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Yeasts and lactic acid bacteria microbiota from *masau* (*Ziziphus mauritiana*) fruits and their fermented fruit pulp in Zimbabwe

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Abstract

Masau are Zimbabwean wild fruits, which are usually eaten raw and/ or processed into products such as porridge, traditional cakes, *mahewu* and jam. Yeasts, yeast-like fungi, and lactic acid bacteria present on the unripe, ripe and dried fruits, and in the fermented *masau* fruits collected from Muzarabani district in Zimbabwe were isolated and identified using physiological and molecular methods. The predominant species were identified as *Saccharomyces cerevisiae*, *Issatchenkia orientalis*, *Pichia fabianii* and *Aureobasidium pullulans*. *A. pullulans* was the dominant species on the unripe fruits but was not isolated from the fermented fruit pulp. *S. cerevisiae* and *I. orientalis* were predominant in the fermented fruit pulp but were not detected in the unripe fruits. *S. cerevisiae*, *I. orientalis*, *P. fabianii* and *S. fibuligera* are fermentative yeasts and these might be used in the future development of starter cultures to produce better quality fermented products from *masau* fruit. Lactic acid bacteria were preliminary identified and the predominant strains found were *Lactobacillus agilis* and *L. plantarum*. Other species identified included *L. bifermentans*, *L. minor*, *L. divergens*, *L. confusus*, *L. hilgardii*, *L. fructosus*, *L. fermentum* and *Streptococcus* spp. Some of the strains of LAB could also potentially be used in a mixed-starter culture with yeasts and might contribute positively in the production of fermented *masau* fruit products.

Keywords: *Masau* fruits; Yeasts; Lactic acid bacteria; Starter cultures; Fermentation

1. Introduction

Ziziphus mauritiana fruit, which is locally called *masau* fruit in Zimbabwe, is one of the wild fruits that are traditionally fermented into beverages through spontaneous and uncontrolled processes. Other wild fruits that are spontaneously fermented include *mapfura* (*Sclerocarya birrea* subspecies *caffra*), *hacha* (*Parinari curatellifolia*) and *mazhanje* (*Uapaca kirkiana*) (Gadaga et al., 1999). However, because the fermentations are spontaneous and uncontrolled, the product microbiota are inconsistent and the fermented products are of variable quality (Halm et al., 1993; Sanni et al., 1994; Gadaga, 2000). Their fermentation mainly relies on the microbiota from the fruit surfaces and to some extent from the utensils used during the fermentation process. The diverse microbial flora on fruit surfaces may play an important role during the spontaneous fermentation process (Fleet, 2003). The type of microorganisms includes coliforms, lactic acid bacteria, yeasts and moulds (Mbugua, 1985). The spontaneous fermentation of grape juice into wine, as observed by Louis Pasteur about 150 years ago, established the broadly accepted view that yeasts have a natural association with fruits and fruit products (Fleet, 2003). *Z. mauritiana* was also reported previously to be among the Zimbabwean indigenous fruits that are associated with high numbers of microbes which contribute to the natural fermentation following ripening (Chivero et al., 2001).

In Zimbabwe, *masau* fruits are spontaneously fermented for 6–7 days and then distilled into a potent spirit called *kachasu* (Tredgold, 1986; Gadaga et al., 1999), that is highly intoxicating and is

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regarded as illegal beverage (Brett et al., 1992). The fermented fruit pulp is not consumed as such, because of its unattractive exterior and smell. *Kachasu* is consumed as a beverage by the local people and its alcohol content varies from producer to producer.

The microbial changes taking place during the spontaneous fermentation of *masau* have not been recorded. The objective of this study, therefore is to isolate and identify yeasts and LAB microbiota that are involved in the spontaneous fermentation of *masau* fruit pulp and on the whole fruit. This will be followed in future by the selection and development of starter cultures to produce better quality fermented products from *masau* fruits.

2. Materials and methods

2.1. Sample collection

The collection and analysis of samples was done during two consecutive years 2004 and 2005 in the month of August when *masau* fruits are in season. Both ripe and unripe *masau* fruits were harvested from Muzarabani (16° 20′ S, 31° 21′ E) district in Northern Zimbabwe. Dried fruit samples were obtained from the local people who ferment the fruits. The fruits were collected into sterile stomacher bags (Stomacher® Lab System, London, UK) and ferried to the laboratory in a cooler box. Fermented samples (200 ml) ($n=7$ for each year) were collected into sterile 250 ml screw capped bottles (Schott Duran, Elmsford, NY) from the local people in Muzarabani. Samples of distilled product made from *masau* fruits were also collected into 250 ml sterile bottles . These samples were also transported to the laboratory in a cooler box and were kept at 5 °C on arrival and processed the following day.

