ADDIS ABABA UNIVERSITY SCHOOL OF GRADUATE STUDIES COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES DEPARTMENT OF MICROBIAL, CELLULAR AND MOLECULAR BIOLOGY

Physico-chemical and Microbiological Analysis of Honey and Tej Collected from Central Ethiopia and Production of Tej using Starter Culture

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As member of the examining board of the final MSc open defense, we certify that we have read and evaluated the Thesis entitled "**Physico-chemical and Microbiological Analysis of Honey and Tej Collected from Central Ethiopia and Production of Tej using Starter Culture"** prepared and submitted by Frehiwot Dagima Gurmecha and recommend that it was accepted as fulfilling the Thesis requirement for the degree of Master of Science in Applied Microbiology.

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DECLARATION

I declare that the thesis hereby submitted by me for the Degree of Masters of Science (MSc) in Biology (Applied Microbiology) to the school of Graduate Studies of Addis Ababa University is my own independent work and has not previously been submitted by me or anybody else at another university. The materials obtained from other sources have been duly acknowledged in the thesis.

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LIST OF ABBREVIATIONS

EC Electrical conductivity

IES Institute of Ethiopian standard

HMF Hydroxymethylfufural

Harmonized Method of the International Honey Commission

IHC International honey commission

HPLC High Performance Liquid Chromatography

MC Moisture Content

DW Dry Weight

H_2O_2 Hydrogen Peroxide

PCA Plate Count Agar

YPD Yeast Peptone Dextrose

AMB Aerobic Mesophilic Bacteria

ASF Aerobic Spore Forming

MRS De Man-Rogasa-Sharpe agar

VRBL Violet red bile-lactose agar

TA Titrable Acidity

SG Specific Gravity

Cfu Colony forming unit

µl microliter

ms millisiernens

mS/c millisiemens per centimeter

millimin milliliters per minute

M Molarity

g/100ml gram per 100 milliliters

w/v weight by volume

w/w weight by weight

nm nanometer

$Meqkg^{-1}$ Milliequivalent per kilometer

ABSTRACT

Tej is the most diverse traditional fermented alcoholic beverage of Ethiopia. It is prepared from honey, water and leaves of Gesho (Rhamnus prinoides). In our country, Tej is commonly fermented by natural microflora. The aim of this study was to investigate the physicochemical, proximate and microbiological properties of honey and Tej from central part of *Ethiopia and its surrounding and production of Tej by using starter culture. pH. specific gravity, moisture content, ash, acidity, electrical conductivity, Hydroxyl methyl furfural, sugars and alcohol contents are the main physicochemical and proximate parameters analyzed in honey and Tej samples. Aerobic spore-forming bacteria, Aerobic Mesophilic bacteria, Enterbacteriaceae, Yeast and Moulds and Total coliforms were the microorganisms assessed for the Tej samples. A total of 20 honey and 30 Tej samples were collected. The mean values of pH and Acidity of Tej and honey ranged from 3.45-4.20 and 1.0-2.7,JorTej and from* 2.96-4.45 *and* 17.87- 52.15 *for honey, respectively, The mean moisture and ash contents of Tej and honey varied from* 86.7- 92.7 *and 0- 0.2,/01' Tej, 80.01-84.75 and 0-0.3 for honey respectively. The mean value of EC of Tej ranged from* 0.44 - 0.76 *and the values of honey ranged from* 0.27-0.92.The *HMF of honey ranged from* 0.0-1.05*, and the mean of alcohol content ranged from 8.62-14.49 for the Tej samples. Yeasts were the dominant microorganisms found in the Tej and they were not defected in honey samples. The yeast counts were ranged from 5.60- 8.00 Cfu/g. Aerobic spore- forming bacteria (ASFB), Total coliform and Enterbacteriaceae were not detected Based on their growth performance under different physiological stresses ten LAB and ten yeast isolates were screened. The Yeast isolates were tentatively identified into four species level, Saccharomyces cerevisie, Saccharomyces daireness, Debaryomyces carsonii. Both the LAB and yeast isolates were combined in different proportions to formulate starter cultures for the production of Tej. Ten formulations were made in different proportions based on their compatibility of the isolates. Using Four Yeast and Ten LAB-Yeast starter culture formulations, Tej was prepared under controlled fermentation conditions. The overall sensory acceptability analysis showed that formulate .F #5, F#2, and F#7 were the best mixed starter cultures for Tej preparation as compared with the control. However, further molecular identification of the isolates into species level and investigation of the microbial dynamics of Tej is recommended for future use of these isolates, The result for physicochemical and proximate analysis were aligned with national and international standards.*

Keywords: *Controlled fermentation, honey, starter culture, Physicochemical and proximate analysis, Tej, Sensory analysis.*

1. INTRODUCTION

1.1. BACKGROUND

Among the many fermented foods, people have been taking in alcoholic beverages since prehistoric times all over the world. This is because fermented products can play an important role, contributing to the livelihoods of rural and perturbing dwellers (Gadaga *et al*., 1999; Jeyaram *et al*., 2009). In Africa, fermented alcoholic beverages are consumed on a variety of occasions, that include marriage, naming, festivals, social gatherings, burial ceremonies, and resolving disputes (Bekele Bahiru *et al*., 2001).

They combined and used the barks or stems of plants as medicines for fever and a variety of other illnesses (Okafor, 1972). Fermented drinks made from cereals are called beers, while those made from fruits are called wines (Pederson, 1979). There are fermented alcohol-filled African drinks available. These include gara, tembo-mnazi, Tanzanian wanzuki gongo, and Egyptian bouza. South African kaffir beer, Kenyan muratna and uragela, and Nigerian palm wine (Belele Bahiru *et al.,* 2001). Tej, Tella, and Areki are traditional fermented drinks from Ethiopia (Tadele Yohannes *et al*., 2013).

Tej is an Ethiopian honey wine made from honey, water, and the leaves of Gesho (*Rhamnus prinoidesy*).It is regarded as the national drink of Ethiopia because it is drunk at social gatherins , weddings, holidays, and cultural events. Its alcohol content ranges from 8 to 14%.Normally, th e components of honey impart the yellow color of Tej; however, in situations where the honey l acks yellow color or sugar is utilized as a substrate, other coloring agents are used to produce Te j. Due to concoction and adulteration practices producers usually are not willing to tell about additives used and their composition (Bekele Bahiru *et al.,* 2001).

Ethiopia is well-known for its varied agroecology and climate. Consequently, the nation emerged as the top producer of wax and honey in Africa (Demoz & Mikula, 2012). Honey has many different health-promoting and therapeutic qualities in addition to nutritionally essential compounds. Nowadays, it is regarded as an important area for traditional medicine worldwide

(Kahraman *et al*., 2010). Numerous reports discuss the value of using honey as a medication (Gul *et al*., 2015). According to Pathare *et al*. (2015), the most essential components of honey include a variety of saccharides, water, proteins, vitamins, organic acids, flavonoids, inorganic substances, trace elements, and unstable compounds like enzymes. Higher dosages of 50–80g of honeyper intake have a number of beneficial nutritional, therapeutic, and preventive qualities (Bogdanov *et al.*, 2008).

1.2. STATEMENT OF THE PROBLEM

Tej is fermented by microorganisms added to the production medium from sources like substrates used to make Tej and fermentation vats or equipment, just like other traditionally fermented drinks. As a result, the fermentation of these products exhibits poor organoleptic properties, microbiological instability, and inconsistent and unpredictable behavior (Holzapfel, 2002). Therefore, there is a chance that these naturally occurring products could be hazardous to humans. So there is a need to develop starter culture and optimize the fermentation process condition to improve and commercialize Tej in Ethiopia.

1.3. SIGNIFICANCE OF THE STUDY

This study can improve awareness for both consumer and producer how to produce good quality Tej within the market with scientific justification. So the local producers will be benefited from the developed single and mixed starter culture and able to produce consistent, safe and good quality Tej. The output of this thesis also helps the community to improve their income by selling high quality Tej and also foster the brands of Ethiopian honey and Tej.

1.4. OBJECTIVES

1.4.1. GENERAL OBJECTIVE

- To assess the Physico-chemical and Microbiological Analysis of Honey and Tej Collected from Central Ethiopia and Production of Tej using Starter Culture

1.4.2. SPECIFIC OBJECTIVES

The specific objective of the study was to:

- Assess the physicochemical property of Tej and honey.
- Assess the Proximate composition of Tej and honey.
- Assess the microbial composition of Tej.
- Screen and identify potential starter cultures from Tej.
- Formulate and use the best stress tolerant isolates *LAB* and Yeasts for Tej production
- Evaluate the sensory attributes of the Tej sample produced under controlled conditions

2. LITERATURE REVIEW

2.1. STARTER CULTURE

Starter cultures are chosen microbial preparations that are used to improve the effectiveness of fermentation operations. Numerous microbial cultures are utilized in the food business to assure the production of safe and high-quality commodities (Pereira *et al*., 2019).The first pure cultures were used in Denmark and Germany in 1890 for the industrial fermentation of milk products (Holzapfel,1997). A preparation of living microorganisms that are deliberately used to assist the beginning of fermentation, producing specific changes in the chemical composition, changing the sensorial properties of the substrate to obtain a more homogeneous product.

The application of starter culture at the home level would prevent contamination with pathogenic bacteria, the decomposition of a poisonous chemical, and the possibility of accidental microflora that creates off-flavor. A starter culture is utilized to minimize fermentation time while maintaining a particular sensory quality. Furthermore, it has control over the starting phase of a fermentation process and may therefore regulate the conclusion of a fermentation process (Holzapfel, 2002). To alleviate the problem of variations in organoleptic quality and microbiological stability observed in African indigenous fermented foods, including Ethiopia, the use of starter cultures would be an appropriate approach for fermentation process control (Kimaryo *et al*., 2000).

The majority of the fermented foods are produced at the household level and hygiene is a major concern in Africa. The use of starter cultures has been an appropriate approach for control, optimization, ensuring product consistency and to a reasonable extent eliminates the problem of food-borne pathogens (Ayad, 2009).. According to Steinkraus (1996) most studies on African fermented foods have focused on isolation and identification of microorganisms involved in the fermentation process. However, there is very liter information on the occurrence and growth of pathogens.

The potential of starter cultures for fermentation on a home scale for most Ethiopian traditionally fermented foods has yet to be thoroughly explored, and they are often natural, with no specified starter cultures utilized to commence it (Mogessie Ashenafi, 2006). Using

functional starter cultures offers greater functionality than traditional starter cultures, and it represents a method of improving and optimizing the substrate fermentation process, resulting in tastier, safer, and healthier products (Leroy, 2006).

2.2. SELECTION CRITERIA FOR STARTER CULTURE DEVELOPMENT8

Starter culture development for lactic fermentations is based on selecting stress resistant and lactic acid-producing *LAB* strains. The lactic fermentation associated stressful conditions include temperature changes, osmotic stress and lactic acid accumulation. These features are required for fast microbial adaptation and growth, ensuring the development of physical properties (texture, viscosity, and body) and taste of manufactured products (Johnson *et al.*, 2013).

The ability of yeasts to ferment carbohydrates into ethanol is the primary selection parameter for developing starter cultures in the alcoholic beverage sector. Selecting yeast strains capable of rapid growth in sugary starting materials, such as cereal starches in beers, whiskies, and sake production, sucrose-rich plants (molasses or sugar juice from sugarcane) in rums, cachaca, and tequila, and fructose-rich fruits in wine, improves ethanol production efficiency. Sugar metabolism should be evaluated under fermentation-related stressful conditions such as high temperatures (35-45°C) and ethanol concentration (more than 20%), and yeast resistance to low pH is an important attribute to make the process less susceptible to bacterial infection (Da Conceicao *et al*., 2015 and Rantsiou *et al*., 2012).

The common selection criteria include: (i) low production of hydrogen sulfide (Hz.S), a byproduct of yeasts' metabolism that is responsible for conferring off-flavors; (ii) low production of acetic acid and other organic acids that may affect sensory quality and ethanol yield; (iii) flocculation capacity, which facilitates downstream processing and reduces cost of cells recovery; and (iv) volatile aroma compounds production, such as higher alcohols, esters, organic acids, sulfur, and aldehydes (Torriani *et al.,* 2011).

2.3. MIXED-STRAIN STARTERS (MSS) TRADITIONAL STARTERS

Mixed-strain starters, acquired by precise choice of natural starters, are preserved cultivated and supplied by starter firms and research organizations. It contains an undefined blend of strains

that diverge in their physiological and technological characteristics (Parente and Cogan, 2004). These undefined strains are grown with few subcultures under controlled environments, the firmness of their composition and function is significantly enhanced with reduced intrinsic variability in contrast to natural strains (Limsowtin *et al.*1996). According to Carminati *et al.*, 2010 stated that traditional cultivation of Mixed strain starters needs numerous shifts to boost the bulk starter by means of minimal quantities of stock cultures that are exchanged using concentrated cultures for inoculation of bulk starter tank,consequently reducing the necessity for shifts inside the factory and the possibility of fluxes in starter components and function.

2.4. DEFINED STRAIN STARTERS (DSS)

Defined strain starters are made up of one or more cultures of dominant strains of the conventional product that are favored, maintained, produced, and provided by specialist firms. Meanwhile, the strains and/or species ratio in DSS is well-defined; their technical function is highly repeatable with an advantageous characteristic. DSS is currently substituting conventional starters (Carminati et al., 2010). Furthermore, because of the lack of natural microbial diversity, retention of the distinguishing traits is hard. However, evaluation of critical characteristics such as growth and acid production, genomic or biochemical traits of individual species can result in a rational blend of strains suitable for culture formulation with appropriate properties (Carrninati et al., 2010). DSS strains are added to the starter at a lower concentration and can be used independently (Powell *et al.*, 2011).

