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Identification of lactic acid bacteria and yeast from Oshikundu using 16S and 26S rDNA gene sequencing

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Abstract

Oshikundu is brewed from fermentation of pearl millet (*Pennisetum glaucum* (L.) R.Br) meal commonly known as *mahangu*, sorghum (*Sorghum bicolor* (L.) Moench)/pearl millet malt, water and/or bran (from pearl millet). Consumption of this brew is daily in many households mostly in the Northern Namibia. *Oshikundu* samples were collected from north central and central region of Namibia. Isolation of lactic acid bacteria (LAB) was carried out on deMan Rogosa Sharpe (MRS), M17 and Rogosa agars, meanwhile malt extract and potato dextrose agars were used for yeast. DNA from isolates was amplified using standard polymerase chain reaction (PCR) and PCRâĂŞproducts were sequenced thereafter. Sequence was multiple aligned through Basic Local Alignment Search Tool (BLAST) and compared to the known DNA sequences in databases found in National Center for Biotechnology Information (NCBI)/GenBank database for LAB and yeast identification from *oshikundu*. Identified LAB were *Lactobacillus plantarum, L. pentosus, L. fermentum, L. acidifarinae, L. spicheri, L. namurensis, L. zymae, L. brevis, Leuconostoc gurlium, L. delbrueckiee subsp. bulgaricus, L. buncheri* and *Pediococcus acidilactici*. Meanwhile for yeast were *Saccharomyces spp.*, and *Pichia*

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kudriavzevii. From the results, it appears that the dominant LAB is mostly heterofermentative and yeast likely to produce ethanol in *oshikundu*. The study contributes to the scanty information of possible LAB and yeast that may be responsible for *oshikundu* fermentation.

Keywords: *Oshikundu, mahangu* (pearl millet), lactic acid bacteria, yeast

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1 Introduction

There is an increasing demand for healthy food, driven by population growth and rising affluence globally. This demand will impose severe challenges on the agro-processing sector, agricultural and social systems (Mace et al., 2013). African dietary ethos consist of fermented and non-fermented food and beverage that are of cereal based products from sorghum, millet, maize, cassava, wild legume seeds, tubers, meat and milk (Tamang and Samuel, 2010). Most food and beverages from sorghum and millet in Africa have undergone a lactic acid fermentation or a malting process or both during production, an important characteristic of these products (Belton and Taylor, 2004). Fermentation being one of the oldest, economical forms of food preservation and production (Blandino et al., 2003; Chelule et al., 2010) is of importance as a technology which is affordable and also combating foodborne diseases (Franz et al., 2014).

Recently, cereals gained a lot of attention as raw material for non-alcoholic and functional beverages production. Due to their high fiber, whole or multi-grain content cereal based beverages has become a greater part of the new better-for-you foods and beverages with desirable benefits (Dongmo et al., 2016). The most desirable benefits resulting from cereals are their high nutritional value and bioactive compound content (Adil et al., 2012). Lactic acid bacteria (LAB) and yeast in fermented food and beverages across Africa from sorghum and millet such as *ogi, doro, injera, uji, togwa, busa, bushera, amgba, bel-saalga, bogbe, dèguè, kenkey, kisra, kamu-zaki, hulumur, mwenge, kisra, kaffir beer* have been reviewed (Blandino et al., 2003; Franz et al., 2014; Adebiyi et al., 2016).

Lactic acid fermented malt based beverages are non-alcoholic, with low pH value (3.5-4.5) and produced by the fermentation of cereals, cereal substrates or blends by LAB strains. Available lactic acid fermented cereal beverages include yogurt-like cereal functional beverages and traditional cereal fermented beverages (Corbo et al., 2014) . Some of these lactic acid fermented malt based beverages serve as food and thirst quencher such as *oshikundu* from Namibia (Embashu, 2014). *Oshikundu* is made from fermentation of pearl millet (*Pennisetum glaucum* (L.) R.Br) meal (*uusila/oufila*) commonly known as *mahangu* in Namibia,

sorghum (*Sorghum bicolor* (L.) Moench)/pearl millet malt, water and/or bran (from pearl millet). This brew is made in many household across the country and found its way at informal market.

