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# Microbial biodiversity of traditional Beninese sorghum beer starter and multi-stage fermentation for beer safety and flavor improvement

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It’s really fascinating for exploring the biotechnological properties of both yeast and lactic acid bacteria. I now understood why Louis Pasteur (1822-1895) said: “**A bottle of wine contains more philosophy than all the books in the world**”.

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**CONTENT**

ACKNOWLEDGEMENTS……………………………………………………………………i

PUBLICATIONS ……………………………………………………………….……….ii

CONFERENCE CONTRIBUTIONS ………………………………………..………..iii

CONTENT ……………………………..……………………………..………..…..iv

ABBREVIATIONS ………………………………..…………………………………vi

SUMMARY ……………………………..……………………………………….....vii

ZUSAMENFASSUNG ……………………………..………………………………viii

1.INTRODUCTION…………………………..………………………………………1

1.1 African fermented beverages: functionality and safety problems ………………....1

1.2 Diversity of cyanide containing foods and sorghum cyanogenic glucoside metabolism……………………………………………………………………..…….4

1.3 Biotechnological properties of lactic acid bacteria occurring in cereal based products.............................................................................................................7

1.3.1 Lactic acid bacteria metabolism pathways and food preservation ………...……7

1.3.2 Food and beverage functionality due to the probiotic LAB and prebiotic substances…………………..……………………………………………………11

1.3.3 Proteolytic and amylolytic lactic acid bacteria and cereal based food saccharification………………..……………………………………………...…12

1.4 Potential biotic and abiotic factors influencing beer organoleptic qualities……….16

1.5 Motivation ………………………………………………………..……………..20

2. RESULTS (Paper publications) ……………………………..…………..…………21

2.1 Summary of results ……………………………..………………………...............21

2.2 β-D-glucosidase as "Key enzyme" for sorghum cyanogenic glucoside (dhurrin) removal and beer bioflavouring ………………………….……………………………25

2.3 Phenotypical and molecular characterization of yeast content in the starter of *Tchoukoutou*, a Beninese African sorghum beer ……………………………….……32

2.4 Polyphasic characterization of lactic acid bacteria isolated from Beninese sorghum beer starter……………………………………..………………………..……46

2.5 Assessment of malting and mash bio-acidification on the turnover of sorghum cyanogenic glucoside and protein hydrolysis improvement ……………………………..…..54

3. Discussion ……………………………..……………………………………………...61

4. References ……………………………………………………………………………76

## ABBREVIATIONS

ATP: Adenosine Tri-Phosphate

CECT: Spanish Type Culture Collection

FAO: Food and Agriculture Organisation

FHB: Fusarium Head Bright

$H_2O_2$: Hydrogen Peroxide

ITS: Internal Transcribed Spacer

LSU: Large Subunit

MALDI-TOF: Matrix Assisted Laser Desorption Ionisation-Time of Flight

MDS: Multidimensional Scaling

MS: Mass Spectrometry

NCBI: National Center for Biotechnology Information

OPP: Oligopeptides

PHB: Para-hydroxybenzaldehyde

PrtP: Protein Transporter

PYF: Premature Yeast Flocculation

RFLP: Restriction Fragment Length Polymorphism

RSM: Response Surface Methodology

VDKs: Vicinal Diketones

WHO: World Health Organization

**SUMMARY**

The traditional African sorghum beers are a sour-alcoholic drink obtained by spontaneous fermentation where a deposit of the previous drink is used as a starter. The cyanide-containing compounds (dhurrin) in sprouted grain of *Sorghum bicolor*, the instability of the beer's organoleptic quality and short shelf life hinders the competitiveness of traditional sorghum beers. The knowledge about the microbial population of the starter, biological mash acidification with selected aryl-β-D-glucosidase producer lactic acid bacteria (LAB) as well as an increase of the beer's aroma-active compounds were the focus to improve traditional African beers' organoleptic quality and solve the safety problem due to sorghum cyanogenic glucoside (dhurrin). To attain this goal, yeast and LAB were isolated from the starter and characterized using a polyphasic approach, the influence of sorghum malting on dhurrin metabolism was evaluated, and the mashing and fermentation processes were improved for significant dhurrin hydrolysis and beer flavour improvement. Our results show that the microbial population of Beninese African beer is very diverse where different yeasts including *Saccharomyces (S. cerevisiae) and non-Saccharomyces (Pichia kudriavzevii*, *Candida ethanolica* and *Debaryomyces hansenii)* co-operate with different species of *Lactobacillus: L. plantarum*, *L. fermentum*, *L. helveticus, L brevis, L. acidophilus* and *L. paracasei*. We also observed that the high amount of dhurrin (244 -180 ppm) is sequestrated in sorghum malts after sprouting and the normal mashing procedure is limited in terms of significant, let alone complete, dhurrin removal. Sorghum bio-acidification with *L. paracasei* ND-34*,* the highest aryl-β-D-glucosidase producer LAB strain, completely removed dhurrin in the brown sorghum wort. Furthermore, an improvement of saccharification characterized by an increase of free amino nitrogen, amino acids and sugars was also observed after this biological acidification. The ale yeast S81 is stressed during sorghum wort fermentation and the uptake of diacetyl and 2,3-pentanedione (vicinal diketones) is limited compared to the strain TO-37 isolated from the traditional starter as *S. cerevisiae.* The latter is able to syntheze important aroma-active components such as isoamyl alcohol, isoamyl acetate and acetyl acetate up to 116 mg/L, 1.8 mg/L and 22.1 mg/L, respectively, from 13 Plato sorghum wort after 72 hours of fermentation at 27 °C. We are confident that our approach based on multi-stage fermentation will help improve sorghum beers qualities.

**ZUSAMENFASSUNG**

Die traditionellen afrikanischen Sorghumbiere sind saueralkoholische Getränke, die durch spontane Fermentation erhalten werden, wobei ein Überrest des vorherigen Getränks als Starter verwendet wird. Die cyanidhaltigen Verbindungen (Dhurrin) im gekeimten Korn von *Sorghum bicolor*, die Instabilität der organoleptischen Qualität und die kurze Haltbarkeit des Bieres behindern die Verbreitung traditioneller Sorghumbiere. Das Wissen um die mikrobielle Population des Starters, die biologische Maische-Ansäuerung mit ausgewählten Aryl-β-D-Glucosidase-produzierenden Milchsäurebakterien (LAB) sowie eine Erhöhung der bieraromatischen Wirkstoffe standen im Vordergrund, um die organoleptische Qualität der traditionellen afrikanischen Biere zu verbessern und das Sicherheitsproblem durch cyanogenes Glucosid des Sorghum (Dhurrin) zu lösen. Um dieses Ziel zu erreichen, wurden Hefe und LAB aus dem Starter isoliert und unter Verwendung eines polyphasischen Ansatzes charakterisiert, der Einfluss von Sorghum-Mälzen auf den Dhurrin-Stoffwechsel wurde bewertet und die Maisch- und Fermentationsprozesse wurden verbessert für eine signifikante Dhurrin-Hydrolyse und Verbesserung des Bieraromas. Unsere Ergebnisse zeigen, dass die mikrobielle Population des afrikanischen Bieres sehr divers ist, wo verschiedene Hefen einschließlich *Saccharomyces (S. cerevisiae)* und Nicht-*Saccharomyces (Pichia kudriavzevii*, *Candida ethanolica* und *Debaryomyces hansenii)* mit verschiedenen Arten von *Lactobacillus* zusammenarbeiten: *L. plantarum*, *L. fermentum*, *L. helveticus*, *L brevis*, *L. acidophilus* und *L. paracasei*. Wir beobachteten auch, dass die hohe Menge an Dhurrin (244-180 ppm) in Sorghummalz nach der Keimung gebunden wird und das normale Maischenverfahren für eine signifikante oder gar vollständige Entfernung von Dhurrin nicht ausreicht. Sorghum-Bio-Ansäuerung mit *L. paracasei* ND-34, dem höchsten Aryl-β-D-Glucosidase-produzierenden LAB-Stamm, entfernte Dhurrin vollständig aus der braunen Sorghumwürze. Darüber hinaus wurde nach dieser biologischen Ansäuerung auch eine Verbesserung der Verzuckerung beobachtet, die durch einen Anstieg von freiem Aminostickstoff, Aminosäuren und Zuckern gekennzeichnet ist. Die Ale-Hefe S81 wird während der Sorghum-Würze-Fermentation gestresst und die Aufnahme von Diacetyl und 2,3-Pentandion (vicinalen Diketonen) ist im Vergleich zu dem vom traditionellen Starter isolierten Stamm TO-37 als *S. cerevisiae* begrenzt. Dieser ist fähig, wichtige aromaaktive Komponenten zu synthetisieren, wie Isoamylalkohol, Isoamylacetat und Acetylacetat, nach einer 72-stündigen Fermentation bei 27 °C bis zu 116 mg/L, 1,8 mg/L bzw. 22,1 mg/L aus 13 Plato-Sorghumwürze. Wir sind

zuversichtlich, dass unsere Ergebnisse helfen werden, traditionelle Biere zu verbessern

# 1. INTRODUCTION

## 1.1 African fermented beverages: functionality and safety problems

Fermentation is one of the oldest and most economical methods of producing and preserving foods. It is also a natural way to destroy undesirable components, to enhance the nutritive value and organoleptic quality of the food, to reduce energy required for cooking and to make a safer product (Simango, 1997). The preparation of fermented foods and beverages first occurred in an artisan way without any knowledge of the role of microorganisms involved before the industrial revolution by the middle of the nineteenth century. Whatever the scientific and technological revolutions, several fermented foods and beverages are still produced based on ancestral knowledge, which is transmitted from one generation to another, especially in developing countries. In all African countries, cereal grains such as sorghum (*Sorghum bicolor* (L) *Moench*), millets (*Pennisetum glaucum*, *Eleusine coracana*) and maize (*Zea mays*) are common raw materials used to produce traditional non-alcoholic and alcoholic beverages. The traditional fermented foods are very diverse, which results from the multitude of different used raw materials, the processing technology and the composition of the food substrate. The latter is an important intrinsic factor of selection of microorganisms, which thereby determine the type of fermentation: alcoholic, lactic acid, acetic acid and alkali fermentation. Spontaneous fermentation is the key characteristic of African traditional fermented foods and beverages, and the biodiversity of the occurring microorganisms during this step is the main factor responsible for the different bouquet of traditional fermented foods and beverages. *Gowe* is a popular non-alcoholic Beninese beverage obtained by spontaneous lactic acid fermentation, sold as a paste product, and consumed as an energy drink after adding water, sugar, ice and milk (Adinsi et al., 2017; Michodjehoun-Mestres, Hounhouigan, Dossou, & Mestres, 2005; Vieira-Dalodé, Jespersen, Hounhouigan, Moller, Nago, & Jakobsen, 2007). Similar cereal-based non-alcoholic beverages obtained by lactic acid fermentation are also present in other African countries and known as *Bushera* in Uganda (Muyanja, Narvhus, Treimo, & Langsrud, 2003), *Borde* in Ethiopia (Kebede, 2007), *Kunu-zaki* in Nigeria (Obadina, Oyewole, and Awojobi, 2008) and *Mahewu* or *Maphulo* in South Africa (Blandino et al., 2003; Gadaga, Mutukumira, Narvhus & Feresu, 1999). Besides African non-alcoholic beverages, the most widespread traditional beverage produced in all African countries is the traditional alcoholic beverage. The processing technique

is different from one locality to another but always involves malting, mashing and spontaneous fermentations (Kayodé, Hounhouigan, & Nout, 2007; Osseyi, Tagba, Karou, Ketevi & Lamboni, 2011). After sorghum malting and mashing, the deposit of previous beer is used as a starter to initiate the fermentation during production of *Tchoukoutou* and other traditional African beers. This traditional starter used by Beninese sorghum beer producers contains different lactic acid bacteria and yeasts which produce lactic acid and alcohol as main metabolites, respectively. The occurring yeast and lactic acid bacteria probably cooperate for the taste and flavor which characterizes *Tchoukoutou*. Outside the desirable organoleptic quality, African sorghum beers have also been positively indexed for their functional qualities due to the health-promoting properties of sorghum: prevention and reduction of oxidative stress and cardio-vascular diseases as well as anti-cancer, anti-diabetic, anti-inflammatory, and anti-hypertensive effects (Althwab, Carr, Weller, Dweikat, & Schlegel, 2015; Taylor, Schober, & Bean, 2006). The brown and red varieties of sorghum are a source of phenolic compounds with a high concentration of anthocyanins, especially luteolinidin and apigenidin (Awika & Rooney, 2004). Another nutritional importance of African sorghum beer is its health benefit due to some specific occurring microorganisms. Besides the use of the beer deposit as a starter to initiate fermentation, it is also used for the treatment of some human diseases. N'tcha et al. (2015) interviewed 90 producers of Beninese sorghum beer (*Tchoukoutou*) of which 80 % reported that the deposit of *Tchoukoutou* is also used for the treatment of diarrhea. African sorghum beer is drunk while the fermentation is ongoing, and consumers also claim that this traditional fermented beer promotes health such as curing diarrhea (Kayodé, Deh, Baba-moussa, Kotchoni, & Hounhouigan, 2012; Schoustra, Kasase, Toarta, Kassen, & Poulain, 2013). Sanni, Franz, Schillinger, Huch, Guigas, & Holzapfel (2013) isolated many lactic acid bacteria from *ogi* and *fufu*, West African traditional fermented foods and observed that many strains were able to produce $H_2O_2$, and were tolerant towards bile and low pH with a good adherence capacity to mucus-secreting epithelial cells. Whatever these potential probiotic properties of African sorghum beer microbiota, there is still a lack of scientific works regarding the reengineering of the traditional fermented food and beverage processing techniques and the domestication of these microorganisms with a potential probiotic properties.

Regardless of the desirable organoleptic quality of African sorghum beers and its health benefits, several potential consumers are waiting for the improvement of its hygienic quality in order to include these lower-cost beverages into their food habits. The materials used for African sorghum beer processing are rudimentary and composed of large jars for mashing, cooking pots, and basket and cloth for filtration (Osseyi, Tagba, Karou, Ketevi, & Lamboni, 2011). The processing techniques are unstandardized and metabolites resulting from conversion are not monitored during fermentation. The sensory characteristics are unstable, and the beer has a short shelf life and cannot be conserved more than 3 days. The contaminants of African sorghum beer can be classified into two different groups: exogenous contaminants (enterotoxins, mycotoxins) and the sprouted sorghum's endogenous cyanogenic glucoside (dhurrin). Even though it is true that below pH 4.5, most pathogenic microorganisms are not able to grow, acid-resistant pathogenic microorganisms exist, which can survive the fermentation process. Nout & Motarjemi (1997) reported that foodborne viruses such as rotavirus responsible for diarrhea have been shown to be relatively acid resistant. Concomitant research work shows the acid resistance of strains belonging to *Escherichia coli* (*E. coli 0157:H7*). Another exogenous contaminant which compromises the safety of African traditional beers is the presence of mycotoxins. Mycotoxins are toxic secondary metabolites produced by fungi and contaminate various agricultural commodities either before harvest or under post-harvest conditions. The climatic conditions, poor harvesting practices, improper storage, poor transportation conditions and poor control during processing, especially during germination, contribute to fungal growth and therefore increase the risk of mycotoxin production. Aflatoxin produced by *Aspergillus flavus* is particularly dire in Africa and other tropical countries (Bankole & Adebanjo, 2003; Lewis et al., 2005; Muthomi, Erke, Ehne, & Utitu, 2002; Wagacha & Muthomi, 2008). Several cases of death were reported from India (more than 106 cases) in 1974, in Nigeria (more than 100 cases) in 2005 and especially in Kenya (215 cases) in 2004 by Azziz-baumgartner et al., (2005) and Krishnamachari, Nagaarajan, Bhat & Tilak, (1975). Ezekiel et al., (2015), Matumba et al., (2014) and Odhav & Naiker (2002) also reported several cases of traditional cereal-derived fermented foods and beverages contaminated by aflatoxin B1, fumonisin (B1, B2, B3) and ochratoxin A. In European countries, Fusarium Head Blight (FHB) is an important disease of barley (*Hordeum vulgare*). *F. graminearum* and *F. culmorum* are the most damaging from the FHB complex, followed by *F. poae* and

*F. avenaceum*, impacting several malting and brewing quality parameters (Schwarz, Schwarz, Zhou, Prom & Steffenson, 2001). Concomitant research works demonstrate that cereal contamination by fungi induces premature yeast flocculation (PYF) during fermentation (Axcell, Van Nierop & Vundla, 2000; Herrera & Axcell, 1991; Lake & Speers, 2016; Nierop, Axcell, Cantrell, & Rautenbach, 2008; Panteloglou, Smart & David, 2012; Shang, Li, Cai, Lu, & Chen, 2014; Verstrepen, Derdelinckx, Verachtert & Delvaux, 2003) as well as beer gushing problems (Beattle, Schwarz, Horsley, Barr & Casper, 1998; Lowe, Ulmer, Sinderen, & Arendt, 2004; Schwarz, Beattle, & Casper, 1996; Wolf-hall, 2007). To limit death caused by food contamination and malnutrition, the FAO/WHO workshop held in Pretoria (South Africa) suggested cereal germination and fermentation to improve the safety and nutritional value of traditional food and beverages (Nout & Motarjemi, 1997). Unfortunately, the toxigenic hydrogen cyanide precursor (dhurrin) is synthetized during sorghum germination (Ahmed, Mahgoub & Babiker, 1996; Haque & Bradbury, 2002; Ikediobi, 1988; Møller, 2010; Panasiuk & Bills, 1984; Traoré, Mouquet, Icard-Vernière, Traoré & Trèche, 2004; Uvere, Adenuga & Mordi, 2000).

### 1.2 Diversity of cyanide containing foods and cyanogenic glucoside metabolism

Cyanogenic glucosides, plant secondary metabolites, account for approximately 90 % of the wide group of plant toxins. The major edible plants in which cyanogenic glucosides occur are bitter almonds (*Prunus dulcis*), sorghum (*Sorghum bicolor*), cassava (*Manihot esculenta*), lima beans (*Phaseolus lunatus*), apricot (*Prunus armeniace*) kernel, peach (*Prunus persica*) kernel and bamboo shoots (*Bambusa arundinacea*) (Haque & Bradbury, 2002). There are approximately 25 cyanogenic glucosides known with the major cyanogenic glucosides found in the edible parts of plants being: amygdalin (almonds); linamarin (cassava, lima beans); lotaustralin (cassava, lima beans); prunasin (stone fruit); taxiphyllin (bamboo shoots); and dhurrin (sorghum). The relation between amino acids and cyanogenic glucosides was clearly demonstrated with incorporation of $^{14}C$-labelled amino acids. The biosynthesis precursors of the cyanogenic glucosides are therefore amino acids such as L-valine for linamarin, L-isoleucine for lotaustralin, L-leucine for osmaronin, L-phenylalanine for prunasin or amygdalin and L-tyrosine for dhurrin (Fig. 1). The non-protein amino acid cyclopentenyl-glycine has been also reported.

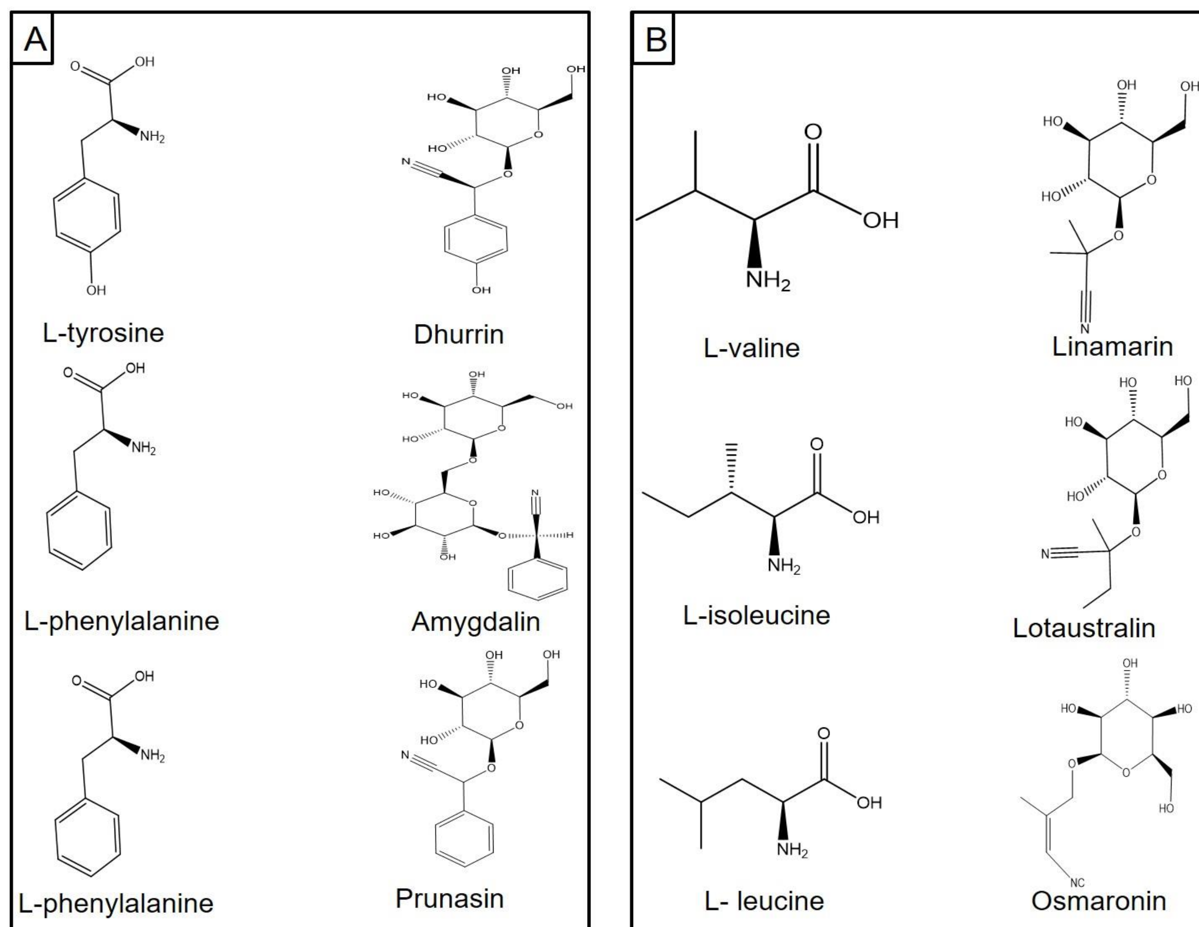


**Figure 1:** Structure of aromatic (A) and aliphatic (B) cyanogenic glucosides and the corresponding precursor amino acids.

The general pathway of biosynthesis of cyanogenic glucosides is given after Tapper & Reay, (1973): the α-amino acids are hydroxylated to form an N-hydroxylamino acid, which is then converted to an aldoxime, this in turn to a nitrile. The nitrile is hydroxylated to form an α-hydroxynitrile, which is glucosylated to form the corresponding cyanogenic glucoside (McFarlane, Lees & Conn, 1975). All known cyanogenic glucosides are β-linked, mostly with D-glucose. Formation of metabolons (macromolecular enzyme complexes) facilitating the channelling of substrates in biosynthetic pathways have been reported by Gnanasekaran et al. (2016); Jensen, Anne, Hamann, Naur, & Lindberg (2011) and Vazquez-albacete et al. (2017) and several cytochrome-P450 enzymes from plants associated with distinct cyanogenic glucosides have been isolated and characterized. After the two-step hydroxylation mediated by cytochrome-P450 of cyanogenic glucoside synthesis, UDP-Glc-Glucosyltransferase catalyses the third step characterized by the transfer of sugar moieties from activated donor

molecules to specific acceptor molecules, forming glucosidic bonds. The term “cyanogenesis” means not only the synthesis or presence of the cyanogenic glucosides but also the enzymatic hydrolysis producing the free hydrogen cyanide and other compounds such as aglycone and sugar. Upon tissue disruption, caused by chewing insect or livestock, the cyanogenic glucosides are brought in contact with specific β-glucosidases, resulting in the formation of sugar and a cyanohydrin (α-hydroxynitrile) which then decomposes spontaneously or in an enzymatic reaction catalysed by hydroxynitrile lyase, resulting in the formation of a ketone or aldehyde (aglycone) and hydrogen cyanide (Fig. 2).

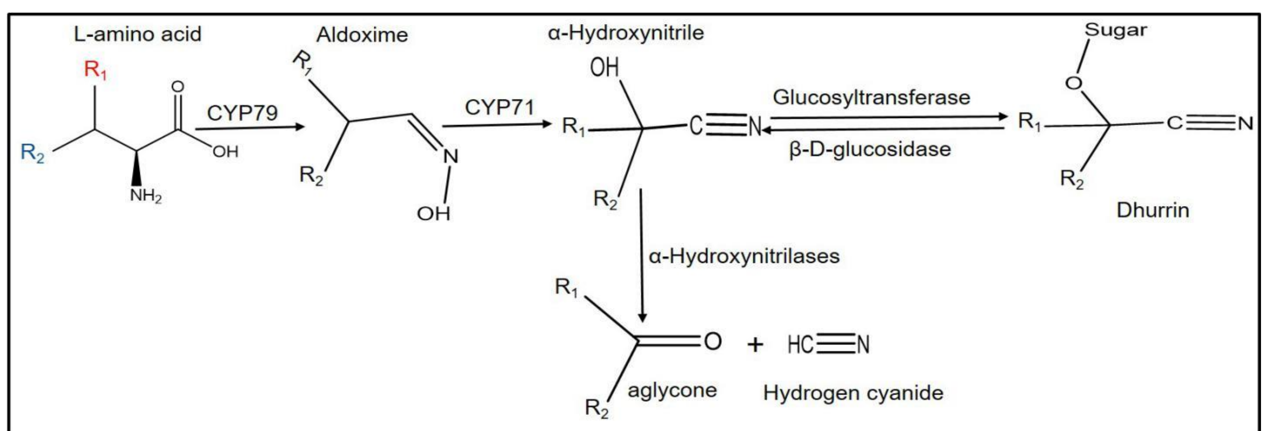


**Figure 2:** Cyanogenesis: General metabolism pathway of cyanogenic glucoside synthesis and its hydrolysis by β-D-glucosidase to hydrogen cyanide and aglycone (ketone or aldehyde) via α-hydroxynitrile. See Table 1 for $R_1$ and $R_2$.

Cyanide-containing foods are responsible for several intoxications linked to the high prevalence of toxico-nutritional neurogical diseases, especially *konzo*, characterized by a distinct neurogical entity with selective upper motor neuron damage, and death in developing countries (Banea, Bradbury, Mandombi, Nahimana, Denton, Foster & Katumbay, 2015; Nzwalo & Cliff, 2011) because of the nutritional status which plays an important role for the detoxification by the organism.

**Table 1:** Diversity of cyanogenic glucosides occurring in foods, feeds, and the precursor L-amino acids.

| Names | Precursor | R1 | R2 | Sugar | Occurrence |
|---|---|---|---|---|---|
| Dhurrin | L-tyrosine | p-Hydroxyphenyl | Hydrogen | glucose | Sorghum |
| Taxiphyllin | L-tyrosine | p-hydroxyphenyl | Hydrogen | glucose | Bamboo shoot |
| Amygdalin | L-phenylalanine | Phenyl | Hydrogen | Gentiobiose | Amonds, apricot, |
| Linamarin | L-valine | Methyl | Methyl | Glucose | Cassava, lima bean |
| Prunasin | L-phenylalanine | Phenyl | Hydrogen | Glucose | Ferns (bracken fern) |
| Linustatin | L-valine | Methyl | Methyl | Gentiobiose | Flax seed, cassava |
| Lotaustralin | L-isoleucine | Methyl | Ethyl | Glucose | Cassava, lima bean |
| Neolinustatin | L-isoleucine | Methyl | Ethyl | Gentiobiose | Flax seed |
| Sambunigrin | L-phenylalanine | Phenyl | Hydrogen | Glucose | Elderberries |

Dhurrin and taxiphyllin are different by the configurations S and R, respectively. The prunasin and sambunigrin also differ by the configurations R and S, respectively. (Lechtenberg & Nahrstedt, 1999).

## 1.3 Biotechnological properties of lactic acid bacteria occurring in cereal-based products

### 1.3.1 Lactic acid bacteria metabolism pathways and food bio-preservation

The contemporary consensus definition considers a typical lactic acid bacteria as Gram-positive, non-spore forming, catalase-negative, devoid of cytochromes, non-aerobic but aerotolerant, nutritionally fastidious, acid-tolerant and strictly fermentative, with lactic acid as the major end-product of sugar fermentation (Franz & Holzapfel, 2011; Klein, Pack, Bonaparte, & Reuter, 1998). Lactic acid bacteria have been intimately associated with human culture and well-being throughout history. In our time, the industrialization of food bio-transformations and the positive contributions of

particular microbes to sensory, quality and safety features of fermented foods have become synonymous with the positive image of lactic acid bacteria. Improved microbiological safety through competitive antagonistic microorganisms or their metabolic products is the main goal of biological food preservation. The biological significance is thought to be that of amensalism, a means of one bacterium gaining advantage over another competing microbe. This can be achieved by changing the environment by acidification or production of "toxins" against competitors (Walter P Hammes & Tichaczek, 1994; Wilhelm H Holzapfel & Wood, 2014; Tannock, 1981). Upon fermentation of hexoses, lactic acid is produced by homofermentation or equimolar amounts of lactic acid, acetic acid/ethanol and $CO_2$ are produced by heterofermentative lactic acid bacteria (Fig.3). It has long been observed that weak acids (lactic acid, acetic acid) have a more powerful antimicrobial activity at low pH than neutral pH (Simon & Blackman, 1949). Between the two acids, acetic acid is the strongest inhibitor and has a wide range of inhibitory activity against yeast, molds and bacteria. This stronger antimicrobial activity of acetic acid is due to its higher pKa (4.87) compared to that of lactic acid (3.08). A mixture of lactic acid and acetic acid has been observed to reduce the growth rate of *Salmonella enterica* ser.var. *typhimurium* more than either acid alone, suggesting a strong synergistic activity between lactic acid and acetic acid (Rubin, 1978) when heterofermentative lactic acid bacteria are used for food preservation. The stronger inhibition effect of propionic acid has also been proven by researchers and is comparable to that of acetic acid (Eklund, 1983). In the presence of oxygen, lactic acid bacteria are able to generate hydrogen peroxide. The bactericidal effect of hydrogen peroxide has been attributed to its strong oxidizing effect on the bacterial cell (Lindgren & Dobrogosz, 1990; Morris, 1976). When Lemoigne (1927) described the antimicrobial properties of diacetyl, Jay (1982) observed that diacetyl is more active against gram-negative lactic acid bacteria and demonstrated that diacetyl reacts with the arginine-binding protein of gram-negative bacteria, thereby interfering with the utilization of this amino acid. There are also several reports on the production of low molecular weight anti-microbial components (reuterin, reutericyclin, 2-pyrrolidone-5-carboxylic acid) by lactic acid bacteria (Dobrogosz, Casas, Pagano, Talarico, Sjöberg & Karlsson, 1989; Gänzle & Vogel, 2003)

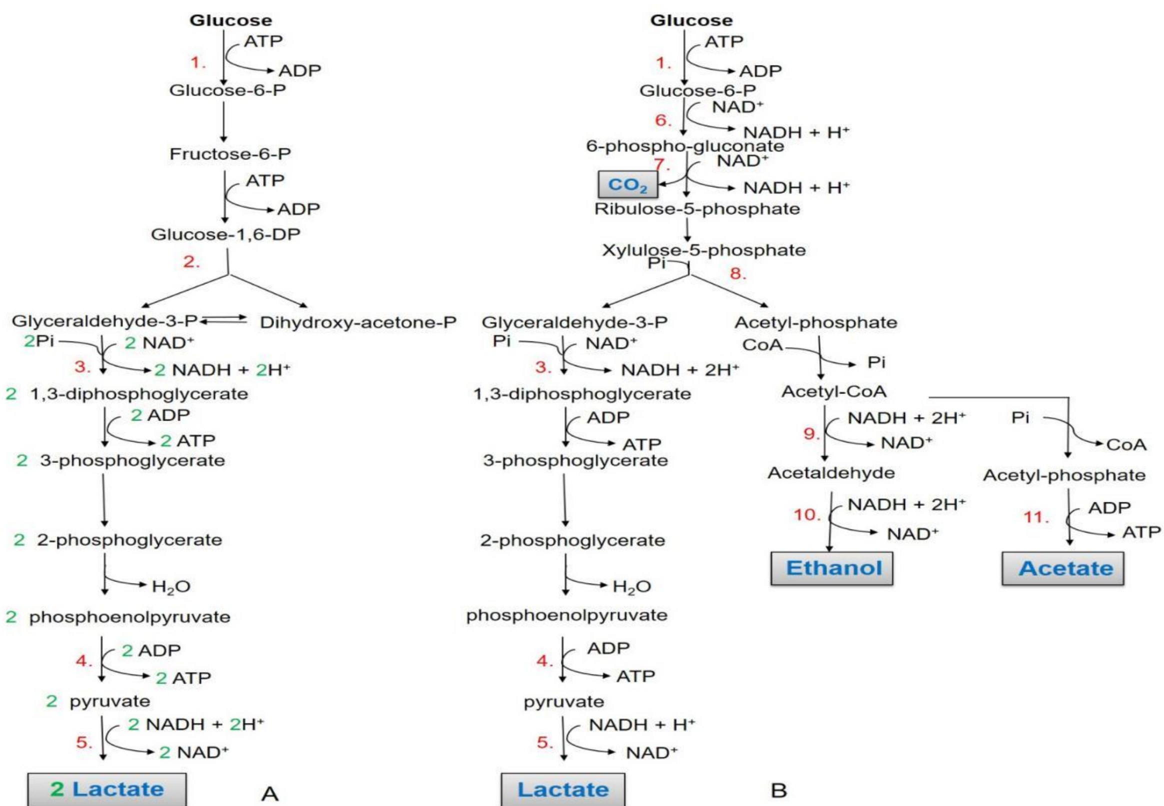


**Figure 3:** Major fermentative pathways of glucose: (A) homolactic fermentation, (Glycolysis-Meyerhof-Parnas pathway), (B) heterolactic fermentation (6-phosphogluconate/phosphoketolase pathway). The enzymes catalysing the reactions are numbered: 1. Glucokinase, 2. Fructose-1,6-diphosphate aldolase, 3. Glyceraldehyde 3-phosphate dehydrogenase, 4. Pyruvate kinase, 5. Lactate dehydrogenase, 7. 6-phosphogluconate dehydrogenase, 8. Phosphoketolase, 9. Acetaldehyde dehydrogenase, 10. Alcohol dehydrogenase, 11. Acetate kinase.