2.2. Microbiological analyses

2.2.1. Isolation of yeasts and LAB from masau fruits and the fermented fruit pulp

Masau fruits (100 g) were aseptically weighed and transferred into sterile 250 ml bottles containing Peptone Physiological Saline (100 ml) [0.8% NaCl (Merck, Darmstadt, Germany), 0.1% neutral peptone (Oxoid, Basingstoke, UK); PPS]. The bottles were vigorously shaken by hand for 10 min. Appropriate serial dilutions were plated in duplicate on Malt Extract Agar (MEA) (Oxoid) containing 0.1% oxytetracycline (Sigma-Aldrich Co., St Louis, MO, USA), and on de Man, Rogosa, Sharpe (MRS) agar (Oxoid) containing 0.1% natamycin (Aldrich Chemical Co., Gillingham, Dorset, England). Similarly, appropriate serial dilutions were also made from the fermented fruit pulp samples and plated in duplicate on MEA containing 0.1% oxytetracycline and MRS agar containing 0.1% natamycin. The MEA plates were incubated at 25 °C for 48 h. MRS agar plates were incubated at 37 °C for 48 h under anaerobic conditions using the BBL® Gas Pak® Anaerobic Systems (Becton Dickinson Company, Maryland, USA) according to the manufacturer's instructions. After the incubation a differential count based on colony colour was made for yeasts of different colony morphology, whereas presumptive LAB colonies were counted on the MRS agar plates.

2.2.2. Purification of yeasts and LAB

Morphologically distinct yeast and LAB colonies were selected and purified by making streak plates on MEA and MRS respectively. The MEA plates were incubated at 25 °C for 48 h and the MRS plates were incubated as mentioned above.

2.2.3. Morphological characterization of yeasts

Yeast strains were streaked on Glucose Peptone Yeast Extract Agar [2% D-glucose (Merck), 0.5% bacto peptone (Oxoid), 0.5% yeast extract (Oxoid), and 2% agar (Oxoid); GPYA] plates and incubated at 25 °C for 7 days and examined for characteristics such as colony appearance, cell shape and presence of filaments (Kurtzman et al., 2003).

2.3. Phenotypical characterization of yeasts

The phenotypic characteristics of the yeast isolates were examined using conventional tests as described by Kurtzman et al. (2003). These included ability to ferment sugars, assimilation of nitrogen compounds, diazonium blue B test, production of acetic acid and growth at different temperatures. The ability of yeasts to ferment sugars was detected by examining the cultures for production of CO_2 gas. A filter sterilized solution of 6% of any of the sugars [2 ml; glucose, galactose, sucrose, maltose, lactose and raffinose (Sigma Chemical Co., St Louis, MO, USA)] was gently mixed with sterile 2% (w/v) yeast extract broth (4 ml) in a test tube containing an inverted Durham tube. A yeast cell suspension (0.2 ml) was then added and the tube was incubated at 25 °C. Observations were made after every two days for 28 days. Assimilation of nitrate (Merck), nitrite (Merck), ethylamine (Acros Organics, New Jersey, USA), L-lysine (Acros), cadaverine (Acros), creatine (Merck), imidazole (Merck) and ammonium sulphate (Merck) by the yeast strains was tested using the auxanogram method. To test for the production of acetic acid, a small amount of yeast inoculum was streaked onto plates of GPYA with 0.5% w/v $CaCO_3$ (Merck). The production of acetic acid was observed as a clear zone around the culture. The growth of yeasts at different temperatures was tested by streaking the culture on a slant of GPYA and incubating for 5 days at the following temperatures: 25 °C, 30 °C, 37 °C, 42 °C and 45 °C. In addition, the ability of the yeast isolates to assimilate carbon compounds was tested using the API 20C kit (Bio-Mérieux, Marcy l'Etoile, France) according to the manufacturer's instructions.

