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Fermentative Microbes of *Khadi*, a Traditional Alcoholic Beverage of Botswana

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Abstract: *Khadi* is a popular traditional alcoholic beverage in rural households in Botswana. The product is produced by fermentation of ripened sun-dried *Grewia flava* (Malvaceae) fruits supplemented with brown Table Sugar. Despite its popularity, its growing consumer acceptance, its potential nutritional value, and its contribution to the socio-economic lifestyle of Botswana, the production process remains non-standardized. Non-standardized production processes lead to discrepancies in product quality and safety as well as varying shelf life. Identification of unknown fermentative microorganisms of *khadi* is an important step towards standardization of its brewing process for entrance into commercial markets. The aim of this study was to isolate and identify bacteria and yeasts responsible for fermentation of *khadi*. Yeasts and bacteria harbored in 18 *khadi* samples from 18 brewers in central and northern Botswana were investigated using classic culture-dependent techniques and DNA sequencing methods. Additionally, we used the same techniques to investigate the presence of bacteria and yeasts on six batches of ripened-dried *G. flava* fruits used for production of the sampled brews. Our results revealed that *Saccharomyces cerevisiae* closely related to a commercial baker's yeast strain sold locally was the most predominant yeast species in *khadi* suggesting a possible non-spontaneous brewing process. However, we also detected diverse non-*Saccharomyces* yeasts, which are not available commercially in retail shops in Botswana. This suggests that spontaneous fermentation is partially responsible for fermentation of *khadi*. This study, presenting the first microbiological characterization of a prominent traditional alcoholic beverage in Botswana, is vital for development of starter cultures for the production of a consistent product towards the commercialization of *khadi*.

Keywords: fermentation; yeast; bacteria; *Grewia flava* fruits; *khadi*; traditional alcoholic beverage

1. Introduction

The African continent has a variety of traditional alcoholic brews from both cereal- and non-cereal-based substrates [1,2]. Africans of diverse cultures, have used a variety of indigenous substrates that underpin production of novel alcoholic drinks [3]. There is, however, a scarcity of information on the microbial composition of these popular brews in sub-Saharan Africa. In Botswana, a number of traditional alcoholic brews, which include *bojwalwa-jwa-setswana*, *sekhokho*, *setopoti*, *bojwalwa-jwa-morula*, *muchema* and *khadi* dominate household and village markets but they have limited production information or completely lack scientific documentation.

Khadi is a popular traditional alcoholic beverage well known for its nutritional value and socio-cultural importance by many in Botswana [4]. *Khadi* is often made from uncontrolled spontaneous fermentation of wild, ripened and sun-dried fruits of *Grewia flava* (family: Malvaceae) shrubs [5]. Instead of using *G. flava* fruits, *khadi* can also be made from other species of *Grewia*, such as *G. occidentalis* and *G. flavascens* known as *moretlwa* and *mokgomphatha* in the local *Setswana* language, as well as from tubers of *Kedrostis hirtella* (family: Cucurbitaceae, *mogakangwaga*) and *Khadia acutipetala* (family: Aizoaceae, *khodi*) [6]. In some cases, substrates listed above can be mixed to produce *khadi* with distinct sensory properties. The fruits are soaked in warm water and supplemented with brown Table Sugar. After cooling, previously fermented *G. flava* fruits are added to the mixture (back-slopping technique) before fermentation commences. The back-slopping process is an inoculum enrichment practice, which involves the use of *G. flava* fruits collected from the previous batch. These fruits then serve as the source of the active starter cultures, and the procedure is repeated in a limited iterative process. Ethanol content of this alcoholic beverage has been reported to range from 1.7% to 5.7% (*v/v*) [7]. The World Health Organization's global status report on alcohol [8] described *khadi* as a 'designer alcohol' because the ingredients could be varied to suit consumers' preferences. It is this unique attribute which gives *khadi* a competitive edge among regional and international alcoholic beverages sold in Botswana.

The major drawbacks of this traditional alcoholic beverage are batch-to-batch differences of product quality, safety and shelf life due to variability in raw materials and non-standardized production methods. The microbiota that drive fermentation also remain unknown. There are no published reports of studies on yeasts and bacteria responsible for *khadi* fermentation. Conventional and non-conventional yeasts are well known to play an important role in alcoholic fermentations, whereas bacteria are known to be involved in enhancing functional properties of wine through non-alcoholic fermentation [2,9–13]. Isolation and identification of microorganisms responsible for fermentation of *khadi* is indispensable for the potential development of standardized fermentation processes. This is important for the improvement of fermentation efficiency and production of a quality beverage that can potentially compete in regional and international markets. The aim of this study was to isolate and identify bacteria and yeasts responsible for fermentation of *khadi*.

2. Materials and Methods

2.1. Sample Collection

During the survey, 250 g of ripened sun-dried *G. flava* fruits and 500 mL *khadi* were randomly collected from local *khadi* brewers. Local fruit harvesters supplied these fruits. Collection of samples was only undertaken in central and northern towns and villages of Botswana (Tonota, Palapye, Serowe, Letlhakane, Mmashoro and Maun) (Figure 1 and Table S1) because *khadi* brewers in the southern part of Botswana use tubers instead of *G. flava* fruits for fermentation of *khadi*. A total of 6 batches of ripened sun-dried *G. flava* fruits and 18 *khadi* samples were collected. Fruit samples were collected aseptically using sterile zip-lock bags whereas *khadi* samples were collected in sterile 500 mL bottles. Samples were transported to the laboratory at a temperature of 0–4 °C in a cooler box with frozen ice packs before analyses. All samples were analyzed at most 6 hours after collection. All the *khadi* samples were purchased from brewers at a stage when they felt that *khadi* was ready for sale and consumption i.e., after the completion of fermentation.

2.2. Enumeration of Yeasts and Bacteria

2.2.1. *Grewia flava* Fruits

A maximum of 5 ripened sun-dried fruits from each of the fruit batches were crushed and re-suspended in 2 mL PBS (phosphate buffered saline). The mixture was then vortexed for 5 min and stored on ice. To isolate yeasts, 1 mL from each sample was pelleted in a micro-centrifuge for 1 min at 14,000× *g*. The pellets were then re-suspended in 200 µL lysozyme solution (10 mg/mL lysozyme;

20 mM Tris-HCl; 2mM EDTA; 1% Triton X-100 (pH 8)) and maintained at room temperature for 30 min to lyse the bacteria [14]. 100 µL of 10-fold serially diluted samples (in 1 × PBS) was spread plated on yeast peptone dextrose (YPD) agar (2% glucose; 1% yeast extract; 2% peptone; and 1.5% agar, at a pH of 6.2) supplemented with a cocktail of antibiotics (100 µg/mL streptomycin, 100 µg/mL penicillin; and 100 µg/mL ampicillin) for selective growth of yeasts before enumeration. The plates were incubated at 30 °C for 48 to 72 hrs. After incubation, colony-forming units (CFU) were counted and expressed as log colony-forming units per milliliter ($\log_{10}$ CFU/mL). For further studies, representatives of morphologically distinct colonies were picked, observed under a microscope (DE/Axioplan 2; Carl Zeiss, Oberkochen, Germany), sub-cultured twice on YPD plates and then cryopreserved at −80 °C in 25% glycerol. To isolate bacteria, the samples were serially diluted and spread on Luria broth (LB) agar (1% NaCl; 1% peptone; 0.5% yeast extract; and 1.5% agar at a pH of 7.4) supplemented with 10 µg/mL of cycloheximide to select against yeasts. After incubation at 37 °C for 24 to 72 hrs, a minimum of 5 representatives of different bacteria and yeast colonies were purified and stored as mentioned above. The experiments were performed in triplicate.

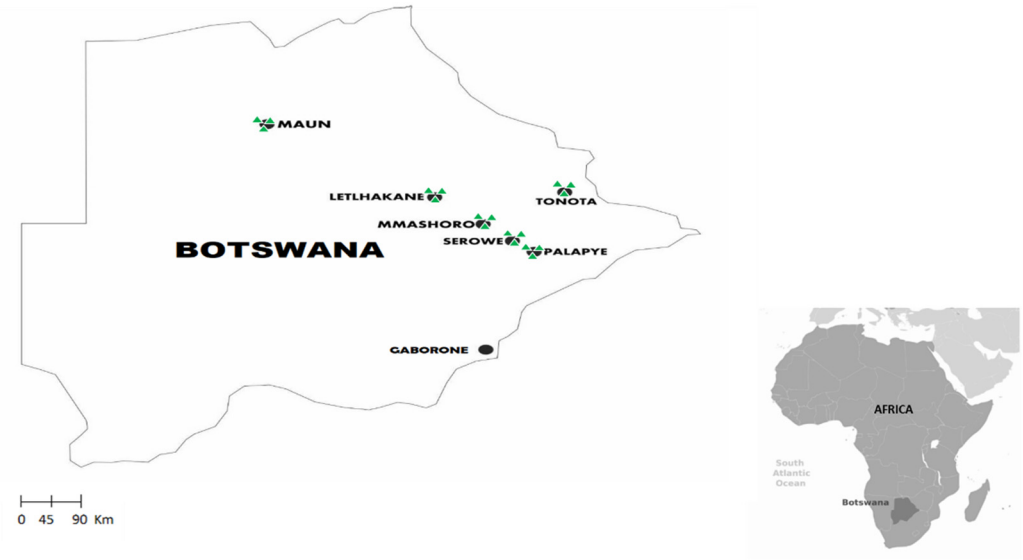

Figure 1. Map of sampling areas across Botswana. The map shows the locations in the central and northern parts of Botswana (black dots) from which ripened sun-dried *G. flava* fruits and *khadi* were collected. The green triangles show the number of replicates per location.

2.2.2. *Khadi*

Aliquots from all *khadi* samples were 10-fold serially diluted in PBS and kept on ice. 100 µL of the aliquots (10^0 to 10^{-6}) were plated on either antibiotic supplemented YPD or antifungal supplemented LB agar media to enumerate yeasts or bacteria, respectively, as described in the section above (2.2.1). De Man, Rogosa and Sharpe agar (MRS agar) plates (CONDA Laboratory, Spain) supplemented with 10 µg/mL of cycloheximide were used to determine and enumerate lactic acid bacteria (LAB). Plates were incubated at 37 °C under partial anaerobic conditions (10% of CO_2) for 72 hrs. Colonies were counted and expressed as log colony-forming units per milliliter ($\log_{10}$ CFU/mL). The experiments were performed in triplicate.

