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Biochemical Properties and Molecular Characterization of Fermented Alcoholic Honey (*Muratina*) Microbes from Mbeere-Kenya

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Abstract

Muratina is an alcoholic beverage (wine) amongst the communities around The Mt. Kenya, obtained from spontaneous fermentation of honey in a gourd with dried *Kigelia africana* (Lam.) Benth.fruits. The wine production is still carried out using traditional technology and has never been scaled up. It serves cultural and social value amongst the communities. The objective of this study was to characterize and document the product process and to evaluate the chemical and microbiological quality of *Muratina*. The production process involves mixing water and honey in a ration of 17L water to 3Kg honey, then allowing it to ferment in a gourd with pre-cured K.africana fruits for 3-5 days at 30°C after which it is ready. *Muratina* has an alcohol content of 19.66± 0.47 (mL/100ml), pH of 4.06 ± 0.12 and titratable acidity of 7.57± 0.45 (g tartaric acid/100 mL). Microbiological analysis of *Muratina* showed aerobic mesophiles at 2.1-5.5 x 103 CFU/mL, LAB at 3.2-7.7 x 104 CFU/mL and yeasts at concentration of 5.6 – 7.0 x 103 CFU/mL. Biochemical analysis of LAB isolates revealed various resistances to ox gal, pH and NaCl indicating their potential use as probiotics. All the isolates tested were able to withstand 3% ox-gal, although none were able to grow at pH 1-3. Identification of LAB was carried out using API 50 CHL and the sequencing of the 16s rRNA while those of yeasts was carried out using API ID 32. The isolates were identified as *Lactobacillus plantarum*, Lactococcus spp and *Pediococcus spp. Saccharomyces cereviseae* was the major yeast isolated. The high alcohol content of Muratina indicates that it has yeasts that could be of commercial value.

Keywords: Honey; *Muratina*; Fermentation; Wine; *Kigelia Africana*

Introduction

Traditional fermentation technologies still play a major role in African communities as a means of food preservation, diversification, source of livelihood and cultural values [1,2]. The existence of many tribes with diverse food preferences and cultural practices has contributed to the existence of these traditional foods technologies passed from generation

to another. Fermentation of honey into wine is one such technology that has remained relevant over years in many communities [2-4].

The tribal and cultural diversity in many African countries Kenya included has played a major role in the maintenance of traditional fermented foods and beverages amongst various tribes. One such product is *Muratina*. *Muratina* is an alcoholic beverage (wine) amongst the communities around The Mt. Kenya (Mbeere, Embu, Kikuyu, Meru, Tharaka, and Kirinyaga), obtained from spontaneous fermentation of honey in a gourd with dried K.africana fruits. The wine production is still carried out using traditional technology and has never been scaled up.

Muratina has a cultural value where it forms a key component in marriage rites e.g "*Uuki wa muragi*"-was a mandatory first wine guard that the father of a boy had to take to the girl's father to report the intention to marry the girl; others like "*Uuki/Njovi/Njohi ya Mwana*" –was a mandatory guard of honey wine which had to be delivered by the boy's father during the first sitting to negotiate the dowry. This wine had to be taken by everyone to bless the marriage irrespective of if you are an alcohol drinker or not, hence the traditional value (This paper-personal interview with elders). This tradition is still practiced to-date although it is fading with many homes adopting Christianity. However the importance of honey wine in these communities cannot be under scored.

Many African traditional fermented products have been extensively studied in terms of their microbial populations, chemical constitution as well as scaling up production to industrial scale [5-8]. However most studies have documented cereal based fermented products [9,10] and very little attention has been given to their non-cereal counterparts such as honey wine and Palm-wine from Kenya [1,11,12]. A related cereal based product from the same community where *muratina* was sampled is *Kimeere* [13]. The objective of this study therefore was to explore the microbial population of fermented honey (*Muratina*) from Mbeere using molecular sequencing of the 16S rDNA. This paper details the traditional production process of *Muratina*, its microbiological quality, isolation of microbes there in and testing their biochemical properties.