2.4. Genotypical identification of yeasts

2.4.1. DNA extraction

DNA was extracted according to the cetyltrimethylammonium bromide (CTAB) method. Yeast cells (500 μl) were transferred to a 1.5ml Eppendorf tube, before adding CTAB buffer (800 μl) (O'Donnel et al., 1997). The suspension was mixed using a vortex mixer and incubated for 1 h at 65 °C, with vigorous shaking at 15 min intervals. The suspension was centrifuged at 21,000 *g* in an Eppendorf bench top centrifuge for 30 min at 4 °C. The supernatant (700 μl) was transferred to a

fresh 1.5 ml Eppendorf tube. Subsequently, chloroform–isoamylalcohol (700 µl, 24:1 by volume) was added, and the solution was shaken vigorously. The solution was then centrifuged at 21,000 *g* for 20 min at 4 °C and the supernatant (500 µl) was transferred to a fresh Eppendorf tube. To this solution, chloroform–isoamylalcohol (500 µl) was added, and the suspension was centrifuged again at 21,000 *g* for 10 min at 4 °C. From this suspension, a portion of the aqueous layer (350 µl) was pipetted and mixed with CTAB buffer (150 µl). To this, ice-cold isopropanol (300 µl, kept at −20 °C) was added, and the DNA was precipitated by centrifugation at 21,000 *g* for 10 min at 4 °C. The pellet obtained was washed with 70% alcohol. It was dried and then suspended in sterile water (100 µl) containing RNase (4 µl; 10 mg/ml) (USB Corp., Cleveland, Ohio). The samples were stored at −20 °C.

2.4.2. PCR amplification and sequencing of Internal Transcribed Spacer (ITS) and Large Subunit (LSU) regions

The sequencing of the ITS 1+2 regions of the rDNA and the D1/D2 domains of the 26s rRNA was done using the procedures described by Gupta et al. (2004). The nucleotide sequences obtained were identified using BLAST.

2.4.3. Amplified Fragment Length Polymorphism (AFLP) analysis

AFLP analysis (Vos et al., 1995) was performed according to the manufacturer's instructions in the AFLP microbial fingerprinting protocol (Applied Biosystems), with some modifications according to the method described by Gupta et al. (2004) for the construction of the dendrogram.

2.5. Physiological identification of LAB

The 35 isolates of LAB were confirmed using the regular Gram stain method, the catalase and oxidase tests, and characterized by their growth at different temperatures and assimilation of carbon compounds. The presence of catalase activity was observed by formation of gas bubbles after suspension of bacterial cells in a droplet of 3% hydrogen peroxide. The oxidase reaction of LAB was determined using BBL DrySlide Oxidase (Difco) according to the manufacturer's instructions. The growth of LAB was tested by inoculating the culture into MRS broth and incubating for 5 days at 15 °C and 45 °C. The carbohydrate fermentation profiles of the LAB isolates were investigated using API 50 CHL strips and API CHL medium according to the manufacturer's instructions (Bio-Merieux). Using the data obtained from the above mentioned tests the LAB were then identified using Intelligent Bacteria Identification System (IBIS) software (Wijtzes et al., 1997).

3. Results and discussion

3.1. Enumeration of yeasts

Yeast strains (107 isolates) were isolated from the unripe, ripe and dried fruits, and the fermented fruit pulp. The yeasts had different morphological characteristics and could be grouped according to the colour of their colonies, namely red/pink and cream/white as shown in Table 1. Generally there was a decrease in the red/pink yeast population and an increase in the cream/white yeast population from the unripe fruit to the fermented fruit pulp. The yeast populations obtained in the ripe fruits are comparable to the literature values (ranging from 2 to 6 log CFU $g^{-1}$of fruit) obtained for yeast populations on different types of ripe fruit surfaces such as apples, grapes and strawberries (Rosini et al., 1982; Dennis and Davis, 1977; Beech, 1993). Fleet (2003) also reported that the yeasts responsible for the fermentation originate from the surface of the fruit, contact with processing equipment and other environmental sources, and developed into communities as dense as 7–8 log CFU g^{-1}, which is similar to yeast population counts (approximately 9.26 log CFU g^{-1}) obtained in the fermented fruit pulp in this study.

Table 1
Yeast counts from *masau* (*Z. mauritiana*) fruits and fermented *masau* fruit pulp from Muzarabani district in Zimbabwe

Fruit sample	Yeasts (log CFU g^{-1})	
	Red/pink	Cream/white
Unripe (n=10)	3.74±0.09 [a]	3.30±0.1
Ripe (n=10)	2.97±0.03	3.93±0.04
Dried (n=10)	1.23±0.04	4.28±0.02
Fermented (n=14)	<4	9.26±0.4

[a] Values are means ± standard deviation of n determinants (two harvests); each determinant was calculated as the average of a duplicate.