2.3. Identification of Yeast and Bacteria Using Polymerase Chain Reaction (PCR)

2.3.1. Yeasts

A single colony from each of the yeast isolates with distinct morphological features was picked and purified by re-streaking on YPD agar plates. These colonies were then re-suspended in 50 µL of

sterile deionized water. The suspension was then boiled at 98 °C for 15 min and then centrifuged for 1 min at 16,000× *g* using a micro-centrifuge. Two µL of the supernatant was then used as a template for polymerase chain reaction (PCR) amplification of a 560 bp DNA fragment of the D1/D2 region of the 26S rDNA using universal PCR primers, NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) as reported elsewhere [15,16]. PCR amplification was performed using ProFlex PCR Systems (Applied Biosystems, USA) in a 20 µL reaction volume containing 2.5 µL 10× PCR buffer, 2.0 µM of each primer, 0.2 mM dNTPs, and 1.25 U *Taq* DNA polymerase (Takara Bio Inc., Japan) using the following cycling conditions: initial denaturation at 98 °C for 3 min; 38 cycles of denaturation (98 °C for 15 s), annealing (54 °C for 1 min), and extension (72 °C for 1 min), a final extension step at 72 °C for 7 min and held at 4 °C until required. Negative controls in which the template DNA in the PCR mixture was replaced with sterile distilled water were also included. All amplicons were purified using a QIAquick PCR product purification kit (Qiagen, GmBH, Germany) according to manufacturer´s instructions. The amplicons were sequenced by Inqaba Biotech (Pretoria, South Africa). SnapGene®Viewer software ver. 4.2.11 (GSL Biotech) sequence editing tool was used to generate contiguous sequences (http://www.snapgene.com). Species identification was done by using nucleotide Basic Local Alignment Search Tool (BLASTn) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) as well as using pairwise identification tool available on the Westerdijk Fungal Biodiversity Institute strain database, (http://www.westerdijkinstitute.nl/). A commercial baker's yeast (*S. cerevisiae*) obtained from a local supermarket was used as the control in this study (Anchor Yeast, South Africa). It should be noted that there are no retail outlets in Botswana that sell commercial brewer's yeast that could have been used as another control for this aspect of the study.

2.3.2. Bacteria

Genomic DNA was extracted using the GenElute ™ Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) according to manufacturer's instructions. The extracted genomic DNA was used for PCR amplification as in Section 2.3.1, except that 16S rDNA was amplified using a different pair of universal primers, pAF (5′-AGAGTTTGATCCTGGCTCAG-3′) and pER (5′-CCGTCAATTCCTTTGAGTTT-3′) [17] and the following cycling conditions; initial denaturation at 98 °C for 30 s, 35 cycles of denaturation (98 °C for 30 s), annealing (47 °C for 30 s), and extension (72 °C for 30 s), a final extension step at 72 °C for 7 min and held at 4 °C ∞. Negative controls were as above. PCR product purification, sequencing and identification of bacteria were done as in Section 2.3.1.

2.3.3. Phylogenetic Analyses

To determine the relationship of the microbial isolates from the *G. flava* fruits and *khadi* samples, we used MEGA 7.0.26 software [18]. The sequences were aligned using MUSCLE within the MEGA software and then the aligned sequences were used to calculate the best maximum likelihood model (Jukes–Cantor, Kimura 2-parameter, Tamura 3-parameter, Hasegawa–Kishino–Yano, Tamura–Nei and General time reversible models) to be used to construct the phylogenetic tree [19].

2.3.4. *In Silico* PCR-Restriction Fragment Length Polymorphism (RFLP) to Profile Yeasts Driving *Khadi* Fermentation

We sought to investigate whether the *khadi* brewers used a strain of a commercial yeast species as starter cultures or whether they depend on naturally occurring yeasts. To do so, we performed an *in silico* PCR-RFLP (restriction fragment length polymorphism) test using the D1/D2 domain of the 26S rDNA amplicons with a SnapGene®Viewer software ver.4.2.11 (GSL Biotech) (http://www.snapgene.com). The inbuilt gel electrophoresis simulation option with a set parameter of 4% agarose gel option was chosen for visualization after an *in-silico* digestion with the following restriction enzymes; *Glu*I, *Hae*III, *Hinf*I and *Rsa*I. pUC19 – *Sau*3AI digest was selected as a molecular weight ladder to compare the sizes of the restricted fragments.

The D1/D2 domain of the 26S rDNA sequences of the commercial yeast species, namely ale yeast (*Saccharomyces cerevisiae*, SafAle T58, Fermentis, France), lager yeast (*Saccharomyces pastorianus*, Lallemand Brewing, Austria) and wine yeast (*Saccharomyces cerevisiae*, Lalvin EC-1118 and RC-212) were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/). These were ran in parallel with commercial baker's yeast (*Saccharomyces cerevisiae*, Anchor Yeast, South Africa). Restriction fragment patterns of non-*Saccharomyces* yeasts found in *khadi* from across Botswana were also investigated using in silico RFLP as detailed above.

2.4. Ethanol Assays

Khadi samples were centrifuged and the resultant cell-free supernatant was used to determine the ethanol content. An enzymatic ethanol assay kit (K-ETOH 08-18, Megazyme, Ireland) was used according to the manufacturer's instructions. The samples were analyzed in triplicate.

2.5. Statistical Analyses

One-way analysis of variance (ANOVA) was used to compare differences among the colony forming units of *khadi* samples (Microsoft Office 2019, USA).

3. Results and Discussion

3.1. Abundance of Yeasts and Bacteria from Grewia Flava Fruits and Khadi

3.1.1. *Grewia flava* Fruits

Grewia flava fruits play an important role in *khadi* fermentation in northern Botswana as the source of the fermenTable Sugars in addition to supplemented table brown sugar. These fruits could also be the source of fermenting microorganisms during spontaneous fermentation. For this reason, we enumerated the yeasts and bacteria found on ripened and sun-dried *G. flava* fruits used to produce *khadi*. We noted that in each area there was a single supplier of locally collected batches of *G. flava* fruits and hence only one batch of fruits per town or village was used for investigation. There were lower numbers of yeasts as compared to bacteria. On average, 1.0×10^3 $\log_{10}$ CFU/mL of yeasts were observed as compared to 3.5×10^3 $\log_{10}$ CFU/mL colonies of bacteria (Table 1). The colonization of fruits by bacteria and yeasts is well described [20] but the low number of both bacterial and yeast colonies observed could be attributed to the storage and processing of the fruits. The drying and storage of the fruits before fermentation could have created a selective environment for proliferation of relative few yeasts and bacteria with specific attributes that allow them to survive in a new environment [21].

Table 1. Abundance of yeasts and bacteria isolated from the ripened sun-dried *G. flava* fruits samples expressed in Log $_{10}$CFU/mL (colony forming units per millimeter).

Sample	$\log_{10}$ CFU/mL	
	Yeasts	**Bacteria**
Letlhakane	3.0	3.6
Maun	3.0	3.5
Mmashoro	0	3.6
Palapye	3.3	3.3
Serowe	3.0	3.6
Tonota	3.0	3.6
Average	2.6	3.5

3.1.2. *Khadi*

After the enumeration of yeasts and bacteria on dried fruits, we sought to perform the same enumeration in the final fermentation product, *khadi*. Interestingly, even though bacteria were found

on dried fruits, none were found in all *khadi* samples investigated. Although the role of bacteria during the progression of fermentation in other alcoholic beverages has been described [22,23], their role during the progression of *khadi* fermentation was not investigated in this study. Since the ripened sun-dried fruits were sampled before fermentation and the final product after fermentation, the absence of bacteria at the end of fermentation could be the due to their role in the early stages of fermentation, their inhibition by the accumulation of ethanol, as well as out-competition by yeasts. The total number of yeasts on the *khadi* samples ranged from 5.1 ± 0.09 $\log_{10}$ CFU/mL (Mmashoro) to 6.4 ± 0.22 $\log_{10}$ CFU/mL (Palapye) (Figure 2). On average, 5.6 ± 0.57 $\log_{10}$ CFU/mL of yeasts were recorded. The yeast CFU recorded from the ripened and dried *G. flava* fruits were lower as compared to those recorded from the *khadi* samples. There was no statistically significant difference in concentrations of yeasts at the end of fermentation among the 18 *khadi* samples (one-way ANOVA; $p < 0.05$).

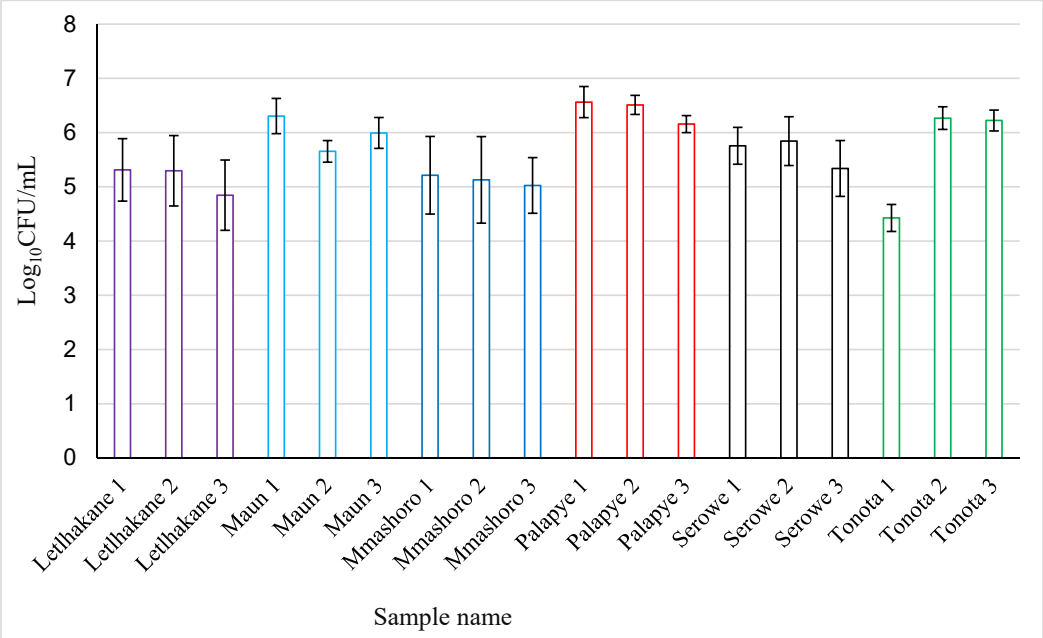

Figure 2. Variability of amounts of yeast ($\log_{10}$ CFU/mL) isolated from *khadi* samples from different parts of the central and northern Botswana. Six samples were collected in triplicate and analyzed individually. Color code: purple = Letlhakane, sky blue = Maun, navy blue = Mmashoro, red = Palapye, Black = Serowe and green = Tonota.