2.5. ALCOHOLIC BEVERAGE

According to Anal (2019) alcoholic fermentation has been practiced since ancient times and is one of the oldest and most important techniques in food processing. The process results in the production of various alcoholic beverages like beers, wines, and distilled liquors, are use yeast and sometimes yeast-like molds, such as *Amylomyces rouxii*, and mold-like yeasts like *Endomycopsis* and bacteria like *Zymomonas mobilis*.

The microorganisms involve the utilization of fermentable sugars from substrates like cereal grains, sugar cane juice, palm sap, fruit juices, diluted honey, or hydrolyzed starch, the result is

mainly ethanol and carbon dioxide production. Alcoholic beverages are part of human dietary culture and have an inseparable relationship with the life of mankind in history. The exact time when mankind started to produce and consume alcoholic beverages are not known but beer is known to have been produced by the Sumerians before 7,000BC (Boekhout, and Robert, 2003).Fermented beverages constitute a major part of the diet of traditional African homes. They are consumed in different occasions such as marriage, naming and rain making ceremonies (Zvauya *et al.*, 1997).

2.5.1. WINE

Wine's characteristic aroma is influenced by a variety of factors, including the grape variety, viticulture and winemaking practices, wine maturation, and storage conditions. The initial conversion of grape must to wine is an alcoholic fermentation primarily produced by one or more strains of yeast, especially *Saccharomyces cerevisiae*. (Carr et al. (2002) state that wine is a mixture of hundreds of complex compounds, many of which contribute to the color, mouth feel, or aromatic properties of this beverage. After the alcoholic fermentation, malolactic fermentation (MLF) is often undertaken, depending on the style of wine that is being produced. MLF is carried out by LAB, most commonly *Oenococcus oeni*, which is acidophilic and indigenous to wine and is generally thought to be best suited to the harsh environment of wine. *Lactobacillus spp.* and *Pediococcus spp.* can also important to ferment malolactic fermentation, but not always to completion (Renouf *et al.*, 2008). However, recent red wine trials have shown that strains of *Lactobacillus plantarum* have the potential to produce efficient malolactic fermentation and also produce desirable sensory attributes .

2.5.2. BEER

Since beer was discovered thousands of years ago, the brewing process has not undergone any major changes. The grain is first malted, dried, and crushed before being combined with hot water to produce wort. The capacity of yeast to create sweet, viscous wort is necessary for

fermentation to take place. The wort's composition and the conditions created in the fermenting vessel have an impact on the biochemical processes that take place during fermentation, which represent the genotype and its phenotypic expression of the yeast strain (Ferreira and Guid, 2018).

According to Michel *et al,* (2016) beers are broadly classified as either ale or lager depending on the yeast used (*Saccharomyces cerevisiae*or *S. pastorianus*, respectively) and fermentation conditions. The modern brewers today embrace the use of novel ingredients including spices, herbs and fruits to alter a beer's flavour, creating what is regarded as craft or specialty beers. Yeast is the most significant contributor for beer flavour. The most common beer is the one that is beer produced from barley malt. However, wheat beer which is produced using fermented wheat considered a special beer type and thereby increased the demand for suitable brewing wheat cultivars (Faltermeier *et al.,* 2014).

2.6. ETHIOPIAN TRADITIONAL ALCOHOLIC BEVERAGES

Ethiopia is a country rich in cultural variety. Various ethnic groups process and consume a wide range of meals and drinks. Many traditional fermented drinks are manufactured on a small scale and are mainly consumed locally. Tella, Tej, Borde, Shamita, Areki, Keribo, and Korefe are examples of fermented drinks. Fermentation procedures of Tej, Tella, Areki, and Korefe like other historically fermented alcoholic drinks rely on the microorganisms which occur in the materials and equipment (Mogessie Ashenafi, 2002; and Getachew Tafere, 2015). These traditional alcoholic beverages are extensively drunk by the country's many ethnic groups as an important element of the local customs of significant social occasions like as public holidays, weddings, funerals, and other types of festivals. (Dersehilign Awegichew *et al.,* 2017).

2.6.1. TELLA

Tella is an Ethiopian traditional beverage made from various components such as barely, com, wheat, sorghum, Teff, and maize, however millet has been utilized in some locations (Alemu Fite *et al*., 1991). The preliminary procedures were identical to those reported by (Samuel Sable and Berhanu Abegaz, 1991). The clay container (Ensera, a traditional pot)

was scrubbed many times with Grawa (*Vernonia amygdalinay* and water) and then smoked with wood from Weyra (*Oleaeurapaea subsp. Cuspidate*) to provide pleasant smell or flavor and to be neat. To produce Tella for an average of six to eight days was required throughout the winter season.

2.6.2. AREKI

Areki is colorless (sometimes colorful) distilled traditional alcoholic beverage in Ethiopia. It is preparation process almost similar with Tella except that fermentation mass in this case is more concentrated (Tadele Yohannes *et al.,* 2013). The component for Areki preparation is Kita (a thin, 5-10 mm thick, pancake-like bread), Bikil (germinated grain), powdered Gesho (*Rhamnus prinoides)*; additional ingredients, Kosso (*Hagenia abyssinica*), Gibeto Lupinus albu or white Lupin), and Tens-Adam (Ruta chalepensisy and its pH between 4.30-4.51 (Gizaw Debebe, 2006). Areki is classified into two traditionally: Terra-Areki and Dagim Areki. The word Dagim in Amharic refers to 'second time' which, indicates that it is distilled second time, and the word Terra in Amharic refers to 'ordinary'. Terra-Areki is a colorless, clear, local alcoholic beverage, which is distilled from a fermentation product of Yereki-tinsis Terra-Areki had 34.09% (v/v) alcohol content and the average alcohol content of Dagim Areki is around 45% (v/v) (Desta Belachew,1977). The alcoholic contents of Areki were measured and found in the range of and 33.95 to 39.9% v/v ethanol, (Tadele Yohannes *et al.,* 2013).

2.6.3. BORDE

Borde is a low-alcohol beverage, despite the fact that its fermentation period is lengthy enough for 4 days to yield in a significant concentration of ethanol (Alemu Fite *et al*., 1991). Borde is made in a spontaneous manner with basic equipment. The beverage is whitish-grey to brown in color, with a bushy consistency and a sweet-sour flavor. Borde is made from cereals such as maize (Zea mays), barley (Hordeum vulgare), wheat (Triticum sativumi, finger millet (Eleusine coracanat, sorghum (Sorghum bicolor), and tef tEragrostis Ie/) and is widely consumed in Ethiopia's southern and western regions (Kebede Abegaz *et al*., 2002b).

According to Kebede Abegaz et al. (2002a), the preparation of Borde is time consuming,

unsanitary, and complicated, requiring grinding, souring, roasting, steam heating, boiling, cooling, mashing, wet-milling and wet-sieving operations. This may provide an opportunity for a variable bio-physicochemical environment that selects for involved microflora that overcome technological barriers in the natural fermentation of Borde. Cooking of the adjuncts at about 90°C can kill vegetative microbial cells, resulting in the hydrolysis of starch and thus creating an environment suitable for the proliferation of microorganisms during traditional fermentation of Borde. The spontaneous fermentation of Borde resulted in undefined quality with a low pH, high counts of aerobic mesophiles bacteria, LAB and yeast (Kebede Abegaz *et al.*,2002b).

2.6.4. TEJ

Tej, also referred to as honey wine, is a home-processed and commercially available beverage made from honey, water, leaves, and occasionally the bark of Gesho (*Rhamnus prinoidesy*). A mixture of honey and sugar is used as ingredients, and natural food coloring is added so that the beverage achieves a yellow color similar to that made from honey. Some Tej makers also add different concoctions such as barks or roots of some plants or secrete herbal ingredients to improve flavor or potency and to draw customers. Because of concoction, adulteration practices, and possibly other factors, producers are typically unwilling to disclose the ingredients they use (Bekele Bahiru *et al*., 2001).

2.7. TEJ PREPARATION

Important components and cleaning agents before the Tej fermentation begins, the fermentation vessels are cleaned by Grawa (*Vernonia amygdalina* Del.) and Weyra (*Oleaeuropaeai*). Weyra splinters are used to smoke the fermentation jars, while grawa leaves are utilized to clean the fermentation vessels. The following list includes some of Tej's key constituents. Rhhamnus prinoides, or Gesho Gesho, also known as Rhamnus prinoides, refers to the leaves or stems of this plant are used to make Tej.

Although the plant is widely grown as a wild plant in practically every region of the nation between 1500 and 2000 meters , it is also skillfully farmed, occasionally even more extensively as a field crop. R. prinoides encompasses over 5000 hectares of land used for ongoing

agricultural production. This woody shrub is used to make traditional alcoholic beverages like Tella and Tej, which are popular domestic libations throughout the nation, just way hops are used from its leaves or stems. Harry (2016).

Tella and tej are traditional fermented drinks from East Africa that employ gesho as a bittering ingredient (Lee *et al.*, 2015). Because it is used extensively in locally produced drinks like Tella and Tej, R. prinoides is grown in Ethiopia. Gesho cultivation is a significant commodity, despite the fact that it is widely found across the nation. (Ararso Nagari and Alemayehu Abebaw (2014) identify two significant gesho manufacturing centers: Tigrai, which is located near Kara Kori in North Shewa, and Sebeta, which is located just west of Addis Ababa.

2.7.1. Honey

Honey has special qualities that make it one of the most sought-after goods. It may be utilized for industrial, medical, and nutritional applications. According to Fatimah Buba *et al*. (2013), honey is a valuable item on the global market that helps many nations gain foreign cash. Since ancient times, honey has been utilized extensively as an edible commodity for cosmetic production as well as for sickness prevention and treatment. A sweet material called honey is made from the secretions that originate from the living portions of plants or from the nectar of flowers. The primary sugars in honey are glucose and fructose, but it also contains vitamins, mineral salts, and enzymes. Honey's high sugar content has been linked to decreased water activity, an acid pH, and bactericidal chemicals (H202 and inhibin). Additionally, honey regularly experiences yeast-driven fermentations, rendering the product unfit for human consumption (Haimanot Abebe, 2011). Tej is made by washing and seasoning the fermentation pot with smoke from burning R. prinoides stems. Water is added to the pot, along with honey, and covered with a cloth to allow the mixture to ferment for two to three days. Wax and top scum are then removed.

To lower the temperature, part of the must—along with cleaned and peeled R. prinoides—is cooked before being added back to the fermentation must. In warmer climates, the pot is covered and fermented constantly for an additional 5 days; in colder climates, it ferments

continuously for 15 to 20 days. After the combination has finished fermenting and is suitable for consumption, it is mixed every day and filtered through cloth to remove sediment and R. prinoides. (Abeba Gobezie and Vogel, 1983).

A high-quality Tej should be yellow, sweet, bubbly, and hazy because of the yeast content, according to Vogel and Abeba Gobezie (1983). Tej's flavor is influenced by the temperature and the region of the nation in which the bees have collected nectar. As a commercial product, tej is typically sold for consumption at the point of manufacturing by each production unit. (Ararso Nagari and Alemayehu Abebaw (2014) state that Shewa and Sebeta, which are located to the west of Addis Ababa, are significant gesho producing locations.

2.7.2. Chemical composition of honey

There are more than 300 bioactive ingredients in natural honey. But the majority of honey's composition is made up of water and sugars, namely fructose and glucose, which make up 95–99% of the dry matter in honey, and around 4-5% of fructooligosaccharides (Ajibola *et al*., 2015). Other sugars found in addition to fructose (38%) and glucose (31%), are maltose, sucrose, maltose, turanose, isomaltose, larninaribiose, nigerose, kojibiose, gentiobiose, and oligosaccharides (Ahmed *et al*., 2018).

2.7.3. Quality Characteristic of Honey

Honey's sensory, physical, chemical, and microbiological qualities are primarily linked to its quality. The honey is defined by the European Council Directive, which also sets minimum quality requirements for honey that is sold as honey or used as a component in goods that are meant to be consumed by humans. The amount of honey that is contaminated with sugars and mislabeled according to its botanical or geographic origin that is sold in the market in 2015.

According to Alvarez-Suarez *et al.* (2010), the primary factors determining honey's properties include its moisture content, electrical conductivity, ash content, reducing and non-reducing sugars, free acidity, diastase activity, and HMF concentration. Because of this, a number of

nations have established stringent requirements for commercial honey, including certain physical characteristics and chemical compositions. Vitamins and bionutrients may be destroyed by thermal treatment, which is used to delay crystallization and eradicate contaminating microorganisms. It can also cause a simultaneous decrease in diastase activity and an increase in HMF content. National and international guidelines are utilized to restrict the usage of heat treatment in order to reduce diastase activity and HMF levels (Tosi *et al*., 2002).

2.8. MICROORGANISMS IN FERMENTED TRADITIONAL BEVERAGES

2.8.1. Lactic acid bacteria

Gram-positive, non-spore-forming, rod- and cocci-shaped, catalase- and oxidase-negative, non-aerobic but aero-tolerant, meticulous, and acid-tolerant are the characteristics of lactic acid bacteria. The primary characteristic of LABs has been their capacity to produce different isomers of lactic acid through the fermentation of glucose. The LAB also separated into two groups: homofermentative and heterofermentative, based on the metabolic routes via which hexoses are broken down. Lactic acid is the primary or only byproduct of glucose fermentation produced by homolactic bacteria, including Pediococcus, Lactococcus, and certain lactobacilli. In addition to producing lactic acid, heterolactics—such as Weisella, Leuconostoc, and certain lactobacilli—also create carbon dioxide, acetic acid, and ethanol through the fermentation of glucose (Carr *et al.*, 2002).

