LWT - Food Science and Technology 216 (2025) 117343

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

The influence of spontaneous microbial fermentation isolates on physicochemical properties and cup quality of wet processed arabica coffee (*Coffea arabica*)

Khadijah Nakyinsige[a,*], Fredrick Mugerwa[a,d], Arthur Tabula[a,b], Diriisa Mugampoza[a], Moses Matovu[c], Michael Bamuwamye[a]

[a] *Department of Food Science and Technology, Faculty of Science, Kyambogo University, P.O.Box 1 Kyambogo, Kampala, Uganda*
[b] *Uganda National Bureau of Standards, Bweyogerere Industrial and Business Park, Plot 2-12 By-Pass Link, P.O. Box 6329, Kampala, Uganda*
[c] *National Agricultural Research Organization, P.O. Box 7065, Kampala, Uganda*
[d] *Uganda Coffee Development Authority, P.O. Box 7065, Kampala, Uganda*

ARTICLE INFO

Keywords:
Coffee
Arabica coffee
Fermentation
Microbial isolates
Starter culture
Cup quality

ABSTRACT

Uganda hardly exports speciality coffee. This is attributed to dependence on spontaneous wet processing which yields inferior quality coffees. 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. The isolates were grouped by cell morphology and biochemical features and confirmation done using polymerase chain reaction. Controlled fermentation of pulped Arabica coffee was carried out. Physicochemical changes namely pectin, total soluble sugars, viscosity, hydrogen potential and titratable acidity were monitored in the fermenting mass using standard methods. Fermented coffee beans were dried, roasted and evaluated for cup quality using the Speciality Coffee Association cupping protocol. A total of 130 microbial isolates were obtained on the different growth media. *Psuedomonas* sp., *Paenibacillus campinasensis*, *Kazachstania exigua* and *Pichia kudriavzevii*, were the predominant species. Mixed cultures significantly ($p < 0.05$) reduced fermentation period by 30% and attained the highest mean cup score (84 points). Pectin content and pH reduced to 0.00% and 3.85, respectively. Total soluble solids, titratable acidity and absolute viscosity increased to 8.92 ^{o}Brix, 3.85 g/L and 88 cP, respectively. There was a strong positive correlation (0.83) between *P. kudriavzevii*, pH and the cup quality. Processors may use starter cultures to improve on quality of Arabica coffee.

1. Introduction

Coffee is such an important trade commodity, coming second after petroleum, globally (Kulapichitr et al., 2019), and first in Uganda where it is the most significant contributor to foreign exchange earnings (International Coffee Organisation, 2019) and the leading source of employment or source of livelihood for rural smallholder farmers. Coffee belongs to the Rubiaceae family, genus *Coffea* (Adepoju et al., 2017). 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 ones (International Coffee Organisation, 2019). Arabica coffee dominates the world coffee market with a 70–80% market share (Kulapichitr et al., 2019). In Uganda, it contributes only 15%, making the country's global contribution to stand at 1% (FAOSTAT, 2021). Arabica coffee's preference due to superior sensory properties (Kulapichitr et al., 2019) necessitates more research in its entire value chain in Uganda.

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). 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

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* Corresponding author. Kyambogo University, P.O.Box 1 Kyambogo, Kampala, Uganda.
E-mail address: hnakyinsige@kyu.ac.ug (K. Nakyinsige).

https://doi.org/10.1016/j.lwt.2025.117343
Received 12 August 2024; Received in revised form 22 December 2024; Accepted 5 January 2025

than dry or semi-dry processed coffee (Avallone et al., 2000).

Starter cultures or starters are individual or mixed microbial cultures used in known concentrations to promote fermentation. 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 (Pereira et al., 2017). 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., 2017). *Pichia 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., 2015). 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 aimed at evaluating 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 processing.

2. Materials and methods

2.1. Collection of samples

Ripe Arabica coffee cherries (550 kg) were harvested by hand picking from randomly selected SL 14 variety coffee trees. 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).

2.2. Sample preparation

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 Uganda Industrial Research Institute (UIRI) Analytical Laboratories for microbial isolation within 6 h. For determination of physicochemical characteristics, and cup quality, Arabica coffee was obtained from a coffee farm in Bushiyi parish, Bulucheke sub county; Bududa District. Bududa is one of the major Arabica coffee producing districts in Uganda.

