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Yeast diversity during tapping and fermentation of palm wine from Cameroon

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ABSTRACT

In the present study, we have investigated the occurrence of yeast flora during tapping and fermentation of palm wine from Cameroon. The yeast diversity was investigated using both traditional culture-dependent and culture-independent methods. Moreover, to characterize the isolates of the predominant yeast species (*Saccharomyces cerevisiae*) at the strain level, primers specific for δ sequences and mini-satellites of genes encoding the cell wall were used. The results confirm the broad quantitative presence of yeast, lactic acid bacteria and acetic acid bacteria during the palm wine tapping process, and highlight a reduced diversity of yeast species using both dependent and independent methods. Together with the predominant species *S. cerevisiae*, during the tapping of the palm wine the other species found were *Saccharomycodes ludwigii* and *Zygosaccharomyces bailii*. In addition, denaturing gradient gel electrophoresis (DGGE) analysis detected *Hanseniaspora uvarum*, *Candida parapsilopsis*, *Candida fermentati* and *Pichia fermentans*. In contrast to the progressive simplification of yeast diversity at the species level, the molecular characterization of the *S. cerevisiae* isolates at the strain level showed a wide intraspecies biodiversity during the different steps of the tapping process. Indeed, 15 different biotypes were detected using a combination of three primer pairs, which were well distributed in all of the samples collected during the tapping process, indicating that a multistarter fermentation takes place in this particular natural, semi-continuous fermentation process.

1. Introduction

Palm wine is made from the fermented sap of tropical plants of the *palmae* family, such as the oil palm (*Elaeis guineensis*), coconut palm (*Phoenix dactylifera*), date palm, nipa palm, kithul palm and raffia palm (*Raphia hookeri*) (Sanni and Lonner, 1993; Ejiofor et al., 1994; Ayogu, 1999; Nwachukwu et al., 2006). It is an alcoholic beverage that is produced and consumed in very large quantities in West Africa, and it is known throughout the major parts of Africa under various names, such as 'mimbo' in Cameroon, 'nsafufuo' in Ghana, an 'emu' in Nigeria (Jespersen, 2003). Thus, in various African countries and beyond, the sap of the palm tree is tapped and allowed to undergo spontaneous fermentation, which promotes the proliferation of yeast species for the conversion of the sweet substrate into an alcoholic beverage containing important nutritional components, including amino acids, proteins, vitamins and sugars (Okafor, 1972). The palm sap is obtained from either the immature male inflorescence (inflorescence tapping) or from the stem (stem tapping). This is commonly practised in Nigeria, Benin and La Cote d'Ivoire (Okafor, 1972). However, in Cameroon and Ghana, the process of tapping palm wine involves first felling or cutting down the tree, leaving the felled tree for a period of about 2 weeks for the sap to concentrate, followed by tapping for up to 8 weeks. If the palm wine is not consumed within a few days, it begins to develop a vinegary taste, which is unacceptable to consumers.

In African societies, palm wine has a significant role in customary practices. With the central role of this alcoholic beverage in traditional society, it is important that the microbiology and biochemistry of its fermentation process are well understood. However, like many other traditional fermented foods in Africa, the production of palm wine is by the age-old method of chance inoculation and uncontrolled fermentation. Therefore, the usual variations in the quality and stability of the product are not unexpected.

Studies over the years have been devoted to the isolation and identification of the microorganisms responsible for various fermentation processes. Yeasts have been reported to be involved in several different types of indigenous fermented foods and beverages. The yeast species most often reported in African indigenous fermented foods and beverages is *Saccharomyces cerevisiae*. However, in indigenous fermentation, *S. cerevisiae* often co-exists with other microorganisms. Lactic acid bacteria (LAB) are recognized as having important roles in the fermentation and

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preservation of a great variety of food and feed, while the roles of acetic acid bacteria (AAB) in the development of the vinegary taste in palm wine have also been investigated (Amoa-Awua et al., 2006). LAB and AAB have been found at high levels, while the yeast species *S. cerevisiae* and *Schizosaccharomyces pombe* have been reported to be the dominant yeast species (Odunfa and Oyewole, 1998); however, other yeast species have also been found, such as *Kloeckera apiculata*, *Candida krusei*, and other *Candida* spp., *Pichia* spp. (Atacador-Ramos, 1996; Amoa-Awua et al., 2006).

In recent years, culture-independent methods based on molecular biology techniques have been developed to study microbial population dynamics. Today, culture-independent methods are particularly attractive, as they provide a good and rapid strategy for yeast detection, and they represent a valid alternative to classical microbiological analyses. In addition, culture-independent analysis offers the possibility of detecting species that may be present in the habitat at viable, but non-culturable, levels (Head et al., 1997; Rappe and Giovannini, 2003; Ercolini, 2004). Indeed, classical microbiological methods based on plate counts, and isolation and biochemical identification have been criticized, since only easily culturable microorganisms can be detected, and members of microbial communities that need elective enrichment are not identified.

In the present study, we have investigated the occurrence of microflora in palm wine during the tapping of felled palm trees using both culture-dependent and culture-independent methods. Although significant progress has been made in defining the microflora associated with the fermentation of palm wine, the molecular characterization at the species and strain levels of the yeast diversity throughout the process of palm wine production is lacking.