LAB can be found in a variety of fermented foods and drinks (Stiles and Holzapfel, 1997). Principal LAB genera include Enterococcus and Carnobacterium. Numerous fermented foods and drinks have yielded the following bacteria: Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus, and Weissella (Salminen *et al*., 2004; Holzapfel and Wood, 2014).

When it comes to some traditional fermented beverages in Ethiopia, Lactobacillus sp. (primarily Lactobacillus plantarum), Leuconostoc, and Pediococcus are the most common fermenting microorganisms and yeast involved in Tej fermentation (Bekele Bahiru *et al.*, 2006). Borde fermentation involves Leuconostoc mesenteroid, Lactobacillus brevis, Lactobacillus viridescens, Pediococcus pentosaceus, Lactobacillus.curvatus, Lactobacillus, Lactobacillus

sanfrancisco, Lactobacillus pontis, and Lactobacillus delbrueckii, and LAB is also involved in Keribo (Reda Nemo and Ketema Bacha, 2020).

2.8.2. Yeast

Yeasts are eukaryotic microorganisms with a diverse range of ecological niches. They mostly inhabit water, soil, air, and plant and fruit surfaces. Their morphological characteristics include round, ellipsoidal, and oval forms. The most significant biotechnology in terms of economic impact is the fermentation of carbon sources to produce alcohol using yeast. The manufacturing of all alcoholic drinks involves yeast, and choosing the right strains of the yeast is crucial for maintaining both the alcohol yield and the sensory quality of the final product (Walker *et al*., 2016).

Breweries have special yeasts on hand for their unique brews. Two varieties of yeast are utilized in the brewing process: S. pastorianus is used as a bottom-fermenting yeast in the lager brewing procedures, while S. cerevisiae is employed as a top-fermenting yeast to generate ales (Libkind *et al*., 2011).Alcoholic fermentation is often carried out by certain S. cerevisiae strains, which also carry out the spontaneous fermentation of juice to generate ciders. This guarantees that the final goods will always be of a high caliber (Lorenzini, 2019).

Teramoto *et al*. (2005) state that yeast strain selection is important since it affects how well sugar is converted to alcohol. Compared to readily acquired commercial strains, yeasts isolated from honey provide several benefits. The yeasts that were identified are resistant to both high sugar concentrations and ethanol. Numerous *Saccharomyces cerevisiae* strains have demonstrated via culture studies that they are suitable for the production of mead (Pereira *et al*., 2014).According to (Bekele Bahiru *et al*. (2006), the most frequent yeasts detected during Tej fermentation are *S. cereviseae, Debaromyces phaffi*, *Kluyveromyces bulgaricus,* and *Kluyveromyces veronae*. In Borde and Shameta fermentation, yeast (*Saccharomyces cerevisiae*) and *Rhodoturula spp*. were discovered (Reda Nemo and Ketema Bacha, 2020).

3. MATERIAL AND METHODS

The study was conducted at Ethiopian Conformity Assessment Enterprise in Biochemical laboratory.

3.1. The Study Areas

Areas selected for sample collection were Weliso, Sebeta, Adama, Addis Ababa (Kirkos, Addis keterna, Kolfe, Nifas silk lafto and Yeka subcities), Alemgena, Sendafa, Sululuta and Holeta.

Table 1,Sampling sites of Tej and honey.

No. Sampling site	**Sampling site**	**Number of samples collected Tej and honey**	**Distance from the capital city (KM)**	**Geographical coordination of the sampling point**	
				Elevation (m.a.s.l)	**Latitude and longitude**
1	Adama	3 tej and 4 honey	99km	1712m	10026'O"N,39°24' O"E.
2	Holeta	3 tej	40km	2291m	903'N 38030'E
3	Sendafa	3 tej	35km	2514m	909'N 3902'E
4	Woliso	4 honey	114km	2063m	8°32'N 37°58'E
5	Sabeta	3 tej and 4 honey	23km	2356m	8.9112°N,38.626 8° E
6	Sululeta	3tej	26km	2624m	9°11 '0"N,38°45'E
7	Alemgena	3tej	19km	2369m	8°55'N38°39'E
8	Addis Ketema	4 honey	Sub city	2454m	901'48" N 38044'24" E
9	Kirkos	3 tej and 4 honey	Sub city	2352m	9°0'15.12"N3 8°45'31.28"E
10	Kolfe	3 tej	Sub city	2367m	38^{o}7172° or 38° 43' 2" E
11	Nifas silk	3 tej	Sub city	2224m	8.9852° N, 38.7111° E
12	Yeka	3 tej	Sub city	2470m	9.0225° N, 38.8000°

3.2. Samples Collection

A total of 50 (30 Tej and 20 honey) samples were randomly collected from different known hotels and vending houses in and around Addis Ababa l000ml for Tej and 500g for honey. The study sites were selected purposively based on the production of honey, which is the main component of honey wine production. The particular brewing houses were selected based on availability, types of product (in terms of quality) and willingness of the local retailers to sell their beverage products. The samples were collected aseptically in sterile flasks (bottles) and transported using icebox to Microbiology laboratory. The samples were kept in the refrigerator at 4°C until used for analysis.

3.3. PHYSICOCHEMICAL CHARACTERISTICS OF TEJ AND HONEY

3.3.1. Determination of Electric Conductivity (EC)

The EC of honey was measured by dissolving, 20 g anhydrous honey, in distilled water. Then transfer the solution quantitatively to a 100ml volumetric flask and making up to volume with distilled water. Forty milliliters of the sample solution was poured into a beaker and the beaker was placed in a thermostatic water bath at 20 "C. The conductivity cell was thoroughly rinsed with the remaining part of the solution and the conductivity cell was immersed into the sample to record the result.The EC of the Tej samples were determined by pipetting, 10 m1 of each Tej sample directly into a beaker and recoding the values by inserting digital multi meter (Bante 900- UK) into separately into the Tej samples (Lee *et al., 2013).*

3.3.2. Determination of the Specific Gravity (SG)

The specific gravity of the Tej samples was determined using a pycnometer. The weights of an empty pycnometer, pycnometer with distilled water, and pycnometer with the samples was measured using a balance.

$$\boldsymbol{Specific\ gravity} = \frac{Weight\ of\ pycnometer\ with\ sample - Weight\ of\ empty\ pycnometer}{Volume\ Distilled\ water}$$

3.3.3. Determination of pH value

Ten gram of honey sample was dissolved in 75 ml of carbon dioxide-free water in a 250ml beaker and thoroughly mixed by stirring it with the magnetic stirrer. Then pH electrode was immersed into the mixed sample and the pH was recorded. The pH of Tej samples was measured by dipping the electrode of a digital pH meter in the samples (Bogdanov, 2009). The calibrations of the pH meter were checked by deionized water and buffer solutions of pH values 4, 7 and 9.

3.3.4. Titratable acidity of Tej and Honey

Five gram of sample were transferred in to a 250 ml conical flask and diluted with distilled water. From the diluted sample, 10ml of supernatant of the solution was taken and each sample was titrated with 0.1M of standard solution of NaOH using 3 drops of 1% phenolphthalein as an indicator. As the color became a faint pink color used to know the volume of NaOH consumed until the end point. The titratable acidity of samples was determined according to Zyauya *et al.,* 1997. The amount of acidity was calculated using a formula

$$\text{Titratable acidity (g/100ml)} = \frac{\text{Amount of NaOH titrated x mol/L of NaOH x 5}}{\text{Volume of sample (ml)}}$$

3.3.5. Hydroxyl methyl furfural of honey samples

HMF content was determined based on the procedure of IES (2021). Accordingly, Weigh 10gm of honey sample in a beaker and dissolve the sample with deionized water Transfer to 50ml volumetric flask and make up to volume with deionized water Filter through 0.45µm syringe filter in to 2ml. The HMF content of the sample is calculated by comparing the corresponding peak areas of the sample and those of the standard solutions, taking into account the dilution. There is a linear relationship between the concentration and the area of the HMF peak. Results are expressed in mg/kg, to 1 decimal place.

$Y=mx+b$

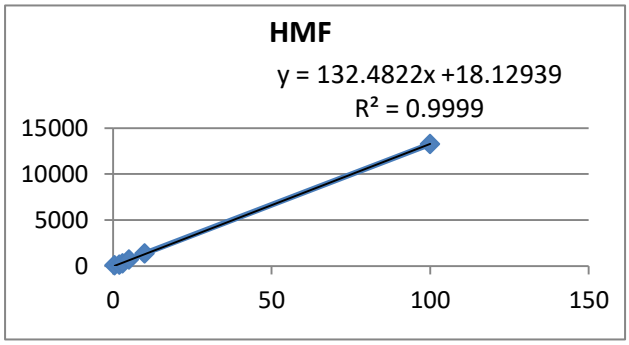
Where, y is the area of sample, m= is the slope found on the calibration graph

x = is the concentration of HMF read on the instrument

b= is the y-intercept from the graph

$$x = \frac{y - b}{m}$$

$$\text{Concentration of HMF} = \frac{(x)\ (\text{dilution volume})}{\text{Test portion}}$$



HMF in Honey	
Conc	Area
0.5	69.92
2	136
3	285
5	700
10	1371.4
100	13263

3.3.6. Total Alcohol Content of Tej samples

The total alcohol content of Tej samples was determined by specific gravity method of AOAC 1904 (Williams, 1984). A hundred ml volumetric flask was filled with sample, which was transferred to a distillation flask to which a condenser was fit. The volumetric flask was repeatedly rinsed with distilled water, and the water was added to the flask. The content of the flask was distilled slowly at a uniform rate from 30 to 60 minute using longer time for higher percentage of alcohol. Then, about 95 ml of distillate was collected in a 100-ml volumetric flask, the flask filled with distilled water and the content was mixed by inversion. The specific gravity of this distillate was determined at 20°C.

3.3.7. Free Acidity of Honey

The free acidity of honey samples was determined according to AOAC (2005). Ten grams of honey samples was dissolved in 75m1 of carbon-dioxide-free distilled water in 250m1 beaker and stirred with a magnetic stirrer. The solution was titrated with 0.l M NaOH solution to final pH of 8.30. The free acidity is expressed as mill equivalents or a mill mole of acid/kg honey, which is calculated as ml of 0.1 M NaOH x 10. The result is expressed to one decimal place as per the procedure of (IHC 2009).

Where acidity = 10 V, V is the volume of 0.1 N NaOH in 10 g of honey.

3.4. PROXIMATE ANALYSIS OF TEJ AND HONEY

3.4.1. Determination of ash content

About 5 g(ml) of each honey and Tej sample was weighed out into previously weighed porcelain crucible. Organic matter was charred by igniting the sample on a hot plate in the fume cupboard. The crucible was placed in the muffle furnace and heated at 600°C for 6 h. After complete ignition to constant weight, the sample was cooled in a desiccator and weighed immediately (Agbagwa,.*et al.,* 2011). The percentage of ash was calculated as:

$$[\%\ \text{Total Ash} = \frac{(\text{weight of residue g})}{(\text{weight of sample g})} \times 100]$$

3.4.2. Determination of moisture content

To determine moisture contents, 50 ml capacity clean oven-dried beakers were weigh and record (W 1). Then, 10 ml (g) of the samples was separately weighed and added into pre-weighed dried beakers (W2) and transfer into an oven to dry at 105°C for 2 h. After 2 h of drying, the samples was relocate to a desiccator and weight (W3) was record (AOAC, 2002).

$$\%\ Moisture\ Content = \frac{W2 - W3}{W2 - W1} x100$$

3.4.3. Determination of sugar content of Tej samples

To determine the honey sugars present in the samples, high-performance liquid chromatography (HPLC) (HPLC-1260 Infinity Series Agilent Technologies, USA) was utilized in accordance with the guidelines set forth by (IHC 2009). Five grams of honey were dissolved in 40 mL of distilled water and filtered using a syringe filter (0.45μm) before chromatographic analysis. The sugar profile was analyzed using an analytical stainless-steel column containing amine-modified silica gel (250 mm × 4.6 mm, 5–7μm particle size). A mobile phase consisting of 80 % acetonitrile and 20 % water was employed using the isocratic method, and the flow rate was 1.3 mL/min. The amount of each sugar was detected by a Refractive Index Detector maintained at a temperature of 30 ◦C following injection of 10μL into the column. In preparation for calibration, sugar standards (fructose, glucose, sucrose, and maltose) mixture which contain 20 mg/mL, 15

mg/mL, 10 mg/mL, 5 mg/mL, and 1.5 mg/mL were weighed, and five-level serial dilutions were prepared in accordance with IHC procedures. Each standard solution was dissolved in 40 mL of HPLC-grade water and mixed with 25 mL of methanol (HPLC grade) in a calibrated 100 mL flask. The standard solution was then filtered through a 0.45-μm nylon membrane filter (syringe filter), and the filtrate was poured into an injection vial. Calibration sugar standard solutions were prepared by pipetting 1.0 mL mixed standard stock solution into five 1.5 mL amber glass vials. Identification of honey sugars was obtained by comparing their retention times with those of the standard sugars (IHC 2009), and triplicate injections were performed. Average peak areas were used for the peak quantification.

3.5. MICROBIAL ANALYSIS OF TEJ

Microbiological Enumeration

Microbiological enumeration was conducted following methods used by Ugbogu and Ekeleme (2015) and Esra and Abdelgadir (2015). Accordingly, 25 ml/g each of well-mixed Tej and honey samples were separately added to 225 ml sterile peptone water; homogenized in a flask at 100 rpm for 10 min in a homogenizer. After homogenization, 1 ml of each Tej and honey sample was aseptically transferred into 9 ml of peptone water and mixed thoroughly using a vortex mixer. The homogenate was serially diluted from 10^{-2} to 10^{-7} and from appropriate dilution (10^{-3} and 10^{-4}) was spread plated in duplicate on pre-solidified sterile agar media.

