

Full Length Research Paper

Bacterial dynamics and chemical changes during the spontaneous production of the fermented porridge (*Calugi*) from cassava and corn

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A traditional fermented porridge made from maize, cassava and rice, named *calugi*, was microbiologically and chemically analyzed. Our results show that aerobic mesophilic bacteria reached values of approximately $7 \log \text{CFU ml}^{-1}$ and were the predominant group; acetic acid bacteria were also observed at the onset of fermentation ($3.82 \log \text{CFU ml}^{-1}$). The population of lactic acid bacteria was $3.7 \log \text{CFU ml}^{-1}$ and remained constant throughout the fermentation. The denaturing gradient gel electrophoresis (DGGE) bands were identified as *Corynebacterium variabile*, *Lactobacillus paracasei*, *L. plantarum*, *L. casei*, *Bacillus* spp. (*Bacillus cereus* group), *B. subtilis*, *Streptomyces* sp., *Enterobacter cloacae*, *Streptococcus parasanguis*, *Streptococcus salivarius*, *Weissella cibaria* and *Weissella confusa*, in addition to uncultivable bacteria. The most abundant carbohydrate in the substrate was maltose (38 g L^{-1} at the beginning of fermentation). Acetic and lactic acids were the principal acids found by high performance liquid chromatography (HPLC) (0.66 g L^{-1} and 3.54 g L^{-1} , respectively). Twenty-one (21) minor compounds were identified by gas chromatography during the fermentation process. The compounds present at the highest concentrations were furfuryl alcohol, nonanoic acid, decanoic acid and 1,1-dietoxyethane. These appeared to be related to the flavor of the *calugi*.

Key words: Indigenous food, cereal fermentation, cassava, corn, volatile compounds, denaturing gradient gel electrophoresis (DGGE), saliva.

INTRODUCTION

Indigenous fermented foods are of worldwide interest and consequently have provided new avenues for scientific research in the past. Several Amerindian tribes in Brazil (*Araweté*, *Kayapó*, *Karajá*, *Javaé*, *Juruna* and *Tapirapé*) use small-scale fermentation to produce foods and beverages with high nutritional value, which also have medical and religious significance (Wagley, 1988). These indigenous groups have little contact with external cultures; therefore, investigating Brazilian indigenous food is not easy (Santos et al., 2012). Nevertheless, the study of these beverages and fermented foods is necessary for describing the empirical knowledge of indigenous people

and to contribute information to improve the food safety of these products.

Some substrates, such as cottonseed, rice, peanut, cassava and sweet potato, are used as raw materials in the fermentation and production of these traditional foods and beverages in Brazil and Africa (Almeida et al., 2007; Oguntoyinbo and Dodd, 2010; Ramos et al., 2010, 2011; Santos et al., 2012; Schwan et al., 2007). Studies characterizing the microbiota of various traditional products revealed a great diversity of bacteria involved in fermentation. According to Almeida et al. (2007) *Lactobacillus*, *Bacillus*, *Corynebacterium* and *Enterobacter*

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were identified in the beverage *cauim*, produced from cassava and rice, in which the species *Lactobacillus pentosus* and *L. plantarum* were the dominant bacteria. Ramos et al. (2010, 2011) showed that the *Lactobacillus* genus was dominant throughout fermentation, and *L. plantarum*, *L. fermentum*, *L. paracasei* and *L. brevis* were the principal microorganisms found during the fermentation of peanuts, cottonseeds and rice. The microbiota of *caxiri*, an indigenous alcoholic beverage based on cassava and sweet potato, was described and found that the bacteria were mainly represented by *Bacillus pumilus*, *Bacillus* spp. (*Bacillus cereus* group) and *B. subtilis* (Santos et al., 2012).

For many years, research on fermented foods and beverages was based on culture-dependent methods, followed by genotypic/phenotypic identification. Later, the culture-independent methods (PCR-DGGE) were added to the culture-dependent methods of identification. DGGE analysis has been widely used to study complex microbial communities originating from food samples, such as *doenjang* (Kim et al., 2009), fermented cassava (Miambi et al., 2003), *gari* (Oguntoyinbo and Dodd, 2010), corn and rice *calugi* (Miguel et al., 2012) and *cauim* made from cottonseeds, rice and peanuts (Ramos et al., 2010, 2011).

Calugi is a non-alcoholic food produced by the Brazilian Indians of the *Javaé* tribe using corn, cassava and rice as the raw materials. The inoculum is the fluid that is obtained by the chewing of sweet potatoes by the women (Almeida et al., 2007). The *calugi* is usually consumed in daily meals by adults and children. To identify and report an empirical fermentation used by Brazilian natives for food production and to avoid the loss of indigenous knowledge with acculturation caused by globalization, samples of *calugi* were collected and studied. Thus, the aims of this work were to evaluate the profile of organic acids, sugars, ethanol and volatile compounds during *calugi* fermentation produced from cassava and corn by gas chromatography-flame ionization detection (GC-FID) and high-performance liquid chromatography (HPLC) and furthermore, evaluate the dynamic bacterial using a culture-independent approach (PCR-DGGE).