3.2. Identification of yeasts

Morphologically distinct colonies of yeasts were picked and identified (Tables 2 and 3) using the methods as described by Deak (1993, 2003), Kurtzman et al. (2003) and Gupta et al. (2004). From the yeast strains isolated, 14 different species were identified namely: *Saccharomyces cerevisiae* (21 strains), *Issatchenkia orientalis* (25 strains), *Pichia fabianii* (12 strains), *Aureobasidium pullulans* (26 strains), *Candida glabrata* (3 strains), *Pichia ciferrii* (3 strains), *Saccharomycopisis fibuligera* (2 strains), *Hanseniaspora opuntiae* (1 strain), *Zygoascus hellenicus* (2 strains), *Cryptococcus flavus* (1 strain), *Cryptococcus magnus* (1 strain), *Candida parapsilosis* (1 strain), *Candida pyralidae* (1 strain) and *Rhodotorula mucilaginosa* (1 strain) (Table 4). Seven of the isolates could not be identified. However, according to the data obtained by sequencing the ITS and LSU regions the closest identification for the isolates 002, 008, 98, 97, 112, 92 and 95 were *Cryptococcus heveanensis*, *Bullera dendrophila*, *Fusarium lichenicola* (mould), *Filobasidium floriforme*, *Cryptococcus chernovii*, *C. flavus* and *Myrothecium roridum* respectively. The red/pink yeasts enumerated in Table 1 represent *A. pullulans*, *C. flavus* and *R. mucilaginosa* which were found dominantly on the unripe fruits; these are all non-fermentative yeasts. The cream/white group that was predominant includes all the other identified yeast species and comprised both

Table 2
Identification of yeasts isolated from *masau* fruits and fermented *masau* fruit pulp from Muzarabani district in Zimbabwe

Substrate	Identity and strain reference number						
	Saccharomyces cerevisiae (135, 124, 141, 38, 146, 131, 126, 168, 165, 143, 142,149, 102, 153, 128, 148, 46, 139, 160, 116, 130)	*Issachenkia orientalis* (132, 94, 151, 123, 110, 42, 3, 152, 100, 166, 144, 105, 51, 27, 150, 137, 91, 129, 30, 32, 52, 59, 138, 140, 125)	*Pichia fabianii* (145, 167, 70, 76, 1a, 2a, 4a, 6a1, 65, 6a2, 8a1, 8a2)	*Saccharomycopsis fibuligera* (3a, 66)	*Hanseniaspora opuntiae* (54)	*Aureobasidium pullulans* (3, 5, 6, 7, 9, 13, 10, 72, 16, 19, 20, 37, 73, 63,75, 119, 109, 99, 104, 56, 61, 74, 57, 58, 64)	*Candida glabrata* (133, 134, 147)
Fermentation of:							
Glucose	+	V	+	+	+	−	V
Sucrose	+	−	+	+	+	−	−
Maltose	+	nd	V	+	+	−	−
Galactose	+	−	−	nd	+	−	−
Raffinose	+	−	−	nd	−	−	−
Assimilation of:							
Glucose	+	+	+	+	+	+	+
Galactose	V	V	V	−	+	V	−
MDG	V	−	V	−	−	V	−
Cellobiose	−	V	+	+	−	+	−
Lactose	−	−	−	−	+	V	−
Maltose	+	−	V	+	+	+	−
Saccharose	+	−	+	+	+	+	−
Trehalose	V	−	+	−	+	+	V
Melezitose	V	−	+	−	+	+	−
Raffinose	V	−	+	−	+	+	−
L-lysine	−	+	+	+	nd	+	V
Cadaverine	−	+	+	+	nd	+	−
Creatine	V	V	+	+	nd	+	V
Growth at 37 °C	+	+	+	+	+	+	+