3.2. Diversity of Microbial Isolates from Grewia Flava Fruits

3.2.1. Yeasts

The diversity of resident communities of yeasts on the dried fruits have the potential to shape the fermentation and the outcome of the alcoholic beverage. We therefore sought to examine the diversity of yeasts on dried *G. flava* fruit surfaces by sequencing the D1/D2 domains of the 26S rDNA. Six species of yeast-like fungi and yeasts dominated the fruit samples, namely *Aureobasidium leucospermi*, *Aureobasidium melanogenum*, *Naganishia diffluens*, *Aureobasidium* spp., *Aureobasidium pullulans* and *Saccharomyces cerevisiae* (Table 2). All of the above species except *S. cerevisiae* have been previously reported to tolerate arid environments [24] typical of the Botswanan climate. The identification of these fruit resident yeasts posed a question of whether they could be responsible for fermentation of *khadi*. Interestingly, *Aureobasidium* spp. have been reported to be among the main non-conventional yeasts known to be involved in spontaneous alcoholic fermentations of wine, beer, tequila, mezcal and cachaça [25,26]. Although basidiomycetous yeasts have been isolated from fruits and fermented products [2,27], their role in *khadi* fermentation remains unknown. *S. cerevisiae*, a well-known fermenting

yeast inhabiting sugar rich environment such as wild fruits, was only isolated from Letlhakane samples. Its rarity and presence in low frequencies on fruits before fermentation commences is not surprising [28–32]. The species eventually dominates fermentation after engineering the ecosystem through its robust make-accumulate-consume fermentative lifestyle irrespective of the presence of oxygen [30–33].

Table 2. Identity of yeasts isolated from ripened and dried *G. flava* fruits.

Species Name	Isolate Number	Collection Number	Accession Number	Identity (%)
Aureobasidium leucospermi	D2	Palapye	KX893326.1	99
Aureobasidium melanogenum	D3	Serowe	MF370933.1	99
Aureobasidium pullulans	D6	Tonota	MF979210.1	99
Aureobasidium spp.	D5	Maun	KT361586.1	99
Naganishia diffluens	D4	Palapye	KU316762.1	99
Saccharomyces cerevisiae	D7	Letlhakane	HQ108377.1	99

3.2.2. Bacteria

Bacteria are known to play a role in enhancing the functional properties of alcoholic beverages such as wine, through non-alcoholic fermentation, producing products such as organic acids which impart complex flavor profiles [22,23]. Bacteria are sympatric to yeasts on sugar rich environments such as fruits [26,34]. We sought to investigate the diversity of bacteria on *G. flava* fruits as they likely contribute to shape the quality of the end product, *khadi*. A total of 21 phylogenetically diverse bacterial isolates (Table 3) were recorded. *Bacillus* species surprisingly dominated the isolates accounting for 76.2% of the identified isolates (16 out of 21). The dominance of *Bacillus* species could be due to the isolation media we used in this study, method of handling and storage of fruits, which probably favored their proliferation or their natural abundance on *G. flava* fruits. It should be noted that ripened *G. flava* fruits are sun dried and packaged in repurposed bags before being sold to local brewers. This suggests that the processing of ripened sun-dried fruits probably selects for ultraviolet (UV)-resistant spores of *Bacillus* species [35] which then dominate the dried fruits isolates. *Acinetobacter lwoffii*, *Desemzia incerta*, *Exiguobacterium indicum* and *Staphylococcus saprophyticus* were the only non-*Bacillus* bacteria isolated from ripened and dried fruits. *A. lwoffii* is associated with skin microbiota probably introduced during the processing of fruits [36,37]. *Staphylococcus saprophyticus* has been reported to inhabit a fruit niche [26,38], hence could be associated with *G. flava* fruits in this study. We did not detect any lactic acid bacteria (LAB) from the fruits.

Table 3. Identity of bacteria isolated from *Grewia flava* fruits based on D1/D2 region of the 16S rDNA.

Species Name	Isolate Number	Collection Number	Accession Number	Identity (%)
Acinetobacter lwoffii	MMB4	Mmashoro	KF818633.1	99
Acinetobacter lwoffii	SB2	Serowe	KF818633.1	99
Bacillus amyloliquefaciens	PB1	Palapye	MG892875.1	99
Bacillus cereus	TB3	Tonota	AB523744.1	99
Bacillus cereus	LB4	Letlhakane	MG021182.1	99
Bacillus methylotrophicus	SB1	Serowe	KM659219.1	99
Bacillus oleronius	LB1	Letlhakane	KY773585.1	98
Bacillus pichinotyi	PB2	Palapye	MG705701.1	98
Bacillus amyloliquefaciens subsp. *plantarum*	MMB2	Mmashoro	JN661699.1	99
Bacillus spp.	MAB3	Maun	KF646681.1	99
Bacillus spp.	TB4	Tonota	KT443870.1	99
Bacillus thuringiensis	SB3	Serowe	KY910253.1	99
Bacillus thuringiensis	LB3	Letlhakane	KY495218.1	99
Bacillus simplex	LB2	Letlhakane	AB547125.1	99
Bacillus subtilis	MMB3	Mmashoro	MH261154.1	99
Bacillus subtilis	SB4	Serowe	MH261154.1	98
Bacillus subtilis	TB1	Tonota	MH261154.1	99
Bacillus velezensis	MMB1	Mmashoro	MH000677.1	99
Desemzia incerta	TB2	Tonota	KF712891.1	99
Exiguobacterium indicum	MAB1	Maun	MH819520.1	93
Staphylococcus saprophyticus	MAB2	Maun	CP022093.2	99

3.3. Diversity of Yeasts from the Fermented Brew, Khadi

Mainly Saccharomycotina and a few Basidomycota yeasts were isolated from all the brews. This observation was not surprising, as Saccharomycotina yeasts are known to engineer the environment that favors their proliferation through fermentation [30]. Alcoholic fermentation is a niche reconstruction lifestyle of Crabtree positive yeasts which creates heat, CO_2 and ethanol to inhibit the growth of competitors [30,33,39]. The limited diversity of yeasts on the dried fruits in comparison to the wide diversity of bacteria prompted further investigation to establish which microorganisms could be responsible for the fermentation of *khadi*. A total of 74 yeast isolates with a wide phylogenetic diversity were recorded from different *khadi* samples (Table 4, Figure 3). The isolates belonged to 13 yeast species (Table 5). Interestingly, most of those yeasts were not isolated from the ripened sun-dried fruits. There are two possibilities to explain this, one is that there was a source of yeasts other than those from the wild fruits, and the other is that those yeasts were rare on the fruit surfaces. *S. cerevisiae* dominated the isolates (51.4%) followed by *Candida ethanolica* (12.2%) and *Saccharomycodes ludwigii* (10.8%) (Tables 4 and 5). The dominance of *S. cerevisiae* at the end of the alcoholic fermentation, despite the dominance of non-conventional yeasts and bacteria, during the early stages of fermentation is well documented [25,30,32]. These results suggest that *khadi* is a product of mixed-culture fermentation with phylogenetically diverse yeasts dominated by *S. cerevisiae* (Table 4).

Mixed-culture fermentations made up of conventional and non-conventional yeasts leads to distinctive aromatic profiles of alcoholic beverages [40–44]. The production of aroma compounds in alcoholic beverages also requires non-conventional yeasts. For example, *Lachancea* spp. are well-known producers of fruity esters, which impart a characteristic fruity flavor to alcoholic brews [45–47]. Fermentative species of the genus *Candida*, such as *C. ethanolica* and *C. sake*, also known to generate unique flavors of a brew, were isolated [48] in brews from Letlhakane, Tonota and Maun. However, the isolation of spoilage yeasts, such as *Brettanomyces* spp. and *Zygosaccharomyces* spp. [49–51], in samples from Serowe and Tonota, respectively, could negatively impact on the quality of a spontaneously fermented brew. *Brettanomyces bruxellensis* has been isolated from beverages such as wines which characteristically develop unpleasant and distinctive aromas due to the production of volatile phenolic compounds and diacetyl. These "Brett taints" are normally associated with the smell of barnyards, burnt plastic, wet animals and horse sweat [50,52–54], but on a positive note, *Brettanomyces* spp. can also be used in the production of Lambic beers [55]. *Brettanomyces* spp. have been reported to produce high amounts of ethyl esters which contribute to the overall fruity or floral character of the beverage [56]. *Zygosaccharomyces bailii* is a wine spoilage yeast associated with grapes whose spoilage is characterized by generation of taints, odors, off-flavors, development of hazes, and excessive gas production [26,49]. Research also shows that *Z. bailii* can improve the flavor complexity of alcoholic beverages either used individually or as a mixed culture with *S. cerevisiae* producing flavor compounds such as alcohols, acids, esters, ketones and aldehydes [57]. A concern for spontaneously fermented brews is the presence of clinically relevant species. *Pichia kudriavzevii* (also known as *Candida krusei* [58]) was the only potentially opportunistic pathogen [58] that was isolated and it was found in a Letlhakane sample. However, *P. kudriavzevii* also has good fermentation characteristics and it has been previously isolated from *taruba*, grapes and *masau* fruits [1,32,59].

Table 4. Identity of yeast isolates from the *khadi* samples based on D1/D2 domains of 26S rDNA.