Among these varieties of antimicrobial substances produced by lactic acid bacteria, bacteriocins are one of the most promising natural food preservatives. Bacteriocins are extracellularly released, ribosomally synthesized, bioactive, small or complex peptides that have a bactericidal or bacteriostatic effect on other species, generally inhibiting the microorganisms that are closely related to the producing strain (Bearso, Bearson & Forster, 1997; Cherrington, Hinton, Mead & Chopra, 1991; Shortt, 1999). All bacteriocins are synthesized as a biologically inactive prepeptide carrying an N-terminal leader peptide attached to the C-terminal propeptide (Hoover & Chen, 2005). The immunity protein provides total protection to the producer of the bacteriocin (Fig. 4). The mode of action against food pathogenic flora occurs through several steps of

recognition involving different domains and structures of this antimicrobial peptide that end by killing the target cell after pore formation. The initial binding is characterized by electrostatic interactions with negatively charged phosphate groups on the target cell membranes.

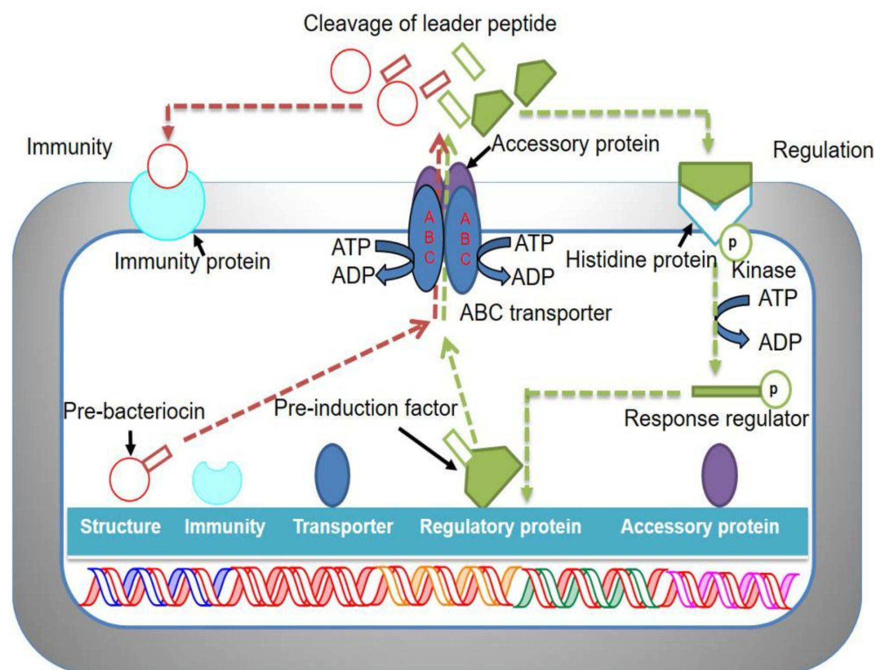


**Figure 4**: Biosynthesis of bacteriocin with immunity of producer and regulation mechanism. Formation of prebacteriocin and prepeptide of induction factor (IF). The processing of the prebacteriocin and pre-IF and translocation by the ABC-transporter result in the release of mature bacteriocin and IF. Histidine protein kinase senses the presence of IF and autophosphorylates. The transfer of the phosphoryl group to the response regulator activates transcription of the regulated genes and the immunity of the producer.

Nisin is the best-known and characterized bacteriocin, the only one with a commercial use. It has been reported that Nisin is effective against more than 90 % of gram-positive beer spoilage microorganisms (Müller-Auffermann, Grijalva & Jacob, 2015). Nisin interferes with the cytoplasmic membrane of the susceptible species by binding to the anionic phospholipids, especially lipid II, which is an essential element in cell wall formation (Fig. 5). Subsequently, nisin permeates and inserts itself into the membrane,

forming an ion channel or a pore. This allows the efflux of intracellular components, such as ATP, potassium, protons and amino acids (Abee, 1995; Gert, Konings, & Driessen, 1999; Montville & Bruno, 1994). Bacteriocins therefore generate great interest in the food industry owing to their properties: they inhibit food spoilage and pathogenic bacteria, are non-toxic, do not interfere with the product taste and are naturally produced.

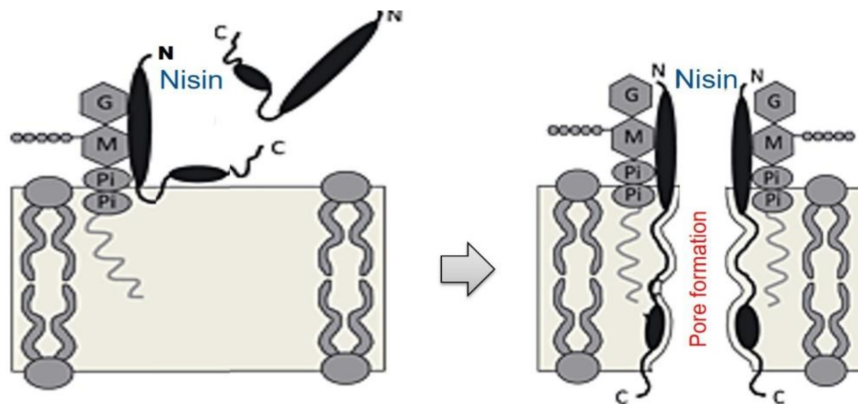


**Figure 5:** Mode of action of nisin. Reported from Müller-Auffermann, Grijalva & Jacob, (2015)

### 1.3.2 Food and beverage functionality due to the probiotic LAB and prebiotic substances

African fermented food and beverage microbiota is not very well characterized. However, the central role of lactic acid bacteria in fermentation is widely acknowledged, and it is accepted that these microorganisms exert beneficial effects through two mechanisms: direct effects of the live microbial cells, known as the "probiotic effect", or indirect effects during fermentation where these microbes act as cell factories for the generation of secondary metabolites with health-promoting properties (Hayes, Stanton, Fitzgerald, & Ross, 2007), as partially described in section 1.3.1. As reported by Vaughan & Vos (2005), Rastall et al. (2005) defined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". At the beginning of the 20$^{th}$ century, potential health-promoting effects associated with several lactic acid bacteria were highlighted by Metchnikoff (1908). Since then, numerous commercial products with such postulated claims abound in the dairy-based food and beverage markets. The health benefit of probiotic microorganisms have been related to inhibiting gut pathogens by interference or antagonism, immune-modulation, anticarcinogenic activities, alleviating lactose

intolerance symptoms, reducing serum cholesterol levels, reducing blood pressure, preventing and decreasing incidence and duration of diarrhea, maintaining mucosal integrity, and being involved in protection against gut cancer (Parvez, Malik, Kang, & Kim, 2006; Reid, 1999)

The consumption of live bacteria is not the only strategy to increase the size of the health-beneficial cell population in the intestinal tract. An alternative approach has been investigated where the commensal bifidobacteria or lactobacilli are selectively promoted by intake of certain nonviable substances, known as prebiotics. Gibson & Roberfroid, (1995) first described a prebiotic as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and / or activity of one or a limited number of bacteria in a colon, and thus improves host health". For a dietary substrate to be classed as a prebiotic, three criteria are therefore required: The substrate must not be hydrolyzed or absorbed in the stomach or small intestine, it must be selective for beneficial commensal bacteria in the colon such as bifidobacteria, and finally the substrate should induce beneficial luminal / systemic effects within the host. Through diet, resistant starch is the quantitatively most important (Cummings & Macfarlane, 1991). Non-starch polysaccharides form the next largest contribution and include plant-derived substrates such as pectin, cellulose, hemicellulose, guar and xylan. The premise behind prebiotics is therefore to stimulate certain indigenous bacteria in the gut rather than introducing exogenous species, as is the case with probiotics.

### 1.3.3 Proteolytic and amylolytic lactic acid bacteria and cereal-based food saccharification

It is a general belief that lactic acid bacteria have a very limited capacity to synthesize amino acids using inorganic nitrogen sources. The growth on chemically defined minimal media is then slow and lactic acid bacteria have adapted by developing systems to efficiently exploit the nitrogen source present. One of the most extensively studied systems in this regard is the proteolytic system. Proteolysis in dairy lactic acid bacteria has been studied in detail and from these studies, the picture emerges that the proteolytic systems of lactococci and lactobacilli are remarkably similar in their components and mode of action. The structural components of proteolytic systems of lactic acid bacteria can be divided into three groups on the basis of their function:

proteinases that break down casein to peptides, peptidases that degrade peptides, and transport systems that translocate the breakdown products across the cytoplasmic membrane. Proteinase is excellularly located and various researchers attempting to define the substrate binding pocket for protein degradation have concluded that proteinase has a broad substrate specificity (Juillard, Le Bars, Kunji, Konings, Gripon & Richard, 1995). To use the amino acids for biosynthesis, products derived from protein degradation traverse the membrane. Transport studies in peptidase-free membrane vesicles of *Lactococcus lactis* have shown that relatively hydrophilic di- and tripeptides are transported by a proton motive force-driven transport mechanism (Smid, Driessen & Konings, 1989). Kunji, Mierau, Hagting, Poolman & Konings (1996) also show that *Lactococcus lactis* possesses a transporter that is specific for oligopeptides (Opp) as well and these oligopeptide transporters are driven by ATP rather than proton motive force. Following the breakdown by PrtP and transport, the protein-derived peptides are further hydrolyzed by peptidase (Fig. 6).

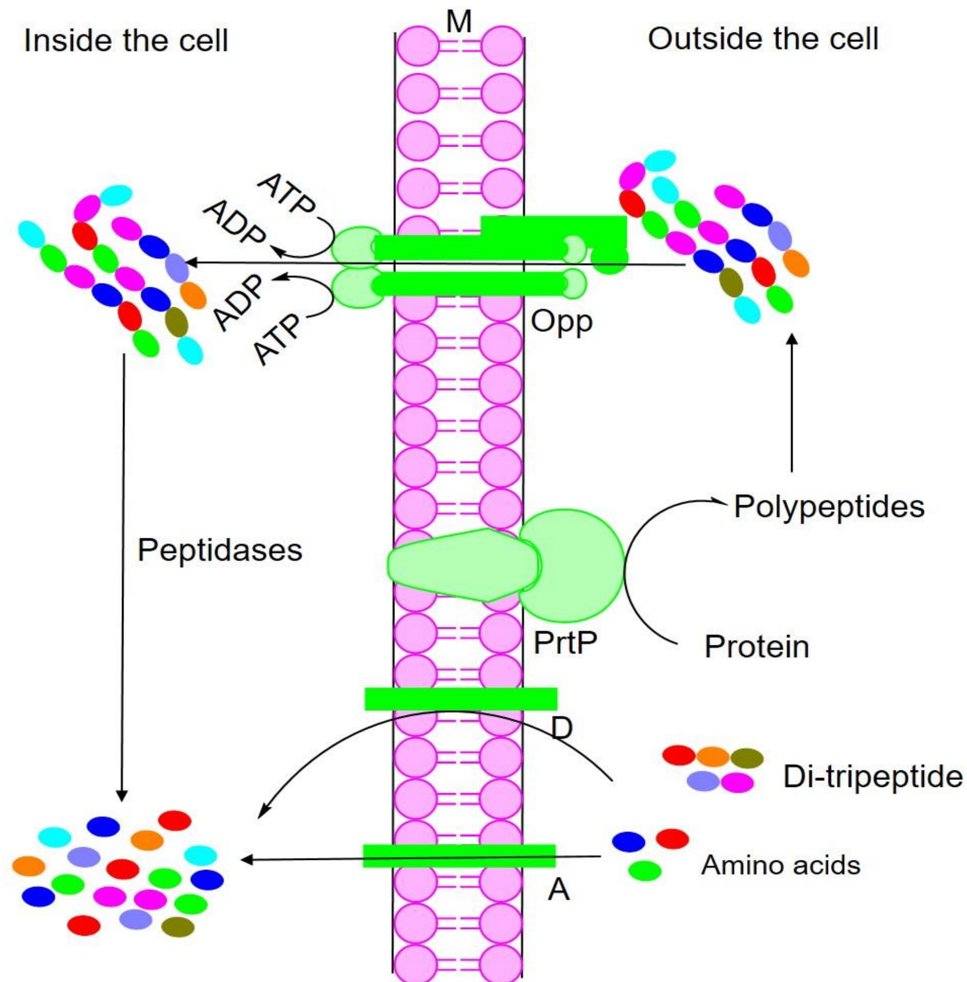


**Figure 6:** Model of the proteolytic pathway in lactic acid bacteria including membrane-anchored proteinase system (PrtP), oligopeptide transport system (Opp), di- and tripeptide transport system (D) and amino acid transport system (A).

Outside of protein, some lactic acid bacteria are also able to decompose starchy material through synthesizing amylases and pullulanase (starch debranching enzymes) during fermentation. Lactic acid, the main metabolite resulting from that fermentation, has a wide range of beneficial uses in the sectors relating to food industries where it is used as acidulant as well as flavouring, emulsifying, and pH buffering agent, or inhibitor of bacterial spoilage (Datta, Tsai, Bonsignor & Moon,1995; Litchfield, 1996). It is also used in other large-scale industrial products. As reported by Reddy, Naveena, Venkateshwar, & Kumar (2008), conventional biotechnological production of lactic acid from starchy materials requires pre-treatment for gelatinization and liquefaction followed by enzymatic saccharification to glucose and subsequent conversion of glucose to lactic acid by fermentation (Anuradha & Suresh, 1999). This two-step process involving consecutive enzymatic hydrolysis and fermentation makes

lactic acid economically unattractive. The direct bio-convection of carbohydrate materials to lactic acid coupling the enzymatic hydrolysis of carbohydrate substrates and microbial fermentation of the derived glucose to lactic acid has been made effective by application of amylolytic lactic acid bacteria, most belonging to the *Lactobacillus* genus (Cheng, Muller, Jaeger & Bajpai, 1991; Naveena, Vishnu & Altaf, 2003; Vishnu & Seenayya, 2002). Because of these high-potential industrial amylase applications and the high world starch availability, amylolytic lactic acid bacteria still receive attention. Several amylolytic lactic acid bacteria have been isolated from different traditional starch fermented foods produced from root cassava, maize, millet, rice and sorghum grain. Amylolytic lactic acid bacteria strains of *Lactobacillus fermentum* were isolated in Benin from Beninese maize sourdough (Ogi and mawe) by Agati, Guyot, Morlon-Guyot, Talamond, & Hounhouigan (1998). Bohak et al., (1998) described a new lactic acid bacteria *(Lactobacillus amylolyticus*) isolated from beer malt and wort with amylase activity. Sanni & Morlon-Guyot (2002) also identified amylolytic lactic acid bacteria such as *Lactobacillus plantarum* and *Lactobacillus fermentum* from various Nigerian traditional amyloceous fermented foods. Petrova, Emanuilova, & Petrov, (2010) identified from *Boza*, a Bulgarian alcoholic maize- and wheat-based beverage, two strains of lactic acid bacteria with amylolytic activity belonging to *Lactobacillus plantarum* and *Lactobacillus pentosus* species. These different research works therefore show the high presence of amylolytic lactic acid bacteria in traditional starchy (cassava and cereal) foods where the occurring lactic acid bacteria lower the pH under 4.5 by organic acid production, thereby inhibiting the food pathogenic microflora. Another importance of amylolytic and proteolytic lactic acid bacteria in developing countries is the reduction of viscosity of highly concentrated starchy cereal gruels, which give them semi-fluid consistency by partial starch and protein hydrolysis. The high viscosity of complementary foods significantly reduce its energy density and is the main factor of an etiological protein energy malnutrition in young children. *Koko*, *Gowe*, *Mawe* and *Ogi* are typical Beninese starchy cereal gruels made from spontaneous lactic acid fermentation, which are used as complementary foods by children or breakfast by adults. Seeing an interest in and the importance of these traditional foods, the reengineering of the processing and the domestication of the occurring potential proteolytic, amylolytic lactic acid bacteria are strongly necessary to increase the nutritional value and solve the traditional foods' safety problems.

## 1.4 Potential biotic and abiotic factors influencing beer organoleptic qualities

Beer, like any fermented food, is a microbial product whose final sensory characteristics strongly depend on the occurring microorganism and the wort composition. The main yeasts strains employed for worldwide beer production are classified into the categories of ale and lager yeasts. Ale yeast (*Saccharomyces cerevisiae*) strains referred to as top-fermenting are more diverse, haploid or polyploid with 3 or 4 copies of each chromosome (Codon, Benitez & Korhola, 1998) whereas the lager yeasts (*Saccharomyces pastorianus*) referred to as bottom-fermenting are the more complex organisms as they result from *S. cerevisiae* and *S. bayanus* hybridization (Turakainen, Kristo & Korhola, 1994). Different other yeasts belonging to non-*Saccharomyces* yeasts have also been used as pur starter or in combination with *S. cerevisiae* to produce some specific beers. With the advances in the production of beer worldwide, more challenges therefore arise each year in the search for new approaches to the development of distinctive beverages. Attempts to obtain products with more complex sensory characteristics have led experts and brewers to prospect for non-conventional yeasts. Besides the widespread use of *Dekkera*/*Brettanomyces* for the production of sour beers, other species are emerging for presenting unusual metabolic features that include the production of fruity esters. *Wickerhamomyces anomalus* and *Torulaspora delbruekii* stand out as the most promising yeasts in brewing processes. Such new tendencies in the use of non-*Saccharomyces* yeasts extend to the production of low-alcohol beers, functional beers, and bioflavoring approaches (Basso & Alcarde, 2016). It has been shown, for example, that *Dekkera bruxellensis* and *Hanseniaspora uvarum* (anamorph, *Kloeckera apiculata*) have the ability to produce many esters with a fruity character. Lately, other wild yeast species such as *D. anomala*, *Hanseniaspora uvarum* and *Debaryomyces spp* have been mentioned as interesting alternatives in brewing. Whatever this diversity of yeast use in brewing, microbiologists and brewers most appreciate their performance on the based on specific beer components. Indeed, all brewing yeasts produce glycerol, vicinal diketones (VDKs), alcohols, esters, short-chain fatty acids, organic acids and diverse sulfur-containing substances (Bokulich & Bamforth, 2013; Dzialo, Park, Steensels, Lievens, & Verstrepen, 2017) (Fig. 7).

**Figure 7**: Metabolism pathways of aroma component synthesis during fermentation by *S. cerevisiae*.

Among these aroma components, aroma-active higher alcohol and esters are of major interest because of their contribution to the aroma in fermented beverages. The levels of each of these components found in beer depends on the fermentation conditions, the wort C: N ratio, time of fermentation and maturation, and the yeast strain. Since Felix Ehrlich, (1907) proposed that higher alcohols are derived from amino acid catabolism, much research has been carried out to understand the key reaction involved in the Ehrlich pathway. Indeed, Sentheshanmuganathan (1960) demonstrated that the formation of higher alcohols from amino acids is mediated by sequential enzymatic reactions, which involve transaminase, decarboxylase and alcohol dehydrogenase. Indeed, amino acid uptake is performed by several amino acid transporters located in the cell membrane of yeast (Jauniaux & Grenson, 1990). The assimilated amino acids are further transaminated to α-keto acids (Hazelwood, Daran, Van Maris, Pronk, & Dickinson, 2008a), which requires four enzymes encoded by the genes BAT1, BAT2, ARO8 and ARO9 (Kispal, Steiner, Court, Rolinski, & Lill, 1996; Pires, Teixeira, Brányik, & Vicente, 2014). Yeast cells then decarboxylate α-keto into

fusel aldehydes followed by a reduction to form fusel/higher alcohols or by oxidation to form fusel acid (Hazelwood, Daran, Van Maris, Pronk & Dickinson, 2008). In *S. cerevisiae*, there are five genes encoding decarboxylases ARO10 and TH13 and three pyruvate decarboxylases (PDC1, PDC5 and PDC6) (Bolat, Romagnoli, Zhu, Pronk, & Daran, 2013; J R Dickinson et al., 1997). The last step of the Ehrlich pathway, in which the aldehydes are converted into their respective alcohol, involves the action of alcohol dehydrogenases encoded by the genes ADH1-5 and the formaldehyde dehydrogenase Sfa1 able to catalyse the conversion of fusel aldehydes into higher alcohols (Dickinson, Salgado, & Hewlins, 2003). Higher alcohols are also formed during upstream biosynthesis of amino acids (Fig. 8). Besides the flavor-active higher alcohols, aroma-active acetate ester and ethyl ester are important as well for the fruity, candy and perfume flavor. Esters are formed intracellularly mainly during primary fermentation by yeast in an enzyme-catalyzed condensation reaction of organic acids and alcohols.

Regardless of the monitoring of fermentation by automatic measurement of wort gravity, CO2 evolution, the pH decrease, ethanol formation, as well as camera based observation of events in fermenter in pursuit of constant fermentation performance, obtaining beer with a stable organoleptic quality (taste and flavor) is still a big brewer's challenge. To achieve this goal, all variable parameters have to be understood. Outside of the housekeeping genes, other genes including those involved in aroma flavor synthesis as well as their regulation are affected by several brewing abiotic factors. Bamforth, (2003) observed that one major variable that receives less detailed analysis and control than others parameters in fermenter is the wort composition. The recent works carried out by Procopio, Sprung, & Becker (2015) help to further understand the impact of wort amino acid composition on beer flavor. The authors observed that an increase of wort leucine, isoleucine, valine, glutamine, cysteine and proline content positively affect the beer's flavor-active ester and higher alcohol content. Lack of amino acids in the wort will therefore induce the synthesis of amino acids, especially the branched amino acids (valine, leucine and isoleucine), which will unfortunately lead to the high presence of diacetyl and pentanedione (vicinal diketones) in beer (Fig. 8). Since valine has a feedback inhibition of enzymes controlling the formation of diacetyl precursors, an increase of wort valine seems to be an approach for diacetyl reduction. An improvement of sorghum protein hydrolysis to

increase the wort amino acids will undoubtedly improve sorghum beer flavor-active components.

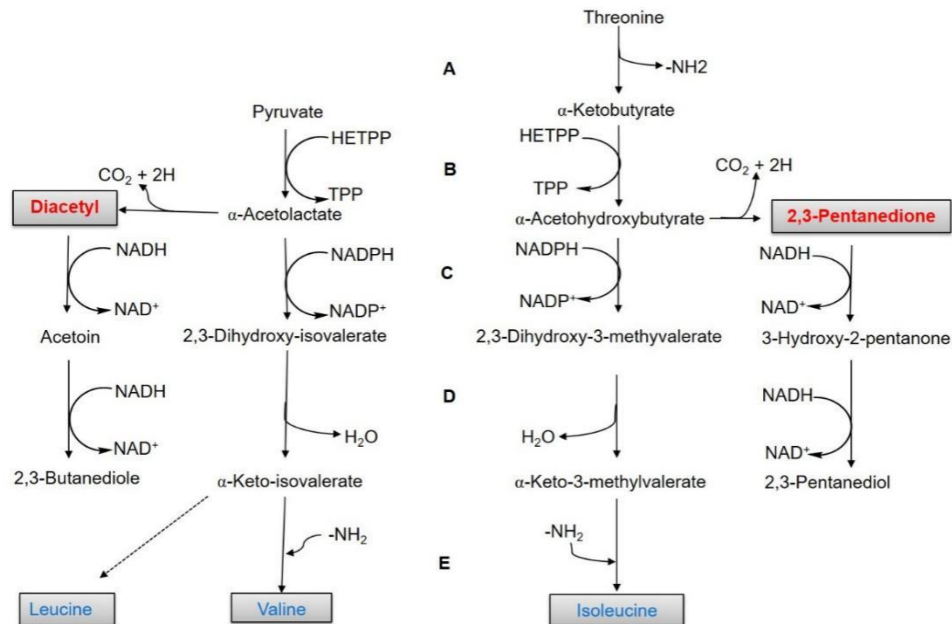


**Figure 8:** Metabolism of relatively undesirable vicinal dicketones (VDKs) formation during branched amine acid (valine, leucine and isoleucine) synthesis. The process involves several enzymes such as: **A**. Threonine deaminase (*IVL1*), **B**: Synthase (*ILV2, ILV6*), **C**: Reductoisomerase (*ILV4, ILV5*), **D**: Dehydratase (*ILV3*) and **E**: Transaminase (*BAT1, BAT2*).

Another approach for beer flavor improvement, which has not yet received a large scientific explanation, is the hydrolysis of glucosidic compounds by β-D-glucosidase. Glucosides are non-volatile compounds composed by one aromatic molecule (aglycone) and one β-D-glucose (glycone). The enzymatic hydrolysis of glucosides releases the sugar molecules, which are further metabolized, and the aglycone, which can express aromatic activity (Winterhalter & Skouroumounis, 1997).

### 1.5 Motivation

African sorghum beer is produced on the basis on unstandardized traditional processing techniques, and the instability of the resulting sorghum beer as well as the presence of high amounts of undigested starch and proteins hinder the marketability. Furthermore, sorghum sprouting induces a tremendous dhurrin synthesis and an inappropriate sorghum mashing program may lead to dhurrin and its toxigenic residue in sorghum beers. Identifying yeast and lactic acid bacteria content in the starter, standardizing the malting and mashing process, and selecting β-D-glucosidase producer lactic acid bacteria and performance yeast for dhurrin removal and sorghum beer flavor improvement are our challenges. To achieve these objectives, we propose:

1. Review the impact of sorghum germination on dhurrin synthesis, the associated toxico-nutritional diseases and the importance of specific β-D-glucosidases on the detoxification process.

2. Identify different yeasts and lactic acid bacteria contained in the traditional starter used for traditional beer production.

3. Optimize sorghum malting conditions and appreciate the impact of sprouting on sorghum cyanogenic glucoside (dhurrin) metabolism

4. Design a standardized mashing program in order to remove toxigenic hydrogen cyanide precursor (dhurrin) and select a proteolytic lactic acid bacteria which expresses β-D-glucosidase for dhurrin and protein hydrolysis improvement

5. Domesticate the isolated *Saccharomyces* yeasts based on flavor-active higher alcohols and esters as well as undesirable aroma components vicinal diketones (VDKs)

# 2. RESULTS (PAPER PUBLICATIONS)

## 2.1 Summary of results

**PART 1:** β-D-glucosidase as "key enzyme" for sorghum cyanogenic glucoside (dhurrin) removal and beer bioflavouring

Sorghum malt used during African beer processing contains a high level of cyanogenic glucoside (dhurrin), up to 1375 ppm. In traditional sorghum malting and mashing, dhurrin is not sufficiently hydrolyzed due to uncontrolled germination and a high gelatinization temperature. The cyanide content of traditional African beers (11 ppm) is higher than the minimum dose (1 ppm) required to form carcinogenic ethyl carbamate during alcoholic fermentation. In the detoxification process, Aryl-β-D-glucosidase (dhurrinase) is the "key component". For significant dhurrin hydrolysis during mashing, optimizing dhurrinase synthesis during malting is a good solution to reduce dhurrin completely to below the harmful dose in the sorghum wort. Lactic acid bacteria which exhibit aryl-β-D-glucosidase prior to alcoholic fermentation may help to reduce ethyl carbamate content in alcoholic beverages. Moreover, some specific β-D-glucosidases have a dual property, being able  to cleave and synthetize glucosides bonds and thereby generating a good precursors for beer bio-flavouring.

**PART 2:** Phenotypical and molecular characterization of yeast content in the starter of *Tchoukoutou*, a Beninese African sorghum beer

---

*Tchoukoutou* is a Beninese African sorghum beer obtained by mixed-fermentation including different yeast and lactic acid bacteria (LAB). Until now, the make-up whole microbial communities of the starter and the main reason for the diversity of this special beer's organoleptic quality (taste and flavor) had remained unknown. A total of 240 yeasts isolated from a *Tchoukoutou* starter have been characterized following the polyphasic approach and using yeast phenotype (morphology and physiology), proteins (MALDI-TOF MS), ITS1-5.8S-ITS4 and D1/D2 of large subunit (LSU) rRNA gene as biomarkers. The microbial ecology of the starter used to produce *Tchoukoutou* is very diverse and belongs to different strains of four species, including *Saccharomyces cerevisiae* (75.17 %) as the dominant yeast, followed by *Pichia kudriavzevii* (17.24 %), *Candida ethanolica* (4.14 %) and *Debaryomyces hansenii* complex (3.45 %). *D. hansenii* complex and *C. ethanolica* are two yeast species which have never yet been isolated from *Tchoukoutou*. Some *S. cerevisiae* with an interesting fermentative profile are able to metabolize lactic acid (lactic acid bacteria metabolite) and therefore may increase the beer pH thereby allowing the growth of LAB for further beer maturation and flavor enhancement during Yeast-LAB mixed-fermentation.

## **PART 3:** Polyphasic characterization of lactic acid bacteria isolated from Beninese sorghum beer starter

*Tchoukoutou* is a Beninese traditional sorghum beer obtained by mixed fermentation including yeast and lactic acid bacteria (LAB). The starter's LAB communities as well as their biotechnological importance remain unknown. Furthermore, the sprouted grain of sorghum bicolor, which is used during the beer processing, contains a cyanogenic glucoside (dhurrin). In order to elucidate Beninese sorghum beer starter LAB microbiota, 69 LAB isolated from traditional starters were characterized using a polyphasic approach including phenotypical characterization (physiology and MALDI-TOF MS) and 16S rRNA gene comparison. Based on the enzymes substrate specificity, LAB expressing aryl-β-D-glucosidase and amylase was indexed as potential candidate for dhurrin removal and saccharification improvement. All isolated bacteria belong to the same genus *Lactobacillus* with different strains of the five species *L. fermentum*, *L. plantarum*, *L. helveticus*, *L. paracasei* and *L. brevis* and diverse metabolic pathways. MALDI-TOF MS is a good method for accurate and high-throughput LAB identification. Several facultative hetero-fermentative LAB such as *L. plantarum* and *L. paracasei* express β-D-glucosidase and amylase. These β-D-glucosidase producers LAB will likely cleave the conjugated glucose of dhurrin, thereby contributing to detoxification if used for controlled sorghum mash bio-acidification.

**PART 4:** Assessment of malting and mash bio-acidification on the turnover of sorghum cyanogenic glucoside and protein hydrolysis improvement.