2.3. 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 27 °C (day -time) and between 18 and 22° (night-time) while relative humidity ranged between 60 and 75 %. The samples were transferred aseptically into sterile sample glass bottles. To 1 mL of each sample of the ferment, 9 mL s 0.1% peptone water was added, followed by 10-fold serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}). The diluted samples were then analyzed for the viable counts using plate count agar (PCA) and Nutrient agar. Analysis was also done for Lactic Acid Bacteria, and yeasts and moulds using surface inoculation on a De Man, Rogosa and Sharpe agar (MRS) and potato dextrose agar (PDA), respectively. Analysis was carried out in triplicate. The culture media plates were aerobically incubated at 30 °C for 48 h for the viable bacteria, 37 °C for 24 h for non-fastidious bacteria, 30 °C for 48 h for lactobacilli and 26 °C 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.

Morphological identification of the cells was done using the Olympus CX43 Biological Microscope (Gem Scientific Ltd, Germany). The Gram staining protocol of Smith and Hussey (2005) (Smith et al., 2005) was used to differentiate between the Gram-positive and Gram-negative bacteria. 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) (Smart et al., 1999) was followed for their presumptive selective identification. Microscopic examination (X110M) was done for viable yeasts cells 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, 2010). 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 °C (Masoud et al., 2004).

2.4. Molecular identification of microbial isolates

2.4.1. 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) (Mahuku, 2004). 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). Deoxy ribonucleic acid extraction involved pipetting 0.5 mL of bacteria or yeast culture suspension into a 1.5 mL micro-centrifuge tube, centrifuged at 12100×*g* rcf for 1 min and the supernatant discarded. Then, 800 μL of 1M NaCl was added to the cell pellet, vortexed for 30 s, centrifuged at 12100×*g* rcf for 1 min and the supernatant 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 12100×*g* rcf. To 500 μL of the supernatant transferred into a new tube, an equal volume of ice-cold isopropanol was added, and incubated at −20 °C overnight (to precipitate the DNA). This was followed by centrifuging for 10 min at 12100×*g* rcf to pellet the DNA. The supernatant was discarded and the pellet was washed with 70 % ethanol (800 μL) and centrifuged at 12100×*g* rcf 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.

2.4.2. Amplification of 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 of 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 1 mM EDTA) at 80 V for 60 min. The gel was then stained with ethidium bromide (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 NA1111-1 KT, 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 were 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 12100×*g* rcf 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 12100×*g* rcf 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 12100×*g* rcf 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 12100×*g* rcf 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.

2.4.3. 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.

2.5. Determination of the physicochemical changes

The predominant isolates were sub cultured in nutrient broth for bacteria and potato dextrose broth for yeast in a rotary incubator shaking at 93 x f rcf at 25 °C for 48 h. The cultures were then standardised against a 0.5 McFarland standard turbidity level (1.5×10^8 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), absolute viscosity, pH and titratable acidity at 12 h intervals over a 72 h period.

2.6. 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) (Castillo-Israel et al., 2015). The fermented coffee (50 g) was weighed out on an analytical balance (Model MSI 104 TS, 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 °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 at 55 °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 following equation:

$$\text{Yield }\% = (\text{Quantity of pectin extract} / \text{mucilage quantity}) * 100$$

2.7. Determination of total soluble solids

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

2.8. Determination of absolute viscosity

Absolute viscosity was determined as centipoise (cP) according to the BS EN ISO 2884-1 method (BS EN ISO 2884-1, 2006). 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. Absolute viscosity readings were displayed on the screen. The spindle was disinfected using 70 % ethanol in between measurements.

2.9. Determination of pH

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

2.10. Determination of titratable acidity

Titratable acidity (TA) was determined according to the AOAC official method 942.15 (AOAC 2000) (AOAC Official Methods of Analysis, 2000, chap. 37). The coffee ferment was diluted with distilled water (10 g–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:

$$\text{Titratable acidity (TA)} = (V \times N \times 1000 \times 0.091) / W$$

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.

