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CARACTERISATION ET AMELIORATION DE LA QUALITE DE LA BIERE TRADITIONNELLE RWANDAISE « IKIGAGE » FABRIQUEE A BASE DE SORGHO

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Résumé

Ikigage est une bière traditionnelle Rwandaise fabriquée à base de malt de sorgho et de certaines plantes locales, principalement les feuilles de *Vernonia amygdalina* « umubirizi ». Cependant, cette bière reste moins attrayante que les bières occidentales du type pils en raison de la mauvaise qualité hygiénique, des variations organoleptiques et de la courte durée de conservation. Ce travail s'est intéressé à la caractérisation de la bière *ikigage* afin d'améliorer sa qualité hygiénique et de réduire les variations organoleptiques en utilisant les matières premières locales. La première partie de notre étude montre que la bière *ikigage* commercialisée au Rwanda est caractérisée par la présence de *Stapylococcus aureus* et de beaucoup de microorganismes d'origine fécale (*Escherichia coli* et Streptocoques fécaux), par la faible teneur en éthanol, par l'acidité élevée et une importante quantité des protéines. Les levures (*Saccharomyces cerevisiae* et *Issatckenkia orientalis*), suivies par les bactéries lactiques (*Lactobacillus fermentum* et *Lactobacillus buchneri),* sont des microorganismes majoritaires impliqués dans la fermentation de la bière *ikigage*. Par ailleurs, un des problèmes majeurs dans le brassage de la bière de sorgho est la faible teneur en sucres fermentescibles due à la faible activité de β-amylase dans le malt de sorgho. L'utilisation de malt (30 %) d'*Eleusine coracana* « uburo » (variété Musama) associée avec la procédure de brassage par décantation augmente la teneur en sucres fermentescibles, particulièrement le maltose, dans le moût de sorgho, et par conséquent la teneur en éthanol dans la bière. La deuxième partie de notre étude montre que *V. amygdalina*, connu pour ses propriétés antibactériennes et son amertume semblables au houblon, contribue à la production de méthyle salicylate, beta-damascenone et de nombreux terpènes (*δ*-3-carene, *β*-farnesene, farnesol, *β*-citronellol, linalool et 1,8-cineole) dans la bière *ikigage*. Ce travail met en évidence la présence de 14 thiols polyfonctionnels dans les bières fabriquées avec les matières premières non occidentales. Parmi eux, 3-méthyl-2-buten-1-thiol, très connu pour son arôme houblonnée agréable, émerge comme l'odorant le plus important dans les bières *ikigage* non houblonnées contenant *V. amygdalina*. *V. amygdalina* contribue également à la production de 1-butanethiol et de 4-sulfanyl-4-méthyl-2-pentanone dans la bière de sorgho *ikigage*. Cependant, contrairement à le houblon, les feuilles de *V. amygdalina* ajoutées durant l'ébullition inhibe la production de 2-sulfanyl éthyl acétate. La troisième partie de notre étude montre que l'utilisation de *S. cerevisiae* en combinaison avec *I. orientalis* et *L. fermentum* comme starter permet de produire une bière ikigage ayant le statut de GRAS (generally recognized as safe) tout en conservant les caractéristiques organoleptiques semblables à celles de la bière traditionnelle « ikigage » locale produite par les paysans.

Mots clés : Sorgho, sucres, fermentation, bière, *ikigage*, qualité hygiénique, qualité organoleptique, *Vernonia amygdalina*, *Umubirizi*, Rwanda.

François LYUMUGABE (2013). Characterisation and improvement of the quality of Rwandese traditional beer « ikigage » made from sorghum; University of Liège, Gembloux Agro-Bio Tech, (Belgium), 208 p., 20 tables, 22 figures.

Abstract

Ikigage is a Rwandese traditional beer made from sorghum malt and local plants, mainly *Vernonia amygdalina* "umubirizi". However, this beer remains less attractive than Western beers of pils type because of poor hygienic quality, variations of organoleptic quality and limited shelf life. The aim of this work is to characterize *ikigage* beer in order to improve its hygienic quality and to reduce the organoleptic variations using the local raw materials. The first part of our study shows that *ikigage* beer marketed in Rwanda is characterized by the presence of *Stapylococcus aureus* and many micro-organisms of fecal origin (*Escherichia coli* and *fecal streptococci*), by the low ethanol content, high total acidity and an important amounts of proteins. The yeasts (*Saccharomyces cerevisiae* and *Issatckenkia orientalis*), followed by the lactic acid bacteria (*Lactobacillus fermentum* and *Lactobacillus buchneri*), are the predominant microorganisms involved in fermentation of *ikigage* beer fermentation. Besides, one of the major problems in sorghum beer brewing is the efficient conversion of the starch extracts into fermentable sugars due to the weak activity of β-amylase in sorghum malt. The use of *Eleusine coracana* "uburo" (Musama variety) malt (30%), associated with mashing decantation procedure, increases the content of sugar fermentable, maltose particularly, in sorghum wort, and consequently ethanol content in the beer. The second part of our study shows that *V. amygdalina*, known for its antibacterial properties and its bitterness similar to hops, contributes to the production of methyl salycilate, beta-damascenone and many terpenes compounds (*δ*-3-carene, *β*-farnesene, farnesol, *β*-citronellol and linalool), in the sorghum beer "ikigage". This work reveals also the presence of 14 polyfunctional thiols in beers brewed with 'non-Western' raw materials. Among them, the well-known hop constituent 3-methyl-2-buten-1-thiol emerged as a key flavour in the unhopped beers containing *V. amygdalina*. *V. amygdalina* also contributes to the production of 1-butanethiol and 4-sulfanyl-4-methyl-2-pentanone in sorghum beer. However, contrary to hops, *V. amygdalina* addition during boiling also appears to strongly inhibit the production of 2-sulfanylethyl acetate. The third part of our study shows that the use of *S. cerevisiae* in combination with *I. orientalis* and *L. fermentum* as starter allows producing *ikigage* beer having the GRAS (generally recognized as safe) statute while preserving the organoleptic characteristics similar to those of local traditional beer "ikigage" produced by peasants.

Keywords: Sorghum, sugars, fermentation, beer, *iIkigage*, hygienic quality, organoleptic quality, *Vernonia amygdalina*, *Umubirizi*, Rwanda.

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Liste des publications et communications scientifiques

1. Publications

Lyumugabe, F., Kamiliza, G., Bajyana, E., Thonart, Ph. (2010). Microbiological and physico-chemical characteristics of Rwandese traditional sorghum beer «ikigage». *African Journal of Biotechnology, Vol. 9(27), pp. 4241-4246*.

Lyumugabe, F., Gros, J., Nzungize, Jh., Bajyana, E., Thonart, Ph. (2012). Characteristics of African traditional beers brewed with sorghum malt – a review. *Biotechnologie, Agronomie, Société et Environnement*, 16*(4), 509-530.*

Lyumugabe, F., Gros, J., Thonart, Ph., Collin, S. (2012). Occurrence of polyfunctional thiols in sorghum beer « ikigage » made with *Vernonia amygdalina* «Umubirizi». *Flavour and Fragrance Journal, 27, 372–377.*

Lyumugabe, F., Bajyana, E., Wathelet J.P., Thonart, Ph., 2013. Volatile compounds of the traditional sorghum beers « ikigage » brewed with *Vernonia amygdalina* «Umubirizi». *Cerevisia, 37 (4), 89 -96.*

Lyumugabe, F., Nzuginze, Jh., Gros, J., Bajyana, E., Thonart, Ph., 2013. Traditional sorghum beer «ikigage» brewing using *Eleusine coracana* «uburo» to increase saccharification. *American Journal of Food Technology* (submitted).

Lyumugabe, F., Nsanzimana, D., Bajyana, E., Thonart, Ph. (2013). Production of Rwandese traditional sorghum beer «ikigage» using *Saccharomyces cerevisae, Issatckenkia orientalis* and *Lactobacillus fermentum* as starter cultures. *Word Journal of Microbiology and Biotechnology (en cours de soumission).*

Lyumugabe, F., Habayo, A., Uyisenga, J.P., Nzazimana, D., Mashako A., Bajyana, E., Thonart, Ph. Selection and characterization of predominant yeast strains involved in fermentation of the Rwandese traditional sorghum beer « ikigage ». *Rwanda Journal (en cours de soumission).*

2. Posters

Lyumugabe, F., Kamiliza, G., Bajyana, E., Thonart, Ph., 2009. Microbiological characteristics of Rwandese traditional beer « ikigage ». *Fourteenth Conference on Food Microbiology, June 18-19, 2009*, Liège (Belgium).

Lyumugabe, F., Bajyana, E., Thonart, Ph., 2009. Charcateristics of sorghum beer « ikigage » and a yeast strain isolated from Rwandese traditional leaven «umusemburo». *International congress of research and innovation for sustainable, October 19-25, 2008*, Butare (Rwanda).

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Liste des abréviations

CWBI: Centre Wallon de Biologie industrielle

SFAR : Student Financing Agency for Rwanda

U/g: Unité/gramme

SDU/g: Sorghum Diastatic Units

Kg: Kilogramme

LAB: Lactic Acid Bacteria

FAN: Free Amino Nitrogen

rDNA: Ribosomal Desoxyribo Nucleic Acid

ITS: internal transcribed spacer

PCR: Polymerase Chain Reaction

GRAS: Generally Recognized as Safe

MRS: Man, Rogosa and Sharpe

L: Litre

CFU/mL: colony-forming units/ milliliter

v/v : volume /volume

w/v: poids /volume

AP: Acidification power

GMS: Glucose Mannose Sensitive

MI: Mannose Insensitive

MS: Mannose sensitive

EBC: European Brewery Convention

HS-SPME: Headspace-Solid Phase Micro Extraction

GC-MS: Chromatographie en phase Gazeuse couplée avec Spectrométrie de Masse

GC- PFPD : Chromatographie en phase Gazeuse couplée avec Détecteur à Photométrie de Flamme Pulsée

NIST : National Institute of Standards and Technology

TABLE DES MATIERES

Introduction générale

Le sorgho [*sorgho bicolore* (L.) Moench], originaire d'Afrique (3000 à 5000 ans avant J.C), appartient à l'ordre des Poales et à la famille de Poacea (Belton et Taylor, 2002). La culture de sorgho s'adapte à tous les sols (secs, détrempés ou à forte salinité) et résiste bien aux agro-écologies chaudes et sèches ; ces qualités lui confèrent un avantage considérable par rapport à d'autres cultures céréalières (ex. orge) dans les régions de la zone tropicale et semi-aride (Asiedu, 1991 ; Fliedel *et al*., 1996).

Après le maïs, le blé, le riz et l'orge, le sorgho est la cinquième céréale la plus cultivée dans le monde. Sa production représente 61,7 millions de tonnes (moyenne 2006-2008), dont 25,6 millions de tonnes pour l'Afrique, où il couvre 4 % des terres arables (CTA, 2011). Le Nigéria et le Soudan sont les plus grands pays producteurs de sorgho en Afrique. Bien que le Rwanda ne soit pas un grand producteur, le sorgho y est la plus importante céréale en termes de production. En 2011, sa production était estimée à 181,535 tonnes/an, sur 129,215 d'hectares (Minagri, annual report 2010/2011).

Les grains de sorgho constituent une source importante d'énergie et de protéines pour des millions d'habitants d'Asie et d'Afrique ; il représente en effet l'alimentation de base des populations les plus pauvres et les plus défavorisées du globe (Aiedu, 1991). L'amélioration de la qualité nutritionnelle de sorgho s'avère d'une grande importance dans ces régions où les populations sont confrontées fréquemment au problème de la malnutrition. La pauvre qualité nutritionnelle de cette céréale dans l'alimentation humaine est due à l'insuffisance de certains acides aminés essentiels tels que l'isoleucine et la lysine (Neucere et Sumrell, 1979), à la pauvre digestibilité en protéine (Hamarker *et al*., 1986) et à la présence importante des tannins (Price et Bultler, 1977). Plusieurs études sur le sorgho ont montré que la fermentation, une technologie très peu coûteuse, augmente la teneur en protéine et améliore sa digestibilité (Chavan *et al*., 1988, 1989 ; Noha *et al*., 2011), fournit une meilleure composition en acides

aminés essentiels (Au et Fields, 1981), diminue la teneur en tannins (Hassan et El Tinay, 1995 ; Osman, 2004) et augmente la teneur en vitamines (El Tinay, 1979; Chavan *et al*., 1989). La fermentation est une approche également idéale pour conserver et améliorer les propriétés organoleptiques d'aliments dans les pays en développement (Holzapfel, 2002).

Depuis les temps immémoriaux, les Rwandais transforment le sorgho en une bière traditionnelle appelée *ikigage* après une étape de fermentation. Cette bière joue un rôle socio-culturel très important au Rwanda. Elle est servie dans les différentes cérémonies et fêtes rwandaises (mariage, funérailles, dons de vaches, etc.). Dans la culture Rwandaise, la bière de sorgho symbolise la complémentarité des sexes et l'entente cordiale entre les peuples. Elle est également appelée le lait de la houe « amata y'isuka »; ce qui lui confère de véritables lettres de noblesses. La bière *ikigage* est également rencontrée dans presque toutes les régions d'Afrique sous des appellations différentes. Elle est connue sous le nom de *Tchoukoutou* au Bénin et au Togo (Kayode et *al*, 2005), *Dolo* au Burkina – Faso (Dicko *et al*., 2006), *Pito* au Ghana, *Burukutu* ou *Otika* au Nigérian (Faparusi *et al*., 1973), *Bili bili* au Tchad (Maoura *et al*, 2005). Les processus de fabrication de ces bières sont très variables et dépendent du savoir-faire de chaque région (Hagglade et Holzapfel, 2004).

Au Rwanda, le procédé traditionnel de la préparation de la bière *ikigage* implique le trempage de grains de sorgho dans de l'eau durant 24h, la germination à la température ambiante durant 72h. Puis viennent le séchage sous le soleil, la production du moût de sorgho « *igikoma* » (infusion à 65° C), et la fermentation (12-24h). La fermentation est initiée par un levain traditionnel dit « *umusemburo* », résultant d'une fermentation spontanée du moût de sorgho, qui est préparé avec des feuilles connues sous le nom d'*umubirizi* «*Vernonia amygdalina »* (Lyumugabe *et al.,* 2010). La bière *ikigage* est non seulement un aliment pour les populations rurales, mais également une source de revenus non négligeables, particulièrement pour les

femmes qui la produisent à l'échelle locale sur fond d'une technologie traditionnelle. Cependant, un certain nombre de contraintes freinent le développement de ce secteur d'activités, et contribuant, ainsi, à la disparition de cette bière, surtout en milieux urbains. D'une part, la production reste strictement artisanale et donne un produit conservable durant seulement 24 heures et dont la qualité hygiénique est souvent mise en doute. Récemment, la bière de sorgho a été mise en cause dans l'intoxication alimentaire de 151 personnes lors d'un mariage au sud du Rwanda (Conférence de presse du Ministre de la Santé, Newtimes, 2012). D'autre part, la faible teneur en éthanol et les variations de caractéristiques organoleptiques rendent *ikigage* moins attrayant que les bières de type pils occidental fabriquées par deux firmes (Bralirwa et Brasserie des Mille Collines) basées au Rwanda. Les prix élevés de leurs produits obligent la population rurale à retourner vers la consommation des bières traditionnelles, malgré des nombreux cas de toxi-infections alimentaires liées à leur consommation. Pour le Gouvernement Rwandais, la mise au point d'une bière traditionnelle de bonne qualité hygiénique est devenue une nécessité.

Un des problèmes majeurs dans l'amélioration du brassage de la bière de sorgho est la faible teneur en sucres fermentescibles (maltose). Cette faible teneur est notamment due à la faible activité de béta- amylase de malt de sorgho (Palmer, 1989; Dufour *et al.*, 1992). La teneur en éthanol reste faible dans le produit fini, facilitant, ainsi, le développement de contaminants potentiellement pathogènes. Le mélange de sorgho avec du malt d'orge (Okafor *et al*., 1980 ; Goode *et al*., 2003), ou l'utilisation d'enzymes commerciales (Dale *et al.*, 1989 ; Bajomo et Young, 1994), ont été proposés comme solutions, pour résoudre ce problème de carence en β-amylase. Cependant, ces approches restent inappropriées dans le contexte du Rwanda en raison du coût élevé de l'orge (une culture non tropicale) et des enzymes commerciales. Malheureusement, à notre connaissance, il n'existe pas d'études portant sur la recherche de solutions locales, comme par exemple, le mélange de sorgho avec d'autres céréales locales.

Par ailleurs, l'utilisation des starters sous forme de cultures pures, ou mixtes, a été proposée comme une meilleure méthode pour améliorer la qualité hygiénique, réduire les variations organoleptiques et l'instabilité microbiologique des aliments fermentés d'Afrique (Kirmaryo et *al*, 2002; Holpzapfel, 2002). L'utilisation d'un starter adéquat améliore le contrôle et l'optimisation du processus de fermentation et la prédictibilité des produits dérivés (Holpzapfel, 1987). L'utilisation des starters pourrait donc être une approche prometteuse pour l'amélioration d'*ikigage* du Rwanda. Cependant, l'utilisation de starter requiert, au préalable, la caractérisation du produit traditionnel à améliorer (Holzapfel, 2002).

L'objectif de ce travail est de caractériser la bière de sorgho « *ikigage* » du Rwanda, afin d'améliorer sa qualité hygiénique, et de réduire les variations organoleptiques, en utilisant les matières premières locales. Pour y parvenir, on a d'abord effectué des analyses microbiologiques et physico-chimiques de la bière traditionnelle « *ikigage* » commercialisée au Rwanda. Un suivi technique et microbiologique a été également effectué aux différentes étapes du processus de fabrication de la bière *ikigage*, en vue de répertorier les microorganismes majoritaires impliqués dans la fermentation de cette bière. Des essais de composition mixte de sorgho et d'*Eleusine coracana* (céréale locale) ont été étudiés, afin d'améliorer la saccharification du moût de sorgho. Les caractéristiques physico-chimiques et aromatiques des bières-pilotes produites avec les souches de microorganismes sélectionnés ont été comparées à celles de la bière traditionnelle produite localement par des paysans rwandais. Afin d'explorer le potentiel de *V. amygdalina* sur le profil aromatique de la bière *ikigage*, des bières-pilotes ont été produites, avec ou sans ajout de *V. amygdalina* durant le brassage de la bière de sorgho. Le profil odorant de ces bières a été comparé à celui de la bière traditionnelle produite localement par des paysans.

Avant d'aborder l'ensemble des résultats et leurs discussions, la synthèse des éléments de la littérature présentera les caractéristiques des bières traditionnelles africaines brassées avec le malt de sorgho, tout en précisant les différences majeures avec les bières occidentales à base de malt d'orge.

Chapitre 1 :

Synthèse bibliographique

Ce travail a fait l'objet de la publication suivante :

Lyumugabe, F., Gros, J., Nzungize, Jh., Bajyana, E., Thonart, Ph. (2012). Characteristics of African traditional beers brewed with sorghum malt – a review. *Biotechnologie, Agronomie, Société et Environnement*, 16*(4), 509-530.*

Préambule au chapitre 1

Les bières traditionnelles à base de sorgho sont produites dans plusieurs pays d'Afrique, mais les processus de fabrication varient en fonction de leur localisation géographique. Elles sont très riches en calories, en vitamines du groupe B comprenant thiamine, acide folique, riboflavine et acide nicotinique, et en acides aminés essentiels, tels que la lysine. Cependant, les bières africaines à base de sorgho sont moins attrayantes que les bières occidentales, en raison de leur qualité hygiénique, de la variation de leurs caractéristiques organoleptiques, et de leur courte durée de conservation.

Le présent chapitre de synthèse bibliographique décrit le processus de fabrication et les caractéristiques microbiologiques, biochimiques et nutritives des bières traditionnelles africaines à base de sorgho, tout en précisant les différences majeures avec les bières occidentales à base de malt d'orge. Il montre, également, le rôle socio-culturel des bières africaines à base de sorgho, et l'état actuel des recherches effectuées pour améliorer la qualité de ces bières.

Characteristics of African traditional beers brewed with sorghum malt – A review

RUNNING TITLE HEADER: African traditional sorghum beer

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Abstract

Traditional sorghum beers are produced in several countries of Africa, but variations in the manufacturing process may occur depending on the geographic localization. These beers are very rich in calories, B-group vitamins including thiamine, folic acid, riboflavin and nicotinic acid, and essential amino acids such as lysine. However, the traditional sorghum beer is less attractive than Western beers because of its poorer hygienic quality, organoleptic variations and shorter shelf life. Research into the microbiological and biochemical characteristics of traditional sorghum beers as well as their technologies have been performed and documented in several African countries. This review aims to summarize the production processes and compositional characteristics of African traditional sorghum beers (*ikigage*, *merissa*, *doro*, *dolo*, *pito*, *amgba* and *tchoukoutou*). It also highlights the major differences between these traditional beers and barley malt beer, consumed worldwide, and suggests adaptations that could be made to improve the production process of traditional sorghum beer.

Keywords: Cereals, Sorghum, Malt, Sugars, Fermentation, Alcoholic, Beers, Africa

Résumé

Les bières traditionnelles à base de sorgho sont produites dans plusieurs pays d'Afrique, mais les processus de fabrication varient en fonction de leur localisation géographique. Elles sont très riches en calories, en vitamines du groupe B comprenant la thiamine, l'acide folique, la riboflavine et l'acide nicotinique, et en acides aminés essentiels tels que la lysine. Cependant, les bières africaines à base de sorgho sont moins attrayantes que les bières occidentales en raison de leur qualité hygiénique, de la variation de leurs caractéristiques organoleptiques et de leur courte durée de conservation. Les recherches sur les caractéristiques microbiologiques, biochimiques et technologiques des bières traditionnelles africaines ont été effectuées et documentées dans plusieurs pays d'Afrique. L'objectif de cette revue bibliographique sur les bières traditionnelles à base de sorgho est de récapituler le processus de fabrication et les caractéristiques des bières traditionnelles africaines (*ikigage*, *merissa*, *doro*, *dolo*, *pito*, *amgba* et *tchoukoutou*) à base de sorgho, tout en précisant les différences majeures avec la bière à base de malt d'orge. Cette revue s'adapte également au progrès accompli dans l'amélioration des bières traditionnelles africaines à base de sorgho.

Mots – clés: Céréales, Sorgho, Sucres, Fermentation, Alcool, Bières, Afrique.

1. INTRODUCTION

Sorghum, unlike barley, is very well adapted to the semi-arid and sub-tropical conditions prevailing over most of the African continent (Agu et al., 1998). Like barley, sorghum belongs to the grass family of Gramineae. In Africa, sorghum grain is the major cereal crop used to produce the traditional "opaque" beers (Novellie, 1976; Asiedu, 1991). However, only certain sorghum varieties (*e.g.* red grain) are specifically used to produce sorghum beers. These beers are known as *ikigage* in Rwanda (Lyumugabe et al., 2010), *tchoukoutou* in Benin and Togo (Kayodé et al., 2005), *dolo* in Burkina- Faso (Dicko et al., 2006), *pito* or *burkutu* in Nigeria and Ghana (Ekundayo, 1969; Faparusi et al., 1973), *amgba* in Cameroon (Chevassus-Agnes et al., 1979), *doro* or *chibuku* in Zimbabwe (Chamunorwa et al., 2002), *merissa* in Sudan (Dirar, 1978), *mtama* in Tanzania (Tisekwa, 1989), *bili bili* in Chad (Maoura et al., 2005) and *kaffir* in South Africa (Novellie et al., 1986).

The manufacturing processes of African traditional sorghum beer essentially involves malting, drying, milling, souring, boiling, mashing and alcoholic fermentation, but variations may occur depending on the geographic localization (Haggblade et al., 2004). These types of beer differ from European (lager) types in the fact that lactic fermentation also occurs during sorghum beer processing. In addition, African traditional sorghum beer is consumed while it is still fermenting, and the drink contains large amounts of fragments of insoluble materials (Rooney et al., 1991). These fragments are mainly starch residues and dextrins that are not digested during mashing and fermentation (Glennie et al., 1986). Sorghum beers bear very little resemblance in appearance to Western beer made with barley. However, some studies have suggested that the use of sorghum malt (instead of barley malt) in lager-beer brewing is unlikely to succeed because of some inherent problems (enzymes, starch characteristics, polyphenols) associated with sorghum (Aisien, 1982; Palmer, 1991; Bajomo et al., 1994).

Several studies into the microbiological and biochemical characteristics of traditional sorghum beers as well as their technologies have been carried out and documented in different African countries (Novellie, 1962; Ekundayo, 1969; Faparusi et al., 1973; Dirar, 1978; Tisekwa, 1989; Chamunorwa et al., 2002; Maoura et al., 2005). A very varied yeast and lactic bacteria acid flora has been found in African sorghum beers, although *Saccharomyces cerevisiae* and heterofermentative *Lactobacillus* usually predominate (Novellie, 1976; Sefa-dedeh et al., 1999; Chamunorwa et al., 2002; Maoura et al., 2005; Kayodé et al., 2007a; Lyumugabe et al., 2010). Traditional African sorghum beers are very rich in calories, B-group vitamins including thiamine, folic acid, riboflavin, and nicotinic acid, and essential amino acids such as lysin (Chevassus-Agnes et al., 1979). The beers are consumed at various festivals and African ceremonies (*e.g.*, marriage, birth, baptism, the handing over of a dowry, etc.) and constitute a source of economic return for the female beer producers. However, in the majority of African countries, traditional sorghum beers are less attractive than Western beers brewed with barley malt because of their poor hygienic quality, low ethanol content, organoleptic variation and unsatisfactory conservation (Novellie et al., 1986; Tisekwa, 1989; Sanni et al., 1999; Lyumugabe et al., 2010). This review aims to summarize the production processes and characteristics of African traditional sorghum beers. It also highlights the major differences between these traditional beers and the familiar barley malt beer, consumed worldwide, and suggests adaptations that could be made to improve the production processes of traditional sorghum beer.

2. MALTING

Malting is the germination of cereal grain in moist air under controlled conditions, the primary objective being to promote the development of hydrolytic enzymes, which are not present in the ungerminated grain. The malting process essentially involves steeping,

germinating and limiting cereal seedling growth, once enzymes have been produced for the degradation of starch and proteins in the cereal grain, but before the exhaustion of the polysaccharide.

2.1. Steeping

The steeping or soaking of cereal grain in water is widely acknowledged as the most critical stage of the malting process (French et al., 1990; Dewar et al., 1997). This is a consequence of the importance of initiating germination such that modification of the endosperm structure will progress at a rate producing malt of the desired quality. During the Western beers brewing process (**Figure 1**), malting begins with the soaking of the barley in water for 2 days at 10-16 °C in order to increase the moisture content to around 45% (Moll, 1991; Waites et al., 2001). Periodically, the water is temporarily drained off and aeration is provided, thus preventing anaerobic conditions that can cause grain embryo damage. In Africa, the traditional sorghum malting process also starts with the soaking of the sorghum grain in water for 10 to 24 h at ambient temperature (Maoura et al., 2009; Lyumugabe et al., 2010), but, in this case, the water is not renewed or aired. The steep-out moisture content of sorghum grain is affected by both steeping time and temperature (Dewar et al., 1997). However, the steeping period at a given time varies according to the sorghum cultivar. A variation in moisture content of 32.4 to 43.4% has been observed after steeping 26 sorghum cultivars for 24 h (Kumar et al., 1992). The steep moisture increases as the steeping temperature rises from 10 to 30 °C, for any given period (Novellie, 1962). The effect of steeping conditions has been extensively investigated in an attempt to increase sorghum malt amylase activity. In 1962, Novellie reported that steeping time had little effect on the final diastatic power of sorghum

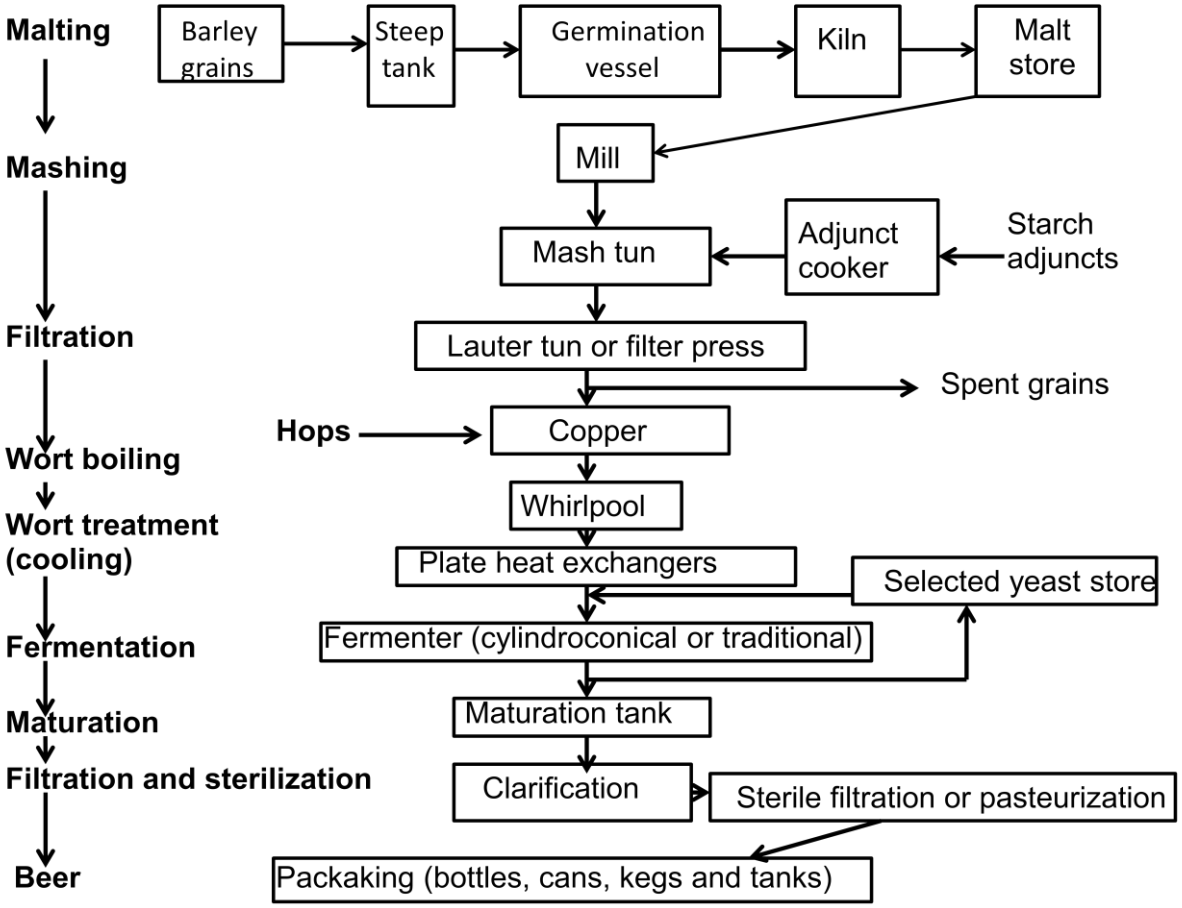

Figure 1. Western beers brewing process of western beers (Moll, 1991; Wait et al., 2001) – Procédé de brassage des bières occidentalles (Moll, 1991; Wait et al., 2001).

malt. Steep moisture of 42 to 48%, attained after steeping sorghum for 18 to 22 h at 30 °C, is optimal for enzymatic activity (Morall et al., 1986). An increase in steep moisture with a steeping time of between 12 and 20 h at 30 °C is accompanied by a corresponding increase in reducing sugar content, and in cold-and hot water extracts (Owuama et al., 1994). The level of increase in these features of the sorghum malt appears to be directly proportional to its diastatic power (Pathirana et al., 1983). A steeping regime, and in particular the use of air-rests and a final warm water (40 °C) steeping period has been shown to enhance sorghum malt quality, including β-amylase activity (Ezeogu et al., 1995). A later study specifically

confirmed the importance of the effect of air-rests on the level of sorghum malt β-amylase activity (Okungbowa et al., 2002). It is likely that the presence of oxygen leads to a more rapid increase in seedling metabolic activity (Dewar et al., 1997). The diastatic power of malt has also been shown to increase with steeping temperature (up 30 °C) and with the level of free amino nitrogen (FAN) (Dewar et al., 1997). Steeping the sorghum grain in dilute alkaline liquor (0.1% of $Ca(OH)_2$, KOH or NaOH) has been shown to significantly enhance the diastatic activity of sorghum malt especially β-amylase activity (Okolo et al., 1996; Okungbowa et al., 2002).

2.2. Germination

After steeping, the sorghum grains are then spread out on a germination device (*e.g.* green plantain leaves or a plastic sheet) to form a layer (2 to 3 cm in thickness) and the grains are kept covered for 2-3 days at ambient temperature (Chevassus-Agnes et al., 1979; Lyumugabe et al., 2010). The layer of grains is sometimes turned over twice a day and the initial moisture level is maintained by spraying with water. This technique is similar to the old germination process that was previously used to produce Western beers, where barley grains were spread out on malting floors to a depth of 10-20 cm for 3-5 days at 16-19 °C (Moll, 1991). However, Western breweries now use various mechanized systems, which have grain beds of about 1 m in depth. These grains beds are aerated with moist cool air and turned mechanically every 8-12 h to aid respiration by the grain and to prevent the build-up of heat; otherwise the grain embryo may become damaged.

Germination involves the outgrowth of the plumule and radicle of the seedling until suitable enzymes (*e.g.* starch degrading enzymes and proteases) have been produced for the malt (Palmer, 1989). During germination, the hormone gibberellic acid (GA), at low concentration (0.1-0.2 ppm), induces the barley aleurone layer to produce endosperm-

degrading enzymes such as α-amylase, protease, pentosanases and endo-beta-glucanase (Palmer, 1989), but this hormone plays no such role in enzyme development in sorghum (Aisien et al., 1983; Palmer, 1989). In sorghum, α-amylase and carboxypeptidases are produced by the scutellum, while endo-β-glucanase, limit dextrinase and endo-protease develop in the starchy endosperm. These contrasts with malting barley where α-amylase, endo-protease, limit dextrinase and endo-β-glucanase develop in the aleurone layer, while carboxypeptidases and β-amylase are found in the starchy endosperm (Aisien et al., 1983; Palmer, 1989). Phosphate, an important mineral found in barley aleurone tissue and in the sorghum embryo (Palmer et al., 1989), may account for differences in the enzyme-producing potentials of the barley aleurone and the sorghum embryo (Agu et al., 1998). The endosperm of malted sorghum retains starch compaction and is not as friable as the barley malt grain. Malting (respiration and root) loss of sorghum malts is about 20%, while the malting loss of barley malt is about 7% after 6 d of growth at 25 °C and 16 °C (Palmer et al., 1989).

Another important physiological difference between sorghum and barley malts is that malted sorghum grains contain low levels of endo-β-glucanase and β-amylase (Aisien et al., 1983). In their experiments, Beta et al. (1995) found that levels of α-amylase activity (25-183 U.g-1) and β-amylase activity (11-41 SDU.g-1) in sorghum malt varied, depending on the sorghum variety. Recently, a comparative study of white sorghum varieties indicated that the F-2-20 varieties from Senegal have an α-amylase activity of 312, 6 U.g-1 and a β-amylase activity of 62, 7 U.g-1 (Khady et al., 2010). However, compared to barley malt, whose β-amylase activity is 400 U.g-1 (Taylor et al., 1993), sorghum malt is not adapted to be used in an efficient brewing industry. Several assumptions explain the weakness of β-amylase activity in sorghum malt. Uriyo et al. (1999) explained this weakness in terms of an interaction between β-amylase and polyphenols during the extraction process, whereas Dufour et al. (1992) showed that β-amylase activity remains weak, even in sorghum varieties with low

polyphenol content. Other authors claim that inhibitors apart from polyphenols would be present, causing partial solubilization of β-amylase or a weak output during malting (Palmer et al., 1989; Agu et al., 1997a). According to Taylor et al. (1993), ungerminated sorghum also does not exhibit β-amylase activity. This is fundamentally different from barley, where the ungerminated grain does exhibit β-amylase activity. It appears that tropical cereal grains such as pearl millet, sorghum and maize possess only the "ubiquitous" form of β-amylase, whereas temperate Triticeae cereals such as barley, wheat and rye also possess the "endosperm specific" form of the enzyme, which is present in these grains at seed maturity (Zeigler, 1999).

Germination of sorghum grains at a temperature of between 25 and 30 °C is recommended for the development of optimum amylase and diastatic power in sorghum malt (Novellie, 1962; Okafor et al., 1980). At 30 °C, 3 to 7 days of sorghum grain germination produces well modified malts with a high diastatic power, and an increase level of hot-water extract, sugar content and free amino nitrogen (Morrall et al., 1986; Lasekan et al., 1995), but the optimal germination time and temperature vary with the sorghum variety (Novellie, 1962; Okafor et al., 1980; Demuyakor et al., 1992). Activity of α-amylase and β-amylase has been shown to develop to a greater extend in the yellow and red sorghum varieties than in white sorghum varieties when germinated at 30 °C (Agu et al., 1997b). However, germinating sorghum at the relatively high temperature of 35 °C or at lower temperatures of between 15 and 20 °C, slows down amylase formation and consequently reduces diastatic power (Morrall et al., 1986). Moreover, germinating sorghum grains heavily infected with moulds produce a malt with low amylase activity (Agu et al., 1999). Microbial infection of Nigerian sorghum grain has been shown to be caused by the presence of *Aspergillus* sp., *Penicillium* sp., *Neurospora* sp., *Rhizopus* sp., *Fusarium* sp., *Curvularia* sp. and *Dreschlera* sp. (Boboye et al., 1994). Formaldehyde (0.1%) can be added to the steep to retard fungal activity (Palmer et

al., 1989). As a result of fungal grain infection, some African traditional opaque beers have been reported to contain different amounts of aflatoxins (Nikander et al., 1991). Recently, studies carried out by Matumba et al. (2011) indicate the presence of aflatoxin (6,6 to 54,6 μg.kg-1) in a sorghum malt from Malawi.

Maltase, or α-glucosidase, which catalyses the hydrolysis of maltose into glucose, is present in ungerminated sorghum and does not increase significantly during malting. Sorghum maltase is a very heavy molecular weight enzyme, whose solubility characteristics differ from those of barley. Sorghum α-glucosidase is soluble in water, but is also active in its insoluble state and adheres strongly to insoluble solids (Novellie, 1982; Taylor et al., 1994). The development of α-glucosidase in sorghum is influenced by length of germination period and temperature (Agu et al., 1997a). In barley, α-glucosidase levels are generally lower than those of sorghum malt, especially at 30 °C and at day 5 of germination (Agu et al., 1997c). The sorghum malt with the highest maltase activity, however, produces the lowest glucose levels in wort, suggesting that maltase is not the dominant enzyme producing sugar during the mashing of sorghum malts (Agu et al., 1997a).

2.3. Kilning

Kilning involves the drying of the green malt in a kiln or oven at a relatively high temperature until the rootlets become friable or brittle. Kilning has the objective of stopping embryo growth and enzyme activity, while minimizing enzyme denaturation, and the process develops flavor and color (melanoidin compounds). In the African traditional sorghum beer brewing process, the germinated sorghum grains are dried under the sun and are stored under protection during the night to avoid rehydration. Generally, this drying step takes 2-3 days depending on sunlight intensity. However, in the Western beer brewing process, the germinated barley grains are kilned *via* a two-stage process. The grain are first of all dried at 50-60 °C and are then cured at 80-110 °C (Moll, 1991).

As part of the sorghum malting process, Novellie (1962) advocated the kilning of the malt up to 50 °C. However, whilst kilning periods at 80 °C may enhance the flavor of the malt, such a temperature may damage the enzymatic activity of the malt (Aisien et al., 1987) and reduce volatile compounds. Kilning malts in two stages, initially to 55 °C and subsequently to 65 °C, has been shown to produce good malts with a considerable reduction in moisture and a higher sugar content than kilning at a single temperature of 65 °C (Owuama et al., 1994). Thus, the two-stage kilning process allows for the greater survival of hydrolytic enzymes and for the malt to acquire its characteristic flavor.

3. MASHING

The objectives of mashing are to form and extract into solution, fermentable sugars, amino acids, vitamins, etc., from malt. Malt normally provides most of the potential fermentable materials and sufficient enzymes to generate a well balanced fermentation medium. African sorghum beer is unique as a fermented beverage in requiring starch, not only as a source of sugar, but as a thickening and suspending agent. Gelatinized starch gives the beer its characteristic creamy body and keeps in suspension the particles of grain and malt that are essential constituents of beer.

One of the problems involved in sorghum beer brewing is the efficient conversion of the starch extracts into fermentable sugars for yeast (*Saccharomyces cerevisiae*). Regarding the relative amounts of fermentable sugars in sorghum and barley worts, the major difference has been found to reside in the glucose content (Palmer, 1989; Dufour et al., 1992). While some studies have found barley malt worts to contain more maltose than glucose (Briggs et al., 1981; Dufour et al., 1992), others have reported that sorghum malt worts contain similar levels of glucose and maltose (Taylor, 1992). The difference observed in the proportions of glucose and maltose sugars in sorghum and barley malt worts has been attributed to the low

levels of β-amylase in sorghum malt (Palmer, 1989). Other authors (Taylor et al., 1994) have attributed the high level of glucose found in sorghum malt wort to the catalytic activity of α-glucosidase, from the maltase family, in hydrolysing maltose into glucose in sorghum malt wort. However, Agu et al. (1997c) showed that there is no direct relationship between the α-glucosidase levels in sorghum or barley malt and the maltose to glucose ratios found in their worts. It is worth noting that, in that study, barley malt developed a higher level of α-glucosidase than did sorghum malt, but that it produced less glucose and several times more maltose in the wort than it was the case in sorghum wort. The main reason for the limitation of maltose production in sorghum malt wort is likely to be inadequate gelatinization of sorghum starch rather than inadequate levels of hydrolytic enzymes (Dufour et al., 1992; Agu et al., 1997c). Results obtained by Agu et al. (1997b) showed that different sorghum varieties malted and mashed under similar conditions presented wide variations in their sugar profiles due to seasonal and processing differences. For example, the authors showed that, when malt is produced at 30 °C, the white (ISCV400) and yellow (SS20) sorghum varieties produce high glucose and maltose levels, while SS9 (red variety) and SS16 (white variety) produce low glucose and higher maltose levels. However, these last two varieties produce higher levels of glucose and maltose when malt is produced at 20 °C. However, the variations caused by seed variety and malting temperature do not alter the greater influence exerted by starch gelatinization on the sugar profile of sorghum worts than on the sugar profile of barley worts (Agu et al., 1998).

Generally, sorghum starch gelatinization temperatures (67-81 °C) are far higher (Akingbala et al., 1982; Beta et al., 2001) than the range quoted for barley starch of 51-60 °C (Lineback, 1984). Furthermore, these temperatures increase the thermal deactivation of the sorghum malt enzyme (Guerra et al., 2009). Consequently, the simultaneous gelatinization and hydrolysis of starch, which occurs during mashing of the barley malt, is problematical in

the case of sorghum malt. Although short mashing (gelatinization) periods at 75 °C followed by a conversion (saccharification) period at 65 °C would improve the development of extracts from sorghum malt, unacceptable extract loss would still occur because of enzyme inactivation and inadequate gelatinization (Palmer et al., 1989). Indeed, relatively high levels of starch extract comparable to those of barley malts have been obtained by using a non-conventional mashing procedure. The procedure involves, decanting active enzyme wort after mashing sorghum malt at 45 °C for 30 min, and gelatinizing the starchy grist residue at 80 to 100 °C before mixing with the wort, to achieve a saccharifying temperature of 63-65 °C (Palmer, 1989; Igyor et al., 2001). The viscosities of sorghum malt worts have been shown to be similar to those of barley malts (Igyor et al., 2001), but the fermentable extracts of these sorghum worts have been shown to be still lower than those of barley malt (Palmer, 1989). These results suggest that small quantities of β-amylase in sorghum wort also affect saccharification.

The drawbacks highlighted above in using sorghum malt in beer brewing led to the approach proposing the use of mixtures of malted barley (30-40%) with sorghum (60-70%) during mashing (Okafor et al., 1980; Goode et al., 2003), or the addition of exogenous enzymes to the unmalted sorghum (Dale et al., 1989; Bajomo et al., 1994). In this last case, the addition of external enzymes is associated with processing difficulties such as α-amino nitrogen (FAN) depletion (Dale et al., 1989). The advantage of including a percentage of malted sorghum as a source of endogenous proteases has also been reported. The addition of malted sorghum avoids the need to add these enzymes, thereby avoiding the poor foam retention associated with commercial proteolytic enzymes (Agu et al., 1998). However, these optimal solutions for reducing the levels of non-fermentable sugars in sorghum worts are inappropriate in an African traditional brewing context because the tropical climate is not conducive to barley cultivation, and commercial enzymes are very expensive. Nevertheless, a 20% (w/v) sweet

potato flour substitution for sorghum malt has been shown to increase the level of β-amylase in sorghum wort (Etim et al., 1992). Pearl millet (*Pennisetum glaucum*) malt also appears to have some advantages compared to sorghum as it has a higher β-amylase activity and higher FAN levels (Pelembe et al., 2004). In Rwandan traditional sorghum beer brewing, the association of sorghum malt and *Eleusine coracana* (*uburo*) or the addition of banana juice (*umutobe*) during sorghum malt mashing increases the fermentable sugars in sorghum wort (Lyumugabe et al., 2010). As β-amylase is the enzyme responsible for the hydrolysis of starch into maltose, the high level of activity of this enzyme in *Eleusine coracana* malt compared with sorghum malt (Taylor, 2009) could explain the increase in fermentable sugars in this wort. However, brewing processes using a mixture of sorghum with local cultures have not been extensively investigated.

Generally, after mashing, the mash is filtered before boiling. During the African traditional beer brewing process, filtration is achieved by simple decantation (Lyumugabe et al., 2010) or *via* a rudimentary press filter made of a nylon cloth stretched over a bowl and raked with a wooden stick (Maoura et al., 2009). In comparison with barley, sorghum malt mashes filter poorly (Aisien et al., 1987). This is clearly related to differences in the qualities of the endosperm cell walls of sorghum and barley. Unlike in barley, the endosperm cell walls in sorghum are not substantially broken down during malting (Glennie, 1984). The cell walls themselves are rich in water-unextractable glucoronoarabinoxylans (Verbruggen, 1996) and sorghum malt appears to be deficient in the wall degrading enzyme endo-β-glucanase (Aisien et al., 1983). This seems to pose a serious filtration problem for sorghum malt mashes, and the addition of exogenous hemicellulolytic enzymes is probably only a short-term solution.

Boiling of wort is performed for several reasons, in particular to bring about the denaturation of malt enzymes and any enzymes supplements, and the sterilization of the wort. Although this stage exists in the brewing process of many African traditional sorghum beers

(*e.g. dolo, tchoukoutou, amgba*) (Chevassus-Agnes et al., 1979; Dicko et al., 2006; Kayodé et al., 2007b), it is absent from the brewing process of traditional sorghum beers (*e.g. ikigage, mtama, impeke*) from East African countries (Tisekwa, 1989; Nzigamasabo et al., 2009; Lyumugabe et al., 2010). In the European beer brewing process, barley wort obtained from the mash is transferred to a "copper" ("kettle") for boiling, along with dried hops or hop extracts. Hops are the flower cones of the female hop vine (*Humulus lupulus*), and they contain α and β acids, primarily humulones and lupulones. These give to beer its bitter flavor, after isomerization of α-acids into iso-α acids during boiling, and they also help inhibit certain beer spoilage bacteria and maintain foam stability. African traditional sorghum beers are generally unhopped. However, several studies have reported the possibility of using African plants (*e.g. Vernonia amygdalina, Gongronema latifolium, Garcinia kola*) instead of hops in African sorghum beers (Ogundiwin et al., 1991; Okoh et al., 1995; Ajebesone et al., 2004; Okoro et al., 2007; Adenuga et al., 2010) because the hop plant is a temperate crop and cannot be successfully grown in Africa tropical countries. *Vernonia amygdalina*, known as "bitter leaf", can be used instead, because it resembles hops in its antimicrobial properties (Mboto et al., 2009; Oboh et al., 2009) and bitter flavor (Ajebesone et al., 2004; Adenuga et al., 2010). Furthermore the addition of extract of *V. amygdalina* leaves to sorghum wort increases the levels of amino acids, mainly isoleucine, leucine and histidine (Lasekan et al., 1999). However, further research needs to be directed in particular towards the contribution of this plant to the organoleptic properties of sorghum beer.

4. FERMENTATION

Fermentation is the important step by which yeast converts the sugars in the wort into ethyl alcohol. In Western breweries, the fermentation process is started by selected yeast strains (*S. cerevisae* or *S. carlsbergensis*) and the fermentation time ranges between 8-15 days at 10-16

°C (Moll, 1991; Waites et al., 2001). In the case of African traditional sorghum beers, sorghum wort is inoculated with a traditional leaven, and fermentation time varies between 10 and 24 h in ambient temperature.

African traditional leaven is a result of the spontaneous fermentation of sorghum malt wort (Kayodé et al., 2005; Lyumugabe et al., 2010). The manufacturing methods of this leaven are diverse in Africa and depend on built-in ingredients. **Table 1** shows the types of microorganisms involved in spontaneous fermentation in traditional sorghum beer brewing. Very varied yeasts and bacteria flora have been found in African sorghum beers, although *S. cerevisiae* and *Lactobacillus* sp. usually predominate (Novellie, 1976; Maoura et al., 2005; Kayodé et al., 2007a; Lyumugabe et al., 2010). Unlike European beer made with barley, African sorghum beers are typical examples of lactic fermentation followed by alcoholic fermentation in which initially, lactic acid bacteria (LAB), and later yeasts, play the dominant role (Novellie, 1982; Holzapfel, 1997; Kayodé et al., 2005; Maoura et al., 2009). Due to their higher growth rate, bacteria typically dominate the early stages of fermentation. A symbiotic relationship could explain the simultaneous presence of yeasts and LAB. LAB create an acid environment favorable to the proliferation of yeasts. These yeasts produce vitamins and increase other factors, such as amino acids, to aid the growth of LAB.

Unlike European beers, where the desired flavor is often critically affected by wild yeasts and other microorganisms, African beers may display a wide variation in tastes and aromas while still being acceptable to the consumer. As in the case of the Belgian beer, Lambic, African sorghum beers are the product of more or less spontaneous fermentation, in that pitching is not practiced. On the other hand, African sorghum beers differ from Lambic in that the Belgian beer is subjected to a very long post-fermentation period during which yeasts of the genus *Brettanomyces* are responsible for creating the typical bouquet of that beer (Van der Walt, 1956).

Table 1. Micro-organisms involved in the fermentation of some African traditional sorghum beers – Microorganismes impliqués dans la fermentation de la plupart des bières traditionnelles africaines à base de sorgho.

Beer name	Predominant microorganisms involved	Country	Reference
Ikigage	*Saccharomyces cerevisiae Issatchenkia orientalis, Lactobacillus fermentum, Lactobacillus buchneri , Lactobacillus* sp.	Rwanda	Lyumugabe et al., 2010
Tchoukoutou	*S. cerevisiae Torulaspora delbrueckii, S. pastorianus, Lactobacillus divergens, Lb. fermentum, Lb. fructivorans , Lb. sp.*	Benin	Kayodé et al., 2005 Kayodé et al., 2007
Bili bili	*S. cerevisiae, K. marxianus, Cryptococcus albidius, Debaryomyces hanseni, Lactic acid bacteria*	Chad	Maoura et al., 2005
Burkutu	*S.cerevisiae, S. chavelieri, Leuconostoc mensenteroides, Candida acetobacter*	Nigeria and Ghana	Blandino et al., 2003 ; Van der Aa Kühle et al., 2001
Pito	*S. cerevisiae, Candida tropicalis, Kloeckera apiculata, Hansenula anomala, Torulaspora delbrueckii, Schizosaccharomyces pombe, K.africanus, Lactobacillus spp,Leuconostoc* spp.	Ghana	Sefa-Dedeh et al., 1999 ; Van der Aa Kühle et al., 2001
Dolo	*S. cerevisiae, Lb. Delbrueckii,Lb ; fermentum Pediococcus acidilactici, Lb.lactis, Lc lactis,*	Burkina faso	Van der Aa Kühle et al., 2001 ; Sawadogo-Lingani et al., 2007 ; Glover et al., 2009
Doro or *Chibuku*	*S. cerevisiae, Lb plantarum, Lb. delbrueckii, Lc. Lactis, Lc. raffinolactis*	Zimbabwe	Jespersen, 2003 ; Chamunorwa et al., 2002;
Kaffir	*S. cerevisiae, Candida krusei, Kloeckera apiculata, Lb fermentum, Lb plantarum, Lb brevis, Lc dextranicum*	Southern Africa	Van Der walt, 1956

5. TYPES OF AFRICAN TRADITIONAL SORGHUM BEER BREWING

Generally, African traditional sorghum beers are brewed with pigmented sorghum varieties (red or brown). The white varieties are always mixed with red sorghum because consumers prefer to drink colored beers which they believe to be healthy (Kayodé et al., 2005). These African sorghum beers are not a clear, sparkling liquid, but opaque with suspended solids (5-7%). The beers have a rather low alcohol content (2-4.5% v/v), a pH of between 3.3 and 4 and a lactic acid rate of about 0.26%. Their color varies from a pale buff to a pinky brown according the ingredients used. Usually, African sorghum beers have a touch of fruitiness added to their fermentation odor. They are beer is consumed in an actively fermenting state and therefore their shelf life is a quite short (24 h-72 h) (Novellie et al., 1986; Tisekwa, 1989; Maoura et al., 2009; Lyumugabe et al., 2010). However, African traditional sorghum beers vary in their denomination and their production processes, according to their geographic localization.