MATERIALS AND METHODS

Calugi preparation

Calugi is prepared by the *Javaé* people who inhabit the region along the *Javaé* River, the smaller affluent of the *Araguaia* River, and who are distributed in villages in the state of Tocantins (located in northern Brazil). This porridge was prepared in the traditional manner using cassava, corn and sweet potato (Figures 1 and 2). To prepare 10 L of *calugi*, approximately 700 g of dried corn (*Zea mays*), 6 kg of cassava tubers (*Manihot utilissima*) and 100 g of sweet potato (*Ipomoea batatas* L.) were used. The cassava tubers were peeled, washed and grated to obtain a wet mass. The resulting mass was squeezed, producing consistent dumplings that were used as the substrate for fermentation. The corn was soaked in water for 30 min and then was macerated using a rustic wooden

mortar and pestle. The resulting corn flour was mixed with 3 L of water and sieved to remove the peel. The cassava and corn masses were mixed with 7 L of water and cooked for 2 h, with stirring every 10 min. The sweet potato was peeled and washed before use. Approximately, 40 min after cooking (when the porridge had cooled), the inoculum (the mastication juice of the sweet potato) was added. Then, the mixture was homogenized and allowed to ferment in open aluminum vessels at the ambient temperature (approximately 30°C). The diagram of traditional production methods (Figures 1 and 2) shows the preparation of the *calugi* porridge and the inoculation process, which are non-sterile. Four fermentations were performed in the same conditions described above for 48 h each.

Sampling

Samples were taken in duplicate. For each sample collected at 12 h intervals, 20 mL of fermenting substrate was added to sterile empty bottles (physicochemical analysis) and 20 mL was added to sterile bottles containing 180 mL of saline peptone diluent (0.1% peptone (Himedia, Mumbai, India), 0.5% NaCl (Merck, Darmstadt, Germany), 0.03% Na₂H₂PO₄ (Merck), 20% glycerol (Himedia) for determination of microbiota and were frozen until analysis. Serial dilutions (10⁻² to 10⁻⁷) were prepared in saline peptone water after mixing in a Stomacher circulator at normal speed for 60 s.

Enumeration, isolation, purification and maintenance of microorganisms

Microorganisms were counted using four different culture media: nutrient agar medium (Merck) was used as a general medium for the viable aerobic mesophilic bacteria (AMB) population, the plates were incubated for three days at 30°C. Violet red bile agar with glucose (VRBG) (Oxoid, Hampshire, England) was used for bile-tolerant Gram-negative *Enterobacteriaceae* (GNE), Acetic acid bacteria (AAB) were enumerated by surface inoculation on GYC agar (5% glucose (Merck), 1% yeast extract (Merck), 3% calcium carbonate (Merck), 2% agar (Merck), pH=5.6), containing 0.1% cycloheximide to inhibit yeasts growth and 50 mg/liter penicillin to inhibit LAB growth. VRBG and GYC agar were incubated at 37°C for 24-48 h and at 25°C for 5-8 days, respectively. De Man Rogosa Sharpe (MRS, Merck) agar containing 0.1% cysteine-HCl was used for LAB count. MRS plates were incubated in acrylic anaerobic jars at 30°C for three to four days. Subsequently, the morphological characteristics of each colony type were recorded and counts made for each type. For each colony type, a number of colonies corresponding to the square root of the number of colonies of that type were restreaked and purified. For long term storage, purified isolates were stored at -80°C.

PCR amplification and DGGE conditions

The initial step in the extraction of total DNA from samples was performed according to the protocol described by Ramos et al. (2010). The DNA was extracted using the QIAamp DNA Mini Kit - DNA Purification from Tissues (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. The samples were stored at -20°C until further use. The DNA from the bacterial community was amplified with primers 338fgc (5'-CGC CCG CCG CGC GCG GCG GGC GGC GGC GGC GCA CGG GGC GAC TCC TAC GGG AGG CAG CAG-3') (the GC clamp is underlined) and 518r (5'-ATT ACC GCG GCTGCT GG-3'), which span the V3 region of the 16S rRNA gene (Ovreas et al., 1997). The PCR mix (25 µL) was composed by: 0.625 U Taq DNA polymerase (Promega, Milan, Italy), 2.5 µL 10X buffer, 0.1 mM dNTP, 0.2 mM