2.11. Sensory evaluation

2.11.1. Preparation of samples for cupping

A kg of dried coffee beans was hulled and processed following the procedure according Speciality Coffee Association of America (SCAA) (Specialty Coffee Association of American, 2015). The moisture content of the beans was measured using a moisture analyzer (model: Belt and Bearings). The roast was completed in 10 min. The samples were immediately air cooled to room temperature, packed in airtight plastic containers and stored in a cool dark place for at least 8 h before cupping. Immediately prior to cupping, samples were ground. A golden cup was brewed by mixing 8.25 g of coffee with 150 mL of hot water. The brew was steeped undisturbed for 5 min before evaluation.

2.11.2. Cupping

The cupping process was carried out with an 8 (eight) panel Q-cuppers, using a ratio of 0.055 g of coffee per mL of water, three (3) cups per sample, and mesh classification. The panel of cuppers were to identify the attributes, notes, and profiles of each one of the samples, according to the Speciality Coffee Association of America (SCAA) cupping protocol (Specialty Coffee Association of American, 2015). The cuppers registered the quantity of primary and secondary defects, attributes, notes and the profile of each of the samples cupped. The evaluated sensory attributes were aroma, aftertaste, acidity, flavour, body, balance (synergistic combination of flavour acidity and body), uniformity, clean cup, sweetness and overall impression. The sum of all these evaluated attributes was computed as the total score.

2.12. 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.January 5, 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–72 h. The significant differences between the starter cultures were evaluated using the Turkey's tests at $p < 0.05$.F.

Principal component analysis (PCA) for the cup score and physicochemical characteristics of the different starter cultures were analyzed using XLSTAT version (2020.January 5, 1072). This 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 (Pires et al., 2017). 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. Results and discussion

3.1. Isolation and identification of predominant microorganisms in spontaneous coffee fermentation

A total of 130 microbial isolates were obtained on the different growth media. Bacteria dominated (61 %; *Pseudomonas* sp. = 30 %, *Paenibacillus campinasensis* = 31 %) over the yeasts (39 %; *Kazachstania exigua* = 30 %, *Pichia kudriavzevii* = 9 %) (Table 1).

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 2 shows the results of presumptive identification of the different isolate grouped on the basis of their phenotypes.

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.

3.2. 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 3.

The 5.8S rRNA genes and 16S rRNA gene of isolates for yeasts and bacteria respectively, were successfully amplified (Fig. 1). After gene amplification, Sanger sequencing yielded sequences for each of the 4 groups of isolates. A BLAST search (Fig. 2) using sequences of the isolate groups in the NCBI nucleotide database established that the isolates were correspondingly *Psuedomonas* sp., *Paenibacillus campinasensis*, *Kazachstania exigua* and *Pichia kudriavzevii*. Isolates B_1, B_2 Y_1, and Y_2 were assigned accession numbers MW14251, MT634699, MT634702, and MT634703 in the NCBI nucleotide database, respectively (Table 4).

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). A broad 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., 2014); (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). Pseudomonas sp. has been reported in caffeine degradation (Baker et al., 2012).

3.3. Physicochemical changes in inoculated coffee wet fermentation

3.3.1. 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 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

Table 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: *Pseudomonas* sp.*;* B_2: *P. campinasensis.*

Table 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: *Pseudomonas* sp.*;* B_2: *P. campinasensis.*

Table 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.

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*, *Psuedomonas* sp. and *P. campinasensis* (0.000%; Table 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 5). Results of the current study were slightly different from the findings of Kulandaivelu (2013) (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., 2015).

Rapid pectin breakdown is necessary to achieve shorter coffee fermentation time (Haile & 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 (Table 6). The mixed starter comprising *K. exigua, P. kudriavzevii, Pseudomonas* sp. and *P. campinasensis* was predicted to have potential to reduce fermentation time to 28 h. On the basis of pectin degradation, the combination of *Pseudomonas* sp. and *P. kudriavzevii* did not show a significant ($p = 020$) effect on the fermentation time (Table 6).