5.1. *Ikigage* of Rwanda

Ikigage or *amarwa* is a traditional alcoholic beverage manufactured in Rwanda with malted sorghum (**Figure 2**). The traditional process of *ikigage* manufacture has been described by Lyumugabe et al. (2010). After washing, red sorghum grains are immersed in water (*kwinika*) for 24 h. The grains are then drained in a bag with a stone top for 48h so that the process of germination is completed and grain rootlets appear (*kumera*). The grains are spread out on a cloth in a wet place. Ash is spread over the cloth and leaves of the eucalyptus or banana tree are laid on top of the ash. The sorghum grains are then spread out on the leaves, to encourage germination. The grains are dried under the sun for at least two days at 29 °C. When the grains are semi-dry, the rootlets are removed (*kuyavunga*). The semi-dry malt grains are ground or crushed. Brewers heat water (20 l) to boiling and add approximately 2 kg of ground

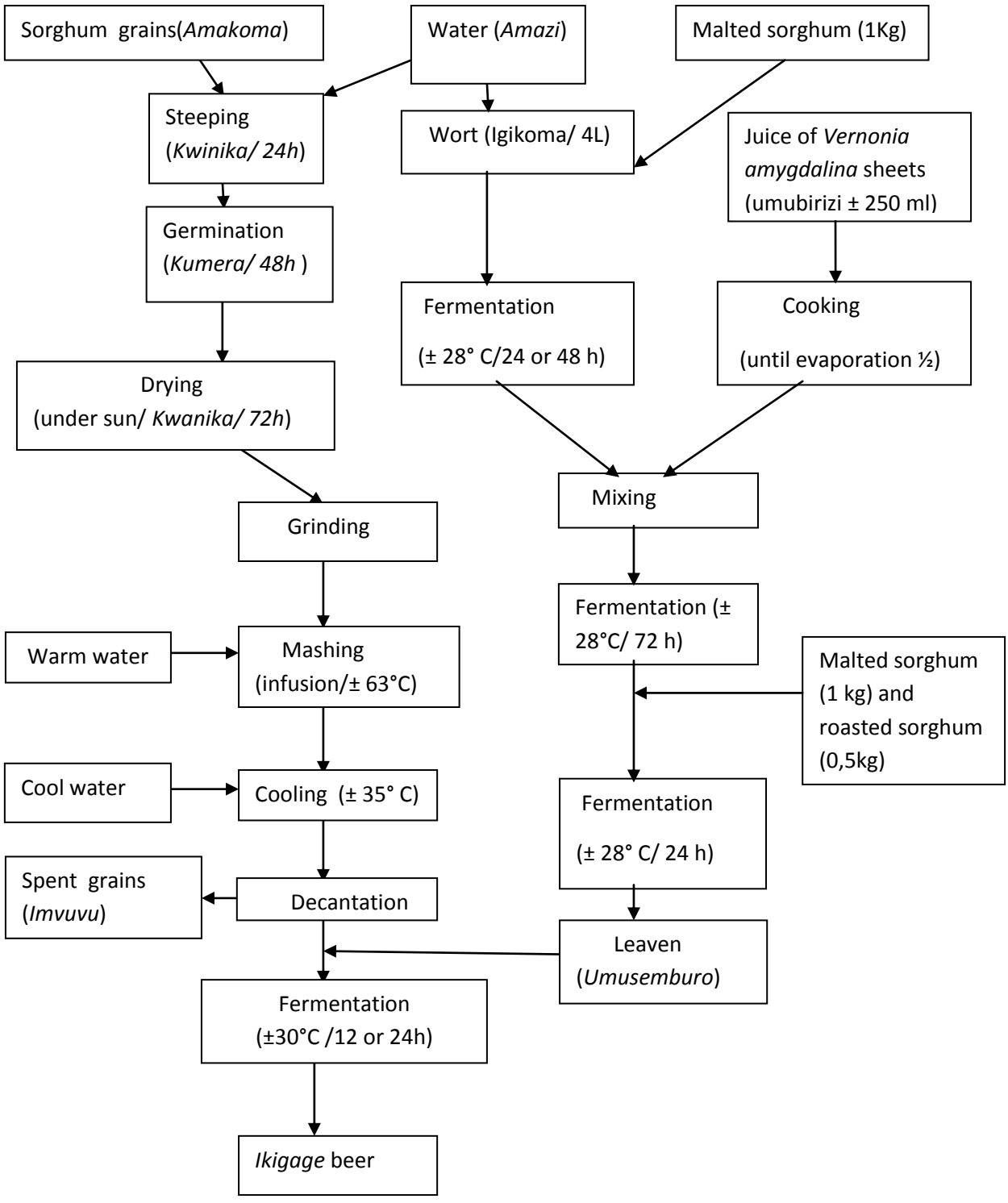

Figure 2. Traditional manufacturing processes of traditional sorghum beer *ikigage* (adapted from Lyumugabe et al., 2010) – Processus traditionnel de la fabrication de la bière de sorgho *ikigag* (adapté à partir de Lyumugabe et al., 2010).

malt grains in order to gelatinize the starch. This solution is then mixed with ground malt (16 kg) in a large container. The mixing temperature is typically between 63 °C and 71 °C. Following the infusion process, cool water is added (40 l) to bring the temperature back to between 34 °C and 40 °C. In some cases, brewers leave a decanter of this mixture to rest for approximately 3 h in order to eliminate the draffs (*imvuzo*). After cooling, the traditional leaven (*umusemburo*) is inoculated in order to start the fermentation process. The fermentation container is covered with leaves of the banana tree, and then by a cloth and a lid. After 12 to 24 h of fermentation, *ikigage* is ready for consumption. The ethanol levels, soluble protein and the pH of *ikigage* are 2.2% (v/v), 9.2 g.l-1 and 3.9 respectively (Lyumugabe et al., 2010).

5.2. *Merissa* of Sudan

Merissa is a traditional alcoholic beverage manufactured in Sudan using malted red sorghum or millet. Dirar (1978) describes a complex procedure (**Figure 3**) for making *merissa* beer. The red sorghum grains are malted, dried and reduced into flour. Ungerminated sorghum is milled into a fine flour and cooked in two equal lots: the first lot is lightly cooked to a greyish brown paste while, the second lot is well cooked to a brown paste. These two lots are then mixed and allowed to cool. The resulting product, "*futtara*", is a gelatinized solid material. One part malt flour is mixed with a quantity of water necessary for good humidification and is incubated at room temperature for 36 h until lactic fermentation occurs. The acid paste obtained (called "*ajeen*") is cooked in a container and mashing is then carried out until the substance takes on a chestnut color, with a high acidification and a caramel flavor. This product (called "*soorij*") is then cooled. Malt (5%), water and an inoculum of a previous *merissa* product are then added to the *soorij* and the mixture is left to ferment for 4-5 h. The resulting product (called "*deboba*") is a vigorously fermenting, thick, dark suspension that is too sour to drink. After cooking, *futtara* is mixed with about 5% malt flour and is successively

added to the *deboba*. After 8-10 h of fermentation, the product, *merissa*, is filtered through a suitable fabric mesh to partially retain the solid particles, while the liquid undergoes full fermentation. The resulting drink has a pH of 4 and an alcohol level of around 5% (v/v).

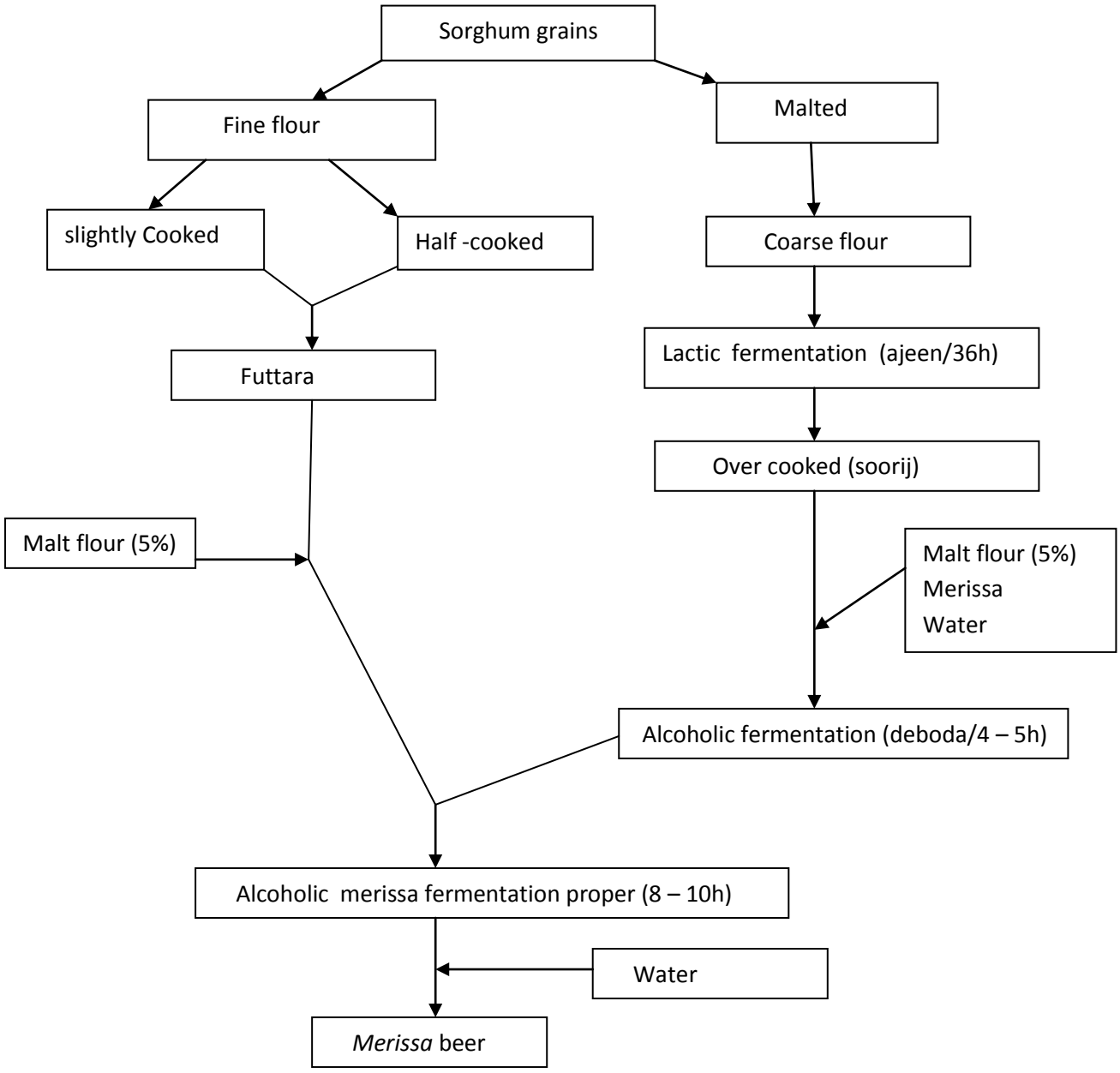

Figure 3. Traditional manufacturing processes of *merissa* beer (Dirar, 1978) – Processus traditionnel de la fabrication de la bière *merissa* (Dirar, 1978).

5.3. *Doro* of Zimbabwe

The traditional sorghum beer of Zimbabwe is known as *doro*, *chibuku*, *hwahwa*, *mhamba* or *uthwala* in different regions of the country (Chamunorwa et al., 2002). The traditional *doro* brewing process (**Figure 4**) has been described by Benhura et al. (1989). The brewing process starts with the malting of red sorghum to produce a substance called *masvusvu*. Sorghum grains are soaked in water for 24 h at room temperature. They are then placed in a sack, washed and left to germinate for 2-3 days at room temperature. After this stage, the seedlings are sun-dried for 3 days and the semi-dry malt grains are ground or crushed. Approximately 24 l of water is mixed with 7 kg of sorghum malt flour. This mixture is heated while stirring until boiling. The *masvusvu* is cooled, diluted in clay pots and left to sour at ambient temperature for about 2 days. On the third day, the soured product (*mhanga*) is boiled for 3-5 h, reducing the original volume by a quarter in the process. The boiled *mhanga* is allowed to stand overnight after which time, more malt flour is added. Typically, the amount of malt added is about half the amount used at the beginning of the brewing process. On the sixth day, some *masvusvu*, two to three times more than the amount cooked on the first day, is prepared and allowed to cool. Meanwhile, a small portion of the *mhanga* is strained and kept separately. The strainings (*masese*), the rest of the unstrained *mhanga* and the fresh portion of cooled *masvusvu* are all mixed together with water to yield *biti*. The mixture is left to ferment for about 2 h and the resulting product, called *madirwa*, is then strained, mixed with the previously strained *mhanga* and left to ferment overnight. The fermentation process takes 5-7 days depending on ambient temperature. Ethanol is thought to be the main alcohol contained in the final *doro* beer (about 4% v/v).

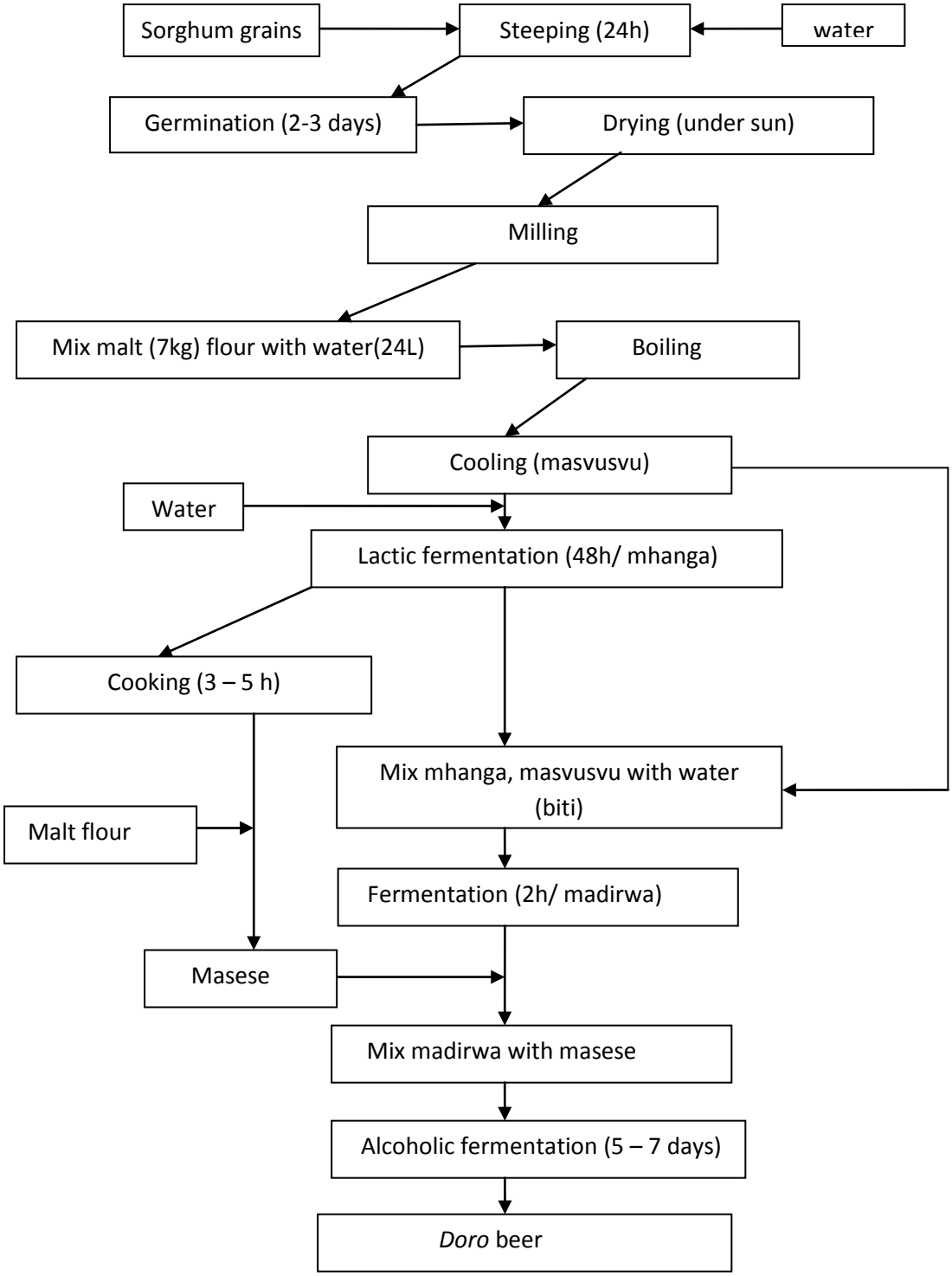

Figure 4. Traditional manufacturing processes of *doro* beer – Processus traditionnel de la fabrication de la bière *doro*.

5.4. *Dolo* of Burkina Faso

Dolo is a popular traditional alcoholic beverage manufactured in Burkina Faso, and is most often made from red sorghum malt (Hilhorst, 1986). The traditional malting process for *dolo* (**Figure 5**) is similar to that described for the *ikigage* beer. The malt obtained is used by the traditional brewers ("*dolotières"*) to prepare the *dolo* beer. Sorghum malt flour is mixed with water (1:10, w/v) and the mixture is then decanted (for approximately 10-12 h) to separate off the enzymatic supernatant phase with precipitate containing starch. Water is added to the precipitate and the mixture is boiled to gelatinize starch, but the supernatant is not boiled (Dicko et al., 2006). After cooling, the precipitate is filtered to separate the soluble components (starch, sugars, proteins, etc.) and the residue (used as animal feed). The filtrate is mixed with previous supernatant and boiled at 65-70 ºC for 12-16 h in order to obtain the wort. This method seems to be a good traditional mashing process for producing sorghum wort with a high fermentable rate for sugars, because the process overcomes the problem of sorghum starch gelatinization and hydrolytic enzyme denaturation. After this stage, the wort is cooked and then cooled overnight to room temperature (30-40 ºC). The cooled wort is inoculated with a traditional leaven to start the fermentation process, leading to the *dolo* beer after 12-24 h (Griffon et al., 20011, cited by Maoura et al., 2009). The final *dolo* beer is opaque, with a red color, an alcohol content of 2-4% v/v and a pH of 4-5 (Dicko et al., 2006).

5.5. *Pito* and *burukutu* of Nigeria

Pito and *burukutu* are traditional Nigerian alcoholic beverages brewed with red or white sorghum malt and/or maize. The brewing process for *pito* (**Figure 6**) has been described by Ekundayo (1969). Briefly, sorghum grains are steeped in water (24-48 h) and then, drained. The grains are then allowed to germinate for four to five days and are sun-dried before grinding. The malt flour is mixed with water and the mixture is then boiled for 3-4 h to form a

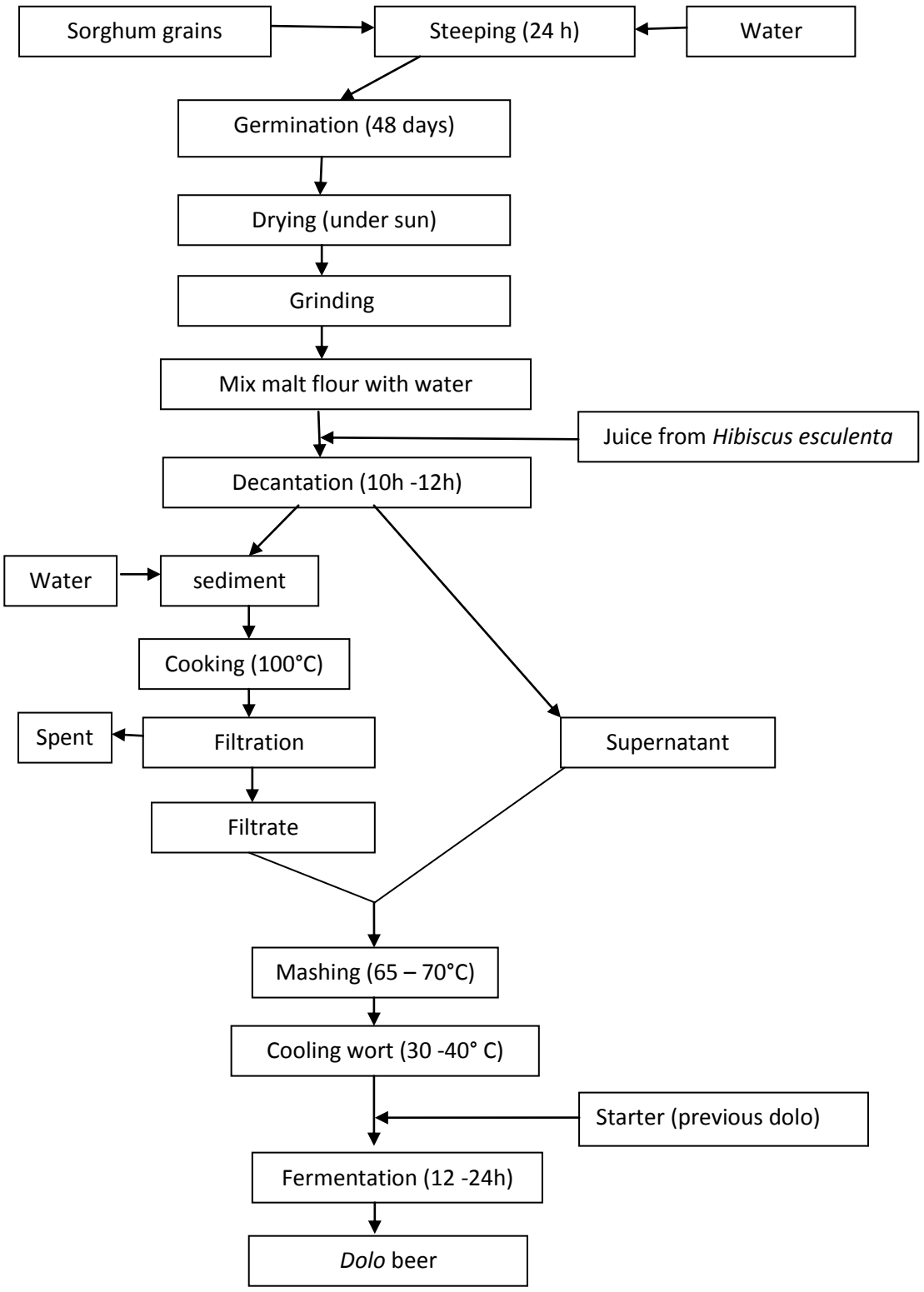

Figure 5. Traditional manufacturing processes of *dolo* beer – Processus traditionnel de la fabrication de la bière *dolo*.

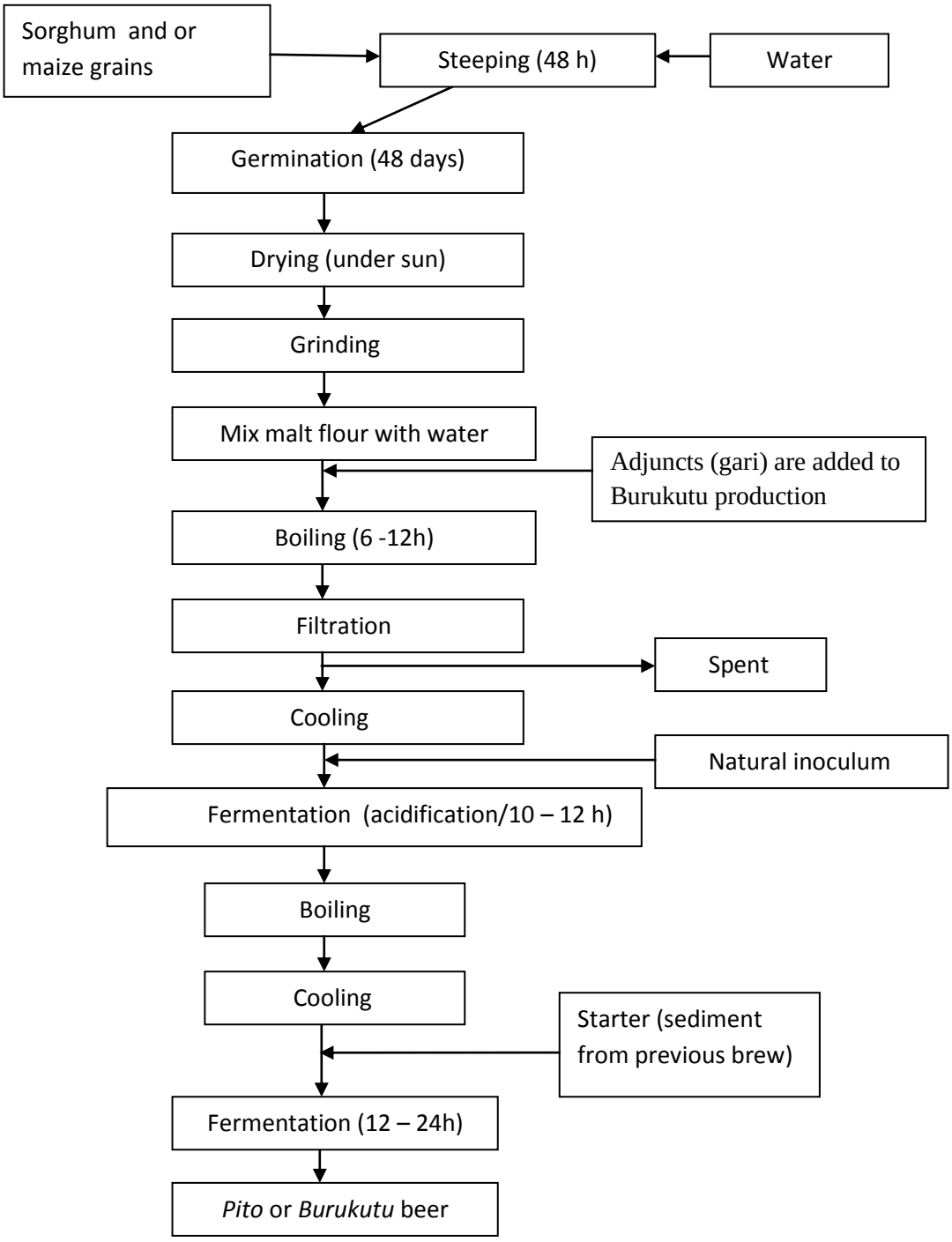

Figure 6. Traditional manufacturing processes of *pito* beer (Asiedu, 1991 ; Achi, 2005) – Processus traditionnel de la fabrication de la bière *pito* (Asiedu, 1991 ; Achi, 2005).

slurry. During the mashing stage of *burukutu* production, adjuncts are added in the form of *gari* (a farinaceous starchy powder produced from cassava, *Manihot esculenta*). However, adjuncts are not added during the production of *pito* (Faparusi et al., 1973; Ekundayo, 1969). After cooling, the paste is filtered and left in spontaneous lactic fermentation (acidification) at room temperature for approximately 12 h. More water is added and the mixture is then cooked for 3 h and cooled to around 20 to 29 °C. Cooled wort is subsequently left to ferment at room temperature for 12-24 h. The two resulting products are: a top clear supernatant, called "*pito"* and a thick brown suspension, called "*burukutu*".

5.6. *Amgba* or *bili bili* of Cameroon

Amgba, well known by the name *bili bili*, is a very popular traditional alcoholic beverage in Cameroon (among the Baya ethnic group). The drink is brewed primarily using sorghum malt (*mouskouari* or *djigari* variety), but millet malt (*fonio* variety) can also be associated with *mouskouari*. The traditional brewing process (**Figure 7**) for this beer has been described by Chevassus-Agnes et al. (1979). The sorghum grains are soaked in water for 12 to 72 h at room temperature in order to obtain a moisture level of 35 to 40%. The grains are then left in a heap in a container or spread out on a germination device (green plantain leaves, beaten soil, rocks) to form a layer (3 to 5 cm in thickness) and are kept covered until rootlets appear. If needed, initial moisture levels are maintained by spraying with water. The germination time is on average 4 days. After this step, the grains are dried under the sun for a maximum 1 day and ground into a fine flour. This malt flour is then mixed with water and sap (*gombo*) from trees. In particular *gombo* from *Triumfetta* sp. seems to improve the flocculation and filtration of the insoluble matter during decantation. This operation using the sap resembles that carried out during the clarification of barley beers in European breweries. However, the sorghum beer clarification process using *gombo* after fermentation has not been extensively investigated. After 1 to 2 h of decantation, the enzymatic supernatant phase is carefully collected, while the

settled residue is cooked until boiling in order to gelatinize the starch. After cooking, the thick mash obtained is mixed with the previous supernatant at 65-70 °C. The mixture is then filtered by decantation or using a traditional device similar to the filter-tank used in industrial Western brewing. Very often, the traditional brewer leaves the filtrate in spontaneous lactic fermentation to acidify the wort. After boiling, the wort is cooled to approximately 30 °C and then inoculated with traditional leaven, "*affouk*" to start fermentation. After 12 to 24 h of fermentation, the resulting *amgba* can be consumed.

5.7. *Tchoukoutou* of Benin and Togo

Tchoukoutou, or *chakpalo* is a traditional alcoholic beverage produced in Benin and Togo principally using sorghum malt (red and brown varieties), but other starch sources, such as millet or maize can be used as adjuncts or as substitutes (Kayodé et al., 2005; Osseyi et al., 2011). *Tchoukoutou* and *chakpalo* are distinguishable by both their appearance and taste. *Tchoukoutou* is an opaque (turbid) and acidic beer while *chakpalo* is a clear and sweet fluid beer. The traditional brewing process (**Figure 8**) for *tchoukoutou* has been described by Kayodé et al. (2005, 2007b). Approximately 27 kg of grains are soaked in water for 9 to 12 h and then left to germinate during 72 to 85 h. After this step, the grains are dried under the sun (7-15 h) and ground into a fine flour. This malt flour is then mixed with water and left in decantation. After decantation (1-2 h), the enzymatic supernatant phase is collected and the residue containing starch is gelatinized by gradual heating until boiling for 2 h. The thick mash obtained is mixed with the previous supernatant phase and left in a state of acidifying (lactic) fermentation (13-14 h). After this stage, the mixture is filtered to obtain the wort. After cooking (6-9 h), cooled wort is inoculated with a traditional leaven (known as *kpètè-kpètè* in the Bariba, Denti and Yoruba languages) in order to start alcoholic fermentation. After 13 to 14 h of fermentation, *tchoukoutou* is ready for consumption. This beer is sour with

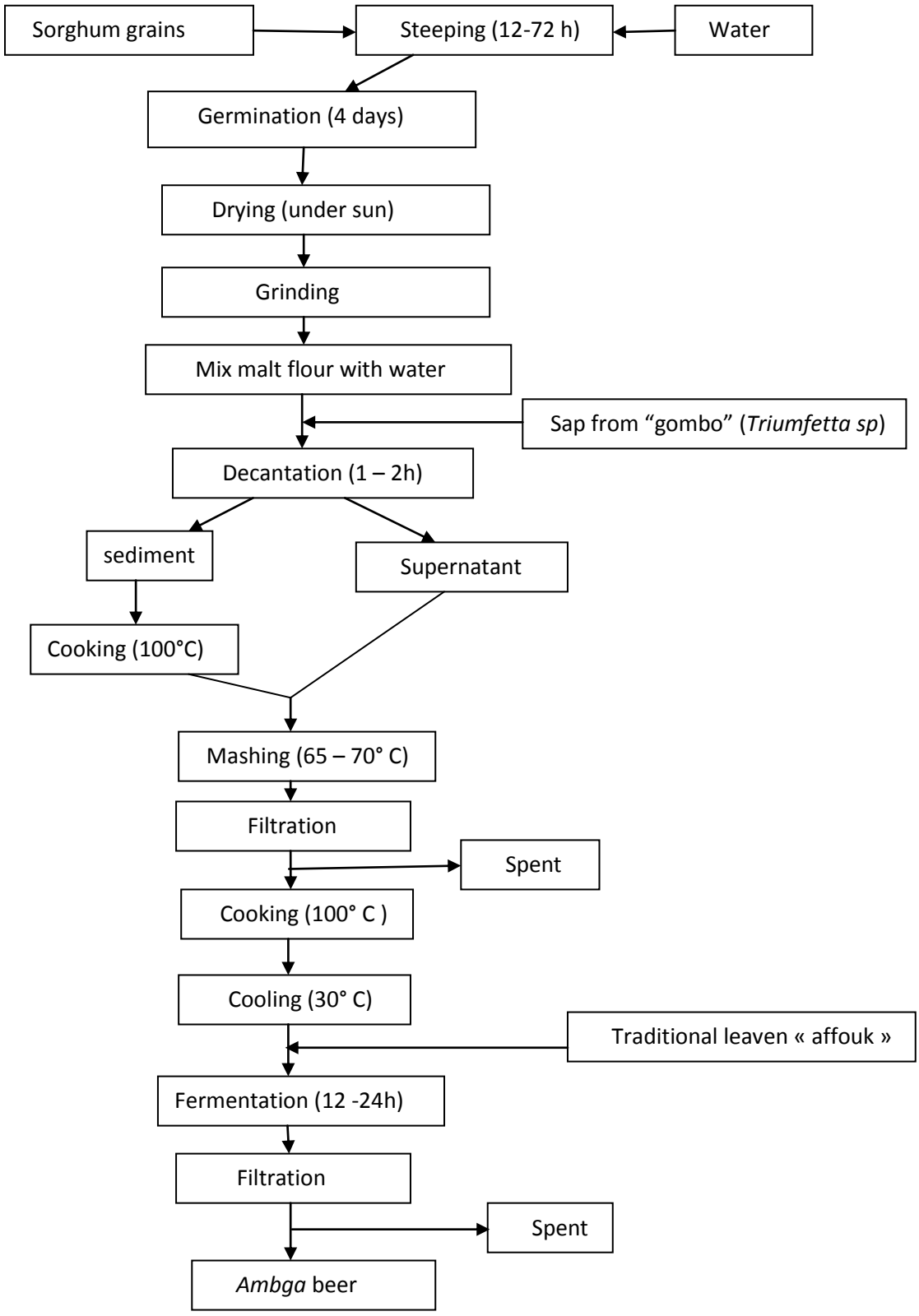

Figure 7. Traditional manufacturing processes of *ambga* beer (Chevassus et al., 1979) – Processus traditionnel de la fabrication de la bière *ambga* (Chevassus et al., 1979).

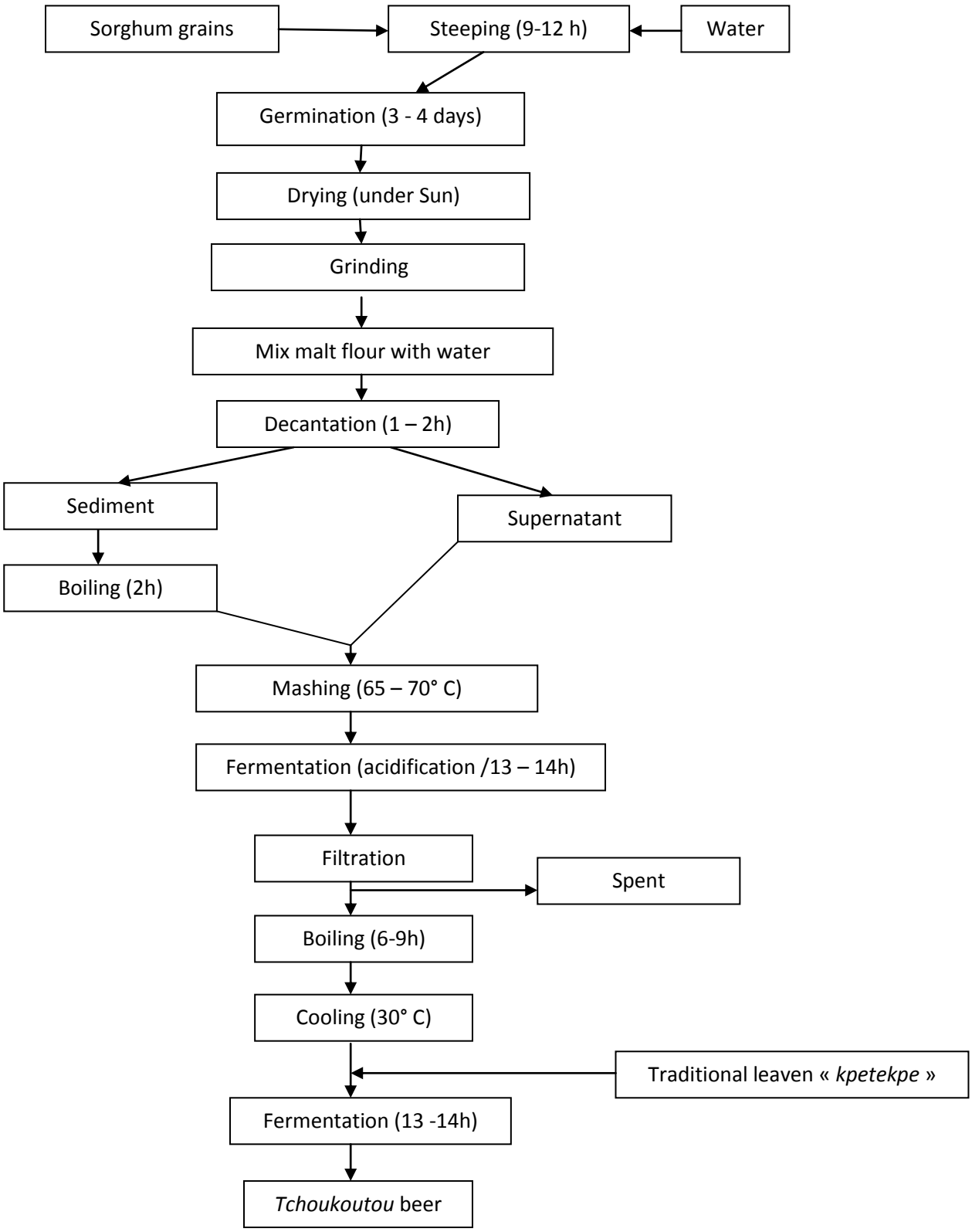

Figure 8. Traditional manufacturing processes of *tchoukoutou* beer – Processus traditionnel de la fabrication de la bière *tchoukoutou*.

This beer is sour with a pH of 3.2: it contains a relatively high but variable level of solids and crude protein (Kayodé et al., 2007b) and has a 4% (v/v) alcohol content (Osseyi et al., 2011).

6. SOCIO-CULTURAL ASPECTS AND NUTRITIONAL STATUS OF SORGHUM BEER

African cereal beers (made from sorghum, millet, maize, etc.) have ancient origins. They may have originated in Egypt or Mesopotamia, where beers were being produced by at least 3,500 BC, and probably much earlier (Briggs et al., 1981). The first mentions of sorghum beer or millet beer come from the Arab travellers who, in the 6th and 7th centuries, praised the merits of beer manufactured in the Sahel region, in particular the *merissa* beer of Sudan (Huetz de Lemps, 2001). The manufacturing of sorghum beers is a tradition preserved by African women brewers and passed down to the next generation. In African tradition, sorghum beer symbolizes the woman, representing silence and a tacit acceptance of the "*entente*" between the peoples. In ancient times, royalties due to the local authorities were paid only in the form of sorghum or sorghum beer. Sorghum beer is called the "milk of the hoe" in Africa (*amata y'isuka* in the Rwandese language), affording the beer noble qualities (De Lame, 1995). Sorghum beer is an ancestral beverage widely used in various festivals and African ceremonies such as marriage, praying for rain, communication with ancestors, births, the handing-over of a dowry, circumcision, burial ceremonies, and the popular annual sorghum festival (Kayodé et al., 2007b; Lyumugabe et al., 2010). In Rwanda or Burundi, dowry handing-over ceremonies start initially with the consumption of traditional sorghum beer. The representatives of the two families greet each other around a clay jug (called an *ikibindi*) filled with sorghum beer because *ikigage* beer symbolizes the complementarity of the sexes (De Lame, 1996). Traditional sorghum beer is also consumed after community work or meetings

of mutual associations, in order to provide energy (Van Liere, 19932, cited by Kayodé et al., 2007b).

Traditional sorghum beer is mainly consumed by the poorest in society, and contributes significantly to the diet of millions of African people (Kayodé et al., 2007b). It is very rich in calories. It is also rich in the B-group vitamins including thiamine, folic acid, riboflavin, and nicotinic acid and is high in essential amino acids such as lysin (**Table 2**).

According to Chevassus-Agnes et al. (1979), the significant dry matter losses during sorghum beer preparation seem to be balanced by the improvement in protein and amino-acid digestibility, mineral availability and vitamin content. Germination increases the digestive availability of essential amino acids, which is preserved in subsequent stages of production (Taylor, 1983). Fe solubility gradually increases during the beer making process (germination and fermentation) and is highly correlated with phytate and reactive phenolic compounds in the product. However, important losses of minerals occur during the beer making process, particularly during the mashing stage; thus, the quantity of Fe available to consumers is restricted (Kayodé et al., 2007a). Phenolic groups and tannins present in sorghum grain impair the grain's nutritional value by sequestering exogenous and endogenous proteins in the form of indigestible complexes (Maoura et al., 2009). On the other hand the beer brewing process removes significant amounts of tannin (Dhanker et al., 1987; Osuntogun et al., 1989). Nevertheless, the nutritional value of sorghum beers is generally higher than that of European barley beers (**Table 3**) due to the presence of yeast, lactic acid bacteria and other suspended material. Due to their low alcohol content and the large quantity of suspended solids, many consumers consider these indigenous fermented sorghum beers to be more of a food than a beverage.

Table 2. Comparison of chemical compositions of sorghum grain, malted sorghum and Cameroonian sorghum beer "amgba"(expressed for 100g dry matter) – Comparaison de la composition chimique de grain de sorgho, malt de sorgho et bière de sorgho du Cameroun "amgba" (exprimé à 100g de matières sèches).

	Grain	malt	Amgba
Calories (kj)	381	380	394
Protein (g)	9,4	9,8	8,7
Lysine (g % proteins)	3,3	3,7	7,2
Lipids (g)	2,8	2,2	0,3
Total sugars (g)	85,6	86,2	86,1
Non – digestible sugars (g)	2,3	3,7	0,3
Ash (g)	2,1	1,7	4,1
Calcium (mg)	11	9,3	20,7
Total phosphorus (mg)	319	327	630
Phytic phosphorus (mg)	166	85	112
Potassium (mg)	391	361	1101
Sodium (mg)	14,5	14,7	26,9
Thiamine (µg)	407*[1]	426*[2]	3441*[3]
Riboflavine (µg)	98	231*[4]	760
Niacin (mg)	4,3	5,3	8

Source: adaptation of Maoura et al. (2009) from Chevassus et al. (1979) – Adaptation de Maoura et al. (2009) à partir de Chevassus et al. (1979) ; Extreme values: [(1)] 170 – 545; [(2)] 168 -565; [(3)] 1693 – 5241; [(4)] 169 – 300 – Valeurs extrêmes : [(1)] 170 – 545; [(2)] 168 -565; [(3)] 1693 – 5241; [(4)] 169 – 300.

Table 3. Nutrients in African and European beers (per 100g portion) – Eléments nutritifs des bières Européennes et africaines (par 100g portion).

	African traditional beers	**European lager beers**
calories (kj)	155	164
Dry matter (g)	7,9	4,0
Insoluble dry matter (g)	3,9	0
Protein (Nx 5,7)	0,6	0,3
Carbohydrate (g)	4,8	3,2
Alcohol (g)	2,9	4,0
Ca (mg)	2,2	6,3
P (mg)	39	40
K (mg)	84	47
Na (mg)	1,1	3
Fe (mg)	2,55	0,1
Vitamin B_1(mg)	0,11	0,003
Vitamin B_2 (mg)	0,05	0,04
Niacin (mg)	0,43	0,71
Vitamin B_{12} (µg)	0,03	-
Pantothenic acid (mg)	0,09	0,18
Vitamin C	0,04	_

Source: Nout (1987)

7. SHELF LIFE OF TRADITIONAL SORGHUM BEER

Traditionally-made sorghum beers have a poor keeping quality. The limited shelf life (stability) of sorghum beers has been reported as the major problem confronting commercial brewers in Sudan (Dirar, 1978), in Tanzania (Tisekwa, 1989), in Nigeria (Sanni et al., 1999) and in Rwanda (Lyumugabe et al., 2010).

Sorghum beer is consumed while it is still fermenting. The wort from which the beer is made is not heated – or otherwise treated prior to the addition of yeast, and the drink therefore always carries a residual microflora originating mainly from its ingredients. The resulting beer is thus microbiologically unstable *i.e.*, infected at varying levels with yeasts and bacteria. Sanni et al. (1999) isolated the following bacteria from deteriorating sorghum beer (*pito* and *burukutu*): *Aspergillus aceti*, *Aspergillus hansenii*, *Aspergillus pasteurianus*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus brevis*, *Alcaligenes*, *Saccharomyces*

cerevisiae, *Micrococcus* spp., *Candida* spp., *Bacillus licheniformis*, *Flavobacterium* spp., *Candida mycoderma*, *Hansenula anomala*, *Saccharomyces diastaticus* (questionable), *Bacillus* spp. and *Rhodotorula* spp.

Sorghum beers spoil rapidly because they are actively fermenting when solid, with organisms in addition to yeasts flourishing in the rich medium. During fermentation, yeasts initially increase in number. Then in the later stage of logarithmic growth the production of ethanol starts and proceeds during the stationary phase. It has been observed that during this time, very little or no increase in the number of contaminating organisms seems to occur. However, at the end of fermentation, the yeasts die, or else they undergo autolysis and their cell constituents are released into the beer. With little or no competition from yeasts for the readily available nutrients, contaminating microorganisms increase rapidly in number and their metabolites change the flavor of the beer. Because of the relatively high temperature of fermentation, these sequential events occur within a short time period. This period does not usually exceed more than 3 days in summer or 5 days in winter before this spoilage occurs. The metabolic activities of mesophilic lactic acid bacteria are primarily responsible for the spoilage. These bacteria, along with other undesirable bacteria (*Acetobacter*), produce acetic acid, volatile off-flavors, fruity odors, and pellicles which render the taste, odor and texture of the beer unacceptable to consumers (Van der Walt, 1956). **Table 4** describes the types of spoilage during the conservation of sorghum beers.

The flash-pasteurization method increases the shelf life of industrial European beers by destroying spoilage microbes. Unfortunately, this process is not applied in traditional sorghum beer-making. Early attempts at pasteurization failed because they led to an unacceptable increase in beer viscosity – through further gelatinization of starch and elimination of amylolytic enzymes – and also eliminated the beer's characteristic effervescence by killing the active yeasts (Novellie et al., 1986). On the other hand,

pasteurization of beer results in the killing of a large proportion of yeast cells, thereby making the B-group vitamins they contain available to human consumers of beer (Van Heerden, 1987). Post-fermentation pasteurization has enabled the shelf life of "tugela gold" sorghum beer to be extended to an extent comparable to that of European barley beers (Haggblade et al., 2004). Recently, Osseyi et al. (2011) were able to obtain stability in the *tchoukoutou* beer for at least 6 months after 3 h of bottle fermentation stopped by pasteurization in a water bath at 75-80 ºC for 15 min.

8. USE OF STARTER CULTURES TO IMPROVE SORGHUM BEER

A starter culture may be defined as a preparation or material containing large numbers of variable microorganisms selected for their properties and their harmlessness, which may be added to accelerate a fermentation process (Holzapfel, 2002).

In Africa, starters are used in the form of traditional leaven, resulting from spontaneous fermentation of the wort. As a result, both the desirable and non-desirable strains contained in the leaven are reintroduced with fermentation, inducing the fermentation of the sorghum wort. For example, the fermentation of the *ikigage* beer is initiated by a traditional leaven (*umusemburo*), which contains *Saccharomyces cerevisae*, *Candida inconspicua*, *Issatchenkia orientalis*, *Candida magnolia* and *Candida humilis*, *Lactobacillus fermentum*, *Lactobacillus buchneri*, *Aspergillus* sp., *Staphyloccocus aureus* and *Escherichia coli* (Lyumugabe et al., 2010). Selected use of a dominant species (*e.g. S. cerevisiae*, *Lactobacillus* sp. or *I. orientalis*) could stabilize the organoleptic quality of this beer, increase its ethanol content and improve its hygienic quality.

The use of starter cultures has been applied successfully to many products, and studies have been undertaken in the development of starter cultures for many other fermented foods from Africa (*kivunde*, *ogi* and *togwa*) (Teniola et al., 2001; Kirmaryo et al., 2002; Mugula et

Table 4. Spoilage of traditional sorghum beer – Détérioration de la bière traditionnelle à base du sorgho.

Micro organisms	Chemical produced	Odors	Other effects
Acetobacter sp	Acetic acid	Vinegar	Pellicle
Pediococcus sp	Lactic acid	-	Ropiness
Lactobacillus sp (Homferm)	Lactic acid	-	Ropiness
Lactobacillus sp (heteroferm)	Lactic acid and acetic acid	-	Ropiness, turbidity
Leuconostoc sp	Diacetyl; 2,3- butanediol	Butter/honey Sweet	-
Zymomonas sp	Ethanol, $C0_2$, acetaldehyde H_2S	Rotten apples Rotten eggs	-
Obesumbacterium sp	Dimethylsulfide	Parsnip, cooked cabbage moldy	-
Candida sp	-	Fruity	Pellicle
Pichia sp	-	-	Pellicle
Rhodotorula sp	-	-	Red coloration
Hansenula sp	-		Pellicle
Saccharomyces sp (wild strains)	Diacetyl	Phenolic, butter/ honey	Superatenuation

Source : Haggblade et al., (2004).

al., 2003). Research on improvement in the quality of traditional sorghum beer has focused on the adaptation of the starter culture. Sefa-Dedeh et al. (1999) used a pure culture of *S. cerevisiae* and a mixture culture comprised of *S. cerevisiae* with *Kloeckera apiculata* or *Candida tropicalis*, to produce in the laboratory a *pito* beer containing a high ethanol content compared to traditional *pito*. By contrast, they also found that a mixture of three cultures (*S. cerevisiae*, *K. apiculata* and *C. tropicalis*) as the starter produced a *pito* beer with a low ethanol content compared to the traditional *pito* beer. Orji et al. (2003) found that *S. cerevisiae* in combination with *Lactobacillus plantarum*, as a starter culture, also led to the

satisfactory production of a *pito* beer with a taste and aroma similar to local *pito* beer, but with a low ethanol content. N'Guessan et al. (2010) successfully used *S. cerevisiae* in combination with *C. tropicalis* as starter cultures for the alcoholic fermentation of the *tchapalo* beer, but further investigations are required before a definitive conclusion. Glover et al. (2009) showed that *dolo* beer produced from starter combinations of one strain of *L. fermentum* and both *S. cerevisiae* strains had a taste and aroma that did not differ significantly from the local *dolo* beer. This kind of research needs to be widened to other types of sorghum beer because the microorganisms involved in spontaneous fermentation are very diverse.

When the starter is adapted to the substrate, its use improves control of the fermentation process and the predictability of its products (Holzapfel, 1997). In addition, it facilitates control over the initial phase of fermentation (Holzapfel, 2002). In the same way, the hygienic quality and acceptability of African traditional foods could be improved with the use of a suitable starter (Gran et al., 2003). The use of starter cultures also reduces the organoleptic variations and the microbiological instability of African fermented foods (Kirmaryo et al., 2002). However, the use of starter cultures does not provide an absolute guarantee against failure of fermentation process, nor does it eliminate the health hazards associated with pathogens, toxinogens, or toxic components or residues (Holzapfel, 2002). The metabolic activities of desirable fermentation microorganisms must be supported by observing the basic principles of Good Manufacturing Practice (GMP).