Justification

This study was inspired by the fact that in Kenya, Honey wine is categorized as illicit brew while in the rest of the world mead demand is in an upward surge. The technology is slowly dying because the government bans its production. As a matter of fact, most young people less than 45 years have no clue how it is brewed. This is contrary to the trend in other parts of the world. In the USA for example, honey wine has become so popular that there is an association "The American Mead Makers Association", an organization whose sole purpose is to promote the brewing and consumption of honey wine in the USA.

Materials and Methods

The Process

The production process involves diluting honey with water (3:17) ratio as showed in Figure 1. This is followed by mixing in a gourd with dried *K. africana* fruits. The fruit comes from a tree commonly called the "sausage tree" (Kigelia africana) due to the long sausage like fruit that it bears. The fruits hang down on a strong like twigs that drop from the tree branches [14]. The mixture is then left to ferment for 3-5 days after which it is filtered and it's ready for consumption.

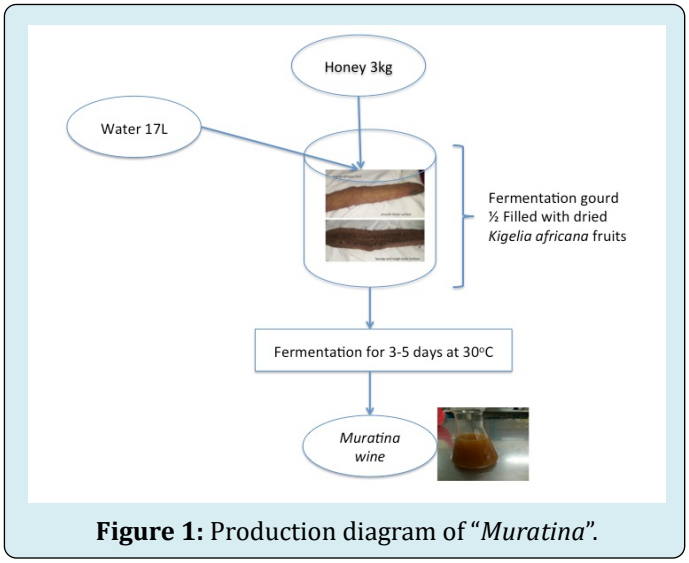

Figure 1: Production diagram of "*Muratina*".

Sample Collection

About 500g-fermented honey was originally transported from Kenya to Germany for personal consumption. In the process, when explaining the product production process to the institute colleagues, it aroused some interest, which led to this study. We conducted routine microbiological and nutritional analysis on the sample. The samples was originally from a pooled sampled of *Muratina* from three homesteads in Kirii, Mbeere North in Embu County at the end of fermentation for a research project in Technical University of Kenya.

Biochemical Analysis

pH and Titratable Acidity:

- pH was measured in triplicates using a bench pH meter (mettler Toledo-five easy) at room temperature by the use of the pH meter.
- T.A was done by volumetric analysis using basic titration as described by Kiribhaga, et al. [1] with slight modification. Briefly 15 ml of the sample was measured into a flask and 100 millilitres of distilled water was added followed by 3 drops of phenolphthalein indicator and then titrated against 0.1N NaOH solution, until colour changed to pink. The volume of 0.1N NaOH used was recorded. This was done in triplicates and results recorded for analysis.

Alcohol Content: Alcohol content was determined following the method described by Biri, et al. [15]. Briefly, 50ml of *Muratina* was measured into a volumetric flask followed by distillation. 45ml of the distillate was collected and made up to 50ml with distilled water. The specific gravity was determined using the specific gravity bottle. The corresponding alcoholic content was calculated using the formula below:-

$$\text{Specific Gravity} = \frac{W_2 - W_1}{W_3 - W_1} \times 100$$

Where,
W1= Weight of empty bottle
W2= Weight of empty bottle+ sample
W3= Weight of empty bottle + sample+ water

This was done in triplicates and results recorded for analysis. The results were confirmed using an alcohol-meter.

Microbiological Methods

Isolation of Microorganisms: Isolation of Total aerobic mesophiles, lactic acid bacteria and yeasts was carried out using routine MBT established protocol. Total aerobic mesophiles were isolated on Medium 17 (M17, Merck, 115108) incubated at 32oC aerobically. Lactic acid bacteria (LAB) were isolated on MRS agar (BIO-RAD, France), after incubation at 37°C for 48h under anaerobic conditions. Yeasts were isolated on Sabouraud Chloramphenicol agar medium (BIO-RAD, France) and plates were incubated at 30°C for 3-5 days as described by Njeru, et al. [7]. The colonies from different media were counted and expressed as cfu/ml of sample. Colonies were selected from the agar media for further characterization and identification. Randomly selected colonies were sub-cultured through streaking method onto respective media three times until pure colonies were achieved. The purity of colonies was controlled using microscope (Axioskop 40 model).

