MARIA GABRIELA DA CRUZ PEDROZO MIGUEL

CARACTERIZAÇÃO FÍSICO-QUÍMICA E MICROBIOLÓGICA DO ALIMENTO INDÍGENA CALUGI

LAVRAS – MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciência dos Alimentos, para a obtenção do título de Doutor.

Orientadora

Dra. Rosane Freitas Schwan

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RESUMO

O calugi é uma bebida fermentada não alcoólica produzida pelos índios Javaé cujo consumo é feito por crianças e adultos, diariamente. Diferentes substratos, tais como mandioca, milho e arroz, podem ser utilizados para seu preparo. No entanto, sua forma de inoculação é a mesma, independente do substrato. O inóculo é preparado por uma índia da tribo, ocorrendo a mastigação da batata-doce e, posteriormente, o líquido remanescente é adicionado aos substratos cozidos. O presente trabalho foi realizado com o objetivo de avaliar a dinâmica microbiana e caracterizar físico-química e nutricionalmente a bebida calugi elaborada com diferentes substratos. Foram avaliados a população de microrganismos e o perfil de metabólitos produzidos em duas diferentes bebidas, sendo uma preparada com mandioca e milho e outra, com arroz e milho. Para a caracterização da bebida, amostras de cada calugi foram coletadas em intervalos de 12 horas. A técnica de PCR-DGGE foi utilizada para avaliação da diversidade microbiana e as técnicas cromatográficas cromatografia líquida de alta performance (CLAE) e cromatografia gasosa (GC) foram empregadas para a caracterização do perfil de metabólitos. A bebida à base de mandioca e milho apresentou, durante 48 horas de fermentação, uma sucessão microbiana, com contagem de 9,65 log UFC/mL de bactérias ácido lática, 6,11 log UFC/mL Enterobactérias, 4,71 log UFC/mL de bactérias ácido acéticas e 4,33 log UFC/mL de bactérias mesofílicas, sendo identificadas nesta bebida as espécies *Corynebacterium variabile*, *Lactobacillus paracasei*, *L. plantarum*, *L. casei*, *Streptomyces* sp., *Bacillus cereus*, *B. subtilis*, *Streptococcus parasanguis*, *S. salivarius*, *Weissella cibaria* e *W. confusa*. Para bebida produzida à base de arroz e milho, suas contagens microbianas foram: 5,06 log UFC/mL de bactérias mesofílicas, 4,69 log UFC/mL de bactérias ácido láticas, 4,37 log UFC/mL de leveduras, 3,29 log UFC/mL de enterobactérias e 3,14 log UFC/mL de bactérias ácido acéticas. Dentre as espécies identificadas por PCR-DGGE destaca-se *Streptococcus salivarius*, *S. parasanguis*, *Bacillus cereus*, *Lactobacillus plantarum*, *Enterobacter cloacae*, *Wesseilla confusa*, *Saccharomyces cerevisiae*, *Pichia fermentans* e *Candida* sp. Foram identificados e quantificados por CLAE os carboidratos glicose, sacarose, frutose e maltose; os alcoóis etanol, metanol e glicerol e os ácidos lático, acético, málico, succínico e ácido cítrico, nas duas bebidas em estudo. Pela técnica de GC foi possível identificar e quantificar 21 compostos na bebida calugi de mandioca e milho e 15 compostos na bebida de arroz e milho. Na bebida calugi de mandioca e milho, os compostos que apresentaram as maiores concentrações durante todo o processo de fermentação foram furfuril álcool (20398.15 µg/L), furfural (1273.98 µg/L), ácido butírico (1222.85 µg/L) e b-citronellol (866.91 µg/L). Para o calugi de arroz e milho, os compostos encontrados em maiores concentrações foram o ácido decanoico

(113.88 µg/L), acetaldeído (69.46 µg/L) e isobutil acetate (42.96 µg/L), que apresentaram maiores concentrações. O conhecimento da dinâmica populacional e do perfil dos metabólitos produzidos durante a fermentação das duas bebidas calugi foi de grande importância para a caracterização e o conhecimento da microbiota, além de contribuir para estudos futuros no intuito de detectar microrganismos com potencial biotecnológico.

Palavras-chave: Calugi. PCR-DGGE. Cromatografia líquida. Cromatografia gasosa. Alimentos fermentados indígenas.

ABSTRACT

Calugi is a non-alcoholic fermented beverage produced by the Javaé Indians, the consumption of which is done by both children and adults, daily. Different substrates, such as cassava, corn and rice, can be utilized for its preparation. Nevertheless, its form of inoculation is the same, independent of the substrate. The inoculum is prepared by a female Indian of the tribe, the chewing of the sweet potato occurring and, afterwards, the remaining liquid is added to the cooked substrates. The present work was conducted with the objective of evaluating the microbial dynamics and characterizing both physicochemically and nutritionally the calugi beverage made from different substrates. The population of microorganisms and the profile of metabolites produced in two different beverages were evaluated, one being prepared with cassava and corn and the other with rice and corn. For the characterization of the beverage, samples of each calugi were collected at 12-hour intervals. The PCR-DGGE technique was utilized fore valuation of the microbial diversity and the high performance liquid chromatography (HPLC) and gas chromatography (GC) techniques were employed for the metabolite profile. The beverage based on cassava and corn presented for 48 hours' fermentation, a microbial succession with count of 9.65 log CFU/mL of lactic acid bacteria, 6.11 log CFU/mL Enterobacteria, 4.71 log CFU/mL of acetic acid bacteria and 4.33 log UFC/mL of mesophylic bacteria, in this beverage the species *Corynebacterium variabile*, *Lactobacillus paracasei*, *L. plantarum*, *L. casei*, *Streptomyces* sp., *Bacillus cereus*, *B. subtilis*, *Streptococcus parasanguis*, *S. salivarius*, *Weissella cibaria* and *W. confuse* being identified. For the beverage produced on the basis of rice and corn, its microbial counts were: 506 log CFU/mL of mesophylic bacteria, 4.69 log CFU/mL of lactic acid bacteria, 4.37 log CFU/mL of yeasts, 3.29 log CFU/mL of enterobacteria and 3.14 log CFU/mL of acetic acid bacteria. Among the species identified by PCR-DGGE, *Streptococcus salivarius*, *S. parasanguis*, *Bacillus cereus*, *Lactobacillus plantarum*, *Enterobacter cloacae*, *Wesseilla confusa*, *Saccharomyces cerevisiae*, *Pichia fermentans* and *Candida* stand out. The carbohydrates glucose, sucrose, fructose and maltose; the alcohols ethanol, methanol and glycerol and lactic, acetic, malic, succinic and citric acids were identified and quantified by CLAE in the two beverages under study. Through the CG technique, it was possible to identify and quantify 21 compounds in the calugi beverage of cassava and corn and 15 compounds in the rice and corn beverage. In the calugi beverage of cassava and corn, the compounds presenting the highest concentrations over the fermentation process were furfuryl alcohol (20398.15 µg/L), furfural (1273.98 µg/L), butyric acid (1222.85 µg/L) and b-citronellol (866.91 µg/L). For the rice and corn calugi, the compounds found at highest concentrations were decanoic acid (113.88 µg/L), acetaldehyde (69.46

μg/L) and isobutyl acetate (42.96 μg/L), which showed the greatest concentrations. The knowledge of the population dynamics and of the profile of the metabolites produced during the fermentation of the two calugi beverages was of great importance to both the characterization and knowledge of the microbiota, in addition to contributing towards future studies with the purpose of detecting microorganisms with biotechnological potential.

Keywords: Calugi. PCR-DGGE. Liquid chromatography. Gas chromatography. Indigenous fermented foods.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

Os alimentos e as bebidas fermentados são amplamente distribuídos geográfica e culturalmente. A fermentação é um dos métodos mais antigos e econômicos de produzir e preservar alimentos. A origem da técnica de fermentação ainda é incerta, porém, historiadores relatam sua utilização na Antiguidade, há mais de 7000 anos.

Atualmente, alguns alimentos fermentados são industrializados e fazem parte da dieta da população de quase todo o mundo, como, por exemplo, pão, queijo, iogurte, vinagre, bebidas fermentadas (cerveja, vinho) e fermentadas/destiladas (uísque, cachaça, tequila).

Os alimentos e as bebidas fermentadas indígenas são produzidos e consumidos, geralmente, em ocasiões comemorativas, por diferentes etnias. Dependendo da região e da etnia, há variações nas matérias-primas e no tempo de fermentação, porém, em muitos casos, um procedimento comum é, durante a mastigação prévia da matéria-prima, a hidrólise do amido pela incorporação da ptialina amilase salivar.

Os hábitos alimentares indígenas incluem o consumo de pratos feitos à base de peixes e quelônios aquáticos e de mandioca, arroz, batata-doce, batata, cará, banana, farinha de mandioca, mel e o calugi. O calugi é um mingau típico das comunidades Karajá/Javaé, preparado com arroz, milho socado e mandioca, acrescidos de porções de saliva. Em seguida, essa mistura é aquecida e fica em repouso durante alguns dias, período em que ocorre a fermentação.

Dentre o grande número de bebidas fermentadas indígenas brasileiras estão o caxiri (bebida fermentada de mandioca e batata doce), o cauim (bebida fermentada produzida a partir dos mais diversos substratos, tais como mandioca,

arroz, amendoim, abóbora, semente de algodão, milho entre outros), o aloá (bebida fermentada de abacaxi), a chicha (bebida fermentada de milho ou de mandioca), a caiçuma ou caissuma (fermentação a partir da pupunha), a tiquira (aguardente obtida da fermentação da mandioca) e o tarubá (bebida fermentada de mandioca).