9. CONCLUSION

Traditional sorghum beers have a socio-cultural and nutritional value in Africa. Compared to the brewing of European beer with barley, the brewing of traditional sorghum beer is characterized by the complexity of the malting process, the speed and short time of alcoholic fermentation, and the existence of lactic fermentation.

In Africa, the association of sorghum with other cereals (*e.g. Eleusine coracana*, *Pennisetum glaucum*, sweet potato) available in Africa could solve the problem of the lack of β-amylase in sorghum malt and provide a means of avoiding the use of the commercial enzymes and barley malt.

The pasteurization of sorghum beer appears most promising for resolving the brewer's perennial principal problem of a shorter shelf life. However, in order for this to happen, research will be needed to ensure the necessary refinements in pasteurization, and factory brewers would need to adopt the pasteurization process as their production standard (Haggblade et al., 2004).

The presence of unspecified microorganisms from traditional leaven complicates the control of the fermentation process and yields products of variable quality. The use of starter cultures seems to be a good method to reduce organoleptic variations and to reduce the risk of contamination with pathogenic organisms. This approach would also increase the chances of preserving of traditional sorghum beer, giving it a longer shelf life. The existing variations in the production processes of African traditional sorghum beer could be incorporated into the development of a large variety of sorghum beers in Africa.

REFERENCES

Achi O.K., 2005. The potential for upgrading traditional fermented foods through biotechnology. *Afri. J. Biotech., 5, 375-380.*

Adenuga W., Olaleye O.N. & Adepoju P.A., 2010. Utilization of bitter vegetable leaves (*Gongronema latifolium, Vernonia amygdalina*) and *Garcinia kola* extracts as substitutes for hops in sorghum beer production. *Afri J. Biotech.,9, 8819-8823.*

Agu, R, C. & Palmer, G. H., 1997a. The effect of temperature on the modification of sorghum and barley during malting. *Process Biochem., 32, 501-507.*

Agu, R. C. & Palmer, G. H., 1997b. Effect of mashing procedures on some sorghum varieties germinated at different temperatures. *Process Biochem., 32, 147-158.*

Agu, R. C. & Palmer, G. H., 1997c. Alpha -glucosidase of sorghum and barley malts. *J. Inst.*

Brew., 103, 25-29.
Agu R.C. & Palmer G.H., 1998. A reassessment of sorghum for lager-beer brewing. *Bioresour. Technol., 66, 253- 261.*

Agu, R. C. & Palmer, G. H., 1999. Development of Micro-organisms during the Malting of Sorghum., *J. inst. Brew.,105, 101 - 106*

Aisien, A. O.,1982. Enzyme modification of sorghum endosperm during seedling growth and malting. *J. Sci. Food and Agric., 33, 754-759.*

Aisien A.O. & Palmer G.H., 1983. The sorghum embryo in relation to the hydrolysis of the endosperm during germination and seedling growth. *J. Sci. Food Agri.,34, 113 – 121.*

Aisien A.O. & Muts G.C.J., 1987. Micro-scale malting and brewing studies of some sorghum varieties, *J. Instit. Brew, 93, 328 - 331.*

Ajebesone, P.E. & Aina, J.O., 2004. Potential African substitutes for hops in tropical beer brewing. *Afric. J. Food Techn., 9, 13 - 16.*

Akingbala J.O., Rooney L.W., Palacios L.G. & Sweat V.E., 1982. Thermal properties of sorghum starches. In: Martin, J.V. (Ed.), International Symposium on sorghum grain quality. *ICRISAT, Patancheru, India, pp 251 - 261.*

Asiedu J.J., 1991. La transformation des produits agricoles en zone tropicale : approche technologiqu*e. Paris :Karthala.*

Bajomo M. F. & Young, T. W., 1994. Fermentation of wort from 100% of raw sorghum and enzyme. *J. Inst. Brew., 100, 79 - 84.*

Benhura M.A. & Chingombe A., 1989. Traditional brewing methods of Zimbabwe. *Zimbabwe Scie. News, 23, 69-70.*

Blandino A., Al- Aseeri, Pandiella S.S., Cantero D., Webb C., 2003. Cereal – based fermented foods and beverages. *Food Res.Inter., 36, 527-547.*

Beta T., Rooney L.W. & Waniska, R.D. 1995. Malting characteristics of sorghum cultivars. *Cereal Chem.,72, 533-538.*

Beta, T., Corke, H., 2001. Genetic and environmental variation in sorghum starch properties. *J. Cereal Sci., 34, 261-268.*

Boboye, B.E. & Adetuyi, F.C., 1994 Fungal population associated with raw materials and intermediate products of lager beer produced from Nigerian sorghum grains. *J. Food Sci. Techn.,31, 148-150.*

Briggs D.E. Hough J.S. Stevens R. & Young T.W., 1981. Malting and brewing science, vol. 1. London: Chapman & Hall.

Chamunorwa A.T., Sara B.F. & Mutukumira A.N., 2002, Identification of Lactic Acid Bacteria isolated from Opaque beer (*Chibuku*) for potential use as a starter culture. *J. Food Techn. Afric., 7, 93-97*
Chevassus-Agnes S., Favier J.C. & Joseph A., 1979. Traditional technology and nutritive value of Cameroon sorghum beers. *CAH Onarest 2, 83-112.*

Dale C.J., Young T.W. & Makinde A., 1989. Extruded sorghum as a brewing raw material. *J. Inst. Brew.,95, 157-164*

De Lame D., 1995. La bière en bouteille et le lait de la houe, parabole d'une colline rwandaise. In: Devisch R ; De Boeck F; Jonckers D (eds), Alimentations, traditions et développements en Afrique intertropicale. Paris : L'Harmattan, pp. 116-153.

De Lame D., 1996. Une colline entre mille ou le calme avant la tempête : Transformations et blocages du Rwanda rural. Annalen menswetenschappen - Annales sciences humaines, vol. 154. Tervuren : KMMA-MRAC. 358 p.

Demuyakor B. & Ohta Y., 1992. Malt characteristics of *sorghum vulgare* varieties from Ghana. *J. Sci. Food Agri., 59, 457 - 462.*

Dewar J., Taylor J.R.N. & Berjak P. 1997. Determination of improved steeping conditions for sorghum malting. *J. Cereal Sci.,26, 129–131.*

Dhanker N. & Chauhan B.M., 1987. Effect of temperature and fermentation time on phytic and polypheno content rabadia² fermented pearl millet food. *J. Food Sci., 52, 828 – 829.*

Dicko M.H., Gruppen H., Traoré1 A. S., Voragen A.G.J. & Van Berkel W.J.H., 2006. Sorghum grain as human food in Africa: relevance of content of starch and amylase activities. *Afri. J. Biotech.,5, 384-395.*

Dirar H.A 1978. A microbiological study of Sudanese Merissa brewing. *J. Food Sci.,43,163-168.*

Dufour J.P., Melotte L. & Srebrnik S., 1992. Sorghum malts for the production of a lager beer. *J. Amer. Soci. Brew. Chem.,50, 110-119.*

Ekundayo JA., 1969. The production of pito, a Nigerian fermented beverage. *J. Food Technol. 4: 217 -225.*

Etim, M.U. & Etokakpan, O.U., 1992. Sorghum brewing using sweet potato enzymic flour to increase saccharification. *World J. Microbiol. Biotech., 8, 509 - 511.*

Ezeogu, L.I. & Okolo, B.N., 1995 Effects of air rest periods on malting sorghum response to final warm water steep. *J. Inst. Brew., 101, 39 - 45.*

Faparusi S.I, Olofinboba M.O. & Ekwundayo J.A., 1973. The Microbiology of burukutu beer Z. *Allg Mikrobiologie. 13: 563-568.*

French B.J. & McRuer G.R., 1990. Malt quality as affected by various steep aeration regimes.

M.B.A.A. *Technical Quarterly, 27, 10–14.*

Glennie C. W.,1984. Endosperm cell wall modification in sorghum grain during germination. *Cereal Chem., 61, 285 – 289.*
Glennie C. W. & Wight, A. W., 1986. Dextrins in sorghum beer. *J. Inst. Brew., 92, 384-386.*

Glover R. L. K., Sawadogo- Lingani H., Diawara B., Jespersen L. & Jakobsen M., 2009. Utilization of Lactobacillus fermentum and Saccharomyces cerevisiae as starter cultures in the production of 'dolo'. *J. Appl. Biosci., 22, 1312 – 1319.*

Goode D. L. Elke K. Arendt E.K. 2003. Pilot Scale Production of a Lager Beer from a Grist Containing 50% Unmalted Sorghum. *J. Inst. Brew.,109, 208-217.*

Gran H.M., Gadaga H.T & Narbhus J.A., 2003. Utilization of various starter cultures in the product of Amasi, a Zimbabwean naturally fermented raw milk product. *Int. J. Food Microbiol., 88, 19-28*

Guerra N.P., Torrado – Agrasar A., Lopez – Macias C . & al., 2009. Use of amylolytic enzymes in brewing. In: Victor . R. Preedy.(Ed.), Beer in health disease prevention. *Academic press, Elsevier , pp 114 – 126.*

Haggblade S & Holzapfel H., 2004. Industrialization of Africa's indigenous beer brewing. In: Streinrous KH KH (2 ed.) industrialization of indigenous fermented foods, New York City: CRC Press.

Hilhorst R., 1986. Bierbereiding in Burkina Faso. *PT/Procestechniek, 41, 93-95.*

Holzapfel W., 1997. Use of starter cultures in fermentation on a household scale. *Food Control, 8, 241-258.*

Holzapfel W., 2002. Appropriate starter culture technologies for small– scale fermentation in developing countries. *Int. J. Food Microbiol.,75, 197-212.*

Huetz de Lemps A., 2001. Boissons et civilisations en Afrique. Presses universitaires de Bordeaux, pessac, France.

Igyor M.A., Ogbonna A.C. & Palmer G.H., 2001. Effect of malting temperature and mashing methods on sorghum wort composition and beer flavour. *Process Biochem., 36, 1039-1044.*

Jespersen L., 2003. Occurrence and taxonomic characteristics of strains of *Saccharomyces cerevisiae* predominant in African indigenous fermented foods and beverages. *FEMS Yeast Res.,3, 191-200.*

Kayodé APP., Aégbidi A., Linnemenn A.R., Nout M.J.R. & Hounhouigana J.D., 2005. Quality of farmer's varieties of sorghum and derived foods as perceived by consumers in Benin. *Ecol. Food Nutr., 44, 271-294.*

Kayodé APP., Hounhouigana J.D. & Nout M.J.R., 2007a. Impact of brewing process operations on phyta, phenolic compounds and in vitro solubility of iron and zinc in opaque sorghum beer. *LWT, 40, 834-841.*

Kayodé APP., Hounhouigana J.D., Nout M.J.R. & Niehof A., 2007b. Household production of sorghum beer in Benin: technological and socio-economic aspects. *Int. J. Cons. Studies, 31, 1470-6431.*

Khady BA, Tine E, Destain J, Ndiaga C. & Thonart PH., 2010. Etude comparative des composes phénolitiques, du pouvoir antioxydant de différentes variétés de sorgho sénégalais et des enzymes amylolytiques de leur malt. *Biotechnol. Agron. Soc., 14, 131-139.*

Kirmaryo V. M., Massawe G.A., Olasupo N.A. and Hololzpfel W.H., 2002. The use of a starter culture in the fermentation of cassava for the production of '' Kivunde'', a traditional Tanzanian food product. *Int.J.Food Microbiol., 56, 179-190*.

Kumar L.S., Daudu M.A., Shetty H.S. & Malleshi, N.G., 1992. Seed mycoflora and malting characteristics of some sorghum cultivars. *J. Cereal Sci.,15, 203-209*.

Lasekan O. O., Idowu M. A. & Lasekan, W., 1995. Effect of germination and degree of grind (coarse/fine)on the extract and sugar content of sorghum malts. *Food Chem., 58, 125-128.*

Lasekan O.O, Lasekan W.O. & Babalola J.O., 1999. Effect of *Vernonia amygdalina* (bitter leaf) extract on brewing qualities and amino acid profiles of stout drinks from sorghum and barley malts. *Food chem.., 64, 507-510.*

Lineback, D.R., 1984. The starch granule: Organization and properties. *Bakers Digest 58, 16–21*.

Lyumugabe L., Kamaliza G., Bajyana E. & Thonart PH., 2010. Microbiological and physico-chemical characteristic of Rwandese traditional beer "*Ikigage*". *Afr. J. Biotech., 9, 4241-4246.*

Maoura N., Mbaiguinam M., Nguyen HV., Gaillardin C. & Pourquie J., 2005. Identification and typing of the yeast strains isolated from bili bili, a traditional sorghum beer of chad. *Afr. J. Biotech., 4, 646-656.*

Maoura N. & Pourquie J., 2009. Sorghum beer: Production, nutritional value and impact upon human health. In: Victor . R. Preedy.(Ed.), Beer in health disease prevention. *Academic press, Elsevier , pp 53 -60.*

Matumba L.,Monjerezi M., Khonga E. B. & Lakudzala D.D., 2011. Aflatoxins in sorghum, sorghum malt and traditional opaque beer in southern Malawi. *Food Control, 22, 266 – 268.*

Mboto C.I., Eja, M. E., Adegoke A.A., Iwatt G.D., Asikong B.E., Takon I., Udo S.M. & Akeh M., 2009. Phytochemical properties and antimicrobial activities of combined effect of extracts of the leaves of *Garcinia kola, Vernonia amygdalina* and honey on some medically important microorganisms. *Afri J. Microbiol. Res.,3,557-559.*

Moll M. 1991. Bières, Techniques et documentation Lavoisier, Apria, Paris

Morrall P., Boyd H.K., Taylor J.R.N. & van Der-Walt W.H., 1986. Effect of germination time, temperature and moisture on malting of sorghum (Sorghum bicolor*). J. Inst. Brew, 92, 439 - 445.*

Mugula J.K, Nnkoa S.A.M., J.A Narvhusb J.A. and Sorhaug T., 2003. Microbiological and fermentation characteristics of togwa, a Tanzanian fermented food. *Inter. J. Food Microbiol., 80, 187-199.*

N'guessan F.K, N'diri D.Y, F. Camara & Djè M.k., 2010. *Saccharomyces cerevisiae* and *Candida tropicalis* as starter cultures for the alcoholic fermentation of tchapalo, a traditional sorghum beer. *World J. Microbiol. Biotechn., 26, 693 – 699.*

Nikander P., Seppälä T., Kilonzo G. P., Huttunen P., Saarinen L. & Kilima, E., 1991. Ingredients and contaminants of traditional alcoholic beverages in Tanzania. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 85, 133 - 135.*

Nout M.J.R., 1987. Composition of foods: African traditional beers. *Laboratory new letter, 8:18.*

Novellie L. 1962. Kaffircorn malting and brewing studies XI. Effect of malting conditions on the diastatic power of kaffircorn malts. *J. Sci. Food Agri., 13, 115 - 120.*

Novellie L., 1976. Beverages from sorghum and millets. In: Proceedings of the international symposium on sorghum and millets for human Food. *International Association for Cereal Chemistry Symposium in Vienna, Tropical Products Institute.*

Novellie L., 1982. Fermented porridge. In: Proceedings of the international symposium on sorghum grain quality. *ICRISAT (eds), Pantacheru, India, pp. 121-128.*

Novellie L. & De Schaepdrijver P., 1986. Modern developments in traditional African beers. In: MR Adams (ed). Progress in Industrial Microbiology. Amestardam: *Elsevier Science., 23, 73 – 157.*

Nzigamasabo A. & Nimpagaritse A., 2009. Traditional fermented foods and beverages in Burundi. *Food Res. Inter., 42, 588–594.*

Oboh F. O.J. & Masodje H.I., 2009. Nutritional and antimicrobial properties of *Vernonia amygdalina* leaves. *Inter. J. Biom., Health Sci., 5, 51-56.*

Ogundiwin J.O. & Ilori M.O., 1991. Development of stout from sorghum malt. *Lebensmittel-Wissenschaft Und- Technologies, 1991, 24, 182-185.*

Okafor N. & Aniche G.N., 1980. Brewing a lager beer from Nigerian sorghum. *Brew. Dist. Inter.,10, 32-35.*

Okoh IA., Babalola GO. & Ilori MO., 1995. Effect of methanol extract of *Vernonia amygdalina* on malting and brewing properties of sorghum. *Tech. Quart. Master Brew. Assoc. Am. 32, 11-14.*

Okolo B.N. & and Ezeogu L.I., 1996. Duration of final warm water steep as a crucial factor in protein modification in sorghum malts, *J. Inst. Brew*., 102**,** 167–177.

Okoro C.C . & Aina, J. O., 2007. Effect of storage on the brewing properties of tropical hop substitutes. *Afri. J. Biotech., 6, 1479- 1483.*

Okungbowa J., Obeta J.A.N. & Ezeogu L.I., 2002. Sorghum b-amylase production: relationship with grain cultivar, steep regime, steep liquor composition and kilning temperature. *J. Inst. Brew., 108, 362–370.*

Orji M.U, Mbata T.I, Anich G.N & Ahonkhai I., 2003. The use of starter cultures to produce "pito", a Nigerian fermented alcoholic beverage. *World J. Microbiol. Biotech., 19, 733 -736.*

Osseyi E.G., Tagba P., Karou S.D., Ketevi A.P. and Lamboni C.R. 2011. Stabilization of the Traditional Sorghum Beer, "Tchoukoutou" using Rustic Wine-Making Method. *Advance J. Food Sci. Techn.,3, 254-258.*

Osuntogun B.A., Adewusi S.R.A. Ogundiwin J.O. & Nwasike C.C., 1989. Effect of cultivar steeping and malting on tannin, total polyphenol and cyanide content on Nigeria sorghum. *Cereal Chem., 66, 87- 89.*

Owuama, C.I. & Okafor, N., 1990. Use of unmalted sorghum as a brewing adjunct. *World J. Microbiol. Biotech., 6, 318 - 322.*

Owuama C.I. & Asheno I., 1994. Studies on malting conditions for sorghum. *Food Chem., 49, 257-260.*

Palmer G. H., 1989. Cereals in malting and brewing. In *Cereal Science and Technology,* ed. Palmer, G. H. pp. 61-242. Aberdeen: Aberdeen University Press.

Palmer G.H., Etokakpan O.U. & Igyor M.A., 1989. Review: Sorghum as brewing material. MIRCEN *J. Appl.Microbiol. Biotech., 5, 265-275.*

Palmer G. H., 1991. Enzymic degradation of the endosperm cell walls of germinated sorghum. *World J. Microbiol. Biotech., 7, 17-21.*

Pathirana R.A., Shivayogasundaram K. & Jayatissa, P.M., 1983. Optimization of conditions for malting of sorghum. *J. Food Sci. Techn., 20, 108-112.*

Pelembe, L.A.M., Dewar, J., Taylor, J.R.N., 2004. Effect of Germination Moisture and Time on Pearl Millet Malt Quality – With Respect to Its Opaque and Lager Beer Brewing Potential. *J. Instit. Brew. 110, 320–325.*

Rooney L. W. & Serna-Saldivar S. O., 1991. Sorghum. In: K. J. Lorenz and K. Kulp (Ed) Handbook of Cereal Science and Technology. Marcel Dekker Inc., New York, USA, pp. 233-270.

Sanni AI., Onilude AA., Fadahusi IF. & Afolabi RO.,1999. Microbial deterioration of traditional alcoholic beverages in Nigeria. *Food Res. Inter., 32, 163-167.*

Sawadogo-Lingani H., Lei V., Diawara B., Nielsen DS., Moller PL., Traoré AS. & Jakobsen M, 2007. The biodiversity of predominant lactic acid bacteria in *dolo* and *pito* wort, for production of sorghum beer. *J. Appl. Microbiol., 103, 765 - 777.*

Sefa-Dedeh S., Sanni AI., Tetteh G. & Sakyi-Dawson E.,1999. Yeasts in the traditional brewing of *pito* in Ghana. *World J. Microbiol. Biotech.,15, 593-597.*

Taylor J. R. N., 1983. Effect of malting on the protein and free amino nitrogen composition of sorghum. *J. Sc. Food Agri., 34, 885 -892.*

Taylor J. R. N., 1992. Mashing with malted grain sorghum. *J. Amer. Soci. Brew. Chem., 50, 13-18.*

Taylor, J.R.N., Robbins, D.J., 1993. Factors influencing beta-amylase activity in sorghum malt. *J. Ins. Brew., 99, 413–416.*

Taylor, J.R.N., 2009. Developments in Africa's cereal crops – potential sustainable resources for brewing in tropical and sub – tropical countries, Institute of Brewing and Distilling, Africa Section12th Scientific and Technical Convention, London.

Taylor J.R.N. & Dewar J., 1994. Role of alpha-glucosidase in the fermentable sugar composition of sorghum malt mashes. *J. Instit. Brew., 100, 417-419.*

Teniola OD. & Odunfa SA., 2001. The effects of processing methods on the level of lysine and methionine and the general acceptability of *ogi* processed using starter cultures. *Int. J. Food Microbiol. 63: 1-9.*

Tisekwa B., 1989. Improvement of traditional manufacturing of sorghum beer (mtama) in Tanzania. *Thesis, Ghent University, Belgium, pp. 2- 18.*

Uriyo M. & Eigel W.E., 1999. Duration of kilning treatment on α-amylase, β-amylase and endo-(1, 3) (1, 4)-β-D-glucanase activity of malted Sorghum (*Sorghum bicolor*). *Process Biochem., 35, 433-436.*

Van der Aa Kühle A., Jespersen L., Glover RLK., Diawara B. & Jakobsen M., 2001. Identification and characterization of *Saccharomyces cerevisiae* strains isolated from West African sorghum beer. *Yeast,18, 1069-1079.*

Van Der Walt J.P., 1956. Kaffircorn malting and brewing studies: Studies on the microbiology of kaffir beer. *J. Sci. food Agri., 7, 105-113.*

Van Heerden IV., Glennie GW., 1987. Availability of B- vitamin in sorghum beer. *Nutri. Rep. Inst., 35, 147-155.*

Verbruggen M.A., 1996. Glucuronoarabinoxylans from sorghum grain, *PhD thesis, Wageningen Agricultural University, Wageningen, Holland.*

Waites M. J., Morgan N. L., Rockey J.S. & Higton G., 2001. Industrial microbiology: an introduction, Blackwell science, London, UK.

Zeigler P., 1999. Cereal beta-amylases. *J. Cereal Sci., 29, 195-204.*

Chapitre 2 :

Caractéristiques microbiologiques et physico – chimiques de la bière traditionnelle « *ikigage* » du Rwanda

Ce travail a fait l'objet de la publication suivante :

Lyumugabe, F., Kamiliza, G., Bajyana, E., Thonart, Ph. (2010). Microbiological and physico-chemical characteristic of Rwandese traditional sorghum beer « ikigage ». *African Journal of Biotechnology, Vol. 9(27), pp. 4241-4246*.

Préambule au chapitre 2

La bière de sorgho « *ikigage* » est une boisson traditionnelle très populaire au Rwanda. Elle est spécialement consommée et appréciée dans différentes fêtes et cérémonies. La bière *ikigage* est considérée à la fois comme un aliment et comme une boisson par la majorité de la population pauvre vivant à la campagne. Au cours de ces dernières années, la qualité sanitaire de cette bière a été mise en doute, en raison, notamment, de nombreux cas de toxi-infections alimentaires. L'amélioration de la qualité hygiénique de la bière de sorgho « ikigage » est devenue une nécessité pour le Rwandais.

L'objectif de ce chapitre a été de caractériser la bière traditionnelle Rwandaise « *ikigage* », afin d'améliorer sa qualité hygiénique et de réduire les variations organoleptiques. Dans ce sens précis, nous avons procédé à des analyses microbiologiques et physico – chimiques de 40 échantillons de la bière *ikigage*. Lesdits échantillons ont été collectés dans différents points de vente de cette bière, dans la Province du Sud du Rwanda. Une enquête auprès de brasseuses traditionnelles, et un suivi technique du processus de fabrication, ont permis d'établir le diagramme de production de cette bière. Les échantillons récoltés aux différents points à risques de contamination ont été également soumis à des analyses microbiologiques. Ces analyses ont concerné le dénombrement de la flore aérobie mésophile totale, de *Staphylococus aureus*, de germes d'origine fécale (*Esherichia coli*, streptocoques fécaux, Coliformes totaux), de levures, de moisissures et de bactéries lactiques. Les microorganismes majoritaires ont été identifiés à partir de leurs caractéristiques phénotypiques et génotypiques.

Microbiological and physico-chemical characteristics of Rwandese traditional sorghum beer "*ikigage*"

RUNNING TITLE HEADER: Characteristic of sorghum beer "*ikigage*"

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Abstract

Samples of traditional sorghum beer *ikigage* was collected in the southern province of Rwanda and analyzed for microbiological and physico-chemical contents. *Ikigage* contained total aerobic mesophilic bacteria (33.55 x 10^6 cfu/ml), yeast (10.15 x 10^6 cfu/ml), lactic acid bacteria (35.35 x 10^4 cfu/ml), moulds (4.12 x 10^4 cfu/ml), *E. coli* (21.90 x 10^3 cfu/ml), fecal streptococci (22.50 x 10^3 cfu/ml), *Staphylococcus aureus* (16.02 x 10^3 cfu/ml), total coliform (32.30 x 10^3 cfu/ml), ethanol, soluble protein, reducing sugars, total acidity, pH and Brix were 2.2% (v/v), 9.2 g/l, 2.3, 1.7%, 3.9 and 11.5 bx, respectively. The yeast was identified by API 20 C test and confirmed by PCR-Sequencing of ITS-5.8S region of rDNA. Seventy yeasts isolated in the samples were found to belong to *Saccharomyces cerevisae*, *Candida inconspicua*, *Issatchenkia orientalis*, *Candida magnolia* and *Candida humilis*. Lactic acid bacteria were identified using the API 50 CHL system. Ten different isolates of lactic acid bacteria belonged exclusively to the genus Lactobacillus: *Lactobacillus fermentum*, *Lactobacillus buchneri*, and *Lactobacillus* sp. The micro-organisms of fecal origin are from the water and the operations postfermentation process. The presence of potential pathogens emphasizes the importance of developing starter cultures with GRAS status for commercialization of *ikigage*.

Key words: Sorghum beer, *Ikigage*, alcohol fermentation, microbiology, physico-chemistry.

1. INTRODUCTION

Ikigage is a traditional alcoholic beverage manufactured in Rwanda with malted sorghum. It is known as *Tchoukoutou* in Benin or Togo, *Dolo* in Burkina-Faso, *Pito* in Ghana, *Burukutu* or *Otika* in Nigeria, *Bili bili* in Tchad and *Mtama* in Tanzania (Ekundayo, 1969; Kayode et al., 2005; Odunfa and Adeyele, 1985; Maoura et al., 2005; Tisekwa, 1989). The manufacturing processes are very variable and dependent on the geographical location (Haggblade and Holzapfel, 1989). These African beers are very rich in calories, vitamin B and essential amino-acids such as lysin (Chevassus et al., 1976).

In Rwanda, *ikigage* is consumed in various festivals and Rwandese ceremonies (e.g., marriage, birth, baptism, dowery, etc.) and constitutes a source of economic return for the women manufacturers. Currently, the manufacture of this beer is declining because of poor hygienic quality, unsatisfactory conservation and poor yield of ethanol and variations of organoleptic quality. The increasing demand for quality and quantity, formulated by the urban consumers requires the focusing on a Rwandese beer of good organoleptic and hygienic quality and well -conditioned. But, the scientific studies of *ikigage* from Rwanda are not in existence. The aim of this present paper is to analysis the microbiological and physico-chemical quality of Rwandese traditional beer "*ikigage".*

2. MATERIALS AND METHODS

2.1. Processing and sampling

Fifty samples of *ikigage* were collected in sterile bottles (500 and 1000 ml) from 10 local sites of marketing in the southern province of Rwanda. Five samples were collected in each site. 32 women manufacturers of *ikigage* in those sites were interviewed to enable us establish the diagram of *ikigage* production. Three independent manufacturers were selected for a follow

up. The samples collected were brought to the laboratory of Microbiology at the National University of Rwanda for analysis.

2.2. Enumeration of microorganisms

Ten milliliter of sample were diluted in 90 ml sterile peptone physiological saline solution (1 g Peptone, 8.5 g NaCl and 1000 ml distilled water) and homogenized. Decimal dilutions were plated. Total plate count aerobic mesophilic bacteria was enumerated on Plate Count Agar (PCA-OXOID) and supplemented with Cycloheximide 0.5%. The plates were incubated at 28°C for 48 to 72 h. Total coliforms and *Escherichia coli* were required on Bubble Lactose Bile with Brilliant green (BLBVB- DIFCO). The tubes provided with the bells of Durham were incubated at 30°C during 24 to 48 h. The positive tubes revealed were required on water peptone without indol and incubated at 44°C for 24 h to detect for *E. coli*. *E.coli* was revealed using Kovac's reagent. *Staphylococcus aureus* was enumerated on Manitol Salt Agar (MSA - Sigma) and revealed by the test of coagulation with the plasma rabbit. The plates were incubated at 37°C for 48 h. Fecal *Streptococcal* was enumerated on Slanet Agar (SL-Merck) supplemented with Cycloheximide at 0.5% after 48 h of incubation at 37°C. Salmonella were analyzed by the procedure of the French association of standardization (AFNOR, V 08-052). Yeasts and moulds were enumerated on YPD-Chloramphenicol (10 g yeast extract, 10 g peptone, 20 g glucose, 20 g agar, 0.5 g chloramphenicol and 1000 ml distilled water) after 48 to 72 h of incubation at 30°C. Lactic acid bacteria were enumerated on Man, Rogosa and Sharpe Agar (MRS - FPP) and supplemented with Cycloheximide 0.5%. The plates were incubated at 37°C for 48 h under anaerobic conditions. Except total coliform and *E. coli*, the enumeration was carried out in double and the plates containing between 30 and 300 colonies were considered. Total coliforms and *E. coli* were enumerated by the method of probable smallest number.

2.3. Isolation and identification of microorganisms

Isolated yeasts were purified by successive sub-culturing on YPD. The yeast strains were identified using the API 20 C kit (Biomérieux) and confirmed by PCR – Sequencing of internal transcribed spacer (ITS) region of rDNA. The ITS1 - 5.8S - ITS2 regions of rDNA were amplified by PCR using the primer ITS1 (5P- TCCGTAGGTGAACCTGCGG-) and ITS4 (5P- TCCTCCGCTTATTGATATGC-) according to White et al (1990). The purified PCR products were directly sequenced using ABI 3130 genetic analyzer. Blast searches of sequences were performed at the National Centre for Biotechnology Information (NCBI) Gen Bank data library.

Lactic bacteria were purified by successive sub-culturing on MRS and further characterized using the API 50 CHL system (BioMérieux) according to the procedure indicated by the manufacturer. The identification of lactic bacteria was done using API taxon 2004 software. Lactobacilli were recognized as Gram-positive and catalase - negative.

The pure colonies of moulds were identified using 10 day old cultures on YPD. Cultures and microscopic characteristics were examined and moulds were classified according to Barnett and Hunter (1972).

2.4. Physico-chemical analysis

The samples were centrifuged at 6000 X g for 10 min, filtered through filter paper and analyzed by standard methods. The pH was measured using a pH meter 781 (Metrohm Herisau). Titratables acidity, expressed as a percentage lactic acid, was determined by titrating the samples with 0.1 N NaOH to the phenolphthalein end point. The brix was measured by a refractometer (ATAGO, Japan). The soluble proteins were determined using the method of Lowry et al., (1951). Reducing sugars were determined by the method of luffschoorl

(Fouassin and Noirfalise, 1981). Ethanol was determined by enzymatic method using the Boehringer Kit (R-Biopharm AG,D-64293 Darmastadt).

3. RESULTS

3.1. Manufacturing process of *ikigage*

The results of the investigation enabled us to describe the various sequences of manufacture of *Ikigage* (Figure 1).

3.1.1. Malting

Generally, the traditional brewers choose *Amakoma* (Sorghum bicolor) to prepare *ikigage*. The malting process involves steeping, germinating and limiting cereal seedling growth.

Steeping

After washing, the grains are immersed in water (*kwinika*) for 24 h. The grains are then drained in a bag with a stone top during 24 hours so that the process of germination is completed and rootlets appear (*kumera*).

Germination

After draining, the grains are spread out on a cloth in a wet place. Ash was put down on the cloth and then leaves of eucalyptus or banana tree, then the grains are spread out to support germination. The intermediate duration of germination is approximately 48 h.

Drying

The grains are dried under the sun for at least two days at 29°C (± 3.2). When the grains were quite dry, the rootlets are removed (kuyavunga).

3.1.2. Mashing

Grinding

The quite dry malt grains are ground or crushed. Certain brewers use a grinding stone to crush the dry malt grains. This method is very traditional and takes several hours to obtain grinding

sufficient for the manufacture of beer. More recently, the malt grains are crushed in crusher machine (hammer mill).

Infusion

The brewers heat water (20 L) to boiling and add approximately 2 kg ground malt grains. Then, warm water (20 L) is mixed with ground malt (16 kg) in a large container. The temperature of mixing is typically between 63 and 71°C.

Cooling and decantation

After the infusion, cool water is added (40 L) to bringtemperature back to between 34 and 40°C. After cooling, certain brewers leave this mixture to rest approximately 3 hours in order to eliminate the draffs "imvuzo". Other brewers do not make the decantation and the dregs are thrown out after sale of *Ikigage*.

3.1.3. Traditional leaven "umusemburo"

Rwandan traditional leaven "*Umusemburo*" is a result of fermentation of malted sorghum. The manufacturing methods of *Umusemburo* can be summarized in four stages. The first stage consists of preparing the wort of malted sorghum (*Igikoma*). Approximately 4 L of water are mixed with 1 kg of flour of malted sorghum. This mixture is incubated in a gourd during 24 to 48 h. The second stage consists of the extraction of the juice of *Vernonia amygdalina* (*Umubirizi*). The sheets are crushed in a mortar and approximately 240 ml of the juice are extracted. This juice is boiled in a pan (*Icyungo*) until complete evaporation. The third stage consists of transvasing *igikoma* in *icyungo* and to add some fresh stems of *Euphorobia tirucalli* (*Umuyenzi*). This mixture is covered and left in fermentation for 72 h. The fourth stage consists of adding two types of flour of malted sorghum. The brewer adds approximately 1 kg of malted sorghum and 0.5 kg of malted sorghum roasted. This mixture is

again covered and left in fermentation for 24 h. After this time, *umusemburo* is ready for use. This leaven can be used immediately or preserved after drying.

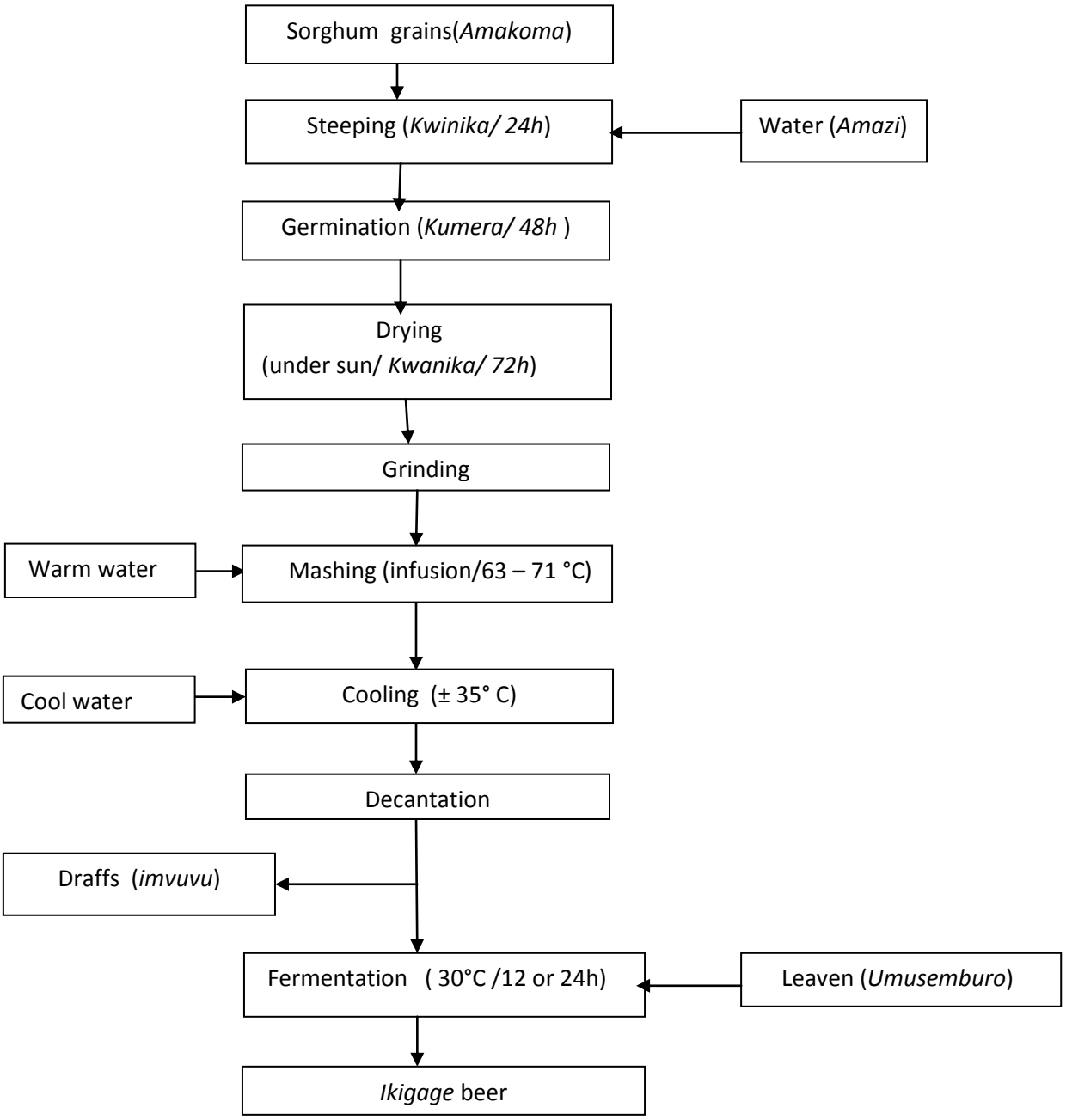

Figure 1. Diagram of manufacturing of Rwandese traditional sorghum beer "*ikigage"*.

The methods of manufacture of this leaven are diversified in Rwanda. Each province or district selects the ingredients to be used, but *V. amygdalina* are used in all areas of Rwanda. The other plants used in the preparation of *umusemburo* are listed in **Table 1**. The simplest, fast and less expensive method consists of mixing 1 L of wine from banana and 1 kg of flour of malted sorghum. This mixture is fermented under sun for 8 h. After this time, *umusemburo* is ready for consumption.

3.1.4. Fermentation

After cooling, one inoculates the traditional leaven "*umusemburo*" to start the fermentation. The fermentation container is covered with leaves of the banana tree, cloth and a lid. After 12 to 24 h of fermentation, *ikigage* is ready for consumption.

3.2. Microbiological and physicochemical analysis of marketed *ikigage*

Table 2 indicates the results of microbiological and physicochemical analysis of *ikigage* marketed in Rwanda. These results show a prevalence of total aerobics mesophilic, yeast and lactic bacteria. One notices also a very low pH and a weak ethanol concentration.

3.3. Microbiological analysis at the various stages of manufacture of *ikigage*

Figure 2 indicates the results of microbiological analysis at the various stages of manufacture of Rwandese traditional beer "*ikigage"*. Total aerobic mesophilic, yeast, lactic bacteria, mould, total coliform and *S. aureus* are present in raw material and leaven used. Total coliform and *S. aureus* disappear after fermentation. *E. coli* and fecal streptococci comes exclusively from water used for cooling and leavens. They are absent in the *ikigage* justafter fermentation.

Table 1. Plants used in the preparation of *Umusemburo* from Rwanda

Scientific name of plants used	Vernacular name	Part used
V. amygdalina	Umubirizi	Leaves
E. tirucalli	Umuyenzi	Stems
M. lutea	Umusave	Leaves
N. tabacum	Itabi	Leaves
S. capsicoïdes	Igitoborwa	fruits
T. diversifolia	Icyicamahirwe	Leaves
V. aemulans	Idoma	Leaves

Table 2. Microbiological and physico-chemical analysis of marketed *Ikigag*e

Microbiological parameters	Amount (cfu/ml)	Physico-chemical parameters	Value
Total aerobic mesophilic	33.55×10^6	pH	3.9 (±0.46
Yeast	10.15×10^6	Total acidity (%)	1.72 (±0.41)
Lactic acid bacteria	35.35×10^4	Brix	11.6 (±1.53)
Moulds	4.12×10^4	Reducing sugars (%)	2.33 (±0.78)
Total coliform	32.30×10^3	Soluble proteins (g/l)	9.22 (±1.04)
E. coli	21.10×10^3	Ethanol (% v/v)	2.2 (±0.46)
S. aureus	16.02×10^3		
Fecal streptococci	22.50×10^3		

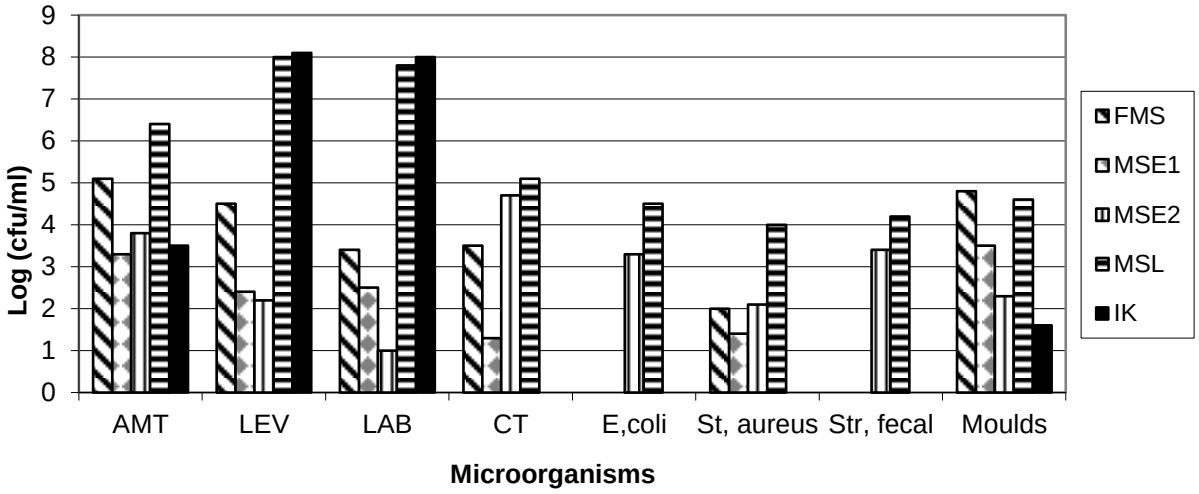

Figure 2. Micro-organisms at the various stages of manufacture of *Ikigage*

***FMS**: Flour of malted sorghum; **MSE1**: Mix malted sorghum and warm water; **MSE2**: MSE1 + cool water; **MSL**: Mix cooled must and leaven; **IK**: ikigage (beer) after fermentation; **AMT**: total aerobic mesophilic; **LEV**: yeasts; LAB: lactic acid bacteria; CT: total coliform*

3.4. Identification of microorganisms

Seventy yeasts isolated in the samples were found to belong to *Saccharomyces cerevisae* (39), *Candida inconspicua* (16), *Issatchenkia orientalis* (7), *Candida magnolia* (3) and *Candida humilis* (5). We isolated ten different lactic acid bacteria strains from *ikigage* belonging exclusively to the genus Lactobacillus: *Lactobacillus fermentum* (4), *Lactobacillus buchneri* (2), *Lactobacillus* sp. (4). Ten mould isolated were found to belong to *Aspergillus niger* (3), *Fusarium* sp. (4) and *Aspergillus* sp. (3).

4. DISCUSSION

Ikigage manufacturing is artisanal and characterized by absence of the instrumental and analytical control. The water quantity used does not take account of final concentration of wort used as in brewery. The mixing is done by hardening and quickly to mitigate temperature change.

Contrary to other traditional processes of manufacture of beers containing sorghum (Kayodeet al., 2005; Odunfa and Adeyele, 1985; Maoura et al., 2005), the phase of cooking does not exist during manufacturing of *ikigage*.

The role of the plants incorporated in the preparation of *umusemburo* from Rwanda is not yet well-known. The traditional brewers of *ikigage* say that *E. tirucalli* prevents the production of the bubbles during the leaven prefermentation and that *V. amygdalina* is used for maturation of leaven. However, the microbiological analysis of various types of *umusemburo* shows that the leaven prepared with *V. amygdalina* and *E. tirucalli* contain many yeasts compared to other types (Nsabimana, 1997; National University of Rwanda, Rwanda, TFE licence). One possible hop substitute is *V. amygdalin*a, known as "bitter leaf". It resembles the hop not only in its bitter flavor but also its antimicrobial properties (Okoh et al., 1995).

Ikigage marketed in Rwanda contains a very low ethanol level, low pH and high total acidity. These results are similar to those obtained by other researchers with various African fermented foods (Odunfa and Adeyele, 1995; Maoura et al., 2006). According to Kazanas and Fields (1981), the high acidity in sorghum beer is explained by presence of lactic acid bacteria. The small ethanol concentration in *ikigage* could be explained by small quantity of fermentable sugars. Novellie (1982) indicates that the lack of fermentable sugars in sorghum malt is a consequence of β-amaylase low content. Indeed, according to the results obtained by Khady et al. (2010), malt of sorghum contains a good activity α–amylase (312.6 ± 11.7 U/g) and a weak activity of β-amylase (62.7± 4.4 U/g). But the improvements are necessary to raise the content β-amylase in malt of sorghum. In old Rwanda, the traditional brewers mixed the malted sorghum and *Eleusine coracana* (*uburo*) malted to produce a beer with high alcohol content. *Uburo* brings β-amylase necessary for the hydrolysis of the maltose starch. However, the small size of these grains could pose engineering problems in the modern breweries.

Ikigage contains a high concentration of soluble proteins. Maoura et al. (2006) obtained results similar with *bili bili* of Tchad. The proteins contribute to the growth of yeasts during fermentation and to the stable foam formation in the beer. The total aerobic mesophilic, yeasts and lactic acid bacteria (LAB) are prevalent in the flora of *ikigage*. They are brought by raw material and leaven used during the production of *ikigage*. This result was also obtained by Kayode et al. (2007) with *tcoukoutu.*

According to Holzapfel (1997), African opaque beers are typical examples of lactic fermentation followed by alcoholic fermentation in which initially, LAB and later yeasts play the dominant role. Due to their higher growth rate, bacteria typically dominate the early stages of fermentation. A symbiotic relation could explain the simultaneous presence of yeast and LAB (Munyaja et al., 2003). LAB creates an acid environment favorable to the proliferation

of yeasts. The yeasts produce vitamin and increase other factors such as amino-acids for the growth of LAB.

The major yeasts isolated from *ikigage* beers are dominated by *Sacharomyces cerevisiae*, known for their role in alcoholic fermentation. Similar findings were obtained by Maoura et al. (2005) for traditional sorghum beer *bili bili*, Kayode et al. (2007) for sorghum beer *tchoukoutou* and Naumova et al. (2003) for sorghum beer *pito*. But, *I. orientalis* was not previously isolated from sorghum beer. This species is unable to ferment maltose. However, *I. orientalis* was identified as being the yeast specie dominant in togwa, Tanzanian fermented food manufactured from sorghum or maize or millet (Mugulu et al., 2003). It was was also isolated in the various fruits and wines (Loveness et al., 2007; Ciani and Maccarelli, 1998). *C. inconspicua* has been isolated from human sputum and tongue and is known to be an opportunistic human pathogen (Maxwell et al., 2003).

Lactobacilli were also isolated from many other African sorghum beers (Kayode et al., 2007; Odunfa and Adeyele, 1985; Nout, 1980; Novellie, 1982). *A. niger*, *Fusarium sp* and *Aspergillus sp* were also isolated by Gassem (1999) from fermented bread produced from sorghum, but, also isolated *penicillium sp.*

The disappearance of total coliform, *E. coli*, *S. aureus* and fecal streptococci microorganisms in *ikigage* is explained by production of acids and fall of pH during fermentation. Their presence in marketed *ikigage* is attributed to post-fermentation processing; water used for dilution, utensils and handling in work environment probably introduces these micro-organisms. The presence of *E. coli* and fecal streptococci indicate a contamination of fecal origin. Generally in food, the standards accept an inferior number with 10^2 cfu/ml and 10^3 cfu/ml, respectively (Guiraud, 1998). The proliferation of *S. aureus* can cause stomach disorders and vomiting for the consumer. However, the danger of intoxication typically occurs between 10^5 and 10^6 cfu/ ml of food (Guiraud, 1998).

5. CONCLUSION

The process of *ikigage* manufacturing is very artisanal and does not take account of the quality rules. The analyzed samples of Rwandese traditional beer, *ikigage* are characterized by absence of Salmonella and presence of many microorganisms of fecal origin, and the final product present a risk to consumers with a weakened immune systems. These micro-organisms come from the operations post- fermentation process. The presence of potential pathogens emphasizes the importance of developing starter cultures with GRAS status for commercialization of *ikigage*.

The major microorganisms involved in the *ikigage* fermentation were *S. cerevisiae* and heterofermentative lactobacilli. The approach using starters made of these microorganisms appears to be a good method for the improvement of *ikigage*, but it requires screening and characterization of the powerful *S. cerevisiae* and heterofermentative lactobacilli strains from the traditional processes. Being adapted to the substrate, a typical starter facilitates improved control of a fermentation process and predictability of its products (Holzapfel, 1997). In addition, starter cultures facilitate control over the initial phase of a fermentation process (Holzapfel, 2002).

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REFERENCES

Barnett HL, Hunter BB (1972). Ilustrated genera of imperfect fundi, 3rd edn, pp. 53-180.

Chevassus AS, Favier JC, Joseph A (1976). Technologie traditionnelle et valeurs nutritives des bières de sorgho du Cameroun. Cah. Nutr. Dièt. 11: 89-95.

Ciani M, Maccarelli F (1998). Oenological properties of non Saccharomyces yeasts aossociated with wine – marking. World J.Microbiol. Biotechnol. 14: 199-203.

Ekundayo JA (1969). The production of pito, a Nigerian fermented beverage. J. Food Technol. 4: 217 -225.

Fouassin A, Noirfalise A (1981). Méthodes d'analyse des substances alimentaires, Université de Liège, Faculté de Médecine, Presses Universitaires, 4 ème éd. pp. 19-35.

Haggblade S, Holzapfel H (1989). Industrialization of Africa's indigenous beer brewing. In: Streinrous KH KH (ed.) industrialization of indigenous fermented, New york, Marcel Dkker Inc, pp. 192-193.

Holzapfel W (1997). Use of starter cultures in fermentation on a household scale. Food Control, 8: 241-258.

Holzapfel W (2002). Appropriate starter culture technologies for small– scale fermentation in developing countries. Int. J. Food Microbiol. 75: 197-212.

Gassem MA (1999). Study of the microorganisms associated with the fermented bread (khamir) produced from sorghum in Gizan region, Saudi Arabia. J. Appl. Microbiol. 86: 221-225.

Guiraud JP (1998). Microbiologie alimentaire. Dunod Ed, Paris, pp. 88-355.

Khady BA, Tine E, Destain J, Ndiaga C, Thonart PH (2010). Etude comparative des composes phénolitiques, du pouvoir antioxydant de différentes variétés de sorgho sénégalais et des enzymes amylolytiques de leur malt. Biotechnol. Agron. Soc. 14: 131-139.

Kazanas N, Fields ML (1981). Nutritional improvement of sorghum by fermentation. J. Food Sci. 46: 819-821.

Kayode APP, Hounhouigan JD, Nout MJR (2007). Impact of brewing process operations on phytate, phenolic compunds and in vitro solubility of iron and zinc in opaque sorghum beer. LWT- Food Sci. Technol. 40: 834-841.

Kayode APP, Aégbidi A, Linnemenn AR, Nout MJR, Hounhouigana JD (2005). Quality of farmer's varieties of sorghum and derived foods as perceived by consumers in Benin. Ecol. Food Nutr. 44: 271-294.