3.5.1. Enumeration of Aerobic Mesophilic Bacteria

A volume of 1 ml of the aliquot from the final dilution was pour plated in duplicate on Plate Count Agar (PCA) and incubated at 32°C for 48 h colony counts were made using colony counter. The enumeration of *Aerobic mesophilic bacteria* colonies were done according to (ISO procedure 4833-1-2015).

3.5.2. Enumeration of Enterobacteriaceae

A volume of 1 ml of the aliquot from the final dilution was pour-plated in duplicates on Violet Red Bile Glucose Agar and incubated at 32°C for 24 h. Then, purple/pink colored colonies surrounded by purple halos was counted as members of *Enterobacteriaceae*. The enumeration

of *Enterobacteriaceae* colonies were done according to (ISO procedure 2152-2-2004).

3.5.3. Enumeration of Aerobic spore-forming bacteria

10 ml of the final dilution was heated in a water bath kept at 80°C for 10 min and cooled rapidly under tap water. Then, a volume of 0.1 ml aliquot from the final dilution was spread-plated in duplicate on PCA plates and incubated at 32°C for 72 h. The enumeration of *Aerobic spore forming* bacteria colonies were counted as aerobic spore forming bacteria according to (ISO procedure 4833-1-2015).

3.5.4. Enumeration of Total Coliform

1 ml of each decimal dilution (10-1 and 10-2) was poured aseptically into sterile plates. Violet red bile-lactose agar (VRBL), melted and cooled in a water bath at 45° C, was added to the inoculum at a rate of 15 ml per dish. After solidification of the first layer, a second 5 ml layer of VBRL was added. Control of the sterility of the medium was carried out in a Petri dish with approximately 15 rn1 of VBRL. The total coliform count was done directly after incubation at 30^{O}C for 24-48 hours. The total coliforms were carried out according to ISO 4831: 2006.

3.5.5. Enumeration of Yeast and Mould

Volumes of 0.1ml of the final dilutions was spread plated on Chloramphenicol-Bromo phenol blue agar consisting of (gil distilled water) yeasts extract (Oxoid) 5.0, glucose 20, chloramphenicol 0.1, Bromophenol-blue om, agar 15, pH 6.0-6.4.The plates were incubated at 25-28°C for 4-5 days. Smooth, non-hairy colonies lacking extensions at margins under stereoscopic microscope was counted as yeasts and each was preserved for further study. The enumeration of yeast and mould was done according to according to standard (ISO procedure 21527-2-2014).

3.6. ISOLATION OF YEAST FROM TEJ

The yeasts were isolated from different Tej and honey samples after dilution followed by plating the appropriate dilution (10^{-3} and 10^{-4}) onto yeast extract peptone dextrose agar (YPDA). Twenty five ml of each of the sample was transferred to 225 ml of sterile peptone water and

successively diluted to 10^{-2} up to 10^{-6}. Aliquot of 0.1 ml from appropriate dilutions (10^{-3} and 10^{-4}) for Tej and (10^{-1}) for honey was spread-plated onto YPDA (Pons *et al*., 1986). The YPDA medium contained (g/l) of yeast extract 10, peptone 20, dextrose 20, and agar 20. The medium was supplemented with 0.1 mg/ml streptomycin sulphate antibiotics to inhibit the growth of bacteria (Osho, 2005). The plates were incubated at 28℃ for 4 - 5 days. Yeast isolates were further purified by sub culturing on YEPD medium by streaking. The pure culture was kept on YPDA agar slant and stored at 4℃ for further study.

3.7. COLONY MORPHOLOGY OF YEAST

From a 48 h young pure culture, a thin smear was prepared on a microscope slide. The culture was observed under light microscope using oil immersion objective. Cell shape and arrangement were used for the morphological identification of yeast.

3.8. ISOLATION OF LAB FROM TEJ AND HONEY

For isolation of LAB, 25 ml Tej or 25 g of honey sample was mixed with 225 ml of separate sterile peptone water (0.1% w/v). From the dilution, 0.1 ml aliquot was spread-plated on pre-dried surface of MRS (de Man, Rogosa, and Sharpe) agar plate in duplicates. The inoculated plates were incubated under anaerobic condition using anaerobic jar at 32°C for 48 hours. Then, 5-10 distinct colonies were randomly picked from countable MRS plates for further purification. The isolated colonies of LAB were transferred into about 5 ml MRS broth and purified by repeated streaking on MRS agar. Pure culture of LAB was then streaked onto MRS agar slant and stored at 4°C for further characterization (Patil *et al.*, 2010).

3.8.1. Cell morphology for LAB

Fresh overnight cultures were wet mounted on microscopic slides and examined under a light microscope using oil immersion objective (1000x). Microbial cell shape and arrangement were used to identify the LAB.

3.9. BIOCHEMICAL CHARACTERIZATION OF LAB AND YEAST

3.9.1. Catalase test

Pure culture of yeast was placed on a clean microscope slide. One drop of 3% hydrogen peroxide (H_2O_2) was put into yeast colony. The gas production (bubbling) indicated positive catalase test.

3.9.2. KOH test

KOH test was made according to Gregersen (1978). One to two drops of 3% KOH solution was dropped on a glass microscope slide that was placed on a dark background. A colony was picked from a young pure culture and stirred (5-10 stirrings) into the KOH with a wire loop. The changing of the KOH solution to viscous solution and formation of a thread of slime following the loop upon rising was considered as a positive test while a watery suspension considered as positive test.

3.9.3. Spore test

Each lactic acid bacteria isolate was grown on MRS agar for 24 - 36 h. Heat-fixed smears of the pure isolate were prepared on separate slides and flooded with 5% malachite green solution and steamed for a minute. The stain was washed off with gently flowing water and counter stained with 2 drops of safranin solutions for 20 second. The slides were allowed to air dry and examined under oil immersion objective (l000x) lens. Endospores contained cells were stained green while vegetative cells stained pink (Cheesbrough, 2006).

3.9.4. Gas production from glucose

Carbon dioxide (CO_2) production from glucose was performed using a modified YPD and MRS broth media with inverted Durham tubes. The 5 ml YPD and MRS broth was inoculated with 100µl of fresh cultures of the yeast and LAB isolates and was incubated at 30 °C for 5 days aerobically; gas production during fermentation was indicated by the uplift of the inverted Durham tubes.

3.9.5. Fermentation of carbohydrates

Fermentation ability of the isolates against various carbohydrate sources were determined in

the carbohydrate fermentation basal medium of Wickerham (Van der Walt, 1971) prepared from 4.5g yeast extract (Oxoid), 7.5 g peptone, sufficient amount of bromothymol blue was added. Sugar (2%) solutions were prepared by dissolving in yeast extract broth and the sugars were filtered by watts man paper. 4ml aliquot of the YEPD and MRS broth dispensed in test tubes. The test tubes contained an inverted Durham's tubes and the sugar solution, according to Van der Walt (1971). After sterilization, 1 ml aliquot of the sugar solution was aseptically added to the test tubes containing YPD and MRS. The sugars used for this test includes: galactose, mannose, fructose, glucose, maltose, glycerol, D-mannitol, sucrose, lactose, soluble starch, and bromothymol blue was used as an indicator. 500µl fresh yeast and LAB culture was inoculated into the test tubes contained different carbohydrates and incubated at 28°C for 24 to 72 h. All negative results obtained within the specified time were further incubated for another 72 h. A blank consisted of inoculated basal medium devoid of any carbon sources was used as a control. The accumulation of gas in the Durham's tubes and change of color was record as positive result.

3.9.6. Assimilation of carbohydrates

Assimilation of carbohydrates were determined according to the method of (Barnette, et at 1979) by the auxanographic technique on nitrogen base agar medium prepare from 5g Caesin peptone (Oxoid), 3g yeast extract (Oxoid), 15g washed agar, l000ml distilled water, pH were adjusted to 6.0 to 6.4. A volume of 15-18 rn1 aliquots dispensed in test tube was sterilized as 121°C for 15 minutes cool to 45°C and inoculate with young yeast and LAB cultures. These was thoroughly mixed and poured into sterile petri dishes to solidify. The sugars and alcohol use for the assimilation study were glucose, galactose, maltose, manitole, rabinose, turanose, and xylose About 5mg of the compounds were place on the dried agar surfaces at the edge of the petri dishes opposite to one another. Three test compounds and Glucose was tested on each petri dish. The petri dishes were incubated at 28°C for up to about 10 days and examine for growth under illuminated colony counter.

3.9.7 Diacetyl

One milliliter aliquot of the culture was mixed with 0.5 ml of 0.5% (w/v) α-naphthol and 0.2 ml KOH for 15s and allowed to stand for 5 min. The appearance of a red ring at the top of the tube was considered positive result for diacetyl production.

3.10. PHYSIOLOGICAL STRESS TOLERANCE TEST

3.10.1. ETHANOL TOLERANCE

Ethanol tolerance of yeast and LAB isolates were assessed by inoculated 5% fresh broth culture of each LAB and yeast isolate into test tube consisted of YEPD broth and MRS broth supplemented with 10, 15 and 20% ethanol (v/v) in triplicates. After inoculation, test tubes were incubated at (32°C for LAB and 28°C for yeast) for 24 h. Samples were taken after 24h and optical density was recorded at 600nm on UV-visible Spectrophotometer. A control flask contained YEPD broth and MRS broth inoculated with yeast and LAB isolate without ethanol respectively (Ali and Khan, 2014).

3.10.2. SALT TOLERANCE

The yeast and LAB isolates were tested against different concentrations of NaCI (5, 10, and 15%) in YEPD and MRS broth respectively and done in triplicates. Fresh culture of each yeast and LAB isolate (5% v/v) was inoculated into YEPD and MRS broth and incubated for 24 h at 28°C and 32°C respectively. A control flask contained the same broth culture without salt was used. Samples were taken after 24 h and optical density were recorded at 600nm using UV- visible spectrophotometer (Ali and Khan, 2014).

3.10.3. TEMPERATURE TOLERANCE

Overnight LAB isolates were incubated in MRS broth for 24 h at 30, 35, 40, and 45°C. While the yeast temperature tolerance was determined by inoculating an overnight pure culture of yeast isolate into test tubes containing YEPD broth at 25,30,35,40 and 45°C temperatures for 24 h. Samples were taken after 24 h and optical density were recorded at 600nm using U'V-visible spectrophotometer (Kurtzman, 1998).

3.10.4. ACID TOLERANCE

The LAB and yeast isolates were subjected to acid tolerance assay at pH 2.5, 3 and 3.5 according to the method described by Ramos et at. (2013). MRS broth and YEPD broth with pH adjusted to 2.5, 3 and 3.5 using 1 N HCl and inoculate overnight pure culture of yeast and LAB isolate into test tubes containing acidic MRS and YEPD broth then incubated for 24 h at 32°C. The assay was performed in triplicate. Samples were taken after 24h and optical density were recorded at 600nm using Uv-visible spectrophotometer.

3.11. STARTER CULTURE DEVELOPMENT

3.11.1. Inter-compatibility test of the isolates

The inter-compatibility of the candidate isolates were determined by cross-streaking each isolate against each other on MRS agar plate and YEPD agar followed by incubation for 48 h at (32°C for LAB and 28°C) for yeast (Anupama, 20 IS). Their growth patterns were observed after incubation. The isolates that showed best compatibility were chosen for starter formulations.

3.11.2. SELECTION OF DOMINANT YEAST AND LAB

The selection of dominate yeast and LAB was based their ability to tolerate acid, ethanol, temperature, salt tolerance and best inter compatibility test.

3.11.3. STARTER CULTURE FORMULATION

The selected isolates were formulated using Design Expert version 7.0.0, 2005, Statistical software. The number of combinations of formulated starter culture depended on the number of compatibility of candidate isolates.

3.11.4. CONTROLLED FERMENTATION

In controlled fermentation, 400ml of sterilized tap water was mixed with 200g of pasteurized honey (honey was pasteurized at 65.5°C for 30 min) in 1000 ml capacity flask (Fig 1). The medium was inoculated with LAB and yeast isolates based on the combinations/formulates obtained from Design Expert software and the mixture was incubated at 25°C for 10 days after

proper shaking. Twenty gram of washed and peeled bark of *R. prinoides* was separately autoclaved and added after 3 days of incubation and the flask was properly shaken. The flask was closed with sterile rubber stoppers and the fermentation process was continued for 9 days at room temperature. Finally, the fermentation broth was filtered through Muslin cloth to remove all sediments and *R. prinoides* . The filtrate was collected by sterilized Tej bottles for sensory analysis. The experiments were replicated two times. This procedure was done for various combinations of LAB and yeasts isolates.