Os indios Javaé são considerados como um dos subgrupos Karajá/Javaé, ou como uma das três etnias que compõem os Karajá. Os Karajá propriamente ditos, os Javaé e os Xambioá, são etnias habitantes das margens do rio Araguaia. Essas três etnias falam o mesmo dialeto, a linguagem karajá (tronco linguístico macro-jê). No entanto, a etnia Karajá, até o momento, é a mais pesquisada. Atualmente, as três etnias (Karajá, Xambioá e Javaé) são consideradas etnias distintas e não subgrupos de um mesmo povo.

As bebidas utilizadas para o estudo são consumidas por adultos e crianças e são elaboradas à base de mandioca e milho, e arroz e milho. No entanto, diversos outros substratos podem ser utilizados. O presente trabalho foi realizado para descrever os metabólitos produzidos durante o processo de fermentação bebida calugi produzida pelos índios Javaé e acompanhar a dinâmica da população microbiana por PCR-DGGE.

2 REFERENCIAL TEÓRICO

2.1 A etnia Javaé

No território brasileiro encontram-se cerca de 227 povos (ou etnias) indígenas falantes de mais de 180 línguas diferentes. A maior parte dessa população distribui-se por diversas aldeias, situadas no interior de 593 terras indígenas, de norte a sul do território nacional (INSTITUTO SÓCIO AMBIENTAL, 2007). A diversidade cultural entre os homens indígenas e não indígenas é distinta, além de serem constatadas também as similaridades e as diferenças entre os povos indígenas de uma mesma região.

No estado do Tocantins, os índios Karajá e Javaé habitam aldeias localizadas no interior da ilha do Bananal e as nove aldeias Karajá (Santa Isabel do Morro, Fontoura, Macaúba, Mirindiba, Tytema, JK, Itxalá, Axiwé e Watau) estão dispostas às margens do rio Araguaia, Das onze aldeias Javaé, dez (São João, Wari-Wari, Canoanã, Cachoeirinha, Barreira Branca, Boa Esperança, Txiodé, Barra do Rio Verde, Txuiri e Boto Velho) estão posicionadas às margens do rio Javaés (braço menor do rio Araguaia) e uma delas (Imotxi), às margens do Riozinho, no interior da ilha do Bananal. As três aldeias Xambioá (Kuherê, Xambioá e Wari Lyty) estão no município de Santa Fé do Araguaia, região do Bico-do-Papagaio, porção baixa do rio Araguaia, posicionadas às suas margens (SALERA JÚNIOR, 2005).

A localização das tribos indígenas Javaé concentra-se ao longo do rio Javaés, o braço menor do Araguaia, formador da ilha do Bananal (Figura 1) e elas distribuem-se nas seguintes aldeias: Barreira Branca, Barreira do Pequi, Boto Velho, Wariwari e Canoanã (RODRIGUES, 2005), nos municípios de Formoso do Araguaia, Cristalândia e Araguaçu. Os Karajá propriamente ditos localizam-se ao longo do meio e do alto curso do Araguaia, formados pela

comunidade de Aruanã (GO), pelas aldeias Santa Isabel do Morro, Fontoura, Macaúba, São Raimundo, no oeste da ilha do Bananal, e também por aldeias menores, como São Domingos. Os Xambioá são tradicionalmente habitantes do baixo curso do Araguaia, próximos à sua foz no Tocantins, ocupando duas aldeias no município de Araguaína, TO (LIMA FILHO, 1994).

Figura 1 Mapa da localização da ilha Bananal – Tocantis
Fonte: Barroso (2012)

Há muito tempo, os Javaé, os Karajá e os Xambioá habitam o vale do rio Araguaia, onde está localizada, na ilha do Bananal, a maior ilha fluvial do mundo. A ilha do Bananal situa-se no estado do Tocantins, em uma área de transição entre o cerrado e a floresta amazônica, e é constituída de inúmeros

rios, lagos, savanas inundáveis (conhecidas regionalmente como "varjão") e matas de galeria. Seu território tem cerca de 2 milhões de hectares e é coberto pelas águas do Araguaia em quase a sua totalidade, durante a estação cheia (INSTITUTO SÓCIO AMBIENTAL, 2007). Os povos Karajá, Javaé e Xambioá são o mesmo povo e se autodenominam Iny. Pertencem ao tronco linguístico macro-jê, família Karajá e língua karajá. Os três grupos falam a mesma língua e vieram migrando do norte, baixo Araguaia, antes de 1500.

A base da subsistência das comunidades Javaé/Karajás é a ictiofauna. A subida das águas no período de chuvas alterna as fontes de recursos alimentares das aldeias, uma vez que dificulta as possibilidades de pesca. Quanto aos produtos da roça, eles cultivam o milho, a abóbora, a mandioca e a melancia. Do cerrado, alimentam-se de pequi e demais frutas sazonais (LIMA, 2004). Os hábitos alimentares incluem o consumo de pratos feitos à base de peixes e quelônios aquáticos e de mandioca, arroz, batata-doce, batata, cará, banana, farinha de mandioca, mel e o calugi, um mingau típico dessas comunidades (SALERA JÚNIOR, 2005).

2.2 Alimentos e bebidas fermentadas produzidos a partir de diferentes substratos

A fermentação é uma das formas mais antigas de processamento e preservação de alimentos no mundo, remontando a mais de 7.000 anos, na Babilônia (SHETTY; JESPERSEN, 2006). Adicionalmente, a fermentação é uma das formas mais fáceis de preservação de alimentos, podendo conferir benefícios nutricionais e organolépticos a alimentos fermentados. A fermentação é efetuada pela microbiota natural das matérias-primas, microrganismos fixados aos equipamentos de fermentação ou adicionados denominados de culturas iniciadoras.

Por definição, fermentação pode também ser descrita como mudanças bioquímicas provocadas pela atividade metabólica de microrganismos e/ou enzimas na oxidação de carboidratos em ambientes aeróbicos, anaeróbicos ou parcialmente anaeróbicos (WALKER, 1998).

Alimentos fermentados podem ser produzidos pela utilização de diferentes matérias-primas. Em todo o mundo, comumente, utilizam-se mandioca, arroz, milho, batata-doce, seiva de palmeira, abóbora, amendoim, feijão e sementes de algodão (RAMOS et al., 2010; WAGLEY, 1988). A mandioca está entre as sete primeiras culturas alimentares do mundo e é natural de países de clima tropical e subtropical, sendo importante fonte de calorias para a população de países pertencentes à Ásia, à África e à América Latina. O carboidrato mais abundante na mandioca é o amido, que compõe cerca de 30% a 50% do peso seco do seu bagaço (PANDEY et al., 2000).

Por esta razão, é comum a utilização deste vegetal para a extração do amido, como também o uso deste vegetal como base para diversos produtos, incluindo comida, alimentação de animais e outros alimentos à base de farinha de mandioca, como bolos e doces (BRAUMAN et al., 1996; WANG et al., 2010). Apesar de apresentar essa grande vantagem, que é a sua riqueza em amido, a mandioca pode apresentar alguns inconvenientes, como baixo consumo de energia, baixo teor de proteínas e potencial toxicidade de cianeto (WANG et al., 2010). Porém, não obstante, a mandioca vem sendo utilizada predominantemente na elaboração de diferentes alimentos, podendo ser também usada para a produção de etanol combustível (ADEOTI, 2010).

Como outras raízes, a mandioca tem baixo teor de proteínas (1%) e o bagaço da mandioca contém compostos como cálcio, fosfatos, vitamina B2 e niacina (VANDENBERGHE et al., 2000). Mesmo que rica em amido e pobre em outros compostos essenciais à saúde humana, há muitos relatos sobre a utilização da mandioca para a elaboração de diferentes alimentos e, em especial,

bebidas indígenas, como gari, fufu, kokonte, cauim, banku, tape ketan e peujeum (AIDOO; ROB-NOUT; SARKAR, 2006; ALMEIDA; RACHID; SCHWAN, 2007; BLANDINO et al., 2003; KOSTINEK et al., 2005; SCHWAN et al., 2007; STEINKRAUS, 1998).

O cauim é uma bebida fermentada de mandioca produzida pelos povos indígenas Tapirapé. Almeida, Rachid e Schwan (2007) e Schwan et al. (2007) realizaram estudos para identificar a microbiota presente na fermentação da bebida cauim e observaram grande diversidade de microrganismos presentes nela. Segundo Almeida, Rachid e Schwan (2007), vários substratos são utilizados pelos Tapirapé para a produção desta bebida, tais como arroz, mandioca, milho, amendoim, semente de algodão, abóbora, buriti, banana e outras frutas. A microbiota envolvida na fermentação da mandioca para a produção do cauim foi descrita, por ambos os autores, como sendo as bactérias pertencentes aos gêneros *Lactobacillus*, *Bacillus*, *Corynebacterium* e *Paenibacillus*. Dentre as leveduras, os gêneros encontrados foram *Saccharomyces*, *Candida*, *Pichia* e *Debaryomyces.*

Um dos alimentos produzidos a partir da fermentação espontânea de mandioca é o chamado de polvilho azedo (LACERDA et al., 2005). Esta farinha é muito utilizada na América Latina, especialmente na Colômbia e no Brasil, na produção de pães de queijo e outros alimentos tradicionais. A microbiota presente durante a fermentação do polvilho azedo é bem diversificada, sendo encontradas bactérias e leveduras de diferentes gêneros, tais como *Lactobacillus*, *Bacillus*, *Bifidobacterium*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Candida*, *Debaryomyces*, *Kluyveromyces, Galactomyces* e *Issatchenkia.*

O sour dough é uma massa produzida no Congo a partir da fermentação da mandioca. Esta massa é muito utilizada como massa base para preparo de outros alimentos (MIAMBI; GUYOT; AMPE, 2003). Durante a fermentação para a produção dessa massa foram identificadas bactérias e leveduras

pertencentes aos gêneros *Lactobacillus*, *Pediococcus*, *Weissella*, *Clostridium*, *Propionibacterium*, *Bacillus*, *Candida* e *Saccharomyces.*

O fufu é outro tipo de alimento fermentado produzido a partir da fermentação da mandioca. As leveduras envolvidas durante a produção deste alimento foram descritas por Oyewole (2001), sendo identificadas, durante as 96 horas de fermentação, leveduras dos gêneros *Pichia*, *Candida*, *Saccharomyces* e *Zygosaccharomyces* e bactérias pertencentes aos gêneros *Streptococcus*, *Geotrichum*, *Corynebacterium*, *Lactobacillus* e *Leuconostoc*. Neste mesmo estudo, o autor estudou a interação entre leveduras e bactérias ácido láticas, sendo utilizado isolados de *Candida krusei* e *Lactobacillus plantarum*. Os resultados demonstraram que a utilização de cultura iniciadora na fermentação de mandioca para a produção de fufu apresenta melhores resultados quando se inoculam leveduras associadas a bactérias ácido láticas.

