 2020). Brewing yeasts are also members of the *Saccharomyces* genus, but two distinct species are used in ale and lager brewing. Similarly, wine yeast starter cultures are mostly *S. cerevisiae* strains, but sometimes non-Saccharomyces starter cultures (e.g., *Candida stellata*) may be used to impart

distinct flavor and aroma characteristics to wine (Dashko *et al.*, 2015). Likewise, in the production of distilled spirits, fermentations of mash sugars into ethanol, and numerous secondary fermentation metabolites will cumulatively contribute for the flavor development of the final product by specific strains of *S. cerevisiae* (Inge, 2003). As a result, selecting the appropriate yeast strain to contribute to the organoleptic qualities of spirits is critical. The first step in the alcoholic fermentation pathway is pyruvate, which is produced by yeast via the EMP pathway, but obtained by *Zymomonas* via the ED pathway (bacteria) (Pfeiffer & Morley, 2014). The pyruvate is then decarboxylated to acetaldehyde in the next step, which is catalyzed by the enzyme pyruvate decarboxylase (Figure 2.1). The regeneration of NAD during the reduction of acetaldehyde into ethanol, which is catalyzed by alcohol deydrogenase, maintains the redox balance of alcoholic fermentation (Saika *et al.*, 2006).

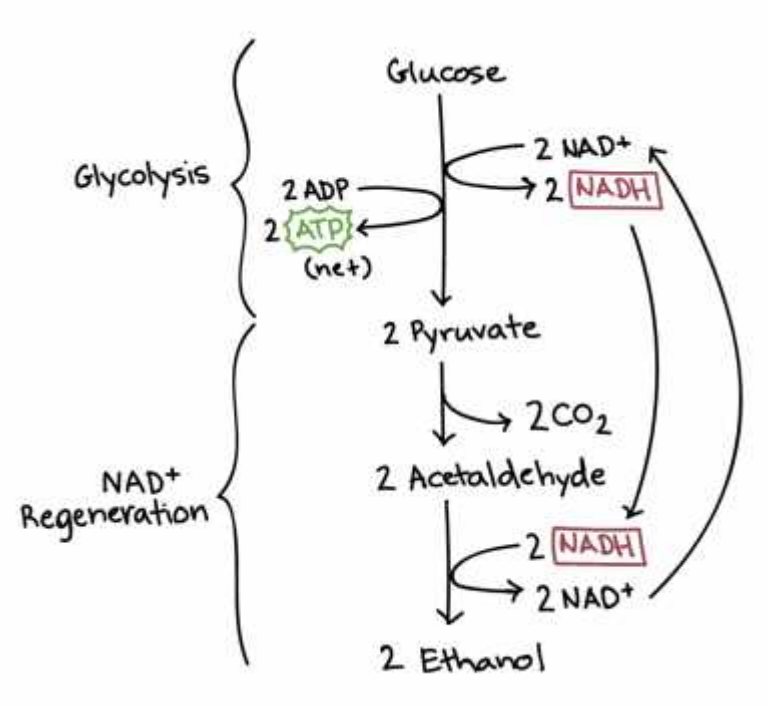

Figure 2. 1 Microbial metabolic pathway of alcoholic fermentation

2.2 Ethiopian traditional beverages

Fermented beverages are an important part of diets in all parts of the world, accounting for roughly one-third of global food consumption and 20–40% (by weight) of personal diets (Mulaw & Tesfaye, 2017). Similarly, Ethiopia produces and consumes about 8 million hectoliters of indigenous fermented alcoholic beverages each year. Alcoholic beverages produced commercially and traditionally have nearly equal market share in the country (CSA, 2016). In addition to its role in social functions, it is an important part of the diet in all parts of the world (Mulaw & Tesfaye,

2017; Negasi *et al.*, 2017). Usually, this traditional fermentation processes are integrated into village life, commonly use locally available raw materials, are inexpensive, and are part of the culture. Furthermore, their lower cost has given them an advantage in the market competition over commercially produced beverages, as it provides a low-cost option for low-income consumers (Lemi, 2020). Since there is not standard production process, during the production of traditional beverages the fermentation period, processing conditions are selected based on the producer's personal judgment (Hotessa & Robe, 2020).

In Ethiopia, diverse range of traditional fermented beverages are produced from a variety of raw materials, including maize, barley, wheat, and honey (Negasi *et al.*, 2017). Ethiopian fermented beverages are processed in a variety of ways, which vary from region to region and product to product. Among these *Borde*, *Tella*, *Shamita*, *Keribo*, *Korefe*, *Tej*, *Cheka*, *Ogol* and *Booka* are very popular indigenous Ethiopian fermented alcoholic beverages (Table 2.2). These beverages have a total alcohol content ranging from 1.53 to 21.7 percent (v/v) (Bahiru *et al.*, 2001b; Elema *et al.*, 2018). All of these alcoholic beverages are made on a small scale and sold by home-based alcohol vendors. Besides, the fermentation systems that produce these traditional alcoholic beverages are known as acid-alcohol fermentation systems (Nemo & Bacha, 2020b). Ethiopian beverages are frequently of poor quality and fail to meet their objectives due to a lack of standardized processes, back-slopping, and starter culture (FAO, 2011). Furthermore, the process of making these fermented beverages is time-consuming and labor-intensive (Abegaz *et al.*, 2002).

Ethiopian traditional alcoholic beverages is done via natural or spontaneous fermentation system (Abegaz, 2007). Because of the nature of this fermentation system, it frequently involves mixed cultures of microbes (Hotessa & Robe, 2020). During fermentation, some of these microbes from the mixed culture were involved in parallel, while others were involved sequentially with a changing dominant biota (Nemo & Bacha, 2021). As a result, the isolation of such microbes for use as a starter culture should not be limited to dominant organisms, but should also include microbes found in lower numbers that may play an important role in the process (Hotessa & Robe, 2020). Raw materials, traditional processing utensils, and back slopping (using a small portion of beverage from a previous fermentation) are common sources of these microorganisms (Bahiru *et al.*, 2006a).

Table 2. 2 Summary of Ethiopian traditional fermented beverages

Category of beverages	Beverages	Raw Materials	Prominent production & consumption regions	References
Beers	*Tella*	Barley *(Hordeum vulgare*), wheat *(Triticum aestivum)*, maize *(Zea mays)*, finger millet *(Eleusine coracana)*, sorghum *(Sorghum bicolor)*, "*teff*" (*Eragrostis tef*), "gesho" (*Rhamnus prinoides*)	Amhara, Oromia, Tigray, SNNP, Addis Ababa	(Andualem *et al.*, 2017; Tekle, Jabasingh, *et al.*, 2019)
	Borde	Maize *(Z. mays)*, barley *(H. vulgare)*, wheat *(T. aestivum)*, finger millet *(E. coracana)*, sorghum *(S. bicolor)*	SNNP	(Abegaz, 2007; Bacha *et al.*, 1998)
	Shamita	Roasted barley *(H. vulgare)* flour, salt, linseed *(Linum usitatissimum)* flour, chili pepper *(Capsicum annuum)*	SNNP, Addis Ababa	(Ashenafi & Mehari, 1995a; Bacha *et al.*, 1999)
	Korefe	Malted and non-malted barley *(H. vulgare)*, "gesho" (*R. prinoides*)	Amhara	(Getnet & Berhanu, 2016)
	Keribo	Barley *(H. vulgare)*, sugar, bakery yeast *(Saccharomyces cerevisiae)*	Oromia, Amhara, Addis Ababa	(Abawari, 2013a, 2013b)
	Cheka	Sorghum *(S. bicolor)*, maize *(Z. mays)*, finger millet *(E. coracana)*, vegetables, root of taro *(Colocasia esculenta)*	SNNP	(Desta & Melese, 2019; Worku *et al.*, 2015, 2018)
	Areke	Barley *(H. vulgare)*, "gesho" *(R. prinoides)*, sorghum	Amhara, Oromia, Tigray, SNNP, Addis Ababa	(Debebe *et al.*, 2016; M. Lee *et al.*, 2015a; Tafere, 2015)
Wine	*Tej*	Honey, "gesho" *(R. prinoides)*	Oromia, Amhara, Tigray, Addis Ababa	(Bahiru *et al.*, 2001a, 2006b)
	Ogol	honey, barks of native tree *(Blighia unijungata)*	Gambella (Majangir)	(Teramoto *et al.*, 2005a)
	Booka	Honey, bladder of cow	Oromia (Gujii)	(Elema *et al.*, 2018)

Source: (Fentie *et al.*, 2020)

The nutritional values of Ethiopian indigenous alcoholic beverages vary widely from low to high levels. The nutritional values of some low-alcohol beverages are higher than those of their raw materials (Abegaz *et al.*, 2002). The main justification advanced for these results by most authors who studied Ethiopian traditional fermented beverages is the presence of live microorganisms in these beverages (Abegaz *et al.*, 2004; Ashenafi & Mehari, 1995b; Bacha *et al.*, 1999). Whereas, the nutritional values of high-alcohol beverages are lower than those of low-alcohol traditional beverages (Bahiru *et al.*, 2001b; Tekle *et al.*, 2019). Only a few microorganisms can withstand the adverse environmental condition of the growth medium as the fermentation progresses to the end. As a result, microorganisms that are unable to adapt to their new environment will be lysed and used as a source of protein for the surviving species' cell maintenance. For the spontaneous, and uncontrolled fermentation systems, this explanation works even better. As a result, the competition for survival among microbial species reduces the nutritional profile of beverages (Cason *et al.*, 2020; Karen, *et al.*, 2020; Lucke, 2015).

Total polyphenols (TP) content and antioxidant activity (AA) are indicators of a beverage's functional properties (Cory *et al.*, 2018). These polyphenols and antioxidants promote health by regulating metabolism and scavenging free radicals (Ganesan & Xu, 2017). Many Ethiopian traditional alcoholic beverages have demonstrated a good TP and AA values (Table 2.3). Among other traditional fermented beverages *Tella* had a higher phenolic content (Shewakena *et al.*, 2017). Despite the fact that there are numerous factors influencing this result, raw materials, particularly the amount of gesho added to the mixture, account for the lion's share of the variance (Debebe *et al.*, 2016).

Identification of the raw materials, traditional processing techniques, microbial characterization, and microbial dynamics on Ethiopian traditional fermented foods have been reported for the last two decades (Hotessa & Robe, 2020; Lemi, 2020). However, the majority of these studies lack completeness in the information needed to modernize the entire process of these beverages. As a result, we believe that future research should focus on rheological properties, microbial metagenomics, starter culture development, shelf-life extension, process modification, kinetics, and optimization.

Table 2. 3 Proximate composition, functional properties, higher alcohol, and fluoride ion contents of Ethiopian alcoholic beverages

Beverages	Nutritional value	Functional properties	Higher alcohol and fluoride ion	References
Tella	Total protein–0.4% Carbohydrate–1.98%	TP (µg mL^{-1})–232.40 AA(µg mL^{-1})–296.00 Folate ($mgcg^{-1}$)–0.093	Fusel oil (ppm)–51 Methanol(ppm)–41.5 Fluoride ion (mg/L)–4.26	(Belete *et al.*, 2017; Fite *et al.*, 1991; Shewakena *et al.*, 2017; Tekle, Jabasingh, *et al.*, 2019)
Borde	Total protein–9.55%, Crude fat–6.88%, Total ash–3.66%	TP (µg mL^{-1})–9.50 AA(µg mL^{-1})–198.5	Fluoride ion (mg/L)–4.95	(Abegaz *et al.*, 2002; Ashenafi & Mehari, 1995a; Belete *et al.*, 2017; Debebe *et al.*, 2016)
Shamita	Total protein–10.37% Crude fat–6.85% Total ash–3.46%	–	Fluoride ion (mg/L)–5.21	(Ashenafi & Mehari, 1995b; Belete *et al.*, 2017)
Korefe	–	TP (µg mL^{-1})–167.60 AA(µg mL^{-1})–278.13	Fluoride ion (mg/L)–1.39	(Belete *et al.*, 2017; Shewakena *et al.*, 2017)
Cheka	Total protein–3.83% Crude fat–1.49% Carbohydrate–16.59% Total ash–0.79%	–	Methanol(ppm)–271.55	(Worku *et al.*, 2018)
Keribo	–	TP (µg mL^{-1})–12.65 AA(µg mL^{-1})–64.66	–	(Debebe *et al.*, 2016)
Tej	Total protein–0.35% Crude fat–0.35% Carbohydrate-3.58% Total ash–0.04% Moisture–82.18%	TP (µg mL^{-1})–197.00 AA(µg mL^{-1})–240.37	Fusel oil (ppm)–205.08 Fluoride ion (mg/L)–6.68	(Bahiru *et al.*, 2001a; Belete *et al.*, 2017; Debebe *et al.*, 2016; Nemo & Bacha, 2020a; Shewakena *et al.*, 2017)
Bokaa	Ash content–0.82% Crude fat –1.43% Total Nitrogen–7.01%	–	–	(Elema *et al.*, 2018)

TP in gallic acid equivalent (GAE)
AA in ascorbic acid equivalent (AAE)
All values are average values
–for values, not available in the literatures

Source: (Fentie *et al.*, 2020)

2.3 Ethiopian honey wine, *Tej*

Tej, Ethiopian honey wine, is a commercially available home-processed Ethiopian traditional beverage (Vogel & Gobezie, 1995). It's a household product, and each producer typically sells its product for consumption at the point of manufacture. *Tej* is commonly used for bartering in Ethiopia, and they play a significant role in dowries, feasts, and marriage ceremonies (Morcos *et al.*, 1996). It is usually prepared from honey, water and Gesho *(R. prenoides).* For commercial purposes, a mixture of honey and sugar is sometimes used by alcohol vendors to make *Tej*. When sugar is used as part of the substrate, a natural food colorant is added to give the beverage a yellow color that is similar to honey (Fite *et al.*, 1991). Some producers might also add various concoctions, such as barks or roots of various plants, or secrete herbal ingredients to improve flavor or potency, as well as to attract customers. Producers are usually unwilling to disclose the additives used and their composition due to blending, adulteration practices, and possibly other factors (Morcos *et al.*, 1996).

2.3.1 Raw materials of *Tej*

2.3.1.1 Honey

Honey (blossom or nectar honey) is defined as "a natural sweet substance produced by honey bees (*Apis mellifera*) from plant nectar, which the bees gather, transform by blending with different substances of their own, deposit, dehydrate, store, and leave to ripen and mature in the honey comb (Codex Alimentarius Commission, 2019). It was used as an essential source of carbohydrates and the only widely available natural sweetener for a long time in human history. Honey is one of the most commonly used products in traditional medicine, due to its therapeutic potential in the treatment of respiratory and gastrointestinal illnesses, the healing of wounds and burns (Al-Mamary *et al.*, 2002). Honey can be classified as mono-floral or multi-floral honey depending on whether the bees forage predominantly on one type of plant or a variety of botanical species (Alvarez-Suarez *et al.*, 2014).

Based on 2018 FAOSTAT data honey production around the globe is around 1,766,420 tons. This quantitative data also shows that the United States is the world's leading honey producer, accounting for 34% of total natural honey production. With a ten-years average of 446,961 tons, China was ranked second on the global honey producer list. Likewise, the last fifteen years Africa's average annual production has been around 175, 451.7 tons. East Africa contributed the lion shear by producing around 100,586.6 tons per year on average (Figure 2.2A). This figure accounts more than

half of the total annual production in the continent. Once again, Ethiopia is the leading honey producer country in the east Africa region (Figure 2.2B). It takes almost half of the total production; to be precise it accounted around 45% of the total production by the region. For the last fifteen years, Ethiopia's annual average natural honey production was 45, 408 tones (Figure 2.2C). In 2017, a total of 66,221 tons were produced, which was a new high (FAO, 2017). Despite the fact that the country has a natural honey production potential of 500,000 tons per year, production has only reached 10% of that (Gebretsadik & Negash, 2016). The honey belt zones of western and southern Ethiopia produce the majority of beekeeping products in terms of quantity. (Figure 2.2D). Apicultural resources abound, especially in the country's south western and south eastern zones, which include Illubabor, Jima, Bale, West Wellega, Benchi Maji, Keffa, , Sidama, and Gedeo zones (Teferi, 2018).

Honey is a viscous, concentrated liquid made up of sugar, protein, minerals, aromatic compounds, pigments, waxes, and pollen grains (Manzanares et al., 2011). Sugar profile, moisture content, acidity, and HMF are the physicochemical properties are usually used to indicate the maturity of honey. Even though oligosaccharides are detected in honey, reducing sugars (65%) are the major carbohydrate present in honey. Higher moisture content (>20%) is a good indication for honey fermentation which makes storage for a longer period of time difficult (Olaitan et al., 2007). Honey is generally acidic, but if it falls below a certain threshold, it could indicate the onset of fermentation (Missio *et al.*, 2015). Other honey physicochemical properties such as, color, ash content, electrical conductivity (EC) and insoluble solids are used to indicate the purity honey (Codex Alimentarius Commission, 2019). EC is another important quality parameter for determining the type of honey, adulteration, and origin of the flora (Belay *et al.*, 2016). The color of honey is mainly dependent on the mineral content of honey (Gairola *et al.*, 2013). Generally, physicochemical, biochemical and antioxidant properties of honey highly depend on the agro- ecological zone, soil conditions, type of honey bees, type of flora, post-harvest processing and storage conditions, type of hives etc (Cimpoiu *et al.*, 2013; Escuredo *et al.*, 2014).

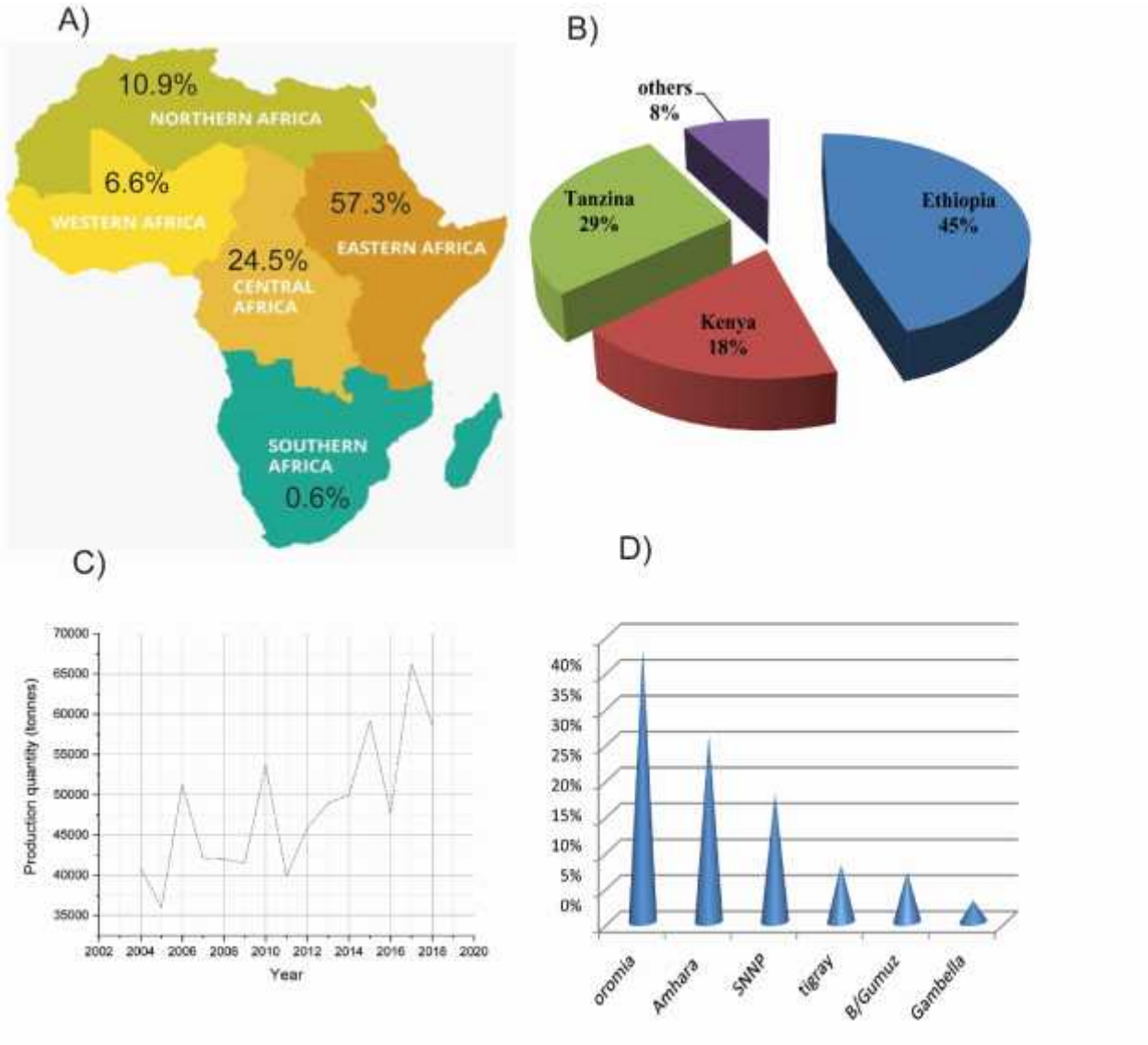

Figure 2. 2 the last fifteen years average honey production in **A**) Africa, **B**) East Africa, **C**) Ethiopia, and **D**) Each region in Ethiopia.

2.3.1.2 Gesho (*R. prinoides*)

To achieve the same bitter flavor that commercial beer uses hop (*Humulus lupulus*), Ethiopian traditional beverage (*Tella* and *Tej*) producers also uses gesho (*R. prinoides*) to get the same result (Amabye, 2015; Berhanu, 2014). It is one of several locally available tropical and subtropical plants being considered as a potential hop replacement for commercial beer production (Berhanu, 2014; Okafor *et al.*, 2016). Actually, the precent soft resine of gesho (*R. prinoides*) plant is higher than hop (*H. lupulus*) (Berhanu, 2014). This soft resin is a major precursor to bitter flavor. Thus gesho (*R. prinoides*) is a good candidate to replace a commercially available hop for the production of

beer. Gesho (*R. prinoides*) is also a source of fermentative microorganisms that additionally regulates the microflora during the fermentation process (Abegaz, *et al.*, 2007).

Gesho (*R. prinoides*) is family of *Rhamnaceae* that includes more than 150 taxa. Only two species, *R. prinoides* and *R. staddo*, are commonly grown in Ethiopia. Actually, *R. staddo* is not commonly used in the preparation of Ethiopian traditional beverage (Zeleke, 2010). Whereas, Gesho (*R. prinoides*) is a perennial crop that can reach a height of 6 meters and is grown at elevations ranging from 1500 to 2500 meters above sea level (Bezabeh, 2017). This plant covered approximately 28,386 ha of land and about 37,162 tons were produced in year 2015 (CSA, 2016).

A substance β-sorigenin-8-O-β-D-glucoside, named ‘geshoidin’ by Abegaz & Kebede, is the responsible naphthalenic compound in gesho (*R. prinoides*) leaves and steam for imparting bitterness for the prepared traditional beverages. Aside from 'geshoidin,' the leaves and stem of gesho contain rhamnocitrin, emodin, musizin, physcion, -sorigenin, chrysophanol, rhamnocitrin, quercetin, and 3-O-methylquercetin (*R. prinoides*) (Abegaz & Kebede, 1995). In additional to imparting a bitter flavor, the majority of the identified compounds have antioxidant and antimicrobial properties. Gesho (*R. prinoides*) has been used as a traditional medicine in many African countries to treat joint pain, stomach complications, fever, common cold, diarrhea, malaria, body weakness, sprains, appetizer and pneumonia, gonorrhea, rheumatism, colic, and ringworm infections (Amabye, 2015).

Table 2. 4 Trends of research conducted on gesho (*R. Prinoides*)

Investigator's name, Year	Title	Sample collection sites	Part of the plant	Major results
(Abegaz & Kebede, 1995)	Geshoidin: A bitter principle of *Rhamus Prinoides* and other constituents of the leaves	Addis Ababa	Fresh and dried leaves	After extraction by methanol and choloroform, compound identification and characterization were preformed ✓ develop a structure and characterized β-sorigenin-8-β-D-glucoside, 'geshoidin' from the extract ✓ Identified additional substances; rhamnocitrin, emodin, musizin, physcion, β-sorigenin, chrysophanol,rhamnocitrin, quercetin, 3-O-methylquercetin
(Amabye, 2015)	Evaluation of Phytochemical, Chemical Composition, Antioxidant and Antimicrobial Screening Parameters of *Rhamnus prinoides* (Gesho) Available in the Market of Mekelle, Tigray, Ethiopia	Mekelle, Ethiopia	leaf	❖ Qualitative determinations were done for gesho phytochemicals; Alkaloids, triterpene, saponins, tannins, phenols, glycosides, resins ❖ The MIC (mg/l) for *E.coli, S. aureus, P.aeroginosa, S. mutans* were 400,200,400 and 200 respectively.
(Amabye, 2015)	Levels of essential and non-essential metals in *Rhamnus Prinoides* (gesho) cultivated in Ethiopia	Tigray, Amhara and Oromia, Ethiopioa leaf and stem	Leaf and stem	The average levels of metals (mg/kg) were determined in gesho from different parts of the country ✓ For the leaves Ca (14,270), Mg (4,454), Cr (12.84), Mn (13.01), Fe (117.45), Co (32.15), Ni (20.05), Cu (39.75), Zn (27.6), Cd (1.96), and Pb (21.35) ✓ For steam Ca (4,638), Mg (4,082), Cr (ND–16.3), Mn (3.07), Fe (73), Co (55.2), Ni

			(14.44), Cu (124.9), Zn (22.8), and Cd (ND–1.56)
(Abegaz, *et al*., 2007)	Electrochemical behavior and voltammetric determination of geshoidin and its spectrophotometric and antioxidant properties in aqueous buffer solutions		❖ Show the possibility of direct determination of geshoidin from the solution without further purification process. ❖ Demonstrate the structural change of geshoidin with the change in pH of the solution ❖ geshoidin has the antioxidant capacity
(Berhanu, 2014)	Microbial profile of *Tella* and the role of gesho (*Rhamnus prinoides*) as bittering and antimicrobial agent in traditional *Tella* (Beer) production	Leaf and stem	❖ A mean percent essential oil, soft resin, hard resin, and total resin for the leaf of gesho was 1.13,15.73,2.73 and 18.46, respectively ❖ A mean percent essential oil, soft resin, hard resin, and total resin for the steam of gesho was 0.60,12.40,4.74 and 17.16 respectively ❖ MIC of gesho extract against clinical and pathogenic bacteria ranged from 97.5 to 780 mg/ml ❖ MBC of gesho extract against clinical and pathogenic bacteria ranged 195 to 780 mg/ml

*** ***MIC***- Minimum Inhibition Concentration, ***MBC***- Minimum bactericidal Concentration

2.3.2 Tej Production process

The raw materials to produce *Tej* are honey, gesho (*R. prenoides*), water and naturally available microbes. The honey used to brew *Tej* is typically collected from wild nests or produced in traditional cylindrical hives. This honey usually contains wax, broken combs, pollen, and bees. The *Tej* producers believe that crude honey is superior to refined honey in the production of Ethiopian honey wine (Vogel & Gobezie, 1995). The pollen acts as a nutrient for the yeast, and residual wax on the surface may help to create the anaerobic environment required for *Tej* fermentation. The main equipment needed to make *Tej* is a covered container and a filtration cloth (Nemo & Bacha, 2020b).

The process of making *Tej* begins with the fermentation pot being smoked with gesho (*R. prenoides*) and olive wood steam. This preparative process is essential to produced *Tej* having a desired smoky flavor. The flow sheet for production of *Tej* is given in Figure 2.2. Usually, one part of honey is mixed with 2 to 5 (v/v) parts of water in the smoked pot. This diluted honey will have a sugar content from 13 to 27%. The mixture is then covered with a clean cloth for 2 to 3 days to complete the first phase fermentation process. Subsequently, the wax and top scum is then removed from the fermenting blend. Then after, some portion of the must is boiled with washed and peeled gesho (*R. prenoides)* and returned to the fermenting must after cooling it to room temperature. The pot is then covered and left to ferment for another 15-20 days to complete the secondary fermentation phase. Finally, the mixture is filtered through a clean cloth to remove sediment and gesho (*R. prenoides*). If the fermentation is successful, the final alcohol content of fully mature *Tej* will range between 7 and 14 percent v/v (Morcos *et al.*, 1996).

2.3.3 Physicochemical and microbiological characteristics of *Tej*

Thus far, limited information is available on the physicochemical properties and microbiological profile of *Tej*. We summarize the physicochemical and microbial profile of *Tej* samples from the published article in chronological order because the results in each reports showed a figure difference even for the same analysis. The first article on *Tej* was published in 2001 by Bahiru *et al.*, who collected approximately 200 *Tej* samples from ten local producers in Addis Ababa, Ethiopia. The report showed pH, titratable acidity alcohol content, proximate composition, fusel oil varied significantly for sample to sample.

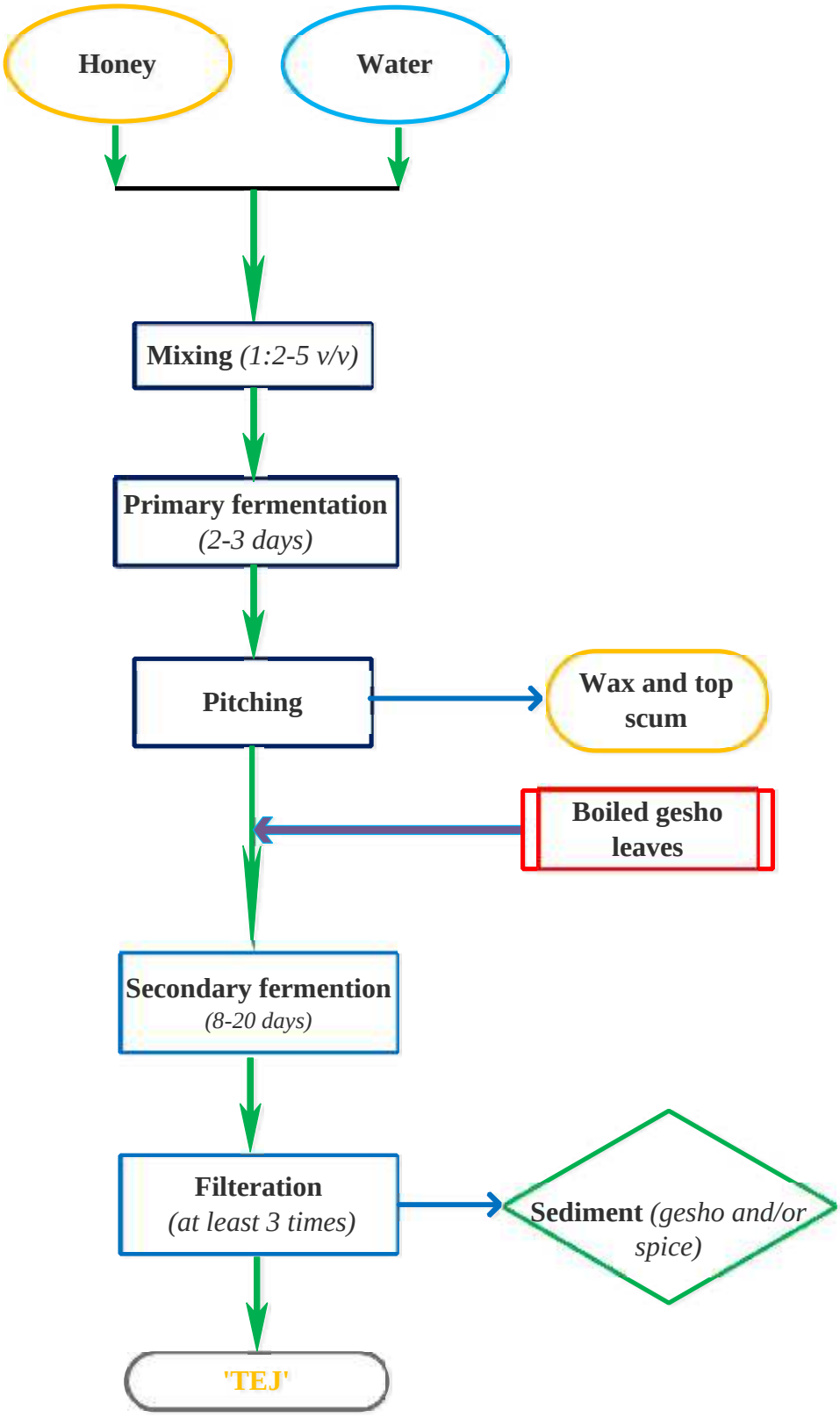

Figure 2. 3 Flow chart of *Tej* production process

The same authors, (Bahiru *et al.*, 2006a), again reported the microbiological profile for the *Tej* samples collected Addis Ababa, Ethiopia. The report aim was to investigate the yeast and lactic acid flora for this specific traditional alcoholic beverage. Based on phenotypic characterization *Saccharomyces cerevisiae* and *Lactobacillus* were the dominate microbes in all of the *Tej* samples.

Yohannes *et al.*, 2013 collected *filter-tella*, *Tej* and *areki* from ten local alcohol vending houses around Jimma, Ethiopia. The study's goal was to examine the alcohol content, pH, turbidity, and sensory properties of the collected samples. Finally, the report showed that the pH of *Tej* varied in the range from 3.66 to 4.45. Furthermore, the alcohol content of the same sample varied in the range from 8.94 to 13.16% (v/v). Similarly, Nemo & Bacha, 2020 also performed the microbial and physicochemical profile on the selected traditional alcoholic beverage. The average physicochemical and microbiological count of 30 *Tej* samples were covered in this report.

2.3.4 Factor affecting honey wine fermentation

High sugar concentration of honey-must is the major factor that negatively affect the fermentation rate of honey wine. This higher sugar concentration creates osmotic stress on the fermentative microorganisms and slowed the fermentation process. Honey concentration greater than 30% had a major effect on the rate of fermentation (Pereira *et al.*, 2015). The fermentation is also influenced by the different types of honey; dark honey contains more minerals than light honey. Pereira *et al.*, (2009) investigated the ability of the *S. cerevisiae* to produce mead using honey from the Tras-os-Montes, Portugal. As expected, dark honey produced better results than clear honey because it contains more minerals and has a higher pH.

Honey is primarily composed of fermentable sugars, but it is deficient in nitrogen, minerals, and growth factors, all of which has a big influence yeast growth and fermentation (Mendes-Ferreira *et al.*, 2010). It affects the honey fermentation rate through either biomass growth and cellular activity (Pereira *et al.*, 2015). Indeed, nitrogen deficiency is one of the most common causes of stuck or sluggish honey fermentation (Gupta & Sharma, 2009). Amino acid catabolism, which affects the formation of aroma compounds, is also influenced by added nutrients (Bisson, 2004).

Table 2. 5 Research trends on physicochemical and microbiological profile of *Tej*

Name of Investigator	Title	Area of Investigation	Major result	Recommendations by the authors (if any)
Bahiru *et al*., 2005	Yeast and lactic acid flora of *Tej*, an indigenous Ethiopian honey wine: Variations within and between production units	➢ Study the microbial flora of *Tej* samples collected from Addis Ababa, Ethiopia	Yeasts & LAB were among the dominant micro-organisms ⟩ Yest flora were composed of *Saccharomyces cerevisiae* (25%), *Kluyvermyces bulgaricus* (16%), *Debaromyces phaffi* (14%), *K. veronae* (10%) ⟩ lactic flora consisted of *Lactobacillus, Streptococcus, Leuconostoc* and *Pediococcus* species	Identification and use of the ***appropriate lactic and yeast strains*** and ***optimization of process*** conditions would be useful to initiate large-scale commercial production of *Te*.
Bahiru *et al.*, 2001	Chemical and nutritional properties of *Tej*, an indigenous Ethiopian honey wine: variations within and between production units	➢ Study the physcio-chemical analysis for the collected samples	⟩ pH values of samples varied between *3.07 and 4.90* ⟩ range for titratable acidity was *0.1 g/l00ml to 1.03 g/l00ml* ⟩ total alcohol content varied between values as low as *2.7% to values as high as 21.7%* ⟩ Fusel oil content of samples ranged between *0.1 g/l00L and 88 g/l00L*	Further work on ***designing of suitable substrates***, ***selection of appropriate cultures*** and ***optimization of process conditions*** would help to produce *Tej* with better keeping quality and acceptable levels of alcohol, fusel oils and other hazardous metabolites.
Yohannes *et al.*, 2013	Preparation and Physico-chemical analysis of some Ethiopian traditional alcoholic beverages	➢ pH, alcohol content and sensory analysis were carried out for unfiltered *Tella, Tej a*nd *areki s*amples	⟩ the pH value of *Tej* ranged from *3.56 to 4.45* ⟩ alcohol contents of *Tej* samples ranged from *8.94 to 13.16* ⟩ sensory evaluation all are under acceptable limit	

Furthermore, deficiencies of minerals and vitamins in the fermentation medium also affect the sensory qualities of honey wine as well as its fermentation performance (Gibson, 2011). For optimal honey-must fermentation, honey must be supplemented with nitrogen and minerals to achieve at least 150 mg L^{-1} YAN and 465 mgL^{-1} potassium ion (K+) (Schwarz et al., 2021). However, it's important to remember that too much nutrient can be just as bad as not enough. Excess nitrogen has been shown to cause cell death in yeast, and any nutrient that is not consumed by the intended microbe is available for spoilage organisms to use (Aranda *et al.*, 2011; Tesnière *et al.*, 2013).

Agitation is also the important factor that should be controlled during the fermentation of honey wine (Steinkraus & Morse, 1966). It is possible to suspend fermentative microorganisms during the fermentation process by using slow agitation. This in turn will significantly reduce the fermentation time and cell autolysis which might the major cause of undesirable flavor (Rollero *et al.*, 2018). Moreover, the pH of the honey and the fermentation broth is influencing the rate of fermentation considerably (Pereira *et al.*, 2015). According to reports, a pH of 3.7-4.6 is ideal for honey fermentation; preferably, a pH of 3.7 was high enough to allow rapid fermentation while remaining low enough to inhibit the growth of undesirable bacteria (Gupta & Sharma, 2009; Mendes-Ferreira *et al.*, 2010).

The size of inoculum has also significant effects on the rate of fermentation during honey wine production (Carrau *et al.*, 2010). Larger inoculum sizes, especially at the start of fermentation, will aid in reducing the lag phase of microbial growth. However, due to the effect of larger inoculum size, the rapid increase in fermentation rate will not last up to the end of fermentation (Cavalcante da Silva *et al.*, 2018). Temperature is another important parameter in honey wine fermentation process (Twilley *et al.*, 2018). The rate of chemical reactions, including those of enzymes in *S. cerevisiae*, have been shown to increase in direct proportion to temperature. Fermenting at higher temperatures, on the other hand, can have a negative impact on the production of desirable aroma compounds. Furthermore, heat treatment and unfavorable storage conditions can degrade the quality of both honey and mead. As a result, temperature is a critical parameter to monitor in order to produce high-quality honey wine (Kahoun *et al.*, 2017; Peng *et al.*, 2015).

2.4 Development of direct fermentation system

To modernize traditional fermentation system into a direct inoculated fermentation a number of steps is need. The primary goal of developing direct fermentation system is to improve the quality and safety of fermented products while preserving their authenticity and uniqueness. It all begin by studying the microbial ecology of the fermented foods. Then microbial isolation, screening, process optimization, and reactor designed will follow processing of microbial ecology study. Furthermore, upstream and downstream processes should also be considered critically for the for the global transition of the state of art of traditional fermentation system to technology-based direct inoculated fermentation system. However, Microbial ecology study and starter culture development is a very critical step that should be done in a paramount importance. Figure 2.4 depicts a strategy for transitioning from spontaneous fermentation to directed fermentation (Navarrete-Bolaños, 2012).

2.4.1 Food microbial ecology study

Microbial ecology studies are aimed to characterize microbial diversity, community structure, and interactions with each other in their natural environments in order to determine changes in microbial populations. The following are the three basic questions that microbial ecology study should answer very well: (i) “who is there?” it is all about identifying the available microorganisms in given food environment. (ii) “who is doing what?” which is the assigning of the identified microorganisms to their functional roles. (iii) “What role do these microorganisms’ activities play in specific ecosystem functions or processes?” (Ercolini, 2013). Culture dependent or culture independent method could be applied to study the microbial ecology of food samples.

Culture-dependent microbial ecology study is a traditional microbiological technique that involves cultivating microorganisms on media and then analyzing their phenotypic or genotypic characteristics (Vaz-Moreira *et al.*, 2011). However, this method is not sufficient to explore the microbial diversity as it does not reveal a clear picture of the community diversity. Since approximately 99% of the microorganisms found in nature cannot be cultured in the laboratory, this method is insufficient for investigating microbial diversity and obtaining a complete picture of community diversity (Kikani *et al.*, 2017). Thus, this method failed to identify the predominant microorganisms in food environment that could not be cultivated using conventional methods.

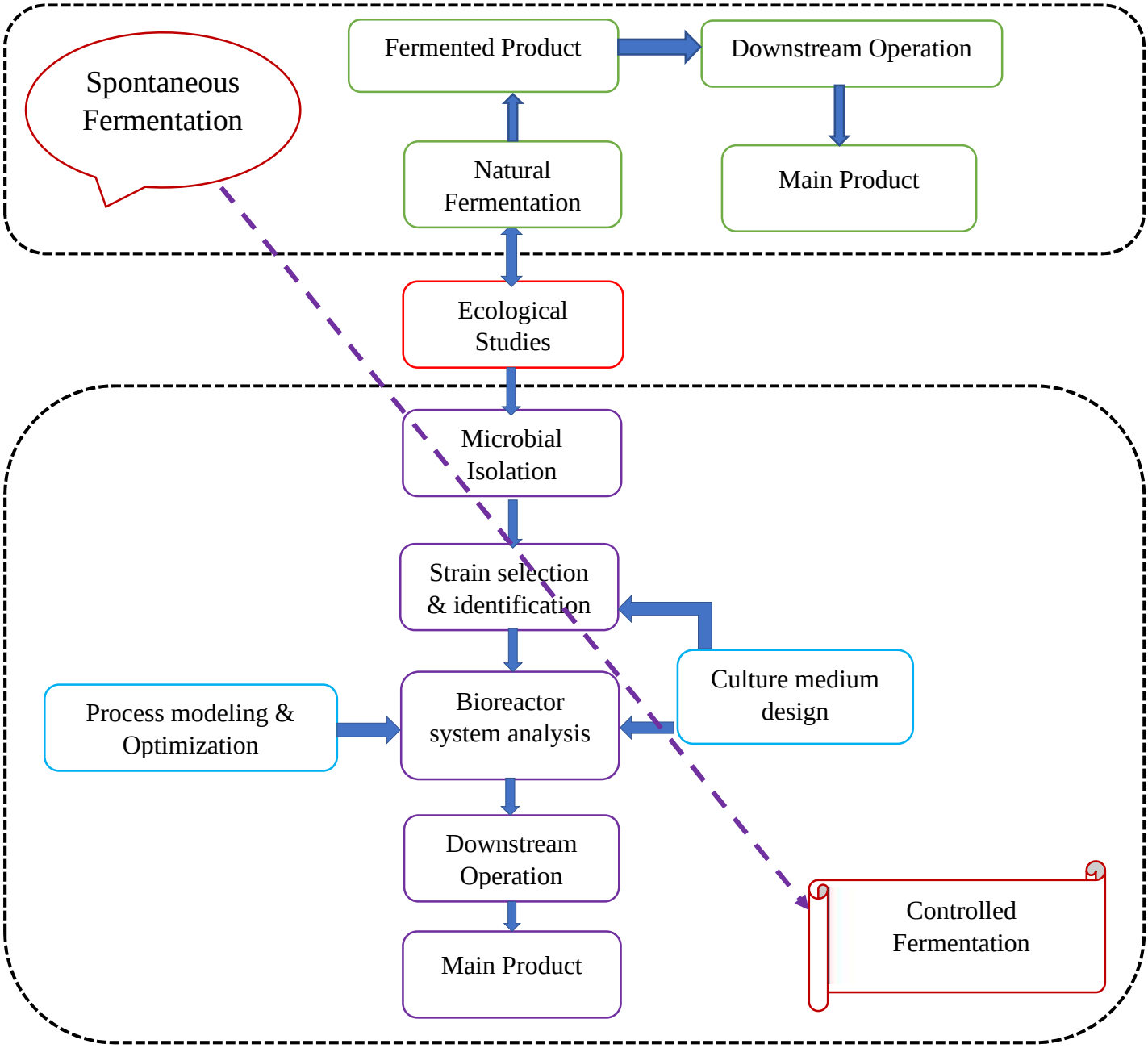

Figure 2. 4 A guideline for the development of direct fermentation system

As a result, if only conventional approaches are used, the genetic information and biotechnological prospects of the majority of organisms living in a given habitat will remain untapped (Aminin *et al.*, 2008).