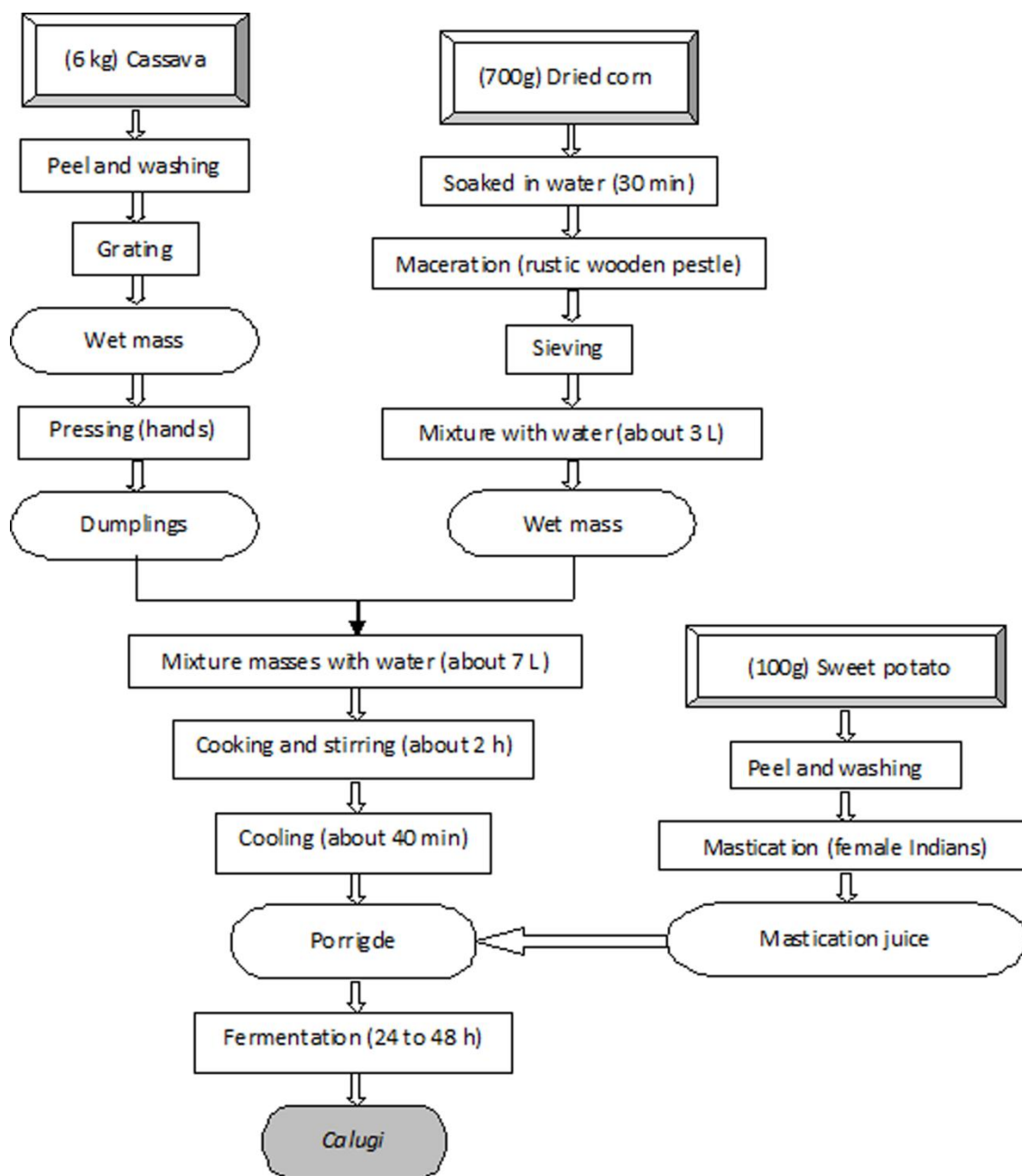


Figure 1. A flow diagram for the processing of cassava roots, dried corn and sweet potato into *calugi*.

of each primer, 1.5 mM MgCl₂ and 1 μL of extracted DNA. The amplification was performed according to Ramos et al. (2010); aliquots (2 μL) of the amplified products were analyzed by electrophoresis on 1% agarose gels before they were used in DGGE analysis.

The PCR products were separated using DGGE (BioRad Universal Dcode Mutation Detection System, USA). Samples were directly loaded into an 8% polyacrylamide gel with 0.5x TAE and a denaturant gradient between 35 and 70%. The gradient was created using polyacrylamide containing 0-100% of the denaturant (7 M urea and 40% of formamide). Electrophoresis was performed at a constant voltage of 70 V for 16 h at a constant temperature of 60°C. After electrophoresis, the gels were stained for 30 min in a

SYBR Green I solution (Molecular Probes, Eugene, UK) (1:10.000 v/v) and analyzed with UV transillumination. The gel images were photographed using Lpix Image (LTB 20x20 HE, LPix®, Brazil).

Identification of DGGE bands

The identity of selected bands in the DGGE profiles was revealed by sequencing. Fragments of selected DNA bands were excised from the gels, reamplified and sequenced by MacroGen Inc. (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer. The sequences were compared to the GenBank database using the BLAST algorithm (National Center for Biotechnology Information,



Figure 2. Steps in the preparation of *calugi*: peeling and removing the unwanted parts (A), washing the roots (B), grating cassava to obtain a wet mass (C), maceration of corn in a rustic wooden pestle (D), sieving (E), mixing with water and sieving to remove the peel (F), inoculation with mastication juice of the sweet potato (G) and fermented *calugi* (H).

Maryland, USA). Using this technique, it is possible to detect uncultivable bacteria (viable but non-culturable bacteria).

Chemical analysis

The pH, starch and protein contents of *calugi* were determined using the methodology proposed by AOAC (2000).

Chromatographic analysis

HPLC analysis

Carbohydrates (glucose, sucrose, maltose and fructose), organic acids (acetic acid, lactic acid, malic acid, citric acid, propionic acid, and succinic acid) and alcohols (ethanol, methanol and glycerol) were identified according to methodology proposed by Duarte et al. (2010). The analyses were carried out using a high performance liquid chromatography system (HPLC) (Shimadzu, model LC-10Ai, Shimadzu Corp., Japan), equipped with dual detection system consisting of a UV-Vis detector (SPD-10Ai) and a refractive index detector (RID-10Ai). A Shimadzu ion exclusion column (Shim-pack SCR-101 H, 7.9 mm x 30 cm) was used operating at 50°C for acids, and at 30°C for ethanol, methanol and glycerol, using 100 mM of perchloric acid as the eluent at a flow rate of 0.6 ml min⁻¹. The acids were detected via UV absorbance (210 nm), while alcohols were detected via RID. For carbohydrates we used the column Supelcosil LC-NH₂ (4.6mm x 25 cm) operating at 30°C with acetonitrile:water (75:25) as mobile phase in a flow rate of 1ml min⁻¹. The sugars were detected via RID. All samples were examined in triplicate, and individual compounds were identified based in the retention time of standards injected at the same conditions and their concentrations were determined using external calibration method. Calibration curves were constructed by injecting different concentrations of the standards under the same conditions for sample analysis and the areas obtained was plotted a linear curve whose equation was used to estimate the concentration of the compounds in the sample.

GC-FID analysis

The extraction of minor volatile compounds was performed according to the methodology proposed by Duarte et al. (2010). Extracts of volatile compounds were analyzed by gas chromatography (GC) using a Shimadzu GC model 17A, equipped with a flame ionization detector (FID) and a capillary column DB Wax (30 m x 0.25 mm i.d x 0.25 µm) (J & W Scientific, Folsom, Calif., U.S.A.). The operating conditions were: the oven temperature was maintained at 50°C for 5 min, raised to 190°C by increments of 3°C min⁻¹ and then kept at 190°C for 10 min. Injector and detector temperature were kept at 230 and 240°C respectively; the carrier gas (N₂) was kept at a flow rate of 1.2 mL min⁻¹. Injections of 1 µL were made in the split mode (1:10). The identification of volatile compounds was done by comparing the retention times of each compounds with those of standard compounds injected at the same conditions. The quantification of the volatile compounds was expressed as 4-nonanol (312 µg L⁻¹ as internal standard) equivalents.

