POTENTIAL OF SPONTANEOUS MICROBIAL FERMENTATION ISOLATES TO IMPROVE FERMENTATION AND CUP QUALITY OF WET PROCESSED ARABICA COFFEE (*Coffea arabica*)

BY

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DECLARATION

I, Fredrick Mugerwa (Reg. No. 17/U/14717/GMFT/PE), declare that this dissertation is original work and has never been submitted to any University or Institution of Higher Learning for any academic award.

…… ……..

Signature Date

APPROVAL

This is to certify that the work presented herein is the student's own, done by him under our supervision and guidance, and is now ready for submission to the Board of Examiners of Kyambogo University.

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DEDICATION

Dedicated to my parents; the late Mr. George Balaba Ssali, Ms. Betty Verediana Nankabirwa and Mrs Regina Kyeyune who have been a source of inspiration in my education.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AOAC – Association of Official Analytical Chemists

BLAST – Basic Local Alignment Search Tool

CBD – Coffee Berry Disease

CFU – Colony Forming Units

CGA – Chlorogenic Acids

CLR – Coffee Leaf Rust

DNA – Deoxy Ribonucleic acid

DRC – Democratic Republic of Congo

FA – Fatty Acids

FAO – Food and Agriculture Organisation of the United Nations

IARC – International Agency for Research on Cancer

ICO – International Coffee Organization

IDRC - Infectious Diseases Research Council

ISO – International Organization for Standardization

ITS – Internal Transcribed Spacer

MRS - De Man, Rogosa and Sharpe agar

NCBI – National Centre for Biotechnology Information

OTA – Ochratoxin A

PCA – Plate Count Agar

PCR – Polymerase Chain Reaction

PDA – Potato Dextrose Agar

pH – Hydrogen Potential

RNA – Ribonucleic acid

SCA – Specialty Coffee Association

TA – Titratable Acidity

TSS – Total Soluble Solids

UCDA – Uganda Coffee Development Authority

UIA – Uganda Investment Authority

UIRI – Uganda Industrial Research Institute

WCR – World Coffee Research

PCA – Principal Component Analysis

ABSTRACT

Spontaneous wet processing of coffee currently practiced in Uganda is constrained by long processing times, losses, and inferior quality coffees. Starter cultures are nowadays used to control fermentation of foods. This study evaluated the predominant yeasts and bacteria in the spontaneous fermentation of Arabica coffee (*Coffea arabica*) from Bugisu and their potential use as starter cultures in the wet fermentation of coffee. A total of 21 samples of the fermenting mass of Arabica coffee (100 ml each) were used for isolation of predominant yeasts and bacteria during spontaneous fermentation. The isolates were grouped by cell morphology and biochemical features and then subjected to polymerase reaction (PCR) for confirmation of species. Genomic DNA of the organisms was extracted and the variable region 3 of 5.8S and 16S rRNA genes for yeast and bacteria respectively were amplified PCR using universal primers ITS1 and ITS4, and P16S and M26S, respectively. Representative isolates from each PCR profile were identified by sequencing using Sanger sequencing and the closest known relatives of the sequences obtained were retrieved from the National Centre for Biotechnology Information data base using a Basic Local Alignment Search Tool (BLAST). The predominant microorganisms were enriched and their effects on the fermentation process were determined. Controlled fermentation of pulped Arabica coffee (100 kg) was carried out. Physicochemical changes namely pectin, total soluble sugars, viscosity, hydrogen potential and titratable acidity were monitored in the fermenting mass using standard AOAC and ISO methods. Coffee beans from the controlled fermentation experiments were roasted at 210^{o}C for 10 min, to medium dark roast profile and evaluated for the cup quality using the Specialty Coffee Association (SCA) cupping protocol. The yeasts *Kazachstania exigua* and *Pichia kudriavzevii*, and the bacteria *Leuconostoc mesenteroides* and *Paenibacillus campinasensi* were the predominant spp. in the in the spontaneous fermentation of *C. arabica* in Bugisu sub region. Mixed cultures performed better than single cultures in promoting fermentation. When all four

organisms were enriched to 12.2 log CFU/ml and applied, the fermentation period was significantly ($p<0.05$) reduced by 30%. There was reduction in the physicochemical parameters of the fermenting mass viz. pectin content from 0.604% to 0.000% and pH from 6.43 to 3.85. Total soluble solids (TSS), titratable acidity (TA) and viscosity increased from 3.05 ^{o}Brix to 8.92 ^{o}Brix, 1.22 g/L to 3.85 g/L and from 18.00 cP to 23.88 cP, respectively. Arabica coffee fermented using mixed cultures attained the highest mean cup score (84 points). There was a strong positive correlation (0.83) between *P. kudriavzevii* and pH of the fermenting mass and the cup quality. Processors should use starter cultures to reduce the processing time and to enhance the cup quality of Arabica coffee.

Keywords: Wet processing, fermentation, pectin degradation, *Coffea arabica*, cup quality.

CHAPTER ONE: INTRODUCTION

1.1 Background

Coffee belongs to the Rubiaceae family, genus *Coffea* (Martins *et al.*, 2020). Although more than 80 coffee species have been identified worldwide, *Cofffea arabica* (Arabica coffee) and *Coffea robusta* var. canephora (Robusta coffee) are the most economically important spp (Adepoju *et al.*, 2017). The importance of coffee stems from its health benefits mainly to relive physical fatigue and increase mental alertness in consumers (Butt and Sultan, 2011). The market share for Arabica coffee on the world market is *ca.* 66% and Robusta coffee accounts for the rest (El-Rahman and Faid, 2014). World coffee production was *ca.* 165 million bags in 2019 with Brazil, Vietnam, Colombia, Indonesia, Ethiopia, Peru, India, Uganda, Honduras and Mexico being the top 10 producers (ICO, 2019). Coffee contributes between 20 and 30% of Uganda's foreign exchange earnings (Bamwesigye and Hlavackova, 2019). Uganda's production comprises 80% Robusta coffee and 20% Arabica coffee (UCDA, 2019). Arabica and Robusta coffees are different in many ways; their conditions of growth, chemical composition and characteristics of the brew made from the ground roasted beans (Gichimu *et al.*, 2014) . Arabica coffee has superior sensory properties in the cup and hence attracts higher premiums (Kulapichitr *et al.*, 2019).

Coffee is one of the most popular beverages in the world, with over 600 billion cups served every year (Pereira *et al.*, 2016). Coffee beverage is the product of the green coffee beans, the seeds in the coffee cherries (Klingel *et al.*, 2020). The ripe coffee cherries undergo postharvest processing which has an impact on the quality and cost of the final product (Hameed *et al.*, 2020). The harvested mature coffee cherries are processed using dry, semi-dry or wet processing methods, to

produce green coffee beans (Evangelista, *et al*., 2014). In dry processing, the cherries are sun or hot air dried to a moisture content of 10 to 12.0% (Pereira *et al.*, 2019). The dry cherries are then cleaned and dehulled to remove the husk and pulp (Klingel *et al.*, 2020). In the semi-dry process, coffee fruits are depulped and fermented on a platform under the sun (Evangelista *et al*., 2014). In the wet method on the other hand, coffee fruits are first depulped mechanically (Pereira *et al.*, 2019). Then, the beans are put in tanks containing water and allowing fermentation to take place for 24 to 72 h to degrade the surrounding mucilage, followed by sun or solar or mechanical drying (Haile and Kang, 2019). The main objective of fermentation is to breakdown the mucilage covering the parchment skin and to improve the bean quality hence the cup quality of the coffee (Kulandaivelu, 2013). The physicochemical changes occurring during coffee fermentation include pectin degradation by pectinase enzymes and the subsequent production of organic acids from the carbohydrates (Kulandaivelu, 2013). Wet processing reduces the processing time from about 35 days to less than 10 days (Pereira *et al.*, 2019). It also produces coffee of superior quality than dry or semi-dry processed coffee (Avallone *et al.*, 2000).

Nowadays, starter cultures are used to control fermentation and subsequently promote coffee quality (Pereira *et al.*, 2017). Fermented foods in Europe, North America, Australia and New Zealand are produced using defined starter cultures while similar foods in Asia and Africa are produced by the spontaneous fermentation process (Tamang *et al.*, 2020). Starter cultures or starters are individual or mixed microbial cultures used in known concentrations to promote fermentation (Laranjo, Potes and Elias, 2019). Studies have characterized spontaneous fermentation, from which strains of yeasts and bacteria have been isolated and used as starters (Martinez *et al.*, 2019). Bacteria, yeast, and fungi break down the sugars and the pectins within the

mucilage and produce alcohols which are subsequently oxidized to organic acids , thus creating a broader spectrum of flavours (Klingel *et al.,* 2020). Because of their favourable contributions towards the flavour in the beverage, yeasts are the most studied and used in coffee fermentation (Martinez *et al*., 2019). *Pichia fermentans* and *Saccharomyces* spp. are the major yeast isolates with great potential for use as starter cultures in coffee processing (Pereira *et al.,* 2016). *P. fermentans* produces higher concentrations of flavour-active ester compounds (viz., ethyl acetate and isoamyl acetate) while *Saccharomyces* spp. is better as a pectinase-producing strain. The activities of these organisms have been indicated to produce coffee with novel and desirable flavour profiles but a standard commercial coffee starter culture is yet to be documented (Pereira *et al.*, 2014). Spontaneous fermentation takes a longer time, and may result into over fermentation of sugars to produce undesirable acidic compounds. These compounds lead to acid flavours and odours in the coffee beans. There is paucity of information on the effects of controlled starter cultures on coffee fermentation and bean quality in Uganda. Therefore, this study evaluated the microorganisms in the spontaneous fermentation of Arabica coffee in Uganda, and the potential application of the predominant isolates as starter cultures in commercial coffee production.

1.2 Statement of the problem

Coffee quality has recently become a high demand for coffee consumers and is directly related to its chemical composition, especially the flavour compounds (Martinez *et al.*, 2019). However, the chemical and volatile profiles of coffee vary depending on several factors such as the method of processing, which involves fermentation in the case of wet processing (Martinez *et al.,* 2019). Limited efforts have been made towards controlling fermentation processes and determining the effect of starter cultures in the development of coffee quality characteristics. Spontaneous

fermentation of *C. arabica* lasts as long as 72 h, which slows down production (Masoud *et al.*, 2004). It can also lead to over-fermentation and the production of propionic (CH_3CH_2COOH) and butyric ($CH_3CH_2CH_2COOH$) acids (Haile and Kang, 2019). These acids contribute towards coffee cup quality defects. Information related to physicochemical and microbial dynamics during coffee fermentation and the effects of fermentation time on coffee cup quality is lacking (Kulandaivelu, 2013). In addition, lack of control of the fermentation process leads to inconsistence in the quality of the final product (Hameed *et al.*, 2020). According to Nsibirwa (2016), Uganda earns low premiums from Arabica coffee due to inconsistency of the quality and the exportation of up to 55% of Arabica coffee as dry green coffee beans. These effects significantly impact on the coffee value chain players in the Bugisu sub-region (UCDA, 2019). Furthermore, spontaneous fermentation can promote the proliferation of toxigenic fungi that produce ochratoxin A; OTA (Silva *et al.*, 2013; Evangelista *et al.*, 2014). The International Agency for Research on Cancer (IARC) has classified OTA as possibly carcinogenic (Group 2B) to humans (IARC, 1993).

1.3 Justification of the study

Uganda's Arabica coffee production accounts for *ca.* 20% of the country's total production of coffee of which 45% is wet processed, with Bugisu sub region producing the highest quantities (UCDA, 2019; Nsibirwa (2016)). Many food products such as yoghurt, bread, wine and cheese are derived from fermentation using defined starter cultures (Mullan, 2014). Application of starter cultures in coffee processing would lead to production of desirable flavour profiles in a shorter fermentation time, so that more coffee can be wet processed per unit time (Pereira *et al.*, 2014). The rejection of coffee due to poor cup quality (sensory quality) leads to loss of revenue for processors and farmers, and also leads to the reduction in the national foreign exchange earnings

(Murthy and Naidu, 2011). The use of starter cultures would lead to consistency and improvement in sensory quality of the final product (coffee beverage; Quintero, 2014; Albala, 2015). Starter cultures ensure controlled fermentation and shorten the fermentation time (Silva *et al.*, 2014). Because wet processing time would be potentially shortened, post-harvest losses due to toxigenic mycotoxins could also be reduced (Haile and Kang, 2019). Ultimately, Uganda's Arabica coffee production (processing) output would increase, moreover of premium quality and price, leading to increased direct incomes for the processors, the country's exports and forex earnings.

1.4 Objectives

1.4.1 General objective

To evaluate the potential of spontaneous microbial fermentation isolates to improve fermentation and cup quality of wet processed Arabica coffee from Bugisu sub region.

1.4.2 Specific objectives

The specific objectives of the study were to determine:

1. The predominant yeast and bacterial species during spontaneous wet processing of Arabica coffee.
2. The effect of the predominant yeasts and bacteria isolates on the physicochemical characteristics (pectin, TSS, viscosity, pH and TA) of the wet fermentation process of Arabica coffee.
3. The effect of the predominant yeast and bacteria isolates on the cup quality of Arabica coffee.
4. The correlation between the physicochemical characteristics of wet fermentation and the cup quality of Arabica coffee.

1.5 Hypotheses

1. Yeasts predominate over bacteria during the wet processing of Arabica coffee.
2. Predominant yeasts and bacteria isolates reduce fermentation time from 72h by 25%.
3. Wet processing using the predominant yeasts and bacteria isolates improves the cup quality of wet processed Arabica coffee.
4. Physicochemical characteristics of the fermentation process of Arabica coffee are positively correlated with its cup quality components.

CHAPTER TWO : LITERATURE REVIEW

2.1 Botanical classification of genus *Coffea*

The coffee tree is part of the sub-kingdom of plants known scientifically as the Angiosperm, or Angiospermae, meaning that the plant reproduces by seeds enclosed in a box-like compartment, the ovary, at the base of the flower (Ferreira *et al.*, 2019). It belongs to the family *Rubiaceae*, subfamily *Ixoroideae*. The current classification of the genus *Coffea* results from recent fusions of several subgenera and genera. According to Leroy and Bridson (1967), two genera existed in this subfamily, *Coffea* L. and *Psilanthus* Hook. f. (an Australasian genus), with the *Coffea* genus being split into two subgenera, *Coffea* and *Baracoffea*. According to Davis *et al.* (2007) and Maurin *et al.* (2007) there is a relationship between the two subgenera. Later, the subgenus *Coffea* and genus *Psilanthus* were merged using molecular and morphological data, leading to the current *Coffea* genus, which is by far the most economically important member of the Rubiaceae family (Davis *et al.*, 2011). The various species of subgenus *Coffea* are largely present on the African continent, although they are mostly restricted to tropical zones when growing in the wild (Farah *et al.*, 2014). There are 41 species from continental Africa, 59 from Madagascar and 4 from nearby islands; Grand Comore, Mascarenes Islands, Mauritius and Réunion (Anthony *et al*., 2010). The merging of subgenus *Coffea* and genus *Psilanthus*, located in Asia and Australasia, resulted into the current 125 species in the genus *Coffea* (Ferreira *et al.*, 2019). The botanical classification of coffee is shown in Fig. 2.1.

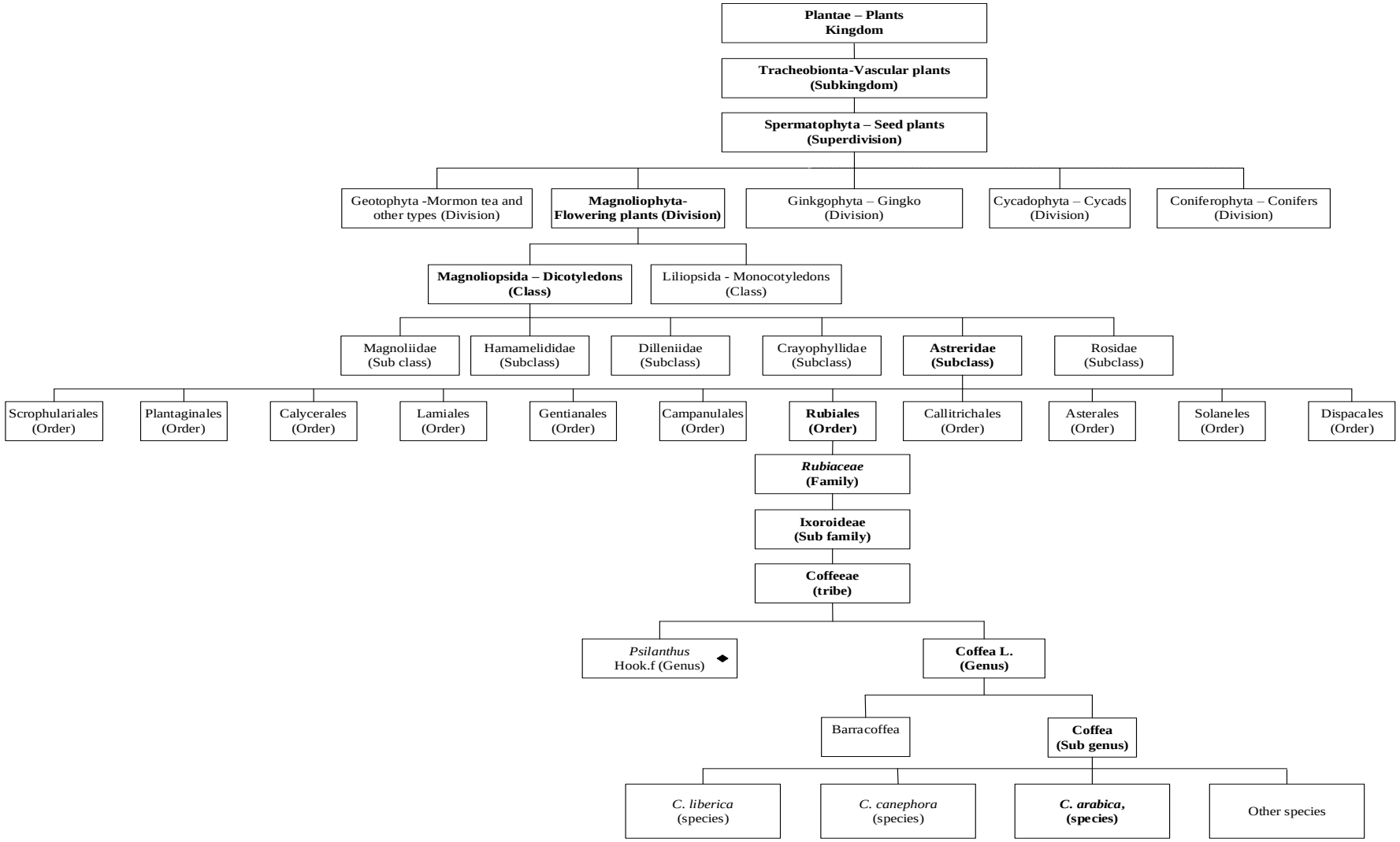

Figure 2.1: Botanical classification of the coffee plant
Source: Anthony *et al.* (2010) and Natural Resources Conservation Service (USDA, 2016).

Coffea arabica, *Coffea canephora* and, to a much less extent, *Coffea liberica*, are the species of commercial importance under the genus (Patay *et al*., 2016). *Coffea arabica* is the most widely cultivated crop in the world (ICO, 2019). *C. arabica* is a tetraploid species (2*n* =4*x* = 44) originating from a natural hybridization between either *C. canephora* and *C. eugenioides* or ecotypes related to these two diploid (2*n* = 2*x* = 22) species (Prakash *et al*., 2002; Leroy *et al*., 2006). Polyploid *C. arabica* species; triploid (3n = 33), pentaploid (5n = 55), hexaploid (6n = 66) and octoploid (8n = 88) plants, have also been reported (Patay *et al*., 2016). Globally, *Coffea arabica* is the species with highest cup quality compared to the other species, but the plant is not as strong and disease resistant as is *C. canephora* species (Mishra and Slater, 2012). *Coffea liberica* Hiern is a diploid species cultivated to a minor extent, mainly because of its sensitivity to diseases, especially *Fusarium xylarioides* (Ferreira *et al.*, 2019). Its seeds tend to have a better cup quality compared to *C. canephora* species, but still inferior compared to *C. arabica* (Alonso-Salces *et al.*, 2009). Triploid hybrids, originating from crosses between *C. arabica* and diploid species, have also been reported (Bizzo *et al*., 2014). These hybrids tend to be robust plants but are almost completely sterile (Ferreira *et al.*, 2019).