A agbelima é um alimento à base de mandioca, no qual as raízes são descascadas, raladas e misturadas a inóculo tradicional. A microbiota predominante durante sua fermentação envolve bactérias ácido láticas, principalmente *Lactobacillus plantarum*, *Lactobacillus brevis* e *Leuconostoc mesenteroides*. A quebra do tecido da mandioca resulta em uma massa de textura suave e esta hidrólise, provavelmente, é realizada por *Bacillus* spp., *Candida krusei*, *C. tropicalis*, *Zygosaccharomyces bailii* e fungos filamentosos, por meio de sua atividade celulolítica. Agbelima é largamente consumida em países como Ghana, Togo e Benin (MANTE; SAKYI-DAWSON; AMOA-AWUA, 2003).

O caxiri é uma bebida alcoólica tradicional fermentada, produzida pelos índios Juruna (Yudjá), habitantes do Parque Indígena do Xingu, localizado no estado do Mato Grosso. Essa bebida é preparada à base de mandioca e batata-doce e é fermentada por microrganismos que estão presentes nas matérias-primas utilizadas para a sua produção (SANTOS, 2010). As bactérias

predominantes no processo fermentativo pertencem aos gêneros *Bacillus*, *Lactobacillus*, *Enterobacter* e *Pediococcus*. *Saccharomyces cerevisiae* foi a levedura dominante e também foram encontrados representantes das espécies *Rhodotorula mucilaginosa*, *Pichia membranifaciens*, *P. guilliermondii* e *Cryptococcus luteolus.*

A batata-doce, assim como a mandioca, é uma raiz tuberosa rica em energia comestível e com baixo teor de proteínas. Este vegetal, rico em carboidratos, tem grandes quantidades de vitaminas, como a C e a B6, além de riboflavina, ácido pantotênico, ácido fólico e a provitamina A; antioxidantes, como ácido fenólico, antocianinas, tocoferol e β-caroteno, e também minerais e cobre (PANDA; PARMANICK; RAY, 2007; TETCHI et al., 2007; WIREKO-MANU; ELLIS; ODURO, 2010). De acordo com a Organização das Nações Unidas para Agricultura e Alimentação - FAO (2007 citado por PANDA; PARMANICK; RAY, 2007), a batata-doce foi a sétima cultura alimentar mais importante do mundo. Tradicionalmente, esta raiz tuberosa pode ser preparada como a batata tradicional, mas também pode ser seca e moída para a produção de farinha, amido ou fécula. A batata-doce é comumente utilizada para a produção de doces e bebidas caseiras, devido à sua rica composição em compostos benéficos à saúde humana (WIREKO-MANU; ELLIS; ODURO, 2010) e ao sabor agradável.

Wireko-Manu, Ellis e Oduro (2010) elaboraram uma bebida não alcoólica à base de bata-doce e concluíram que as suas propriedades estão dentro das variedades de propriedades de sucos de frutas e bebidas já existentes e comercializadas, podendo servir ao propósito apresentado. As fontes mais comuns de amido são batata, trigo, mandioca, milho e arroz (BLANDINO et al., 2003; TETCHI et al., 2007). No entanto, além de fontes de carboidratos, os grãos, como o arroz e o milho, são importantes fontes de proteínas, vitaminas,

[Eve1] Comentário: Que propósito??

minerais e fibras e são comumente aplicados na alimentação, após serem fermentados.

Os alimentos preparados a partir da fermentação de grãos mais comuns são à base de arroz, trigo, milho e sorgo, e são conhecidos em muitas partes do mundo. Abriouel et al. (2006) identificaram microrganismos presentes em dois alimentos fermentados tradicionalmente consumidos na África, poto poto e o dégué. Na África, é bastante comum o consumo de alimentos fermentados produzidos a partir da fermentação de cereais, como é o caso do poto poto, que é um alimento produzido pela fermentação de milho e é consumido, nas famílias congolesas, por crianças que estão na fase de desmame.

O dégué e o poto poto são alimentos fermentados usando o milheto como substrato e estes são consumidos pelas famílias da Bukina Faso. Em ambos os casos, a fermentação é espontânea e sem nenhum controle da microbiota envolvida durante a mesma. Abriouel et al. (2006) identificaram as espécies de bactérias ácido láticas como dominantes durante o processo de elaboração das bebidas, sendo encontradas *Lactobacillus plantarum*, *L. paraplantarum*, *L. acidophilus*, *L. casei*, *Bacillus* e *Escherichia coli* nas amostras de poto poto e *L. fermentum*, *L. casei*, *L. brevis*, *L. gassei* e *Enterococcus* nas amostras de dégué.

A hawaijar é uma bebida tradicional indígena fermentada à base de soja com *flavour* e viscosidade característicos e é comumente consumida em Manipur, na Índia. Os microrganismos dominantes desta bebida foram identificados, tendo sido encontradas espécies do gênero *Bacillus* (*B. cereus*, *B. subtilis*, *B. Licheniformis*) e *Staphylococcus* (*S. Aureus* e *S. Sciuri*) (JEYARAM et al., 2008). Segundo Tamang (2005), no norte da Índia, uma variedade de alimentos doces fermentados é produzida a partir de soja e consumida por pessoas da etnia da região. Alimentos, como kinema, são consumidos nos morros de Dargilling, em Sikkim; turangbai, na Megalaya e hawaijar, em

Manipur; aakhuni, em Nagaland; bekangum em Mizoram e pruyaan, em Arunachal Pradesh.

[Eve2] Comentário: Favor conferir. Tirei as aspas e coloquei o nome dos alimentos em minúscula e dos locais em maiúscula. Estava muito confuso.

O doenjang é uma pasta de soja produzida na Coreia. Tradicionalmente produzida pela fermentação da soja, tem sido consumida, por séculos, como uma fonte de proteína e ingrediente aromatizante naquele país. Durante a sua produção, uma variedade de bactérias, leveduras e fungos filamentosos foi encontrada. Dentre eles, estão bactérias ácido láticas, como *L. Plantarum* e *L. sakei*, e outros grupos, como *B. subtilis*, *B. licheniformis*, *Leuconostoc mesenteroide* e *Enterococcus faecium*, além de leveduras e fungos filamentosos, como *Debaryomyces hansenii*, *Candida krusei*, *Candida etchellsii*, *Candida intermedia*, *Pichia anomala*, *Zygosaccharomyces rouxii*, *Mucor plumbeus* e *Aspergillus oryzae* (KIM et al., 2009).

Como se pode observar, é de grande importância, tanto para comunidade científica como para a população consumidora de alimentos e bebidas fermentadas a partir de diferentes substratos, o conhecimento da microbiota presente durante o processo de elaboração do produto, como também a caracterização físico-química destes subtratos utilizados como matéria-prima, uma vez que é essencial para a compreensão da dinâmica da microbiota presente. A identificação dos organismos presentes durante a fermentação dos diferentes alimentos fermentados à base de mandioca, batata-doce, milho e arroz, permite compreender os diferentes papéis exercidos pelos diferentes microrganismos na formação do alimento. Isso pode ser útil para aprimorar sua qualidade e permitir sua comercialização. Uma forma de alcançar este objetivo é o desenvolvimento de culturas iniciadoras que substituam o inóculo tradicional de cada alimento.

2.3 Análises de metabólitos

Há alguns anos, técnicas foram desenvolvidas no intuito de identificar microrganismos presentes em diferentes amostras e ambientes. Estas técnicas visam o entendimento do papel de determinado microrganismo em um ecossistema e são conhecidas, nos dias de hoje, como métodos dependentes (isolamento seletivo e condições de incubação) ou independentes de cultivo (eletroforese desnaturante em gel de gradiente, DGGE).

Consequentemente, outras áreas tecnológicas, como genômica, transcriptômica, proteômica e metabolômica, emergiram e desenvolveram métodos que proporcionaram uma adequada abordagem para esse tipo de investigação (VUYST et al., 2009). Atualmente, têm-se utilizado estas técnicas para estudar a dinâmica das comunidades microbianas ou a dominância de espécies em alimentos fermentados. Técnicas como a cromatografia líquida de alta eficiência (CLAE ou HPLC), cromatografia gasosa associada à espectrometria de massa (GC-MS), cromatografia líquida acoplada à espectrometria de massa (LC-MS) e eletroforese capilar acoplada à espectrometria de massa (CE-MS) são utilizados cada vez mais, comprovando a eficácia do seu uso para a identificação de metabólitos microbianos, permitindo, assim, associá-los aos organismos presentes em diferentes alimentos.