	Pichia ciferrii (115, 164, 169)	*Cryptococcus flavus* (111)	*Cryptoccus magnus* (71)	*Zygoascus hellenicus* (121, 122)	*Candida parapsilosis* (93)	*Candida pyralidae* (90)	*Rhodotorula mucilaginosa* (117)
Fermentation of:							
Glucose	+	−	−	+	+	−	−
Sucrose	+	−	−	+	−	−	−
Maltose	nd	nd	−	nd	−	nd	−
Galactose	−	nd	−	+	−	−	−
Raffinose	−	nd	−	nd	nd	nd	−
Assimilation of:							
Glucose	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+
MDG	−	+	+	−	+	−	−
Cellobiose	−	+	+	+	+	−	−
Lactose	−	+	+	−	−	−	−
Maltose	+	+	+	+	+	−	+
Saccharose	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+
Melezitose	+	+	+	−	+	−	+
Raffinose	+	+	+	−	+	−	+
L-lysine	+	nd	nd	+	−	+	nd
Cadaverine	−	nd	+	+	−	+	nd
Creatine	+	nd	+	+	−	+	nd
Growth at 37 °C	+	nd	−	W	W	W	+

Notes: nd = no data, w = weak, + = positive, − = negative test, v = variable, MDG = methyl-D-glucose. Results for macro and micro-morphology, acetic acid production, diazonium blue B test, glycerol, xylose, adonitol, xylitol, inositol, sorbitol, *N*-acetyl-glucosamine, imidazole, ethylamine, growth at 42 °C and 45 °C are not included in the table.

Table 3
The diversity of yeasts isolated from *masau* (*Z. mauritiana*) fruits and fermented *masau* fruit pulp from Muzarabani rural community in Zimbabwe

Yeast species	No of isolates detected in fruits			
	Unripe	Ripe	Dried	Fermented
Fermentative:				
S. cerevisiae	–	1 [a]	–	20
I. orientalis	–	3	–	22
P. fabianii	–	–	8	4
S. fibuligera	–	–	1	1
H. opuntiae	–	–	–	1
Non-fermentative:				
A. pullulans	19	1	6	–
C. glabrata	–	–	–	3
P. ciferrii	–	–	3	–
Cr. magnus	–	1	–	–
Cr. flavus	1	–	–	–
Z. hellenicus	–	2	–	–
C. parapsilosis	–	1	–	–
C. pyralidae	–	1	–	–
R. mucilaginosa	1	–	–	–

– = not found.
[a] Values are combined data from the two harvests.

fermentative and non-fermentative yeasts. These results represent yeast isolates selected from the two harvests and it was observed that *H. opuntiae*, *Cr. magnus*, and yeasts 002 and 008 were only encountered in the samples collected in 2004. *C. glabrata, P. ciferrii, Cr. flavus, C. parapsilosis, C. pyralidae, R. mucilaginosa, Z. hellenicus*, and the yeasts 92, 95, 97, 98 and 112 were found only in the samples collected in 2005. The reason why certain yeast species were not found in year one and others in year two could be that they were missed during the selection since they occurred less frequently when compared to the other yeast species found in both harvests. All the other species were common in both years. However, the comparison could not be done in terms of CFU's because in 2004 less of the cream colonies were picked for identification since they appeared the same on solid media, and in 2005 more cream colonies were picked for identification.

The fruit surface presents an environment of limited nutrient availability, depending on the concentration of sugars, organic acids and amino acids, which leach from the underlying tissue. Many factors affect the populations and community structure of yeasts that are present on any one type of fruit, including fruit cultivars, geographic location, and fruit developmental stage (Fleet, 2003). In this study the unripe fruit surface was colonised mainly by *A. pullulans*. Clark et al. (1954) and also Chand-Goyal and Spotts (1996), reported *A. pullulans*'s relative abundance as highest among the yeast/yeast like fungi colonising the surface of pears and apples. This fungus also causes russeting of apples (Okagbue and Siwela, 2002; Gildemacher et al., 2006). *A. pullulans* has also been isolated from tropical plant leaves, marula fruits, flowers, pulp and juices in Zimbabwe (Okagbue et al., 2001). It is an important microorganism in applied microbiology and biotechnology because of the extracellular enzymes it produces, that have industrial applications (Deshpande et al., 1992; Okagbue et al., 2001). Other yeast species which were identified on the unripe fruits included *Cr. flavus* and *R. mucilaginosa*. The ripe fruits were colonised by a wide range of yeasts species (Table 3) comprising fermentative as well as non-fermentative yeasts. On the ripe *masau* fruits, *S. cerevisiae* was found less frequently than in the fermented fruit pulp. The gradual transition from non-fermentative species in unripe fruits, through mixed populations in ripe fruit, towards predominantly fermentative species in fermented pulp is evident, and we assume that this is strongly related with the evolving degree of maturity and ensuing chemical composition and softening of consistency. This phenomenon is similar as in grape fermentation for wine production (Rosini et al., 1982; Heard and Fleet,