2.2 Anatomy of coffee

The coffee fruit (coffee cherry) is mainly divided into two parts, pericarp and seed (Klingel *et al*., 2020). The pericarp is the three outer layers of the fruit: the exocarp (skin), the mesocarp (mucilage) and the endocarp (parchment; Fig 2.2). The coffee seed or bean comprises a silver skin, endosperm, and an embryo (Ferreira *et al.*, 2019). Coffee seed sizes vary depending on the variety; however, on average they are 10 mm long and 6 mm wide (Klingel *et al*., 2020). The exocarp, also known as peel, skin or epicarp, is the outermost layer of the coffee fruit. It is formed by a layer of parenchymal cells (Ferreira *et al.*, 2019). Its colour changes depending on the maturity of the fruit. At the beginning of the development of the fruit, it is green due to

the presence of chlorophyll. The colour after ripening, depending on the coffee variety, is red or yellow. The red colour comes from anthocyanin pigments, while the yellow colour is attributed to luteolin (Klingel *et al*., 2020). The mesocarp also known as mucilage, is the pulp of the coffee fruit. In the green coffee fruit, the mesocarp is rigid, as the fruit matures the pectolytic enzymes break the pectic chains, resulting in an insoluble hydrogel very rich in sugars and pectins (Ezike, 2017). In the wet processing method, the mucilage is removed by controlled fermentation. In the honey processing method, the mucilage is left adhering to the bean during drying. While in the dry processing method, the mucilage along with the exocarp, is left intact during drying (Haile and Kang, 2019). Studies have shown that the mucilage/water ratio of the mesocarp increases as the altitude increases (Pérez-Sariñana and Saldaña-Trinidad, 2017). The endocarp, hull or parchment is the innermost layer of the pericarp and is the layer that covers and protects the bean, has a pale-yellow colour and is of a hard and fragile consistency when it has dried. It is formed from 3 to 7 layers of sclerenchyma cells (Nemesa, 2000). Pectin is the thin layer that joins the pulp and outer skin of the cherry to its desired caffeine-rich inner contents (Pérez-Sariñana *et al*., 2017).

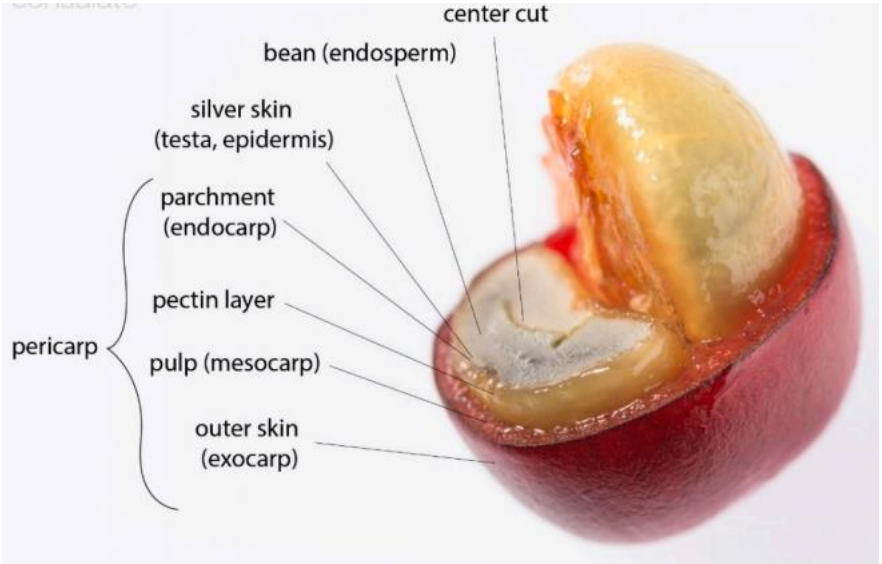

Figure 2.2: Cross section of a ripe coffee cherry with its different layers

Source: Klingel *et al*. (2020)

The pericarp, is either removed during initial processing or just before the coffee is exported as green coffee. Silver skin (testa, epidermis) is the last layer of the cherry before the bean, it is mostly removed during processing, although parts usually remain and are removed as the bean is roasted. In certain regions where coffee is grown, the silver skin can be a darker colour, producing the bean; endosperm (Klingel *et al*., 2020).

2.3 Origin and Distribution of subgenus *Coffea* in Africa

Coffea species have colonized many types of forests throughout a wide elevational distribution on the African continent (Ferreira *et al.*, 2019). Up to 70% of species in the sub genus *Coffea* are present in humid and evergreen forests, and at least 13% are adapted to seasonally dry forests in continental Africa (Anthony *et al*., 2010). The other 17% of the species are adapted to various other types of forests, including humid evergreen forests, gallery forests, seasonally dry (evergreen to deciduous) forests, savannah woodlands and shrublands (Anthony *et al*., 2010). In Madagascar, 67% of the species grow only in humid evergreen forests, 17% grow only in seasonally dry forests and the remaining species grow in both types of forests (Ferreira *et al.*, 2019). Coffee trees are naturally found from sea level up to 2500 m, but no species grow throughout this entire range (Noirot *et al.*, 2016). The largest number of endemic species in Africa is present between 200 and 1000 m above sea level, including *C. canephora* and *C. liberica* sub sp Dewevrei (Hameed *et al.*, 2020). This broad range is mainly caused by variations in latitude. In Uganda, an equatorial country where the minimum temperatures are warm and relatively stable, *C. canephora* grows above 1000 m (UCDA, 2019). The altitude range for *Arabica coffee* optimum growth is 1200 to 1950 m, with average growth occurring at 1575 m (Ferreira *et al.*, 2019). The elevation range is observed both on the continent and on islands, although the number of species that grow over 1000 m above sea level is higher in continental Africa (Ferreira *et al.*, 2019).

2.4 The most famous Arabica coffee varieties in Bugisu sub region

According to the World Coffee Research (WCR), there are dozens of widely cultivated Arabica coffee varieties around the world, and each is unique in its performance and adaptation to local conditions (World Coffee Research, 2018). However, Arabica coffee varieties include *C. arabica* var. typica and *C. arabica* var. bourbon which are considered to be the major coffee varieties (Pruvot-Woehl *et al*., 2020). All other varieties are a product of these two. *C. arabica* var. typica is the base from which many coffee varieties have been developed. Typica coffee plants have a conical shape with a main vertical trunk and secondary verticals that slant slightly (Ferreira *et al.*, 2019). The plants are tall, 3.5 to 4 m high, with lateral branches forming 50 to 70° angles with the vertical stem. Typica coffee is characterized by very low production, susceptibility to disease, but has excellent cup quality (World Coffee Research, 2018). Bourbon coffee plants on the other hand, produce 20 to 30% more coffee than Typica, but have a less harvest compared to most coffee varieties (World Coffee Research, 2018). The plants have less of a conical shape than Typica coffee plants, but have more secondary branches. The angles between the secondary branches and the main stem are smaller, and the branch points on the main stem are closely spaced (Pruvot-Woehl *et al*., 2020). Bourbon coffee leaves are broad and wavy on the edges. The fruits are relatively small and dense, and the cherries mature quickly. The cup quality is excellent and similar to that of Typica coffee. Although the importance of varieties was not high on the coffee industry agenda until recently, coffee breeders have always been active in creating improved varieties for both Robusta and Arabica coffee (Pruvot-Woehl *et al*., 2020).

Uganda's Arabica coffee of premium quality is from Bugisu sub region, on the western slopes of Mountain Elgon. It was first introduced to the country in 1912 by A. Whyte then, head of Scientific Department in Uganda. French missionaries from Ethiopia later introduced Arabica

Bourbon (UCDA, 2019). Production and disease resistance generally govern the types of coffee beans that a farmer chooses to plant (UCDA, 2019). The main varieties are the Bourbon descendants SL14 on a larger scale and SL28, KP162, KP423 and the Bugisu local (Nyasaland) on a smaller scale (World Coffee Research, 2018). Hybrid varieties include Ruiru II and Catimor129. SL comes from Scott Laboratories, a company that the Kenyan government hired in the 1930s to create a productive and resistant coffee variety (World Coffee Research, 2018). Ruiru II variety was released by SL in 1985. The variety name has the prefix "Ruiru" referring to the location of the Kenyan Coffee Research Station where it was developed (Hindorf and Omondi, 2011) . KP423 was selected from Kent, a Bourbon-related population from south-western India and was introduced to Tanzania and elsewhere in the 1920s as part of an intensive selection program at the Lyamungu Research Station in Tanzania, focused on quality and production (World Coffee Research, 2018). The various Kent-derived varieties originate with a selection made by Mr. L. P. Kent in 1911 from a single tree growing in his Doddengooda Estate in Mysore, south-western India (World Coffee Research, 2018).

According to World Coffee Research, 2018, the SL28 cultivar was selected on a single tree basis from the Tanganyika Drought Resistant variety in 1931. The variety is suited for coffee growing zones at 2000 to 3000 ft above sea level. It has intensely citrusy, sweet and multidimensional flavor profiles and is one of the most expensive Arabica coffees on the world market (World Coffee Research, 2018). SL28 and KP162 are highly susceptible to coffee berry disease (CBD) and coffee leaf rust (CLR) therefore not recommended for planting. SL14 is more susceptible to CLR than to CBD which is the vice versa of KP423. Ruiru II is not only resistant to the CBD and CLR but is also compact thus allowing farmers' intense production per unit area (Hindorf and Omondi, 2011). KP423 is less susceptible to the lamiine stem borer (*Monochamus leuconotus*) moreover, it yields almost five-fold more coffee cherries than the

SL28 and KP162 varieties (Egonyu *et al.*, 2015). The *Monochamus leuconotus* (Coleoptera: Cerambycidae), which mainly attacks Arabica coffee, is endemic to Africa and has been reported in Angola, Burundi, Cameroon, Democratic Republic of Congo, Ethiopia, Kenya, Malawi, Mozambique, Rwanda, South Africa, Tanzania, Zambia, Uganda and Zimbabwe (Pascoe, 1869). KP423 was released in Tanzania in the 1940s, and made its way to Uganda, where it is an important variety in Arabica-growing regions (World Coffee Research, 2018)..

2.5 Growth and production of coffee

2.5.1 Conditions required for cultivation of Arabica coffee

Arabica coffee can grow well on well-drained volcanic soils in warm, temperate environments (Ferreira *et al.*, 2019). For best root establishment, it requires a fertile, well aerated, free draining, slightly acidic, deep soil with reasonable humus content, in non-water-logged areas (UCDA, 2019). Arabica coffee grows well in cool temperature environments (Winston *et al.*, 2005). Temperatures higher than 24°C cause plant stress which leads to cessation of photosynthesis while mean temperatures less than 15°C limit plant growth (Nievola *et al.*, 2017). Arabica and Robusta coffees ideally grow at elevations between 1,200 and 2,500 m, and between 900 and 1,500 m above sea level (Table 2.1). High elevations (1,500 to 2,500 m) produce superior bean and cup quality due to slow ripening caused by cooler weather associated with higher altitudes (UCDA, 2019). Arabica coffee requires a good amount and well distributed rainfall over a period of at least nine months and throughout the berry development stage (Nievola *et al.*, 2017). This rainfall pattern is required to induce uniform flowering and good fruit formation (UCDA, 2019).

Table 2.1: Optimum conditions for the cultivation of Arabica and Robusta coffee.

Parameter	Arabica	Robusta
Temperature (°C)	15 to 24	24 to 30
Annual rainfall (mm)	1,200 to 1,800	1,200 to 1,800
Altitude (m)	1,200 to 2,500	900 to 1500
Soil pH	5.5 to 6.5	5.5 to 6.5

Source: UCDA Arabica and Robusta coffee handbooks (2019)

In Uganda, Arabica coffee is grown in the highland areas on the slopes of Mount Elgon in the East and Mt. Rwenzori and Mt. Muhabura in the South Western Region (1200 to 2,500 m above sea level). Arabica coffee is grown in Bududa, Manafwa, Mbale, Sironko, Kapchorwa, Bukwo, Kween and Budadiri districts (UCDA, 2019). In the Western region, Arabica coffee is grown in Ntoroko, Kasese, Kisoro, Kanungu, Kabale, Rukungiri, Kamwenge, Mitooma, Ibanda, Kabarole, Buhweju, Bunyangabu, Rubirizi and Rubanda districts (UCDA, 2019). Arabica coffee is also found in the West Nile districts of Zombo, Nebbi and Pakwach (UCDA, 2019). There are two coffee harvest seasons (main and fly crop seasons) for both Arabica and Robusta coffee (UIA, 2007). The main harvesting season for Arabica coffee ranges from April to June for Western Uganda and October to February for the Eastern and West Nile regions.

2.5.2 Coffee production

World coffee production has been increasing over the years and was estimated at 170,937 million (60 kg) bags (ICO, 2019). Brazil is the leading producer, followed by Vietnam and Colombia. Uganda is ranked the 8th largest producer, and accounts for 3% of the total world coffee production (ICO, 2020). World coffee production increased by about 11% from 2012 to 2019, while Uganda's production increased by 20% in the same period (ICO, 2019). Table 2.2 shows the trend of world coffee production during the period 2012 to 2019.

Table 2.2: Coffee production (‘000 60 Kg bags) of the ten leading coffee producing countries compared against the total World production for the period 2011 to 2018.

Country	2012/13	2013/14	2014/15	2015/16	2016/17	2017/18	2018/19
World	152,535	154,095	149,753	153,988	159,916	158,074	170,937
Brazil	55,418	54,689	53,305	52,871	56,788	52,740	62,925
Viet Nam	23,402	27,610	26,500	28,737	25,540	27,888	31,174
Colombia	9,927	12,124	13,333	14,009	14,634	13,824	13,858
Indonesia	13,070	12,901	10,946	12,585	11,541	10,852	9,418
Ethiopia	6,233	6,427	6,575	6,714	7,297	7,454	7,776
Honduras	4,686	4,583	5,268	5,786	7,457	7,560	7,328
India	5,403	5,075	5,450	5,830	6,161	5,813	5,302
Uganda	3,914	3,633	3,744	3,650	4,962	4,597	4,704
Mexico	4,327	3,916	3,636	2,772	3,635	4,485	4,351
Peru	4,450	4,106	2,883	3,304	4,223	4,279	4,263

Source: ICO (2020)

2.6 Health benefits of coffee

Coffee is a complex mixture of more than 800 volatile compounds wherein, caffeine and chlorogenic acids are the most common compounds (Nieber, 2017). Caffeine reduces risk of developing neurodegenerative disease while chlorogenic acids (CGA) play an antioxidant role (Hayat *et al.*, 2015). Caffeine also affects adenosine receptors and its withdrawal is accompanied with muscle fatigue and allied problems in persons addicted to coffee (Hayat *et al.*, 2015). Coffee shows protective or adverse effects on various systems like the skeletal system, the reproductive system, the nervous system, the cardiovascular system, the

homocysteine levels, and the cholesterol levels (Butt and Sultan, 2011). It relieves physical fatigue and increases mental alertness, prevents Parkinson's disease, gallstones, type 2 diabetes, gastrointestinal and lung diseases, and cancers such as breast cancer (George *et al.*, 2008). It plays a role in treating headaches, low blood pressure, obesity and attention deficit-hyperactivity disorders (Kolb *et al.*, 2020). There is also evidence that decaffeinated coffee may, in some respect, have similar benefits as regular coffee, indicating that besides caffeine, other components contribute to the health protecting effects (Nieber, 2017). Coffee contains other bioactive compounds such as the diterpenes cafestol and kahweol, and diterpenoid alcohols which have been associated with many potential health benefits (Gökcen and Şanlier, 2017). Moderate amounts of cafestol and kahweol are antimicrobial and anti-inflammatory and also act as a safeguard against some malignant cells by modulating the detoxifying enzymes (Miranda *et al.*, 2017). Diterpenoids have antioxidant, anticarcinogenic and antimutagenic activities (Nieber, 2017).

2.7 Coffee cup quality

The International Organization for Standardization (ISO) describes quality as "the ability of a set of inherent characteristics of a product, system or process to fulfil requirement of customers and other interested parties" (Leroy *et al.*, 2006). These inherent characteristics can be called "attributes" (Sittipod *et al.*, 2019). One of the most important criteria used for assessing coffee quality is based on sensory analysis and is referred to as cup quality (Franca *et al.*, 2005 ; Donfrancesco *et al.*, 2014). Cup quality characteristics are attributes of coffee that can be distinguished by senses and can be assessed organoleptically by professional coffee tasters (Q graders; Sittipod *et al.*, 2019). The discernment is based on established terminologies for cup quality analysis including acidity , body, cup cleanness and flavour of the brew (Dessalegn *et al.*, 2007). Arabica coffees which attain a minimum total score (cup score) rating of 80 points

are sold as specialty coffees in high end markets (Lingle and Menon, 2017). Coffees scoring between 60 and 80 points are categorised as commodity coffees whereas coffees scoring below 60 points are unacceptable for trading (Sittipod *et al.*, 2019). Lowor *et al*. (2007) showed that aliphatic carboxylic acids such as pyruvic acid, lactic acid and 3-methylbutyric acid are the main acids found in coffee and coffee infusions (Lowor *et al*., 2007). These acids impart an acid pH that leads to changes in the character of the flavour as well as the acidity. The chemical base and organoleptic qualities of a coffee are determined by the quality of the green coffee bean, which in turn, depends on the growing region and type of processing (Illy and Viani, 2005).

2.8 Factors affecting coffee bean quality

2.8.1 Moisture

The International Coffee Organization (ICO) recommends moisture content of between 8 and 12.5% w/w (target of 11% w/w) within which coffee quality attributes are maintained during storage (McCormick, 2002). Moisture content less than 8% is likely to result in irreversible colour damage and loss of cup quality (Lingle and Menon, 2017). On the other hand, a moisture content higher than 12.5% w/w leads to mould growth and the subsequent production of Ochratoxin A during storage, and the loss of sensory quality as well (FAO, 2006). With regard to relative humidity, a humidity greater than 80% allows for water condensation thereby promoting insect infestation and fungal growth (Pardo *et al.*, 2006). Chemical reactions and microbial developments are also considerably accelerated.

2.8.2 Temperature and light

Coffee needs to be stored at temperatures ranging between 20 and 25^{o}C (Ross *et al.*, 2006). Temperature has a profound effect on the rate of bean respiration (DaMatta, 2004). Shelf life

is shortened at lower altitudes by approximately three months but the natural shelf life can be as long as 8 months for green coffee beans stored at > 1400 m above sea level (Clarke, 2012). In general, the higher the altitude the temperature falls. The duration and intensity of light exposure have also been reported to affect coffee quality (Avelino *et al.*, 2005). Light intensity together with the environmental temperature positively influence the physical quality, sugar content and phenolic composition of the coffee bean (Somporn *et al.*, 2012).

2.8.3 Harvest and post-harvest management practices

The method and time of harvest have been reported to affect coffee quality (Hicks, 2002). Traditional hand-picking produces the best quality green coffee beans by decreasing the percentage of defects (Taveira *et al.*, 2015). Coffee harvested at the end of the season also gives beans with higher maturity levels than those picked at the start (Vaast *et al.*, 2008). Generally, post-harvest processing activities contribute about 60% of the quality of the green coffee beans (Haile and Kang, 2019). The overall coffee quality is influenced by factors contributing to changes in the physicochemical properties and sensorial attributes (Kreuml *et al*., 2013). Post-harvest operations include pulping, processing, drying, hulling, cleaning, sorting, grading, storage, roasting and grinding have an effect on coffee cup quality (Haile and Kang, 2019). Performing post-harvest processes in a controlled manner can help to maintain the physicochemical properties of coffee (Taveira *et al.*, 2015).

2.9 Coffee bean chemical composition

Green coffee beans contain high proportions of polysaccharides, monosaccharides, lipids, sterols, fatty acids (FA), phenolic acids, polyphenols, alkaloids, proteins, free amino acids (alanine, arginine, asparagine, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine), vitamins

and minerals (Parras *et al.*, 2007). Free sugars such as sucrose, glucose, fructose, arabinose, galactose, and mannose are also present (Belay, 2011). Sugars, especially sucrose, act as precursors to several aroma substances that affect both the flavor and aroma of the beverage (A. Farah *et al.*, 2006). The lipid fraction is particularly important for the formation of aromas and flavors, flavor retention and foam stability in the coffee beverage (Speer and Kölling-Speer, 2006). Coffee also contains and is a good source of polyphenolic compounds. Chlorogenic acids are the main phenolic compounds found in green coffee beans (Jeszka-Skowron *et al.*, 2016). They belong to hydroxycinnamic acid classes and chiefly consists of caffeic acid (3, 4-dihydroxycinnamic acid), ferulic acid (3-methoxy-4-hydroxycinnamic acid), p-coumaric acid (4-hydroxycinnamic acid), and sinapic acid (3, 5-dimethoxy-4-hydroxycinnamic acid; Belay, 2011). Chlorogenic acids play an important role in the formation of the flavor of roasted coffee and have a marked influence on the coffee cup quality. Caffeine (1,3,7-trimethylxanthine; ($C_8H_{10}N_4O_2$) is a purine alkaloid that constitutes the natural content of coffee beans (Gichimu *et al.*, 2014). Caffeine is the most known component of coffee beans. It presents a characteristic bitter taste reported to be important to coffee flavor (Jeszka-Skowron *et al.*, 2016). Trigonelline ($C_7H_7NO_2$) is another bitter alkaloid (pyridine in nature) and one of the precursors of aroma in coffee that degrades up to 90% during roasting, forming mainly niacin, pyridines, and some pyrroles that are important aroma compounds (Dorsey and Jones, 2017). The various constituents along with components of coffee are shown in Table 2.3.