Dentre as diversas técnicas para estudos relacionados à metabolômica, a cromatografia gasosa associada à espectrometria de massa (GC-MS) vem sendo bastante empregada para determinar o perfil metabólico de microrganismos em diferentes amostras (GULLBERG et al., 2004). A técnica de GC-MS apresenta baixo custo em comparação com outros métodos de análise (por exemplo, CE-MS, LC-MS e RMN) e tem alta reprodutibilidade e alta resolução (KOPKA, 2006).

Outra técnica utilizada para a avaliação dos metabólitos da microbiota de alimentos é a cromatografia líquida acoplada à espectrometria de massa (LC-MS). Esta técnica tem sido utilizada para determinar os perfis metabólicos (GU; JONES; LAST, 2007; PLUMB et al., 2002) e é uma solução robusta, sensível e seletiva para análises quantitativas e qualitativas. Yoshida et al. (2009) utilizaram LC-MS em seus estudos com alimentos fermentados japoneses, com o objetivo de demonstrar as vantagens desta técnica para caracterizar o perfil metabólico desses alimentos.

A cromatografia líquida de alta eficiência (CLAE) é bem conhecida e trata-se de uma técnica analítica mais desenvolvida e vem sendo empregada em diversas áreas (DUARTE, 2011). Atualmente, trabalhos vêm sendo realizados associando o uso da HPLC às diferentes técnicas de LC e GC, a fim de determinar todos os metabólitos microbianos presentes em um ambiente para compreender a dinâmica da ecologia microbiana do mesmo.

Em seus estudos com a microbiota envolvida na fermentação de centeio, Weckx et al. (2010) puderam avaliar, por meio das técnicas de HPLC, LC-MS e GC-MS, o perfil dos metabólitos produzidos nesta fermentação, com o objetivo de inferir sobre a dinâmica e a dominância da comunidade, visto que esse processo fermentativo ocorre de maneira espontânea.

O perfil metabólico, durante a fermentação de cheonggukjang (uma pasta fermentada a partir de soja, tradicionalmente consumida na Coreia), foi estudado por Park et al. (2010). Estes autores utilizaram a técnica de GC-MS para elucidar as alterações dos metabólitos produzidos durante a fermentação e concluíram que a técnica utilizada permitiu determinar o perfil dos metabólitos produzidos durante a fermentação, bem como suas mudanças durante o processo, sendo determinados os níveis de aminoácidos, ácidos graxos e ácidos orgânicos.

A caracterização de compostos metabólicos produzidos durante a fermentação de vinho de frutas foi objetivo do trabalho realizado por Duarte et al. (2010). Nesta pesquisa, os autores produziram vinho de cacau, cupuaçu, gabiroba, jaboticaba e umbu e determinaram, por meio de GC-MS, compostos voláteis menores produzidos durante a fermentação e, por GC-FID, compostos voláteis maiores. Os autores obtiveram excelentes resultados dos compostos produzidos nas bebidas, utilizando as técnicas mencionadas.

No intuito de validar a técnica de GC-MS, em estudo de metabólitos microbianos, Koek et al. (2006) utilizaram linhagens referências de *B. subtilis*, *Escherichia coli* e *Propionibacterium freudenreichi* e avaliaram o perfil dos metabólitos produzidos (aminoácidos, ácidos orgânicos e açúcares). Os autores concluíram que o método pode ser aplicado para análises de metabólitos de grande número de microrganismos, bem como para estudos metabolômicos de plantas e animais.

Muitos estudos foram realizados combinando análises em cromatografia de gás-espectrometria de massa (GC-MS) e análise de componentes principais (PCA), para obter perfis metabólicos de vários alimentos (HAMZEHZARGHANI et al., 2005; SCHAUER; ZAMIR; FERNIE, 2005). A PCA é uma análise estatística que permite resumir as informações obtidas pelos métodos analíticos citados em número reduzido de componentes principais. Dessa forma, é possível compreender o efeito dos compostos que atuam diretamente para a formação de *flavour* e do aroma de alimentos fermentados (RODRIGUEZ-CAMPOS et al., 2011).

Os metabólitos que serão formados pelos microrganismos em alimentos fermentados dependerão da composição físico-química da matéria-prima. Durante a fermentação de grãos, cereais, frutas e outros alimentos, muitos compostos voláteis são formados. Compostos como ácidos orgânicos, compostos carbonílicos, álcoois, acetatos e ésteres etílicos são metabólitos microbianos que

contribuem para a formação do sabor e o desenvolvimento do aroma do produto final da fermentação de alimentos. Ácidos orgânicos, como acético diacetil e butírico, tornam produtos à base de cereais mais apetitosos e agradáveis ao paladar humano (BLANDINO et al., 2003).

2.4 Uso de métodos moleculares para estudos de diversidade microbiana

Vários meios de cultivo seletivos têm sido utilizados para a detecção e o isolamento de microrganismos, entretanto, a técnica dependente de cultivo clássica nem sempre é eficiente para a identificação (HOLZAPFEL et al., 1997). A preparação dos meios de cultura seletivos é trabalhosa e tem a limitação de não ser absolutamente seletiva. Além disso, uma desvantagem é o fato de que nem todos os microrganismos podem crescer em meio de cultura, tornando-se impossível isolar e identificar significante número de espécies microbianas (THEUNISSEN et al., 2005). O uso de técnicas moleculares na microbiologia de alimentos tem oferecido várias melhorias no campo da detecção e da identificação microbiana (COCOLIN et al., 2004). Essas técnicas, muitas vezes, são implementadas para estabelecer a diversidade microbiana de uma amostra complexa de alimentos (GONZALEZ et al., 2003).

Nos últimos anos, devido ao uso de ferramentas moleculares, os conhecimentos sobre a diversidade microbiana em comunidades complexas têm aumentado drasticamente. As técnicas moleculares proporcionam uma ferramenta excepcional para a detecção, a identificação e a caracterização de microrganismos envolvidos em diversos ambientes e ecossistemas de alimentos. Diferentes autores (COCOLIN et al., 2007; ERCOLIN, 2004; MUYZER; SMALLA, 1998) têm descrito a aplicação da técnica de DGGE para estudos no campo da ecologia microbiana. No início dos anos 1990, Muyzer, Waal e Uitterlinden (1993) desenvolveram a técnica eletroforese em gel de gradiente

desnaturante (DGGE) com a proposta de caracterizar o perfil dos microrganismos presentes em amostras ambientais, em que eram analisados produtos de PCR, de acordo com suas sequências de nucleotídeos e o tamanho dos produtos diferentes. Isso possibilita não só a avaliação da diversidade genética das comunidades microbianas, bem como, associada ao sequenciamento dos produtos amplificados, a identificação das espécies presentes na comunidade (MONTEIRO, 2007).

Na análise de DGGE, *amplicons* de PCR de mesmo tamanho, mas com diferentes sequências, podem ser separados (MUYZER; SMALLA, 1998) e essa técnica tem sido aplicada extensamente para estudar a dinâmica microbiana em ambientes complexos como o solo (EDENBORN; SEXSTONE, 2007) e os mais diversos tipos de ambientes. Este método foi usado também para investigar a diversidade do fermento nos alimentos e em bebidas, tais como vinho (COCOLIN; BISSON; MILLS, 2000), salsichas (RANTSIOU et al., 2005), fermento de panificação (MEROTH; HAMMES; HERTEL, 2003), café (VILELA et al., 2010), cacau (NIELSEN et al., 2007), kefir (MAGALHÃES et al., 2010; MIGUEL et al., 2010) e cauim (RAMOS et al., 2010).

Cocolin, Bisson e Mills (2000) utilizaram essa técnica para caracterizar a diversidade de leveduras presentes na fermentação de vinho e concluíram que o uso de PCR-DGGE promoveu melhor visão da diversidade microbiana presente durante a fermentação, sem a necessidade do uso de técnicas tradicionais. A técnica de PCR-DGGE também foi utilizada por Nielsen et al. (2007) para confirmar a microbiota envolvida durante a fermentação de cacau de Gana. Estes autores utilizaram técnica dependente e independente de cultivo para acompanhar a dinâmica populacional da fermentação do fruto de cacau e concluíram que essa combinação foi favorável para a identificação.

Estudos têm associado o uso de técnica dependente e independente de cultivo. Esta associação entre técnicas foi realizada por Miambi, Guyot e Ampe

(2003) para avaliar a comunidade bacteriana presente durante a fermentação de mandioca. Verificou-se a ocorrência de dez diferentes bandas, porém, após o sequenciamento das mesmas através do rRNA 16S, mostrou-se a ocorrência de cinco diferentes espécies (*L. plantarum*, *L. fermentum*, *L. delbrueckii*, *B. cereus* e *Clostridium acetobutylicum*). Ramos et al. (2010) utilizaram técnica dependente e independente de cultivo para caracterizar a dinâmica populacional da microbiota de bebida fermentada indígena e concluíram que por ambas as técnicas foi possível observar que há mudança na comunidade microbiana durante o processo de fermentação, sendo ressaltado também quais espécies de bactérias e leveduras foram predominantes durante a fermentação.