---

Sorghum germination induces the synthesis of a toxigenic hydrogen cyanide (HCN) precursor, dhurrin, and the normal sorghum mashing program is limited in dhurrin removal. Furthermore, the lack of proteases in sorghum malt and a protein aggregate forming during cooking limit protein hydrolysis. To detoxify the wort, a new mashing program was designed to optimize dhurrin hydrolysis. To improve dhurrin and protein hydrolysis, three strains of lactic acid bacteria were applied for mash acidification. The results show that the degradation of the synthesized dhurrin occurs only in the shoots and rootlets when the larger amount of this cyanogenic glucoside is still accumulated in malt seed tissues. Pre-heating the mash to 40°C prior to decantation significantly reduces dhurrin content of the wort and improves the proteolytic activity. The mash bio-acidification with *L. paracasei* (ND-34) which removes dhurrin completely in brown wort considerably reduces it to 2.34 ± 0.08 mg/L in white wort and significantly improve protein hydrolysis.

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Review

# β-D-Glucosidase as "key enzyme" for sorghum cyanogenic glucoside (dhurrin) removal and beer bioflavouring

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ABSTRACT

Sorghum malt used during African beer processing contains a high level of cyanogenic glucoside (dhurrin), up to 1375 ppm. In traditional sorghum malting and mashing, dhurrin is not sufficiently hydrolyzed due to uncontrolled germination and a high gelatinization temperature. The cyanide content of traditional African beers (11 ppm) is higher than the minimum dose (1 ppm) required to form carcinogenic ethyl carbamate during alcoholic fermentation. In the detoxification process, aryl-β-D-glucosidase (dhurrinase) is the "key component". For significant dhurrin hydrolysis during mashing, optimizing dhurrinase synthesis during malting is a good solution to reduce dhurrin completely to below the harmful dose in the sorghum wort. Lactic acid bacteria which exhibit aryl-β-D-glucosidase prior to alcoholic fermentation may help to reduce ethyl carbamate content in alcoholic beverages. Moreover, some specific β-D-glucosidases have a dual property, being able to cleave and synthesize glucosides bonds and thereby generating good precursors for beer bioflavouring.

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**Contents**

1. Introduction .......... 217
2. Toxico-nutritional diseases due to cyanogenic glucoside in food and cases of epidemiological study .......... 218
3. Dhurrin residue and ethyl carbamate formation .......... 219
4. Dhurrin biosynthesis and efficiency of malting and mashing in dhurrin removal .......... 219
5. Influence of microbial β-glucosidase in dhurrin removal and beer quality .......... 221
   5.1. Microbial β-D-glucosidase, substrate specificity and abiotic factors influencing its activity .......... 221
   5.2. Efficient β-D-glucosidase production may induce beer bio-flavoring .......... 221
   5.3. Higher and specific β-glucosidase content may induce an increase of beer alcohol .......... 222
6. Conclusion .......... 222
   Acknowledgements .......... 222
   Transparency document .......... 222
   References .......... 222

## 1. Introduction

African sorghum beers are traditional drinks which are produced on the basis of ancestral knowledge transmitted from one generation to next. This special beer is called *Tchoukoutou* in Benin and Togo, *Dolo* in Burkina-Faso, *Amgba* in Cameroon, *Pito* in Ghana and *Burukutu* or *Otika* in Nigeria. Compared to European lager beers, it is very rich in calories, B-group vitamins (thiamine, folic acid, riboflavin, and nicotinic acid), and essential amino acids such as lysine (Lyumugabe et al., 2012). Present in popular bars and its own market, sorghum beer is a refreshing drink much appreciated by a large number of consumers (Osseyi et al., 2011). Regardless of where it is produced, African sorghum beer is a sour drink obtained by uncontrolled and spontaneous mixed-fermentation using the

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traditional starter containing different yeast species and lactic acid bacteria. African sorghum beer is fermented at high temperature without maturation and cannot be conserved for more than three days. Furthermore, it is consumed while still fermenting, and the drink contains large amounts of solids such as undigested carbohydrates and proteins because of the non-standardized malting conditions. In addition, the high gelatinization temperature may denaturate enzymes. Improvised means used for filtration, i.e. basket and cloth, strongly decrease lautering performance. The limited shelf-life of African beers has been reported as the major problem which traditional beer producers encounter in almost all African countries such as Benin, Nigeria, Sudan, Ghana, Tanzania and Rwanda (Kayodé et al., 2007; Lyumugabe et al., 2010; Osseyi et al., 2011). Furthermore, the permanent threat of exogenous fungal contamination compromises the safety of African sorghum beer (Odhav and Naiker, 2002; Ezekiel et al., 2015; Matumba et al., 2014). In addition to these challenges, the raw material used during beer processing, sprouted grains of *Sorghum bicolor*, contains a cyanogenic glucoside called dhurrin (Ahmed et al., 1996). Several toxico-nutritional diseases have been correlated with dietary cyanide, for example *konzo* or "tied leg", tropical ataxic neuropathy, goiter and cretinism (Dillon et al., 2000; Oluwole et al., 2000; Nzwalo and Cliff, 2011; Banea et al., 2015). Moreover, this residue of cyanogenic glycoside is very poisonous as it binds to cytochrome C oxidase and stops cell respiration, a key process in energy metabolism. The residue hydrogen cyanide of this secondary metabolite may be a precursor for the carcinogenic ethyl carbamate (EC). Since cyanide is a toxic compound, different physico-chemical and biological methods are used to remove the toxigenic cyanogenic glucoside residue. These reactions also occur during beer brewing processes and can, if efficient, contribute to reducing dhurrin and its residue. This review focuses on the toxico-nutritional diseases induced by cyanide-containing food and the potential risk due to formation of carcinogenic EC. The efficiency of aryl-β-D-glucosidase in sorghum beer detoxification and bio-flavouring is also evaluated.

## 2. Toxico-nutritional diseases due to cyanogenic glucoside in food and cases of epidemiological study

In certain underprivileged areas of Africa where sorghum and cassava are the main sources of energy, proteins, vitamins and minerals, food intoxication is a serious problem. Thus, the raw material used during African beer processing, namely sprouted grains of *Sorghum bicolor*, contains the cyanogenic glycoside dhurrin. Although dhurrin itself is not harmful, the toxigenic hydrogen cyanide is generated by the action of enzymes from the intestinal microbiota (Carter et al., 1980). This cyanide is responsible for several ill effects within the body. For one, the cyanide-derived thiocyanate impacts iodine availability, thereby causing goiter and cretinism. Several studies have been conducted in animals to confirm this toxic effect. Olusi et al. (1979) fed a rat with cassava and observed decreasing hemoglobin as well as body weight. A similar study conducted by Philbrick et al. (1979) on rats led to a depression of thyroid function with thyroid gland abnormality. Dillon et al. (2000) showed a high prevalence of goiter (33.5%) in the Senegal oriental area, which is characterized by mountains with challenging climatic conditions (Sahelo Sudanian) allowing only to cultivate sorghum and millet.

For another, apart from this anti-nutritional property, cyanide may also bind to the ferric ion of cytochrome C oxidase because of its trivalent state, blocking the last step of the respiratory chain. The resultant internal asphyxiation mainly compromises the central nervous system and the myocardium due to their high oxygen demand. Among others, this causes Konzo, a toxico-nutritional neurological disease which still remains a health problem in several developing countries. Recently, Banea et al. (2015) reported 144 cases of konzo out of 4588 people evaluated with high urinary thiocyanate content ($>$ 300 mol/L) in the Bandundu province of the Democratic Republic of Congo (RDC). Amongst them, 5% of the suffering children were severely disabled and could not walk, and 27% were moderately disabled and needed one or two crutches.

The long-term consumption of cyanide-containing food due to low income can compromise the health of the population, especially of those suffering from chronic protein malnutrition. To understand the influence of the nutritional status on cyanide intoxication, Umoh et al. (1986) administered pure linamarin at a dose of 300 mg/kg bw in food to a group of wistar rats. In contrast to the control, the kwashiorkor rats excreted less thiocyanate, indicating that their protein deficiency prolongs the time of metabolism and hence increases the toxicity of cyanogenic glucosides in the body.

Apart from goiter, cretinism and *konzo*, tropical ataxic neuropathy (TAN) characterized by sensory polyneuropathy, sensory ataxia, bilateral optic atrophy and bilateral sensorineural deafness, reportedly caused by cyanide-containing food, is also an issue in several African countries (Oluwole et al., 2000). Similar dysfunctions have been observed in the case of a 67-year-old woman, in whom sural nerve biopsy specimen revealed a mixed pattern of demyelination and axonal degeneration after administration of three tablets of laetrile (amygdalin equivalent to 25–75 mg of cyanide) for cancer treatment (Kalyanaraman et al., 1983). Based on such evidence, death from cyanide poisoning is believed to result from central nervous system depression, subsequent to inhibited brain cytochrome oxidase activity (Way, 1984).

Since several factors influence hydrolysis of cyanogenic glycosides and the influence of the nutritional status is confounding, human case studies, especially regarding the acute toxicity dose, are difficult. However, on the basis of several cases of food intoxication, the human acute toxicity dose of cyanide has been estimated. By analyzing cyanide content in tissues and gastrointestinal tract contents in victims of fatal poisoning as well as comparative kinetics with dogs, Gettler and Baine (1938) observed that death occurred after absorption of an average of 1.4 mg hydrogen cyanide/kg body weight and estimated the lowest fatal absorbed dose to be 0.54 mg hydrogen cyanide/kg body weight.

Inhaled or percutaneously absorbed hydrogen cyanide passes immediately into the systemic circulation and higher levels are generally found in liver, lungs, blood, and brain. In a man who died after inhaling hydrogen cyanide, the tissue HCN levels were 0.75, 0.42, 0.41, 0.33, and 0.32 mg/100 g of tissue in lung, heart, blood, kidney, and brain, respectively (Gettler and Baine, 1938; Ballantyne, 1983). Lundquist et al. (1985) evaluated the cyanide content of whole blood (0.13 μmol/L), erythrocytes (0.24 μmol/L), and plasma (0.02 μmol/L) of 10 non-smoking humans, demonstrating that the major portion is sequestered in erythrocytes. This realization puts into perspective several epidemiological studies based on human plasma cyanide content to assess risk of cyanide intoxication. Measuring whole blood or at least erythrocyte cyanide may improve such risk assessment. Using readings of whole blood cyanide, Hail and Rumack (1986) observed that the first symptoms like tachycardia or flushing are to be expected in the range of 0.5–1.0 mg/L; levels above 1.0 mg/L are to be classified toxic, and levels exceeding 2.5–3.0 mg/L were reported to be associated with life-threatening symptoms or death. A case was reported by Sahin (2011) of a 28-months-old girl with sudden onset of unconsciousness and seizure after ingestion of approximately ten apricot seeds. Her blood pressure was 92/42 mm H, her whole blood cyanide was more than 3 mg/L, and she died after 22 days of hospitalization.

The issue of foods containing cyanogenic glucosides deserves

attention as the blood cyanide content after ingestion will strongly depend on the cyanogenic glucoside, the pH value of the stomach, and the bacterial flora of the gut. In this respect, Abraham et al. (2016) conducted an epidemiologic study in 12 healthy adult humans who consumed persipan paste, linseed, bitter apricot kernels and fresh cassava with each "meal" containing an equivalent of 6.8 mg of cyanide. Even if lower peak cyanide levels are to be expected in the blood after consuming food containing cyanogenic glucosides compared to free cyanide, high blood cyanide content was still attained with some of these foods, especially cassava and bitter apricot kernels, in which the bound cyanide is as bioavailable as free cyanide. From assessing the risk of these foods, 0.075 mg/kg of body weight was proposed as an acute reference dose.

Since it has been demonstrated that a part of hydrogen cyanide is converted by rhodanese or thiosulfate sulfurtransferase, these mitochondrial enzymes are used as an antidote for in vivo detoxification. Unfortunately, the Km of rhodanese is approximately 3 mM, indicating slow turnover (Billaut-Laden et al., 2006). Furthermore, several rhodanese-encoding TST genes decrease rhodanese activity as well as the level of TST gene expression, thereby compromising the detoxification rate (Billaut-Laden et al., 2006). In vitro detoxification of food containing cyanogenic glucosides by adequate processing is therefore a good approach to limit cyanide intoxication (Nambisan, 2011). The detoxification of cyanogenic glucoside containing foods using traditional processing is limited and processors are the first intoxicated (Okafor et al., 2002). The maximum dose of hydrocyanic acid recommended in alcoholic beverages by the New Zealand Food Safety Authority (NZFSA) and in Switzerland is 1 ppm. If this level is exceeded, ethyl carbamate may be formed during alcoholic fermentation.

## 3. Dhurrin residue and ethyl carbamate formation

Ethyl carbamate (EC), or urethane, is a harmful substance which can be formed from various substances inherent in food and beverages, including hydrogen cyanide resulting from the degradation of cyanogenic glucosides (Lachenmeier et al., 2010), urea and citrulline originating from incomplete arginine hydrolysis by yeast or lactic acid bacteria (Jiao et al., 2014), or food preservatives such as diethyl pyrocarbonate (Ryu et al., 2015). Among these undesirable compounds, cyanate has been reported as the ultimate precursor in most cases by reacting with ethanol to form ethyl carbamate (Fig. 1). In 2010, Lachenmeier et al. observed a high concentration of ethyl carbamate (2.34 ppm) in Tiquira, a Brazilian spirit produced from cassava (*Manihot esculenta*). Later, a similar research was carried out by Ryu et al. (2015), who observed that, unlike other Korean alcoholic beverages, *Maesilju* contains a high level of ethyl carbamate (151.06 ppm). The authors explain the high EC concentration in these different alcoholic beverages by cyanogenic glucosides contained in *Maesil* (amygdalin) and in cassava (linamarin, lotaustralin) used to produce the Korean liquor and Brazilian spirit, respectively. The hydrogen cyanide released from these glucosides similarly to dhurrin is then oxidized to cyanate, increasing the likelihood of ethyl carbamate formation. Even though the ethyl carbamate concentration of African sorghum beers remains unknown, the traditional sorghum brewing process offers optimal conditions for EC formation. Hydrogen cyanide is highly volatile, so that sorghum malting may potentially serve to remove this free cyanide. Ikediobi et al. (1988), however, observed free cyanide (3.5 ppm–11 ppm) in *Burukutu*, a Nigerian traditional sorghum beer, showing that dhurrin removal is limited in traditional malting and mashing. Thus, the cyanide content in African sorghum beer exceeds the minimum dose (1 ppm) required to form EC. Furthermore, due to uncontrolled fermentation which occurs in the presence of oxygen, cyanide may be oxidized to cyanate, which then reacts with ethanol to ethyl carbamate (Fig. 1).

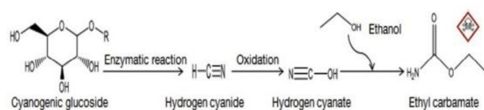


**Fig. 1.** Pathway of forming undesirable, carcinogenic ethyl carbamate, which induces DNA depurination, between dhurrin residue hydrogen cyanide and ethanol during alcoholic fermentation.

African traditional and commercial sorghum beers as well as Mozambique commercial cassava beer (*Impala*) and Ecuadorian traditional beer (*Chicha*) made from cassava may be regarded as a potential risk for consumers due to sprouted sorghum's dhurrin and cassava's linamarin and lotaustralin. Hydrolysis of these cyanogenic glucosides during alcoholic fermentation may lead to dramatic ethyl carbamate formation. Ethyl carbamate is recognized by the International Agency for Research on Cancer (IARC) as a probable carcinogen in humans and several animals. Studies have shown that it may cause increased tumor incidence in several tissue sites including lung, liver, and blood vessels. This multi-site carcinogen may be also oxidized to vinyl carbamate by cytochrome P-450 2E1 and further converted into vinyl carbamate epoxide, which can bind covalently to DNA and induce damage (Sakano et al., 2002). To avoid such carcinogenic substances, many physical, chemical, enzymatic, and metabolic engineering methods have been investigated to reduce EC in alcoholic beverages. Concerning EC derived from cyanate, two different approaches have been proposed, addressing the reduction of either the main precursor cyanide or the propensity of cyanides to react and form cyanates. In this context, Lachenmeier (2005) supplemented mash with a patented copper salt to precipitate cyanide. Later, Hashguchi et al. (2010) applied potassium metabisulfite or an oxygen absorber to inhibit oxidation and observed the reduction of Ume liquor's ethyl carbamate contents by up to 27% or 47%, respectively. On the other hand, removing dhurrin and its residue prior to alcoholic fermentation should be a good solution to detoxify sorghum beers by limiting EC formation. Thus, using lactic acid bacteria which express aryl-β-D-glucosidase to bio-acidify sorghum mash may help to limit EC formation. Furthermore, in order to ensure significant dhurrin breakdown during sorghum mashing, optimizing β-glucosidase bio-activation during sorghum germination may help to reduce the cyanogenic glucoside content.

## 4. Dhurrin biosynthesis and efficiency of malting and mashing in dhurrin removal

The main objective of sorghum malting is to promote bioactivation of hydrolytic enzymes which are not present in the non-germinated sorghum. Unfortunately, a tremendous increase of dhurrin is observed during sorghum germination. Panasiuk and Donald (1984) showed that dhurrin sequestrated in sorghum (*Sorghum bicolor* (L.) *Moench*) varies between 2 and 29 ppm while the cyanide contents of the same cultivars' sprouts obtained after 3 days of germination contain 258–1030 ppm. The same trend has been shown by Ikediobi et al. (1988), who observed that the cyanide content of non-germinated sorghum seeds is around 22.5 ppm depending on the sorghum cultivar but increases during germination to reach 1376 ppm and 963 ppm in the cultivars *L187* and *Mori*, respectively, after 6 days of germination. These observations led to the conclusion that most of the dhurrin content in sprouted sorghum is synthesized during sorghum malting (germination) and that the potential dhurrin synthesis depends on the sorghum

variety. This early dhurrin synthesis may be due to several important functions attributed to secondary metabolism, including sugar and nitrogen transport as well as pivotal roles in organizing chemical defense systems in plants and in plant-insect interactions (Ganjewala et al., 2010). The highly increased dhurrin synthesis during sprouting may be explained by the increased metabolism associated with germination: starch, protein, and lipids are broken down by appropriate hydrolytic and oxidative enzymes to provide substances and energy to synthesize new cellular mass necessary for the developing shoots and rootlets. In the presence of the appropriate enzyme system (enzymes of cytochrome-P450 and UPD-glycosyltransferase), tyrosine from seed protein and glucose from seed starch are channeled into the biosynthetic pathway of dhurrin. After synthesis, dhurrin is hydrolyzed following bio-activation of a specific β-glucosidase (Møller, 2010). β-Glucosidases and α-hydroxynitrilase are the most important enzymes of catabolic pathways in plants: β-glucosidase converts dhurrin to the corresponding α-hydroxynitrile, which then dissociates spontaneously or catalyzed by hydroxynitrilase (Fig. 2). Thus, aryl-β-D-glucosidase is the "key-component" in this removal of sorghum cyanogenic glucoside. The germination of sorghum is crucial and helps to promote amylases and proteases for starch and protein hydrolysis, respectively. If efficient, it may also hydrolyze sorghum anti-nutritional compounds such as oxalate, trypsin inhibitor activity (TIA), and phytate, and improve mineral availability and accessibility (Ogbonna et al., 2012). Regarding the problem of cyanogenic glucosides, spatial distribution must be taken into account: After bio-activation, dhurrin is unequally distributed among the sorghum organs rootlets, shoots and seeds, with highest level observed in shoot and rootlets (Ahmed et al., 1996; Haque and Bradbury, 2002; Traoré et al., 2004). Based on this, researchers seem to promote a single approach that involves removing the sprouted sorghum rootlets and shoots. Traoré et al. (2004) observed that deculming or degerming, i.e. removing shoot and rootlets, reduces sorghum malt cyanide contents by up to 74%. However, this approach, which was adopted by traditional African sorghum beer producers, is not without problems. Sorghum degerming reduces sorghum malt protein by 10%, mineral by 27% and amylase by 25% (Traoré et al., 2004). Still, removing the rootlets is beneficial for the overall brewing process: If not removed, their high-molecular-weight proteins are not digested by yeast during fermentation, and they may affect beer filterability, stability of taste, and brightness of color. In addition, the rootlets are very hygroscopic (Guido and Moreira, 2014) and increase water uptake, thereby compromising malt storage and favoring growth of mold. The latter not only causes the beer to contain mycotoxins and results in the traditional gushing problem, but also induces premature yeast flocculation during fermentation as fungi degrade the husk (Axcell et al., 2000; Panteloglou et al., 2000). Another reason to remove the rootlets is the harsh bitterness and unpleasant "cooked vegetable" taste in beer contributed by dimethyl sulfide (DMS), which compromises palatability. This results from thermal degradation of its well-known precursor *S*-methyl methionine (SMM) during kilning, wort boiling and whirlpool operations (White and Wainwright, 1976, 1977; Dickenson, 1983; Pimenta et al., 1998; Scheuren et al., 2014). Dethier et al. (1991) determined the SMM content in the different parts of the grain and reported that the shoot, followed by the rootlets, is the major contributor to SMM in germinated grain. However, in several African countries, especially in Nigeria and South Africa, large quantities of sprouted sorghum are used for lager beer production without deculming. Yet industrial sorghum beer brewers might profit from deculming because it helps to significantly reduce dhurrin and its residue as well as the DMS precursor SMM and thus to improve the beer's organoleptic quality, i.e. taste and flavor. Although traditional sorghum beer producers degerm sorghum malt, the free cyanide content in African sorghum beer (11 ppm) is higher than the limit prescribed for alcoholic beverages (1 ppm). This indicates that degerming alone is insufficient to reduce cyanide: A deficit of dhurrinase (aryl-β-glucosidase) or conditions (temperature and pH) unsuitable for this enzyme may limit detoxification. Cicek and Esen (1998) purified two different types of β-glucosidase (Dhr1 and Dhr2) from sorghum corresponding to precursors 565 and 514 amino acids, respectively. Mao and Anderson (1967) observed that β-glucosidase II (Dhr2), contrary to β-glucosidase I, is able to hydrolyze dhurrin. However, the bio-activation parameters of β-glucosidase II (dhurrinase II) during sorghum germination and the conditions for its optimal activity, and thus significant dhurrin hydrolysis, still remain unknown. It is, nevertheless, evident that the detoxification rate will strongly depend on dhurrinase II of sprouted sorghum. If β-glucosidase II, known to be specific for dhurrin, is deficient, this may limit the detoxification by hydrolyzing only a fraction of the dhurrin content in sprouted sorghum. After bio-activation of dhurrinase during sorghum malting, mashing disrupts the tissues so that dhurrin and dhurrinase are no longer separated. Here, conditions which optimize β-glucosidase activity should be used to induce significant dhurrin hydrolysis. The badly controlled traditional sorghum mashing is conducted at high temperature (100 °C) and unsuitable pH due to spontaneous bio-acidification of the mash (Fig. 3), decreasing the activities of β-glucosidase and other hydrolases such as amylases, limit-dextrinase and proteases. Simultaneously optimizing the activities of α-amylase, β-amylase, protease and β-glucosidase during malting and mashing may be limited by the specific optimal conditions of these hydrolases or due to dhurrinase II deficiency in sprouted sorghum. The microbial aryl-β-glucosidase

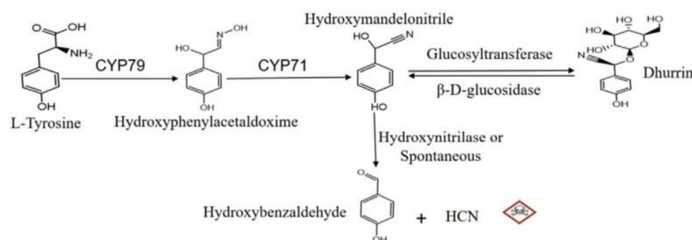


**Fig. 2.** Sequential biosynthesis of sorghum cyanogenic glucoside (dhurrin) from the precursor amino acid (L-tyrosine) and its successive hydrolysis by β-glucosidase and α-hydroxynitrilase to generate the toxigenic volatile hydrogen cyanide (dhurrin residue).

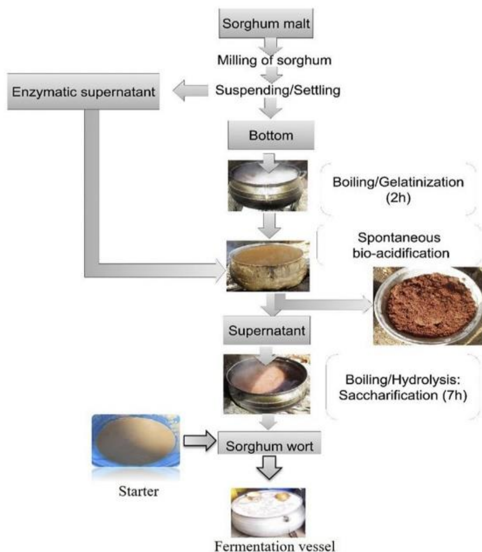


**Fig. 3.** Different steps of sorghum malt mashing and fermentation during Beninese African sorghum beer production. The gelatinization and saccharification occurs at high temperature which may induce the denaturation of the hydrolytic enzymes. The spontaneous bio-acidification of sorghum mash which occurs before saccharification may involve amylolytic lactic acid bacteria. The mash pH obtained after this uncontrolled spontaneous acidification may not be the appropriate optimum pH of β-glucosidase.

could be a good alternative to significantly hydrolyze the remaining dhurrin.

## 5. Influence of microbial β-glucosidase in dhurrin removal and beer quality

Simultaneously optimizing the activity of amylase, protease and β-glucosidase during sorghum malting may be impossible due to the enzymes' distinct optimal conditions. Furthermore, sorghum plus the brown pericarp used as raw material for traditional sorghum beer contains a high amount of tannins. These polyphenols may inhibit β-D-glucosidase activity, thereby limiting dhurrin removal. In this context, the selection of yeast and lactic acid bacteria which express β-D-glucosidase should be a good alternative to improve dhurrin hydrolysis, reducing the glucoside to below the maximum dose (1 ppm). The β-glucosidase-encoding genes are present in the genomes of some lactic acid bacteria, of which most belong to the genus *Lactobacillus* (Mtshali et al., 2010). Selecting such potential lactic acid bacteria, followed by further evaluating β-glucosidase gene expression under acidic brewing conditions, may accomplish dhurrin removal and beer bio-flavoring as well as increase the beer's nutritional value. Through hydrolyzing plant metabolite gluco-conjugates, β-glucosidase-producing lactic acid bacteria could significantly contribute to the dietary and sensory attributes of fermented food: De-glucosylation can release attractive flavor compounds from glycosylated precursors, and lactic acid bacteria increase the bioavailability of health-promoting plant metabolites sequestrated in brewer's raw materials as well as degrade dietary toxins (Michlmayr and Kneifel, 2014).

### 5.1. *Microbial β-D-glucosidase, substrate specificity and abiotic factors influencing its activity*

The turnover of undesirable dhurrin requires a specific β-glucosidase that is able to catalyze the breakdown at an aryl non-reduced terminus. Indeed, three classes of β-glucosidases have been defined on the basis of substrate specificity: aryl-β-glucosidases (class 1), true cellobiases (class 2) and enzymes with broad substrate specificity (class 3), which have various abilities to cleave beta (1,4; 1,6; 1,2) and some alpha (1,3; 1,4; 1,6) glucosidic bonds (Krisch et al., 2010). Several factors such as pH, temperature, alcohol, glucose and some specific minerals are brewing abiotic factors which may have a negative or positive impact on β-glucosidase activity. Thus, selecting an aryl-β-D-glucosidase able to hydrolyze dhurrin under these brewing conditions will have a great importance. During fermentation, *Lactobacillus plantarum* lowers cassava cyanide content from 197.19 mg/g to 4.09 mg/g within 24 h of inoculation (Tefera et al., 2014). Unfortunately, hydrolysis of dhurrin and linamarin in *sorghum bicolor* and *Manihot esculenta,* respectively, involves specific β-glucosidases such as dhurrinase and linamarase because of these glucosides' aryl and alkyl glucosidic bonds. However, Sestelo et al. (2004) purified an aryl-β-D-glucosidase with a high activity at pH 5 from *Lactobacillus plantarum.* Saha and Bothast (1995) also purified a β-D-glucosidase which is optimally stable at pH 5 from *Candida peltata.* Several researchers isolated lactic acid bacteria strains belonging to *Lactobacillus plantarum* and *Lactobacillus paracasei* from African traditional fermented food, which are able to hydrolyze aryl-β-D-glucosidic bonds such as those in salicin, esculin and amygdalin. Additionally, Prasad and Dhanya (1991) applied *Saccharomyces cerevisiae* NCIM 3181 for sorghum wort fermentation and observed a reduction of dhurrin up to 84.6%. These observations indicate that most microbial β-D-glucosidases are acidic, belong to class 3 and are able to cleave aryl or alkyl glucosidic bonds. Even though most microbial β-D-glucosidases have an acidic optimum pH, showing their possible application for sorghum mash or wort bio-acidification to significantly hydrolyze dhurrin, Gueguen et al. (1995) observed that glucose and ethanol inhibit their activity. On the other hand, Saha and Bothast (1995), Riou et al. (1998) observed an increase of microbial β-D-glucosidase in the presence of glucose and ethanol. To explain how glucose affects β-D-glucosidase activity, Yang et al. (2015) point out that several glucose binding sites exist and conclude that the number or location and the relative affinity or preference of these sites, which depend on the enzyme's structural characteristics, determine the variable glucose dependence of β-D-glucosidase activity. It is now clear that β-D-glucosidases are highly diverse and, according to the binding site of glucose, their activity can be inhibited or stimulated. Facing this problem of glucose inhibiting β-D-glucosidase activity, Guo et al. (2016) used site-directed mutagenesis of only two amino acid residues at the entrance to the active site and observed that the β-D-glucosidase produced by the mutant cell is stimulated by glucose. This intensive work thus shows that microbial β-D-glucosidase may potentially be able to significantly hydrolyze dhurrin under sorghum brewing conditions.

### 5.2. *Efficient β-D-glucosidase production may induce beer bio-flavoring*

That off-flavor develops in African sorghum beer after only three days shows the instability of these beers' chemical compounds, which affects its organoleptic qualities. Glucosidic compounds are plant secondary metabolites sequestrated in grains or hops, and they can be cleaved acidically or enzymatically. Chevance et al. (2002) observed an increase of β-damascenone during beer

ageing, which is partially due to acidic hydrolysis of glucosidic precursors especially when the pH is lower than 4.2. However, several authors opt for the enzymatic release of glucose conjugates as the acidic hydrolysis modifies the aglycone responsible of beer flavor enhancement. In winemaking, β-glucosidase plays a key role in the enzymatic release of aroma compounds from glucosidic precursors present in fruit juices (Steensels et al., 2015). *Bretanomyces curtersii* strain LD72, which is a β-glucosidase-positive yeast as opposed to *Saccharomyces cerevisiae* (β-glucosidase-negative), shows a pronounced activity towards glucosides with aliphatic alcohols, aromatic compounds and terpenoid alcohols (Daenen et al., 2008). Even though the main objective during sorghum wort detoxification is to hydrolyze dhurrin, the resulting aglycone now deserves attention because of its biofunctionality and aroma properties. Several aroma precursors are stored in cereal grains and hops in the form of glucose bonds. To valorize these odorless compounds and improve beer flavor, several researchers have applied β-D-glucosidase to remove the conjugated glucose. Kollmannsberger et al. (2006) observed an increase of odoriferous compounds such as linalool and β-damascenone from enzymatic hydrolysis of hops odorless β-D-glucoside of linalool and 3-hydroxy-β-damascenone, respectively. Isoflavones found in soybean have phytoestrogen properties that relieve menopausal symptoms and can help prevent several chronic diseases and certain types of cancer. However, they are mainly in the inactive form of glucosides in soy-based foods. The deglycosylation of glucosides could therefore be achieved by β-D-glucosidase and generate a good precursor for a desirable aroma or bioactive compounds. Besides the cleavage ability, some β-D-glucosidases have biosynthetic properties and may, by this reverse activity, generate aroma active in beer bioflavoring. Gunata et al. (1994) observed that the β-D-glucosidase isolated from *Candida molischiana*, *Aspergillus niger* as well as almond catalyze the synthesis of several primary monoterpernes such as geraniol, nerol and citronellol using cellobiose as carbohydrate donor. Due to this synthetic ability, β-D-glucosidases with transglycosylation activity constitute a point of focus for several researchers. Since a link between the synthetic property and the stimulation of enzyme activity by glucose has been shown, a site-directed mutagenesis has become the common application to obtain mutant cells able to produce a β-D-glucosidase with transglycosylation properties.