Species Name	Isolate Number	Collection Number	Accession Number	Identity (%)
		***Saccharomyces* yeasts**		
Saccharomyces cerevisiae	Z2	Letlhakane 1	KX428522.1	99
Saccharomyces cerevisiae	L3	Letlhakane 1	MG773372.1	99
Saccharomyces cerevisiae (2)	L6 and L10	Letlhakane 2	MG017580.1	99
Saccharomyces cerevisiae	L7	Letlhakane 2	LC336457.1	99
Saccharomyces cerevisiae	L8	Letlhakane 3	MF979228.1	99
Saccharomyces cerevisiae	L11	Letlhakane 3	HM191639.1	99
Saccharomyces cerevisiae	LMA1	Letlhakane 2	MF979228.1	99
Saccharomyces cerevisiae	LMA3	Letlhakane 2	KY109286.1	99
Saccharomyces cerevisiae	LMA4	Letlhakane 1	CP022977.1	99
Saccharomyces cerevisiae	LMA5	Letlhakane 2	KM234472.1	99
Saccharomyces cerevisiae	LMB1	Letlhakane 2	KX119942.1	99
Saccharomyces cerevisiae	MA1	Maun 1	KX428522.1	99
Saccharomyces cerevisiae	MA6	Maun 2	HM107789.1	99
Saccharomyces cerevisiae	AC1MIII	Mmashoro 3	MG641152.1	99
Saccharomyces cerevisiae (2)	AC2MIII and BC1MII	Mmashoro 3 and 2	HM101473.1	99
Saccharomyces cerevisiae	AC3MIII	Mmashoro 3	MF406146.1	99
Saccharomyces cerevisiae	AC5MIII	Mmashoro 3	JN225410.1	99
Saccharomyces cerevisiae	BC4MII	Mmashoro 2	JX141335.1	99
Saccharomyces cerevisiae	MIAC1	Mmashoro 1	KM103041.1	99
Saccharomyces cerevisiae	MIAC3	Mmashoro 1	MF406146.1	99
Saccharomyces cerevisiae	MIAC2	Mmashoro 1	KM103042.1	99
Saccharomyces cerevisiae	MIAC5	Mmashoro 1	MK027354.1	99
Saccharomyces cerevisiae	P10	Palapye 1	KY109242.1	99
Saccharomyces cerevisiae	P11	Palapye 1	HQ443692.1	99
Saccharomyces cerevisiae	P13	Palapye 3	HM101472.1	99
Saccharomyces cerevisiae (2)	P14 and P16	Palapye 2	KX098507.1	99
Saccharomyces cerevisiae	P17	Palapye 3	GU080046.1	99
Saccharomyces cerevisiae (2)	S2 and S7	Serowe 1	GU080049.1	99
Saccharomyces cerevisiae	S6	Serowe 2	GU080046.1	99
Saccharomyces cerevisiae	T5	Tonota 1	MF406147.1	99
Saccharomyces cerevisiae	T6	Tonota 1	HM101472.1	99
Saccharomyces cerevisiae	T8	Tonota 1	JX141335.1	99
Saccharomyces cerevisiae	T9	Tonota 2	MK027354.1	99
Saccharomyces cerevisiae	T11	Tonota 2	MF498873.1	99
Saccharomyces cf. *cerevisiae/paradoxus* (2)	AC4MIII and MIAC4	Mmashoro 1 and 3	KY109333.1	99
		Non-*Saccharomyces* yeasts		
Brettanomyces bruxellensis	S5	Serowe 2	KY107600.1	99
Candida ethanolica	LMB2	Letlhakane 3	KY283163.1	99
Candida ethanolica (2)	LMB4 and LMC1	Letlhakane 1	FM180545.1	99
Candida ethanolica	LMC4	Letlhakane 1	JX880409.1	99
Candida ethanolica (5)	T10, T14 T15, T17, T18	Tonota 2 and 3	JX880409.1	99
Candida sake (2)	MA2 and MA3	Maun 1	JX880410.1	99
Curvibasidium pallidicorallinum	MA7	Maun 3	JX188149.1	99
Pichia kudriavzevii	L13	Letlhakane 3	AY529504.1	99
Pichia kudriavzevii	S8	Serowe 3	AY529504.1	91
Lachancea fermentati	MA4	Maun 2	KY108224.1	99
Lachancea fermentati	MA8	Maun 3	KM234440.1	99
Lachancea fermentati	P18	Palapye 3	KM234440.1	99
Pichia kudriavzevii	L1	Letlhakane 1	KM234442.1	99
Pichia kudriavzevii (2)	L2 and L4	Letlhakane 2	KF214396.1	99
Pichia manshurica	P15	Palapye 2	MK034750.1	99
Rhodotorula nothofagi	Z1	Letlhakane 1	KJ794722.1	99
Saccharomycodes ludwigii (2)	L9 and L12	Letlhakane 3	FM180540.1	99
Saccharomycodes ludwigii (2)	S1 and S4	Serowe 1	FM180540.1	99
Saccharomycodes ludwigii (4)	T7, T13, T12, T16	Tonota 1 and 2	FM180540.1	99
Schizosaccharomyces pombe	P8	Palapye 2	KY296084.1	99
Zygosaccharomyces bailii	T1	Tonota 1	GU080052.1	99
Zygosaccharomyces bailii	T4	Tonota 1	KY296086.1	99

Note: The number in the brackets shows isolates with the name and same accession number.

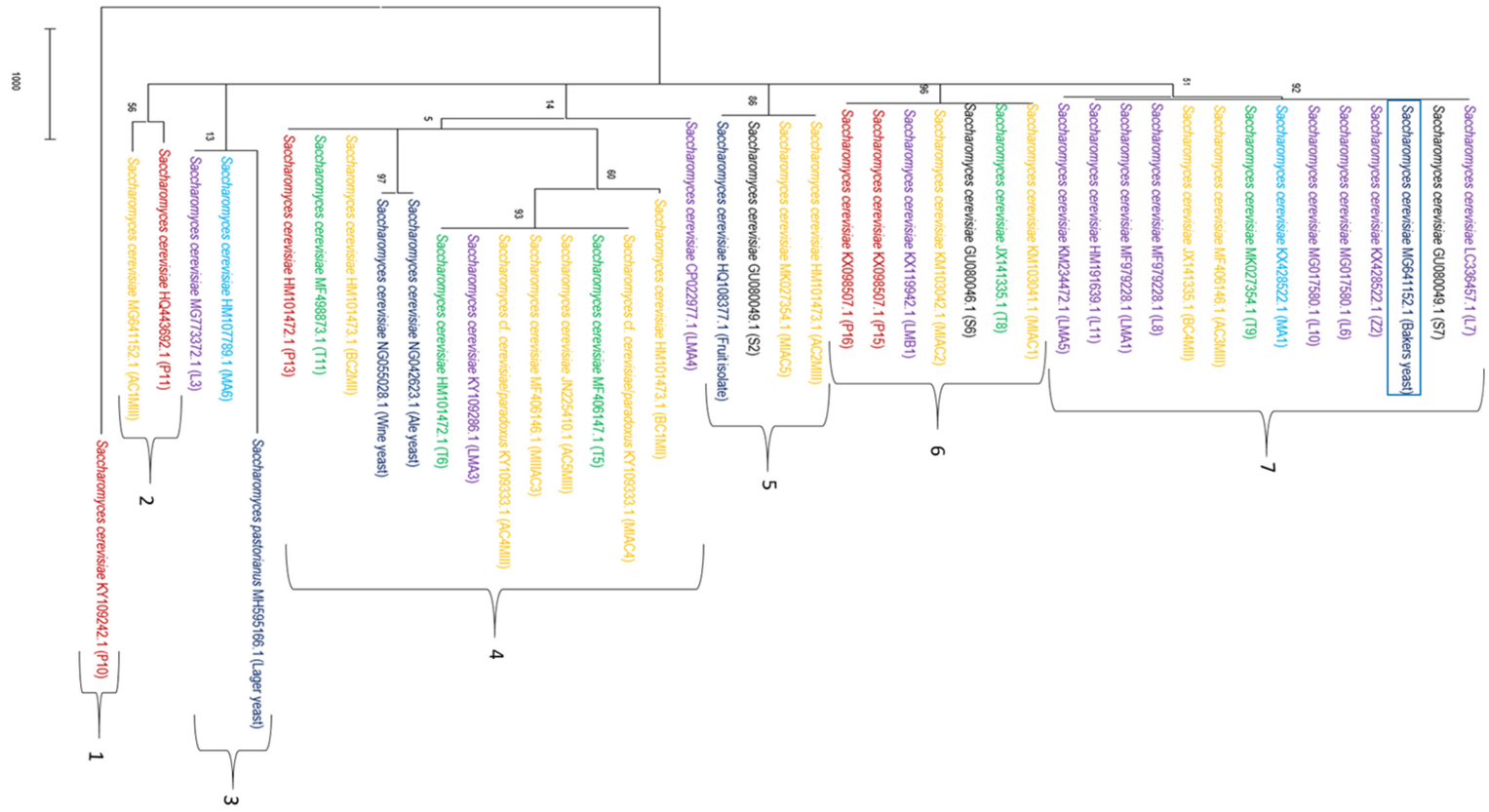

Figure 3. Maximum likelihood analysis of the D1/D2 domains of 26S rDNA of *Saccharomyces* yeasts isolated from *khadi* samples. The evolutionary relationship between isolates was inferred using the maximum likelihood method based on the Hasegawa–Kishino–Yano model [19]. Evolutionary analyses were conducted in MEGA7 [18]. Color code: purple = Letlhakane, sky blue = Maun, yellow = Mmashoro, red = Palapye, Black = Serowe, green = Tonota and navy blue = Controls.

Table 5. Diversity of yeasts from the *khadi* samples.

Species	Number of Isolates Sequenced	%	Letlhakane	Maun	Mmashoro	Palapye	Serowe	Tonota
Saccharomyces cerevisiae	38	51.4	12	2	10	6	3	5
Candida ethanolica	9	12.2	4					5
Saccharomycodes ludwigii	8	10.8	2				2	4
Pichia kudriavzevii	5	6.75	4				1	
Zygosaccharomyces bailii	2	2.7						2
Candida sake	2	2.7		2				
Saccharomyces cf. *cerevisiae/paradoxus*	2	2.7			2			
Lachancea fermentati	3	5.4		2		1		
Curvibasidium pallidicorallinum	1	1.4		1				
Schizosaccharomyces pombe	1	1.4				1		
Brettanomyces bruxellensis	1	1.4					1	
Rhodotorula nothofagi	1	1.4	1					
Pichia manshurica	1	1.4				1		
Total	74	100	23	7	12	9	7	16

One of the expected features of a fermented brew is the presence of *Saccharomyces* yeasts. The phylogenetic tree based on the Hasegawa–Kishino–Yano model in Figure 3 shows the relationship among the *S. cerevisiae* isolates isolated from the *khadi* samples. The tree (Figure 3) suggests that *S. cerevisiae* isolates isolated from different locations are diverse as they cluster into 7 different groups. The results suggest that some brewers could be using commercial ale, baker's yeast, lager and/or wine strains instead of spontaneous fermentation. For example, Group 7 (Figure 3) isolates clustered with a control commercial baker's yeast, which is readily available in local retail shops. In addition, it was noted that some isolates clustered with commercial ale and wine yeasts as shown in Group 4, which are not available in the local supermarkets of Botswana, while two isolates from Maun (MA6) and Letlhakane (L3) clustered in a separate group with the commercial lager yeast. These clusters suggest that commercial yeasts strains have been used as a starter culture. Brewers prefer the addition of starter cultures to circumvent the negative outcomes of spontaneous fermentation, which produces an inconsistent quality of fermented products. Contrastingly, we noted that *S. cerevisiae* strain isolated from *G. flava* fruits (from Letlhakane) clustered with *S. cerevisiae* isolates from *khadi* from Serowe and Mmashoro in Group 5, suggesting that use of spontaneous fermentation could be popular among these two locations. Use of dried fruits from the same supplier in these three locations could also be a possible explanation for the isolation of yeasts that were closely related to isolates from the fruits.