No intuito de se estudar a microbiota presente na bebida fermentada calugi, consumida pelos povos indígenas da tribo Javaé, aldeia Canuanã, o presente estudo foi realizado com o objetivo de identificar, por método independente de cultivo e caracterizar a dinâmica populacional dessa bebida. Aplicou-se a técnica independente de cultivo PCR-DGGE nos diferentes tempos de fermentação da bebida, como também as técnicas de HPLC e GC-FID, para identificar e quantificar os metabólicos produzidos durante a fermentação.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1

Bacterial dynamics and physicochemical changes during the traditional production of *Calugi*, an indigenous Brazilian fermented food

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ABSTRACT

Calugi is a traditional fermented food made by the Brazilian *Javaé* people. It is a porridge produced using different substrates: maize, cassava and rice. The saliva and mastication juice of sweet potato containing microorganisms is used as the inoculum. The main microbial groups associated with the fermentation of *calugi* under traditional conditions have been studied for the first time. Aerobic mesophilic bacteria reached values of approximately 7 log CFU/ml and were the predominant group; high counts of acetic acid bacteria were also observed at the onset of fermentation. The population of lactic acid bacteria remained constant throughout the fermentation. A microbial succession was observed during *calugi* fermentation using denaturing gradient gel electrophoresis (DGGE) of the small subunit rRNA gene. The DGGE bands were identified as *Corynebacterium variabile*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Bacillus* spp. (*Bacillus cereus* group), *Bacillus 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/liter at the beginning of fermentation); this sugar was released throughout the fermentation by the action of amylolytic enzymes. Acetic and lactic acids were the principal acids produced and were correlated with the flavor of the *calugi*. Twenty-one 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*.

Keywords: Indigenous food, Cereal fermentation, Cassava, Corn, volatile compounds, DGGE.

INTRODUCTION

Indigenous fermented foods are of worldwide interest and consequently have provided new avenues for scientific research in the past. However, few studies have been conducted on these Brazilian foods (55). 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 (63). These indigenous groups have little contact with external cultures; therefore, investigating Brazilian indigenous food is not easy (55). 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 security 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 (2, 40, 47, 51, 52, 55, 57). Studies characterizing the microbiota of various traditional products revealed a great diversity of bacteria involved in fermentation. According to Almeida et al. (2), *Lactobacillus, Bacillus, Corynebacterium* and *Enterobacter* were identified in the beverage *cauim*, produced from cassava and rice, in which the species *Lactobacillus pentosus* and *Lactobacillus plantarum* were the dominant bacteria. Ramos et al. (51, 52) showed that the *Lactobacillus* genus was dominant throughout fermentation, and *L. plantarum*, *Lactobacillus fermentum*, *Lactobacillus paracasei* and *Lactobacillus 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* (55).

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. When applied to a specific environment, PCR-DGGE provides a profile representing the genetic diversity of the microbial community. DGGE analysis has been widely used to study complex microbial communities originating from food samples and other environments, such as *doenjang* (28), fermented cassava (34), *gari* (40), coffee (61), kefir grains (35) and *cauim* made from cottonseeds, rice and peanuts (51, 52).

To identify and report for the first time 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. *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 substrates are cooked separately for approximately 2 h. After cooking, the resulting porridge is allowed to cool and an inoculum is added to initiate the fermentative process, which usually takes 24 h to 48 h. The inoculum is the fluid that is obtained by the chewing of sweet potatoes by the women (2). The *calugi* is usually consumed in daily meals by adults and children.

There is no information in the literature about the microbial succession, the compounds produced and the chemical changes that occur during *calugi* fermentation. Therefore, the objectives of this work were to evaluate the profile of organic acids, sugars, ethanol and volatile compounds during *calugi* fermentation and to identify the dominant microbiota 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.

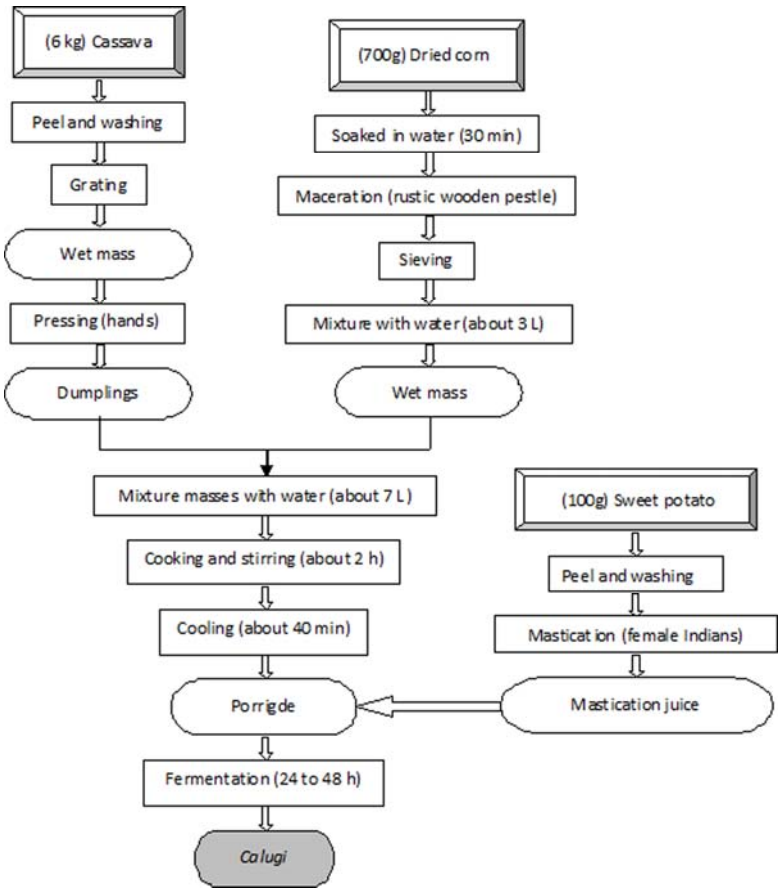

Figure 1 A flow diagram for the processing of cassava roots, dried corn and sweet potato into *calugi*

Calugi was prepared in the traditional manner using cassava, corn and sweet potato (Fig. 1 and Fig. 2). To prepare 10 liters 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 liters of water and sieved to remove the peel. The cassava and corn masses were mixed with 7 liters 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 at the ambient temperature (approximately 30 ºC). The diagram of traditional production methods (Fig. 1 and Fig. 2) shows the preparation of the *calugi* porridge and the inoculation process, which are non-sterile. These are the only raw materials added to the porridge after the cooking and cooling steps.

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)

Sampling

Samples were taken by 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% $Na_2H_2PO_4$ (Merck), 20% glycerol (Himedia)) for determination of microbiota and were frozen until analysis. Serial dilutions (10^{-2}-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 five 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 3 days at 30 °C. VRBG (violet red bile agar with glucose) (Oxoid, Hampshire, England) was used for bile-tolerant Gram-negative *Entorobacteriaceae* (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 3-4 days and at 25 °C for 5–8 days, respectively. MRS (De Man Rogosa Sharpe, Merck) agar containing 0.1% cysteine-HCl under anaerobic conditions was used for LAB count. MRS plates were incubated in acrylic anaerobic jars at 30 °C for 3–4 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 Miguel et al. (35). The DNA was extracted using the QIAamp DNA Mini Kit - DNA Purification from Tissues (Qiagen, Hilden, Germany), in accordance with the of 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 GGG GCG GGG GCA CGG GGG 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 (43). 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 of each primer, 1.5 mM $MgCl_2$ and 1 µl of extracted DNA. The amplification was performed according to Ramos et al. (51). 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 ºC. 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. DNA fragments from selected bands were excised from the gels, reamplified and the fragments 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, Maryland, USA).

Chemical analysis

The pH, starch and protein contents of *calugi* were determined using the methodology proposed by AOAC (8).

Chromatographic analysis

HPLC analysis

Carbohydrates (glucose, sucrose, maltose and fructose), organic acids (acetic acid, lactic acid, malic acid, citric acid, tartaric acid, oxalic acid, propionic acid, butyric acid and succinic acid) and alcohols (ethanol, methanol and glycerol) were identified according to methodology proposed by Duarte et al. (19). The analyses were carried out using a high performance liquid chromatography system (HPLC) (Shimadzu, model LC-10A*i*, Shimadzu Corp., Japan), equipped with dual detection system consisting of a UV-Vis detector (SPD-10A*i*) and a refractive index detector (RID-10A*i*). A Shimadzu ion exclusion column (Shim-pack SCR-101 H, 7.9 mm x 30 cm) was used operating at 50 ºC for acids, and at 30 ºC for ethanol, methanol and glycerol, using 100 Mm of perchloric acid as the eluent at a flow rate of 0.6 ml/min. The acids were

detected via UV absorbance (210 nm), while alcohols were detected via RID. For carbohydrates was used the column Supelcosil LC-NH_2 (4.6mm x 25 cm) operating 30 ºC with acetonitrile:water (75:25) as mobile phase in a flow rate of 1ml/min. The sugars were detected via RID. 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. All samples were examined in quadruplicate.

GC-FID analysis

The extraction of minor volatile compounds was performed according to the methodology proposed by Duarte et al. (19). 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 (30m x 0.25mm i.d x 0.25µm) (J & W Scientific, Folsom, Calif., U.S.A.). The operating conditions were: the oven temperature was maintained at 50 ºC for 5 min, raised to 190 ºC by increments of 3 ºC/min and then kept at 190 ºC for 10 min. Injector and detector temperature were kept at 230 ºC and 240 ºC respectively; the carrier gas (N_2) was kept at a flow rate of 1.2 ml/min. 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/liter 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 evolution 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 AMB increased from 3.92 log CFU/ml to 6.95 log CFU/ml at the beginning (12 h). In the later stages of fermentation, the AMB counts showed a declining tendency, decreasing to 4.47 log CFU/ml. The population of AAB decreased from 3.82 log CFU/ml at the onset of fermentation to 1.5 log CFU/ml at 24 h, and their viable numbers did not change significantly after this time. The LAB load was approximately 3.7 log CFU/ml in all stages of fermentation. GNE were detected on VRBG agar, and the counts ranged from >1 log CFU/ml to 2 log CFU/ml.