The social aspects and processing methods were reported by Embashu et al. (2013). The brewing of *oshikundu* takes place at home on a small scale, with recipe passed on from generation to generation (Embashu et al., 2013). *Oshikundu* is perceived as a non-alcoholic and possibly a lactic acid bacteria fermented beverage, thus it is consumed by everyone in a household. However, Embashu (2014) documented alcohol below 2% v/v in *oshikundu* and gentle bubble during fermentation which would suggest possible yeast fermentation as well. Although pearl millet is the main staple and commercial crops for the majority of people in Northern Namibia, there is no sufficient documentation on their nutritional, fermentation process and microbiota associated with their fermented products. The current study aims at isolating and identifying the LAB and yeast associated with *oshikundu* fermentation by 16S and 26S rDNA gene sequencing and multiple alignments through Basic Local Alignment Search Tool (BLAST).

2 Materials and methods

2.1 Sampling

Oshikundu samples (500 mL) were collected in 500 mL sterile plastic bottles from the Northern-Central (Omusati, Oshana, Oshikoto) and Central (Khomas) region in Namibia, transported in a portable fridge and stored below 4°C until further microbiological analysis.

2.2 Isolation of lactic acid bacteria and yeast

Lactic acid bacterial isolation was done following work by Tanguler and Erten (2012), and Väkeväinen et al. (2018) with few modifications, no supplement or antibiotic was used on the isolation medium. *Oshikundu* samples were diluted ($10-1$ to $10-6$) in peptone water buffered (Biolab, Merck, Germany) and mixed thoroughly using a vortex. The 100 μL aliquots of these mixtures were inoculated on deMan Rogosa Sharpe (MRS) (De Man et al., 1960), M17 (Therzaghi and Sandine, 1975) and Rogosa agar and incubated aerobically at 37°C for 24 hours. After the first incubation period, colonies were further purified by successive streaking on MRS (Biolab, South Africa), M17 (Merck, Germany) and Rogosa agar (Biolab, South Africa) respectively. White/milky in colour colony were then subjected to enzymatic test (catalase) before DNA extraction. Catalase test was done by dissolving

colony from each selective media in a drop of 3% hydrogen peroxide placed on a clean slide (Harrigan, 1998) and only isolates that gave a negative reaction to catalase test were extracted for DNA. Meanwhile, yeast isolation was done following work by Liu et al. (2017) with modifications, no supplement or antibiotic were used on the isolation medium and malt extract and potato dextrose gar were used instead of yeast extract peptone dextrose. Using serial dilution ($10-1$ to $10-3$) samples in sterile peptone water buffered (Biolab, Merck, Germany) and mixed thoroughly using a vortex. To 100 μL aliquots of the mixture was inoculated on malt extract agar (MEA) and potato dextrose agar (PDA) before aerobic incubation at $25°C$ for 48 hours. Colonies were further successive streak on respective agar before DNA isolation.

2.3 Lactic acid bacteria and yeast DNA extraction

DNA extraction from yeast (11 isolates) and bacteria colonies (28 isolates) was done following standard phenol/chloroform method (Neumann et al., 1992) improved by Cheng and Jiang (2006). Bacteria and yeast, colonies were transferred into a clean 1.5 mL micro centrifuge tubes. The colonies were suspended in 200 μL TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), and 100 μL Tris-saturated phenol (pH 8.0) was added to all sample tubes before vortex-mixing for 60 seconds to lyse cells. For yeast samples, the suspension was sonicated for 10 minutes at $5°C$ after the addition of tris-saturated phenol. The samples were subsequently centrifuged at 4000 rpm for 5 min at $4°C$ to separate the aqueous phase from the organic phase. The 160 μL upper aqueous phase was transferred to a clean 1.5 mL micro centrifuge tube. To this 40 μL TE buffer was added to make 200 μL, mixed with 100 μL chloroform and centrifuged for 5 min at 4000 rpm at $4°C$. Lysate was purified by chloroform extraction until a white interface was no longer present. The 160 μL upper aqueous phase was transferred to a clean 1.5 ml micro centrifuge tube and 40 μL TE was added. Chloroform 100 μL volumes was added to the tube, mixed well and centrifuged for 5 min at 4000 rpm at $4°C$. A volume of 150 μL upper aqueous phase was transferred to a clean 1.5 mL micro centrifuge tube. The aqueous phase contained purified DNA and was stored at $-20°C$ for further use. The quantity and purity of extracted DNA from bacteria and yeast samples (1.5 μL) was analysed on a ND-Nanodrop (2000c, Thermo Fisher, Germany) spectrophotometer.