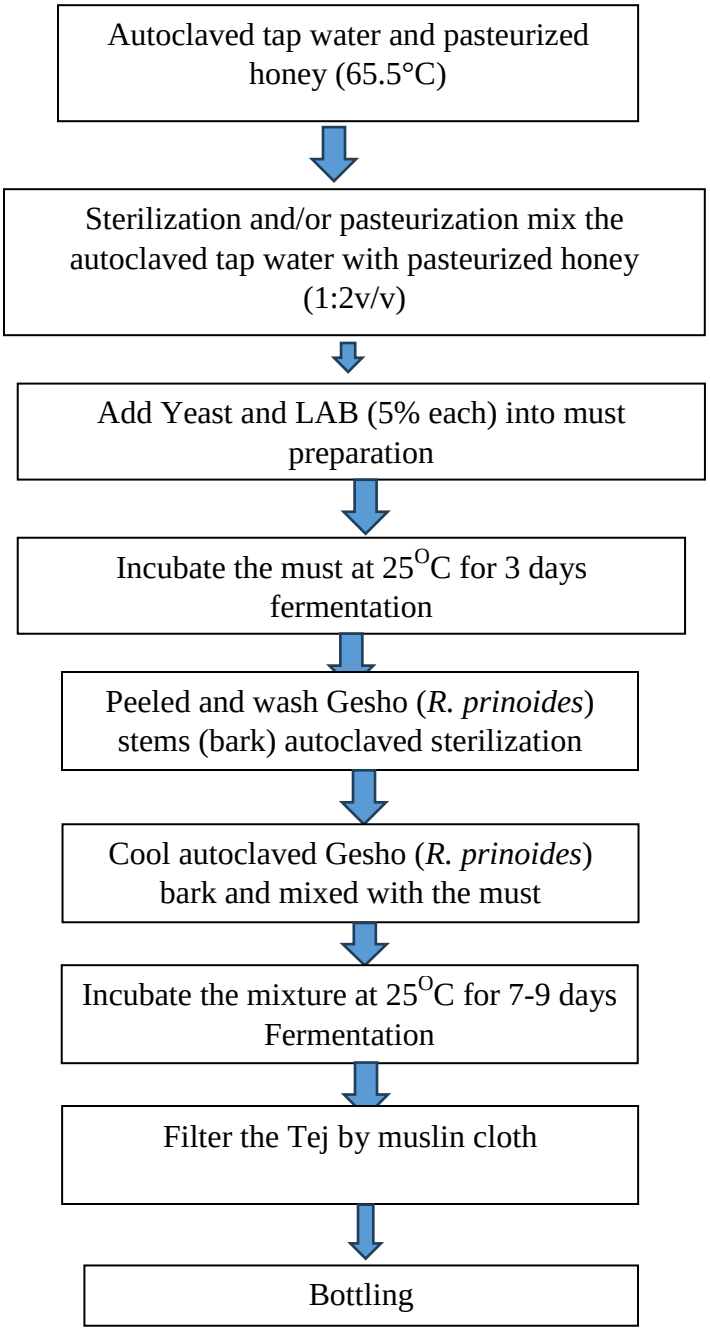


Figure 1: Laboratory preparation of Tej using mixed starter culture

3.11. SENSORY EVALUATION

A ten panel group consisting of staffs of the Faculty of Chemical and Food laboratory was used in assessing the taste, flavor, appearance and overall acceptability of Tej prepared using starter cultures. This was done based on a five point hedonic scale; l= Dislike very much, 2 = dislike, 3= neither like nor dislike, 4= like slightly, 5= Like very much.

3.12. STATISTICAL ANALYSIS

The result obtained from physicochemical, proximate, and microbial analyses of Tej and honey samples was analyzed by IBM SPSS version 26.0 software. Significant difference between the mean was tested at 95% confidence intervals. Statically tests for the treatment was using one way of ANOYA and P-value of less than 0.05 ($P<0.05$) was consider statically significance.

4. RESULTS AND DISCUSSION

4.1. RESULTS FOR HONEY SAMPLES

4.1.1. Moisture Content

The moisture contents of the honey samples analyzed in the present study ranged between 80.5 - 84.7% (Table l), The overall mean 82.58 ± 1.59 moisture content of the study area's honey was higher than the country's average (20.6%) reported by (Adgaba, 1999). Consistent with honey standards set by the Institute of Ethiopian Standards, the moisture content of the study area's honey is above the Grade category (ES, Honey specification, 1202:2021). The maximum acceptable limit for moisture content of Ethiopian honey is 20% (Adgaba, 2007) while the maximum acceptable moisture content of honey reported by the International Honey Commissionis 20% (Bogdanov, 2002).

The moisture content of honey depends on various factors like the harvesting season, the degree of maturity that honey reached in the hive, type of hive used, and environmental temperature The moisture content of honey samples obtained from Addis ketema (Table 1) were significantly ($P<0.05$) higher than honey collected from the study sites. The variation observed in moisture content among honey samples obtained from the Addis ketema may be due to the difference in bee-hive handling practiced by the beekeepers. There is no significant moisture content differences ($p>0.05$) between honey samples obtained from the Kirkos and Woliso sites. In this study the moisture content of 99.9% of honey samples was above 20%. The moisture content of honey is affected by various factors, including environmental conditions, beekeeper practices during harvest, handling, and storage, and the degree of maturity reached in the hive. Other factors, such as weather conditions, handling practices, adulteration with water, and water content of the nectar in the original plant, also contribute to the increase in moisture content (Nigussie *et al*., 2012; Finola *et al*., 2007; Ajlouni & Sujirapinyokul, 2010).)

4.1.2. pH Value

Most honey is acidic in nature and has low pH value. The collected honey samples had a pH value ranging from 2.96-4.45 with an average of 3.92, which is within the acceptable range (between 3.2 and 4.5) set by Bogdanov (2009). No significant differences ($p>0.05$) in pH were observed between honey samples obtained from Adama, Sebeta, Woliso, Kirkose and Addis Ketema (Table. 1). This parameter is very important during the extraction and storage of honey because it influences the texture, stability and shelf life of honey. The results of this study were comparable with report of (Nemo and Bacha (2021).

4.1.3. Hydroxymethyl furfural

HMF content of the honey samples analyzed in this study ranged from 0.13 to 0.38mg/kg (Table.l). The average HMF content of honey collected from five site (Aama, Sebeta, Woliso, Kirkos and Addis Ketema) were statistically significant ($p<0.05$). The amount of HMF in honey is one of the important indicators of honey quality. In fresh honey, HMF is present only in trace amounts and its concentration increases with storage and prolonged heating of honey (Bogdanov, 2011). Diez, (2002) reported that the hydroxymethylfurfural content of honey is an indicator of its freshness. The HMF content of all the analyzed honey samples fell within the range reported by the Ethiopian Quality and Standards Authority (IES, 2021) and international Honey Commission (Bogdanov, 2002).

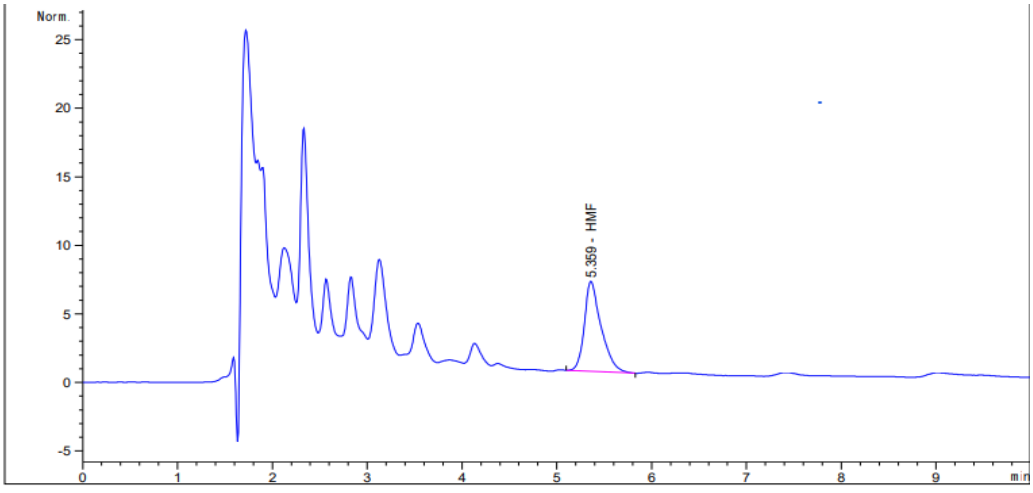


Figure 2 Representative HMF of honey samples.

4.1.4. Reducing Sugar Content of honey

Reducing sugar content of the samples analyzed in this study ranged from 5.54 to 10.01g/100g There is no significant difference ($p<0.05$) in the content of sucrose and maltose between honey samples obtained from Sebeta and Woliso site (Table. 1). Sugars are the main constituents of honey comprising about 95% of honey dry weight (Bogdanov, 2011). Reducing and non-reducing sugars together, account for 85-95% of the carbohydrate in honey. However, the mean glucose, fructose and turanose content of honey samples collected from all sites (Adama, Sebeta, Woliso, Kirkose,and Addis ketema) were significantly different ($p<0.05$) (Table. 1). Added sugar in honey can be detected by measuring its sucrose content. The sucrose content of honey mainly depends on the botanical origin of nectar and according to International Regulatory Standards it should not exceed 5% (g/100g).

4.1.5. The free acidity

Free acidity level of honey samples analyzed in the present study, ranged from 26.6 to 35.2 meq/kg (Table.1). The values of free acidity obtained from the study sites were lower than the 50.4 meq/kg obtained in Algeria and comparable with l7.6 meq/kg reported in Azerbadjan. The acidity of honey samples obtained from the five sites (Table. 1). There is no significant differences ($p>0.05$) observed between the study sites. None of the samples exceeded the honey acidity limit suggested by national (40 meq/kg) (Adgaba, 1999) and international standards (50 meq/kg) (Bogdanov, 2002) .The current samples showed acceptable acidity levels for both national and the Codex Alimentarius criteria. Such results indicate the freshness and conservation of the honey samples and absence of unwanted fermentation in the analyzed honey samples. The acidity of honey is important for taste (Bogdanov, 201I). The main organic acid found in honey is gluconic acid. Honey also contains minor components such as formic, acetic, citric, lactic, maleic, malic, oxalic, pyroglutarnic and succinic acids (Bogdanov, 2011).

4.1.6. Ash Content

Ash content of the honey samples obtained from the study area ranged from 0.07-0.1g (Table. 1). (Bogdanov,2011). There is no significant difference ($p>0.05$) in ash content was observed between honey samples obtained from the five sites (Table. 1) The ash content of all the analyzed honey samples was comparable to the values of 0.1- 0.5%

reported from Algeria. The ash content of all the analyzed honey samples fell within the acceptable range reported by the Ethiopian Quality and Standards Authority (QSAE, 2005) and 0.5% maximum limit reported by the International Honey Commission (Bogdanov, 2002).

4.1.7. Electrical Conductivity

EC of honeys is closely related to the concentration of mineral salts, organic acids, and proteins found in it. The studied honeys show electrical conductivities varying within 0.45-0.64ms/cm (Table 1). It is a good criterion for determining the botanical origin of honey. This parameter is also used for the classification of monofloral honeys. There is highly significant difference ($p>0.05$) in the electrical conductivity of homey samples observed in five sites (Table 1). The electrical conductivity of all the analyzed honey samples fell in the range reported by the Ethiopian Quality and Standards Authority (QSAE, 2005) and 0.8ms/cm maximum limit reported by the International Honey Commission (Bogdanov, 2002).

Table 1. Physicochemical properties and proximate analysis of honey produced in the central and around central Ethiopia.

Source value n=5	Adama	Sebeta	Woliso	Kirkos	Addis Ketema	Ethiopian standard	Codex Alimentarius standard	EU standard
pH	4.11	4.09	3.99	3.99	3.44	-	-	-
MC (%)	82.9	82.9	80.5	83.6	84.7	<20	<20	<20
Ash (%)	0.1	0.1	0.07	0.1	0.07	<0.6	<0.6	<0.6
EC	0.49	0.64	0.45	0.50	0.56	<0.8	<0.8	<0.8
FA	35.2	30.9	33.3	30.9	26.6	<40	<50	<50
Glucose (g/100g)	6.38	6.32	6.43	6.34	6.33	-	-	-
Fructose (g/100g)	5.59	5.56	5.59	5.58	5.62	-	-	-
Turanose (g/100g)	8.92	8.88	8.96	8.92	8.91	-	-	-
Sucrose (g/100g)	8.18	8.17	8.20	8.18	8.18	<8	<8	<8
Maltose (g/100g)	9.96	9.94	9.96	9.99	9.98	-	-	-
HMF (mg/kg)	0.15	0.33	0.38	0.13	0.23	<40	<40	<40

4.2. PHYSICOCHEMICAL PROPERTIES OF TEJ

4.2.1. Titratable Acidity and pH

TA and pH are the two most imperative physicochemical parameters for all type of alcoholic beverages (Tyl and Sadler, 2017). The pH values of the collected Tej samples ranged from 3.45 to 4.20. Similarly, TA for these samples ranged from 1.00 to 2.70g/l. The lower and higher pH and TA values were recorded for the samples collected from Holeta and Nifas silk areas respectively (Table 2). The possible reasons for these differences in pH and TA could be due to

degree of fermentation, type of honey, variable stay /production/ storage time, and/or kind of microbes involved during the course of fermentation. (Nemo & Bacha, 2020) also reported a similar result for Tej samples. In this study, a statistically significant difference ($p < 0.05$) was observed for pH value between the collected Tej samples (Table 2). Nevertheless, no statistically significant difference ($p>0.05$) was observed for the group mean TA value across the samples collected from different areas (Table 2). Based on these pH and TA values, it would not be incorrect to classify Tej under mild acidic alcoholic beverage. Ethanol and organic acid which were produced by the species of *Saccharomyces, Zymomonas* and *Lactobacillus* had great contribution for the results. Favorably, nowadays sour honey wine is getting a good consumer acceptance (Peepall *et al.,* 2019).

Table 2. pH and Titratable acidity of Tej samples

STUDY SITE	**pH**			**TA**		
	Mean	Min	Max	Mean	Min	Max
Sebeta	3.85	3.71	3.96	1.96	1.80	2.30
Nifas silk	3.79	3.62	4.00	2.06	1.30	2.70
Kolfe	4.10	3.95	4.20	1.27	1.09	1.40
Almgena	3.67	3.61	3.73	1.14	1.05	1.21
Kirkos	3.67	3.56	3.73	1.32	1.03	1.81
Sendafa	3.86	3.73	4.10	1.32	1.02	1.54
Sululta	3.79	3.77	3.84	1.23	1.14	1.30
Adama	3.58	3.50	3.63	1.31	1.13	1.46
Holeta	3.67	3.45	3.80	1.37	1.25	1.51
Yeka	3.68	3.52	3.90	1.69	1.00	2.46
Total	3.77	3.45	4.20	1.47	1.00	2.70

4.2.2. Electric conductivity

Due to the advancement of novel food processing technologies, electric conductivity (EC) of foods and beverages is becoming a very vital parameter (Jha, *et al.,* 2011). Sugars, ash, organic acids and proteins contents of the food materials have a big influence on the values of electrical conductivity (Acquarone *et al.,* 2007). The mean Ec values recorded for the collected Tej samples were ranged from 0.44 to 0.76 mS/cm (Table 3). Statistically significant EC value variation ($p < 0.05$) was also observed among the Tej samples (Table 3). The type and amount of honey, the unstandardized composition of raw materials used to prepare Tej also would cause variation in the final important nutrients and the way Gesho *(R. prinoidesi)* is added to the honey water mixture could be the possible reasons for this Ee value variations. Similar EC values for the Tej samples were also reported by (Nerno and Bacha, 2020).