Statistical analysis

Principal component analyses were performed using the software XLSTAT 7.5.2 (Addinsoft's, New York, N.Y., U.S.A.) for the group data of the microbial counts, the carbohydrates present in the porridge and the metabolites produced during *calugi* fermentation.

RESULTS

Microbial count

The dynamics of the microbial groups responsible for the fermentation of cassava, corn and sweet potato to produce *calugi* was analyzed. Throughout the *calugi* fermentation process, the predominant microorganisms were aerobic mesophilic bacteria (AMB). The population of AMB increased from 3.92 log to 6.95 log CFU mL⁻¹ at the beginning (12 h) of fermentation. However, in the late stages of fermentation, the AMB counts showed a declining tendency, decreasing to 4.47 log CFU mL⁻¹. The population of AAB decreased from 3.82 log CFU mL⁻¹ at the onset of fermentation to 1.5 log CFU mL⁻¹ at 24 h, and after this time, there were no significant changes. The LAB count was approximately 3.7 log CFU mL⁻¹ in all stages of fermentation. The Gram-negative *Enterobacteriaceae* were detected on VRBG agar, and the counts ranged from >1 log CFU mL⁻¹ to 2 log CFU mL⁻¹.

Microbial community by PCR-DGGE

Additionally to the microbial counts, PCR-DGGE was also used to analyze the profile of bacteria population in the cassava, corn and sweet potato porridge. The DGGE fingerprint is shown in Figure 3, and the results of the band sequencing are reported in Table 1. The sequencing for the identification of the bands revealed a 96 - 100% similarity with sequences in the GenBank database. In the first sampling time (T0), the identified bacteria were *Corynebacterium* variable (band 1), *L. paracasei*, *L. plantarum*, *L. casei* (bands 2, 3 and 4), uncultured *Geobacteraceae* bacterium (band 5), *Bacillus* spp. (*B. cereus* group) (band 6), uncultured bacteria (bands 7, 8 and 9), *Streptomyces* sp. (bands 10 and 12), *Bacillus* sp. (band 11), *W. cibaria*, *W. confusa* (bands 15 and 16), and *S. salivarius* (band 18).

The species *W. cibaria* and *W. confusa*, in addition to *E. cloacae*, *B. subtilis*, and *S. salivarius* (bands 13, 17 and 18), were detected after 12 h and could be observed until 36 h of fermentation. After 48 h of fermentation, a lower diversity was observed: *S. parasanguis* (band 14), *Bacillus* spp. (*B. cereus* group) (band 6), *W. cibaria*, *W. confusa* and *S. salivarius* (bands 13, 17, and 18) were identified. The bands corresponding to *L. casei* (band 4), uncultured bacterium (band 5), *B. cereus* (band 6), uncultured bacteria (bands 7 and 8), *W. cibaria* (band 15), *W. confusa* (band 16) and *S. salivarius* (band 18) were found at all sampling times during the fermentation process.

Physicochemical and chromatographic analysis

The pH value dropped from 6.20 to 4.03, indicating the spontaneous acidification of the product. The protein

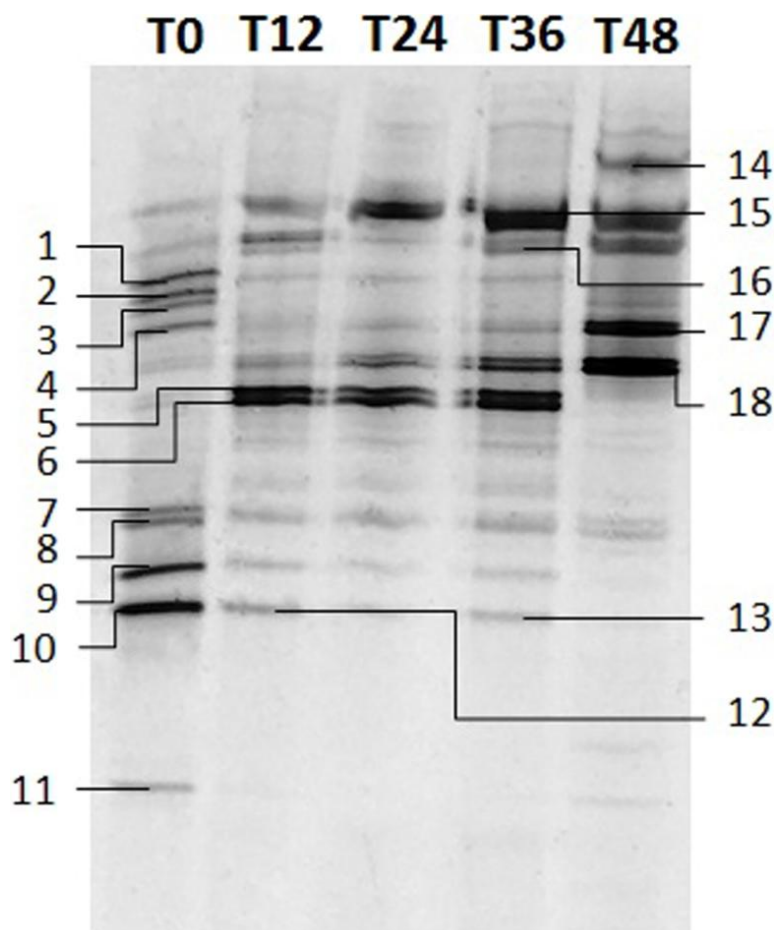


Figure 3. Denaturing Gradient Gel Electrophoresis profiles of bacterial V3 regions of the 16S rRNA gene amplified from *calugi* beverage throughout the 48 h of fermentation. The identification of the bands is reported in Table 1. Bands indicated by numbers were excised and after reamplification they were subjected to sequencing.

content increased, and decreasing concentrations of soluble starches were found throughout the fermentative process (Table 2).