### 5.3. *Higher and specific β-glucosidase content may induce an increase of beer alcohol*

The traditional starter culture used during sorghum wort fermentation contains yeast and lactic acid bacteria which compete and share fermentable sugar. Since this sugar is mainly converted to lactic acid and to ethanol by lactic acid bacteria and yeasts, respectively, the resulting beer contains a less alcohol. Beninese sorghum beer producers claim that consumers can be divided into two different clusters by their preferred alcohol content: the first class encompasses mostly the elderly and women who want beer with a lower alcohol content. The second class consists mainly of young people who want traditional beer with high alcohol. In response to this consumer demand, some *Tchoukoutou* producers enhance their beer's alcohol concentration with distilled alcohol from palm wine. Unfortunately, this may affect beer quality because palm wine possesses a specific aroma profile, so that the resulting beers are not accepted by consumers who recognize *Tchoukoutou* by its specific organoleptic quality (flavor and taste). The controlled mixed-fermentation with selected yeast and lactic acid bacteria which exhibit β-D-glucosidase could be a good solution to satisfy this important consumer class by increasing alcohol content and conserve desirable organoleptic qualities: β-glucosidase is able to generate glucose from cellobiose resulting from synergic and sequential hydrolysis of cellulosic material by endoglucanase and exo-cellobiohydrolase. To increase alcohol content, acidophilic β-glucosidase is required under the acidic fermentation conditions due to the co-presence of lactic acid bacteria and yeast. Lactic acid bacteria producing β-glucosidase with transglycosylation property may therefore have a great importance for increasing African sorghum beer alcohol content.

## 6. Conclusion

The increasing prevalence of toxico-nutritional diseases due to the consumption of cyanide-containing food in African tropical localities shows the limits of badly controlled traditional methods in the detoxification process. Aryl-β-D-glucosidase is a well-suited candidate to remove sorghum cyanogenic glucoside and for beer bioflavouring. Detoxifying sorghum mash prior to alcohol fermentation is necessary to avoid ethyl carbamate formation. The bio-activation of β-glucosidase (dhurrinase) during sorghum malting, followed by shoot and rootlet removal, will significantly contribute to reducing dhurrin in sprouted sorghum. Several lactic acid bacteria isolated from the starter used for spontaneous sorghum wort fermentation express acidic aryl-β-D-glucosidase. The application of these endogenous *Tchoukoutou* lactic acid bacteria for controlled sorghum wort bio-acidification may help to reduce sorghum beer cyanide content to below the recommended maximum dose (1 ppm). Even though the activity of several β-D-glucosidases is inhibited by glucose, glucose-tolerant β-D-glucosidases are known. Furthermore, some specific β-D-glucosidases are stimulated by glucose. The latter can also be obtained by site-directed mutagenesis. Before selecting lactic acid bacteria or mutant cells with transglycosylation properties to remove dhurrin and bio-flavor beer, sorghum brewers can yet successfully apply lactic acid bacteria isolated from African sorghum beer starter for controlled sorghum mash bio-acidification before saccharification to avoid the negative action of glucose. The gelatinization of sorghum mash which traditionally occurs after this biological mash acidification will help to remove the free cyanide as the highly volatile hydrogen cyanide.

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ORIGINAL PAPER

# Phenotypical and molecular characterization of yeast content in the starter of "*Tchoukoutou,*" a Beninese African sorghum beer

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**Abstract** *Tchoukoutou* is a Beninese African sorghum beer obtained by mixed fermentation including different yeast and lactic acid bacteria (LAB). Until now, the make-up of the starter's whole microbial communities and the main reason for the diversity of this special beer's organoleptic quality (taste and flavor) have remained unknown. A total of 240 yeasts isolated from a *Tchoukoutou* starter have been characterized following the polyphasic approach and using yeast phenotype (morphology and physiology), proteins (MALDI-TOF MS), ITS1-5.8S-ITS4 and D1/D2 of the large subunit (LSU) rRNA gene as biomarkers. The microbial ecology of the starter used to produce *Tchoukoutou* is diverse and belongs to different strains of four species including *Saccharomyces cerevisiae* (75.17 %) as the dominant yeast, followed by *Pichia kudriavzevii* (17.24 %), *Candida ethanolica* (4.14 %) and *Debaryomyces hansenii* complex (3.45 %). *D. hansenii* complex and *C. ethanolica* are two yeast species which have never yet been isolated from *Tchoukoutou*. Some *S. cerevisiae* with an interesting fermentative profile are able to metabolize lactic acid (lactic acid bacteria metabolite) and therefore may increase the beer pH, thereby allowing the growth of LAB for further beer maturation and flavor enhancement during Yeast-LAB mixed fermentation. The co-presence of the non-*Saccharomyces* with *S. cerevisiae* in sorghum beer starter depends for the agroecology zones.

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## Introduction

*Tchoukoutou* is an African opaque sorghum beer obtained by mixed fermentation including different yeasts and lactic acid bacteria. This traditional beer is present in all African countries and is also called *pito* or *burukutu* in Ghana and Nigeria, *kefir* beer in South Africa, *pombe* in Tanzania and "Seven days" beer in Zambia [1, 2]. African sorghum beer is very rich in calories, B-group vitamins (thiamine, folic acid, riboflavin, nicotinic acid) and amino acids such as lysine [3]. Present in popular bars and a dedicated local market, sorghum beer is a refreshing drink much appreciated by a large number of consumers. In different local *Tchoukoutou* markets and *Cabaret* (beer gardens), consumers discriminate producers by the organoleptic quality (flavor and taste) and alcohol content of their beer. As observed by Kayode et al. [4] in Benin and Schoustra et al. [5] in Zambia, producers and consumers claim that the traditional sorghum beer offers health benefits such as preventing and curing diarrhea. The health benefits as well as good aroma and flavor of *Tchoukoutou* are due to the combination of yeast and the LAB content in this traditional starter. The LAB content may also cooperate with yeasts for the bio-protection of the beer by producing organic acid and bacteriocins [6]. During the mashing processes of producing *Tchoukoutou* and other African beers, the sorghum mash pH is decreased by spontaneous LAB acidification. This bio-acidification, if efficient, should improve amylase, protease, endo-$\beta$-glucanase and phosphatase activities [7, 8], lautering

performance [9], organoleptic qualities, mineral bioavailability [10] and vitamin production [11].

African sorghum beers are not standardized, and in addition to the variation of organoleptic qualities, several safety problems limit the marketability of these drinks. Indeed, *Tchoukoutou,* because of its instability as well as short shelf life resulting from uncontrolled and unlimited fermentation, is only consumed in the areas where it is produced. Furthermore, African sorghum beer is consumed while it is still fermenting, and the drink contains many fragments of insoluble materials, like undigested starch and protein, which still remain in the beer. This is likely due to the inappropriate gelatinization time, temperature and the rudimentary materials (basket and cloth) used for filtration. The growth of mold during germination is also a permanent exogenous risk that compromises the safety of African sorghum beer. Indeed, aflatoxin produced by *Aspergillus flavus* is the most frequently occurring mycotoxin identified in South African traditional beer [12], in Nigeria's opaque beer [13] and in Malawi's traditional maize beer [14]. In addition to this exogenous contaminant, the raw material used during beer processing, i.e., sprouted grains of *sorghum bicolor*, contains a cyanogenic glycoside [15–18]. If this secondary metabolite of sorghum grain remains in the beer, it generates poisonous hydrogen cyanide by the action of enzymes of the intestinal microbiota after consumption by humans [19]. Because of these safety problems, which hinder the marketability of African sorghum beer, a controlled mixed fermentation may be a potential key to upgrading this traditional beer. This challenge may help producers to move from an ancestral, neolithic household process to industrial production using the endogenous *Tchoukoutou* microorganisms to guarantee its particular organoleptic quality. This will solve the safety problem, increase the shelf life and make this beer as competitive as Berliner Weißbier, Belgian lambic beer and American coolship ales, which are also obtained by mixed fermentation including yeast and LAB. For this purpose, selecting performant yeast and LAB that produce aryl-ß-D-glucosidase is a good approach. Through this approach, dhurrin (sorghum cyanogenic glucoside) and other sorghum glucose conjugate compounds are hydrolyzed thereby contributing to sorghum beer detoxification and flavor enhancement, respectively. To achieve this goal, the first challenge is the identification of different yeasts and LAB which occur during this spontaneous mixed fermentation. In this study, following a polyphasic approach, the different yeasts in the traditional starter used for *Tchoukoutou* production are isolated, discriminated and classified based on their phenotype, proteins and DNA sequence analysis. Furthermore, based on their metabolic profile, yeasts with interesting biotechnological properties are selected.

## Materials and methods

### Sample collection, yeast isolation and purification

Thirty-six samples of *Tchoukoutou* starter were collected in 12 localities of two agroecological zones with high production of African sorghum beer in Benin. Yeast strains were isolated from the starter using the standard serial method. A hundred microliters of the dilution ($10^{-4}$–$10^{-8}$) was plated using the spread plate method on oxytetracycline glucose yeast agar (yeast extract 5 g/l, glucose 20 g/l, agar 15 g/l). The selectiveness of the medium was improved by adding 1 % of oxytetracycline per liter of OGYA medium for LAB inhibition. The inoculated plates were incubated at 27 °C for 72 h. The pure yeast colonies were obtained by picking single colonies and streaking them on the same medium following the quadrant streaking technique. The plates were incubated at 27 °C for 48 h.

### Phenotypical characterization

#### *Morphological and physiological characterization*

Two hundred and forty pure yeast isolates were characterized on the basis of their morphological and physiological characteristics following the methods described by Yarrow [20]. The morphological and physiological data of the online CBS database (www.cbs.knaw.nl) and the key procedure described by Back [21] were used for yeast identification. The results of this phenotypical characterization were labeled as negative (−), positive (+) or weak (w), and R software was used for heat map construction.

#### *MALDI-TOF analysis based on yeast proteome mass spectrum*

A hundred and forty-five yeasts, selected from 240 yeasts which were isolated and phenotypically characterized, were submitted to MALDI-TOF test using the ethanol extraction method. After the growth of the different yeast strains on YPG agar for 48 h at 27 °C, a single yeast colony was suspended in 300 µL of ultrapure water, and then, 900 µL of absolute ethanol was added. The proteome of the precipitated cell material was extracted with formic acid and acetonitrile. One microliter of this yeast extract supernatant was dropped on the target, allowed to dry, overlaid with 1 µL of a matrix solution (α-cyano-4-hydroxy-cinnamic acid). The measurement was performed on a Microflex LT spectrometer (Bruker Daltonik). The Bruker database (Biotyper 3.1) and an in-house database (Technische Mikrobiologie, Weihenstephan) were used for the automatic online yeast identification. According to the similarity between the applied yeast proteome mass spectrum and the mass spectrum of the target yeast strain in the database, the

score value is generated. The identification is highly probable at species level when the score value is between 2.300 and 3.000. No reliable identification is observed when the score value is below 1.699. The applied yeast strain being scored from 1.700 to 2.000 limits the identification to genus level. On the basis of the yeast mass spectra, groupings were visualized by similarity calculations and multidimensional scaling (MDS) proposed by Usbeck et al. [22].

### Molecular characterization

The overnight culture was obtained by growing the yeast strain in YPG medium at 27 °C. The DNA of this overnight culture was isolated according to the method described by Hanna and Xiao [23].

*Amplification of ITS region and RFLP analysis*

The PCR was performed with $ITS_1$ (5′-TCCGTAGGT-GAACCTGCGG-3′) and $ITS_4$ (5′-TCCTCCGCTTATT-GATATGC-3′) primers to amplify the ITS region [24]. This amplified DNA of 50 yeasts which were characterized on the basis of MALDI-TOF analysis was randomly selected from different sub-clusters obtained after multidimensional scaling (MDS). It was digested with three endonucleases *HindIII*, *HinfI* and *HaeIII,* as per the instruction of the manufacturer (New England Biolabs, Frankfurt, Germany). The digested PCR–DNA was run on 2 % agarose gel at constant voltage (130 V) for 80 min and visualized by UV. The yeasts were identified on the basis of their restriction patterns using the Spanish Type Culture Collection CECT database (www.yeast-id.org).

*Amplification of D1/D2 of 26S rDNA and sequencing*

The D1/D2 domain of the large subunit ribosomal RNA gene was amplified with NL1 (5′-GCATATCAATAAGC GGAGGAAAAG-3′) and NL4 (5′GGTCCGTGTTTCAA GACGG-3′) primers [25]. The amplified PCR–DNA of D1/D2 of 22 yeasts which were randomly selected from different RFLP patterns was purified following the manufacturer's (PeqLab, Erlangen) instructions, and it was sequenced with $NL_1$ primer (5′-GCATATCAATAAGCGG AGGAAAAG-3′). The different yeast strains were identified using the NCBI database (Blastn).

## Results

### Metabolic spectrum as biomarker for yeast classification and identification

The yeast content of the traditional starter, which is used for *Tchoukoutou* production, varies from $10^8$ to $10^9$ CFU/g of liquid starter. The colonies of isolated yeasts are creamy-white and yellowish, butyrous in texture, farinose and of smooth appearance. The different yeasts exhibited round, oval and cylindrical shapes at microscopic observation. The fermentation test discriminated the isolated yeasts into different fermentative groups with distinct metabolic profiles. After the classification of the isolated yeasts based on their phenotypic characteristics, two principal clusters were obtained (Fig. 1). The online yeast identification based only on their morphological and physiological characteristics generates ambiguous and inconclusive results. The key procedure described by Back [21] has been used to discriminate *S. cerevisiae* (lysine agar negative) from the non-*Saccharomyces* yeast (lysine agar positive). Cluster 2 probably consists of only the non-*Saccharomyces* yeasts (lysine agar positive) while cluster 1 consists of *S. cerevisiae* (lysine agar negative) with the exception of the yeast strain BE-227. Most of the lysine and crystal violet negative yeast strains do not ferment melibiose. They therefore have the same fermentative aptitude as *S. cerevisiae* top-brewing yeast [21]. Twenty-five of the isolated yeasts including *S. cerevisiae* and non-*Saccharomyces* are able to

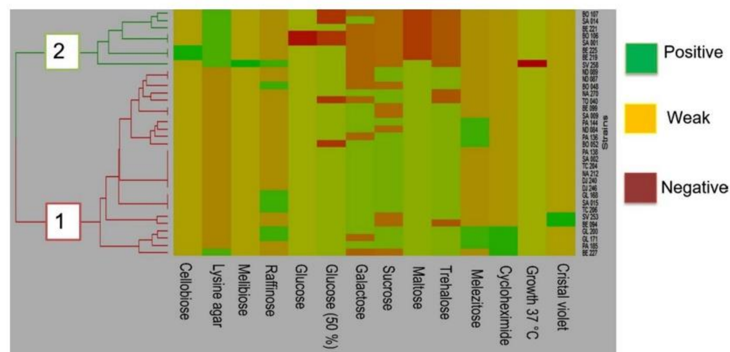


**Fig. 1** Yeast phenome heat map showing the repartition of the isolated yeasts based on their fermentative aptitude and capacity to grow under stress conditions using the Ward method and Euclidean distance. The color play ranges from *green* to *red* via *yellow* when the reaction is positive, weak or negative

use lactic acid as a substrate. Two of the isolated yeasts fermented cellobiose and therefore express $\beta$-D-glucosidase (cellobiase). Most (99.58 %) of the isolated yeasts were able to grow at 37 °C (Table 1).

### Proteome as biomarker for yeast discrimination and identification

A hundred and forty-five yeasts with the largest differences in their metabolic profiles were selected and submitted to MALDI-TOF analysis. After this analysis, 92 yeasts were identified, and among them, 69.51 % were identified at species level (score value $\geq$ 2.3) as *P. kudriavzevii* and *S. cerevisiae,* and 30.49 % were identified at genus level as *Saccharomyces* sp. and *Debaryomyces* sp. After similarity calculations and multidimensional scaling (MDS) based on the yeast proteome mass spectra, the 145 yeasts were distributed to four clusters (Fig. 2). All the yeasts identified as *S. cerevisiae* and *Saccharomyces* sp. as well as 45 non-identified yeasts were grouped in cluster A and those yeasts that were identified as *P. kudriavzevii* belonged to cluster B. Cluster C consisted of only the yeasts belonging to the *Debaryomyces* genus and two unidentified yeasts. The outcluster yeast which does not belong to cluster C was also identified as *Debaryomyces*. Six of the yeasts that were unidentified because they scored below 1.77 were grouped in cluster D. These yeasts have finally been identified as *C. ethanolica* on the basis of the combination of the partial domains 1 and 2 of the large subunit ribosomal RNA gene and the restriction patterns of the 5.8S-ITS region. The different mass spectra generated from the different groups obtained after MALDI-TOF MS analysis were different and discriminated the isolated yeasts to genus and species levels (Fig. 3).

### Yeast molecular characterization on the basis of ITS1-5.8S-ITS4 region and D1/D2 of large subunit rRNA gene

Fifty isolated yeasts were randomly selected from the different protein patterns (MALDI-TOF MS) and submitted to the restriction fragment length polymorphism (RFLP) analysis. After this RFLP analysis with three different endonucleases (*HindIII*, *HinfI* and *HaeIII*), only *HinfI* (Hf) and *HaeIII* (He) digested the ITS1-5.8S-ITS4 region into 2, 3, 4 or 5 fragments according to the strain as shown by the restriction patterns obtained after electrophoresis on agarose gel (Fig. 4). Six different restriction patterns were obtained after RFLP analysis. The restriction profiles 14-Hf (250 and 200 bp) and 14-He (300 and 150 bp) were obtained after the digestion with *HinfI* and *HaeIII,* respectively, and correspond to *C. ethanolica* yeast species. All the yeasts grouped in cluster D, which were obtained after their classification on the basis of protein patterns (MALDI-TOF MS analysis), have the same restriction patterns. The same observation was made with the different yeasts grouped in clusters B and C. In fact, the isolated yeast strains 337 and 375 have the same restriction profile that corresponds to *P. kudriavzevii* while those of 233, 219 and 221 (Fig. 4b) belong to *D. hansenii* complex yeast species. The different yeast strains in clusters B, C and D, which were obtained after yeast classification based on their protein patterns, are therefore homogeneous at species level. The RFLP analysis subdivided the *S. cerevisiae* yeast into different groups corresponding to different genotypes (Fig. 4a and c). The whole strain selected from the three different restriction patterns and belonged to cluster A which includes identified and non-identified yeasts was successfully identified as *S. cerevisiae* on the basis of D1/D2 domain. The ambiguous results corresponding to *C. ethanolica* and *P. deserticola* with a similarity of 100 and 99, respectively, were obtained when the d1 and d2 domain sequence of yeast belonging to cluster D was used. The yeasts grouped in clusters C and D, on the basis of MALDI-TOF analysis, were, respectively, identified as *D. hansenii* and *C. ethanolica* by using 5.8S-ITS region (Table 2).

## Discussion

The yeast characterization on the basis of their morphological and physiological (fermentation and assimilation) traits does not enable to discriminate the non-*Saccharomyces*. The variability of the fermentative and assimilation profiles of the isolated strains, all belonging to *S. cerevisiae* yeast species, limits their identification. However, all yeasts which were identified as *S. cerevisiae* are lysine negative and, contrary to the non-*Saccharomyces* yeasts (lysine positive), show a distinct fermentative profile (Fig. 1). In contrast to the isolated yeasts' morphology and physiology, the yeast proteome obtained after MALDI-TOF MS analysis seems to be a good biomarker enabling yeast discrimination and grouping. The restriction patterns of the 5.8S-ITS sequence and comparing the D1/D2 domain of 26 ribosomal LSU sequence show the homogeny inside the different clusters generated on the basis of protein patterns. Despite the accuracy of MALDI-TOF analysis for yeast grouping, the absence of *C. ethanolica* in the used database hinders its identification. Kurtzman [26], therefore, proposed the expansion of the databases with additional yeast species to achieve complete yeast identification. The MALDI-TOF MS analysis based on microorganism proteome prior to molecular characterization may yet help to accurately group yeast. This rapid yeast grouping may be a good approach for an accurate, high throughput and inexpensive alternative to a large number of yeast characterizations and

**Table 1** Physiological characteristics of yeasts isolated from Beninese African sorghum beer starter

| Strain codes | Fermentation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Glucose | Galactose | Sucrose | Maltose | Cellobiose | Trehalose | Lactose | Melibiose |
| SA-001 | + | − | − | − | − | − | − | − |
| SA-002 | + | + | + | + | − | + | − | − |
| SA-009 | + | + | − | + | − | + | − | − |
| SA-014 | + | + | − | − | − | − | − | − |
| SA-015 | + | + | + | + | − | + | − | − |
| TO-040 | + | − | + | + | − | − | − | − |
| BO-048 | + | − | − | + | − | + | − | − |
| BO-052 | + | + | + | + | − | + | − | − |
| ND-084 | + | + | − | + | − | + | − | − |
| ND-087 | + | − | + | + | − | + | − | − |
| ND-089 | + | − | + | + | − | + | − | − |
| BE-094 | + | + | − | + | − | − | − | − |
| BE-099 | + | + | − | + | − | + | − | − |
| BO-106 | + | − | − | − | − | − | − | − |
| BO-107 | + | − | − | − | − | − | − | − |
| PA-136 | + | − | + | + | − | + | − | − |
| PA-138 | + | + | + | + | − | + | − | − |
| PA-144 | + | + | + | + | − | + | − | − |
| GL-168 | + | + | + | + | − | + | − | − |
| GL-171 | + | − | + | + | − | + | − | − |
| PA-185 | + | + | + | + | − | + | − | − |
| GL-200 | + | + | + | + | − | + | − | − |
| TC-204 | + | + | + | + | − | + | − | − |
| TC-206 | + | + | + | + | − | + | − | − |
| NA-212 | + | + | + | + | − | + | − | − |
| DJ-240 | + | + | + | + | − | + | − | − |
| BE-219 | + | − | − | − | + | − | − | − |
| BE-227 | + | − | − | + | − | + | − | − |
| BE-221 | + | − | − | − | − | − | − | − |
| NA-270 | + | + | + | + | − | − | − | − |
| NA-278 | + | + | − | + | − | + | − | − |
| DJ-246 | + | + | + | + | − | + | − | − |
| SV-253 | + | + | − | + | − | + | − | − |

**Table 1** continued

| Strain codes | Assimilation of carbon and Nitrogen | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Glucose | Galactose | Sorbose | Sucrose | Maltose | Celllobiose | Trehalose | Lactose | Melibiose | Raffinose | Melibiose | Inulin | Starch | Xylose |
| SA-001 | + | + | − | + | + | − | − | − | − | + | + | + | − | − |
| SA-002 | + | − | − | + | + | + | − | − | − | − | − | + | − | − |
| SA-009 | + | + | − | + | + | − | − | − | − | − | − | − | − | − |
| SA-014 | + | + | − | + | + | − | + | − | − | − | + | + | + | − |
| SA-015 | + | − | − | + | + | − | − | − | − | − | − | − | − | − |
| TO-040 | + | − | + | + | + | − | + | − | + | + | + | + | − | + |
| BO-048 | − | + | − | + | + | + | − | + | − | − | − | − | − | − |
| BO-052 | + | − | − | + | + | − | − | − | − | − | + | + | − | − |
| ND-084 | + | + | − | + | + | − | + | − | + | + | + | + | + | − |
| ND-087 | + | − | w | + | + | − | − | − | − | − | + | − | − | − |
| ND-089 | + | − | + | + | + | − | + | − | − | + | + | + | + | − |
| BE-094 | + | + | − | + | + | − | + | − | − | + | + | + | + | − |
| BE-099 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| BO-106 | + | + | − | + | + | − | + | − | − | w | + | + | + | − |
| BO-107 | + | + | w | + | + | − | − | − | − | − | + | + | − | − |
| PA-136 | + | − | − | − | + | − | − | − | − | − | − | − | + | − |
| PA-138 | + | − | − | w | − | + | − | − | − | − | − | − | − | − |
| PA-144 | + | + | − | + | + | − | − | − | − | + | + | + | + | − |
| GL-168 | + | + | + | + | + | − | + | − | + | + | + | + | + | − |
| GL-171 | + | − | − | − | + | − | + | + | − | − | w | − | − | − |
| PA-185 | + | + | − | − | + | − | − | − | − | − | − | − | − | − |
| GL-200 | + | + | − | + | + | − | − | − | − | − | + | w | + | − |
| TC-204 | + | + | − | − | + | − | − | − | − | − | − | − | − | − |
| TC-206 | + | + | − | + | + | − | − | − | − | + | − | + | − | − |
| NA-212 | + | − | − | + | + | + | − | − | − | − | − | + | − | − |
| DJ-240 | + | + | − | − | + | − | − | + | − | − | w | − | − | − |
| BE-219 | + | + | + | + | + | + | + | − | + | + | + | + | + | + |
| BE-227 | + | + | + | + | + | + | + | w | + | + | + | + | + | w |
| BE-221 | + | + | − | + | + | − | − | − | − | + | + | + | − | − |
| NA-270 | + | − | − | + | + | − | + | − | − | − | + | − | − | − |
| NA-278 | + | − | − | − | + | − | + | + | − | − | − | − | + | |
| DJ-246 | + | + | + | + | + | w | + | − | + | − | + | − | + | w |
| SV-253 | + | + | − | + | + | − | − | − | − | − | − | − | − | − |

**Table 1** continued

| Strains codes | Assimilation of carbon and Nitrogen | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | L-arab-inose | D-arab-inose | Ribose | Rham-nose | Erythri-tol | Mannitol | Sorbitol | Methyl glucoside | Salicin | Lactic acid | Succinic acid | Inositol | Esculin | Mannose | Lysine agar | Nitrate |
| SA-001 | − | − | − | − | − | − | − | − | − | + | + | − | − | + | + | + |
| SA-002 | − | − | − | − | − | − | − | − | − | − | − | − | − | + | − | + |
| SA-009 | − | − | − | − | − | − | − | − | − | − | − | − | − | + | − | + |
| SA-014 | − | − | − | − | − | − | − | + | − | + | + | + | − | + | + | + |
| SA-015 | − | − | − | − | − | − | − | − | − | − | − | − | − | + | − | + |
| TO-040 | − | − | − | − | − | − | − | − | − | − | − | − | + | + | − | + |
| BO-048 | − | − | − | − | − | − | − | − | − | − | − | − | − | + | − | + |
| BO-052 | − | − | − | − | − | − | − | − | − | − | − | − | − | + | − | + |
| ND-084 | − | − | − | − | − | − | − | − | − | + | − | − | + | + | − | + |
| ND-087 | − | − | w | − | − | − | − | − | − | − | − | − | − | + | − | + |
| ND-089 | − | − | + | − | − | − | − | − | − | − | − | − | − | + | − | + |
| BE-094 | − | − | − | − | − | + | − | − | − | + | − | − | + | + | − | + |
| BE-099 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | − | + |
| BO-106 | − | − | − | − | − | − | − | w | − | + | + | − | − | + | + | + |
| BO-107 | − | − | − | − | − | − | − | + | + | − | − | − | + | − | + | + |
| PA-136 | − | − | − | − | − | − | − | − | − | − | − | − | − | + | − | + |
| PA-138 | − | − | − | − | − | − | − | − | − | − | − | − | − | + | − | + |
| PA-144 | + | − | − | − | − | − | − | − | − | − | − | − | + | + | − | + |
| GL-168 | + | − | + | − | − | − | − | − | + | − | − | − | + | + | − | + |
| GL-171 | − | − | − | − | − | − | − | w | − | − | − | + | w | + | − | + |
| PA-185 | − | − | − | − | − | − | − | − | − | − | − | − | − | + | − | + |
| GL-200 | − | − | − | − | − | − | − | − | − | + | − | − | − | + | − | + |
| TC-204 | − | − | − | − | − | − | − | − | − | − | − | − | − | + | − | + |
| TC-206 | − | − | − | − | − | − | − | − | − | − | − | − | − | + | − | + |
| NA-212 | − | − | − | − | − | − | − | − | − | − | − | − | − | + | − | + |
| DJ-240 | − | − | − | − | − | − | − | − | − | − | − | − | − | + | − | + |
| BE-219 | − | + | + | − | − | + | + | + | + | + | + | − | + | + | + | + |
| BE-227 | − | w | + | − | + | − | + | − | − | − | + | − | + | + | + | + |
| BE-221 | − | − | − | − | − | − | − | − | − | − | − | − | + | + | + | + |
| NA-270 | − | − | − | − | − | − | − | − | − | − | − | − | − | + | − | + |
| NA-278 | − | − | − | − | − | − | − | − | − | − | − | + | − | + | − | + |
| DJ-246 | − | w | + | − | − | − | − | + | − | − | − | W | + | + | − | + |
| SV-253 | − | − | − | − | − | − | − | − | − | − | − | − | − | + | − | + |

Table 1 continued

| Strain codes | Growth | | | |
|---|---|---|---|---|
| | Growth at 37 °C | Cycloheximide | 50 % Glucose | Cristal violet |
| SA-001 | + | − | + | − |
| SA-002 | + | − | + | − |
| SA-009 | + | − | + | − |
| SA-014 | + | − | − | − |
| SA-015 | + | − | + | − |
| TO-040 | + | − | − | − |
| BO-048 | + | − | + | − |
| BO-052 | + | − | − | − |
| ND-084 | + | − | + | − |
| ND-087 | + | − | + | − |
| ND-089 | + | − | + | − |
| BE-094 | + | − | + | + |
| BE-099 | + | − | + | − |
| BO-106 | + | − | − | − |
| BO-107 | + | − | − | − |
| PA-136 | + | − | + | − |
| PA-138 | + | − | + | − |
| PA-144 | + | − | + | − |
| GL-168 | + | − | + | − |
| GL-171 | + | − | + | − |
| PA-185 | + | + | + | − |
| GL-200 | + | + | + | − |
| TC-204 | + | − | + | − |
| TC-206 | + | − | + | − |
| NA-212 | + | − | + | − |
| DJ-240 | + | − | + | − |
| BE-219 | + | − | + | − |
| BE-227 | + | + | + | − |
| BE-221 | + | − | + | − |
| NA-270 | + | − | + | − |
| NA-278 | + | − | + | − |
| DJ-246 | + | − | + | − |
| SV-253 | + | − | + | + |