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

[a]Bands are numbered as indicated on the DGGE gel shown in Fig. 3
[b]Acession number of the sequence of the closest relative found by BLAST search

Microbial community by PCR-DGGE

To determine the diversity of bacteria in the cassava, corn and sweet potato porridge, PCR-DGGE analysis was employed. The V3 region of the 16S rRNA gene was amplified. The DGGE fingerprint is shown in Fig. 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. At the DNA level, using the universal primer for bacteria (Fig. 3 and Table 1), eight bands that corresponded 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 present at all five fermentation times. Great diversity was present at the initial time of fermentation (T0); the identified species 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 present after 12 h and could be observed until 36 h of fermentation. No differences in these times were detected. 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 present. The bacterial DGGE profile indicated the presence of LAB throughout the fermentation process.

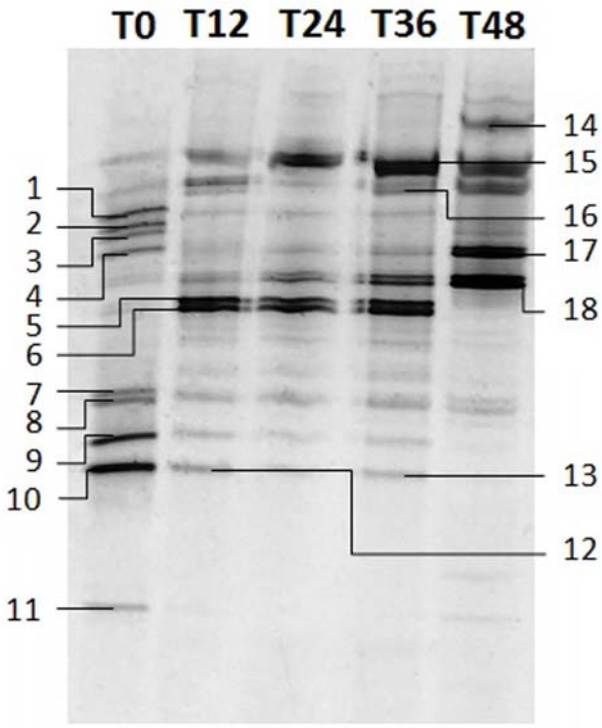

Figure 3 Denaturing Gradient Gel Electrophoresis profiles of bacterial V3 regions of the 16S rRNA gene amplified from *calugi* beverage throughout the 48 h of fermentation. The identification of the bands is reported in Table 1. Bands indicated by numbers were excised and after reamplification they were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

Physico-chemical analysis

The pH value dropped from 6.20 to 4.03, indicating the spontaneous acidification of the product. The protein content increased, and decreasing concentrations of soluble starches were found throughout the fermentative process (Table 2).

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

Parameters	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[b]	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
Tartaric acid	nd	nd	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
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
Oxalic acid	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00

[a-] Determined by HPLC analysis. [b-] Not detected. The data are means of four replicates. ± Standard deviation

Chromatographic analysis

HPLC Analysis

Maltose, glucose, sucrose and fructose were detected during fermentation. Maltose was the most abundant disaccharide throughout the fermentation process, and its concentration reached a peak of 55.70 g/liter at 12 h of fermentation (Table 2). The higher values of maltose (T12 and T36) are

directly related to starch hydrolysis. The decrease observed in maltose concentrations (T24 and T48) was probably due to microbial consumption.

Glucose was found during the fermentation time in concentrations varying from 1.29 to 0.17 g/liter. The highest concentration of glucose was observed after 48 hours of fermentation, which may be due to amylase or microbial activities. Fructose was found at low concentrations in the sample at T0, prior to beginning the fermentation. An increase in the fructose concentration in sample T12 was observed, but later, this carbohydrate was detected in decreasing amounts. A high value for sucrose was also found at the beginning of *calugi* fermentation. However, the sucrose content continuously decreased until 24 h of fermentation, after which it was no longer detected; this carbohydrate was probably hydrolyzed by enzymatic activity, releasing glucose and fructose. As shown in Table 2, at 12 h, the peaks of glucose and fructose are coincident with the highest decrease in sucrose levels.

The acids present at greater concentrations at the beginning of *calugi* fermentation were citric acid (0.87 g/liter), malic acid (0.92 g/liter) and acetic acid (1.70 g/liter). Acetic acid was the main metabolite produced at the onset of fermentation, and its concentration increased, reaching 5.47 g/liter at 12 h. The concentration then decreased throughout fermentation, to approximately 0.65 g/liter 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/liter at 48 h. During the fermentation period, the amounts of malic, succinic and citric acids decreased, while the concentrations of oxalic and propionic acids remained stable. Tartaric acid was present from 24 h and was detected at low concentrations.

The alcohols identified by HPLC during *calugi* fermentation were ethanol, methanol and glycerol (Table 2). Ethanol production during *calugi* fermentation was low (0.4 g/liter) at the end of the fermentation process. The

glycerol concentration increased after 12 h, and at the end of the process, was 3.0 g/liter. Methanol was present in the T0 sample; at 12 h, it increased, but then remained constant (approximately 0.10 g/liter) until the end of the fermentation process.

GC-FID Analysis

Twenty-one compounds were identified and quantified in the *calugi* porridge, including two alcohols, three acetates, five acids, five aldehydes, two ethyl esters, three terpenoids and one acetal (Table 3). The volatile acids identified were butyric, hexanoic, octanoic, nonanoic and decanoic acids. Butyric acid was present only at 0 h, while hexanoic acid was present at 36 h and octanoic acid was detected from 24 h of the fermentation process. Nonanoic and decanoic acids were the most abundant compounds at the beginning of fermentation; they were found at concentrations of 580.18 and 475.91 µg/liter, respectively, and decreased during fermentation.

Furfuryl alcohol and 1,3 butanediol were the volatile alcohols identified. Furfuryl alcohol was found during the entire fermentation process at decreasing concentrations. The highest amount of this compound was 20,398.15 µg/liter at the initial time of fermentation, and it could not be detected after 36 h (Table 3). Five aldehydes were detected during the *calugi* processing: acetaldehyde, hexanal, octanal, furfural and decyl aldehyde. The furfural compound was detected throughout fermentation, and its highest concentration (1,273.98 µg/liter) was found in the T0 sample. Acetaldehyde was detected from 12 h of fermentation. Isobutyl acetate was present at 48 h (28.83 µg/liter) and ethyl acetate was identified from 12 h of fermentation.

Table 3 Concentration of volatile compounds (μg/liter) identified during *calugi* fermentation

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

Nd – not detected

Two ethyl esters were present in the *calugi* preparation; these compounds were identified as ethyl butyrate and diethyl malate. The latter was found in considerable amounts (115.47 µg/liter) at 0 h of fermentation. Another group of compounds identified was the terpenes (3); α-terpeniol, b-citronellol and guaiacol were the representatives of this group. These compounds were identified only at the beginning of fermentation. The acetal (1,1-dietoxyethane) was detected from 0 h and increased throughout the process.

Multivariate analysis

A principal component analysis (PCA) was performed to determine the relationship between the microbial population as determined by plating and the consumption of the substrates and some fermentation products to show which compounds may be directly related to a specific microbial group (Fig. 4). This analysis included microbial counts in MRS, GYC, Nutrient Agar, and VRBG; the carbohydrates consumed (sucrose, glucose, fructose and maltose); and the physicochemical changes in the starch content and acid production (lactic, acetic, malic and citric).

Principal component analysis (PCA) showed that the most distant vectors from zero correspond to a variation with the greatest influence on the value of the main component. Principal components 1 and 2 together explained 88.02% of the variation that occurred between the different fermentation times. The lower left quadrant (negative side of PC1) 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 (negative side of PC2) shows 12 h of *calugi* fermentation, correlating the count in GYC medium (highest population of AAB) with the production of acetic acid and the peaks of glucose and fructose. After 24 h of fermentation, the production of lactic acid was increased (lower right quadrant). At the 36 and 48 h time points

(upper right quadrant), there is an increase in the concentration of lactic acid and maltose with larger populations in the VRBG and MRS media.