Maltose was the most abundant disaccharide throughout the fermentation process, and its concentration reached a peak of 55.70 g L^{-1} at 12 h of fermentation (Table 2). Glucose was found during the fermentation with the highest concentration (1.29 g L^{-1}) measured at 12 h of fermentation. Fructose concentration increased within 12 h, but later, this carbohydrate was detected in decreasing amounts. Sucrose content was continuously consumed during fermentation and after 24 h it was no longer detected.

The acids detected at the beginning of *calugi* fermentation were citric acid (0.87 g L^{-1}), malic acid (0.92 g L^{-1}) and acetic acid (1.70 g L^{-1}). Acetic acid was the main metabolite produced at the onset of fermentation, and its concentration reached 5.47 g L^{-1} at 12 h. However, just 0.65 g L^{-1} of this acid was detected at 48 h. An increased

production of lactic acid occurred until the end of fermentation; this was observed after 24 h, reaching 3.54 g L^{-1} at 48 h. During the fermentation period, the amounts of malic, succinic, citric and propionic acids decreased, while the concentrations of oxalic acid remained stable. Tartaric acid was found from 24 h and was detected at low concentrations.

The ethanol production during *calugi* fermentation was low (0.4 g L^{-1}) at the end of the fermentation process. The glycerol concentration increased after 12 h and at the end of the process, it was 3.0 g L^{-1} . The methanol content at 12 h was 0.23 g L^{-1} and 0.1 g L^{-1} at the end of the fermentation.

GC-FID Analysis

Twenty-one (21) compounds were identified and quantified during the *calugi* fermentation, including two alco-

Table 1. Identification based on BLAST comparison in GenBank, of the bands obtained by PCR-DGGE using universal primers for bacteria.

Band(s) ^a	Close relative	% similarity	Source ^b
1	<i>Corynebacterium variabile</i>	97	CP 002917.1
2	<i>Lactobacillus paracasei</i>	99	FJ 861111.1
3	<i>Lactobacillus plantarum</i>	100	EF 426261.1
4	<i>Lactobacillus casei</i>	99	EF 348441.1
5	<i>Uncultured Geobacteraceae bacterium</i>	96	EF 668416.1
6	<i>Bacillus cereus</i>	100	HQ 333012.1
7	<i>Uncultured bacteria</i>	98	DQ 635035.1
8	<i>Uncultured bacteria</i>	97	AB 241206.1
9	<i>Uncultured bacteria</i>	99	FN 780902.1
10, 12	<i>Streptomyces sp.</i>	99	HM 018105.1
11	<i>Bacillus sp.</i>	98	AY 176766
13	<i>Enterobacter cloacae</i>	97	HM 438951.1
14	<i>Streptococcus parasanguis</i>	99	HQ 333012.1
15	<i>Weissella cibaria</i>	100	HM 369807.1
16	<i>Weissella confusa</i>	100	HM 032145.1
17	<i>Bacillus subtilis</i>	97	GI 10967638.1
18	<i>Streptococcus salivarius</i>	99	GU 425986.1

^aBands are numbered as indicated on the DGGE gel shown in Figure 3. ^bAccession number of the sequence of the closest relative found by BLAST search.

Table 2. Physical and chemical changes in *calugi* during 48 hours of fermentation.

Parameter	Fermentation time (hours)				
	0	12	24	36	48
pH	6.20 ± 0.13	5.02 ± 0.05	4.59 ± 0.05	4.32 ± 0.10	4.03 ± 0.10
Protein (%)	13.38 ± 0.01	15.13 ± 0.04	15.31 ± 0.07	15.75 ± 0.01	16.36 ± 0.00
Starch (%)	3.51 ± 0.01	3.46 ± 0.01	3.35 ± 0.08	3.14 ± 0.01	2.97 ± 0.00
Compounds (g/L)^a					
Glucose	0.60 ± 0.03	1.29 ± 0.29	0.17 ± 0.01	0.38 ± 0.01	0.53 ± 0.10
Fructose	0.40 ± 0.05	2.11 ± 0.60	0.79 ± 0.06	0.70 ± 0.01	0.32 ± 0.06
Sucrose	2.79 ± 0.23	0.71 ± 0.16	nd	nd	nd
Maltose	38.00 ± 3.76	55.70 ± 1.61	37.81 ± 2.02	53.44 ± 3.17	49.01 ± 1.24
Glycerol	0.02 ± 0.03	0.13 ± 0.00	2.53 ± 0.01	2.83 ± 0.04	2.98 ± 0.05
Methanol	0.14 ± 0.02	0.23 ± 0.00	0.09 ± 0.01	0.09 ± 0.00	0.10 ± 0.00
Ethanol	0.26 ± 0.01	0.26 ± 0.00	0.36 ± 0.00	0.38 ± 0.01	0.40 ± 0.01
Acetic acid	1.70 ± 0.11	5.47 ± 0.19	0.66 ± 0.01	0.61 ± 0.00	0.66 ± 0.10
Lactic acid	0.00 ± 0.00	0.07 ± 0.00	2.74 ± 0.03	3.20 ± 0.00	3.54 ± 0.70
Citric acid	0.87 ± 0.06	0.73 ± 0.05	nd	nd	nd
Malic acid	0.92 ± 0.07	0.79 ± 0.03	0.14 ± 0.00	0.13 ± 0.00	0.10 ± 0.05
Succinic acid	0.44 ± 0.02	0.40 ± 0.01	0.17 ± 0.00	0.14 ± 0.00	0.14 ± 0.02
Propionic acid	0.64 ± 0.05	0.48 ± 0.02	0.62 ± 0.01	0.60 ± 0.02	0.60 ± 0.10