The occurrence of non-conventional yeasts with a wide diversity further suggests that local brewers also depend on spontaneous fermentations other than inoculating with a commercial strain of a yeast species (Table 4). Our assumption is based on the fact that there are no commercial non-conventional yeasts sold in Botswana. The brewers´ back-slopping technique could have been responsible for increasing the frequency of non-conventional yeasts to detectable levels. The use of fruits from the same supplier, therefore, could have been the reason for the sharing of starter cultures among brewers, hence the similarity in some cultures.

3.4. Saccharomyces and Non-Saccharomyces Yeasts Responsible for Fermentation of Khadi

Yeasts play a pivotal role (ethanol production) in fermentation of all alcoholic beverages [60]. Although *S. cerevisiae* species were found in low frequencies on *G. flava* fruits before fermentation, the species dominated the isolates from the fermented product, *khadi*. All samples were collected from spontaneously fermented brews or from back-slopped brews and, therefore, the fermenting *Saccharomyces* spp. could be those that were introduced from the ripened sun-dried fruits or previously fermented fruits (inoculum), as well as those resident on the brewing equipment and the brown Table Sugar as reported elsewhere [61].

The diversity of *S. cerevisiae* species isolated from *khadi* in comparison to the fruits prompted us to further investigate adulteration of the brewing process using readily available commercial *S. cerevisiae* species sold by retail stores in Botswana. The brewers may add commercial baker's yeasts during *khadi* production to give it 'strength' and 'enhance its capacity' to intoxicate, instead of relying on spontaneous fermentations [62]. An investigation into the genetic diversity of the *Saccharomyces* spp. using *in-silico* PCR-RFLP suggests that most of the isolates were not genetically distinct from the commercial baker´s yeast (Figure 4a). To be specific, restriction fragment patterns of 67.5% of the species of this species (27 out of 40 isolates) digested with *Hae*III matched a characteristic restriction fragment pattern of the commercial baker´s yeast strain (Figure 4a and see also Figure S1 and Table S4). On the other hand, 13 unique restriction fragment patterns from Mmashoro (AC1MIII, AC2MIII, AC3MIII, AC5MIII, MIAC2 and MIAC3), from Palapye (P10, P14, P16 and P17) and from Letlhakane (LMA4, LMA5 and Z2), which neither matched the *S. cerevisiae* isolate from fruits nor the commercial baker´s, ale, lager and wine species, were observed (Figure 4a). This suggests that there are other sources of fermenting yeasts unaccounted for in this study. One source of yeasts could be equipment used for brewing and pre-processing. Another possibility is that the yeasts were present on the fruit surfaces at lower frequencies to be isolated using the plate count method. The latter is not surprising because different studies have shown that *S. cerevisiae* is usually found in low frequencies during the beginning

of spontaneous fermentations as compared to other yeasts, but accounts for the highest densities at the end of fermentation [30,33]. On the other hand, there is a possibility that some colonies that had similar macro and microscopic morphology to the *S. cerevisiae* isolate from fruits were overlooked in an effort to minimize potentially repetitive sequencing.

Digestion with another enzyme, *Glu*I, suggests that there are subgroups within the species (such as *C. ethanolica, C. sake, P. kudriavzevii* and *Saccharomycodes ludwigii*) (Figure 4b). There was no restriction fragment pattern of isolates from *khadi* that matched the pattern of the *S. cerevisiae* isolate from *G. flava* fruits. There are several possible scenarios that can explain the absence of this strain as concluded from the restriction fragment patterns after fermentation. That is, the strain could have existed in frequencies too low to be picked amongst other isolates, especially if back slopping was practiced, because the back-slopped inocula (including the wild isolate *S. cerevisiae* isolate from the fruits) could have outcompeted it during the fermentation. In spite of the clustering of some isolates with the commercial baker's yeast (Figure 3), the prevalence of strains of *S. cerevisiae* with a distinct restriction fragment pattern from that of the commercial baker's yeast suggests that Mmashoro and Letlhakane are the only areas that rely on spontaneous fermentation for brewing of *khadi* (Figure S1). The *in-silico* PCR-RFLP results further revealed that none of the *khadi* isolates had a similar restriction fragment pattern to the selected ale and lager brewing yeasts and wine yeasts used in the industry, even though there were similarities in the sequencing data (Figure 3). These yeasts are not available in the local supermarkets of Botswana as compared to the readily available baker's yeast explaining why they were not prevalent.

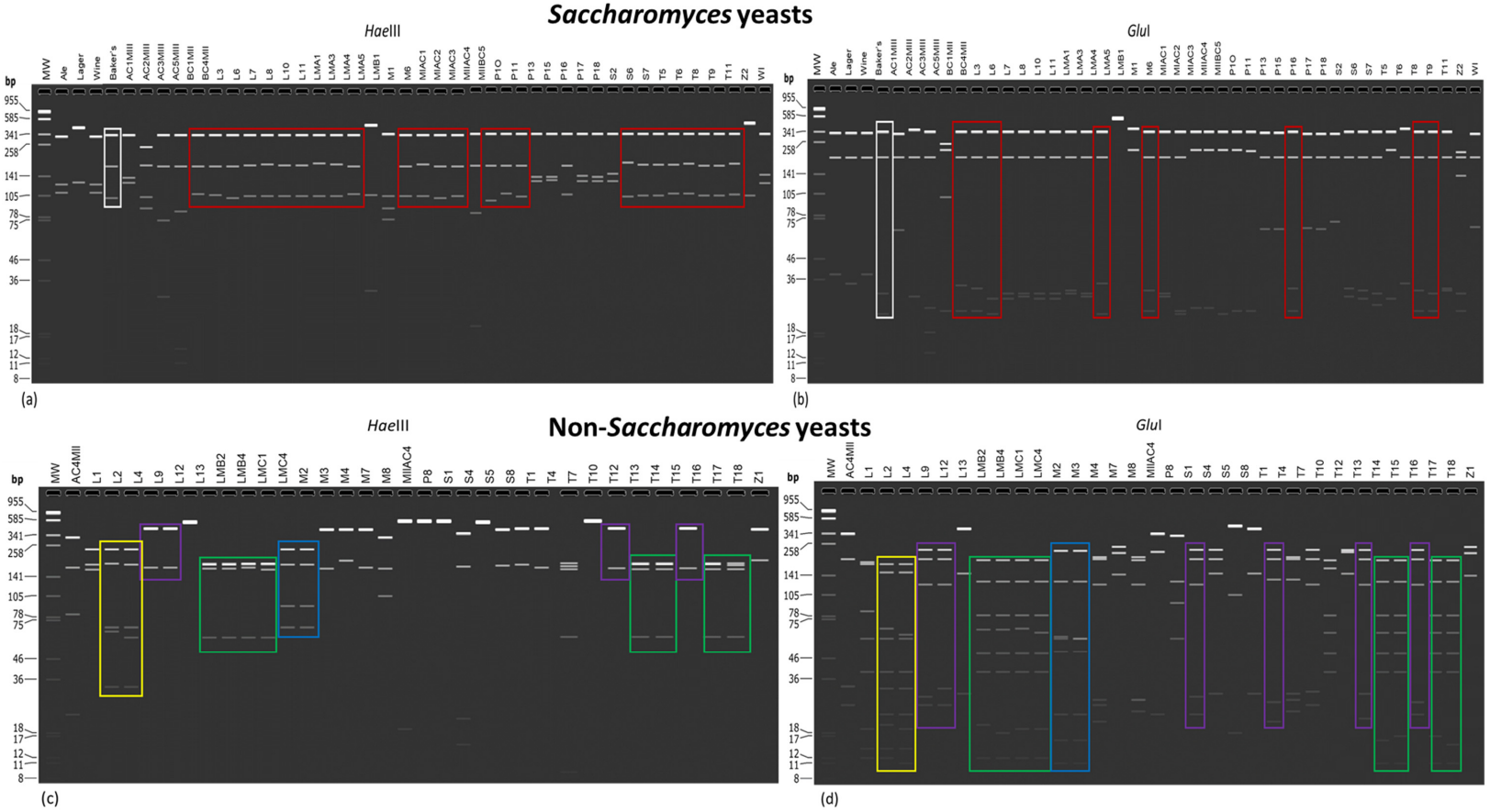

Figure 4. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for yeast isolates from *khadi*. (**a**) and (**b**) show restriction fragment patterns of *Saccharomyces* yeasts obtained using restriction enzymes *Hae*III and *GluI*. The red boxes on (a) and (b) show restriction fragments patterns of isolates that are similar to that of the control commercial baker's yeast (white box). WI is the wild *S. cerevisiae* isolate from *G. flava* fruits. (**c**) and (**d**) show restriction fragments patterns of non-*Saccharomyces* yeasts obtained using restriction enzymes *Hae*III and *Glu*I. The blue, green, purple and yellow boxes on (c) and (d) show matching restriction fragment patterns for some isolates.