2. Materials and methods

2.1. Samples

Fresh palm wine samples were obtained during the first five days of tapping of the oil palm tree (*E. guineensis*) growing in plantations in Odza (Cameroon, Central Africa), in the Summer (December–January) of 2006–2007. The sap samples were collected at 24-h intervals during the natural fermentation, from zero time to the fifth day (A, B, C, D, E, and F). They were collected in plastic containers, through a bamboo tube that was inserted in an incision made into the interior of a felled tree. Approximately 50 ml of palm sap samples were tapped, placed aseptically in sterile plastic bags, and transferred for analysis in the laboratory at the Department of SAIFET, Food, Industrial and Environmental Microbiology Section, Polytechnic University of Marche, Italy, in ice boxes (4 °C).

Within 24–36 h of their collection and transfer to the laboratory, 1 ml liquid samples were placed in tubes containing 9 ml sterile water, and then standard procedures for serial dilutions and microbiological examination were carried out in duplicate.

2.2. Media and direct microorganism isolation procedures

The yeast and bacteria isolation and enumeration were performed in different media: (i) for yeasts: WL nutrient agar (Oxoid, Basingstoke, UK); (ii) for LAB: MRS agar (Oxoid) supplemented with cycloheximide 0.005% (Sigma–Aldrich, St. Louis, USA), to suppress the growth of yeast; (iii) for AAB: GEY agar composed of 2% D-glucose, 5% ethanol, 1% yeast extract and 2% $CaCO_3$ supplemented with cycloheximide 0.005% (Sigma–Aldrich), to suppress the growth of yeast.

The LAB were enumerated by surface inoculation on MRS agar plates incubated anaerobically in jars, while the AAB were incubated under aerobic conditions. All of the plates were incubated at 30 °C for 3–8 days. After this, the colonies of yeasts and bacteria were counted, selected according to their macro-morphological and micro-morphological aspects, and isolated in proportion to their frequencies (Martini et al., 1996). Representative colonies (10–15) were picked randomly from the plates and subjected to the identification procedures. Isolates of bacteria (LAB or AAB) were confirmed using the catalase test: the presence of catalase activity was assessed by the formation of gas bubbles after the suspension of bacterial cells in a droplet of 3% hydrogen peroxide on MRS.

2.3. Yeast identification

In all of the yeast isolates, after an initial micro-morphological characterization, the DNA was extracted. Pure yeast cultures were pre-grown on YPD agar at 25 °C for 3 days. The cell mass was then transferred to screw-cap tubes with 300 μl reaction buffer, containing 0.1 M Trizma, pH 8.0, 50 mM EDTA, 1% SDS and 0.3 g glass beads (∅ 0.45–0.50 mm) (Sigma–Aldrich). The tubes were vortexed 3 times for 1 min each at the highest setting. The mixtures were then boiled for 10 min and transferred to ice for 3 min. For each sample, 20 μl 1 M Tris–HCl, pH 8.0, 15 μl 0.5 M EDTA, pH 8.0, 50 μl 10% SDS and 200 μl 5 M potassium acetate were added. The samples were then incubated for 30 min in ice, and finally centrifuged at 18,000 × *g* for 10 min. The supernatants were transferred to new screw-cap tubes, with the addition of an equal volume of ice-cold isopropanol. The precipitated nucleic acids were collected after an incubation of 5 min, and the pellet was washed in 500 μl ice-cold 70% ethanol, and centrifuged at 18,000 × *g* for 5 min. The DNA obtained was dried overnight, and resuspended in 100 μl 0.1 M TE buffer, pH 8.0, and the DNA template was then incubated at 45 °C for 15 min. After this incubation, the samples were stored at −20 °C and analysed within 2 weeks.

The isolates were identified using the internal transcribed spacer (ITS)-PCR procedure, using the primers ITS1: 5′-TCC GTA GGT GAA CCT GCG G-3′; and ITS4: 5′-TCC TCC GCT TAT TGA TAT GC-3′. The PCR mixture and the thermocycling protocol conditions were applied as described by Esteve-Zarzoso et al. (1999). The amplified DNA was digested without further purification, using three restriction endonucleases: CfoI, HaeIII and HinfI (AB Fermentas Int. INC, Canada). The amplified products (ITS-PCR) and their restriction fragments were separated on a horizontal electrophoresis apparatus (Bio-Rad, Hercules, USA) in 1.5% and 2.5% (w/v) agarose gels, respectively, both in 0.5 × TBE buffer. The gels were stained with ethidium bromide and visualized under UV light (UV source GelDoc 1000, Bio-Rad). The fragment sizes were estimated by comparisons with a DNA standards marker (GeneRuler 100-bp DNA Ladder) (AB Fermentas), and the restriction patterns were compared with previously published studies (Esteve-Zarzoso et al., 1999, 2001; Sabate et al., 2002; de Llanos Frutos et al., 2004; Arroyo-Lòpez et al., 2006).

2.4. Chemical analysis

The reducing sugars, alcohol content and volatile acidity of the palm wine samples were also determined, following the official methods for wines of the European Community (2000). The pH measures were performed directly, using a pH meter (Jenway, Barloword Scientific Ltd, UK).

2.5. Yeast population detection from a matrix by PCR–DGGE

PCR–denaturing gradient gel electrophoresis (DGGE) analysis was performed using the methods described by Cocolin et al.