Loveness K, Nyanga LK, Matinus JR, Nout, MJR, Tendekayi H, Gadaga TH, Theelen B, Boekhout T, Zwietering MH (2007). Yeasts and lactic acid bacteria microbiota from masau (Ziziphus mauritiana) fruits and their fermented fruit pulp in Zimbabwe, Int. J. Microbiol. 120: 159-166.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with Folin Phenol reagent, J. Biol. Chem. 193: 153-179.

Maoura N, Mbaiguinam M, Gaillardin C, Pourquie J (2006). Suivi technique, analytique et microbiologique de la bili bili, bière traditionnelle Tchadienne, Afr. Sci. 02: 69-82.

Maoura N, Mbaiguinam M, Nguyen HV, Gaillardin C, Pourquie J (2005). Identification and typing of the yeast strains isolated from bili bili, a traditional sorghum beer of chad. Afr. J. Biotechnol. 4: 646-656.

Maxwell MJ, Messer SA, Hollis RJ, Boyken L, Tendolkar S, Diekema DJ, Pfaller MA, IFSPG (2003). Evaluation of Intestine Method for Determining fluconazole and voriconazole MICs for 279 clinical
isolates of *Candida* species infrequently isolated from blood. J. Clin. Microbiol. 41: 1087-1090.

Munyaja MBK, Narvhus JA, Treimos J, Langsrud T (2003). Isolation, characherization and identification of lactic acid bacteria from bushera: Ugandan traditional fermented beverage, Int. J. Food Microb. 8: 201-210.

Naumova ES, Korshunova IV, Jespersen L, Naumov GI (2003). Molecular genetic identification of *Saccharomyces* sensu strict strains from African sorghum beer, FEMS Yeast Res. 3(2): 177-184.

Nout MJR (1980). Microbiological aspects of traditional manufacture of Busaa, a Kenyan opaque maize beer Chem. Mikrobiol. Technol.Lebensm. 6: 137-142.

Novellie L (1982). Fermented porridge. In: Proceedings of the international symposium on sorghum grain quality. ICRISAT (eds), Pantacheru, India, pp. 121-128.

Nsabimana D (1997). Levain traditionnel Rwandais : essai d'utilisation en fermentation alcoolique et panaire. Travail de fin d'Etudes, Université Nationale du Rwanda, pp. 22-37.

Odunfa SA, Adeyele S (1985). Microbiological changes during the traditional production of ogi-baba, a west african fermented Sorghum gruel. J. Cereal Sci. 3: 173-180.

Okoh IA, Babalola GO, Ilori MO (1995). Effect of methanol extract of *Vernonia amygdalina* on malting and brewing properties of sorghum. Tech. Quart. Master Brewer's Assoc. Am. 32: 11-14.

Tisekwa B (1989). Improvement of traditional manufacturing of sorghum beer (mtama) in Tanzania. Thesis, Ghent University, Belgium, pp. 2- 18.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenitics. In: Innis N, Gelfand D, Sninsky J and White T (Eds). PCR protocol: a Guide to methods and Applications, Academic press, London, pp. 315-322.

Chapitre 3 :

Sélection et caractérisation des souches de levures performantes impliquées dans la fermentation de la bière traditionnelle « *ikigage* » du Rwanda

Ce travail fera l'objet de la publication suivante :

Lyumugabe, F., Habayo, A., Uyisenga, J.P., Nsanzimana, D., Mashako A., Bajyana, E., Thonart, Ph. Selection and characterization of predominant yeast strains involved in fermentation of the Rwandese traditional sorghum beer "*ikigage*". A soumettre dans Rwanda Journal (Etudes Rwandaises).

Préambule au chapitre 3

Le chapitre précédent a indiqué la prédominance de levures et la faible teneur en éthanol dans la bière traditionnelle du Rwanda *ikigage*. Les levures jouent un rôle majeur dans la fermentation alcoolique, en transformant les sucres en éthanol. De ce fait, la sélection de souches performantes permet d'augmenter la teneur en éthanol. Par ailleurs, l'utilisation de souches locales sélectionnées est très importante, parce que ces souches sont acclimatées aux conditions environnementales locales. Elles assurent également la maintenance des propriétés sensorielles caractéristiques du produit local. Compte tenu de l'importance de l'utilisation de souches de levures prédominantes, nous avons procédé à la sélection de souches et à la caractérisation de levures performantes impliquées dans la fermentation de la bière *ikigage*.

Les souches de levures ont été isolées au cours de la fermentation du moût de sorgho déclenchée par l'ajout du levain traditionnel Rwandais *umusemburo*. Ces souches ont été caractérisées et identifiées. Les souches majoritaires isolées à la fin de la fermentation ont été soumises aux tests de sélection par l'acidification et résistance à l'éthanol. Les souches sélectionnées ont été utilisées dans la fermentation du mout de sorgho afin d'évaluation leurs potentialités fermentaires dans un milieu naturel. Elles ont été également classifiées selon leurs caractéristiques phénotypiques de floculation.

Selection and characterization of predominant yeast strains involved in fermentation of the Rwandese traditional sorghum beer *ikigage*

RUNNING TITLE HEADER: Selection and characterization of yeast strains from *ikigage*

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Abstract

The present study proposes the characterization and the selection of powerful yeasts involved in the fermentation of the Rwandese traditional sorghum beer *ikigage.* The yeast strains isolated at different stages of *ikigage* fermentation were identified by API 20 C test and confirmed by PCR-Sequencing of ITS-5.8S region of rDNA. The selection of yeasts was accomplished by acidification power test, tolerance test to alcohol and the efficiency of ethanol production in natural medium as well as their flocculation capacity and their flocculation phenotypes assessed using spectrophometric method. A total of 127 yeast strains isolated at different steps of *ikigage* fermentation were identified. These strains belong to species of *Saccharomyces cerevisiae*, *Issatckenkia orientalis*, *Candida humilis*, *Candida incuspicua*, *Candida magnoliae*, *Candida krusei* and *Pichia membranifaciens*. *S. cerevisiae* and *I. orientalis* were predominant yeast strains. Seven strains belonging to *S. crevisiae* (6) and *I. orientalis* (1) were selected because of their high acidification power and ethanol tolerance. *I. orientalis* RG1 strain was able to survive in 15% ethanol and fast convert glucose into ethanol while *S. crevisiae* strains (RV6, RB2 and RK1) produced high final ethanol content. The yeast strains selected are non-flocculent (RK1), low flocculent (RV6 and RB2) and medium flocculent (RG1) strains. *S. cerevisiae* RV6, RB2 and RK1 were Newflo phenotype while *I. orientalis* RG1 strain was MI phenotype. Except RK1, which can cause problems of beer clarification, these yeast strains were found suitable for improving the quality of *ikigage* beer. However, further researches are needed to establish the flavour of *ikigage* beer produced from these yeast strains.

Key words: Sorghum beer, *Ikigage*, Alcoholic fermentation, Yeast, Selection

1. INTRODUCTION

The African traditional alcoholic beverages such as *ikigage, pito, dolo, tchoukoutou* and *burukutu* are prepared from sorghum malt. These spontaneously fermented beverages apart from serving as inebriating drinks, are also important in fulfilling social obligations (e.g., marriage, birth, baptism, dowery, etc.) and constitutes a source of economic return for the women manufacturer.

In Rwanda, during *ikigage* manufacturing, the malted wort sorghum is inoculated by a traditional leaven "*umusemburo*" as fermentation starter, which preparation is described by Lyumugabe *et al.* (2010). After 12 to 24 h of fermentation, *ikigage* is ready for consumption. The traditional methods of *umusemburo* production involve the preparation of malted wort sorghum (*igikoma*), the addition of local plants (e.g. *Vernonia amygdalina*) and the spontaneous fermentation of *igikoma*. The microorganisms involved in this fermentation are dominated by yeasts and Lactic acid bacteria (Lyumugabe *et al.*, 2010). However, yeasts are mainly responsible for alcohol content of the alcoholic beverages; *Saccharomyces cerevisiae* was found to predominate in African sorghum beers (Demuyakor *et al*., 1991; Jespersen, 2003; Naoumova *et al.*, 2003; Lyumugabe *et al*., 2010; Kayode *et al*., 2011).

Selecting the best yeast strain with the desired brewing characteristics has always been brewer's dream. The use of selected local yeasts is believed to be much more effective, since these yeasts are presumed to be more competitive because better acclimated to environmental conditions. Moreover, the selection of suitable local yeasts assures the maintenance of the typical sensory properties of the fermented products produced in any given region (Querol *et al*.,1992, Degre, 1993). The criteria for yeast selection will vary according to the requirements of the brewing equipment and the beer style, but they are likely to include the comit the speed of fermentation, yeasts stress tolerance, appropriate flocculation avoiding incomplete

attenuation, rate of attenuation at the desired temperature, balanced flavour compounds, efficient conversion of wort sugars to alcohol and genetic stability (Goldammer, 2008).

Currently, the manufacture of *ikigage* beer is declining because of poor hygienic quality, unsatisfactory conservation and poor yield of ethanol and variations of organoleptic quality (Lyumugabe *et al*., 2010). To improve the quality of this beer as it has done for traditional fermented foods such as *Kivude* (Teniola *et al*., 2001) or *Orgi* (Mugula *et al*., 2003), the approach using the predominant yeast strains as single or mixed starter cultures should be considered for a more predictable fermentation outcome. The present study proposes the characterization and the selection of powerful yeasts involved in the fermentation of Rwandese traditional sorghum beer *ikigage*. The selection of yeasts was accomplished by acidification power test, tolerance test to alcohol and the efficiency of ethanol production in natural medium. The study of flocculation capacity of selected yeasts and their flocculation phenotypes was also performed.

2. MATERIAL AND METHODS

2.1. Samples

A total of 12 samples of traditional leaven "*umusemburo*" were obtained from local producers of traditional sorghum beer "*ikigage*" in the northern (Ruhengeri and Byumba sites) and southern (Tumba and Muhanga sites) province of Rwanda. The local producers of *umusemburo* (one per site) were selected on the basis of frequency of their production. The samples were collected in screw-capped bottles, packed in an insulated icebox, transported to the laboratory and analyzed immediately for microbiological analysis.

2.2. Enumeration of microorganisms

Duplicate aliquots of *umesmburo* (10 ml) were homogenized with 90 ml sterile peptone physiological saline solution (1g Peptone, 8.5 g NaCl and 1000 ml distilled water). The homogenate was decimal diluted and the relevant dilutions surface plated. Total accounts aerobic mesophilic flora, lactic acid bacteria, yeasts and moulds were enumerated as described by Lyumugabe *et al*. (2010).

2.3. Wort fermentation using *umusemburo*

Malted sorghum worts collected from the traditional local brewers were dispensed into a 1000 ml Erlenmeyer flask, equipped with a gas trap and autoclaved at 121° C for 15 min. After cooling to about 45°C, 50 ml of *umusemburo* were added with 500 ml of malted sorghum wort and incubated at 30°C for 48h. After each 8h, the growth of total counts aerobic mesophilic flora, lactic acid bacteria, yeasts and moulds were evaluated during the fermentation. The yeasts isolated during fermentation were characterized and identified.

2.4. Characterization and identification of yeast isolates

Isolation was done by spread-plating 0,1 ml of suitable dilution of sample on YPD-Chloramphenicol (10 g yeast extract, 10 g peptone, 20 g glucose, 20 g agar, 0.5 g chloramphenicol and 1000 ml distilled water) after 48 to 72 h of incubation at 30°C. For characterization and identification of yeasts, isolates from *ikigage* fermentation were purified by successive sub-culturing on YPD. Preliminary confirmation was based on microscopic observation. The isolates were tested for the fermentation of glucose, sucrose, maltose, lactose and raffinose according the description of Van Der Walt and Yarrow (1984). The assimilation of nitrogen source (nitrate, ethylamine hydrochloride, L-lysine and cadavarine) was also tested. Physiological and biochemical characterization of the isolated yeasts were also

determined by investigating the assimilation reactions of sugars using the API 20 C Aux. kit (BioMérieux, Lyon, France) according to the manufacturer's instructions. For the spore morphology, cells were grown on sodium acetate agar and Gorodkowa medium were used to induce the sporulation of yeasts whilst the hyphae growth was also determined by using RAT medium. The identification of yeasts according phenotypic characteristic was done using API taxon 2004 software.

Yeasts identified by phenotypic tests were confirmed by PCR – Sequencing of internal transcribed spacer (ITS) region of rDNA. The ITS1 - 5.8S - ITS2 regions of rDNA were amplified by PCR using the primer ITS1 (5P- TCCGTAGGTGAACCTGCGG-) and ITS4 (5P- TCCTCCGCTTATTGATATGC-) according to White *et al* (1990). The purified PCR products were directly sequenced using ABI 3130 genetic analyzer. Blast searches of sequences were performed at the National Centre for Biotechnology Information (NCBI) Gen Bank data library.

2.5. Acidification power test

The method of Kara *et al*. (1998) was used to determine the acidification power of yeasts strains. The pH meter was calibrated using the two-buffer method before each series of assays. Deionised water pH was adjusted to approximately 6.5 pH for AP studies. Sterile deionised water (15 ml) was placed in a 50 ml conical centrifuge tube containing a conical stir bar. The pH of the water was monitored for 5 min with constant stirring. At the end of 5 min, a pH reading was recorded (AP0) and 5 ml of concentrated yeast slurry (10^9 cells/ml) was added to the centrifuge tube. The yeast suspension was allowed to stir for 10 min, after which the pH was recorded (AP10). Immediately after the recording of the AP10, 5 ml of 20% glucose solution was added to the yeast suspension and allowed to incubate for 10 min. At the

end of 10 min the final pH reading was recorded (AP20). The acidification power was calculated by subtracting the AP20 from the AP0 reading.

2.6. Wort fermentation using the yeast strains selected

The wort (SB) used is a mixture of sorghum malted wort (70%) from local brew and banana juice (30%). Worts were dispensed into a 100 ml and 500 ml Erlenmeyer flask and sterilized by autoclaving at 121° C for 15 min. After cooling to about 45° C, 0, 1 ml of yeast strain selected was cultivated on 25 ml of wort in 100 ml Elernemeyer flask at 30° C for 24 h and then the aliquots of yeast culture were inoculated in 500 ml of wort. Initial cell number of yeast was adjusted to 10^7cfu/ml. Erlenmeyer flask (1000 ml), equipped with a gas trap, was incubated at 30° C for 3 days. The yeast growth and ethanol were appreciated every 15 h.

2.7. Ethanol tolerance

Yeast strains were inoculated into 100 ml conical flask containing 40 ml of YPD liquid and incubated at 30° C for 48h. 1 ml of 48h old yeast culture were then inoculated into 100 ml containing 40 ml of YPD liquid supplemented with 5, 10 and 15 % of ethanol. Samples were taken after 24 and 48 h of incubation, diluted in 0.1% peptone broth when necessary followed by spread-plating (0.1 ml) on YPD- Agar plates. The colonies were enumerated after incubation at 30 °C for 48 h and colony forming unit (Cfu/ml) obtained.

2.8. Flocculation study

Flocculation rate

The flocculation rate was determined by spectrophotometric method (Hautcourt *et al.*, 1999). The yeast strains were inoculated in 10 ml of wort SB, sterilized by autoclaving at 121° C for 15 min, and incubated at 30° C for 72 h. The cell cultures obtained by the procedure described

above were centrifuged and the cells were re-suspended in 5ml of Helm's tampon (0,51 g calcium sulfate, 6,8g sodium acetate, 4,05g acetic acid, 4% ethanol, 1L distillated water, pH =4,5) and in 10 ml EDTA solution (46,5g EDTA, 0,35 sodium dihydrogenophasphate, pH=7,3 , 250ml distillated water). The degree of flocculation of the different strains was determined in terms of the ratio between the optical density at 620nm of the culture suspension in Helm's tampon (OD_B) and culture suspension in EDTA solution ((OD_A-OD_B/OD_A) x 100). The following flocculation scale was established: ratio ≤ 5%, 0 (no flocculent); ratio between 5% and 20% (low flocculent); ratio between 20% and 50% (medium flocculent); ratio ≥ 50% (high flocculent).

Flocculation inhibition by sugars

The flocculent strains were cultivated at 30° C in sterile YPD medium for 72 h under shaking. The cultures were centrifuged at 5000 g for 5 min at room temperature and washed once with distilled water. Cells were suspended to a final density of 10^6 cfu/ml in 10 ml of Helm's tampon supplemented with glucose or mannose 1M. The cell suspension was maintained at room temperature for 20 min, and flocculation capacity in the presence of these sugars was determined. Flocculation of a strain of yeast is inhibited by sugar if the value of its rate in medium containing sugars solution is lower than the rate of the same strain suspended in calcium.

3. RESULTS AND DISCUSSION

3.1. Microbial population during *ikigage* fermentation

The results of the microbial content of traditional leaven "*umusemburo*" from Rwanda are indicated in **Table 1.** These results showed the predominance of yeasts and lactic acid bacteria in *umusemburo* leaven.

The evolution of microbial population during the fermentation of malted wort sorghum initiated by *umusemburo* leaven in shown in **Figure 1**. Total mesophilic aerobic flora and moulds increase after 8h and disappear respectively after 32 and 40h of fermentation. Yeasts and lactic acid bacteria resist until the end of fermentation, although the progressive reduction of lactic acid bacteria start after 40h. The disappearance of total flora and moulds would be due on the one hand to the acidity of sorghum wort during fermentation and on the other hand with the scarcity of oxygen. The increase of total acidity and decrease of pH during *ikigage* fermentation is accompagned with increase of lactic acid bacteria.

Yeasts and lactic acid bacteria are the predominant microorganisms involved in most African traditional foods and beverage fermentations (Odunfa, 1985; Mugula *et al*., 2003). The quantities relatively higher of yeasts were also reported in traditional "*ikigage*" beers (Lyumugabe *et al*., 2010), "*pito*" beers (Glover *et al.,* 2005) and "*tchoukoutou*" beer (Kayodé *et al.,* 2011) from several African countries. This suggests the existence of a double fermentation (alcoholic and lactic) indicated by several authors (Novellie, 1976; Munyaja *et al.*, 2003).

3.2. Yeast strains isolated during *ikigage* fermentation

A total of 127 yeast strains were isolated during *ikigage* fermentation and subjected to morphological, fermentation and assimilation tests. The profiles of carbohydrate and fermentation and assimilation of yeasts isolates are shown in **Table 2** and **3**. None of the yeast isolates can ferment lactose, but they were able to ferment glucose (89 %), sucrose (87,7 %), maltose (51,6 %) and raffinose (24,2 %). 41,7 % of yeasts isolates were also able to assimilate ethylamine, L-lysine and cadaverine whereas none of the isolates can assimilate nitrate. Based on their assimilation of carbon compounds, 12 assimilation profiles were distinguished (62,3 %), galactose (56 %) maltose (51,6%) and glycerol (40,7 %).

Table 1. Microbial content of traditional leaven *umusemburo* from Rwanda

Sample origin	Total mesophilic aerobic flora (cfu/ml)	Yeasts (cfu/ml)	Lactic acid bacteria (Cfu/ml)	Moulds (Cfu/ml)
Byumba	78,2. 10^6	61, 2. 10^6	81, 3. 10^6	2,6. 10^5
Muhanga	119,7. 10^6	114,9.10^6	59,2. 10^6	5, 2.10^5
Ruhengeri	210, 5.10^6	107,3.10^6	11, 2.10^6	5,5. 10^5
Tumba	134, 8.10^6	75,8. 10^6	58, 2.10^6	5,5. 10^5
Mean	134,8 .10^6	89,8 .10^6	52, 6. 10^6	3,7. 10^5

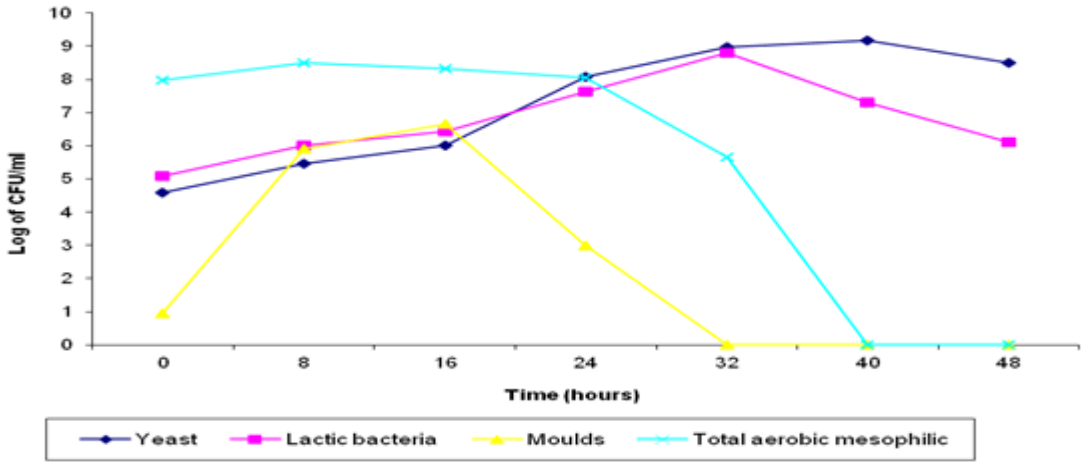

Figure 1. Evolution of microbial popualion of *umusemburo* during ferementation

A minor part of the isolates were able to assimilate raffinose (29,7 %), N-acetyl – glucosamine (18,7 %), trehalose (14,3 %), methyl D – glucopyranoside (8,8%) and calcium 2-cetogluconate (6, 6 %). None of them assimilated arabinose, xylose, adonitol, xylitol, inositol, sorbitol, cellobiose, lactose and melezitol.

According to their phenotypic characters and also molecular identification (**Table 4**), the yeasts isolated during *ikigage* fermentation were found to belong to *S. cerevisiae, I. orientalis, C. humilis, C. incuspicua, C. magnoliae, C. krusei* and *P. membranifaciens*. The percentages

of each species at various stages of *ikigage* fermentation are shown in **Table 5**. When the *ikigage* fermentation were initiated, we found *S. cerevisiae* (41,8 %), *C. incospicua* (21 %), *C. magnolia* (15,8 %), *P. membranifaciens* (15,8 %) and *C. krusei* (10,5 %). *S. cerevisiae* was the most frequent species at the various stages while *I. orientalis* and *C. humilis* were observed after 8h of fermentation. At the end of fermentation, only *S. cerevisiae* (61 %), *I. orientalis* (29, 3 %), *C. humilis* (7 %) and *C. incospicua* (3,7 %) were found in the fermented product. These results indicate clearly the dominance of *S. cerevisiae* (53,5 %), follow - up by *I. Orientalis* during the *ikigage* fermentation process. The dominance of *S. cerevisiae* is a well indication of occurrence of the alcoholic fermentation during fermentation of *ikigage* beer. Other similar studies realized by reported *S. cerevisiae* as being the predominant yeasts species associated in the alcoholic fermentation of *pito* (Sefa-Deheh *et al*., 1999), *dolo* (Konlani *et al*., 1996; Van der Aa Kuhle *et al*., 2001) and *tchoukoutou* (Koyodé *et al.*, 2011). These authors reported that *S. cerevisiae* was often associated with other yeast species which may contribute to the organoleptic characters of African sorghum beers. However, while agreeing on the dominance of *S. cerevisiae*, the occurrence of the non – Saccharomyces species seems to vary. *Candida tropicalis, Torulaspora delbrueckii, Kloeckera apiculata, Hansenula anomala, Schizosaccharomyces pombe* and *Kluyveromyces africanus* were found in sorghum beers from Togo, Burkina Faso and Ghana (Demuykor *et al.*, 1991; Konlani *et al*., 1996; Sefa-Deheh *et al*., 1999). In *Burukutu* from Nigeria, Sanni *et al.* (1993) reported also the presence of *H. anomala, kloeckera apiculata, C. tropicalis, C. krusei, C. castelli, Geotricum candidum, P. membraefaciens* and *klyveromyces africanus*. According to these authors, *C. tropicalis* was the predominant non – *Saccharomyces*. Except *P. membraefaciens* and *C. krusei*, these yeasts species were not found in this work. We found the presence of *I. Orientalis, C. magnolia, C. humulis* and *C. incospicua* during *ikigage* fermentation.

Table 2. Carbohydrate assimilation profiles of yeasts isolated during *ikigage* fermentation

	*A	B	C	D	E	F	G	H	I	J	K	L	Total (%)
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	100
Glycerol	+	+	-	-	+	+	-	-	-	-	-	-	40,7
Calcium 2-ceto-gluconate	-	-	-	-	-	-	+	-	-	-	-	-	5,5
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	0
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	0
Adonitole	-	-	-	-	-	-	-	-	-	-	-	-	0
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	0
Galactose	-	+	+	-	+	-	-	+	+	-	+	-	56
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	0
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	0
Methyl D – glucopyranoside	-	-	-	-	-	-	-	-	-	-	+	-	8,8
N-acetyl – glucosamine	+	-	-	-	-	+	-	-	-	-	-	-	18,7
Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	0
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	0
Maltose	-	-	+	+	+	-	-	+	-	+	+	-	51,6
Sucrose	-	+	+	+	+	-	+	+	+	+	+	-	62,3
Trehalose	-	-	-	-	-	-	-	-	+	-	+	-	14,3
Melezitol	-	-	-	-	-	-	-	-	-	-	-	-	0
Raffinose	-	-	-	-	-	-	-	+	+	+	-	-	29,7
Number of isolates (%)	7	17,3	11,8	10,2	7	5,5	6,3	8	8	8,6	8	2,4	

***A:** isolates L3, N1, K4, R3, A4, M2, N2, R8, R9; **B:** isolates RG1, F2, L6, RG2, R6, G7, V8, RT1, G6, B7, M6 ; A5, N3 ; G9, V11, A6, A7, M8, F5, F9, K12, T15; **C :** isolates RV6, RK1, G10, K8, RN4, RL1, K3, L5, R2, F1, T2, L11, L12, F7, V12 **; D :** isolates RT8 , B8, A8, RN6, RF3, V4, RT4, A1, N10, V3, L13, B5, N9 ; **E :** isolates RV2, F8, F6,RG3,RK5,V10, F4, L10, T7 ; **F:** isolates R4, T12, R7, R5, B3, L9, K7; **G:** isolates T6, T9, G4, T3, A10, R11, M7, M11; **H:** isolates RB2, V1, T14, T13, RA3, RB1, RN7, K6, V9, K10 ; **I:** isolates RR1, RT5, M5, G5, RT4, F10, A9, R10, R12, N8; **J:** isolates RM1, RM3, RM4, V5, RV7, A2, B4, K9, K11, G12, V13 ; **K :** isolates L7, M9, M10, B6, L8, L4, L2, K2, N5, G8. **L**: isolates T10, T11, G11.

Table 3. Fermentation characteristics and nitrogen assimilation profile of yeast isolated during *ikigage* fermentation

Isolates	**Fermentation of carbohydrate**					**Assimilation of nitrogen**			
	Glucose	Lactose	Maltose	Sucrose	Raffinose	Nitrate	Ethylamine	L-lysine	Cadaverine
L3, N1, K4, R3, A4, M2, N2, R8, R9	-	-	-	-	-	-	+	+	+
RB2, V1, RA3, RB1, RN7, K6, V9, RM1, RM3, RM4, V5, RV7, A2, B4, RF3, RK5, RK1, T14, T13, K10, K11, G12, V13, K9	+	-	+	+	+	-	-	-	-
RR1,RT5, M5, G5, RT4, F10,A9,R12, N8	+	-	-	+	+	-	-	-	-
RV6,G10,K8, RN4, RL1, K3, L5, R2, F1, T2, RT8 , RN6, V4, RT4, A1, V3, B5, RV2,RG3,V10, F4, L10, L7, B6, L8, L4, L2, K2, N5, G8, L11, L12, F7, V12, B8, A8, N10, N9, L13, F8, F6, M9, M10	+	-	+	+	-	-	-	-	-
RG1, L6, RG2, R6, G7, V8, RT1, G6, B7, M6 ; A5, N3 ; G9, V11, R4, T12, R7, R5, B3, L9, K7, T6, T7, T9, G4, T3, F2, K12, T15, F9, A7, M8, A6, F5, T12, A10, R11, M7	+	-	-	+	-	-	+	+	+
T10, T11, G11	+	-	-	-	-	-	+	+	+
Frequency (%)	89	0	51,6	87,7	24,2	0	41,7	41,7	41,7

Table 4. Yeasts identified according to sequences of 5.8S rDNA-ITS region

Yeast isolates	Number of nucleotides compared	Percent homology with GenBank	GenBank accession number of corresponding sequence	Species
RB2	615	100	AM262829	*S. cerevisiae*
RL1	544	98	AB279747	*S. cerevisiae*
RK1	399	99	AY796193	*S. cerevisiae*
RG1	545	99	AF262033	*I.orientalis*
RN4	552	99	AM262830	*S. cerevisiae*
RT1	397	97	AF417255	*I.orientalis*
RG2	447	98	AB365318	*I.orientalis*
RR1	470	98	AY493349	*C. humilis*
RT5	470	98	AY493349	*C. humilis*
RV6	445	99	AM262824	*S. cerevisiae*
RT8	499	97	AB280539	*S. cerevisiae*
RM1	455	98	EU145764	*S. cerevisiae*

Table 5. Distribution of yeast species (%) isolated during *ikigage* fermentation

	Fermentation time						
	0h	8h	16h	24h	32h	40h	48h
S. cerevisiae	41,8	50	47	50	66,6	63,2	61,5
I. orientalis	-	5,5	17,7	18,7	30,1	26,3	29,3
C. krusei	5,5	9,6	5,7	-	3,3	-	-
C. magnoliae	15,8	16,6	0	12,5	-	-	-
C. humilis	-	7	17,6	12,5	-	7,5	7
C. incospicua	21	7,9	11,7	6,2	-	3	3,7
P. membranifaciens	15,8	3,1	-	-	-	-	-

Contrary to Westen African sorghum beers, where *Candida tropicalis* is predominant yeast strains after *S. crevisiae*, *I. orientalis* was predominant non-*Saccharomyces cerevisiae* yeast involved in fermentation of Rwandese sorghum beer. *I. orientalis* was also

more dominant yeast specie in togwa, Tanzanian fermented food manufactured from sorghum (Mugulu *et al*., 2003).

3.3. Selection of yeast strains

Acidification power

Fourteen strains of majority yeast species (*S. cerevisiae* and *I. orientalis*) isolated at the end of *ikigage* fermentation were pre-selected and subjected to acidification power test. The results of acidification power test are represented in **Figure 2**. On the 23 yeast strains tested in this work, 7 (RB2, RK1, RV6, RN4, RT8, RL1, RG1) are highly active with good fermentation

potential (AP value between 1,5 and 3), while 6 (RG2, RR1, RT5, RT8, RM3, RN7) with AP values between 1 and 1,5 indicated a reduced metabolic activity and 10 yeast strains indicate low metabolic competence (AP value below 1) that could result in sluggish fermentations.

Acidification power indicates glycolytic activity and endogenous reserves of the yeast cell to maintain a fixed ratio between intracellular and extracellular hydrogen ion concentrations (Sigler *et al.*, 1981; Opekarova *et al.*, 1982). It is closely related to viability and fermentation performance of yeast strains (Mathieu *et al*., 1991; White *et al*., 2003; Gabriel *et al*., 2008).

Ethanol tolerance

The effect of different concentrations of ethanol on the growth of 7 yeast strains (RB2, RK1, RV6, RN4, RT8, RL1, RG1) previousily selected was studied at 30 °C. The results obtained (**Figure 3**) show that all yeast strains studied resist very well to the concentration of 5% ethanol. There was a progressive decrease in the viable counts of all the isolates when the concentration of the ethanol was increased. *S cerevisiae* strains RV6, RB2 and RK1, and I. *orientalis* strains RG1 exhibited good tolerance at 10% ethanol. Only *I. orientalis* RG1 was able to survive in 15% ethanol. Similar results have been reported by Day *et* al. (1975) for yeast brewing. They have found that the ability of different yeasts to tolerate high levels of ethanol varies widely. But, *Saccharomyces* strains brewing are fairly uniform in their response to ethanol, tolerating ethanol concentrations of 7 to 13%. *I. orientalis* is very well known as first yeast with high ethanol tolerance (Okuma *et al*., 1995; Isono *et al.*, 2012). The plasma membrane composition was identified as being central to the ethanol tolerance of yeast strains, with yeast responding to increased ethanol concentration, in a dose-dependent manner, by increasing the unsaturation index, and hence fluidity, of their membranes (Beaven *et al*., 1982 ; Odumeru *et al*., 1993. Alexandre *et al*., 1994).

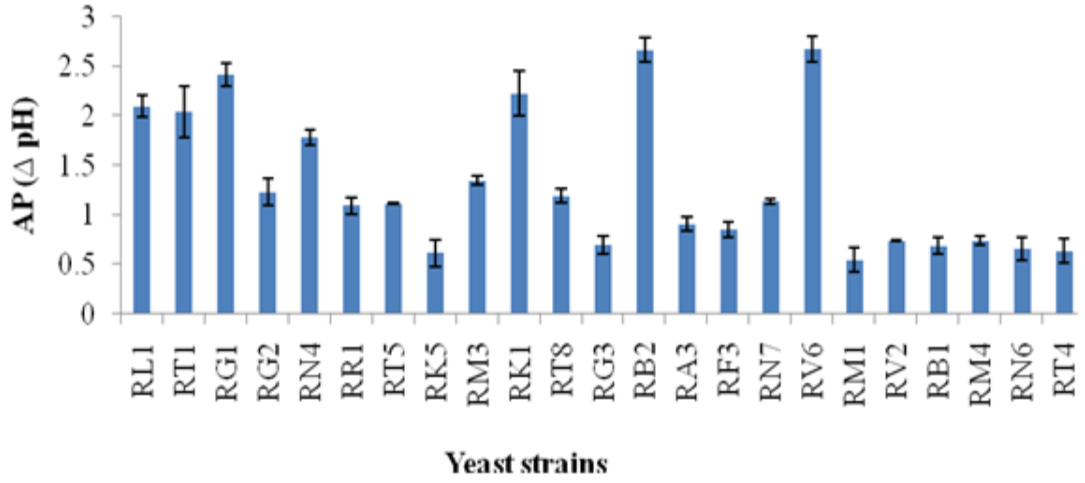

Figure 2. Acidification power of yeast strains pre-selected from ikigage fermentation

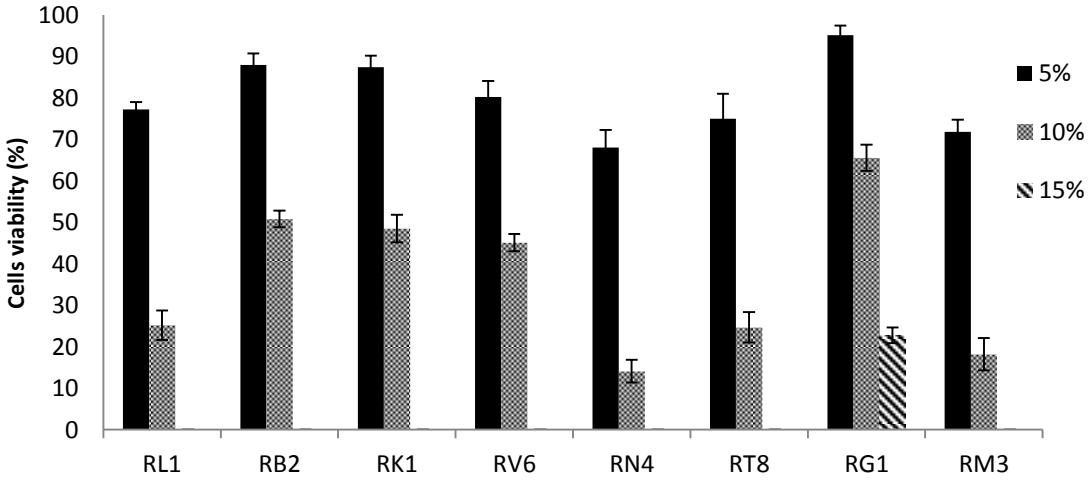

Figure 3. Effect of different concentrations of ethanol on growth of *S. cerevisiae* strains (RL1, RB2, RK1, RV6, RN4, RT8 and RM3) and *I. orientalis* RG1 strain at 30 °C after 48h.

Although ethanol tolerance and ethanol production ability are not directly correlated (Benitez *et al*., 1983), *S. cerevisiae* RK1, RV6 and RB2 strains and *I. orientalis* RK1 strain can be employed for optimization of *ikigage* beer processing with higher ethanol content. However, fermentable sugars in sorghum wort are limited, addition of adjunct or development of

sorghum with improved malting characteristics will be necessary before *ikigage* beer with increased alcohol percentage can be produced.

Ethanol production

The ethanol produced by each yeast strains is shown in **Figure 4**. The final ethanol content of *I. orientalis* strain RG1 (2, 38 % v/v) was lower than those of *S. cerevisiae* RV6, RK1 and RB2 strains (5,45 - 6,7% v/v), but *I. orientalis* produced high ethanol concentration after 15 and 30h. Ethanol production rates reflected the cell growth pattern. The faster cell growth of RG1 after 15 and 30h coincided with faster ethanol production rate because high ability of this strain to assimilate and fermente glucose. Recently, Isono *et al*. (2012) demonstrated that *I. orientalis* strains have a very high ability to ferment glucose to ethanol under high stress conditions (high temperature, such as acid, salt) comparatively to *S. cerevisiae.* Lower final ethonal concentration can be explained by not maltose assimilation and fermentation by *I. orientalis.* This also explains the weak attenuation limit (**Figure 5**) compared to *S. cerevisiae* RV6, RB2, RK1. Hewever, high limit attenuation was obtained with *S. cerevisiae* RV6 strains.

Flocculation

The results of floculation ability yeast strains and characteristics of flocculent strains are indicated in **Table 6**. The flocculation degree of RV6, RB2, RK1 and RG1 was 14.7, 18.8, 1.5

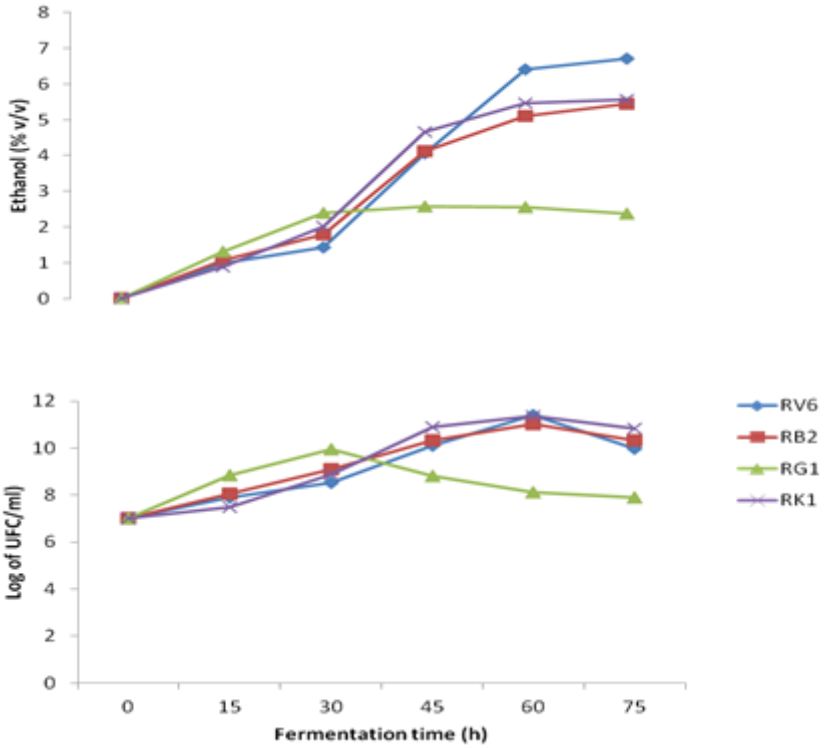

Figure 4. Ethanol production and yeast cell growth during fermentation

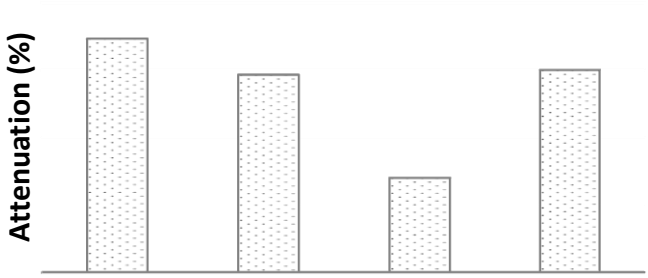

Figure 5. Attenuation of selected yeast strains isolated from umusemburo leaven

Table 6. Flocculation characteristics of yeast strains isolated from *umusemburo* leaven

	RV6	RB2	RG1	RK1
Flocculation rate (%)	14,7	18,8	29,2	1,45
Flocculation inhibition by sugar	Newflo or GMS	Newflo or GMS	MI	Newflo or GMS

and 29,2%, respectively. According to Gilliland (1951) classification, RK1 is non - flocculent strain (≤ 5%) while RB2 and RV6 are low flocculent strains (between 5 and 20 %). RG1 is considered as medium flocculent strain (between 20 and 50%).

The flocculation can be defined as the reversible phenomenon wherein yeast cells adhere in clumps and either sediment rapidly from the medium in which they are suspended or rise to the medium's surface (Stewart *et al,* 1981). Flocculating is a very important property in itself in the brewing industry as well as in biotechnological applications due to the fact that it is often the primary method of yeast separation.

Premature yeast flocculation cause significant financial and logistical problems to the brewer due to incomplete conversion of sugars to alcohol resulting in higher residual extract and lower alcohol than specification demands, flavour abnormalities, disruption of process cycle times and potential issues with the re-use of the yeast in subsequent fermentations (Axell, 2003; Koizumi, 2006; 2009). While non – flocculation of yeast cause many problems for beer clarification (Speers *et al*., 2006). From this point of view, RV6, RB2 and RG1 are considered as good yeast strains for *ikigage* production.

Moreover, the flocculation involves lectin – like protein – carbohydrate recognition and interaction in a manner of calcium – dependent and sugar sensitive (Speers and Ritcey, 1995). The lectins are proteins in the walls of flocculent cells able to bind to carbohydrates on neighboring cells. These are specific proteins that require the presence of calcium to maintain their active conformation. Calcium has the property to activate the lectin while decreasing the repulsion between cells and increasing the hydrophobicity of cell surfaces. The fixing of calcium ions on lectin site adjacent to the binding site for sugars passes them to their active form (Stratford, 1992; Yu-Lai *et al*., 1998; Stan *et al*., 2000). Three flocculation phenotypes

have been described according to sugar specificity: Flo 1, flocculation of yeast was only inhibited by mannose (MS); the NewFlo phenotype, inhibited by glucose and mannose (GMS); and the mannose insensitive MI phenotype, following Stratford (1989) and Masy *et al.*, 1992. The majority of brewery yeast strains belong to the NewFlo phenotype (Soares and Vroman, 2003). In our wort, the *S. cerevisiae* RV6, RB2 and RK1 were glucose – mannose sensitive while *I. orientalis* RG1 strain was mannose insensitive (**Table 6**). According to Masy *et al*. (1992), flocculation of yeasts classified as mannose insensitive could be produced by hydrophobic interactions or specific interactions not involving mannans (protein–protein, protein–lipid).

4. CONCLUSION

The present study proposes the characterization and the selection of powerful yeasts involved in the fermentation of *ikigage.* The yeasts involved in *ikigage* fermentation belong to species of *S. cerevisiae, I. orientalis*, *C. humilis*, *C. incuspicua*, *C. magnoliae*, *C. krusei* and *P. membranifaciens*. *S. cerevisiae,* followed by *I. orientalis,* was predominant at the end of fermentation. Seven strains belong to *S. crevisiae* (6) and *I. orientalis* (1) were selected because their high acidification power and ethanol tolerance. *I. orientalis* RG1 strains was able to survive in 15% ethanol and fast convert glucose into ethanol while *S. crevisiae* strains (RV6, RB2 and RK1) produced high final ethanol content. The yeast strains selected are non – flocculent (RK1), low flocculent (RV6 and RB2) and medium flocculent (RG1) strains. *S. cerevisiae* RV6, RB2 and RK1 were Newflo phenotype while *I. orientalis* RG1 strain was MI phenotype. Except RK1, which can cause problems of beer clarification, these yeast strains revealed suitable for improving the quality of *ikigage* beer. However, further researches are needed to establish the flavour of *ikigage* beer produced from these yeast strains.

REFERENCES

Alexandre H., Rousseaux I. & Charpentier C., 1994. Relationship between ethanol tolerance, lipid composition and plasma membrane fluidity in *Saccharomyces cerevisiae* and Kloeckera apiculata. FEMS *Microbiol Lett., 124, 17–21.*

Axcell, B. C., 2003. Impact of wort composition on flocculation. In: Brewing Yeast Fermentation Performance, 2nd ed., K. A. Smart, Ed., Blackwell Science: Oxford, pp120-128.

Beaven MJ, Charpentier C & Rose A (1982) Production and tolerance of ethanol in relation to phospholipid fatty-acyl composition in *Saccharomyces cerevisiae* NCYC 431. *J. Gen. Microbiol., 128, 1447–1455.*

Benitez T., Del Castllo L., Aguilera A., Conde J. & Cerdaolmedo E. 1983. Selection of wine yeast for growth and fermentation in the presence of ethanol and sucrose. *Applied and Environ. Microbiol., 45, 1429 – 1436.*

Day A., Anderson E. & Martin P.A., 1975. Ethanol tolerance of brewing yeasts. In European *Brewing Convention, Proceedings of the congress, Elsevier Scientific publishing Co., Amsterdam, pp 377 – 391*.

Degre´ R., 1993. Selection and commercial cultivation of wine yeast and bacteria. In: Fleet, G.H. (Ed.), Wine Microbiology and Biotechnology. *Harwood Academic Publishers, Chur, pp. 421–447.*

Demuyakor B. & Ohta Y., 1991. Characteristics of pito yeasts from Ghana. *Food microbiology, 8, 183 – 193.*

Gabriel P., Dienstbie R.M., Matoulková D., Kosař k. & Sigler K. 2008. Optimised acidification power test of yeast vitality and its use in brewing Practice. *J. Inst. Brew. 114, 270–276.*

Gilliland R.B., 1951. The flocculation characteristics of brewing yeast during fermentation. *Proc. Eur. Brewing convention congr., Brighton, pp. 35 – 58.*

Glover RLK., Abaidoo RC., Jakobsen M. & Jespersen L., 2005. Biodiversity of *Saccharomyces cerevisae* isolated from a survey of *pito* production sites in various parts of Ghana. *Syst. Appl. Microbiol., 28: 755-761*.

Goldamer T. 2008. The brewer's handbook. 2nd edition, Apex Publishers, USA, 496p

Jespersen J., 2003. Occurrence and taxonomic characteristics of strains of Saccharomyces cerevisiae predominant in African indigenous fermented foods and beverages. *FEMS Yeast Research 3, 191 – 200.*

Yu – Lai J. & Speers R.A., 1998. Flocculation of *Saccharomyces cerevisiae*. *Food Res. Inter., 31, 421 – 440.*

Isono N, Hayakawa H., Usami A., Mishima T. & Hisamatsu M., 2012. A comparative study of ethanol production by Issatchenkia orientalis strains under stress conditions. *J. Biosc. Bioeng., 113, 76- 78.*

Kara B.V., Simpson W.J. & Hammond J.R.M., 1998. Prediction of the fermentation performance of brewing with the acidification power test. *J. inst. Brew., 94, 153 – 158.*

Kayodé A. P. P., Vieira-Dalodé G., Kotchoni S. O., Linnemann A. R., Hounhouigan J. D., Van Boekel M. A. J. S. & Nout M. J. R., 2011. Diversity of yeasts involved in the fermentation of tchoukoutou, an opaque sorghum beer from Benin. *Afri. J. Microbiol. Res., 5, 2737-2742.*

Koizumi H., 2008. Barley malt polysaccharides inducing premature yeast flocculation and their possible mechanisms. *J. Am. Soc. Brew. Chem., 66, 137-142.*

Koizumi H., 2009. Structural features of barley malt polysaccharides inducing premature yeast flocculation. *J. Am. Soc. Brew. Chem.,67, 129-134.*

Konlani S, Delgenes JP, Moletta R, Traore A, Doh A., 1996. Isolation and physiological characteristics of yeasts involved in sorghum beer production. *Food Biotechnol., 10, 29-40.*

Lyumugabe L., Kamaliza G., Bajyana E. & Thonart PH., 2010. Microbiological and physico-chemical characteristic of Rwandese traditional beer "*Ikigage*". *Afr. J. Biotechnol., 9, 4241-4246.*

Masy C.L., Henquinet A., Mestdagh M.M., 1992. Flocculation of Saccharomyces cerevisiae: inhibitions by sugars. *Can. J. Microbiol. 38, 1298–1306.*

Mathieu C., Van den Bergh L. & Iserentant D., 1991. Prediction of yeast fermentation performance using the acidification power test. *Proceedings of the European Brewery Convention Congress, Lisbon, IRL Press: Oxford, pp. 273-280.*

Mugula J.K, Nnkoa S.A.M., J.A Narvhusb J.A. and Sorhaug T., 2003. Microbiological and fermentation characteristics of togwa, a Tanzanian fermented food. *Inter. J. Food Microbiol., 80, 187-199.*

Muyanja CMBK., Narvhus JA., Treimo J., Langsrud T., 2003. Isolation, characterization and identification of lactic acid bacteria from bushera: A Ugandan traditional fermented beverage. *Int. J. Food Microbiol., 80, 201-210.*

Naoumova ES., Korshunova IV., Jespersen L. & Naumov GS., 2003. Molecular genetic identification of Sacharomyces sensu stricto strains from African sorghum beer. *FEMS Yeast Research, 3, 177 – 184.*

Novellie L., 1976. Beverages from sorghum and millets. In: Proceedings of the international symposium on sorghum and millets for human Food. *International Association for Cereal Chemistry Symposium in Vienna, Tropical Products Institute.*

Odumeru JA., D'Amore T., Russell I. & Stewart GG., 1993; Alterations in fatty acid composition and trehalose concentration of Saccharomyces brewing strains in response to heat and ethanol shock. *J. Ind. Microbiol. Biotechnol., 1, 113–119.*

Odunfa SA. & Adeyele S., 1985. Microbiological changes during the traditional production of ogi-baba, a west african fermented Sorghum gruel. *J. Cereal Sci., 3, 173-180.*

Okuma Y, Endo A, Iwasaki H, Ito Y, Goto S., 1986. Isolation and properties of ethanol-using yeasts with acid and ethanol tolerance. *J. Ferment. Technol., 64, 379–382.*

Opekarova M. & Sigler K., 1982. Acidification Power: Indicator of Metabolic Activity and Autolytic Changes in *Saccharomyces cerevisiae. Folia Microbiol., 27, 395-403.*

Querol A., Barrio E., Huerta T. & Ramon D., 1992. Molecular monitoring of wine fermentations conducted by dry yeast strains. *Appl. Environ. Microbiol. 58, 2948–2952.*

Sanni AI. & Lönner C., 1993. Identification of yeast isolated from Nigerian traditional alcoholic beverages. *Food Microbiol., 10, 517-523.*

Sefa-Dedeh S., Sanni AI., Tetteh G. & Sakyi-Dawson E., 1999. Yeasts in the traditional brewing of *pito* in Ghana. *World J. Microbiol., 15, 593-597.*

Sigler K., Knotkova A. & Kotyk A., 1981. Factors governing substrate-induced generation and extrusion of protons in the yeast *Saccharomyces cerevisiae. Biochim. Biophys. Aeta 643, 572.*

Soares E.V. & Vroman, A., 2003. Effect of different starvation conditions on the flocculation of *Saccharomyces cerevisiae*. *J. Appl. Microbiol., 95, 325–330.*

Speers, A., Ritcey, L.L., 1995. Towards an ideal flocculation assay. *J. Am. Soc. Chem. 53, 174–177.*

Speers A., Wan Y. Q., Yu-Lai Jin Y.L. & Stewart R.J., 2006. Effects of fermentation parameters and cell wall properties on yeast flocculation. *J. Inst. Brew. 112, 246–254.*

Stratford M., 1989. Evidence for two mechanisms of flocculation in *Saccharomyces cerevisiae. Yeast, 5, 441–445.*

Stratford M. 1992. Lectin-mediated aggregation of yeasts–yeast flocculation. Biotech. *Genet Eng. Rev., 10, 283–341*.