Non-conventional yeasts could be responsible for fermentation of *khadi* rather than conventional *Saccharomyces* yeasts alone. The fermentative lifestyle of species of the genus *Saccharomyces* is not unique among yeasts of the *Saccharomycetaceae* family [39,60,63], as it may have coincided with the origin of angiosperms, about 125 million years ago [63,64]. Therefore, other non-conventional yeasts in the *Saccharomycetaceae* family, which evolved in the same time period, are also capable of making and accumulating ethanol. In this study we identified a number of non-conventional yeasts (Tables 4 and 5) which could have also been responsible for fermentation of *khadi.* Therefore, we further investigated the genetic diversity using in silico PCR-RFLP of non-*Saccharomyces* yeasts to reveal unique species responsible for fermentation of different brews. We observed that among the genetically diverse yeasts, there were several unique yeasts that could be responsible for *khadi* fermentation (Figure 4c,d). We noted that *C. ethanolica* strains isolated from Tonota (T14, T15, T17 and T18) had matching restriction fragment pattern with *C. ethanolica* strains from Letlhakane (LMB2, LMB4, LMC1 and LMC4) after digestion with *Hae*III. These two locations are 200 km apart and therefore sharing of the back slopped broth or dried fruits is highly unlikely although the ability of yeasts to be dispersed over long distances by insects is well documented [65,66]. Therefore, this explains that these species could be naturally occurring on *G. flava* fruits in a large territory. In agreement to our assertions, strains from a different species, *Saccharomycodes ludwigii* from *khadi* from the same locations Tonota (T13, and T16) and Letlhakane (L9 and L12) also had a similar restriction fragment pattern when digested using *Hae*III (Figure 4c). Similar results can be observed when running the same experiment using *GluI* (Figure 4c,d) as seen in Table S3. This suggests the primary source of the fermenting yeasts could have been the fruits and not the fermentation vessels or the equipment used. It is noteworthy that non-*Saccharomyces* yeasts are not commercially available in Botswana, hence their role in spontaneous fermentation of *khadi* is supported by the above statement. There has been a rapid expansion of craft breweries that utilize non-*Saccharomyces* yeasts in the modern brewing industry [67–69]. This strategy has gained special attention due to the demand for innovative and aromatically diverse beers associated with changing global lifestyles [70,71]. Non-*Saccharomyces* yeasts have been demonstrated to produce a wide range of aromatic compounds and other metabolites that are known to improve the sensorial profiles of alcoholic beverages [41,43,68]. This work further increases the attention to exploit these yeasts to define *khadi* traits towards the development of this traditional beverage. The characterization of these non-*Saccharomyces* yeasts (not presented in this paper) should be explored to understand how these diverse yeasts reported here could enrich and diversify aromas and flavors. Such studies would pave the way to improvement of the sensory complexity of *khadi* towards its entry into regional and international markets.

3.5. Variation in Alcohol Content among Khadi Samples

The ethanol concentration among the *khadi* samples investigated ranged from 0.8 ± 0.11 to 8.7 ± 0.11% (*v/v*) (Figure 5a). The highest ethanol content was recorded from Maun 1 whereas Maun 3 had the lowest ethanol content. Maun 1 has the highest ethanol concentration and its fermentation was carried out by the combination of *S. cerevisiae* and *C. sake*. The lowest ethanol concentration was from Maun 3 and its fermentation was carried by *Lachancea fermentati* and *Curvibasidium pallidicorallinum* which is an ethanol-tolerant species. On average, ethanol content of *khadi* was 5.3 ± 2.55% (*v/v*). This value is comparable to the average, 3.7% (*v/v*), previously reported by Mapitse et al. [7]. Commercial beers range from 3 to 6% (*v/v*) [72] meaning *khadi* ranges closely to commercial beers. The diversity of fermenting yeasts among *khadi* samples could be one of the main reasons of the discrepancies of amounts of ethanol accumulated. The biochemical composition of the dried *G. flava* fruits and the physiological qualities of the yeasts may also limit the ethanol content which depends on the carbohydrate/sugar composition of different batches of fruits. Species from the genus *Saccharomyces* produce higher amounts of ethanol as compared to other genera and their non-conventional counterparts [63]. In addition, the use of uncontrolled and partially aerobic fermenting vessels could also account for the discrepancies in alcohol content among *khadi* brews as oxygen levels determine alcoholic fermentation patterns [73]. Lower

oxygen partial pressures increase the glycolytic flux towards a fermentative metabolism, whereas the opposite is true for environments with high oxygen concentrations [60,74]. It is worth noting that fermentation time is not standardized in the brewing of *khadi.* Another variable that could account for discrepancies in ethanol concentration is time to completion of fermentation. Time to harvest is not standardized in the brewing of *khadi*. The amount of ethanol at the end of fermentation of *khadi* is, therefore, stage- and condition-specific.

3.6. The pH of Khadi, an Important Factor in Preservation of the Brew

The pH of the *khadi* samples ranged from 2.6 to 3.6 (Figure 5b, also see Table S5b). Sample Letlhakane 2 and Serowe 2 had the least acidity as compared to all samples, whereas sample Palapye 3 was the most acidic with a pH of 2.6. The average pH of all the samples was 3.1 ± 0.31 which is in agreement with studies reported by Mapitse et al. [7] who reported an average pH of 3.1 ± 0.15. pH influences the overall physiological characteristics of alcoholic beverages, such as taste, color and microbiological stability. Most importantly, pH also strongly influences sanitation of the product, an attribute important for increasing shelf life of the brew [75]. Other than the presence of ethanol, the acidic pH is likely the other reason why there were no bacteria isolated from *khadi*. pH is the primary determinant of the community structure of bacteria, with a large number of bacteria known to be inhibited by low pHs [76–78]. Although there are other factors, such as mineral content and titratable acidity, among others that should be considered to make valid conclusions on the effects of an acidic brew, the information on the relative acidity of the brew would be important for commercialization of *khadi.*

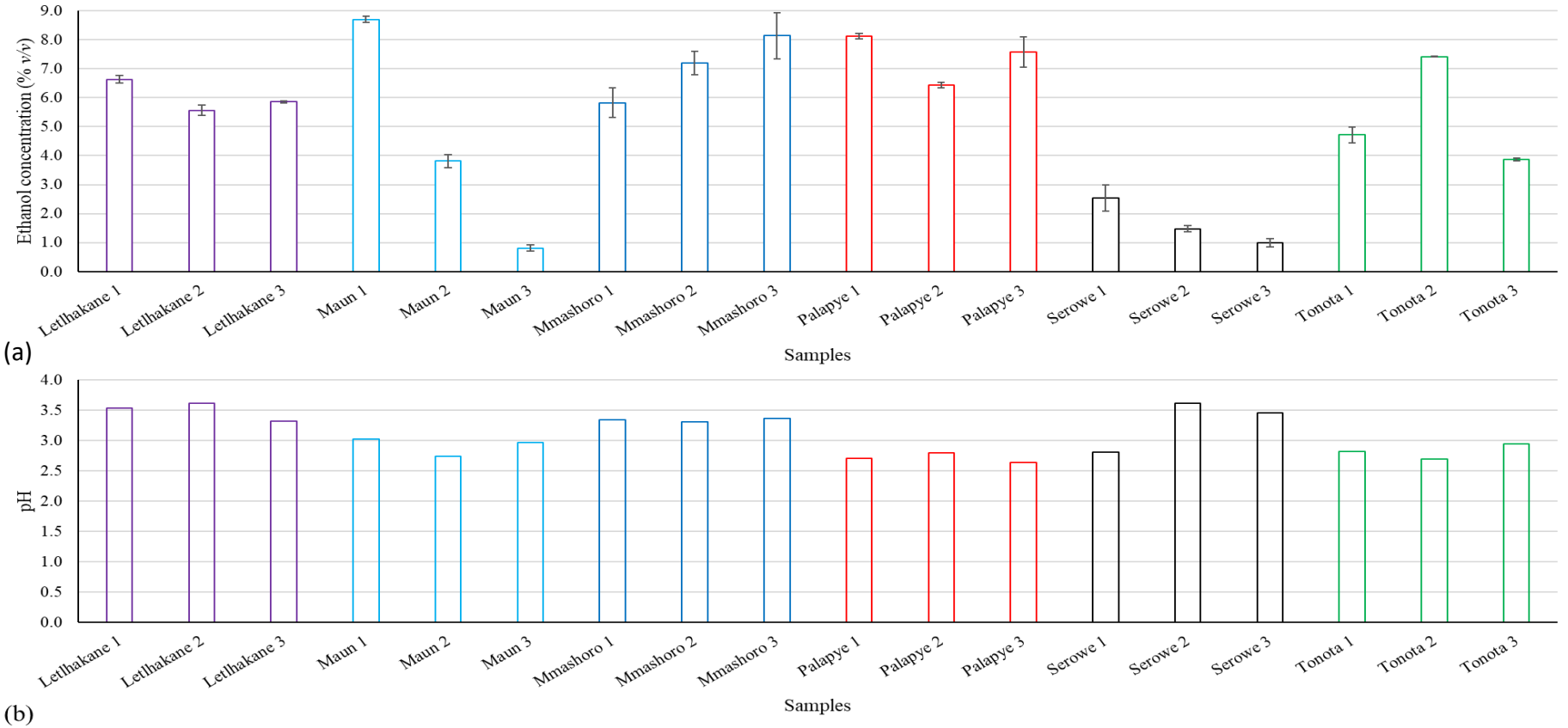

Figure 5. The physio-chemical properties of the *khadi* samples. (**a**) Variable average ethanol content and (**b**) average pH of the *khadi* samples used in the study. Color code: purple = Letlhakane, sky blue = Maun, navy blue = Mmashoro, red = Palapye, Black = Serowe and green = Tonota.

4. Conclusions

This study presents the first microbiological characterization of *khadi*, a popular traditional alcoholic beverage in Botswana. Our work suggests that some *khadi* brewers practice spontaneous fermentation, whereas others use a commercial baker's yeast. Further studies to test for the functionality of the mixed consortia and resultant aromatic profiles are needed for development of starter cultures for consistent product quality towards commercialization of *khadi*.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/2311-5637/6/2/51/s1. Figure S1: PCR-RFLP for yeast isolates from khadi. Table S1: The coordinates of the khadi sampling areas. Table S2: The yeast enumeration results in log10CFU/mL from khadi. Table S3: PCR-RFLP sizes of all S. cerevisiae strains isolated in this work. Table S4: PCR-RFLP sizes of all non-Saccharomyces strains isolated in this work. Table S5: The ethanol assay test results for the khadi samples.