Biochemical Tests for Colonies: Pure colonies were tested for their morphology and biochemical characteristics. Briefly the following tests were done using routine MBT established protocols and as described by Njeru, et al. [7].

- Morphological Identification –Microscopic
- API 50 CHL identification for LAB and API-System ID 32 for yeasts
- Gram Staining
- Catalase test
- Oxidase test
- KOH-test
- Bile salt tolerance at 1%, 2% and 3%,
- Low pH tolerance at pH 1, 2 and 3
- NaCl test (3.5% and 6.5%)
- Temperature test (15°C and 45°C)

DNA Isolation & 16S rDNA Sequencing

LAB Isolates were grown overnight at 37°C in MRS broth (Merck KGaA, Darmstadt, Germany). From this culture; 5ml was sampled from which total DNA was extracted following the protocol and purified using PCR clean kit according to manufacturer's instructions. DNA was confirmed and visualized on agarose gel.

Primers & Sequencing

Primers (27F-1492R) used for specific amplification of bacterial 16S rRNA genes have been described previously [16] and 16S rRNA amplicons were sequenced by GATC-Biotech (Eurofins Genomics).

Multiple Sequence Alignment and Phylogeny Construction

16SrRNA sequences from the isolated LAB together with reference sequences of LAB obtained from National Center for Biotechnology (NCBI) GenBank were subjected to Multiple Sequence Alignment (MSA) using MUSCLE algorithm [17] in MEGA X [18]. Consequently, a neighbour joining tree phylogenetic tree from the MSA was constructed in MEGA

X using p-distance as the substitution model and bootstrap resampling as a test of phylogeny; number of bootstrap iterations were set at 1000 [19]. Phylogeny construction helped in identification and showing the relationships of *Muratina* LAB isolates.

Species Identification

The nucleotide sequences of the 16S rRNA genes from all LAB isolates were subjected to sequence similarity search in the GenBank data library of 16S ribosomal RNA sequences using nucleotide BLAST program (blastn) on the NCBI website http://www.ncbi.nlm.nih.gov/18 LAB isolates' 16S rRNA were also subjected to sequence similarity search in other bacterial and Archeal 16SrRNA databases recommended for taxonomic identification of bacteria which include Ribosomal Database Project (RDP) [20] and SILVA RNA database [21]. Based on sequence similarity results from the three databases, it was possible to identify LAB bacteria from *Muratina*. 16S rRNA sequence similarity 98% has been accepted as a threshold for bacterial species demarcation of which Similarity Index/Percentage Identity of >98 indicates that the query sequence is of same bacterial species and below 98 % indicates that the query sequence of a different strain or species [22].

Results

pH, Titratable Acidity and Alcohol Content

Results for pH, Titratable acidity and percentage alcohol content of Muratina are shown in Table 1 below as 4.06 ± 0.12., 7.57± 0.45 and 19.66± 0.47 respectfully.

Analysis	Mean
pH	4.06 ± 0.12
Titratable acidity (g tartaric acid/100 mL)	7.57± 0.45
Ethanol (mL/100 mL)	19.66± 0.47

Table 1: Biochemical characteristics of *Muratina*.

Microbiological Analysis

The results for Aerobic mesophiles, Lactic acid bacteria and Yeasts are shown in Table 2. Aerobic mesophiles ranges from 2.1-5.5 x 103 CFU/mL, Lactic acid bacteria 3.2-7.7 x104 CFU/mL and Yeasts ranged from 5.6-7.0x 103 CFU/mL. The morphological characteristics of various isolates are tabulated in Table 3. The samples microbiological analysis shown a significant difference from the beginning when samples were fresh to the end at 96hours as shown in Table 2.