2.4 DNA amplification using Polymerase Chain Reaction (PCR)

DNA that was extracted from bacteria was amplified using standard PCR by two set of primers: Ec338f (5'-ACT CCT ACG GGA GGC AGC AGC AG-3') corresponding to nucleotide positions 349 to 368 and Ec518r (5'-ATT ACC GCG GCT GCT GG-3') targets the V3 region of the 16S ribosomal DNA with expected 400 bp corresponding to nucleotide

positions 529 to 545 and acetic acid and lactic acid bacteria primers:WBAC1 (5'-GTC GTC AGC TCG TGT CGT GAG A-3') corresponding to nucleotide positions 1069 to 1090 and WBAC2 (5'-CCC GGG AAC GTA TTC ACC GCG-3'), corresponding to nucleotide positions 1374 to 1394 on the *L. plantarum* 16S rDNA gene sequence (GenBank accession number AJ271852) for both set of the above primers (Lopez et al., 2003) targets the V7 to V8 regions of the 16S rDNA genes which produce amplicons of approximately 330 bp (Camu et al., 2007; Di Cagno et al., 2014). The PCR reactions for WBAC1 and WBAC2 were run for 30 cycles: Initial denaturation at $95°C$ for 5 min and final extension at $72°C$ for 5 minutes, denaturation was at $95°C$ for 60s, annealing at $67°C$ for 30s and extension at $72°C$ for 60s (Lopez et al., 2003). While for Ec338f and Ec518r the PCR reactions were run for 25 cycles: Initial denaturation at $94°C$ for 5 min and final extension at $72°C$ for 10 minutes, denaturation was at $94°C$ for 60s, annealing at $52°C$ for 60s and extension at $72°C$ for 60s (Amper et al., 1999; Omar and Amper, 2000; Lopez et al., 2003).

The DNA extracted from yeast was amplified by PCR using the forward primer NL1 (5'-GCC ATA TCA ATA AGC GGA GGA AAA G-3') and the reverse primer LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3') targets the D1 to D2 region of the 26 ribosomal DNA corresponding to nucleotide positions 266 to 285 on the S. cerevisiae 26S RNA gene (GenBank accession number M19229) (Cocolin et al., 2000). The reactions were run for 30 cycles: Initial denaturation at $95°C$ for 5 min and final extension at $72°C$ for 7 minutes, denaturation was at $95°C$ for 60s, annealing at $52°C$ for 45s and extension at $72°C$ for 60s (Cocolin et al., 2000). The PCR was set to hold at $4°C$. The reaction master mix for both bacteria and yeast was performed in a total volume of $25\,\mu L$ for each reaction with the following reagents: PCR master mix $12.5\,\mu L$ (1x), forward and reverse primers ($100\,\mu M$) for yeast and bacteria, $2.5\,\mu L$ ($0.5\,\mu M$) and $1.0\,\mu l$ ($0.2\,\mu M$) each respectively, DNA template $5\,\mu L$ ($\leq 500\,ng$) and nuclease free water $2.5\,\mu L$. PCR products were run on 2% agarose gels, stained with ethidium bromide and the DNA bands on the agarose gel were visualized using a UV trans-illuminator.

The un-purified PCR-products were sent for sequencing to Inqaba biotech (Pretoria, South Africa). The obtained sequences from Inqaba biotech (Pretoria, South Africa) were edited with Chromas lite (Technelysium Pty Ltd, Australia). The cleaned sequences in Federal Acquisition STreamlining Act (FASTA) format was subjected to Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990) for similarity identification. Multiple alignment and sequence similarity with that of available sequences of reference strains from GenBank database found in National Centre for Biotechnology Information (NCBI) (`www.ncbi.nlm.nih.gov`).

Figure 1: PCR products for lactic acid bacteria separated using gel electrophoresis on a 2% agarose gel. M: 100bp DNA ladder, lane C,D,G,H,I,J,L,M and N (Ec338f-Ec518r primer), O,P,Q,R,S,T,U,V,W,X,Y,Z (WBAC1-WBAC2 primer).