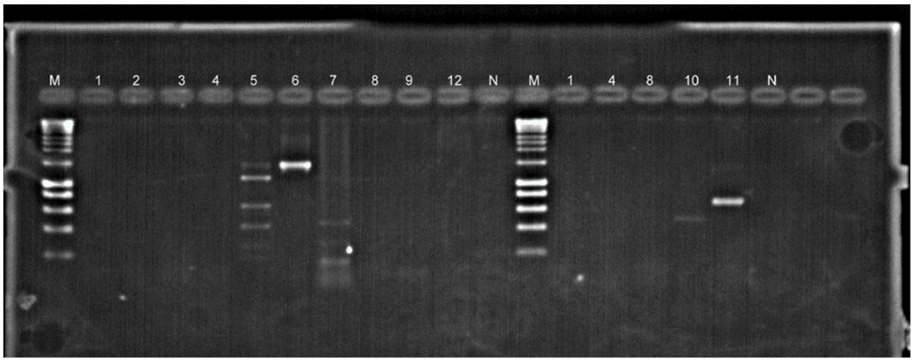

Fig. 1. Agorose gel showing amplified 16S rRNA gene and 5.8S ITS gene of bacteria (5 and 6) and yeasts (10 and 11), respectively. M = 1 kb plus ladder (Thermo scientific), N = no template negative control.

Fig. 2. Agorose gel showing amplified 16S rRNA gene and 5.8S ITS gene of bacteria (5) and yeasts (10 and11), respectively. M = 1 kb Plus ladder (Thermo Scientific), N = no template negative control.

Table 4
Identified isolates obtained from spontaneous fermentation of Arabica coffee.

Isolate	Count	Band Size	5.8S/16SrRNA gene sequence spp	Homology[a]	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

Y_1: *K. exigua*; Y_2: *P. kudriavzevii*; B_1: *Pseudomonas* sp.*;* B_2: *P. campinasensis.*

[a] Percentage of identical nucleotides of the closest relative found in the GeneBank database.

3.3.2. Changes in total soluble solids (^{o} Brix)

Total soluble solids (TSS) in the fermenting mass varied between 3.03 °Brix and 9.40 °Brix (Table 7). 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 7). The highest potential to increase TSS of the fermenting mass, at 24 h of fermentation, was observed for *K. exigua*, *P. kudriavzevii*, *Pseudomonas* sp.*and P. campinasensis* (7.30 °Brix) and *K. exigua*, *P. kudriavzevii* and *Pseudomonas* sp. (7.08 °Brix). These TSS values were significantly different ($p < 0.05$) from the TSS of the other treatments (Table 7). Starter culture combination *K. exigua*, *P. kudriavzevii*, *Pseudomonas* sp. and *P. campinasensis* had the highest TSS at the different fermentation times (0 h–72 h), followed by *K. exigua*, *Pseudomonas* sp. *and P. campinasensis*. At 72 h, mixed starters *K. exigua*, *P. kudriavzevii*, *P. campinasensis* and *Pseudomonas* sp., *K. exigua, L. mesenteroides* and *P. campinasensis, P. kudriavzevii* and *Pseudomonas* sp., and *K. exigua* and *P. kudriavzevii* had the highest total soluble solids (8.92 ^{o}Brix, 8.83 ^{o}Brix, 7.98 ^{o}Brix and 7.78 ^{o}Brix, respectively) which were significantly different ($p < 0.05$) from the rest of the treatments, Table 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) (Quintero, 2014) who reported that early attainment of ^{o}Brix of 8.0–9.0 during fermenting is necessary to produce the desired level of sweetness in the coffee beverage.

3.3.3. Changes in absolute viscosity

Absolute viscosity of the fermenting mass ranged from 18.00 cP to 25.20 cP (Table 8). Generally, there was no significant difference ($p > 0.05$) between the absolute viscosity of the treatments inoculated with the different starter cultures from 0 h to 36 h (Table 8). After 48 h of fermentation, viscosity was highest for the non-inoculated experiment (control; Table 8). At 48 h the absolute 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), *Pseudomonas* sp. and *P. campinasensis* (23.20 cP), *K. exigua* and *Pseudomonas* sp. (23.80 cP), and *P. kudriavzevii* and *Pseudomonas* sp. (23.38 cP) at 72 h. Lower absolute viscosity was observed when the starter *K. exigua*, *Pseudomonas* sp.and *P. campinasensis* was used, from 48 h to 72 h. These findings were in agreement with the findings of Avallone et al. (2002) (Avallone et al., 2000) who reported that *Pseudomonas* sp.and *Kazachstania* sp. do not produce endopectolytic enzymes which catalyse the breakdown of pectic substances leading to enhanced absolute viscosity in the fermenting mass.