Media/Analysis	Range	Sign, Diff
M17 (Aerobic Mesophiles)	2.1-5.5 x 103 CFU/mL	P=0.0380
MRS (Lactic Acid Bacteria)	3.2-7.7 x 104 CFU/mL	P=0.0150
Sabouraud Chloramphenicol agar (Yeasts)	5.6 – 7.0 x 103 CFU/mL	P=0.0247

Table 2: Microbiological analysis.

Isolate number	Origin	Description
03190524 01	MRS, *Muratina*, 10^-2	Small, white, raised around
03190524 02	MRS, *Muratina*, 10^-2	Small, white, raised around
03190524 03	MRS, *Muratina*, 10^-2	Uneven, bulged, tall, greasy
03190524 04	MRS, *Muratina*, 10^-2	Small, white, round, slightly dull
03190524 05	MRS, *Muratina*, 10^-3	Small, white, round, slightly dull
03190524 06	MRS, *Muratina*, 10^-3	Tiny, round, slightly white, transparent border
03190524 07	MRS, *Muratina*, 10^-3	Small, white, raised around
03190524 08	MRS + 20% Glucose, *Muratina*, 10^-3	Tiny, round, slightly white, transparent border
03190524 09	MRS + 20% Glucose, *Muratina*, 10^-3	Small, white, raised around
03190524 10	MRS + 20% Glucose, *Muratina*, 10^-3	Tiny, round, slightly white, transparent border
03190524 11	M17 + 20% Glucose, *Muratina*, 10^-2	Large, slightly uneven, white bacilli
03190524 12	M17 + 20% Glucose, *Muratina*, 10^-2	Large, uneven, white to transparent, shiny - bacilli
03190524 13	M17 + 20% Glucose, *Muratina*, 10^-2	Large, white, uneven, wrinkled - bacilli
03190524 14	YGC, Muratina, 10^-2	White, round, raised in the middle

03190524 15	YGC + 20% Glucose, *Muratina*, 10^-2	White, round, raised in the middle
03190524 16	YGC + 20% Glucose, *Muratina*, 10^-2	White, round, raised in the middle
03190524 17	YGC + 20% Glucose, *Muratina*, 10^-2	White, round, raised in the middle
03190524 18	YGC + 20% Glucose, *Muratina*, 10^-2	White, round, pointed in the middle + slightly yellowish beige, medium in size
03190524 19	YGC + 20% Glucose, *Muratina*, 10^-2	Small, round, white, flat
03190524 20	YGC, *Muratina*, 10^-3	Large, beige, round, center pointed

Table 3: Colony Characteristics.

Observations

The colonies on the M17 agar were large and irregular with a blunt surface. Partly the colonies formed strong mucus that dripped down during incubation as shown in the Figure 1 below. The microscopic image showed that they are aerobic spore formers as shown in Figure 2 and 3 below.

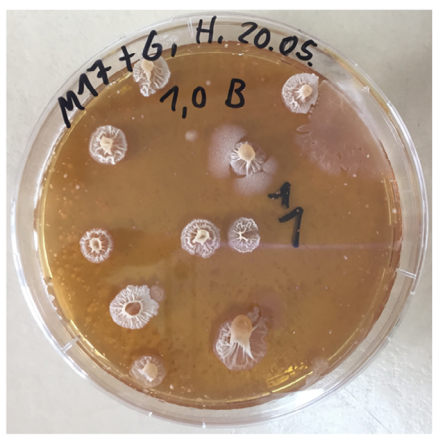

Figure 2: Colonies on M17 agar plates.

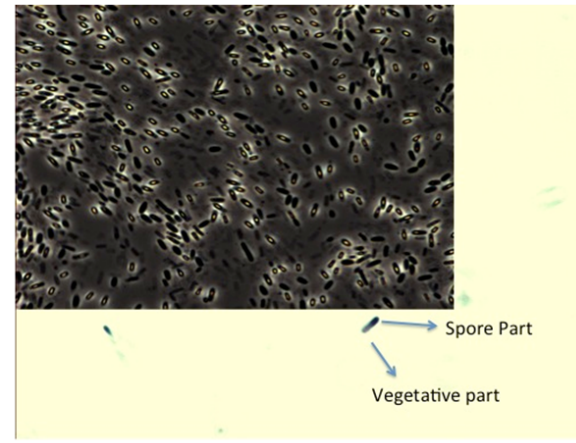

Figure 3: Microscopic image of colonies from M17 agar-Sporeformers as observed under phase contrast microscope (Axioskop 40 model).