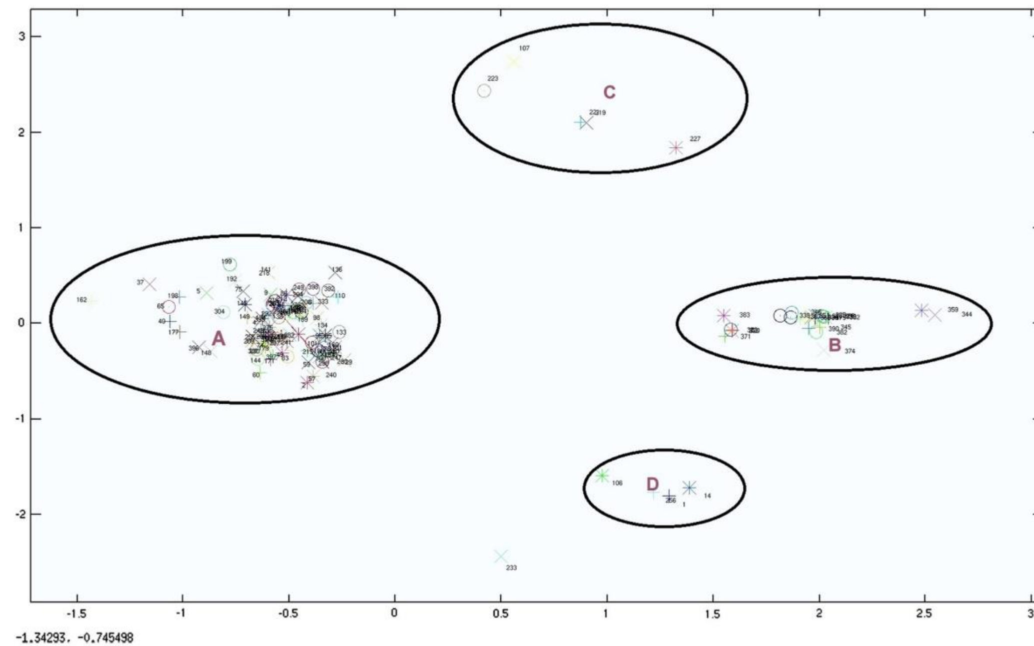


**Fig. 2** Multidimensional scaling (MDS) based on protein patterns showing the repartition of different yeasts isolated from the starter of African sorghum beer. *S. cerevisiae* yeasts were separated from non-*Saccharomyces* yeasts. The non-*Saccharomyces* yeasts were distributed into three different groups belonging to *Pichia*, *Candida* and *Debaryomyces* genus. *Saccharomyces cerevisiae* is the dominant yeast (*A*) followed by *P. kudriavzevii* (*B*), *D. hansenii* (*C*) and *C. ethanolica* (*D*)

classifications. However, grouping the *D. hansenii* complex yeast is difficult and its identification is limited to genus level. The discrimination of yeast belonging to *D. hansenii* complex such as *D. fabryi*, *D. macquariensis*, *D. nepalensis*, *D. propopidis*, *D. subglobosus*, *D. tyrocola*, *D. vietnamensis*, *D. vindobonensis* and solving the limit of the 26S rRNA gene to differentiate these ascomycetous yeasts still remains a challenge. According to the work carried out by Prista et al. [27] and Martinez et al. [28], the *Debaryomyces* is very sensitive to the presence of some ionic substances such as sodium and potassium, which induce a variation of their proteome. This high susceptibility of *D. hansenii* complex, causing a small variation of the growth conditions to strongly affect their proteome, will probably preclude obtaining a protein pattern similar to that content in the used library for a reliable *Debaryomyces* yeast identification. Comparing the D1/D2 of the 26S rDNA sequence does not enhance the result obtained with yeast identification on the basis of the protein patterns. The limits of differentiating yeast belonging to *D. hansenii* complex due to the high similarity among their D1/D2 domain of the 26S rRNA gene was also observed by Kurtzman et al. [29], Groenewald et al. [30] and Martorell et al. [31]. Sequence analysis of the D1/D2 domain also indicated that *C. ethanolica* and *P. deserticola* are closely related, and they were both identified with a similarity of 100 and 99 %, respectively. The 5.8S-ITS region seems to be more discriminative and, contrary to the D1/D2 domain of 26 LSU, allows the differentiation of *C. ethanolica* from *P. deserticola*. Indeed, the polymorphic restriction endonuclease *HaeIII* generated different restriction patterns corresponding to two DNA fragments (110, 310 bp) for *C. ethanolica* (Table 2) and three DNA sequences (80, 100, 280 bp) for *P. deserticola* according to the restriction patterns obtained from the used CECT database. The *Debaryomyces* yeasts were successfully identified to species level as *D. hansenii* on the basis of the restriction patterns generated from the digestion of the 5.8S-ITS region with *HinfI* and *HaeIII*. This result is likely due to the absence of the others *Debaryomyces* yeasts in the used CECT database. The RFLP analysis of the 5.8S-ITS sequence is limited and does not allow the differentiation of some closely related *Debaryomyces* yeasts. Martorell et al. [31] shows the limit of the RFLP analysis of the 5.8S-ITS gene to differentiate to

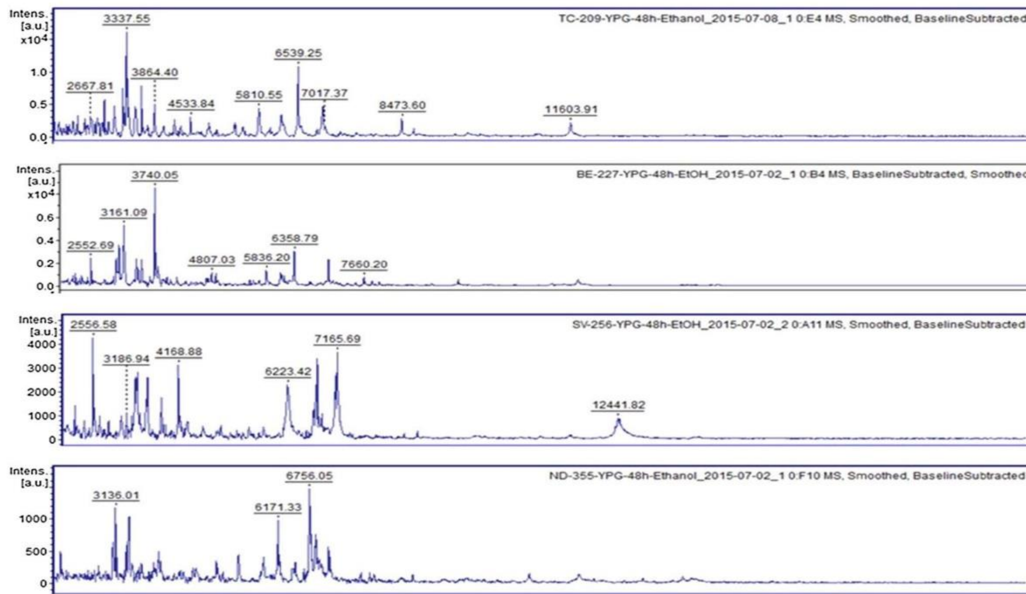


**Fig. 3** Proteome mass spectrum belonging to each of the four groups of identified yeast: *S. cerevisiae* (TC-209), *D. hansenii* (BE-227), *P. kudriavzevii* (ND-355) and *C. ethanolica* (SV-256) obtained after matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) using Bruker software, α-cyano-4-hydroxy-cinnamic acid as matrix after the ethanol extraction method

species level some *Debaryomyces* yeast such as *D. castellii*, *D. courdertii*, *D. nepalensis*, *D. polymorphus*, *D. pseudopolymorphus*, *D. robertsiae*, *D. udenii* and *D. vanrifiae*. The recent work of Wrent et al. [32] also shows the limit of the RFLP of the 5.8S-ITS region to discriminate the *D. hansenii* complex such as *D. hansenii*, *D. fabryi* and *D. subglobosus*. After RFLP of the 5.8S-ITS region, the polymorphic endonucleases *HaeIII* and *HinfI*, therefore, lead to restriction patterns which discriminated the isolated *Candida, Pichia* and probably *Debaryomyces* yeasts to species level and *S. cerevisiae* to strain level (Fig. 4). Indeed, the yeast strains belonging to *S. cerevisiae* have been distributed in three different patterns corresponding to three different genotypes of these species. This biodiversity of the *S. cerevisiae* component in *Tchoukoutou* starter has been confirmed by their phenotype, characterized by different metabolic profiles (Fig. 1, sub-clusters I) and protein patterns (Fig. 2) even if the D1/D2 sequence analysis does not evidence any high diversity among these isolated *Saccharomyces* yeasts. Yet the polyphasic approach that integrates yeast phenotype, protein patterns generated by MALDI-TOF MS, and DNA sequence comparison therefore provides reliable information about relationships among yeasts and helps to avoid a mis-identification. Based on this polyphasic approach, it became evident that *S. cerevisiae* is present in the whole starter and is the dominant or only yeast species (55–100 %) of 11 starters out of the 12 localities where the starters were collected (Fig. 5). This predominance of *S. cerevisiae* yeast was also observed by Kayode et al. [33], by Djegui et al. [34] and Greppi et al. [35]. The outgrowth of non-*Saccharomyces* yeasts by *S. cerevisiae* was also observed during wine production [36]. As observed by Csoma et al. [37], it seems that *S. cerevisiae*, according to its genome, is more flexible than some other yeasts, which allows this dominant yeast species to more efficiently adapt to the continuously changing environment during fermentation. *S. cerevisiae* yeast contained in the Beninese traditional sorghum beer starter have different and interesting fermentative aptitudes. Indeed, some specific strains of *S. cerevisiae* ferment sucrose and therefore express invertase. Djegui et al. [34] indexed *S. cerevisiae* with invertase gene as a performant strains for the brewing process. In addition to invertase properties, many isolated yeast strains belonging to *S. cerevisiae* species show a good fermentative profile. They ferment the mains sorghum's wort sugar and are able to use lactic acid as one of their substrates. These *S. cerevisiae* yeast species may increase the beer pH during yeast and LAB symbiotic interaction, thereby allowing the growth of LAB that may contribute to further beer maturation and enhance the beer flavor and

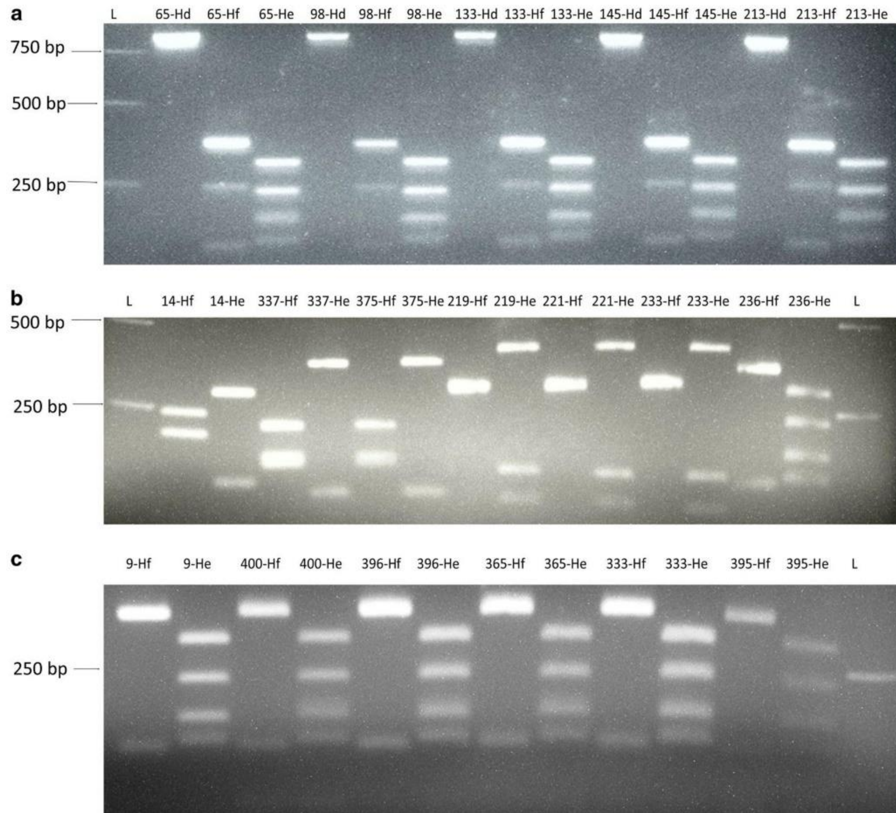


**Fig. 4** Restriction patterns obtained after digestion of the amplified PCR product of the ITS1-5.8S-ITS4 domain of selected yeasts with the restriction endonuclease *HindIII* (Hd), *HinfI* (Hf) and *HaeIII* (He). All the restriction patterns of **a** and **b** belong to *S. cerevisiae*. *C. ethanolica* (14), *P. kudriavzevii* (337, 375) and *D. hansenii* (233, 219, 221). The 250-bp ladder (L) was used during this analysis

**Table 2** Restriction patterns of the isolated non-Saccharomyces yeasts obtained with the application of endonucleases *HinfI* and *HaeIII* for the digestion of the 5.8S-ITS region and the corresponding yeast species in the Spanish Type Culture Collection (CECT) database

| Strains | Amplified DNA (bp) | Restriction patterns (bp) | | Identification | Match (%) |
|---|---|---|---|---|---|
| | | *HinfI* | *HaeIII* | | |
| 14 | 450 | 200 + 250 | 110 + 310 | *C. ethanolica* | 100 |
| 219, 221, 233 | 650 | 325 + 325 | 90 + 150 + 420 | *D. hansenii* | 100 |

nutritive value. The co-presence of *S. cerevisiae* with *P. kudriavzevii* is observed in the starters collected from Toucountouna (26.67 %), N'dali (45 %) and from Natitingou where *P. kudriavzevii* is the dominant yeast (61.11 %). *P. kudriavzevii* has been identified from several West African traditional beverages made from fermented cereals. N'guessan et al. [38] observed its co-presence with *S. cerevisiae* in *Tchapolo*, the Ivory Coast traditional sorghum beer. *P. kudriavzevii* is also the dominant yeast species isolated from *Dolo*, traditional sorghum beer produced in several localities of Burkina Faso [39]. Annan et al. [40] used *P. kudriavzevii* in combination with *S. cerevisiae* to enhance

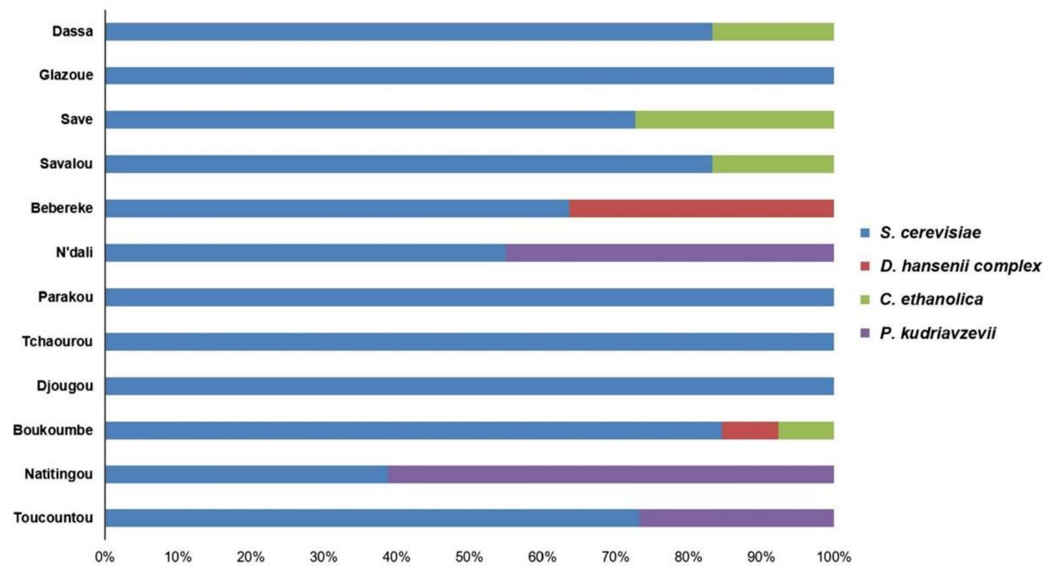


**Fig. 5** Diversity of yeast population of the traditional starter collected in 12 localities of Beninese sorghum beer production

the taste and flavor of *Koko*, a Nigerian cereal-based African fermented food. *C. ethanolica* and *D. hansenii* complex have never been identified from West African sorghum beer. Recently, Visintin et al. [41] observed that *C. ethanolica* occurs during fermentation of West African cocoa beans and indexed this yeast species to possess several enzymes that may impact the quality of the final product. *S. cerevisiae* and *C. ethanolica* were the two different yeast species isolated from the starter collected in Dassa, Savalou and Save. The beer produced in central Benin is also different from that produced in the north of Benin by it organoleptic quality (taste and flavor) and lower alcohol content. The non-*Saccharomyces D. hansenii* complex yeasts were identified in the starter collected in two localities in northeast Benin. Some strains of *D. hansenii* may have a great importance and cooperate with *S. cerevisiae* for the specific aroma compound profile of the Beninese sorghum beer. In fact, the strains BE-219 and BE-225 identified as *D. hansenii* express cellobiase ($\beta$-glucosidase) and may be a potential candidate for beer flavor enhancement. Specific $\beta$-glucosidase is able to hydrolyze the aryl-$\beta$-D-glucosides from the non-reducing terminus and generates a good precursor from the secondary gluco-conjugate metabolite sequestrated in the cereal grain for beer bioflavouring. *D. hansenii* is present in many habitats, but has been most isolated from a traditional cheese. Indexed by Romero et al. [42] as a contaminant of intermediate moisture foods, Gori et al. [43] observed the heterogeneity of this yeast species with regard to the cheese flavoring. Indeed, Gori et al. [43] isolated from a cheese, *D. hansenii* producing high alcohols (2-methyl-1-propanol, 3-methyl-1-butanol, 3-methyl-3-buten-1-ol) and aldehydes (2-methylpropanal, 3-methylbutanal). Even if spontaneous fermentations are ubiquitous in many developed countries in Africa, Asia and South America and constitute the only way of food preservation, the controlled fermentation with the selected performant yeast and LAB from the African traditional starter should be a good alternative to solve African sorghum beer safety problems, increase its shelf life and guarantee the same organoleptic quality for the consumers. The selection of performant yeast and LAB from this traditional starter still remains a challenge for the industrial production of *Tchoukoutou* as opposed to the existing household production.

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**Compliance with ethical standards**

**Conflict of interest** None.

**Compliance with ethics requirements** This article does not contain any studies with human and animals subjects.

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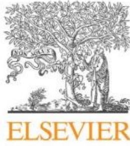
LWT - Food Science and Technology 80 (2017) 51–58



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# Polyphasic characterization of lactic acid bacteria isolated from Beninese sorghum beer starter

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*Tchoukoutou* is a Beninese traditional sorghum beer obtained by mixed fermentation including yeast and lactic acid bacteria (LAB). The starter's LAB communities as well as their biotechnological importance remain unknown. Furthermore, the sprouted grain of *Sorghum bicolor*, which is used during the beer processing, contains a cyanogenic glucoside (dhurrin). In order to elucidate Beninese sorghum beer starter LAB microbiota, 69 LAB isolated from traditional starters were characterized using a polyphasic approach including phenotypical characterization (physiology and MALDI-TOF MS) and 16S rRNA gene comparison. Based on the enzymes substrate specificity, LAB expressing aryl-β-D-glucosidase and amylase were indexed as potential candidates for dhurrin removal and saccharification improvement. All isolated bacteria belong to the same genus *Lactobacillus* with different strains of the five species *L. fermentum*, *L. plantarum*, *L. helveticus*, *L. paracasei* and *L. brevis* and diverse metabolic pathways. MALDI-TOF MS is a good method for accurate and high-throughput LAB identification. Several facultative hetero-fermentative LAB such as *L. plantarum* and *L. paracasei* express β-D-glucosidase and amylase. These β-D-glucosidase producers LAB will likely cleave the conjugated glucose of dhurrin, thereby contributing to detoxification if used for controlled sorghum mash bio-acidification.

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## 1. Introduction

African sorghum beer is a traditional drink produced on the basis of ancestral knowledge which is transmitted from one generation to the next. This special beer is called *Tchoukoutou* in Benin and Togo, *Pombe* in Tanzania, *Dolo* in Burkina-Faso, *Amgba* in Cameroon, *Pito* in Ghana, "seven days" beer in Zambia, and *Burukutu* or *Otika* in Nigeria. The production of these special beers follows three main steps such as the malting, mashing and fermentation. However, the manufacturing processes are very variable and depend on the geographical location. The typical traditional Beninese sorghum beer processing is characterized by multi-stage fermentation where sorghum mash is spontaneously acidified by lactic acid bacteria before the main sorghum wort mixed fermentation with the traditional starter (beer deposit) containing different yeast and lactic acid bacteria (Tokpohozin, Fischer, Sacher, & Becker, 2016a). Consumers recognize this special beer by its taste and specific flavor. The aroma compound profile of African traditional beer results from a symbiotic interaction of different yeast species, predominantly *Saccharomyces cerevisiae* (Greppi et al., 2013; Tokpohozin et al., 2016b) cooperating with various species of lactic acid bacteria (LAB). The beer resulting from mixed fermentation with this cocktail of microorganisms is sour with a pH of 3.2–3.5, 2–4% (v/v) alcohol, and unhoped; it is drunk while fermentation continues, without prior maturation. Additional to the resultant short shelf-life, two contaminants pose a severe health risk to consumers: for one, mycotoxins contributed from exogenous sources (Ezekiel et al., 2015; Matumba et al., 2014; Odhav, 2002), for another, the cyanogenic glucoside dhurrin in the raw material of beer processing, namely sprouted grains of *Sorghum bicolor* (Ahmed, Mahgoub, & Babiker, 1996; Traoré, Mouquet, Icard-Vernière, Traoré, & Trèche, 2004). If this secondary metabolite of sprouted sorghum grain remains in the beer, it generates poisonous hydrogen cyanide by the action of enzymes produced by the intestinal microbiota after consumption (Carter, McLafferty, & Goldman, 1980). Several toxico-nutritional diseases are reportedly associated with dietary cyanide, for example "*konzo*" or "tied leg", tropical ataxic neuropathy, goiter, and cretinism (Banea et al., 2015).

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Moreover, as observed by Ryu et al. (2015) and Lachenmeier et al. (2010), this cyanide is the ultimate precursor for the multi-site formation of carcinogenic ethyl carbamate (urethane). However, hydrolyzing dhurrin prior to consumption prevents these adverse effects, so that LAB which express the β-D-glucosidase required for such cleavage are potential candidates for detoxifying sorghum wort. The enzyme, which catalyzes hydrolysis of the β-D-glucosidic bond, may additionally generate good precursors for beer bio-flavoring from sequestered glucosides. To discover which strains of LAB may contribute here, the first challenge is identification: Following a polyphasic approach, we isolated, characterized, and identified LAB in the traditional starter used during *Tchoukoutou* production. Based on their metabolic profile, LAB with interesting biotechnological properties were indexed.

## 2. Materials and methods

### 2.1. Sampling

Fifteen samples of *Tchoukoutou* starter (beer deposit) were collected at May 2015 from five localities (Toucountouna, Natitingou, Djougou, Parakou, N'dali) of traditional sorghum beer production in North Benin (Fig. 1) in sterile plastic bottles. To stop the fermentation, the starters were placed in a cooler on ice.

### 2.2. Lactic acid bacteria isolation and purification

Lactic acid bacteria were isolated from the starter using the standard serial method. A hundred microliters of the dilution ($10^{-4}$ to $10^{-8}$) were plated using the spread plate method on MRS agar. The medium's selectiveness was improved by adding 1 mg of cycloheximide to 100 mL of MRS medium to inhibit yeasts. The inoculated plates were incubated at 28 °C for 72 h. Pure cultures were obtained by picking single colonies and streaking them on NBB agar medium following the quadrant streaking technique. Yellow colonies were selected as lactic acid bacteria. These isolated cultures of lactic acid bacteria were stored in glycerol at −80 °C.

### 2.3. Physiological characterization of lactic acid bacteria

Sixty-nine isolated lactic acid bacteria were phenotypically characterized using different substrates of carbon, including monosaccharides (pentose and hexose), disaccharides, polysaccharides, glucosides, polyalcohols and amino acids as described by Back (2000). Minimal Sharpe medium (10 g/L peptone, 5 g/L yeast extract, 2 g/L potassium hydrogen phosphate, 2 g/L diammonium hydrogen citrate, 5 g/L sodium acetate, 0.1 g/L magnesium sulfate, 0.005 g/L manganese sulfate, 1 g/L Tween 80) was prepared and chlorophenol red (0.04 g/L) was added as indicator of pH change. This minimum medium was supplemented with 1 percent of sugar substrate and the pH was adjusted to 5.8. A color change to yellow during fermentation was appreciated as a positive result; for glucose, maltose, and gluconate, gas production was additionally detected with inverted Durham tubes. For esculin splitting specifically, loss of bluish fluorescence under UV light (366 nm) and a color change from brownish to black indicated a positive result (Edberg, Gam, Bottenbley, & Singer, 1976). After 10 days' incubation, Nessler's reagent (Merck KGaA, Darmstadt,

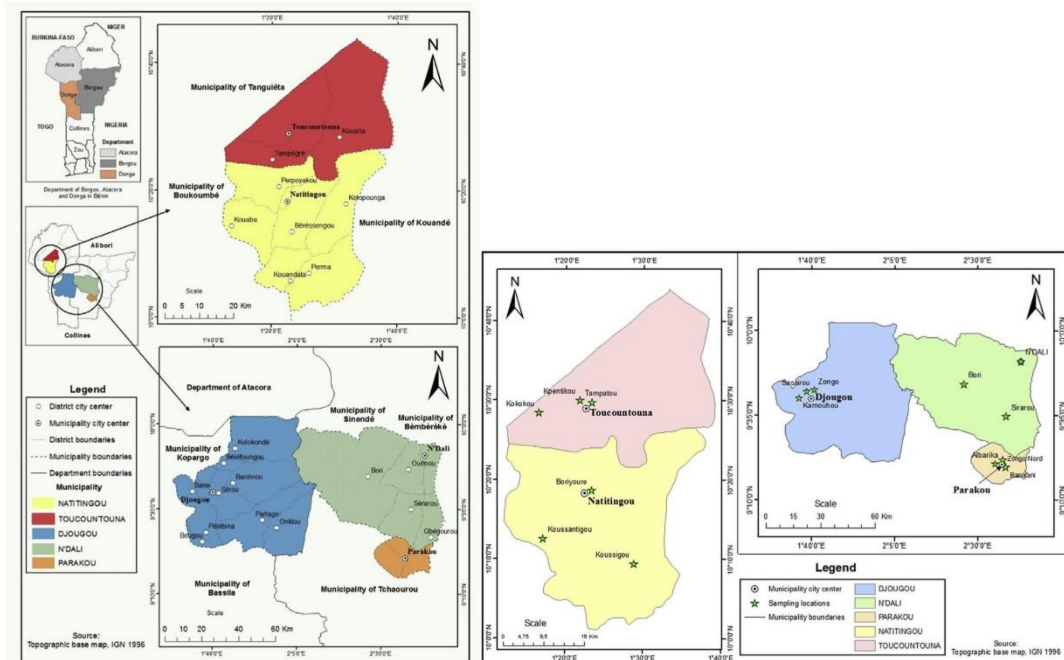


**Fig. 1.** Carte of situation showing the 5 principal zones (Toucountouna, Natitingou, N'dali, Djougou, Parakou) and the corresponding localities where sorghum starter were collected. The carte of situation and the carte showing the localities where traditional sorghum beer starter were collected were realized on the based of the data of the topographic IGN 1996 of Benin using the ArcGis software version 10.1.

Germany) was used to detect production of ammonia from arginine as described by Hahn, Bockemühl, and Döll (1976). The results of this phenotypical characterization were labeled as negative (−), positive (+), or weak (w), and 0, 100, and 50 were respectively attributed as quantitative data to construct a heatmap with R software.

### 2.4. Molecular characterization

#### 2.4.1. Lactic acid bacteria DNA extraction

Thirty-five lactic acid bacteria with different metabolic profiles were selected for molecular analysis. The overnight culture was obtained by growing these strains in MRS broth medium at 27 °C. The genomic DNA was isolated according to the phenol-chloroform method (Moore, Arnscheidt, Krüger, Strömpl, & Mau, 2004). Three milliliters of 48 h' cell growth were centrifuged (5000 rpm, 3 min) and the cell pellet was two times washed with 1 mL TE buffer ($10^{-3}$ mol/L Tris-HCl, $10^{-3}$ mol/L EDTA, pH 8). The pellet was suspended in 1 mL TE buffer and 630 μL of suspension was transferred to a 1.5 mL reaction tube containing 70 μL of 10% (w/v) SDS and incubated for 1 h at 37 °C. After adding 700 μL phenol, samples were vortexed for 30 s and reaction tubes were centrifuged for 10 min at 12,000 rpm. The supernatant was transferred to a tube containing 700 μL phenol: chloroform: isoamyl alcohol (25:24:1), vortexed (30 s) and centrifuged (10 min, 12,000 rpm). This step was repeat with chloroform: isoamyl alcohol (24:1). Four hundred and fifty microliters of the supernatant were mixed with 50 μL sodium acetate (3 mol/L) and 1 mL of saturated ethanol. Samples were centrifuged (15,000 rpm, 10 min) and the supernatant was removed. The DNA pellet was carefully suspended in 700 μL solution containing 670 μL isopropanol and 30 μL sodium acetate (3 mol/L). After centrifugation (15,000 rpm, 10 min), the supernatant is removed and the precipitated DNA was washed with 50 μL of 75% ethanol. The DNA pellet was dried for 20 min at 50 °C and then suspended in 50 μL bi-distilled water, 3 μL Rnase A (10 mg/mL) was added and incubated at 50 °C for 1 h.

#### 2.4.2. Amplification and sequencing of 16S rRNA gene

The PCR reaction was performed with 27-F (5′-AGAGTTTGATCCTGGCTCAG -3′) and 1492-R (5′-TACGGCTACCTTGTTACGACTT-3′) primers to amplify the 16S ribosomal RNA gene in 50 μL of reaction mix containing 10 μL of 5× reaction buffer, 1 μL of $10^{-3}$ mol/L dNTPs, 1 μL each of $10^{-3}$ mol/L forward and reverse primer, 0.25 μL of One Taq polymerase and a variable volume of genomic DNA template containing 100 ng of DNA; the volume was adjusted to 50 μL with bi-distilled water. The PCR reaction was conducted for 32 cycles with denaturation at 94 °C, annealing at 55 °C, and elongation at 68 °C. The amplified DNA was purified with the Cyclepure kit following the manufacturer's (PeqLab Erlangen) instructions and sequenced with 27-F and 1492-R primers.

#### 2.4.3. Phylogenetic analysis

The sequences obtained from one LAB strain with forward or reverse primer were trimmed and assembled using index-based assembly with the EzTaxon database. The characterized LAB were phylogenetically analyzed using MEGA 6 software with direct identification and comparison to the closest-related strain in the NCBI (National Center for Biotechnology Information) database (NCBI Blast n, Bacteria and Archaea database).

### 2.5. MALDI-TOF MS analysis based on LAB proteome mass spectrum

The sixty-nine isolated LAB were submitted to MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry) analysis using the on-plate extraction method by direct transfer of lactic acid bacteria. After 3 days' growth on MRS agar at 27 °C, a single colony of lactic acid bacteria was smeared on the spot of the target. Each spot was overlaid with 1 μL of formic acid and dried, followed by 1 μL of α-cyano-4-hydroxy-cinnamic-acid (matrix solution) and again drying. The measurement was performed on a Microflex LT spectrometer (Bruker Daltonik). The Bruker database (Biotyper) was used for automatic online LAB identification. The identification level (genus, species) was determined based on score value as described by Tokpohozin et al. 2016b: According to the similarity between the proteome mass spectrum of the applied LAB and that of the strain targeted in the database, the score value is generated. Identification is highly probable at species level when the score value is between 2.300 and 3.000. No reliable identification is possible when the score value is below 1.699. The applied strain being scored between 1.700 and 2.000 limits the identification to genus level.

## 3. Results

### 3.1. Phenotypical characterization

The LAB content of the traditional liquid starter used for Beninese sorghum beer production is between $1.1 \times 10^8$ and $2.8 \times 10^9$ CFU/g. The fermentation test discriminated the 69 isolated lactic acid bacteria into 43 different profiles within three metabolic pathways (obligate homo-fermentative, facultative hetero-fermentative and obligatory hetero-fermentative LAB). Several LAB identified as homo-fermentative or facultative hetero-fermentative were able to ferment cellobiose as well as compounds containing an aryl-D-glucosidic bond, namely salicin, esculin and amygdalin; thus, they express β-D-glucosidase. Of these isolates, 53 percent were able to hydrolyze the polysaccharides starch and dextrin, indicating additional expression of amylase. Among the strains producing both β-D-glucosidase and amylase, strains ND-130 and ND-32 belonging to cluster III ferment melibiose (Fig. 2) and therefore express α-galactosidase. Overall, the sub-cluster III groups LAB with interesting biotechnological properties expressing β-D-glucosidase, amylase and α-galactosidase.

### 3.2. Molecular characterization: 16S rDNA sequence comparison

LAB with different metabolic profiles were compared in terms of their 16S rDNA sequence. The identified LAB belong to the genus *Lactobacillus*, and the 16S rDNA comparison led to *L. brevis*, *L. plantarum* and *L. fermentum* being successfully identified. However, unambiguously assigning LAB to *L. casei* and *L. helveticus* was limited for two reasons: for one, strains of *L. helveticus* are highly biodiverse, and for another, there are some species that are closely related to *L. casei* and *L. helveticus* (Fig. 3); both factors interfere with certain differentiation.

### 3.3. MALDI-TOF MS

Identifying LAB was based on the mass spectrum generated by MALDI-TOF MS. After this analysis, most LAB were successfully identified at species level as *L. fermentum*, *L. plantarum*, *L. paracasei* and *L. brevis* (score value > 2.3) (Fig. 4). Several LAB were only assigned at genus level due to the lower score value ($1.7 \leq$ score value $< 2.3$); however, *L. helveticus* was proposed as the probable species. The lower score value again stems from the biodiversity within this species, similarly to the limited classification by 16S rDNA. A number of strains that belong to the *L. helveticus* clade based on the 16S rRNA gene remain unidentified; here, identification at species level and differentiation from closely related species requires more suitable biomarkers.