4.2.3. Alcohol Content

The objective of alcoholic fermentation is production of ethanol from a given carbon source (Tamang, 2010) .The mean ethanol content of Tej samples collected from the different areas were in the range of 8.62-14.49g/100mL (Table 3). This wide range in the alcohol content could be due to the variable standard and composition of raw materials, type and abundance of fermentative microorganisms, conditions of fermentation process, period of fermentation, and storage conditions after fermentation (Rodriguez-Bencomo *et al.,* 2012).

Almost all of the collected Tej samples had shown a statistically significant difference ($p <0.05$) in the value of alcohol content (Table 3). One of the samples from Kirkose area had recorded maximum ethanol content (14.49 g/l00 mL). In addition to, the other possible justifications which were already raised before, this higher alcohol content could be due to the co-dominance of Saccharomyces and Zymomonas in the specified sample. In fact, all of the samples dominated by these two genera showed higher alcohol content (Table 3). However, the sample from Nifas silk show lower ethanol content (8.62 g/l00 mL) compared to other Tej samples. The possible reason for this might be due to the short fermentation time and low other raw materials to water ratio during production. In comparison to the other beverages, the mean alcoholic content of Tej in this study is above that of Tej (13 to 13.73) , from Addis Ababa as reported by Alemu Fite et .01.(1991), and it falls in the range for that of meads, (6.6 to 14.2) (Steinkraus, 1983); Coconut toddy (Bassir, 1968), and other

samples of Tej (Steinkraus, 1983). But, the mean alcohol content of Tej, in this study is above the mean alcohol content of honey wines in the US market (6.4 to 16.6) (Steinkraus, 1983), (7 to 13) as reported by Belachew Desta (1977).

4.2.4. Specific gravity

Specific gravity of the selected Tej showed that, the mean SG (g/100 ml) of Tej samples collected from the different areas was in the range of (0.9-1.0). In this study, a statistically no significant difference ($p>0.05$) was observed for specific gravity value between the collected Tej samples (Table 3) and also the results were comparable with ES 6841:2021.

Table 3. EC, AC and SG of Tej Samples.

Study Sites	**EC**			**AC (g/100g)**			**SG(g/100g)**		
	Mean	**Min.**	**Max.**	**Mean**	**Min.**	**Max.**	**Mean**	**Min.**	**Max.**
Sebeta	0.580	0.530	0.617	10.85	9.53	12.00	1.00	1.00	1.00
Nifas silk	0.669	0.601	0.741	9.63	8.62	10.33	0.96	0.90	1.00
Kolfe	0.594	0.529	0.651	13.40	11.61	14.41	0.96	0.90	1.00
Almgena	0.528	0.500	0.561	13.41	12.45	14.01	1.00	1.00	1.00
Kirkos	0.449	0.389	0.484	13.50	12.02	14.49	0.99	0.99	1.00
Sendafa	0.732	0.674	0.838	12.42	12.24	12.72	0.99	0.99	1.00

Sululta	0.762	0.699	0.836	13.17	12.76	13.42	1.00	1.00	1.00
Adama	0.701	0.579	0.873	11.13	10.92	11.46	0.93	0.90	1.00
Holeta	0.657	0.582	0.702	10.33	9.71	11.10	1.00	1.00	1.00
Yeka	0.701	0.603	0.885	9.73	9.01	10.21	0.99	0.99	1.00
Total	-	-	-	11.76	8.62	14.49	0.98	0.90	1.00

Note: EC= Electrical conductivity, AC=Alcohol content and SG=Specific gravity

n=number of samples.

4.2.5. Moisture Content

The mean moisture content (g/100 ml) of Tej samples collected from the different sampling areas were in the range of (84.15-95.00). The values are comparable with (95.78) with the values reported by Nemo and Bacha (2021). Tej samples collected from Yeka site had the lowest moisture content and samples from Nifas silk had higher moisture content than samples collected other sampling sites. Statistically significant difference ($p < 0.05$) in the moisture content was observed between the collected Tej samples (Table 4). The moisture content of Tej samples was higher than Borde, Grawa and Tella (Nemo and Bacha, 2021) and (Sahle and Abegaz, 1991).

4.2.6. Ash Content

The proximate composition analysis of the Tej showed that, the mean ash content (g/100 ml) of Tej samples collected from the different areas were in the range of (0.0-0.2). Its lower than the values reported by Nemo and Bacha (2021). The mean of ash content of Tej was 0.4. From ten study sites seven sites (Nifas silk, Kolfe, Alemgena, Kirkos, Sendafa, Sululuta and Adama had the lowest ash content which is (0.0) and Tej samples from Sebeta site had higher ash content which is 0.2 than other study sites. In this study, a statistically significant difference ($p < 0.05$) was observed for ash content value between the collected Tej samples (Table 4). Processing methods and temperature applied during boiling the content would be the possible reason for the minimal variation.

Table 4. MC and AC of Tej samples.

Study Sites	MC (%)			Ash (%)		
	Mean	**Min.**	**Max.**	**Mean**	**Min.**	**Max.**
Sebeta	92.70	91.20	94.25	0.16	0.10	0.20
Nifas silk	92.31	89.30	95.00	0.06	0.00	0.10
Kolfe	89.95	87.90	92.75	0.03	0.00	0.10
Almgena	89.21	88.65	90.15	0.06	0.00	0.10
Kirkos	90.45	88.65	91.85	0.03	0.00	0.10
Sendafa	88.50	87.00	91.00	0.06	0.00	0.10
Sululta	87.98	86.35	89.30	0.03	0.00	0.10
Adama	91.50	90.65	92.15	0.06	0.00	0.10
Holeta	87.98	86.35	89.30	0.03	0.00	0.10
Yeka	86.71	84.15	88.75	0.10	0.10	0.10
Total	89.82	84.15	95.00	0.07	0.00	0.20

Note: MC= Moisture Content and AC= Ash Content

4.3. MICROBIOLOGICAL ANAYLSIS OF TEJ AND HONEY

4.3.1. Aerobic mesophilic bacterial

The mean value of Aerobic mesophilic bacteria counts of Tej samples was 5.96 ± 1.12log cfu/ml. this study had larger colony count of *AMB* than report of Nemo and Bacha (2020). And no significant difference ($p > 0.05$ was observed between the samples in *AMB* counts. *AMB* is an indicator of quality and have indirect contribution towards a safety assessment of ready-to-eat food; however, it gives useful information about the remaining shelf-life of the food products (Centre for Food Safety, 2022). Contamination of these Tej samples could result from pre- or post-processing contamination from the handlers.

The Local beverage vendors are often unlicensed, untrained in food safety, food hygiene and sanitation, and they work under unclean conditions (FAO, 2014). In this study, the *Aerobic spore-forming bacteria* (ASFB), total coliforms and *Enterbacteriaceae* counts were not

detected. Similarly, Bahiru *et al.,* (2001) also reported the absence or very low detection of ASFB among the examined samples. The absence of Enterobacteriaceae in Tej samples were also reported from Debre Markos (Getachew *et al.,* 2022). The absence of coliform, *Enterobacteriaceae* and Aerobic spore formers is mainly correlating with the high acidity and relative safety of the samples.

Table 5.Mean value of AMB

Study Sites	***AMB* (log cfu/ml))**		
	Mean + std.	**Min.**	**Max.**
Sebeta	5.50±0.79	4.60	6.10
Nifas silk	4.20±2.55	2.00	7.00
Kolfe	6.83±0.56	6.20	7.30
Almgena	6.83±0.11	6.70	6.90
Kirkos	5.33±0.72	4.50	5.80
Sendafa	6.93±0.05	6.90	7.00
Sululta	6.43±0.64	5.70	6.90
Adama	5.83±0.11	5.70	5.90
Holeta	6.00±0.17	5.80	6.10
Yeka	5.73±0.56	5.10	6.20
Total	5.96±1.12	2.00	7.30

4.3.2. Yeast and Molds

Yeast and molds mean counts (log cfu/ ml) was 6.56±0.59, the current study indicated that all Tej samples had no significant differences between the mean values of yeast. This result was align with the report of Nemo and Bacha, (2020) reported that yeast were the dominant microbes in Tej samples.

According to the spss outputs (table 6), the mean yeast and mold statistically significant yeast and mold value variation ($p < 0.05$) was also observed among the Tej. This significant variation in yeast and mold count is mainly indicates the fermentation status and higher amount of yeast

content in the raw materials before production.

Table 6. Mean value of Yeast and Mold.

Study Sites	***YM*(log cfu/ml))**		
	Mean +std.	**Min.**	**Max.**
Sebeta	6.60±0.60	5.90	7.00
Nifas silk	6.00±0.00	6.00	6.00
Kolfe	6.13±0.61	5.60	6.80
Almgena	5.80±0.26	5.60	6.10
Kirkos	6.56±0.66	5.80	7.00
Sendafa	6.53±0.56	5.90	7.00
Sululta	7.33±0.58	6.90	8.00
Adama	6.80±0.26	6.60	7.10
Holeta	6.90±0.17	6.80	7.10
Yeka	6.93±0.37	6.50	7.20
Total	6.56±0.59	5.60	8.00

Note: *YM= Yeast* and *Molds*

4.4. CHARACTERIZATION AND IDENTIFICATION OF THE ISOLATED YEASTS AND LAB

For the identification of the Yeast and LAB isolates microscopic observation and physiological tests were done.

4.4.1. Microscopic observations of the isolated yeasts

A total of 150 yeasts were isolated from 30 Tej samples however, only ten yeast isolates that showed best growth performance in all physiological stresses (ethanol, acid, temperature and salt tolerances) were listed in Table 7. The cultural characteristics of all potential yeast isolates were smooth, white, dry and circular in margin, color, texture and shape, respectively. The cultural characteristics of 4c, 5d, 13a, 25c and 27a yeast isolates were flat and 6a, 7a, 23a, 28a

and 29a isolates were raised in elevation. All the potential yeast isolates were oval and cellular morphology.

Table 7. Cultural, Morphological, and Biochemical characteristics of yeast isolates'

Isolate code	**Cultural characteristics**			**Cell shape**			**Catalase**
	Margin	**Color**	**Elevation**	**Texture**	**Shape**	**Shape**	
4c	Smooth	White	raised	Dry	Circular	Oval	+
5d	Smooth	White	raised	Dry	Circular	Oval	+
6a	Smooth	White	flat	Moist	Circular	Oval	+
7a	Smooth	White	flat	Dry	Circular	Oval	+
13a	Smooth	White	raised	Dry	Circular	Oval	+
23a	Smooth	White	flat	Dry	Circular	Oval	+
25a	Smooth	White	raised	Dry	Circular	Oval	+
27a	Smooth	White	raised	Dry	Circular	Oval	+
28a	Smooth	White	flat	Dry	Circular	Oval	+
29a	Smooth	White	flat	Dry	Circular	Oval	+

4.4.2. Microscopic observations of the isolated LAB

A total of 400 *LAB* were isolated from 30 Tej samples however, only ten *LAB* isolates that showed best growth performance in all physiological stresses (ethanol, acid, temperature and salt tolerances) were listed in Table 8. All the isolates were gram positive or KOH negative, catalase negative and non-spore formers. The cultural characteristics of all *LAB* margin were smooth. Two *LAB* isolates color were white and the rest were grey. About 40% of LAB textures were moist and 60% were dry. The shapes of all LAB isolates were circular in cultural characteristics and had streptococci cell arrangement in morphological characteristics under microscope except AAUL3 *LAB* isolate which was diplococcal (Table 8).

Table 8: Cultural, Morphological, and Biochemical characteristics of LAB *isolates.*

Isolate code	Cultural characteristics				Cell shape		Biochemical test		
	Margin	**Color**	**Elevation**	**Texture**	**Shape**	**Shape**	**Catalase**	**KOH**	**Spore**
AAUL 1	Smooth	White	raised	Dry	Circular	Streptococci	-ve	-ve	-ve
AAUL 2	Smooth	White	raised	Moist	Circular	Streptococci	-ve	-ve	-ve
AAUL 3	Smooth	White	flat	Moist	Circular	Diplococcic	-ve	-ve	-ve
AAUL 4	Smooth	White	raised	Dry	Circular	Streptococci	-ve	-ve	-ve
AAUL 5	Smooth	White	raised	Dry	Circular	Streptococci	-ve	-ve	-ve
AAUL 6	Smooth	White	raised	Dry	Circular	Streptococci	-ve	-ve	-ve
AAUL 7	Smooth	White	raised	Dry	Circular	Streptococci	-ve	-ve	-ve
AAUL 8	Smooth	White	raised	Dry	Circular	Streptococci	-ve	-ve	-ve
AAUL 9	Smooth	Grey	flat	Moist	Circular	Streptococci	-ve	-ve	-ve
AAUL 10	Smooth	Grey	flat	Moist	Circular	Streptococci	-ve	-ve	-ve

Note: AAUL=Addis Ababa University LAB isolate; AAUL7, AAUL8 ....AAUL l0 are LAB isolate negative = no production.