Culture-independent methods are based on a direct analysis of DNA or RNA for better characterization of the whole microbial communities, and their metabolic activities in a particular food product. This method provides crucial information on complete understanding of microbial diversity and phylogeny. There are two types of culture-independent community analysis

approaches: (i) partial community analysis and (ii) whole community analysis. Partial community analysis is based on direct extraction of genetic materials (total DNA or RNA) from food samples, which is then amplified using polymerase chain reaction (PCR) techniques. Whole community analysis approach uses high-throughput sequencing or Next-generation DNA sequencing (NGS) (Stefani *et al.*, 2015). This method is a hundred times faster and less expensive than the traditional Sanger method and it is already the most powerful culture-independent method for analyzing all genetic information existed in total DNA/RNA extracted from food samples as well as in pure cultures (Ercolini, 2013). Genomic material extraction, library preparation, sequencing, and bioinformatic analysis are the major steps conducted in next generation sequencing (Figure 2.4) The metagenomic data with other "omics" (transcriptomics, functional genomics, proteomics, and metabolomics) provide a deeper insight to the microbial ecology of foods environment (Roy & LaPointe, 2016).

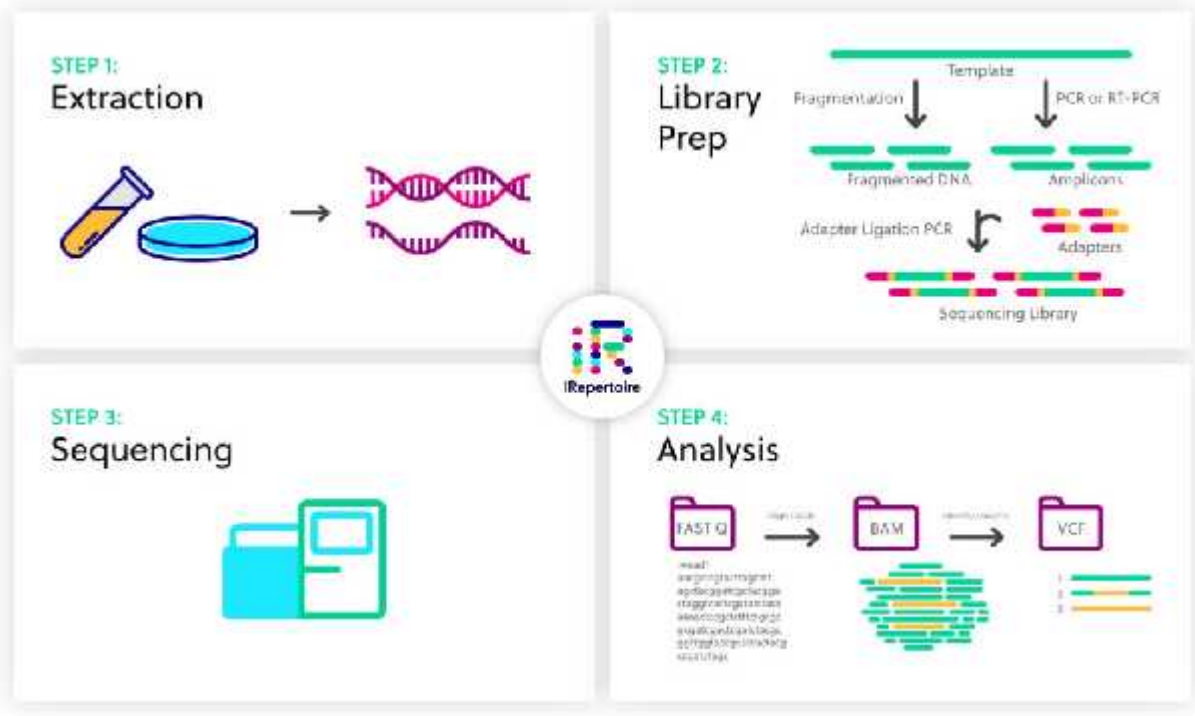

Figure 2. 5 Workflow of next generation sequencing

2.4.2 Selection of starter culture

Competitive activities among a variety of microorganisms are a common phenomenon in spontaneous fermentation systems (Capozzi *et al.*, 2017). Those who are best adapted to the substrate as well as the technical control parameters finally take control of the process. Other

contaminating microbes (e.g. *Enterobacteriaceae*) may be inhibited by the production of metabolites (e.g. organic acids), which may provide an additional advantage during fermentation (Holzapfel, 2002). However, unpredictability and uncontrollability nature of spontaneous fermentation is the main drawback of this fermentation system (Cuvas-Limon *et al.*, 2021). Despite these disadvantages, some brewers and winemakers in various regions still prefer spontaneous fermentations because they believe that premium quality wine is produced, and will continue to be produced, using the "traditional" technique based on spontaneous fermentation (Navarrete-Bolaños, 2012). Inoculated fermentation, on the other hand, is the preferred method for large-scale wineries where rapid and reliable fermentations are required for consistent beverage flavor and quality.

Modern starter cultures are selected either as single or multiple strains, specifically for their adaptation to a substrate or raw material (Rodríguez-Lerma *et al.*, 2011). The selection of appropriate starter strains should consider their interactions in mixed cultures, as well as their behavior under controlled conditions (Smid & Lacroix, 2013). Adaptation to a specific substrate, differences in growth rate, antimicrobial properties, ability to degrade anti-nutritional factors, flavor, and quality attributes are some other selection criteria for microbial strains that will be used as starter cultures for food fermentation (Holzapfel, 2002). Thus, the number of strains chosen should be based on the evaluation of as many output functions as needed, according to the desired final characteristics of the product (Navarrete-Bolaños, 2012). In general, a single pure culture is linked to one feature, while a mixture of cultures is linked to multiple features.

The proper design of the fermentation medium and the adjustment of good processing parameters, on the other hand, can support the good performance of the selected starter culture. There is a good chance that the chosen strain will perform well in a nitrogen-supplemented fermentation medium, especially for substrates like honey (Pereira *et al.*, 2015). Aside from that, fermentation conditions like agitation speed, pH, temperature, and aeration have a significant impact on the performance of the chosen starter culture (Ajala *et al.*, 2020). Thus, understanding, analyzing, and integrating the upstream, starter culture development, and downstream processes is critical when developing direct fermentation from a given spontaneous fermentation system (Navarrete-Bolaños, 2012).

2.5 Biological process modeling

In broad definition mathematical modeling is the process developing a model (mathematical) that used to expresses any phenomena in a mathematical term. Thus, a biological process model is a

collection of mathematical equations and constants that are typically solved on a computer or machine to make quantitative predictions about certain aspect (s) of a biological process (Brian & Keith, 1997). Model can be developed in two ways: it can be developed from the scratch as new model or it can be adopted, extended and further developed (Goulet, 2016). In either way, the developed model should be as simple as possible. The first step in model development is a concise definition of the aim of the model (Mu *et al.*, 2006). The process description and model requirements are the next step in model creation. Following the creation of the first version model, the model will be parameterized through simulation and comparison to experimental data. Finally, model adequacy checking will be performed to determine whether the model adequately describes the process (Herwig *et al.*, 2021).

2.5.1 Model types

Process models is categorized in to mechanistic and non-mechanistic models (Figure 2.5). Mechanistic models are the models which describe any biological models based on the deep process knowledge (González-Figueredo *et al.*, 2019). Whereas the non-mechanistic mode is the black box model in which it is entirely based on input-output data (Himmelblau, 2000). The other model type which is called hybrid model (gray box model) is based on both process knowledge and input output data (Acuña *et al.*, 1999). This type of model gives an advantage to get the best side of the two models.

2.5.1.1 Mechanistic Models

Mechanistic models are classified as structured or unstructured. Unstructured models treat cells as if they were a uniform black box, akin to a simple balance room. in this model extracellular variables are the only ones that influence cell growth and behavior (Moser *et al.*, 2021). Besides, this model has a lower complexity and fewer model parameters, making parameter identification easier (Kostov *et al.*, 2012). Furthermore, unstructured models are simple to create, manage, and are ideal for reproducing microbial growth quantitatively (Mu *et al.*, 2006). These models are especially useful in the early stages of process development when there is a limited amount of data (Moser *et al.*, 2021). These kinds of models are limited in their ability to explain different growth patterns and transitions among growth phases due to their simplicity. In most cases, unstructured models only consider single effects (such as product inhibition or substrate limitation) (Muloiwa *et al.*, 2020). However, to handle multiple effects extension of the model is needed. These additions increase the number of model parameters and, as a result, it also increases the model's complexity. This may

result in the loss of model simplicity, which was the primary benefit of unstructured models (Cheirsilp *et al.*, 2007).

Structured models, on the other hand, divide the cell in different compartments, each of which contains a different component with a specific task. As a result, cells are not considered homogeneous (Okpokwasili & Nweke, 2005). This complicates models and expands the number of model parameters. Although changes in cell properties can be described in these kinds of models, all cells are the same and change at the same rate. These model structures are simple enough while also providing the foundation for a description of the most important effects for efficient process optimization based on biological knowledge (Moser *et al.*, 2021). Segregated and non-segregated models are further subdivided from structured and unstructured models (Goulet, 2016). Segregated models take into account cell populations in which not all cells in the same metabolic state are uniform. This enables the simulation of cell populations with varying growth and production characteristics (González-Figueredo *et al.*, 2019). Over time, these segregated cell populations can change independently of one another. In non-segregated models, all cells are assumed to be identical. Segregated models are much more complex than non-segregated models, and they limit the models' generality (Muloiwa *et al.*, 2020).

2.5.1.2 Non-Mechanistic Models

Non-mechanistic models are data-driven models that rely solely on mathematical correlations for their construction. Because of the generic structure of non-mechanistic models, they take less time than mechanistic models (Stosch *et al.*, 2014). Moreover, non-mechanistic models only take into account perturbations that are significant above a user-defined threshold. The task of defining this signal-to-noise ratio is both important and difficult. Non-mechanistic models are still difficult to validate and are more specific to the process. Extrapolation outside of the data range used to create a model is rarely possible. As a result, they can only be related to other processes in a limited way, if at all (Schubert *et al.*, 1994). As a result, this model structure is lacking in the process' predictive capacity, particularly for data outside of the trained data. Artificial neural networks (ANN) is one of the most common model of this kind (Ławryńczuk, 2008). They are made up of an input layer and an output layer that are linked by one or more hidden layers (Grahovac *et al.*, 2016). Sigmoidal switch functions with adjustable thresholds describes these connections. The "learning" process determines the thresholds (Karakuzu *et al.*, 2006). The learning process necessitates a large amount of data in order to produce reliable results. If there aren't enough experimental data, mechanistic

models can be used to generate these data (Grahovac *et al.*, 2016). Model adaptation takes time, but artificial neural networks have quick computing times after that. Process knowledge and insight aren't used in ANNs. ANNs were frequently used for bioprocess control and optimization (Nagy, 2007). They are purely mathematical correlations, so no mechanistic insight into the process is possible (Karim *et al.*, 1997). Only significant effects and relationships in the data sets available for the learning process can be described by ANN. ANNs, strictly speaking, allow for the description of processes but not the prediction of outcomes (Chen *et al.*, 2004).

2.5.1.3 Hybrid Models

Hybrid models combine mechanistic and non-mechanistic models. Hybrid models combine different types of knowledge in this model (Stosch *et al.*, 2014). One type is a priori knowledge obtained through mechanistic modeling in the form of material and energy balances, and kinetic laws (Schubert *et al.*, 1994). Data-driven knowledge, in the form of heuristics and process data from completed cultivations, is the other type of knowledge (Doherty *et al.*, 1997).

Hybrid modeling aims to strike a balance between the benefits and drawbacks of mechanistic and non-mechanistic models (Galvanauskas *et al.*, 2004). While mechanistic models are capable of providing predictive results, their development is time consuming and requires detailed process knowledge. Non-mechanistic, data-driven models, on the other hand, are easy to create and apply, but they only have good descriptive properties within the range of the data they are based on (Stosch *et al.*, 2014). When compared to purely data-driven modeling approaches, the main advantage of hybrid models is that they can achieve higher accuracy, more efficient model development, and better extrapolation properties. The most difficult challenge is implementing hybrid models and their parameterization algorithms, which is said to be error-prone and labor-intensive (Galvanauskas *et al.*, 2004).

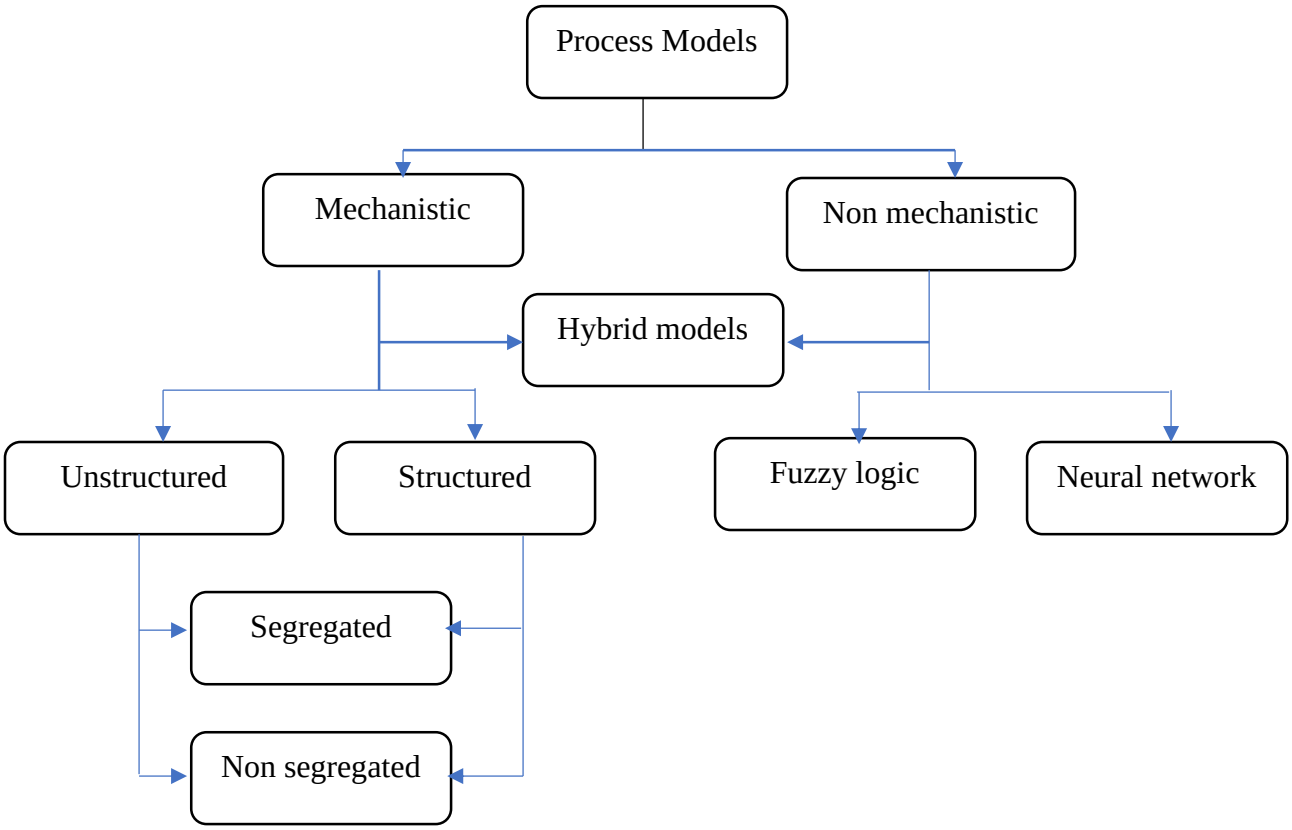

Figure 2. 6 Classification of models

2.5.2 Mechanistic, unstructured, non-segregated kinetic models

Other than some non-mechanistic models, most of the fermentation kinetic models which are used to describe microbial growth, substrate consumption, and product formation are mechanistic unstructured non-segregated kinetic model (Cheirsilp *et al.*, 2007; Jin *et al.*, 2012; Kostov *et al.*, 2012; Mu *et al.*, 2006). Besides, most of the microbial reactions is performed under batch fermenter and this model is best in describing this kind of reactions in the reactor (Arellano-Plaza *et al.*, 2007; Jin *et al.*, 2012; Mu *et al.*, 2006). The models used to express the aforementioned issues, may derived from two basic things: (i) based of substrate concentration (Muloiwa *et al.*, 2020) or (ii) based on cell concentration (Berkman *et al.*, 1990). Based on these two concepts other extensions of inclusion for substrate inhibition, product inhibition may incorporate as an extension to the above basic model (Kostov *et al.*, 2012).

Model based on the cell concentration is mainly expressed by the logistic model. This model is usually expressed by the following formula;

$$\frac{dX_v}{dt} = KX_v\left(1 - \frac{X_v}{X_{v\infty}}\right) \quad 2.1$$

Where X_v is viable cell count, t is fermentation time, k is intrinsic growth rate constant (h^{-1}), $X_{v\infty}$ is maximum cell concentration (cell L^{-1})

$$X_v(t) = \frac{X_{v\infty}}{1 + \left(\frac{X_{v\infty} - X_{v0}}{X_{v0}}\right)e^{-kt}} \quad 2.2$$

Whereas the model based on the substrate or product or Monod based model is expressed as follows

$$\frac{dX_v}{dt} = \mu X_v - K_d X_v \quad 2.3$$

$$\frac{dS}{dt} = -\left[Y_{x/s}^{-1} + Y_{p/s}^{-1}\right]\mu X_v - m_s X_v \quad 2.4$$

$$\frac{dP}{dt} = qX_v \quad 2.5$$

Where μ is specific growth rate (h^{-1}), K_d is death rate (h^{-1}), m_s is maintenance rate (cell L^{-1}), q is specific product formation, S is substrate (g/L), P is product (g/L), $Y_{p/s}$ and $Y_{x/s}$ yield coefficients [g/g] of product and biomass obtained from the substrate consumed.

However, these specific growth and specific product formation rates are highly dependent on substrate limitation, substrate inhibition, and product inhibition of the process.

Table 2. 6 Summary of unstructured kinetic models

Model Name	μ	q	References
Monod	$\mu=\frac{\mu_{max}S}{K_s+S}$	$q=\frac{q_{max}S}{K_s+S}$	(Monod, 1949)
Blackman	$\mu=\frac{\mu_{max}S}{K_s}$	$\mu=\frac{q_{max}S}{K_s}$	(Blackman, 1905)
Haldane	$\mu=\frac{\mu_{max}S}{K_s+S+\frac{S^2}{K_i}}$	$q=\frac{q_{max}S}{K_s+S+\frac{S^2}{K_i}}$	(Haldane, 1965)
Tesseir	$\mu=\mu_{max}(1-e^{SK_i})$	$q=q_{max}(1-e^{SK_i})$	(Teissier, 1942)
Moser	$\mu=\frac{\mu_{max}S^n}{K_s+S^n}$	$q=\frac{q_{max}S^n}{K_s+S^n}$	(H. Moser, 1958)
Contois	$\mu=\frac{\mu_{max}S}{XK_s+S}$	$q=\frac{q_{max}S}{XK_s+S}$	(Contois, 1959)
Logarithmic	$\mu=a+bln(S)$	$q=a+bln(S)$	(Westerho *et al.*, 1982)
Aiba-Edwards	$\mu=\mu_{max}\frac{S}{K_s+S}\exp(-\frac{S}{K_I})$	$q=q\frac{S}{K_s+S}\exp(-\frac{S}{K_I})$	(Aiba *et al.*, 1968)
Powell	$\mu=\frac{(\mu_{max}+m)S}{K_s+S}-m$	$q=\frac{(q_{max}+m)S}{K_s+S}-m$	
Verhulst	$\mu=\mu_{max}\left[1-\frac{X}{X_m}\right]$	$q=q_{max}\left[1-\frac{X}{X_m}\right]$	(Verhulst, 1838)
Luong	$\mu=\mu_{max}\frac{S}{K_S+S}\left(1-\frac{S}{S_n}\right)^n$	$\mu=\mu_{max}\frac{S}{K_S+S}\left(1-\frac{S}{S_n}\right)^n$	(Luong, 1987)
Webb	$\mu=\frac{\mu_{max}S\left(1+\frac{S}{K_i}\right)}{S+K_s+\left(\frac{S^2}{K_i}\right)}$	$q=\frac{q_{max}S\left(1+\frac{S}{K_i}\right)}{S+K_s+\left(\frac{S^2}{K_i}\right)}$	(Webb, 1963)
Yano and Koga	$\mu=\frac{\mu_{max}}{K_s+S+\frac{S^2}{K_1}+\frac{S^3}{K_1^2}}$	$q=\frac{q_{max}}{K_s+S+\frac{S^2}{K_1}+\frac{S^3}{K_1^2}}$	(Yano & Koga, 1969)

Source: (Muloiwa *et al.*, 2020)

2.6 Concluding remarks

Food fermentation was one of the first human-created food processing techniques. This process can be classified into several types based on different perspectives. Depending on the process and/or the formation product, this process can be classified as alcohol, lactic, alkaline, or acetic fermentation. Depending on the process, it could be spontaneous or direct inoculated fermentation. It could also be solid state or submerged state fermentation, depending on the state in which the process is carried out. The process of converting a given substrate into ethanol with the help of yeast (*S. cerevisiae*) and/or bacteria (*Z. mobilis*), which is the focus of our research, is known as alcoholic fermentation.

Traditional beverage consumption and production is a very popular practice in Ethiopia. These beverages have equal market share with a large scale produced alcoholic beverages in the country. *Tella* and *Tej* take the lion's share of the credit. *Tej*, Ethiopian honey wine, is produced through a spontaneous fermentation system using honey and gesho (*R. prinoides*) as a raw material. When compared to other traditional beverages, the processing steps and equipment required to make *Tej* are relatively simple and easily accessible. According to previous literature, more than 80% of all honey produced is used to make this traditional beverage. Given the country's honey production potential (500,000 tons per year), modernizing this traditional beverage will have a significant economic impact. Thus far, limited reports are available on Ethiopian honey win, *Tej*. These literatures are entirely focused on the characterization of the final product. Almost all of these reports, however, recommended that further study should be conducted into developing appropriate fermentation medium, selecting desirable fermenting microorganisms, and optimizing processing conditions in order to achieve the ultimate aim of large-scale production.

The first step in developing a direct fermentation system for any given spontaneous fermentation process is to conduct an ecological study during the fermentation period as well as the final product. Information on the microbial structure was the primary focus of this ecological study. The physicochemical properties observed as a result of the presence of the encountered microbes, on the other hand, are part of this study. Following that, the next critical step is isolation, screening, identification and selection presumptive starter cultures. Another cornerstone task for the development of the aforementioned process is designing of a compatible fermentation medium and understanding the effect of processing parameters on the selected microbial strains. Last but not least, the downstream process should be given equal consideration during the process's development.

Modeling biological processes is different and more complicated than normal reaction kinetic modeling. Many studies have attempted to model biological processes using the background model (WBM), the input-output data model (BBM), or a combination of the two (GBM). Each of these model types has procs and cons. Because the fermentation process involves so many different parameters, it is nearly impossible to handle and describe all of the fermentation parameters in the developed model. However, when compared to other models, mechanistic (WBM), unstructured, non-segregated models have a good predictive capacity for limited data and for processes with little knowledge.

CHAPTER 3

General Materials and Methods

3.1 Study area description

Study samples were collected from three different locations in Ethiopia: Addis Ababa (AA), Debre Markos (DM), and Bahir Dar (BD). The locations were chosen based on their honey production potential, *Tej* consumption culture, accessibility, and suitability for sample collection. Addis Ababa, which is located at 9°1′48″N, 38°44′24″E and has an elevation of 2,355 meters, was one of the locations where samples were collected. This city serves as both the country's capital and its largest metropolitan area. Debre Markos is located at 10° 20' 0.996"N, 37° 43' 47.82"E and has an elevation of 2,446 meters. Bahir Dar, the capital city of the Amhara region, is located at 11°35'37.1"N, 37°23'26.77"E and has an elevation of 1,800 m. Figure 3.1 illustrates a study area map with the enlarged top-right side depicting the northwestern part of Ethiopia and the bottom-right side displaying Addis Ababa (capital), Ethiopia. Besides, the small green dots on the map represent the specific locations where *Tej* samples were collected.

3.2 Sample collection, transportation, and storage

Samples of *Tej*, honey, and gesho (*R. prinoides*) were collected from three Ethiopian cities: Addis Ababa (AA), Bahir Dar (BD), and Debre Markos (DM) (Figure 3.1). To be more specific, samples were collected within a 35-kilometer radius of each of the aforementioned sites. Based on their willingness and availability, each sample was obtained from a local alcoholic beverage vendor. A total of 21 *Tej* samples were collected from various households in the aforementioned locations for studying physicochemical and microbiological profiling of the samples. Aside from that, these samples were collected all at once from respective areas. For the fermentation dynamics study, one household was chosen from each sample collection area, and samples were collected sequentially until the fermentation time was completed. All samples were collected in screw cap plastic bottles aseptically. They were then transported to the analysis sites, Addis Ababa Science and Technology University and Kyungpook National University, using an insulated ice box with a freezing pack. The samples required for further analysis were kept in a freezer at -20^0C.

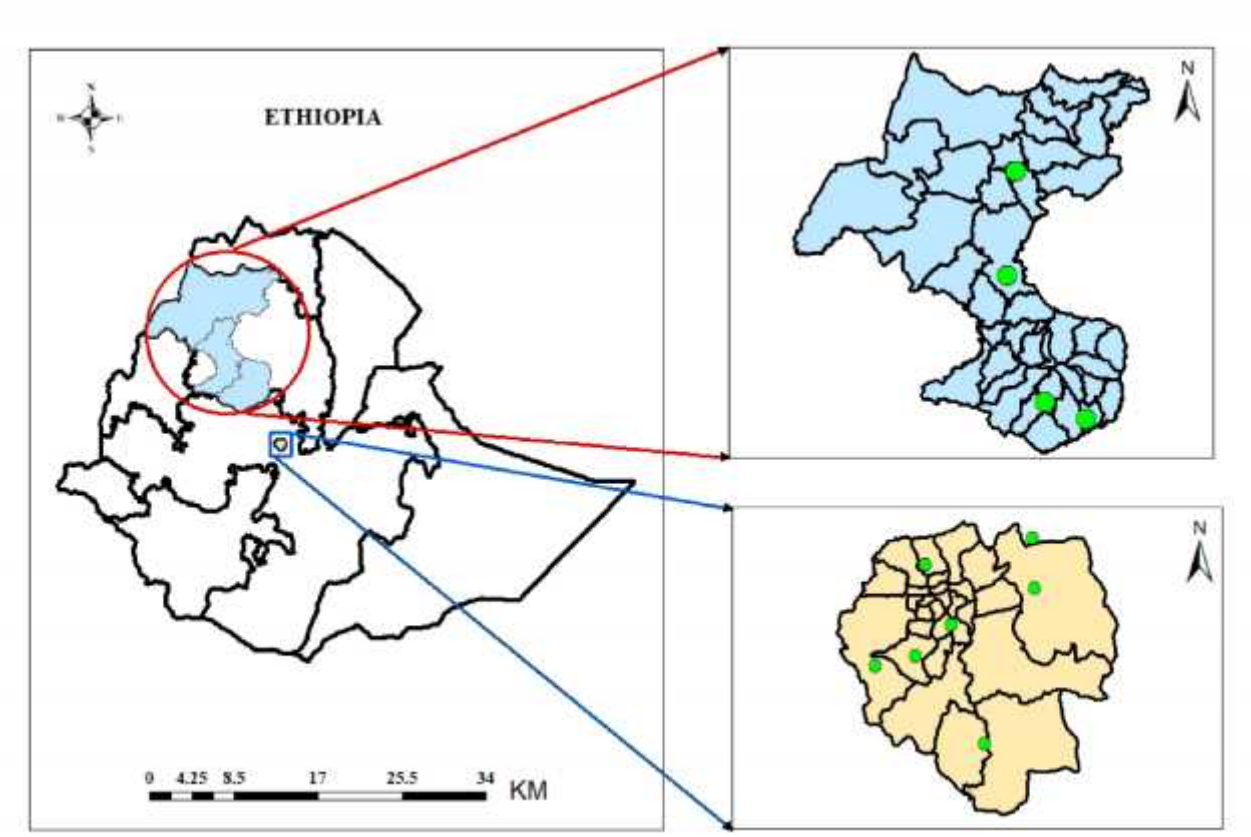

Figure 3. 1 The study area map pinpointed the precise location of the assessments and samples collected.

3.3 Physicochemical analysis

3.3.1 pH

Both pH and electric conductivity were measured by following standard procedure developed by OIV, (2020). A digital pH/Conductivity meter (Thermo Fisher Scientific, ORION STAR A215, USA) was used for the measurement of both pH and electric conductivity. Before measuring the pH, it was calibrated with buffer solutions of pH 4.01, 7.01, and 10.1. At 21°C, the rinsed pH electrode is dipped in a beaker containing 30 ml *Tej* samples. The same instrument was used to measure electric conductivity after it had been calibrated with 1413 and 12900 S/cm conductivity standards. The rinsed conductivity electrode was then immersed into a 30 ml beker containing *Tej* samples at temperature of 21°C. Both measurements' readings were taken in triplicate.

3.3.2 Titratable acidity (TA)

TA was calculated once more using the official methods of OIV, (2020). In a 250 mL Erlenmeyer flask, 30 mL of distilled water, 1 mL of bromothymol blue indicator solution, and 10 mL of the sample were mixed together. The prepared solution was then titrated against 0.1 mol/L sodium hydroxide (NaOH) solution until a color change from blue to green was observed. Then, to use this solution as a reference for end point determination, add 5 mL of pH 7 buffer solution in to the previously titrated sample. In a similar way, other test samples were titrated against 0.1 mol/L NaOH solution, and the volume of the titrant was measured in triplicate for each sample. The total acidity in milliequivalents per liter is then expressed as follows:

$$A=10*\text{volume of NaOH} \quad (3.1)$$

3.3.3 Sugar profiling

Tej's glucose, fructose, and sucrose content were quantified using high performance liquid chromatography-evaporative light scattering detection (HPLC-ELSD), following the method developed by Ma *et al.*, (2014) with minor modifications. To begin, a standard stock solution of glucose, fructose, and sucrose was made by diluting each standard with double distilled water (ddH_2O) to a final concentration of 12,000 mg L^{-1}. Then, using ddH_2O, standard solutions with concentrations of 800, 1000, 1200, 1400, 1600, 1800, 2000, 2200, and 2500 mg L^{-1} were prepared from the stock solution. Approximately 5 mL of *Tej* from each sample was first centrifuged at 3200 rpm for 30 minutes at 4^{o}C. The centrifuged sample's supernatant was double filtered sequentially through 0.45 and 0.22 m pore size membranes. All *Tej* samples were sugar profiled using a Shimadzu HPLC system. This HPLC system was outfitted with an LC-20AT quaternary gradient pump, a YMC-Pack Polyamine II (250x4.6 mml.D.S-5m,12nm) column, and an ELSD-LT II (Shimadzu, Japan) detector with Shimadzu LC solution software. At a column temperature of 30^{o}C, an aliquot of 10µL was injected into the HPLC-ELSD instrument. Each run lasted 45 minutes in total. At a flow rate of 1.0 mL min^{-1}, an isocratic mobile phase of Acetonitrile (ACN): Water (75:25) was used. At a flow rate of 2 L min^{-1}, nitrogen (N_2) was used as a nebulizer gas. Finally, calibration curves for each sugar standards were plotted by peak area versus concentration. The linearity was assessed by linear regression analysis using the least square regression method. The peak area of each sample was then compared to the sugar standard concentrations on the standard curve.

3.3.4 Ethanol quantification

The level of ethanol was determined using the standard method developed by OIV (2020). A 200 mL aliquot of *Tej* was added to a volumetric flask. The measuring cylinder was then rinsed with three 20-mL aliquots of distilled water. Another 200mL volumetric flask was used to collect the distillate, and 20 mL of distilled water was added to it. Throughout the distillation process, this collection flask was immersed in a cold-water bath. The distillate collection volumetric flask was then filled to the mark with a distilled water. The weight of this volumetric flask is then measured using a four-digit sensitive weighting balance, and the density of this distillate is calculated. Finally, the alcohol content was calculated from the standard table of aqueous organic solutions by using the density and temperature of the distillate.

3.4 Statistical data analysis

The mean values of all physicochemical data presented in this study were compared for significant difference ($p < 0.05$) by first checking for normality and homoscedasticity (Levene's test), then one way analysis of variance (ANOVA) using the Duncan test (Granato *et al.*, 2014). The vegan package was used to compute the alpha diversity indices of Chao1, Shannon, Evenness, and InvSimpson for microbiome data (Oksanen *et al.*, 2017). For the analysis of beta diversity, the Unifrac distance was used to measure the differences between groups based on sample collection areas (Chen *et al.*, 2012). The mathematical models proposed were simulated using the MTLAB 2020b software package. For simulation, the Runge-Kutta-45 integration algorithm was used. For parameter estimation, non-linear regression was used with weighted least squares error minimization between predicted and experimental data.

CHAPTER 4

Traditional Processing Methods, Physicochemical Properties, and Microbiological Profile of Ethiopian Honey Wine, *Tej*

Abstract

Ethiopian honey wine, *Tej*, is a naturally fermented traditional alcoholic beverage made primarily of honey and "gesho" (*R. prinoides*). At the moment, there is limited amount of information available on the processing methods and characterization of *Tej*. As a result, the purpose of this chapter is to reveal the traditional processing techniques, physicochemical properties and microbiological diversity of *Tej* samples collected from different areas of Ethiopia. To achieve these goals, assessments, spectrophotometric methods, electrochemical and chromatographic techniques, and high-throughput sequencing were used. Based on the assessment results, from area to area, as well as from household to household, the raw materials, type of utensils, and processing method used by producer of *Tej* are largely similar. Despite a statistical difference in the exact values of physicochemical properties among the collected *Tej* samples, the titratable acidity and pH values of the samples ranged from 1.81 to 8.65 g/L, and 2.8 to 3.8, respectively. Similarly, the samples' alcohol and sugar contents ranged from 6.36 to 11.34 g/100 mL and from 0.37 to 31.6 g/L, respectively. Furthermore, the samples' ABTS and DPPH values ranged from 27.4 to 73.1%, and 37.9 to 81.0%, respectively. Moreover, a few fermentative microorganisms dominated the microbial community structure of the *Tej* samples. The genera *Lactobacillus* (53.15%) and *Zymomonas* (38.41%) dominated the bacterial community structure. The fungal community structure, on the other hand, was entirely dominated by the genus *Saccharomyces* (99.66%). Additionally, *Zymomonas, Lactobacillus*, and *Saccharomyces* were the observed core microbiome for the collected *Tej* samples. Furthermore, when the comparison base is the area of sample collection, there were no statistically significant differences between bacterial and fungal communities in alpha diversity analysis. However, Unweighted Unifrac beta diversity analysis revealed a statistically significant difference between bacterial communities. In general, the observed shared physicochemical characteristic features and dominance by a specific group of microorganisms could be interpreted as a boon for the development of inoculated fermentation system for this traditional alcoholic beverage.

Keywords*:* Physicochemical property, Spontaneous fermentation, Antioxidant activity, *Tej*, *Saccharomyces, Lactobacillus*, *Zymomonas,*

4.1 Introduction

In Ethiopia, the consumption and production of traditional alcoholic beverages is a very popular practice (Lemi, 2020). *Tej*, *Tella*, *Borde, Keribo*, *Booka*, *Shamita*, *Korefe, Cheka,* and *Ogol* are some of Ethiopia's most popular traditional fermented beverages (Fentie *et al.*, 2020). These beverages are manufactured at the household level and consumed in the vicinity of the manufacturing facility. Ethiopian honey wine, *Tej*, is made from the spontaneous fermentation of honey. The preferred raw materials for the production of *Tej* are raw honey and "gesho" (*R. prinoides*) (Vogel & Gobezie, 1995). It contains more alcohol than any other non-distilled Ethiopian traditional alcoholic beverage (Nemo & Bacha, 2020a; Yohannes *et al.*, 2013).

The basic processing steps for *Tej* making are much more consistent across the country. It all starts with a 1:3 (v/v) mixture of raw honey and water. The honey-to-water ratio can sometimes reach 1:4 (v/v) (Vogel & Gobezie, 1995). This blend is then fermented for 3–4 days with naturally available microbes found in substrate (honey) and utensils to complete the first fermentation phase (Bahiru *et al.*, 2001a). After a few days, the mixture is filtered and mixed with boiled "gesho" (*R. prinoides*) leaves and stems. It is primarily used to add a bitter flavor to Ethiopian honey wine (Tekle *et al.*, 2019). Fresh leaves of "gesho" (*R. prinoides*) may be used instead of the boiled once in some parts of the country. The mixture is allowed to ferment naturally for an additional 8–20 days. Finally, it will be served to consumers following the completion of secondary fermentation and the removal of solid residues through filtration with a clean cloth (Bahiru *et al.*, 2006).

Ethiopian traditional alcoholic beverages collected from various local producers (vendors) had inconsistencies in their microbial profile, physicochemical properties, and sensorial characteristics (Tafere, 2015). For example, the pH, titratable acidity, alcohol content, and proximate composition of *Tej* samples differed significantly (Bahiru *et al.*, 2001). To date, no publication has addressed the effect of geographical location differences on the physicochemical properties of *Tej* samples. The samples differ not only in terms of physicochemical properties, but also in terms of microbiological profile. Yeast and lactic acid bacteria dominated the microorganisms' detected profiles of *Tej* samples using microbial cultivation techniques (Bahiru *et al.*, 2006). Furthermore, a high load of Enterobacteriaceae and aerobic mesophilic bacteria were reported in *Tej* samples collected from the Jimma district of Ethiopia (Nemo & Bacha, 2020b). Diaz *et al.*, (2019) discovered *Zymomonas* and *Lactobacillus* in a single *Tej* sample collected from Addis Ababa, Ethiopia. However, the detailed

bacterial and fungal community structure of spontaneously fermented *Tej* remains a significant gap that needs to be filled.

To modernize *Tej* processing from natural to direct inoculated fermentation, previous reports should be supported by extensive background knowledge of microbiome and physicochemical properties. Furthermore, the functional assay of *Tej*, which may have been inherited from honey or "gesho" (*R. prinoides*), has not yet been studied. As a result, the purpose of this chapter is to reveal the fungi and bacterial communities, physicochemical property, and antioxidant activity of *Tej* samples collected from various areas of Ethiopia.

4.1Materials and Methods

4.2.1 Survey data collection

Using an open-ended questioner, the assessment of traditional processing methods, quality characteristics, and shelf stability of *Tej* in the previously mentioned study areas (Section 3.1) was carried out (Appendix 1). This question was aimed squarely at the producers. The assessment was given in Amharic at their respective manufacturing and/or vending locations. A total of 35 producers were chosen at random for interviews based on their willingness to collaborate.

4.2.2 Sample collection

Sample collection, transportation was done according to the methods described in section 3.2

4.2.3 Physicochemical analysis

4.2.3.1 pH

pH measurements were performed according to the methods described in section 3.3.1

4.2.3.2 Titratable acidity (TA)

TA measurements were performed according to the methods described in section 3.3.2

4.2.3.3 Sugar profiling

Sugar profiling was done by using the method described in section 3.3.3

4.2.3.4 Ethanol quantification

Ethanol quantification were performed according to the methods described in section 3.3.4

4.2.4 Antioxidant determination

4.2.4.1 DPPH radical scavenging assay

The DPPH assay for *Tej* samples was determined using the method developed by Blois, (1958). A centrifuged sample of approximately 20 µL is added to DPPH (D9132-Sigma Aldrich) ethanol solution (150 µM DPPH in 180 µL ethanol). Simultaneously, a blank solution was made by combining 180 µL DPPH and 20 µL ethanol. After incubating both the blank and sample solutions for 15 minutes at 37°C, optical density (TECAN microplate reader, Switzerland) was measured at 517 nm. Finally, the DPPH assay was expressed using the formula below;

$$\text{DPPH activity (\%)} = \frac{\text{Blank OD-Sample OD}}{\text{Blank OD}} \text{x100}$$

4.2.4.2 ABTS radical scavenging assay

The ABTS assay for the *Tej* samples collected from different locations were determined by following the methods developed by Re *et al.* (1999). ABTS radical cation ($ABTS^{+}$) was first diluted by buffer of 5 mM phosphate at pH of 7.4 to 0.7±0.02 absorbance at the wave length of 734 nm. This diluted solution (197µL) was then mixed with a well centrifuged sample (3µL). Finally, the optical density value was measured at 734 nm wave length and the results were expressed as follows;

$$\text{ABTS activity (\%)} = \frac{\text{Blank OD-Sample OD}}{\text{Blank OD}} \text{x100}$$

4.2.5 Microbiome analysis

4.2.5.1 DNA extraction

To obtain the highest cell concentration, 40mL of *Tej* samples were centrifuged. The DNA for each sample was then extracted from the sediment of the centrifuge using the QIAamp PowerSoil Pro Kit (QIAGEN, Germany) in accordance with the manufacturer's instructions (QIAGEN, 2021). The details of DNA extraction procedures is illustrated in the Figure 4.1 below. The extracted DNA concentration was then determined using a Qubit 2.0 Fluorometer (Life Technologies, USA). Finally, high-quality DNA was kept at -20°C for further analysis.

4.2.5.2 16S rRNA gene sequencing

The Nextera Library Preparation Kit's barcode set was used to create an amplicon sequencing library for each *Tej* sample (Illumina Inc., USA). Bacterial hypervariable regions (HV4–HV5) were amplified using universal primers 515F (GTGNCAGCMGCCGCGGTAA) and 907R (CCGYCAATTYMTTTRAGTTT) on a Mastercycler Nexus GSX1 (Eppendorf, Germany) (Kang et al., 2021). The first stage of PCR amplification consisted of 5 minutes of pre-denaturation at 95°C, followed by 15 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, waiting 30 seconds at 72°C for extension, and finally a 5-minute final extension at 72°C (Jung et al., 2020). Under the same conditions, the second PCR amplification was performed, but the number of cycles was increased to 25. The QIAquick gel extraction kit was then used to purify the amplified, barcoded, and pooled DNA (QIAGEN, Germany). Amplified PCR products were multiplexed at 100 ng/L for each *Tej* sample into a single product by determining the concentration of DNA in each sample with a Qubit 2.0 fluorometer (Life Technologies, USA). The downstream procedures used the AMPure XP (BECKMAN COULTER Inc., USA) to select the target specific amplified and barcoded DNA of around 550 bp of size. The volume of PCR amplified product from the second step was adjusted to 1:1 (v/v) for AMPure XP beads. The prepared library was then kept at -20°C until it was used in subsequent experiments. The prepared amplicon library's quality and quantity were double-checked using an Agilent 2100 Bioanalyzer (Agilent, USA). Finally, the prepared amplicon library was run on the Illumina MiSeq (Illumina-MiSeq-USA) platform in accordance with the manufacturer's instructions. The amplicon sequencing was done with a 250 single-end configuration, and the base calling and image analysis were done with the MiSeq Control Software (MCS).

4.2.5.3 Internal transcribed spacer (ITS) sequencing

A forward and reverse primers of ITS86F (GTGAATCATCGAATCTTTGAA) and ITS4 (TCCTCCGCTTATTGATATGC) were used to target for amplification of fungal internal transcribed (ITS2) regions (Turenne *et al.*, 1999; White *et al.*, 1990).