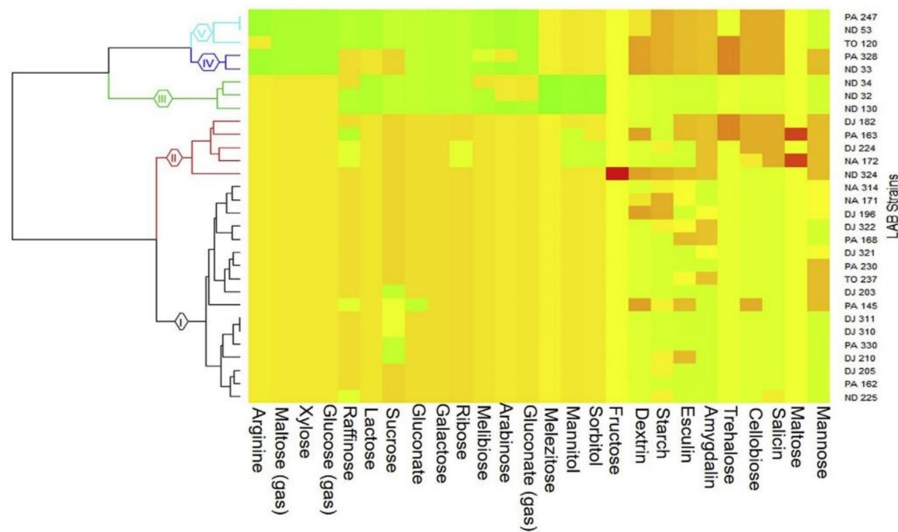


**Fig. 2.** Heat map showing the repartition of the isolated lactic acid bacteria by their metabolic pathways using R software and Ward method. The color play ranges from green to red via yellow when the reaction is positive, weak or negative. The sub-clusters I and II group obligate homofermentative lactic acid bacteria, sub-cluster III group facultative heterofermentative lactic acid bacteria and sub-clusters IV and V group obligate heterofermentative lactic acid bacteria. The facultative heterofermentative LABs in sub-cluster III and some obligate homofermentative LABs in sub-cluster I ferment cellobiose and glucosidic substrates (salicin, amygdalin and esculin) and polysaccharides (dextrin and Starch) and therefore express β-D-glucosidase and amylase. The LABs strains belonging to sub-cluster III (ND-32, ND-34, ND-130) which express amylase and β-D-glucosidase also ferment melibiose and therefore express α-galactosidase. The sub-cluster III groups facultative hetero-fermentative lactic acid bacteria which have an interesting biotechnological properties. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

## 4. Discussion

The phenotypical characterization of the LABs on the basis of their physiological traits, i.e. their sugar spectrum, does not enable reliable identification at species level: The results are redundant, and it is a limiting factor that sugar spectra of strains belonging to the same species *L. helveticus* are highly diverse. Moreover, Hammes and Vogel (1995) observed that several taxa classified by phenotypical characteristics were phylogenetically not coherent. Some obligate homo-fermentative lactic acid bacteria (NA-172, DJ-224) belonging to the subcluster II (Fig. 2) ferment ribose (pentose) showing a typical heterofermentative lactic acid bacteria characteristic. Biddle and Warner (1993) also found that some strains of *Lactobacillus acidophilus*, normally considered to be strictly homofermentative, was able to ferment ribose. Therefore, the sugar spectrum alone does not allow to meaningfully distinguish and thus reliably identify LAB strains, since some obligate homofermentative lactic acid bacteria ferment pentose and the metabolic profiles of strains of the same species can be very different. Therefore, to improve the certainty of identification over using sugar spectra as the sole biomarker, we require more discriminative biomarkers.

This is true of, for one, the molecular characterization via comparing the 16S rDNA sequence, leading to successfully identifying *L. fermentum*, *L. brevis* and *L. plantarum* (Fig. 3). Furthermore, analyzing and comparison of 16S rRNA gene sequence to an existing in the NCBI database help to reveal the phylogenetic neighborhood of the isolated lactic acid bacteria strains. However, differentiating *L. paracasei* and *L. helveticus* from the closely related *L. casei* and thermophile obligate homofermentative lactic acid bacteria (*L. gallinarum, L. acidophilus, L. amylovorus, L. crispatus, L. ultunensus*) respectively, remains a challenge, even more so as the diversity of metabolic profiles within *L. helveticus* reflects in their 16S rRNA gene (Fig. 3). Gatti, Trivisano, Fabrizi, Neviani, and Gardini (2004) have already described the phenotypic and genotypic diversity of this thermophile, obligate homofermentative LAB isolated from Italian cheese. In our work, several isolated LAB were very close to *L. helveticus* in the phylogenetic tree obtained from the 16S rRNA gene (Fig. 3), but Holzapfel and Wood, 2014 points out that since many LAB share more than 97% of their 16S rRNA gene sequences, no similarity threshold value to distinguish between species has been assigned. Generally, classification and nomenclature of closely related LAB, especially those belonging to the *L. casei* taxonomic group, are controversial and pit the judgments of several authors against each other. In an attempt to remedy this, the Judicial Committee on systematics of prokaryotes has published an opinion on this issue, and researchers are now seeking other biomarkers in addition to the 16S rDNA sequence to discriminate closely related LAB.

In contrast to metabolic profiles and 16S rDNA, the proteome obtained from MALDI-TOF MS analysis appears to be a suitable biomarker enabling discrimination and identification of such closely related LAB. Based on this chemo-taxonomy and using protein spectra obtained from MALDI-TOF MS as a biomarker, we successfully identified *L. fermentum*, *L. brevis*, *L. plantarum* as well as *L. paracasei*. Similar to our approach, Sato, Kitahara, Ohkuma, Hotta, and Tamura (2012) applied MALDI-TOF MS to discriminate closely related LAB belonging to the *L. casei* group and observed that the ribosomal proteins encoded in the S10-spc-alpha operon (S10-GERMS) differentiate *L. casei* from other strains of *L. paracasei*. As such, ribosomal protein is the most targeted biomarker in MALDI-TOF MS. However, the diverse metabolic profiles of *L. helveticus* likely impact ribosomal protein; indeed, identification of *L. helveticus* based on the whole-cell protein mass spectrum

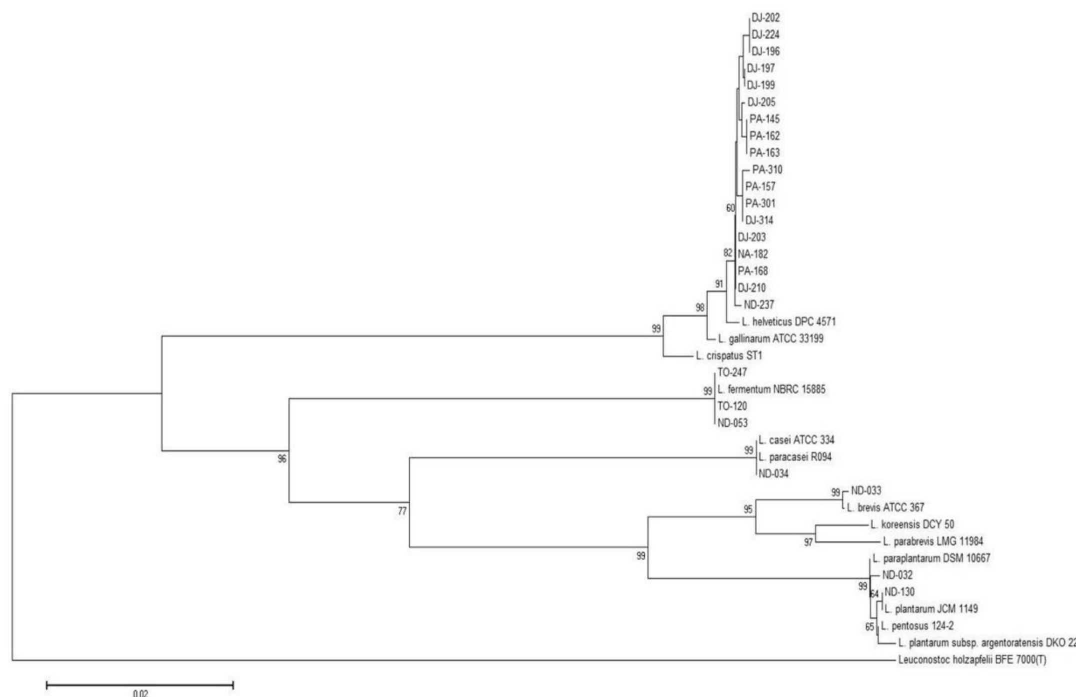


**Fig. 3.** 16S rDNA sequence-based phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (100 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Larkin et al., 2007) and are in the units of the number of base substitutions per site. The 16S rDNA sequences were compared to the closely related lactic acid bacteria belonging to the genus *Lactobacillus* in the NCBI database and *Leuconostoc holzapfelii* BFE 7000(T) was used as an out-group. Type strain sequences of *L. gallinarum, L. crispatus, L. fermentum, L. casei, L. paracasei, L. brevis, L. koreensis, L. parabrevis, L. paraplantarum, L. plantarum, L. pentosus* and *L. helveticus* were added to the alignment and used for tree construction.

generated lower score values (1.703–1.933) and was thus limited to genus level. Nevertheless, *L. helveticus* is the probable species as assigned by the applied Bruker software. Considering that Gatti et al. (1997) observed a protein of 50 kDa which is characteristic for *L. helveticus* and helps to differentiate this LAB from other closely related species, we can attribute the lower score value not only to the biodiversity within *L. helveticus*, but also the absence of entire *L. helveticus* strains in the database. Expanding the database with other strains of *L. helveticus*, especially those isolated from African fermented foods, may help to enhance the score value.

Our results show that MALDI-TOF MS is a good method for accurate, high-throughput, and inexpensive identification of LAB. Previously, Tokpohozin, et al. (2016b) have already used this method for a rapid microorganisms grouping. However, especially the biodiversity within *L. helveticus* combined with the absence of its diverse strains in the proteome database demands analysis of 16S rDNA in addition to MALDI-TOF MS in order to achieve reliable identification: Even provided that growth and measurement conditions are kept constant to prevent proteome variations, strains of the same species may be too diverse, and we cannot entirely exclude that factors influencing cell physiology as well as efficiency of matrix and analyte ionization (protonation) during the measurement affect the proteome spectrum. The polyphasic characterization is therefore a good approach to meaningfully discriminate and identify microorganisms.

On the basis of MALDI-TOF MS and 16S rDNA, we found that *L. plantarum*, *L. paracasei*, *L. fermentum*, *L. brevis* and *L. helveticus* occur during the fermentation of *Tchoukoutou*. The LAB's metabolic profile is important as well and helps to group the isolated LAB according to their metabolic pathway (Fig. 2). Obligate homofermentative LAB assigned as *L. helveticus* by 16S rDNA and MALDI-TOF MS were grouped into sub-clusters I and II corresponding to their metabolic profile (Fig. 2).

Overall, the microbial community of Beninese sorghum beer starter is diverse, and this biodiversity depends on locality: The thermophile homofermentative LAB belonging to *L. helveticus* clade, present in all starters, are dominant in starter collected in Parakou, Natitingou and Djougou, while those collected in N'Dali and Touncoutouna are dominated by *L. plantarum* and *L. fermentum*, respectively. *L. brevis* is only found in the starter from Djougou, and *L. paracasei* in that from N'Dali and Natitingou (Fig. 5). Similar to our work, Hounhouigan, Nout, Nago, Houben, and Rombouts (1993) observed that *Lactobacillus* spp. is a dominant lactic acid bacteria (94%) involved in the processing of mawe, a Beninese fermented maize dough. However, others LAB species belonging to *Lactococcus*, *Pediococcus* and *Leuconostoc* genus were identified. The similar LAB population was identified from *bushera*, a sorghum non-alcoholic beverage produces in Uganda (Muyanja, Narvhus, Treimo, & Langsrud, 2003). It is therefore evident that in additional to agro-ecological zone, several factors such as the

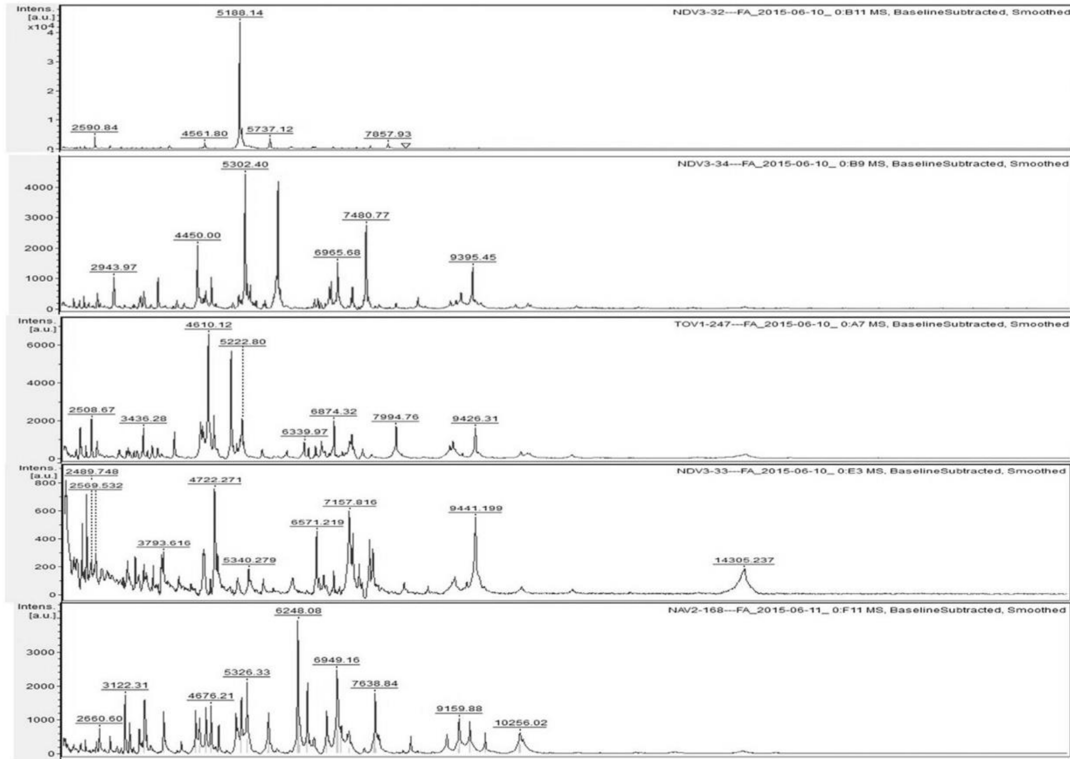


**Fig. 4.** Specificity of whole cell proteome mass spectrum belonging to the isolated lactic acid bacteria such as *L. plantarum* (NDV3-32), *L. paracasei* (NDV3-34), *L. fermentum* (TOV1-247), *L. brevis* (NDV3-33) and *L. helveticus* or closely related the mophile lactic acid bacteria (NAV2-168). The different proteome spectra were obtained by Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) using the α-cyano-hydroxy cinnamic acid as matrix and direct formic acid extraction method. The isolated lactic acid bacteria were automatical online identified using Bruker database.

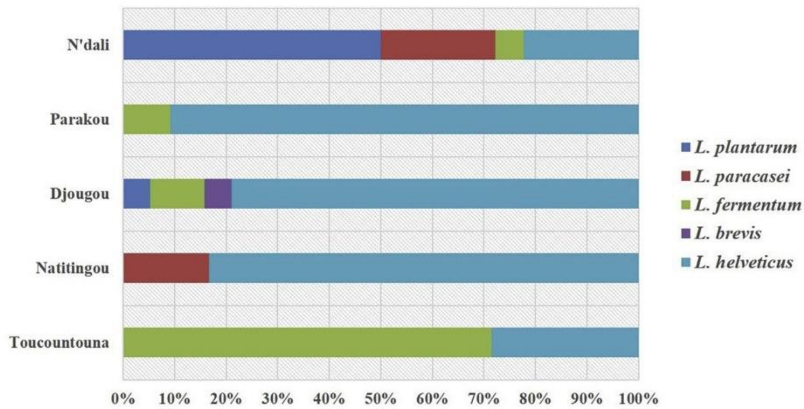


**Fig. 5.** Biodiversity of lactic acid bacteria Beninese West-African sorghum beer starter showing the repartition of the isolated bacteria in the five localities where the starters were collected.

processing techniques (malting, mashing), the sorghum wort composition, the metabolites resulting from fermentation (organic acid, alcohol) and the pH determine the *Tchoukoutou* starter microbiota.

The biodiversity of the isolated LAB impacts their biotechnological properties. Some strains isolated as *L. plantarum*, *L. paracasei* and *L. helveticus* express amylase and β-D-glucosidase. On the other hand, and in contrast to work carried out by Agati, Guyot, Morlon-Guyot, Talamond, and Hounhouigan (1998), no *L. fermentum* strain expressed amylase; likewise, LAB identified as *L. brevis* did not express β-D-glucosidase as observed by Michlmayr, Schümann, Barreira Braz Da Silva, Kulbe, and Del Hierro (2010). This indicates that some biotechnological properties are strain dependent. Significantly, the isolated *L. helveticus*, *L. plantarum* and *L. paracasei* hydrolyze several glucosidic compounds (salicin, esculin, amygdalin) and are promising candidates to remove dhurrin from sprouted sorghum (Tokpohozin et al., 2016a). The germination of sorghum induces a tremendous increase of this cyanogenic glucoside, so that the beer's total cyanide content (11 mg/kg) exceeds the recommended dose (1 mg/kg); African sorghum beer producers can now apply the isolated *L. plantarum*, *L. paracasei* and *L. helveticus* to hydrolyze dhurrin.

As an additional benefit, applying these autochthonous African sorghum beer LAB for controlled bio-acidification of sorghum mash may help to improve the beer's organoleptic qualities, especially flavor. The latter furthermore profits from hydrolases cleaving the glucose moiety off of hop- and cereal-derived, aroma-active precursors: Kollmannsberger, Biendl, and Nitz (2006) observed an increase of odoriferous compounds such as linalool and β-damascenone from enzymatic hydrolysis of the respective odorless linalool and 3-hydroxy-β-damascenone β-D-glucosides in hop. Besides the hydrolytic activity, some β-D-glucosidases are also biosynthetic and may, by this reverse activity, generate aroma-active substances. Thus, Gunata, Vallier, Sapis, Baumes, and Bayonove (1994) observed that β-D-glucosidase isolated from *Candida molischiana* and *Aspergillus niger* as well as almond catalyze the synthesis of several primary monoterpenes such as geraniol, nerol and citronellol. In these same LAB of sub-cluster III (Fig. 2), hydrolysis of melibiose and raffinose is also an interesting property which may help to remove from beer oligosaccharides responsible for flatulence.

## 5. Conclusion

The polyphasic approach takes into account the isolated lactic acid bacteria phenotype (physiology, proteome), genotype (16S rDNA) and integrates it in a consensus for a reliable classification and identification. The lactic acid bacteria of the starter used during African sorghum beer production belong to the genus *Lactobacillus* including L. *plantarum*, *L. paracasei*, *L. brevis*, *L. fermentum* and *L. helveticus*. Some LAB belonging to the *L. helveticus* clade remain unidentified even after MALDI-TOF MS analysis; differentiating these unidentified LAB therefore requires a more suitable biomarker. Several LAB belonging to *L. plantarum*, *L. paracasei* and *L. helveticus* express both β-D-glucosidase and amylase as well as α-galactosidase. The selection of LAB with high expression of β-D-glucosidase and amylase under the conditions of sorghum brewing can help to significantly improve saccharification, dhurrin detoxification, and bio-flavoring.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.lwt.2017.02.004.

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# Assessment of malting and mash bio-acidification on the turnover of sorghum cyanogenic glucoside and protein hydrolysis improvement

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ABSTRACT

Sorghum germination induces the synthesis of a toxigenic hydrogen cyanide (HCN) precursor, dhurrin, and the normal sorghum mashing program is limited in dhurrin removal. Furthermore, the lack of proteases in sorghum malt and the formation of protein aggregate during cooking limit protein hydrolysis. To detoxify the wort, a new mashing program was designed to optimize dhurrin hydrolysis. To improve dhurrin and protein hydrolysis, three strains of lactic acid bacteria were applied for mash acidification. The results show that the degradation of the synthesized dhurrin occurs only in shoots and rootlets when the larger amount of this cyanogenic glucoside is still accumulated in malt seed tissues. Pre-heating the mash to 40 °C prior to decantation significantly reduces dhurrin content of the wort and improves the proteolytic activity. The mash bio-acidification with *L. paracasei* (ND-34) which removes dhurrin completely in brown wort considerably reduces it to 2.34 ± 0.08 mg/L in white wort and significantly improves protein hydrolysis.

## 1. Introduction

Sorghum is the fifth most produced cereal grain after barley crops in the world, and its use as raw material for traditional sour beers and industrial beer processing in several developing countries is now increasing. Sorghum grains have been indexed as a potential raw material to produce functional foods (Althwab, Carr, Weller, Dweikat, & Schlegel, 2015; Taylor, Schober, & Bean, 2006). It is a source of phenolic compounds, and some cultivars of *Sorghum bicolor* contain a high concentration of anthocyanins, especially luteolinidin and apigenidin (Awika, Rooney, & Waniska, 2004). As reported by Awika and Rooney (2004), available epidemiological evidence shows that consumption of sorghum-derived foods reduces the risk of certain types of cancer in humans compared to other cereals. Furthermore, malting improves the nutritional value of sorghum foods by decreasing anti-nutritional compounds and increasing the mineral availability. This process also promotes several enzymes, such as amylase, protease, limit dextrinase, phosphatase, and glucanase.

Yet whatever the importance of sorghum sprouting, the germination also induces an increase of sorghum cyanogenic glucoside, called dhurrin (Traoré, Mouquet, Icard-Vernière, Traoré, & Trèche, 2004). Dhurrin is the most studied cyanogenic glucoside, the metabolism of which has received increasing scientific explanation. To ensure pivotal plant functions such as sugar transport and natural chemical defense (Ganjewala, Kumar, & Ambika, 2010), substrates are channeled to the precursor amino acid L-tyrosine (Møller, 2010) by the formation of macromolecular enzyme complexes (Jensen, Anne, Hamann, Naur, & Lindberg, 2011) for dhurrin synthesis. The toxigenic hydrogen cyanide (HCN) may be generated by the enzyme actions in the intestinal microbiota (Carter, McLafferty, & Goldman, 1980), if dhurrin is not removed before food consumption. Several toxico-nutritional diseases correlate with dietary cyanide, for example, konzo or "tied leg," tropical ataxic neuropathy, goiter and cretinism (Banea et al., 2015). It has also been shown that cyanide may be oxidized to cyanate, which then reacts with ethanol to the multisite carcinogenic ethyl carbamate (EC) (Lachenmeier et al., 2010) responsible for DNA damage (Sakano, Oikawa, Hiraku, & Kawanishi, 2002). To detoxify sorghum wort and preclude the presence of the ultimate EC precursor (cyanate) during alcoholic fermentation, Tokpohozin, Fischer, Sacher, and Becker (2016) show the importance of dhurrin degradation by LAB-derived aryl-β-D-glucosidase prior to alcoholic fermentation. Contrary to cassava foods, no epidemiological study was carried out to know whether the consumption of sorghum malt derived foods results in any adverse physiological effects. Umoh, Maduagwu, and Amole (1986) observed that the long-term consumption of cyanide-containing food can compromise the health of the population, especially of those suffering from chronic protein malnutrition. Therefore, care should be taken with the sprouted sorghum because of the remaining bound cyanide. Additionally, the

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lack of hydrolases in sorghum malt is an important problem limiting saccharification (starch and protein hydrolysis). Several working groups demonstrate the lower enzyme content of sprouted sorghum compared to barley malt (Espinosa-Ramírez, Pérez-carrillo, & Serna-Saldívar, 2013). Taylor and Robbins (1993) estimate that sorghum malt β-amylase content compared to that of sprouted barley is lower by 25%. Furthermore, the formation of strong protein cross-links or protein structures during cooking reduces the efficiency of starch and protein conversion (Duodu, Taylor, Belton, & Hamaker, 2003). Lactic acid bacteria possess complex proteolytic enzymes, capable of hydrolyzing food proteins to peptides and amino acids (Mierau, Kunji, Venema, & Kok, 1997). This contributes to the texture, taste and aroma of fermented products (McSweeney & Sousa, 2000).

The main challenges of this study are the detoxification of sorghum wort and the improvement of protein hydrolysis. To achieve these objectives, we first evaluated the influence of malting two West-African sorghums on dhurrin synthesis and their degradation to *p*-hydroxybenzaldehyde. Further, we designed a new sorghum mashing program, including biological mash acidification with the selected β-glucosidase producer lactic acid bacteria strains to improve the detoxification and proteolytic activity.

## 2. Material and methods

### 2.1. Sorghum seeds collection and malting conditions

Two traditional landraces sorghums, white and brown called *Dobi Kpika* and *Dobi Swan*, respectively, were collected from a research and experimentation site of Alafiarou (Borgou/Benin). The two landraces sorghums' seeds were malted in 1 kg micro-malting systems. The steeping degree of the brown and white sorghums was adjusted to 42% and 43%, respectively, following a standard steeping procedure (MEBAK 1.5.3). When the steeping degree was reached, the germination was conducted in micro-climate chambers controlled at 27 °C for three days. The obtained green malt was kilned at 50 °C for one day.

### 2.2. Determination of sorghum malt amylase activity

Sorghum malt was milled and the amylase activities in brown and white sorghum flour extracts were measured using Ceralpha and Betamyl-kits (Megazyme, Ireland). Specifically, α-amylase was determined using the Ceralpha kit (K-Cera), while β-amylase was carried out using the Betamyl-kit (Megazyme, Ireland).

### 2.3. Lactic acid bacteria β-D-glucosidase activity

#### 2.3.1. Cell growth conditions

Lactic acid bacteria identified as *L. plantarum* (ND-32, ND-130) and *L. paracasei* (ND-34), which phenotypically express β-D-glucosidase (Tokpohozin, Waldenmaier, Fischer, & Becker, 2017), were selected to measure extracellular and cell-bound β-D-glucosidase activity. The preculture was prepared by inoculating 50 mL of Sharpe medium (10 g/L of peptone, 5 g/L of yeast extract, 2 g/L of potassium hydrogen phosphate, 2 g/L of diammonium hydrogen citrate, 5 g/L of sodium acetate, 0.1 g/L of magnesium sulfate, 0.005 g/L of manganese sulfate and 1 g/L of Tween 80) containing 10 g/L of cellobiose with one colony of the selected lactic acid bacteria strains. After overnight cell growth, 50 mL of the same medium were inoculated with 1 mL of the cell suspension and incubated at 28 °C for 20 h of cell growth. To follow the cell growth, 300 μL of the inoculated medium were distributed in a 96-wells microplate and the cell kinetics were followed for 24 h with Citation H5.

#### 2.3.2. β-glucosidase activity determination

To determine the β-D-glucosidase activity, 1 mL of cell suspension was centrifuged at 10,000 rpm for 1 min at 4 °C. The supernatant was separated and the cell pellets were twice washed with 1 mL of the corresponding citrate-phosphate (CP) buffer (pH 5, 5.6 and 6). The washed pellets were suspended in 0.5 mL of the appropriate buffer. For each reaction, 100, 50, and 25 μL of either supernatant or bacterial suspension were mixed with $10^{-3}$ mol/L β-NPG (4-Nitrophenyl β-D-glucoside; Sigma-Aldrich; Hamburg, Germany) in CP buffer to an adjusted volume of 500 μL to determine the cell-bound and extracellular β-D-glucosidase activities. The reaction mix was incubated for 30 min at different temperatures (20–70 °C). Samples containing cells were centrifuged at 10,000 rpm for 1 min at 4 °C. To stop the reaction after 30 min, 100 μL of the reaction mix was transferred to a microplate well containing 100 μL of 0.2 mol/L Tris-hydromethyl-aminomethane (pH = 12). An enzyme standard curve was prepared with β-glucosidase from almonds (Sigma-Aldrich, Germany) and the OD was measured at 405 nm using a Synergy H4 Hybrid Multi-Mode microplate reader. The cell pellets were collected and dried at 105 °C for 24 h, and the cell-bound β-D-glucosidase activity measured was normalized by dry cell weight.

### 2.4. Sorghum mashing process

#### 2.4.1. Estimation of sorghum gelatinization temperature

The brown and white sorghum malt starch gelatinization temperature was estimated following EBC method as described by Slack and Wainwright (1980).

#### 2.4.2. Sorghum malt mashing by decantation procedure

The brown and white sorghum malts were milled and sorghum mashing was conducted in 300 mL laboratory small scale with 71 g of sorghum grains. After milling, 200 mL of distilled water were first added and the mixture then homogenized. Seventy milliliters of the decanted supernatant were removed and the starch was heated to 55 °C for 30 min and then gelatinized at 80 °C. After gelatinization, the mash was cooled to 50 °C and the removed supernatant was added for protein hydrolysis before heating to 65 °C for starch hydrolysis. After saccharification, the mash temperature was increased to 80 °C for enzyme denaturation (Fig. 1a). As alternative treatment, the mixture malt-distilled water was pre-heated to 40 °C for 30 min prior to the decantation (Fig. 1b).

#### 2.4.3. Biological acidification of sorghum mash

The gelatinized sorghum mash was cooled to 28 °C and bio-acidified to pH 5.1 near the optimal hydrolase activities (β-D-glucosidase, amylases, proteases) with each of the three lactic acid bacteria strain. To obtain young pre-culture cells, lactic acid bacteria were propagated in minimum medium (Sharpe medium plus 10 g/L starch). After 36 h of incubation, the suspension was centrifuged and washed. The cooled sorghum mashes were inoculated to Log (CFU/ml) = 7 with a pre-culture of each young cell lactic acid bacteria. The mashes obtained with the different lactic acid bacteria strains were heated to 50 °C for protease activity and then to 65 °C for starch hydrolysis. After this saccharification, the mash temperature was increased to 80 °C for enzyme denaturation as described in Fig. 1c. The obtained mashes were centrifuged at 3000 rpm for 4 min and filtered, before a total of 100 mL of distilled water was sequentially added for sorghum mash draff washing.

### 2.5. Quantification of dhurrin and p-hydroxybenzaldehyde (pHB)

#### 2.5.1. Dhurrin and p-hydroxybenzaldehyde extraction

Dhurrin and its hydrolysate *p*-hydroxybenzaldehyde were extracted from various samples including unmalted brown and white sorghum grains, steeped sorghum grains, green sorghum malts as well as deculmed brown and white sorghum malts. Sorghum samples were ground to fine powder, and 10 mL of 80% methanol was first pre-heated to 60 °C and added to 0.5 g of sorghum flours. The tubes containing the mixture were heated to 60 °C in a water bath for 1 h and then

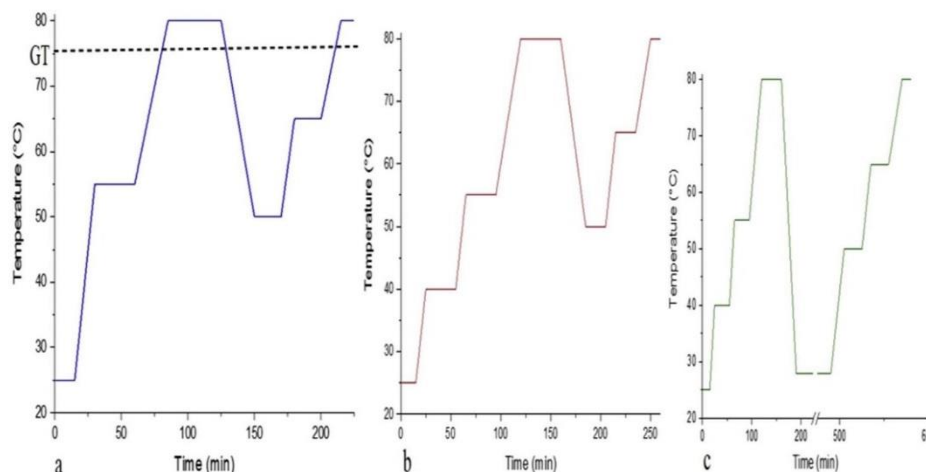


**Fig. 1.** Designed sorghum mashing programs including a standard sorghum mashing by decantation (a) and sorghum mashing pre-heating (b) or mash biological acidification with lactic acid bacteria (c) for dhurrin hydrolysis and saccharification improvement.

centrifuged at 3000 rpm for 4 min, and the supernatant was ultra-filtrated with a 0.45 μm filter.