Among the ten LAB isolates, six of them (AAULl, AAUL2, AAUL4, AAUL5, AAUL9 and AAULIO) were hetero-fermentative, while the rest of the isolates (AAUL3, AAUL6, AAUL7, and AAUL8) were homo-fermentative group of LAB. According to their biochemical and cellular morphology (Table 8), the ten LAB isolates were tentatively grouped into three genera: Lactobacillus (20%), Leuconostoc (40%) and Lactococcus 40%).

Table 9.Grouping of LAB genera based on biochemical characteristics cultural, morphological and gas production.

Isolate Code	**LAB Type**	**Gas production**	**Cellular Shape**	**Genera**	**Diacetyl**
AAUL 1	Hetero	+	Rod	*Lactobacillus*	+
AAUL 2	Hetero	+	Cocci	*Leuconostoc*	+
AAUL 3	Homo	-	Cocci	*Lactococcus*	+
AAUL 4	Hetero	+	Red	*Lactobacillus*	+
AAUL 5	Hetero	+	Cocci	*Leuconostoc*	+

AAUL 6	Homo	-	Cocci	*Lactococcus*	+
AAUL 7	Homo	-	Cocci	*Lactococcus*	+
AAUL 8	Homo	-	Cocci	*Lactococcus*	+
AAUL 9	Hetero	+	Cocci	*Leuconostoc*	+
AAUL 10	Hetero	+	Cocci	*Leuconostoc*	+

Note: "-"= *no gas production;* "+"= *gas production*

4.4.3 Physiological test of the isolated yeasts

Physiological properties of the dominant yeast isolates of Tej at the time of consumption are shown in Table 10. All the yeast isolates were fermentative. All of them were found to be positive for the fermentation of glucose, sucrose, maltose, and raffinose; negative for lactose, trehalose, starch, dextrin and xylose. They assimilate glucose, sucrose, maltose, trehalose and raffinose; while lactose, starch, dextrin and xylose were not assimilated by them. The results of the microscopically observations and the physiological tests showed that the dominant yeasts isolated from the Tej were all *Saccharomyces cerevisiae*, *Saccharomyces daireness*, *Debaryomyces carsonii* and *Saccharomyces pastorianus* . As fermentation progresses, wild yeasts die due to their sensitivity to ethanol concentration of 2 to 6 % (v/v) while *S. cerevisiae* proliferates and completes the fermentation.

Yeasts of the genus *Saccharomyces* were considered by Vogel and Abeba Gobezie (1977) to be responsible for the conversion of sugars to ethanol in Tej. High sugar concentrations and high specific growth rates trigger alcoholic fermentation by *Saccharomyces cerevisiae* even under fully aerobic conditions (Van hoek *et al.,* 1998). Similar to findings in this study, the dominant organisms in Phillipine basi were Saccharomyces, Endomycopsis and lactic acid bacteria (Sanchez, 1977; Tanimura *et al.,* 1978).

Table 10. Fermentation and Assimilation.

Species of yeast Isolates		Fermentation										Assimilation									
Code		Galactose	Maltose	A/rabinnose	Sucrose	Rabinose	Xylose	D-Manitol	Turanose	D-Xylose	D-Galactose	D-glucose	Maltose	Lactose	Sucrose	D-Fructose	D-Mannose	D-Manitol	Glycerol	D-Xylose	D-Galactose
4c	*saccharomyces cerevisiae*	–	+	–	+	+	+	–	–	–	+	+	+	–	+	+	+	–	+	+	+
5d	*NI*	+	–	–	+	+	+	–	–	–	–	+	–	–	+	+	V	+	+	+	–
6a	NI	+	+	–	+	–	+	–	–	–	+	+	+	–	+	+	–	+	–	+	+
7a	*Saccharomyces*	+	+	–	+	–	+	–	–	–	+	+	+	+	+	+	–	+	–	–	+
	pastorianus																				
13a	NI	+	+	–	+	+	+	–	–	–	+	+	+	–	+	+	+	–	+	–	+
23a	*saccharomyces daireness*	–	–	–	–	–	–	–	–	–	–	+	+	–	+	+	+	+	V	V	+
25c	*NI*	+	+	–	+	+	+	–	–	–	+	+	+	–	+	+	+	–	V	–	+
27a	*Debaryomyces carsonii*	+	+	–	+	+	+	–	–	–	–	+	+	+	+	+	–	+	–	+	+
28a	NI	–	+	–	+	+	+	–	–	–	–	+	+	+	+	+	+	+	–	+	+
29a	NI	-	+	+	+	+	+	–	+	+	+	+	+	+	v	+	+	+	+	-	-

V = variable, "+" = positive, "– "= negative "NI"= not identified, Source: Barnette *et al.,* (1979).

4.5. PHYSIOLOGICA STRESS TEST OF YEAST AND LAB

4.5.1. Ethanol

Ethanol tolerance of yeast isolates were summarized in Figure 2. All yeast isolates shown different levels of tolerance against various concentrations of ethanol. Generally, most yeast isolates were better survived in 10% ethanol concentration, followed by 15% and 20%. With OD600 values ranged from 1.0-1.9%, 0.30-1.40% and 0.30-1.00%, respectively. The highest l0% ethanol tolerance was observed by 28a followed by 27a and 29a.

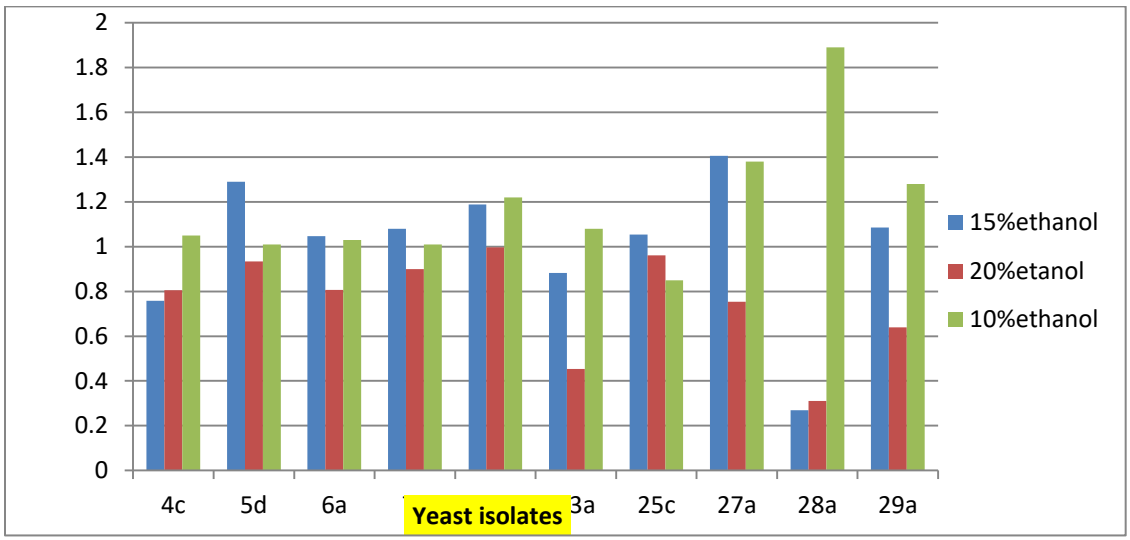


Figure 3.Ethanol tolerance Yeast isolated at 24h of incubation.

4.5.2. pH Value

The physiological stress tolerance characteristics of yeast isolates were determined by different concentrations of pH, various levels tolerance was observed among the yeast isolates. The 00600 ranges of 1.1 to 1.8 was observed at pH 3.5. The lowest pH tolerance ranges were recorded at pH 2.5, followed by pH 3.0. Tills results indicated that as the pH value increase, the growth performance of the yeast isolates were also increased. According, to Vinlcius *et al.,* 2012, all of the cocoa yeast isolates tolerated the pH range of a typical cocoa fermentation process (from pH 2.0 to pH 5.0). So the current study

comparable.

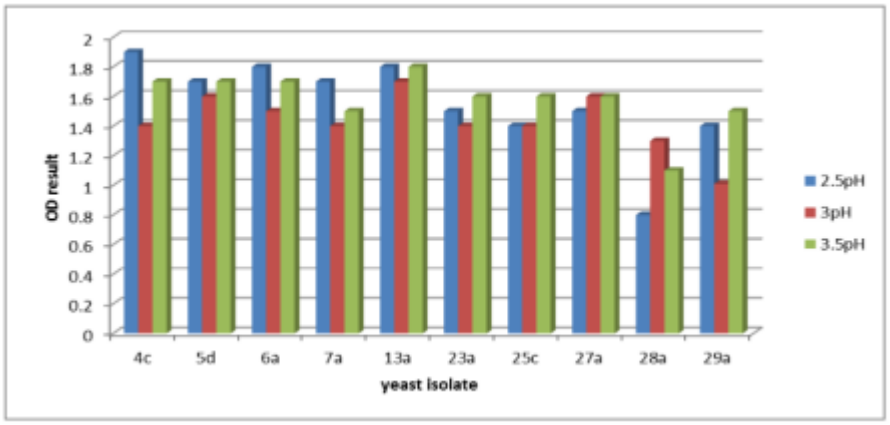


Figure 4.pH tolerance of yeast isolates at 24h of incubation.

4.5.3. Temperature

The temperature tolerance of yeast isolate at 25°C range from 1.2 to 1.8OD600 (Figure 4).5d, 6a, 23a, 27a have high OD600 (l.8) result in 30°C range 0.6- 2.0OD600 result 4c have high OD result and followed by 6a, 13a, 27a and 29a ..With regard to temperature tolerance, the yeast isolates better survived with OD600 ranges of 2.2 to 5.6 at temperature of 25°C, 30°C and 35°C than 40°c and 45°c. However, at 40 and 45°C, varied survivals were recorded. Generally, most yeast isolates had better survived in 40°C than 45°C. Overall, the yeast isolates in this study showed better performance in slats and temperature tolerate than the commercial yeast strain (*S. pastorianusy*).

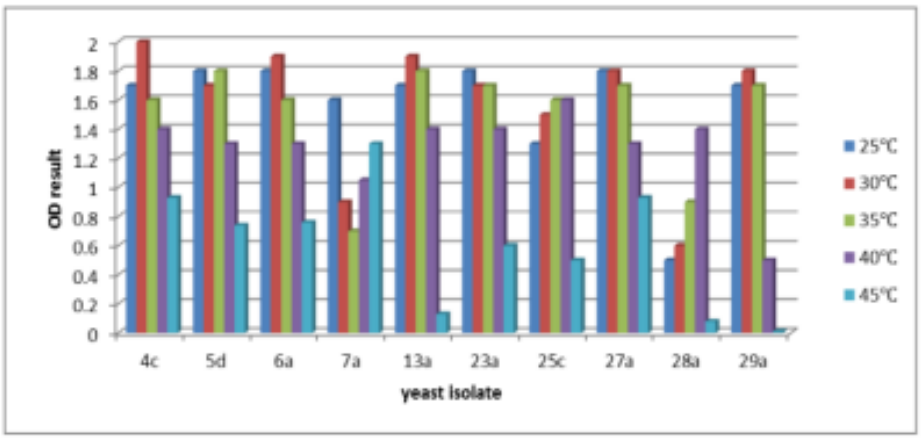


Figure 5.Temperature tolerance of yeast isolates at 24h of incubation.

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4.5.4. SALT

All the yeast isolates were tested for survival against various concentrations of salt. As the results indicated in Figure 5, at 5% salt concentration, most yeast isolates survived in the ranges of 0.2 to 1.1 OD600. But at 10% slat concentration all yeast isolates were less than 0.8 OD600.

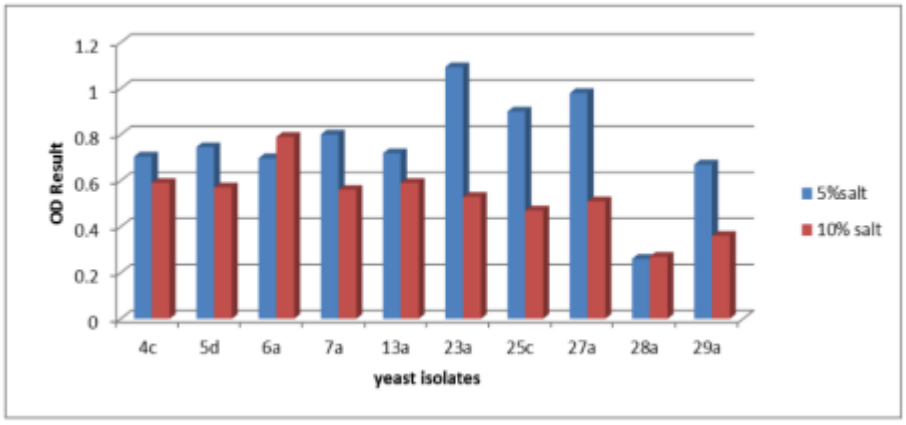

Figure 6.Salt tolerance of yeast isolates at 24h of incubation.

Ten LAB isolates were determined by different concentrations of ethanol (10, 15 and 20%), pH (2.5,3.0, and 3.5), temperature (35, 40, and 45°C), and salt (5 and 10%). All LAB isolates grown at 10,15 and 20% ethanol (v/v) with 00600 values ranged from 0.30-0.98,0.31-1.50 and 1.10- 4.35, respectively. The highest l0% ethanol tolerance was observed by AAUL9 followed by AAULS and AAUL6. With regard to pH, various levels tolerance was observed among the LAB isolates. The 00600 ranges of 0.9 to 2.8 was observed at pH 3.5. The lowest pH tolerance ranges were recorded at pH 2.5, followed by pH 3.0. This results indicated that as the pH value increase, the growth performance of the LAB isolates were also increased (Fig. 6).