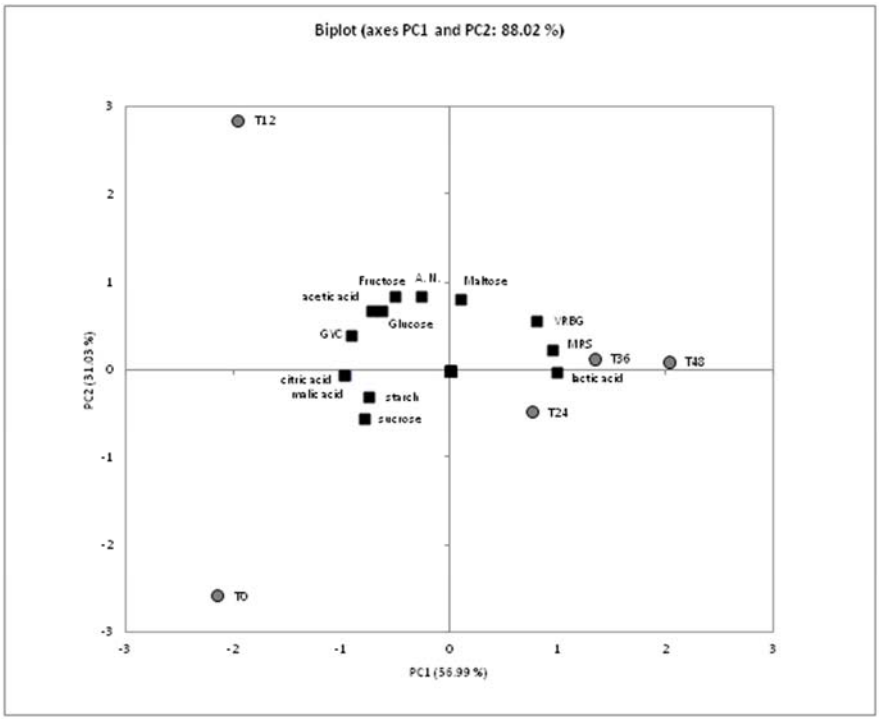

Figure 4 Principal component analysis (PCA) of enumeration and physical parameters during *calugi* fermentation

DISCUSSION

Traditional and rudimentary fermented foods and beverages prepared from substrates rich in fermentable carbohydrates, based on cereals and tubers such as rice, wheat, corn, sorghum, cassava or sweet potato, are consumed in many parts of the world. The microbiota of these products is quite complex and not widely known. Bacteria and yeasts have been reported in several different types of indigenous fermented foods and beverages (2, 21, 47, 51, 57). These

microorganisms are responsible for the physical and chemical changes in these products.

The study of the ecology of *calugi*, a cassava, corn and sweet potato fermented porridge, was carried out by traditional microbiological methods, including plate counts and culture-independent methods (PCR-DGGE). Combining these methods, we found that *Lactobacillus*, *Weissella* and *Bacillus* species were the dominant bacterial groups. Representatives of the *Corynebacterium, Streptomyces, Streptococcus* and *Enterobacteriaceae* groups were also found. Ampe et al. (7) suggested that microbial succession is determined by the sensitivities of microorganisms to the very acidic conditions that develop during the process.

The media MRS, GYC, Nutrient agar and VRBG were used for bacterial enumeration because they were previously reported to be efficient for the isolation of bacterial groups from fermented cassava products (2, 40). The culture-dependent approach indicated that AMB was the dominant microbial group. Ramos et al. (52) 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 (2, 21, 52).

In this study, representative organisms 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 (2, 3, 15, 47, 55).

The bacteria *Bacillus* spp. (*B. cereus* group) produced stronger bands in DGGE from 12 h until 36 hours of *calugi* fermentation. The disappearance of these bands after 36 h coincides with the reduction of the AMB population. The inhibition of *Bacillus* spp. (*B. cereus* group) growth and sporulation could be

attributed to the suboptimal pH, the presence of organic acids and competitive constraints (53). This finding was also reported by Ramos et al. (52). This group of pathogenic bacteria is ubiquitous and commonly found in soil, plant materials and in unprocessed foods (2).

Members of the *Bacillus* group secrete a wide range of degradative enzymes, including amylases and proteases, which may be important in the fermentation process. Amoa-Awua and Jakobsen (3) and Amoa-Awua et al. (5) demonstrated one of the possible roles of *Bacillus* species in cassava fermented products: the ability of some of these species (e.g., *B. subtilis*) to hydrolyze the tissues of cassava through the production of cellulases.

Strains of *Corynebacterium* can also secrete amylase, and the species *C. variabile* was found at time 0 h in the fermentation of *calugi*, indicating that this fermentative microorganism originated from the specific inoculum. In agreement with this result, Ramos et al. (52) found that most species of *Corynebacterium* present in a peanut and rice fermentation were contained in the chewed sweet potato, suggesting that the inoculum was the source of these bacteria in the fermented product.

The species of the genera *Bacillus* and *Corynebacterium* are known for their ability to hydrolyze starch (46), 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 (2, 48). The high concentration of maltose observed throughout the fermentation process may be due to the amount of starch in cassava, which is approximately 30-50% of its dry weight (49). 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. Similar results in Zambian munkoyo were reported by Foma et al. (21) using *Rhynchosia insignis*

roots and by Almeida et al. (2) and Tou et al. (60) in several fermented cereal-based beverages (cauim and ben-saalga). Almeida et al. (2) reported that some bacteria isolated during cassava fermentation could secrete both amylases and proteases; therefore, they could be contributing to the starch hydrolysis of cassava and also to the increased protein content observed here. Soluble protein values varied from 13.38 to 16.36% during the fermentation. The small increase in soluble protein was similar to that reported by Almeida et al. (2). Those authors explained that the increase in protein content could be due to the presence of proteolytic organisms capable of hydrolyzing the proteins in this fermentation. Another possibility is that the highest protein content may be correlated with the increase in the microbial biomass during the fermentation process and the consequent secretion of protein molecules (32).

Interestingly, for the first time, *Streptomyces* sp. was detected in cassava and corn fermentations. This actinobacteria was detected by DGGE at 0 h of fermentation, indicating that it probably originated from the inoculum. *Streptomyces* species are heterotrophic feeders, and they can utilize both simple and complex molecules as nutrients. Although proteases and amylases are mainly fungal and eubacterial products, the possibility of using *Streptomyces* for enzyme production has been investigated (62). *Streptomyces* species liberate extracellular enzymes, and approximately three-quarters of *Streptomyces* species may produce antibiotics (25).

In cassava fermented foods, a high number of lactic acid bacteria have been found by thers, for example, Oguntoyinbo and Dodd (41), Almeida et al. (2) and Lacerda et al. (31). In such fermentations, endogenous grains of amylases generated fermentable sugars that serve as a source of energy for the LAB. *L. plantarum* and *L. paracasei* were detected mainly in sample T0, which indicates that these LAB might have originated from the fermentation vessel or the inoculum, the ambient, or had been associated with the tubers. Ramos et al.

(51) studied a traditional inoculated beverage with the mastication juice of sweet potato and observed that the microbial populations present in the saliva and just before the addition of the inoculum were different, except for *L. fermentum* and *L. paracasei*, which were present in the saliva. Therefore, the saliva is an important inoculum due to amylase and to the presence of some strains of LAB.

S. parasanguis was detected by DGGE at 48 h of fermentation, indicating that this species is probably a contaminant of the process and possibly comes from the people who prepared the porridge. Amoa-Awua et al. (4) also reported the occurrence of *Streptococcus* spp. in cassava fermentations. *W. cibaria, W. confusa, S. salivarius* and *L. casei* were found during all fermentation times and seem to be mainly responsible for the production of lactic acid and acetic acid. In addition to the involvement of *L. plantarum* and *W. confusa* in the process, which contribute to the acidification process, the involvement of *W. cibaria* in *calugi* fermentation could be specific to this type of fermentation. *W. confusa* has also been isolated from maize-derived *ogi* (39). During the entire processing of the *calugi* fermentations, the numbers of lactic acid bacteria remained constant, indicating high microbial stability. In agreement with our results, *L. plantarum*, *L. paracasei*, *L. casei*, and *W. confusa* were reported to be present at lower levels during the fermentation of *agbelima* (4), *gari* (30) and *attieke* (15).

The use of culture-independent techniques has enabled us to determine the LAB succession during the fermentation of *calugi*. The results showed that the dynamics of LAB are time dependent. Different strains of LAB were detected with the progression of fermentation, and there was a decrease in the diversity of bands over time. This is likely to be due to minor components of the original microbiota being selected by the acid or other nutritional conditions. 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. The diverse strains of LAB identified in this work have been reported to have different desirable functionalities during the fermentation of vegetal starch (34). The 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 (51), cottonseeds and rice (52), cassava dough (34) and *togwa* (36). These species were also predominant in Nigerian *ogi* (42).

There was a slight increase in the ethanol concentration after 24 h; this increase probably resulted from the LAB activity that can convert sugars into lactic acid, acetic acid, fumaric acid, ethanol and carbon dioxide. During the fermentation process, lactic acid bacteria contributed the most to the progressive acidification of the product and the pH decreased. 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 reduction in pH has been reported to be due to the production of acids by fermenting microorganisms (56). The final pH of *calugi* was approximately 4.0; these low pH values were also found in *cauim* fermentation (2), in *fufu* production (20, 45) and in a cottonseed and rice beverage (52).

The carbohydrates (glucose, fructose and sucrose) were detected in small amounts of no more than 3 g/liter. Chelule et al. (14) reported sucrose consumption by acid lactic bacteria, some species of *Bacillus* and yeasts during fermentation of *amahewu*, a maize-based porridge. The highest values of glucose and fructose were observed at 12 h of fermentation and declined after 24 h, being used by the microorganisms as carbon and energy sources for the production of metabolites such as alcohols and acids.

The slight increase in acidity may be attributed to alcohols and organic acids. Organic acids identified in *calugi* fermentations include citric, succinic, acetic, lactic, malic, tartaric, propionic and oxalic acids. Banigo and Muller (9) identified the main acids in *ogi* as lactate, butyrate, acetate and formate. The production of these acids during the fermentation of maize dough porridge and their ability to inhibit a variety of organisms has been reported (33). Lactic, acetic, malic, succinic and formic acid have been reported in cereal-based fermented traditional beverages in Nigeria (54). Propionic acid was detected at low concentrations in *calugi* fermentation; these results are similar to those reported by Onyango et al. (44) in maize, fingermillet, cassava, maize–fingermillet and cassava fingermillet fermented *uji*. Organic acids occur in fermented products as a result of hydrolysis, biochemical metabolism and microbial activity; quantitative determination of organic acids is important in the study of fermented foods for technical, nutritional, sensorial and microbial reasons (59).