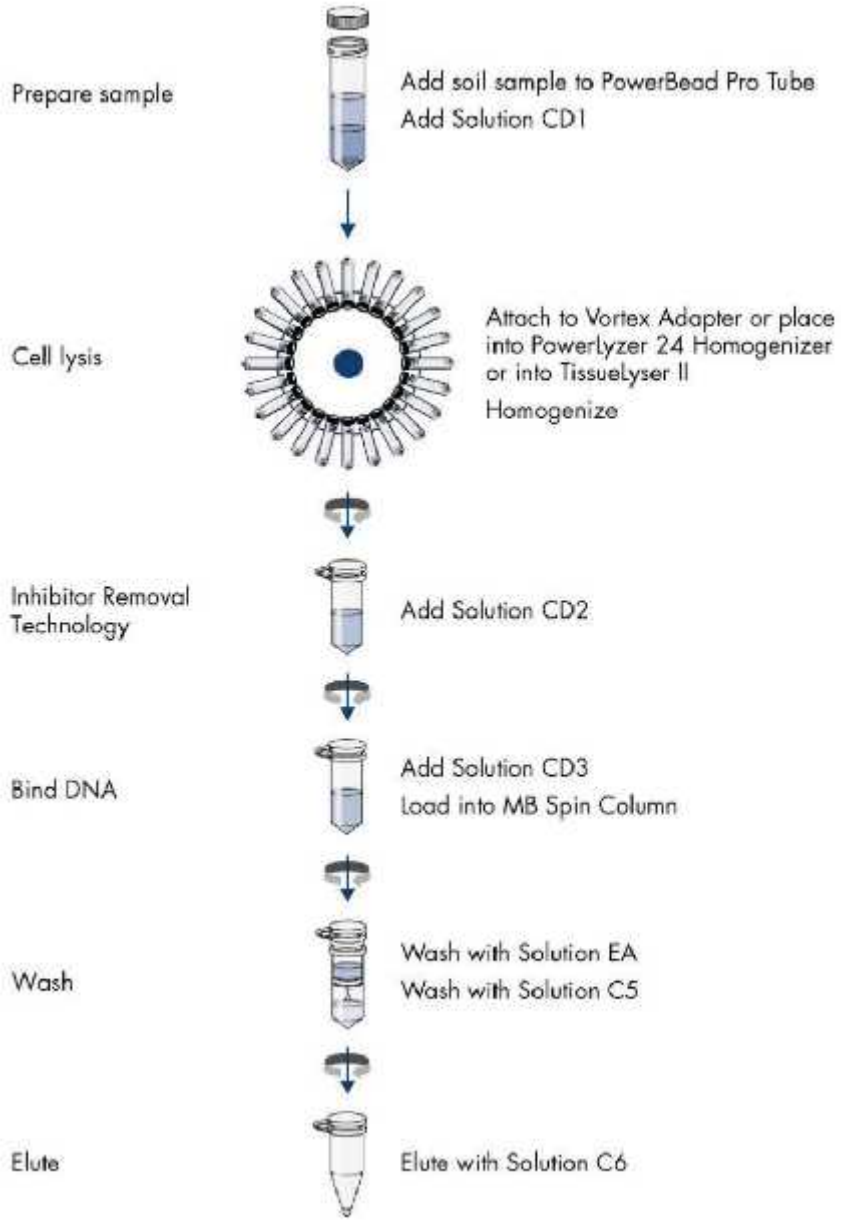

Figure 4. 1 DNA extraction procedures from the centrifuged *Tej* samples (QIAGEN, 2021)

The first PCR amplification was performed at 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, and 72 for 5 minutes (Jung *et al.*, 2020). The second PCR was also carried out under the same conditions as the first. All other steps of multiplexing, purification, pooling, and quality checking were performed in a similar way as it was done for 16SrRNA amplicon sequencing. Following the preparation of the library sequence, as well as checking the quality and quantity of the amplicons, it was finally subjected for sequencing using Illumina Miseq platform.

4.2.5.4 Bioinformatics analysis

Quantitative Insights into Microbial Ecology 2 (QIIME2) version (v) 2020.11 was used to process the raw data of single end readings generated by the Illumina-Miseq platform (Bolyen *et al.*, 2019). DADA2 was used to perform quality filtering, trimming both sides, and denoising of the sequence in order to obtain amplicon sequence variants (ASV) (Callahan *et al.*, 2016). A quality filter was applied to ASVs with an abundance mean depth of 0.1% and a Q score of less than 30. Taxonomic identification was performed using classify-sklearn classifier for 99% similarity against UNITE and SILVA 132 reference data bases for fungi and bacteria, respectively, after removing chloroplast, mitochondrial, and unassigned ASVs. After that, the phylogenetic tree was created using the sepp and mafft algorithms for bacteria and fungi, respectively. Finally, to normalize the different sequencing depths of each sample, the sequences among the samples were rarefied.

4.2.6 Statistical Analysis

In this experiment, triplicates of the physicochemical and antioxidant analyses measurements were performed. For microbiome data statistical analyses and visualization, the web-based Calypso and R Studio 4.0.3 were used. The vegan package was used to calculate the alpha diversity indices for the Chao1, Shannon, Evenness, and InvSimpson indices (Oksanen *et al.*, 2017). After checking the data for normality and homoscedasticity (Levene's test) (Granato *et al.*, 2014), one way analysis of variance (ANOVA) was used to determine for statistical significance difference a $P<0.05$). The Unifrac distance metrics was used to measure the differences between groups by taking sample collection areas as bases in the analysis of beta diversity.

4.3 Results and Discussion

4.3.1 Assessment on traditional processing techniques of *Tej*

4.3.1.1 Types and proportion of raw materials

Even though the proportions of the ingredients vary from household to households, all producers use honey, gesho, and water as raw materials (Table 4.1). Despite the fact that the proportion of honey to water ratio depends on the moisture content of honey, 63% of respondents answered 1:3 to 1:4 honey to water ratio is used to make a good quality *Tej*. Furthermore, all of the respondents preferred a multifloral honey (yellow honey) with its comb to produce *Tej*. Basically, they believed that mono-floral honey takes longer to ferment, but once the fermentation is completed, the product

is very popular with customers. Moreover, honey harvested a long before is preferred by 40% of *Tej* producers. The age of the honey is not a major consideration for the remaining producers.

Due to the ever-increasing price of honey, some producers substituted sugar for honey during the *Tej* making process. Small *Tej* vending houses, in particular, use this technique to deal with low-income customers. These producers use 40 kg of sugar to produce 100 L of *Tej* (Table 4.1). Malt is the other ingredient that is only used by producers who use sugar as the major fermenting substrate. This malt is typically made from wheat, but it can also be made from maze and finger millet. For every 100 L of *Tej* produced, approximately 10 kg of malt powder is added (Table 4.1). Some producers, who use sugar as the primary substrate, also use a yellow colorant to produce a deep yellow *Tej*.

Table 4. 1 List of ingredients and their respective proportion to produce *Tej*

Raw materials	**No of respondent**	**Percentage**	**Proportion to produce 100 L *Tej***	**Remark**
Honey	All producers use these ingredients		33 kg	For honey based *Tej*
Gesho				
Water			67 kg	For honey based *Tej*
Sugar	13	37	40 kg	All Producers use sugar as a
Malt	13	37	10 kg	substrate use these raw
Colorant	9	25	Spoonful	materials

Regardless of production methods, gesho (*R. prinoides*) is the other main ingredient used by all producers, to impart a bitter flavor to the produced *Tej*. Gesho (*R. prinoides*) leaves and stems can be added to the honey-must mixture after or before it has been boiled. The boiled gesho was preferred over the fresh gesho one by producers who use sugar as a primary fermenting substrate. Since a shorter fermentation time prevented complete extraction, boiling will aid in overcoming this major disadvantage by extracting the major bittering compound from stems and leaves of gesho (*R. prinoides*) before it is added to the honey-must mixture. For producers who follow this procedure, approximately 5 kg of gesho stems are boiled with sufficient water, to produce 100 L of *Tej*. However, for entirely honey-based *Tej*, since fermentation took longer, adding gesho without boiling might not have a major effect on the extraction of bittering components from gesho. To produce 100 L of *Tej*, these producer uses an average of 2 kg gesho. Nevertheless, please keep in

mind that these figures are an average based on the respondent's responses to how much gesho is used in the production of *Tej*.

4.3.1.2 Major utensils and indigenous processing methods

Tej is made with readily available equipment and utensils that are small in quantity and inexpensive when compared to other traditional fermented beverages (Tekle *et al.*, 2019). Respondent producers use barrels made of wood (56%), metal (14%), and plastic (30%) for the main fermentation step (Figure 4.3 A & B). Furthermore, during the manufacturing process, all of the producers use a larger wood stick to mix all of the ingredients. Metal pots, cheese cloth, knives, and traditional sieves, are the other most commonly used utensils for not only to make *Tej* but also to other food processing. Nonetheless, Table 4.2 lists the equipment that are frequently used to make *Tej*.

The indigenous processing techniques used to produce *Tej* can be divided into two categories based on the type of fermentable substrate (Figure 4.2). Regardless of production technique, all producers included in this assessment study begin their process by washing the main fermentation vat (one of the three-barrel types listed above) using "grawa" (*Vernonia amygdalina*). Some producers even wash their equipment and utensils with wood ash to clean them and reduce the possibility of contamination. The properly washed equipment and utensils will further be smoked by “weira” (*Olea africana*) to improve the sensorial qualities of the final product.

Honey *Tej*, which 63% of respondents produced, uses honey as the sole fermenting substrate. Honey will first be mixed with water in a 1:3 to 1:4 ratio (Figure 4.2). Actually, the proportion of this mixture is determined by the moisture content of the honey. However, all of the respondents answered within this range as a mixing ratio. Similar range of honey to water ratios were reported by Vogel & Gobezie, (1995). The mixture is then allowed to ferment for four days. After the specified time has passed, it will be filtered to remove the larger particle size solid residues from the blend. The gesho (*R. prinoides*) leaves will then be vigorously mixed with the previously prepared blend. The mixture is then allowed to complete secondary fermentation for one additional week during the warm season or more than three weeks during the cold season. However, approximately 30% of respondents stated that if the honey is mono-floral honey, they call it dark honey, secondary fermentation may take more than two months. Please keep in mind that, the blend must be agitated once per day during this secondary fermentation period. The product will then be filtered and served to the customer as an actively fermented product, if not it will undergo additional

maturation step to be served as dry *Tej*. About 15% of this group's producers use a previous batch of fermented *Tej* as a back sloping to increase the rate of fermentation. Furthermore, during the cold seasons, a small volume (5 L) of the fermenting mixture is boiled and added back to the fermentation medium (100 L) to raise the overall temperature, which helps to increase the rate of fermentation.

Table 4. 2 Some of utensils and equipment used to make *Tej*

Utensils and equipment	**Operation**
Metal Barrel	For mixing, fermentation, storage
Wood barrel	For mixing, fermentation, storage
Plastic barrel	For mixing, fermentation, storage
Metal pot	For boiling gesho
Traditional sieve	For separating residual solids form fermenting honey-must
Cheese cloth	For further separating residual solids form fermenting honey-must
Wood stick	For mixing
Knives	For reducing size of the gesho stems
Traditional flat tray (“*Sefed*”)	For screening and cleaning.

Some of *Tej* vendors, approximately 37% the respondent in this particular survey, use cane sugar as the primary substrate for fermentation. Cane sugar, malt powder, honey, gesho (*R. prinoides*) and water were the main ingredients used to producer this type of *Tej* (Figure 4.2). The mixture is then allowed to ferment for three to four days as a primary fermentation. Then, boiled gesho stems will be added to this blend and fermented for an additional 3 to 4 days. After completing this secondary fermentation, the mixture will be filtered, and honey will be added on top of this blend, which will then be allowed to ferment for another 2 to 3 days.

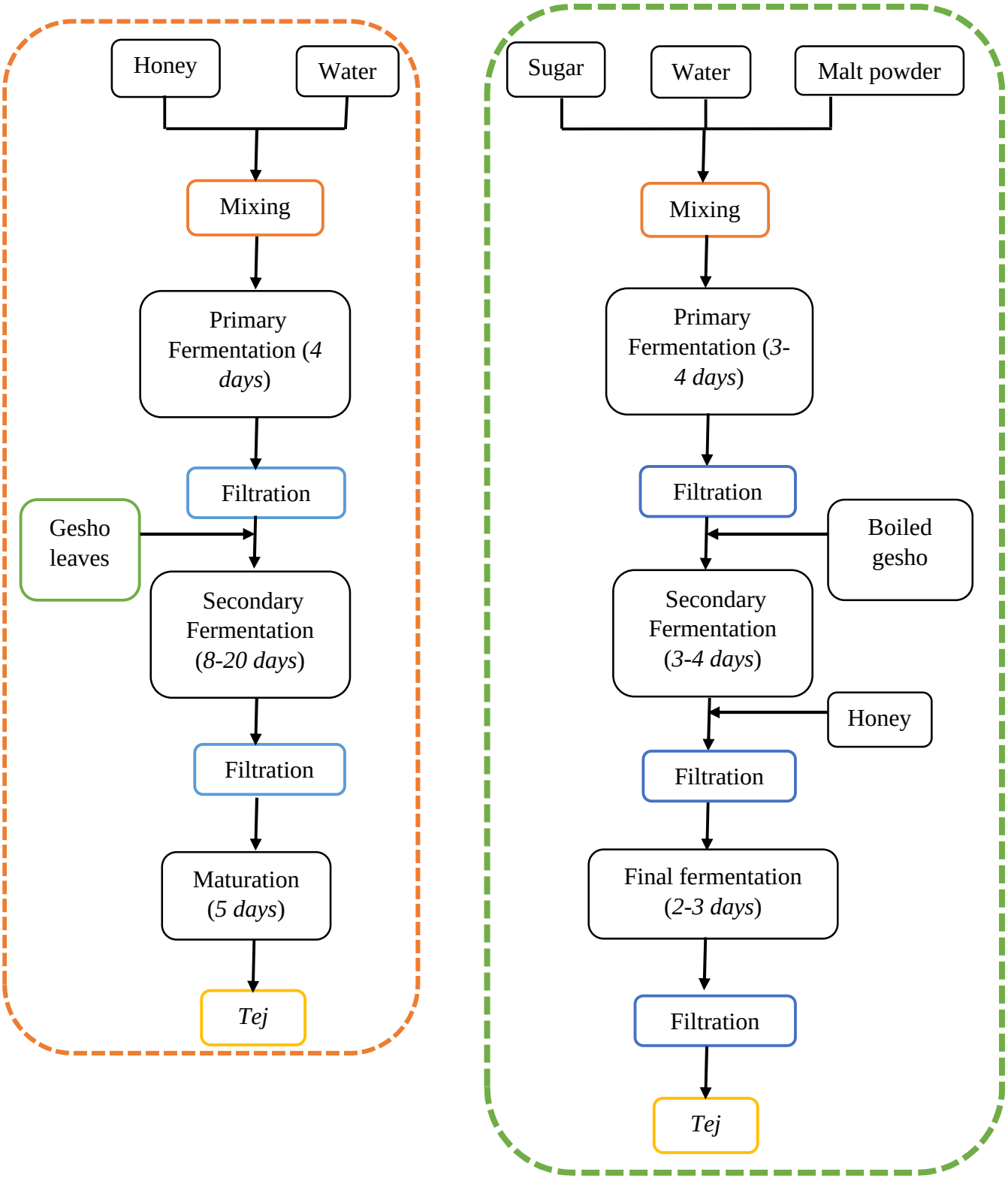

Figure 4. 2 Process flow chart to produce *Tej* from different ingredients

4.3.1.3 Quality attributes and shelf stability of *Tej*

Excellent quality *Tej* appears as a yellow, less viscous liquid and with a known test of gesho (*R. prinoides*) and ethanol. *Tej* can typically be stored at room temperature for 4 to 5 days. The vendors, in particular, have a special tanker that is closed from the top and has a get valve from the bottom to drain the product (Figure 4.3C). This reduces the intensity of the product's exposure to excess air. Furthermore, 40% of the respondents said *Tej* could be stored for more than six months. It must, however, be stored in a clean tank, avoided frequent exposure to air, strictly avoided contact with water, filtered at regular intervals, and mix it with a small amount of honey every two weeks.

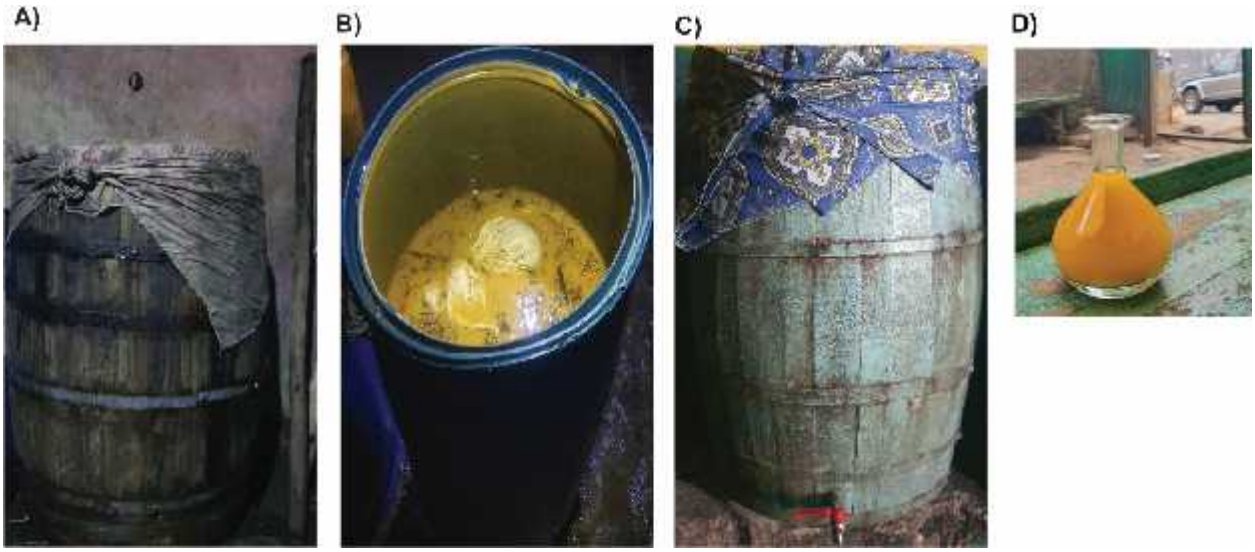

Figure 4. 3 Picture of barrels used for **A**) primary and secondary fermentation **B**) final fermentation **C**) retailing purposes, and **D**) is *Tej* served to the customer

4.3.2 Physicochemical properties

Titratable acidity (TA) and pH are among the important physicochemical parameters for many of alcoholic beverages (Tyl & Sadler, 2017). The pH of the *Tej* samples were ranged from 2.8 to 3.8. Similarly, TA levels in these samples ranged from 1.81 to 8.63 g tartaric acid/L. The *Tej* samples collected from the DM and AA areas had a higher and lower pH and TA values, respectively (Table 4.3). The difference in TA and pH could be due to the degree of fermentation, the type of honey used, and/or the kind of microbes present during the fermentation process. Nemo & Bacha, (2020) had reported a similar finding on the acidity of *Tej* samples that was collected from Jima district. A similar pH and TA values was observed for honey wine produced by a direct inoculated fermentation using *S. cerevisiae*) strains (Pereira *et al.*, 2019).

In this study, pH and TA values of the collected *Tej* samples showed a statistically significant difference at $p < 0.05$ (Table 4.3). Despite this, no statistically significant difference ($p < 0.05$) in the group mean pH value was found across the samples collected from various locations. In comparison to the samples from the BD area, the samples from the AA and DM areas had a significantly higher group TA value ($p < 0.05$) (Table 4.3). *Tej* could easily be classified as a mild acidic alcoholic beverage based on these pH and TA values. *Saccharomyces*, *Zymomonas,* and *Lactobacillus* produced ethanol and organic acid, which had a significant impact for these results. Thankfully, sour honey wine is becoming increasingly popular among consumers (Peepall *et al.*, 2019).

Electric conductivity (EC) of beverages and foods is becoming a very important parameter as a result of the advancement of novel food processing technologies (Jha *et al.*, 2011). The contents of ash, sugars, Proteins, and organic acids in food materials have a significant impact on the EC values (Acquarone *et al.*, 2007). The mean EC values for the *Tej* samples were ranged from 0.333 to 0.724 mS/cm (Table 4.3). There was also statistically significant EC value variation ($p < 0.05$) was observed among the *Tej* samples (Table 4.3). Samples collected from DM (0.662 ± 0.13) had higher and significantly different electrical conductivity values based on area wise comparison. The way "gesho" (*R. prinoides*) added to the honey water mixture, as well as the type and amount of honey used, could all be factors in the EC value variations. Nemo & Bacha, (2020) reported a similar electrical conductivity value for the *Tej* samples.

Table 4. 3 Physio-chemical and antioxidant properties of Ethiopian honey wine, *Tej*

Samples	Physicochemical Properties							Antioxidant Properties	
	pH	TA (g/L)	EC (mS/cm)	Fructose(g/L)	Glucose(g/L)	F/G	Ethanol (g/100 mL)	DPPH (%)	ABTS (%)
	Values for each collected *'Tej'* samples								
A_1	3.24±0.010^{i}	6.24±0.01^{h}	0.520±0.01^{de}	11.51±0.01^{b}	3.58±0.01	3.22	9.01±0.02^{j}	52.55±0.128^{fgh}	47.05±0.647^{l}
A_2	2.83±0.015^{m}	8.65±0.02^{a}	0.499±0.01^{e}	3.89±0.08^{i}	0.61±0.01	6.38	9.27±0.02^{i}	63.10±0.067^{c}	49.47±0.554^{i}
A_3	3.00±0.015^{l}	7.94±0.03^{b}	0.509±0.01^{de}	3.60±0.02^{j}	0.95±0.01	3.79	8.12±0.02^{o}	45.49±0.048k	50.36±0.620^{h}
A_4	3.52±0.015^{d}	4.59±0.03^{l}	0.567±0.01^{c}	1.68±0.03^{o}	ND	-	8.49±0.03^{m}	51.14±0.579^{ghi}	53.83±0.352^{f}
A_5	3.47±0.010^{e}	4.49±0.04^{m}	0.520±0.01^{de}	2.66±0.05^{n}	0.52±0.01	3.23	10.40±0.01^{f}	59.21±0.391^{d}	70.20±0.385^{b}
A_6	3.35±0.021^{g}	4.12±0.05^{n}	0.524±0.02^{d}	3.85±0.05^{i}	0.75±0.01	5.13	10.82±0.02^{c}	48.44±0.875^{ijk}	47.79±0.322^{kl}
A_7	3.13±0.015^{j}	6.07±0.11^{i}	0.571±0.01^{c}	6.42±0.03^{f}	1.40±0.01	4.59	8.72±0.03^{k}	54.04±0.766^{g}	59.24±0.594^{d}
B_1	3.30±0.015^{g}	1.83±0.09^{t}	0.358±0.01^{gh}	9.90±0.05^{c}	6.55±0.01	1.51	11.34±0.01^{a}	39.81±0.446^{l}	27.37±0.133^{n}
B_2	3.27±0.015^{g}	3.73±0.08^{o}	0.458±0.01^{f}	3.23±0.02^{l}	0.97±0.01	3.33	10.24±0.02^{g}	81.00±0.003^{a}	64.93±0.664^{c}
B_3	3.21±0.015^{h}	6.81±0.07^{f}	0.333±0.01^{i}	25.05±0.04^{a}	1.87±0.01	13.40	7.21±0.01^{q}	57.74±0.446^{d}	49.90±0.222^{hi}
B_4	3.50±0.021^{d}	5.86±0.14^{i}	0.342±0.02^{hi}	3.38±0.06^{k}	0.98±0.01	3.45	6.36±0.01^{s}	48.12±0.637^{ijk}	42.43±0.276^{m}
B_5	3.69±0.015^{b}	1.99±0.11^{r}	0.460±0.01^{f}	7.77±0.02^{e}	2.61±0.01	2.98	6.83±0.03^{r}	52.57±0.368^{fgh}	49.40±0.084^{i}
B_6	3.59±0.015^{c}	4.75±0.07^{k}	0.582±0.01^{c}	4.12±0.04^{h}	0.84±0.01	4.90	10.56±0.02^{e}	70.31±0.893^{b}	51.92±0.244^{g}
B_7	3.72±0.006^{b}	1.81±0.08^{t}	0.368±0.02^{g}	8.41±0.01^{d}	0.85±0.01	9.89	9.03±0.02^{j}	53.89±0.290^{fg}	73.10±0.100^{a}
D_1	3.75±0.015^{a}	1.88±0.07^{s}	0.372±0.01^{g}	0.80±0.01^{q}	0.20±0.01	4.00	8.43±0.01^{n}	49.69±0.551^{hij}	56.70±0.135^{e}
D_2	3.27±0.015^{g}	6.65±0.06^{g}	0.717±0.02^{a}	2.87±0.01^{m}	ND	-	7.76±0.02^{p}	57.34±0.848^{de}	49.69±0.188^{hi}
D_3	3.09±0.015^{j}	6.92±0.09^{d}	0.703±0.02^{a}	4.50±0.03^{g}	0.65±0.01	6.92	8.67±0.01^{l}	37.87±0.575^{l}	47.33±0.557^{l}
D_4	3.42±0.015^{f}	3.36±0.13^{q}	0.724±0.01^{a}	0.37±0.01^{r}	0.07±0.01	5.29	10.77±0.02^{d}	45.92±0.006^{k}	52.23±0.311^{g}
D_5	3.10±0.015^{j}	6.97±0.08^{c}	0.716±0.01^{a}	0.95±0.01^{p}	ND	-	8.73±0.02^{k}	54.63±0.419^{ef}	48.63±0.369^{j}
D_6	3.06±0.006^{k}	6.90±0.09^{e}	0.676±0.03^{b}	3.88±0.02^{i}	0.75±0.01	5.17	10.02±0.01^{h}	48.67±0.051^{ijk}	48.46±0.163^{jk}
D_7	3.39±0.015^{f}	3.47±0.06^{p}	0.725±0.03^{a}	0.85±0.04^{q}	0.093±0.01	9.14	11.01±0.02^{b}	47.66±0.551^{jk}	50.40±0.152^{h}
	Mean values for samples collected from the same areas								
AA	3.22±0.242^{a}	6.01±1.68^{a}	0.530±0.03^{b}	4.80±3.13^{b}	1.12±1.11	4.29	9.26±1.00^{a}	53.42±6.100^{ab}	53.99±8.272^{a}
BD	3.47±0.204^{a}	3.82±1.97^{b}	0.414±0.09^{c}	8.84±7.23^{a}	2.10±1.97	4.21	8.80±2.00^{a}	57.63±13.872^{a}	51.29±14.821^{a}
DM	3.28±0.250^{a}	5.17±2.07^{a}	0.662±0.13^{a}	2.03±1.60^{b}	0.25±0.30	7.96	9.34±1.25^{a}	48.83±6.285^{b}	50.49±3.157^{a}

A_1- A_7, B_1-B_2, D_1-D_6 are sample collected from Addis Ababa (AA), Bahir Dar (BD) and Debre Markos (DM), respectively. TA-Titratable Acidity (g of tartaric acid per litter), EC-electrical conductivity, DPPH-2,2-diphenyl-1-picrylhydrazyl, ABTS-3-ethylbenzothiazoline-6-sulfonic acid diammonium salt, ND – Not detected, F/G – Mean fructose/Mean glucose

Glucose and fructose dominated the sugar profile of the *Tej* samples collected from different locations of Ethiopia (Table 4.3). The dominance of these mono-saccharide sugars in honey wine is not surprising result given that glucose and fructose account for 85 to 95% of total sugar in honey (Finola *et al.*, 2007). The dominance of these simple sugars especially at the start of fermentation, has a big advantage on avoiding saccharification process which is the necessary processing step for polysaccharide dominate substrate. However, if the concentration of sugars exceeds a certain threshold limit, the process may become stuck or sluggish due to osmotic stress on fermentative microorganisms (Pereira *et al.*, 2013). The glucose and fructose levels of *Tej* samples were ranged from 6.55 g/L to below detectable (ND) limit, and 0.95 g/L to 25g/L, respectively (Table 4.3). *Tej* can thus be classified as a less sweet honey wine because its sugar content is less than 100 g/L (Pereira *et al.*, 2009). There are two possible explanations for the lower sugar content in the samples. The first is due to proper substrate utilization during the fermentation period. Another reason could be a higher dilution of honey prior to the start of fermentation.

All *Tej* samples collected from various locations of Ethiopia had shown a higher fructose to glucose ratio (Table 4.3). The fermentative microorganisms' preference for glucose over fructose during the fermentation period is most likely responsible for this higher FGR values in all of the samples. In general, samples collected in the BD area had a higher sugar content than samples collected in the AA and DM areas (Table 4.3). Even though the type and amount of honey, as well as the conditions of the fermentation process, will have an effect on this result, the difference in preparative steps in each respective area has a significant contribution to the difference in areal based comparison of sugar content. This is especially true for *Tej* samples collected from the DM region, where a water-to-honey ratio of 1:4 is commonly used during the preparation process. Furthermore, the sucrose content of all *Tej* samples was below the detectable limit. The matured honey used as an ingredient in the production of *Tej* could be the likely cause of the low sucrose content. This is because the sucrose content of honey will be converted to simple (monosaccharides) sugars as it matures (Ramalhosa *et al.*, 2011).

Alcoholic fermentation's primary goal is to produce ethanol from available carbon source substrate (Tamang, 2010). The average ethanol content of *Tej* samples in the aforementioned areas ranged from 6.36 to 11.34 g/100 mL (Table 4.3). This broad range in alcohol content could be due to the fermentation process conditions, type and abundance of fermentative microorganisms, length

of fermentation period, and post-fermentation storage conditions (Rodríguez-Lerma *et al.*, 2011). Almost all of the *Tej* samples had a statistically significant difference in alcohol content for $P < 0.05$ (Table 4.3). Especially, one of the samples from the BD area had the highest ethanol content (11.34 g/100 mL). This higher ethanol content might be due to the co-dominance of *Saccharomyces* and *Zymomonas* in the specified sample, in addition to the other possible explanations already mentioned (Figure 4.4B). In fact, all of the *Tej* samples dominated by the aforementioned two genera had shown a higher alcohol level content (Table 4.3; Figure 4.4B). Meanwhile, when compared to other *Tej* samples, sample B3 had a lower ethanol content (6.36 g/100 mL). This could be due to the predominance of *Acetobacter* in this particular sample. This bacteria's species is a very well-known microorganism that degrades ethanol to acetic acid in the presence of oxygen (Bartowsky & Henschke, 2008). Nonetheless, there was no statistically significant difference ($P < 0.05$) observed in alcohol content for the comparison based on sample collection area (Table 4.3). Previous studies by Nemo & Bacha, (2020) and Bahiru *et al.*, (2001) found a similar level of alcohol content in *Tej* samples.

4.3.3 Antioxidant activity

To reduce oxidative stress in the human body, our daily foods and beverages should boost our physiological antioxidant activity (Floegel, 2011). The most popular colorimetry antioxidant assays (DPPH and ABTS) were used in this research to analyze the antioxidant activity of *Tej* samples. The average total antioxidant activity of *Tej* samples was 53.6% for DPPH and 51.92% for ABTS (Table 4.3). Based on this finding, we can conclude that all of the *Tej* samples had a high antioxidant capacity. Indeed, the source of this antioxidant activity is primarily honey, with a minor contribution from the added "gesho" (*R. prinoides*) (Aazza *et al.*, 2013). Additionally, the predominance of *Saccharomyces* and *Lactobacillus* in all of the collected *Tej* samples, which are known microbial strains that are capable of producing flavonoid and polyphenol through a complex microbial enzymatic system, could be the possible source of bioactive compounds (Svensson *et al.*, 2010; Verni et al., 2019).

The ability of the *Tej* sample to donate hydrogen atoms to DPPH reactive species in a homogeneous environment was examined using the DPPH radical assay. For the *Tej* samples in this study, the highest and lowest DPPH values were 81% and 37.9%, respectively. *Tej* Samples collected from the same area as well as from different area showed a statistically significantly different ($p < 0.05$) DPPH values (Table 4.3). When compared on the basis of collection area, samples from DM

(49.02%), and AA (53.42%) areas had a significantly lower ($p < 0.05$) DPPH value as compared to samples from BD (57.63%). Even though there could be a variety of explanations for these differences, the geographical location where honey was obtained and the way "gesho" (*R. prinoides*) was added to the honey-must may have played a major role in the outcomes. In the DM area, for example, "gesho" (*R. prinoides*) leaves are usually added to the honey-must without boiling. This could make it more difficult to extract bioactive compounds from the added gesho (*R. prinoides*) leaves. Furthermore, differences in the concentration and type of fermentative microorganisms involved during fermentation may contribute to the differences in antioxidant activity values among the *Tej* Samples. This argument was bolstered by Leonard *et al.*, (2021) review paper on the influence of fermentation on the bioactive and phenolic compound profiles of plant-based foods.

ABTS scavenging activity of the analyzed *Tej* samples were ranged from 27.4% to 73.1% (Table 4.3). A sample collected from the BD area yielded both the lowest and highest ABTS values. Although the ABTS values differed statistically between the samples, there was no statistically significant difference between the samples based on collected areas comparison ($p > 0.05$) (Table 4.3). Similar antioxidant activity results for ABTS and DPPH were reported for a honey wine from Poland (Socha *et al.*, 2015).

4.3.4 Microbial community structures

Despite the unfavorable living conditions, some bacteria and fungi genera coexist in fully matured *Tej* samples (Figure 4.4). Relative abundance of bacterial and fungal communities of *Tej* samples collected from Bahir Dar (BD), Addis Ababa (AA), and Debre Markos (DM) areas at phylum, family, genus, and species level is illustrated in Figure 4.4. Each bar in this figure represents either a fungal or bacterial community structure of each sample from respective areas. The ingredients and utensils used in the preparation process could be a source of these fermentative microorganisms (Bahiru *et al.*, 2006). 16SrRNA and ITS amplicon sequencing were used to examine the bacterial and fungal community structures of 21 *Tej* samples. The Illumina MiSeq platform was used to sequence the amplified hypervariable (HV_4-HV_5) regions of bacteria and fungi.

4.3.4.1 Bacterial community structure

By comparing our Illumina MiSeq sequenced data with SILVA database, phylogenetic analysis and taxonomic assignment of the bacterial community was performed for the microbes that had a 97% similarity. According to our findings, *Proteobacteria* and *Firmicutes* were the most common phyla

found in *Tej* samples (Figure 4.4A). *Firmicutes* was more prevalent in samples collected from the AA (76.79%) area than in samples collected from the DM (34.39%) and BD (33.89%) areas. *Proteobacteria*, on the other hand, was the most common phylum in DM (62.47%) and BD (64.36%) samples, compared to AA (21.91%) samples (Figure 4.5). For Korean traditional rice beer "Makgeolli," a similar phylum of *Proteobacteria* and *Firmicutes* dominance was also reported (Jung *et al.*, 2020).

For those *Tej* samples dominated by the *Firmicutes* phylum, *Lactobacillus* was the most common genus (Figure 4.4A). Particularly, *Tej* samples from AA area were highly predominated by *Lactobacillus* species (Figure 4.5A). In fact, the presence of this genus aids in the development of distinct sensory characteristics of the produced alcoholic beverage. This is as a result of the fact that *Lactobacillus* species have the ability to produce extracellular secondary metabolites like ketones, organic acids, and alcohols (Fuochi *et al.*, 2019). Furthermore, the higher TA values observed on the *Tej* samples that were collected from the AA area could be due to this *Lactobacillus* species dominance, as organic acids have the ability to do so (Table 4.3). Moreover, one of the samples (A4) was completely dominated by the *Fructobacillus* genus. This species of this genus is fructophilic by their nature which they prefer fructose over glucose as major carbon source (Endo & Dicks, 2014). In comparison to other samples taken from the same area, this sample had a lower fructose level (Table 4.3). By applying a phenotypic microbial cultivation method analysis, a *Tej* sample collected from AA, Ethiopia, was found to have a similar lactic acid bacterial dominance (Bahiru *et al.*, 2006).

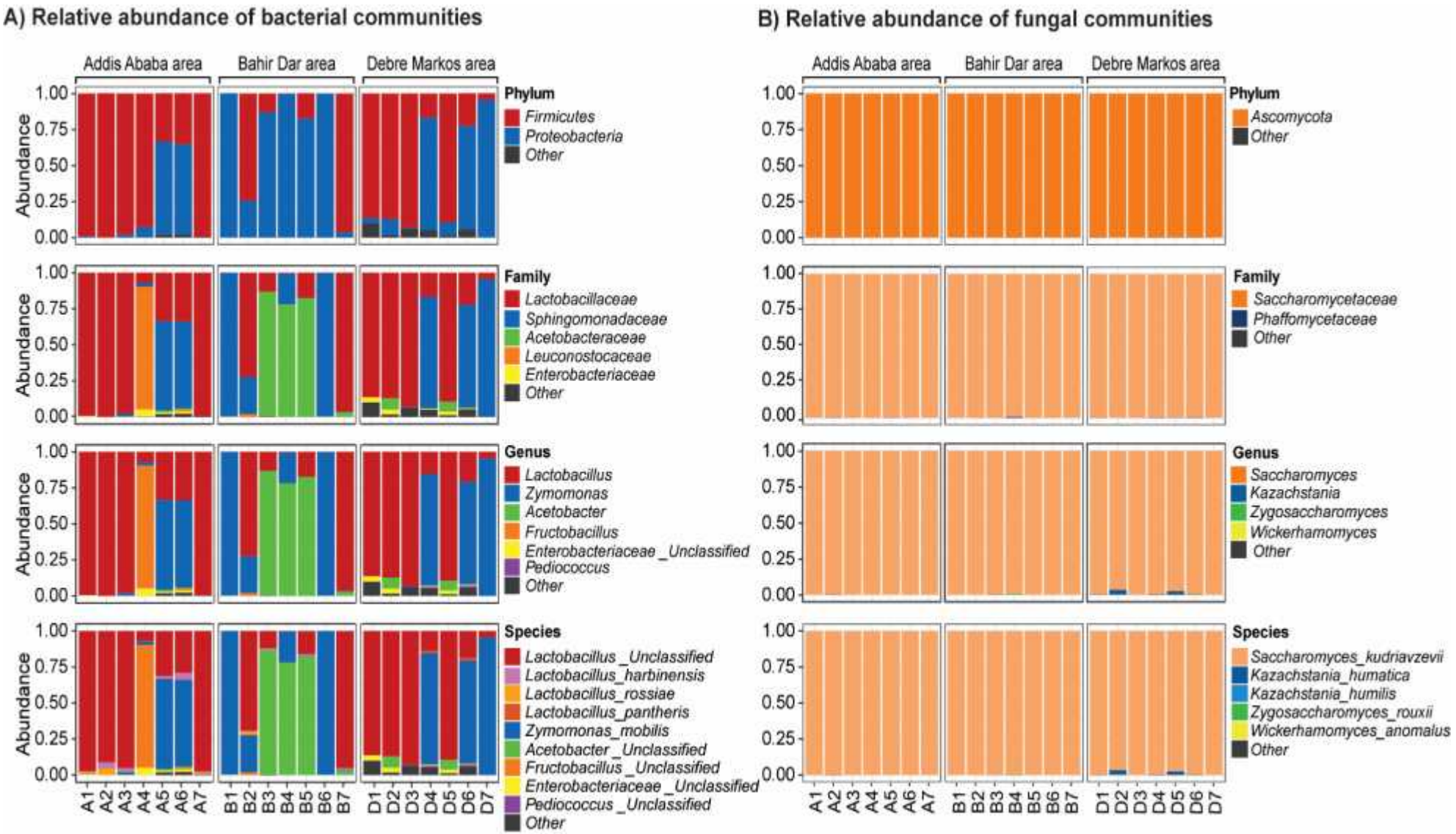

Figure 4. 4 Relative abundance of **A**) bacterial, and **B**) fungal communities of *Tej* samples collected from AA, BD and DM areas at phylum, family, genus, and species level. Each bar represents either a bacterial or fungal community structure of an independent sample.

Acetobacter and *Zymomonas* were the most common genera for *Tej* samples that were dominated by Proteobacteria phylum (Figure 4.4A). *Acetobacter* was found in greater abundance in the samples collected from the BD area (49.19%) than in the samples collected from the AA (1%) and DM (1.8%) areas. The ability of these bacteria to convert ethanol to acetic acid in the presence of oxygen is one of their most distinguishing characteristics. Higher acetic acid content degrades the quality of alcoholic beverages by producing a very sour taste (Bartowsky *et al.*, 2003). When compared to samples collected from AA and DM, BD *Tej* samples dominated by *Acetobacter* had shown a higher pH value (Table 4.3). *Acetobacter* proliferation was found to be similar in wine samples with a higher pH value (Joyeux et al., 1984). For those *Tej* samples predominated by *Proteobacteria* phyla, *Zymomonas* was the other most abundant genus of all (Figure 4.4A). In comparison to the samples from BD (20.17%) and AA (20.6%), the samples from DM had shown a higher dominance in the genus of *Zymomonas* (59.28%) (Figure 4.5). This bacterium have a good ability to produce bioethanol that has a fruity odor (Li *et al.*, 2019; Rogers *et al.*, 1982). Another distinguishing feature of *Zymomonas* is its preference of sucrose, glucose, and fructose over other polysaccharide sugar as carbon sources (Li *et al.*, 2019). Given the abundance of these mono and disaccharides in honey, *Zymomonas* dominance in spontaneously fermented honey wine is not surprising. A similar predominance of *Zymomonas* was also observed for other African spontaneously fermented traditional alcoholic beverages (Diaz *et al.*, 2019). Moreover, the dominance of this genus was also observed in Mexican alcoholic beverage, “Pulque” (Chacón-Vargas *et al.*, 2020).

The other member of *Protobacteria* found in *Tej* samples is *Enterobacteriaceae*, which was discovered (abundance >1%) in samples taken from the DM (1.38%) area (Figure 4.5C). The bacterial diversity of *Tej* samples from AA, BD and DM areas are illustrated in Figure 5.3 A, B & C, respectively. One of the *Tej* samples from AA (A4), in particular, had a noticeable abundance of *Enterobacteriaceae* (Figure 4.4A). In fact, the vast majority of bacteria in this family are pathogenic (Paterson, 2006). In addition, the presence of *Enterobacteriaceae* in any food indicates poor handling (Amorim & Nascimento, 2017). As a result, the DM area samples had undoubtedly been mishandled during and/or after fermentation. For the *Tej* samples form Jimma area, Nemo & Bacha, (2020) also found about 2 log CFU mL^{-1} *Enterobacteriaceae* count.

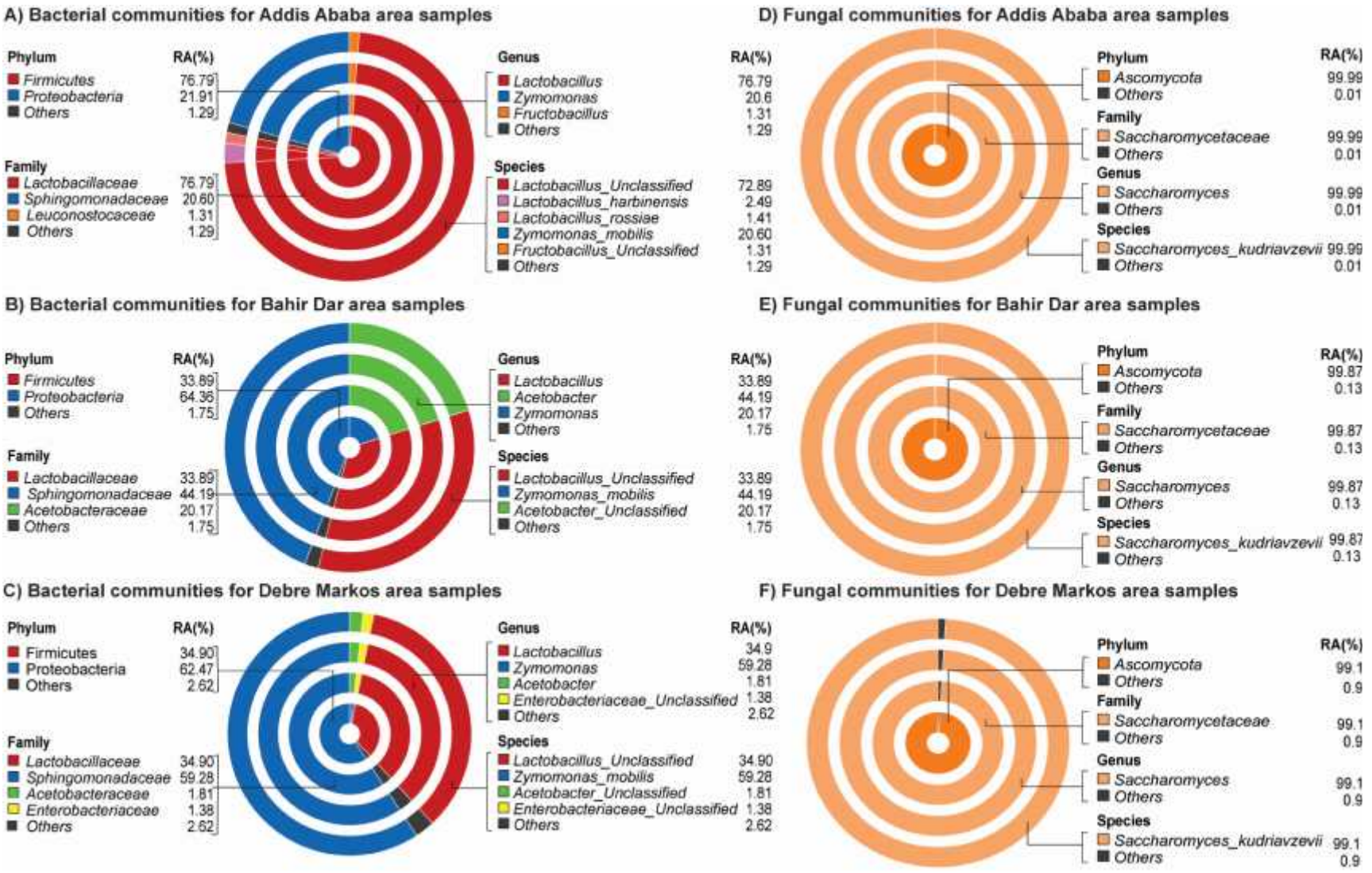

Figure 4. 5 Doughnut chart for illustration of **A**), **B**), and **C**) bacterial, and **D**), **E**), and **F**) fungal community structure based on the collection areas. Each doughnut chart is composed from the microbiome data of seven samples collected from respective areas.