Table 2.3: Chemical composition of green coffee

Constituent		Components
Soluble carbohydrates	Monosaccharides	Fructose, glucose, galactose, arabinose (traces)
	Oligosaccharides	Sucrose, raffinose, stachyose
	Polysaccharides	Polymers of galactose, mannose, arabinose, glucose
Insoluble polysaccharides	Hemicelluloses	Polymers of galactose, arabinose, mannose
	Cellulose, β(1–4) mannan	
Lignin		Cross-linked phenolic polymers
Acids and phenols	Volatile acids	
	Non-volatile aliphatic acids	Citric acid, malic acid, quinic acid
	Chlorogenic acids	Mono-, dicaffeoyl- and feruloylquinic acid
Lipids	Waxes	
	Oil	Main fatty acids; Palmitic acid, stearic acid, oleic acid, linoleic acid
N compounds	Free amino acids	Main amino acids: Glutamic acid, aspartic acid, asparagine
	Proteins	
	Caffeine	Traces of theobromine and theophylline
	Trigonelline	
Minerals		Calcium (Ca), potassium (K), iron (Fe), phosphorus (P), nickel (Ni), magnesium (Mg), and chromium (Cr)

Source : Kulapichitr *et al.* (2019)

2.10 Effect of roasting on cup quality

Roasting refers to the thermal process of transformation of dried green coffee beans to roasted beans (Hameed *et al.*, 2018). The beans are commonly roasted in large commercial roasters, placing beans in large metal cylinders and blowing hot air on them (Chun and Sen, 2020). Roasting gradually raises the temperature of the beans to between 180 and 230°C (Amri *et al.*, 2020). This triggers the release of steam, causing the beans to swell as well as darken in colour and develop roasted flavors (Bicho *et al.*, 2012). The time-temperature roasting profile is used to satisfy the changing consumer demand for coffees with high flavor complexity (Wang and Lim, 2015). The high temperature and pressure inside the beans during roasting triggers a large number of chemical reactions leading to the formation of several volatile and non-volatile compounds that in part determine coffee aroma and flavour characteristics (Bonnlander *et al*., 2005). The chemical reactions that happen during roasting include; Maillard and Strecker reactions, degradation of proteins, polysaccharides, trigonelline and chlorogenic acids (Buffo and Cardelli-Freire, 2004). Maillard reaction is a non-enzymatic browning reaction between nitrogen-containing substances on the one hand (proteins, peptides, amino acids, serotonine and trigonelline) and reducing carbohydrates, hydroxy-acids and phenols on the other, to form amino aldoses and amino ketones by condensation (Buffo and Cardelli-Freire, 2004). Strecker degradation is a reaction between an amino acid and an α-dicarbonyl compound with the resultant formation of an amino ketone that condenses to form nitrogen heterocyclic compounds or reacts with formaldehyde to form oxazoles (Buffo *et al*., 2004). Thermal degradation of chlorogenic acids during roasting results into phenolic substances that contribute to astringency and bitterness (Buffo *et al.*, 2004). The steps involved in the Maillard degradation reaction are summarized in Figure 2.3.

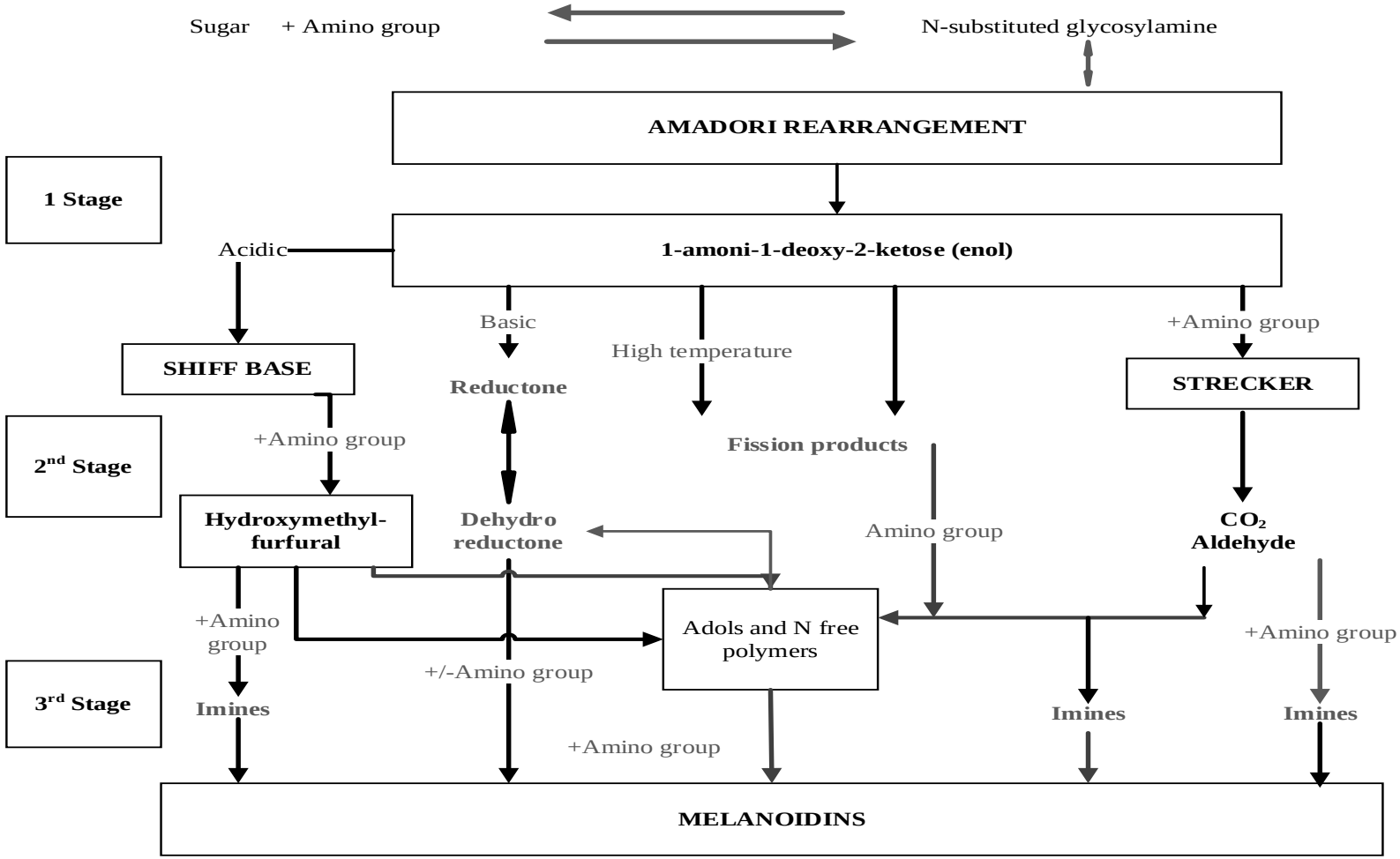

Figure 2.3: Hodge scheme of Maillard reactions (Hodge 1953)

Source: Cheung and Mehta (2015)

2.11 Coffee processing

Processing plays a crucial role in coffee quality determination and must begin immediately after harvesting the beans to prevent the pulp from fermenting and deteriorating (Taveira *et al.*, 2015). The harvested coffee beans are either processed by the dry, semi dry or wet methods, and these vary in complexity depending on the expected quality of the coffee. Figure 2.4 below illustrates the coffee processing methods.

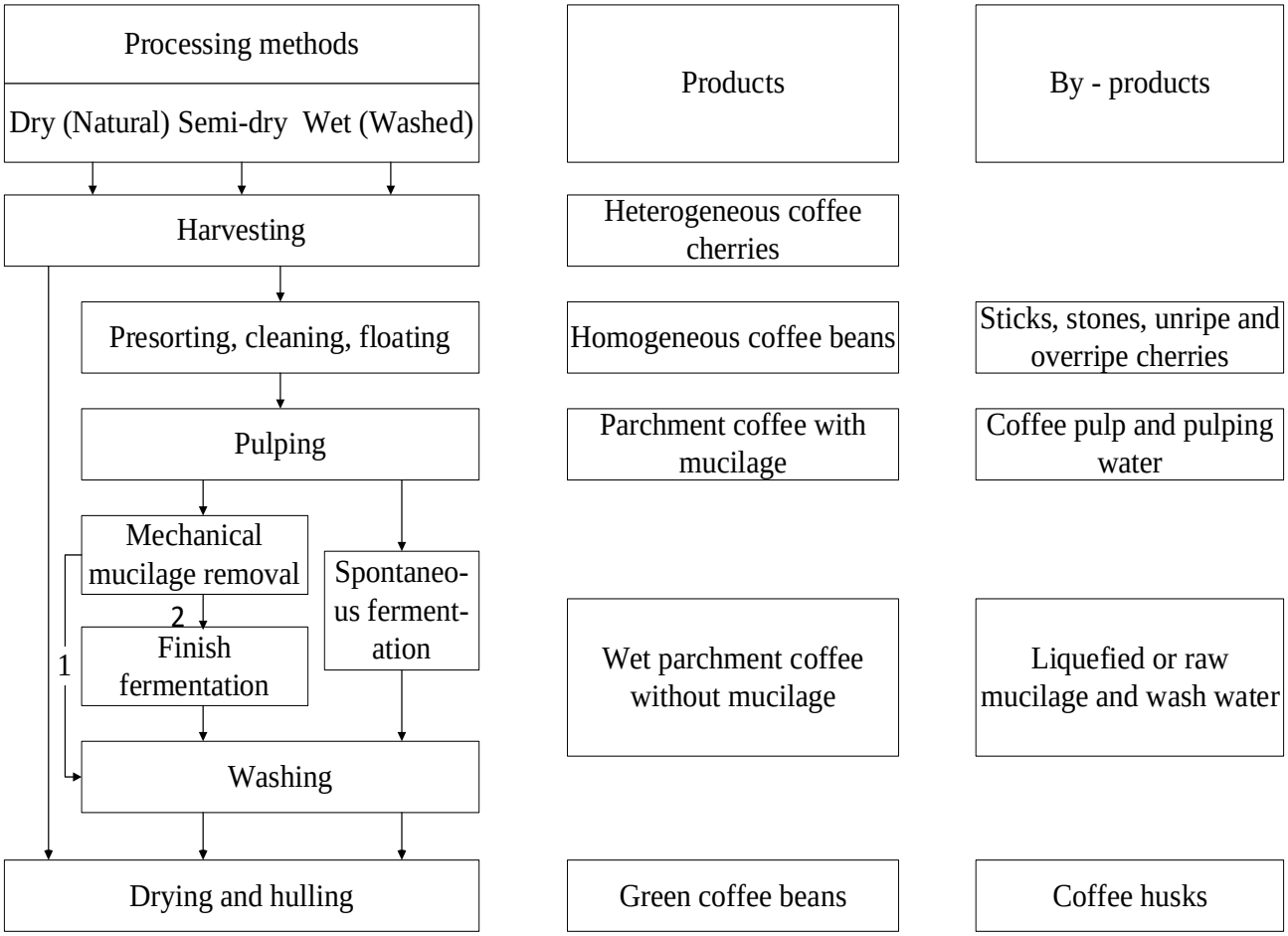

Figure 2.4: General scheme of coffee processing and preparation of green coffee beans.

Source : Haile and Kang (2016)

2.11.1 Dry processing

Drying coffee in the sun is a simple natural process which is usually time consuming. It is the part of the postharvest process that enables the removal of excess moisture to a level that is safe for long time storage without any impact on aroma or taste of the final beverage (Haile and Kang, 2016). It is less labour intensive, cheaper, simpler and produces "natural" coffees. During sun-drying, the fresh beans are dried immediately after harvesting in order to stabilize and preserve the quality (Haile and Kang, 2016).

Drying is an important and critical step in coffee processing, and may affect the quality of the final product (FAO, 2006). It removes the surface moisture that could potentially initiate a harmful post-fermentation reaction if allowed to remain. Because of this, the thickness or depth of the layer of beans being sun dried must not exceed 4 cm, when on the drying material. Behailu *et al.* (2008) noted that coffee dried at a drying depth of 5 cm gives low cup quality. The duration of drying is dependent on the climate, temperature, sun's intensity, relative humidity, ventilation and the nature of the drying material (Enyan, 2011). Sun-drying is the predominantly used method in Uganda. Although dry processed coffee has greater body, it is less aromatic and its acidity is significantly lower than that of washed coffee (Gashaw *et al.*, 2018). Currently, over 80% of Uganda's coffee is dry processed implying that it is of low quality and attracts a low price on the world market (You and Bolwig, 2006).

2.11.2 Semi-dry processing

Semi-dry processing bridges the gap between washed and natural coffees as it generally possesses some of the body and sweetness of a natural coffee while retaining some of the acidity of a washed coffee (Hameed *et al.*, 2018). The semi-dry (pulped natural or honey dry) processing is an intermediate process between wet and dry processing, and is largely used in

Indonesia and Brazil (Poltronieri and Rossi, 2016). The process reduces acidity and increases body (Mohammedsani, 2017). Like in wet processing, the coffee exocarp and significant portion of mesocarp are separated mechanically, using pulping machines (Ribeiro *et al.*, 2017). The coffee beans, still coated with mucilage, are then stored for up to one day. However, unlike wet-processing method, the sticky part (mucilage) remains and is allowed to dry on the parchment instead of complete removal (Mohammedsani, 2017). The parchment coffee is then dried in the sun to attain a moisture content of 10 to 12% (Ghosh and Venkatachalapathy, 2014). However, careful drying is crucial to the success of this method since honey dried coffee beans still contain a layer of fermentable sugar for use by yeasts and bacteria (Ghosh *et al*., 2014). The processor must rake the green coffee beans 2 to 3 times per hour to prevent mold and fungal infections (Mohammedsani, 2017). Once the beans have attained the desired moisture level, they are dry milled to remove the "*parchment*" layers. Semi-dry coffees often have a syrupy body with enhanced sweetness, round acidity and earthy undertones (Ribeiro *et al.*, 2017).

2.12 Wet processing

Approximately half of the world coffee harvest is processed by the wet method in which the coffee berry is subjected to mechanical and biological operations in order to separate the bean or seed from the exocarp (skin), mesocarp (mucilagenous pulp) and the endocarp (parchment; Padmapriya *et al.*, 2013). The resulting product, washed or parchment coffee, can have its parchment easily hulled to produce the dry green coffee bean (Padmapriya *et al.*, 2013). Parchment coffee is obtained by pulping to remove the pulp, which is the main by-product constituting 43% of the fresh weight of the coffee fruit (Saenger *et al*., 2001). The mucilage of between 0.5 and 2 mm thickness is removed in tanks by natural/conventional fermentation. Mucilage is an obstacle to further drying of the beans because of the high pectin content. Wet

processing produces the “washed” or “mild” coffees and this involves more capital outlay, more water and more care than the dry method (Hicks, 2002). During fermentation, mucilage texture changes from viscous and slippery to watery and fluid (Avallone *et al.*, 2001). In wet or washed coffee processing, the ripened fruit is squeezed during pulping, allowing the soft pulpy part of the bean with the skin to be separated. This method is considered to produce a higher quality product which fetches a high market price since it preserves some intrinsic qualities of the bean better and also the resulting green coffee beans are more homogeneous, moreover with minimal defects (Subedi, 2011). The fermentation process can be optimized by monitoring the pH and brix of the fermenting mass. In general, the pH is reduced by about 30% and the brix increased up to between 8 and 15 °Brix (Quintero, 2014).

2.13 Coffee fermentation

One of the key processing activities in the production of coffee is the fermentation process (Martinez *et al.*, 2019). This process is important for degrading the mucilage surrounding the bean and for aroma development. Coffee fermentation refers to the microbial action of yeasts and bacteria breaking down the sugars in the coffee mucilage, which leads to acid production thereby adding to the complexity of a coffee (Martinez *et al.*, 2019). Fermentation can either be spontaneous (uncontrolled) or inoculated (controlled). Spontaneous fermentation is the fermentation that naturally occurs when the wild yeasts and bacteria present in coffee are encouraged to propagate (Martinez *et al.*, 2019). However, long fermentation times synonymous with spontaneous fermentation result in not only a microbial shift (from *Leuconostoc* to acid-tolerant lactobacilli) but also changes in the chemical composition of green coffee beans and distinct sensory attributes like acidity, body and sweetness in the brewed cup (Zhang, *et al*., 2019; Kulandaivelu, 2013). Presently, coffee fermentation in Uganda relies on the naturally occurring microbiota of the fresh raw materials. However,

controlled coffee fermentation by the use of starter cultures may guarantee standardized quality and reduce the economic loss to the producer (Pereira, 2015). Controlled fermentation can be carried out using pure (single) or mixed culture (Pereira *et al.*, 2017). Thus, starter culture development is crucial and is done by selecting microorganisms that have certain characteristics, such as mucilage degradation ability, tolerance to stress during fermentation, the ability to suppress the growth of pathogenic fungi, and a positive impact on the sensory quality of the coffee (Haile and Kang, 2019). The use of starter cultures shortens fermentation time, increases food safety and contributes towards the standardization of product properties (Laranjo *et al*., 2019).

2.14 Microbiota of wet processed coffee

Previous studies have identified over 50 yeasts and bacterial species that are present during coffee fermentation (Evangelista *et al.*, 2015). Microorganisms play a major role in coffee fermentation by degrading its mucilage by producing different enzymes, acids and alcohols (Haile *et al*., 2019). It is also during fermentation that precursors of flavour-enhancing compounds are naturally produced by the fermentative microbiota present in coffee fruits (Gonzalez-Rios *et al*. 2007). Different types of microorganisms interact with natural coffee mucilage in diverse ways, some simultaneously and others in succession (Poltronieri and Rossi, 2016). The molecular identification of microorganisms associated with coffee wet processing was performed by Masoud *et al.*(2004). The microorganisms involved in coffee fermentation are yeasts and bacteria (Haile and Kang, 2019). Coffee bean fermentation processes need to be well controlled to ensure the development of microorganisms that give a high-quality beverage with good coffee aroma (Bressani, *et al*., 2018). However, Arabica coffee processed by the wet process but from different regions, showed differences in the microbiota during fermentation, and consequently, differences in the volatile compounds and beverages (Bressani, *et al*., 2018).

2.14.1 Bacteria

An early study on the action of microorganisms present in coffee fermentation was published by Pederson and Breed (1946) using samples of coffee from Colombia and Mexico. The authors isolated cocci and microaerophilic bacteria such as *Leuconostoc mesenteroides, Lactobacillus plantarum, Lactobacillus brevis,* and *Streptococcus faecalis* as facilitating the lysis of mucilage but not its detachment. Agate and Bhat (1966) isolated and identified *Streptococcus, Pseudomonas, Flavobacterium,* and *Proteus* from depulped coffees in India and stated that these bacteria are not pectinolytic and therefore not involved in the process of fermentation of depulped fruits. The most common genera of bacteria present during coffee fermentation are *Lactobacillus, Bacillus, Arthrobacter, Acinetobacter, Klebsiella* and *Weissella* (Evangelista *et al*., 2014). Lactic acid bacteria showed relative prevalence of over 60% at all sampling times (Junqueira *et al*., 2019). These micro-organisms produce pectatelyase which is unable to depolymerize esterified pectins of mucilage without previous de-esterification. Inoculation with pectolytic bacteria allows microbiological control of the fermentation process. In semidry processing, 15 species, including *Acinetobacter, Bacillus cereus, B. macerans, B. megaterium, B. subtilis, Enterobacter agglomerans, Erwinia herbicola, Escherichia coli, Klebsiella pneumoniae, Lactobacillus brevis, L. plantarum, Lactococcus lactis, Leuconostoc mesenteroides,* and *Serratia,* have been identified using traditional and molecular methods (Pereira *et al*., 2017).

2.14.2 Yeast

Yeasts are microscopic fungi consisting of single oval cells that reproduce by budding, and are capable of converting sugars into alcohol and carbon dioxide (Junqueira *et al*., 2019). The population of yeasts in depulped coffees is greater than that of bacteria at the beginning of the fermentation period. The density of yeasts is close to 10^4 CFU/g and this increases to 10^7 CFU/g

during fermentation (Masoud *et al*., 2004). *Saccharomyces, Pichia, Candida, Rhodotorula, Hanseniaspora* and *Kluyveromyces* are the most common yeast genera that have been isolated (Evangelista *et al*., 2014; Junqueira *et al*., 2019). Yeasts originating from spontaneously fermenting coffee beans including *Pichia* spp. are the most frequent isolates, followed by *Candida* spp. and *Saccharomyces* spp. (Pereira *et al.*, 2016). According to Pereira et al. (2016), *P. fermentans* produces the highest concentrations of flavor-active ester compounds (viz., ethyl acetate and isoamyl acetate), while *Saccharomyces* spp. is a better pectinase-producing strain. Inoculation of a single culture of *P. fermentans* and co-culture of *P. fermentans* and *Saccharomyces* spp. enhances the formation of volatile aroma compounds during the fermentation process. Pereira *et al.* (2014) reported *Pichia fermentans, Pichia kluyveri* and *Candida* species as the most frequent isolates in fermented coffee from Brazil. *Candida glabrata, C. quercitrusa, Saccharomyces* spp., *Pichia guilliermondii, Pichia caribbica, Hanseniaspora opuntiae* and *P. fermentans* were found with lower frequency (Poltronieri and Rossi, 2016). However, Poltronieri and Rossi (2016) reported *Pichia kluyveri* and *Pichia anomala* as the predominant yeasts isolated from Arabica coffee fermented in East Africa (). *Kluyveromyces marxianus*, *Candida pseudointermedia, Issatchenkia orientalis, Pichia ohmeri* and *Torulaspora delbrueckii* occur in lower concentrations (Poltronieri *et al*., 2016). *Pichia kluyvery, P. anomala*, *Hanseniaspora uvarum* have an inhibitory effect on the growth of *Aspergillus ochraceus*, a fungus that is potentially toxigenic and is often isolated in coffee fruits and beans. Thus, yeasts play a dual role during the processing of coffee fruits: they facilitate fermentation and biological control against undesirable flora.