^aDetermined by HPLC analysis; ^bNot detected; The data are means of four replicates; ± standard deviation.

hols, three acetates, five acids, five aldehydes, two ethyl esters, three terpenoids and one acetal (Table 3). The identified volatile acids were butyric, hexanoic, octanoic, nonanoic and decanoic acids. Butyric acid was measured

only at 0 h, while octanoic and hexanoic acid were detected at 24 and at 36 h of fermentation, respectively. Nonanoic and decanoic acids were the most abundant compounds at the beginning of fermentation with concen

Table 3. Concentration of volatile compounds ($\mu\text{g/liter}$) identified during *calugi* fermentation.

Group	Compound ($\mu\text{g/L}$)	Fermentation time (h)				
		0	12	24	36	48
Alcohols (2)	Furfuryl alcohol	20398.15 \pm 12.8	230.35 \pm 7.3	126.35 \pm 5.4	58.29 \pm 3.0	nd
	1,3 butanediol	759.32 \pm 3.2	nd	nd	nd	nd
	Ethyl acetate	nd	15.93 \pm 4.4	8.78 \pm 2.7	28.53 \pm 0.7	9.16 \pm 0.3
Acetates (3)	Isobutyl acetate	nd	53.06 \pm 8.0	148.25 \pm 5.2	114.30 \pm 19.8	28.83 \pm 0.8
	Phenylethyl acetate	783.83 \pm 2.6	nd	nd	nd	nd
	Butyric acid	1222.85 \pm 4.5	nd	nd	nd	nd
	Hexanoic acid	nd	nd	nd	24.11 \pm 0.2	nd
Acids (5)	Octanoic acid	nd	nd	63.58 \pm 3.7	35.04 \pm 4.6	39.87 \pm 9.0
	Nonanoic acid	580.18 \pm 3.7	80.33 \pm 2.2	258.05 \pm 7.6	22.89 \pm 0.7	36.26 \pm 1.8
	Decanoic acid	475.91 \pm 7.4	27.17 \pm 0.3	141.03 \pm 7.4	33.98 \pm 6.8	51.75 \pm 9.7
	Acetaldehyde	nd	55.17 \pm 6.0	45.10 \pm 4.9	102.39 \pm 5.9	56.51 \pm 4.5
	Hexanal	nd	11.59 \pm 0.9	nd	nd	nd
Aldehydes (5)	Octanal	nd	50.36 \pm 6.2	nd	nd	nd
	Furfural	1273.98 \pm 5.5	68.81 \pm 1.3	nd	nd	nd
	Decyl aldehyde	246.73 \pm 4.3	16.77 \pm 3.7	nd	nd	nd
	Ethyl butyrate	nd	6.42 \pm 0.3	nd	nd	nd
Ethyl esters (2)	Diethyl malate	115.47 \pm 7.4	nd	nd	nd	nd
	α -Terpeniol	143.49 \pm 2.4	nd	nd	nd	nd
Terpenes (3)	b-Citronellol	866.91 \pm 6.0	77.03 \pm 6.8	nd	nd	nd
	Guaiacol	126.09 \pm 7.8	nd	nd	nd	nd
Others (1)	1,1-dietoxyethane	36.13 \pm 1.1	18.29 \pm 6.3	72.21 \pm 4.3	89.12 \pm 5.2	82.14 \pm 4.3

Nd, not detected.

trations of 580.18 and 475.91 $\mu\text{g L}^{-1}$, respectively.

The furfuryl alcohol was found during the entire fermentation process at decreasing concentrations. The highest amount of this compound was 20,398.15 $\mu\text{g L}^{-1}$ at the initial time of fermentation (Table 3). Five aldehydes were identified during the *calugi* fermentation: acetaldehyde, hexanal, octanal, furfural and decyl aldehyde. Furfural was detected throughout fermentation, and its highest concentration (1,273.98 $\mu\text{g L}^{-1}$) was found in the T0 sample. Acetaldehyde and ethyl acetate were found from 12 h of fermentation. Isobutyl acetate was detected (28.83 $\mu\text{g L}^{-1}$) at 48 h of fermentative process.

Two ethyl esters were detected in the *calugi*; these compounds were identified as ethyl butyrate and diethyl malate. The latter was found in considerable amounts (115.47 $\mu\text{g L}^{-1}$) at 0 h of fermentation. In the group of terpenes, α -terpeniol, b-citronellol and guaiacol were identified. These compounds were detected only at the beginning of fermentation.

Multivariate analysis

The principal component analysis (PCA) was performed to determine the relationship between the microbial population, the consumption of the substrates and some fermentation products to show which compounds may be

directly related to a specific microbial group (Figure 4). Principal components 1 (PC1) and 2 (PC2) together explained 88.02% of the total variance. The lower left quadrant (negative side of PC1 and PC2) shows 0 h of *calugi* fermentation. This sample was characterized by the presence of citric and malic acids, sucrose and starch at the beginning of the process. The upper left quadrant (positive side of PC2) shows 12 h of *calugi* fermentation, correlating the count in GYC medium showing the highest population of AAB with the production of acetic acid and the peaks of glucose and fructose. After 24 h of fermentation (positive side of PC1), the population of LAB (MRS count) was correlated with the production of lactic acid (Figure 4).