Table 4
Identification of yeasts isolated from *masau* (*Z. mauritiana*) fruits and fermented *masau* fruit pulp by D1/D2 domains of 26s rRNA (LSU) and ITS sequence analysis

Species and group no.	No of strains	ITS accession no.	LSU accession no.	% similarity of ITS sequences	% similarity of LSU sequences
S. cerevisiae					
Group 1 [a]	5	AB212260.1	AJ746340.1	99	100
Group 2	7	AB212260.1	AJ746340.1	99	99
Group 3	3	AB212260.1	AJ746340.1	99	100
Group 4	3	AB212260.1	AY601161.1	99	99
Group 5	2	DQ167471.1	AJ746340.1	99	99
Group 6	1	AB212260.1	Z7332.1	99	99
I. orientalis					
Group 1	9	AY939808.1	AY707865.1	100	100
Group 2	5	AY939808.1	AY601160.1	99	99
Group 3	3	AY939808.1	AY707865.1	100	99
Group 4	5	AY939808.1	AY707865.1	99	100
Group 5	3	AY939808.1	AY707865.1	99	99
P. fabianii					
Group 1	1	AF335967.1	AF335967.1	100	100
Group 2	1	AF335967.1	AF335967.1	99	99
Group 3	2	AF335967.1	AF335967.1	100	99
Group 4	7	AF335967.1	AF335967.1	99	100
Group 5	1	AF335967.1	AF335967.1	99	99
S. fibuligera					
Group 1	1	AF335940.1	U40088.1	99	100
Group 2	1	U410409.1	U40088.1	99	99
A. pullulans					
Group 1	13	AY225166.1	AF050239.1	99	100
Group 2	4	AY225166.1	AF050239.1	99	100
Group 3	3	AY225166.1	AF050239.1	99	100
Group 4	4	AY139394.1	AF050239.1	99	100
Group 5	1	Ay225166.1	AF050239.1	99	100
Group 6	1	AY625057.1	AY18811.1	99	96
C. glabrata					
Group 1	2	AY939749.1	AY198398.1	99	99
Group 2	1	nd	AJ617300.1	nd	100
P. ciferrii	3	nd	U74587.1	nd	100
Cr. flavus	1	AF444338.1	AF075497	nd	99
Cr. magnus	1	AF444450.1	AY362182.1	99	100
Z. hellenicus	2	AY447022.1	AY447006.1	99	100
C. parapsilosis	1	AJ49821.1	AY391843.1	99	99
C. pyralidae	1	AY013715	AY498864.1	98	99
H. opuntiae	1	nd	AY267820.1	nd	100
R. mucilaginosa	1	DQ386306.1	AB02610.2	98	99

nd = no data.
[a] The groups are based on the sequencing consensus similarity.

1985; Fleet, 2003). Difficulties to detect or isolate *S. cerevisiae* from either immature or mature grapes, have been reported elsewhere (Martini et al., 1996; Van der Westhuizen et al., 2000). *P. fabianii* was recorded highest on the dried fruits probably because it is able to thrive under reduced water activity. The fermented fruit pulp harboured a predominance of *S. cerevisiae*, *I. orientalis* and *P. fabianii* and other minor species like *S. fibuligera*, *C. glabrata* and *H. opuntiae*. These