2.15 Development of starter culture for the wet fermentation of Arabica coffee

Starter cultures consist of microorganisms that are inoculated directly into food materials in order to bring about desired and predictable changes in the finished product (Durso and

Hutkins, 2003). These changes may include enhanced preservation, improved nutritional value, modified sensory qualities, and increased economic value (Haile and Kang, 2019). In the food industry, starter cultures are widely known for use in cheese, yogurt, beer and wine production (Tamang *et al.*, 2020). Starter cultures improve the quality of fermented foods by providing better fermentation control and predictability of the final product (Evangelista *et al*., 2014). Fermented foods and beverages have long been manufactured without the use of starter cultures (Tamang *et al.*, 2020). Although many fermented foods can be made without a starter culture, the addition of concentrated microorganisms, in the form of a starter culture, provides a basis for ensuring that products are manufactured on a consistent schedule, with consistent product qualities (Durso and Hutkins, 2003). Modern large-scale industrial production of fermented foods and beverages demands consistent product quality and predictable production schedules, as well as stringent quality control to ensure food safety.

The use of starter cultures in controlled coffee fermentation ensures the best quality of coffee and increased economic benefits for all players along the coffee value chain (Tamang *et al*., 2020). Starter cultures are selected as single or multiple strains. Microorganisms that facilitate the degradation of mucilage must be capable of secreting pectin lyase and polygalacturonase enzymes (Martinez *et al.*, 2019). According to Corsetti *et al*. (2012), microorganisms selected for starter cultures should have certain characteristics, such as being non-pathogenic, non-toxigenic, and adaptable to the raw materials and the fermentation process. They should also improve sensory quality, prolong shelf life, reduce processing time and repress development of food-related pathogenic microorganisms such as toxigenic moulds (Haile and Kang, 2019).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Materials

All chemicals used were of analytical grade. Hydrochloric acid (HCl; specific gravity, 1.18 g/mL), sodium hydroxide (NaOH; 99%) and absolute ethanol (EtOH; 99%) were purchased from BDH laboratories (Uganda). Nutrient agar, plate count agar (PCA) used to cultivate viable microbes, potato dextrose agar (PDA) used to cultivate yeasts and molds, and De Man, Rogosa and Sharpe agar (MRS) that was used to cultivate lactic acid bacteria were purchased from Palin Diagnostics International (Uganda). Ampicillin powder purchased from Sigma Aldrich LLC (St. Louis, MO, USA), was used to suppress the growth of bacteria in PDA. Lysis buffer (10 mM Tris-HCl, 2 mM EDTA, 1% SDS) and ammonium acetate ($NH_4CH_3CO_2$) for DNA extraction were purchased from Bioneer (South Korea). 5.8S and 16S primers and PCR master mix for microbial DNA amplification were also obtained from Bioneer.

3.2 Equipment

Sample weight was taken on a Mettler Toledo analytical balance MSI 104TS model manufactured in United States of America. An LG hot air oven 050B model manufactured in China was used to dry residual pellets of the pectin extract. Changes in physicochemical properties (pH, ^{o}Brix and Viscosity) of the fermenting mass were determined using a Palin test pH meter Model 800 manufactured by Palin International (England), a portable refractometer Model PAL3 manufactured by ATAGO (USA) and a Viscometer Model 74,000 supplied by Swastik Systems and Services (India), respectively. The moisture content of green coffee beans was determined using a Moisture Analyser Model 6070 manufactured by Agripro Sinar Technology (United Kingdom). Green coffee beans were roasted in the Probat B4 coffee

sample drum roaster manufactured by Probat (Deutschland) while the roast profile of the beans was determined on a M-Basic Agtron Scale manufactured by Gourmet (USA).

3.3 Methods

3.3.1 Sampling and sample treatment

A total of 21 spontaneously fermenting Arabica coffee samples (liquid fraction and beans) of 100 ml each were drawn aseptically from fermentation tanks of Kyagalanyi Coffee Limited's wet processing stations in Bugisu sub region in the districts of Bududa, Mbale and Sironko. The samples were taken every 12 h over a 72 h period and transferred into sample sterile glass bottles, and then placed in ice boxes containing ice blocks before transportation to the laboratory for microbial isolation within 6 h. For determination of physicochemical characteristics of the microbial isolates on the fermenting coffee mass and effect on cup quality, Arabica coffee was obtained from Mr. Nasambi Siragi's coffee farm in Bushiyi parish, Bulucheke sub county; Bududa District. Bududa is one of the major Arabica coffee producing districts in Uganda. The activities undertaken in the study are summarized in Fig. 3.1

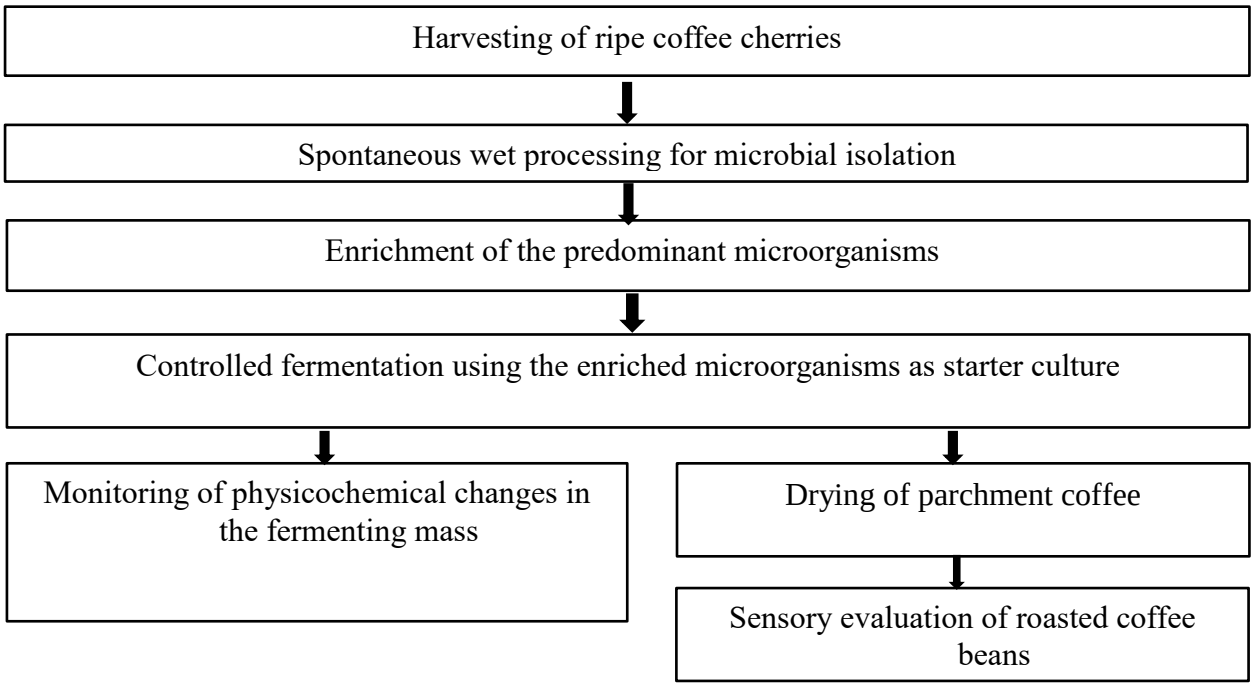

Figure 3.1: An illustration of the study to evaluate the potential of spontaneous fermentation isolates to improve the fermentation process and cup quality of wet processed Arabica coffee.

Ripe Arabica coffee cherries (550 kg) were harvested by hand picking from randomly selected SL 14 variety coffee trees (Kulandaivelu, 2013). The coffee cherries were packaged in jute bags and transferred to the Manafwa Washing Station (Kyagalanyi Coffee Limited) for pulping. The pulped coffee (100 kg) was packaged in sterile polypropylene bags, iced and transported in cool boxes to the Uganda Industrial Research Institute (UIRI) Analytical Laboratories for physicochemical experimentation within 6 h. The experiments started immediately upon arrival at the laboratory. Fermentation was carried out in plastic buckets containing parchment (1 kg): distilled water (2 L) in accordance with (Pereira *et al.*, 2014).

3.3.2 Isolation of predominant microorganisms in spontaneous fermentation

Samples (liquid fractions and parchment) of spontaneously fermenting coffee biomass (100 ml) were taken from fermentation tanks at 12 h intervals over a 72 h period. The temperature of the fermentation tanks environment was between 23 and 26ºC (day -time) and between 18 and 22º (night-time) while relative humidity ranged between 60 and 75% (Kulandaivelu, 2013). The samples were transferred aseptically into sterile sample glass bottles. To 1 ml of each sample of the ferment was added 9 ml sterile 0.1% peptone water, in triplicate, followed by 10-fold serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}; Masoud *et al*., 2004). The diluted samples were then analysed for the viable counts using plate count agar (PCA) and Nutrient agar. Analysis was also done for Lactic Acid Bacteria, and yeasts and molds using surface inoculation on a De Man, Rogosa and Sharpe agar (MRS) and potato dextrose agar (PDA), respectively. The culture media plates were aerobically incubated at 30ºC for 48 h for the viable bacteria, 37 for 24 h for non-fastidious bacteria, 30ºC for 48 h for lactobacilli and 26ºC for 72 h for yeast and moulds. Colony characteristics were observed and representative single colonies were isolated and sub cultured on the respective media.

The microbial population was estimated as colony forming units (CFU) per ml. CFU/ml in the original sample was calculated by multiplying the CFU on the countable plate by the 1/Final dilution factor (FDF). Morphological identification of the cells was done using the Olympus CX43 Biological Microscope manufactured by Gem Scientific Ltd (The Federal Republic of Germany).The Gram staining protocol according to (Smith and Hussey, 2005) was used to differentiate between the Gram-positive and Gram-negative bacteria. This involved application of a primary stain (crystal violet) for 1 min to the heat-fixed smear, washing in a stream of tap water for 2 s, followed by addition of a mordant (Gram's Iodine) for 1 min, rapid decolorization with acetone for 15 s, and lastly, counterstaining with Safranin for 1 s, before washing in a stream of tap water and blot drying. The results of Gram staining were observed under oil immersion using a microscope (X110M). Gram-positive bacterial cells were observed to stain a purple colour while the Gram-negative stained red. In the case of yeasts, the Methylene Blue staining technique previously used by Smart *et al.* (1999) was followed for their presumptive selective identification. This technique involved smearing a yeast suspension on a clean microscope slide. The smear was allowed to dry at room temperature and fixed by passing several times through the flame of a Bunsen burner. The fixed film was then stained with aqueous methylene blue (1% w/v) for 4 min, rinsed with water and stained for 2 min in tannic acid (5% w/v). The film was then rinsed with water and blotted dry before microscopic examination for observation of viable yeasts cells (X110M) which stained bright or dark blue.

Bacteria were further classified as catalase-positive or catalase-negative based on the reaction of microbial cells with a solution of 30% hydrogen peroxide (H_2O_2). The catalase test was performed using the slide (drop) method (Reiner, 2013). The catalase test was performed by collecting a small amount of a colony from each isolate, using sterile inoculating loops, after 24 h incubation. The colony was placed on a microscope slide, covered with a petri dish. Using

a Pasteur pipette, one drop of H_2O_2 was then placed on the transferred colony. Positive reactions were considered for effervescence (bubble formation) observations. A magnifying glass was used to observe weak positive reactions. The isolates were then grouped according to their phenotypic characteristics (cell morphology and biochemical features). The isolates were preserved using glycerol in proportions of 20: 80 glycerol to culture broth and stored in an ultra-low temperature freezer at -80^{o}C ultra (Masoud *et al*., 2004).

3.3.3 Molecular identification of microbial isolates

3.3.3.1 Deoxy ribonucleic acid (DNA) extraction

Deoxy ribonucleic acid (DNA) material was extracted from the selected isolates of yeasts and bacteria according to the method described by Mahuku (2004). DNA extraction involved pipetting 0.5 ml of bacteria or yeast culture suspension into a 1.5 ml micro-centrifuge tube, centrifuged at 13000 rpm for 1 min and the supernatant was discarded. Then 800 µl of 1M NaCl was added to the cell pellet, vortexed for 30s, centrifuged at 13000 rpm for 1 min and the supernatant was discarded. This process was repeated twice and the resultant pellet was retained in the tube. To the pellet was added 500 µl of TES buffer (0.2M Tris-HCl [pH8], 10 mM EDTA [pH8], 0.5M NaCl, 1% SDS and proteinase K 50 µg/µl), vortexed and incubated at 65°C for 30 min. Thereafter one-half volume (250 µl) of 7.5M ammonium acetate was added (to precipitate proteins), and the sample was vortexed and incubated on ice for 10 min followed by centrifuging for 15 min at 13000 rpm. To 500 µl of the supernatant transferred into a new tube, an equal volume of ice-cold isopropanol was added, and incubated at minus 20°C overnight (to precipitate the DNA). This was followed by centrifuging for 10 min at 13000 rpm to pellet the DNA. The supernatant was discarded and the pellet was washed with 70% ethanol (800 µl) and centrifuged at 13000 rpm for 5 min. The supernatant was discarded and the pellet was then left to air dry for 6 h. The dry DNA pellet was resuspended in nuclease free water,

quantified using a Nanodrop 2000/2000c spectrophotometer (Thermo scientific, USA) and was then diluted to 200 ng/µl using Tris-EDTA (TE) buffer and stored at -20°C for later amplification of target essential genes. The extracted DNA was used in downstream Polymerase Chain Reactions (PCR) to amplify the 16S rRNA gene and 5.8S rRNA gene of bacteria and yeasts, respectively.

3.3.3.2 Amplification of the 5.8S rRNA and 16S rRNA genes

In the case of yeasts, the internal transcribed spacer 5.8S rRNA gene was amplified using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5′-CCTCCGCTTATTGATATGC-3′; Masoud *et al.*, 2004). For bacteria, the 16S rRNA gene (variable region 3, the most conserved region) was amplified using P16S (5'CCAGCAGCCGCGGTAATACG-3') and M26S (5'-ATCGGCTACCTTG TTACGACTTC3') primers (Adriko *et al.*, 2014). The PCR reaction mix comprised 12.5 µl of 2X Gotaq premix (Bioneer), 0.625 µl of 10 M forward and reverse primer each, 2 µl of 50 ng/µl DNA sample and this was topped up to 25 µl using nuclease free water. The PCR cycle conditions for amplifying the 5.8S rRNA gene were; 4 min initial denaturation at 95°C, 35 cycles of 20 s of denaturation at 95°C, 30 s annealing at 55°C, 30 s extension at 72°C and 5 min of final extension at 72°C. The PCR cycle conditions for amplifying the 16s rRNA gene were; 4 min initial denaturation at 95°C, 35 cycles of; 20s denaturation at 95°C, 30 min annealing at 60°C, 40 min extension at 72°C and 5 min of final extension at 72°C. The PCR products (amplicons; 200 to 700 bp) and a 1 kb plus DNA ladder (Thermo Scientific) were separated by agarose gel electrophoresis using a 1.5% agarose gel in 1X TAE buffer (40 mM Tris, 20 mM acetate and 1mM EDTA) at 80 volts for 60 min. The gel was then stained with ethidium bromide manufactured by Thermo fisher scientific (UK) at 0.5 µg/ml final concentration and the bands visualized using a Gel documentation machine (UVP 97-0664-02,

Fisher Scientific, Norway). The bands were then excised using a surgical blade, after which they were purified using a Gel Extraction kit Gene Elute Model NA1111-1KT manufactured by Sigma-Aldrich (German). The DNA fragment of interest was excised from an agarose gel using a clean, sharp scalpel, placed in a 1.5 ml Eppendorf tube and weighed. Three gel volumes of the gel solubilization solution was added to the slice (for example for 100 mg of gel was added 300 ml of gel solubilization solution). The gel mixture was then incubated at 60°C until the gel was completely dissolved. Binding columns were prepared by placing them in 2 ml collection tubes, adding 500 µl of column preparation solution to each of them and centrifuging at 12000 rpm for 1 min. One gel volume of isopropanol was added in to the solubilized gel mixture and this mixture was pipetted into a prepared binding column and centrifuged at 12000 rpm for 1 min. The binding column was removed from the 2 ml collection tube and the flow through liquid was discarded. Then it was returned into the collection tube and 700 µl of wash solution was added, centrifuged at 12000 rpm for 1 min and the flow through liquid was discarded too. Lastly the binding column was transferred to a fresh collection tube and 50 µl of elution solution was added at the centre of the membrane, incubated for 1 min at room temperature followed by centrifuging at 12000 rpm for 1 min. The DNA contained in the flow through was then quantified using a Nano-drop and then stored at -20°C before sending them for sequencing.

3.3.3.3 Deoxy ribonucleic acid sequencing and species identification

The amplified PCR products/amplicons were sequenced at the Infectious Diseases Research Council (IDRC, Uganda). Sanger sequencing was done, and ab1 DNA sequence files obtained were aligned using the Bio-edit version 7.2 sequence alignment editor and converted to FASTA file format. The aligned sequences were then compared to the GenBank/nucleotide data base

using the Basic Local Alignment Search Tool (BLAST) program (National Centre for Biotechnology Information, Bethesda, MD) for the identification of isolates.

3.4 Determination of the physicochemical changes in the wet fermenting biomass

The predominant isolates were sub cultured in nutrient broth for bacteria and potato dextrose broth for yeast in a rotary incubator shaking at 100 rpm at 25°C for 48 h. The cultures were then standardised against a 0.5 McFarland standard turbidity level (1.5 x 10^8 CFU/ml or 12.2 log CFU/ml equivalent). The standardized culture broth was inoculated (10 ml) onto the surface of the coffee fermenting mass. The fermenting mass was monitored for changes in pectin degradation, total soluble solids (TSS), viscosity, pH and titratable acidity at 12 h intervals over a 72 h period.

3.4.1 Determination of pectin degradation of the mucilage

The pectin degradation of the mucilage was determined, as pectin content during fermentation, according to the method previously described by Castillo-Israel *et al.* (2015). The fermented coffee (50 g) was weighed out on an analytical balance (Model MSI 104TS, Mettler Toledo, USA). The coffee was transferred into a 500 ml beaker and 0.01 N Hydrochloric acid (200 ml) was added. The mixture was heated with continuous stirring at 90^{o}C for 45 min. The mixture was filtered on a 1 mm mesh. To the filtrate was added twice its amount, absolute ethanol (95%) in a 500 ml conical flask. The mixture was left to stand for 40 min. The resultant precipitate was filtered through a nylon cloth and washed with ethanol (55%). The precipitate was again washed with 75% ethanol. The remaining residue was dried (55^{o}C) in an oven for 48 h. The pectin extract was cooled, weighed and stored under cool and dry conditions. The yield was computed according to the equation 1:

Yield % = (Quantity of pectin extract/mucilage quantity) *100 (1)

3.4.2 Determination of total soluble solids

Total soluble solids (TSS) were determined as °Brix according to the ISO 2173:2003 - Refractometric method using an ATAGO portable refractometer.

3.4.3 Determination of viscosity

Viscosity was determined as centipoise (cP) according to the BS EN ISO 2884-1:2006 method. A digital rotational viscometer was used. The spindle of the viscometer was inserted into the centre of the liquid fraction of the fermenting coffee mass in a plastic bucket. Viscosity readings were displayed on the screen. The spindle was disinfected using 70% ethanol in between measurements.

3.4.4 Determination of pH

The pH changes of the coffee fermenting biomass according to AOAC (1998) official method 973.41 using a digital pH meter Model PE.136 manufactured by ELICO Ltd (India). pH was monitored by sampling 100 g of the coffee ferment and taking measurements at 25°C.

3.4.5 Determination of titratable acidity

Titratable acidity (TA) was determined according to the AOAC official method 942.15 (AOAC 2000). The coffee ferment was diluted with distilled water (10 g to 25 ml) and titrated to pH 8.1 by 0.1N sodium hydroxide (NaOH) using phenolphthalein indicator. The TA, expressed as lactic acid in g/L of the ferment, was calculated according to equation 2:

Titratable acidity (TA) = (V × N × 1000 × 0.091) /W (2)

where: N is the normality of NaOH, 0.091 is the conversion factor for lactic acid, V is the volume (mL) of NaOH required and W is the mass (g) of coffee ferment used.

3.5 Sensory analysis of coffee brew obtained from controlled fermentation of roasted *Coffea arabica* beans

Sensory evaluation was carried out at the Uganda Coffee Development Authority (UCDA) cupping laboratory. The methodology followed the analysis protocol guidelines of the Specialty Coffee Association (SCA). Two groups of tasters, each with three expert Q-graders were used (Pereira *et al*., 2017). Parchment coffee (500 g) was sundried to *ca.* 12% moisture content (ICO, 2019). It was hulled to obtain green coffee beans. The green coffee beans (180 g) were roasted to medium dark roast profile based on 45 and 55 Agtron colour scale at 210^{o}C in a Probat PRZ 4 Barrel sample Roaster manufactured by Probat (Germany). Roasting was done for *ca*. 10 min within 24 h of cupping. The roasted coffee was rapidly cooled and allowed to degas for 12 h. The cool roasted coffee (14 g), was ground to particle size 3 (slightly coarse grind) and infused in 200 ml of hot (93^{o}C) potable water in 5 white ceramic cups. The coffee grinds were allowed to steep undisturbed for 4 min. For sample uniformity evaluation, five cups of each sample were prepared. The brews were assessed to determine sensory characteristics. The entire cup was presented to the cuppers, including the grounds, which formed a "crust" on top. Following crust removal using 30 g stainless steel spoons, the liqueur was tasted by aspiration into the mouth and nose. The coffee brew was assessed for fragrance/aroma, flavour, aftertaste, acidity, body, balance, sweetness, uniformity, clean cup, overall impression, and finally, the overall score. Assessment was done following the Specialty Coffee Association (SCA) 16-point scale that represents levels of quality from 6 to 9.75, with quarter point increments. These levels are: good (6.00 to 6.75), very good (7.00 to 7.75), excellent (8.00 to 8.75) and outstanding (9.00 and above; SCA, 2015). The cup score was

determined by adding up all the components (Sittipod *et al.*, 2019). The procedure was repeated for all the samples until consistent results were obtained.