Most *Lactobacillus*, *Fructobacillus*, *Acetobacter*, and *Pediococcus* species were unclassified to species level due to short read length. Despite this fact, *Lactobacillus rossiae*, *Lactobacillus harbinensis*, *Zymomonas mobilis*, and *Lactobacillus pantheris* were the species level identified bacterial communities (Figure 4.4A). For samples collected from the AA area, *L. harbinensis* and *L. rossiae* were found in lower abundance (Figure 4.4A). *L. harbinensis* are hetrofermentative lactic acid bacteria which can produce lactate, ethanol, and acetate (Miyamoto *et al.*, 2005). *L. rossiae* strains, which are commonly found in fermented meat, flour, and vegetables, are also obligately heterofermentative lactic acid bacteria (De Angelis *et al.*, 2014). Although these species were not found in samples from the DM and BD areas, heterofermentative *Lactobacillus* is very much expected from such spontaneously fermented alcoholic beverages. *L. pantheris* is another facultative anaerobic homofermentative lactic acid bacteria (Liu & Dong, 2002) found in AA samples (Figure 4.4A). These species' strains have also been isolated from a traditional Turkish fermented fruit drink (Sagdic *et al.*, 2014). *Z. mobilis* is predominate bacterial species for the samples from DM and BD areas with a relative abundance (RA %) of 59.28% and 44.19%, respectively (Figure 4.5). Even at low biomass concentrations, this gram negative, facultative anaerobic bacteria can produce a significant amount of bioethanol (He *et al.*, 2014a).

In general, the findings showed that *Lactobacillus* (52.14%) and *Zymomonas* (38.41%) were the most common bacterial genera found in *Tej* samples (Figure 5.4A). These culturable bacteria have the potential to be used as a mixed starter culture for honey wine fermentation due to an increase in market acceptance of sour honey wine (Peepall *et al.*, 2019). Furthermore, *L. harbinensis*, *Fructobacilus*, and *L. pantheris* were the unique genera and species for the collected *Tej* samples from AA area. Despite the fact that *Acerobacter* is a contaminant, it was the shared genera for the *Tej* samples from DM and BD areas (Figure 4.5). Moreover, the bacterial community which is classified as others categories in Figure 4.4, which have a relative abundance of < 1%, is tabulated in Table 4.3. *Aeromonas*, *Enhydrobacter*, *Gluconobacter*, *Leuconostoc*, *Pediococcus*, *Staphylococcus*, *Weissella* etc were some of the species observed in a *Tej* samples which have a relative abundance lower than 1%. However, still *Unclassified_Lactobacillus* and *Enterobacteriaceae_Unclassified* microbes was observed in category (Table 4.3).

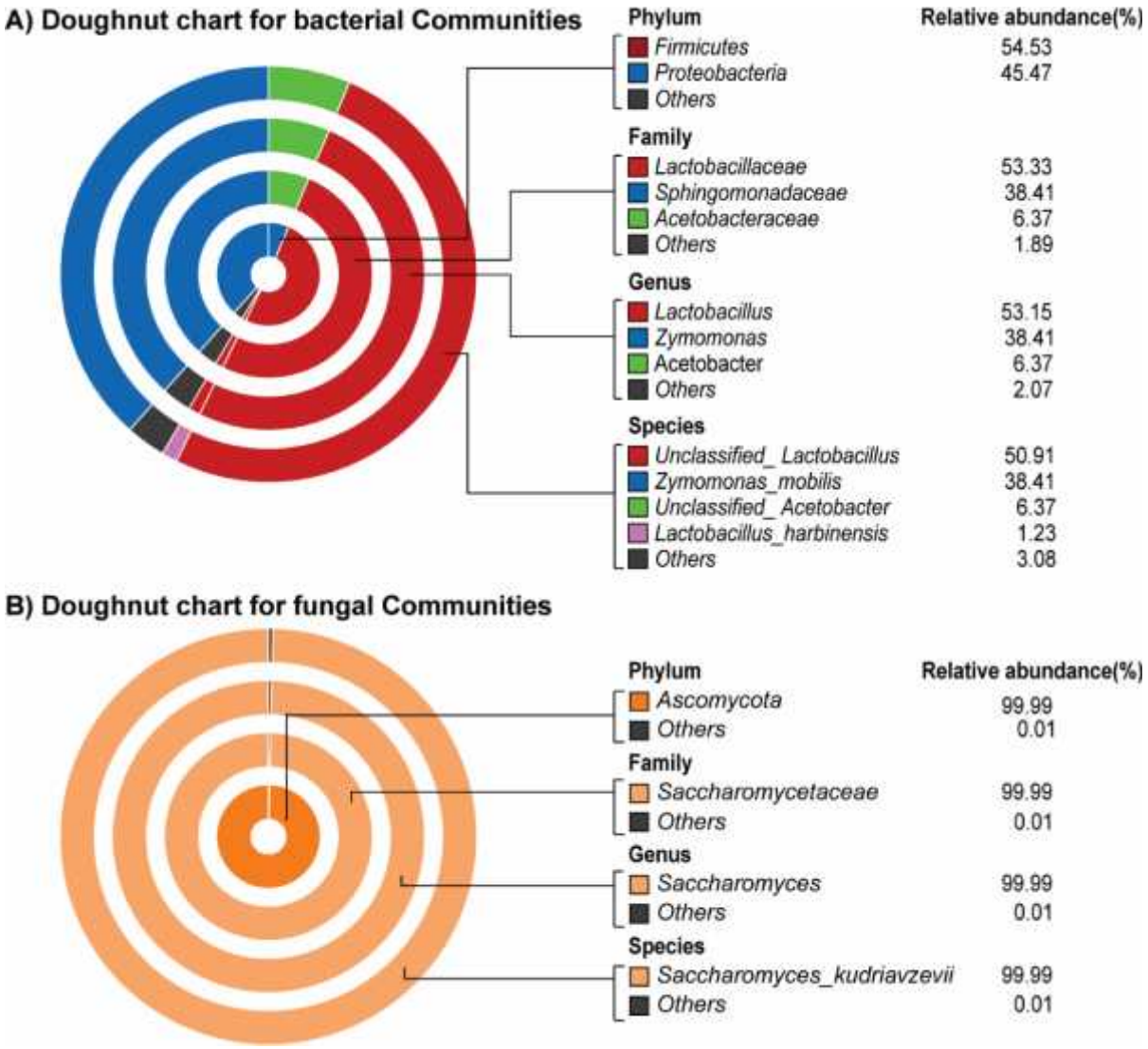

Figure 4. 6 Doughnut chart to illustrate **A**) bacterial community structure, and **B**) fungal community structure for all collected *Tej* samples. Each doughnut chart is composed from twenty-one samples microbiome data.

4.3.4.2 Fungal community structure

A single phylum of *Ascomycota* dominated the fungal community structure of *Tej* samples (99.99%t) (Figure 4.6B). In addition, the genus *Saccharomyces*, *Zygosaccharomyces*, *Kazachstania*, and *Wickerhamomyces* were identified in the *Tej* samples (Figure 4.4B). In all of the collected samples, *Saccharomyces* was by far the most prevalent fungal genus (> 99.1%) (Figure 4.4B). Traditional palm wine produced in Cote d'lvoire showed a similar dominance of *S. cerevisiae* (87%) (Djeni *et al.*, 2020). However, our findings differ from those of a previous study that focused on *Tej's* microbiology (Bahiru *et al.*, 2006). The authors discovered that *S. cerevisiae* only contributed

about 25% of the total yeast community in the *Tej* samples, followed by *Kluyvermyces bulgaricus* (16%), *Debaromyces phaffi* (14%), and *Kluyvermyces veronae* (10%). However, keep in mind that the results presented above were entirely based on the phenotypic microbial cultivation method. *S. kudriavzevii* was identified as the dominant species in collected *Tej* samples, with 98% similarity to the UNITE reference data base. In fact, *S. kudriavzevii* is a hybrid species that shares 23 to 100% of its genome with *S. cerevisiae* (Peris *et al.*, 2018). Furthermore, it is cryophilic by their nature (10–25°C) and has a high capacity to produce glycerol. *S. kudriavzevii* is the next promising non-*S. cerevisiae* species that could be used as a starter culture for wine fermentation due to these promising properties (Alonso-del-Real *et al.*, 2017).

Kazachstania was the most common genus found in wine grapes and sourdough (Carbonetto *et al.*, 2020; Jood *et al.*, 2017). *Kazachstania* species were observed in lower abundance in *Tej* samples collected from DM. The specific *Kazachstania* species observed in *Tej* samples from the aforementioned area were *K. humilis* and *K. humatica*. Generally, the species of *Kazachstania* is not advisable to use as an exclusive single strain starter culture for wine fermentation (Jood *et al.*, 2017). Co-cultivation of *Kazachstania* species with *S. cerevisiae*, on the other hand, would aid the produced beverage in the development of good aroma and flavor (Dashko *et al.*, 2015). From Linear discriminant analysis (LefSe) result, K. *humilis* were the different and significantly higher species found in the samples collected from DM areas (Figure 4.7B).

Zygosaccharomyces was the other observed genus in Ethiopian honey wine. This fructophilic microorganism can grow in environments with low pH and high sugar concentrations (Wang *et al.*, 2016). As a result, the involvement of *Zygosaccharomyces* strains in naturally fermented Ethiopian honey wine is entirely normal. Nonetheless, the use of *Zygosaccharomyces* species as a starter culture in wine fermentation remains a contentious issue (Escott *et al.*, 2018). *Wickerhamomyces* strains were also found in the B4 *Tej* sample (Figure 4.4B). This species has good characteristics for producing good aroma and preventing the formation of haze (Wang *et al.*, 2020). Furthermore, *W. anomalus* has the potential to act as a biological control for successful fermentation (Padilla *et al.*, 2018).

The use of non-*Saccharomyces* in conjunction with *Saccharomyces* yeast is gaining popularity these days (Carbonetto *et al.*, 2020). This is due to the ability of non-*Saccharomyces* yeast species to improve the aroma profile of wine by producing acetate ester which has fruity flavor (Gamero *et al.*, 2020). Although the non-*Saccharomyces* species encountered in this study were not found in

greater abundance (1%), believes that incorporating one of these species as a mixed culture may aid in the development of a direct *Tej* fermentation system without sacrificing the wholesomeness of Ethiopian honey wine (Figure 4.6B).

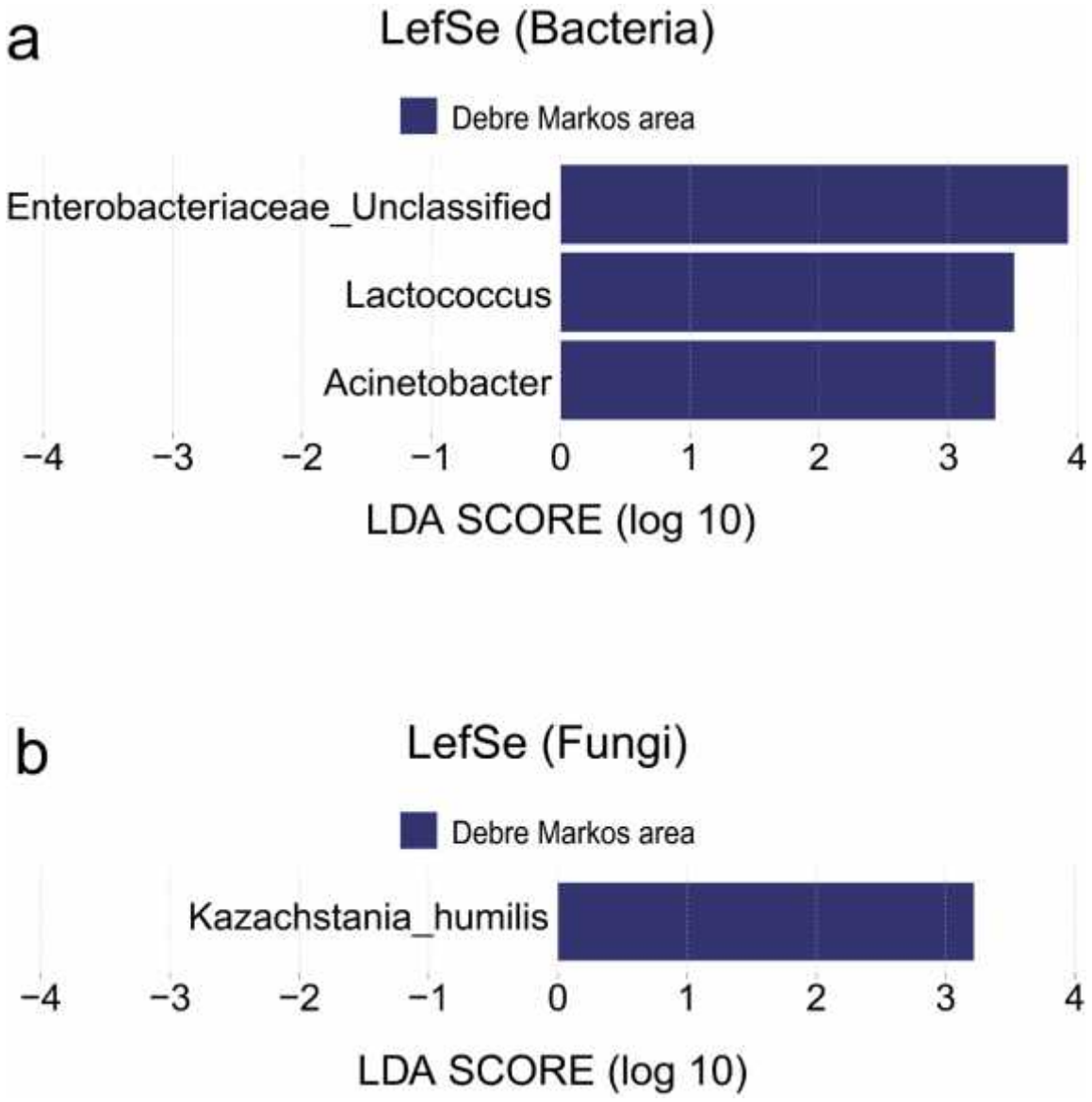

Figure 4. 7 Linear discriminant analysis effect size (LefSe) for **A**) bacteria and **B**) fungi communities

1 Table 4. 4 Bacterial and fungal community structure at the relative abundance < 1% (classified as others)

Bacterial Community structure at the relative abundance of < 1% (grouped as others)						
S/N	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**RA (%)**
1	*Proteobacteria*	*Gammaproteobacteria*	*Aeromonadales*	*Aeromonadaceae*	*Aeromonas*	0.00023
2	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Enhydrobacter*	7.10E-06
3	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	*Enterobacteriaceae_Unclassified*	0.00666
4	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Leuconostocaceae*	*Fructobacillus*	0.00705
5	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Leuconostocaceae*	*Fructobacillus*	7.34E-05
6	*Proteobacteria*	*Alphaproteobacteria*	*Acetobacterales*	*Acetobacteraceae*	*Gluconobacter*	0.00016
7	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Lactobacillales_Unclassified*	*Lactobacillales_Unclassified*	2.13E-05
8	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*	0.00011
9	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*	0.00018
10	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*	0.00218
11	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*	0.00771
12	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Lactococcus*	0.00202
13	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Leuconostocaceae*	*Leuconostoc*	0.00242
14	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Pediococcus*	0.00161
15	*Firmicutes*	*Bacilli*	*Staphylococcales*	*Staphylococcaceae*	*Staphylococcus*	5.68E-05
16	*Firmicutes*	*Negativicutes*	*Veillonellales-Selenomonadales*	*Veillonellales-Selenomonadales_Unclassified*	*Veillonellales-Selenomonadales_Unclassified*	0.00012
17	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Leuconostocaceae*	*Weissella*	0.00025
Fungal Community structure for the relative abundance of <1% (grouped as others)						
S/N	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**RA (%)**
1	*Ascomycota*	*Saccharomycetes*	*Saccharomycetales*	*Saccharomycetales_fam_Incertae_sedis*	*Candida*	4.49E-06
2	*Ascomycota*	*Saccharomycetes*	*Saccharomycetales*	*Phaffomycetaceae*	*Cyberlindnera*	5.39E-05
3	*Ascomycota*	*Saccharomycetes*	*Saccharomycetales*	*Saccharomycetaceae*	*Kazachstania*	0.00233
4	*Ascomycota*	*Saccharomycetes*	*Saccharomycetales*	*Saccharomycetaceae*	*Kazachstania*	0.00048
6	*Ascomycota*	*Saccharomycetes*	*Saccharomycetales*	*Saccharomycetaceae*	*Torulaspora*	4.49E-05
7	*Ascomycota*	*Saccharomycetes*	*Saccharomycetales*	*Phaffomycetaceae*	*Wickerhamomyces*	0.00043
8	*Ascomycota*	*Saccharomycetes*	*Saccharomycetales*	*Saccharomycetaceae*	*Zygosaccharomyces*	0.00011

2

However, there has been no prior experience with the use of non-*S. cerevisiae* strains for honey wine fermentation, either in the form of a mixed or single strain culture.

4.3.5 Alpha and beta diversity

The species richness and diversity of microorganisms analyzed in *Tej* samples were demonstrated using the alpha diversity indices: chao1, evenness, observed, shannon, and Invsimpson. Actually, the alpha and beta diversity analyses were performed after clustering the *Tej* samples based on collection areas. As a result, the primary goal of these diversity analyses was to demonstrate the effect of geographical location on the microbial diversity of the collected samples. The exact values of alpha diversity indices for each *Tej* sample are articulated in Table 4.5.

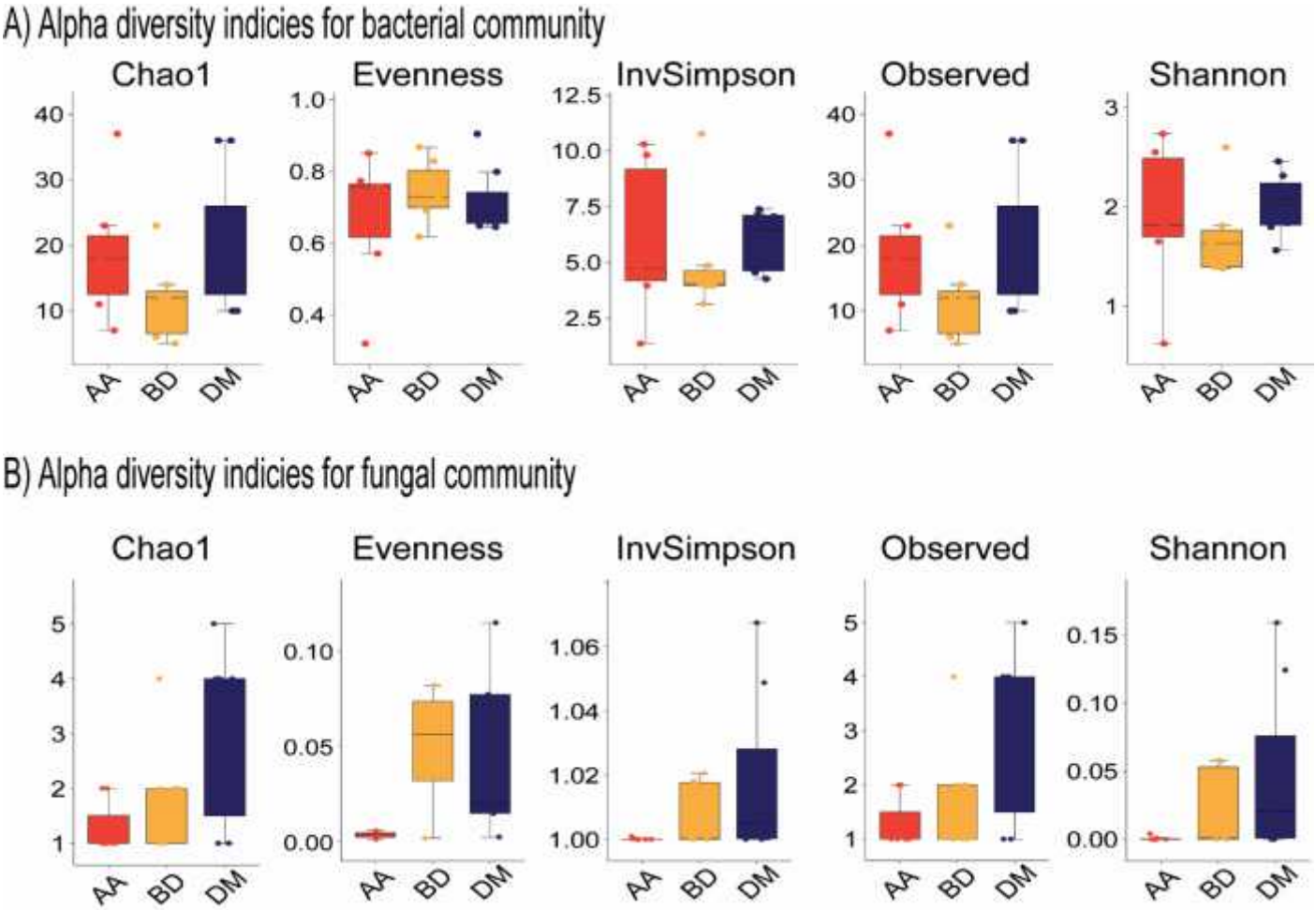

Figure 4. 8 The most common alpha diversity indices for **A**) bacterial **B**) fungal communities of *Tej* samples

Table 4. 5 Alpha diversity of bacteria and fungi communities

	Alpha diversity indices for bacteria						**Alpha diversity indices for fungi**					
Locations	**Chao1**	**Shannon**	**Simpson**	**Evenness**	**Invsimp**	**Obs**	**Chao1**	**Shannon**	**Simpson**	**Evenness**	**Invsimp**	**Obs**
A1	20	2.549232	0.902917	0.850955	10.30043	20	1	0	0	0	1.00	1
A2	11	1.817178	0.78934	0.757822	4.746981	11	2	0.000968	0.000189	0.001397	1.000189	2
A3	14	1.745972	0.772441	0.661589	4.39446	14	1	0	0	0	1.00	1
A4	7	0.623537	0.264767	0.320435	1.360114	7	1	0	0	0	1.00	1
A5	23	2.425679	0.882881	0.773619	8.53834	23	2	0.004083	0.000943	0.005891	1.000944	2
A6	37	2.734643	0.898213	0.757326	9.824424	37	1	0	0	0	1.00	1
A7	18	1.650703	0.747359	0.571104	3.958186	18	1	0	0	0	1.00	1
Average	19 ± 9.8	2 ± 0.7	0.75±0.22	1 ± 0.2	61 ± 3.4	18.57±9.78	1.29±0.49					1.29±0.49
B1	5	1.396404	0.747856	0.867635	3.965989	5	2	0.056851	0.020163	0.082019	1.020578	2
B2	23	2.599736	0.907108	0.829131	10.76516	23	1	0	0	0	1.00	1
B3	14	1.631996	0.750932	0.618401	4.014962	14	2	0.001395	0.000283	0.002013	1.000283	2
B4	7	1.377463	0.681323	0.707876	3.137976	7	4	0.058188	0.017796	0.041973	1.018118	4
B5	12	1.717067	0.77267	0.690999	4.398897	12	1	0	0	0	1.00	1
B6	6	1.393619	0.747154	0.777794	3.954984	6	2	0.049155	0.016924	0.070916	1.017216	2
B7	12	1.810743	0.794263	0.728697	4.860575	12	1	0	0	0	1.00	1
Average	11 ± 6.21	1.7 ± 0.43	0.8 ±0.07	0.7 ± 0.09	5.01 ± 2.59	11.29±6.21	1.86±1.07					1.86±1.07
D1	15	2.164121	0.858454	0.799143	7.064865	15	2	0.001806	0.000377	0.002606	1.000377	2
D2	16	1.835379	0.786576	0.661973	4.685511	16	4	0.159168	0.063211	0.114815	1.067477	4
D3	10	2.083656	0.860267	0.90492	7.156523	10	1	0	0	0	1	1
D4	36	2.313813	0.845374	0.645682	6.467207	36	4	0.020706	0.005178	0.014936	1.005205	4
D5	16	1.798805	0.780191	0.648782	4.549399	16	5	0.12428	0.046534	0.07722	1.048805	5
D6	36	2.457644	0.864651	0.685819	7.388298	36	4	0.027907	0.007337	0.020131	1.007391	4
D7	10	1.56241	0.765524	0.678546	4.264823	10	1	0	0	0	1	1
Average	20±11.32	2.03± 0.31	0.82±0.04	0.72±0.10	5.94±1.38	19.86±11.32	3.00±1.63					3.00±1.63
	p-value						p-value					
A Vs B	0.122	0.479	0.82	0.333	0.491	0.122	0.223	0.060	0.059		0.059	0.223
A Vs D	0.824	0.753	0.421	0.549	0.876	0.824	0.021	0.084	0.104		0.107	0.021
B Vs D	0.104	0.131	0.122	0.579	0.42	0.104	0.147	0.395	0.379		0.368	0.147

A_1- A_7, B_1-B_2, D_1-D_6 are *Tej* sample collected from Addis Ababa (AA), Bahir Dar(BD) and Debre Markos(DM), respectively

The Chao 1 values of the samples were used to estimate the species richness. The collected *Tej* samples had a lower bacterial species richness (Figure 4.8A; Table 4.5). Although there is no significant difference ($P > 0.05$) in Chao1 richness for a sample based on collection areas, samples from AA (19.8) and DM (20.32) had higher Chao1 richness than the sample from BD (11 6.21). (Table 4.5). Intuitively, this lower bacterial richness in *Tej* samples could be due to the two major reasons listed below. The first reason could be due to competition among microbial communities during spontaneous fermentation. The other reason could be due to the *Tej's* inherent physicochemical properties. This is primarily due to its lower pH, higher substrate concentration, and ethanol toxicity (Smid & Lacroix, 2013). Diaz *et al.*, (2019) also found that other African alcoholic beverages had lower bacterial richness. Essentially, the dominance of a specific species in naturally fermented food products could be viewed as a blessing for the modernization of spontaneous fermentation system.

Shannon and Invsimpson indices were used to assess the uniformity of *Tej* samples in bacterial community ecology. All of the *Tej* samples had a diverse range of bacterial species (Figure 4.8A). This is most likely due to the strong interaction stability of the surviving bacterial communities. Furthermore, neither the Shannon nor the Invsimpson indices showed a statistically significant difference ($P > 0.05$). To be more specific, the Shannon index for samples collected from AA (2 ± 0.7) and DM (2.03 ± 0.31) areas was higher than the Shannon index for samples collected from BD (1.7 ± 0.43) area (Figure 4.8A; Table 4.5).

Since the fungal communities *Tej* samples were dominated by *Saccharomyces* (Figure 4.8B), it is to be expected to observe a lower Chao1 values. Actually, it is a well-known fact that ethanol-producing yeast species are the most common microorganisms in any alcoholic beverages. A comparison of Chao 1 values between AA and DM area samples, on the other hand, revealed a statistically significant difference ($P < 0.05$) (Figure 4.8B).

A non-parametric statistical analysis of similarity was used to conduct a beta diversity analysis of both bacterial and yeast species in *Tej* samples from various areas. In the Principal Co-ordinate Analysis of Unweighted UniFrac distance (PCoA) plot for the bacterial communities, a visible cluster distance ($P < 0.04$) was observed (Figure 4.9A). This plot had a total variance of 45.8%. For fungal communities, however, there was no visible cluster distance ($P > 0.05$) in unweighted UniFrac distance (PCoA) plot (Figure 4.9B). This is because *Saccharomyces* species predominated in all of the samples. Furthermore, this dominance causes some points to overlap in a two-

dimensional PCoA plot. Furthermore, no significant difference (P > 0.05) was observed in the weighted UniFrac PCoA plot for both bacterial and fungal communities (Figure 4.9C & D).

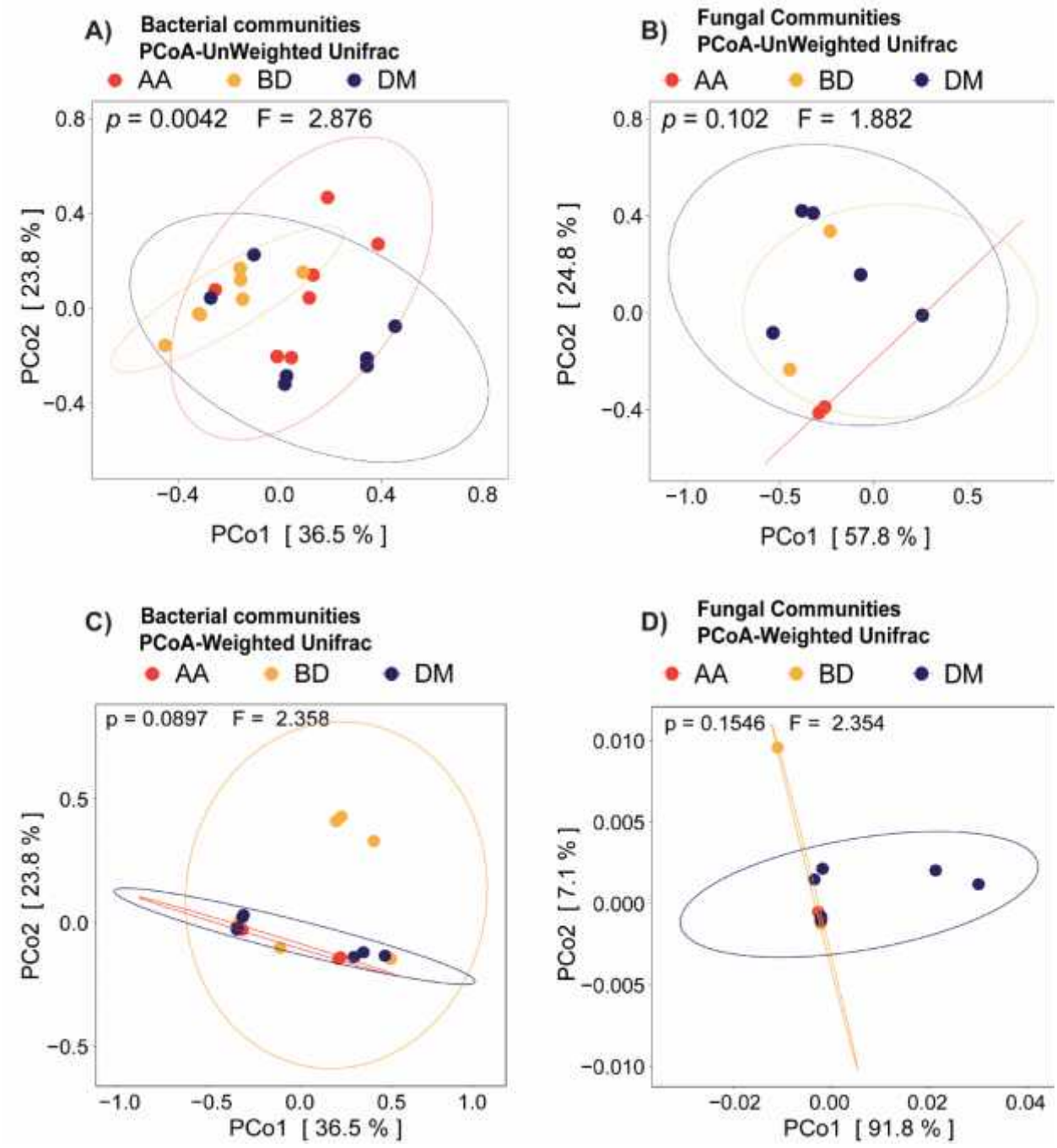

Figure 4. 9 Principal Co-ordinate Analysis of Unweighted UniFrac distance (PCoA) plots demonstrating the beta diversity of **A**) & **C**) bacterial and **B**) & **D**) fungal communities.

Thus, the bacterial communities differed only when the absence and presence of taxa were considered (Figure 4.9A). The quantitative beta diversity analysis, on the other hand, revealed that

all of the samples from any of the three areas had a roughly comparable dominant tax (Figure 4.9C & D). This finding of no significant difference in abundance-based beta diversity measurement is encouraging for the development of inoculated *Tej* fermentation processes. This is due to the fact that the majority of the quality parameters of fermented products are heavily reliant on the most abundant tax.

4.4 Conclusions

The spontaneous fermentation process that occurs during the production of traditional foods in general, and traditional beverages in particular, is neither predictable nor controllable. Thus, switching to inoculated fermentation is the only way to ensure consistent product quality while also improving process optimization and control. Ethiopian honey wine, *Tej*, is a naturally fermented alcoholic beverage made primarily of honey and gesho. However, the majority of honey could be replaced with cane sugar to lower the product's price and make it more accessible to low-income consumers. In general, *Tej* samples showed a high alcohol content, low residual sugar, and good antioxidant activity. The precise physicochemical and antioxidant activity values, on the other hand, differed between samples from the same or different areas of collection. Similarly, the structure of the bacterial community differed significantly between samples collected from different locations of Ethiopia. Despite this variation in the bacterial community, certain genera of lactic acid bacteria predominated in all of the samples. Furthermore, the genus *Saccharomyces* dominated the fungal communities of *Tej* samples. Because of the fermentative microbial dominance, all samples had a lower species richness. Meanwhile, a significant difference in beta diversity was observed in the bacterial communities of *Tej* samples. In summary, this chapter provides background information that will aid in the modernization of the current spontaneous *Tej* fermentation process. However, a better understanding of *Tej* fermentation dynamics is still needed to get a complete picture of the production process.

CHAPTER 5

Fermentation Dynamics of Spontaneously Fermented Ethiopian Honey Wine, *Tej*

Abstract

Tej, Ethiopian spontaneously fermented honey wine, is a popular alcoholic beverage. However, detailed information needed to modernize the manufacturing process of this traditional beverage has yet to be gathered. As a result, the goal of this chapter is to reveal the physicochemical and microbial dynamics that occur during the whole period of *Tej* fermentation. *Tej* samples were collected every 48 hours from three households in three different Ethiopian cities to achieve this goal. The average pH of all samples decreased from 3.84 to 3.26 during the fermentation period. whereas, the titratable acidity (TA) increased from 0.28 to 3.09 g/L on average. Similarly, during the fermentation period, the average ethanol content increased from non-detectable to 12.02 g/100 mL. The average sugar content, on the other hand, decreased as the fermentation progressed from 372.44 to 110.1 g/L. Generally, pH, glucose, and fructose content decreased as fermentation progressed, while TA and alcohol content increased. As the fermentation progressed to the final stages, antioxidant activity, increased from 27.85% to 49.97% for DPPH and 15.77% to 49.69% for ABTS assay. The antioxidant activity measured by the ABTS assay, in particular, was significantly higher than the antioxidant activity measured by the DPPH assay. Furthermore, as *Tej* fermentation progressed, the bacterial communities shifted to *Lactobacillus* dominance. Besides, *Saccharomyces* species exclusively dominated the fungal communities throughout the fermentation period. Microbial diversity also reduced toward the end of fermentation as a result of these dominances. Especially, the beta-diversity analyses of both bacterial and fungal communities moved to one direction as the fermentation gradually ended. Moreover, during *Tej* fermentation periods, quantitative increases in both microbial communities were observed. Fungal gene copy numbers, on the other hand, were half as high as bacterial gene copy numbers. Finally, the species of *Lactobacillus* and *Saccharomyces* could be used as mixed starter culture candidates in inoculated *Tej* fermentation systems.

Keywords*:* Dynamics, *Tej*, Physicochemical, Microbiota, Antioxidant

5.1 Introduction

In Ethiopia, traditional fermented beverages are produced and consumed in significant quantities at households' level (Fentie *et al.*, 2020). Ethiopian alcoholic beverages have at least the same domestic market share as commercial alcoholic beverages (Mulaw & Tesfaye, 2017). Because of the spontaneous fermentation process, these beverages frequently have acidic alcoholic properties (Nemo & Bacha, 2020). *Tej* is a popular fermented alcoholic beverage in the country that is primarily made from raw honey (Bahiru *et al.*, 2001). Approximately 80% of the honey produced in the country is used to make this beverage (Gebremedhin *et al.*, 2013). Indeed, *Saccharomyces cerevisiae*-inoculated fermentation systems have long been used in other parts of the world to produce honey wine (Balogu & Towobola, 2017). Ethiopian honey wine, on the other hand, is still made using spontaneous fermentation systems (Bahiru *et al.*, 2006).

Raw honey and "gesho" (*R. prinoides*), as well as naturally occurring microorganisms, are the basic ingredients used to make high-quality *Tej* (Lemi, 2020). he first step in making *Tej* is to mix water and honey in of 3:1 ratio. After 2–3 days of primary fermentation, the blend is filtered through a clean cheesecloth. After that, the honey water filtrate is combined with the previously boiled and cooled "gesho"(*R. prinoides*) leaves and stems. Fresh "gesho"(*R. prinoides*) leaves are now being used in some communities instead of dried and boiled leaves and stems. Some producers also add a small amount of malt powder to the boiled "gesho" (*R. prinoides*) leaves (Nemo & Bacha, 2021). This mixture, regardless of the ingredients, goes through a secondary fermentation for at least 8–21 days. After the secondary fermentation period is completed, this mixture is filtered and served to the consumer as the finished product, *Tej*.

Excellent quality *Tej* has a cloudy appearance, a yellow color, and a sweet flavor (Vogel & Gobezie, 1995). Unfortunately, due to the spontaneous nature of fermentation, it is often difficult to achieve these key quality characteristics (Lemi, 2020). To achieve consistent product quality, the natural fermentation process must be replaced with inoculation fermentation system (Steinkraus, 2004), so getting the knowledge on the dynamic behaviors of the system is critical to achieve the aforementioned objective. Thus, understanding the dynamics of microbial communities and exometabolomic profiles will undoubtedly aid in gaining a complete picture of any spontaneous fermentation system (Tamang & Thapa, 2006).

Previous research on *Tej* had solely focused on the final product's characterization. Bahiru *et al.* (2001) investigated the nutritional and physicochemical quality of *Tej* samples that was collected from Addis Ababa, Ethiopia. The microbiological profiling of *Tej* samples collected from the same area was done by the same authors, Bahiru *et al.*, (2006). Similarly, Yohannes *et al.*, (2013) investigated the physicochemical properties of *Tej* alongside with other traditional fermented beverage samples collected from Jimma, Ethiopia. Recently, Nemo & Bacha (2020) conducted a similar microbial count and physicochemical analysis of *Tej* and other traditional alcoholic beverage samples collected from Jimma district. In general, these studies revealed that the physicochemical properties of the samples collected from various households and areas differed significantly. Nonetheless, during the fermentation process, the final product is the result of various dynamic interactions among microbes and produced metabolic products (Weckx *et al.*, 2010). As a result, characterization of the final product alone does not reveal the entire story of the spontaneous fermentation system. To our knowledge, there is a paucity of information in the literature specifically about *Tej* fermentation dynamics. To fill this gap, we looked at changes in antioxidant activities, physicochemical properties, and microbial community dynamics over time during the natural fermentation of Ethiopian honey wine, *Tej*.

5.2 Materials and Methods

5.2.1 Sample collection and transportation

Sample collection, transportation was done according to the methods described in section 3.2

5.2.2 Physicochemical analysis

5.2.2.1 pH

pH measurements were performed according to the methods described in section 3.3.1

5.2.2.2 Titratable acidity (TA)

TA measurements were performed according to the methods described in section 3.3.2

5.2.2.3 Sugar profiling

Sugar profiling was done by using the method described in section 3.3.3

5.2.2.4 Ethanol quantification

Ethanol quantification were performed according to the methods described in section 3.3.4

5.2.3 Antioxidant activity

5.2.3.1 DPPH assay

DPPH assay was performed by following the methods described in section 4.2.4.1

5.2.3.2 ABTS assay

ABTS assay was done by using the method briefly described in section 4.2.4.2

5.2.4 Microbiome analysis

5.2.4.1 DNA extraction

DNA was extracted by exact method which is described in section 4.2.5.1

5.2.4.2 16S rRNA sequencing

16SrRNA sequencing was done according to the methods described in section 4.2.5.2

5.2.4.3 Internal transcribed spacer (ITS) sequencing

ITS region amplification and sequencing were done according to the method described in section 4.2.5.3

5.2.4.4 Quantitative PCR

With minor modifications, quantitative PCR (qPCR) was carried out according to (Park *et al.*, (2009). First, Escherichia coli DH5α was renovated using the one copy 16SrDNA, and ITS fragment pTOP TA V2 cloned vector. Following the manufacturer's instructions, the plasmid DNA was then extracted using the GeneJET Plasmid Miniprep kit (Thermo Scientific, USA). The concentrations of this DNA were then measured using a Qubit 2.0 fluorometer (Life Technologies) after it was serially diluted from 10^{-1} to 10^{-9}. A 10 µL PCR reaction mixture composed from 1 µL DNA template, 5 µL MG 2X qPCR Master mix (SYBR Green), 0.3 µL universal forward and reverse primer (Table 6.1), and 3.4 µL sterile diluted water. The following qPCR conditions were used; initial denaturation 95°C for 5 min followed by 40 cycles of denaturation 95°C for 30 s, annealing 57°C (bacteria)/62°C(fungi) for 30 s, and final extension 72°C for 30 s. The standard curves were then generated for both serially diluted bacterial and fungi plasmid DNA. Finally, the gene copy numbers of samples bacteria and fungi DNA concentrations were calculated by interpolation from the generated standard curve.