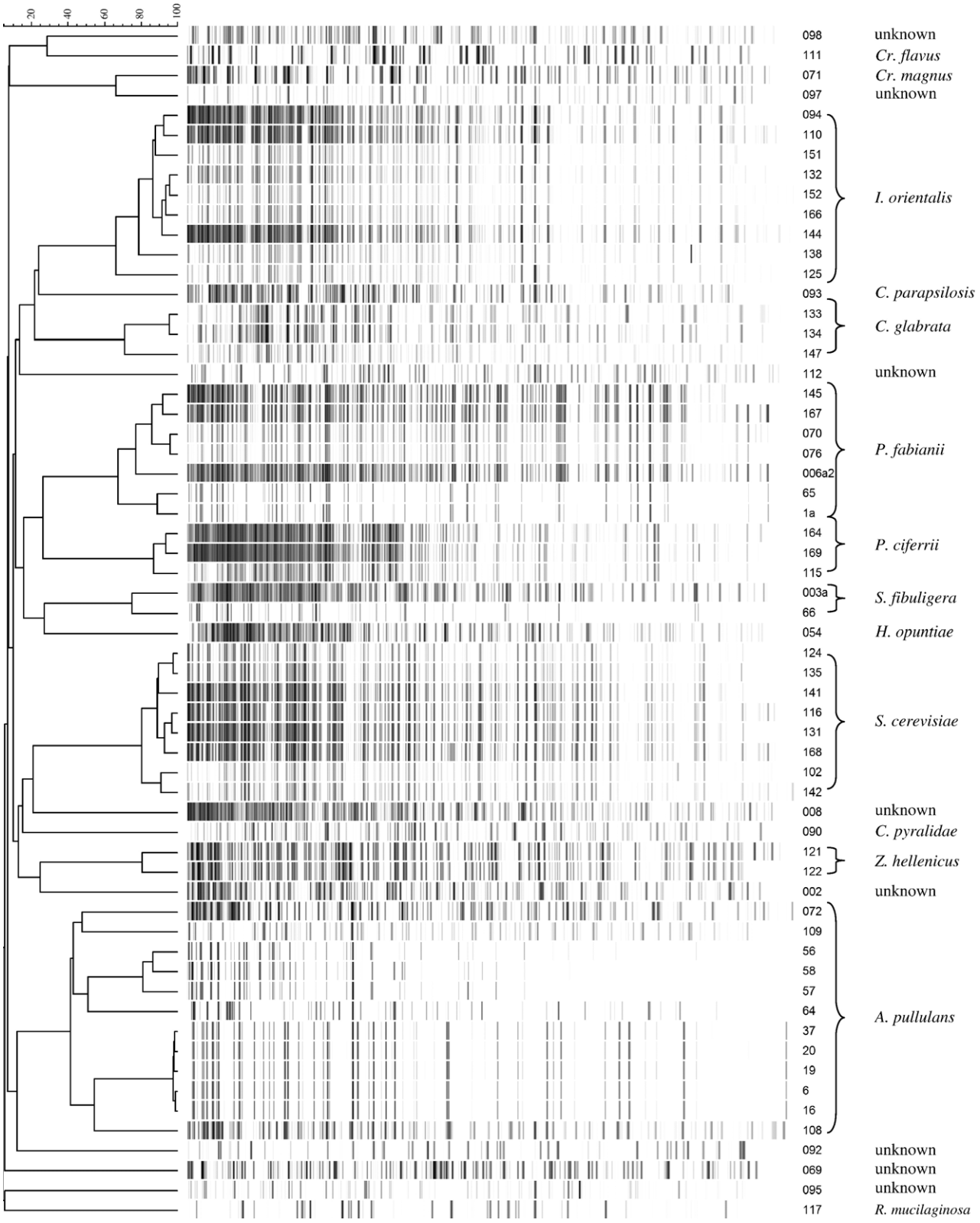

Fig. 1. AFLP dendrogram representing the identified and unidentified yeast species isolated from *masau* (*Z. mauritiana*) fruits and fermented fruit pulp.

are all fermentative yeasts, which have also been reported as predominating in traditional fermented foods such as Korean *nuruk*, Nigerian *fufu* and Indian *idli* (Nout, 2003).

The yeast species were grouped based on their physiological properties and molecular data obtained from sequencing of D1/D2 domains and ITS 1+2 regions as well as AFLP fingerprinting. The sequencing results in Table 4 show the accession number and percentage similarity for both the LSU and ITS of the different yeast species. The molecular identification techniques were used in combination with conventional methods of identification in order to confirm the identities and to assess relatedness among the yeast species. A dendrogram based on the AFLP analysis representing all yeasts species isolated is depicted in Fig. 1. The AFLP patterns clearly show that each species forms a distinct cluster. Common bands as well as different bands were observed thus showing the DNA polymorphisms among the isolates of the same yeasts species. This is particularly clear for isolates belonging to *A. pullulans*.