3.6 Principle Component Analysis (PCA)

Principle Component Analysis (PCA) was done to determine the contribution of each of the physicochemical characteristics of the different starter cultures on the coffee cup quality over a 72 h fermentation period. A PCA biplot was constructed to show clusters of the isolate combinations, their physicochemical characteristics and cup quality components (Wood *et al.*, 2018). Eigenvalues and Pearson (n) correlation matrix were used to establish clusters and explain the variability observed in the individual clusters and to identify the cup quality components that were associated most with the physicochemical characteristics of the starter cultures. Correlation coefficients (r) of physicochemical characteristic of starter culture with cup quality components were determined. The coefficients were rated as follows: r=0.40-0.59: moderate, 0.6-0.79: strong and 0.8-1: very strong correlation (Wood *et al.*, 2018). The correlations were used to generate information that would guide food processors to focus on particular physicochemical characteristic(s) of starter culture that would produce the best consumer cup quality.

3.7 Statistical analysis

All experiments were done in triplicate and the results expressed as the mean ± standard deviation of the mean. A one-way ANOVA was done using XLSTAT version (2020.5.1.1072) to identify significant differences between the physicochemical characteristics of the fermenting mass and the cup quality of the fermented coffees. A 2-way ANOVA was also done to establish the interactions of time and starter culture on the cup quality of fermented coffee over a time period of 0 h to 72 h. Principal component analysis for the cup score and

physicochemical characteristics of the different starter cultures were analyzed using XLSTAT version (2020.5.1.1072). The significant differences between the starter cultures were evaluated using the Turkey's tests at $p<0.05$.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Isolation and identification of predominant microorganisms in spontaneous coffee fermentation

A total of 130 microbial isolates were obtained on the different growth media (Table 4.1). Bacteria dominated (61%; B_1 = 30%, B_2 = 31%) over the yeasts (39%; Y_1 = 30%, Y_2 = 9%).

Table 4.1: Enumeration (log CFU/ml) of predominant yeast and bacteria from spontaneous *C. arabica* fermentation

Fermentation time	PCA	NA	PDA	Mean	PDA	Mean	NA	MRS	Mean	PCA	NA	Mean
	Y_1	Y_1	Y_1	Y_1	Y_2	Y_2	B_1	B_1	B_1	B_2	B_2	B_2
0	7.49	6.43	8.37	7.43	8.61	8.61	10.59	9.76	10.17	7.49	6.51	7.00
12	10.86	7.50	10.13	9.49	9.79	9.79	10.42	10.09	10.25	9.27	7.70	8.49
24	10.60	6.23	9.66	8.83	9.66	9.66	10.94	10.18	10.56	7.64	5.48	6.56
36	10.70	6.34	9.73	8.92	9.68	9.68	10.83	10.62	10.72	7.75	5.71	6.73
48	10.84	6.57	10.20	9.21	10.06	10.06	10.94	10.65	10.79	7.71	6.41	7.06
60	10.70	7.21	10.53	9.48	10.40	10.40	10.94	8.62	10.78	7.82	6.59	7.20
72	10.82	7.26	10.58	9.56	10.82	10.82	10.78	7.69	10.73	8.70	6.74	7.72

n=21; Values are means of three independent replicates; PCA: Plate Count Agar; NA: Nutrient Agar; MRS: Man, Rogosa and Sharpe; PDA: Potato Dextrose Agar; Y_1: *K. exigua;* Y_2: *P. kudriavzevii*; B_1: *L. mesenteroides;* B_2: *P. campinasensi*.

The colonies observed on PCA and NA were creamish white in colour; their elevation was raised and flat; circular, rhizoid and spindle in form; large, medium and small in size; glistening

and dull on surface with entire margins. Cells from these colonies appeared as Gram positive spherical, diplo, scanty, chained and clustered cocci under the microscope. Scanty gram-negative rods and cocci were also observed. In as far as MRS is concerned, the colonies were observed to be small in size, greyish, round and slimy with smooth margins. Cells from colonies on MRS appeared as distinct, chained and clumped Gram-positive rods. Colonies on PDA were observed to be grey and cream in colour, small (0.8 mm) and medium (1.5 mm), budding and ovoid in shape, rough edged and dry with entire margins. Cells from these colonies presented dark blue spherical, oval, budding and clustered cells. Table 4.2 shows the results of presumptive identification of the different isolate grouped on the basis of their phenotypes.

Table 4.2: Phenotypic characteristics of the selected isolate groups for presumptive identification

Isolate Group	Methylene Blue	Gram Stain			Form	Catalase	Oval	Presumptive species
		-ve/ +ve	Cocci	Rods/ Bacilli				
Y_1	+	n/a	n/a	n/a	Budding	-	+	*Kazachstania* spp
Y_2	+	n/a	n/a	n/a	Budding	-	+	*Pichia* spp.
B_1	n/a	+	+	-	Clustered	-	n/a	Lactic acid bacteria (*Lactobacillus* spp., *Leuconostoc* spp.)
B_2	n/a	+	-	+	Clumped	-	n/a	*Paenibacillus* spp.

Y_1: *K. exigua*; Y_2: *P. kudriavzevii*; B_1: *L. mesenteroides ;* B_2: *P. campinasensi*.

The majority (71%) of bacteria were Gram-positive while 29% were Gram-negative. Most bacteria isolates were characterised as catalase negative Gram-positive rods hence the resulting biochemical characterisation of lactic acid bacteria. On the other hand, the observed catalase

negative isolates which stained blue on PDA were characterised as yeasts. The isolate groups were then subjected to PCR for genotypic speciation.

4.1.1 Amplification, sequencing of the 5.8S and 16S rRNA genes and identification of species

From the DNA extracted from the samples, genes were amplified and the DNA quantity and quality were determined. The results are shown in Table 4.3.

Table 4.3: Quality and quantity of DNA extracted from the selected microbial isolates

Isolate No.	Isolate type	DNA (ng/µl)	260/280 bp	260/230 bp
1	Bacteria	119.6	2.13	0.49
2	Bacteria	266.8	2.02	1.35
3	Bacteria	1311.3	2.00	1.62
4	Bacteria	177.0	2.09	0.64
5	Bacteria	1664.9	1.97	2.07
6	Bacteria	10.8	1.90	0.56
7	Bacteria	1784.1	1.92	1.92
8	Bacteria	178.7	2.00	0.61
9	Bacteria	100.0	2.09	1.75
10	Yeast	1754.6	2.02	1.85
11	Yeast	4810.8	1.78	1.89
12	Bacteria	305.2	1.80	1.52

bp: base pairs.

The 5.8S rRNA genes and 16S rRNA gene of isolates for yeasts and bacteria respectively, were successfully amplified (Appendix I). After gene amplification, Sanger sequencing yielded sequences for each of the 4 groups of isolates. A BLAST search (Appendix II) using sequences of the isolate groups in the NCBI nucleotide database established that the isolates were correspondingly *Kazachstania exigua*, *Leuconostoc mesenteroides*, *Paenibacillus campinasensis* and *Pichia kudriavzevii*. Isolates Y_1, Y_2, B_1, B_2 and were assigned accession numbers MT634702, MW142501, MT634699 and MT634703 in the NCBI nucleotide database, respectively (Table 4.4).

Table 4.4: Identified isolates obtained from spontaneous fermentation of Arabica coffee

Isolate	Count	Band Size	5.8S/16SrRNA gene sequence spp	Homology[1]	Gene Accession No.
Y_1	39	268	*K. exigua*	99.63	MT634702
Y_2	12	271	*P. kudriavzevii*	98.54	MT634703
B_1	40	202	*L. mesenteroides*	98.12	MW142501
B_2	39	493	*P. campinasensis*	100.00	MT634699

[1]Percentage of identical nucleotides of the closest relative found in the Gene-Bank database.
Y_1: *K. exigua*; Y_2: *P. kudriavzevii*; B_1: *L. mesenteroides ;* B_2: *P. campinasensi.*

A BLAST and phylogenetic analysis in the isolation and identification of local ethanolic yeasts inhabiting coffee processing environments in Tanzania revealed the presence of *Pichia kudriavzevii* yeasts (Hamadi *et al*., 2014). *K. exigua* has been reported in coffee processing wastewater (Pires *et al*., 2017). Species of *Leuconostoc*, such as *L. mesenteroides*, *L. pseudomesenteroides* and *L. citreum*, have been reported as predominant LAB in coffee fermentations performed in Mexico, Colombia, India and Taiwan (Carvalho *et al*., 2018; Pothakosa *et al*., 2020). A broad bacterial diversity of bacteria have been isolated from coffee fermentations in the different processing methods and identified in genera such as *Bacillus*,

Paenibacillus, Acinetobacter, Streptococcus, Pseudomonas, Flavobacterium, Proteus, Aerobacter, Escherichia, Hafnia, Klebsiella, Tatumella, Paracolobactrum and *Serratia* (Evangelista *et al.*, 2015; Pereira *et al.*, 2015). Generally, these bacteria are detected in freshly extracted beans and are thought to originate from the exocarp (skin plus pulp), water, the surfaces of fermentation tanks and soil. A number of these bacteria have the ability to degrade pectin, especially the *Bacillus* species (Pereira *et al*., 2015).

4.2 Physicochemical changes in inoculated coffee wet fermentation

4.2.1 Pectin degradation

Results of pectin degradation indicated that generally, there was a reduction in the pectin content with increasing fermentation time. Pectin content ranged from 0.000 % to 0.604 % as indicated in Table 4.5. In comparison to the control, fermentation at 36 h produced significantly ($p<0.05$) the highest pectin degradation across all starter cultures. There were no significant differences ($p>0.05$) in pectin degradation by the different starters before 24 h. However, at 36 h, the pectin degradation potential of both the single and mixed starter cultures was significantly different ($p<0.05$) from the control. The highest degradation potential, at 36 h, was observed for starter culture $Y_1Y_2B_1B_2$: *K. exigua*, *P. kudriavzevii*, *L. mesenteroides* and *P. campinasensi* (0.000% ; Table 4.5), which was not significantly ($p>0.05$) different from the other treatments. At 60 h, pectin was observed to have been degraded completely in all the treatments. Only the control experiment attained a significantly different ($p<0.05$) pectin content (0.011%; Table 4.5). Results of the current study were slightly different from the findings of Kulandaivelu (2013) who reported that complete pectin degradation during inoculated fermentation of Arabica coffee in India took 20 h. Differences in the observed pectin content with the different starter cultures could probably be due to differences in the pectolytic activity of the different microorganisms applied (Pereira *et al.*, 2014).

Table 4.5: Changes in Pectin degradation (%) during fermentation of Arabica coffee inoculated with different single and mixed microbial isolates

Starter	Fermentation time (h)						
	0	12	24	36	48	60	72
Control	0.601±0.00 [a]	0.037±0.00[a]	0.129±0.00[a]	0.102±0.00[a]	0.091±0.00[a]	0.011±0.00[a]	0.000±0.00[a]
Y_1	0.519±0.05[a]	0.159±0.08[a]	0.028±0.04[a]	0.001±0.00[b]	0.000±0.00[b]	0.000±0.00[b]	0.000±0.00[a]
Y_2	0.570±0.14[a]	0.120±0.02[a]	0.058±0.01[a]	0.002±0.00[b]	0.001 ± 0.00[b]	0.000±0.00[b]	0.000±0.00[a]
Y_1Y_2	0.560±0.03[a]	0.130±0.03[a]	0.055±0.01[a]	0.002+0.00[b]	0.000 ± 0.00[b]	0.000±0.00[b]	0.000±0.00[a]
B_1	0.528±0.01[a]	0.138±0.25[a]	0.066±0.09[a]	0.001±0.00[b]	0.000 ± 0.00[b]	0.000±0.00[b]	0.000±0.00[a]
B_2	0.532±0.08[a]	0.127±0.06[a]	0.061±0.07[a]	0.006±0.00[b]	0.003 ± 0.00[b]	0.000±0.00[b]	0.000±0.00[a]
B_1B_2	0.542±0.08[a]	0.133±0.06[a]	0.064±0.07[a]	0.005±0.00[b]	0.000 ± 0.00[b]	0.000±0.00[b]	0.000±0.00[a]
Y_1B_1	0.522±0.05[a]	0.121±0.08[a]	0.028±0.04[a]	0.001±0.00[b]	0.000±0.00[b]	0.000±0.00[b]	0.000±0.00[a]
Y_1B_2	0.521±0.05[a]	0.147±0.08[a]	0.021±0.04[a]	0.001±0.00[b]	0.000±0.00[b]	0.000±0.00[b]	0.000±0.00[a]
Y_2B_1	0.538±0.03[a]	0.125±0.03[a]	0.012±0.01[a]	0.001±0.00[b]	0.008 ± 0.00[b]	0.000±0.00[b]	0.000±0.00[a]
Y_2B_2	0.344±0.05[a]	0.102±0.00[a]	0.011±0.00[a]	0.001±0.00[b]	0.000 ± 0.00[b]	0.000±0.00[b]	0.000±0.00[a]
$Y_1B_1B_2$	0.575±0.11[a]	0.136±0.08[a]	0.065±0.07[a]	0.004±0.00[b]	0.002 ± 0.00[b]	0.000±0.00[b]	0.000±0.00[a]
$Y_1Y_2B_1B_2$	0.604±0.11[a]	0.110±0.01[a]	0.014±0.00[a]	0.000±0.00[b]	0.000 ± 0.00[b]	0.000±0.00[b]	0.000±0.00[a]

n=91. Values are means of three independent replicates. Values with different superscript letters in a column are significantly different ($p<0.05$). Y_1: *K. exigua*; Y_2: *P. kudriavzevii*; B_1: *L. mesenteroides ;* B_2: *P. campinasensi* ; Control: un-inoculated.

Rapid pectin breakdown is necessary to achieve shorter coffee fermentation time (Haile and Kang, 2019). A statistical regression performed to predict the fermentation time varied significantly ($p<0.05$) depending on the starter culture type. On the basis of pectin degradation, fermentation time ranged between 28 h and 40 h (Appendix III). The mixed starter comprising *K. exigua, P. kudriavzevii, L. mesenteroides* and *P. campinasensi* was predicted to have potential to reduce fermentation time to 28 h. On the basis of pectin degradation, the combination of *L. mesenteroides* and *P. kudriavzevii* did not show a significant ($p = 020$) effect on the fermentation time (Appendix III).

4.2.2 Changes in total soluble solids (°Brix)

Total soluble solids (TSS) in the fermenting mass varied between 3.03 °Brix and 9.40 °Brix (Table 4.6). Generally, there was no significant difference ($p>0.05$) between the TSS of the treatments inoculated with different starter cultures at fermentation time 0 h and 12 h (Table 4.6). The highest potential to increase TSS of the fermenting mass, at 24 h of fermentation, was observed for *K. exigua, P. kudriavzevii, L. mesenteroides and P. campinasensi* (7.30 °Brix) and *K. exigua, P. kudriavzevii* and *L. mesenteroides* (7.08 °Brix). These TSS values were significantly different ($p<0.05$) from the TSS of the other treatments (Table 4.6). Starter culture combination *K. exigua, P. kudriavzevii, L. mesenteroides* and *P. campinasensi* had the highest TSS at the different fermentation times (0 h to 72 h), followed by *K. exigua, L. mesenteroides and P. campinasensi*. At 72 h, mixed starters *K. exigua, P. kudriavzevii, P. campinasensi* and *L. mesenteroides, K. exigua, L. mesenteroides* and *P. campinasensi, P. kudriavzevii* and *L.* mesenteroides, and *K. exigua* and *P. kudriavzevii* had the highest total soluble solids (8.92 °Brix, 8.83 °Brix, 7.98 °Brix and 7.78 °Brix, respectively) which were significantly different ($p<0.05$) from the rest of the treatments, Table 4.4). Differences in TSS could be attributed to variations in the rate of microbial breakdown of sugars to organic acids (Quintero, 2014). These

findings were in agreement with those of Quintero (2014) who reported that early attainment of ^{o}Brix of 8.0 to 9.0 during fermenting is necessary to produce the desired level of sweetness in the coffee beverage.

Table 4.6: Changes in Total soluble solids (°Brix) of the fermenting mass of Arabica coffee after inoculation with different single and mixed microbial isolates

Starter	Fermentation time (h)						
	0	12	24	36	48	60	72
Control	3.22+0.10^{b}	3.80+0.09^{a}	4.40+0.12^{b}	4.80+0.10^{bc}	6.60+0.08^{a}	8.00+0.10^{b}	8.50+0.07^{abc}
Y_1	3.05+0.07^{b}	3.65+0.28^{a}	4.00+0.00^{b}	3.90+0.00^{c}	3.95+0.00^{c}	4.18+0.11^{c}	4.63+0.18^{d}
Y_2	3.15+0.02^{b}	4.39+0.44^{a}	5.16+0.27^{ab}	4.45+0.11^{c}	4.29+0.05^{bc}	4.49+0.05^{c}	4.61+0.02^{d}
Y_1Y_2	3.25+0.07^{b}	4.20+0.14^{a}	4.88+0.25^{ab}	5.08+0.18^{bc}	6.45+0.07^{a}	7.05+0.07^{b}	7.78+0.18^{bc}
B_1	3.03+0.04^{b}	3.80+0.07^{a}	3.95+0.07^{b}	4.00+0.28^{c}	4.00+0.14^{c}	4.25+0.28^{c}	4.70+0.49^{d}
B_2	3.08+0.11^{b}	3.95+0.21^{a}	4.00+0.00^{b}	3.80+0.14^{c}	4.85+0.49^{b}	5.15+0.28^{c}	5.50+028^{d}
B_1B_2	3.10+0.11^{b}	3.85+0.21^{a}	4.00+0.00^{b}	3.80+0.14^{c}	4.85+0.49^{b}	5.15+0.28^{c}	5.50+028^{d}
Y_1B_1	3.05+0.07^{b}	3.65+0.28^{a}	4.00+0.00^{b}	3.90+0.00^{c}	3.95+0.00^{c}	4.18+0.11^{c}	4.63+0.18^{d}
Y_1B_2	3.13+0.11^{b}	3.95+0.21^{a}	4.00+0.00^{b}	3.80+0.14^{c}	4.85+0.49^{b}	5.15+0.28^{c}	5.50+028^{d}
Y_2B_1	3.15+0.14^{b}	3.75+0.49^{a}	4.15+0.21^{b}	4.10+0.21^{c}	7.00+0.07^{a}	7.43+0.11^{b}	7.98+0.18^{abc}
Y_2B_2	3.10+0.14^{b}	3.60+0.57^{a}	4.45+1.20^{b}	3.65+0.35^{c}	7.03+0.04^{a}	7.42+0.09^{b}	7.71+0.21^{c}
$Y_1B_1B_2$	3.23+0.04^{b}	4.53+0.32^{a}	7.08+0.18^{a}	6.57+0.68^{ab}	7.18+0.04^{a}	8.08+0.53^{b}	8.83+0.11^{ab}
$Y_1Y_2B_1B_2$	3.10+0.14^{b}	3.90+0.11^{a}	7.30+0.14^{a}	7.20+0.00^{a}	7.20+0.39^{a}	9.40+0.67^{a}	8.92+0.15^{a}

n=91. Values are means of three independent replicates. Values with different superscript letters in the same column are significantly different ($p<0.05$). Y_1 : *K. exigua* ; Y_2 : *P. kudriavzevii* ; B_1 : *L. mesenteroides ;* B_2 : *P. campinasensi* ; Control : un-inoculated.

4.2.3 Changes in Viscosity

Viscosity of the fermenting mass ranged from 18.00 cP to 25.20 cP (Table 4.7). Generally, there was no significant difference ($p>0.05$) between the viscosity of the treatments inoculated with the different starter cultures from 0 h to 36 h (Table 4.7). After 48 h of fermentation, viscosity was highest for the non-inoculated experiment (control; Table 4.7). At 48 h the viscosity of the control was not significantly ($p>0.05$) different from that of *K. exigua* (23.88 cP) as well as *P. kudriavzevii* (23.98 cP), *P. campinasensi* (23.80 cP), *L. mesenteroides* and *P. campinasensi* (23.20 cP), *K. exigua* and *L. mesenteroides* (23.80 cP), and *P. kudriavzevii* and *L. mesenteroides* (23.38 cP) at 72 h. Lower viscosity was observed when the starter *K. exigua*, *L. mesenteroides* and *P. campinasensi* was used, from 48 h to 72 h. These findings were in agreement with the findings of Avallone *et al.* (2002) who reported that *L. mesenteroides* and *Kazachstania* sp. do not produce endopectolytic enzymes which catalyse the breakdown of pectic substances leading to enhanced viscosity in the fermenting mass.