Table 5. 1 List of primers used for amplicon sequencing and quantitative real-time PCR (qRT–PCR) amplification

Primers purpose	Target	Designation	Sequence (5 to 3)	References
Amplicon sequencing	Bacteria	515F 907R	GTGNCAGCMGCCGCGGTAA CCGYCAATTYMTTTRAGTTT	(Parada et al., 2016)
	Fungi	ITS1	CTTGGTCATTTAGAGGAAGTA	(Gardes & Bruns, 1993)
		ITS2	GTGAATCATCGAATCTTTGAA	(Turenne et al., 1999)
qRT–PCR amplification	Bacteria	Eub338	ACTCCTACGGGAGGCAGCAG	(Lane, 1991)
		Eub518	ATTACCGCGGCTGCTGG	(Muyzer et al., 1993)
	Fungi	Fu18S1	GGAAACTCACCAGGTCCAGA	(Borneman & Hartin, 2000)
		Nussu1536	ATTGCAATGCYCTATCCCCA	

5.2.4.5 Bioinformatics analysis

Bioinformatic analysis were performed by using the method described in section 4.2.5.4

5.2.5 Statistical analysis

Statistical analysis was also performed according to the methods described in section 4.2.6

5.3 Results and Discussion

5.3.1 Physicochemical property dynamics

Acidity is one of the important physicochemical properties to monitor during the production of beverages, especially alcoholic beverages (Holzapfel, 2015). The sensory attributes, overall fermentation process, and shelf-stability of the beverages are all influenced by this parameter (Tôrres *et al.*, 2011) Regardless of the households from which samples were taken, as fermentation progressed to the end, the acidity of all *Tej* samples increased significantly ($P < 0.05$), with mean pH values falling from 3.58 – 2.98, 3.78 – 3.1, and 4.16 – 3.7 for AA, BD, and DM samples, respectively (Figure 5.1A). Similarly, as fermentation progressed, the mean TA for BD, AA, and DM samples increased from 0.23 – 4.01, 0.23 – 3.00, and 0.38 – 2.25, respectively (Figure 5.1B). The continuous production of organic acids, particularly lactic acid formation from *Lactobacillus*, was the main source of increase in the acidity levels during *Tej* fermentation system (Chidi *et al.*, 2018). During laboratory-scale *Tej* fermentation, a similar increase in acidity was also observed (Nemo & Bacha, 2021). *Lactobacillus* species produce lactic acid, which results in a steeper gradient of acidity increase, especially at the start of fermentation (Figure 5.1A and B). Moreover, honey's

lower buffering capacity may aggravate the larger acidity gradient especially at the start of fermentation, even if the organic acid concentration is lower (Tôrres *et al.*, 2011). Although all *Tej* samples showed a similar trend of increasing acidity, there was a significant ($P < 0.05$) difference in the final pH and TA values for samples collected from various households. This was especially true, for the samples collected from households in the DM area (Figure 5.1A and B). Variations in processing conditions, raw materials, and microbial community structure and quantities during spontaneous fermentations may have contributed to this.

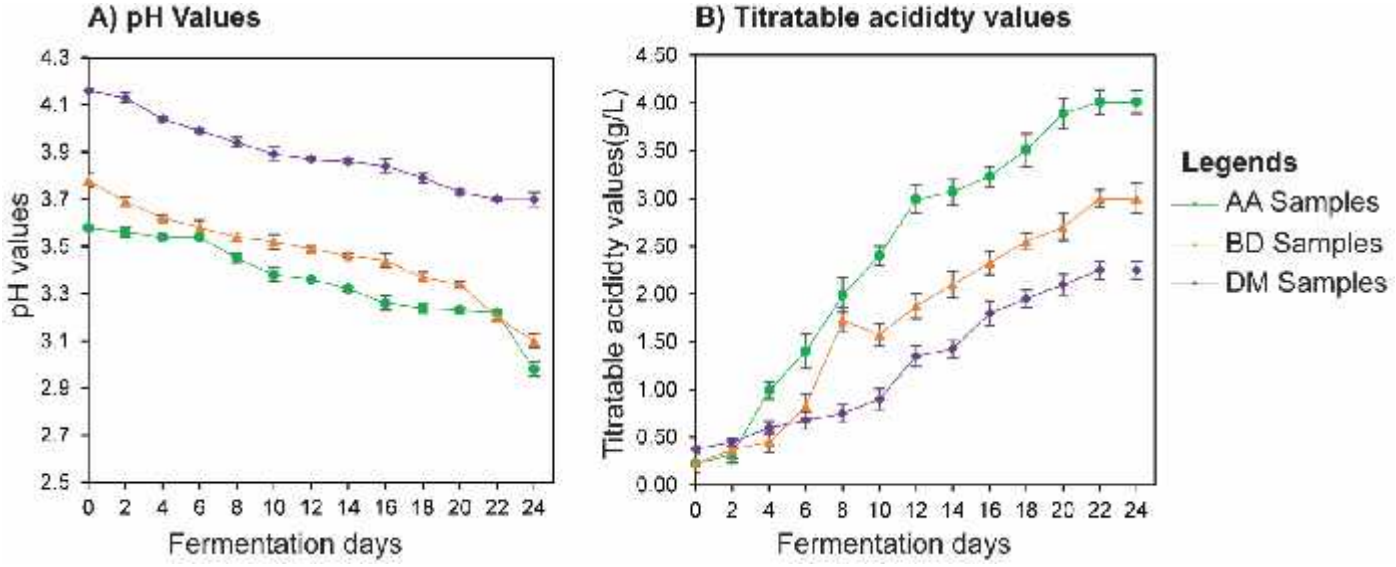

Figure 5. 1 Progressive changes in **A**) pH, and **B**) Titratable acidity during the spontaneous fermentation of *Tej*. Each dot in the line graph represents pH and Titratable acidity values from the three study area locations.

Throughout the entire *Tej* fermentation period, the fermentation medium was dominated by simple sugars of glucose and fructose (Figure 5.2A and B). Even at the start of the fermentation, the levels of sucrose in all samples were below detectable limits. Mature honey, which is usually used as the raw material for *Tej*, could be the primary cause of the low sucrose levels, as it will eventually turn to simple sugar as it ages (Belay *et al.*, 2017). During the fermentation period, mean glucose levels in the BD, AA, and DM samples decreased significantly ($P < 0.05$) from 167.22 – 55.98 g/L, 146.4 – 44.26 g/L, and 159.6 – 34.1 g/L, respectively (Figure 6.2A). Similarly, all through fermentation, average fructose levels in BD, AA, and DM household samples reduced significantly ($P < 0.05$) from 205.22 – 102 g/L, 204.84 – 84 g/L, and 191.94 – 76 g/L, respectively (Figure 5.2C). Microbial growth and exo-metabolite production are primarily reason for the decreased sugar level (Pereira *et al.*, 2017). However, glucose uptake was much faster than fructose consumption rate at

the start of the fermentation (Figure 5.2A and B), 2- and 3-fold decreases were observed in fructose, and glucose, respectively. It's possible that the fermentative microorganisms involved in *Tej* fermentation prefer glucose over fructose, which could explain this result. Similar fructose and glucose uptake rates were observed for honey wine fermentation with different di ammonium phosphate supplement concentrations (Cristian *et al.*, 2015). After day 16, glucose consumption rates in our study decreased and then plateaued (Figure 5.2A and B). This could have been due to the negative effects of fermentative microbes' primary and secondary metabolites (Iglesias *et al.*, 2014). The increase in ethanol content, which is a known microbial growth inhibitor, may have played a major role in the outcome. Furthermore, in terms of household comparisons, BD samples consumed more sugar at a faster rate, whereas DM samples consumed sugar at a slower rate throughout the fermentation period (Figure 5.2A and B). The difference in microbial community structure, which is inherited from spontaneous *Tej* fermentation, was the primary cause for this household differences (Marshall & Mejía-Loríio, 2011).

The main goal of any alcoholic beverage fermentation is to use fermentative microorganisms to convert available sugars to ethanol (Buglass, 2010). To achieve this phenomenon, intrinsic biochemical reactions is required with a favorable environment (Steinkraus, 2004). A carbon source like honey, which has a lower buffer capacity, low pH, deficiencies in essential nutrients, and high osmotic pressure may exacerbate the situation (Pereira *et al.*, 2017). However, all of the *Tej* samples in this study showed a progressive alcohol level increase, especially after the eighth fermentation day (Figure 5.2C). The extended lag growth phase of microbial growth may have contributed to the late start in alcohol production (Aranda *et al.*, 2019). After 24 days of fermentation, BD samples had shown a maximum alcohol content of 12.02 ± 0.02 g/100 mL; additionally, the alcohol production rate during the fermentation period was also higher for this sample (Figure 5.2C). *Lactobacillus*, *Zymomonas*, and *Saccharomyces* dominated this sample, which may have contributed to this finding (Figure 6.4). However, after the twentieth day of fermentation, all samples' ethanol production decreased, then plateaued (Figure 5.2C). This type of result could be caused by the death and/or inactivation of microbes at this stage of fermentation (Martínez-Moreno *et al.*, 2012).

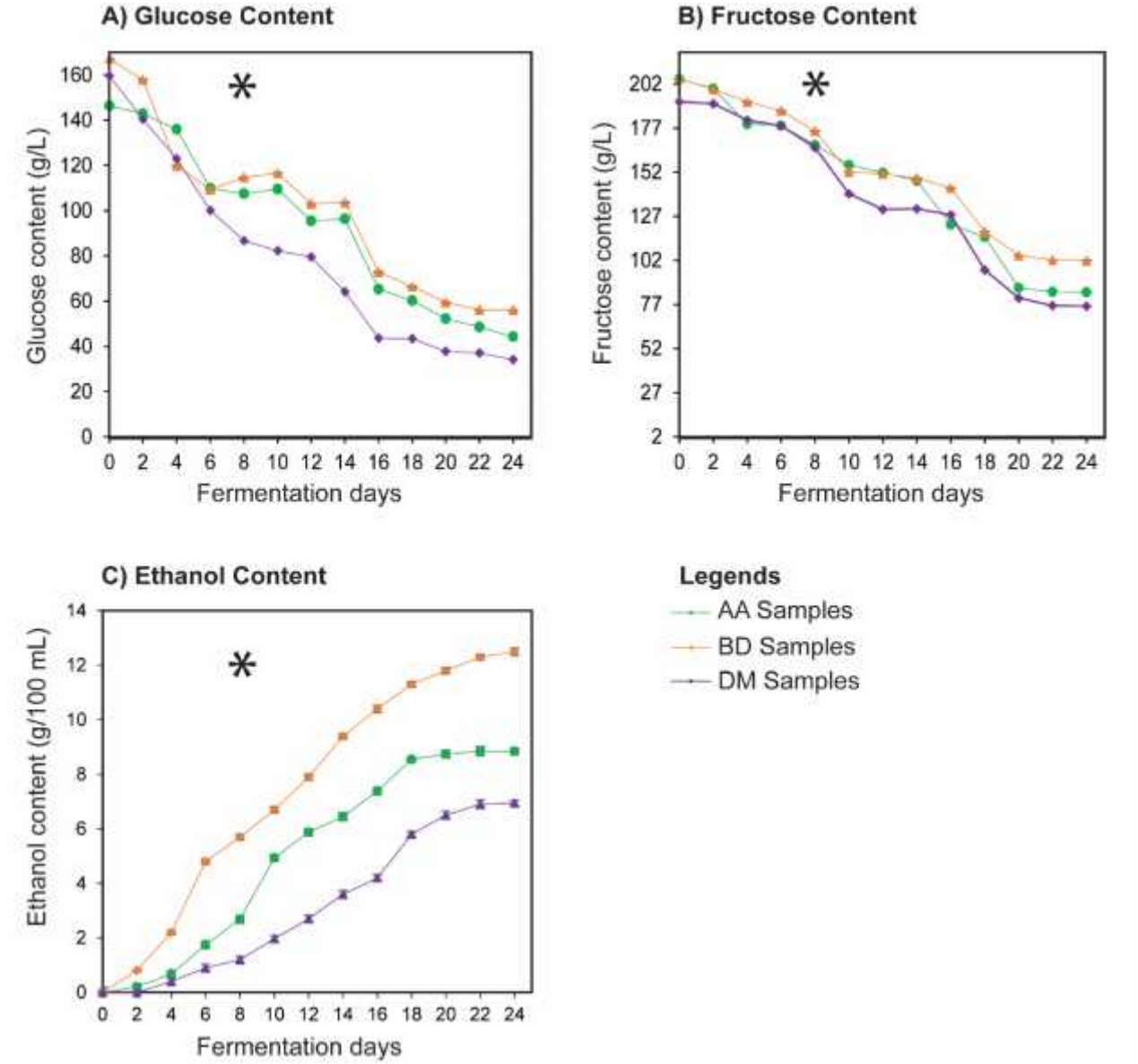

Figure 5. 2 Substrate consumption and product formation dynamics during spontaneous *Tej* fermentation. Each dot in the line graph represents **A**) glucose, **B**) fructose, and **C**) ethanol levels from the three study area locations. The stars (*) in the figure is to indicate the time at which secondary fermentation began.

5.3.2 Antioxidant activity dynamics

In this particular study, both colorimetric antioxidant assays, DPPH and ABTS (+), were used to track the development of *Tej's* functional properties. Cumulative antioxidant activities for ABTS and DPPH assays increased significantly ($P < 0.05$) during fermentation periods, rising from 15.77 ± 2.18 – 49.69 ± 1.06% for ABTS, and 27.85 ± 2.19% to 49.97 ± 1.06 % for DPPH (Figure 6.3A and B). Honey contains antioxidant activity in the form of *β*-carotene, ascorbic acid, *α*-tocopherol,

catalase, and peroxides (Chua *et al.*, 2013). Furthermore, Chen *et al.*, (2020) found that "gesho" (*R. prinoides*) has a high antioxidant capacity. As a result, these two raw materials could be the important sources of antioxidant activity in *Tej* samples. Between days 3 and 12 of the fermentation period, *Tej* antioxidant capacity increased rapidly (Figure 5.3). This was most likely due to bioactive compounds being extracted from "gesho" (*R. prinoides*). Both antioxidant activity assays showed nearly constant values after 12 days, until the last day of fermentation (Figure 5.3). Cornelian cherry fruit juice supplemented honey wine had similar antioxidant capacity results previously (Adamenko *et al.*, 2018).

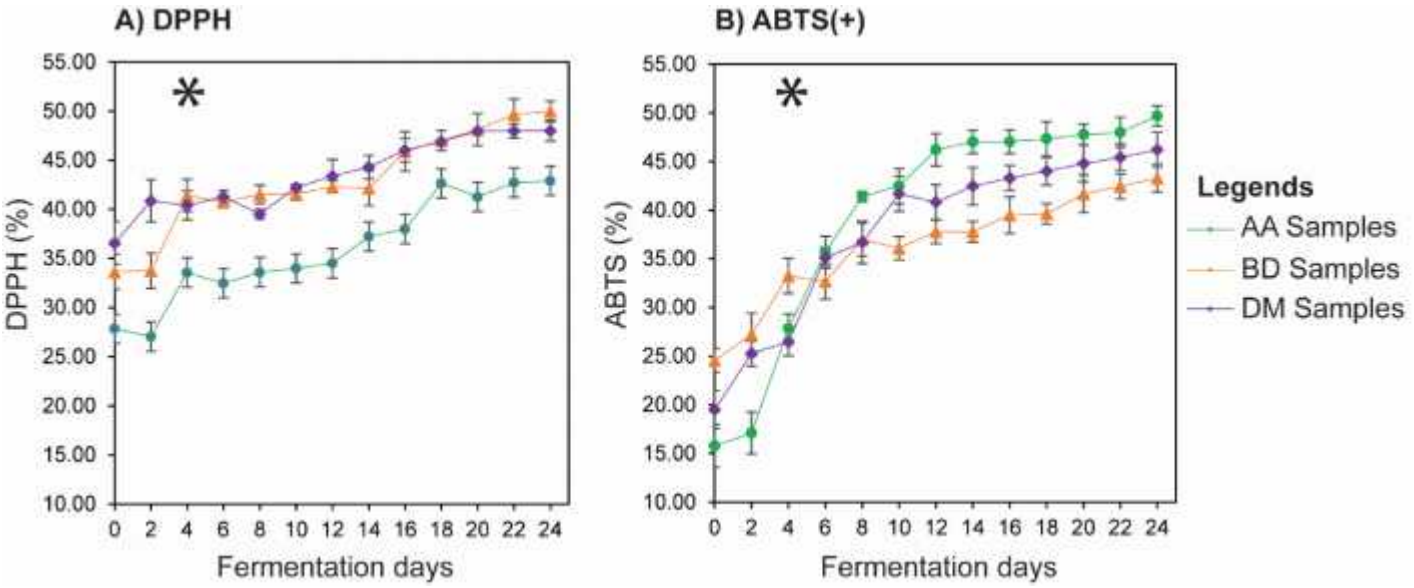

Figure 5. 3 Antioxidant activity dynamics during spontaneous *Tej* fermentation. Each dot in the line graph represents **A**) DPPH, and **B**) ABTS (+) values from the three study area locations. The stars (*) in the figure is to indicate the time at which gesho is added to the honey-water mixture.

The DPPH and ABTS values during *Tej* fermentation showed a gentle and a strong increment, respectively (Figure 5.3). The lower water solubility of DPPH radicals may have contributed for this variation (Arnao, 2001). Meanwhile, time-dependent antioxidant activity differences were observed in *Tej* samples from various households (Figure 5.3). For example, DM samples showed a significant increase ($P < 0.05$) in ABTS levels after the third day of fermentation. As a result, the way "gesho" (*R. prinoides*) was added to the honey-must mixture, different floral sources, environmental factors, and processing conditions may have all played a role in the variations in antioxidant capacity found at households' level (Akalın *et al.*, 2017).

5.3.3 Microbial community dynamics

Certain bacterial and fungal species dominated the microbial community structures in *Tej* fermentations (Figure 5.4). Continuous microbial metabolite production, high sugar levels, microbe competition, and anaerobic conditions during fermentation processes may have all played a role in this dominance (Holzapfel, 2015). Thus, as fermentation progressed to the end, particular group of microorganisms that can withstanding the competition between microorganisms and unfavorable conditions will dominate and survive till the end of fermentation.

Firmicutes and *Proteobacteria* phyla dominated bacterial communities during *Tej* fermentation (Figure 5.4A). *Firmicutes* were by far the most common phylum (> 80%), especially near the end of fermentation. Proteobacteria was the next most abundant phylum, especially during the early and/or late stages of fermentation (Figure 5.4A). *Lactobacillus*, *Clostridium_sensu_stricto_1*, *Paeniclostridium*, *Romboutsia*, *Peptostreptococcaceae*, *Paenibacillus*, *Lachnospiraceae*, *Bacillus*, *Enterococcaceae*, and *Oceanobacillus* were the some of the observed genera and families and under the phyla of *Firmicutes*. Similarly, *Zymomonas*, *Pseudomonas*, *Acetobacter*, *Enterobacteriaceae*, and *Comamonadaceae* were the observed families and genera under the phyla of *Proteobacteria* (Figure 5.4A). In actual fact, these two phyla are the most likely bacterial phyla that could be observed in fermented beverages with the expected differences in their relative abundance (Cason *et al.*, 2020). Other traditional alcoholic beverages have reported a similar finding (Diaz *et al.*, 2019).

Throughout the fermentation period, the *Ascomycota* phylum dominated all fungal communities. The genera *Saccharomyces*, *Candida*, *Torulaspora*, and *Zygosaccharomyces* were found under the *Ascomycota* phyla (Figure 5.4C). This phylum contains the most diverse and numerous fungal species (Taylor *et al.*, 2015). In Indian traditional alcoholic beverage starters, there was a similar *Ascomycota* dominance (Sha *et al.*, 2019).

During the *Tej* fermentation, *Lactobacillus* was the dominate bacterial genus, especially near the end of fermentation period (Figure 5.4A). The relative abundance of this species increased from 24% to 100%. *Lactobacillus* species have a high capacity for producing compounds that aid in the development of distinct flavors in alcoholic beverages (Fuochi *et al.*, 2019). Although *Lactobacillus* was found in a higher abundance in all of the samples, the increment patterns varied among the household samples (Figure 5.4A). *Lactobacillus* is also said to have dominated the final fermented

Tej product as well as the Korean traditional beverage, *Shindari* (Bahiru *et al.*, 2006a; Cha *et al.*, 2014).

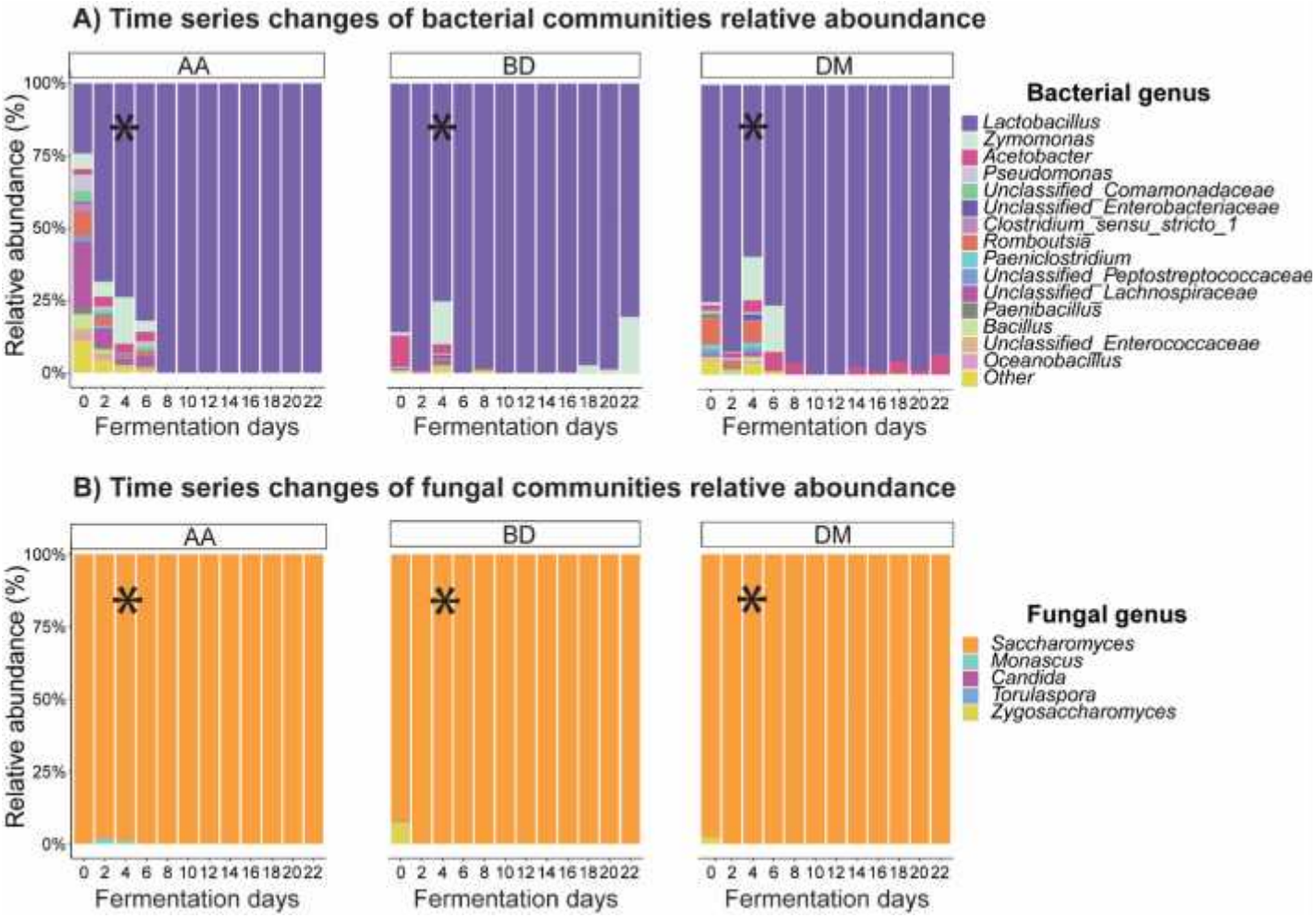

Figure 5. 4 Time series changes of relative abundance of **A**) bacteria, and **B**) fungal communities during *Tej* fermentation period. The stars (*) in the figure is to indicate the time at which gesho is added to the honey-water mixture.

Zymomonas was one of the frequently observed genera during *Tej* fermentation period (Figure 5.4A). This genus had a higher relative abundance on day four of fermentation, which could have been due to the addition of "gesho" (*R. prinoides*) on day three. However, by the tenth day of fermentation, BD samples had a 19.4% relative abundance of *Zymomonas*, whereas AA and DM samples were dominated exclusively by *Lactobacillus* (Figure 5.4A). Indeed, species of this genus, particularly *Zymomonas mobilis*, are commonly found in most of African traditional alcoholic beverages (Diaz *et al.*, 2019). This may be due to the species' ability to produce higher levels of alcohol from mono- and disaccharide sugars (He *et al.*, 2014).

During *Tej* fermentation period, the *Acetobacter* genus was discovered at the start of the fermentation, on day 4, and/or at the end of the fermentation (Figure 5.4A). In the presence of oxygen, this bacterium converts ethanol to acetic acid (Bartowsky *et al.*, 2003). During fermentation periods, the relative abundance of this genus decreased from 10.8 – 0.1%, 1.0 – 0.0%, and 4.0 – 1.0% in BD, AA, and DM samples, respectively (Figure 5.4A). These reductions in relative abundance were most likely caused by a lack of ethanol and oxygen at the start and during the fermentation periods, respectively (Motlhanka *et al.*, 2018). Competition from fermentative microorganisms may also have played a role in this outcome. These decreases also implied that contamination could have occurred during the preparation and/or addition of raw materials.

Pathogenic *Enterobacteriaceae* and *Pseudomonas* microbes were also observed at the raw material addition step during Tej making process (Figure 5.4A). After day six of fermentation, however, they were not found in any of the samples. This could be explained by the unfavorable conditions created by the metabolic products of dominant fermentative microorganisms. A similar decrease in pathogenic microorganisms was also observed during the *Tej* fermentation period by applying the traditional microbial plating techniques (Nemo & Bacha, 2021).

Throughout the *Tej* fermentation period, *Saccharomyces* was the most prevalent fungal genus (Figure 5.4B). In fact, this genus is the main microbe responsible for sugar to ethanol conversion. As a result, the predominance of *Saccharomyces* during alcoholic beverage fermentation is a common occurrence during successful alcoholic fermentation (Walker & Stewart, 2016a). Other *Zygosaccharomyces*, *Monascus*, *Torulaspora* and *Candida* species were detected at low relative abundance levels early in the fermentation process, but *Saccharomyces* eventually took over the dominance (Figure 5.4B). During the spontaneous fermentation of Catalanesca grapes, a similar Saccharomyces dominant status was observed (Di Maro *et al.*, 2007).

To sum up, *Saccharomyces* and *Lactobacillus* were the predominant genera encountered during the whole *Tej* fermentation period. The microbiota of traditional wineries in each household is thought to play a significant role in the exclusive dominance of certain microbes. Both genera are the most common microbes found in a variety of naturally fermented alcoholic beverages (Jung, *et al.*, 2012). Furthermore, the majority of species in these genera fall into the "generally recognized as safe (GRAS)" category (Vida-Plavec & Berlec, 2020).

5.3.4 Microbial diversity dynamics

To characterize microbiome diversity changes during *Tej* fermentation, bacterial and fungal α-diversity analyses were performed. For all household samples, the α-diversity indices Chao1, Shannon, InvSimpson, Evenness, and Simpson were calculated (Table 5.2, Figure 5.5A and B). The Chao1 index was used to show that species richness occurred during the fermentation of *Tej*. As the fermentation neared completion, both bacterial and fungal community richness had decreased, regardless of collection households (Table 5.2). The dominance of specific microbes could be the primary cause of the Chao1 index's steady decline. The Shannon and Simpson indices were also used to examine changes in microbial diversity in bacteria and fungi communities (Table 5.2, Figure 5.5A and B). As the fermentation progressed to the end, the bacterial and fungal community richness decreased gradually and sharply, respectively.

On the fourth day of fermentation, however, there was a noticeable increase in bacterial community diversity. This observation could have been caused by the addition of "gesho" (*R. prinoides*) and anaerobic fermentation conditions. Evenness was also used to show the number of different species in a given sample (Haig *et al.*, 2015). During the *Tej* fermentation process, both bacterial and fungal communities showed less evenness. The dominance of *Lactobacillus* and *Saccharomyces* as fermentation advanced to the end may have contributed to this lower evenness value.

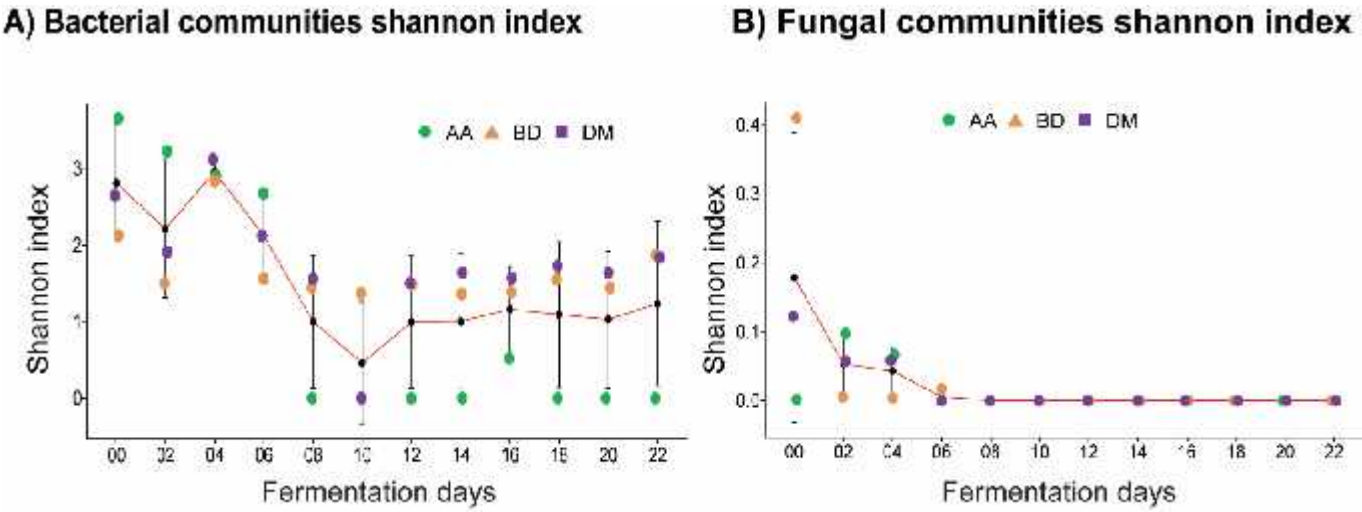

Figure 5. 5 The shannon index for **A**) bacterial, and **B**) fungal communities during the *Tej* fermentation period. Each dotes on the graph represent the *Tej* samples collected from respective study areas.

Unweighted UniFrac Principal Co-ordinate Analysis (PCoA) plot was used to demonstrate the β–diversity of microbial communities occurred during the whole *Tej* fermentation period (Figure 5.6 A and B). The plot basically shows the level of difference in microbial tax for the *Tej* samples collected from those three households during the fermentation period. As the fermentation period progressed, the PCoA plot for bacterial communities revealed a visible cluster and a shift in one direction. As the samples were compared based on the fermentation time, a visible cluster distance ($P < 0.0025$) with a cumulative variance of 77% was observed for bacterial communities (Figure 5.6A). This is because, as the fermentation period progressed to the end, the *Lactobacillus* genera dominated the bacterial communities of the fermentation medium.

Table 5. 2 Alpha diversity indices of time dependent *Tej* samples collected from three households

Bacterial communities' alpha diversity													
Indices	**Locations**	**Fermentation days**											
		0	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**16**	**18**	**20**	**22**
Chao1	AA	58	66	53	30	1	1	1	1	2	1	1	1
	BD	40	14	44	13	16	14	13	13	14	19	14	15
	DM	57	24	41	20	10	1	10	14	15	18	10	15
Evenness	AA	0.90	0.77	0.73	0.79	NA	NA	NA	NA	0.75	NA	NA	NA
	BD	0.58	0.57	0.75	0.61	0.52	0.52	0.58	0.53	0.53	0.53	0.55	0.69
	DM	0.65	0.60	0.84	0.71	0.68	NA	0.65	0.62	0.58	0.60	0.71	0.68
Simpson	AA	0.96	0.93	0.92	0.89	0.00	0.00	0.00	0.00	0.34	0.00	0.00	0.00
	BD	0.83	0.74	0.91	0.76	0.72	0.70	0.74	0.70	0.71	0.73	0.72	0.81
	DM	0.86	0.79	0.93	0.83	0.74	0.00	0.73	0.76	0.75	0.77	0.76	0.79
InvSimpson	AA	27.83	13.82	12.67	9.51	1.00	1.00	1.00	1.00	1.51	1.00	1.00	1.00
	BD	5.79	3.82	11.65	4.23	3.57	3.38	3.79	3.37	3.42	3.72	3.52	5.17
	DM	7.38	4.69	13.99	5.74	3.83	1.00	3.76	4.19	4.01	4.28	4.19	4.69
Fungal communities' alpha diversity													
Indices	**Locations**	**Fermentation days**											
		0	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**16**	**18**	**20**	**22**
Chao1	AA	3	3	3	1	2	1	1	1	1	1	1	1
	BD	11	4	2	5	1	1	1	1	1	1	1	1
	DM	4	3	2	1	1	1	1	1	1	1	1	1
Evenness	AA	0.00	0.09	0.06	NA	0	NA	NA	NA	NA	NA	NA	NA
	BD	0.17	0.00	0.01	0.01	NA	NA	NA	NA	NA	NA	NA	NA
	DM	0.09	0.05	0.08	NA	NA	NA	NA	NA	NA	NA	NA	NA
Simpson	AA	0.00	0.04	0.02	0.00	0	0	0	0	0	0	0	0
	BD	0.15	0.00	0.00	0.00	0	0	0	0	0	0	0	0
	DM	0.04	0.02	0.02	0.00	0	0	0	0	0	0	0	0
InvSimpson	AA	1.00	1.04	1.02	1.00	1	1	1	1	1	1	1	1
	BD	1.18	1.00	1.00	1.00	1	1	1	1	1	1	1	1
	DM	1.05	1.02	1.02	1.00	1	1	1	1	1	1	1	1

AA – Addis Ababa, BD – Bahir Dar, DM – Debre Markos

Furthermore, AA and DM household samples had shown a visible cluster of distance that differed from BD household samples. In the PCoA plot, fungal communities also showed a visible cluster distance ($P < 0.0146$), with a cumulative variance of 95.6% for a time series of samples during *Tej* fermentation (Figure 5.6B). As the fermentation period came to an end, fungal communities, like bacterial communities, were moving in one direction. This specific observation was largely influenced by the dominant *Saccharomyces*. Similarly, for qualitative analysis of fungal taxes based on household comparisons, there was no discernible cluster of distance. In general, the time-dependent β-diversity analyses revealed that as the fermentation progressed, both the bacterial and fungal communities moved in the same direction (Figure 5.6A and B).

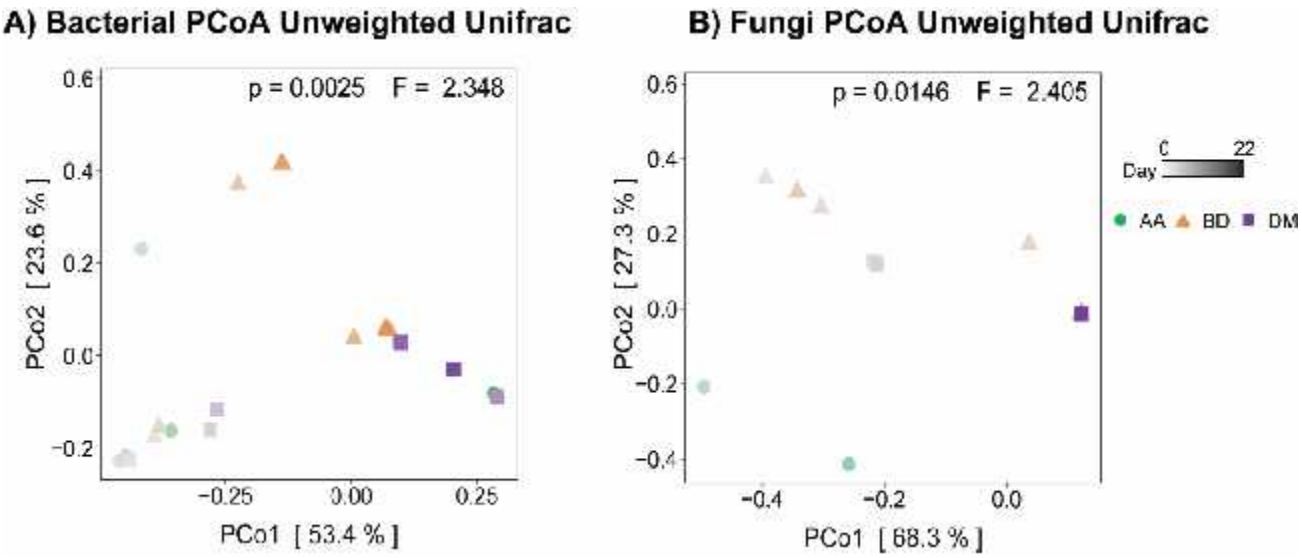

Figure 5. 6 Principal coordinate analysis (PCoA) of Unweighted Unifrac distances demonstrated β-diversity for **A**) bacterial, and **B**) fungal communities during *Tej* fermentation periods.

5.3.3 Quantitative microbial dynamics

To get a complete picture of microbial kinetics during spontaneous fermentation, qualitative analysis of microbial community structures must be accompanied by quantitative analyses (Kim *et al.*, 2015). qPCR was used in this study to determine the absolute quantification of total bacteria and yeast during *Tej* fermentation period. As it is demonstrated in Figure 5.7A & B, based on the thrashhold line, almost all of the sample had been amplified well during the qPCR running test. In a qualitative assay, the crossing of this statistical noise threshold is used to call a sample positive, and the cycle number at which it occurs is used to generate a standard curve and quantify the starting template in

a quantitative PCR. Similarly, the melting curve for both bacterial and fungi DNA showed a positive result in amplification of the targeted region using the selected primers (Figure 5.7C & D). For fungi, a standard curve with the equation Y = 3.5261X + 4.7208, R = 0.9954 was developed, and for bacteria, a standard curve with the equation Y = 2.267X + 12.951, R = 0.9877 was developed (Y = cycle threshold; X = log10 DNA concentration at ng/L).

Total bacteria and fungi increased in number during fermentations (Figure 5.8). For BD, AA, and DM household samples, total bacteria gene copy numbers increased from 2.53×10^5–1.53×10^{10}, 2.92×10^7–1.25×10^{10}, and 2.56×10^5–7.5×10^9 ng/L, respectively. Similarly, for BD, AA, and DM household samples, total fungi gene copy numbers increased from 4.29×10^6–2.68×10^{10}, 9.5×10^6–1.91×10^{10}, and 4.16×10^6–1.1×10^{10} ng/L, respectively (Figure 5.7). These sharp increases in total bacterial and fungal gene copy numbers were fueled by the growth of *Lactobacillus* and *Saccharomyces* fermentative microbes (Figure 5.4). Previous research on *Tej* fermentation dynamics revealed similar increases in lactic acid bacteria and yeast, ranging from 4.01 – 8.88 log CFU/mL and 4.16 – 9.41 log CFU/mL, respectively, during Tej fermentations. (Nemo & Bacha, 2021).

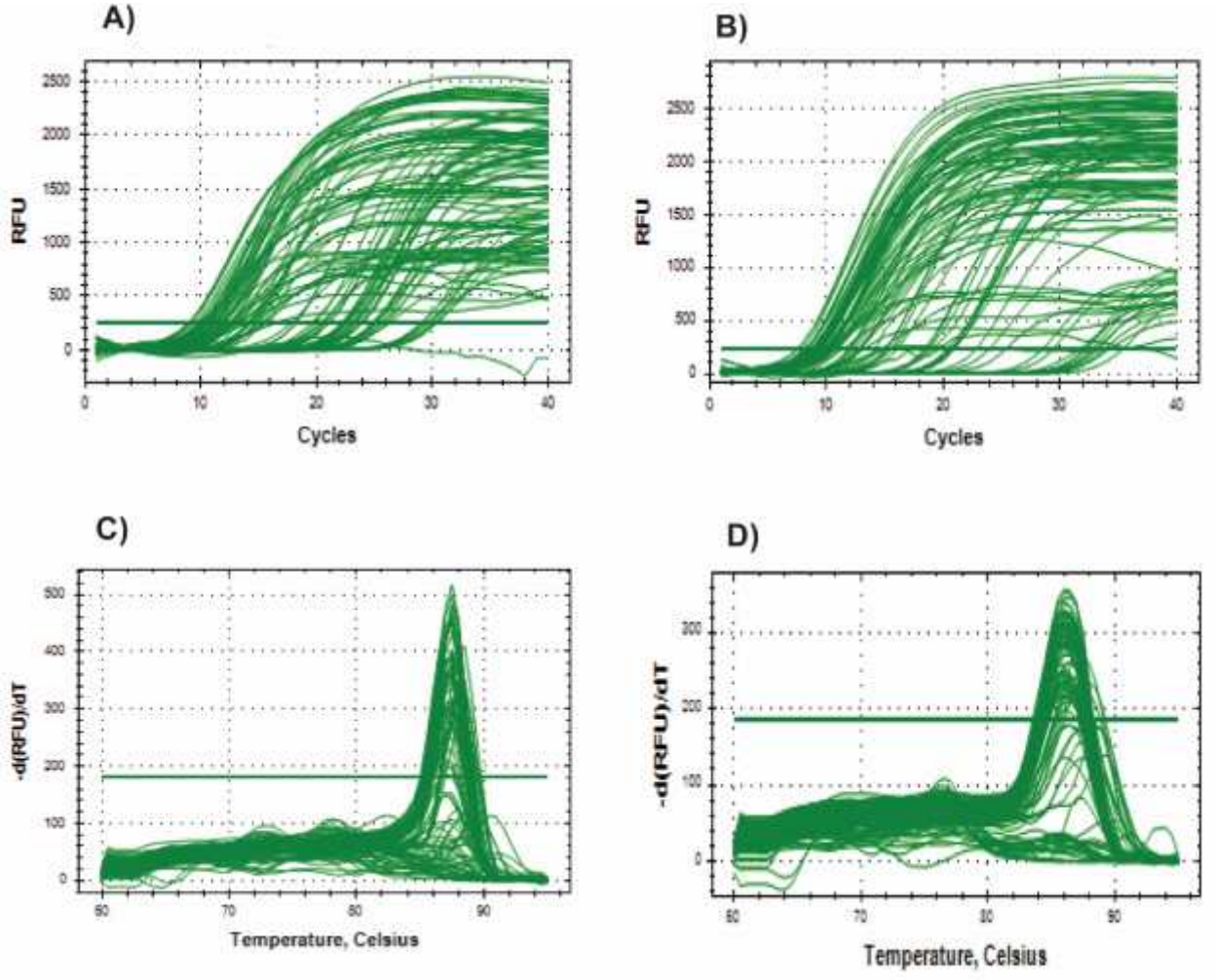

Figure 5. 7 qPCR amplification curve of **A**) bacteria, **B**) fungi, and qPCR melting curve of **C**) bacteria, **D**) fungi

The gene copy numbers of fungi and bacteria were nearly constant across all household samples after tenth day of fermentation (Figure 5.8). This situation most likely reflected microbial flora stationary growth phases; microbes may have entered this phase due to a lack of essential nutrients, increased cell-to-cell interactions, and toxic metabolite accumulation (Jaishankar & Srivastava, 2017). During the fermentation of Korean kimchi, a similar pattern was also observed for bacteria and fungi gene copy numbers (Park *et al.*, 2009). During the stationary phases of our study, the bacterial to fungal ratios for AA, BD, and DM household samples were 0.6, 0.5, and 0.7, respectively (Figure 5.8).