DISCUSSION

In this study, the bacterial population was slightly different compared to rice and corn *calugi* (Miguel et al., 2012). Ramos et al. (2011) also found high counts of mesophilic bacteria in a non-alcoholic indigenous fermented beverage produced using cottonseeds and rice. Mesophilic bacteria are commonly found in fermented foods and beverages from cereals and tubers (Almeida et al., 2007; Miguel et al., 2012). The population found in fermented porridge from cassava and corn was lower than that

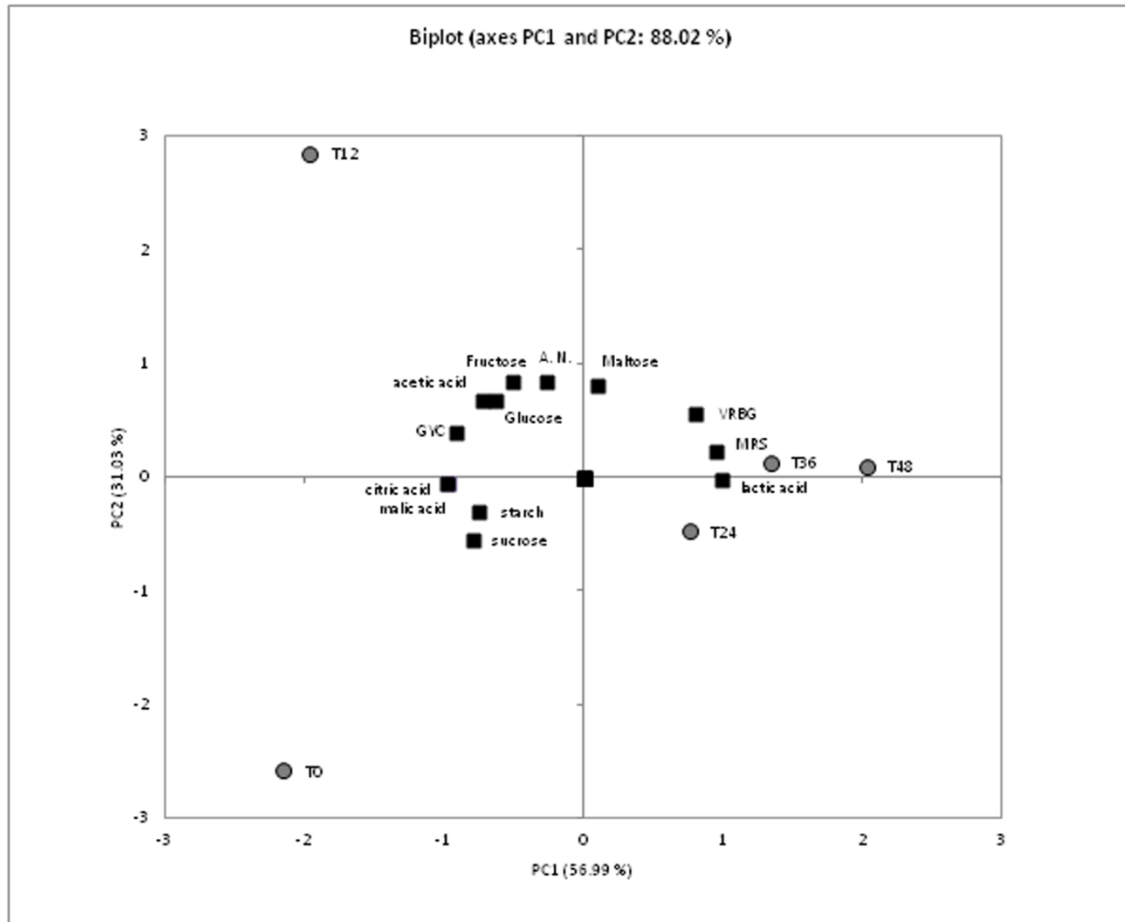


Figure 4. Principal component analysis (PCA) of enumeration and physical parameters during *calugi* fermentation.

reported by Almeida et al. (2007) and Ramos et al. (2010). This difference could be due to the inoculum and the different substrates used for the beverage preparation.

The use of culture-independent techniques has enabled us to determine the LAB succession during the fermentation of *calugi*. Different strains of LAB were detected with the progression of fermentation, and there was a decrease in the diversity of bands over time. Interesting fact can be observed in Table 2 where in the lactic acid also increased during fermentation. The analysis of the PCR-DGGE V3 regions revealed that the facultative heterofermentative *L. plantarum*, *L. casei* and *L. paracasei* were dominant at the initiation of the fermentation, but that at the later stages, the obligatory heterofermentative and homofermentative *W. cibaria*, *W. confusa*, *S. salivarius* and *S. parasanguis* were the dominant LAB. Species *L. plantarum*, *L. paracasei*, *L. casei*, and *W. confusa* were identified by the DGGE technique in other cereal-based foods such as a rice and peanut beverage (Ramos et al., 2010), cottonseeds and rice (Ramos et al., 2011), cassava dough (Miambi et al.,

2003) and *togwa* (Mugula et al., 2003). Microorganisms from *B. subtilis*, the *Bacillus* spp. (*B. cereus* group) and other *Bacillus* sp. were the main mesophilic microorganisms detected during fermentation. These species have been detected in other indigenous fermentations of cassava in Brazil and Africa (Almeida et al., 2007; Amo-Awua and Jakobsen, 1995; Assanvo et al., 2006; Padonou et al., 2009; Santos et al., 2012).

For the first time, *Streptomyces* sp. was detected in cassava and corn fermentations. This actinobacteria are heterotrophic feeders, and they can utilize both simple and complex molecules as nutrients (Yang and Wang, 1999). In addition to the above mentioned species, *E. cloacae* was the GNE detected by the PCR-DGGE method, bacteria from these genera were also shown to be present in indigenous beverages in Brazil (Miguel et al., 2012, Ramos et al., 2010, 2011; Santos et al., 2012).