#### 2.5.2. Quantification of dhurrin and its hydrolysate (pHB)

After extraction and filtration, bi-distilled water was added to the extract at a ratio of 1:1 (v/v) for the quantification of dhurrin and *p*-hydroxybenzaldehyde in different sorghum samples. The quantification was also carried out in sorghum worts by adding methanol to the wort at the same ratio. The pure dhurrin and *p*-hydroxybenzaldehyde were prepared by mixing a methanol-water solution at a ratio of 1:1 (v/v). The pure dhurrin and *p*-hydroxybenzaldehyde used in this study were provided by Sigma-Adrich, Germany. Quantification by high performance liquid chromatography was conducted using the modified method developed by Johansen, Holm, Erik, Christian, and Hansen (2007). The samples' water content was appreciated on the basis of the EBC method.

### 2.6. Determination of sorghum wort extract, free amino nitrogen

The Anton Paar alcolyser was used to determine the acidified and normal brown as well as white sorghum wort extract. The free amino nitrogen of the different samples was measured following the EBC ninhydrin method (Lie, 1973).

### 2.7. Determination of sorghum wort color and total polyphenol

The colors and the total polyphenol of different acidified and non-acidified brown and white sorghum worts were determined using the spectrophotometry EBC method.

### 2.8. Statistical analysis

All experiments were repeated at least three times. Data were analyzed using analysis of variance and means compared by Tukey test. The statistical level of significances was set at $P \leq .05$.

## 3. Results and discussion

### 3.1. Brown and white sorghum malts amylase content

The α-amylase activity of sorghum malts generated under the optimal malting conditions were 46 and 70 U/g in white and brown sorghum, whereas their β-amylase activities were 225 and 247 U/g, respectively. The brown sorghum contains more amylase compared to the white one. The particularity of these two West-African sorghums is their high β-amylase content which differs from that reported by Beta, Rooney, and Waniska (1995) and Hassani, Zarnkow, and Becker (2014). In their research, the maximum of β-amylase of South African sorghum grains correspond to 41 and 60 U/g. A West-African sorghum grain with a high β-amylase (110 U/g) and low α-amylase (less than 50 U/g) content was also reported by Agu and Palmer (1997). Similar to our work, Traoré et al. (2004) reported 56 U/g as the optimal α-amylase of red sorghum collected from Ouagadougou (West-Africa). This difference of sorghum amylase content may probably be related to the different malting conditions, the diversity of sorghum varieties with geographic locations, as well as the method used for these hydrolases quantification. Whatever the observed high β-amylase content in these West-African sorghum, it is still very lower compared to barley malt β-amylase (1024 U/g) content (Wang, Zhang, Chen, Shen, & Wu, 2003).

### 3.2. Influence of sorghum malting on dhurrin metabolism

The dhurrin content of unmalted brown and white sorghum corresponds to 34.8 ± 3.12 and 44.13 ± 0.00 μg/g, respectively (Fig. 2). The sprouting of sorghum grains induced a tremendous increase of sorghum cyanogenic glucoside (dhurrin). The dhurrin content rises during steeping and germination and reaches 318.27 ± 10.33 and 222.96 ± 0.68 μg/g in brown and white sorghums, respectively. The brown malt dhurrin content was higher compared to the white sorghum malt, showing that in addition to the germination time, the dhurrin content of the sprouted grains depends on sorghum variety. During the synthesis, dhurrin is partially converted to *p*-hydroxybenzaldehyde, showing that sorghum sprouting induces dhurrinase (β-D-glucosidase) synthesis (Fig. 3). Our results show an increase of *p*-hydroxybenzaldehyde during steeping and germination, reaching 331.7 ± 5.63 and 467.3 ± 3.03 μg/g in brown and white green sorghum malts, respectively. Regardless of dhurrin and dhurrinase synthesis, the dhurrin degradation during malting seems to be located in the sprouted sorghum shoots and rootlets, while a large amount is accumulated in sprouted seed tissues. Indeed, *p*-hydroxybenzaldehyde was quasi absent in malts when shoots and rootlets are removed (Fig. 2). This deculming (shoots and rootlets removal) also reduced the

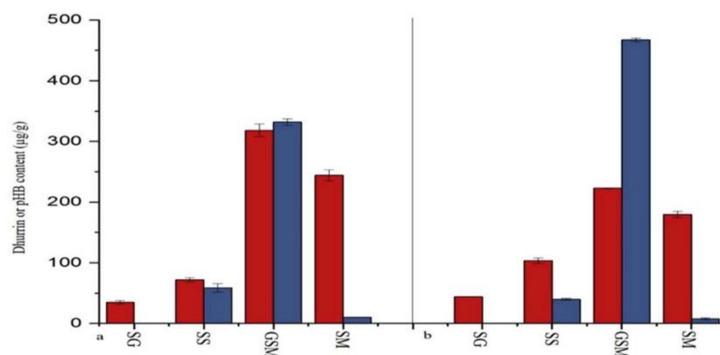


**Fig. 2.** Graphs showing dhurrin and its hydrolysate *p*-hydrobenzaldehyde (pHB) in brown (a) and white (b) unmalted sorghum grains (SG), steeped sorghum grains (SS), green sorghum malt (GSM) and kilned sorghum malts (SM). Red and blue colors were used for dhurrin and *p*-hydroxybenzaldehyde (pHB), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

remaining accumulated dhurrin by 20% and 24% in white and brown cultivars, respectively. Even though deculming decreases dhurrin content in malt, the accumulated dhurrin is still high with 244.20 ± 9.09 and 180 ± 5.17 µg/g in brown and white sorghum malts, respectively. Yet despite the malt's high dhurrin content, detoxification of germinated sorghum-derived foods has not received specific attention.

### 3.3. Standardization of sorghum mashing program for significant sorghum wort detoxification and protein hydrolysis

The gelatinization temperatures of the brown and white sorghum malts are 74.5 and 75.0 °C, respectively. Due to these high temperatures, sorghum malts are mashed by decantation where the enzymatic supernatant is removed and the starch gelatinizes at 80 °C. Sorghum mashing, because of the occurring heat treatment and the presence of dhurrinase, is a potential process to reduce sorghum wort dhurrin. After the application of this normal decantation procedure, the dhurrin contents of brown and white sorghum worts'were 4.38 ± 0.64 and 11.045 ± 0.417 mg/L, respectively. These results clearly show that the application of the normal decantation procedure is limited in terms of dhurrin hydrolysis. To significantly reduce sorghum wort dhurrin content, the mixture of milled sorghum malt and water was pre-heated to 40 °C prior to decantation for dhurrin hydrolysis and a good enzyme extraction. Pre-heating to 40 °C for 30 min before decanting the enzymatic supernatant induced significant reduction ($p < .05$) of the brown and white sorghum wort dhurrin contents to 1.35 ± 0.03 and 5.20 ± 0.65 mg/L, respectively (Fig. 4). To further improve dhurrin degradation during the mashing process for significant detoxification, lactic acid bacteria expressing β-D-glucosidase have been applied for mash acidification. Conditions for optimal β-D-glucosidase activity as well as those of sorghum malt hydrolase (amylase, limit dextrinase, protease) have be considered during the acidification for significant dhurrin, starch, and protein hydrolysis. Our results show that the optimal cell-associated β-D-glucosidase activities were 113.51, 77.94 and 41.67 U/g of dry cell weight for *L. paracasei* (ND-34), *L. plantarum* (ND-32) and *L. plantarum* (ND-130), respectively (Fig. 5a). These optimal activities were obtained at 40 °C and pH = 5.6. The content of cell biomass obtained with *L. plantarum* (ND-32, ND-130) is higher than that of *L. paracasei* (ND-34) and already reaches the stationary phase after 20 h of cell growth. Contrary to the strain ND-34, the growth kinetics of ND-32 and ND-130 are characterized by a long latence (8 h) and exponential phase (Fig. 5b). Even though it is evident that ND-34 cell growth could be improved by changing growth conditions, the relationships between cell growth and β-D-glucosidase synthesis have to

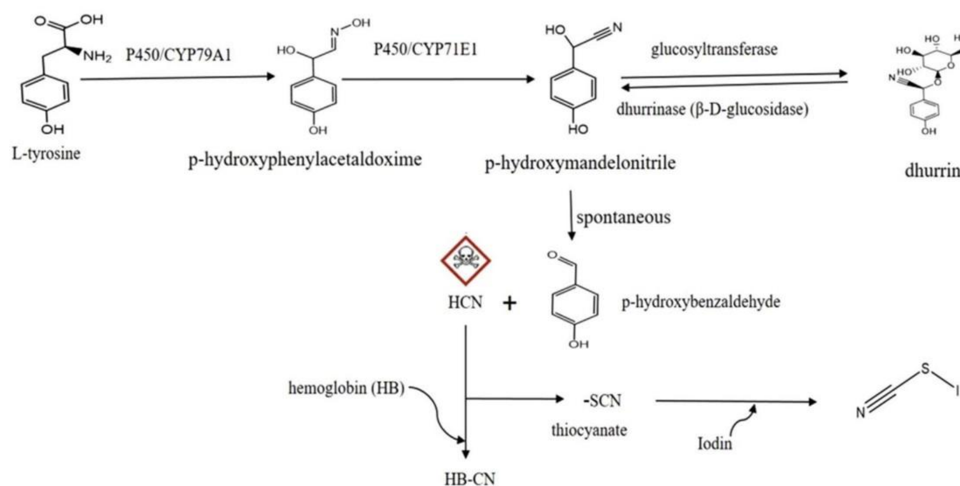


**Fig. 3.** The mechanism of dhurrin (*p*-hydroxymandelonitrile-β-D-glucopyranoside) biosynthesis during sorghum germination from the precursor amino acid (L-tyrosine) and its conversion to *p*-hydroxybenzaldehyde via hydroxymandelonitrile by β-D-glucosidase (dhurrinase). Cyanide can also be converted to thiocyanate and then acts as an anti-nutritional compound by complexing iodine, causing goiter and cretinism if it is not removed before food consumption.

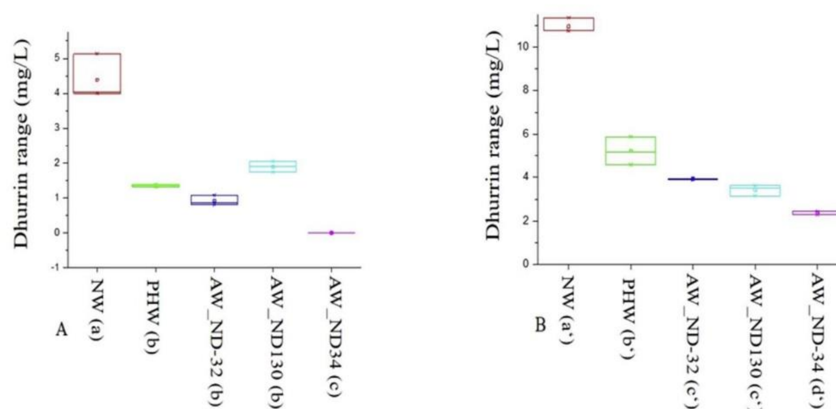


**Fig. 4.** Graphs showing the influence of sorghum mash pre-heating and biological acidification with *L. plantarum* (ND-32), *L. plantarum* (ND-130) and *L. paracasei* (ND-34) on dhurrin content in brown sorghum wort (A) and white sorghum wort (B). The difference in the letters (a - c or a'- d') in bracket indicate a significant difference ($p < .05$). NW = Normal wort; PHW = Pre-heated wort; AW_ND-32 = Acidified wort with the strain ND-32; AW_ND-34 = Acidified wort with the strain ND-34; AW_ND-130 = Acidified wort with the strain ND-130. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

be first understood. The strain ND-34, isolated as *L. paracasei*, is a high β-D-glucosidase producer. This high activity was also present at various temperatures ranging from 30 to 50 °C and pH = 5 to 6, showing that the acidification of sorghum mashes to pH = 5 can not inhibit the activity of β-D-glucosidase. This pH is also near the optimum of sorghum malt hydrolases. During saccharification, the acidified sorghum mash could also be heated at 50 °C for both dhurrin and protein hydrolysis. However, because of its structure, the hydrolysis of dhurrin requires aryl-β-D-glucosidase. Tokpohozin et al. (2017) demonstrated that the selected lactic acid bacteria strains were able to ferment aryl-β-D-glucosidic compounds such as amygdalin, salicin, and esculin. The application of *L. paracasei* (ND-34) for mash acidification completely removed dhurrin in the brown sorghum and significantly reduced it ($p < .05$) in white sorghum wort to 2.34 ± 0.08 mg/L (Fig. 4). This concentration theoretically corresponds to 0.2 mg/L of HCN if dhurrin is completely hydrolyzed. This estimated cyanide concentration is much lower compared to the maximum dose (1 mg/L) recommended in alcoholic beverages by the New Zealand Food Safety Authority and in Switzerland. Considering the efficiency of sorghum wort detoxification by significant dhurrin removal, *Lactobacillus paracasei* ND-34 is the performant lactic acid bacteria strain. The significant dhurrin reduction in white sorghum wort and its complete removal in brown sorghum wort by pre-heating to 40 °C prior to decantation followed by biological acidification is related to the dhurrinase and β-D-glucosidase contained in sorghum malt and produced by lactic acid bacteria, respectively. Outside sorghum, cassava-derived foods have also been negatively indexed because of cassava root linamarin and lotaustralin (cyanogenic glucosides) content. The use of *L. paracasei* ND-34 in the place of uncontrolled spontaneous acidification occurring during cassava food processing could help to cleave the conjugate glucose of cassava (*Manihot esculenta*) linamarin and lotaustralin. After enzymatic hydrolysis of these sorghum and cassava cyanogenic glucosides, the labile

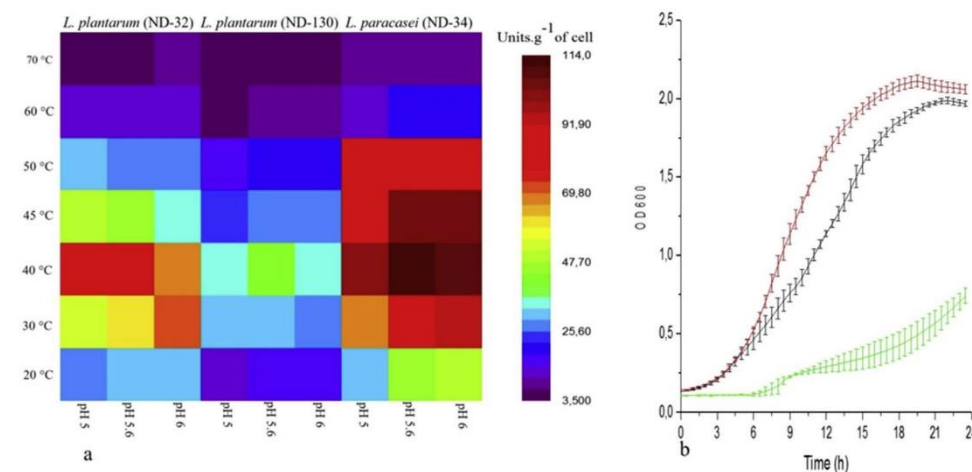


**Fig. 5.** a)Heatmap showing the influence of temperature and pH on the activity of β-D-glucosidase produced by *Lactobacillus plantarum* (ND-32, ND-130) and *Lactobacillus paracasei* (ND-34) isolated from Beninese African sorghum beer (*Tchoukoutou*) starter towards *p*-nitrophenyl-β-D-glucoside. The lactic acid bacteria isolated as *L. paracasei* (ND-34) produce a high activity of β-D-glucosidase stable at various temperatures and pH. The graph b shows the kinetics of lactic acid bacteria cell growth. The red, black and green color were used for the ND-32, ND-130 and ND-34, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

**Table 1**
Sorghum worts characteristics: extract (degree Plato), free amino nitrogen (FAN), *p*-hydroxybenzaldehyde (pHB) total polyphenol, color and pH.

| Different sorghum worts | Extract (°P) | FAN (mg/L) | pHB (mg/L) | Color | Polyphenol (mg/L) | pH |
|---|---|---|---|---|---|---|
| **Brown sorghum wort** | | | | | | |
| Normal wort | 17.1 ± 0.3 | 222.4 ± 2.5a | 19.84 ± 2.20 | 11.53 ± 0.57 | 46.74 ± 8.11 | 5.80 ± 0.09 |
| Pre-heated wort | 16.7 ± 1 | 236.6 ± 4.5b | 19.81 ± 2.54 | 11.08 ± 0.32 | 46.74 ± 1.72 | 5.68 ± 0.04 |
| Acidified wort (ND-34) | 16.5 ± 1.1 | 273.1 ± 2.7c | 22.03 ± 0.21 | 11.25 ± 1.15 | 76.26 ± 5.93 | 5.11 ± 0.007 |
| **White sorghum wort** | | | | | | |
| Normal wort | 16.5 ± 0,2 | 226.6 ± 9.4a‘ | 8.34 ± 0.94 | 8.28 ± 0.31 | 35.26 ± 1.25 | 5.71 ± 0,03 |
| Pre-heated wort | 17.6 ± 0.4 | 284.0 ± 13b‘ | 7.69 ± 0.98 | 8.50 ± 0.16 | 27.88 ± 5.73 | 5.54 ± 0.09 |
| Acidified wort (ND-34) | 17.5 ± 0.5 | 305.3 ± 1.9c‘ | 2.88 ± 0.17 | 6.48 ± 0.98 | 16.4 ± 2.32 | 5.15 ± 0.01 |

The wort free amino nitrogen value affected with different letters a, b, c or a‘, b‘and c‘ indicate a significant different ($p < .05$). The letters a, b, c and a’, b’, c’ were used to differentiate the two tables.

cyanohydrin can spontaneously be converted and the resulting toxigenic volatile HCN can be removed by the processing heat treatments.

Yet even if the sorghum safety problem because of dhurrin has been solved, the wort extract, especially the free amino nitrogen content, is important as well since it affects yeast fermentability. After the application of the normal decantation procedure, the free amino nitrogen contents of brown and white worts were 222.46 ± 2.53 and 226.62 ± 9.48 mg/L, respectively (Table 1). Pre-heating sorghum mashes to 40 °C for 30 min increased free amino nitrogen content. The pre-heating, thus, helps for malt enzyme extraction for efficient protein hydrolysis during saccharification. As reported by Duodu et al. (2003), several exogenous and endogenous factors limit protein digestibility in sorghum. Indeed, indigestible tannin-protein crosslink (Chibber, Mertz, & Axtell, 1980), phytate-protein complexes (Elkhalil, Tinay, Mohamed, & Elsheikh, 2001) or polypeptide-carbohydrate linkage (Fry, 1982) forming during sorghum mashing limit protein hydrolysis. Proteolysis is important for lactic acid bacteria, which have limited abilities for amino acid synthesis. In addition to dhurrin hydrolysis, the biological mash acidification with *L. plantarum* and *L. paracasei* significantly improved protein hydrolysis ($p < .05$). The pre-heating at 40 °C following by mash acidification with *L. paracasei* ND-34 increased brown and white sorghum protein hydrolysis up to 23% and 34%, respectively, after only 5 h of mash acidification. Other alternatives were also proposed by researchers to improve sorghum wort free amino nitrogen content. Taylor and Boyd (1986) added proteolytic enzymes to the mash to increase sorghum wort free amino nitrogen content. Heredia-Olea, Cortés-Ceballos, and Serna-Saldívar (2017) applied *Aspergillus orizae* during sorghum malting and observed an increase in the wort free amino nitrogen by 24%.

The significant increase of sorghum wort free amino nitrogen characterized by wort amino acids and peptides undoubtedly increases the yeast fermentability, thereby improving the beer flavor. Additional to amino acids, phenolic aldehydes have also been indexed to contribute to the flavor of fermented food. Because of the bioactive properties of several aglycones resulting from sequestrated glucosidic compounds, researchers now apply β-D-glucosidase to improve wine flavor (Wang, Zhang, Li, & Xu, 2013). The aglycone *p*-hydroxybenzaldehyde (pHB) resulting from dhurrin hydrolysis is abundant in the brown sorghum worts (Table 1) because of extensive dhurrin hydrolysis during mashing. The dhurrin hydrolysate *p*-hydroxybenzaldehyde is unstable. Bvochora, Danner, Miyafuji, Braun, and Zvauya (2005) observed its decrease during alcoholic fermentation, but an increase of *p*-hydroxybenzoic acid and *p*-hydroxybenzoyl alcohol was also observed during opaque sorghum beer production. Besides these monophenolic compounds, sorghum worts, especially the brown sorghum, contain a high quantity of polyphenol (Table 1). Some polyphenols have been negatively indexed to inhibit the yeast growth and enzyme activities during fermentation (Daiber, 1975). In spit of such negative effect, the health benefit and the importance of polyphenols regarding beer stability is very well known. The selection of performant yeast from African sorghum beer starter could help to avoid this potential phenolytic negative effect. Tokpohozin, Lauterbach et al. (2016) isolated from African sorghum beer starter several *S. cerevisiae* with interesting fermentative profiles. The application of these authoctonous sorghum beer starter yeasts could help to avoid the probable negative effect caused by some polyphenols.

## 4. Conclusion

A high amount of dhurrin is accumulated in sorghum seed tissues during germination. According to sorghum malt dhurrin content, an appropriate mashing program for its significant reduction should be followed. Unfortunately, the standard sorghum mashing program is limited in sorghum wort detoxification with the presence of dhurrin in sorghum wort. Our designed new mashing program characterized by sorghum mash pre-heating and mash bio-acidification should be followed during sorghum beer processing to significantly detoxify sorghum wort and improve protein hydrolysis. *L. paracasei* ND-34, the authoctoneous African sorghum beer starter lactic acid bacteria, can also be applied to detoxify other cyanide-containing foods to limit food intoxication, which still remains a current problem in several developing countries.

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## 3. Discussion

The production of African sorghum beer still occurs on the basis of ancestral knowledge and several safety problems compromise this special African sorghum beer's marketability. The re-engineering of the processing techniques is strongly necessary to improve the traditional beer's safety, nutritional value, stability and organoleptic quality. Isolation, characterization and selection of the performant yeasts and lactic acid bacteria with interesting properties from the traditional starter used to initiate fermentation were our first challenge. Yeasts and lactic acid bacteria were therefore characterized using different biomarkers: phenotype (morphology, physiology), cell proteome (MALDI-TOF) and sequence comparison. The sugar spectrum helped to separate the *Saccharomyces* yeast from the non-*Saccharomyces* yeasts. After applying these phenotypical tests, the different lactic acid bacteria were also very distinct by their metabolic pathways: obligate homofermentative, obligate hetero-fermentative and facultative hetero-fermentative lactic acid bacteria. However, the phenotypical characteristics (metabolic profiles) are limited in meaningfully discriminating yeasts and lactic acid bacteria for a reliable identification. The results are redundant, and it is a limiting factor that sugar spectra of strains belonging to the same species *L. helveticus* are highly diverse. Moreover, Hammes & Vogel (1995) observed that several taxa classified by phenotypical characteristics were phylogenetically not coherent. Some obligate homo-fermentative lactic acid bacteria (NA-172, DJ-224) ferment ribose, showing a typical hetero-fermentative lactic acid bacteria characteristic. Biddle & Warner (1993) also found that some strains of *Lactobacillus acidophilus*, normally considered to be strictly homofermentative, are able to ferment ribose. Therefore, the sugar spectrum alone does not allow to meaningfully distinguish and thus reliably identify lactic acid bacteria, since some obligate homofermentative lactic acid bacteria ferment pentose and the metabolic profiles of strains of the same species can be very different. Yeast and lactic acid bacteria identification based only on phenotype therefore led to inconclusive results with mis-identification. To improve the result of yeast and lactic acid bacteria identification, Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) was applied for proteome analysis and specific gene sequences were compared for better differentiation. The application of MALDI-TOF MS helps to group the isolated yeasts in different clusters with a successful

identification of *Saccharomyces cerevisiae* and *Pichia krudriavzevii* to species level and *Debaryomyces* to genus level. Comparing the D1/D2 of the 26S rDNA sequence does not enhance the result obtained with yeast identification on the basis of the protein patterns. The limits of differentiating yeast belonging to *D. hansenii* complex due to the high similarity among their D1/D2 domain of the 26S rRNA gene was also observed by Groenewald, Daniel, Robert, Poot, & Smith, (2008) and Kurtzman & Robnett, (1997). Sequence analysis of the D1/D2 domain also indicated that *C. ethanolica* and *P. deserticola* are closely related, and they were both identified with a similarity of 100 and 99%, respectively. The 5.8S-ITS region seems to be more discriminative and, contrary to the D1/D2 domain of 26 LSU, allows the differentiation of *C. ethanolica* from *P. deserticola*. However, the RFLP analysis of the 5.8S-ITS sequence is limited and does not allow the differentiation of some closely related *Debaryomyces* yeasts. Martorell, Fernández-Espinar, & Querol (2005) show the limits of RFLP analysis of the 5.8S-ITS gene to differentiate to species level several *Debaryomyces* yeast such as *D. castellii*, *D. courdertii*, *D. nepalensis*, *D. polymorphus*, *D. pseudopolymorphus*, *D. robertsiae*, *D. udenii* and *D. vanrifiae*. The work of Wrent, Rivas, Gil de Prado, Peinado, & de Silóniz (2015) made a similar observation pertaining to the *D. hansenii* complex such as *D. hansenii*, *D. fabryi*, and *D. subglobosus*.

Concerning lactic acid bacteria, we successfully identified *L. fermentum*, *L. brevis* and *L. plantarum* by molecular characterization via comparison of the 16S rDNA sequence. However, differentiating *L. paracasei* from the closely related *L. casei* and *L. helveticus* from thermophile obligate homofermentative lactic acid bacteria such as *L. gallinarum*, *L. acidophilus*, *L. amylovorus*, *L. crispatus*, *L. ultunensus* is not possible with this 16S ribosomal encoding gene. In our work, several isolated lactic acid bacteria were very close to *L. helveticus* according to their 16S rRNA gene, but Holzapfel & Wood (2014) point out that since many lactic acid bacteria share more than 97% of their 16S rRNA gene sequences, no similarity threshold value to distinguish between species has been assigned. In contrast to the comparison of metabolic profiles (sugar spectrum) and 16S rRNA-encoding genes, the proteome obtained from the whole cell by MALDI-TOF MS analysis appears to be a suitable biomarker enabling discrimination and identification of such closely LAB. Based on this chemotaxonomy (LAB whole proteome), we successfully identified *L. fermentum*, *L. Brevis*, *L. plantarum* as well as *L. paracasei*

and *L. helveticus*. An improvement of the Bruker database used helped to later identify *L. acidophilus*. It is therefore evident that MALDI-TOF MS is a good method for accurate, high-throughput, and inexpensive identification of yeasts and lactic acid bacteria. However, the comparison of the DNA sequences of the isolated yeasts and lactic acid bacteria to an existing entry in the National Center for Biotechnology Information (NCBI) is important as well and helps to confirm the result generated by yeast and lactic acid bacteria identification based on their proteome. The polyphasic characterization using the microorganism's metabolic profile, proteome and specific DNA sequences as biomarkers is therefore a good approach which brings into consensus the different results and helps to solve the specific limit of each method in order to reliably discriminate, classify and identify yeasts and lactic acid bacteria. Based on this polyphasic characterization, we can assert that the microbiota of the starter used for *Tchoukoutou* production is diverse with *S. cerevisiae* the dominant yeast species followed by *P. kudriavzevii*, *D. hansenii* and *C. ethanolica*. These *Saccharomyces* and non-*Saccharomyces* yeasts cooperate with different species of lactic acid bacteria belonging to the *Lactobacillus* genus such as *L. fermentum*, *L. brevis*, *L. plantarum*, *L. paracasei* and several thermophile homofermentative lactic acid bacteria strains belonging to *L. helveticus* and *L. acidophilus* species. That non-*Saccharomyces* are dominated by the *Saccharomyces* yeasts shows that *S. cerevisiae* yeast strains are probably more flexible to the stress conditions, which allows these potent yeasts to better adapt to the continuously changing environment during fermentation. Muyanja et al., (2003) also show that *Lactobacillus* compared to other lactic acid bacteria tolerate more acidic conditions. The substrate composition and the metabolites resulting from fermentation are therefore intrinsic factors which determine the microbiota of traditional fermented foods.

The isolated yeasts identified as *S. cerevisiae* have different metabolic profiles showing the diversity at strain level. Several strains of this yeast species are able to ferment glucose, maltose, maltotriose, sucrose and fructose. Due to their diversity, these *S. cerevisiae* could be domesticated for different types of beer production. After *S. cerevisiae* yeasts, the high presence of *P. kudriavzevii* in the traditional starter used for *Tchoukoutou* production has also been observed. Greppi et al. (2015) isolated *P. kudriavzevii* (known as *Candida krusei*) from a traditional Beninese non-alcoholic beverage and observed that this yeast species expresses phytase. Phytate is the anti-

nutritional compound contained in sorghum and its hydrolysis by the occurring phytase producer yeast *P. kudriavzevii* will enhance mineral availability. Annan, Poll, Sefa-Dedeh, Plahar, & Jakobsen (2003) also used this non-conventional yeast *P. kudriavzevii* in combination with *S. cerevisiae* to enhance the flavor and taste of *koko*, a West-African porridge. Regarding possible probiotic effects, consumers and producers of African sorghum beer claim that the beer offers a health benefit and helps to cure diarrhea. Indeed, the probiotic effect of *P. kudriavzevii* isolated from Beninese and Nigerian fermented non-alcoholic beverages has been proven by Greppi et al., (2017) and Ogunremi, Sanni, & Agrawal, (2015). Among the occurring lactic acid bacteria, *Lactobacillus plantarum*, *L. paracasei*, *L. helveticus* and *L. acidophilus* have been positively indexed for several biotechnological properties such as probiotic (Klein et al., 1998), bacteriocin (Klaenhammer, 1993; Leroy & De Vuyst, 2004), and phytase production (De Angelis et al., 2003; Sreeramulu, Srinivasa, Nand, & Joseph, 1996) as well as the capacity of synthetizing several oligosaccharides of nutritional importance. Additional to these properties, our results show that the lactic acid bacteria isolated as *L. plantarum* (ND-32, ND-130) and *L. paracasei* (ND-34) exhibit interesting enzyme activities such as amylase, protease and especially β-D-glucosidase. The latter is highly expressed by *L. paracasei* (ND-34) compared to other isolated strains. However the presence of these β-D-glucosidase producers' lactic acid bacteria in the traditional starter use for African sorghum beer production and the heat treatment occurring during sorghum malt mashing, it has been shown the presence of dhurrin (sorghum cyanogenic glucoside) in traditional sorghum beer (Ikediobi, 1988). To contribute of solving this safety problem, the sorghum malting conditions were optimized and the influence of the optimal malting conditions on dhurrin synthesis were appreciated. The variation of malting conditions strongly influenced the malt hydrolases (α-amylase and β-amylase) content (Fig. 9).

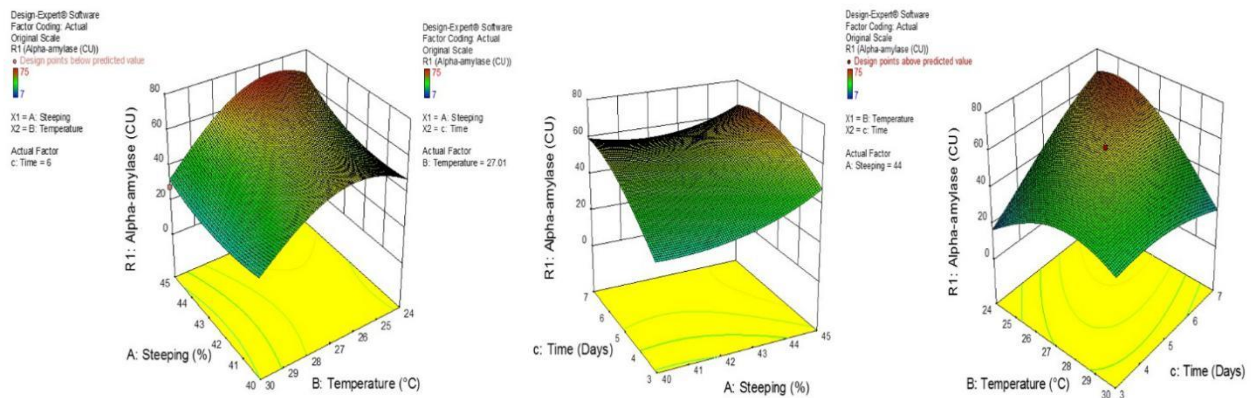


**Figure 9:** Response surface methodology (RSM) showing the influence of malting conditions on white sorghum malt α-amylase content.