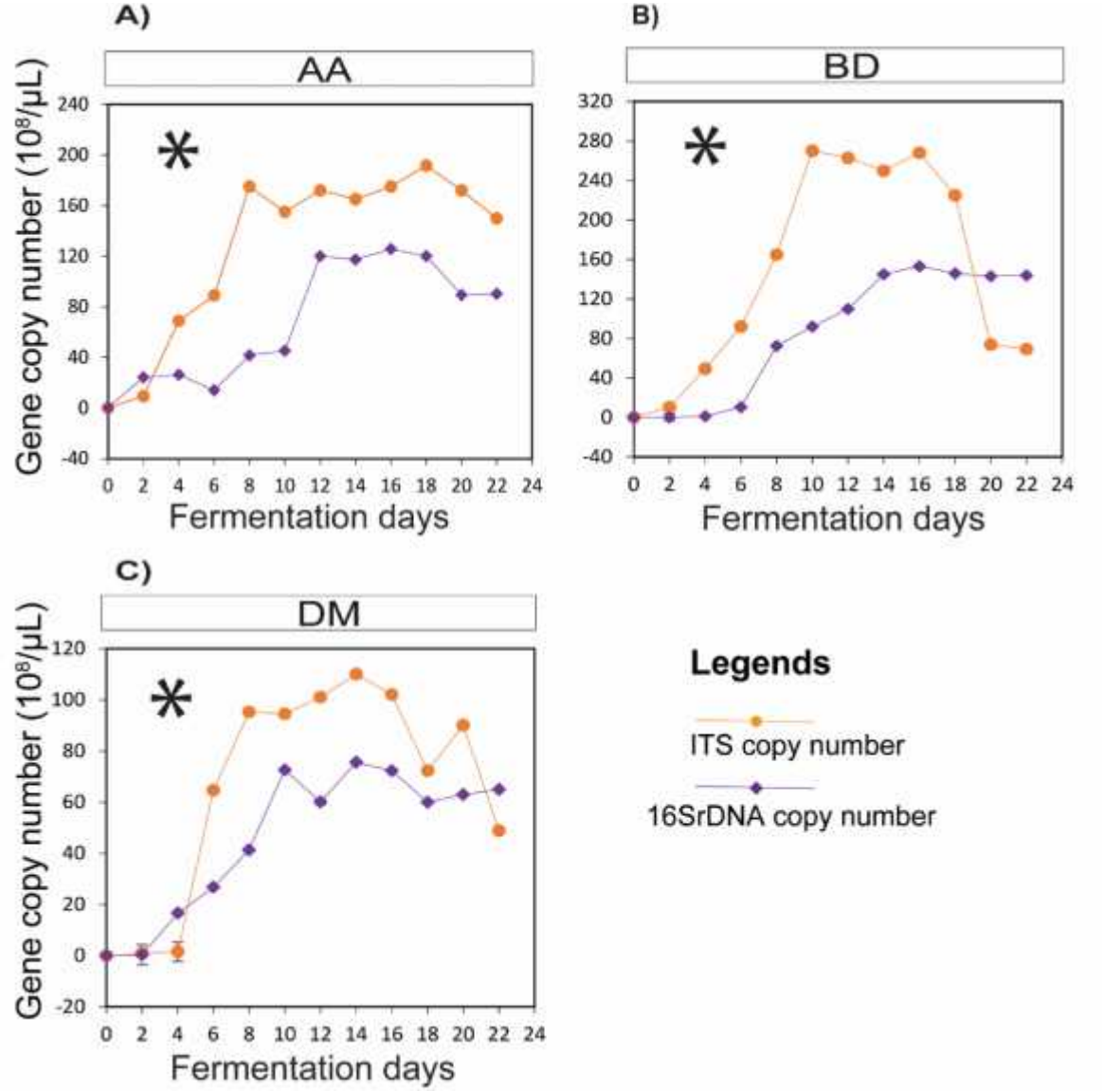

Figure 5. 8 Time series changes of bacterial and fungal gene copy number for the *Tej* samples collected from **A**) AA, **B**) BD, and **C**) DM areas. The stars (*) in the figure is to indicate the time at which gesho is added to the honey-water mixture.

In general, the spontaneous nature of fermentation processes most likely caused a small quantitative variation in different household samples. Despite this, qualitative and quantitative data revealed that the *Lactobacillus* and *Saccharomyces* genera were compatible and performed well during successful *Tej* fermentation processes (Figure 5.4). Furthermore, bacteria-to-fungus ratios revealed that bacterial levels were nearly 50% lower than fungal levels. *Saccharomyces* may have grown faster than *Lactobacillus* due to their ability to withstand stressful environmental conditions of honey-must. During the alcoholic fermentation of Brazilian Cauim, similar results were also observed (Ramos *et al.*, 2010).

5.4 Conclusions

Fermentation dynamics research is essential for modernizing any spontaneous fermentation system. This study shed light on the interaction of physicochemical and microbial properties during the *Tej* fermentation period, which will be useful in future modernization efforts. Most importantly, regardless of sample collection households, the *Lactobacillus* and *Saccharomyces* genera dominated all time-course study samples, especially after fourth day of fermentation. Physicochemical characteristics matched those of these dominant fermentative microbes, and they also matched those of previous *Tej* reports. As a result, both genera could be seriously considered as mixed starter cultures for the fermentation of Ethiopian honey wine, *Tej*, without sacrificing the major wine's distinctive characteristics.

CHAPTER 6

Development of Mixed Starter Culture for the Fermentation of Ethiopian Honey Wine, *Tej*

Abstract

Ethiopian honey wine is one of the country's most popular spontaneously fermented traditional alcoholic beverages. However, the final product of this natural fermentation system is frequently of poor and inconsistent quality. Furthermore, it makes the process difficult to predict, control, and correct. Thus, the main aim of this chapter was to develop a direct fermentation system for Ethiopian honey wine, *Tej*. After isolating fermentative microbial strains from *Tej* samples, they were subjected to intensive screening to fit to its purpose. Later, phenotypic and genotypic characterization and inoculation of isolates to honey-must were performed sequentially. Finally, microbial interaction and physicochemical analysis, including volatile compounds profiling, were done for the inoculated samples. A total of 91 and 83 presumptive Lactic acid bacteria and yeast were isolated from *Tej* samples, respectively. After intensive screening only 6 and 8 isolates that had shown a good fermentative performance were subjected to genotypic characterization. The isolates were identified as *Saccharomycetaceae* and *Lactobacillaceae* based on ITS and 16SrRNA sequencing results. Furthermore, when all *Lactobacillus* isolates were analyzed using Randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR), there was no visible clonal difference between strains of the same species. Likewise, only 3 of 6 *S. cerevisiae* strains showed similar allelic diversity when tested using the Microsatellite multiplex PCR method. These strains were analyzed further using phenotypic microarray and demonstrated a good ability to tolerate osmotic stress and a lower pH environment while requiring minimal micro-nutrients. These strains were used to make *Tej* once more, this time with a different combination of isolated strains as the starter culture. *Tej* sample produced by mixed culture inoculation of *Saccharomyces* and *Lactobacillus* species showed similar physicochemical, volatile compounds, and sensory attributes values with that of the control sample. Thus, a mixture of *Saccharomyces* and *Lactobacillus* strains could be used as a starter culture to produce Ethiopian honey, *Tej*, without sacrificing of its major quality attributes.

Keywords: *Tej*, Starter culture, Volatile compound, Physicochemical properties, Sensory attributes

6.1 Introduction

Traditional fermented foods and beverages are made from locally available raw materials using native microorganisms, indigenous knowledge and locally available utensils (Steinkraus, 2004). In Ethiopia, the production and consumption of traditional foods and beverages is a very common practice (Lemi, 2020). Ethiopian honey wine, *Tej*, is one of the popular traditional alcoholic beverages in the country (Fentie *et al.*, 2020). It is typically a household commercial product sold for consumption at the point of production (Bahiru *et al.*, 2006; Fentie *et al.*, 2020). Like other traditional alcoholic beverages, *Tej* is produced by spontaneous fermentation of crude honey (Bahiru *et al.*, 2001b; Vogel & Gobezie, 1995). Due to the absence of direct inoculation, the fermentative microorganisms responsible for converting honey-water mixture to ethanol during *Tej* fermentation are thought to be found in raw materials and utensils (Lemi, 2020). This spontaneous honey fermentation could be the primary cause of inconsistency in the quality of *Tej* produced by different households (Nemo & Bacha, 2020). The honey quality difference might also contribute to this variation in the physicochemical properties of the final product (Bahiru *et al.*, 2001b).

Traditional *Tej* producers prefer crude honey instead of purified honey for the making a good quality *Tej* (Vogel & Gobezie, 1995). This crude honey is first mixed with water at a proportion of 1:3. Because of the increase in the price of honey, some producers substitute some portion of honey with cane sugar during *Tej* making (Bahiru *et al.*, 2006b). The mixture is then left in mixing tank for an additional 3–4 days to complete its primary stage fermentation. It will then be filtered using clean cloth to remove sediment and suspended particles. Subsequently, leaves and stems of "gesho" (*R. prinoides*) are added to the previously fermented honey-water mixture. "gesho" (*R. prinoides*) is primarily added to mixture to enhance the flavor of final product (Bahiru *et al.*, 2006b). Again, some *Tej* makers may also add barks from a selected tree and/or herbal ingredients to add a distinctive flavor to *Tej* (Vogel & Gobezie, 1995). The mixture is again allowed to ferment for an additional 8–21 days in anaerobic conditions to complete the final fermentation stage. After the mentioned fermentation period, it is now ready for consumption as a final product. Customers typically prefer *Tej*, which has a yellow color, a sweet taste, and a turbid appearance (Bahiru *et al.*, 2001b).

So far, limited studies had been conducted towards to the development of direct inoculated *Tej* fermentation system. The very first full article on *Tej* by Bahiru *et al.*, (2001) reported the physicochemical properties of *Tej* samples collected from Addis Ababa, Ethiopia. This report shows a considerable variation in some physicochemical properties for *Tej* samples collected from various

households. After five years, the same authors reported the yeast and lactic acid flora of *Tej* by using phenotypic characterization method (Bahiru *et al.*, 2006b). Similarly, *Lactobacillus* and *Saccharomyces* were the dominant taxa for the *Tej* samples collected from different part of the country. However, the dominance level was substantially different within and across the samples collected from different households. Furthermore, as the fermentation period drew close to an end, the microbial diversity shifted towards a specific microbial dominance (Fentie *et al.*, 2022). Furthermore, *Tej* exhibited good antioxidant activity, possibly inherited from honey and "gesho" (*R. prinoides*). The antioxidant capacity of *Tej* was also increased as the fermentation progressed to completion (Fentie *et al.*, 2022).

Previous reports on the physicochemical and microbial ecology of *Tej* did not provide enough information to modernize this spontaneous fermentation system (Bahiru *et al.*, 2001, 2006; Nemo & Bacha, 2020). The selection of starter culture strains and process variable optimizations are still open gaps to be filled for the complete transformation of this process. However, inoculated fermentation using single strain cultures might cause the fermented products to lose their peculiarity (Navarrete-Bolaños, 2012). The main reason for this drawback is that the basis for selecting single strains is to minimize or maximize only one output function of the process (Barrajón *et al.*, 2011). Using a mixed culture is the best option for dealing with the drawbacks caused by both spontaneous and single strain inoculated fermentation systems (Ciani *et al.*, 2010). Besides, using commercially available strains as a starter culture may result in substrate incompatibility, inability to adapt to stressful fermentation conditions, and/or loss of distinctive final product characteristics (Barrajón *et al.*, 2011; Ciani *et al.*, 2010). Thus, the purpose of this study was to isolate, screen, and characterize potential mixed starter cultures from *Tej* samples collected from various households, with the ultimate goal of developing an inoculated fermentation system for this traditional alcoholic beverage. Furthermore, we analyzed the physicochemical and volatile compounds to assess whether the isolated strain inoculations achieved the intended quality attributes of the final product.

6.2 Materials and Methods

6.2.1 Sample collection, transportation and storage

Sample collection, transportation was done according to the methods described in section 3.2

6.2.2 Enumeration and isolation of presumptive starter cultures

Yeast enumeration and isolation were carried out using YPD agar (Qvirist *et al.*, 2016). Chloramphenicol (100 µg/mL) was added to the growth media after autoclaving and cooling to 50 °C to inhibit bacterial growth. Each *Tej* sample (1 mL) was mixed with 9 mL of sterile saline solution (0.85% NaCl) to produce a serially diluted inoculating sample. After inoculation, the petri dish was incubated under aerobic condition at a temperature of 30 °C for a period of 48 h. At the same time, presumptive *Lactobacillus* enumeration and isolations were done via MRS agar medium and incubated under the anaerobic condition at 30 °C for 72 h. The macroscopic features (shape, size, pigment, surface, elevation, and opacity) of yeast and *Lactobacillus* colonies of different types were streaked on the same respective mediums. About 15 colonies of each representative type were re-streaked on the same medium to obtain pure colonies.

6.2.3 Phenotypic and physiological characterization

Presumptive *Lactobacillus* isolates were subjected to gram reaction, catalase activity, and indole tests according to methods described by Saarisalo *et al.*, (2007). Then gram positive, catalase, and indole negative bacterial together with yeast isolates were subjected to purpose-oriented screening. The ability of isolates to ferment carbohydrate and to produce carbon dioxide (CO_2) was conducted using Durham tubes according to standard protocol (Reiner, 2012). Then, both isolates (presumed yeast and *Lactobacillus*) with the ability to ferment glucose, and sucrose and produce CO_2 were passed on to the next stage of the screening process. The ability to grow at high sugar concentrations was tested by applying 10%, 18%, and 25% glucose on growth media (Jermini *et al.*, 1987). The microbial isolates with high sugar concentration tolerance were subjected to a third test, which was the ability to grow at lower pH levels of 3.5, 4.0, and 4.5. Both bacteria and yeast isolated with the ability to grow at a lower pH were subjected to a temperature sensitivity test. Thus, all isolates screened using the methods mentioned above were further tested for their ability to grow at temperatures of 15 °C, and 35 °C. Isolates that performed well in the above purpose-oriented screening were further subjected to genotypic characterization.

6.2.4 Genotypic identification and characterization

After extracting DNA from the isolated strains, ITS and 16sRNA amplicon sequencing were performed for the identification of yeast and *Lactobacillus* isolates, respectively. The identified *Lactobacillus* strains were then subjected to RAPD-PCR analysis by applying the methods used by

Kostinek *et al.*, (2005). Similarly, all *S. cerevisiae* isolates were subjected to microsatellite multiplex PCR analysis using the method developed by Vaudano & Garcia-Moruno, (2007). Three microsatellite loci, SCPTSY7, SC8132X, and YOR267C were used for their high degree of polymorphism. Both RAPD-PCR and microsatellite multiplex PCR products were separated by using 1.8% (w/v) agarose gel electrophoresis system. After pattern processing and cluster analysis, a dendrogram was developed by using UPGMA method.

6.2.5 Phenotypic Microarray analysis

PM assay for our isolates was performed by following manufacturer protocol of PM Technology (Biolog, Hayward, CA, USA). PM micro-panels are 96-well microplates with different substrates in each well. This study used PM5, PM9, and PM10 assays to determine nutrient requirements, osmotic/ionic stress responses, and sensitivity to different pH environments. Each well of the panels contains the required minimal medium components, specific dye, and unique substrate. The DNA amplicon sequenced bacterial and yeast isolates from *Tej* samples were chosen for this analysis. All test strains were first incubated overnight at a temperature of 30 °C on SMA (Standard Methods Agar, BioMérieux) medium. The cells were then collected from the SMA medium with a sterile cotton swab and dispersed in a sterile capped tube containing 20 mL of the inoculation fluid (IF-0, Biolog Inc.). Using a Biolog turbidimeter, the cell concentration was adjusted to 81% transmittance after all the bubbles created during cell dispersion had settled. After that, PM5, PM9, and PM10 plates were inoculated with the cell suspension (100 µL per well) and incubated at a temperature of 33 °C for about 120 h in the Omnilog Incubator/Reader (Biolog Inc., Hayward, USA). The color changes in the wells were measured once every 15 min, allowing for both amplification and quantification of the phenotype using OmniLog® data collection Software v 1.2 (Biolog Inc.).

6.3.6 Mixed culture fermentation

6.3.6.1 Starter culture preparations

Three Lactobacillus isolates were first inoculated into a 250 mL conical flask with MRS broth and incubated at 30 °C for 24 h under anaerobic conditions. Similarly, three yeast isolates were inoculated into a 250 ml Erlenmeyer flask with YPD broth and incubated at a temperature of 30 °C for 18 h. Following the incubation period, the growing mediums were centrifuged at 3500 rpm for 10 min to obtain a higher cell concentration. It was then washed twice using sterile water and

resuspended again in saline solution (0.85% NaCl). Finally, the concentration of the suspended inoculums was adjusted to achieve the required final microbial concentrations.

6.3.6.2 Inoculated *Tej* fermentation

Tej was made in the lab by using inoculated fermentation and the same procedures as when it was made traditionally (Fentie *et al.*, 2020; Vogel & Gobezie, 1995). Honey was first mixed with water at 1:3 ratio. Then, this mixture was pasteurized at 65 ^{o}C for 10 min. The pasteurized honey-water mixture was then cooled and aseptically transferred to 50 mL conical flask with a top screw cap. The previously prepared inoculums were then mixed with different strain combinations to create eight *Tej* samples. The first *Tej* sample (TS1) was prepared by inoculating *S. cerevisiae* (3 log cfu/mL) to honey-must mixture. The second sample (TS2) was inoculated with *S. cerevisiae* (3 log cfu/mL) and *P. fermentans* (2 log cfu/mL). The third sample (TS3) was inoculated with *S. cerevisiae* (3 log cfu/mL), *P. fermentans* (1 log cfu/mL), and *W. anomalus* (2 log cfu/mL). The fourth sample (TS4) was inoculated with *S. cerevisiae* (3 log cfu/mL) and *W. anomalus* (2.5 log cfu/mL). The fifth sample (TS5) was inoculated by *S. cerevisiae* (3 log cfu/mL) and *L. hilgardii* (2 log cfu/mL). The sixth test sample (TS6) was inoculated with *S. cerevisiae* (3 log cfu/mL) and *L. parabuchneri* (2 log cfu/mL). Similarly, sample seven (TS7) was inoculated with *S. cerevisiae* (3 log cfu/mL) and *L. paracasei* (2 log cfu/mL). The last experimental *Tej* sample (TS8) was prepared by inoculating a honey-water mixture with *S. cerevisiae* (3 log cfu/mL), *L. hilgardii* (2 log cfu/mL), *L. parabuchneri* (1 log cfu/mL) and *L. paracasei* (1 log cfu/mL). After inoculation, they were allowed to finish their primary fermentation stage for about 4 days at room temperature (26 °C). At this time, 1.5 g of boiled and sterilized gesho leaves and stems were added to the fermentation mediums (50 mL). The *Tej*-making process was completed after allowing these mixtures to spend for 20 more days.

6.2.5 Physicochemical analysis

6.2.5.1 Sugar profiling

Sugar profiling was done by using the method described in section 3.3.3

6.2.5.2 Ethanol quantification

Ethanol quantification were performed according to the methods described in section 3.3.4

6.2.5.3 Lactic acid quantification

The lactic acid concentration in the *Tej* samples were quantified using spectrophotometric method developed by Borshchevskaya *et al.*, (2016). A calibration curve was first constructed using serially

diluted known concentration of DL-lactic acid stock solution (89%, ρ = 1.2 g/mL, Sigma-Aldrich). The absorbance of each serially diluted lactic acid stock (50 L) solution was measured at 390 nm after it was mixed with 2 mL of a 0.2% solution of iron (III) chloride ($FeCl_3.6H_2O$). Similarly, the *Tej* samples were first centrifuged to remove the cellular particles from the fermented aliquot. The separated supernatant was then diluted 20-fold using deionized water. Subsequently, the test samples (50 µL) were mixed with 2 mL of a 0.2% solution of iron (III) chloride, and the absorbance at 390 nm was measured compared to the reference solution (2 mL of a 0.2% solution of $FeCl_3.6H_2O$). The reaction and measurements were conducted at a temperature of 25 °C ± 5 °C. Finally, the lactic acid concentration was calculated using a calibration curve that accounted for the 20-fold dilution of the *Tej* sample.

6.2.6 Volatile aromatic compound profiling

Volatile aromatic compounds were profiled using a headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME-GC-MS). *Tej* samples produced using various inoculum combinations were examined for volatile aromatic compounds by adapting the methods used by Ravasio *et al.*, (2018). Briefly, 2.5 mL of the *Tej* sample and internal standard of 2-octanol (10 µL) was placed in a 20 mL head space extraction glass vials. Samples were then equilibrated for 10 min at 40 °C and 250 rpm. The extract was then adsorbed for 30 min at 40 °C using 50 µm layer DVB/CAR/PDMS fiber. After extraction, the fibers were drawn into the needle and transferred to the injection of the GC-MS system for desorption at 250 °C for 5 min. Chromatographic separation was performed with a TRACE TR-5 GC column (30 m,0.25 mm×0.25µm), using helium as carrier gas at 1.0 mL/min. The oven temperature program was set as follows: 50 °C to 220 °C, at 2 °C /min, then raised to 240ºC, at 10 °C /min and held for 10 min. The total run time was 91 min. The mass spectrometer (quadrupole) operated in full scan mode, detecting fragments in a mass range of 35 to 500 m/z. The Internal standard was used to semi-quantify volatile aromatic compounds following the method used by Pino and Barzola-Miranda, (2020). The final identification of aromatic compounds was conducted by comparing mass spectra with the NIST 14 library and chemical standards.

6.2.7 Sensory Analysis

The acceptability of *Tej* samples fermented by direct inoculation of isolated strains was assessed using consumer-based sensory analysis. Twenty-four experienced consumers were chosen to evaluate the sensory attributes of the test samples. The evaluations were conducted in a sensory

panel room at 25 °C. About 100 mL *Tej* samples were served to the panelist using transparent plastic cup. The samples were evaluated thrice after being blind-coded with three-digit random numbers. The panelists were then asked to rate each sample, with a seven-point hedonistic scale, for the (a) color (b) turbidity (c) alcoholic aroma (d) honey like aroma (e) astringency and (f) overall acceptance.

6.2.8 Statistical analysis

All experimental analysis were conducted in triplicates. The collected data were then checked for normality and homoscedasticity (Levene's test)(Granato *et al.*, 2014). Subsequently, means of the samples data were compared by Duncan's multiple comparison test. The statistical significance ($P < 0.05$) was later determined by a one-way analysis of variance (ANOVA). The data analysis, CCA and PCoA plots were performed using RStudio 4.0.3 software.

6.3 Results and Discussion

6.3.1 Isolation, characterization and screening of starter cultures

Ethiopian honey wine, *Tej*, is produced by spontaneous fermentation process (Bahiru *et al.*, 2006b; Vogel & Gobezie, 1995). However, this kind of spontaneous fermentation has a main draw back on the predictability and control of the process (Navarrete-Bolaños, 2012). To shift from spontaneous to direct fermentation, without losing any of its major quality attributes, studying the microbial ecology and development of starter culture is the major tasks (Steinkraus, 2004). The previous studies on *Tej* samples had clearly point out the microbial ecology was dominated by the species of *Saccharomyces* and *Lactobacillus* (Diaz *et al.*, 2019). Thus, development of starter culture composed from a mixture of *Lactobacillus* and *Saccharomyces* species will help to achieve a direct *Tej* fermentation system without sacrificing the good qualities of the final product.

Based on macroscopic feature differences, 91 bacterial and 83 fungal colonies were isolated from *Tej* samples using de Man, Rogosa, and Sharpe (MRS) and yeast extract peptone dextrose (YPD) media (Figure 6.1). The catalase activity, gram reaction, and indole test to characterize the bacterial isolates. Indole negative, gram-negative, and catalase-negative characteristics were found in 67 of the 91 isolates. These bacterial and fungal isolates were subjected to further physiological tests. Isolates that could ferment carbohydrates and produce gas advanced to the next level of screening, which included the ability to grow at lower pH, varying temperatures, and higher sugar concentrations (Table 6.1). As a result, the number of isolates was reduced as the screening

progressed to the end. A total of 34 presumptive heterofermentative *Lactobacillus* that could ferment carbohydrates and produce gas (Figure 6.1) were screened from 67 bacterial isolates. Similarly, 49 fungal isolates that had a good ability to ferment carbohydrates were screened from the first isolated pool (Table 6.1). Subsequently, 27 and 31 bacterial and fungal isolates with a good ability to proliferate at lower pH (4.5) growth media were again screened. In this study, the isolates with an optical density (OD) value greater than 1.0 at 600 nm wavelength were considered as higher pH tolerant microbes. From the prior pool of isolates, 21 and 20 bacterial and yeast isolates were screened for their good ability to grow in extreme temperatures (Table 6.1). Finally, the ability of the isolates to grow at a higher sugar concentration was performed by using glucose concentrated MRS and YPD media. A total of 6 and 8 bacterial and yeast isolates with desirable characteristics were selected and subjected to genotypic identification and characterization.

Table 6. 1 Physiological tests for purpose-oriented screening of bacterial and yeast isolates from *Tej* samples

Physiological tests	Characteristic results	Number of isolates	Percent of isolates
Bacterial isolates		91	
Gram reaction	Negative	82	90.11%
Indole test	Negative	89	97.80%
Catalase activity	Negative	78	85.71%
Carbohydrate fermentation test and gas production			
Glucose	Positive	50	54.94%
Sucrose	Positive	41	45.05%
Ability to grow at a lower pH (4.5)	++	27	29.67%
Ability to grow in different temperature			
15 °C	++	25	27.47%
35 °C	+	23	25.27%
Ability to grow at high sugar (glucose) concentration			
10%	++	20	21.97%
15%	++	14	15.38%
20%	+	11	12.09%
Fungal isolates		83	
Carbohydrate fermentation test			
Glucose	Positive	68	81.92%
Sucrose	Positive	62	74.69%
Ability to grow at a lower pH (4.5)	++	31	37.35%
Ability to grow in different temperature			
15 °C	++	28	33.73%
35 °C	+	24	26.37%
Ability to grow at high sugar (glucose) concentration			
10%	++	19	22.89%
20%	++	13	15.66%
30%	+	10	12.05%

+ isolates that had recorded 0.8–1.0 OD values at 600 nm after 24 h incubation period.

++ isolates that had recorded greater than 1.0 OD values at 600 nm after 24 h incubation period.

Figure 6. 1 Pictures of **A**) Yeast and Lactic acid bacteria isolates, and **B**) Durham tube carbohydrate fermentation

6.3.2 Identification of isolates

Genotypic identification of bacterial and yeast isolates was performed using 16S rRNA gene and ITS amplicon sequencing. The morphological features and genotypic identification of the isolates are presented in Table 6.2. In addition, the phylogenetic tree based on the 16S rRNA gene and ITS amplicon sequenced data of the isolates together with the closely related species is illustrated in Figure 6.2. All bacterial isolates had shown greater than 98.5% similarity with the National Center for Biotechnology Information (NCBI) nucleotide sequence database (Table 6.2). Moreover, all isolated bacteria were a family of *Lactobacillaceae* (Figure 1A& B). *Lactobacillus hilgardii*, *Lactobacillus paracasei,* and *Lactobacillus parabuchneri* were the identified species from 6 bacterial isolates. The source of these isolates was *Tej* samples collected from different locations (Table 6.2). For instance, *L. parabuchneri* strains were isolated from *Tej* samples collected from Debre Markos, Ethiopia. Similarly, the rest two of *Lactobacillaceae* isolates were identified as *L. paracasei*. These strains were isolated from *Tej* samples collected from Addis Ababa and Bahir Dar, Ethiopia (Table 6.2). Usually, lactic acid bacteria are introduced to wine after the completion of alcoholic fermentation or at the start of malolactic fermentation (Bauer & Dicks, 2017). However, in Ethiopian honey wine, *Lactobacillus* and *Saccharomyces* are involved in the fermentation from the beginning up to the end of the fermentation period (Fentie *et al.,* 2022). Thus, isolating *Lactobacillus* strains from *Tej* samples was not a surprising result. Especially, strains of *L. hilgardii* had been commonly involved in the alcoholic fermentations (Escalante *et al.*, 2004). Besides, all of the isolates were under the heterofermentative *Lactobacillus* category. The *Lactobacillus* under this category has the ability to produce lactate, ethanol, and carbon dioxide from a given carbon source (Ayivi *et al.*, 2020). Furthermore, some of the *Lactobacillus* species enhance the flavor of the produced alcoholic beverage (Mishra *et al.*, 2010). Similar lactic acid bacterial isolates for the *Tej* sample were obtained by applying phenotypic microbial characterization techniques (Bahiru *et al.*, 2006b).

Based on ITS sequence data, identified isolates were the species of *Saccharomycetaceae* (Figure 6.2A). *Saccharomyces cerevisiae*, *Pichia fermentans*, and *Wickerhamomyces anomalus* were the identified species from the collected *Tej* samples (Table 6.2). Six out of eight isolates were identified as *S. cerevisiae* strains. The strains of this species were isolated from *Tej* samples collected from all source locations. The other identified isolate using ITS sequence was *P. fermentans* strain. This strain was isolated from *Tej* samples collected from Bahir Dar and Addis Ababa, Ethiopia.

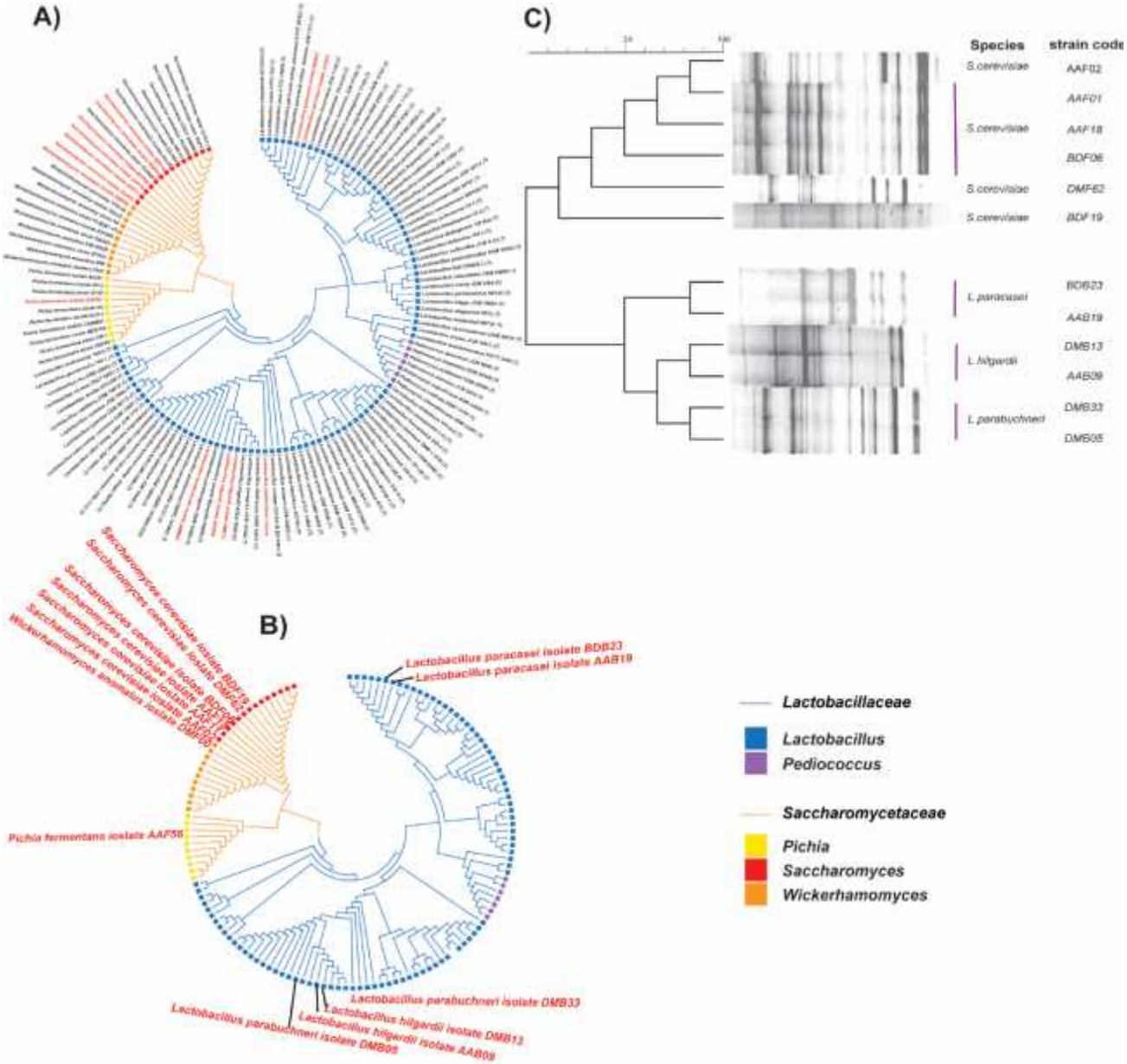

Figure 6. 2 Phylogenetic tree of **A**) & **B**) Lactobacillus and Yeast isolates and **C**) phylogenetic relationship between the isolates

The remaining isolate, *W. anomalus* strain, was isolated from *Tej* samples collected from Debre Markos (Table 6.2). Since they are the most responsible species for converting carbohydrates to ethanol, *S. cerevisiae* is the most expected strain in every alcoholic beverage (Kouame, *et al.*, 2020). Similar *S. cerevisiae* isolates were obtained from *Tej* and other Ethiopian traditional beverages (Bahiru *et al.*, 2006b; Teramoto *et al.*, 2005b). Although it is uncommon to use *W. anomalus* and *P. fermentans* isolates as the sole starter culture for alcoholic beverage fermentation, co-culturing

them with *S. cerevisiae* to improve the flavor of the alcoholic beverage is currently attracting the interest of many scientists (Clemente-Jimenez *et al.*, 2005; Lee & Park, 2020; Padilla *et al.*, 2018). Thus, the isolated *Saccharomycetaceae* strains have a good potential to be used as a starter culture for the fermentation of Ethiopian honey. However, *W. anomalus and P. fermentan*s were not isolated from all sample collection areas. This could be due to the inherent microbial variability of the samples caused by the spontaneous nature of fermentation, or it could also be due to the stringent screening procedure (Bahiru *et al.*, 2006b).

Furthermore, phylogenetic relationship and genetic diversity analysis were conducted for the isolated strains of the same species. Randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) was used to achieve these objectives for *Lactobacillus* isolates. The *Lactobacillus* isolates clonal relationship were studied by using RAPD-PCR DNA fingerprinting. Because it amplifies fragments of genomic DNA, RAPD is one of the most powerful tools for studying genetic variation in living organisms (Saxena *et al.*, 2014). However, there is still a loophole of this method on the reproducibility due to mismatch in annealing (Sesena *et al.*, 2004). All strains of *Lactobacillus* species had clustered very narrowly together with very uniform DNA fragment patterns (Figure 6.2C). Especially strains of the same species did not show a high level of polymorphism. The primary justification that could be forwarded for this result could be due to intensive screening for harsh environment might cause isolated strains to have a similar allelic diversity. The luck of reproducibility, accuracy and application of limited number of primers during RAPD-PCR amplification could also be the other factor for this particular result (Saxena *et al.*, 2014; Sesena *et al.*, 2004).

The phylogenetic relationship and diversity of isolated fungi strains was also studied by using microsatellite multiplex PCR. Microsatellites, also known as simple sequence repeats (SSRs), are short repeats of DNA sequence motifs found in many genomes (Legras *et al.*, 2005). All *S. cerevisiae* isolates were subjected to the above-mentioned analysis, with the exception of *P. fermentans* and *W. anomalus*, which were isolated only once. *T*he strains of *S. cerevisiae* showed a higher level of polymorphism (Figure 6.2C). Among six *S. cerevisiae* strains three of them had shown high level of polymorphism. The rest three *S. cerevisiae* strain isolates had shown a difference in the pattern of amplified DNA fragments on agarose gel electrophoresis.

Table 6. 2 Morphological characteristics and genotypic identification of bacterial and fungal isolates

Isolate strain code	Source	Morphological characterization						Genotypic identification		GenBank accession number
		Shape	Size	Pigment	Surface	Elevation	Opacity	Species	Percent Identity	
Bacterial isolates										
DMB13	DM	Round	Medium	White	Glistening	Flat	Opaque	*L. hilgardii*	99.80	AB911494.1
DMB33	DM	Round	Medium	White	Smooth	Flat	Opaque	*L. parabuchneri*	99.67	CP018796.1
AAB09	AA	Punctiform	Small	Creamy	Smooth	Pulvinate	Opaque	*L. hilgardii*	99.61	AB911494.1
BDB23	BD	Punctiform	Small	Creamy	Glistening	Flat	Opaque	*L. paracasei*	99.31	KR816165.1
DMB05	DM	Irregular	Medium	Creamy	Glistening	Convex	Translucent	*L. parabuchneri*	99.97	FJ476125.1
AAB19	AA	Punctiform	Small	White	Smooth	Flat	Opaque	*L. paracasei*	99.87	MK966340.1
Yeast isolates										
AAF02	AA	Round	Large	White	Smooth	Umbonate	Opaque	*S. cerevisiae*	99.85	MT355081.1
AAF11	AA	Round	Large	White	Glistening	Umbonate	Opaque	*S. cerevisiae*	98.79	MT355081.1
AAF18	AA	Round	Medium	Creamy	Smooth	Raise	Translucent	*S. cerevisiae*	99.36	MT355081.1
DMF00	DM	Round	Medium	Creamy	Smooth	Flat	Opaque	*W. anomalus*	98.36	MT321267.1
BDF06	BD	Round	Large	White	Smooth	Pulvinate	Opaque	*S. cerevisiae*	99.35	MT355081.1
AAF56	AA	Round	Medium	White	Dull	Convex	Opaque	*P. fermentans*	98.52	MT645425.1
DMF62	DM	Round	Large	Creamy	Glistening	Flat	Opaque	*S. cerevisiae*	99.61	LC215450.1
BDF19	BD	Round	Large	White	Smooth	Umbonate	Translucent	*S.cerevisiae*	99.43	KY105065.1

AA – Addis Ababa, BD – Bahir Dar, DM – Debere Markos

The inherent high genetic diversity of *S. cerevisiae* strains could be the possible major reason for these variations. However, the number of analyzed loci still had the influence on the genetic diversity of the strains (Saeed *et al.*, 2016). Thus, it is worth to know that, the current result could have been different if more than three loci of the total genome had been amplified. *S. cerevisiae* with the strain code AAF18 was chosen for further analysis and inoculation honey-must in this study based on the majority in the similarity of presence. Since strains of the same species of *Lactobacillus* showed a high level of genome similarity, we used a strain code DMB13, DMB33, and DMB23 to be used as mixed culture inoculum for honey-must fermentation.

6.3.3 Phenotypic properties of isolates

The characterization of isolates was further strengthened by phenotypic microarray (PM) analysis. PM 5, PM 9, and PM 10 microplates were used to achieve this purpose. The result of phenotypic microarray is illustrated by heat map in Figure 6.3. The larger magnitude in the figure is indicated by dark red, while the magnitude gradually falls to light red, white, light blue, and finally dark blue, with the latter denoting a lesser magnitude. The nutritional requirement of the isolates was determined using PM 5 microplate. PM 9 and PM 10 were utilized to evaluate the reaction of the isolates to osmotic stress and extreme pH environment. The isolated *S. cerevisiae* showed the best metabolism at the medium supplemented with D-biotin, nicotinamide, and (5)4-amino imidazole-4(5)-carboxamide (Figure 6.3A). While *P. fermentans* isolate metabolism was best at the medium supplemented with inosine + thiamine, thiamine and pyrophosphate. This high metabolism could be due to the micronutrient (vitamins and minerals) requirements of microbe for the proper metabolism of cells (Santos Júnior *et al.*, 2021). However, *W. anomalus* and almost all of *Lactobacillus* isolates didn’t have any special preference over the nutrient supplements available on PM 5 (Figure 6.3A). Moreover, *Saccharomycetaceae* and *Lactobacillus* isolates had exhibited an excellent osmotic tolerance ability. All of *Saccharomycetaceae* isolates had recorded a good metabolism at different concentration of sodium nitrite, sodium lactate, sodium formate, sodium sulfate, ethylene glycol and potassium chloride. This tolerance could be due to the adaptation of isolates to higher osmotic pressure experienced during honey wine fermentation. Nevertheless, lower metabolism had recorded for these isolates at the growth medium containing high sodium chloride (NaCl) concentration (Figure 6.3B). Similarly, all *Lactobacillus* isolates responded well to an increased sodium nitrite, sodium sulfate, and ethylene glycol concentrations. Like *Saccharomycetaceae*

isolates, all the *Lactobacillus* isolates had difficulties for proper metabolism at a higher NaCl concentration. (Figure 6.3).

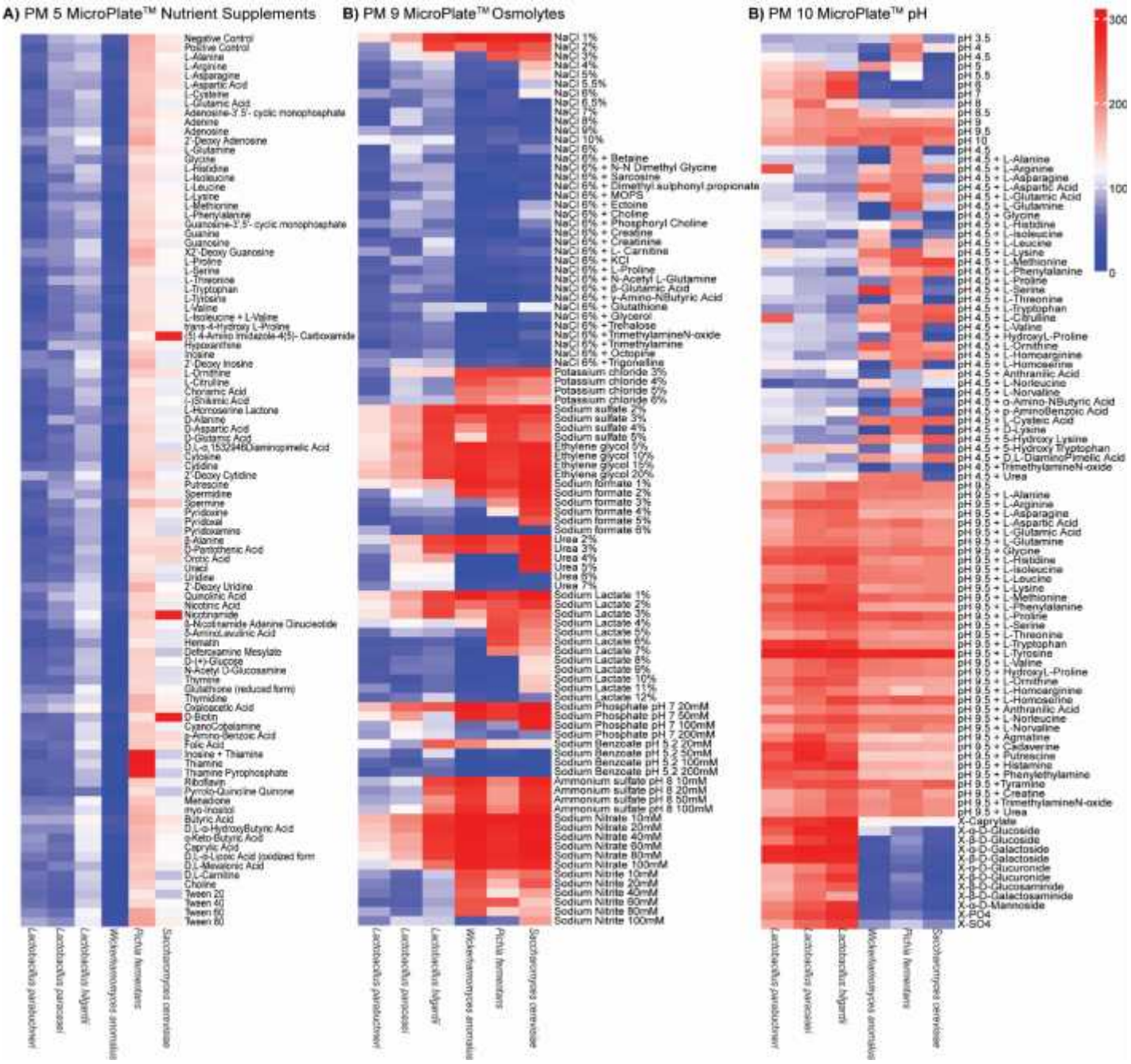

Figure 6. 3 Heat map of the phenotypic microarray results of isolates using different **A**) Nutrient supplements **B**) Osmolytes, and **C**) pH microplates.

However, a lower magnitude of metabolism for all isolates was observed for the growth medium containing greater than 6% NaCl concentration. This result could be due to the cellular metabolic shift of isolated microbes from hyperplasia to hypertrophy at higher NaCl concentration

(Stubblefield & Mueller, 1960). Since honey-must has a lower pH by itself, and the pH continues to drop as fermentation progresses, tolerance to acidic stress is one of the essential characteristics of isolates to look for when choosing a starter culture. However, a lower pH can charge the biological molecules, which later can negatively impact both structure and function of cells (Lund *et al.*, 2020). Thus, PM 10 plate were used to assess the response of isolates towards to the change in pH. Phenotypic microarray pH characterization revealed that *Saccharomycetaceae* isolates were more adaptable to lower pH environment than *Lactobacillus* species. This response could be due to the better membrane stability of fungi over bacteria. Especially for a pH lower than 4.5, the magnitude of metabolism for *Lactobacillus* isolates was very low. The isolates of *Lactobacillus* species responded well for the medium having pH values greater than or equal to 4.5 (Figure 6.3C). Compared to other *Saccharomycetaceae* isolates, *P. fermentans* responded relatively better to different pH environments. A similar *Pichia* isolates tolerance over the other Saccharomyces isolates was observed in the previous study (Sun *et al.*, 2020). Although they are not the same as *P. fermentans*, *S. cerevisiae* and *W. anomalus* had also demonstrated acceptable tolerance to a different pH condition (Figure 6.3C).