3 Results & discussion

3.1 LAB and Yeast PCR

After DNA isolation and quantification (section 2.3), the isolated DNA was used in the standard PCR (section 2.4) to amplify the targeted gene fragments for LAB and yeast. The LAB PCR (Figure 1) product analysed through gel electrophoresis on a 2% agarose gel shows the presence of two different amplicons; the amplicons generated with Ec338f-Ec518r primer combination was between 400-300 bp (lane C,D,G,H,I,J,L,M and N) and while that which was amplified with WBAC1-WBAC2 primer combination (O,P,Q,R,S,T,U,V,W,X,Y,Z) for LAB was 350-300 bp. Meanwhile the yeast PCR results (Figure 2) in lane A, D, G, H, I and K show amplified PCR products with 300-200 bp size amplified using the NL1-LS2 primer. The length of base pair given by the amplification of Ec338f-Ec518r and WBAC1-WBAC2 primer combination is in agreement for the expected base pair as reported by Lopez et al. (2003).

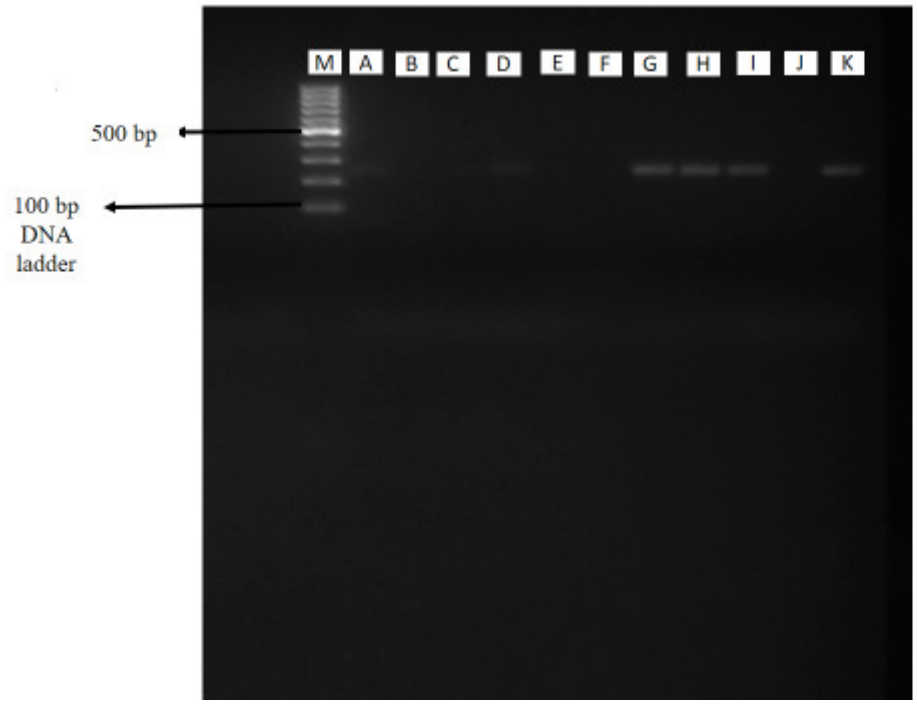

Figure 2: PCR products for yeast separated using gel electrophoresis on a 2% agarose gel. M: 100bp DNA ladder, lane A, D, G, H, I and K amplified PCR products with 300-200 bp size amplicons with NL1-LS2 primer.

3.2 LAB and Yeast identification

Identification of LAB and yeast in *oshikundu* was done through multiple alignment of the sequence by comparing to the GenBank (figure 3). The identified specie from oshikundu that $\geq 98\%$ similarity includes *L. plantarum, L. pentosus, L. acidifarinae, L. paraplantarum, L. spicheri, L. namurensis, L. zymae, L. fermentum, L. brevis, Leuconostoc gurlium, L. delbrueckii subsp bulgaricus, L. buncheri* and *Pediococcus acidilactici* (figure 4). The LAB *L. fermentum, L. pentosus, L. plantarum*, in *oshikundu* has been previously reported by Embashu (2014) using API 50 CH/CHL medium, in which *L. delbrueckii subsp. delbreckii* was reported meanwhile *L. delbrueckii subsp. bulgaricus* was identified in this study. Similar LAB *L. plantarum* and *L. brevis* (Lopez et al., 2003), *L. fermentum* and *P. acidilactici* (Vieira-Dalodè et al., 2007) were also reported in fermented product using different identification molecular methods.