Pure colonies were isolated by streaking in relevant media and controlled using a phase contrast microscope until clean colonies were achieve as shown in the Figure 4 below.

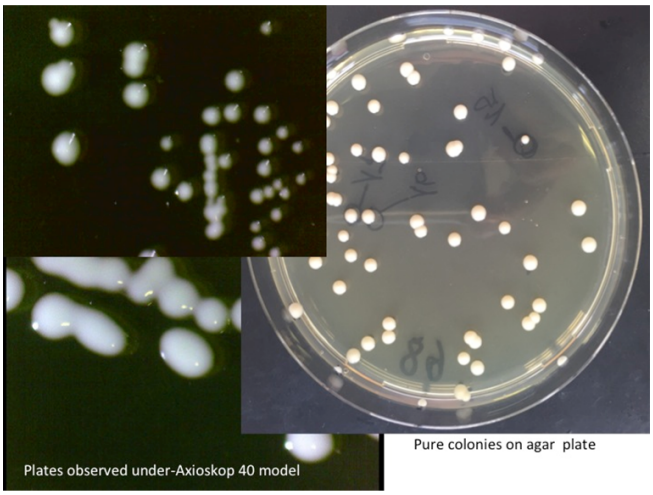

Figure 4: Clean colonies from different plates as observed under phase contrast microscope model (Axioskop 40 model).

Biochemical Characterization of LABs

Pure colonies from MRS agar were subjected to Gram staining, Oxidase test, Catalase test, growth in media adjusted to various pH, NaCl and Ox-bile concentrations and results recorded in the Table 4 below.

Isolate No**.	Gram- Staining*	Oxidase	Catalase	Growth in MRS with additive							
				pH			NaCl (%)		Ox – bile (%)		
	Gram- Staining	Oxidase - test	3% H_2O_2	1	2	3	3.50%	6.50%	1%	2%	3%
1	+	-	-	-	-	-	+	+	+	+	+
3	+	-	-	-	-	-	+	+	+	+	+
6	+	-	-	-	-	-	+	+	+	+	+
8	+	-	-	-	-	-	+	+	+	+	+
10	+	-	-	-	-	-	+	+	+	+	+
11	+	-	-	-	-	-	+	+	+	+	+
13	+	-	-	-	-	-	+	+	+	+	+

Table 4: Biochemical Characterization of LAB.
*KOH rapid test / Gram staining **Isolate numbers start with 03190524 as in Table 4
LAB: Lactic acid bacteria, A: Anggeraja, B: Enrekang, C: Cendana, +: Weak growth, ++: Medium growth, +++: Strong growth, -: No growth
Isolate No. Acc. Identity

Identification of LAB

Based on sequence similarity search in GenBank, RDP and SILVA databases, LAB isolates were identified based on Similarity Indices which were above 98 % which was considered a perfect match. LAB bacteria identified belonged to four genera which include: *Pediococcus*, *Lactobacillus*, *Lactococcus* and *Enterococcus*. Phylogenetic tree constructed shows the relationship between the LAB isolates together with "the perfect match"/reference species (REF) retrieved from the online 16S rRNA databases. Phylogenetic tree constructed is shown in Figure 5 below while the species identified tabulated in Table 5.