6.3.4 Interaction between dominate species

Following the extensive characterization of isolated microorganisms, it had been discovered that they had a strong chance of being employed as a starter culture for *Tej* fermentation. However, their growth in honey-based media and interaction with each other was the important concept to be covered for developing a starter culture. Fermentative yeast and *Lactobacillus* count were performed five times throughout the fermentation period of *Tej*. Eight *Tej* samples were produced by inoculating isolated *Lactobacillus* and *Saccharomycetaceae* species in various combinations as a mixed starter culture. Other than pasteurization and inoculation with a defined starter culture, all the processes were conventional. The *Tej* samples from one to four were entirely inoculated by the isolates of *Saccharomycetaceae*. Other samples from five to eight were inoculated by the isolates of both *Lactobacillus* and *Saccharomycetaceae* strains. *Tej* that was spontaneously fermented and widely regarded as the best Ethiopian honey wine was used as a control sample. The details of the production process and the nomenclature of the samples are described in the methods section. Plate count methods for total fermentative yeast and *Lactobacillus* were used to study the interaction of the inoculated microbes during the fermentation period. The total fermentative yeast growth rate for the TS1 sample had the highest exponential growth rate compared to other *Tej* samples inoculated

with *Saccharomycetaceae* only (Figure 6.4A). The availability of ample nutrients and little microbial competition causes a higher fermentative yeast count for the TS1 sample, especially at the early fermentation stage (Peleg & Corradini, 2011). Besides, this sample reaches a stationary phase after 14 days of fermentation. Sample TS3 started at a higher cell count of 6 logs CFU/mL and reached the maximum (8 logs CFU/mL) after 14 days of fermentation. A similar increment of fermentative yeast for sample TS3 could be due to a higher initial microbial count of inoculum and higher availability of the required nutrient (Egli, 2015; Peleg & Corradini, 2011). Sample TS2 and TS4 showed a gentle growth rate compared to TS1 and TS3. Especially the TS4 sample showed a slower growth rate than other yeast-only inoculated samples. Surprisingly, the slower growth rate of fermentative yeast for sample TS4 could be caused by either mutual inhibition or the slow growth rate of one of the inoculums. However, this and TS2 samples showed an active growth rate even after 21 days of fermentation (Figure 6.4A).

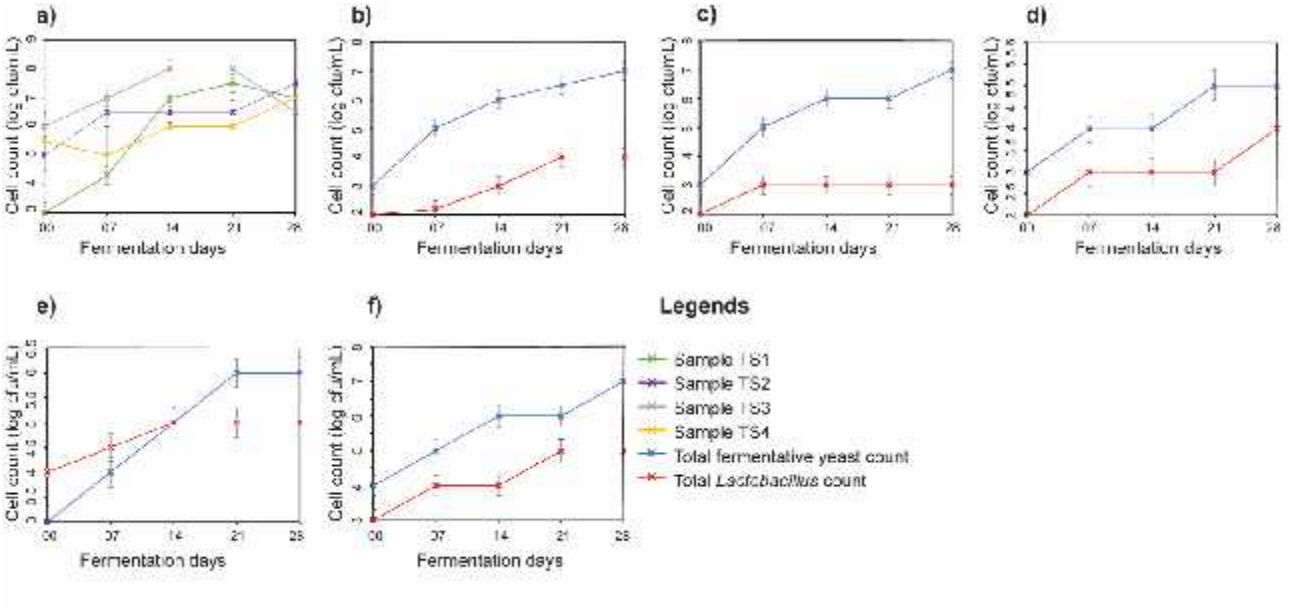

Figure 6. 4 Microbial growth curve of **a)** total fermentative yeast for TS1, TS2, TS3 and TS4 **b)** total fermentative yeast and *Lactobacillus* for sample TS5 **c)** total fermentative yeast and *Lactobacillus* for sample TS6, **d)** total fermentative yeast and *Lactobacillus* for sample TS7 and **e)** total fermentative yeast and *Lactobacillus* for sample TS8 **f)** total fermentative yeast and *Lactobacillus* for the control sample

Sample TS5, inoculated with a mixed culture of *S. cerevisiae* and *L. hilgardii* strains, grew rapidly in total fermentative yeast and *Lactobacillus* cell count. However, the growth rate of fermentative yeast was by far higher than that of *Lactobacillus*. The cell count of *Lactobacillus* was plateaued at 4 logs CFU/mL after 21 days of fermentation. In contrast, the cell count for total fermentative yeast reaches 7 logs CFU/mL at the end of fermentation (Figure 6.4B). A similar higher fermentative yeast count was observed for the TS6 sample compared to its co-inoculated *Lactobacillus* count. After seven days of fermentation, the *Lactobacillus* cell count enters to stationary phase with a cell count of 3 logs CFU/mL. In contrast, the total fermentative yeast of this sample showed a higher growth rate and reached a maximum cell count of 7 logs CFU/mL at the end of its fermentation period (Figure 6.4C). A proportional growth rate was observed for yeast and *Lactobacillus* during the TS7 *Tej* sample fermentation period. Nonetheless the growth rate of yeast was higher than the growth rate of *Lactobacillus* (Figure 6.4D). At the end of fermentation, this sample recorded 5 and 4 logs CFU/mL count for total fermentative yeast and *Lactobacillus* cell counts, respectively. Sample TS8 started its fermentation with a higher *Lactobacillus* cell count (4 logs CFU/mL) and continued its dominance up to 14 days of fermentation. However, the cell count of fermentative yeast overtook this dominancy after 14 days. At the end of the fermentation period, the total fermentative yeast and *Lactobacillus* count reached 6 and 5 logs CFU/mL, respectively (Figure 6.4E). The fast growth rate of *Saccharomyces* was observed compared to that of *Lactobacillus* in a mixed culture inoculated *Tej* fermentation. This fast growth rate could be caused either by the inhibition of *Lactobacillus* by *Saccharomyces* species or due to the inherent fast growth rate characteristics of *Saccharomyces* (Jin *et al.*, 2019; Thomas et al., 2001). However, the growth rate of *Lactobacillus* itself followed almost a similar pattern as it was discussed in the previous study on *Tej* microbial dynamics (Fentie *et al.*, 2022). Similar studies on the co-inoculation of *Saccharomyces* and *Lactobacillus* had revealed a production of good quality characteristics alcoholic beverage (Dysvik *et al.*, 2020). The control sample also showed a similar microbial growth pattern to the previous mixed culture inoculated *Tej* samples. Specifically, the total fermentative yeast was a bit higher (4 logs CFU/mL) than the other inoculated test *Tej* samples. Nevertheless, the final cell concentration for both cell counts was similar to other test samples (Figure 6.4F).

6.3.5 Physicochemical characteristics

The final product physicochemical and volatile compound analysis of *Tej* samples was performed to assess whether or not the inoculated fermentation had met the intended goal. These

physicochemical properties of *Tej* samples produced by inoculation of different mixed culture combinations is presented in Table 6.3. Significantly higher ($P < 0.05$) glucose (2.97 g/L) level was observed for TS1 sample. While the TS8 sample (1.97 g/L) and control sample (1.95 g/L) had significantly lower glucose levels ($P < 0.05$). Similarly, the fructose concentration of the TS2 sample was much higher, while the TS8 sample fructose level was significantly lower ($P<0.05$). Generally, the control and mixed culture fermented samples showed lower sugar content as compared to other inoculated samples (Table 6.3). In terms of residual sugar concentration, all test samples fell within the range observed in previous survey studies for *Tej* samples (Bahiru *et al.*, 2001; Nemo & Bacha, 2020). This result is essentially a positive testimony for the ability of the inoculum to utilize carbon sources from honey for cellular growth and the production of exo-metabolic products (Pereira *et al.*, 2013). Likewise, the ethanol level of the samples showed a significant difference for the test samples. The control *Tej* sample had a significantly higher ($P<0.05$) ethanol level (10.43 g/100 mL) as compared to other samples. Whereas TS2 sample had significantly lower ($P < 0.05$) ethanol level of 7.87 g/100mL as compared to other inoculated samples (Table 6.3). In comparison to samples inoculated with solely yeast culture, samples inoculated with a mixed culture of *Lactobacillus* and *S. cerevisiae* had a greater ethanol content (Table 6.3). This could be due to the co-inoculation of heterofermentative *Lactobacillus* isolates, resulting in additional ethanol production (Basso *et al.*, 2014).

Table 6. 3 The physicochemical properties of fermented honey wine inoculated with various *Saccharomycetaceae* and *Lactobacillaceae* strain combinations

Samples	**Physicochemical properties**					
	Glucose	Fructose	Ethanol	Lactic acid	pH	TA (g/L)
TS1	2.97 ± 0.01^{a}	4.20 ± 0.01^{b}	8.70 ± 0.02^{f}	ND	3.02±0.01	0.96±0.04
TS2	2.71 ± 0.01^{c}	4.65 ± 0.01^{a}	7.87 ± 0.02^{i}	ND	3.34±0.01	1.14±0.03
TS3	2.10 ± 0.01^{g}	4.02 ± 0.01^{e}	8.00 ± 0.03^{g}	1.09±0.03	3.66±0.01	1.31±0.04
TS4	2.79 ± 0.01^{b}	3.99 ± 0.01^{f}	9.22 ± 0.02^{c}	0.98±0.01	3.55±0.01	0.88±0.04
TS5	2.35 ± 0.01^{f}	4.14 ± 0.01^{c}	8.95 ± 0.02^{d}	2.87±0.02	2.93±0.01	4.33±0.05
TS6	2.57 ± 0.01^{e}	4.09 ± 0.01^{d}	7.96 ± 0.02^{h}	2.67±0.02	3.19±0.01	4.97±0.05
TS7	2.68 ± 0.01^{d}	3.94 ± 0.01^{g}	8.81 ± 0.03^{e}	2.22±0.01	3.00±0.01	5.10±0.09
TS8	1.97 ± 0.01^{h}	3.47 ± 0.01^{i}	9.75 ± 0.03^{b}	3.14±0.02	2.84±0.01	4.46±0.04
Control	1.95 ± 0.01^{h}	3.86 ± 0.01^{h}	10.43 ± 0.02^{a}	2.93±0.02	3.15±0.01	6.37±0.05

All values are mean ± SD (standard deviation)

Units for glucose, fructose, lactic acid and TA – g/L, Ethanol – g/100 mL

Statistical significance difference is compared vertically

TS1 – *S. cerevisiae*, TS2 – *S. cerevisiae* and *P. fermentans*. TS3 – *S. cerevisiae*, *P. fermentans*, and *W. anomalus*. TS4 – *S. cerevisiae* and *W. anomalus*, TS5 – *S. cerevisiae* and *L. hilgardii*, TS6 – *S. cerevisiae* and *L. parabuchneri*. TS7 – *S. cerevisiae* and *L. paracasei*. TS8 – *S. cerevisiae*, *L. hilgardii*, *L. parabuchneri* and *L. paracasei*.

Moreover, the lactic acid levels of fermented honey wines were in the range from not detectable limit to 3.14 g/L. With the exception of sample TS8, which had a very high lactic acid concentration, honey wines inoculated with a mixed culture of *Lactobacillus* and *S. cerevisiae* had roughly the same concentration of lactic acid (Table 6.3). Moreover, TS3 and TS4 samples also showed the same lactic acid concentration, probably due to the lactic acid-producing yeast or due to lactic acid bacteria trace contamination. Similar lactic acid production by yeast was observed during the fermentation of beer and mead (Osburn *et al.*, 2018; Peepall *et al.*, 2019). Similarly, the titratable acidity was lower for the samples that were inoculated with yeast combinations as compared to samples inoculated with *S. cerevisiae* and *Lactobacillus*. Besides, the spontaneously fermented control sample had shown a higher titratable acidity (6.37 g/L).

6.3.6 Volatile compound and sensory attributes

The volatile compound analysis will help to objectively investigate the influence of different inoculum combinations on fermented *Tej* samples. The volatile compound of the honey wine is derived either from raw materials or from fermentative microorganisms (Chitarrini *et al.*, 2020). The analysis of volatile compounds in honey wines inoculated with different combinations of strains was performed using headspace solid-phase micro extractor gas chromatography-tandem with a mass spectrophotometer (HS-SPME-GC-MS). Esters, alcohols, carbonyl compounds, alkanes, and acids were the major compounds identified and quantified by using 2-octanol as internal standard from direct inoculated honey wines samples. The most common major volatile compounds in the honey wines samples, inoculated with various microbial strains, were alcohols and esters. Alcohols were the most dominant volatiles compounds observed in all fermented honey wines samples (Table 6.4). The subtotal value of these compounds ranged from 728.9 mg/L (control sample) to 577.56 mg/L (TS3 sample). Ethanol was the most abundant volatile compound identified and quantified in all samples. The mean value of this compound ranged from 321.20 mg/L to 448.80 mg/L (Table 6.4). In this study, phenylethyl alcohol was the second most dominant volatile compound in all fermented honey wine samples. The value of Phenylethyl alcohol ranged from 219.79 mg/L to 274.50 mg/L (Table 6.4). Like honey wine samples treated with different fining agents (Pascoal et al., 2019), alcohol was also the most dominant volatile compound in all of *Tej* samples. The dominant alcohols (ethanol and phenylethyl alcohol) were the compounds responsible for the vinous odor and pungent taste of alcoholic beverages (Zhang *et al.*, 2015). Furthermore, higher alcohols (1-Propanol, 2-methyl-, 2,3-Butanediol, and 2-Heptanol) in the tested samples also contributes to

the good flavor of honey wine (Pascoal *et al.*, 2019). These higher alcohol content and proportion played a significant role in improving the wine taste (Styger *et al.*, 2011).

About 13 ester compounds found in all samples, including the control, were identified and quantified. Especially, the ethyl ester of octanoic acid, dodecanoic acid, benzoic acid, and hexanoic acid was the common ester observed for all samples (Table 6.4). Nevertheless, esters and alcohols were the most dominant volatile compounds in all test and control samples0. Since honey wine is an alcoholic beverage, the presence of alcohol and alcohol derivative volatile compounds is expected to observe (Chitarrini *et al.*, 2020; Pereira *et al.*, 2019). During alcoholic fermentation and slow aging, alcohol acetyltransferase and other enzymes could catalyze the formation of esters from an activated fatty-acyl CoA compound and alcohol (Erten *et al.*, 2007). Besides, isoamyl acetate was the major compound for TS1, TS2, TS3, TS6, and control samples. In contrast, this compound was below the detectable limit for the TS7 and TS8 samples. Isoamyl acetate, the most abundant ester in *Tej* samples, is the compound responsible for fruity and floral notes and is typically derived from yeasts (Liu *et al.*, 2019). Moreover, Octanoic acid, ethyl ester, the second most abundant ester found in all samples, give pear, brandy, and lentil flavor to the produced *Tej* (Panighel & Flamini, 2014). This volatile compound is usually the product of the fermentation process (Begala *et al.*, 2002). Esters, which contribute to the fruity aroma of wines, are generally good indicators of fermented beverages' young age (Ferreira *et al.*, 2000).

Besides esters and ethanol, other volatile compounds including alkanes, volatile acids, carbonyl groups independently and interactively play a none dimensioning effect for the test and flavor of honey wine (Nan *et al.*, 2021). The other volatile compound found in the majority of the samples was alkanes, with average mean values ranging from 13.14 mg/L (TS3 sample) to 43.29 mg/L (TS5 sample). For all fermented honey wine samples, tetradecane was the dominant volatile compound under the alkanes group (Table 6.4). Although cyclopentasiloxane, dodecamethyl- compound was not detectable in the TS3 sample, it was the most abundant compound in all samples compared to other compounds in the alkanes category (Table 6.4). Benzaldehyde, which was observed in all samples, was the major volatile compound in the carbonyl group. Furfural, observed in some of the samples, was another minor compound in this category. Volatile acids, which ranged from 16.64 mg/L to 29.87 mg/L, were the other significant volatile compounds found in the fermented honey wine sample (Table 6.4).

Table 6. 4 Volatile compounds found in the majority of honey wine samples inoculated with various *Saccharomycetaceae* and *Lactobacillaceae* strains.

Volatile compounds (mg/L)	Honey wine test samples								
	TS1	TS2	TS3	TS4	TS5	TS6	TS7	TS8	Control
Esters									
Hexadecanoic acid, ethyl ester	1.64	2.18	1.82	2.84	2.71	1.99	2.09	2.09	2.27
Acetic acid, 2-phenylethyl ester	1.54	1.42	1.38	1.55	1.72	1.56	1.43	1.45	1.49
Octanoic acid, ethyl ester	13.77	16.99	14.87	15.94	16.40	14.72	15.00	17.01	21.28
Decanoic acid, ethyl ester	0.99	1.23	1.31	1.43	1.32	1.14	1.31	1.24	
Dodecanoic acid, ethyl ester	9.13	12.60	12.34	12.93	13.42	8.20	13.73	11.76	22.63
Methyl salicylate	2.56	2.96	3.29	3.10	3.26	2.55	3.20	3.21	3.58
1-Butanol, 3-methyl-/Isoamyl acetate	29.83	31.07	25.96	3.04	34.14	6.76			35.59
Hexanoic acid, ethyl ester	8.30	7.46	8.16	8.34	10.20	8.28	8.74	8.50	8.78
Ethyl 9-hexadecenoate	1.01	1.08		1.32	1.33	0.92	1.00		1.03
Ethyl 9-decenoate	1.19	1.36	1.24	1.46	1.45	1.10	1.29	1.34	1.66
Ethyl acetate	0.78	0.61	1.13		1.52	0.68		0.81	0.69
Alcohol									
Ethanol	411.20	399.20	321.20	378.40	455.20	430.80	448.40	382.80	440.80
Phenylethyl Alcohol	268.33	244.28	219.79	225.92	270.95	255.00	226.15	243.82	274.50
2-Heptanol	8.72	8.59	9.02	9.18	11.15	8.56	9.42	9.57	
2,3-Butanediol	8.49	9.45	8.97	9.85	11.21	11.00	10.03	12.16	1.22
1-Propanol, 2-methyl-	11.78	11.39	11.92	11.97	14.21	12.00		12.47	12.38
Carbonyl compounds									
Benzaldehyde	20.16	21.90	21.45	22.42	24.31	18.98	19.39	19.76	19.06
Anisaldehyde	7.95	7.51			9.02	8.80	9.09	9.07	8.75
Furfural	0.72		0.98			0.74		0.77	0.75
Alkanes									
Cyclohexasiloxane, dodecamethyl-	15.62	17.32		16.19	14.10	14.12	15.04	16.63	15.55
Cyclooctasiloxane, hexadecamethyl-	1.12	1.06	0.94	0.82	1.01		1.33	0.82	0.73
Cyclopentasiloxane, decamethyl-	0.98	0.97	2.12	0.97	0.94	0.93		0.97	0.92
Tetradecane	9.20	7.79	10.09	7.89	15.26	13.38	13.79	10.64	9.23
Dodecane	7.60	6.88		7.26	11.99	9.88	11.13	8.98	8.43
Acids									
Silanediol, dimethyl-	11.94	10.69	16.43	12.18	13.21	10.21	8.96	13.36	13.26
Benzene	11.02	11.56	13.44	12.37	14.46	12.02	7.68	12.36	12.50
Other Compounds									
2,4-Di-tert-butylphenol	38.82	42.09	28.34	41.22	44.15	36.34	33.50	38.21	38.35
15-Crown-5	1.04	0.62		1.22	0.77		0.86		
12-Crown-4	0.75	0.72	0.91	0.69			0.83	0.73	0.69
α-Terpineol	7.04	6.17			6.98		7.69	6.94	7.20

All values are the means of the duplicates

Table spaces left open is for the values below the detectable limit

TS1 – *S. cerevisiae*, TS2 – *S. cerevisiae* and *P. fermentans*. TS3 – *S. cerevisiae*, *P. fermentans*, and *W. anomalus*. TS4 – *S. cerevisiae* and *W. anomalus*, TS5 – *S. cerevisiae* and *L. hilgardii*, TS6 – *S. cerevisiae* and *L. parabuchneri*. TS7 – *S. cerevisiae* and *L. paracasei*. TS8 – *S. cerevisiae*, *L. hilgardii*, *L. parabuchneri* and *L. paracasei*.

Silanediol, dimethyl-, and Benzene were the volatile acids observed in all *Tej* samples. The volatile phenol 2,4-Di-tert-butyl-phenol, the other significant volatile compound observed in all of the samples, ranged from 28.34 mg/L (TS5 sample) to 42.09 mg/L (TS8 sample) (Table 6.4). The volatile compounds that were not observed in the majority of the samples were then plotted on the canonical correspondence analysis (CCA) plot. The compounds such as Benzaldehyde, 3,4-dimethyl-, 1-Butanol, 3-methyl-, 2-Pentanol, Formate, Benzoic acid, Hexadecane were only observed in one or two of fermented honey wine samples (Figure 6.5A). The majority of these compounds plotted on CCA are volatile compounds derived from microbial fermentation process (Englezos *et al.*, 2018). Furthermore, a Bray-Curtis principal coordinate analysis (PCoA) plot was utilized to assess dissimilarity between the honey wine tests and control samples based on the detected volatile components. Based on volatile compounds, TS5, TS8, and control samples clustered together on the PCoA plot, especially along the PCoA1 axis (Figure 6.5B). The clustered sample is a good indication of the similarity of the compounds for the samples mentioned above because the PCoA plot was generated from total volatile compounds (Wang et al., 2015). Moreover, a subjective analysis of honey wine test samples was performed using a seven-point hedonic sensory score test. Color, turbidity, alcohol aroma, astringency, honey-like aroma, and sourness were the tested sensory attributes of the samples. In this analysis, samples had a cluster pattern on the radar plot of a seven-point hedonic scale score for alcoholic aroma, astringency, sourness, and overall acceptance of sensory quality attributes. These sensory attributes are actually the results of volatile compounds present in the samples (Verzera *et al.*, 2008). The color of all test and control *Tej* samples had scored almost a similar result with no significant difference ($P > 0.05$) (Figure 6.5C). Nevertheless, there was a significant difference for other sensory attributes between the test samples ($P < 0.05$). Furthermore, TS5, TS6, TS7, and control samples showed a cluster pattern for most sensory attributes (Figure 6.5C). Specifically, these samples were made from honey-must fermentation inoculated with a mixed culture (*Saccharomyces* and *Lactobacillus*).

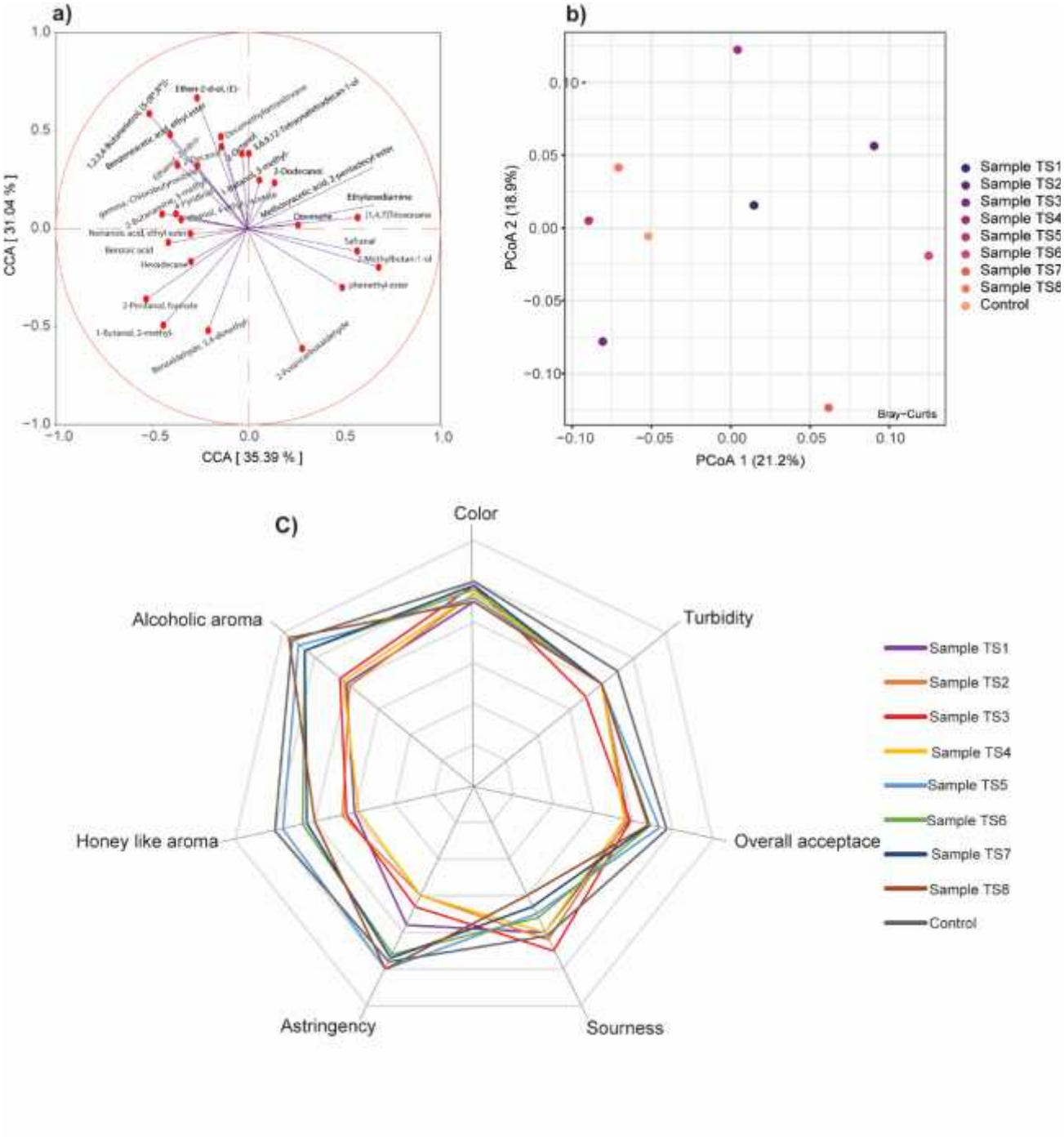

Figure 6. 5 The volatile compound and sensory properties of honey wine samples inoculated with various strain combinations **A**) CCA plot of minor volatile compounds, **B**) PCoA plot with Bray-Curtis dissimilarity, and **B**) Radar plot for the sensory analysis.

6.4 Conclusions

The isolated strains of *Saccharomycetaceae* and *Lactobacillaceae* for the purpose of starting honey wine fermentation had a good tolerance to osmotic pressure, a lower pH environment, and required minimal micro-nutrition. These isolates, with different combinations, were applied to honey-must to assess its ability to produce a good quality *Tej*. Generally, test samples inoculated with a mixed culture of *Saccharomyces* and *Lactobacillus* strains had lower residual sugar content and higher ethanol level. Besides, the volatile compounds and sensory attributes for these samples had shown a good similarity with the control sample. Particularly, TS5 and TS8 samples, inoculated by different combinations of *S. cerevisiae*, *L. hilgardii*, *L. parabuchneri*, and *L. paracasei* had a close quality attribute resemblance with that of the control sample. Thus, Ethiopian honey wine can be fermented using a direct inoculation system with either *S. cerevisiae* and *L. hilgardii* or *S. cerevisiae* and other *Lactobacillus* isolates (*L. hilgardii*, *L. parabuchneri*, and *L. paracasei*) without losing its major quality attributes.

CHAPTER 7

Kinetic Modeling for Nitrogen Limited Mixed Culture Honey Wine Fermentation System

Abstract

Stuck or sluggish fermentation is more likely phenomenon to occur when honey-must is fermented to make alcoholic beverages. This is primarily due to the fermentation medium's lower nitrogen content. As a result, the purpose of this chapter is to discuss the effect of nitrogen concentration on fermentation kinetics and to assess the possibility of developing a predictive kinetic model for a nitrogen-influenced mixed culture honey wine fermentation system. As a result, five nitrogen concentrations were added to honey-must: 100, 140, 180, 220, and 300 mg/L. The predictive abilities of three kinetic models (Monod, Teissier, and Ghose and Tyagi) were then compared. Finally, the model was validated at two extreme nitrogen supplement concentrations (100 mg/L and 300 mg/L). This was accomplished by simulating these models to estimate the optimal model parameter, and then performing model validation using the previously estimated parameter to determine the model's goodness of fit against the collected experimental data. As the nitrogen supplement concentration increases, both Yeast and Lactic acid bacteria strains showed fast growing rate by decreasing the lag phase. Besides, higher ethanol level was obtained as the concentration of nitrogen was increase to 300 mg/L. However, honey-must supplemented with nitrogen at the concentration of 180 mg/L shown optimum ethanol level and cell concentration. Furthermore, Monod model have a good predict capability for LAB growth rate, and sugar utilization kinetics. Teissier model, on the other hand, showed a good predictive ability for Yeast growth kinetics, nitrogen consumption rate, and ethanol production rate. Generally, the curve fitting and model validation revealed that, substrate limited models had better capture the nitrogen influenced mixed culture honey wine fermentation system. Moreover, the fermentation time of honey-must can be shorten by supplementation of nitrogen with an optimum concentration

Keywords: Nitrogen, kinetic modeling, data fitting, simulation, model validation

7.1 Introduction

Ethiopian honey wine, *Tej*, is one of the most commonly consumed traditional alcoholic beverages in country (Bahiru *et al.*, 2006; 2020; Lemi, 2020). It is mainly made from crude honey, and gesho (*R. prinoides*) (Bahiru *et al.*, 2001b). The detailed production process had intensively covered in the previous studies (Vogel & Gobezie, 1995). Like other traditional fermented alcoholic beverages, *Tej* is also produced by spontaneous fermentation system (Bahiru *et al.*, 2006). However, this spontaneous fermentation makes the product to have inconsistent quality, stuck and/or sluggish fermentation, off flavor development and sometimes failure to produce ethanol at the end of fermentation period. High osmotic stress, deficiency of important micro-nutrients, high level of ethanol, and extremely lower pH forces honey wine fermentation to face the aforementioned problems (Mendes-Ferreira *et al.*, 2010). For the better control of the process, upgrading this process to direct inoculation fermentation system is mandatory.

Previous research on Ethiopian traditional beverages in general, and *Tej* in particular, has focused on the final product's characterization (Lemi, 2020). The physicochemical properties of *Tej* samples collected from different areas of Ethiopia showed a considerable variation (Bahiru *et al.*, 2001b). However, the samples alcohol content was greater than 8.5% (v/v) (Bahiru *et al.*, 2001; Nemo & Bacha, 2020). Besides, the sample collected from a certain area of Ethiopia had shown a considerable antioxidant capacity (Fentie *et al.*, 2022). Furthermore, the microbial diversity of *Tej* were dominated by the species of *Saccharomyces* and *Lactobacillus* (Bahiru *et al.*, 2006a; Fentie *et al.*, 2022). The dominance of these species was also observed throughout the fermentation period (Fentie *et al.*, 2022). As a result, the first priority task for modernizing the process would be to develop a mixed culture inoculum that resembles this traditional fermentation system. Additionally, understanding the biomass growth, product formation and substrate utilization kinetics will further assist the upgrading of the process.

Mathematical model is one of the useful tools used to describe any process quantitatively (Goulet, 2016). This quantitative expression provides important information for design, analysis, economic calculations, operations and control of any biological process (Echiegu, 2015; Kostov *et al.*, 2012). However, due to the complexity of the biological process, accurately modeling the fermentation process is difficult (Formenti *et al.*, 2014; Goulet, 2016). The black box model (BBM), which is entirely derived from input-output experimental data, is the most commonly used model for capturing the dynamic behavior of biological processes (Sewsynker-Sukai *et al.*, 2017). The white

box model (WBM), which is based on the first principal model, is another type of model used to quantitatively describe biological processes (González-Figueredo *et al.*, 2019; D. Wang *et al.*, 2004). In previous studies, both BBM and WBM were used to model biological processes in general, and wine fermentation in particular (Arellano-Plaza *et al.*, 2007; Román *et al.*, 2011; Sipos *et al.*, 2020; Zentou *et al.*, 2019). Especially, mechanistic Unstructured nonsegregated model is frequently used to describe the fermentation kinetics (Ardestani, 2014; Arellano-Plaza *et al.*, 2007; Kostov *et al.*, 2012). This model considered cell and its components as a single species in the solution. The kinetic behavior of fermentation process is best explained by its kinetic parameters (Arellano-Plaza *et al.*, 2007). These parameters are usually calculated by using the biomass growth, product formation, and substrate utilization rates of the given fermentation process (Phisalaphong *et al.*, 2006). These parameters in turn are used to construct mathematical model used to adequately describe biological process (Cheirsilp *et al.*, 2007).

Modeling of mixed culture honey-must fermentation is even difficult as compared to modeling of many chemical reactions (Cheirsilp *et al.*, 2007). Harsh fermentation environment, deficiency of essential micronutrients, and interaction between the inoculated microbes complicate the development of accurate model for above mentioned fermentation process. Thus far, limited research had been conducted on the kinetic modeling and optimization of honey wine fermentation (Cuenca *et al.*, 2021). Most of these prior studies uses response surface methodology to achieve this optimization purpose (Gomes *et al.*, 2013; Srimeena *et al.*, 2014). To the best of our knowledge, there is no prior study on the kinetic modeling of batch mixed culture honey wine fermentation system. Thus, the aim of this chapter is to analyze the effect of nitrogen supplement on the mixed culture (*S. cerevisiae* and *L. hilgardii*) honey wine fermentation system. Furthermore, different kinetic models were simulated to for the best fit to the experimental data to determine the kinetic parameters for the given nitrogen limited fermentation system. These models were ultimately aimed to predict the biomass growth, product formation and substrate utilization rate even before the honey-wine fermentation commenced.

7.2 Materials and Methods

7.2.1 Propagation of microorganisms

Both strains of microorganisms used as starter cultures in this study were isolated from *Tej* samples collected from various locations across Ethiopia. *S. cerevisiae AAF02* was recovered from the stock

solution by streaked on yeast extract peptone dextrose agar (YPD) incubate for at 30 °C for a period of 24 h. Similarly, *L. hilgardii AAB09* strain was initiated by streaking on de Man, Rogosa, and Sharpe (MRS) agar and incubated for a period of 48 h at 30 °C. A loopful of these strains were again inoculated to the respective broth and incubated with the same conditions mentioned above. Finally, they were then centrifuged, washed and suspended in sterile saline solution (0.85% NaCl).

7.2.2 Inoculation and fermentation

First honey was mixed with water at 1:3 ratio. It was then pasteurized at 65 °C for a period of 15 min holding time. Filter sterilized diammonium phosphate (DAP) solution was then added to the previously pasteurized and cooled honey-must mixture with the final concentration of 100 mg/L, 140 mg/L, 180 mg/L, 240 mg/L, and 300 mg/L. The propagated strains of were then inoculated to the fermentation medium with a final concentration of 10^3 and 10^2 cfu/mL for *S. cerevisiae AAF02* and *L. hilgardii AAB09*, respectively. With the exception of pasteurization, nitrogen source supplementation, and inoculation with a defined starter culture, all other processing steps to make this honey wine were identical to those used in the past (Vogel & Gobezie, 1995). The fermentation was carried out in a 50 ml screwed cap conical flask to make sampling easier during the process. Samples were taken every six hours for period of 120 hours for microbial and assimilated nitrogen kinetics. Whereas sugar, ethanol and lactic acid concentrations were measured every 12 hours during the whole fermentation period.

7.2.3 Analytical methods

7.2.3.1 Microbial count

The microbial growth was monitored in triplicate by using microbial cultivation method. The growing medium and incubating conditions were exactly the same as stated in section 7.2.2. The grown colonies within the range from 25 to 250 were counted and reported with its respective dilutions factor.

7.2.3.2 Sugar profiling

Sugar profiling was done by using the method described in section 3.3.3

7.2.3.3 Ethanol quantification

Ethanol quantification were performed according to the methods described in section 3.3.4

7.2.3.4 Lactic acid quantification

Lactic acid quantification was performed according to the methods described in section 6.2.5.3

7.2.3.4 Yeast assimilated nitrogen (YAN) quantification

Yeast assimilated nitrogen (YAN) was measured by formaldehyde titration methods used by Pereira et al., (2013).

7.2.4 Fitting the model data

Model fitting were performed by using MATLAB 2020b software. Nonlinear regression function was used for parameter estimations. The function will accept the empirical data, the exact time points (X) at which the experimental data was collected, the simulated results at X, and initial guess of parameter values. The system was then integrated, which takes initial parameter values and time points as input to perform the integration. Then evaluation of the system solution was obtained from the function at X.

7.2.5 Model validation

Model validation was carried out once more using the MATLAB 2020b software. At this point, the models were evaluated for their accuracy in predicting the microbial growth rate, substrate consumption, and product formation for mixed culture honey-must fermentation system by plugging the previously estimated parameters into ordinary differential equations to generate the necessary data. This data is then compared with the measured experimental data using *goodnessOfFit*(.) function from MATLAB software. This function takes as inputs test data, simulated data from the model, and a cost function. The goodness of fit is determined by the cost function. This cost function is the error between predicted and input test data. Normalized Mean Square Error (NMSE) were used for these statistical calculations by using the following equation:

$$\text{NMSE}=1-\frac{\|\text{actual} - \text{predicted}\|^2}{\|\text{actual} - \text{mean of actual}\|^2} \qquad 7.1$$

Where: NMSE $\in [-\infty, 1]$, where $-\infty$ indicates a bad fit and 1 a perfect fit

7.3 Results and Discussion

7.3.1 Nitrogen influenced fermentation rates

The main purpose of fermentation is conversion of sugar to ethanol with the help of fermentative microorganism (Walker & Stewart, 2016b). However, under certain conditions, fermentation may take longer to complete (sluggish fermentation) or may leave a higher sugar concentration (stuck fermentation) (Alexandre & Charpentier, 1998; Malherbe *et al.*, 2016). Lower oxygen and nitrogen level, higher ethanol and sugar level, and extreme pH and temperature are the main cause for the occurrence of abnormalities in the fermentation process (Blateyron & Sablayrolles, 2001). From the aforementioned factors, nitrogen limitation takes the lion-share contribution on creating abnormal fermentation especially for the honey fermentation (Almeida *et al.*, 2020). Thus, it is common practice to supplement the honey-must with nitrogen, preferably early in the alcoholic fermentation process (Mendes-Ferreira, Oliveira, *et al.*, 2015). This is because assimilable nitrogen highly related to microbial growth and metabolism.

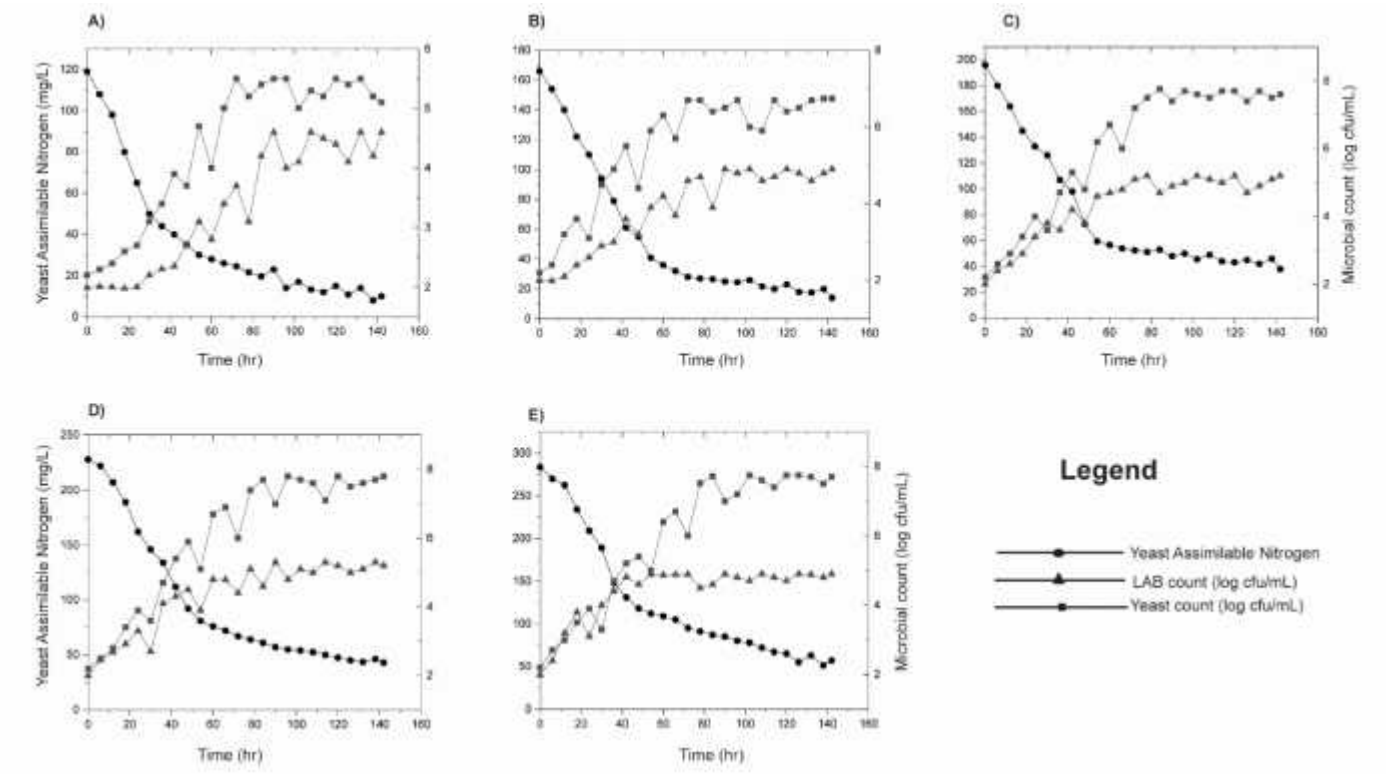

Figure 7. 1 Yeast and LAB growth kinetics, and YAN utilization kinetics for honey-must fermentation medium supplemented diammonium phosphate (DAP) at a concentration of **A**) 100 mg/L, **B**) 140 mg/L, **C**) 180 mg/L, **D**) 220 mg/L, and **E**) 300 mg/L

The microbial growth rate, and nitrogen utilization rate for different nitrogen supplement concentration is illustrated in Figure 7.1. The fermentation must that was supplemented with 300 mg/L DAP had a higher final fermentative yeast count (7.7 log cfu/mL). In contrast, the fermentative yeast count was lower (5.1 log cfu/L) in the fermentation must that was supplemented with DAP at a concentration of 100 mg/L. Furthermore, an increase in DAP supplementation resulted in a continuous increase in the fermentative yeast count (Figure 7.1). These findings, however, contradicted the other finding on the fermentation of grape wine supplemented with different DAP concentrations. They found that increasing the YAN concentration above 140 mg/L had no effect on the growth of fermentative microorganisms (Mendes-Ferreira *et al.*, 2004). Furthermore, increasing the nitrogen level above this point may result in the production of undesirable metabolites (Ribeiro *et al.*, 2003). The precise nitrogen requirement, however, is determined by the type of fermenting strains, processing conditions, and the quality of the nitrogen supplement. As a result, the deviation in our result could be due to one of the aforementioned causes.