Stan S. & Despa F. 2000. On the doublet formation in the flocculation process of the yeast cells. *Biosystems, 57, 139 – 145.*

Stewart, G.G., Russell, I., 1981. Yeast flocculation. *In: Pollock, J.R.A. (Ed.), Brewing Science. Academic Presspp., Toronto, pp. 61–92.*

Teniola OD. & Odunfa SA., 2001. The effects of processing methods on the level of lysine and methionine and the general acceptability of *ogi* processed using starter cultures. *Int. J. Food Microbiol. 63: 1-9.*

Van der Aa Kühle A, Jespersen L, Glover RLK, Diawara B, Jakobsen M., 2001. Identification and characterization of *Saccharomyces cerevisiae* strains isolated from West African sorghum beer. *Yeast,18, 1069-1079.*

White TJ, Bruns T, Lee S, Taylor J., 1990. Amplification and direct sequencing of fungal ribosomal DNA genes for phylogenitics. In: Innis N, Gelfand D, Sninsky J and White T (Eds). PCR protocol: a Guide to methods and Applications, Academic press, London, pp. 315-322.

White L.R., Richardson A.J., Schiewe A. J. & White C.E. 2003. Comparison of yeast viability/vitality methods and their relationship to fermentation performance. In: Smart K. (Second edition). Brewing yeast fermentation performance, Blackwell science, oxford, Uk, pp. 138 - 148.

Chapitre 4 :

Amélioration du brassage de la bière de sorgho « ikigage » en utilisant *Eleusine coracana* « uburo » pour augmenter la saccharification

Ce travail fait l'objet de la publication suivante :

Lyumugabe, F., Nzuginze, Jh., Gros, J., Bajyana, E., Thonart, Ph., 2013. Traditional sorghum beer « *ikigage* » brewing using *Eleusine coracana* « *uburo* » to increase saccharification. *American Journal of Food Technology* (submitted).

Préambule au chapitre 4

Au cours des précédents chapitres, nous avons, à plusieurs reprises, souligné que le problème d'insuffisance de sucres fermentescibles dans le moût de sorgho est une entrave majeure à l'amélioration de la teneur en éthanol dans la bière de sorgho. Plusieurs travaux ont rapporté que ce problème est dû à la faible activité de β-amylase dans le malt de sorgho. Les solutions proposées dans la littérature (association de sorgho avec le malt d'orge ou utilisation d'enzymes commerciales exogènes) ne sont pas adaptées au contexte traditionnel du Rwanda, en raison du coût élevé de l'orge et des enzymes commerciales. Malheureusement, à notre connaissance, il n'existe pas d'études portant sur la recherche de solutions locales, comme par exemple, le mélange de sorgho avec d'autres céréales locales. C'est dans ce contexte que nous avons évalué les potentialités de l'utilisation d'*Eleusine coracana* (*uburo*) pour augmenter la saccharification durant le brassage de la bière de sorgho**.**

L'activité amylasique, la teneur en azote aminé libre (FAN) et en protéines de malts des différentes variétés (Musama, Mwamba, N161) d'*Eleusine coracana* (*uburo*) du Rwanda ont été déterminées et comparées à celles du malt de sorgho (variété *Kigufi*). Des essais de composition mixte de malt de sorgho et d'uburo ont été étudiés, afin d'améliorer la sacharification du moût de sorgho. La gélatinisation de l'amidon requiert des hautes températures susceptibles d'augmenter la désactivation des amylases ; d'où la problématique de l'utilisation simultanée de la gélatinisation et saccharification durant le brassage de la bière de sorgho. Nous avons évalué les caractéristiques brassicoles du moût produit par la méthode Rwandaise de brassage (infusion à 65° C) et la méthode de brassage par décantation, utilisée en Afrique de l'ouest. Cette dernière comprend l'étape de gélatinisation de l'amidon après décantation et puis celle de la saccharification après addition du surnageant contenant des enzymes hydrolytiques (α et β-amylases).

Traditional sorghum beer "Ikigage" brewing using *Eleusine coracana* "*Uburo*" to increase saccharification

RUNNING TITLE HEADER: Improvement of sorghum brewing using *Eleusine coracana*

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Abstract

Sorghum is a cereal widely used in African traditional beers production. But on the major problems of sorghum brewing is the insufficiency of saccharification due mainly to low β-amylase activity of this cereal. Three *Eleusine coracana* "*Uburo*" (Musama, N161 and Mwamba varieties) from Rwanda were studied with a view to improve the saccharification during red sorghum (Kigufi variety of Rwanda) malt mashing. The β-amylase activities of Musama (301,6 U/g), N161 (227,2) and Mwamba (112,6) malts were much higher than in kigufi sorghum malt (73,3 U/g). The mashing of the mixture of sorghum malt (70%) with *Uburo* (Musama variety) malt (30%) allowed to produce more fermentable sugars, particulary maltose, reaching amounts 2-fold higher than when pure sorghum malt was used. Moreover, when the decantation method is applied, the fermentable sugars content draws a 100 % increase by comparison to the traditional mashing method (infusion). However, the free amino nitrogen the worts obtained by decantation mashing procedure was slightly lower than that of the worts obtained by traditional mashing procedure, but was still within the range needed for yeast growth. To improve brewing of traditional sorghum beer in African context, *Eleusine coracana* malt could be used in place of barley or extraneous enzyme. More research would be needed to establish the flavor of the final beer produced from this study.

Key words: Sorghum beer, Alcoholic fermentation, *Eleusine coracana*, *Uburo*, Saccharification

1. INTRODUCTION

Sorghum grain constitutes the major cereal crop used to produce traditional African alcoholic beverages named "opaque beers" (Novellie, 1976; Asiedu, 1991). Unlike barley, classified in the same *Graminea* family, sorghum is very well adapted to the semi-arid and sub-tropical conditions prevailing over most of the African continent (Agu *et al*. 1998a). The resulting beers are known as *ikigage* in Rwanda (Lyumugabe et al., 2010), as *tchoukoutou* in Benin or Togo (Kayode *et al*., 2005), as *dolo* in Burkina-Faso (Dicko et al., 2006), and many other equivalents in Nigeria, Ghana, Sudan, Chad, South Africa (Lyumugabe *et al*., 2010) with variations in manufacturing processes (Haggblade and Holzapfel, 1989).

In Rwanda, *ikigage* beer is appreciated in various festivals and Rwandese ceremonies (e.g., marriage, birth, baptism, dowery, etc) and constitutes a source of economic return for the women manufacturers. However, like the most of African sorghum beers, *ikigage* is less attractive as convenience beverage than western beers brewed with barley malt because of poor hygienic quality and low ethanol content (Lyumugabe *et al.*, 2010). The low conversion of sorghum starch into fermentable sugars for yeast (*Saccharomyces cerevisiae*) is incriminated. The low levels of β-amylase activity in sorghum malt can explain the low saccharification (Palmer, 1989; Dufour *et al.*, 1992). Other studies showed that the main reason is likely to be inadequate gelatinization of sorghum starch rather than inadequate levels of hydrolytic enzymes (Agu *et al*., 1997a; Dufour *et al*., 1992).

Decantation mashing procedure is widely applied in West Africa. By removing the "enzymatic supernatant" and increased gelatinization temperature, this process helps to enhance starch gelatinization while preserving the enzymatic activity pool. Relatively high levels of starch extracts comparable to those of barley malts have been obtained by using this

method (Palmer 1989; Igyor *et al*., 2001). Still lower fermentable sugars yield of these sorghum worts suggested insufficient β-amylase levels (Palmer, 1989). The use of commercial enzymes (Dale *et al*., 1989; Bajomo and Young, 1994; Goode *et al*., 2003a) or the mixtures of malted barley with sorghum (Okafor *et al*., 1980; Goode *et al.*, 2003b) during mashing were proposed as a solution to increase the β-amylase activity level. However, these optimal solutions for reducing the levels of non-fermentable sugars in sorghum wort are incongruous in an African traditional brewing context because tropical climate is not conducive to barley cultivation, and commercial enzymes are not sustainable for reasonable production cost. Brewing processes using mixture of sorghum with local cereals have not been extensively investigated.

Eleusine coracana, named commonly finger millet because the plant head resembles the hand fingers, is an annual cereal cultivated in Eastern and Southern Africa as well as Southern Asia (De wet *et al*., 1984). Since ancient time the grains are used or brewing African opaque beers (Nout and Davies, 1982; Gadaga *et al*., 1999; Muyanja *et al*., 2003; Lyumugabe *et al*., 2010), but little is known about its real interest in brewing. This study assesses the use of *Eleusine coracana* (called "uburo" in Rwandese language) in sorghum brewing in order to increase the fermentable sugars of sorghum wort and ethanol content of traditional sorghum beer. In this study α- and β-amylase levels, free amino nitrogen and proteins content of three *Eleusine coracana* "uburo" (Musama, N161 and Mwamba varieties) and red sorghum (Kigufi variety) from Rwanda were evaluated. Traditional mashing procedure (infusion) and decantation mashing procedure developed for sorghum malt were employed and the produced worts were assessed for their brewing qualities.

2. MATERALS AND METHODS

2.1. Plant materials

The red sorghum grains (Kigufi variety) and *Eleusine coracana "*uburo*"* grains (Musama, N161 and Mwamba varieties) used in this study were obtained from Rubona and Musanze stations of Rwanda Agriculture Board (RAB). Several varieties of sorghum occur in Rwanda, among them, *kigufi* is most used to prepare traditional sorghum beer *ikigage*.

2.2. Malting procedure

After removal of broken kernels and debris, the selected grains (1 kg) were steeped in distilled water (2 L) at 25° C for 24 h. Before and after steeping, grains were sterilized by immersion in sodium hypochlorite solution (1% wt/v). After rinsing with sterile distilled water as described elsewhere (Ezeogu *et al.*, 1995), the grains were germinated at 30 ° C for 72 h and then kilned at 50° C for 24 h. The shoots and rootlets were removed manually and the malt kernels were fine milled.

2.3. Malt analyses

Alpha and beta amylase activity

Alpha and Beta amylase activity were determined on malt by using specific colorimetric methods developed by Megazyme international (Ireland Ltd, Irlande): AMYLAZYME (Azurine-crosslinked amylose = AZCL-Amylose) for α-amylases and BETAMYL for ß-amylase. The results are expressed in Cerapha units per gram corresponding to enzymes quantity necessary to release a p-nitrophénol (µmol/minute/g of dry matter). Dry matter contents (DM) were determined by oven drying at 105° C to constant weight.

Protein and free amino nitrogen

Protein content in grain was determined with the Kjeldahl method using a Vapodest 30s (Gerhardt, Königswinter, Germany) and by multiplying the results with the 6.25 coefficient.

The extraction of free amino nitrogen (FAN) in malt was carried out as described by Pelembe *et al*. (2002). The FAN was determined by European Brewery Convention method 8.10 (EBC analytica, 2004) using glycine as reference amino acid.

2.4. Wort production

The wort was produced by Rwandese traditional mashing procedure described elsewhere (Lyumugabe *et al*., 2010) and by decantation mashing procedure developed for sorghum (Palmer, 1989; Agu and Palmer, 1996; Igyor *et al.,* 2001).

Traditional mashing procedure

This mashing is a slightly modification of Rwandese traditional procedure. Fifty gram milled malt were added to 1500 mL distilled water and boiling at 80° C for 30 minutes and then cooled below 65° C at which 250 g milled malt were added. The mash was stirred and the temperature is maintained at 65° C for 60 min and then cooled to 30° C.

Decantation mashing procedure

Three hundred grams of milled malt were mixed with 1500 mL distilled water at 45° C and left in decantation during 30 min. Thereafter, 750 mL of the clear "enzymatic supernatant" was removed while mash residues were heated at 90° C for 30 min. to gelatinize malt starch. After cooling below 50° C, the clear ''enzymatic supernatant'' was re-added and then the mixture was brewed according to the following mashing program: 60 minutes at 63° C, 10 minutes at 75° C and cooled to 30° C.

2.5. Wort analyses

Extract, fermentability and free amino nitrogen

The specific gravity was measured in triplicate using the pycnometer method at 20° C. Wort extracts was calculated according to European Convention of Brewing method 8.3 (EBC

analytica, 2004). Fermentability was determined also according to European Convention of Brewing method 8.6 (EBC analytica, 2004). The Ninhydrin method was used to estimate free amino nitrogen present in wort (EBC analytica, 2004).

Sugars and starch

The sugars composition of wort samples was performed by HPLC on an Agilent 1100 series apparatus (Agilent Technologies, Massy, France) equipped with a refractometric detector. The samples, previously filtered through a 0.2 µm acetate membrane, were eluted with 0.1% H_3PO_4 at 0.5 mL/min. Sugars were separated on a C-610-H ion exchange column (300mm x 7.8 mm, supelco, Bellefonte, PA) preceded by a pre-column H (5cm x 4.6 mm, supelco, Bellefonte, Pennsylvania, United States). Qualitative analysis of starch was done on wort using the iodine colour complex reaction. The iodine starch complex leading to blue-black colour, indicate the presence of starch.

Viscosity and colour

Viscosity was determined by European Convention of Brewing method 8.4 (EBC analytica, 2004) using glass capillary viscometer. Colour was also determined using procedure recommended by European Convention of Brewing.

Fermentation

Filtered wort (500 mL) was pitched with yeast *Saccharomyces cerevisiae* (RV6) in a sterile 1000 mL Erlenmeyer flask equipped with a gas trap. Yeast strain RV6, isolated from Rwandese traditional leaven, was obtained from the Walloon Center of Industrial Biology (CWBI), Gembloux, Belgium. Yeast was propagated in 10 mL of YPD (Yeast extract, Peptone, Dextrose) broth and incubated at 30° C for 24 h. The inoculum was prepared by transferring propagated yeast to sterilized 10 mL YPD (15 h incubation) then placed into 50 mL YPD broth (24 h incubation). Fifteen milliliters of the resulting culture were placed in

100 mL wort and incubated for 24h. Finally, experimental worts (500 mL) were pitched with 10^6 yeast cells/mL and the fermentation was carried out at 30° C for 72 h. Ethanol was determined by enzymatic method using the Boehringer Kit (R-Biopharm AG,D-64293 Darmastadt).

3. RESULTS AND DISCUSSION

3.1. Biochemical properties of sorghum and *uburo* malts from Rwanda

Starch hydrolytic activities of grains assessed by colorimetric methods show important differences between malts from Rwandese sorghum (kigufi variety) and eleusine (musama, N161 and mwamba varieties)(**Figure 1**). The higher level of α–amylase activity is given for kigufi sorghum variety (268.2 U/g) and the lowest levels in the mwamba eleusine variety (43 U/g). The high α-amylase potential of sorghum has been observed by several authors (Aisien, 1982, Aisien and palmer, 1983). The α–amylase levels here reported for the Kigufi sorghum reveal to be in similar ranges than the levels previously pointed out by other authors for sorghum malts (Dufour *et al*., 1992; Beta *et al*., 1995; Agu et palmer, 1997b, Letsididi *et al*., 2008; Khady *et al*., 2010) and barley malt (Brennan *et al*., 1997; Kramer *et al*., 2001). The here studied Kigufi sorghum variety exhibited α–amylase levels in similar range to the enzyme levels previously reported sorghum malts (Dufour *et al*., 1992; Beta *et al*., 1995; Agu et palmer, 1997b, Letsididi *et al*., 2008; Khady *et al*., 2010) and barley malt (Brennan *et al*., 1997; Kramer *et al*., 2001).

Concerning the β-amylase activity, Kigufi sorghum variety (73.3 U/g) exhibited a lower level than in eleusine malts from Musama (301.6 U/g), N161 (227.2 U/g) and Mwamba (112 U/g) varieties. Similar trends were previously reported in comparison of β-amylase activity levels

in *uburo* malts (81 - 608 U/g) and sorghum malt (17 - 57 U/g) (Taylor and Robins, 1993; Taylor, 2009). In this study, the β-amylase activity level of sorghum Kigufi variety is slightly higher than the activity levels reported by Taylor and Robins (1993) using same method, but remains lower when compared to the certain sorghum cultivars from Botswana (Letsididi *et al.*, 2008) and Nigeria (Agu and Palmer, 1996).

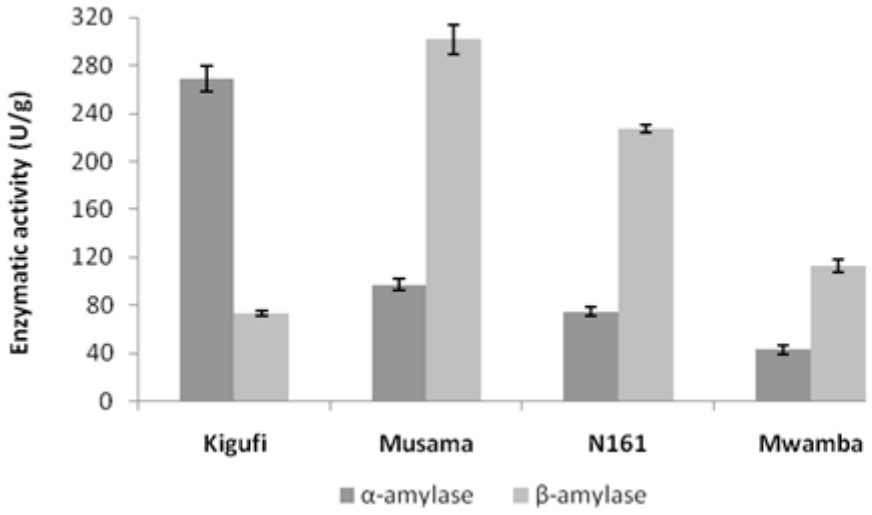

Figure 1: Enzymatic activity of sorghum (Kigufi variety) and eleusine (Musama, N161 and mwamba varieties) malts from Rwanda

Table 1: Free amino nitrogen and proteins contents of sorghum (Kigufi variety) and eleusine (Musama, N161 and Mwamba varieties) from Rwanda

	Kigufi	Musama	N161	Mwamba
Proteins (%)	10.9 (± 0.5)	10.5 (± 0.3)	10.8 (± 0 .4)	9.6 (± 0.3)
FAN (mg/L)	76.7 (± 2.7)	121.0 (± 3.6)	98.9 (± 5.8)	128.0 (± 7.2)

However, β-amylase activity of eleusine malts used in this wort was considerably higher than the reported for sorghum and sometimes slightly lower than reported for barley (414 betamyl units/g) by Taylor and Robins (1993). The generation of maltose in wort being essential for a

good fermentation (Zeigler, 1999), the high saccharification activity potential of those Rwandese eleusine varieties is very significant with respect to its potential for beer brewing.

The results in **Table 1** did not show a great difference between total protein contents of sorghum (11 %) and eleusine malts (10-11 %). However, eleusine malts contained larger amounts of free amino nitrogen (99 - 128 mg/L) when compared to sorghum malt (77 mg/L).

In sorghum malt, the lower nutrient content can be explained by insufficient proteolysis due to the vitreous nature of sorghum endosperm structure (Klopfenstein and Hoseney, 1995) or by the too high specificities of endopeptidase in the proteins decomposition (Agu and palmer, 1999). As a consequence, low protein hydrolysis into free amino nitrogen and short chain peptides is obtained (Taylor and Evans, 1989).

3.2. Use of sorghum and *uburo* in mashing and fermentation

Decantation mashing procedure involves the partition of the wort by removal of enzymatic supernatant in order to conduct adapted gelatinization temperature to those tropical cereals (80 - 100 °C) while preserving the hydrolytic enzyme pool integrity. Enzymatic part is then reintroduced in the cooled wort for infusion mashing (65 °C for 60 minutes and 75 °C for 10 minutes). In order to assess the interest of this method with the Rwandese varieties and the introduction of eleusine malts in sorghum malt for brewing, the decantation mashing and Rwandese traditional mashing (infusion at 65° C) are here compared with different combination of the two malted cereals.

The results of effect of mashing method and *uburo* on sorghum wort properties are shown in **Table 2**. The higher β-amylase activity of *uburo* was confirmed by a significant increase of wort extract when used 30% *uburo* (9.5% sugar density (w/w) with traditional infusion mashing versus 8.3% sugar density (w/w) with pure sorghum malt). Those values were even

higher when decantation mashing was applied (13% sugar density (w/w) with mixture of sorghum and *uburo* malt versus 9.4% sugar density (w/w) with pure sorghum malt). Other studies (Igyor *et al.,* 2001) reported also the higher extract rate of worts from decantation mashing procedure most probably due to better gelatinization of sorghum starch. However, the results in **Table 2** showed also that the FAN contents in worts obtained by traditional infusion mashing procedure is slightly higher compared to those obtained by decantation mashing procedure. This can be explained by the temperature of infusion mashing (65° C) increasing the activity of proteolytic enzymes (Briggs *et al*., 2004) while the decantation mashing method probably denatured and precipitated the proteins of wort, rendering them less susceptible to proteolytic enzyme degradation (Agu and Palmer, 1998b).

The preservation of the enzymatic pool from high temperature treatment results into higher fermentable sugars level, maltose particularly (**Table 3).** These results confirm also the influence of β-amylase, from *uburo* malt, in the production of maltose with a twofold increase with respect of the same mash composition (e.g. 13.4 g/l with pure sorghum versus 25.7 g/l with 30% *uburo* malt in infusion mashing method). The decantation mashing method permitted a twofold gain in maltose content from the same malted cereal combination, leading to a fourfold increase when this method is applied with 30 % *uburo* malt (53.3 g/l) in comparison with pure sorghum in traditional infusion method (13.4 g/l). However, a slight diminution of glucose content is observed with the use of *uburo* malt. The dilution of the α-amylase pool by diminution of sorghum part can explain this observation.

Table 2. Effect of mashing method and *uburo* on sorghum wort properties

	Traditional infusion mashing				Decantation mashing			
	100% sorghum	90 % sorghum and 10% *uburo*	80 % sorghum and 20% *uburo*	70% sorghum and 30% *uburo*	100% sorghum	90 % sorghum and 10% *uburo*	80 % sorghum and 20% *uburo*	70 % sorghum and 30% *uburo*
Extract (° EBC)	8.3	8.3	8.7	9.5	9.4	9.7	11.4	13.0
Fermentability (%)	68.8	-	77.7	76.2	72.0	-	77.8	78.8
FAN (mg/L)	138.2	145.2	172	192.3	129.9	141.0	163.4	183.1
Colour (° EBC)	7.1	7.7	11.1	11.2	9.1	9.0	18.2	18.9
Viscosity (cp)	1.32	1.32	1.34	1.37	1.46	1.47	1.52	1.57
Iodine test	+	+	+	+	+	+	+	+

* Results are presented as mean of triplicate experiment.

Table 3. Sugar profile (g/L) of worts from sorghum and *uburo* malts

	Traditional infusion mashing		Decantation mashing	
	100% sorghum	70% sorghum and 30% *uburo*	100% sorghum	70% sorghum and 30% *uburo*
Glucose	9.3	7.0	18.5	15.1
Maltose	13.4	25.7	28.2	53.3
Maltotriose	5.0	12.4	9.9	23.5
Fructose	1.3	1.8	2.1	3.6

Table 4. Characteristics of beers made with sorghum and *uburo* malts

	Traditional infusion mashing		Decantation mashing	
	100% sorghum	70% sorghum and 30% *uburo*	100% sorghum	70% sorghum and 30% *uburo*
pH	4,6	4;5	4.5	4.4
Specific gravity	1.006	1.009	1.007	1.013
Ethanol (% v/v)	2.3	3.1	3.3	4.7
FAN (mg/L)	51.0	64.2	48.0	41.4
Colour (° EBC)	6.2	6.0	11.0	19.7

The higher values of fermentable sugars obtained from the mixture 70% sorghum and 30 % *uburo* malts mash in the decantation procedure rather than in the traditional infusion mashing resulted in the production of higher alcohol of the fermented wort (4,7 % v/v with decantation procedure versus 3.1 % v/v with infusion mashing, **Table 4**). However, the ethanol contents

(3.3 % v/v) of beers product from pure sorghum malt mashed by decantation procedure remain slightly higher than beer product from traditional infusion mashing or Rwandese ikigage beers from peasants (Lyumugabe *et al*., 2010).

4. Conclusion

This study shows that the β-amylase activity is higher in *Eleusine coracana* "*Uburo*" malts than in sorghum malt. The mixture of sorghum malt (70%) with *Uburo* (Musama variety) malt (30%) mashed by decantation method produced sufficient extract, fermentable sugars, free amino nitrogen and ethanol during fermentation than 100% sorghum malt. Althrough the decantation mashing procedure proved able to increase by two – fold the fermentable sugars of wort (maltose), free amino nitrogen was found slight low level than traditional infusion mashing method, but still in sufficient level for a proper fermentation (leading to a 50% increase in ethanol). To improve brewing of traditional sorghum beer in Africa context, *Eleusine coracana* malt could be used in place of barley or extraneous enzyme. More research would be needed to establish the flavor of the final beer produced from this study.

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REFERENCES

Agu, R.C., Palmer, G.H., 1996. Enzymic breakdown of endosperm. of sorghum at different malting temperatures. *J. Inst. Brew., 102,415-418.*

Agu, R.C., Palmer, G.H., 1997a. Alpha -glucosidase of sorghum and barley malts. *J. Inst. Brew., 103, 25-29.*

Agu, R.C., Palmer, G.H., 1997b. Effect of mashing procedures on some sorghum varieties

germinated at different temperatures. *Proc. Biochem., 32, 147-158.*

Agu, R.C., Palmer, G.H., 1998a. A reassessment of sorghum for lager-beer brewing. *Bioresour. Technol*., 66, *253- 261.*

Agu, R.C., Palmer, G.H., 1998b. Effect of mashing with commercial enzymes on the properties of sorghum worts. *World J. Microbiol. Biotechnol., 14,43- 48.*

Agu, R.C., Palmer, G.H., 1999. Comparative development solution nitrogen in the malts of barley and sorghum. *Proc. Biochem., 35, 497-502*.

Asiedu, J.J., 1991. La transformation des produits agricoles en zone tropicale : approche technologique. *Paris :Karthala*.

Aisien, A.O., 1982. Enzyme modification of sorghum endosperm during seedling growth and malting. *J. Sci. Food Agric., 33, 754-759.*

Aisien, A.O., Palmer, G.H., 1983. The sorghum embryo in relation to the hydrolysis of the endosperm during germination and seedling growth. *J. Sci. Food Agric., 34, 113-121.*

Bajomo, M. F. and Young, T. W., 1994. Fermentation of wort from 100% of raw sorghum and enzyme. *J. Inst. Brew., 100, 79 - 84.*

Beta, T., Rooney, L.W. and Waniska, R.D., 1995. Malting Characteristics of sorghum cultivars. *J. Cereal Chem.,* 72, 533-538.

Brennan, C.S., Amor, M.A., Harris, N., Smith, D., Cantrell, I., Griggs, D., Shewry, P.R., 1997. Cultivar differences in modification patterns of protein and carbohydrate reserves during malting of barley. *J. Cereal Sci. 26, 83–93.*

Briggs, D.E., Boulton C.A., Brookes, P.A., Stevens, R., 2004. Brewing science practice. *Woodhead publishing,* Cambridge, 10 – 606.

Dale, C.J., Young, T.W., Makinde, A., 1989. Extruded sorghum as a brewing raw material. *J. Inst. Brew., 95, 157-164.*

De wet J.M.J., Prasada Rao K.E., Brink D.E., Mengesha M.H., 1984. Systematics and evolution of *Eleusine coracana* (Gramineae). *Amer. J. Bot., 71, 550-557.*

Dicko, M.H., Gruppen H., Traoré, A. S., Voragen A.G.J. & Van Berkel W.J.H. 2006. Sorghum grain as human food in Africa: relevance of content of starch and amylase activities. *Afr. J. Biotech., 5, 384-395.*

Dufour, J.P., Melotte, L. & Srebrnik, S., 1992. Sorghum malts for the production of a lager beer. *J. Ame. Soc. Brew. Chem., 50, 110-119*

EBC –analytica, 2004. European Brewery Convention methods, Fifth Edition.

Ezeogu, L.I., Okolo, B.N., 1995. Effects of air rest periods on malting sorghum response to final warm water steep, *J. Inst. Brew., 101, 39 - 45.*

Gadaga, T.H., Mutukumira, A.N., Narvhus, J.A., Feresu, S.B., 1999. A review of traditional fermented foods and beverages of Zimbabwe. *Inter. J. Food Microbiology, 53, 1 –11.*

Goode D.L., Halbert C., Arendt E.K. 2003a. Optimization of mashing conditions when mashing with unmalted sorghum and commercial enzymes. *J. Am. Society of brewing chemists, 61, 69 – 78.*

Goode, D.L. Elke, K., Arendt, E.K., 2003b. Pilot Scale Production of a Lager Beer from a Grist Containing 50% Unmalted Sorghum. *J. Inst. Brew., 109, 208-217.*

Haggblade, S., Holzapfel, H., 2004. Industrialization of Africa's indigenous beer brewing. *In: Streinrous KH KH (2 ed.)* industrialization of indigenous fermented foods, New York City: CRC Press.

Kayodé, APP., Aégbidi, A., Linnemenn, A.R., Nout, M.J.R., Hounhouigana, J.D., 2005. Quality of farmer's varieties of sorghum and derived foods as perceived by consumers in Benin. *Ecol. Food Nutr. 44: 271-294.*

Khady, BA., Tine, E., Destain, J., Ndiaga C., Thonart, PH., 2010. Etude comparative des composés phénolitiques, du pouvoir antioxydant de différentes variétés de sorgho sénégalais et des enzymes amylolytiques de leur malt. *Biotechnol. Agron. Soc, 14, 131-139.*

Klopfenstein, C.F., Hoseney, R.C., 1995. Nutritional properties of sorghum and the millets. *In D.A.V. Dendy (Ed),* sorghum and millets: chemistry and Technology, *Ame. Assoc. Cereal chem*. Incn St. Paul,MN, 125 -168.

Kraemer, J.E.G., Mundstock, E.C., Molina, S.C., 2001. Development expression of amylases during Barley malting. *J. Cereal Sci*., 33, *279-288.*

Igyor, M.A., Ogbonna, A.C., Palmer, G.H., 2001. Effect of malting temperature and mashing methods on sorghum wort composition and beer flavour. *Proc. Biochem., 36, 1039–1044.*

Letsididi R., Bulawayo B., Kebakile M. & Ezeogu L.I., 2008. Evaluation of indigenous Botswana sorghumcultivars with respect to their diastatic power, α-amylase, β-amylase, and limit dextrinase potentials for malting. *J. Am. Soc. Brew. Chem., 66, 29-36.*

Lyumugabe, F., Kamaliza, G., Bajyana, E., Thonart, PH., 2010. Microbiological and physico-chemical characteristic of Rwandese traditional beer "*Ikigage*". *Afr. J. Biotech., 9, 4241-4246.*

Munyaja, MBK., Narvhus, JA., Treimos, J., Langsrud, T., 2003. Isolation characherization and identification of lactic acid bacteria from bushera: Ugandan traditional fermented beverage. *Int. J. Food Microbiology, 8, 201-210.*

Novellie, L., 1976. Beverages from sorghum and millets. In: Proceedings of the international symposium on sorghum and millets for human Food. *Inter. Associ. Cereal Chem.,* Symposium in Vienna, Tropical Products Institute.

Nout, M. J. R., Davies, B. J. 1982. Malting characteristics of finger millet, sorghum and barley. *J. Inst.Brew., 88, 157-163.*

Okafor, N., Aniche, G.N., 1980. Brewing a lager beer from Nigerian sorghum. *Brewing and Distilling Inter., 10, 32-35.*

Palmer, G.H., 1989. Cereals in malting and brewing. In *Cereal Science and Technology,* ed. Palmer, G.H. Aberdeen: Aberdeen University Press, 61-242.

Pelembe, L. A. M., Dewar, J. and Taylor, J. R. N. 2002. Effect of malting conditions on pearl millet malt quality. *J. Inst. Brew., 108, 7–12.*

Taylor, J.R.N., Evan, D.J., 1989. Action of sorghum protease on the protein bodies of sorghum starchy endosperme. *J. bontany, 40, 763 -768.*

Taylor, J.R.N., Robins D.J., 1993. Factors influencing beta-amaylase activity in sorghum malt. *J. Inst. Brew., 99, 413-416.*

Taylor, J.R.N., 2009. Developments in Africa's cereal crops- potential sustainable resource for brewing in tropical and susb-tropical countries. *Institute of brewing and distilling*, Africa Section 12th scientific and technical convention. The society: London.

Zeigler, P., 1999. Cereal beta-amylases. *J. Cereal Sci., 29, 195–204.*

Chapitre 5 :
Composés volatils de la bière traditionnelle de sorgho « ikigage »

brassée avec *Vernonia amygdalina* « umubirizi »

Ce travail a fait l'objet de la publication suivante :

Lyumugabe, F., Bajyana, E., Wathelet J.P., Thonart, Ph., 2013. Volatile compounds of the traditional sorghum beers *ikigag* brewed with *Vernonia amygdalina* "*umubirizi*". *Cerevisia, 37 (4), 89-96.*

Preambule au chapitre 5

Au cours du premier et du second chapitre, nous avons indiqué que les feuilles de *Vernonia amygalina* sont utilisées au cours de la fabrication de la bière traditionnelle rwandaise brassée à base du sorgho *ikigage*. La littérature indique que l'amertume et les propriétés antibactériennes de cette plante sont analogues à celles du houblon. Mais le profil aromatique de bières produites avec *V. amygdalina* n'a jamais été étudié. Dans ces conditions, nous avons comparé la bière traditionnelle *ikigage* des paysans rwandais à des bières-pilotes fabriquées avec ou sans *V. amygdalina* à la place du houblon, afin d'évaluer l'impact de *V. amygdalina* sur le profil aromatique de la bière *ikigage*. Nous avons, par ailleurs, évalué le potentiel aromatique des souches de levures performantes sélectionnées (RV6 et RB2). Les bières-pilotes ont été brassées à base du malt de sorgho (70%) et d'eleusine (30%), en utilisant la méthode de brassage par décantation. L'extraction et l'analyse de composés volatiles ont été réalisées par la méthode HS-SPME et à l'aide de la chromatographie en phase gazeuse couplée au spectromètre de masse.

Volatile compounds of the traditional sorghum beers "ikigage" brewed with *Vernonia amygdalina* "umubirizi"

RUNNING TITLE HEADER: Volatile compounds of Sorghum beer

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Abstract

Volatile compounds in traditional sorghum beer *ikigage* brewed with *Vernonia amygdalina* were investigated using Headspace-Solid Phase Micro Extraction (HS-SPME) and gas chromatography - mass Spectrometry (GC/MS). A total of 75 volatile compounds were identified in the traditional *ikigage* made by Rwandese peasants (using *V. amygdalina* leaves during the preparation of traditional leaven) and pilot *ikigage* brewed with and without addition of *V. amygdalina* in the boiling kettle (instead of hops). Traditional sorghum beer brewed with *V. amygdalina* was characterized by the presence of higher levels of esters, alcohols and fatty acids. Ethyl acetate, ethyl caprylate, ethyl caproate and ethyl caprate are the main ester components with higher concentrations. Higher alcohols were mainly composed of propan-1-ol, 2-methyl propan-1-ol, 3-methylbutan-1-ol, 2-methylbutan-1-ol and 2-phenylethanol. Acetic, caproic and caprylic acids were the most important fatty acids. Volatile phenols such as 2-methoxy-4-vinylphenol, 4-vinylphenol and 2-methoxyphenol were also detected in the sorghum beer *ikigage* and may contribute to its phenolic note. *V. amygdalina* provides the terpenes compounds (*δ*-3-carene, *β*-farnesene, farnesol, *β*-citronellol and linalool), methyl salycilate and beta-damascenone in the sorghum beer. Like in hopped beers, these compounds might play a significant role in the overall flavor and aroma of the sorghum beer *ikigage*. *V. amygdalina* leaves emerge as an interesting hops substitute for tropical beers, but complementary data are still needed to understand which volatile compounds or precursors preexist in *V. amygdalina*.

Keywords: Volatile compounds; aroma; sorghum beer; *ikigage*; *Vernonia amygdalina*

INTRODUCTION

Aroma substances are important components in beer as they largely contribute to the quality of the final product. A great number of volatile compounds belonging to heterogenous chemical groups, including alcohols, esters, acids, carbonyl compounds, terpenic substances and sulfur, have been identified in western beers made with barley malt and hops (Meilgaard, 1975; Schieberle, 1991; Vanderhaegen *et al*., 2003). These different substances are proved to influence the beer aroma and flavor to a very different degree. Addition of hops during beer brewing imparts also a unique "hoppy" aroma to beer, due to the complexity of hop oils, which contains in excess of 300 components (Hughes and Baxter, 2001).

African traditional beers such as *ikigage, pito, dolo, tchoukoutou* and *burukutu* are prepared from sorghum malt and are unhopped. These beverages, apart from serving as inebriating drinks, are also important in fulfilling social obligations (e.g. marriage, birth, baptism, dowery, etc.). In the process of *ikigage* brewing, the fermentation is conducted by traditional leaven "umusemburo" made from sorghum malted wort *igikoma* with *Vernonia amygdalina* (Lyumugabe *et al*., 2010).

Several studies reported the possibility of using *V. amygdalina* instead of hops in African sorghum beers (Okoh *et al*., 1995; Ajebesone and Aina, 2004; Okoro and Aina, 2007). *V. amygdalina*, known as "bitter leaf", is a green shrub found all over Africa and belongs to the Asteraceace family. It mimics hops not only by its antimicrobial properties (Mboto et al., 2009; Oboh and Masodje, 2009) but also by its bitter taste (Ajebesone and Aina, 2004; Adenuga *et al.*, 2010).

Several studies have been undertaken over the past years to identify the micro-organisms involved in traditional sorghum beers (Jespersen, 2003; Muyanja *et al*., 2003; Naumova *et al.*,

2003; Maoura *et al*., 2005; Lyumugabe *et al*., 2010; Kayodé *et al*., 2011), but the aroma profile of these beers has not been extensively investigated. The aim of this study is to define the profile of volatile compounds of traditional sorghum beer *ikigage* brewed with *V. amygdalina*. Headspace-Solid Phase Micro Extraction (HS-SPME) and gas chromatography - mass spectrometry (GC/MS) were applied to analyze the volatile compounds. Traditional *ikigage* made by Rwandese peasants (using of *V. amygdalina* during the preparation of traditional leaven) was compared to pilot *ikigage* brewed with or without addition of *V. amygdalina* (instead of hops) in the boiling kettle and–fermented with the dominant yeast strains selected from traditional leaven (referred to as RV6 and RB2).

MATERIALS AND METHODS

Ikigage beer samples

Production of pilot beers

The production process for pilot beers with (RV6.V and RB2.V) and without (RV6 and RB2) *V. amygdalina* was described by Lyumugabe *et al*. (2012). 200g malt (60g *Eleusine coracana* and 140g red sorghum) from peasants were mashed in 1000 mL distilled water at 45 °C for 30 min. Thereafter, 150 mL of the clear "enzymatic supernatant" was removed, while the remaining mash was heated at 90 °C, held at the same temperature for 30 min, and cooled below 50 °C while the clear ''enzymatic supernatant'' was re-added. This mixture was then brewed according to the following mashing procedure: 1h at 63 °C, 10 min at 75 °C. The filtration was very poor, so the mash was centrifuged at 4000 x g for 5 min and then the filtrate was heated until boiling for 1h. For the production of RV6.V and RB2.V pilot beers, leaves of *V. amygdalina* (2 g/L) were added 10 min before the end of boiling. Fermentation was conducted with *Saccharomyces cerevisiae* strains (RV6 and RB2) selected from Rwandese traditional leaven *umusemburo* in an Erlenmeyer flask, equipped with a gas trap.

Yeast was pitched at 10^7 Ufc/ml and fermentation carried out at 30 °C for 3 days. After centrifugation (13.000g, 25 min), the beer was kept at -20 °C until extraction and detection of volatile compounds.

Beers made by Rwandese peasants

Samples of traditional beer *ikigage* were collected from Tumba market site, in the Southern Province of Rwanda. They were taken in bottles of 500 mL and stored in the freezer at -20 °C and then extracted immediately after opening. The traditional brewing process of *ikigag*e has been described by Lyumugabe *et al*. (2010). *V. amygdalina* is only used during the production process of traditional "*umusemburo*" leaven.

Reagents

DL-octan-3-ol and absolute ethanol were purchased from Acros Organics (Geel - Belgium) and Sigma-Aldrich (Belgium), respectively. The pure reference compounds (propan-1-ol, 2-methylpropan-1-ol, 2-phenethyl alcohol, hexan-1-ol, decan-1-ol, ethyl acetate, ethyl butyrate, ethyl caproate, ethyl caprate, ethyl caprylate, methyl salicylate, capric acid, caproic acid, caprylic acid, dimethyl sulfide) used were also purchased from Sigma-Aldrich (Belgium).

Gas chromatography - mass spectrometry

Analyses were carried out using an Agilent 7890 GC system equipped with a 5975C inert XL EI/CI mass selective detector (Agilent Technologies, Santa Clara, CA, USA), Thermal Desorption Unit (TDU, Gerstel), PTV inlet (CIS 4, Gerstel) and MPS 2 with headspace and DHS option (Gerstel). An HP-5 MS column (30 m x 0.25 mm ID) with a film thickness of 0.25 μm was applied to extract volatile compounds from the headspace of above-prepared glass vial. The GC was equipped with a split-splitless injector which was held at 250° C. After starting at 30 °C, the oven temperature was raised in 3 steps after 2 min: 30-70 °C at

10 °C/min followed by 1 min at 70° C, 70-220° C at 4° C/min and 220-280 °C at 20° C/min, and was finally held at 280° C for 6 min. During these programs, a constant flow rate (1.0 mL/min) of the carrier gas (Helium) was maintained. Mass spectra were obtained by electronic impact (EI) scan mode (low mass: 30.0; high mass: 500.0; threshold: 150) and temperature source (230° C) was generated.

HS-SPME procedure

After opening the bottle, beer samples were immediately degassed in an ultrasonic bath for 10 min at 5 °C. 10 mL of beer were enclosed in a 20 mL SPME glass vial together with 2.5 g of sodium chloride and 5 µL of the internal standard 3-octanol (100 mg/L in absolute ethanol) and then equilibrated at 40 °C for 10 min under agitation (Gerstel Agitator/Stirrer). After this period, the 50/30 µm Divinylbenzene / Carboxen / Polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco Inc., Bellefonte, PA) was exposed in the headspace of the vial for 30 min and the extracted analytes from fiber were automatically desorbed in injection port of the GC-MS system at 250 °C.

Identification and semi-quantification

The identification was achieved by comparing mass spectra obtained from the sample with those from the NIST and Pal1600k.L libraries Database or from the pure standards injected in the same conditions and by comparing the kovats index estimated for each compound on both chromatographic column with the values given in the literature (Pino et al., 2010; www. pherobase.com/database/kovats).

Selective ion monitoring was used for integrations of all chromatogram peaks and the semi-quantitative concentrations of volatiles in *ikigage* were calculated according to the method proposed by Zhao *et al*. (2009), as follows:

Semi - quantitative concentrations = (peak area/ IS peak area) × IS concentration

Sensory evaluation

Descriptive sensory analysis was performed by trained panels (7 persons) that have a great experience in the sensory evaluation of beers. Previously, the descriptive attributes of most representatives of beers were defined in first test and terms related to odor (fruity, spicy, flowery/green, sulfury, malty, phenolic and lambic) were determined. Each evaluation was conducted in individual tasting booths at room temperature (20 °C). The intensity of the descriptors was rated on a scale from 0 to 5; a score of zero indicated that a descriptor was not perceived and a score of 5 indicated the highest intensity.

RESULTS AND DISCUSSION

A total of 75 volatile compounds (**Table 1**) were identified in traditional beer *ikigage* made by Rwandese peasants (using *V. amygdalina* during the preparation of leaven) and pilot *ikigage* beers brewed with and without addition of *V. amygdalina* in the boiling kettle (instead of hops). The majority of compounds identified in these beers were esters (32 compounds) followed by alcohols (12 compounds), carbonyls (9 compounds), acids (7 compounds) and terpenes (7 compounds). Moreover, three phenols, three sulfurs and two furans were identified.

Ester compounds

Esters were found in important amounts in all *ikigage* samples analyzed. Among them, ethyl acetate, ethyl caprylate, ethyl caproate, ethyl caprate and isoamyl acetate represent the major esters within analyzed beers. Higher concentration of ethyl caprylate, ethyl caproate and ethyl caprate was found in pilot beer *ikigage* brewed with RB2 strain while ethyl acetate was

found in higher concentration in beer *ikigage* made by peasants and pilot beer *ikigage* brewed with RV6 strain. Ethyl lactate was solely found in *ikigage* made by peasants. Ethyl acetate and ethyl lactate are generally considered as bacterial volatile compounds (Damiani *et al*., 1996). In fact, the traditional fermentation of Rwandese sorghum beer *ikigage* is conducted by a mixture of lactic bacteria and yeasts (Lyumugabe *et al.*, 2010), explaining the higher concentration of ethyl acetate and ethyl lactate in *ikigage* made by peasants compared to pilot *ikigage* produced only with dominant *S. cerevisiae* strains. Important amounts of ethyl acetate, ethyl lactate, ethyl caprylate and ethyl caprate also characterized Belgian beers (lambic and gueuze) issued from spontaneous fermentation of barley wort (Spaepen *et al*. 1978). The high temperature fermentation and yeast strains associated with the production of *ikigage* can explain the high concentration of ethyl caprylate and ethyl caprate (Suomalainen, 1981).

Compounds related to V. amygdalina

Methyl salicylate, beta-damascenone and some terpenes compounds (δ-3-carene, β-farnesene, farnesol, β-citronellol and linalool) revealed specific to *ikigage* containing *V. amygdalina* (**Figure 1**). Higher concentrations of methyl salicylate were obtained when *V. amygdalina* is added during boiling step. As for hops, glycosidically bound methyl salicylate potentially present in *V.amygdalina* could be released during brewing process and fermentation (Kollmannsberg *et al*., 2006; Daenen, 2008). Methyl salicylate is also considered as major flavor compounds of root beers (Burdock, 1995) and tea leaves (Abraham *et al*., 1976).

Beta-damascenone is an important odorant in various western hopped beers (Schieberle, 1991; Lermusieau *et al*., 2001; Chevance *et al*., 2002). As observed in hops by Kollmannsberger *et al*. (2006) and Daenen (2008), beta-damescenone could result from the degradation of β-D-glucoside of 3-hydroxy-β-damascone from *V. amygdalina*. The wild

strains RB2 and RV6 could have specific ability to cleave the glycoside bound. However, beta-damescenone has been also found in unhopped wort and beers (Lermusieau *et al*., 2001; Fritsch and Schieberle, 2005).

Despite their generally low concentrations, terpenes are regarded as important hop-derived beer flavors (King and Dickinson, 2003; Kishimoto *et al*., 2005; Takoi *et al*., 2010; Nance and Setzer, 2011). Linalool, *β*-farnesene and geraniol have been described in essential oils from *V. amygdalina* (Asawalam and Hassanali, 2006; Ogunbinu *et al*., 2009). In hops, linalool and *β*-citronellol occur as glucosidically bound flavour precursors (Kollmannsberger *et al*., 2006; Takoi *et al*., 2010). Citronellol results from geraniol issued from yeasts reduction activity during fermentation (King and Dickinson, 2003). Like in hopped beers, these compounds detected in beers containing *V. amygdalina* might play a significant role in overall flavor and aroma of *ikigage*.

Higher alcohols

Propan-1-ol, 2-methylpropan-1-ol, 3-methylbutan-1-ol, 2-methylbutan-1-ol and 2-phenylethanol are most important amounts in *ikigage*. The higher concentrations of these compounds were observed in traditional sorghum beer produced by peasants. These fermentation-derived products contribute most significantly to the alcohol-like or solvent-like aroma and to the warm mouth feel of the beer. They are also known to be important as precursors of corresponding esters (Pinho *et al*., 2006). Two different metabolic pathways are hypothesized to be involved in the production of these higher alcohols: anabolic pathways implicated in *de novo* synthesis of branched-chain amino-acids through their biosynthetic pathway from glucose, and the catabolism (Ehrlich pathway) of valine, leucine, isoleucine, and 2-phenylalanine (Oshita *et al*., 1995; Hazelwood, 2008; Pietruska *et al*., 2010). The amino-acids released probably by the proteolytic activity of lactic acid bacteria (Spicher and

Nierle, 1988) present in *ikigage* (Lyumugabe *et al*., 2010) and their use by yeast could explain important amounts of high alcohols observed in traditional *ikigage* made by peasants.

Short and Medium Chain Fatty Acids

Acetic acid was the major fatty acid found in all *ikigage* samples analyzed, but the highest concentration was observed in traditional *ikigage* made by peasants. Caprylic and capric acids were also detected in all samples while propionic acid and butyric acid and its isomer were only detected in traditional *ikigage* made by peasants. Important amount of these organic acids in *ikigage* can be attributed to the activity of Lactobacilli heterofermentative (*Lactobacillus fermentum* and *Lactobacillus brukneri*), dominant lactic acid bacteria in *ikigage* (Lyumugabe *et al*., 2010). Caprylic and capric acids are responsible for aroma in beers described as "cheesy" or "goaty", but generally their concentration is not high enough to affect the beer flavor negatively. Off-flavors due to these acids normally arise from excess formation during fermentation, not from other causes such as infection or raw materials (Meilgaard, 1975; Clapperton, 1978). These organic acids also confer a "sour" taste to beers (Montanari *et al*., 1999). They are produced by yeast metabolism during fermentation and accumulate in beer. The multienzyme complex fatty acid synthase leading to the biosynthesis of fatty acids requires the formation of Acetyl-CoA (Nykänen and Suomalainen, 1983), which is produced by oxidative decarboxylation of pyruvic acid. Although the formation of these acids is somewhat affected by the composition of wort, it depends mostly on the yeast strain used for fermentation (Clapperton, 1978).