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References

1. Nyanga, L.K.; Nout, M.J.R.; Smid, E.J.; Boekhout, T.; Zwietering, M.H. Fermentation characteristics of yeasts isolated from traditionally fermented *masau* (*Ziziphus mauritiana*) fruits. *Int. J. Food Microbiol.* **2013**, *166*, 426–432. [CrossRef] [PubMed]
2. Nyanga, L.K.; Nout, M.J.R.; Gadaga, T.H.; Theelen, B.; Boekhout, T.; Zwietering, M.H. Yeasts and lactic acid bacteria microbiota from *masau* (*Ziziphus mauritiana*) fruits and their fermented fruit pulp in Zimbabwe. *Int. J. Food Microbiol.* **2007**, *120*, 159–166. [CrossRef] [PubMed]
3. Tishkoff, S.A.; Reed, F.A.; Friedlaender, F.R.; Ranciaro, A.; Froment, A.; Hirbo, J.B.; Awomoyi, A.A.; Bodo, J.; Doumbo, O.; Ibrahim, M.; et al. The Genetic Structure and History of Africans and African Americans. *J. Sci.* **2009**, *324*, 1035–1044. [CrossRef] [PubMed]
4. Finlay, J.; Jones, R.K. *Alcohol Consumption and the Nature of Alcohol Related Problems in Botswana: A Premiliminary Report*; Michigen State University: East Lansing, MI, USA, 1982.
5. Leger, S. Overview of bushmen's uses of several plants present at the Kalahari Meerkat Project. In *The Hidden Gifts of Nature-A Description of Today's Use of Plants in West Bushmanland (Namibia)*; German Development Service: Wächtersbach, Germany, 1997; pp. 2–5.
6. Van Wyk, B.E.; Gericke, N. *People's plants: A Guide to Useful Plants of Southern Africa*; Briza Publications: Johannesburg, South Africa, 2000.
7. Mapitse, R.; Okatch, H.; Moshoeshoe, E. Analysis of Volatile Compounds in *Khadi* (an Unrecorded Alcohol Beverage) from Botswana by Gas Chromatography-Flame Ionization Detection (GC-FID). *South Afr. J. Chem.* **2014**, *67*, 184–188.
8. WHO. *Global Status Report on Alcohol*; World Heal Organization: Geneva, Switzerland, 2004; pp. 4–6.
9. Swiegers, J.H.; Bartowsky, E.J.; Henschke, P.A.; Pretorius, I.S. Yeast and bacterial modulation of wine aroma and flavour. *Aust. J. Grape Wine Res.* **2005**, *11*, 139–173. [CrossRef]
10. Freire, A.L.; Ramos, C.L.; Schwan, R.F. Microbiological and chemical parameters during cassava based-substrate fermentation using potential starter cultures of lactic acid bacteria and yeast. *Food Res. Int.* **2015**, *76*, 787–795. [CrossRef]
11. Gadaga, T.H.; Mutukumira, A.N.; Narvhus, J.A.; Feresu, S.B. A review of traditional fermented foods and beverages of Zimbabwe. *Int. J. Food Microbiol.* **1999**, *53*, 1–11. [CrossRef]
12. Adekoya, I.; Obadina, A.; Adaku, C.C.; De Boevre, M.; Okoth, S.; De Saeger, S.; Njobeh, P. Mycobiota and co-occurrence of mycotoxins in South African maize-based opaque beer. *Int. J. Food Microbiol.* **2018**, *270*, 22–30. [CrossRef]
13. Lyumugabe, F.; Gros, J.; Nzungize, J.; Bajyana, E.; Thonart, P. Characteristics of African traditional beers brewed with sorghum malt: A review. *Biotechnol. Agron. Soc. Environ.* **2012**, *16*, 509–530.

14. Lee, Y.; Choi, Y.; Lee, S.; Park, J.; Shim, J.; Park, K.; Kim, W. Screening Wild Yeast Strains for Alcohol Fermentation from Various Fruits. *Mycobiology* **2011**, *39*, 33–39. [CrossRef]
15. Kurtzman, C.P.; Robnett, C.J. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie Leeuwenhoek* **1998**, *98*, 331–371. [CrossRef] [PubMed]
16. Lõoke, M.; Kristjuhan, K.; Kristjuhan, A. Extraction of genomic DNA from yeasts for PCR based applications. *Biotechniques* **2011**, *50*, 325–328. [CrossRef] [PubMed]
17. Ouoba, L.I.I.; Diawara, B.; Amoa-Awua, W.K.; Traoré, A.S.; Møller, P.L. Genotyping of starter cultures of Bacillus subtilis and Bacillus pumilus for fermentation of African locust bean (*Parkia biglobosa*) to produce Soumbala. *Int. J. Food Microbiol.* **2004**, *90*, 197–205. [CrossRef]
18. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* **2018**, *33*, 1870–1874. [CrossRef] [PubMed]
19. Tamura, K.; Nei, M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* **1993**, *10*, 512–526. [CrossRef]
20. Kurtzman, C.P.; Fell, J.W.; Boekhout, T.; Robert, V. Methods for isolation, phenotypic characterization and maintenance of yeasts. In *The Yeasts*; Elsevier B.V.: Amsterdam, The Netherlands, 2011; Volume 1, pp. 87–110. ISBN 9780444521491.
21. Fleet, G.H. Yeasts in fruit and fruit products. In *Yeasts in Food*; B. Behr's Verlag GmbH & Co.: Heidelberg, Germany, 2003; pp. 267–287. ISBN 9781845698485.
22. Valente, A.; Sanches-Silva, A.; Albuquerque, T.G.; Costa, H.S.; Transfer, T.; Conference, I.I.; Kozhahmetova, Z.; Kasenova, G.; Sedláček, I.; Nováková, D.; et al. Microbiological and chemical parameters during cassava based-substrate fermentation using potential starter cultures of lactic acid bacteria and yeast. *Int. J. Food Microbiol.* **2017**, *5*, 1–11. [CrossRef]
23. Styger, G.; Prior, B.; Bauer, F.F. Wine flavor and aroma. *J. Ind. Microbiol. Biotechnol.* **2011**, *38*, 1145–1159. [CrossRef]
24. Fotedar, R.; Kolecka, A.; Boekhout, T.; Fell, J.W.; Al-Maliki, A.; Zeyara, A.; Al Marri, M. Fungal diversity of the hypersaline Inland Sea in Qatar. *Bot. Mar.* **2018**, *61*, 595–609. [CrossRef]
25. Varela, C. The impact of non-Saccharomyces yeasts in the production of alcoholic beverages. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 9861–9874. [CrossRef]
26. Barata, A.; Malfeito-Ferreira, M.; Loureiro, V. The microbial ecology of wine grape berries. *Int. J. Food Microbiol.* **2012**, *153*, 243–259. [CrossRef]
27. Moubasher, A.-A.H.; Abdel-Sater, M.A.; Soliman, Z.S.M. Diversity of floricolous yeasts and filamentous fungi of some ornamental and edible fruit plants in assiut area, egypt. *Curr. Res. Environ. Appl. Mycol.* **2018**, *8*, 135–161. [CrossRef]
28. Cousin, F.; Le Guellec, R.; Schlusselhuber, M.; Dalmasso, M.; Laplace, J.-M.; Cretenet, M. Microorganisms in fermented apple beverages: Current Knowledge and Future Directions. *Microorganisms* **2017**, *5*. [CrossRef]
29. Pretorius, I.S. Tailoring wine yeast for the new millennium: Novel approaches to the ancient art of winemaking. *Yeast* **2000**, *16*, 675–729. [CrossRef]
30. Goddard, M.R. Quantifying the complexities of *Saccharomyces cerevisiae*'s ecosystem engineering via fermentation. *Ecol. Soc. Am.* **2008**, *89*, 2077–2082.
31. Mortimer, R.; Polsinelli, M. On the origins of wine yeast. *Res. Microbiol.* **1999**, *150*, 199–204. [CrossRef]
32. Ponzzes-Gomes, C.M.P.B.S.; De Mélo, D.L.F.M.; Santana, C.A.; Pereira, G.E.; Mendonça, M.O.C.; Gomes, F.C.O.; Oliveira, E.S.; Barbosa, A.M., Jr.; Trindade, R.C.; Rosa, C.A. *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts in grape varieties of the São Francisco Valley. *Braz. J. Microbiol.* **2014**, *45*, 411–416. [CrossRef]
33. Dashko, S.; Zhou, N.; Compagno, C. Why, when and how did yeast evolve alcoholic fermentation? *FEMS Yeast Res.* **2014**, *14*. [CrossRef]
34. Zhou, N.; Bottagisi, S.; Katz, M.; Schacherer, J.; Friedrich, A.; Gojkovic, Z.; Swamy, K.B.S.; Knecht, W.; Compagno, C.; Piškur, J. Yeast-bacteria competition induced new metabolic traits through large-scale genomic rearrangements in *Lachancea kluyveri*. *FEMS Yeast Res.* **2017**, *17*. [CrossRef]
35. Cortesão, M.; Fuchs, F.M.; Commichau, F.M.; Eichenberger, P.; Schuerger, A.C.; Nicholson, W.L.; Setlow, P.; Moeller, R. *Bacillus subtilis* spore resistance to simulated mars surface conditions. *Front. Microbiol.* **2019**, *10*, 1–16. [CrossRef]