3.3.4. Changes in hydrogen ion concentration (pH)

The pH of the coffee fermenting mass decreased from 6.48 to 3.85 (Table 9). 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

Table 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: *Pseudomonas* sp.*;* B_2: *P. campinasensis*; Control: un-inoculated.

Table 6
Regression of dependent variables for prediction of the fermentation time.

Variable	Class	Model ($y = b_\circ + b_1 * t$) y	b°	b_1	Adjusted R^2 value	Significance (P-value)	Time (h)
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
°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: *Pseudomonas* sp.; B_2: *P. campinasensis*.

constant between 36 h and 60 h. The highest potential to reduce pH of the fermenting mass was observed for treatments with *Pseudomonas* sp., *Pseudomonas* sp., and *P. campinasensis*, and *K. exigua* and *Pseudomonas* sp. (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 9). The results of this study were in agreement with the findings of Quintero (2014) (Quintero, 2014), who reported that during the natural fermentation of Arabica coffee, the pH decreased in the range of 3.9–4.2 after 18–30 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)].

3.3.5. 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 10). Starter cultures *K. exigua* and *Pseudomonas* sp., *K. exigua*, *Pseudomonas* sp., *K. exigua*, *P. kudriavzevii, Pseudomonas* sp. and *P. campinasensi K. exigua* and *P. campinasensis, K. exigua* and *Pseudomonas* sp. and *P. campinasensis*, *Pseudomonas* sp. and *P. campinasensis* 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) (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

Table 7
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: *Pseudomonas* sp.; B_2: *P. campinasensis*; Control: un-inoculated.

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 (Haile & Kang, 2019).

Table 9
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: *Pseudomonas* sp.; B_2: *P. campinasensis*; Control: non-inoculated.

Table 8
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: *Pseudomonas* sp.; B_2: *P. campinasensis*; Control: non-inoculated.

Table 10
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 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: *Pseudomonas* sp.; B_2: *P. campinasensis*; Control: non-inoculated.

3.4. 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 11). 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*, *Pseudomonas* sp. and *P. campinasensis* producing the highest mean cup score (84.034 points; Fig. 3) 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) (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

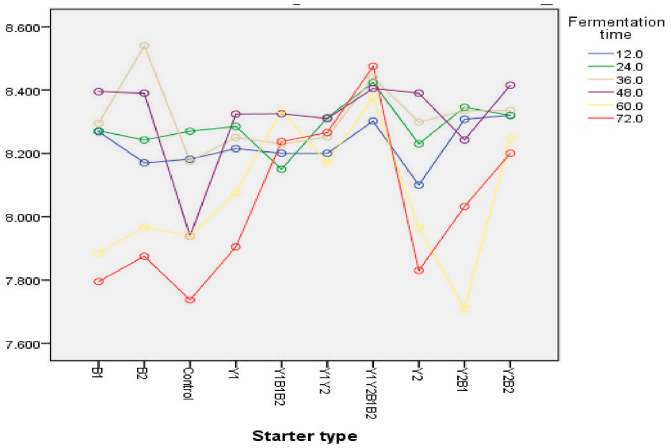

Fig. 3. Comparison of coffee beverage mean cup scores against the different starter culture treatments during fermentation. Y_1: *K. exigua*: Y_2: *P. kudriavzevii*; B_1: *Psuedomonas;* B_2: *P. campinasensis*; Control: non-inoculated.

Table 11
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: *Pseudomonas* sp.; B_2: *P. campinasensis*; Control: un-inoculated.

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); (Haile & Kang, 2019)].

3.5. Principal component analysis (PCA)

Correlation between cup quality and physicochemical characteristics of the fermented coffees using different starter cultures was done using PCA. 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 (Fig. 4). Principal component (F1 52.71%) contrasted single starter cultures (*Pseudomonas* sp., *P. campinasensis*, *P. kudriavzevii* and *K. exigua*) that were correlated with high pH and absolute 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, Pseudomonas* sp. and *P. campinasensis, K. exigua, Pseudomonas* sp. and *P. campinasensis*, *P. kudriavzevii* and *P. campinasensis*, *P. kudriavzevii* and *Pseudomonas* sp., *K. exigua* and *P. kudriavzevii*) that were correlated with high °Brix of the fermenting mass.