Table 1. Volatile compounds of sorghum beers *ikigage*

Compounds	RI	ID[a]	Concentration in beers (µg/L calculated by internal standard equivalent)				
			IKp	RV6.V	RB2.V	RV6	RB2
Esters							
Ethyl acetate	609[b]	MS/RIL	733	188	302.7	123.3	253.6
Propyl acetate	712	MS/RIL	113.9	1.0	-	-	-
Ethyl isobutyrate	740	MS	0.5	0.7	0.6	0.8	0.6
Isobutyl acetate	773	MS/RIL	35.1	3.4	10.7	2.8	6.3
Isoamyl formate	795	MS	3.1	-	2.7	-	-
Ethyl butyrate	801[b]	MS/RIL	7.0	1.6	1.5	1.5	1.4
Ethyl lactate	815	MS/RIL	175.2	-	-	-	-
Ethyl isovalerate	871	MS	27.6	-	0.8	-	0.8
Isoamyl acetate	877	MS/RIL	133.6	41.9	73.9	30.1	133.2
2-Methylbutyl acetate	879	MS/RIL	12.6	-	-	49.2	44.7
Ethyl valerate	900	MS	31.4	-	7.7	-	7.9
Ethyl caproate	998[b]	MS/RIL	153.8	56.3	220.3	45.2	172.5
Hexyl acetate	1017	MS/RIL	1.1	1.7	17.3	1.9	2.8
Ethyl heptanoate	1096	MS/RIL	3.1	43.3	14.4	15.7	2.4
Ethyl benzoate	1169	MS/RIL	0.9	-	-	-	-
Heptyl acetate	1111	MS	0.2	13.2	8.2	13.7	11.0
Methyl salicylate	1192[b]	MS/RIL	7.8	69.5	43.0	-	-
Ethyl caprylate	1196[b]	MS/RIL	143.5	62.8	268.8	47.9	346
Octyl acetate	1209	MS	-	-	9.4	-	8.2
Isobutyl caprylate	1386	MS	1.6	0.6	1.8	0.4	2.9
2-Phenethyl acetate	1257	MS/RIL	3.2	2.6	3.8	2.6	8.6
Ethyl nonanoate	1294	MS/RIL	2.4	9.3	24.8	11.5	18.8

Table 1 continued

Compounds	RI	ID[a]	IKp	RV6.V	RB2.V	RV6	RB2
Ethyl dec-9-enoate	1382	MS/RIL	7.3	-	-	-	-
Ethyl caprate	1395[b]	MS/RIL	24.3	35.6	100.5	15.8	160.4
3-Methylbutyl octanoate	1447	MS	3.5	-	1.5	-	2.2
Ethyl laurate	1597	MS/RIL	1.1	3.6	5.7	21.3	26.4
Isopropyl laurate	1631	MS	-	0.7	3.7	1.8	1.5
3-Methylbutyl decanoate	1648	MS	1.0	3.5	3.8	1.0	2.2
Phenylethyl valerate	1493		5.0	-	2.7	-	3.1
Ethyl merystate	1797	MS/RIL	2.5	1.8	0.7	1,2	1,5
Ethyl palmitate	> 1900	MS	0,7	1,7	0,3	1.2	0.9
Isopropyl palmitate	> 1900	MS	3.5	0.7	-	0.5	-
Alcohols							
Propan-1-ol	<600[b]	MS	740.9	90.7	23.4	212	114.5
2-Methylpropan-1-ol	<600[b]	MS	294.3	170.4	95.6	24.8	86.7
3-Methylbutan-1-ol	704	MS/RIL	429.5	142	158.7	140.9	137.8
2-Methylbutan-1-ol	709[b]	MS/RIL	22.5	81.6	31.9	25.5	85.6
2,3- Butanediol	804	MS/RIL	39.9	27.0	-	16.3	-
Hexan-1-ol	871[b]	MS/RIL	0.6	14.2	9.9	13.0	6.9
Heptan-1-ol	971	MS/RIL	46.3	4.3	4.3	3.8	4.0
Octan-1-ol	1072	MS/RIL	4.3	11.6	6.9	4.7	13.4
2-Phenethylethanol	1115[b]	MS/RIL	85.3	21.6	34.0	14.3	34.7
Nonan-1-ol	1172	MS/RIL	4.2	11.0	6.5	6.0	6.6
2-Nonanol	1099	MS/RIL	-	54.0	-	42.2	6.9
Decan-1-ol	1273[b]	MS/RIL	2.1	17.5	1.5	12.7	1.9

Table 1 continued

Compounds	RI	ID[a]	IKp	RV6.V	RB2.V	RV6	RB2
Carbonyls							
Acetaldehyde	<600	MS	554.2	199.3	235.3	214.6	351.4
2,3-Butanedione	<600	MS	10.1	15.9	-	16.3	-
2-Methylpropanal	<600	MS	39.1	24.4	27.0	21.3	53.1
3-Methylbutanal	<600	MS	10.2	20.1	58.9	12.5	43.4
1-Hexanal	797	MS/RIL	2.2	0.7	1.7	1.5	1.5
Benzaldehyde	959	MS	2.3	-	1.0	-	-
2- Nonanone	1090	MS/RIL	22.3	11.3	3.2	13.0	6.5
Gamma-nonalactone	1363	MS	3.2	-	1.1	-	-
Beta-damascenone	1385	MS	1.0	1.6	2.5	-	-
Acids							
Acetic acid	700	MS/RIL	1660	34.0	66.0	47.2	31.8
Propionic acid	772	MS	8.8	-	-	-	-
Isobutyric acid	771	MS	0.9	-	-	3.0	-
Butyric acid	772	MS	1.3	-	-	-	-
Caproic acid	1064[b]	MS/RIL	28.3	-	11.6	-	-
Caprylic acid	1186[b]	MS/RIL	8.4	0.5	17.1	0.7	13.6
Capric acid	1380[b]	MS/RIL	2.3	0.2	1.3	1.1	1.7
Terpenes							
Delta 3- carene	1011	MS/RIL	0.8	1.5	1.0	-	-
Limonene	1026	MS/RIL	0.6	6.2	6.8	-	0.2
1,8-Cineole	1030	MS/RIL	0.4	-	-	-	-
Linalool	1097	MS/RIL	trace	1.3	1.0	-	-

Table 1 continued

Compounds	RI	ID[a]	IKp	RV6.V	RB2.V	RV6	RB2
β-Citronellol	1222	MS	0.1	1.2	0.6	-	-
(E,E)Alpha- farnesene	1500	MS/RIL	0.1	0.3	0.3	-	-
(2Z,6E)- Farnesol	1719	MS/RIL	Trace	0.6	0.5	-	-
Furans and phenols							
2,5-Dimethylfuran	797	MS/RIL	-	1.2	0.8	0.8	0.9
2-Pentylfuran	989	MS/RIL	0.7	3.6	3.1	4.9	3.5
2-Methoxyphenol	1087	MS	22.2	-	-	-	-
4-Vynilphenol	1233	MS	0.6	4.4	2.8	3.9	2.0
2-Methoxy-4-Vinylphenol	1315	MS/RIL	18.2	41.4	38.0	34.4	39.6
Sulfurs							
Dimethyl sulfide	<600[b]	MS/RIL	23.1	5.4	16.2	3.2	-
3-(methylthio)-1-propanol	986	MS	-	1.6	3.0	0.3	Trace
3-(methylthio)-1-propyl acetate	1565	MS	-	-	-	2.6	3.1

IKp: traditional sorghum beer *ikigage* made by Rwandese peasants (using *V. amygdalina* during the preparation of traditional leaven). RV6.V and RB2.V: pilot beers brewed with *V. amygdalina* in the boiling kettle, instead of hops; RV6 and RB2: pilot beers brewed without *V. amygdalina***.** [a] ID: identified by mass spectra (MS) and by comparison of retention index (RI on HP-5ms) calculated and retention index from literature (RIL).
[b] identification confirmed by pure standard injection. Trace: when the concentration is lower than 0,1 µg/L.

Phenolic compounds

Some phenolic compounds were found in all *ikigage* samples. Higher concentrations of 2-methoxy-4-vinylphenol and 4-vinylphenol were observed in pilot *ikigage*, while 2-methoxyphenol was only detected in traditional sorghum beer *ikigage* made by peasants. These volatile phenols may originate from the sorghum malts and/or yeast activity during brewing process. Sorghum contains important amounts of p-coumaric acid and ferulic acid

(Hahn *et al*., 1983; Dykes and Rooney, 2006) potentially transformed into highly flavor-active corresponding volatile phenols during the process of either thermal (Fiddler *et al*., 1967) or enzymic decarboxylation by specific yeast strains (Vanbeneden *et al*., 2008a). Vanbeneden *et al*. (2008a) also reported that at mashing temperatures of 90 °C and 100 °C, 4-vinylguaiacol (2-methox-4-vinylphenol) concentrations increased with the heating time. The decantation mashing temperature (90 °C hold for 30 min) used during the production pilot *ikigage* can explain the higher concentrations of those volatiles than the amounts observed in traditional sorghum beer *ikigage* made by peasants. Phenolic compounds are undesirable when present in excessive concentration in pilsner beers (Vanbeneden *et al*., 2008b), but some volatile phenols such as 2-methoxy-4-vinylphenol are known as essential flavor contributor to the characteristic aroma of Belgian white beers (made with unmalted wheat) (Back *et al*., 2000) and German Weizen beers (made with malted wheat) (Wackerbauer *et al*., 1982). These phenolic compounds may participate to the characteristic flavor (smoked,

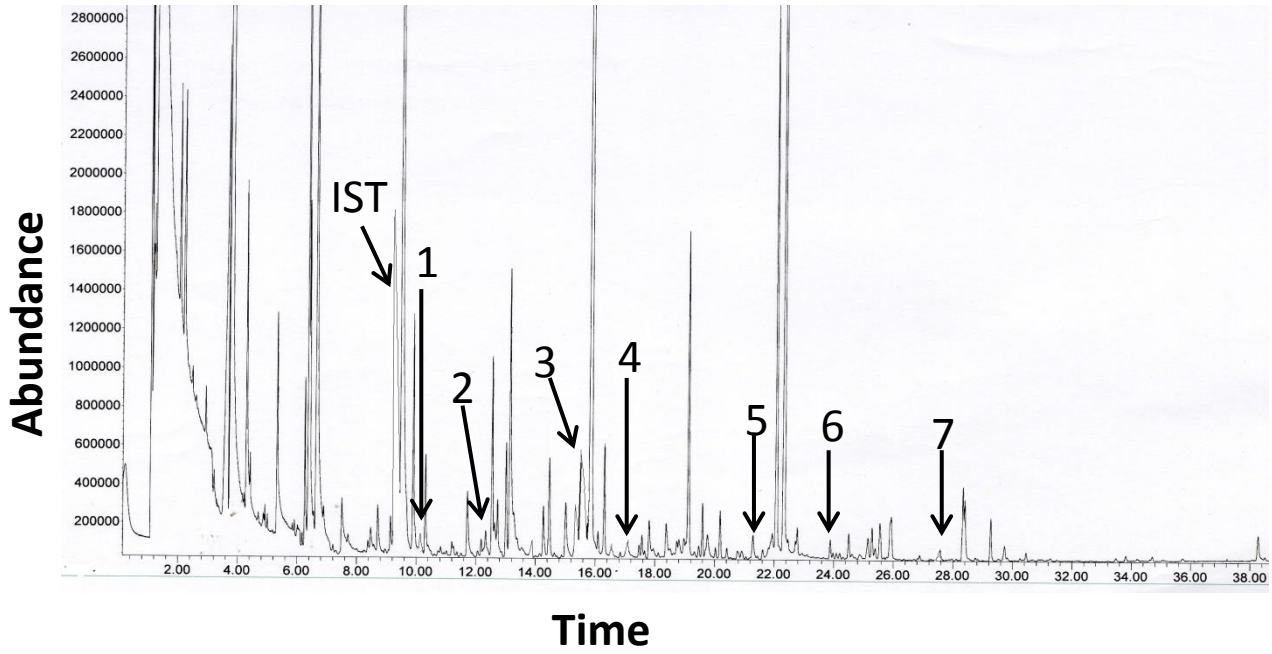

Figure 1. Volatile compounds detected only in ikigage beers made with *V. amygdalina*
(IST: internal standard; **1**: delta 3-carene; **2**: linalool; **3**: methyl salicylate; **4**: beta- citronellol; **5**: beta-damascenone; **6**: Alpha- farnesene; **7**: (2Z6E)- farnesol).

phenol or medicinal-like, dentist-like) noticed by the panelists for the Rwandese sorghum beer *ikigage* IKT (**Figure 2**).

Sulfur compounds

Some sulfur compounds were also found in *ikigage*. Dimethyl sulfide (DMS) and 3-(methylthio)-1-propanol were detected in all *ikigage* samples analyzed. Dimethyl sulfide, with its cooked cabbage flavor, is formed by the thermal decomposition of S-methylmethionine during kiln drying of the malt and wort preparation (Annes and Bamforth, 1982). However, the enzymatic conversion of dimethyl sulfoxide (DMSO) to DMS by brewing yeast is important and, under some circumstance, may be the major source of DMS in beer (Hansen *et al*., 2002; Gijs *et al*., 2003). 3-(Methylthio)-1-propanol and its acetate are derived from the sulfur amino acid methionine following Ehrlich pathway (Etschmann *et al*., 2008) and both impart a powerful odor reminiscent of soup, meat, onions, and potatoes (Arctander, 1969). However, 3-(methylthio)-propyl acetate was only detected in pilot *ikigage* brewed without *V. amygdalina*. Recently, strong decrease of the Ehrlich-derived 2-sulfanyethyl acetate has been described in pilot *ikigage* where *V. amygdalina* was added during boiling (Lyumugabe *et al*., 2012). Complementary data are needed to understand the mechanism involved in the inhibition of the Elhrlich pathway by *V. amygdalina* leaves.

Furans

2-Pentylfuran was found in all *ikigage* samples while 2,5-dimethylfuran was found only in pilot *ikigage*. These heterocyclic compounds have always been associated with the Maillard reaction as this pathway is one of the main pathways generating furan. 2-Pentylfuran was previously found in roasted sorghum malt (Lasekan *et al*., 1997) and pilsner beers (Da Silva *et al*., 2008). 2,5-Dimethylfuran was also found in Shochu koji, traditional Japanese alcoholic beverage made from rice (Yoshizaki *et al*., 2010). The presence of 2,5-Dimethylfuran in pilot

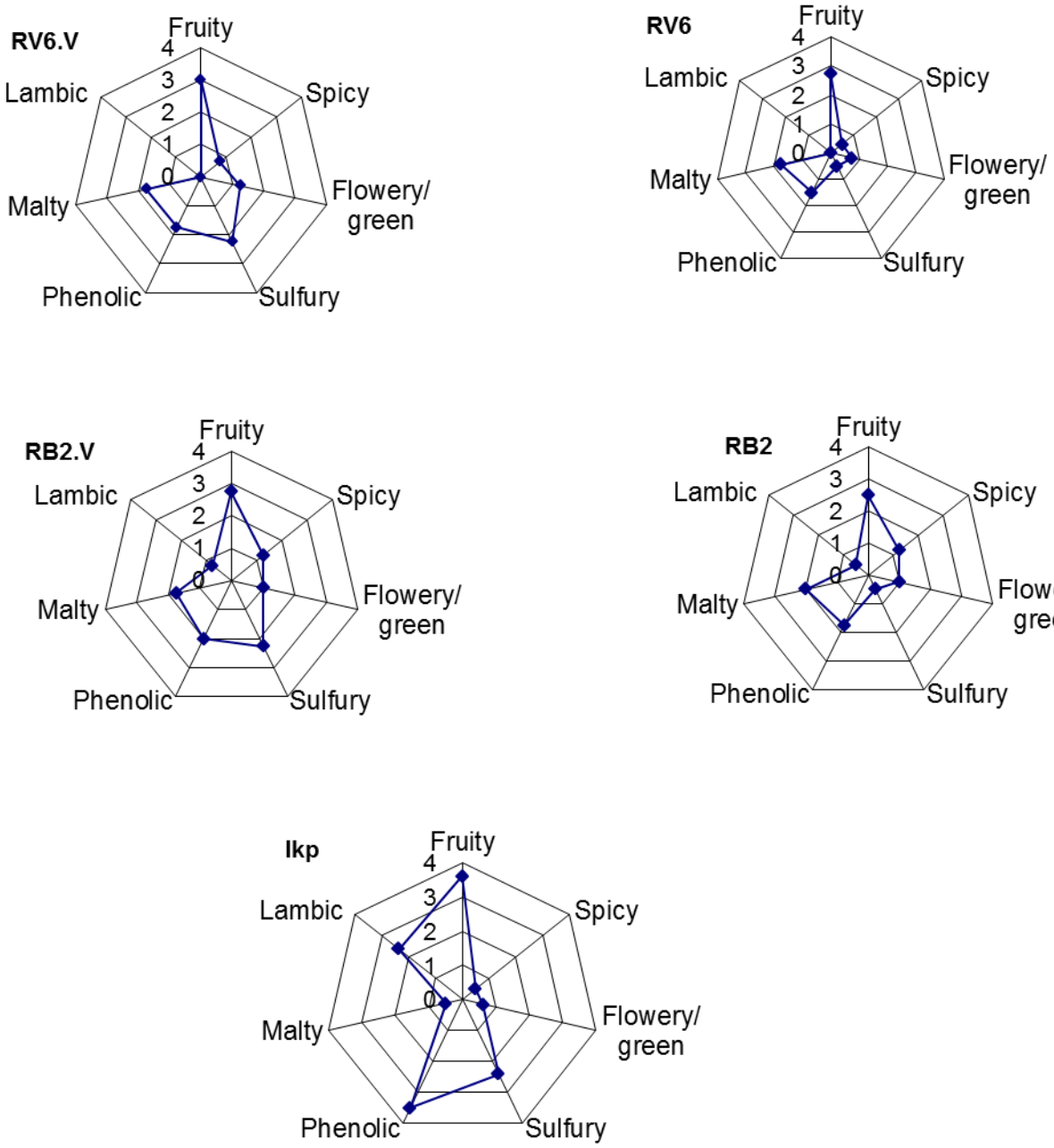

Figure 2. Aroma profile of sorghum beer *ikigage* beers containing *V. amygdalina* (RV6.V, RB2.V and IKp) and without *V. amygdalina* (RV6 and RB2). RV6.V and RV6 are pilot beers produced with selected *S. cerevisiae* strain RV6. RVB2.V and RB2 are also pilot beers produced with Selected *S. cerevisiae* strain RB2. IKp is a traditional sorghum beer *ikigage* made by Rwandese peasants.

ikigage can be attributed to the decantation mashing process applying high temperature for a long period.

Sensory characteristics of sorghum beer ikigage

Figure 2 shows the main sensory attributes of every beer as an average intensity of the individual panelist's scores. The results showed that all *ikigage* samples analyzed were characterized by fruity, phenolic and flowery notes in accordance with important amounts of esters and phenols observed in this work. *Ikigage* brewed with *V. amygdalina* was characterized by high score of sulfury odorant. But the latter attribute cannot be explained by the sulfur compounds detected in *ikigage* containing *V. amygdalina.* This leaves room to suspect the presence of other small volatile compounds such as thiols. Recently, using a specific extraction method of thiols and different gas chromatographic detectors (olfactometry, pulsed-flame photometric detector-PFPD and mass spectrometry), Lyumugabe *et al*. (2012) identified 14 polyfunctional thiols in the sorghum beer *ikigage*. Among them, 3-methyl-2-buten-1-thiol emerged as a key-flavor in *ikigage* containing *V. amygdalina*. These polyfunctional thiols can contribute to the sulfury note of this beer.

Lambic note of traditional sorghum beer made by peasants is probably due to the presence of several wild yeasts and other microorganisms involved in the fermentation resulting in this beer. In Belgian Lambic beer, the yeasts of genus *Brettanomyces* are partially responsible for the typical bouquet. But, the yeast species belonging to this genus were not yet identified in Rwandese traditional sorghum beer (Lyumugabe *et al.*, 2010). Other yeasts species would have been at the origin of Lambic character of *ikigage*.

4. CONCLUSION

Traditional sorghum beer *ikigage* brewed with *V. amygdalina* were characterized by the presence of higher levels of esters, higher alcohols and fatty acids. The higher concentrations of esters were ethyl acetate, ethyl caprylate, ethyl caproate and ethyl caprate. Higher alcohols were mainly composed of 1-propanol, 2-methylpropan-1-ol, 3-methylbutan-1-ol, 2-methylbutan-1-ol and 2-phenylethanol. Acetic, caproic and caprylic acids were important fatty acids. The volatile phenols such as 2-methoxy-4-vinylphenol, 4-vinylphenol and 2-methoxyphenol were also detected in *ikigage* and contribute to the phenolic note of this beer. *V. amygalina* provides terpenes compounds (δ-3-carene, *β*-farnesene, farnesol, *β*-citronellol, linalool and 1,8-cineole), methyl salicylate and beta-damascenone in *ikigage*. Like in hopped beers, these compounds detected in beers containing *V. amygdalina* might play a significant role in overall flavor and aroma of *ikigage*. *V. amygdalina* leaves emerge as an interesting hops substitute for tropical countries, but complementary data are still needed to understand which volatile compounds or precursors preexist in *V. amygdalina*.

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References

Abraham, K.O., Shankaranarayana, M.L., Raghaven, B., Natarajan, CP.**,** 1976. Determination of methyl salicylate in black tea. Mikrochimica Acta, 65, 11-15.

Adenuga, W., Olaleye, O.N., Adepoju P.A., 2010. Utilization of bitter vegetable leaves (Gongronema latifolium, Vernonia amygdalina) and Garcinia kola extracts as substitutes for hops in sorghum beer production. Afri. J. Biotech.,9, 8819-8823.

Ajebesone, P.E., Aina, J.O., 2004. Potential African substitutes for hops in tropical beer brewing. Afri. J. Food and Techn., 9, 13-16.

Annes, B. J., Bamforth, C.W., 1982. Dimethyl sulphides - a review. J. Inst. Brew., 88, 244-52.

Arctander, S., 1969. Perfume and Flavor Chemicals. Volume II, Aroma Chemicals, The Author, Montclair N.J., USA.

Asawalam, E.F., Hassanali, A., 2006. Constituents of the essential oil of Vernonia amygdalina as maize weevil protectants. Trop. Subtrop. Agrosyst., 6, 95-102.

Back, W., Diener, B., Sacher, B., 2000. Hefeweizenbier - Taste spectrum and technology. Brauwelt Inter., 18, 112-119.

Burdock, G.A., 1995. *Fenaroli's Handbook of Flavor Ingredients, Volume II,* 3rd edn. Boca Raton, FL: CRC Press.

Chevance, F., Guyot – Declerck, C., Dupont, J., Collin, S., 2002. Investigation of the beta-damascenone level in fresh and aged commercial beers. J. Agric. Food Chem., 50, 3818-3821.

Clapperton, J. F., 1978. Fatty acids contributing to caprylic flavour in beer. The use of profile and threshold data in flavour research. J. Inst. Brew., 84**,** 107-112.

Daenen, L., 2008. Exploitation of the flavour potential of hop and sour cherry glycosides by Saccharomyces and Brettanomyces glycoside hydrolase activities. PhD Thesis, K.U. Leuven, Belgium.

Damiani, P., Gobbetti, M., Cossignani, L., Corsetti, A., Simonetti, M. S., Rossi, J., 1996. The sourdough microflora. Characterization of hetero- and homofermentative lactic acid bacteria, yeasts and their interactions on the basis of the volatile compounds produced. Lebensm. Wiss. u. Technol., 29**,** 63-70.

Da silva, G.A., Augustino, F. Poppi, R.J., 2008. Explora analysis of the volatile profile of beers by HS-SPME-GC. Food Chem., 111, 1057-1063.

Dykes, L., Rooney, L.W., 2006. Sorghum and millet phenols and antioxidants. J. Cereal Sci., 44, 236-251.

Etschmann, M.M.W., Kötter, P., Hauf, J., Bluemke, W., Entian, K.D. Schrader, J., 2008. Production of the aroma chemicals 3-(methylthio)-1-propanol and 3-(methylthio)-propylacetate with yeasts. Appl Microbiol Biotechn., 80, 579-587.

Fiddler, W., Parker, W. E., Wasserman, A. E., Doerr, R. C., 1967. Thermal decomposition of ferulic acid. J. Agric. Food Chem., 15, 757-761.

Fritsch, H. T., Schieberle, P., 2005. Identification based on quantitative measurements and aroma recombination of the character impact odorants in a Bavarian pilsner-type beer. J. Agric. Food Chem., 53, 7544-7551.

Gijs, L, Vermeleun, C, Collin, S., 2003. Review- occurrence et voies de formation des arômes soufrés dans la bière : les sulfures et les polysulfures. Cerevisiae, 28, 37-46.

Hahn, D.H., Faubion, J.M., Rooney, L.W., 1983. Sorghum phenolic acids, their high performance liquid chromatography separation and their relation to fungal resistance. Cereal Chem., 60: 255-259.

Hansen, J., Bruun, SV., Bech LM., Gjermansen, C., 2002. The level of MXR1 gene expression in brewing yeast during fermentation is a major determinant for the concentration of dimethyl sulfide in beer. FEMS yeast Res., 5, 82-90.

Hazelwood, L. A., 2008. The Ehrlich pathway for fusel alkohol production: a century of research on *Saccharomyces cerevisiae* metabolism. Appl. Environ. Microbiol., 74, 2259-2266.

Hughes, P.S., Baxter E.D., 2001. Beer: Quality, Safety and Nutritional Aspects, Royal Society of Chemistry, UK, p. 40.

Jespersen, L., 2003. Occurrence and taxonomic characteristics of strains of *Saccharomyces cerevisiae* predominant in African indigenous fermented foods and beverages. FEMS Yeast Res., 3, 191-200.

Kayodé, A. P. P., Vieira-Dalodé, G., Kotchoni, S. O., Linnemann, A. R., Hounhouigan, J. D., Van Boekel, M. A. J. S., Nout, M. J. R., 2011. Diversity of yeasts involved in the fermentation of tchoukoutou, an opaque sorghum beer from Benin. Afri. J. Microbiol., Res., 5, 2737-2742.

King, A. Dickinson, J.R., 2003. Biotransformation of hop aroma terpenoids by ale and lager yeasts. FEMS Yeast Res., 3, 53-62.

Kishimoto, T., Wanikawa, A., Kagami, N. & Kawatsura, K., 2005. Analysis of hop – derived terpenoids in beer and evaluation of their behavior using the stir bar-sorptive extraction method with GC – MS. J. Agric. Food Chem., 53, 4701-4707.

Kollmannsberger, H., Biendl, M., Nitz, S., 2006. Occurrence of glycosidically bound flavor compounds in hops, hop products and beer. Monatsschrift für Brauwissenschaft, 59, 83-89.

Lasekan, O., Lasekan, W., Idowu, M. A., 1997. Flavour volatiles of malt beverage from roasted sorghum. Food Chem., 58, 341-344.

Lermusieau, G., Bulens, M., Collin, S., 2001. Use of GC-olfactometry to idendify the hop aromatic compounds. J. Agric. Food Chem., 49, 3867-3874.

Lyumugabe, L., Kamaliza, G., Bajyana, E., Thonart, PH., 2010. Microbiological and physico-chemical characteristic of Rwandese traditional beer "*Ikigage*". Afr. J. Biotechnol., 9, 4241-4246.

Lyumugabe, F., Gros, J. Thonart, P., Collin, S., 2012. Occurrence of polyfunctional thiols in sorghum beer "ikigage" made with *Vernonia amygdalina* "umubirizi". Flav. Frag. J., 27, 372-377.

Maoura, N., Mbaiguinam, M., Nguyen, HV., Gaillardin, C., Pourquie, J., 2005. identification and typing of the yeast strains isolated from bili bili, a traditional sorghum beer of chad. Afri. J. Biotechn., 4, 646-656.

Mboto, C.I., Eja, M. E., Adegoke, A.A., Iwatt, G.D., Asikong, B.E., Takon, I., Udo, S.M., Akeh M., 2009. Phytochemical properties and antimicrobial activities of combined effect of extracts of the leaves of *Garcinia kola, Vernonia amygdalina* and honey on some medically important microorganisms. Afri J. Microbiol. Res., 3,557-559.

Meilgaard, M. C., 1975. Flavor chemistry of beer. Part I: Flavor interaction between principal volatiles. Tech. Q. Master Brew. Assoc. Am., 12, 107-117.

Montanari, L., Perretti, G., Natella, F., Guidi, A., Fantozzi, P., 1999. Organic and phenolic acids in beer. Lebensm.-Wiss. U.-Technol. 32, 535-539.

Muyanja, CMBK., Narvhus, JA., Treimo, J., Langsrud, T., 2003. Isolation, characterization and identification of lactic acid bacteria from bushera: A Ugandan traditional fermented beverage. Inter. J. Food Microbiol., 80, 201-210.

Nance, M.R., Setzer, W.N., 2011. Volatile components of aroma hops (*Humulus lupulus* L.) commonly used in beer brewing. J. Brew. Dist., 2, 16-22.

Naumova, ES., Korshunova, IV., Jespersen, L., Naumov, GS., 2003. Molecular genetic identification of Sacharomyces sensu stricto strains from African sorghum beer. FEMS Yeast Res., 3, 177-184.

Nykänen, L. Suomalainen, H., 1983. Formation of aroma compounds by yeast. In: Aroma of Beer, Wine and Distilled Alcoholic Beverages, Akademie-Verlag: Berlin.

Oboh, F.O.J., Masodje H.I., 2009. Nutritional and antimicrobial properties of *Vernonia amygdalina* leaves. Inter. J. Biomed. Health Sci., 5, 51-56.

Ogunbinu, AO, Flamini, G, Cioni PL, Ogunwande, IA, Okeniyi, SO., 2009. Essential oil constituents of *Eclipta prostrate* (L.)L.and *Vernonia amygdalina* Delile. Nat. Prod. Com., 4, 421-424.

Okoro, C.C . Aina, J. O., 2007. Effect of storage on the brewing properties of tropical hop substitutes. Afri J. Biotech., 6, 1479-1483.
Okoh, IA, Babalola, GO, Ilori, MO., 1995. Effect of methanol extract of *Vernonia amygdalina* on malting and brewing properties of sorghum. Tech. Quart. Master Brewer's Assoc. Am., 32, 11-14.

Oshita, K., Kubota, M., Uchida, M., Ono, M., 1995. Clarification of the relationship between fusel alcohol formation and amino acid assimilation by brewing yeast using 13C-labeled amino acid. In: *Proceedings of the European Brewing Convention*. 387-394. Bruxelles

Pinho, O., Ferreira Isabel, M.P.L.V.O., Santos Lucia, H.M.LM., 2006. Method optimization by solid-phase microextraction in combination with gas chromatography with mass spectrometry for analysis of beer volatile fraction. J. Chrom., 1121, 145-153.

Pietruska, M. Piekech-Przybylska, K., Szopa, J.S., 2010. Synthesis of higher alcohols during fermentation of rye mashes. Food Chem. Biotech., 74, 51-64.

Pino, J.A., Marquez, E., Quijano, C. E., Castro, D., 2010. Volatile compounds in noni (*Morinda citrifolia* L.) at two ripening stages**.** Ciênc. Tecnol. Aliment., Campinas, 3, 183-187.

Spaepen, M., Vanoevelen, D., Verachtert H., 1978. Fatty – Acids and Esters produced during spontaneous fermentation of Lambic and Gueuze. J. Inst. Brew., 84, 278-282.

Spicher, G., Nierle, W., 1988. Proteolytic activity of sourdough bacteria. Appl. Microbiol. Biotech., 28, 487-492.

Schieberle, P., 1991. Primary odorants of pale lager beer. Z. Lebensm. Unters. Forsch., 193, 558-565.

Suomalainen, H., 1981. Yeast esterases and aroma esters in alcoholic beverages. J. Inst. Brew., 87, 296-300.

Takoi, K., Koie, K., Itoga, Katayama, Y., Shimase, M., Nakayama Y., Watari J., 2010. Biotransformation of hop-derived monoterpene alcohols by lager yeast and their contribution to the flavor of hopped beer. J. Agric. Food Chem., 58, 5050-5058.

Vanbeneden, N., Gils, F., Delvaux, F. and Delvaux, F. R., 2008a. Formation of 4-vinyl and 4-ethyl derivatives from hydroxycinnamic acids: Occurrence of volatile phenolic flavour compounds in beer and distribution of Pad1-activity among brewing yeasts. Food Chem., 107, 221-230.

Vanbeneden, N., Van Roey, T., Willems, F., Delvaux, F., Delvaux, F.R. 2008b. Release of phenolic flavour precursors during wort production: Influence of process parameters and grist composition on ferulic acid release during brewing. Food Chem., 111, 83-91.

Vanderhaegen, B., Neven, H., Coghe, S., Vestrepen, K.J., Derdelinckx, G., Verachtert, H., 2003. Bioflavoring and beer refermentation. Appl. Microbiol. Biotech., 62, 140-150.

Wackerbauer, K., Kramer, P., Siepert, J., 1982. Phenolische Aromastoffe in Bier. Brauwelt, 15, 618-626.

Yoshizaki, Y., Yamato, H., Takamine, K., Tamaki, H., Kiyoshi Ito., Sameshima, Y., 2010. Analysis of Volatile Compounds in Shochu Koji, Sake Koji, and Steamed Rice by Gas Chromatography-Mass Spectrometry. J. Inst. Brew., 116, 49-55.

Zhao, Y.P., Xu, Y., Li, J.M., Fan, W.L., Jiang, W.G., 2009. Profile of volatile compounds in 11 brandies by headspace solid-phase microextraction followed by gas chromatography-mass spectrometry. J. Food Sci. 74, 90-99.

Chapitre 6 :

Occurrence des thiols polyfonctionnels dans la bière de sorgho « ikigage » brassée avec *Vernonia amygdalina* « umubirizi »

Ce travail a fait l'objet de la publication suivante :

Lyumugabe, F., Gros, J., Thonart, Ph., Collin, S. (2012). Occurrence of polyfunctional thiols in sorghum beer « *ikigage* » made with *Vernonia maygdalina* « *Umubirizi* ». *Flavour and Fragrance Journal, 27, 372–377.*

Préambule au chapitre 6

Le chapitre précédent a révélé que la bière de sorgho *ikigage* brassée avec *Vernonia amygdalina* est caractérisée par une note soufrée. Il n'existe pas de corrélation entre cette note soufrée et les composés volatiles détectés par la méthode SPME-GC-MS. Dans cette bière, nous avons suspecté l'existence de thiols polyfonctionnels. La littérature indique que les thiols polyfonctionnels détectés souvent à l'état de trace ont un impact sur les caractéristiques aromatiques des bières occidentales fabriquées à base d'orge et de houblon. Cependant, les thiols polyfonctionnels n'ont jamais été identifiés dans les bières fabriquées avec les matières premières non-occidentales.

Dans ce contexte, notre recherche a porté sur les thiols polyfonctionnels de la bière traditionnelle de sorgho « *ikigage* » brassée avec *V. amaygdalina.* Les thiols ont été extraits par la méthode spécifique avec de l'acide *p*-hydroxymercuribenzoique. Les extraits ont été ensuite analysés avec differents détecteurs chromatographiques (olfactometrie, pulsed–flame photometric detector (PFPD), spetrométrie de masse). La méthode AEDA (Aroma Extract Dilution Analysis) a été appliquée, afin d'évaluer la relative contribution des traces non détectées par les détecteurs habituels.

Occurrence of polyfunctional thiols in sorghum beer "ikigage" made with *Vernonia amygdalina* "umubirizi"

RUNNING TITLE HEADER: Polyfuntional thiols in sorghum beer

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Abstract

Several polyfunctional thiols have been previously identified in beers made from barley and hops. These compounds have not been investigated in beers brewed with 'non-Western' raw materials. Here we have performed a thiol-specific extraction with p-hydroxymercuribenzoic acid on a traditional *ikigage* sorghum beer from Rwandese peasants (use of *Vernonia amygdalina* just for yeast propagation), and on two pilot beers with addition (or not) of *V. amygdalina* in the boiling kettle, instead of hops. Gas chromatography–olfactometry, gas chromatography–mass spectrometry and gas chromatography with pulsed flame photometric detection analyses of the extracts enabled us to identify 14 polyfunctional thiols. The well-known hop constituent 3-methyl-2-buten-1-thiol emerged as a key flavour in the unhopped beers containing *V. amygdalina* (flavour dilution>262 144). The addition of *V. amygdalina* during boiling also resulted in the presence of 1-butanethiol, but the production of 2-sulfanylethanol and 2-sulfanylethyl acetate was inhibited. Complementary data are required to understand how *V. amygdalina* leaves are able to impact upon the Ehrlich pathway leading to cysteine and homocysteine-derived thiols.

Keywords: Polyfunctional thiols; Aroma; Sorghum beer; *Ikigage*; *Vernonia amygdalina*

INTRODUCTION

In Sub – Saharan Africa, traditional sorghum beer is a popular alcoholic beverage for African ceremonies. Sorghum beer is known as *ikigage* or *amarwa* in Rwanda[1], *tchoukoutou* in Benin and Togo[2], *pito* in Ghana and Nigerian[3], *bili bili* in Chad[4] and *impeke* in Burundi[5]. These beers made from sorghum malt or millet are unhopped.

Hop is a temperate crop that cannot be successfully grown in tropical African countries. Several studies reported the possibility of using *Vernonia amygdalina* instead of hop in African sorghum beers[6-10]. *V. amygdalina*, known as "bitter leaf", is a green shrub found all over Africa and belonging to the *Asteraceace* family. It mimics hop not only by its antimicrobial properties[11,12] but also by its bitter taste.[10,13,14]

In European beers made of malted barley and hop, many studies have evidenced the key-role of polyfunctional thiols.[15-17] Characterized by extremely low threshold values, these compounds are also important contributors to the characteristic aroma of various fruits[18], and wines[19]. GC-O and GC-PFPD analyses of thiol specific beer extracts (*p*HMB) have led to identifying 2-sulfanylethanol (**2** in **Figure 1**), 3-methyl-2-buten-1-thiol (**4**), 3-sulfanylpropanol (**5**), 2-sulfanylethyl acetate (**6**), 4-sulfanyl-4-methylpentan-2-one (**9**), 3-sulfanylpropyl acetate (**11**), 3-sulfanylhexanol (**15**), 2-sulfanyl-3-methylbutanol and 3-sulfanyl-3-methylbutanol[16]. Among these compounds, the onion-like 2-sulfanyl-3-methylbutanol[20] is suspected of having arisen through the reaction of hop allylic alcohol with yeast hydrogen sulfide[21]. In these "western-like" beers, Takoi observed a strong effect of the hop variety, claiming that this ingredient could be the main source of certain polyfunctional thiols.[22] Recently, Gros *et al*. identified 41 free thiols in several hop varieties and suspected them of being released from bound precursors through wort boiling and fermentation.[23]

Surprisingly, polyfunctional thiols have not yet been studied in beers brewed with non western raw materials. Here we have performed a thiol-specific extraction with *p*-hydroxymercuribenzoic acid (*p*HMB)[24] on a traditional *ikigage* sorghum beer from Rwandese peasants (IKT; use of *Vernonia amygdalina* for yeast propagation), and on two pilot beers made with (RV6.V) or without (RV6) addition of *V. amygdalina* (instead of hop) in the boiling kettle (use of a dominant strain selected from traditional leaven, referred to as RV6). Thiols were analyzed with different gas chromatographic detectors (olfactometry, pulsed–flame photometric detector-PFPD and mass spectrometry), and compared to various references available in our combinatorial libraries[25-28]. The well-known AEDA methodology (Aroma Extract Dilution Analysis)[29] was applied in order to assess the relative contribution of traces undetected by usual detectors.

MATERIALS AND METHODS

Reagents

p-Hydroxymercuribenzoic acid (*p*HMB), hydrochloride L-cysteine monohydrated and HCl 37% were purchased from Sigma-Aldrich (Bornem, Belgium). Dichloromethane (99.9%) obtained from Romil (Cambridge, UK) was distilled before use. 4-Methoxy-2-methylbutan-2-thiol was obtained from Oxford Chemicals (Oxford, U.K.). Milli-Q water was used (Millipore, Bedford, Ma). NaOH and Na_2SO_4 99% were supplied by Janssen (Geel, Belgium). A strongly basic Dowex resin 1 X 2, Cl^- form (Sigma-Aldrich, Bornem, Belgium) was stored in hydrogen chloride (0.1M). Anhydrous sodium sulfate was obtained from Merck (Darmstadt, Germany) and tris(hydroxymethyl)aminomethane (TRIS) from USB (Cleveland, Ohio, USA). 1-Butanethiol (**1**), 2-sulfanylethan-1-ol (**2**), 3-sulfanylpropan-1-ol (**5**), 2-sulfanylethyl acetate (**6**) and 3-sulfanylpropyl acetate (**11**) were purchased from Sigma-Aldrich (Bornem, Belgium) (full chemical structures are given in **Figure 1**). 4-Methoxy-2-

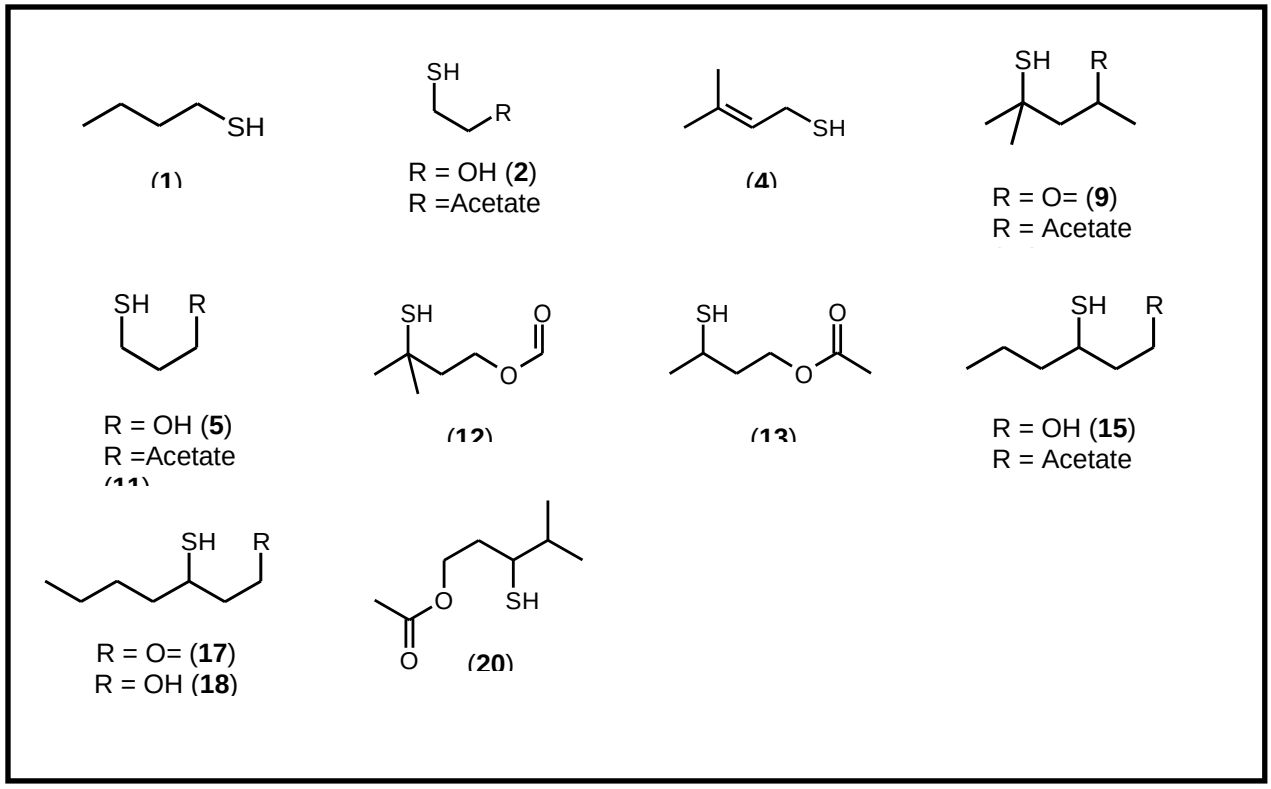

Figure 1. Chemical structures and numbering of thiols

methylbutan-2-thiol, 3-sulfanylhexan-1-ol (**15**) and 3-methyl-2-buten-1-thiol (**4**) were obtained from Oxford Chemicals (Oxford, UK). 4-Sulfanyl-4-methylpentan-2-one (**9**) was from Frutarom (Hartlepool, UK). 3-Sulfanyl-3-methylbutyl formate (**12**) was obtained from Endeavour (Northamptonshire, UK).

Reference compounds synthesized in our laboratory

3-Sulfanylbutyl acetate (**13**), 3-sulfanyl-2-methylpentyl acetate (**20**), 4-sulfanyl-4-methyl-2-pentyl acetate (**16**) and 3-sulfanylhexyl acetate (**19**) were synthesized in our laboratory prior to that work (reagents and complete procedure in[27]). 3-Sulfanylheptanal (**17**) and 3-sulfanylheptanol (**18**) had been previously produced according to Vermeulen et al.[26,28]

Ikigage beer samples

Production of pilot beers with or without Vernonia amygdalina leaves (RV6 and RV6.V)

The production process for pilot beers is described in **Figure 2**. Two hundred grams of malt (60g *Eleusine coracana* and 140g red sorghum) from peasants were mashed in 1000 ml distilled water at 45°C for 30 min. Thereafter, 150 ml of the clear "enzymatic supernatant" was removed, while the remaining mash was heated at 90°C, held at the same temperature for 30 min, and cooled below 50°C at which time the clear ''enzymatic supernatant'' was re-added[30]. This mixture was then brewed according to the following mashing program: 1h at 63°C, 10 min at 75°C. The wort was centrifugated and then heated to boiling for 50 min. For production of the RV6.V pilot beer, leaves of *V. amygdalina* (2 g/L) were added after 10 min boiling. Fermentation was conducted with selected yeast strains (*Saccharomyces cerevisiae* RV6) from Rwandese traditional "*umusemburo*" leaven in a light-protected Erlenmeyer flask, equipped with a gas trap. Yeast was pitched at 10^7 Ufc/ml and fermentation carried out at 30°C for 3 days. After centrifugation (13 000g, 25 min), the beer was kept at -20°C until thiol specific extraction.

Beers from Rwandese peasants (IKT)

Traditional *ikigage* beer was collected from the Tumba market site, in the Southern Province of Rwanda. Samples were taken from opaque tank, in bottles (500 ml), preserved from light with aluminium sheets, stored in the freezer (- 20°C) and then extracted immediately after opening. The traditional process of manufacture of *ikigag*e has been described by Lyumugabe et *al*.[1] *V. amygdalina* is used only during the production process of traditional "*umusemburo*" leaven.

Extraction of polyfunctional thiols

This extraction method is based on the specific affinity of thiols for *p*-hydroxymercuribenzoic acid (*p*HMB)[24]. Beer (500 mL) was stirred with distilled CH_2Cl_2 (200 mL) for 30 minutes. After decantation (± 15 minutes), the lower phase and the interfacial emulsion were centrifuged for 20 minutes at 4000 rpm. The organic phase was then extracted by 2 × 20 mL of a *p*HMB solution (360 mg of *p*HMB, 24.6 g of Tris in 1 L of Millipore water) for 5 and 10 minutes, respectively. The combined aqueous phases were loaded into a strongly basic anion exchanger column (Dowex 1WX2-100 resin from Aldrich Chemicals), washed beforehand by NaOH 2 M, HCl 2 M, and in between rinsed by ultrapure water. Then 50 mL of sodium

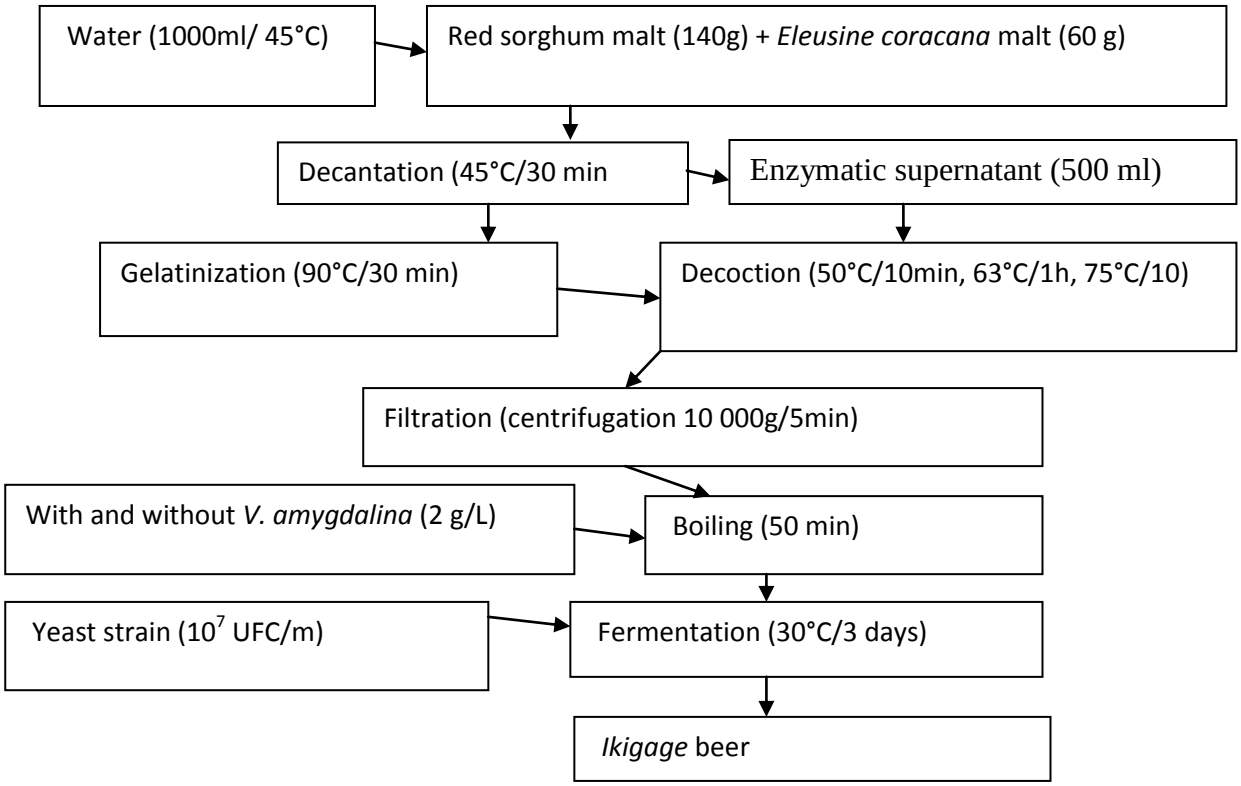

Figure 2. Production process of pilot ikigage beers (RV6.V and RV6)

acetate buffer (0.1 M, pH 6) was poured on the resin to remove impurities. Volatile thiols were released by percolating a purified cysteine solution (640 mg of hydrochloride L-cysteine monohydrated in 60 mL of Millipore water – this solution was washed with 2 × 5 mL of distilled CH_2Cl_2 before use). The eluate containing the volatile thiols was collected and extracted by four and three mL of distilled CH_2Cl_2 using magnetic stirring (5 minutes each time). The organic phases were pooled, dried on anhydrous Na_2SO_4, and finally concentrated in a Kuderna to 250 µL, to be stored at –80°C. 4-Methoxy-2-methyl-butan-2-thiol (Oxford Cemicals, U.K.) added at the first extraction step – and thiazole 99% (Aldrich Chemicals) – added before the final concentration step – were used respectively as internal and external standards (IST and EST).

Gas chromatography–olfactometry

One microlitre of the p-HMB extract was analysedwith a Chrompack CP 9001 gas chromatograph (Antwerpen, Belgium), equipped with a splitless injector maintained at 250° C; the split vent was opened 0.5 min post-injection. Compounds were analysed with a wallcoated open tubular (WCOT) apolar CP-Sil5-CB (50 m x 0.32 mm i.d., 1.2 µm film thickness) or polar FFAP CB (25 m x 0.32 mm i.d.) capillary column. The carrier gas was nitrogen and the pressure was set at 50 kPa (CP-Sil5-CB). The oven temperature was programmed to rise from 36° C to 85° C at 20° C/min, then to 145° C at 1° C/min, and finally to 250° C at 3° C/min and held for 30min. In order to assess the olfactory potential of the extract, the column was connected to a GC-O port (Chrompack) maintained at 250° C. The effluent was diluted with a large volume of air (20 ml/min) pre-humidified with an aqueous copper (II) sulfate solution. The extractwas analysed immediately after extraction by three trained panellists. Complete AEDA was performed on the p-HMB extracts by one operator, with the CP-Sil5-CB column. The extracts were diluted stepwise with dichloromethane (1 + 1 by volume). The dilution factor (FD) is defined as the highest dilution at which the compound

could still be detected (FD= 2^n with n+1=number of dilutions applied on the extract until no detection by GC-O). The precision of this AEDA analysis is n ± 1 (factor 2 between FD values).

Gas chromatography–electronic impact mass spectrometry

Mass spectra (m/z = 40 to 380) were recorded at 70 eV on a ThermoFinnigan Trace MS mass spectrometer connected to a ThermoFinnigan Trace GC 2000 gas chromatograph (Manchester, U.K.) equipped with a splitless injector and a 50 m x 0.32 mm i.d., wall-coated open tubular (WCOT) apolar CP-Sil 5 CB MS capillary column (film thickness: 1.2 µm). The oven temperature program was the same as that described for GC-O. Spectral recording was automatic throughout elution; Xcalibur software was used (Manchester, U.K.).

Gas chromatography with pulsed flame photometric detection

Two microlitres of the *p*-HMB extract were analysed on a ThermoFinnignan Trace GC 2000 gas chromatograph equipped with a splitless injector maintained at 250° C and connected to a O.I. Analytical Pulsed Flame Photometric Detector, model 5380 (College Station, Texas, U.S.A.). The injections were carried out in the splitless mode at 250° C, the split being turned on after 0.5 min. The carrier gas was helium at a pressure of 90 kPa. At the detector, the following parameters were selected: 250° C as the temperature, 600 V as the voltage, 18 ms as the gate width, 6 ms as the gate delay, 580 mV as the trigger level and 3.70 Hz as the pulse frequency. The oven temperature program was the same as that described for GC-O.

Identification of the compounds

Mass spectrometry identifications were done by comparing the mass spectra obtained from each sample with those obtained with pure or synthesized compounds injected under the same conditions and/or present in the NIST library. The retention indices (retention times normalized with respect to adjacently eluting n-alkanes – decimal numeral system) were

determined by injection onto two capillary columns (CP-Sil5-CB and FFAP-CB) connected to the FID or the olfactometric detector. In the case of PFPD detections (interesting for traces giving no GS-MS peak), injection of injection of thioesters allowed translation into the alkane-related decimal numeral system. GC-O and GC-PFPD identifications were checked by co-injection of the extracts with commercial standards and standards issued from combinatorial syntheses achieved according to Vermeulen et al. [25-28].

Quantification of the compounds

Quantifications were achieved by GC-PFPD with the CP-Sil5-CB column. For commercially available thiols, complete calibration curves relative to the IST were used. For commercially unavailable thiols, quantifications are given in internal standard (IST) equivalent (4-methoxy-2-methyl-2-butanethiol added in the sample at 0.67 ng/l).

