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Full Length Research Paper

Microbiological and physico-chemical characteristic of Rwandese traditional beer "*Ikigage*"

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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 either *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 post-fermentation 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.

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).

The African indigenous beers containing millet or sorghum 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".*

MATERIALS AND METHODS

Processing and sampling

Fifty samples of *ikigage* were collected in sterile bottles (500 and 100 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

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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.

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 in water peptone without indol and were incubated at 44°C for 24 h to detect for *E. coli*. *E. coli* were 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.

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- TCCGTAGGTGAA CCTGCGG-) 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).

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

RESULTS

Manufacturing process of *Ikigage*

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

Malting

Generally, the traditional brewers choose *Amakoma* (Sorghum bicolor) to prepare *ikigage*.

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).

Mixing

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).

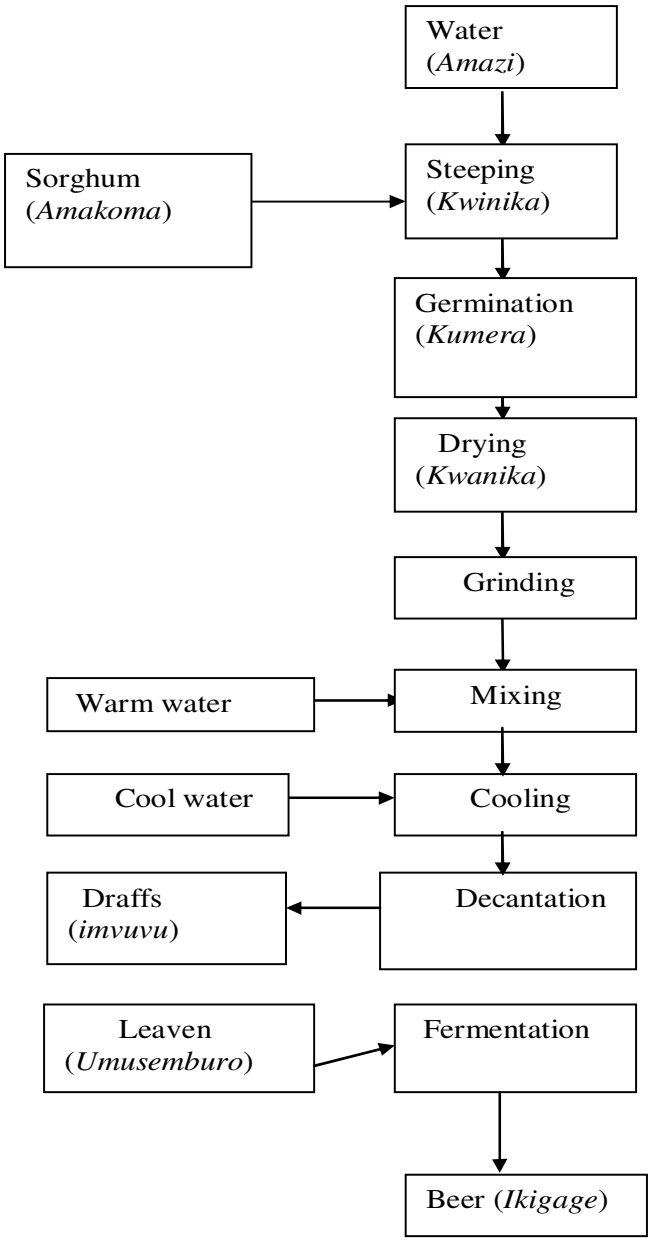

Figure 1. Diagram of manufacturing of Rwandese traditional beer (*Ikigage*).

Decoction

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 decoction, cool water is added (40 L) to bring temperature 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*.

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.

The methods of manufacture of this leaven are diversified in Rwanda. Each province or district selects the ingredients to be used. *V. amygdalina* and *E. tirucalli* are used in all areas of Rwanda. But, other plants can be used in the preparation of *umusemburo* (Table 1).

Currently, 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.

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.

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

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

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

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

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 bacteria	35.35×10^4	Brix	11.6 (±1.53)
Mould	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)
Fecal streptococci	22.50×10^3		

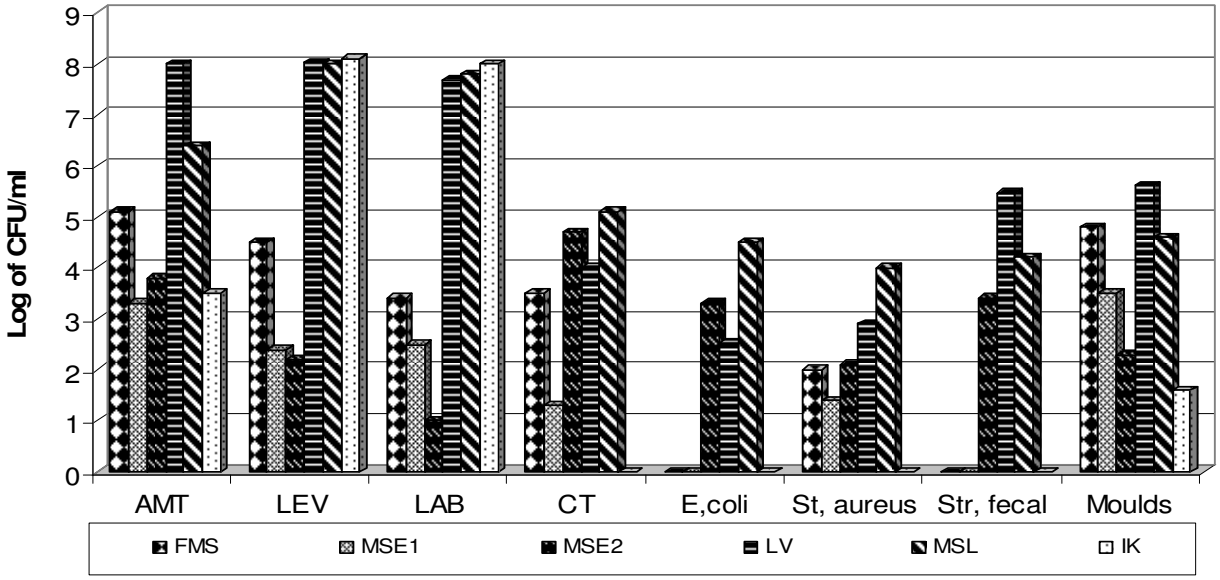

Figure 2. Micro-organisms at the various stages of manufacture of *Ikigage.* FMS: Flour of malted sorghum; MSE1: Mix malted sorghum and warm water; MSE2: MSE1 + cool water; LV: Levean; MSL: Mix cooled must and leaven; IK: *Ikigage* (beer) after fermentation; AMT: total aerobic mesophilic; LEV: yeasts; LAB: lactic acid bacteria; and CT: total coliform.

very low pH and a weak ethanol concentration.

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* just

after fermentation.

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).

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 pre-fermentation 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 (*Uburu*) malted to produce a beer with high alcohol content. *Uburu* brings beta 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, 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 involved in the *ikigage* fermentation are dominated by *Sacharomyces cerevisiae*. They are known for their role in alcoholic fermentation. Similar findings were obtained by Maoura et al. (2005) for traditional beer *bili bili*, Kayode et al. (2007) for Tchoukoutou and Naumova et al. (2003) for Pito. But, these authors did not isolate *I orientalis* in beer manufactured containing malted sorghum. This species is unable to ferment maltose. It would come from banana wine used in the preparation of leaven (*Umusemburo*). *I. orientalis* was 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 know 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).

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 comercialization 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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