Isolates No.	Species Identity	Similarity Index	GenBank Accession No. of Reference species 16S rRNA
77BF71	Enterococcus durans	99.73	NR_113257.1 Enterococcus durans
77BF60	Lactococcus lactis	99.01	NR_113960.1 Lactococcus lactis
77BF61	Pediococcus pentosaceus	99.32	NR_042058.1 Pediococcus pentosaceus
77BF63	Lactococcus lactis	99.03%	NR_113960.1 Lactococcus lactis
77BF64	Pediococcus pentosaceus	98.11%	NR_042058.1 Pediococcus pentosaceus
77BF65	Pediococcus pentosaceus	98.94%	NR_042058.1 Pediococcus pentosaceus
77BF66	Lactococcus lactis	99.57%	NR_113960.1 Lactococcus lactis
77BF67	Pediococcus pentosaceus	99.13%	NR_042058.1 Pediococcus pentosaceus
77BF68	Lactobacillus plantarum	99.37%	NR_104573.1 Lactobacillus plantarum
77BF69	Lactococcus lactis	98.89%	NR_113960.1 Lactobacillus lactis
77BF70	Lactobacillus fermentum	98.60%	NR_113335.1Lactobacillus fermentum
77BF72	Pediococcus pentosaceus	99.14%	NR_042058.1 Pediococcus pentosaceus
77BF73	Lactobacillus fermentum	98.94%	NR_113335.1 Lactobacillus fermentum
77BF74	Pediococcus pentosaceus	98.87%	NR_042058.1 Pediococcus pentosaceus
77BF75	Pediococcus pentosaceus	98.96%	NR_042058.1 Pediococcus pentosaceus
77BF78	Lactococcus lactis	98.86%	NR_116443.1 Lactococcus lactis
77BF79	Lactococcus lactis	99.78%	NR_113960.1 Lactococcus lactis

Table 5: LAB isolates Identified through Similarity search.

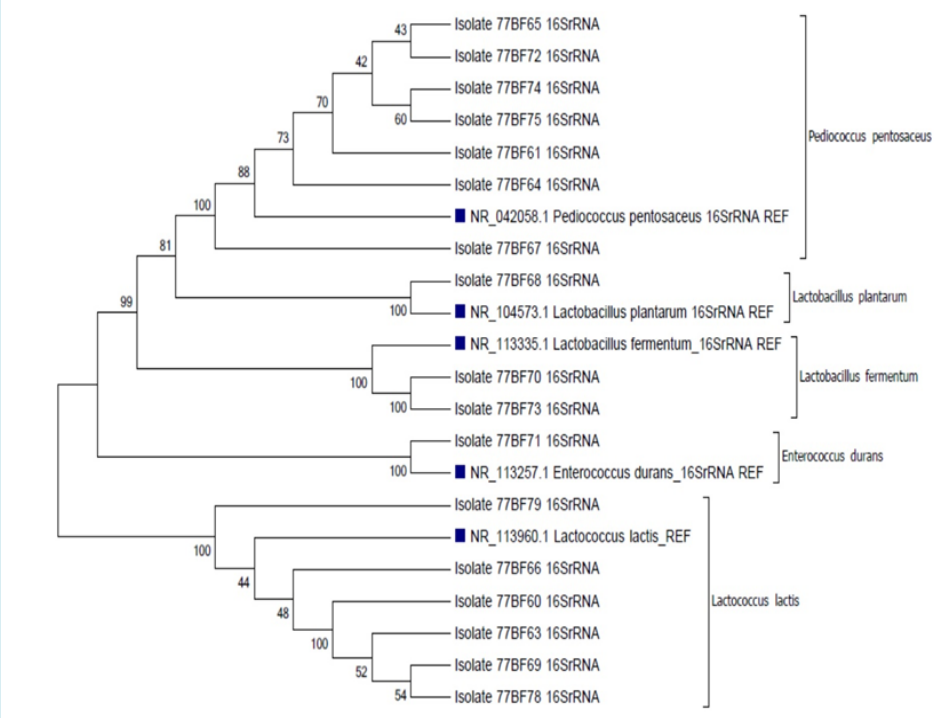

Figure 5: Phylogenetic tree constructed shows the relationship between the LAB isolates together with "the perfect match"/ reference.

Based Similarity index, isolate 77BF61, 77BF64, 77BF65, 77BF77, 77BF72, 77BF64 and 77BF75 were identified as *Pediococcus pentosaceus*, 77BF68 was identified as *Lactobacillus plantarum*, 77BF70 and 77BF73 was identified as *Lactobacillus fermentum*, 77BF71 was identified as *Enterococcus durans* while 77BF60, 77BF63, 77BF66, 77BF69, 77BF78 and 77BF79 were identified as *Lactococcus lactis*. Phylogenetic tree also shows that based 16S rRNA, *Pediococcus* genus is more closely to Lactobacillus genus compared to *Enterococcus* and *Lactococcus* genus. From the tree, *Pediococcus* and Lactobacillus species form a monophyletic group which is well supported by a bootstrap value of 99.