Table 4.7: Changes in viscosity (cP) of the fermenting mass of Arabica coffee inoculated with different single and mixed microbial isolates

Starter	Fermentation (h)						
	0	12	24	36	48	60	72
Control	18.00±0.03^{a}	18.20±0.05^{a}	22.50±0.06^{a}	23.50±0.04^{a}	24.30±0.07^{a}	25.20±0.10^{a}	24.30±0.08^{a}
Y_1	18.23±0.25^{a}	19.43±0.39^{a}	21.45±0.35^{ab}	22.00±1.84^{b}	21.83±0.60^{ab}	21.08±0.25^{d}	20.48+0.18^{abc}
Y_2	18.17±0.28^{a}	19.25±0.78^{a}	21.53±0.32^{ab}	24.48±0.04^{a}	23.88±0.88^{a}	24.83±0.25^{b}	23.98±0.18^{a}
Y_1Y_2	18.15±0.21^{a}	19.28±1.31^{a}	19.98±1.45^{ab}	21.65±1.48^{b}	19.13±0.04^{b}	18.53±0.04^{d}	18.03±0.04^{c}
B_1	18.13±0.14^{a}	19.10±0.18^{a}	20.59+1.47^{ab}	23.33+0.92^{a}	21.99±0.05^{ab}	21.44±0.27^{e}	20.88+0.11^{abc}
B_2	18.07±0.07^{a}	19.50±0.71^{a}	20.08±0.74^{ab}	23.03±1.66^{a}	22.63±0.88^{ab}	23.60±0.64^{c}	23.80±0.49^{a}
B_1B_2	18.17±0.08^{a}	19.40±0.72^{a}	20.02±0.74^{ab}	23.01±1.66^{a}	22.63±0.88^{ab}	23.60±0.54^{c}	23.20±0.49^{b}
Y_1B_1	18.07±0.07^{a}	19.50±0.71^{a}	20.08±0.74^{ab}	23.03±1.66^{a}	22.63±0.88^{ab}	23.60±0.64^{c}	23.80±0.49^{a}
Y_1B_2	18.10±0.14^{a}	19.10±0.12^{a}	20.69+1.47^{ab}	23.13+0.92^{a}	21.90±0.05^{ab}	21.24±0.27^{e}	20.78+0.10^{abc}
Y_2B_1	18.31±0.14^{a}	18.45±0.49^{a}	19.38±0.04^{ab}	20.73±0.18^{c}	21.80±0.18^{ab}	22.53±0.67^{d}	23.38±0.11^{b}
Y_2B_2	18.05±0.35^{a}	18.18±0.25^{a}	18.60±0.14^{b}	21.10±1.05^{c}	21.10±0.14^{ab}	21.38±0.74^{e}	21.93±1.66^{ab}
$Y_1B_1B_2$	18.13±0.1^{a}	18.85±0.21^{a}	19.58±0.39^{ab}	22.05±0.64^{b}	19.13±1.65^{b}	18.43±1.16^{g}	17.90±1.55^{c}
$Y_1B_1B_2Y_2$	18.18±0.2^{a}	19.25±0.28^{a}	20.25±0.35^{ab}	22.98±0.74^{b}	20.63±1.24^{ab}	19.45±0.57^{f}	18.78±0.39^{bc}

n=91. Values are means of three independent replicates. Values with different superscript letters in the same column are significantly different ($p<0.05$). Y_1: *K. exigua*; Y_2: *P. kudriavzevii*; B_1: *L. mesenteroides;* B_2: *P. campinasensi*; Control: non-inoculated.

4.2.4 Changes in hydrogen ion concentration (pH)

The pH of the coffee fermenting mass decreased from 6.48 to 3.85 (Table 4.8). Generally, there was no significant difference ($p>0.05$) in the pH of the fermenting mass due to inoculation of the different starter cultures from 0 h to 24 h while their effect on pH remained almost constant between 36 h and 60 h. The highest potential to reduce pH of the fermenting mass was observed for treatments with *L. mesenteroides*, *L. mesenteroides*, and *P. campinasensi*, and *K. exigua* and *L. mesenteroides* (3.85, 3.85, 3.85, respectively) at 36 h of fermentation which was significantly different ($p<0.05$) from the rest of the starters (Table 4.8). The results of this study were in agreement with the findings of Quintero (2014), who reported that during the natural fermentation of Arabica coffee, the pH decreased in the range of 3.9 to 4.2 after 18 to 30 h. The observed decrease in pH over the different fermentation times by individual starter cultures could be explained by differences in microbial degradation of mucilage into simpler sugars, which is later hydrolysed by microbial enzymes yielding acid components in the fermenting coffee mass as well as acidification due to lactic acid bacteria (Kulandaivelu, 2013; Evangelista *et al.*, 2014).

Table 4.8: Changes in hydrogen ion concentration (pH) of the fermenting mass of Arabica coffee after inoculation with different single and mixed microbial isolates

Starter	Fermentation time (h)						
	0	12	24	36	48	60	72
Control	6.20+0.00^{a}	4.70+0.00^{a}	4.30+0.00^{a}	4.30+0.00^{a}	4.20+0.00^{ab}	4.00+0.00^{ab}	4.00+0.00^{b}
Y_1	6.38+0.18^{a}	5.08+0.18^{a}	4.18+0.04^{a}	3.98+0.11^{ab}	3.975+0.11^{ab}	3.93+0.04^{ab}	4.13+0.11^{ab}
Y_2	6.40+0.11^{a}	5.51+0.23^{a}	4.56+0.34^{a}	4.28+0.14^{a}	4.28+0.14^{a}	4.21+0.09^{a}	4.34+0.05^{a}
Y_1Y_2	6.30+0.00^{a}	4.85+0.07^{a}	4.15+0.07^{a}	3.90+0.00^{ab}	3.90+0.00^{b}	3.90+0.00^{b}	4.10+0.07^{ab}
B_1	6.33+0.04^{a}	4.95+0.07^{a}	4.08+0.04^{a}	3.85+0.00^{b}	3.85+0.00^{b}	3.85+0.00^{b}	4.15+0.00^{ab}
B_2	6.16+0.09^{a}	5.19+0.09^{a}	4.43+0.32^{a}	4.18+0.18^{ab}	4.18+0.18^{ab}	4.14+0.16^{ab}	4.21+0.05^{ab}
B_1B_2	6.43+0.04^{a}	5.25+0.07^{a}	4.08+0.04^{a}	3.85+0.00^{b}	3.85+0.02^{b}	3.85+0.03^{b}	4.18+0.00^{ab}
Y_1B_1	6.43+0.04^{a}	4.91+0.07^{a}	4.18+0.04^{a}	3.85+0.00^{b}	3.95+0.00^{b}	3.81+0.00^{b}	4.25+0.00^{ab}
Y_1B_2	6.48+0.08^{a}	5.08+0.28^{a}	4.58+0.04^{a}	3.98+0.11^{ab}	4.06+0.11^{ab}	3.98+0.04^{ab}	4.14+0.11^{ab}
Y_2B_1	6.30+0.00^{a}	5.05+0.07^{a}	4.20+0.00^{a}	4.00+0.00^{ab}	4.00+0.00^{ab}	3.95+0.07^{ab}	4.15+0.07^{ab}
Y_2B_2	6.30+0.00^{a}	4.90+0.07^{a}	4.20+0.07^{a}	4.05+0.11^{ab}	4.05+0.11^{ab}	4.05+0.11^{ab}	4.20+0.04^{ab}
$Y_1B_1B_2$	6.30+0.00^{a}	5.08+0.32^{a}	4.30+0.28^{a}	3.90+0.00^{ab}	3.90+0.00^{b}	3.90+0.00^{b}	4.15+0.07^{ab}
$Y_1B_1B_2Y_2$	6.20+0.14^{a}	4.95+0.07^{a}	4.10+0.00^{a}	3.90+0.00^{ab}	3.90+0.00^{b}	3.90+0.07^{b}	4.08+0.04^{b}

n=91. Values are means of three independent replicates. Values with different superscript letters in the same column are significantly different ($p<0.05$). Y_1: *K. exigua*; Y_2: *P. kudriavzevii*; B_1: *L. mesenteroides*; B_2: *P. campinasensi* ; Control: non-inoculated.

4.2.5 Changes in titratable acidity (TA)

Titratable acidity can be used as a measure of the degree of fermentation in wet coffee fermentation (Gopinandhan, 2018). Titratable acidity as lactic acid varied from 1.15 g/L to 8.49 g/L for the different starter cultures in the present study at the varying time intervals (Table 4.9). Starter cultures *K. exigua* and *L. mesenteroides*, *K. exigua*, *L. mesenteroides*, *K. exigua*, *P. kudriavzevii*, *L. mesenteroides* and *P. campinasensi K. exigua* and *P. campinasensi*, *K. exigua* and *L. mesenteroides* and *P. campinasensi*, *L. mesenteroides* and *P. campinasensi* produced the highest TA (3.85 g/L, 3.85 g/L, 8.45 g/L, 3.35 g/L, 3.32 g/L) at 24 h of fermentation which was not significantly different ($p>0.05$) from the TA of the rest of the starters. The results of the current study were close to those reported by Gopinandhan (2018) which were in the range of 2.96 g/L to 4.81 g/L for Arabica coffee in India. Increase in titratable acidity is attributed to the microbial degradation of pectin to simple sugars which are latter metabolised by microbial enzymes to yield acids such lactic acid and pyruvic acid in the fermenting coffee mass (Kulandaivelu, 2013). The findings of this study therefore suggest that it is possible to reduce fermentation time from 72 to 24 h to attain the desired acidity within the coffee. However, over fermentation leads to a high acidity which is a poor sensory quality attribute in processed coffee (Lopez *et al.*, 2006; Haile and Kang, 2019).

Table 4.9: Changes in titratable acidity (g/L) of the fermenting mass of Arabica coffee after inoculated with different single and mixed microbial isolates

Starter	Fermentation time (h)						
	0	12	24	36	48	60	72
Control	1.22±0.00^{b}	1.91±0.00^{b}	2.76±0.0^{b}	3.12±0.00^{bc}	4.97±0.00^{b}	6.91±0.00^{ab}	6.91±0.00^{ab}
Y_1	1.30±0.14^{b}	2.49±0.05^{a}	3.85±0.06^{a}	4.75±0.11^{a}	6.31±0.07^{ab}	8.06±0.08^{ab}	8.06±0.08^{ab}
Y_2	1.30±0.00^{b}	1.12±0.01^{cd}	1.30±0.31^{c}	2.31+0.05^{cd}	3.02±0.24^{c}	3.99+0.24^{c}	3.99±0.24^{c}
Y_1Y_2	1.27±0.02^{b}	1.06±0.01^{d}	1.74±0.01^{c}	2.14±0.08^{d}	2.79±0.09^{c}	4.34±0.08^{c}	4.34±0.08^{c}
B_1	1.15±0.00^{b}	1.94±0.07^{b}	3.35±0.01^{a}	4.62±0.08^{a}	6.66±0.09^{a}	8.48+0.08^{a}	8.48±0.08^{a}
B_2	1.26±0.05^{b}	1.92±0.05^{b}	2.73±0.02^{b}	4.97±0.00^{a}	6.81±0.05^{a}	8.42±0.10^{a}	8.42±0.10^{a}
B_1B_2	1.26±0.05^{b}	1.90±0.05^{b}	2.83±0.02^{b}	4.96±0.00^{a}	6.81±0.05^{a}	8.42±0.10^{a}	8.32±0.10^{a}
Y_1B_1	1.30±0.16^{b}	2.49±0.05^{a}	3.85±0.07^{a}	4.85±0.11^{a}	6.61±0.07^{ab}	8.41±0.08^{a}	8.46±0.08^{a}
Y_1B_2	1.27±0.05^{b}	1.91±0.05^{b}	2.78±0.02^{b}	4.97±0.00^{a}	6.87±0.05^{a}	8.45±0.10^{a}	8.46±0.10^{a}
Y_2B_1	1.30±0.00^{b}	1.76±0.05^{b}	1.30±0.11^{c}	3.71±0.05^{b}	5.07±0.04^{b}	6.44±0.16^{b}	6.44±0.16^{b}
Y_2B_2	1.27±0.04^{b}	1.84±0.05^{b}	1.32±0.08^{c}	3.90±0.09^{b}	5.59±0.05^{ab}	7.31±0.10^{ab}	7.31±0.10^{ab}
$Y_1B_1B_2$	1.22±0.05^{b}	1.66±0.02^{bc}	2.74±0.16^{b}	4.90±0.10^{a}	6.84±0.09a	8.28±0.08^{a}	8.28±0.08^{a}
$Y_1B_1B_2Y_2$	1.25±0.00^{b}	1.94±0.10^{b}	3.32±0.07^{a}	4.57±0.08^{a}	6.38+0.09^{a}	8.49±0.12^{a}	8.49±0.12^{a}

n=91. Values are means of three independent replicates. Values with different superscript letters in the same column are significantly different ($p<0.05$); Y_1: *K. exigua*; Y_2: *P. kudriavzevii*; B_1: *L. mesenteroides;* B_2: *P. campinasensi*; Control: non-inoculated.

4.3 Cup quality evaluation

There were no significant differences ($p>0.05$) between the different treatments for all the cup quality components assessed during the study, except for aroma and cup score. The quality of coffee beverages was determined by sensory perception. The findings of this study showed that the coffee beverage cup quality ranged from 80.211 points and 84.034 points, implying a coffee of high quality. Generally, the inoculated fermentations produced beverages with higher sensory scores compared to the un-inoculated control (Table 4.8). On the other hand, beverages prepared from fermentations of mixed starters were scored higher, in the range of 81.475 points to 84.034 points compared to those of pure cultures which ranged from 81.356 to 82.158 points. This therefore indicates a greater potential of utilizing combined starter cultures which has also been reported to aid in flavour and aroma development thereby enhancing the cup quality of fermented coffee beverages (Kulandaivelu, 2013). There was a significant difference ($p<0.05$) between the different cup scores with a starter culture combination of *K. exigua*, *P. kudriavzevii*, *L. mesenteroides* and *P. campinasensi* producing the highest mean cup score (84.034 points; Appendix IV) which was significantly higher than the one produced by the control (80.211 points). The decrease in flavour rating could be attributed to increase in the fungal population in the fermenting mass (Kulandaivelu, 2013). This is probably due to microbial associations and the slow degradation of the mucilage. The findings of this study were in agreement with a study by Pereira *et al*. (2015), who obtained good quality cup characteristics in fermented Arabica coffee within the same ranges as obtained in the present study. The improvement in the cup quality of coffee during fermentation could be due to microbial degradation of the different polysaccharides such as pectin, cellulose, and starch within the coffee mucilage producing metabolites including alcohols, acids, esters and ketones within the coffee bean hence enhancing the perceived sensory quality in fermented coffee beverages (Kulandaivelu, 2013; Puerta, 2015; Lee *et al*., 2015; Kang, 2019).

Table 4.10: Scores of cup quality components (in points) of coffee brew obtained from inoculated fermentation of Arabica coffee beans with different single and mixed microbial isolates

Starter	Aroma	Flavor	Acidity	Body	Uniformity	Clean cups	After taste	Balance	Sweetness	Overall	Cup score
Control	7.499^{ab}	7.361^{a}	7.179^{a}	7.462^{a}	9.335^{a}	9.485^{a}	7.432^{a}	7.300^{a}	9.779^{a}	7.378^{a}	80.211^{b}
Y_1	7.441^{b}	7.445^{a}	7.356^{a}	7.360^{a}	9.905^{a}	9.933^{a}	7.398^{a}	7.438^{a}	10.000^{a}	7.408^{a}	81.684^{ab}
Y_2	7.669^{ab}	7.554^{a}	7.427^{a}	7.463^{a}	9.533^{a}	9.267^{a}	7.477^{a}	7.450^{a}	10.000^{a}	7.517^{a}	81.356^{b}
Y_1Y_2	7.608^{ab}	7.641^{a}	7.576^{a}	7.613^{a}	9.457^{a}	9.439^{a}	7.574^{a}	7.683^{a}	9.731^{a}	7.558^{a}	81.879^{ab}
B_1	7.724^{ab}	7.644^{a}	7.343^{a}	7.602^{a}	9.651^{a}	9.268^{a}	7.467^{a}	7.523^{a}	9.986^{a}	7.584^{a}	81.792^{ab}
B_2	7.440^{b}	7.593^{a}	7.640^{a}	7.441^{a}	9.572^{a}	9.466^{a}	7.705^{a}	7.678^{a}	9.962^{a}	7.663^{a}	82.158^{ab}
B_1B_2	7.430^{b}	7.693^{a}	7.630^{a}	7.442^{a}	9.576^{a}	9.486^{a}	7.715^{a}	7.478^{a}	9.862^{a}	7.163^{a}	81.475^{ab}
Y_1B_1	7.241^{b}	7.645^{a}	7.336^{a}	7.370^{a}	9.925^{a}	9.933^{a}	7.388^{a}	7.458^{a}	10.000^{a}	7.518^{a}	81.814^{ab}
Y_1B_2	7.661^{ab}	7.551^{a}	7.423^{a}	7.460^{a}	9.543^{a}	9.267^{a}	7.477^{a}	7.450^{a}	10.000^{a}	7.517^{a}	81.356^{b}
Y_2B_1	7.620^{ab}	7.571^{a}	7.654^{a}	7.529^{a}	9.800^{a}	9.470^{a}	7.525^{a}	7.429^{a}	9.600^{a}	7.423^{a}	81.621^{ab}
Y_2B_2	7.711^{ab}	7.648^{a}	7.429^{a}	7.567^{a}	9.724^{a}	9.845^{a}	7.494^{a}	7.499^{a}	9.983^{a}	8.007^{a}	82.905^{ab}
$Y_1B_1B_2$	7.635^{ab}	7.618^{a}	7.352^{a}	7.382^{a}	10.122^{a}	10.219^{a}	7.587^{a}	7.345^{a}	10.000^{a}	7.456^{a}	82.766^{ab}
$Y_1Y_2B_1B_2$	7.888^{a}	7.751^{a}	7.504^{a}	7.646^{a}	10.000^{a}	10.000^{a}	7.847^{a}	7.770^{a}	10.000^{a}	7.629^{a}	84.034^{a}

n=130. Values are means of three independent replicates. Values with different superscript letters in the same column are significantly different ($p<0.05$). Y_1: *K. exigua*; Y_2: *P. kudriavzevii*; B_1: *L. mesenteroides;* B_2: *P. campinasensi*; Control: un-inoculated.

4.3.1 Interaction between fermentation time/starter culture and cup score

From the 2-way ANOVA, the effect of the interactions of fermentation time and starter culture on the cup quality was established for optimum and efficient processing conditions. The best scores for aroma were achieved with *L. mesenteroides* at 48 h and the combination of *K. exigua, P. kudriavzevii, L. mesenteroides* and *P. campinasensi* at 72 h (Appendix V), both of which were significantly different ($p<0.05$) from the rest of the interactions. The highest score of acidity was achieved from the interaction of *P. campinasensi* and 24 h fermentation time which was significantly different ($p<0.05$) from the rest of the interactions. Body was highest with the time and starter culture interaction of *K. exigua* and *P. kudriavzevii* at 24 h, *P. campinasensi*, 36 h and *K. exigua, P. kudriavzevii, L. mesenteroides* and *P. campinasensi* at 60 h which were not significantly different ($p>0.05$) from the rest of the interactions (Appendix V). For the cup quality component of uniformity, there were no significant differences ($p>0.05$) between all the interactions.

Interactions of starter culture *K. exigua, P. kudriavzevii, L. mesenteroides* and *P. campinasensi* with fermentation time of 48 h and 72 h and starter culture *P. campinasensi* with 36 h and 48 h produced the best scores for aftertaste. The best balance in the cup was achieved with interactions of *K. exigua, P. kudriavzevii, L. mesenteroides* and *P. campinasensi* with 72 h, *K. exigua, L. mesenteroides* and *P. campinasensi* with 48 h and the non-inoculated sample (control) with 24 h.

Cup score was optimised with *P. campinasensi* and a combination of *K. exigua, P. kudriavzevii, L. mesenteroides* and *P. campinasensi* for fermentation at 36 h and 72 h (Appendix V). When single starter *P. campinasensi* interacted with 36 h, the highest potential to improve cup quality (85.400 points) was observed, followed by the interaction of mixed starter *K. exigua, P.*

kudriavzevii, *L. mesenteroides* and *P. campinasensi* at 72 h (84.750 points). These scores were significantly different ($p<0.05$) from those of the other interactions.

4.4 Principal Component Analysis (PCA)

4.4.1 Correlation between cup quality and physicochemical characteristics of the fermented coffees using different starter cultures

A biplot of the physicochemical characteristics of the different starter cultures and their respective cup quality scores over a 72-h fermentation period was plotted (Figure 4.1).