Despite the high population of AAB found by plating, DGGE was not able to detect the acetic acid bacteria, which may be due to nucleic acid extraction efficiency and selective amplification of 16S rRNA genes (Miambi et al., 2003, Ramos et al., 2011). This finding illustrates the

intrinsic limitation of DGGE analysis in visualizing only the predominant species of a microbial community (Muyzer and Smalla, 1998). Considering specific limitations of both cultivation-independent and cultivation-dependent methods, a polyphasic approach for broad-coverage biodiversity studies of complex ecosystems is recommended (Scheirlinck et al., 2008).

There was a slight increase in the ethanol concentration after 24 h; this increase probably resulted from the LAB activity of heterofermentative (such as *L. plantarum*, *L. casei* and *L. paracasei*) during fermentation of calugi, this group of organisms convert sugars into lactic acid, acetic acid, fumaric acid, ethanol and carbon dioxide. There was a drop in the pH of the porridge after 12 h of fermentation; this finding is consistent with the production of organic acids (Table 2), the presence of LAB and *Bacillus* spp. (*B. cereus* group). The final pH of calugi was approximately 4.0; the production of organic acids in fermented food commonly reduces the pH to values below 4.0, which ensures the microbiological safety of the product because some pathogens do not survive at this pH (Blandino et al., 2003). Low pH values (pH 4.0) were also found in *cauim* fermentation (Almeida et al., 2007), in *fufu* production (Fagbemi and Ijah, 2006; Oyewole, 2001) and in a cottonseed and rice beverage (Ramos et al., 2011). In acidic condition, the product is safer for consumption, because some pathogens do not survive at low pH.

The species of the genera *Bacillus* and *Corynebacterium* are known for their ability to hydrolyze starch (Oyewole and Odunfa, 1989), and these bacteria could be responsible for the starch hydrolysis that often occurs in cassava. Maltose can be directly related to the conversion of starch to maltose/glucose by the enzymatic action of amylases common in microbial metabolism (Almeida et al., 2007, Panda et al., 2006). Starch hydrolysis by amylolytic enzymes contained in the roots of sweet potatoes produces mainly maltose sugar; the amylase from this tuber may have aided in the continuous release of maltose during fermentation.

Organic acids identified in calugi fermentations include citric, succinic, acetic, lactic, malic, tartaric, propionic and oxalic acids. The lactic acid and acetic acid are the dominant organic acids, which can be explained by the presence of LAB during the entire fermentation process. The production of the lactic and acetic acids during the fermentation of maize dough porridge and their ability to inhibit a variety of organisms has been reported (Mensah et al., 1991).

In the present study, the amount of citric acid was reduced, and from 24 h, was no longer detected; citric acid may have been metabolized to acetic acid, carbon dioxide and lactic acid. The lactic acid and acetic acid are the dominant organic acids. The multivariate analysis (PCA) allowed the correlation of the amounts of organic acids with the population of specific groups of microorganisms as detected by plating. The finding that

lactic and acetic acids are produced during calugi fermentation is in agreement with the results of Giraud et al. (1998), who concluded that heterofermentative lactic acid fermentation occurs in most of the spontaneously fermented cassava products in Africa. Propionic acid was detected at low concentrations in calugi fermentation. In addition, these organic acids may interact with other substances such as alcohols and aldehydes, which produce additional flavor compounds during the fermentation process (Liu et al., 2011). Volatile compounds were detected during the fermentation of calugi porridge, including alcohols, acetates, aldehydes, ethyl esters, terpenes and acetal (Table 3). Cassava and corn calugi had higher number of compounds identified in relation to rice and corn calugi; this may be due to the difference between the substrates for the preparation of porridge, since there were also differences observed for carbohydrates, alcohols and acids analyzed (Miguel et al., 2012). The compounds isobutyl acetate, nonanoic and decanoic acids were the main volatiles produced at 24 h of fermentation. However, at 36 h, the more abundant compounds were acetaldehyde and 1,1-dietoxyethane.

The presence of aromas representative of diacetyl, acetic acid and butyric acid make fermented cereal based products more appetizing (Blandino et al., 2003). According to some authors, ethyl butyrate is characterized as having a fruity aroma, similar to papayas and apples, butter and sweetish (Czerny et al., 2008). Lactate and acetate are important flavor enhancers in fermented cereals (Onyango et al., 2000), and the latter has been reported to act as a flavor enhancer, sensitizing consumers toward other aromatic compounds in products such as sourdough, the effect being related to its actual concentration (Gobbetti and Corsetti, 1997).

Furfuryl alcohol is a product of furfural reduction by many bacteria under aerobic conditions (Boopathy, 2009), which explains the high concentrations of both compounds at the initial time of fermentation of the calugi and decreased concentrations of the compound after 48 h. This compound is commonly formed during fermentation of cereals such as corn, rice, sorghum and others (Banigo and Muller, 1972).

The terpene content is considered a positive quality factor of the product because terpenes contribute to its aroma, serving to differentiate it from other beverages, and supply floral nuances to the product (Dragone et al., 2009). The 1,1-dietoxyethane compound is an acetyl that has been used as a solvent, as an intermediate in chemical synthesis for the protection of the carbonyl group in ketones and aldehydes, and in the fragrance industry.

Conclusion

In conclusion, this report is important for characterizing

the microbiota, the metabolites produced and the physicochemical changes that occur during fermentation of *calugi* porridge, produced by the *Javaé* people. Understanding the microbial interactions and identifying the compounds responsible for the flavor and aroma during fermentation processes is required to deliver safe products, with desirable properties, and moreover, to maintain these traditions.

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