Brown and white sorghum steeping to 42 % and 43 %, respectively, followed by germination for a total of 5 days at 27 °C are the appropriate malting conditions promoting high synthesis of β-amylase. At the optimal malting conditions, the β-amylase activities of the used white and brown sorghum malts are 240 U/g and 261 U/g, respectively (Table 2). The particularity of these two West-African sorghum are their high β-amylase content which differe from that reported by Beta, Rooney, & Waniska, (1995) and Hassani et al., (2013) who reported the maximum of β-amylase corresponding to 41 U/g and 60 U/g, respectively, in South African sorghum grains. This observation is likely due to the difference agroecological zones, the diversity of sorghum varieties and the difference malting conditions. Whatever this high β-amylase, it is still very lower compared to β-amylase activities (1024 U/g) of barley malt (Wrent et al., 2015). In addition to enzymes synthesis during sorghum malting, the germination of sorghum also induced cyanogenic glucoside (dhurrin) synthesis. Cyanogenic glucosides are however secondary metabolite. This early dhurrin synthesis have been explained by important functions attributed to this secondary metabolite: Sugar and nitrogen transport, organization of chemical plant defense (Ganjewala, Kumar & Ambika, 2010). Concomitant to our result, Ahmed et al. (1996); Traoré et al. (2004) and Uvere et al. (2000) also observed dhurrin biosynthesis during sorghum sprouting. After biosynthesis, dhurrin is unequally distributed among the sprouted sorghum organs rootlets, shoot and seeds, with highest levels observed in shoots and rootlets.

**Table 2:** Influence of different malting conditions on amylase activities of two different West-African landraces of sorghum seeds.

| Sorghum malting conditions | | | α-amylase U/g (CU) | | β-amylase U/g (BU) | |
|---|---|---|---|---|---|---|
| Steeping (%) | Temperature (°C) | Times (days) | Brown | White | Brown | White |
| 40 | 24 | 3 | 52 | 7 | 123 | 69 |
| 45 | 24 | 3 | 46 | 26 | 118 | 79 |
| 42 | 24 | 5 | 77 | 35 | 156 | 80 |
| 40 | 24 | 7 | 102 | 75 | 129 | 91 |
| 45 | 24 | 7 | 96 | 61 | 177 | 84 |
| 42 | 27 | 3 | 61 | 31 | 130 | 168 |
| 42 | 27 | 4 | 24 | 28 | 108 | 120 |
| 41 | 27 | 5 | 81 | 51 | 188 | 97 |
| 42 | 27 | 5 | 74 | 62 | 261 | 191 |
| 43 | 27 | 5 | 85 | 53 | 146 | 93 |
| 44 | 27 | 5 | 84 | 54 | 125 | 147 |
| 41 | 27 | 6 | 94 | 49 | 154 | 240 |
| 40 | 30 | 3 | 56 | 29 | 114 | 121 |
| 41 | 30 | 5 | 76 | 31 | 80 | 121 |
| 45 | 30 | 6 | 76 | 28 | 177 | 179 |
| 40 | 30 | 7 | 62 | 23 | 123 | 116 |
| 45 | 30 | 7 | 81 | 28 | 155 | 59 |

Based on this observation, deculming (shoots and rootlets removal) should be adopted by sorghum brewers to reduce the beer dhurrin content. Furthermore, sorghum malt rootlets removal is beneficial for the overall brewing process and help limiting the development of mould during storage and the presence of undigested high-molecular-weight proteins in wort. Sorghum germination also induces dhurrinase (β-D-glucosidase) synthesis and the degradation of dhurrin to it hydrolysate p-hydroxybenzaldehyde (pHB) was observed. The high amount of pHB in sorghum shoots and rootlets and its quasi-absence in sorghum malt shows that the degradation of dhurrin by dhurrinase during sorghum sprouting is located in shoots and rootlet when high amount of undigested dhurrin (180-244 µg/g) is accumulated in sorghum malt seeds tissues. Yet despite the malt‘s high dhurrin content, detoxification of sorghum-derived foods has not received specific attention. Sorghum mashing, because of the occurring heat treatments and the presence of dhurrinase, is a potential process to reduce sorghum wort dhurrin. Sorghum malts were mashed using decantation procedure, and the brown and white sorghum worts‘ dhurrin contents were 4.38 ± 0.64

mg/L and 11.045 ± 0.417 mg/L, respectively. These results clearly show that the application of the normal decantation procedure is limited in terms of dhurrin hydrolysis. Since the β-D-glucosidase is also present in malted sorghum, it is therefore important to optimize the conditions for significant dhurrin hydrolysis. Sorghum mash pre-heating to 40 °C for 30 min before decanting the enzymatic supernatant induces significant reduction ($p < 0.05$) of the brown and white sorghum wort dhurrin content to 1.35 ± 0.03 mg/L and 5.20 ± 0.65 mg/L, respectively. Since Umoh, Maduagwu, & Amole (1986) observed that the long-term consumption of cyanide-containing food could compromise the health of the population, especially of those suffering from chronic protein malnutrition; care should be taken with the sorghum wort because of the remaining dhurrin content. The application of β-D-glucosidase during fermentation is a good alternative to improve dhurrin hydrolysis. However, it must be ensured that no spurious reaction would occur because cyanate resulting from dhurrin hydrolysis can react with ethanol for ethyl carbamate formation. Furthermore, no *Saccharomyces* yeasts isolated from the traditional starter expressed β-D-glucosidase. Dhurrin hydrolysis during alcoholic fermentation seems not to be a good option. To further improve dhurrin degradation during the mashing process for significant sorghum wort detoxification, lactic acid bacteria expressing β-D-glucosidase have been applied for mash acidification. The application of *L. paracasei* (ND-34) completely removes dhurrin in the brown sorghum wort and significantly reduces it ($p < 0.05$) in white sorghum wort to 2.34 ± 0.08 mg/L. Considering the efficiency of sorghum wort detoxification by significant dhurrin removal, *Lactobacillus paracasei* ND-34 is the performant lactic acid bacteria strain. The significant dhurrin reduction in white sorghum wort and its complete removal in brown sorghum wort by pre-heating to 40 °C prior to decantation followed by biological acidification is due to dhurrinase and β-D-glucosidase contained in sorghum malt and produced by lactic acid bacteria, respectively. The catalytic mechanism of β-glucosidases has been reported by Vinther et al. (2008) and has been shown to involve two-conserved glutamic acid residues which serve as catalytic nucleophile and a general acid/base catalyst. At the initial step in catalysis, the nucleophile performs a nucleophilic attack at the anomeric carbon ($c_1$ of glucose), which results in formation of glucose-enzyme intermediate. In this process, aglucone departure is facilitated by protonation of the glucosidic by the acid catalysts. During this second catalytic step (deglucosylation), a water molecule is activated by the catalytic base to serve as a nucleophile for hydrolysis of the glucosidic bond and

release of the glucose (Davies & Henrissat, 1995). In additional to dhurrin hydrolysis for sorghum wort detoxification, β-D-glucosidase has biotechnological and industrial importance where the aglucone resulting from cereal and hops glucosidic hydrolysis could act as aroma precursor for beer bioflavoring. The monophenolic compound, p-hydroxybenzaldehyde, resulting from dhurrin hydrolysis is present in sorghum wort after enzymatic dhurrin hydrolysis. The fate of this aglucone during fermentation and the impact of the derived compounds on the beer flavor and taste still need scientific explanation. Even if the influence of the aglucone resulting from glucosidic compounds hydrolysis on beer sensory quality is not known, it is very well known that amino acids are precursors for several relevant aroma-active higher alcohols and esters (Bolat et al. 2013; Felix Ehrlich, 1907; Hazelwood et al. 2008; Kispal et al. 1996; Pires et al. 2014; Sentheshanmuganathan, 1960), and the expression and regulation of different genes encoding for enzymes catalyzing their synthesis are influenced by amino acids (Hazelwood et al. 2008b; Procopio et al. 2015). Unfortunately, protein digestion during saccharification is limited when mashing with sorghum malt (Duodu et al. 2003). The low malt protease content combined with intermolecular disulfide bonds (Ng'Andwe & Hall, 2008) in the poly- and oligomers of kafirin – prolamins that comprise 93% of sorghum protein (Espinosa-ramírez & Serna-saldívar, 2016); indigestible tannin-protein crosslink (Chibber & Mertz, 1980); phytate-protein complexes (Elkhalil et al. 2001); or polypeptide-carbohydrate linkage (Fry, 1982) that forms during mashing limits protein hydrolysis, thereby compromising the wort's amino acid content. In addition to dhurrin hydrolysis, the pre-heating of sorghum mash prior to decantation increased the worts FAN content. This increase of the FAN after mash pre-heating shows that pre-heating helps improve enzyme extraction to avoid their degradation during gelatinization, and their addition during saccharification helps ameliorate the process, especially protein digestion. After sorghum mash pre-heating followed by bio-acidification help increase the wort FAN and branched amino acids (valine, leucine, isoleucine) by up to 27 % and 50 %, respectively (Table 3).

**Table 3:** Influence of sorghum mash pre-heating and biological acidification on wort FAN and branched amino acid composition

| Amino acids | Standard wort | Pre-heated wort | Acidified (ND-32) | Acidified (ND-34) | Acidified (ND-130) |
|---|---|---|---|---|---|
| **Brown sorghum wort** | | | | | |
| **FAN** | 195.14 ± 5.62[a] | 214.33 ± 4.04[b] | 237.45 ± 2.52c | 248.24 ± 2.04c | 231.91 ± 4.46c |
| **Val** | 79.07 ± 0.42a | 101.47 ± 5.15b | 124.18 ± 0.99c | 131.41 ± 2.70c | 128.97 ± 2.86c |
| **Leu** | 86.93 ± 0.45a | 54.47 ± 0.83b | 110.16 ± 12.99c | 110.12 ± 4.07c | 114.84 ± 0.09c |
| **Ile** | 56.69 ± 0.91a | 70.61 ± 0.18b | 82.5 ± 0.40c | 83.93 ± 2.11c | 87.94 ± 0.98c |
| **Total BAA** | 222.69 ± 1.78a | 226.55 ± 6.17a | 316.84 ± 14.39b | 325.46 ± 8.87b | 331.75 ± 3.92b |
| **White sorghum wort** | | | | | |
| **FAN** | 206.02 ± 8.62a’ | 242.09 ± 11.08b’ | 230.29 ±4.08c’ | 261.75 ± 1.63d’ | 179.46 ± 2.02e’ |
| **Val** | 90.01 ± 13.36a’ | 120.82 ± 0.39b’ | 117.52 ± 1.68b’ | 132.10 ± 2.91c’ | 143.39 ± 0.00c’ |
| **Leu** | 98.42 ± 9.62a’ | 112.15 ± 0.47b’ | 109.98 ± 079b’ | 125.61 ± 5.50c’ | 128.72 ± 5.07c’ |
| **Ile** | 59.35 ± 9.61a’ | 84.01 ± 1.30b’ | 78.69 ± 1.34b’ | 90.28 ± 0.59c’ | 97.78 ± 1.50c’ |
| **Total BAA** | 247.78 ± 32.59a’ | 316.99 ± 2.16b’ | 306.19 ± 3.81b’ | 347.99 ± 9.00b’ | 369.89 ± 6.58c’ |

FAN: free amino nitrogen; Val: Valine; Leu: Leucine; Ile: Isoleucine; BAA: branched amino acids. The wort free amino nitrogen and amino acid values affected with different letters a, b, c, d and e or a’, b’, c’, d’ and e’ in the same row indicate a significant difference ($p < .05$).

The screened lactic acid bacteria *L. plantarum* ND-32, ND-160 and especially *L. paracasei* ND-34 are therefore proteolytic lactic acid bacteria which induce both dhurrin hydrolysis and protein digestion improvement. Even if proteolytic is a typical lactococci property where it express this proteolytic property for casein (milk protein) degradation, this important property has been extended to lactobacilli. Therefore, the mash acidification to pH=5 with proteolytic lactic acid bacteria has two positive effects: Firstly, the optimal mashing pH=5 boosts sorghum malt enzyme activities during mashing, and secondly, this biological acidification prior to saccharification allowed the proteolytic lactic acid bacteria to produce protease for protein digestion due to the lack of amino acids. In addition to this lower-cost approach, several others alternatives were proposed by researchers to improve sorghum protein digestion. In order to achieve

level of FAN considered adequate for a good fermentation, Taylor & Boyd, (1986) added proteolytic enzymes during sorghum mashing and improve protein hydrolysis. Ng'Andwe & Hall, (2008) suggested the addition of potassium metabisulphite and protease during sorghum mashing for the reduction of intermolecular disulphide bonds in the kafirins polymers and oligomers to increase protease accessibility for protein hydrolysis improvement. However all these challenges make to improve sorghum mash protein digestion, the wort amino acid content is still less than the known 12 °P barley wort amino acid composition. It is thus evident that low sorghum wort amino acid content will compromise aromagenesis by yeast during fermentation. The wort of our West African sorghum, by virtue of its high proline content (Fig. 10), possesses an amino acid profile different from that reported by Taylor (1982) from South African sorghum wort, where asparagine and glutamine are the two most abundant amino acids. This prevalence of proline in sorghum wort is quite intriguing. Note that, though proline itself is not converted to higher alcohols following the Ehrlich pathway, it can be transformed into the highly assimilable amino acid glutamate. As such, Procopio et al. (2013) also elevated higher alcohols and esters by increasing the wort proline content.

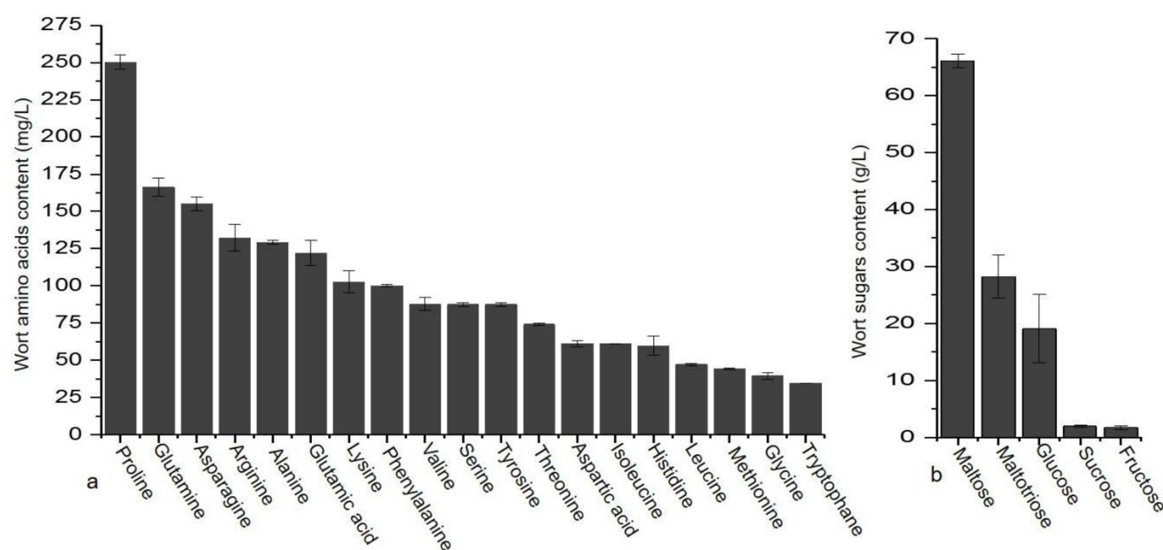


**Fig.10:** Composition of 13 °P brown sorghum wort regarding amino acids (a) and fermentable sugars (b).

The relevant aroma-active higher alcohols isoamyl alcohol, amyl alcohol, and isobutanol result from the metabolism of leucine, isoleucine, and valine, respectively

(Ehrlich, 1907). The isolated yeast strains produced high amounts of amyl alcohol, isoamyl alcohol, isoamyl acetate, and ethyl acetate when the fermentation was conducted at 27 °C as opposed to industrial ale yeast S81 (Table 4). These new *S. cerevisiae* yeasts are therefore well adapted to West-African tropical temperature and the lower amino acid content of sorghum wort. By contrast, the TUM68 yeast S81 generally requires higher amounts of leucine, isoleucine, valine, histidine, glutamine and proline to effectively synthesize aroma-active compounds (Procopio et al., 2013). This limited synthesis of isoamyl alcohol and amyl alcohol by industrial ale yeast is probably due to sorghum wort's typically low content of branched amino acids, particularly leucine and isoleucine. The high peak value of VDKs obtained during the fermentation of sorghum wort with industrial ale yeast TUM68 (Fig. 11) supports this hypothesis and shows that the ale yeast is more sensitive to low amino acid content than the isolated *Saccharomyces* yeasts; the lack of branched amino acids thus exacerbates VDK synthesis which unfortunately leads to high concentrations of diacetyl and pentanedione in beer. However, this is manageable as the VDK content is not only yeast strain dependent, but also reduced by an increase of wort gravity from 11 °P to 13 °P and of the fermentation temperature from 20 °C to 27 °C (Fig. 12).

**Table 4:** Influence of temperature on aroma-active components acetaldehyde, higher alcohols and esters of the young sorghum beers obtained by different *Saccharomyces cerevisiae* yeasts from 11 °P sorghum wort.

| Aroma components (mg/L) | FT (°C) | TO-37 | BE-98 | DA-132 | GL-198 | PA-286 | TUM 68 |
|---|---|---|---|---|---|---|---|
| Acetaldehyde | 20 | 51.353±0.488 | 50.301±4.384 | 45.067±0.118 | 29.345±0.031 | 18.732±0.214 | 21.522±2.260 |
| | 27 | 106.345±5.721 | 39.045±2.603 | 79.753±1.525 | 25.991±4.001 | 15.350±1.205 | 17.191±2.892 |
| **Higher alcohol** | | | | | | | |
| Isoamyl alcohol | 20 | 30.741±0.380 | 37.311±2.125 | 32.921±0.752 | 57.721±1.749 | 33.444±0.691 | 43.473±1,243 |
| | 27 | 51.996±6.297 | 44.282±2.803 | 53.765±4.251 | 63.181±2.561 | 30.288±3.184 | 41.137±0.293 |
| 2-methyl propanol | 20 | 14.183±0.093 | 14.136±0.355 | 11.691±0.058 | 21.664±0.266 | 13.121±0.151 | 42.111±4.967 |
| | 27 | 28.933±1.155 | 21.478±1.022 | 26.341±0.159 | 33.189±6.061 | 17.714±1.198 | 29.713±3.641 |
| 2-methylbutanol | 20 | 10.967±0.091 | 11.819±0.226 | 10.339±0.096 | 21.839±0.065 | 11.144±0.057 | 17.131±0.921 |
| | 27 | 21.322±1.111 | 16.840±0.466 | 19.475±0.972 | 25.975±0.315 | 18.622±0.586 | 9.996±1.886 |
| Propanol | 20 | 11.132±0.101 | 10.145±0.014 | 9.371±0.023 | 20.334±0.025 | 13.811±0.100 | 17.121±0.031 |
| | 27 | 19.939±1.539 | 15.320±0.055 | 19.168±0.162 | 27.637±1.377 | 20.584±0.917 | 8.511±0.884 |
| Total HA | 20 | 67.023±0.665 | 73.411±2.72 | 64.322±0.93 | 121.558±2.105 | 71.52±0.999 | 119.836±7.162 |
| | 27 | 122.19±10.102 | 97.92±4.346 | 118,759±5.544 | 149.982±10.314 | 87.208±5.885 | 89.357±6.704 |
| **Esters** | | | | | | | |
| Isoamyl acetate | 20 | 0.1850±4x10$^{-3}$ | 0.1715±2x10$^{-4}$ | 0.1868±2x10$^{-3}$ | 0.2430±4x10$^{-4}$ | 0.2450±2x10$^{-4}$ | 0.9640±6x10$^{-2}$ |
| | 27 | 0.1681±3x10$^{-4}$ | 0.2525±2.10$^{-3}$ | 0.3700±7x10$^{-4}$ | 0.1693±9x10$^{-4}$ | 0.2155±3.10$^{-3}$ | 0.1470±2.10$^{-3}$ |
| Ethyl acetate | 20 | 7.224±0.051 | 7.658±0.329 | 7.375±0.185 | 8.940±0.267 | 11.233±0.442 | 17.121±0.031 |
| | 27 | 18.230±1.872 | 12.970±1.948 | 16.557±0.388 | 8.799±2.196 | 13.739±1.54 | 7.584±1.055 |
| 2-methyl propyl acetate | 20 | 0.0224±10$^{-5}$ | 0.0182±4x10$^{-6}$ | 0.0153±10$^{-5}$ | 0.0218±4x10$^{-6}$ | 0.0265±2x10$^{-5}$ | 0.0273±10$^{-6}$ |
| | 27 | 0.0547±10$^{-4}$ | 0.0315±7x10$^{-5}$ | 0.0387±10$^{-5}$ | 0.0215±10$^{-5}$ | 0.0236±4x10$^{-5}$ | 0.0251±2.10$^{-5}$ |
| 2-methyl butyl acetate | 20 | 0.0550±10$^{-3}$ | 0.0314±3x10$^{-5}$ | 0.0366±2x10$^{-4}$ | 0.0467±10$^{-5}$ | 0.0400±3x10$^{-6}$ | 0.0727±2x10$^{-3}$ |
| | 27 | 0.0749±3x10$^{-4}$ | 0.0405±10$^{-4}$ | 0.0533±4.10$^{-5}$ | 0.0241±4x10$^{-5}$ | 0.0473±8x10$^{-4}$ | 0.0291±3x10$^{-4}$ |
| Ethyl hexanoate | 20 | 0.1102±10$^{-3}$ | 0.0675±3x10$^{-5}$ | 0.0810±10$^{-4}$ | 0.0646±2x10$^{-5}$ | 0.0972±2x10$^{-4}$ | 0.0571±6x10$^{-5}$ |
| | 27 | 0.0925±5x10$^{-4}$ | 0.0800±3x10$^{-4}$ | 0.0840±4x10$^{-4}$ | 0.0700±4x10$^{-6}$ | 0.0915±2x10$^{-4}$ | 0.0878±10$^{-3}$ |
| Ethyl butanoate | 20 | 0.0710±9x10$^{-4}$ | 0.0374±10$^{-5}$ | 0.0428±7x10$^{-5}$ | 0.0343±10$^{-5}$ | 0.0422±3x10$^{-6}$ | 0.0280±10$^{-5}$ |
| | 27 | 0.0247±3x10$^{-5}$ | 0.0500±10$^{-4}$ | 0.0492±2x10$^{-5}$ | 0.0215±10$^{-5}$ | 0.0416±10$^{-5}$ | 0.0232±10$^{-5}$ |
| Total esters | 20 | 7.6676±0.0579 | 7.984±0.3292 | 7.7375±0.1874 | 9.3504±0.2674 | 11.6839±0.4424 | 18.2701±0.093 |
| | 27 | 18.6449±1.8732 | 13.4245±1.951 | 16.7822±0.3888 | 9.1054±2.1969 | 14.1585±1.544 | 7.8962±1.0583 |

FT= Fermentation temperature. The fermentation was conducted in duplicate

**Table 5:** Young sorghum beer flavour components acetaldehyde, higher alcohols and esters from 13 °P sorghum wort.

| Aroma components | TO-37 | DA-132 | GL-198 | TUM 68 |
|---|---|---|---|---|
| Acetaldehyde (mg/L) | 8.1764±0.6921 | 11.9626±0.0973 | 7.3136±1.0815 | 9.1912±2.4782 |
| **Higher alcohol (mg/L)** | | | | |
| Isoamyl alcohol | 116.2590 ± 0.3852 | 105.0576 ± 2.1234 | 109,4419 ± 0.7118 | 74.6816 ± 1.2427 |
| 2-methyl propanol | 37.1285 ± 0.0910 | 33.8276 ± 0.3508 | 30.6498 ± 0.0602 | 59.7284 ± 1.2090 |
| 2-methylbutanol | 30.7633 ± 0.3284 | 26.4097 ± 0.7100 | 32.2739 ± 0.2873 | 25.4179 ± 1.0771 |
| Propanol | 19,8221 ± 0.1071 | 19.4428 ± 0.0174 | 18.6998 ± 0.0269 | 25.0941 ± 0.0301 |
| **Total** | 203.9729 ± 0.9117 | 184.7377 ± 3.2016 | 191.0654 ± 1.0862 | 184.922 ± 3.5589 |
| **Esters (mg/L)** | | | | |
| Isoamyl acetate | 1.8368± 0.0283 | 1.4056 ± 0.0210 | 1.3228 ± 0.0174 | 1.2388± 0.0535 |
| Ethyl acetate | 22.0679 ± 0.7468 | 20.3508 ± 0.3605 | 18.0123 ± 0.2399 | 15.6713 ± 0.4405 |
| 2-methyl propyl acetate | 0.1005 ± 0.0016 | 0.1322 ± 0.0037 | 0.0633 ± 0.0400 | 0.1444 ± 0.0017 |
| 2-methyl butyl acetate | 0.1906 ± 0.0034 | 0.1419± 0.0012 | 0. 8336 ± 0.0031 | 0.1582 ± 0.0036 |
| Ethyl hexanoate | 0.1403± 0.0047 | 0. 1447± 0.0028 | 0.1200 ± 0.0015 | 0.0514 ± 0.0070 |
| Ethyl butanoate | 0.1403± 0.0015 | 0.1134 ± 0.0045 | 0.0707 ± 0.0028 | 0.0719 ± 0.0082 |
| **Total** | 24.3361 ± 0.7816 | 22.2886 ± 0,3937 | 20.4227 ± 0.3062 | 17.336 ± 0.5145 |

The fermentation was conducted in duplicate.

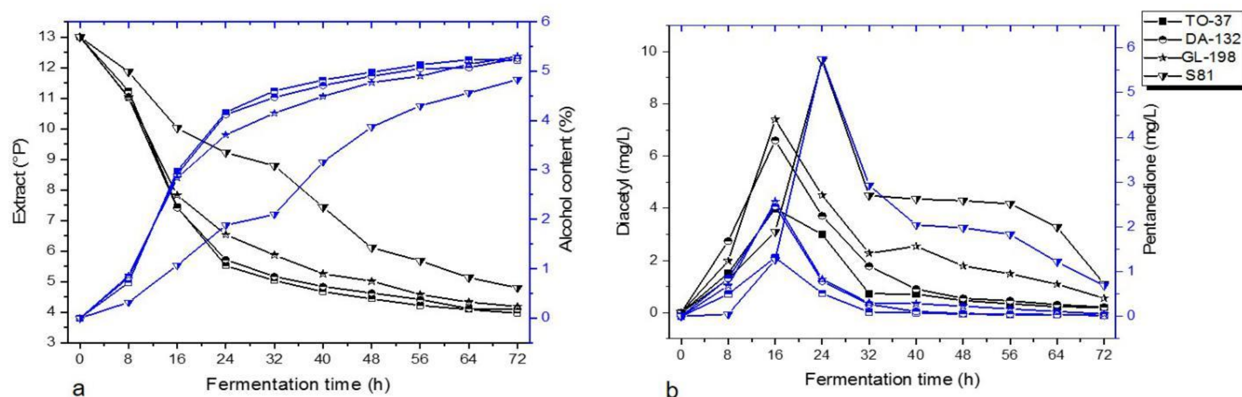


**Fig. 11:** Decrease of extract and an increase of alcohol (a) as well as diacetyl and pentanedione production and their uptake during fermentation of sorghum wort by different *S. cerevisiae* yeast strains.

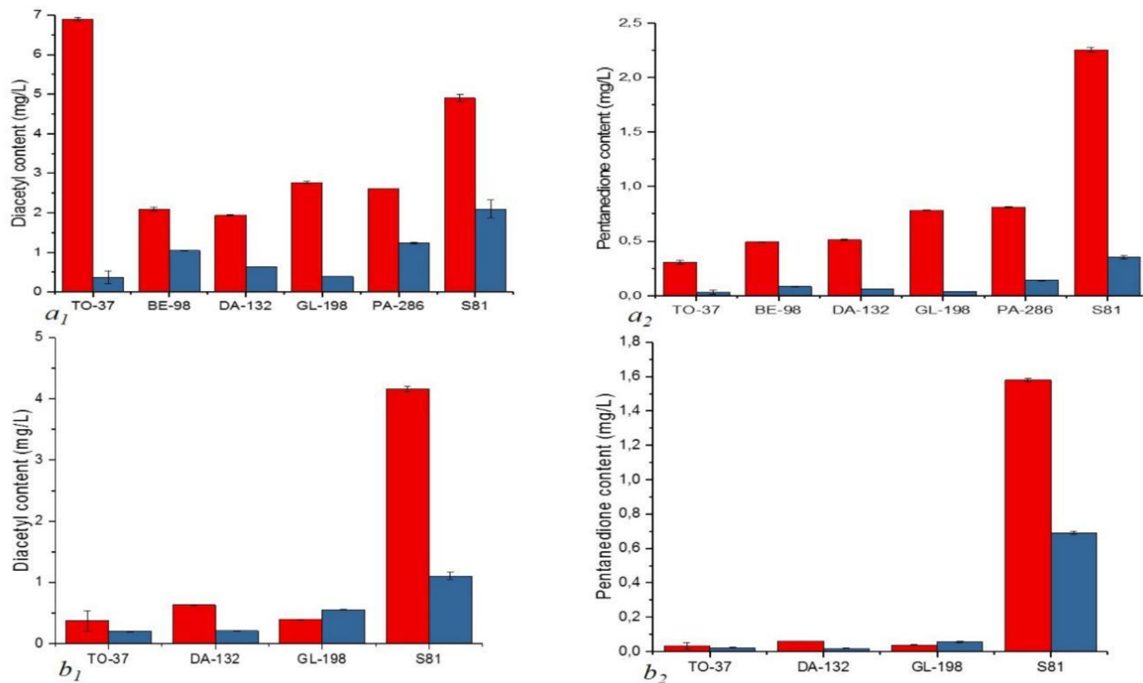


**Fig.12:** Influence of fermentation temperature and wort gravity on the young beer's vicinal diketone content. Red and blue graphs correspond to VDKs obtained at 20 °C and 27 °C, respectively, for $a_1$ and $a_2$, and for 11 °P and 13 °P sorghum wort, respectively, for $b_1$ and $b_2$.

The influence of strain may arise from varying levels of enzyme expression implicated in catalyzing conversion of 2,3-butanedione and 2,3-pentanedione to the corresponding alcohols. The high fermentation temperature and an increase of wort gravity likely increased the yeast population, thereby improving uptake of excreted VDKs. Elevated wort gravity increases the amino acid content, including that of branched amino acids, thus further limiting VDK synthesis. It is also well known that an improved valine content inhibits enzymes controlling the formation of diacetyl precursor α-acetolactate; for instance, Krogerus & Gibson, (2013) increased the valine content and observed a decrease of beer VDK content. Even though TUM68 scavenges much of the accumulated VDKs during fermentation, the VDKs in young beer obtained from this ale yeast still exceeds the lower threshold value of diacetyl and pentanedione and the recommended level in beer. While an increase of fermentation temperature helps reduce VDKs, it negatively affected aromagenesis by industrial *S. cerevisiae* yeast. Therefore, industrial *Saccharomyces* yeast is suboptimal for sorghum wort fermentation overall, seeming to be well adapted to barley wort and being exigent regarding amino acids for efficient growth and aroma component synthesis. By

contrast, the *Saccharomyces* yeasts isolated from African sorghum beer starter seem well adapted to sorghum wort composition. Applied to sorghum wort fermentation, they may help generate beer with high amounts of higher alcohols and esters. Furthermore, an increase of fermentation temperature from 20 °C to the tropical West-African agro-ecological temperature (27 °C) improves the synthesis of aroma components and reduces VDK content. Altogether, while the beer's sensory characteristics result from the entire aroma bouquet – not only synthesized by yeast but also generated by hopping – and aromagenesis by yeast during fermentation could be affected by serial repitching, the high expression of higher alcohols and esters is an exciting prospect for designing appropriate yeasts for sorghum wort fermentation.

In definitive, our new approach based on sorghum mash biological acidification (lactic acid fermentation) to improve sorghum cyanogenic glucoside (dhurrin) and protein hydrolysis followed by sorghum wort fermentation (alcoholic fermentation) with the selected potent new yeast able to produce from sorghum wort high amount of aroma active components higher alcohols and esters is a new approach. We are confident that sorghum beer brewers will adopt our new approach and the selected *L. paracasei* ND-34 and *S. cerevisiae* TO-37 will be used for multi-stage fermentation during industrial sorghum beers production to improve sorghum beer safety and sensory characteristics.

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