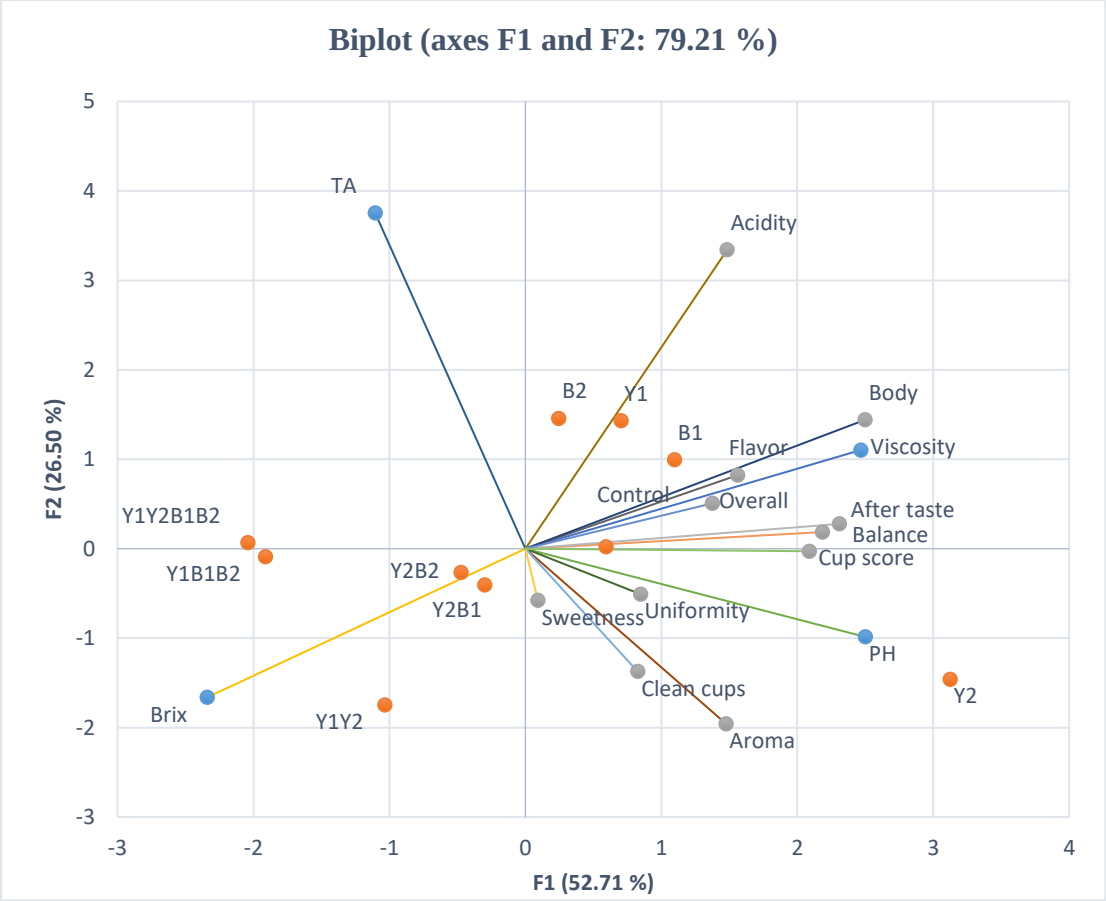

Figure 4.1: Principal component analysis of the cup quality and physicochemical characteristics of the fermenting mass using different starter cultures over a 72-h period.

Principal component (F1 52.71%) contrasted single starter cultures (*L. mesenteroides*, *P. campinasensi*, *P. kudriavzevii* and *K. exigua*) that were correlated with high pH and viscosity

of the fermenting mass which was associated with aroma, flavour, acidity, body, balance, uniformity, sweetness, clean cup, after taste, overall acceptability and cup score, with mixed starter cultures (*K. exigua*, *P. kudriavzevii*, *L. mesenteroides* and *P. campinasensi*, *K. exigua*, *L. mesenteroides* and *P. campinasensi*, *P. kudriavzevii* and *P. campinasensi*, *P. kudriavzevii* and *L. mesenteroides*, *K. exigua* and *P. kudriavzevii*) that were correlated with high ^{o}Brix of the fermenting mass.

The second component (F1 26.50 %) contrasted both mixed and single starter cultures (*K. exigua*, *L. mesenteroides* and *P. campinasensi*, *P. kudriavzevii* and *P. campinasensi*, *P. kudriavzevii* and *L. mesenteroides*, *K. exigua* and *P. kudriavzevii*, and *P. kudriavzevii*) which were correlated with high pH and ^{o}Brix of the fermenting mas, that were associated with sweetness, clean cup, aroma, uniformity and cup score with single starters (*L. mesenteroides*, *P. campinasensi*, *K. exigua*) that were correlated with high TA and viscosity which was associated with acidity, flavour, body overall, aftertaste and balance.

According to PCA, the pH of the coffee beverage determined the perceived sweetness, clean cup, cup uniformity, aroma, flavour, body, aftertaste, balance and cup score ($r = 0.340$, $r = 0.481$, $r = 0.437$, $r = 0.675$, $r = 0.694$, $r = 0.747$, $r = 0.759$, $r = 0.768$ and $r = 0.825$, respectively) which was associated with starter *P. kudriavzevii* (Appendix VI; Fig 4.1). The perceived body, balance and after taste of the coffee beverage is highly determined by the viscosity of the fermented coffee due to microbial activity of the single starter *L. mesenteroides*.

Starter culture *K. exigua*, *P. kudriavzevii*, *L. mesenteroides* and *P. campinasensi* was associated with titratable acidity (TA) and yet TA had negative correlations with the cup quality except for ^{o}Brix, flavour, acidity, body and sweetness which had weak positive correlations (0.019,

0.178, 0.519, 0.076 and 0.046, respectively) (Fig 4.1; Appendix VI). Combined starter cultures *K. exigua*, *P. kudriavzevii* and *L. mesenteroides, P. kudriavzevii* and *L. mesenteroides, P. kudriavzevii* and *P. campinasensi*, and *K. exigua* and *P. kudriavzevii* were highly associated with ^{o}Brix (Fig. 4.1). According to the PCA, ^{o}Brix had strong negative correlations with acidity, body and balance (r = -0.638, r = -0.680 and -0.638; appendix VI). Single starter cultures *L. mesenteroides, P. campinasensi, K. exigua* produced the highest viscosity in the fermentation process (Fig 4.1). PCA also showed that viscosity had a strongly positive correlation with the body, overall acceptability and cup score (r = 0.768, r = 0.697 and r = 0.677, respectively).

The PCA further elaborated that pH was highly associated with the perceived aroma, flavor, body, aftertaste, balance and cup score (r = 0.675, r = 0.694, r = 0.747, r = 0.759, r = 0.768 and r = 0.825 respectively) while acidity was negatively correlated with ^{o}Brix which could be due to the interference of the sugar acid balance (Silva *et al.*, 2014), but had a strong correlation with the body and viscosity (r = -0.698, r = 0.674 and r = 0.768). PCA further indicated that the uniformity of a coffee cup was associated with a strong positive correlation with a clean cup and cup score (r = 0.902 and r = 0.728, respectively), while overall acceptability was explained by a high viscosity, low ^{o}Brix and low TA (r = 0.697, r = -0.099 and -0.007). Cup score was explained by pH, flavor, uniformity, body, viscosity, clean cup, after taste and aroma (r = 0.825, r = 0.825, r = 0.728, r = 0.719, r = 0.677, r = 0.676, r = 0.675 and r = 0.637) but negatively correlated with ^{o}Brix and TA (r = -0.240 and r = -0.097, respectively). Overall, a very strong positive correlation (0.825) was observed between pH of the fermenting mass and the cup quality score. The pH also had a strong positive correlation with *P. kudriavzevii.*

CHAPTER FIVE: CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The potential use of starter cultures for coffee fermentation was studied. The prospect of applying starter cultures in coffee fermentation was to reduce fermentation time, improve process control and sensory quality of the final product. From the results of the study, it can be concluded that *Kazachstania exigua, Pichia kudriavzevii, Leuconostoc mesenteroides* and *Paenibacillus campinasensi* are the predominant spp. during the spontaneous wet processing of Arabica coffee in Bugisu sub region. Inoculation of fresh parchment Arabica coffee beans with *K. exigua, P. kudriavzevii, L. mesenteroides* and *P. campinasensi* in combination at a concentration of 12.2 log CFU/ml at a temperature of 18^{o}C to 26^{o}C and relative humidity 60% to 75% reduces coffee wet fermentation time by 30%. Coffee beverages prepared from wet processed coffee by use of mixed cultures are of better cup quality than those from single cultures. The mixed isolates *K. exigua, P. kudriavzevii, L. mesenteroides* and *P. campinasensi* were the best in improving the cup quality. *P. kudriavzevii* was very strongly associated with pH of the ferment, which determines beverage sweetness, clean cup, uniformity, aroma, flavour, body, aftertaste, balance and cup score. The use of starter cultures in coffee wet fermentation can transform an inconsistent process into an economically valuable proposition.

5.2 Recommendations

It is recommended that:

1. Coffee processors use starters in coffee fermentation since they reduce processing time moreover the end product quality is improved.
2. Better fermentation of Arabica coffee is possible with starter combinations of *K. exigua, P. kudriavzevii, L. mesenteroides* and *P. campinasensi*.
3. This will increase the country's production output of specialty coffees thereby enhancing the branding and penetration of Uganda coffee on the world market.
4. Further study studies are recommended in respect of:
    a. Scaling up this study to include the application of more recent molecular methods such as omics technologies, which have never been used in coffee studies. This will open up new horizons in the industrial production of high quality coffee.
    b. Other studies focusing on cup quality using electronic nose and electronic tongue to compliment sensory evaluation including detection of aroma volatiles produced by the different organisms, as single and mixed cultures, are also suggested.

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Appendix I: PCR analysis of genomic DNA from different bacteria and yeasts in spontaneous coffee fermentation using gene specific primers.

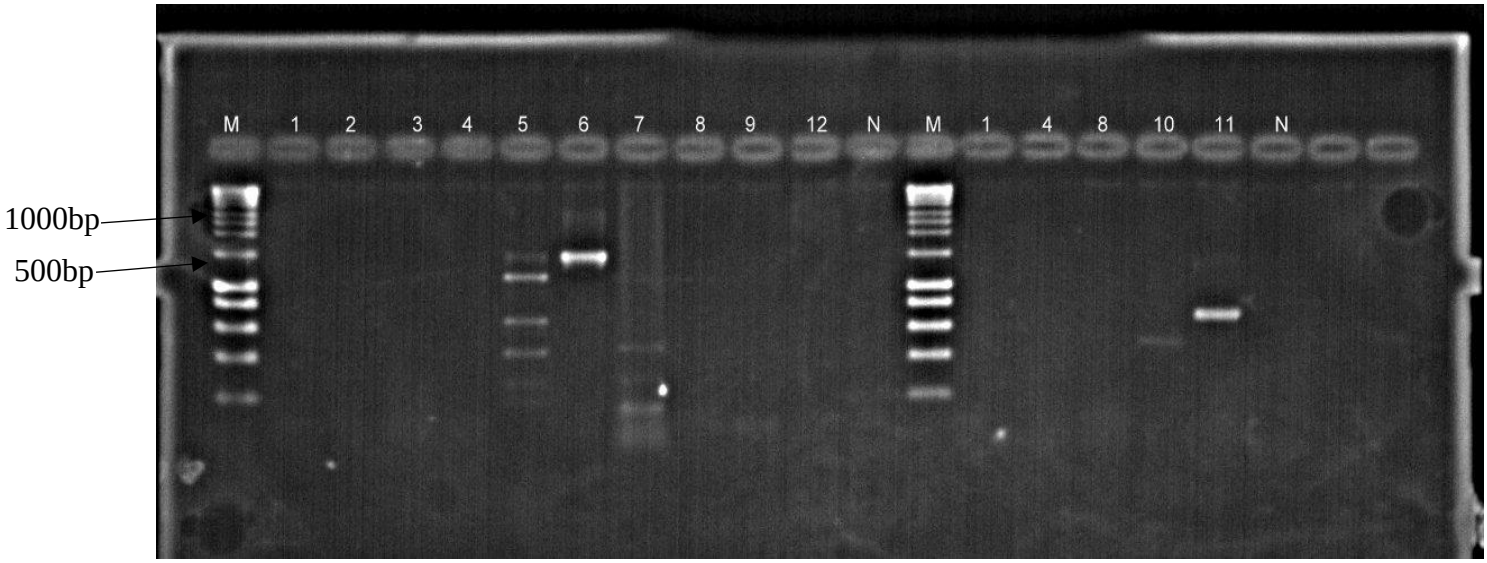
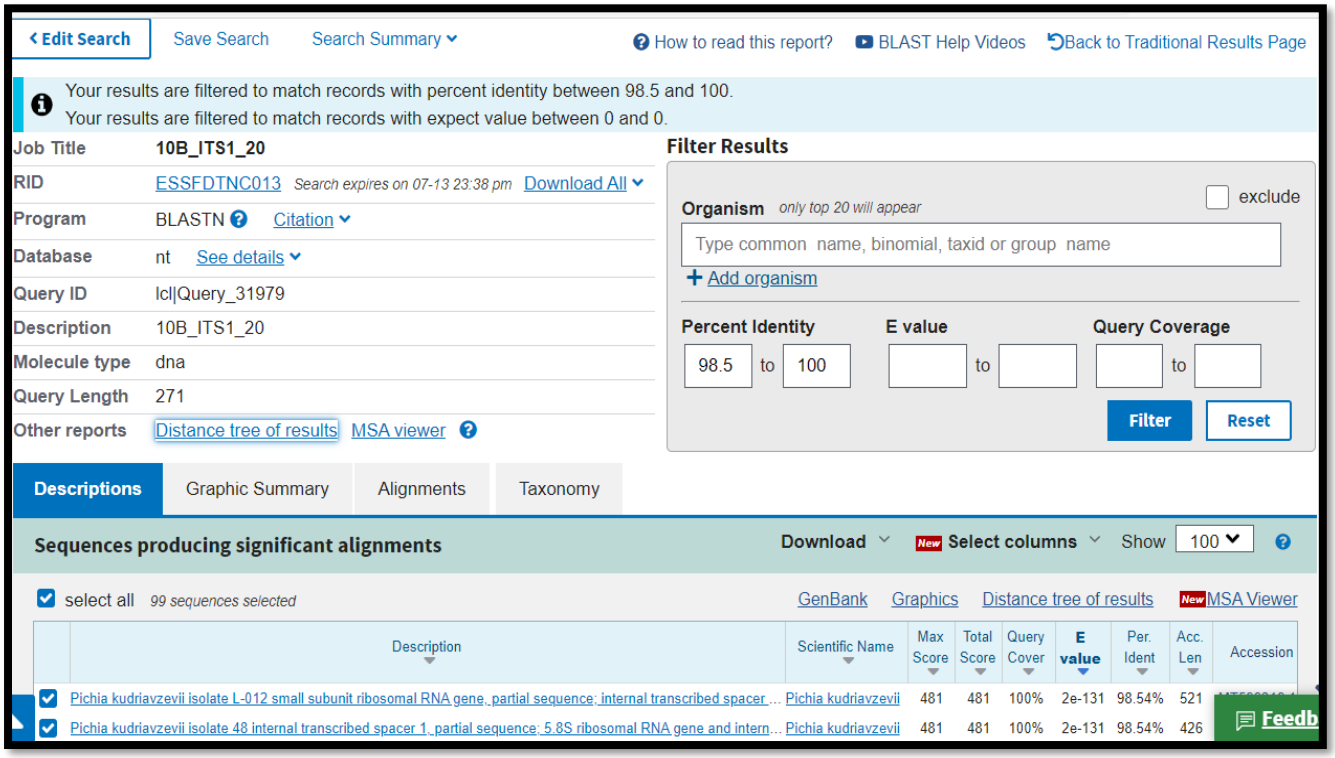
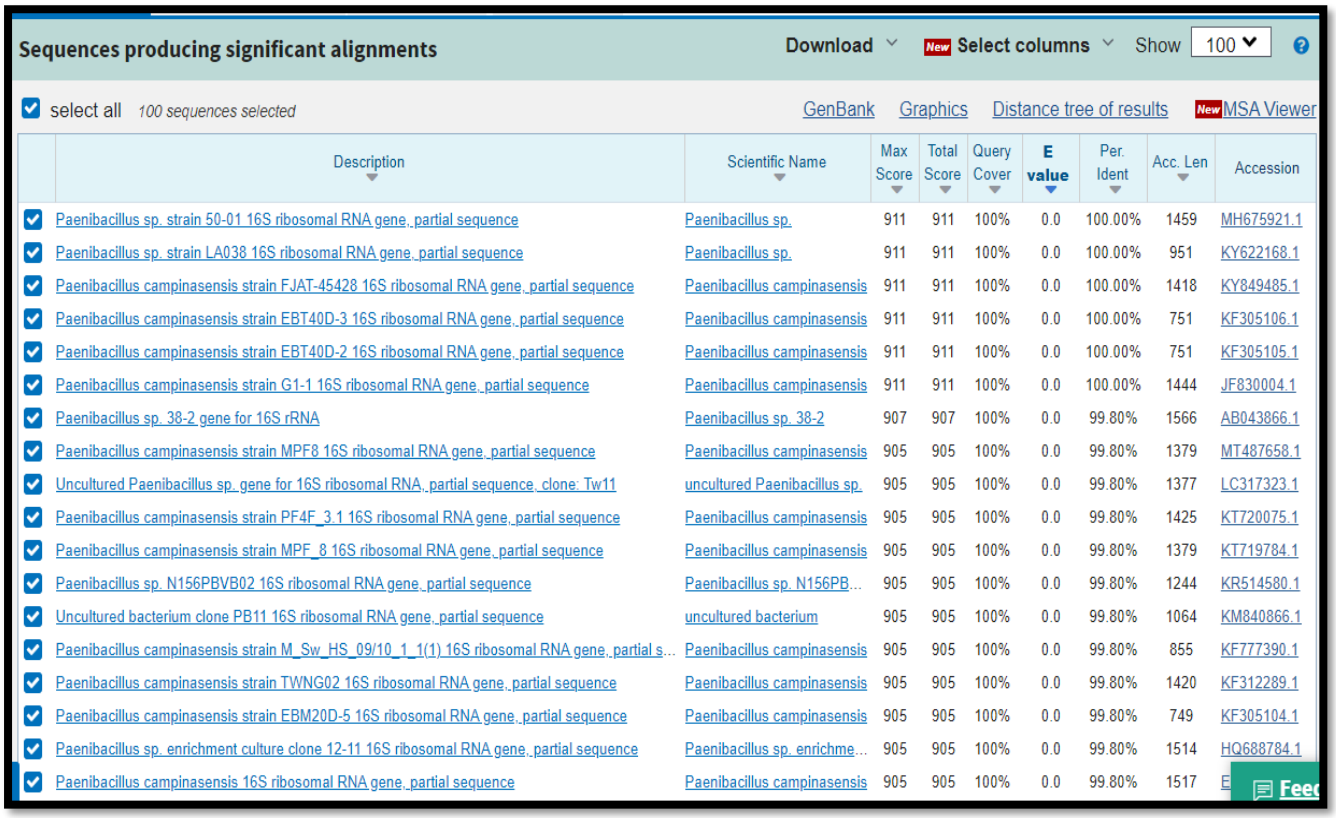


Fig. Agorose gel showing amplified 16S rRNA gene and 5.8S ITS gene of bacteria (5and6) and yeasts (10and11), respectively. M =1kb plus ladder (Thermo scientific), N = no template negative control.

Fig. Agorose gel showing amplified 16S rRNA gene and 5.8S ITS gene of bacteria (5) and yeasts (10and11), respectively. M =1kb Plus ladder (Thermo Scientific), N=no template negative control.