36. Regalado, N.G.; Martin, G.; Antony, S.J. *Acinetobacter lwoffii*: Bacteremia associated with acute gastroenteritis. *Travel Med. Infect. Dis.* **2009**, *7*, 316–317. [CrossRef]
37. Pal Singh, N.; Sagar, T.; Nirmal, K.; Rajender Kaur, I. Pyogenic liver abscess caused by *Acinetobacter lwoffii*: A case report. *J. Clin. Diagn. Res.* **2016**, *10*, DD01–DD02. [CrossRef]
38. Ugbogu, O.C.; Ogodo, A.C. Microbial Flora, Proximate Composition and Vitamin Content of Juices of Three Fruits Bought from a Local Market in Nigeria. *Int. J. Chem. Eng. Appl.* **2015**, *6*, 440–443. [CrossRef]
39. Rozpedowska, E.; Hellborg, L.; Ishchuk, O.P.; Orhan, F.; Galafassi, S.; Merico, A.; Woolfit, M.; Compagno, C.; Piškur, J. Parallel evolution of the make–accumulate–consume strategy in *Saccharomyces* and *Dekkera* yeasts. *Nat. Commun.* **2011**, *2*, 302. [CrossRef]
40. Lencioni, L.; Romani, C.; Gobbi, M.; Comitini, F.; Ciani, M.; Domizio, P. Controlled mixed fermentation at winery scale using *Zygotorulaspora florentina* and *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* **2016**, *234*, 36–44. [CrossRef] [PubMed]
41. Andorrà, I.; Berradre, M.; Mas, A.; Esteve-Zarzoso, B.; Guillamón, J.M. Effect of mixed culture fermentations on yeast populations and aroma profile. *Lwt Food Sci. Technol.* **2012**, *49*, 8–13. [CrossRef]
42. Ciani, M.; Comitini, F.; Mannazzu, I.; Domizio, P. Controlled mixed culture fermentation: A new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Res.* **2009**, *10*, 123–133. [CrossRef] [PubMed]
43. Dashko, S.; Zhou, N.; Tinta, T.; Sivilotti, P.; Lemut, M.S.; Trost, K.; Gamero, A.; Boekhout, T.; Butinar, L.; Vrhovsek, U.; et al. Use of non-conventional yeast improves the wine aroma profile of *Ribolla Gialla*. *J. Ind. Microbiol. Biotechn.* **2015**, *42*, 997–1010. [CrossRef]
44. Gamero, A.; Quintilla, R.; Groenewald, M.; Alkema, W.; Boekhout, T.; Hazelwood, L. High-throughput screening of a large collection of non-conventional yeasts reveals their potential for aroma formation in food fermentation. *Food Microbiol.* **2016**, *60*, 147–159. [CrossRef]
45. Porter, T.J. Biochemical Characterization and Evaluation of the Oenological Attributes of *Lachancea* Species. MSc Thesis (MScAgric), Stellenbosch University, Stellenbosch, South Africa, 2017.
46. Cordero-Bueso, G.; Esteve-Zarzoso, B.; Cabellos, J.M.; Gil-Diaz, M.; Arroyo, T. Biotechnological potential of non-*Saccharomyces* yeasts isolated during Biotechnological potential of non- *Saccharomyces* yeasts isolated during spontaneous fermentations of Malvar (*Vitis vinifera* cv. L.). *Eur. Food Res. Technol.* **2012**, *236*, 2564–2568. [CrossRef]
47. Romano, P.; Suzzi, G.; Domizio, P.; Fatichenti, F. Secondary products formation as a tool for discriminating non- *Saccharomyces* wine strains Strain diversity in non- *Saccharomyces* wine yeasts. *Antonie Van Leeuwenhoek* **1997**, *77*, 239–242. [CrossRef]
48. Maturano, Y.P.; Assof, M.; Fabani, M.P.; Nally, M.C.; Jofré, V.; Rodríguez Assaf, L.A.; Toro, M.E.; Castellanos De Figueroa, L.I.; Vazquez, F. Enzymatic activities produced by mixed Saccharomyces and non-*Saccharomyces* cultures: Relationship with wine volatile composition. *Antonie Van Leeuwenhoek* **2015**, *108*, 1239–1256. [CrossRef] [PubMed]
49. James, S.A.; Stratford, M. Spoilage yeasts with emphasis on the genus Zygosaccharomyces. In *Yeasts in Food*; B. Behr's Verlag GmbH & Co.: Heidelberg, Germany, 2003; pp. 171–191. ISBN 9781845698485.
50. Schifferdecker, A.J.; Siurkus, J.; Andersen, M.R.; Joerck-Ramberg, D.; Ling, Z.; Zhou, N.; Blevins, J.E.; Sibirny, A.A.; Piškur, J.; Ishchuk, O.P. Alcohol dehydrogenase gene *ADH3* activates glucose alcoholic fermentation in genetically engineered *Dekkera bruxellensis* yeast. *Appl. Microbiol. Biotech.* **2016**, *100*, 3219–3231. [CrossRef] [PubMed]
51. Alonso, A.; Belda, I.; Santos, A.; Navascués, E.; Marquina, D. Advances in the control of the spoilage caused by *Zygosaccharomyces* species on sweet wines and concentrated grape musts. *Food Control* **2015**, *51*, 129–134. [CrossRef]
52. Chatonnet, P.; Dubourdieu, D.; Boidron, J.N. The Influence of Brettanomyces/Dekkera sp. Yeasts and Lactic Acid Bacteria on the Ethylphenol Content of Red Wines. *Am. J. Enol. Vitic.* **1995**, *46*, 463–468.
53. Licker, J.L.; Acree, T.E.; Henick-Kling, T. What Is "Brett" (*Brettanomyces*) Flavor?: A Preliminary Investigation. *ACS Symp.Ser.* **1998**, 96–115. [CrossRef]
54. Hittinger, C.T.; Rokas, A.; Bai, F.Y.; Boekhout, T.; Gonçalves, P.; Jeffries, T.W.; Kominek, J.; Lachance, M.A.; Libkind, D.; Rosa, C.A.; et al. Genomics and the making of yeast biodiversity. *Curr. Opin. Genet. Dev.* **2015**, *35*, 100–109. [CrossRef]

55. Schifferdecker, A.J.; Dashko, S.; Ishchuk, O.P.; Piškur, J. The wine and beer yeast *Dekkera bruxellensis*. *Yeast* **2014**, *31*, 323–332. [CrossRef]
56. Capece, A.; Romaniello, R.; Siesto, G.; Romano, P. Conventional and Non-Conventional Yeasts in Beer Production. *Fermentation* **2018**. [CrossRef]
57. Xu, Y.; Zhi, Y.; Wu, Q.; Du, R.; Xu, Y. *Zygosaccharomyces bailii* is a potential producer of various flavor compounds in Chinese Maotai-flavor liquor fermentation. *Front. Microbiol.* **2017**, *8*, 1–9. [CrossRef]
58. Douglass, A.P.; Offei, B.; Braun-galleani, S.; Coughlan, A.Y.; Ortiz-merino, A.; Byrne, K.P.; Wolfe, K.H.; Martos, A.A.R. Population genomics shows no distinction between pathogenic *Candida krusei* and environmental *Pichia kudriavzevii*: One species, four names. *PLoS Path.* **2018**, *14*, e1007138. [CrossRef]
59. Ramos, C.L.; De Sousa, E.S.O.; Ribeiro, J.; Almeida, T.M.M.; Santos, C.C.A.D.A.; Abegg, M.A.; Schwan, R.F. Microbiological and chemical characteristics of tarubá, an indigenous beverage produced from solid cassava fermentation. *Food Microbiol.* **2015**, *49*, 182–188. [CrossRef] [PubMed]
60. Piškur, J.; Rozpedowska, E.; Polakova, S.; Merico, A.; Compagno, C. How did *Saccharomyces* evolve to become a good brewer? *Trends Genet.* **2006**, *22*, 183–186. [CrossRef] [PubMed]
61. Boekhout, T.; Phaff, H.J. Yeast biodiversity. In *Yeasts in Food*; B. Behr's Verlag GmbH & Co.: Heidelberg, Germany, 2003; pp. 1–38. ISBN 9781845698485.
62. Von Rudloff, A.; Mogkatlhe, L.; Mookodi, G. Botswana: Summary of nationwide research, in International Center for Alcohol Policies (ed.), Producers, Sellers, and Drinkers: Studies of Non-commercial Alcohol in Nine Countries. *Glob. Actions Harmful Drink.* **2012**, 13–18. Available online: http://iardwebprod.azurewebsites.net/getattachment/9725694a-dbdb-4f27-b581-5211004e0b3c/icap-producers-sellers-and-drinkers-noncommercial-alcohol-monograph.pdf (accessed on 8 May 2020).
63. Hagman, A.; Sall, T.; Compagno, C.; Piskur, J. Yeast "Make-Accumulate-Consume" Life Strategy Evolved as a Multi-Step Process That Predates the Whole Genome Duplication. *PLoS ONE* **2013**, *8*. [CrossRef] [PubMed]
64. Sun, G.; Dilcher, D.L.; Wang, H.; Chen, Z. A eudicot from the Early Cretaceous of China. *Nature* **2011**, *471*, 625–628. [CrossRef] [PubMed]
65. Baselga, I.; Zafra, O.; Pérez, L.E.; Francisco-Alvarez, R.; Rodriguez-Tarduchy, G.; Santos, C. An AFLP based method for the detection and identification of indigenous yeast in complex must samples without a microbiological culture. *Int. J. Food Microbiol.* **2017**, *241*, 89–97. [CrossRef]
66. Steensels, J.; Daenen, L.; Malcorps, P.; Derdelinckx, G.; Verachtert, H.; Verstrepen, K.J. *Brettanomyces* yeasts-From spoilage organisms to valuable contributors to industrial fermentations. *Int. J. Food Microbiol.* **2015**, *206*, 24–38. [CrossRef]
67. Alfeo, V.; Todaro, A.; Migliore, G.; Borsellino, V.; Schimmenti, E. Microbreweries, brewpubs and beerfirms in the Sicilian craft beer industry. *Int. J. Wine Bus. Res.* **2019**, *32*, 122–138. [CrossRef]
68. Callejo, M.J.; González, C.; Morata, A. Use of Non-Saccharomyces Yeasts in Bottle Fermentation of Aged Beers. In *Brewing Technology*; Intech open: London, UK, 2017. [CrossRef]
69. Iattici, F.; Catallo, M.; Solieri, L. Designing New Yeasts for Craft Brewing: When Natural Biodiversity Meets Biotechnology. *Beverages* **2020**, *6*, 3. [CrossRef]
70. Michel, M.; Meier-Dörnberg, T.; Jacob, F.; Methner, F.J.; Wagner, R.S.; Hutzler, M. Review: Pure non-*Saccharomyces* starter cultures for beer fermentation with a focus on secondary metabolites and practical applications. *J. Inst. Brew.* **2016**, *122*, 569–587. [CrossRef]
71. Ravasio, D.; Carlin, S.; Boekhout, T.; Groenewald, M.; Vrhovsek, U.; Walther, A.; Wendland, J. Adding Flavor to Beverages with Non-Conventional Yeasts. *Fermentation* **2018**, *4*, 15. [CrossRef]
72. Bamforth, C.W. Nutritional aspects of beer-A review. *Nutr. Res.* **2002**, *22*, 227–237. [CrossRef]
73. Merico, A.; Galafassi, S.; Piškur, J.; Compagno, C. The oxygen level determines the fermentation pattern in *Kluyveromyces lactis*. *FEMS Yeast Res.* **2009**, *9*, 749–756. [CrossRef]
74. Pronk, J.T.; Steensma, H.Y.; Van Dijken, J.P. Pyruvate metabolism in *Saccharomyces cerevisiae*. *Yeast* **1996**, *12*, 1607–1633. [CrossRef]
75. Jukić, M.; Mastanjević, K.; Lukinac, J.; Vulin, Z.; Mastanjević, K.; Krstanović, V. Beer–The Importance of Colloidal Stability (Non-Biological Haze). *Fermentation* **2018**, *4*, 91. [CrossRef]
76. Hirshfield, I.N.; Terzulli, S.; O'Byrne, C. Weak organic acids: A panoply of effects on bacteria. *Sci. Prog.* **2003**, *86*, 245–269. [CrossRef] [PubMed]

77. Yu, H.; Huang, G.H.; Zhang, X.D.; Huang, G.H.; Li, Y. Inhibitory effects of organic acids on bacteria growth during food waste composting. *Compos. Sci. Util.* **2010**, *18*, 55–63. [CrossRef]
78. Chelule, P.; Mokoena, M.; Gqaleni, N. Advantages of traditional lactic acid bacteria fermentation of food in Africa. *Curr. Res. Technol. Educ. Top. Appl. Microbiol. Microb. Biotechnol.* **2010**, *2*, 1160–1167.