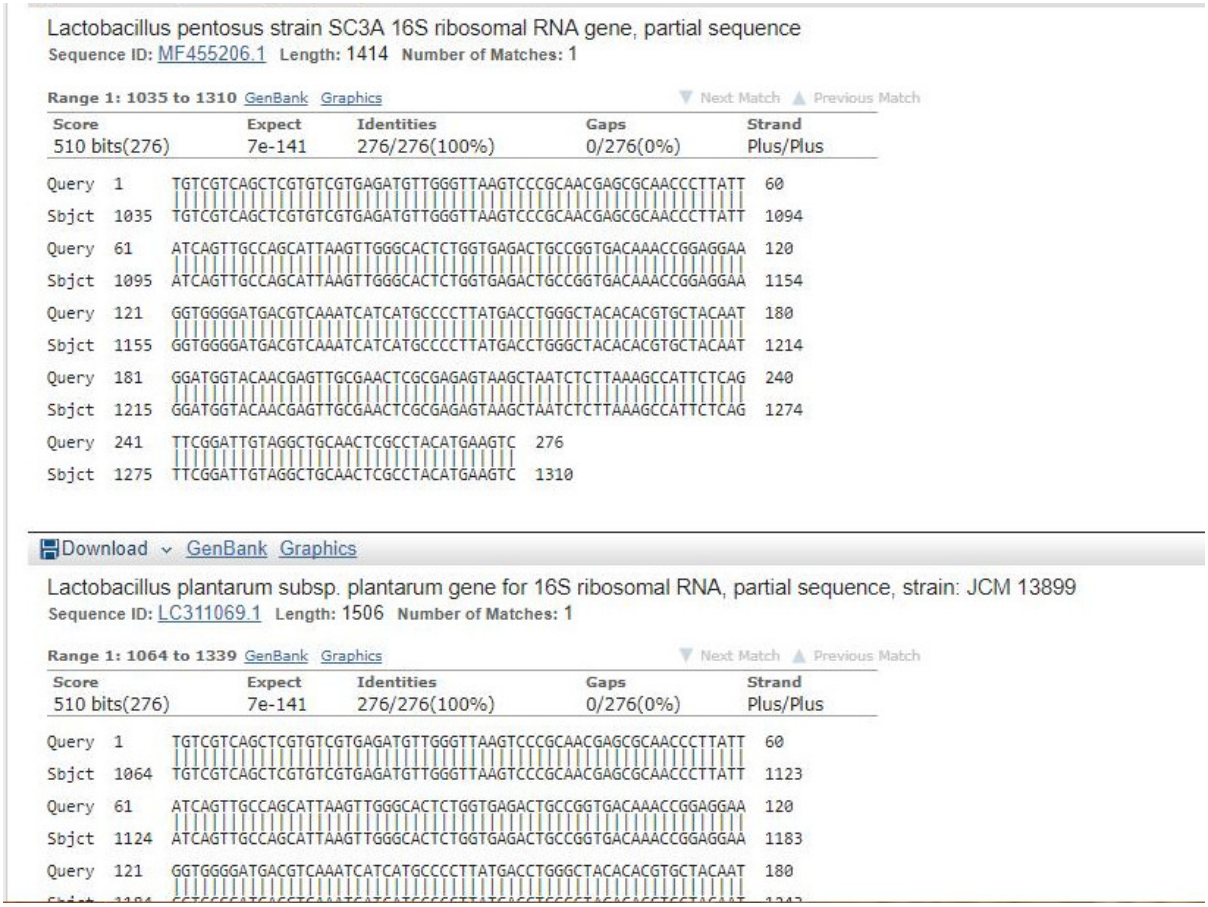

Figure 3: Multiple alignment and sequence similarity with that of available sequences (from *oshikundu* isolates) of reference strains from GenBank database of NCBI.

The LAB from *oshikundu* was clustered in four groups (figure 4) based on their similarity. The overall sequence similarity within different strains of a species ranged between 0.99 to 0.7 (boot strapping). The closest (0.99) was obtained in group III with a lower similarity obtained in divergence of group IV (figure 4). There were a low number of changes per 100 nucleotides site which was 0.2 (20%) (dissimilarity) among the groups, which showered a 0.99 value (boot strapping) in the divergence blanches. Given this, a high similarity value (0.96 and 0.91) in group I and II, may suggest a strong exclusion of any other to the node clusters.