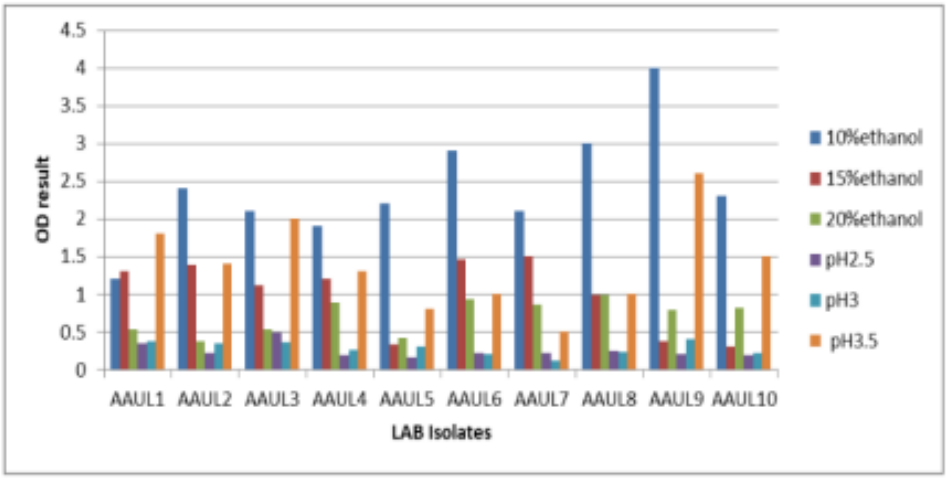

Figure 7.Ethanol and pH tolerance of LAB isolates at 24h of incubation.

The temperature tolerance levels of LAB indicated in Figure 7. Comparing the three selected temperatures, isolates had better survived at 30°C.At 30°C the highest OD600 was recorded by isolate, AAUL9 (6.9), followed by AAUL2 (6.0). Almost the same tolerances were observed by AAUL3, AAUL6 and AAUL8 which was around 00600 of 4.50. LAB had high 00600 record at 45°C next to 30°C and at 40°C and 35°C the results were varied. The salt tolerance test ranges from 2.0 to 4.2 00600 was recorded at 5% salt concentration. At 10% salt concentration almost all LAB isolates were survived below 0.2 00600.

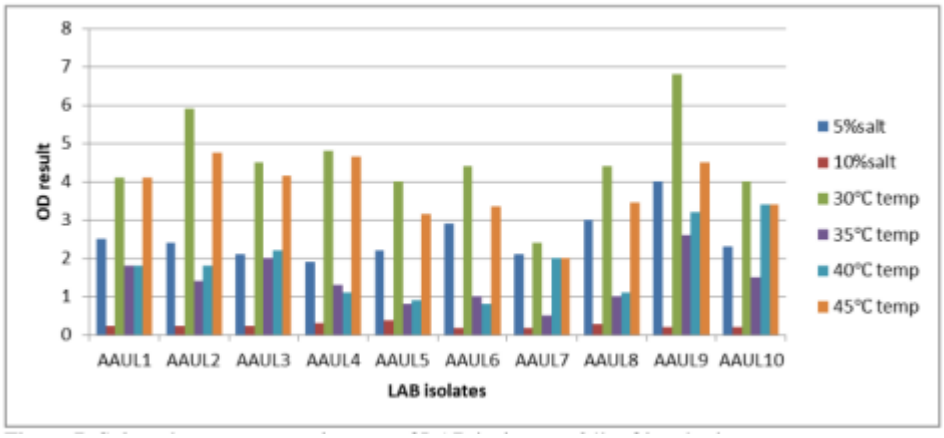

Figure 8.Salt and temperature tolerance of LAB isolates at 24h of incubation.

4.6. STARTER CULTURE DEVELOPMENT

4.6.1. Selection of dominant yeast and LAB

Among the LAB and yeast isolates, four best yeast isolates (4C, 7A, 23A and 27A) and four best LAB isolates (AAUL7, AAUL8, AAUL9 and AAULl0) were selected for evaluating their potential as starter culture based on their best overall growth performance at physiological stress conditions. In addition, compatibility test was used as the second selection criteria for development of starter culture.

4.7. COMPATIBILITY TEST

All the four LAB and four yeast isolates were cross strickled on MRS agar plats and all isolates found to be compatible as indicated in Figure 8. None of the isolates had antagonistic effect against each another. Moreover, (4C, 23A, 27A and 7A) and (AAUL7, AAUL8, AAUL9 and AAULl0) isolates had shown more compatible than others.

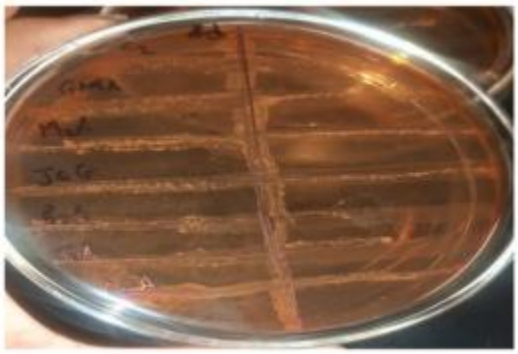

Figure8. Compatibility test

4.8. FERMENTATION OF TEJ USING MIXED STARTER CULTURE

The isolates (LAB and Yeast) were mixed in different combinations and proportions using Design of expert version 13.0 software (2020). Totally fifteen combinations/formulates of starter cultures were made in different proportions (Table 11). These formulates were used to prepare Tej under controlled fermentation process. Spontaneous fermentation was used as control.

Table 11.Formulations of the starter culture.

Formulates	AAUL1 Ml	AAUL2 Ml	AAUL3 Ml	AAUL4 Ml	4CY1 Ml	7AY2 Ml	23AY3 Ml	27AY4 Ml	Total starter inoculates 5%
F #1	2.5	0	0	0	2.5	0	0	0	5%
F #2	2.5	0	0	0	0	0	0	2.5	5%
F #3	0	0	2.5	0	0	2.5	0	0	5%
F #4	0	2.5	0	0	0	2.5	0	0	5%
F #5	0	0	0	2.5	0	0	0	2.5	5%
F #6	0	0	2.5	0	0	0	0	2.5	5%
F#7	0	0	0	2.5	0	0	2.5	0	5%
F#8	0	0	0	2.5	0	0	0	2.5	5%
F#9	0	0	2.5	0	2.5	0	0	0	5%
F#ALL	0.625	0.625	0.625	0.625	0.625	0.625	0.625	0.625	5%
Control									no starter

Note: Control = spontaneously fermented Tej; LAB isolates= AAUL7, AAUL8, AAUL9 and AAUL10; Yeast isolates =4CYl, 7AY2, 23AY3 and 27AY4 and formulated starter culture= Fl, F2 ... Control.

4.9. SENSORY ANALYSIS

The sensory evaluation of controlled fermentation of Tej samples were tested by 10 panelists. The 10 panelists were evaluated all prepared Tej filtrates using 5 point hedonic scales (l=dislike extremely, 2= dislike moderately, 3= neither like nor dislike, 4= like moderately, and 5= like extremely) for the selected descriptive characteristics such as odor, color, texture, and overall acceptability. The score sheet was prepared and gave to each panelist. The highest overall sensory acceptability values of 4.5, 4.4 and 4.3 were observed by formulates #5, #6,and #8 respectively and least overall sensory acceptance mean score of 3.8 was observed by formulate 2 (Table 12). The overall sensory acceptances mean score value of control (spontaneously fermented Tej) was indicated as F1.

Table 12.Sensory evaluation of controlled fermentation vs spontaneous fermentation of Tej.

Product	**Color**	**flavour**	**Texture**	**Overall acceptability**
Combination	**Mean**	**Mean**	**Mean**	**Mean**
F#1(allcombination)	3.7	4.1	4.4	4.3
F#2 y1	4	4	3.9	4.2
F#3y2	3.8	3.9	4.1	4.3
F#4y3	3.9	4.6	4.2	4.1
F#5y4	4	4	4.3	4.5
F#6 L1y1	4	4.3	4.2	4.5
F#7 L1Y4	3.8	4.3	4.2	4.4
F#8 L4Y4	3.6	3.9	4.5	4.2
F#9 L3Y2	3.7±0.6	4.3±0.9	4.2±0.4	4.3±0.6
F#10 L2Y2	3.7±0.6	4.2±0.6	4±1.1	4.4±0.5
F#11 L4Y3	3.7±1.0	4.2±0.9	4.2±0.6	4.3±0.4
F#12 L3Y1	3.6±0.9	4.3±06	4.1±1.1	4.1±0.8
F#13 L3Y4	3.8±0.6	3.8±1.0	4.2±1.0	4.2±0.7
F#14 L2Y4	3.8±0.9	3.9±0.8	4.2±1.0	4.2±0.7
Control (spontaneous prepared Tej)	4.1±0.8	4.4±0.9	4.1±1.1	4.2±0.9

Note: Formulate #1 = all combinations (four LAB and four Yeast).

5. CONCLUSION

The physicochemical parameters were within the limits imposed by national and international standards. For the proximate analysis except moisture and sucrose content all the quality parameters analyzed for honey produced in the five sites complied with both national and international standards. This suggests that honey produced in the study areas generally had good quality.

Generally the most dominant microorganisms observed in this study were yeast and lactic acid bacteria in the various Tej samples collected from central part of Ethiopia and its surrounding but absent in honey samples. All yeast isolates were found to have better tolerance to low pH, high ethanol, high temperature and high salt concentration.

Potential LAB genera of *Lactobacillus, Leuconostoc* and *Lactococcus* were isolated and *Saccharomyces cerevisiae, Saccharomyces daireness, Debaryomyces carsonii* and *Saccharomyces pastorianus* the potential yeast species were isolated from Tej.

The use of these formulated starter culture improve the quality of Tej and the overall sensory acceptability analysis showed that formulate:F #5, F#2, and F#7 were the best mixed starter cultures for Tej preparation compared to the control. This study demonstrated that LAB and yeast formulates showed better production for Ethiopian honey wine (Tej).

Test results of the study indicated that most of the honey samples collected and tested for major honeys quality parameters met the requirements of honey standards. However, the few samples failed to meet the standard, which was mainly due to inappropriate processing and adulteration of the honey. Both adulteration and alteration of natural properties were observed. Therefore, it requires awareness creation on how to render the honey and what type of processing devices should be used. Control mechanisms also need to be designed for adulteration.

LAB and yeast isolates were tested for their ability to produce diacetyl.According to Ogunbanwo *et al*.(2012) *Lactobacillus brevis* produce the highest quantity of lactic acid and diacetyl closely follow by *Lactobacillus fermentum*. Diacetyl and higher alcohols, as the crucial flavor compounds, have been found to associate with the sensory properties of the wine. ILV2, ILV3 and ILV5 genes in isoleucine–valine (ILV) biosynthetic pathway related to diacetyl and higher alcohols have been reported in *Saccharomyces cerevisiae* Ping Li *et al*. (2020). Fouad *et al*. (2022) reported that classical fermentation the first peak of the diacetyl concentration normally appears early. The concentration of diacetyl is then reduced when the yeast assimilates diacetyl and converts it enzymatically into acetone then metabolized. Diacetyl is thought to be a by-product of amino acid biosynthesis involved in relationship between amino acid biosynthesis and the formation of acetolactate, the diacetyl precursor.

6. RECOMMENDATION

- Further identification of the microorganisms using molecular methods and analysis of the metabolites present in the Tej samples fermented under household level and under controlled conditions using starter culture.

- The production processes should further be optimized and the quality of the product must be assessed in large volumes.

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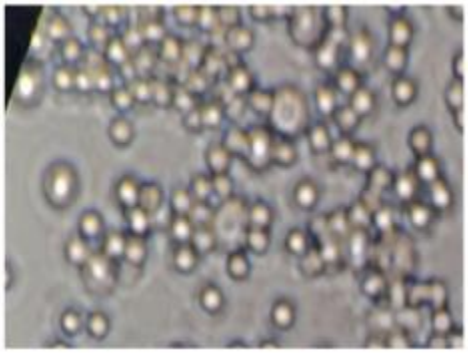
8. APPENDIXES

Appendix 1.Honey sample in the plastic container.

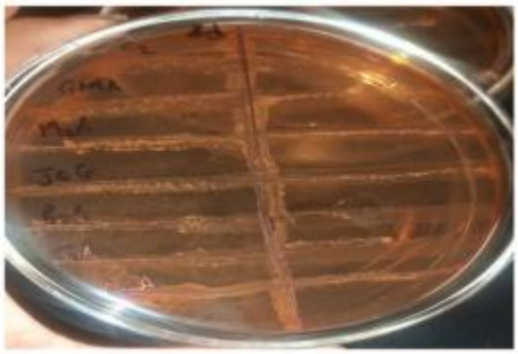
Appendix 2.Microscopical view of yeast.



Appendix 3.Catalase Test of the laboratory experiment.

Appendix 4. catalase Test of the laboratory experiment.

Appendix 5. Compatibility Test of the experiment.



Appendix 6. *Must* fermentation after 3 days.

Appendix 7.Autoclaved Gesho bark.

Appendix 8. Filtration using muslin clothes during Tej preparation.

Appendix 9. Laboratory instrument used for measuring alcohol content.

Appendix 10. Pictures of Titratable Acidity of lab work.

Appendix 11.Measuring Brix used for sugar content of Tej.

Appendix 12. Sensory evaluation format of Tej.

Panelists code _____

Sample code	Sensory Attribute	Score					
		1	2	3	4	5	Remarks
P1	Color						
	Flavor						
	Odor						
	Texture						
	Overall acceptability						

N.B: 1= Dislikeextremely, 2= Dislike moderately, 3= neither like nor dislike

4= Like moderately and 5= Like extremely