Analyses of humulones and lupulones by reverse phase high-performance liquid chromatography

Humulones and lupulones were analysed in *V. amygdalina* leaves according to the method recommended by the European Brewing Convention (EBC 7.4.1)[31]. Humulones and lupulones were extracted by a diethylether/methanol/chlorhydric acid (84:15.75:0.25, v/v) mixture. The HPLC (Waters 600 pump) was equipped with a Grace Smart RP 18 (250 x 4 mm, 5 µm) column and a Perkin-Elmer LC75 detector (314 nm) (Zaventem, Belgium).

RESULTS AND DISCUSSION

Despite the absence of hop, many polyfunctional thiols were detected in the traditional and pilot ikigage beers (**Table 1**). As depicted in **Figure 3**, GC-PFPD analyses of the p-HMB extracts enabled us to identify six thiols in RV6.V beers [1-butanethiol (**1**), 2-sulfanylethanol (**2**), 3-sulfanylpropanol (**5**), 3-sulfanylpropyl acetate (**11**), 3-sulfanyl-3-methylbutyl formate (**12**) and 3-sulfanylheptanal (**17**)]; four in RV6 beers [(**2**), 2-sulfanylethyl acetate (**6**), (**11**) and

(**12**)]; and six thiols in IKT beers [(**2**), (**5**), (**6**), (**11**),(**12**) and 3-sulfanylhexanol (**15**)]. Other polyfunctional thiols [3-methyl-2-buten-1-thiol (**4**), 4-sulfanyl-4-methylpentan-2-one (**9**), 3-sulfanylbutyl acetate (**13**), 4-sulfanyl-4-methyl-2-pentyl acetate (**16**), 3-sulfanylhexyl acetate (**19**) and 3-sulfanyl-2-methylpentyl acetate (**20**)] were detected by GC-O only. Nice GC-MS peaks made it possible to confirm the identification of compounds (**1**), (**2**), (**6**), (**11**) and (**12**).

GC-O analyses (AEDA methodology) evidenced 3-methyl-2-buten-1-thiol (**4,** MBT, skunky, garlic, old tea) as the most odorant thiol in the extracts from *ikigage* beers produced with *V. amygdalina* (RV6.V and IKT). Compared to the reference RV6, 3-methyl-2-buten-1-thiol (**4**) proved much more persistent (FD > 262144) when *V. amygdalina* was used, either during boiling (RV6.V) or for yeast propagation (IKT). In hopped western beers, the skunky off-flavour 3-methyl-2-buten-1-thiol is known to arise through the photochemical degradation of isohumulones[32]. In light-protected beer, 3-methyl-2-buten-1-thiol is found at lower concentration[33], giving rise to pleasant hoppy flavors[34]. In this case, it arises through nucleophilic substitution by hydrogen sulfide on 3-methyl-2-buten-1-ol[16]. The amount of allylic alcohol is correlated to hop bitterness[35,36]. Reports on previous studies carried out in Nigeria mentioned the presence of hop bitter compounds in *V. amygdalina*[13]. In our HPLC analyses, we failed to detect any humulones or lupulones in Rwandese *V. amygdalina*. The source of this isoprenyl moiety thus remains to be identified (saponines or other plant metabolites).

Table 1. Polyfunctional thiols in *ikigage* beers with (RV6.V and IKT) or without *Vernonia amygdalina* (RV6)

N°	Retention indices		Substance	Odor (GC –O)	PFPD concentration in µg/L in beer (FD GC-O AEDA given in parentheses)		
	CP-Sil5-CB	FFAP			**RV6.V**	**RV6**	**IKT**
1	686		1-butanethiol	Rot/egg	0,7* (16)	0	0
2	755	1501	2-sulfanylethanol	Roasted/sewage	0,7* (2)	3,1* (4)	4,0* (4)
3	788		unknown	Cabbages/rubber	0,2 (32)	- (8)	0
4	815	1140	3-methyl-2-buten-1-thiol	Skunky/garlic/old tea/plastic	- (> 262144)	- (16384)	- (> 262144)
5	840	1665	3-sulfanylpropanol	Roasted/potato	Trace (65536^{+})	- (4096^{+})	Trace (65536^{+})
6	872	1444	2-sulfanylethyl acetate	Roasted/plastic	0	10,6* (8)	11,8* (8)
7	900		unknown	Fatty/sauce	0	0	1,3 (8)
8	905		unknown	Flowered	0	0	0,7 (32)
9	921	1386	4-sulfanyl-4-methylpentan-2-one	Cat/blackcurrant	- (256)	0	- (4096)
10	939		unknown	Flowered/rubber/caramel	- (2)	0,2 (32)	- (8)
11	984	1569	3-sulfanylpropyl acetate	Roasted	Trace (1)	0,3* (2)	1,5* (4)
12	991	1527	3-sulfanyl-3-methylbutyl formate	Blackcurrant	0,8* (64)	1* (64)	0,6* (64)
13	1031	1542	3-sulfanylbutyl acetate	Nettle/sesame	- (16)	- (4)	- (4)
14	1044		unknown	Mushroom/soup	- (4)	- (4)	3,5 (32)
15	1106	1860	3-sulfanylhexanol	Rhubarb	- (2)	0	0,4 (8)
16	1111	1528	4-sulfanyl-4-methyl-2-pentyl acetate	Sesame/toast	- (64)	- (64)	- (16)
17	1113	1668	3-sulfanylheptanal	Candy/citrus/almond	Trace (64)	- (2)	- (32)
18	1189	1956	3-sulfanylheptanol	Cola/strawberry	- (16)	- (2)	- (64)
19	1219	1735	3-sulfanylhexyl acetate	Greenery/candy	- (64)	0	- (32)
20	1227	1702	3-sulfanyl-2-methylpentyl acetate	Blackcurrant	- (8)	- (16)	- (16)

RV6.V: Pilot beer made with *V. amygdalina*; **RV6**: Pilot beer made without *V. amygdalina*; **IKT:** Beer from Rwandese peasants; **Trace** when the compound is detected under the Quantification Limit (0.15 µg/L); **- :** when not detected (<0.05 µg/L) by PFPD. ***:** identifications confirmed by mass spectrometry (full scan monitoring). $^{+}$Coelution on CP-Sil5-CB with traces of co-extracted methional.

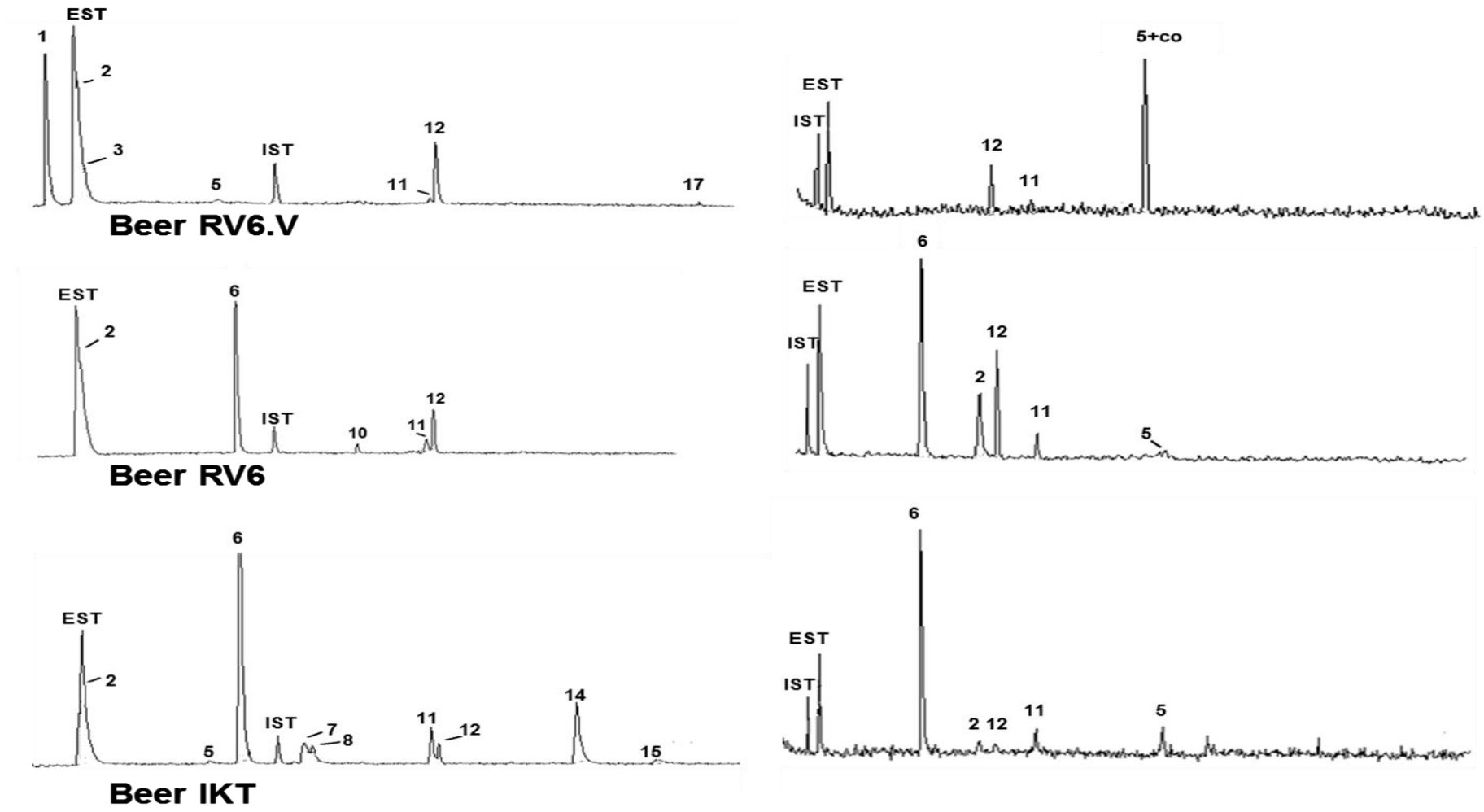

Figure 3. GC-PFPD chromatogram (**a:** CP-Sil5-CB ; **b:** FFAP) of *p*HMB extracts obtained from *ikigage* beer**.** Co = coeluent of 3-sulfanylpropanol on the FFAP column (RI on CP-Sil5-CB > 1200; m/z = 55, 83, 141, 41, 82).

Together with low amounts of 2-sulfanylethanol (**2**) and 3-sulfanylpropyl acetate (**11**), the most surprising result turned out to be the total absence of 2-sulfanylethyl acetate (**6**) in RV6.V (never observed before in usual hopped beers, where (**6**) is often the major PFPD peak[16,23]). This compound was found at the expected concentration in the IKT and RV6 beers. Addition of *V.amygdalina* in the boiling kettle proved able to inhibit the production of 2-sulfanylethanol and 2-sulfanylethyl acetate. Complementary data are needed to understand how V. amygdalina leaves added through boiling can have such a strong impact on cysteine transformation via the Ehrlich pathway.[16]

1-Butanethiol (**1**) emerged as another thiol distinguishing the pilot ikigage beer containing *V. amygdalina* (RV6.V). It is known to contribute to some meat, sea food and cheese flavours.[37] Compound (1) was previously identified in American and Mexican beers,[38] but turned out to be absent in all following studies focusing on beer thiols.[16,22,23] This compound could arise through nucleophilic substitution by hydrogen sulfide on butan-1-ol.

4-Sulfanyl-4-methylpentan-2-one (**9**, catty/blackcurrant) was found in both unhopped beers containing V. amygdalina (FD = 4096 in IKT and 256 in RV6.V). This compound has been identified as a key contributor to the blackcurrant/Muscat-like aroma in Tomahawk and Cascade hopped beers.[17,23] As in the case of p-HMB extracts of Japanese beer[22] and fresh hopped lager beers,[16] only GC-O enabled us to detect it in p-HMB extracts of ikigage beers. On the other hand, Kishimoto,[17] Takoi[22] and Gros[23] were able to quantify it by PFPD or GC-MS in hop samples. In wine, (9) has been shown to be issued from the biolysis of a cysteine conjugate S-4-(methylpentan-2-one)-l-cysteine and from Michael addition of hydrogen sulfide on mesityl oxide.[39,40]

3-Sulfanyl-3-methylbutyl formate (**12**, blackcurrant) was found in all three beers (**Figure 3b**). This compound is usually not detected in fresh industrial lager beers[16] but it can appear

through ageing.[41] It is also found in roasted seeds such as coffee[42,43] and white sesame,[44] where it results from the reaction between formic acid and 3-sulfanyl-3-methylbutanol in the presence of oxygen and heat.[42,44] The absence of hop polyphenols in the *ikigage* beers may explain the occurrence of this oxidationderived molecule.

In conclusion, *V. amygdalina* leaves are emerging as an interesting hop substitute for tropical African countries,[6–14] but the thiol profile of the resulting beers will be significantly different. Even when only used for leaven preparation, *V. amygdalina* brings some plant-derived polyfunctional thiols to beer. *V. amygdalina* addition during boiling also appears to strongly inhibit the production of 2-sulfanylethyl acetate. Complementary data are needed to understand which thiol precursors pre-exist in the plant, and by which mechanisms V. amygdalina might impact the yeast Ehrlich pathway.

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References

1. F. Lyumugabe, G. Kamaliza, E. Bjyana, Ph. Thonart, Afr. J. Biotechnol. 2010, 9, 4241.

2. A. P. P. Kayode, A. Aégbidi, A. R. Linnemenn, M. J. R. Nout, J. D. Hounhouigana, Ecol. Food Nutr. 2005, 44, 271.

3. J. A. Ekundayo, J. Food Technol. 1969, 4, 217.

4. N. Maoura, M. Mbaiguinam, H. V. Nguyen, C. Gaillardin, J. Pourquie, Afr. J. Biotechnol. 2005, 4, 646.

5. A. Nzigamasabo, A. Nimpagaritse, Food Res. Int. 2009, 42, 588.

6. N. Okafor, G. N. Anichie, Brew. Dist. Int. 1983, 13, 20.

7. J. O. Ogundiwin, M. O. Ilori, Lebensm. Wiss. Technol. 1991, 24, 182.

8. I. A. Okoh, G. O. Babalola, M. O. Ilori, Technol. Quart. Master Brew. Assoc. Am. 1995, 32, 11.

9. O. O. Lasekan, W. O. Lasekan, J. O. Babalola, Food Chem. 1999, 64, 507.

10. P. E. Ajebesome, J. O. Aina, Afr. J. Food Technol. 2004, 9, 13.

11. C. I. Mboto, M. E. Eja, A. A. Adegoke, G. D. Iwatt, B. E. Asikong, I. Takon, S. M. Udo, M. Akeh, Afr. J. Microbiol. Res. 2009, 3, 557.

12. F. O. J. Oboh, H. I. Masodje, Int. J. Biomed. Health Sci. 2009, 5, 5.

13. C. C. Okoro, J. O. Aina, Afr. J. Biotechnol. 2007, 6, 1479.
14. W. Adenuga, O. N. Olaleye, P. A. Adepoju, Afr. J. Biotechnol. 2010, 9, 8819.

15. C. Vermeulen, S. Lecocq, S. Collin, In Proceedings of the 29th European Brewery Convention Congress. Dublin, Ireland. Fachverlag Hans Carl: Nürnberg, Germany, ISBN: 798-90-70143-24-4, 2003, 91, 930–940.

16. C. Vermeulen, I. Lejeune, T. T. H. Tran, S. Collin, J. Agric. Food Chem. 2006, 54, 5061.

17. T. Kishimoto, A. Wanikawa, K. Kono, K. Shibata, J. Agric. Food Chem. 2006, 53, 8855.

18. H. K. Engel, R. Tressl, J. Agric. Food Chem. 1991, 39, 2249.

19. T. Tominaga, A. Furrer, R. Henry, D. Dubourdieu, Flavour Fragr. J. 1998, 13, 159.

20. A. Olsen, Carlsberg Res. Commun. 1988, 53, 1.

21. J. Gros, S. Nizet, S. Collin, Acta Hortic. 2009, 848, 273.

22. K. Takoi,M.Dequeil, S. Shinkaruk, C. Thibon, K.Maeda, K. Ito, B. Benetau, D. Dubourdieu, T. Tominaga, J. Agric. Food Chem. 2009, 57, 2493.

23. J. Gros, S. Nizet, S. Collin, J. Agric. Food Chem. 2011, 59, 8853.

24. T. Tominaga,M. L. Murat, D. Dubourdieu, J. Agric. Food Chem. 1998, 46, 1044.

25. C. Vermeulen, J. Pellaud, L. Gijs, S. Collin, J. Agric. Food Chem. 2001, 49, 5445.

26. C. Vermeulen, S. Collin, J. Agric. Food Chem. 2002, 50, 5654.

27. C. Vermeulen, S. Collin, J. Agric. Food Chem. 2003, 51, 3618.

28. C. Vermeulen, C. Guyot-Declerck, S. Collin, J. Agric. Food Chem. 2003, 51, 3623.

29. W. Grosch, Flavour Fragr. J. 1994, 9, 147.

30. M. A. Igyor, A. C. Ogbonnab, G. H. Palmer, Process Biochem. 2001, 36, 1039.

31. Analytica EBC. Hops (7.4.1), Fachverlag Hans Carl: Nürnberg, 1998.

32. C. S. Burns, A. Heyerick, D. de Keukeleire, M. D. E. Forbes, Chem. Eur. J. 2001, 7, 4554.

33. D. Callemien, S. Dasnoy, S. Collin, J. Agric. Food Chem. 2006, 54, 1409.

34. G. Lermusieau, S. Collin, J. Am. Soc. Brew. Chem. 2003, 61, 109.

35. H. Goldstein, P. Ting, A. Navarro, D. Ryder. In Proceedings of the 27th European Brewery Convention; Cannes, France. IRL Press Ltd.: Oxford, England, 1999; 53–62.

36. J. Gros, S. Collin, Cerevisia 2012, 37, 10.

37. J. A. Maga, Crit. Rev. Food Sci. Nutr. 1976, 7, 147.

38. M. C. Meilgaard, MBAA Technol. Q. 1975, 12, 151.

39. T. Tominaga, C. Peyrot des Gachons, D. Dubourdieu, J. Agric. Food Chem. 1998, 46, 5215.

40. R. Schneider, F. Charrier, A. Razungles, R. Baumes, Anal. Chim. Acta 2006, 563, 58.

41. P. Schieberle, Z. Lebensm.-Unters. Forsch. A 1991, 193, 558.

42. I. Blank, A. Sen, W. Grosch, Z. Lebensm. Unters. Forsch. 1992, 195, 239.

43. W.Holscher,O. G. Vitzthum,H. Steinhart, J. Agric. Food Chem. 1992, 40, 655.

44. H. Tamura, A. Fujita, M. Steinhaus, E. Takahisa, H. Watanabe, P. Schieberle, J. Agric. Food Chem. 2011, 59, 10211.

Chapitre 7.

Production de la bière de sorgho «ikigage» en utilisant *Saccharomyces cerevisae, Issatckenkia orientalis* et *Lactobacillus fermentum comme* starters

Ce travail fera l'objet de la publication suivante :

Lyumugabe, F., Nsanzimana, D., Bajyana, E., Thonart, Ph. (2013). Production of Rwandese traditional sorghum beer « *ikigage* » using *Saccharomyces cerevisae, Issatckenkia orientalis* and *Lactobacillus fermentum* as starter cultures. A soumettre dans *Word Journal of Microbiology and Biotechnology*.

Préambule au chapitre 7

Au cours du second et troisième chapitre nous avons montré que *Saccharomyces cervisiae* et *Issatckenkia orientalis* sont des levures majoritaires impliquées dans la fermentation de la bière traditionnelle Rwandaise « ikigage ». Nous avons également montré que les bactéries lactiques étaient associées à cette fermentation ; *Lactobacillus fermentum* étant l'espèce majoritaire. Cependant, la bière *ikigage* est caractérisée également par la présence de *Staphylococcus aureus* et une importante quantité de microorganismes d'origine fécale. L'utilisation des starters sous forme de cultures pures, ou mixtes, a été proposée comme une meilleure méthode pour améliorer la qualité hygiénique, réduire les variations organoleptiques et l'instabilité microbiologique des aliments fermentés d'Afrique.

Dans ce contexte, on a envisagé d'utiliser *S. cervisiae* en combinaison avec *I. orientalis* et *L. fermentum* comme starters, afin de produire la bière *ikigage* ayant le statut de GRAS tout en conservant les caractéristiques organoleptiques semblables à celles de la bière locale. Les caractéristiques microbiologiques, physico-chimiques et organoleptiques des bières-pilote ont été comparées à celles de la bière locale produite par les paysans. Les bières-pilotes ont été brassées à base du malt de sorgho (70%) et d'eleusine (30%), en utilisant la méthode de brassage par décantation (Chapitre 4). Compte tenu de son impact sur le profil aromatique de la bière *ikigage* (chapitre 5 et 6), *Vernonia amygdalina* a été également utilisé durant le brassage des bières-pilotes.

Production of Rwandese traditional sorghum beer « *ikigage* » using *Saccharomyces cerevisae, Issatckenkia orientalis* and *Lactobacillus fermentum* as starter cultures

RUNNING TITLE HEADER: Production of sorghum beer *ikigage* using starters

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Abstract

This study was carried out to evaluate the potential of the use of predominant yeast strains (*Sacharomyces cerevisiae and Issatkenkia orientalis*) and Lactic acid bacteria (*Lactobacillus fermentum*) of Rwandese traditional sorghum beer "*ikigage* as starter cultures to improve *ikigage* beer. *S. cerevisiae* in co – culture with *I. orientalis* produced higher ethanol than *S. cerevisiae* alone or *I. orientalis* alone. *L. fermentum* has an influence on taste sour of *ikigage* beer and contributes also to generate ethyl acetate, ethyl lactate and higher alcohols such as 3-methylbutan-1-ol, 2-methylbutan-1-ol and 2-methylpropan-1-ol of this beer. *I. orientalis* contributed to the production of ethyl butyrate, ethyl caprylate, isobutyl butyrate and their corresponding acids, and to the generation of phenyl alcohols in *ikigage* beers. The association of *S. cerevisiae* with *I. orientalis* and *L. fermentum* produced *ikigage* beer with taste, aroma and mouth feel more similar to *ikigage* beers brewed locally by peasants. It is recommended to use *S. cerevisiae* in association with *I. orientalis* and *L. fermentum* as stater cultures to produce *ikigage* beer having the uniform organoleptic characteristics and a high ethanol content. This method also reduces the risk of contamination of the brew with food sanitary indicator and pathogenic microorganisms and will increase the chance of preservation of *ikigage* beer. However, complementary studies on *ikigage* beer conservation are needed.

Keywords: Sorghum beer; *Ikigage*; Starter cultures; *Saccharomyces cerevisiae; Issatckenkia orientalis; Lactobacillus fermentum.*

1. INTRODUCTION

Fermented foods make up an important contribution to the human diet in many countries because fermentation is an inexpensive technology which preserves food, improves its nutritional value and enhances its sensory properties (Murty and Kumar, 1995; Steinkraus, 1996). Fermentation also has the potential of enhancing food safety by controlling the growth and multiplication of a number of pathogens in foods. Its importance in modern-day life is underlined by the wide spectrum of fermented foods marketed both in developing and industrialized countries (Holzapfel, 2002).

In sub – Saharan African countries, traditional fermented beverages such as *ikigage* (Lyumugabe *et al*., 2010), *tchoukoutou* (Kayode *et al*., 2005), *dolo* (Dicko *et al*., 2006), *pito* (Ekundayo, 1969), *bili bili* (Maoura *et al*. 2005) and *merissa* (Dirar, 1978) are prepared from sorghum and/or millet malts. The fermentation of these beverages is uncontrolled and the microorganisms that intervene come from the raw materials, equipment and local environments or from a residue of previous fermentation batch. These microorganisms, by virtue of their metabolic activities, play an active role in physical, nutritional and organoleptic modification of starting material (Aidoo, 1994). However, the wide variety of microorganisms present in a spontaneously fermented food gives a product with widely varying quality.

The use of starter cultures was proposed like a suitable approach to improve the African traditional fermented food (Kirmaryo et *al.*, 2000; Holpzapfel, 2002). When the starter is adapted to the substrate, its use improves control of a fermentation process and predictability of its products (Holzapfel, 1997). In addition it facilitates the control over the initial phase of fermentation (Holzapfel, 2002). In the same way, hygienic quality and the acceptability of African traditional food could be improved with the use of adequate starter (Gran *et al*., 2003). The use of starter cultures also reduces the organoleptic variations and the

microbiological instability of African fermented food (Kirmaryo *et al*, 2002). The use of S. *cerevisiae* in combination with *Lactobacillus plantarum* as starter cultures was also used succefully for production of *"pito*" sorghum beer having testa and aroma similar to local *pito* beer (Orji *et al*., 2003). Recently, N'Guessan *et al*. (2010) used succefully *S. cerevisiae* and *Candida tropicalis* as starter cultures for the alcoholic fermentation of "*tchapalo"* sorghum beer.

Ikigage is a popular traditional fermented beverage in Rwanda and specially appreciated in various festivals and ceremonies (e.g., marriage, birth, baptism, dowery, etc). But *ikigage* beer is declining because of poor hygienic quality, organoleptic variations and unsatisfactory conservation (Lyumugabe *et al*., 2010). The fermentation of this beer is starting by traditional leaven "*umusemburo*", resulting of a previous spontaneous fermentation of sorghum wort. The dominant microorganisms involved in this fermentation have been identified as being yeasts (*Saccharomyces cerevisiae* and *Issatckenkia orientalis*) and lactic acid bacteria (*Lactobacillus fermentum*) (Lyumugabe *et al*., 2010). The aim of the present study was to investigate the use of *S. cerevisiae*, *I. orientalis* and *L. fermentum* as starter cultures to produce *ikigage* beer of acceptable and consistent quality. Microbiological, physico – chemical, volatile compounds and organoleptic characteristics of pilot *ikigage* beers were compared to commercial *ikigage* beers from peasants.

2. MATERIEL AND METHODS

2.1. Malting

The red sorghum grains (Kigufi variety) and *Eleusine coracana "uburo"* grains (Musama variety) were obtained from Rubona and Musanze stations of Rwanda Agriculture Board (RAB). The grains were sorted manually to remove broken kernels and debris and then used

for malting. The grains selected for malting (5 kg) were steeping in distilled water (10 L) at 25° C for 24h. Before and after steeping, grains were sterilized by immersion in sodium hypoclorite solution (1% wt/v). After rinsing with sterile distilled water as described elsewhere (Ezeogu *et al*., 1995), the grains were germinated at 30° C for 3 days and then kilned at 50° C for 24h. The shoots and rootlets were removed manually and the malt kernels were ground in a hammer mill to pass through a sieve of pore size 1.0 mm.

2.2. Wort production

The wort was produced by decantation mashing procedure developed for sorghum (Palmer, 1989; Igyor et al. 2001). 3,5 kg of milled malt (70% sorghum and 30% *Eleusine coracana*) were mixed with 12 L distilled water at 45° C and left in decantation during 30 min. Thereafter, 6 L of the clear "enzymatic supernatant" was removed while a mash residue was heated at 90°C for 30 min to gelatinize malt starch. After cooling below 50°C, the clear ''enzymatic supernatant'' was re-added and then the mixture was brewed according to the following mashing program: 1h at 63° C, 10 min at 75° C and cooled to 30° C. The filtration was very poor so the mash was centrifuged at 4000 x g for 5 min and then the filtrate was heated until boiling for 1h. The leaves of *Vernonia amygdalina* (2 g/L) were added 10 min before the end of boiling.

2.3. Yeast and lactic acid bacteria strains

Two yeast strains (*Saccharomyces cerevisiae* RV6 and *Issatckenkia orientalis* RG1*)* and lactic acid bacteria strain (*Lactobacillius fermentum* CWBI – B552) used as starter cultures in this work were belonged to the culture collection of Walloon Center of Industrial Biology (CWBI), Gembloux, Belgium. RV6 and RG1 are the selected yeasts isolated from Rwandese traditional leaven "*umusemburo*".

2.4. Preparation of Starter cultures

S. cerevisiae RV6 and *I. orientalis* RG1 strains were each sub - cultured on Yeast extract dextrose peptone (YEPD) agar at 30° C for 48h and then by successive sub – culturing on YEPD broth at 30 ° C for 24h and 18h, respectively. While *L. fermentum* CWBI – B552 strain culture was sub - cultured at 37ºC for 48 h on Man-Rogosa-Sharpe (MRS) agar followed by two successive rounds of sub-culturing in MRS broth with incubation at 37ºC for 24 hours and 16 hours, respectively. Yeast and LAB strains were each harvested by centrifugation at 4000 x g for 20 min and pellets were added in 50 ml of sterile sorghum worts and then incubated at 30ºC for 24h in order to initiate fermentation. The cell concentrations were checked using a Bürker counting cell.

2.5. Fermentation

Seven or ten liters of sterile wort were transferred into plastic fermenter (30 L, Brewferm, Belgium), equipped with airlock bubbler and tap, and pitched with starter cultures to obtain 10^6 cfu/ml followed by the incubation at 30° C for 3 days. In parallel, 2L sterile wort transferred into sterile Erlenmeyer flasks (5 L) were pitched with *L. fermentum* culture to obtain 10^7 cfu/ml, and then incubated at 30° C for 22h and again boiled (30 min) and cooled. Four fermentation systems were constituted as follows:

- 10L wort was inoculated with *S. cerevisaie* alone or *I. orientalis* alone;
- 10L wort was inoculated with *S. cerevisaie* (60%) in combination with *L. fermentum* (40%);
- 10L wort was inoculated with *S. cerevisaie* (60%) in combination with *I. orientalis* (40%);
- 7L wort was pitched with *S. cerevisaie* (60%) in combination with *I. orientalis* (40%), and the mash (3L) from lactic fermentation was added after 24h.

2.6. Enumeration of micro organisms

Duplicate aliquots of *ikigage* beer (10 ml) were diluted in 90ml sterile peptone physiological saline solution (5 g peptone, 8.5 g NaCl, and 1000 ml distilled water, pH = 7.0) and homogenised. Decimal dilutions were plated. Total counts of aerobic mesophilic flora, yeasts, Lactic acid bacteria (LAB), coliform, *Escherichia coli*, fecal streptococci and *Staphylococcus aureus* were enumerated as described by Lyumugabe *et al*. (2010).

2.7. Physicochemical analysis

The pH was measured using a pH meter 781 (Metrohm Herisau). Titratables acidity, expressed as a percentage lactic acid, was determined by titrating the samples with 0.1 N NaOH. Ethanol was determined by enzymatic method using the Boehringer Kit (R-Biopharm AG,D-64293 Darmastadt). The reducing sugars (glucose, maltose and maltotriose) were determined by High-Performance Liquid Chromatography (HPLC) on an Agilent 1100 series apparatus (Agilent Technologies, Massy, France) equipped with a refractometric detector. Sugars were separated on a C-610-H ion exchange column (300mm x 7,8 mm, supelco, Bellefonte, PA) preceded by a pre-column H (5cm x 4,6mm, supelco, Bellefonte, PA).

2.8. Determination of volatile compounds

The analysis of the volatile compounds of *ikigage* beers was performed with Headspace solid phase microextraction (HS -SPME) and an Agilent 7890 GC system equipped with a 5975 C. inert XL EI/ CI. Mass Selective Detector (Agilent Technologies, Santa Clara, CA, USA). The samples analyzed were extracted using a 50/30 µm divinylbenzene / carboxen / polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco, Inc., Bellefonte, PA). 10 ml of beer were enclosed in a 20mL SPME glass vial together with 2.5 g of sodium chloride and 5 µL of the internal standard 3-octanol (100 mg/L in absolute ethanol). The samples were equilibrated at 40° C for 10 min and then the fiber was exposed in the headspace of the vial

for 30 min and the extracted analytes from fiber were automatically desorbed in injection port of the GC – MS system at 250° C. A HP – 5 MS column (30 m x 0,25 mm I.D) with a film thickness of 0,25 μm was applied to extract the volatile compounds from the headspace of above-prepared glass vial. The GC was equipped with a split – splitless injector which was held at 250° C. After starting at 30° C, the oven temperature was raised in 3 steps after 2 min: 30 – 70 °C at 10° C/min followed by 1 min at 70° C; 70 – 220° C at 4° C/min and 220 – 280° C at 20°C/min and was finally held at 280° C for 6 min. During this program a constant flow rate (1.0 mL/min) of the carrier gas (Helium) was maintained. Mass spectra were obtained by electronic impact (E.I) scan mode (Low mass: 30.0; High mass: 500.0; Threshold: 150) and temperature source (230 ° C) were generated. The identification was achieved by comparing mass spectra obtained from the sample with those from the NIST and Pal1600k.L libraries Database and by comparing the kovats index estimated for each compounds on both chromatographic column with the values given in the literature (Pino *et al*., 2010; *www. pherobase. com/database/kovats*). Selective ion monitoring was used for the integrations of all chromatogram peaks and the semi-quantitative concentrations of volatiles in ikigage beers were calculated according to the method proposed by Zhao *et al*. (2009).

2.9. Sensory evaluation of *ikigage*

The *ikigage* samples including the local brew were evaluated by a panel of 7 members (biotechnology students of National university of Rwanda), who were familiar with Rwandese traditional sorghum *ikigage* beers. The order of presentation of samples was randomized and the panelists were asked to rate samples for taste, aroma and mouth feel compared to Rwandese traditional *ikigage* beer using a 9 points hedonic scale ranging from 1 (not different) and 9 (extremely different).

3. RESULTS AND DISCUSSION

3.1. Physico-chemical and microbiological characteristics of sorghum beer *ikigage*

The pH, total acidity (expressed as lactic acid), reducing sugars and ethanol content of pilot *ikigage* beers produced with starter cultures are given in **Table 1**. Pilot *ikigage* beers produced with *S. cerevisiae* in combination with *L. fermentum* or *S. cervisiae* in combination with *I. orientalis* and *L. fermentum* have pH and total acidity more similar to commercial *ikigage* beer from peasants than those produced without association of *L. fermentum*. Higher ethanol content and lower reducing sugars were observed in *ikigage* produced with *S. cerevisiae* in combination with *I. orientalis* following by *ikigage* produced with *S. cerevisiae* alone and ikigage obtained by the association of *S. cerevisiae* with *I. orientatilis* and *L. fermentum*. Except *I. orientalis*, all starter cultures used produced higher ethanol content than that observed in local *ikigage* beers from peasants. Contrary to *ikigage* beers from peasants, *S. aureus* and the food sanitary microorganisms were absents in pilot *ikigage* beers made with starter cultures.

The production of lactic acid by LAB can explain the lower and higher total acidity values observed in *ikigage* beers produced by peasants and those produced with association of *L. fermentum.* The similar total acidity level and low pH value were also observed by Orji *et al*. (2003) in Nigerian traditional sorghum *pito* beers produced by *Lactobacillus plantarum* in combination with *S. cerevisiae* and by *Pediococcus halophilus* in combination with *Candida tropicalis.* Light increase of ethanol content obtained with *S. cerevisiae* in combination with *I. orientalis* compared to *S. cerevisiae* alone shown that *I. orientalis* could have to play an influence, for example by facilitating the synthesis of α-glucosidase by the fast glucose consumption, on maltose and maltotriose fermentation by *S. cerevisiae*. Lower ethanol content obtained with *I. orientalis* alone could be explained by the no maltose fermentation by

this yeast strain. Similar studies on fermentation of grape wort (Kim *et al*., 2008) reported also that *I. orientalis* produced a low ethanol content compared to *S. cerevisae*, but when the co-fermentation of *S. cerevisaie* and *I. orientalis* were used, the ethanol production consumption reducing sugars consumption were similar to those obtained by *S. cerevisiae* alone.

Table 1. Physicochemical and microbiological characteristics of sorghum beers *ikigage*

	SC	SCLF	IO	SCIO	SCIOLF	IKp
pH	5,04	4,01	4,87	4,56	4,16	3,9
Titrable acidity (%)	0,86	1,12	0,81	0,89	1,07	1,37
Reducing sugars (g/l)	24,10	26,33	75,37	17,40	20,78	24,34
Ethanol (% v/v)	4,34	3,04	1,49	4,56	3,99	2,25
Yeast (cfu/ml)	$6{,}5.\ 10^7$	$12{,}4.\ 10^5$	$9{,}8.\ 10^6$	$32{,}4.10^7$	$67{,}2.\ 10^5$	$10{,}15.10^6$
LAB (Cfu/ml)	--	$2{,}4.\ 10^5$	--	--	--	$35{,}35.10^4$

SC: *S. cerevisiae* alone; **IO**: *I. orientalis* alone; **SCIO**: *S. cerevisiae* in combination with *I. orientalis*; **SCLF**: *S. cerevisiae* in combination with *L. fermentum*; **SCIOLF**: *S. cervisiae* in association with *I. orientalis* and *L. fermentum*; **Ikp**: traditional *ikigage* beer from local peasants.

3.2. Volatile compounds of sorghum beer *ikigage*

The results of GC-MS analyses of the volatile compounds obtained from the pilot *ikigage* beers produced with starter cultures and traditional sorghum beers *ikigage* made by peasants are outlined in **Table 2**. Traditional sorghum beer *ikigage* made by peasants differs from those produced in laboratory with starter cultures by relatively high concentrations of certain alcohols (propan-1-ol, 2-methylpropan-1-ol and 2,3-butanediol), esters (ethyl acetate, isobutyl acetate, propyl acetate, ethyl lactate and ethyl valerate), acids (acetic acid and heptanoic acid) and carbonyls (acetaldehyde). These compounds were also found in great quantity in the ikigage beers produced with *S. cerevisiae* in association with *L. fermentum* and *I. orientalis*.

High concentrations of 3-methylbutan-1-ol, 2-methylbutan-1-ol, ethyl acetate, acetic acid and ethyl lactate were also observed in the *ikigage* beers produced with *S. cerevisiae* in combination of *L. fermentum* without association of *I. orientalis.* However, *ikigage* beers produced with *I. orientalis* alone or in combination with *S. cerevisiae* were characterized by high concentration of ethyl butyrate, ethyl caprylate, isobutyl butyrate, ethyl nonanoate, ethyl dec-9-enoate, 3-methylbutyl decanoate, capric acid, butyric acid and 2-phenyl alcohols. These compounds were also found in great quantity in the *ikigage* beers produced with *S. cerevisiae* in association with *L. fermentum* and *I. orientalis*. High concentrations of 3-methylbutan-1-ol, 2-methylbutan-1-ol, ethyl acetate, acetic acid and ethyl lactate were also observed in the *ikigage* beers produced with *S. cerevisiae* in combination of *L. fermentum* without association *of I. orientalis.* However, *ikigage* beers produced with *I. orientalis* alone or in combination with *S. cerevisiae* were characterized by high concentration of ethyl butyrate, ethyl caprylate, isobutyl butyrate, ethyl nonanoate, ethyl dec-9-enoate, 3-methylbutyl decanoate, capric acid, butyric acid and 2-phenyl alcohols. The esters are responsible of the fruity-flowery aromas in fermented beverages (Brankir *et al*., 2008), and were identified in many fermented beverages (Vallejo-cordoba *et al*., 2004, Verstrepen *et al*., 2003; Brankir *et al.*, 2008). Ethyl acetate (and its corresponding acid) and ethyl lactate were also considered as some of the main bacterial volatile compounds (Damiani *et al*., 1996), and the high concentrations were observed in the *ikigage* beers produced by association of triple strains (*S. cerevisiae, I. orientalis* and *L. fermentum*). As Belgian Lambic and gueuze beers (Spaepen *et al.* 1978), ethyl acetate, ethyl lactate and ethyl caprylate can be the characteristic compounds of *ikigage* beer. The important amounts of ethyl butyrate (and its corresponding acid) in this work, manly when *I. orientalis* is used, were also observed in a Bavarian pilsner-type beer, where this odorant was suggested one of the key contributors to the overall aroma (Fritsch and Schieberle, 2005).

Table 2. Volatile compounds of sorghum beers *ikigage*

Compounds	RI	ID	Concentration in beers (µg/L calculated by internal standard equivalent)					
			IKp	SC	IO	SCIO	SCLF	SCIOLF
Esters								
Ethyl acetate	609	MS/RIL	749,3	286,8	38,6	151,5	338	512,7
Propyl acetate	712	MS/RIL	160	1,0	-	-	-	-
Ethyl isobutyrate	740	MS	-	-	69,2	18,2	-	7,5
Isobutyl acetate	773	MS/RIL	49,3	3,4	15,5	11,6	0,3	-
Ethyl butyrate	801	MS/RIL	9,8	1,6	468,7	153,4	-	2,0
Ethyl lactate	815	MS/RIL	205	-	-	-	82,2	31,5
Ethyl isovalerate	871	MS	38,9	-	-	-	-	-
Isoamyl acetate	877	MS/RIL	47,2	41,9	40,3	72,5	67,7	79,0
2-Methylbutyl acetate	879	MS/RIL	17,7	-	-	-	-	-
Ethyl valerate	900	MS	44,1	-	-	-	8,2	0,6
Ethyl caproate	998	MS/RIL	75,6	56,8	12,1	95,1	43,4	66,8
Hexyl acetate	1017	MS/RIL	1,8	1,7	0,1	1,9	1,7	1,3
Ethyl heptanoate	1096	MS/RIL	4,4	43,3	5,9	35,2	0,2	7,7
Ethyl benzoate	1169	MS/RIL	1,3	-	-	-	-	-
Methyl salicylate	1192	MS/RIL	11	69,5	40,8	69,2	60,5	47,8
Ethyl caprylate	1196	MS/RIL	201, 8	62,8	664,8	227	47, 6	88,8
Isobutyl caprylate	1386	MS	2,3	-	-	-	-	-
phenethyl acetate	1257	MS/RIL	4,5	2,6	-	0,6	0,6	1,0
Ethyl nonanoate	1294	MS/RIL	3,4	9,3	275	161,6	56,0	92,4
Ethyl dec-9-enoate	1382	MS/RIL	10,3	-	38,2	19,7	-	-

Table 2 continued

Compounds	**RI**	**ID**	**IKp**	**SC**	**IO**	**SC+IO**	**SC+LF**	**SC+IO+LF**
Ethyl caprate	1395	MS/RIL	34,1	35,7	9,4	42,0	5,5	22,0
3-Methylbutyl octanoate	1447	MS	4,8	-	-	-	-	-
Ethyl laurate	1597	MS/RIL	1,6	3,6	4,8	4,4	4,0	0,9
Isopropyl laurate	1631	MS	0,9	0,6	-	0,7	-	-
3-Methylbutyl decanoate	1648	MS	1,3	-	2,2	1,6	-	-
Ethyl merystate	1797	MS/RIL	2,5	1,7	2,8	4,7	1,0	1,3
Ethyl palmitate	> 1900	MS	1,0	1,7	5,4	2,4	1,3	1,1
Isopropyl palmitate	> 1900	MS	5,0	0,7	5,3	-	-	-
Alcohols								
Propan-1-ol	<600	MS	1041,6	890,7	275,1	810,3	385,1	443,3
2-Methylpropan-1-ol	<600	MS	194,3	170,4	53,3	90,2	156,9	77,4
3-Methylbutan-1-ol	704	MS/RIL	603,8	1042	772,6	948,7	1233,1	1055,3
2-methylbutan-1-ol	709	MS/RIL	22,5	181,6	155,3	182,9	356,7	113,0
2,3- butanediol	804	MS/RIL	56,2	38,1	-	30,4	-	45,9
Hexano-1-l	871	MS/RIL	18,8	24,0	0,3	14,7	17,2	21,3
Heptan-1-ol	971	MS/RIL	65,1	72,0	9,4	21,3	12,2	61,2
Octan-1-ol	1072	MS/RIL	4,3	21,7	16,4	33,3	0,4	3,0
2-Phenethyl alcohol	1115	MS/RIL	120	21,6	84,5	62,0	21,9	32,3
Nonan-1-ol	1172	MS/RIL	-	11,0	16,8	42,5	14,4	9,9
Nonan-2-ol	1099	MS/RIL	-	54,1	-	18,0	-	7,1
Decan-1-ol	1273	MS/RIL	-	17,5	0,8	5,2	-	6,2

Table 2 continued

Compounds	**RI**	**ID**	**IKp**	**SC**	**IO**	**SCIO**	**SCLF**	**SCIOLF**
Acids								
Acetic acid	700	MS/RIL	2333,7	633,2	82,1	433,1	941,1	1015,2
butyric acid	775	MS/RIL	6,1	-	14,0	9,8	-	1,0
Propionic acid	772	MS	12,4	-	-	-	-	4,4
Heptanoic acid	1064	MS/RIL	63,1	-	26,2	28,0	-	13,5
Caprylic acid	1186	MS/RIL	11,2	0,5	8,2	6,6	8,1	16,9
Capric acid	1380	MS/RIL	3,7	-	65,1	49,0	12,3	12,9
Carbonyl								
Acetaldehyde	<600	MS	76,2	21,3	36,3	6,7	18,8	66,3
2-Butanone	<600	MS	-	88,7	-	-	-	-
3-Methylbutanal	<600	MS	34,4	39,1	43,2	31,5	23,1	8,3
2-methylbutanal	<600	MS	11,3	20,1	18,3	12,5	11,4	0,4
1-Hexanal	797	MS/RIL	4,2	7,5	0,6	5,2	0,2	6,0
Phenylacetaldehyde	1048	MS/RIL	0,5	-	-	-	-	-
2- Nonanone	1090	MS/RIL	-	22,2	-	-	-	-
Nonanal	1101	MS/RIL	-	9,6	10,5	3,3	8,0	0,3
2-Decanone	1203	MS	-	1,22	-	-	-	-

*****Identified by mass spectra (**MS**) and by comparison of retention index (**RI**) calculated and retention index from literature (RIL). **SC:** *S. cerevisiae* alone; **IO**: *I. orientalis* alone; **SCIO**: *S. cerevisiae* in combination with *I. orientalis*; **SCLF**: *S. cerevisiae* in combination with *L. fermentum*; **SCIOLF**: *S. cervisaie* in association with *I. orientalis* and *L. fermentum*; Ikp: traditional *ikigage* beer from local brewer.

The alcoholic compounds also contribute to the strong and pungent smell and taste of beer, and their formation is linked to yeast protein synthesis (Gilmare *et al.*, 2008). The high concentration of 3-methylbutan-1-ol, 2-methylbutan-1-ol and 2-methylpropan-1-ol, observed in *ikigage* beers from peasants and those produced when *L. fermentum* was associated, were also found in fermented dough with mixed culture of *L. fermentum* and *S. cerevisiae* (Annan *et al*., 2003). This observation can be explained by the amino acids released by the proteolytic activity of lactic acid bacteria and their use by *S. cerevisiae* (Spicher and Nierle, 1988). Phenethyl alcohol, known to have intense odour of roses and a burning taste, is produced by enzymatic conversion of phenylalanine by yeast cells, particularly by *S. cerevisiae* (Stam *et al*, 1998), but in this wort, *I. orientalis* seems to produce high concentration of this compound more than *S. cerevisiae.*

3.3. Sensory evaluation of ikigage

The results of sensory evaluation of *ikigage* beers produced with starter cultures are indicated in **Figue 1**. These results shown that *ikigage* beers produced with *S. cerevisiae* in association with *I. orientalis* and *L. fermentum*, following those produced with *S. cerevisia* in co-cultures with *L. fermutum,* have the taste, aroma and mouth feel much more similar to *ikigage* beers brewed locally by peasants. *S. cervisiae* in co-cultures with *I. orientalis* produced ikigage beers with aroma also more comparable to local ikigage beers than *S. cerevisiae* or *I. orientalis* alone. The lack sour taste in the *ikigage* produced without *L. fermentum* may explain their unacceptability by the consumer panelists. The *ikigage* beers are characterized sour taste due to acidity produced during fermentation, mainly by *L. fermentum*.

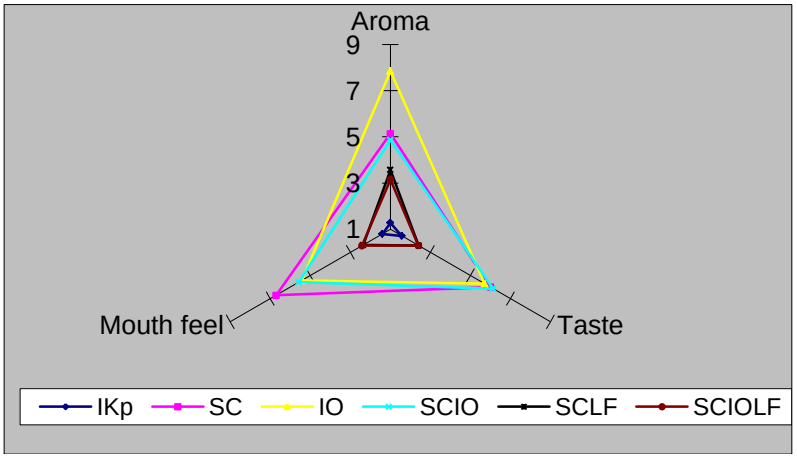

Figure 1. Sensory evaluation of sorghum beers *ikigage*

SC: *S. cerevisiae* alone; **IO**: *I. orientalis* alone; **SCIO**: *S. cerevisiae* in combination with *I. orientalis*; **SCLF***: S. cerevisiae* in combination with *L. fermentum*; **SCIOLF**: *S. cervisiae* in association with *I. orientalis* and L. fermentum; **Ikp**: traditional *ikigage* beer from local brewer. 1: not different; 9: extremely different.

However, the acidity produced by *I. orientalis* may have contributed also to the taste and mouth feel of *ikigage* beers. By comparing the aroma scores with the volatile compounds observed in this work, it evidence that esters and alcohols have contributed to the similarity between aroma of *ikigage* beers produced with the mixture cultures and those produced locally by peasants. Other studies reported also that the sour taste of Nigerian sorghum *pito* beers (Ekundayo, 1969, Orji *et al*., 2003) and *kaffir* sorghum beer of South Africa (Novellie, 1962) was due to the lactic acid produced by the lactic acid bacteria during the fermentation

4. CONCLUSION

The present study has provided information on the use of *S. cerevisiae, I. orientalis* and *L. fermentum* as starter cultures for the production of Rwandese traditional sorghum beers "*ikigage*". *S. cerevisiae* in co – culture with *I. orientalis* produced higher ethanol than *S. cerevisiae* alone or *I. orientalis* alone. *L. fermentum* has an influence on sour taste of *ikigage* beer and contributes also to generate ethyl acetate, ethyl lactate and higher alcohols such as 3-methylbutan-1-ol, 2-methylbutan-1-ol and 2-methylpropan-1-ol of this beer. *I. orientalis*

contributed to the production of ethyl butyrate, ethyl caprylate, isobutyl butyrate and their corresponding acids, and to generation of phenyl alcohols in ikigage beers. The association of *S. cerevisiae* with *I. orientalis* and *L. fermentum* produced *ikigage* beer with taste, aroma and mouth feel much more similar to *ikigage* beers brewed locally by peasants. It is recommended to use *S. cerevisiae* in association with *I. orientalis* and *L. fermentum* as starter cultures to produce *ikigage* beer having the uniform organoleptic characteristics and a high ethanol content. This method also reduces the risk of contamination of the brew with food sanitary indicator and pathogenic microorganisms and will increase the chance of the conservation of ikigage beer. However, complementary studies on *ikigage* beer conservation are needed.