Appendix II: Representative BLAST images obtained for *Pichia Kudriavzevii and Penobacillus campinasensis.*





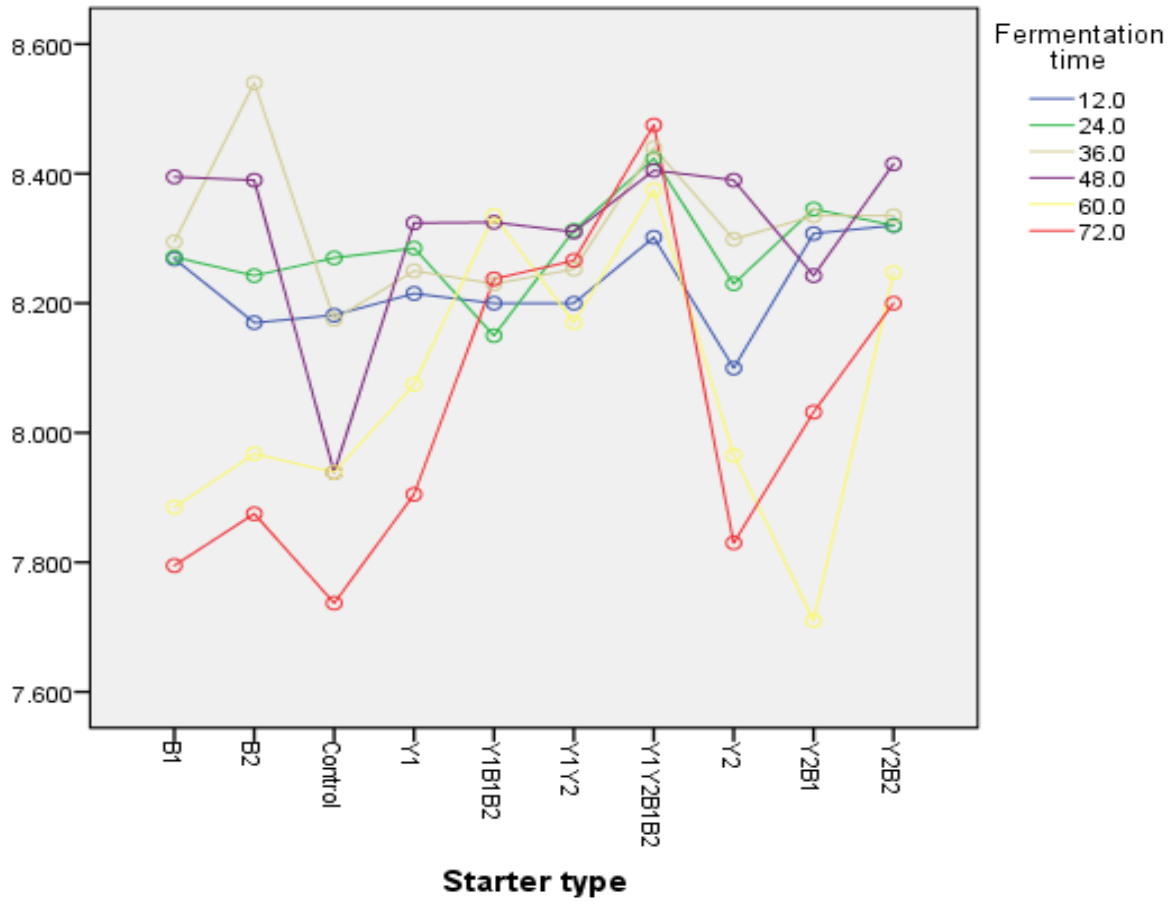
Appendix III: Regression of dependent variables for prediction of the fermentation time

Variable	Class	Model ($y = b_\circ + b_1 * t$)			Adjusted R^2 value	Significance (P-value)	Time (h)
		y	$b_\circ$	b_1			
Pectin (%)	Control	0.00	0.56	-0.01	0.88	0.04	40
	Y_1	0.00	0.39	-0.01	0.62	0.01	30
	Y_2	0.00	0.45	-0.02	0.69	0.01	30
	Y_1Y_2	0.00	0.41	-0.01	0.64	0.02	29
	B_1	0.00	0.51	-0.01	0.75	0.00	36
	B_2	0.00	0.43	-0.01	0.67	0.01	31
	B_1B_2	0.00	0.43	-0.01	0.67	0.01	31
	Y_1B_1	0.00	0.41	-0.01	0.62	0.01	32
	Y_1B_2	0.00	0.47	-0.01	0.67	0.01	34
	Y_2B_1	0.00	0.35	-0.01	0.58	0.02	29
	Y_2B_2	0.00	0.24	-0.01	0.13	0.20	27
	$Y_1B_1B_2$	0.00	0.46	-0.02	0.67	0.01	31
	$Y_1B_1B_2Y_2$	0.00	0.43	-0.02	0.53	0.02	28
^{o}Brix	Control	8.50	-0.14	0.20	0.91	0.00	43
	Y_1	8.50	0.29	0.14	0.78	0.00	60
	Y_2	8.50	1.20	0.13	0.02	0.29	58
	Y_1Y_2	8.50	0.29	0.18	0.98	0.00	45
	B_1	8.50	0.32	0.14	0.71	0.00	59
	B_2	8.50	0.20	0.15	0.84	0.00	55
	B_1B_2	8.50	0.22	0.19	0.84	0.00	44
	Y_1B_1	8.50	0.29	0.18	0.84	0.00	46
	Y_1B_2	8.50	0.20	0.16	0.84	0.00	52
	Y_2B_1	8.50	-0.28	0.19	0.88	0.00	45
	Y_2B_2	8.50	-0.30	0.19	0.80	0.00	46
	$Y_1B_1B_2$	8.50	0.61	0.20	0.74	0.00	39
	$Y_1Y_2B_1B_2$	8.50	0.21	0.23	0.88	0.00	37
Hydrogen ion concentration (pH)	Control	4.00	0.28	0.07	0.52	0.00	52
	Y_1	4.00	0.29	0.05	0.78	0.00	79
	Y_2	4.00	5.77	-0.03	0.65	0.00	65
	Y_1Y_2	4.00	5.38	-0.03	0.55	0.00	53
	B_1	4.00	5.52	-0.02	0.63	0.00	63
	B_2	4.00	5.40	-0.03	0.53	0.00	52
	B_1B_2	4.71	5.40	-0.03	0.53	0.00	25
	Y_1B_1	4.71	5.76	-0.03	0.65	0.00	39
	Y_1B_2	4.71	5.76	-0.03	0.65	0.00	39
	Y_2B_1	4.00	5.38	-0.03	0.52	0.00	55
	Y_2B_2	4.00	5.47	-0.03	0.58	0.00	57
	$Y_1B_1B_2$	4.00	5.49	-0.06	0.58	0.00	26

	$Y_1B_1B_2Y_2$	4.00	5.36	-0.07	0.56	0.00	21
Titratable acidity (g/L)	Control	3.7	0.84	0.09	0.94	0.00	33
	Y_1	3.7	1.46	0.10	0.95	0.00	23
	Y_2	3.7	0.77	0.05	0.89	0.00	64
	Y_1Y_2	3.7	0.72	0.05	0.88	0.00	60
	B_1	3.7	0.84	0.11	0.97	0.00	25
	B_2	3.7	0.80	0.12	0.96	0.00	25
	B_1B_2	3.7	0.76	0.12	0.96	0.00	26
	Y_1B_1	3.7	0.75	0.12	0.96	0.00	26
	Y_1B_2	3.7	0.80	0.12	0.96	0.00	25
	Y_2B_1	3.7	0.66	0.09	0.85	0.00	36
	Y_2B_2	3.7	0.51	0.10	0.86	0.00	32
	$Y_1B_1B_2$	3.7	0.72	0.12	0.96	0.00	25
	$Y_1B_1B_2Y_2$	3.7	0.86	0.11	0.97	0.00	25

Y_1: *K. exigua*; Y_2: *P. kudriavzevii*; B_1: *L. mesenteroides;* B_2: *P. campinasensi*

Appendix IV: Comparison of coffee beverage mean cup scores against the different starter culture treatments during fermentation.



Y_1 : *K. exigua* : Y_2 : *P. kudriavzevii* ; B_1 : *L. mesenteroides ;* B_2 : *P. campinasensi* ; Control : non-inoculated

Appendix V: Two-way ANOVA for the effect of time and starter culture on cup quality

Time*Starter	Aroma	Acidity	Body	Uniformity	After taste	Balance	Overall	Cup score
$36*B_2$	7.800^{abc}	7.900^{ab}	7.900^{a}	10.000^{a}	7.900^{ab}	7.900^{a}	8.000^{b}	85.400^{a}
$36*Y_1Y_2B_1B_2$	7.900^{abc}	7.850^{abcd}	7.700^{a}	10.000^{a}	7.750^{ab}	7.750^{a}	7.750^{bc}	84.400^{abc}
$48*Y_1Y_2B_1B_2$	7.950^{ab}	$7.650^{abcdefg}$	7.650^{a}	10.000^{a}	7.900^{ab}	7.600^{a}	7.650^{bc}	84.050^{abc}
$48*Y_2$	7.850^{abc}	$7.650^{abcdefg}$	7.750^{a}	10.000^{a}	7.600^{ab}	7.600^{a}	7.650^{bc}	83.900^{abc}
$48*B_1$	8.000^{a}	$7.500^{abcdefghi}$	7.650^{a}	10.000^{a}	7.600^{ab}	8.000^{a}	7.600^{bc}	83.950^{abc}
$24*Y_1Y_2B_1B_2$	7.875^{abc}	$7.375^{cdefghi}$	7.737^{a}	10.000^{a}	7.875^{ab}	7.987^{a}	7.563^{bc}	84.237^{abc}
$48*B_2$	7.200^{c}	7.700^{abcdef}	7.700^{a}	10.000^{a}	7.900^{ab}	7.900^{a}	8.000^{b}	83.900^{abc}
$24*Y_1Y_2$	7.875^{abc}	$7.600^{abcdefg}$	7.950^{a}	9.600^{a}	7.875^{ab}	7.900^{a}	7.475^{bc}	83.125^{abc}
$24*Y_2B_1$	7.875^{abc}	$7.600^{abcdefg}$	7.550^{a}	10.000^{a}	7.575^{ab}	7.575^{a}	7.630^{bc}	83.455^{abc}
$60*Y_1Y_2B_1B_2$	7.850^{abc}	$7.550^{abcdefgh}$	7.800^{a}	10.000^{a}	7.650^{ab}	7.400^{a}	7.550^{bc}	83.750^{abc}
$60*Y_1B_1B_2$	7.650^{abc}	$7.600^{abcdefg}$	7.600^{a}	10.000^{a}	7.550^{ab}	7.550^{a}	7.700^{bc}	83.350^{abc}
$72*Y_1Y_2B_1B_2$	8.000^{a}	7.250^{efghi}	7.500^{a}	10.000^{a}	8.333^{a}	8.333^{a}	7.470^{bc}	84.750^{ab}
$48*Y_1$	7.650^{abc}	7.700^{abcdef}	7.690^{a}	10.000^{a}	7.800^{ab}	7.600^{a}	7.150^{bc}	83.240^{abc}
$48*Y_1Y_2$	7.600^{abc}	7.750^{abcde}	7.600^{a}	9.550^{a}	7.600^{ab}	7.650^{a}	7.700^{bc}	83.100^{abc}
$36*Y_2B_1$	7.700^{abc}	7.700^{abcdef}	7.500^{a}	10.000^{a}	7.500^{ab}	7.600^{a}	7.550^{bc}	83.350^{abc}
$48*Y_1B_1B_2$	7.600^{abc}	$7.500^{abcdefghi}$	7.500^{a}	10.000^{a}	7.500^{ab}	8.100^{a}	7.500^{bc}	83.250^{abc}
$36*Y_1Y_2$	7.775^{abc}	$7.600^{abcdefg}$	7.550^{a}	9.600^{a}	7.475^{ab}	7.700^{a}	7.875^{bc}	82.525^{abc}
$12*Y_1Y_2B_1B_2$	7.750^{abc}	7.350^{defghi}	7.487^{a}	10.000^{a}	7.575^{ab}	7.550^{a}	7.843^{bc}	83.017^{abc}
$36*Y_2$	7.775^{abc}	$7.563^{abcdefgh}$	7.500^{a}	10.000^{a}	7.500^{ab}	7.400^{a}	7.687^{bc}	82.987^{abc}
$72*Y_1Y_2$	7.910^{abc}	7.250^{efghi}	7.800^{a}	9.550^{a}	7.600^{ab}	7.650^{a}	7.470^{bc}	82.660^{abc}
$24*Y_1$	7.400^{abc}	$7.500^{abcdefghi}$	7.600^{a}	10.000^{a}	7.550^{ab}	7.550^{a}	7.600^{bc}	82.850^{abc}
$12*Y_2B_1$	7.595^{abc}	7.875^{abc}	7.725^{a}	10.000^{a}	7.425^{ab}	7.400^{a}	7.380^{bc}	83.075^{abc}
$48*Y_2B_2$	7.696^{abc}	$7.433^{abcdefghi}$	7.554^{a}	9.900^{a}	7.471^{ab}	7.462^{a}	9.950^{a}	83.062^{abc}
$36*B_1$	7.787^{abc}	$7.463^{abcdefghi}$	7.562^{a}	10.000^{a}	7.362^{b}	7.225^{a}	7.737^{bc}	82.948^{abc}
$36*Y_2B_2$	7.696^{abc}	$7.433^{abcdefghi}$	7.554^{a}	9.900^{a}	7.471^{ab}	7.462^{a}	7.650^{bc}	83.062^{abc}
$24*B_2$	7.245^{bc}	7.940^{a}	7.262^{a}	10.000^{a}	7.875^{ab}	7.575^{a}	7.312^{bc}	82.426^{abc}
$24*Y_2B_2$	7.696^{abc}	$7.433^{abcdefghi}$	7.554^{a}	9.900^{a}	7.471^{ab}	7.462^{a}	7.550^{bc}	83.062^{abc}
$12*Y_2B_2$	7.696^{abc}	$7.433^{abcdefghi}$	7.554^{a}	9.900^{a}	7.471^{ab}	7.462^{a}	7.550^{bc}	83.062^{abc}

12*B_1	7.562^{abc}	7.313^{efghi}	7.687^{a}	10.000^{a}	7.500^{ab}	7.312^{a}	7.625^{bc}	82.686^{abc}
36*Y_1	7.650^{abc}	7.450^{abcdefghi}	7.150^{a}	10.000^{a}	7.250^{b}	7.600^{a}	7.750^{bc}	82.500^{abc}
24*B_1	7.737^{abc}	7.688^{abcdef}	7.612^{a}	10.000^{a}	7.312^{b}	7.100^{a}	7.387^{bc}	82.711^{abc}
48*Y_2B_1	7.450^{abc}	7.450^{abcdefghi}	7.600^{a}	10.000^{a}	7.450^{ab}	7.500^{a}	7.475^{bc}	82.425^{abc}
72*Y_2B_2	7.696^{abc}	7.433^{abcdefghi}	7.554^{a}	9.900^{a}	7.471^{ab}	7.462^{a}	7.470^{bc}	83.062^{abc}
12*Control	7.713^{abc}	7.253^{efghi}	7.425^{a}	10.000^{a}	7.688^{ab}	7.125^{a}	7.563^{bc}	82.215^{abc}
60*Y_2B_2	7.696^{abc}	7.433^{abcdefghi}	7.554^{a}	9.900^{a}	7.471^{ab}	7.462^{a}	7.400^{bc}	83.062^{abc}
72*$Y_1B_1B_2$	7.937^{ab}	7.313^{efghi}	7.250^{a}	10.000^{a}	7.500^{ab}	7.312^{a}	7.470^{bc}	82.374^{abc}
60*B_2	7.687^{abc}	7.438^{abcdefghi}	7.625^{a}	9.200^{a}	7.562^{ab}	7.812^{a}	7.000^{c}	79.674^{abc}
36*$Y_1B_1B_2$	7.562^{abc}	7.250^{efghi}	7.662^{a}	10.000^{a}	7.812^{ab}	7.287^{a}	7.250^{bc}	82.298^{abc}
60*Y_2	7.650^{abc}	7.550^{abcdefgh}	7.550^{a}	9.200^{a}	7.550^{ab}	7.450^{a}	7.550^{bc}	79.650^{abc}
24*Control	7.750^{abc}	7.150^{ghi}	7.200^{a}	10.000^{a}	7.450^{ab}	8.300^{a}	7.200^{bc}	82.700^{abc}
24*Y_2	7.650^{abc}	7.250^{efghi}	7.600^{a}	10.000^{a}	7.300^{b}	7.550^{a}	7.400^{bc}	82.300^{abc}
72*Y_2B_1	7.550^{abc}	7.900^{ab}	7.400^{a}	9.600^{a}	7.850^{ab}	7.250^{a}	7.470^{bc}	80.320^{abc}
12*$Y_1B_1B_2$	7.600^{abc}	7.450^{abcdefghi}	7.500^{a}	10.000^{a}	7.600^{ab}	7.200^{a}	7.100^{bc}	82.000^{abc}
12*B_2	7.350^{abc}	7.050^{hi}	7.000^{a}	10.000^{a}	7.550^{ab}	7.600^{a}	7.550^{bc}	81.700^{abc}
12*Y_1Y_2	7.200^{c}	7.450^{abcdefghi}	7.500^{a}	10.000^{a}	7.400^{ab}	7.550^{a}	7.350^{bc}	82.000^{abc}
12*Y_1	7.550^{abc}	7.150^{ghi}	7.550^{a}	10.000^{a}	7.350^{b}	7.450^{a}	7.500^{bc}	82.150^{abc}
36*Control	7.600^{abc}	7.150^{ghi}	7.650^{a}	10.000^{a}	7.500^{ab}	7.100^{a}	7.250^{bc}	81.750^{abc}
72*B_2	7.650^{abc}	7.600^{abcdefg}	7.350^{a}	8.000^{a}	7.450^{ab}	7.500^{a}	7.470^{bc}	78.750^{abc}
72*Y_2	7.650^{abc}	7.300^{efghi}	7.250^{a}	8.000^{a}	7.600^{ab}	7.450^{a}	7.470^{bc}	78.300^{abc}
60*Y_1Y_2	7.200^{c}	7.500^{abcdefghi}	7.450^{a}	10.000^{a}	7.400^{ab}	7.100^{a}	7.450^{bc}	81.700^{abc}
60*B_1	7.500^{abc}	7.200^{fghi}	7.600^{a}	9.200^{a}	7.250^{b}	7.450^{a}	7.550^{bc}	78.850^{abc}
24*$Y_1B_1B_2$	7.500^{abc}	7.250^{efghi}	7.000^{a}	10.000^{a}	7.500^{ab}	7.250^{a}	7.250^{bc}	81.500^{abc}
72*B_1	7.500^{abc}	7.500^{abcdefghi}	7.200^{a}	8.000^{a}	7.250^{b}	7.450^{a}	7.470^{bc}	77.950^{bc}
48*Control	7.583^{abc}	7.389^{bcdefghi}	7.417^{a}	9.333^{a}	7.250^{b}	7.333^{a}	7.306^{bc}	79.388^{abc}
60*Control	7.583^{abc}	7.389^{bcdefghi}	7.417^{a}	9.333^{a}	7.250^{b}	7.333^{a}	7.306^{bc}	79.388^{abc}
12*Y_2	7.437^{abc}	7.250^{efghi}	7.125^{a}	10.000^{a}	7.312^{b}	7.250^{a}	7.312^{bc}	80.998^{abc}
60*Y_1	7.450^{abc}	7.250^{efghi}	7.150^{a}	10.000^{a}	7.100^{b}	7.250^{a}	7.300^{bc}	80.750^{abc}
72*Control	7.400^{abc}	7.000^{i}	7.250^{a}	8.000^{a}	7.500^{ab}	7.400^{a}	7.470^{bc}	77.370^{bc}

60*Y_2B_1	7.550^{abc}	7.400^{bcdefghi}	7.400^{a}	9.200^{a}	7.350^{b}	7.250^{a}	7.250^{bc}	77.100^{c}
72*Y_1	7.200^{c}	7.000^{i}	7.300^{a}	9.200^{a}	7.400^{ab}	7.300^{a}	7.470^{bc}	79.050^{abc}

Y_1 : *K. exigua* : Y_2 : *P. kudriavzevii* ; B_1 : *L. mesenteroides ;* B_2 : *P. campinasensi* ; Control : non-inoculated.

Appendix VI: Correlation Matrix of cup quality components with physicochemical characteristics

Variables	Brix	Viscosity	pH	TA	Aroma	Flavor	Acidity	Body	Uniformity	Clean cups	After taste	Balance	Sweetness	Overall	Cup score
Brix	**1**	-0.571	-0.465	0.019	0.025	-0.174	**-0.698**	**-0.680**	0.125	0.156	-0.577	**-0.638**	0.295	-0.099	-0.240
Viscosity	-0.571	**1**	0.515	-0.100	0.387	0.544	0.603	**0.768**	0.365	0.248	0.486	0.520	0.034	**0.697**	**0.677**
pH	-0.465	0.515	**1**	-0.373	**0.675**	**0.694**	0.233	**0.747**	0.437	0.481	**0.759**	**0.768**	0.340	0.376	**0.825**
TA	0.019	-0.100	-0.373	**1**	-0.478	0.178	0.519	0.076	-0.081	-0.265	-0.146	-0.156	0.046	-0.007	-0.097
Aroma	0.025	0.387	**0.675**	-0.478	**1**	**0.634**	-0.019	0.451	0.448	0.399	0.493	0.322	0.138	0.368	**0.637**
Flavor	-0.174	0.544	**0.694**	0.178	**0.634**	**1**	0.510	**0.781**	0.408	0.268	**0.672**	**0.646**	0.425	0.535	**0.825**
Acidity	**-0.698**	0.603	0.233	0.519	-0.019	0.510	**1**	**0.674**	-0.015	-0.277	0.593	0.559	-0.079	0.221	0.343
Body	**-0.680**	**0.768**	**0.747**	0.076	0.451	**0.781**	**0.674**	**1**	0.198	0.163	**0.676**	**0.740**	0.071	0.596	**0.719**
Uniformity	0.125	0.365	0.437	-0.081	0.448	0.408	-0.015	0.198	**1**	**0.902**	0.225	0.033	0.204	0.206	**0.728**
Clean cups	0.156	0.248	0.481	-0.265	0.399	0.268	-0.277	0.163	**0.902**	**1**	0.119	0.016	0.224	0.271	**0.676**
After taste	-0.577	0.486	**0.759**	-0.146	0.493	**0.672**	0.593	**0.676**	0.225	0.119	**1**	**0.909**	0.191	0.150	**0.675**
Balance	**-0.638**	0.520	**0.768**	-0.156	0.322	**0.646**	0.559	**0.740**	0.033	0.016	**0.909**	**1**	0.319	0.283	0.618
Sweetness	0.295	0.034	0.340	0.046	0.138	0.425	-0.079	0.071	0.204	0.224	0.191	0.319	**1**	0.430	0.460
Overall	-0.099	**0.697**	0.376	-0.007	0.368	0.535	0.221	0.596	0.206	0.271	0.150	0.283	0.430	**1**	0.605
Cup score	-0.240	**0.677**	**0.825**	-0.097	**0.637**	**0.825**	0.343	**0.719**	**0.728**	**0.676**	**0.675**	0.618	0.460	0.605	**1**