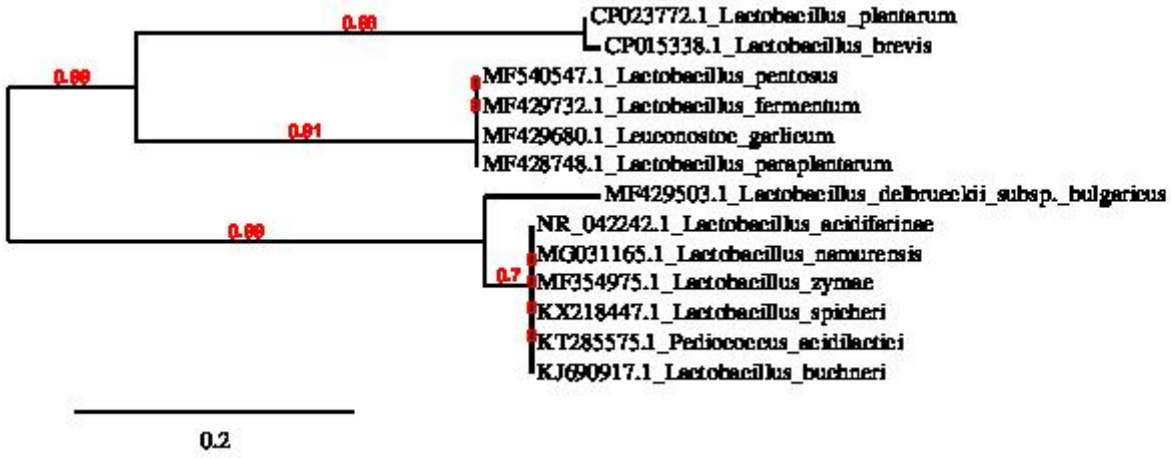

Figure 4: Phylogenetic tree for the identified LAB strains showing the genetic relatedness of LAB from *oshikundu*, obtained from multiple alignment and sequence similarity with that of available sequences of reference strains from GenBank database of NCBI, with number of changes per 100 nucleotide sites 0.2.

Meanwhile for yeast only *Saccharomyces cerevisiae, Saccharomyces paradoxus* and *Pichia kudriavzeii* were identified. *S. cerevisiae* (Stringini et al., 2009; Greppi et al., 2013) from similar fermented product have beer also reported, although using different molecular methods. The pH of *oshikundu* was reported to be between pH 3.33-3.60 by Embashu (2014). Generally, yeast grow on a wide range of pH but a pH 4-4.5 is favoured and LAB grow at pH lower than 4 (Taylor, 2016). According to the review by Blandino et al. (2003) some of the fermented beverages, food, weaning food for baby, gruel made from sorghum and millet to be fermented by *Lactobacillus, Leuconostoc* and *Saccharomyces spp.* that are similar fermenting microorganisms found in *oshikundu*. These millet and sorghum fermented products includes boza (*Lactobacillus, Saccharomyces cerevisiae, Leuconostoc*), buza (*Lactobacillus, Saccharomyces*), burukutu (*Saccharomyces cerevisiae, S. chavelieri, Leuconostoc mesenteroides, Candida, Acetobacter*), chikokivana (*Saccharomyces cerevisiae*), doro (yeasts and bacteria), kaffir beer (yeasts, LAB) merissa (*Saccharomyces*), ogi (*Ped. pentosaceus, L. fermentum, L. plantarum, Saccharomyces cerevisiae, Candida kruseii*) (Franz et al., 2014) and sorghum beer (LAB and yeast) (Blandino et al., 2003).

The identified bacteria from *oshikundu* of which are hetero-fermentative (Adams and Moss, 2008; Prückler et al., 2015) that are likely to produce multiple by-products such as lactic acid, ethanol/carbon dioxide (CO_2), acetic acid and possibly other organic acids during *oshikundu* fermentation. The Saccharomyces cerevisiae may also produce ethanol and carbon dioxide (CO_2) during fermentation of *oshikundu*. The bubbling and ethanol ($< 2\%\ v/v$) reported by Embashu (2014) could possibly be the end product of fermentation by the identified yeast: *Saccharomyces spp.* and *Pichia kudriavzevii* hetero-fermentative bacteria such *Leuconostoc*

L. brevis, L. fermentum, L. plantarum, L. pentosus (Adams and Moss, 2008; Prückler et al., 2015). Nonetheless, it remains unclear whether the alcohol reported by Embashu (2014) is the by-product of hetero-fermentative bacteria or yeast (*Saccharomyces*) in *oshikundu* or product of both, thus further investigations are required.

4 Conclusion

Like most of the traditional fermented products, *Oshikundu* brew appears to be the result of fermentation by LAB and *Saccharomyces cerevisiae*. Nonetheless it also appears that hetero-fermentative LAB is responsible for its alcoholic content. The interaction between LAB and yeast in *oshikundu* need more investigation.

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