References

Aido KE., 1994. Application of biotechnology to indigenous fermented foods. *Proc. Tehnol. Dev. Countries, 12, 83 – 94.*

Alves R.F., Fascimento A. & Nogueira J. 2005. Characterization of the aroma profile of Madeira wine by sorptive extraction techniques. *Analytica Chem. Acta, 546, 11 – 12.*

Annan N.T., Poll L. & Sefa –Dedeh S., 2003. Influence of starter culture combinations of *Lactobacillus fermentum, Saccharomyces cerevisiae* and *Candida krusei* on aroma in Ghanaian maize dough fermentation. *Eur. Food. Res. Technol., 216, 3377 – 384.*

Branyik T, Vicente AA, Dostalek P. & Teixeira JA., 2008. A review of flavour formation in continuous beer fermentations. *J.Inst Brew., 114, 3-13.*

Damiani P., Gobbetti M., Cossignani L., Corsetti A., Simonetti M. S. & Rossi, J., 1996. The sourdough microflora. Characterization of hetero- and homofermentative lactic acid bacteria, yeasts and their interactions on the basis of the volatile compounds produced. *Lebensm. Wiss. u. Technol. 29, 63–70.*

Dicko M.H., Gruppen H., Traoré1 A. S., Voragen A.G.J. & Van Berkel W.J.H., 2006. Sorghum grain as human food in Africa: relevance of content of starch and amylase activities. *African Journal of Biotechnology, 5, 384-395.*

Dirar H.A., 1978. A microbiological study of Sudanese Merissa brewing. *J. Food Sci.,43, 163-168.*

Ekundayo JA., 1969. The production of pito, a Nigerian fermented beverage. *J. Food Technol., 4, 217 -225.*

Ezeogu, L.I. & Okolo, B.N., 1995. Effects of air rest periods on malting sorghum response to final warm water steep. *J. Inst. Brew., 101, 39 - 45.*

Fritsch H.T. & Schieberle P., 2005. Identification based on quantitative measurements and aroma recombination of the character impact odorants in a Bavarian Pilsner – type beer. *J. Agric. Foods chem., 53, 7544 – 7551.*

Gilmare A.S., Fabio A. and Ronei J. P., 2008. Exploratory analysis of the volatile profile of beers by HS- SPME- GC. *Food chem., 111, 1057 - 1063.*

Gran H.M., Gadaga H.T & Narbhus J.A., 2003. Utilization of various starter cultures in the product of Amasi, a Zimbabwean naturally fermented raw milk product. *Int. J. Food Microbiol., 88, 19-28.*

Holzapfel WH., 2002. Appropriate starter culture technologies for small– scale fermentation in developing countries. *Int. J. Food Microbiol, 75,197-212.*

Holzapfel W., 1997. Use of starter cultures in fermentation on a household scale. *Food Control, 8, 241-258.*

Kim D., Hong Y. & Park H., 2008. Co-fermentation of grape must by Issatchenkia orientalis and Saccharomyces cerevisiae reduces the malic acid content in wine. *Biotech. Lett., 30, 633–1638.*

Kirmaryo V. M., Massawe G.A., Olasupo N.A. and Hololzpfel W.H., 2002. The use of a starter culture in the fermentation of cassava for the production of '' Kivunde'', a traditional Tanzanian food product. *Int. J. Food Microbiol., 56, 179-190.*

Igyor M.A., Ogbonna A.C. & Palmer G.H., 2001. Effect of malting temperature and mashing methods on sorghum wort composition and beer flavour. *Process Biochemistry, 36, 1039–1044.*

Lyumugabe L., Kamaliza G., Bajyana E. & Thonart PH., 2010. Microbiological and physico-chemical characteristic of Rwandese traditional beer "*Ikigage*". *Afr. J. Biotechnol., 9, 4241-4246.*

Maoura N., Mbaiguinam M., Nguyen HV., Gaillardin C. & Pourquie J., 2005. Identification and typing of the yeast strains isolated from bili bili, a traditional sorghum beer of chad. *Afr. J. Biotech., 4, 646-656.*

Murty D.S. and K.A. Kumar., 1995. Traditional uses of sorghum and millets. In sorghum and millets: Chemistry and Technology, (D.A.V. Dendy, ed), American Association of Cereal Chemists, St. Paul, MN, USA. 185 - 221.

N'guessan F.K, N'diri D.Y, F. Camara & Djè M.k., 2010. *Saccharomyces cerevisiae* and *Candida tropicalis* as starter cultures for the alcoholic fermentation of tchapalo, a traditional sorghum beer. *World J. Microbiol. Biotech, 26, 693 – 699.*

Novellie L., 1962. Kaffircorn malting and brewing studies XI. Effect of malting conditions on the diastatic power of kaffircorn malts. *J. Sci. Food Agri., 13, 115 - 120.*

Orji M.U, Mbata T.I, Anich G.N & Ahonkhai I., 2003. The use of starter cultures to produce "pito", a Nigerian fermented alcoholic beverage. *World J. Microbiol. Biotechnol, 19, 733 – 736.*

Palmer G. H., 1989. Cereals in malting and brewing. In *Cereal Science and Technology,* ed. Palmer, G. H. pp. 61-242. Aberdeen: Aberdeen University Press.

Pino J.A., Marquez E., Quijano C. E. & Castro D., 2010. Volatile compounds in noni (*Morinda citrifolia* L.) at two ripening stages**.** *Ciênc. Tecnol. Aliment., Campinas, 3, 183-187.*

Spaepen M., Vanoevelen D. & Verachtert H., 1978. Fatty – Acids and Esters produced during spontaneous fermentation of Lambic and Gueuze. *J. Inst. Brew., 84, 278 – 282.*

Spicher, G., Nierle, W., 1988. Proteolytic activity of sourdough bacteria. *Appl. Microbiol. Biotech., 28, 487– 492.*

Stam H., Hoogland M., & Laane C., 1998. Food flavours from yeast. In B. J. B. Wood, Microbiology of fermented foods (2nd ed.). London, UK: Blackie Academic & Professional

Steinkraus, K.H., 1996. Handbook of Indigenous Fermented Foods, 2nd ed, Marcel Dekker, New York.

Vallejo - Cordoba B., Gonzalez - Cordova A.F. & Estrada – Montoya M., 2004. Tequila volatile characterization and ethyl ester determination by solid phase microextraction gas chromatography/ mass spectrometry analysis. *J. Agric.Foods Chem., 52, 5567 – 5571.*

Verstrepen K.J., Derbeilinckx GY., Dufour JP., Winderickx J., Thervelein J.M., ISAK S. Pretorius I.S. and Delvaux F.R., 2003. Flavor active esters: Adding fruitiness to beer. *J. Biosc. Bioeng., 96, 110 – 118.*

Zhao, Y.P., Xu, Y., Li, J.M., Fan, W.L. & Jiang, W.G., 2009. Profile of volatile compounds in 11 brandies by headspace solid-phase microextraction followed by gas chromatography-mass spectrometry. *J. Food Sci. 74, 90–99.*

Chapitre 8 :

Discussion générale, conclusions et perspectives

DISCUSSION GENERALE

La faim et la malnutrition demeurent un grand fléau et touchent près de huit cent millions de personnes dans les pays en développement (FAO, 1997). Dans ces pays, la valorisation des produits locaux grâce à l'utilisation de techniques moins couteuses devrait aider à lutter contre l'insécurité alimentaire. La fermentation est une méthode utilisée par l'homme depuis des millénaires pour rendre des aliments plus nutritifs et plus digestes (Holzapfel, 2002).

Au Rwanda, le sorgho est transformé en une bière locale appelée *ikigage* après une étape de fermentation. Cette bière est considérée à la fois comme un aliment et une boisson par la majorité des Rwandais, surtout vivant dans les campagnes. Cependant, la mauvaise qualité hygiénique et les variations organoleptiques rendent *ikigage* moins attrayant que les bières occidentales du type pils fabriquées au Rwanda à base du malt d'orge importé d'Europe. Mais en raison du faible pouvoir d'achat des consommateurs, la bière de sorgho reste très consommée par la population rurale malgré des nombreux problèmes hygiéniques liés à sa consommation.

Ce travail a porté sur la caractérisation de la bière traditionnelle Rwandaise « ikigage » en vue d'améliorer sa qualité hygiénique et de réduire les variations organoleptiques en utilisant les matières premières locales. Il est composé de sept chapitres reprenant chacun un axe important des recherches menées dans le cadre de ce travail :

- Revue bibliographique sur les bières africaines fabriquées à base de sorgho (Chapitre 1) ;
- Caractéristiques microbiologiques et physico-chimiques de la bière traditionnelle Rwandaise « ikigage » (Chapitre 2) ;
- Sélection et caractérisation des souches de levures performantes impliquées dans la fermentation de la bière traditionnelle « ikigage » du Rwanda (Chapitre 3);

- Amélioration du brassage de la bière de sorgho « ikigage » en utilisant *Eleusine coracana* « uburo » pour augmenter la saccharification (Chapitre 4);

- Composés volatils de la bière traditionnelle « *ikigage* » brassée avec *Vernonia amygdalina* « umubirizi » (Chapitre 5)

- Occurrence des thiols polyfonctionnels dans la bière de sorgho « ikigage » brassée avec *Vernonia amygdalina* « umuburizi » (Chapitre 6) ;

- Production de la bière de sorgho « ikigage » en utilisant *Sacharomyces cerevisiae* en combinaison avec *Issatckenkia oriatilis* et *Lactobacillus fermentum* comme starters (Chapitre 7).

Caractéristiques microbiologiques et physico-chimiques de la bière traditionnelle du Rwanda « ikigage »

Une enquête auprès de 32 brasseuses traditionnelles et un suivi technique du processus de fabrications nous ont permis d'établir le diagramme de production de la bière traditionnelle du Rwanda « ikigage ». Le procédé de maltage, brassage et de fermentation de la bière *ikigage* reste toujours encore empirique. La fermentation est initiée par un levain traditionnel appelé *umusemburo* ; résultat d'une fermentation spontanée du moût de sorgho préparée avec les feuilles de *Vernonia amygdalina*.

Les méthodes de fabrication d'*umusemburo* sont très diversifiées au Rwanda et dépendent des ingrédients incorporés durant la préparation, mais la méthode incorporant *V. amygdalina* est très repandue au Rwanda. D'autres études ont rapporté que *V. amygdalina* peut substituer le houblon dans les pays tropicaux en raison de ses propriétés antimicrobiennes et de son amertume (Ajebesone *et al*., 2004; Oboh *et al*., 2009; Mboto *et al*., 2009 ; Adenuga *et al*., 2010). Cependant, l'impact de *V. amygdalina* sur le profil aromatique des bières de sorgho

n'avait jamais été étudié auparavant. Cette étude a été abordée dans le cadre de ce présent travail (chapitres 5 et 6) et nous y reviendrons encore plus loin.

L'analyse microbiologique et physico-chimique des 40 échantillons de la bière traditionnelle « *ikigage* » commercialisée au Rwanda a montré d'une part que la bière *ikigage* est caractérisée par la présence de levures, de bactéries lactiques, de moisissures et d'une importante quantité des microorganismes d'origine fécale (*E. coli* et Streptocoques fécaux) et de toxi-nfection alimentaire (*Staphylococcus aureus*), et d'autre part, par une teneur en éthanol faible, une acidité totale élevée et une importante quantité des protéines.

Les levures suivies par les bactéries lactiques sont des microorganismes prédominants dans la bière traditionnelle *ikigage* du Rwanda. Septante souches de levures identifiées appartiennent aux espèces *S. cerevisiae* (55.7%), *Candida inconspicua* (22,8 %), *I. orientalis* (10 %), *Candida humilis* (7,1 %) et *Candida magnolia* (4,8 %). Dix souches de bactéries lactiques identifiées appartiennent exclusivement au genre *Lactobacillus* (*L. fermentum*, *L. buchneri, Lactobacillus* sp). *C. incospicua* est une espèce dont la présence est souvent associée au manquement d'hygiène corporelle et peut se révéler pathogène (Maxwell *et al*., 2003). *S. cerevisiae* et les lactobacilles ont été isolés également dans d'autres bières africaines à base de sorgho (Novellie, 1982 ; Odunfa and Adeyele, 1985; Naumova *et al*., 2003 ; Maoura *et al*., 2005 ; Kayode *et al*. 2007), mais *I. orientalis* n'avait pas été isolé auparavant dans ces bières de sorgho.

Au cours de la production de la bière de sorgho, le moût subit une fermentation lactique avant la fermentation alcoolique (Kazanas and Fields, 1981, Muyanja *et al*., 2003). L'acide produit au cours de cette fermentation lactique abaisse le pH; ce qui peut expliquer la disparition de *S. aureus*, coliformes totaux, *E. coli* et Streptocoques fécaux à la fin de la fermentation. Leur présence dans les bières *ikigage* commercialisées peut être attribuée à une contamination post-

fermentation (ustensiles, eau, environnement de travail, etc.); ce qui prouve que ces germes peuvent survivre ou se développer dans la bière de sorgho lorsqu'elle est contaminée après la fermentation. Cependant, cet environnement acide est favorable au développement des levures qui transforment les sucres fermentescibles en alcool et en dioxyde de carbone durant la fermentation. L'éthanol produit contribue à empêcher le développement de bactéries indésirables ou pathogènes dans la bière (Moll, 1991). Malheureusement, la teneur en éthanol dans les bières de sorgho est très faible en raison de la faible quantité des sucres fermentescibles dans le moût; due principalement à la faible activité des β-amylases dans le malt de sorgho (Novellie, 1982, Palmer, 1989, Doufor *et al*., 1992). Dans ce contexte, la sélection des souches de levures performantes et l'amélioration de la qualité du moût s'avèrent nécessaire pour augmenter la teneur en éthanol dans la bière de sorgho.

Sélection et caractérisation des souches de levures performantes impliquées dans la fermentation de la bière traditionnelle « ikigage » du Rwanda

Au cours du chapitre 2, nous avons montré la prédominance des levures dans la bière traditionnelle du Rwanda « ikigage ». Les levures jouent un grand rôle dans la fermentation alcoolique où elles produisent de l'éthanol avec peu de sous-produits. La sélection des souches de levures performantes permet d'améliorer le rendement de la production et de la productivité de l'éthanol. L'utilisation des souches locales sélectionnées est beaucoup plus efficace puisque ces levures sont plus concurrentielles car elles acclimatées aux conditions environnementales locales. Par ailleurs, la sélection des levures locales assure la maintenance des propriétés sensorielles caractéristiques du produit fermenté de chaque région (Degré, 1993). Compte tenu de cette dominance et du rôle majeur des levures dans la fermentation alcoolique, nous avons procédé à la sélection des souches de levures performantes impliquées dans la fermentation de la bière *ikigage* du Rwanda.

Au total 127 souches de levures ont été isolées aux différents stades de la fermentation du moût de sorgho inoculé par le levain traditionnel « umusemburo ». 23 souches de levures majoritaires isolées à la fin de la fermentation ont été pré-sélectionnées. Parmi ces souches, 7 souches appartenant aux *S. cervisiae* (codées RB2, RK1, RV6, RN4, RT8, RL1) et *I. orientalis* (codées RG1 et RG2) ont été sélectionnées sur base de leur pouvoir acidifiant élevé. L'acidification est étroitement liée à la viabilité et à la performance fermentaire des souches de levures (White *et al*., 2003; Gabriel *et al*., 2008). Elle est un critère préliminaire de prédilection du pouvoir fermentaire et par conséquent de criblage des souches de levures brassicoles (Mathieu *et al*., 1991).

Ces souches ont été soumises au test de tolérance à l'éthanol (5%, 10% et 15%) à 30° C après 2 jours d'incubation. Toutes les souches testées résistent bien jusqu'à la concentration de 10% d'éthanol, mais les souches appartenant à l'espèce *I. orientalis* ont toléré jusqu'à la concentration de 15% d'éthanol. Cependant, les concentrations employées sont supérieures à celles observées dans les bières de sorgho commercialisées au Rwanda (moins de 3% d'éthanol). Bien qu'il n'existe pas une corrélation directe entre la tolérance en éthanol et la capacité de la production d'éthanol (Benitez *et al*., 1983), ces informations sont importantes pour la sélection des souches de levures qui peuvent être utilisées pour l'optimisation de la production de la bière *ikigage* avec une teneur en éthanol élevée. Cependant, l'augmentation de la teneur en éthanol devrait passer par l'augmentation de sucres fermentescibles dans le moût de sorgho.

Des fermentations contrôlées du moût de sorgho avec les souches de levures sélectionnées précédemment afin d'évaluer leur capacité de production d'éthanol dans un milieu naturel. Comme la teneur en sucres fermentescibles dans le moût de sorgho est faible, le jus de banane a été ajouté comme adjuvant pour apporter les hydrates de carbone directement assimilables

par les levures. Des hautes teneurs en éthanol (5,45-6,7% v/v) ont été obtenues dans les bières produites avec les souches de *S. cerevisiae* RV6, RB2 et RK1. La faible teneur en éthanol (2, 38 % v/v) observée dans la bière produite avec *I. orientalis* RG1 s'explique par le fait que cette espèce de levure ne fermente pas le maltose. Cependant, l'importance de la levure de bière ne se limite pas seulement à la production de l'éthanol. *I. orientalis* pourrait contribuer à la formation des arômes caractéristiques de la bière *ikigage* (Chapitre 7). L'étude des caractéristiques aromatiques n'a pas été effectuée dans cette partie du travail parce que les fragrances caractéristiques de banane dominent dans les bières de sorgho produites avec le jus de banane (Dahouenon *et al*., 2012); moins préférées par les consommateurs Rwandais qui veulent conserver la différence entre la bière de sorgho « ikigage » et le vin de banane «urwagwa», une boisson traditionnelle Rwandaise produite à partir d'une fermentation spontanée de jus de banane mélangé avec le malt de sorgho. Dans ce contexte, le mélange de sorgho avec une autre céréale locale a été envisagé au chapitre 4 pour augmenter la teneur en sucres fermentescibles dans le moût de sorgho.

La floculation est une propriété très importante des sélections des levures dans les industries brassicoles. Si la levure flocule très prématurément, elle ne transforme pas suffisamment les sucres en éthanol et conduit donc à un rendement très faible. Si elle flocule trop tardivement, la bière sera très difficile à clarifier. D'après la classification de Gilliland (1951), la souche RK1 est poudreuse (flocule trop tardivement) tandis que les souches RB2 et RV6 floculent faiblement et la souche RG1 est d'une floculation moyenne. D'après la classification de Stratford (1989) et de Masy *et al*. (1992), les souches RB2, RV6 et RK1 sont des phénotypes Newflo1 ou GMS c'est -à- dire que la floculation est inhibée par le glucose et le mannose. A l'opposé, la souche RG1 n'est pas inhibée par le mannose, elle est classée dans le phénotype MI. Généralement, les souches brassicoles ont une floculation moyenne ou faible (Gilland, 1951) et du phénotype newflo (Masy *et al*., 1992). Les souches du phénotype MI sont très

prédominants dans les cidreries (Suarez *et al*., 2008), mais elles peuvent être employées dans la production des bières si elles ne sont pas poudreuses ou hyper-floculentes. Bien que la souche RK1 soit poudreuse toutes ces souches peuvent être utilisées dans la production des bières traditionnelles Africaines à base de sorgho, appelée bières opaques en raison de leur turbidité.

Amélioration du brassage de la bière de sorgho « ikigage » en utilisant *Eleusine coracana* « uburo » pour augmenter la saccharification

Au cours des précédents chapitres, nous avons souligné à plusieurs reprises que le problème d'insuffisance des sucres fermentescibles dans le moût de sorgho est une entrave majeure pour augmenter la teneur en éthanol dans la bière de sorgho. Plusieurs travaux ont rapporté que ce problème est dû à la faible activité de β-amylase dans le malt de sorgho (Palmer, 1989; Dufour *et al*., 1992). Les solutions proposées (association de sorgho avec le mat d'orge ou l'utilisation des enzymes exogènes commerciales) dans la littérature ne sont pas adaptées au contexte traditionnel du Rwanda, en raison du coût élevé de l'orge et des enzymes commerciales. C'est dans ce contexte que nous avons étudié la possibilité de mélanger le malt de sorgho avec une céréale locale appelée *uburo* (*Eleusine coracana*) pour augmenter la saccharification durant le brassage de la bière de sorgho.

Nous avons comparé les propriétés brassicoles des malts de trois variétés d'*uburo* (Musama, N161 et Mwamba) avec une variété locale de sorgho (Kigufi) très utilisée dans la production de la bière *ikigage* au Rwanda. L'activité de β-amylase dans le malt de la variété Musama est 4 fois supérieure à celle du malt de sorgho (73,3 U/g); c'est pour cette raison que la variété Musama a été choisie dans la présente étude. Par contre, l'activité de α-amylase est plus élevée dans le malt de sorgho par rapport aux malts d'*uburo*. Sur la base des activités amylasiques, le mélange de sorgho et *uburo* (Musama) parait idéal pour améliorer la

saccharification, mais la méthode de brassage utilisée reste un paramètre très important dans l'hydrolyse de l'amidon en sucres fermentescibles. C'est dans ce cadre qu'ont été comparées la méthode de brassage par infusion (65° C), qui est utilisée au Rwanda (**Figure 2**, chapitre 1), et la méthode de brassage par décantation, qui est largement utilisée en Afrique de l'ouest. Cette dernière méthode comprend l'étape de la gélatinisation de l'amidon après décantation et puis celle de la saccharification après addition du surnageant contenant des enzymes hydrolytiques (**Figure 2**, chapitre 6).

Les résultats de notre étude montrent que quelque soit la méthode utilisée, le moût issu du mélange de malt de sorgho (70%) avec le malt d'*uburo* (30%) contient plus de sucres fermentescibles, en particulier le maltose, que le moût produit avec 100% malt de sorgho. Toutefois, la méthode de brassage par décantation produit beaucoup plus de sucres fermentescibles que la méthode de brassage par infusion. Ces résultats s'expliquent d'une part par l'important apport de β-amylase provenant du malt d'*uburo*, et d'autre part par la température (90° C) de gélatinisation utilisée durant le brassage par décantation. Igyor *et al*. (2001) ont indiqué que les températures de 80 à 100° C sont adéquates à la gélatinisation de l'amidon du sorgho. Ces températures augmentent la désactivation thermique des amylases (Guerra *et al*., 2009), d'où la problématique de l'utilisation simultanée de la gélatinisation et saccharification dans le brassage de sorgho sans addition des enzymes exogènes. L'action des enzymes hydrolytiques est limitée si l'amidon n'est pas gélatinisé; ce qui explique la faible quantité de sucres fermentescibles dans le moût obtenu par la méthode d'infusion. La méthode de décantation semble être bien indiquée pour le brassage de la bière de sorgho dans le contexte Africain. Cependant, la concentration en azote aminé libre (FAN) dans le moût obtenu par cette méthode est légèrement inférieure à celle du moût obtenu par la méthode d'infusion, mais reste suffisante dans la mesure où la teneur en éthanol est au moins deux fois supérieure à celle de la bière locale.

Composés volatils de la bière traditionnelle de sorgho « ikigage » brassée avec *Vernonia amygdalina* « umubirizi »

Nous avons indiqué au cours du premier et second chapitre que *V. amygalina* est utilisé au cours de la fabrication de la bière traditionnelle Rwandaise « *ikigage* » à base de sorgho. La littérature indique que l'amertume et les propriétés antibactériennes de cette plante sont semblables à celles du houblon (Ajebesone *et al*., 2004; Mboto *et al*., 2009 ; Adenuga *et al*., 2010), mais l'impact de *V. amygdalina* sur le profil aromatique de la bière de sorgho n'a jamais été étudié auparavant. Dans ce contexte, nous avons comparé la bière traditionnelle « *ikigage* » des paysans avec les bières pilotes fabriquées avec ou sans *V. amygdalina* à la place du houblon afin d'explorer l'effet de *V. amygdalina* sur le profil aromatique de la bière de sorgho « *ikigage* ». Les bières pilotes ont été brassées par la méthode de décantation en utilisant les malts de sorgho (70%) et d'eleusine (30%) comme matières premières et les souches de levures sélectionnées (RV6 et RB2) comme starters.

Un total des 75 composés volatils appartenant au groupe des esters (32), des alcools (12), des carbonyles (9), des acides organiques (7), des terpènes (7), soufrés (3), phénols (3) et des furanes ont été identifiés dans la bière *ikigage* provenant des paysans et les bières pilotes fabriquées avec ou addition of *V. amygdalina* durant l'ébullion.

Cette étude a révélé la présence de méthyle salicylate, de nombreux terpènes (δ-3-carene, β-farnesene, farnesol, β-citronellol, Linalol et 1,8-cineole) et de béta-damascenone dans les bières contenant *V. amygadalina*. Des quantités importantes de Méthyle salicylate ne sont pas souvent asscociées à la bière houblonnée. Cependant, le houblon contient les glycosides qui peuvent être hydrolysés et libérer le méthyle salicylate au cours du brassage et de la fermentation (Kollmannsberg *et al*., 2006, Daenen, 2008). Des concentrations élevées de méthyle salicylate dans la bière *ikigage* peuvent être dues à la quantité importante de

glycosides dans les feuilles de *V. amygadilina* et/ou à l'activité glycosidasique des souches de levures isolées de l'*umusemburo*. Des terpènes identifiés dans la bière *ikigage* sont généralement considérés comme des arômes dérivés du houblon (King *et al*., 2003; Takoi et al., 2010; Nance *et al*., 2001). La présence de ces arômes terpéniques dans les bières *ikigage* non houblonnées peut dériver des feuilles de *V. amygdalina*. Linalool, 1,8-cineole, β-farasene et geraniol ont été détectés dans les feuilles de *V. amygdalina* (Asawalam and Hassanali, 2006; Ogunbindu *et al*., 2009). Comme dans le cas du houblon (Kollmannsberger *et al*., 2006), la présence de béta-damascenone dans les bières *ikigage* contenant *V. amygdalina* seulement peut être attribuée à l'hydrolyse de β-D-glucoside de 3-hydroxy-β-damascone au cours du brassage.

Des concentrations élevées d'éthyle acétate (et son acide), propan-1-ol, 2-méthylpropan-1-ol, 3-méthylbutan-1-ol, 2-méthylbutan-1-ol et 2-phénylethanol dans la bière *ikigage* des paysants par rapport aux bières-pilotes peuvent être associées aux bactéries lactiques (*L. fermentum*, *L. buchneri*, *Lactobacillus* sp) présentes dans la bière traditionnelle des paysans. Les bières pilotes ont été produites avec uniquement les souches de levures dominantes (*S. cervisiae*) isolées du levain *umusemburo*. Ce qui explique également la présence d'éthyle lactate uniquement dans la bière *ikigage* des paysans. Ethyle acétate et éthyle lactate sont considérés comme des composés volatils typiques des bactéries lactiques (Dimiani *et al.*, 1996). Généralement, les alcools supérieurs sont synthétisés par les levures via la voie anabolique à partir du glucose ou via la voie catabolique à partir de leurs acides aminés correspondants. Les acides aminés libérés probablement par l'activité protéolytique des bactéries lactiques et ensuite utilisés par les levures pourraient expliquer les concentrations élevées des alcools dans la bière *ikigage* provenant des paysans. L'association des bactéries lactiques (ex. *L. fermentum*) avec les souches de levures sélectionnées pourrait être idéale dans la production de la bière *ikigage*.

Les bières *ikigage* sont caractérisées également par des quantités importantes d'éthyle caprylate, éthyle caproate et éthyle caprate et leurs correspondants acides. Cependant, les concentrations sont légèrement élevées dans les bières-pilotes produites avec les souches de levures sélectionnées. Ethyle caprylate et éthyle caprate sont également considérés comme des composées volatils caractéristiques de l'arôme des bières Lambic et Gueuze (Spaepen *et al*. 1978). La température de fermentation élevée et les souches de levures sont des facteurs qui peuvent influencer l'augmentation de la concentration éthyl caprylate et éthyl caprate dans la bière (Suomolainen, 1981; Soerens *et al*., 2008) ; ce qui explique les concentrations élevées de ces composés dans les bières *ikigage* puisque ces bières ont été fermentées à 30 °C.

Ce travail révèle également la présence de 2-méthoxy-4-vinylphénol et 4-vinylphénol dans les bières *ikigage*, mais des concentrations plus élevées ont été observées dans les bières pilotes. 2-méthoxyphénol a été détecté uniquement dans la bière *ikigage* des paysans. Ces composés proviennent probablement du malt de sorgho et/ou de l'activité des levures durant le processus de préparation de la bière *ikigage*. Durant le brassage ou fermentation, les acides p-cumarique et férulique du malt de sorgho (Hahn *et al*., 1983; Brovchora *et al*., 2005) peuvent être transformés à des composés volatils tels que le 2-méthoxy-4vinylphénol et 4-vinylphénol. Les températures de brassage de 90 - 100° C favorisent l'augmentation du 2-méthox-4-vinylphénol dans la bière (Vanbeneden *et al*., 2008). La température utilisée pour gélatiniser l'amidon du sorgho peut justifier les concentrations élevées de 2-méthox-4-vinylphénol dans les bières-pilotes. Ces composés peuvent être des constituants de l'arôme caractéristique de la bière *ikigage* comme dans les bières blanches Belges (Back *et al*., 2000) et des bières de Weizen d'Allemagne (Wackerbauer *et al*., 1982).

L'analyse sensorielle a montré que la bière traditionnelle des paysans et les bières pilotes produites avec les souches de levures sélectionnées sont caractérisées par les notes fruitées,

phénoliques et fleuries. Ces résultats prouvent l'impact des esters et des composés phénoliques sur les arômes caractéristiques de la bière *ikigage*. Par contre, le haut score de la note soufrée obtenu seulement dans les bières brassées avec *V. amygdalina* n'est pas en corrélation avec des composés soufrés (diméthyl sulfide, 3-(methylthio)-1-propanol et 3-(methylthio)-1-propyl acétate) trouvés dans ces bières. Dans ce contexte, nous avons suspecté l'existence des thiols polyfonctionnels non détectés par la méthode d'extraction SPME-GS - MS.

Occurrence des thiols polyfonctionnels dans la bière de sorgho « ikigage » brassée avec *Vernonia amygdalina* « umuburizi »

Au cours du chapitre précédent nous avons suspecté l'existence des thiols polyfonctionnels dans la bière *ikigage* faite avec *V. amygdalina*. Les thiols polyfonctionnels souvent détectés à l'état de trace ont un impact sur les caractéristiques aromatiques des bières occidentales fabriquées à base d'orge et de houblon (Vermeulen *et al*., 2003 ; 2006). Cependant, à notre connaissance, les thiols polyfonctionnels n'ont jamais été identifiés dans les bières fabriquées avec les matières non-occidentales. Dans ce contexte, nous avons mené une recherche sur les thiols polyfonctionnels de la bière traditionnelle Rwandaise « ikigage » fabriquée à base de sorgho et de *V. amaygdalina* en utilisant des techniques d'extraction et détections appropriées.

Cette étude a révélé l'existence de 14 thiols polyfonctionnels dans la bière *ikigage* provenant des paysans et des bières pilotes fabriquées avec ou sans *V. amygalina*. Parmi ces thiols, le 3-méthyl-2-buten-1-thiols a émergé comme l'odorant le plus important dans les bières de sorgho contenant *V. amygdalina*. Dans les bières occidentales, le 3-méthyl-2-buten-1-thiol donne une odeur houblonnée très agréable (Lermusieau *et al*., 2003) et sa formation est souvent associée aux composés amers d'houblon (humulones et lupulones) (Goldstein *et al*., 1999 ; Gros *et al*., 2009) via la substitution nucléophile par le sulfure d'hygrogène sur le 3-

méthyl-2-buten-1-ol. (Vermeulen *et al*., 2006) lorsque les bières sont protégées de la lumière. Mais dans notre étude, les humilones et lupulones n'ont pas été trouvés dans les feuilles *V. amygdalina*. L'origine du 3-méthyl-2-buten-1-thiol dans la bière *ikigage* non houblonnée contenant *V. amygdalina* reste à déterminer. Il pourrait provenir des saponines ou des autres métabolites de la plante.

Cette étude a montré également la faible concentration de 2-sulfanyléthanol et de 3-sulfanylpropyl acétate, et l'absence totale de 2-sulfanyléthyl acétate dans les bières produites avec addition de *V. amygdalina* durant l'ébullition. Cette observation n'a jamais été faite sur les bières houblonnées où ils sont parmi les principaux thiols polyfonctionnels. Le 2-sulfanyléthanol, le 2-sulfanyléthyl acétate et le 3-sulfanylpropyl acétate résultent principalement de la dégradation de la cystéine via la voie d'Ehrlich (Vermeulen et al., 2006). *V. amygdalina* pourrait avoir un effet inhibiteur au niveau de la voie Ehrlich contrairement au houblon.

Cependant, *V. amaygalina* favorise la production du 1-butanethiol dans la mesure où il a été détecté uniquement dans les bières contenant du *V. amygdalina*. La littérature rapporte que ce thiol contribue à l'arôme des viandes, des fruits de mer, des fromages et des bières américaines et mexicaines (Meilgaard, 1975). Mais il n'a pas été détecté dans les récentes études menées sur les thiols polyfonctionnels des bières houblonnées. Le 1-Butanethiol pourrait résulter de la substitution nucléophile par le sulfure d'hydrogène sur le butan-1-ol provenant de *V. amygdalina.*

Production de la bière de sorgho « ikigage » en utilisant *Saccharomyces cerevisiae, Issatckenkia orientalis* et *Lactobacillus fermentum* comme starters

Dans les chapitres 2 et 3 nous avons montré que les levures (*S. cerevisiae* suivi par *I. orientalis*) et les bactéries lactiques (*Lactobacillus fermentum*) sont des microorganismes majoritaires impliqués dans la fermentation de la bière traditionnelle Rwandaise « *ikigage* ». Dans ce contexte, nous avons envisagé de produire la bière *ikigage* en utilisant *S. cerevisiae* en combinaison avec *I. orientalis* et *L. fermentum* comme starters. Les caractéristiques microbiologiques, physico – chimiques et organoleptiques de la bière pilote ont été comparées à celles de la bière locale produite par les paysans.

Des résultats obtenus, il ressort que la teneur en éthanol des bières pilote produites avec *S. cerevisae* seule ou en combinaison avec *I. orientalis* et *L. fermentatum* est deux fois plus élevée que celle d'*ikigage* produit localement par les paysans. Cependant, la teneur en éthanol de cette bière locale a été plus élevée que celle de la bière pilote produite avec *I. orientalis* seule. La teneur en acidité totale des bières produites avec *S. cerevisiae* seule ou en combinaison avec *I. orientalis* est très faible par rapport à la bière *ikigage* locale. Mais quand *L. fermentum* est associé, l'acidité totale ainsi que le pH deviennent plus semblables à la bière locale suite à la production de l'acide lactique.

Cette étude montre également que le profil des composés volatils (particulièrement les esters, alcools et acides) de la bière *ikigage* produite avec *S. cerevisiae* en combinaison avec *I. orientalis* et *L. fermentum* est similaire à celui de la bière *ikigage* locale. *I. orientalis* contribue à la production de l'acide butyrique (et son isomère) et 2-phénylethanol tandis que *L. fermentum* contribue à la production de l'éthyle lactate, de l'éthyle acétate ainsi que son acide, et à l'augmentation de la concentration de certains alcools tels que le 2-méthylpropan-1-ol, 3-méthylbutan-1-ol et 2-méthylbutan-1-ol.

L'analyse sensorielle a montré qu'il n'existe pas des différences significatives entre la bière *ikigage* commercialisée au Rwanda et les bières pilotes produites avec *S. cerevisiae* en combinaison avec *I. orientalis* et *L. fermentum*. Cependant, les scores élevés obtenus avec la bière *ikigage* produite avec *S. cerevisiae* seule montrent que cette bière n'est pas semblable à celle produite par les paysans. *I. orientalis* et *L. fermentum* semblent conférer à la bière *ikigage* sa saveur et son arôme caractéristiques.

CONCLUSION ET PERSPECTIVES

Ce travail est une contribution à l'amélioration de la qualité de la bière traditionnelle Rwandaise « ikigage » fabriquée à base de sorgho. Il a permis de caractériser la bière ikigage et de mettre au point une bière *ikigage* améliorée en utilisant les matières premières locales.

La bière traditionnelle « ikigage » du Rwanda est caractérisée, d'une part, par la faible teneur en éthanol, l'acidité élevée et une importante quantité de protéines. Et d'autre part, par la présence de *S. aureus* et de beaucoup de microorganismes d'origine fécale, notamment *E. coli* et Streptocoques fécaux. Ces microorganismes présentent un risque majeur pour les consommateurs ayant un système immunitaire faible. Les levures et les bactéries lactiques hétérofermentaires (*L. fermentum, L. buchneri* et *Lactobacillus sp)* sont des microorganismes majoritaires de la bière *ikigage*. *S. cerevisiae* et *I. orientalis* sont des levures majoritaires impliquées dans la fermentation de la bière de sorgho. Les souches de *S. cerevisiae* (RV6 et RB2) et *I. orientalis* (RG1) sélectionnées peuvent être utilisées comme starter pour la production de la bière traditionnelle Rwandaise « ikigage » ayant le statut de GRAS. Cependant, des études sur la résistance de ces souches aux différentes techniques de conservation sont nécessaires.

Dans le cas du Rwanda, l'utilisation d'*Eleusine coracana* « *uburo* » (variété Musama) associée à l'emploi de la méthode non -conventionnelle de brassage par décantation est bien indiquée pour augmenter la teneur en sucres fermentescibles dans le moût de sorgho et par conséquent la teneur en éthanol dans la bière *ikigage*. Le malt d'*uburo* apporte une quantité importante de β-amylase et la méthode de décantation permet de gélatiniser l'amidon tout en préservant l'efficacité des enzymes hydrolytiques. Cependant, des études portées sur l'amélioration de la filtration du moût devront être effectuées.

L'utilisation de *S. cerevisiae* en combination avec *I. orientalis* et *L. fermentum* comme starter permet d'améliorer la teneur en éthanol de la bière de sorgho « ikigage » tout en conservant les caractéristiques organoleptiques semblables à celles de la bière traditionnelle « *ikigage* » locale. Elle réduit également les risques de contamination de la bière par les microorganismes potentiellement pathogènes et pourrait ainsi contribuer à augmenter la durée de conservation de la bière *ikigage*. Cependant, d'autres études complémentaires devront s'orienter vers la conservation de cette bière.

La bière *ikigage* est caractérisée par la présence de nombreux esters, alcohols et acides. Parmi les esters, éthyle acétate, éthyle caprylate, éthyle caproate et éthyle caprate sont les plus importants. Les principaux alcools sont notamment propan-1-ol, 2-méthylpropan-1-ol, 3-méthylbutan-1-ol, 2-méthylbutan-1-ol et 2-phényl alcohol. Les acides acétique, caproique et caprylique sont les principaux acides de la bière *ikigage*. Elle est caractérisée également par la présence de certains composés phénoliques tels que 2-méthoxy-4vinylphénol, 4-vinylphénol et 2-méthoxyphénol. Ces composés contribuent à la note fruitée, fleurie et phénolique caractéristique de la bière *ikigage*.

V. amygdalina, très connu pour son amertume et ses propriétés antibactériennes semblables au houblon, apporte également à la bière de sorgho de nombreux terpènes (3-carene, β-farnesene, farnesol, β-citronellol, Linalol et 1,8-cineole), méthyle salcylate et béta-damascenone. Comme dans les bières houblonnées, ces composés pourraient jouer un rôle significatif dans l'arôme global de la bière *ikigage*.

Ce travail révèle également pour la première fois la présence de 14 thiols polyfonctionnels dans les bières fabriquées avec les matières premières non occidentales. Parmi eux, le 3-méthyl-2-buten-1-thiol, très connu pour son arôme houblonnée agréable, a émergé comme le composé le plus important dans les bières *ikigage* non houblonnées contenant *V. amygdalina*. *V. amygdalina* contribue également à la production du 1-butanethiol et de 4-sulfanyl-4-méthyl-2-pentanone dans la bière *ikigage*.

Les feuilles de *V. amygdalina* émergent comme un substituant intéressant du houblon dans les pays tropicaux. Mais contrairement au houblon, elle inhibe la production de 2-sulfanyl éthyle acétate lorsqu'elle est ajoutée durant l'ébullition. Des études complémentaires sont nécessaires pour déterminer les composés volatiles ou leurs précurseurs présents dans les feuilles de *V. amygdalina*, et pour comprendre le mécanisme d'action de *V. amygdalina* au niveau de la voie de l'Ehrlich.

REFERENCES BIBLIOGRAPHIQUES

Adenuga W., Olaleye O.N. & Adepoju P.A., 2010. Utilization of bitter vegetable leaves (*Gongronema latifolium, Vernonia amygdalina*) and *Garcinia kola* extracts as substitutes for hops in sorghum beer production. *Afri J. Biotech.,9, 8819-8823.*

Ajebesone, P.E. & Aina, J.O., 2004. Potential African substitutes for hops in tropical beer brewing. *African J. Food and Techn., 9, 13 – 16.*

Asiedu J.J., 1991. La transformation des produits agricoles en zone tropicale : approche technologique. *Paris :Karthala*.

Au, P.M. & Fields, M.L. 1981. A research note on nutritive quality of fermented sorghum. J. *Food Sci., 46: 652-654.*

Back, W., Diener, B., & Sacher, B. 2000. Hefeweizenbier – Taste spectrum and technology. *Brauwelt Inter., 18, 112–119.*

Bajomo M. F. & Young, T. W., 1994. Fermentation of wort from 100% of raw sorghum and enzyme. *J. Inst. Brew., 100, 79 – 84.*

Belton P.S. & Taylor J.R.N., 2002. Pseudocereals and Less Common Cereals: Grain Properties and Utilization Potential. Springer, Berlin.

Benitez T., Del Castllo L., Aguilera A., Conde J. & Cerdaolmedo E. 1983. Selection of wine yeast for growth and fermentation in the presence of ethanol and sucrose. *Appl. Envir. Microbiol., 45, 1429 – 1436.*

Chavan, J. K., Kadam, S. S. & Beuchat L. R., 1989. Nutritional improvement of cereals by fermentation**.** *Critical reviews in food science and nutrition, 28, 349–400.*

Chavan, U.D., Chavan J.K. & Kadam S.S., 1988. Effect of fermentation on soluble protein and *in vitro* protein digestibility of sorghum, green gram and sorghum-green gram blend. *J. Food Sci., 53, 1574–1575.*

Chevassus-Agnes S., Favier J.C. & Joseph A., 1979. Traditional technology and nutritive value of Cameroon sorghum beers. *CAH Onarest 2, 83-112*

CTA., 2011. Sorgho, grain d'avenir. *Spore, 151, p20*. http://spore.cta.int

Daenen L., 2008. Exploitation of the flavour potential of hop and sour cherry glycosides by Saccharomyces and Brettanomyces glycoside hydrolase activities. PhD Thesis, K.U. Leuven, Belgium

Dahouenon-Ahoussi E., Degnon R.G., Adjou E.S. & Dominique C.K., 2012. SohounhloueStabilisation de la bière produite à partir de matières amylacées locales (Sorghum bicolor et Musa acuminata) par adjonction de l'huile essentielle de Cymbopogon citratus. *J. Appl. Biosci., 51, 3596 – 3607.*

Dale C.J., Young T.W. & Makinde A., 1989. Extruded sorghum as a brewing raw material. *J. Instit. Brewing, 95, 157-164.*

Damiani P., Gobbetti M., Cossignani L., Corsetti A., Simonetti M. S. & Rossi, J., 1996. The sourdough microflora. Characterization of hetero- and homofermentative lactic acid bacteria, yeasts and their interactions on the basis of the volatile compounds produced. *Lebensm. Wiss. u. Technol. 29, 63–70*

Degre´ R., 1993. Selection and commercial cultivation of wine yeast and bacteria. In: Fleet, G.H. (Ed.), Wine Microbiology and Biotechnology. *Harwood Academic Publishers, Chur, pp.*

421–447.

Dicko M.H., Gruppen H., Traoré1 A. S., Voragen A.G.J. & Van Berkel W.J.H., 2006. Sorghum grain as human food in Africa: relevance of content of starch and amylase activities. *Afri. J. Biotechnol., 5, 384-395.*

Dufour J.P., Melotte L. & Srebrnik S., 1992. Sorghum malts for the production of a lager beer. *J. Amer. Soci. Brew. Chem.,50, 110-119.*

El Tinay A.H., Abdel Gadir A.M. & El Hidai M., 1979. Sorghum fermented kisra bread: Nutritive value of kisra. *J. Sci. Food and Agric., 30, 859-863.*

FAO, 1997. Agriculture food and nutrition for Africa : a resource book for teachers of agriculture. Food and Agriculture Organization of United Nation. Rome. 135p.

Faparusi S.I, Olofinboba M.O. & Ekwundayo J.A., 1973. The Microbiology of burukutu beer Z. *Allg Mikrobiologie. 13: 563-568.*

Fliedel G., Marti A. & Thiedaut., 1996. Caractérisation du sorgho. CIRAD-CA, Montpellier

Gabriel P., Dienstbie R.M., Matoulková D., Kosař k. & Sigler K. 2008. Optimised acidification power test of yeast vitality and its use in brewing Practice. *J. Inst. Brew., 114, 270–276.*

Gilliland R.B. 1951. The flocculation characteristics of brewing yeast during fermentation. *Proc. Eur. Brewing convention congr., Brighton, pp. 35 – 58.*

Goldstein, H.; Ting, P.; Navarro, A.; Ryder, D., 1999; Water-Soluble Hop Flavor Precursors and Their Role in Beer. *27th European Brewery Convention*; Cannes, France.

Goode D. L. Elke K. Arendt E.K. 2003. Pilot Scale Production of a Lager Beer from a Grist Containing 50% Unmalted Sorghum. *J. Inst. Brew.,109, 208-217.*

Gros, J., Nizet, S.; Collin, S., 2009. Hop allylic alcohols are precursors of sulfur-containing odorants in fresh beer. *Acta Hort.***,** *848, 273-278.*

Guerra N.P., Torrado – Agrasar A., Lopez – Macias C . & al. 2009. Use of amylolytic enzymes in brewing. In: Victor . R. Preedy.(Ed.), Beer in health disease prevention. *Academic press, Elsevier , pp 114 – 126.*

Haggblade S & Holzapfel H., 2004. Industrialization of Africa's indigenous beer brewing. In: Streinrous KH KH (2 ed.) industrialization of indigenous fermented foods, New York City: CRC Press.

Hamaker B.R., Kirleis A.W., Mertz E.T. & Axtell J.D., 1986. Effect of cooking on the protein profiles and in vitro digestibility of sorghum and maize. *J. Agric. Food Chem., 34, 647.*

Hassan I.A.G. & El Tinay A.H., 1995. Effect of fermentation on tannin content and *in vitro* protein digestibility of two sorghum cultivars. *Food Chem., 53, 149-151.*

Holzapfel W., 1997. Use of starter cultures in fermentation on a household scale. *Food Control, 8, 241-258.*

Holzapfel W., 2002. Appropriate starter culture technologies for small– scale fermentation in developing countries. *Int. J. Food Microbiol. 75, 197-212*.

Kayodé APP., Aégbidi A., Linnemenn A.R., Nout M.J.R. & Hounhouigana J.D., 2005. Quality of farmer's varieties of sorghum and derived foods as perceived by consumers in Benin. *Ecol. Food Nutr., 44, 271-294.*

Kazanas N. & Fields ML., 1981. Nutritional improvement of sorghum by fermentation. *J. Food Sci., 46, 819-821.*

Kirmaryo V. M., Massawe G.A., Olasupo N.A. & Hololzpfel W.H., 2002. The use of a starter culture in the fermentation of cassava for the production of '' Kivunde'', a traditional Tanzanian food product. *Int.J.Food Microbiol., 56, 179-190*.

King A. & Dickinson J.R., Biotransformation of hop aroma terpenoids by ale and lager yeasts. *FEMS Yeast Res., 3, 53 – 62.*

Kollmannsberger H., Biendl M. & Nitz S., 2006. Occurrence of glycosidically bound flavor compounds in hops, hop products and beer. Monatsschrift für Brauwissenschaft, 59, 83 – 89.

Igyor M.A., Ogbonna A.C. & Palmer G.H. 2001. Effect of malting temperature and mashing methods on sorghum wort composition and beer flavour. *Process Biochemistry, 36, 1039–1044.*

Lermusieau, G. & Collin, S., 2003. Volatile sulfur compounds in hops and residual concentrations in beers. *J. Am. Soc. Brew.Chem., 61,* 109-113.

Lyumugabe L., Kamaliza G., Bajyana E. & Thonart PH., 2010. Microbiological and physico-chemical characteristic of Rwandese traditional beer "*Ikigage*". *Afr. J. Biotechnol., 9, 4241-4246.*

Maoura N., Mbaiguinam M., Nguyen HV., Gaillardin C. & Pourquie J., 2005. Identification and typing of the yeast strains isolated from bili bili, a traditional sorghum beer of chad. *Afr. J. Biotechnol., 4: 646-656.*

Masy C.L., Henquinet A., Mestdagh M.M., 1992. Flocculation of Saccharomyces cerevisiae: inhibitions by sugars. *Can. J. Microbiol. 38, 1298–1306.*

Mathieu C., Van den Bergh L. & Iserentant D. 1991. Prediction of yeast fermentation performance using the acidification power test. *Proceedings of the European Brewery Convention Congress*, Lisbon, IRL Press: Oxford, pp. 273-280.

Mboto C.I., Eja, M. E., Adegoke A.A., Iwatt G.D., Asikong B.E., Takon I., Udo S.M. & Akeh M., 2009. Phytochemical properties and antimicrobial activities of combined effect of extracts of the leaves of *Garcinia kola, Vernonia amygdalina* and honey on some medically important microorganisms. *Afri J. Microbiol. Research, 3,557-559.*

Minagri., 2012. Annual report: Crop assessment 2011. Republic of Rwanda

Munyaja MBK, Narvhus JA, Treimos J, Langsrud T., 2003. Isolation, characherization and identification of lactic acid bacteria from bushera: Ugandan traditional fermented beverage, *Int. J. Food Microbiol., 8, 201-210.*

Naumova ES, Korshunova IV, Jespersen L, Naumov GI., 2003. Molecular genetic identification of *Saccharomyces* sensu strict strains from African sorghum beer. *FEMS Yeast Res., 3,177-184.*

Neucere N.J. & Sumell G., 1979. Protein fractions from five varieties of grain sorghum: amino acid composition and solubility properties *J. Agric. Food Chem.*, 27, 809–812

Noha A., Mohammed I. A., Mohamed A. & Elfadil E. B., 2011. Nutritional Evaluation of Sorghum Flour (*Sorghumbicolor L. Moench)* During Processing of Injera. *Inter. J. Biological and Life Sciences 7, 35 – 39.*

Novellie L.,1982. Fermented porridge. In: Proceedings of the international symposium on sorghum grain quality. ICRISAT (eds), Pantacheru, India, pp. 121-128.

Oboh F. O.J. & Masodje H.I., 2009. Nutritional and antimicrobial properties of *Vernonia amygdalina* leaves. *Inter. J. Biomedical and Health Science, 5, 51 – 56.*

Odunfa SA. & Adeyele S., 1985. Microbiological changes during the traditional production of ogi-baba, a west african fermented Sorghum gruel. *J. Cereal Sci., 3, 173-180.*

Ogunbinu AO, Flamini G, Cioni PL, Ogunwande IA, Okeniyi SO., 2009. Essential oil constituents of *Eclipta prostrate* (L.) and *Vernonia amygdalina* Delile. *Natural product communication, 4, 421-424.*

Okafor N. & Aniche G.N., 1980. Brewing a lager beer from Nigerian sorghum. *Brew. Dist.g Inter. J., 10, 32-35.*

Okoh IA., Babalola GO. & Ilori MO., 1995. Effect of methanol extract of *Vernonia amygdalina* on malting and brewing properties of sorghum. *Tech. Quart. Master Brewer's Assoc. Am. 32, 11-14.*

Okoro C.C . & Aina, J. O., 2007. Effect of storage on the brewing properties of tropical hop substitutes. *Afri. J. Biotech., 6, 1479- 1483*.

Osman M. A. 2004. Changes in sorghum enzyme inhibitors, phytic acid, tannins and in vitro protein digestibility occurring during Khamir (local bread) fermentation. *Food Chem., 88, 129-134.*

Palmer G. H., 1989. Cereals in malting and brewing. In *Cereal Science and Technology,* ed. Palmer, G. H. pp. 61-242. Aberdeen: Aberdeen University Press.

Price M. L. & Butler L. G., 1977. Rapid Visual Estimation and Spectrophotometer Determination of Tannin content of sorghum grain. *J. Agric. Food Chem., 25, 1268-1273.*

Stratford M. 1989. Evidence for two mechanisms of flocculation in *Saccharomyces cerevisiae*. *Yeast, 5, 441–445.*

Soares E.V. & Vroman, A. 2003. Effect of different starvation conditions on the flocculation of Saccharomyces cerevisiae. *J. Appl. Microbiol., 95, 325–330.*

Spaepen, M., Vanoevelen, D., Verachtert H., 1978. Fatty – Acids and Esters produced during spontaneous fermentation of Lambic and Gueuze. J. Inst. Brew., 84, 278 – 282.

Suomalainen H., 1981. Yeast esterases and aroma esters in alcoholic beverages. *J. Inst. Brew., 87, 296 – 300.*

Takoi K., Koie K., Itoga, Katayama Y., Shimase M., Nakayama Y., Watari J., 2010. Biotransformation of hop-derived monoterpene alcohols by lager yeast and their contribution to the flavor of hopped beer. *J. Agric. Food Chem., 58, 5050 – 5058.*

Vermeulen, C.; Lecocq, S.; Collin, S. 2003. Polyfunctional Thiols and Drinkability of Beer. *29th European Brewery Convention Congress*; Dublin, Ireland.

Vermeulen, C.; Lejeune, I; Tran T.T.H. & Collin, S., 2006. Occurrence of Polyfunctional Thiols in Fresh Lager Beers. *J. Agric. Food. Chem., 54, 5061 – 5068.*