The biodiversity seen in our isolates of *S. cerevisiae* has also been observed in other studies on African indigenous fermented foods and beverages (Jespersen, 2003). Not all the strains had assimilation profiles typical for *S. cerevisiae* and this could probably be explained by the polymorphisms seen in Fig 1. Some of the strains were shown to be weak in fermenting both sucrose and galactose. Variability was also shown in acetic acid production, assimilation of carbon compounds (galactose, inositol, methyl α-D-glucoside, *N*-acetyl-glucosamine, saccharose, melezitose and raffinose) and growth at 42 °C and 45 °C. In total, six assimilation profiles were distinguished.

3.3. Lactic acid bacteria

Yeasts are not the only organisms of importance in the microbiology of fruit and fruit products. Bacteria, especially lactic acid bacteria (LAB) and acetic acid bacteria, are prominent in the spoilage of some fruits and fruit products, and certain species of LAB can have positive contribution in the production of wines (Fleet, 2003). In most indigenous fermentation processes, yeasts occur in association with LAB (Sanni, 1993; Gobbetti et al., 1994; Caplice and Fitzgerald, 1999; Fleet, 1999; Jespersen, 2003; Nout, 2003). This was also observed in this study whereby LAB and yeasts were found co-existing in *masau* fruits and the fermented fruit pulp.

Table 5
Lactic acid bacteria from *masau* (*Z. mauritiana*) fruits and fermented *masau* fruit pulp

Fruit	Lactic acid bacteria[a] (log CFU g^{-1})
Unripe (n=10)	<2
Ripe fruits (n=10)	2.99±0.07[b]
Dried fruits (n=10)	2.91±0.11
Fermented samples (n=14)	9.21±0.58

[a]Grown on MRS agar with 0.1% natamycin, incubated anaerobically, Gram-positive and catalase-negative, [b]Values are means±standard deviation of n determinants (two harvests) and each determinant was calculated as the average of duplicate counts.

LAB populations found on ripe and dried fruits were approximately 2.99 and 2.91 log CFU g^{-1} (Table 5). Just like the yeasts, the LAB multiply during fermentation and therefore larger numbers of LAB were found in fermented fruit pulp compared with the unprocessed fruits (Table 5).

We characterized only a number of representative and predominant LAB, collected from fruits harvested in two consecutive seasons; of each fruit type 10 samples were analysed in duplicate. LAB isolates from both years showed similar phenotypic properties.

The LAB found in ripe fruits were preliminary identified as *Lactobacillus agilis* (2 strains), *L. minor* (2 strains), *L. confusus* (1 strain) and *L. fructosus* (1 strain). In dried fruits *L. minor* (1 strain), *L. divergens* (2 strains) and 2 unidentified strains were found. The fermented fruit pulp harboured mostly *L. agilis* (9 strains) and *L. plantarum* (6 strains). The other species found in fermented fruit pulp included *L. bifermentans* (2 strains), *L. divergens* (2 strains), *L. fermentum* (1 strain), *L. hilgardii* (1 strain), *L. minor* (1 strain), *Streptococcus* spp. (2 strains) and 1 unidentified strain. The LAB results are preliminary being based on phenotypic properties, and need to be confirmed by molecular identification methods.

L. plantarum has been reported to be involved in many cereal-based African fermented foods such as *Ogi*, *Uji* and *Mahewu* (Halm et al., 1993; Sanni, 1993; Holzapfel, 1997; Oyewole, 1997) as well as other fermented foods (Hammes and Tichaczek, 1994; Caplice and Fitzgerald, 1999; Leisner et al., 1999). Certain strains of *Lactobacillus plantarum* and *L. agilis* have been reported to have probiotic effects (Lee and Salminen, 1995).

4. Conclusion

This study showed that there is a transition from the predominance of non-fermentative yeasts to fermentative yeasts species as the *masau* fruit matures until it is fermented. *A. pullulans,* a non-fermentative yeast-like fungus was dominant on the unripe fruit surface. The ripe and dried fruits carried a mixture of non-fermentative and fermentative yeasts. The fermentative yeasts *S. cerevisiae*, *I. orientalis* and *P. fabianii* were dominant in the fermented *masau* fruit pulp. These yeasts should be tested for their functionality during the fermentation, and some might be useful as starter culture to produce better quality fermented *masau* fruit products. The yeasts were found co-existing with LAB in the ripe fruits and the fermented fruit pulp; likewise, the functionality of LAB in the fermentation should be investigated, in view to develop mixed yeast-LAB starter cultures to produce a fermented fruit product of stable and consistent quality.

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