Discussion & Conclusion

Production Process

The production process of *Muratina* has been briefly been described in Figure 1. The curing of *Kigelia africana* fruits that act as microbes immobilizer has been studied and documented before [23]. Through the curing process it is presumed that there is natural selection of microbes which tends towards a pure culture immobilized in the *Kigelia africana* fruits. This is indicated by the presence of few strains compared to other spontaneously fermented products studied before [7,23]. In this study less than 10 strains were isolated with yeast strain being only Saccharomyces cereviseae. To maintain high temperatures of 30°C, the brewers either remove the gourd out in the sun when it's sunny or they place the gourd near the fire place during the process of fermentation. The product is ready in 3-5 days after which it is filtered and consumed. The optimization results can be seen in the high alcohol content of almost 20%, which is comparable to commercial wines. This process is similar to other traditionally fermented wines studied before [1,3,24-26].

Chemical Characteristics

Muratina has an alcohol content of 19.66± 0.47 (mL/100ml) , pH of 4.06 ± 0.12 and titratable acidity of 7.57± 0.45 (g tartaric acid/100 mL). The alcoholic content of *muratina* seems higher than other traditional wines studied before while the pH and titratable acidity were comparable [1,24-29]. This suggests that the yeast cultures from *Muratina* could possess high alcohol content tolerance and hence very good candidates for starter cultures in wine manufacture.

Microbiological Quality

Microbial diversity in *muratina* was observed to be very low. Only 4 strains of LAB and only one strain of yeast (Saccharomyces cerevisiae) were isolated. This could be due

to the immobilization of these strains in *Kigelia africana* fruit. Strains of Saccharomyces cerevisiae and Lactobacillus plantarum have been reported before as responsible for spontaneous fermentation of wines [4,24,25,30]. The lack of diversity has been reported before by Gangl, et al. [3], this could be due to the fact that not many microorganisms are adapted to the high alcohol content in wines.

With alcohol content of approximately 20%, it is expected that only strains with high osmotic potential can survive. Lactobacillus and Lactococcus has been isolated before in high alcohol content media [31]. It is able to survive higher alcohol content. The ability of yeasts to withstand harsh environment such as high osmotic pressure and high alcohol content makes them key fermenting microorganisms of most traditional brews and commercial ones too [32-35]. The presence of Kigelia African fruits could the primary source of Lactobacillus *spp*. since it is predominantly found in plants as the name suggests. It has also been isolated in wines and suggested to play a role in malolactic fermentation in wine making [36-38]. Hence the presence of LAB strains is very consistent with earlier studies. LAB plays a major role in malolatic fermentation, a process that is responsible for aroma in wines [36-40]. The microbial balance in *muratina* could explain its popularity even if it is spontaneously fermented. It tends to have balanced characteristics of flavor/aroma and alcohol content. The microbial balance between the yeasts and lactic acid bacteria could play a role in this.

The strains isolated from *Muratina* also showed high resistance to bile salts which suggests that they could be good candidates as probiotics. The probiotic potential of these identified strains need be further investigated. Strains isolated from wine have been tested for probiotic potential before [25,41,42]. It would hence be worthwhile to carry on more studies and identify more suitable candidates from these *muratina* isolates.

Conclusion

The study has showed that microbial populations mi *muratina* shows a very low diversity towards few strains which seem to play specific roles in the *muratina* fermentation. In our study, *Saccharomyces cerevisiae* was the major fermenting microorganism in combination with LABs. This is a very unique natural selection where the yeast *S. cerevisiae* produces ethanol and LAB *spp* is responsible for malo-lactic fermentation. Our studies show that *muratina* has high alcohol content comparable to other commercial brands. This suggests that the yeast strains there in could be good candidates for starter culture development. We however recommend further studies with the isolated strains to test their performance individually and in combination. There is also need to have a microbiome study to capture all the microbes taking part in the fermentation of *muratina*.

This property indicates that isolates from honey wine could survive under gastric conditions and hence could be good candidates for probiotics. Presence of high numbers of *Saccharomyces cerevisiae* yeast strains indicated that the yeast was able to colonize and become dominant. The isolates could be purified and used as starter cultures since they produce and survive in high alcohol content. The role of *Kigelia africana* fruit in the fermentation of *muratina* also need to be investigated.

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