Lactic acid bacterial (LAB) cells also showed an increase in final microbial cell concentration up to 180 mg/L DAP supplement concentration (Figure 7.1). There was no discernible trend of increase in the final cell concentration of LAB after this level of ammonia concentration. However, the LAB cell concentration was still lower than that of the yeast cell concentration. This phenomenon was also observed in the quantification of total bacteria and total yeast by quantitative-PCR performed during the spontaneous fermentation of (Fentie *et al.*, 2022). This could be due to either the Yeast's strong inhibitory effect on the LAB or the harsh environment created during *Tej* fermentation.

Besides, the ammonia supplement concentration has an effect on the shortening the lag phase of microbial growth (Figure 7.1). To be more specific, the lag phase was shortened when the fermentation medium was supplemented with DAP concentrations greater than 140 mg/L. Not only was the lag phase affected by ammonium supplement concentration, but so was the exponential growth rate. The greater the ammonium concentration, the greater the exponential microbial growth rate (Figure 7.1 B–E). Because nitrogen is the only limiting substrate during honey fermentation, when there is excess nitrogen on the fermentation medium, the microbes proliferate more quickly (Oliveira, *et al.*, 2015). After these exponential growth phases were completed, all fermentation mediums with different ammonium concentrations entered the stationary phase.

Regardless of the initial ammonium concentration, yeast assimilable nitrogen (YAN) has decreased as the fermentation has progressed to the end (Figure 7.1). However, higher rate of utilization was

observed for fermentation medium supplemented with diammonium phosphate at the concentration of 100 mg/L (Figure 7.1A). Furthermore, nitrogen utilization was higher at the start of fermentation and reached a nearly constant level after the microbial growth phase was switched from exponential to stationary. This could be because the microbes' desire to consume nitrogen decreases as they reach the stationary phase (Torrea *et al.*, 2011). However, the fermentation medium supplemented with diammonium phosphate at the concentration of 300 mg/L showed a progressive decrease in YAN even after the microbes had entered to stationary phase (Figure 7.1E). This could be due to high biomass concentration for this particular fermentation medium.

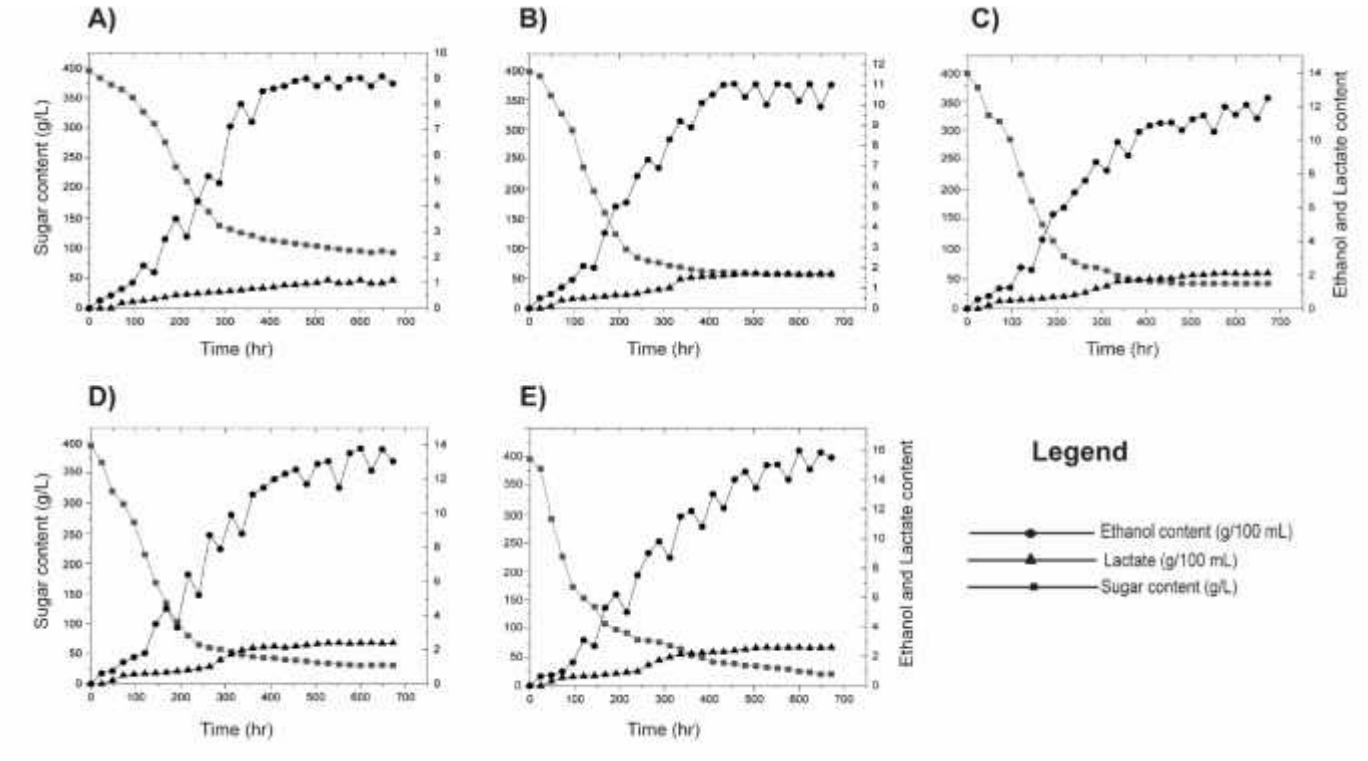

Figure 7. 2 Ethanol and Lactate production, and Sugar utilization kinetics for honey-must fermentation medium supplemented diammonium phosphate (DAP) at a concentration of **A**) 100 mg/L, **B**) 140 mg/L, **C**) 180 mg/L, **D**) 240 mg/L, and **E**) 300 mg/L

Almost immediately after the end of the exponential microbial growth phase, ethanol production and sugar consumption began (Figure 7.1 & 2). Higher ethanol concentration (15.5 g/100 mL) was obtained for the fermentation medium supplemented with diammonium phosphate at the concentration of 300 mg/L (Figure 7.2E). Conversely, the lower ethanol level was (8.8 g/100 mL) was obtained for the honey-must supplanted with diammonium phosphate at a concentration of 100

mg/L (Figure 7.2 A). Nevertheless, a continual increment of ethanol level was observed for an increasing in diammonium phosphate concentration. The higher yeast and LAB concentrations in these fermentation mediums could be the likely cause of the higher ethanol content. Similar phenomenon was also observed for the fermentation of honey wine with different nutrient supplement (Twilley *et al.*, 2018).

Higher nitrogen concentrations resulted in a faster rate of sugar utilization. The residual sugar in fermentation medium supplemented with 100 mg/L diammonium phosphate was higher (92.5g/L) than in fermentation medium supplemented with 300 mg/L diammonium phosphate (Figure 7.2A). Higher biomass concentration and conversion factor may have a significant impact on this result. Lactate concentrations were lower in almost all of the samples supplemented with different nitrogen concentrations than ethanol concentrations. This could be due to the lower LAB in comparison to the yeast concentration. Furthermore, the hetro-fermentative nature of the inoculated LAB may contribute to the higher ethanol content than lactate content. Nonetheless, the lower lactate concentration may have a positive effect on the final product's sensorial quality. However, the nitrogen concentration has still an effect on the final lactate concentration. For an initial nitrogen concentration of 300 mg/L, a higher lactate concentration (2.6 g/100 mL) was obtained (Figure 7.2 E). Base on the final ethanol content, cell concentration and residual sugar level diammonium phosphate supplemented at the concentration of 180 mg/L was chosen for kinetic modeling study.

7.3.2 Model development

Simple models are required to provide a solid foundation for the design of fermentation processes, economic calculations, and fermentation process control. Modeling necessitates simplifications of the complex biological system, which is also a major goal of modeling (Annuar *et al.*, 2008). The majority of the fermentation models that have been developed are biochemically knowledge-based models (Jin *et al.*, 2012; Phisalaphong *et al.*, 2006; Zentou *et al.*, 2019). These unstructured kinetic models have advantages in describing the biological phenomena occurred during the fermentation process (Wang *et al.*, 2004). Likewise, in this study, unstructured kinetics models were also applied to examined for better describing the dynamic behavior of batch mixed culture honey wine fermentation. These models were aimed to describe *S. cerevisiae* and *L. hilgardii* growth rate, nitrogen and sugar consumption rate, ethanol and lactic acid production rate of this mixed culture fermentation system. The following models were proposed with an assumption of 1) all products formations are none growth associated 2) consumption of nitrogen is not associated with product

formation. It was assumed that it was solely used for microorganism growth. 3) sugar consumption is entirely associated with the product formation 4) *L. hilgardii* strains were assumed as not participated in ethanol production. 5) due to the honey-must high sugar concentration, competition for sugar by fermenting microbes were neglected 6) maintenance requirements for the limiting substrate could also assumed to be small to be neglected

$$d_{v,l}/d = \mu_l X_{v,l} \quad 7.2$$

$$dP_i/dt = q_i X_i \quad 7.3$$

$$dN/dt = -\mu_Y X_Y/Y_{Y/N} - \mu_L X_L/Y_{L/N} \quad 7.4$$

$$dS/dt = \mu_Y X_Y/Y_{e/S} - \mu_L X_L/Y_{l/S} \quad 7.5$$

Where, *i* was used to indicate the muti-microbes or muti-product expression. The other nomenclatures: X, P, N, and S were used to denote cell, product, nitrogen and sugar concentrations, respectively. Similarly, μ and *q* were used to denote the specific microbial growth rate and specific product formation rate, which had a non-linear mathematical dependance of product and substrate. Table 7.1 demonstrated the possible dependance which needs to be checked by curve fitting. The constants: $Y_{Y/N}$, $Y_{L/N}$, $Y_{e/S}$, and $Y_{l/S}$ were again used to denote yield coefficients [g/g] used to specify the microbial (yeast and LAB) growth and product (ethanol and lactic acid) formations from the respective consumed substrates. Furthermore, in our pervious experiments, the growth of *S. cerevisiae* was not significantly affected by the co-culturing with *L. hilgardii*. However, inhibitory effect was observed on *L. hilgardii* due to the presence of *S. cerevisiae*. This observation was also demonstrated in the previous culture independent microbial quantifications. Thus, the following expression was incorporated to the model equations to properly express the effect of *L. hilgardii* inhibition

$$1- \varepsilon X_v \quad 7.5$$

7.3.3 Fitting the model to experimental data

Due to microbial interaction and competition, modeling the mixed culture fermentation system using only the first principal model might not adequately capture the kinetics. Most of the time, finding an adequate model for the above-mentioned fermentation system is extremely difficult (Mu *et al.*, 2006). However, when there is enough nutrient and a proven interaction between the microbial

strains, modeling will be less complicated than in the preceding case (Cheirsilp *et al.*, 2007). To describe the kinetic behavior of any fermentation process, several approaches can be used. The most common are kinetic models that are influenced by substrate limitation, substrate inhibition (due to high concentration), and product inhibition (Muloiwa *et al.*, 2020). In this study, however, the basic models were systematically chosen only for substrate limitation and product inhibition. This is because the strains used to ferment honey-must have been shown to have a higher sugar tolerance. Thus, two substrates limiting (Monod and Teissier) and one product inhibition (Ghose and Tyagi) model were used to evaluate the model's adequacy to describe the fermentation of honey-must (Table 7.1). Each models' parameters were estimated by using non-linear regression between the simulated data and experimental data collected during biomass growth, substrate utilization, and product formation. These simulated data is obtained by integrating the system ordinary differential equation (ODE) by using the Ruge-Kutta 4th and 5th algorithm (Zentou *et al.*, 2019).

Table 7. 1 Kinetic models dependance on substrate limitation and product inhibition for *S. cerevisiae* and *L. hilgardii*

S. cerevisiae					
S/N	**Model name**	**μ_Y**	**q_Y**	**Effect to capture**	**Reference**
1	Monod	$\mu_{max,Y}\frac{N}{K_{sx,Y}+N}$	$q_{pmax}\frac{S}{K_{sp,E}+S}$	Substrate limitation	(Monod, 1949)
2	Teissier	$\mu_{max,Y}\left(1-\exp(-\frac{N}{K_{sx,Y}})\right)$	$q_{pmax}\left(1-\exp(-\frac{S}{K_{sp,E}})\right)$	Substrate limitation	(Teissier, 1942)
3	Ghose and Tyagi	$\mu_{max,Y}\left(1-\frac{P}{P_{xmax}}\right)$	$q_{pmax}\left(1-\frac{P}{P_{pmax}}\right)$	Product inhibition	(Ghose & Tyagi, 1979)
L. hilgardii					
		μ_L	**q_L**		
1	Monod-Cheirsilp	$\mu_{max,L}(1-\varepsilon_\mu X_y)\frac{N}{K_{sx,L}+N}$	$q_{pmax}\frac{S}{K_{sp,L}+S}$	Substrate limitation	(Cheirsilp et al., 2007; Monod, 1949)
2	Teissier-Cheirsilp	$\mu_{max,L}(1-\varepsilon_\mu X_y)\left(1-\exp(-\frac{N}{K_{sx,L}})\right)$	$q_{pmax}\left(1-\exp(-\frac{S}{K_{sp,L}})\right)$	Substrate limitation	(Cheirsilp et al., 2007; Teissier, 1942)
3	Ghose and Tyagi-Cheirsilp	$\mu_{max,L}(1-\varepsilon_\mu X_y)\left(1-\frac{P}{P_{xmax}}\right)$	$q_{pmax}\left(1-\frac{P}{P_{pmax}}\right)$	Product inhibition	(Cheirsilp et al., 2007; Ghose & Tyagi, 1979)

ε is the effect factor of *S. cerevisiae* on *L. hilgardii* growth rate, since it had inhibitory effect, it was expressed by $(1-\varepsilon X_Y)$

After obtaining of optimized parameters by simulation and nonlinear regression the proposed models were compared for the best fit for the mixed culture microbial honey-must fermentation

(Figure 7.3). Most of these models face a difficulty to capture the microbial growth dynamics especially at the exponential growth phase (Figure 7.3A & B). This result was particularly very true for the yeast growth (Figure 7.3A). The most likely explanation for this result could be the model structure that assumes nitrogen as sole substrate for microbial growth and/or the ignorance of microbial growth maintenance parameter. Besides, the viable cell count that we used in this study, which differs from previous studies that typically used a dry cell mass, may also contribute for these lower model adequacies for the aforementioned three models (Arellano-Plaza *et al.*, 2007). At later stage of fermentation, stationary phase, the substrate limited models (Monod and Teissier) had shown a better fit for microbial growth kinetics than their counter part of product inhibition model (Ghose and Tyagi) (Table 7.2). This is a indication that products inhibition have a lower impact especially when it comes with to the microbial growth kinetics. The root cause might be due to the low product concentration during the active growth phase of the microbes. Similar finding was also observed nitrogen limited grape wine fermentation (Cramer *et al.*, 2002).

Table 7. 2 Kinetic parameters obtained by numerical calculations using the proposed kinetic models.

Rate kinetics	**Kinetic Parameters**	**Proposed Kinetic models**					
		Monod		Teissier		Ghose and Tyagi	
		Values	NMSE	Values	NMSE	Values	NMSE
Yeast growth kinetics	$\mu_{max,Y}$ (hr^{-1})	0.453	0.78	0.846	0.81	4.519	0.53
	$K_{sx,Y}$ (g /L)	0.0241		0.018		1.471	
LAB growth kinetics (Includes Cheirsilp model)	$\mu_{max,L}$(hr^{-1})	0.163	0.97	0.573	0.87	3.168	0.74
	$K_{sx,L}$ (g /L)	0.019		0.037		1.693	
Ethanol production kinetics	$q_{max,Y}$ (hr^{-1})	0.362	0.75	0.426	0.95	2.673	0.56
	$K_{sp,Y}$ (g /L)	25		18		10	
Lactate production Kinetics	$q_{max,L}$(hr^{-1})	0.846	0.69	1.46	0.53	2.694	0.50
	$K_{sp,L}$(g /L)	10		12		23	
Nitrogen consumption kinetics	$Y_{Y/N}$ (g/g)	21	0.89	32	0.98	22	0.79
	$Y_{L/N}$ (g/g)	8		5		18	
Sugar consumption Kinetics	$Y_{e/S}$ (g/g)	0.849	0.97	2.64	0.89	5.74	0.77
	$Y_{l/S}$ (g/g)	0.097		1.34		2.67	
		Monod-Cheirsilp		Teissier-Cheirsilp		Ghose and Tyagi-Cheirsilp	
Inhibition effect	ε_{μ}	0.355		0.155		0.473	

The growth of LAB was relatively simulated with a good fit by the Monod-Cheirsilp model. This model is the extension of Monod model by including inhibition effect cause by fast growth of Yeast cells (Cheirsilp *et al.*, 2007; Monod, 1949). The model actually captured both the exponential and stationary phase of LAB growth in the honey-must fermentation medium (Figure 7.3 B). Hence, the substrate limitation and yeast inhibition adequately capture the LAB growth with a good fit (NMSE=0.97). Over all, the substrate limitation model had shown a relatively better fit than the product inhibition model for both microbial strain growth kinetics during honey-must fermentation (Table 7.2).

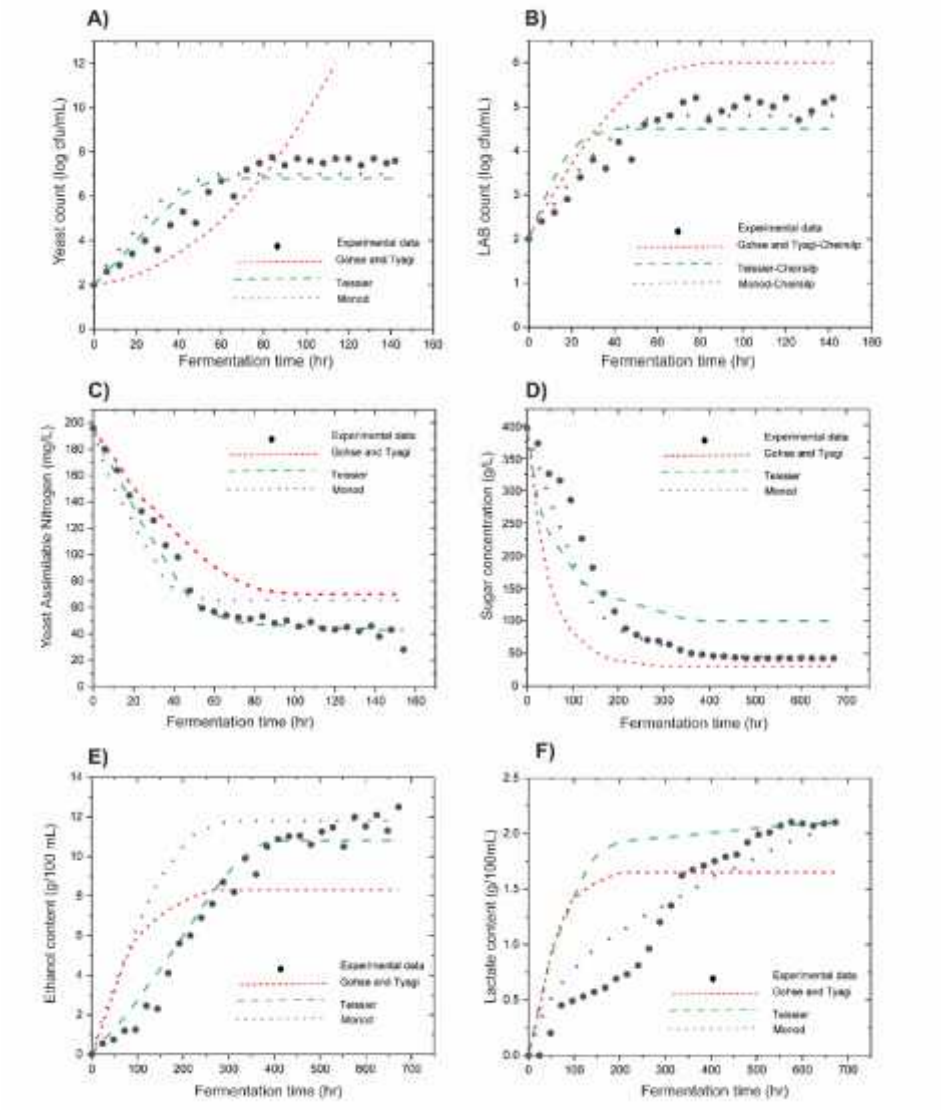

Figure 7. 3 Nitrogen supplemented Tej fermentation kinetic model comparisons for **A**) Yeast growth, **B**) Lactic acid bacteria (LAB) growth, **C**) Yeast assimilable nitrogen (YAN) consumption, **D**) Sugar utilization, **E**) Ethanol production, and **F**) Lactate formation

Substrate utilization kinetic model fitting, like microbial growth kinetics, was accomplished through simulation and regression of the ordinary differential equation (Eq 7.4 & 7.5) in conjunction with the aforementioned three models. However, unlike the microbial growth kinetic model, the simulation for substrate utilization kinetics fit the experimental data better (Figure 7.3C & D). Particularly, Teissier model had shown a better fit for nitrogen utilization kinetics as compared to other models (Table 7.2). This could be basically due to the inherit nature of the model which has the ability to capture the dynamics YAN consumption rate at a lower microbial inhibition (Dutta, 2015). On the other hand, Monod model-based simulation had shown a better fit for sugar utilization kinetics (Figure 7.3D). In this study, for the purpose of simplicity, utilization of sugar was exclusively given for the production ethanol and lactate. The best fit by Monod could be again due to the inherent structure of the model. Usually, Monod performs well for the process that has: 1) high substrate concentrations 2) no substrate inhibition 3) matured cell growth (Muloiwa *et al.*, 2020). Since our fermentation system had a higher sugar concentration together with a fully matured microbial culture, the good fit (NMSE = 0.97) obtained by simulating Monod based differential equation is not a surprising result. Similar to microbial growth the product inhibition-based model (Ghose and Tyagi) had a lower fit to the experimental data for both nitrogen as well as sugar consumption kinetic modeling (Figure 7.3C).

Table 7. 3 Normalized mean square error values of the model validated models

Process kinetics	**NMSE values**	
	100 mg/L	300 mg/L
Yeast growth kinetics (*Teissier model*)	0.79	0.90
LAB growth kinetics (*Monod-Cheirsilp model*)	0.69	0.98
Nitrogen utilization kinetics (*Teissier model*)	0.97	0.91
Sugar consumption kinetics (*Monod model*)	0.89	0.97
Ethanol production kinetics (*Teissier model*)	0.99	0.98
Lactate production kinetics (*Monod model*)	0.53	0.62

The model comparisons between the proposed three models were again performed for ethanol and lactate product formation kinetics. The results showed that Teissier model provided a good fit (NMSE = 0.95) for the optimized kinetic parameters to the ethanol production experimental data (Figure 7.3E). Nevertheless, even this model had a problem on capturing the production of ethanol at early stage of production. On the other hand, lactate production kinetics was relatively fitted by

using Monod model (Figure 7.3F). However, still Monod model had a lower level of adequacy for capturing the dynamics of lactate production. The lower NMSE (0.69) value of Monod model for lactate production is the good testimony for this argument (Table 7.2). This could be due to the lower level of lactate production during *Tej* fermentation process. Nonetheless, the models based on substrate limitation still plays a pivotal role in the better simulating the substrate utilization kinetics (Table 7.2). In addition to the model structures, the particular microbial strains behaviors for tolerance of higher ethanol and sugar concentration could also contributed for this result (Mahanta *et al.*, 2014).

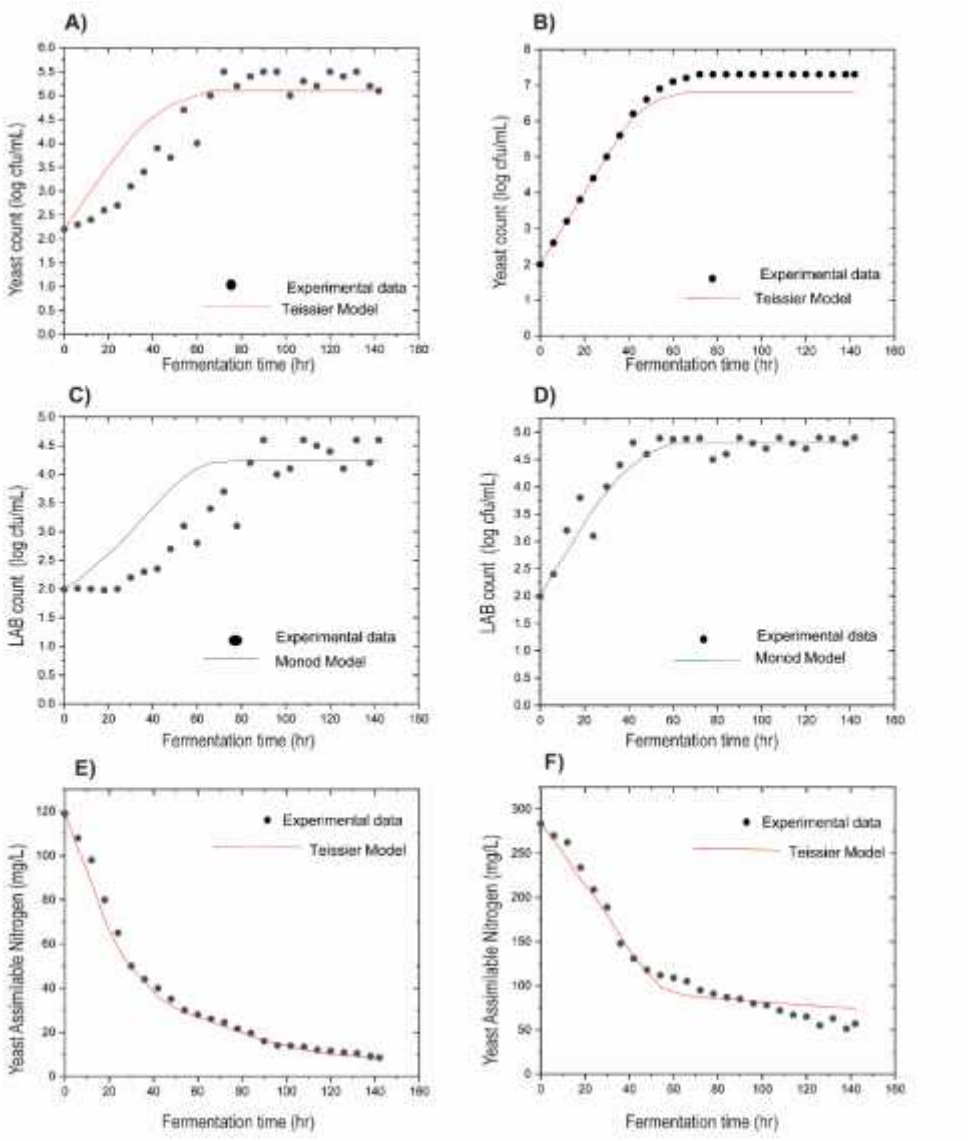

Figure 7. 4 Model validation for Yeast growth rate at nitrogen concentration of **A**) 100 mg/L, **B**) 300 mg/L, LAB growth rate at nitrogen concentration of **C**) 100 mg/L, **D**) 300 mg/L, YAN utilization at nitrogen concentration of **E**)100 mg/L, and **F**) 300 mg/L

7.3.4 Model validation

After the optimization of parameter, the best fit models were selected from the pools based on NMSE values for further model validation stage. Thus, Teissier model was selected for validation to predict Yeast growth, nitrogen utilization and ethanol production kinetics. Monod model, on the other hand, used to predict the LAB growth, and sugar utilization kinetics. The validity of these models was checked by using the prior optimized parameter values listed in Table (7.2) and the experimentally collected data for predicting microbial growth, substrate consumption and product utilization for honey-must fermentation which were supplemented with nitrogen concentration at 100, and 300 mg/L.

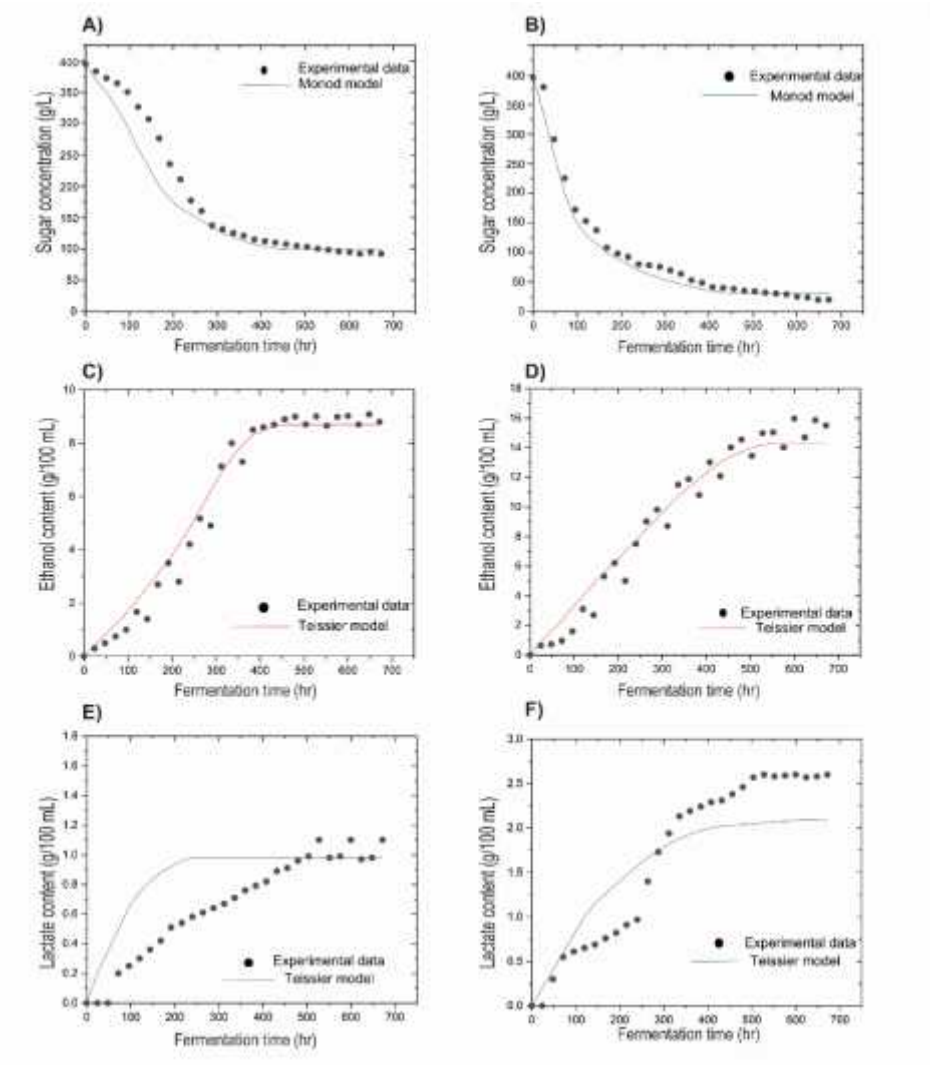

Figure 7. 5 Model validation for sugar consumption rate at nitrogen concentration of **A**) 100 mg/L, **B**) 300 mg/L, ethanol production rate at nitrogen concentration of **C**) 100 mg/L, **D**) 300 mg/L, Lactate formation at nitrogen concentration of **E**)100 mg/L, and **F**) 300 mg/L

The validation of Teissier model for yeast growth, showed lacks of good fit (NMSE = 0.79) for the yeast growth kinetics for the fermentation medium supplemented with 100 mg/L (Figure 7.4A). This could be due to the longer lag period observed in this fermentation system due to the lower nitrogen concentration. Furthermore, the Teissier model has inherent limitations in describing fermentation systems with a longer lag phase (Jin et al., 2012). The same result and justification were observed for LAB growth rate prediction (NMSE = 0.69) by Monod-Cheirsilp model for the fermentation medium supplemented with 100 mg/L (Figure 7.4C). Besides, Monod model didn't capture the lactate production rate (NMSE = 0.53) for neither of the two-nitrogen concentration fermenting honey-must. Actually, these data do have a problem even during the data fitting section of this chapter (Figure 7.3F). Thus, it is expected to have a very lower NMSE for the given model simulation at specified parameter. Other than the aforementioned kinetics, all other rates were adequately predicted by using either one of the two model with a good fit (Figure 7.4 & Table 7.3).

7.4 Conclusions

Supplementation of honey-must with different concentration of nitrogen had a big influence of the increasing the fermentation rate of honey-must. As the concentration of nitrogen increases so does the total microbial count and ethanol level of the final product. The fermentation medium supplemented with 180 mg/L nitrogen showed an optimum cell concentration and ethanol level. The developed unstructured kinetic model had adequately captured the substrate utilization and ethanol formation rates. Furthermore, LAB growth rate at fermentation medium supplemented by 300 mg/L had shown a good fit. However, the predictive kinetic model had faced difficulties to capture the growth rate of both Yeast and LAB at a lower concentration of nitrogen. This is mainly caused by a longer lag period for a lower nitrogen concentration. Besides the model fail to describe the lactate production rate due to the lower production volume. In general, supplementing the honey-must with a nitrogen is one of decreasing the probability of occurrence of stuck and/or sluggish fermentation. Moreover, substrate limited unstructured kinetic models adequately describes the rate of substrate consumption and product formation during mixed culture honey wine fermentation.

CHAPTER 8

General Conclusions and Recommendations

8.1 Conclusions

Alcoholic fermentation is the process of converting a certain carbon source in to ethanol by using fermentative microorganisms. *Tej* is one of the popular spontaneously fermented Ethiopian alcoholic beverages usually made from honey and gesho. The production process varies from location to location, as well as from household to household. For example, instead of honey, some producers use cane sugar as the primary fermentative substrate. Besides of sugar cane, these producers use malt powder and colorant to make this traditional beverage. Furthermore, some producers use fresh gesho leaves rather than boiled gesho stems and leaves. Excellent quality *Tej*, according to producers and consumers, is manifested as yellow in color, and less viscous liquid with a known test of gesho (*R. prinoides*) and ethanol.

Spontaneously fermented *Tej* samples collected from different location and households showed a considerable variation in their physicochemical properties. Acidity, residual sugar level, ethanol level, and antioxidant values, for example, differed between *Tej* samples from different sources. In addition, there were differences in the bacterial community structure between the samples. Essentially, this distinction was much more overserved for area-based comparisons than for household comparisons. Regardless of this variation, the *Lactobacillus* and *Zymomonas* species dominated the entire sample. However, there was no difference in the fungal community structure between the samples. They were all completely dominated by *Saccharomyces* species.

The physicochemical and microbiological qualities of *Tej* had changed over time during the successful fermentation process. As the fermentation progressed to the end, the acidity, ethanol level, and antioxidant capacity of *Tej* increased, while the sugar level decreased. All of these physicochemical parameter dynamics indicate that a successful fermentation process is taking place. *Tej*'s bacterial community dynamics had also changed, going from a diverse to a more dominated microbial strain. The fungal community, however, was once again dominated by *Saccharomyces* species during the whole fermentation period. As a result of this microbial dynamics study, it was discovered that these two kingdoms can coexist from the start of fermentation to the end. Furthermore, bacterial communities are half as large as fungal communities throughout the fermentation process.

Lactic acid bacteria and yeast strains were isolated from *Tej* samples with the expectation that they could be used as starter cultures for Ethiopian honey wine fermentation. The isolated yeasts were genotypically identified as *S. cerevisiae, P. fermentans, W. anomalus*. Similarly, the isolated LAB were genotypically identified as *L. hilgardii*, *L. paracasei*, and *L. parabuchneri*. These isolates have shown a good tolerance for low pH, and high osmotic pressure. Because of these characteristics, they can be considered as an ideal strain to use as mixed starter culture for the fermentation of *Tej*. A comparison study for the set of starter culture mixtures revealed that the more the starter culture is mixed, the more it resembles the physicochemical parameters of the control sample. Samples inoculated with *S. cerevisiae* and *L. hilgardii*, in particular, had a striking similarity to the control samples. As a result, these strains can be considered a serious candidate for use as a mixed starter culture for honey-must fermentation.

The addition of nitrogen to the honey-must had a significant impact on the fermentation time. The rate of honey wine fermentation was increased as the concentration of supplemented nitrogen increased. However, as the nitrogen concentration increased, so did the final alcohol content and cell concentration, even after the required fermentation period has been completed. Models of substrate limitation and product inhibition were used to fit the experimental data and predict microbial growth, substrate consumption, and product formation rates. Tessier and Monod, two substrate limiting models, showed ag good fit for the 180 mg/L nitrogen supplemented honey wine fermentation. However, these models are limited in their ability on adequately capturing the dynamic behavior of lower concentration nitrogen supplemented honey-must fermentation.

In general, upgrading spontaneous *Tej* fermentation to direct inoculated fermentation system was unavoidable in order to produce consistent quality of the final product. While *Tej* ferments naturally, both bacterial and fungal communities play an important role. As a result, in order to produce *Tej* through a direct inoculated fermentation system without compromising its major quality parameters, the inoculum must be composed from both bacteria and fungi strains. In this study, *Tej* produced by inoculating a mixed culture of *S. cerevisiae* and *L. hilgardii*, in particular, had shown a quality parameter similar to the control sample.

8.2 Recommendations

This study, which was entirely focused on the development of a direct fermentation system for spontaneously fermented Ethiopian honey wine, yielded several significant findings. Nonetheless, due to the breadth of the subject, several gaps remain to be filled in order to ensure a smooth transition of this process. The following are some of the most critical gaps that must be filled in the near future.

- Evaluation of the physicochemical and microbiological diversity of *Tej* samples collected from different sociocultural societies and geographical locations other than those covered in this study.
- Isolation of more microbial strains from various sources that have a high ability to perform under harsh environmental conditions and could be used as a starter culture for honey wine fermentation.
- Examining the effect of the method and amount of gesho added to the honey-must mixture, as well as processing parameters (temperature, mixing rate, substrate concentration, inoculum volume, and so on) on the fermentation system and *Tej* quality parameters.
- Evaluation of the isolated strains' safety, technological importance, function properties, commercial viability, and mixed culture strain proportions to produce a high-quality product *Tej*
- Design of *Tej* fermentation medium with an emphasis on micronutrient supplementation to increase the likelihood of successful fermentation
- Evaluating the impact of upstream and downstream processes on the quality *Tej*

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Appendices

Appendix 1- List of publications

Fentie, E. G., Emire, S. A., Demsash, H. D., Dadi, D. W., & Shin, J.-H. (2020). Cereal- and fruit based Ethiopian traditional fermented alcoholic beverages. *Foods*, *9*(12), 1–16.

Fentie, E., Jeong, M., Emire, S., Demsash, H., Kim, M. A., Jeon, H.-J., Lee, S.-E., Tagele, S., Park, Y.-J., & Shin, J.-H. (2022). Physicochemical properties, antioxidant activities and microbial communities of Ethiopian honey wine, *Tej*. *Food Research International*, *152*.

Fentie, E. G., Jeong, M., Emire, S. A., Demsash, H. D., Kim, M. A., Jeon, H.-J., Lee, S.-E., Tagele, S. B., Park, Y.-J., & Shin, J.-H. (2022). Microbiome dataset of spontaneously fermented Ethiopian honey wine, *Tej*. *Data in Brief*, *42*, 108022.

Fentie, E. G., Jeong, M., Emire, S. A., Demsash, H. D., Kim, M. A., & Shin, J.-H. (2022). Fermentation dynamics of spontaneously fermented Ethiopian honey wine, *Tej*. *LWT*, *155*, 112927.

Appendix 2 - Survey to traditional processing methods of *Tej*

Survey to traditional processing methods of 'Tej'

The information obtained from this survey is used for the partial fulfillment of PhD dissertation entitled with 'Development of advanced fermentation system for the production of alcoholic beverage '*Tej*' with suspended mixed culture'. The aim of this questioner is to collect relevant information on raw materials and indigenous processing methods, quality characteristics and shelf stability of Ethiopian honey wine '*Tej*'. The research team would like to say thank you in advance for your effort and time to fill this questioner.

Name _____ Location _____

I. Personal Information

1. Age

   20-35 ☐
   35-50 ☐
   50-65 ☐

2. Gender

   Female ☐
   Male ☐

3. Role

   Producer ☐
   Consumer ☐
   Exporter ☐
   Other _____

II. Raw materials and processing methods

1. What type of honey is very suitable for the for ‘Tej’ production? what makes different form other type of honey?

_____

_____

_____

_____

2. What are the raw materials other than honey are used for the production of ‘*Tej*’?

_____

_____

_____

_____

_____

3. In what proportion these ingredients are used for ‘*Tej*’ production?

_____

_____

_____

_____

_____

4. What are the steps applied to prepare ‘*Tej*’ in your localities?

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_____

5. What are the major equipments used in the production of ‘*Tej*’?

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6. What are the factors that affect the time of '*Tej*' fermentation?

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III. Quality characteristics of '*Tej*'

1. What are the good quality characteristics of '*Tej*'?

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2. What are the major causes for 'off flavor" development in '*Tej*'

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_____

3. Is 'Turbidity' a major problem in the final product ('*Tej*')? If 'Yes' what are the major cause for this problem?

_____

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_____

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_____

4. What remedial actions should be taken to get clear product.

_____

_____

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_____

IV. Shelf stability of '*Tej*'

1. How long '*Tej*' can be kept in good quality?

_____

_____

_____

_____

2. In what conditions '*Tej*' should be kept to extend its shelf life?

_____

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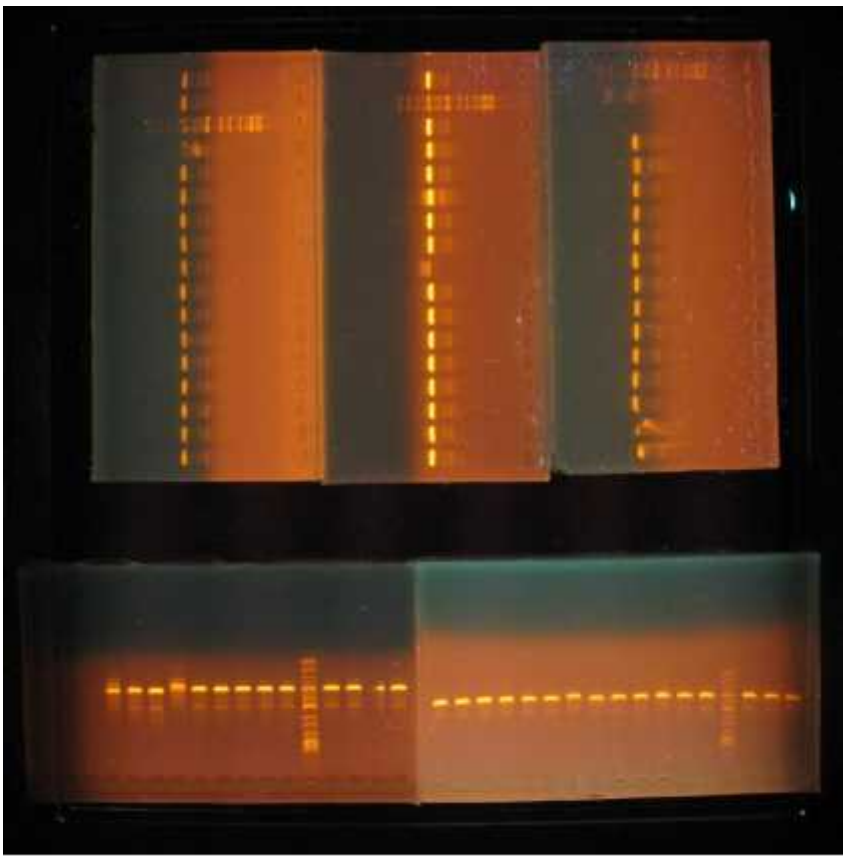
Appendix 3 - Research work

A) Gel electrophoresis



B) Cryopreservation

C) Illumia MiSeq sequencer

D) Morphology of an isolate