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

The pH of the coffee beverage was correlated with 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* (Fig. 4). 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 *Pseudomonas* sp. Starter cultures *K. exigua*, *P. kudriavzevii*, *Pseudomonas* sp. and *P. campinasensis* were associated with titratable acidity (TA) and yet TA had negative correlations with the cup quality except for °Brix, flavour, acidity, body and sweetness which had weak positive correlations (0.019, 0.178, 0.519, 0.076 and 0.046, respectively). Combined starter cultures *K. exigua*, *P. kudriavzevii* and *Pseudomonas* sp., *P. kudriavzevii* and *Pseudomonas* sp., *P. kudriavzevii* and *P. campinasensis*, and *K. exigua* and *P. kudriavzevii* were highly associated with °Brix. However, °Brix had strong negative correlations with acidity, body and balance (r = −0.638, r = −0.680 and −0.638). Acidity was negatively correlated with °Brix which could be due to the interference of the sugar acid balance [35], but had a strong correlation with the body and viscosity (r = −0.698, r = 0.674 and r = 0.768). 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 °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.*

4. Conclusion

The prospect of applying starter cultures in coffee fermentation was to reduce fermentation time, improve process control and sensory quality of the final product. *Kazachstania exigua*, *Pichia kudriavzevii*, *Pseudomonas* sp. and *Paenibacillus campinasensis* were the predominant spp. during the spontaneous wet processing of Arabica coffee in Bugisu sub region. Starter cultures reduced coffee wet fermentation time by 30%. Use of mixed cultures resulted in better cup quality. *Pichia 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. However, the current study did not identify all the isolates in the fermenting coffee mass and concentrated on the four most dominant ones. Further studies may go ahead and identify all microbial isolates. Future researches should concentrated on studying the optimal industrial conditions to further reduce fermentation time using the identified starters.

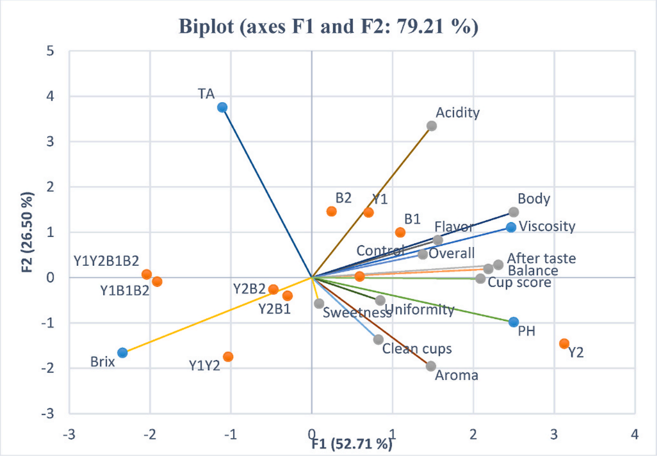

Fig. 4. Principal component analysis of the cup quality and physicochemical characteristics of the fermenting mass using different starter cultures over a 72-h period.

CRediT authorship contribution statement

Khadijah Nakyinsige: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fredrick Mugerwa:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Arthur Tabula:** Writing – original draft, Validation, Methodology, Formal analysis, Conceptualization. **Diriisa Mugampoza:** Writing – review & editing, Resources, Methodology, Formal analysis, Data curation, Conceptualization. **Moses Matovu:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Michael Bamuwamye:** Conceptualization, Investigation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing.

Ethics statement

The study was conducted following research ethics guidelines of Clarke International University Research Ethics Committee (CIU-REC): CLARKE-2024-1088.

Declaration of competing interest

The authors declare thatv they have no known competing financial interests or personnal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Kyagalanyi Coffee Limited is acknowledged for providing the facilities for fermentation and access to farmers in Manafwa, Bududa and Sironko. Uganda Industrial Research Institute and Infectious Diseases Research Council are acknowledged for their tremendous support in laboratory experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2025.117343.

Data availability

Data is available at the National Centre for Biotechnology Information gene bank. The accession numbers in the in the NCBI nucleotide database are MW14251, MT634699, MT634702, and MT634703.

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