Cassava contains many compounds such as calcium, phosphorus, vitamin B2 and niacin, which may correlate with the concentration of citric acid at the beginning of the fermentation (49). Citric acid can be metabolized to acetic acid, carbon dioxide and lactic acid. Accordingly, in the present study, the amount of citric acid was reduced, and from 24 h, was no longer detected. This result may be associated with the increase in the concentration of acetic and lactic acids at 12 h of fermentation.

The results from this study showed that 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. These results showed that the production of some acids is related to some groups of microorganisms (21, 64). Lactic acid production increased up to the end of fermentation (24 h), reaching

3.54 g/liter at the end of fermentation. High concentrations of lactate have also been reported for fermentations of maize and cassava during the production of *pozol* (6) and *cauim* (2). A significant production of acetate was also observed by Santos et al. (55) in fermenting cassava and sweet potatoes during the production of the alcoholic beverage *caxiri*. The finding that lactic and acetic acids are produced during *calugi* fermentation is in agreement with the results of Giraud et al. (22), who concluded that heterofermentative lactic acid fermentation occurs in most of the spontaneously fermented cassava products in Africa.

Acetic acid was produced intensively during the first 12 h of fermentation, reaching a concentration of 5.47 g/liter; however, at 24 hours, there was a noticeable decrease (0.66 g/liter). The loss of acetic acid during fermentation might be due to the consumption of alcohol by the bacteria and the natural evaporation of the volatile constituents (alcohol and acetic acid). In some cases, the loss of acetic acid may be higher due to the transformation of acetic acid to water and carbon dioxide by *Acetobacter* spp (17).

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 (34, 52). Several isolates were collected in GYC agar plates, but did not produce a detectable DGGE band in *calugi* DNA fingerprinting. This finding illustrates the intrinsic limitation of DGGE analysis in visualizing only the predominant species of a microbial community (38). Considering specific limitations of both cultivation-independent and cultivation-dependent methods, a polyphasic approach for broad-coverage biodiversity studies of complex ecosystems is recommended (58).

The tolerance to low pH by LAB along with the propensity of lactic acid bacteria to produce a variety of antimicrobial substances creates unfavorable

conditions for the growth of pathogens as well as toxigenic and spoilage organisms. The presence of LAB in *calugi* porridge might play a role in the low counts of pathogenic microorganisms despite the rudimentary preparation process. GNE was detected at low counts after 12 h of fermentation, and the population remained constant at approximately 2 log CFU/ml. *E. cloacae* was the GNE detected by the PCR-DGGE method; its band became stronger at 36 h, which coincided with the largest population as determined by plating (approximately 2.6 log CFU/ml). Ramos et al. (52) reported that the presence of LAB in a spontaneous peanut and rice fermentation might play a role in the reduced survival of bacterial pathogens despite the use of saliva as the inoculum. On the other hand, cooking corn and cassava masses may be considered as a sterilization procedure and might explain the low contamination levels during fermentation. Almeida et al. (2) performed a microbiological analysis of chewed sweet potato and revealed the presence of *E. cloacae* in it and in samples after inoculation. Achi (1) reported the presence of *Enterobacter* spp. during the fermentation of *obiolor* and attributed their presence to the fact that they are soil organisms and are found mostly as food contaminants. Bacteria from these genera were also shown to be present in indigenous beverages in Brazil (51, 52, 55).

Microorganisms adapted to substrate conditions by virtue of their metabolic activities contribute to the development of characteristic properties such as taste, aroma, visual appearance, texture, shelf life, nutritional value and the safety of fermented foods (27). Many of these desirable attributes may also be associated with spontaneous food fermentations, the outcome and quality of which may not always be predictable or controllable. This is a major problem typically associated with traditionally fermented foods, mainly in Africa and in South America. Their preparation generally relies on inoculation type (i.e., either

natural contamination or back-slopping), and the result is often a product of inconsistent quality, poor hygiene, poor nutritional value and short shelf life.

Volatile compounds are linked to the fermentation and processing of food (41). During cereal fermentation, several volatile compounds are formed, which contribute to the complex flavors of the products (13). Some volatile compounds were detected during the fermentation of *calugi* porridge, including alcohols, acetates, aldehydes, ethyl esters, terpenes and acetal (Table 3). The compounds isobutyl acetate, nonanoic and decanoic acids were the main volatiles produced at 24 h of fermentation in concentrations of 148.25, 258.05 and 141.03 µg/liter, respectively. However, at 36 h, the more abundant compounds were acetaldehyde and 1.1-dietoxyethane (102.39 and 89.12 µg/liter, respectively). Accordingly, there was a relatively lower concentration of volatile compounds in the millet-based product (36). Large variations in volatile organic compounds were also observed between individual household bushera samples (37). Some of the flavor compounds may originate from the unfermented substrates.

The presence of aromas representative of diacetyl, acetic acid and butyric acid make fermented cereal based products more appetizing (10). According to some authors, ethyl butyrate is characterized as having a fruity aroma, similar to papayas and apples, butter and sweetish (16). Lactate and acetate are important flavor enhancers in fermented cereals (44), 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 (24). Acetates are the result of the reaction of acetyl-CoA with higher alcohols, which are formed through the degradation of amino acids or carbohydrates. These compounds contribute positively to the overall wine quality, and most of them have a mature flavor and fruity aroma that contribute to the “fruity” and “floral” sensorial properties (50).

The *L. plantarum*, a species found in *calugi* samples, has the ability to produce aromatic compounds that can give a pleasant fruity aroma. LAB are thought to be responsible for producing a series of aldehydes and esters (26) and thereby impart a pleasant aroma to the final product. It has also been reported that products prepared under spontaneous fermentation show instability because of inconsistency in the natural inoculum, thus causing variation in fermentation parameters from one batch to another (29).

The proteolytic activity of fermentation microorganisms, often in combination with malt enzymes, may produce precursors of flavor compounds, such as amino acids, which may be deaminated or decarboxylated to aldehydes, and these may be oxidized to acids or reduced to alcohols (23). The resulting methyl alcohols are reported to be flavor enhancers (24). Furfuryl alcohol is a product of furfural reduction by many bacteria under aerobic conditions (11), 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 (10).

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 (18). The 1,1-dietoxyethane compound is an acetal 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. This compound can be produced by the acid-catalyzed reaction of ethanol and acetaldehyde (12).

In conclusion, this is the first study reporting the microbiological and physicochemical characterization of *calugi* porridge produced by the *Javaé* people. The potential of PCR-DGGE as a molecular tool to identify the microbiota potentially involved in *calugi* production was demonstrated. Our

results emphasize the dependence of cassava fermentation on the microbiota of the traditional inoculum. In addition, the identification of the dominant microorganisms at each processing stage of *calugi* contributes to a better understanding of the fermentation process. Species of the genera *Bacillus, Lactobacillus* and *Weissela* were the predominant microorganisms present in the beverage. The results obtained in this work are important for characterizing the microbiota, the metabolites produced and the physicochemical changes that occur during fermentation of *calugi*.

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ARTIGO 2

Physico-chemical and microbiological characterization of corn and rice 'calugi' produced by Brazilian Amerindian people

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ABSTRACT

'Calugi' is a fermented porridge that is produced from corn and rice by Javaé Amerindians. Samples of this porridge were chemically and microbiologically characterized. The microbial population was composed of yeasts, aerobic mesophilic bacteria (AMB), lactic acid bacteria (LAB), acetic acid bacteria (AAB) and some enterobacteria. The population of lactic acid bacteria (LAB), acetic acid bacteria (AAB) and yeasts increased slightly during 'calugi' fermentation. During the initial fermentation period (12 and 24 h), counts of the bacterial population (LAB, AMB and enterobacteria) and yeast increased. After 48 h of fermentation, the population of mesophilic bacteria was 5.06 log CFU/mL; lactic acid bacteria (LAB), 4.69 log CFU/mL; yeast, 4.37 log CFU/mL; Enterobacteria, 3.29 log CFU/mL and acid acetic bacteria (AAB), 3.14 log CFU/mL. During the fermentation process, *Lactobacillus plantarum, Streptococcus salivarius, S. parasanguis, Weissella confusa, Enterobacter cloacae, Bacillus cereus* and *Bacillus* sp. and the yeasts *Saccharomyces cerevisiae*, *Pichia fermentans* and *Candida* sp. were detected by PCR-DGGE. The LAB were dominant during the process and were likely most responsible for the reduction in pH value, which permitted yeast growth. Carbohydrates (70.48 g/L – maltose), alcohols (1.70 g/L – glycerol) and acids (4.56 g/L – acid lactic) were identified by HPLC. Fifteen volatile compounds were identified and quantified by GC-FID. From the fermentation, acetaldehyde, 1-1-dietoxyethane, isobutyl acetate, ethyl acetate, furfuryl alcohol and nonanoic acid were identified, with maximum concentrations of 457.02 µg/L, 73.96 µg/L, 54.03 µg/L, 24.82 µg/L, 755.82 µg/L, 61.85 µg/L, respectively.

1. Introduction

In several countries, foods and beverages are largely produced from cereals, and the preparation of many indigenous or traditional fermented foods and beverages remains a home art (Blandino, Al-Aseeria, Pandiellaa, Cantero, & Webb, 2003).

Spontaneous food fermentations are performed by proto-cooperation between yeast, lactic acid bacteria (LAB), acetic acid bacteria (AAB) and fungi (Blandino, Al-Aseeria, Pandiellaa, Cantero, & Webb, 2003). The symbiosis between bacteria and yeasts has been reported in the fermentation of traditional beverages such as hawaijar (Jeyaram et al., 2008), doenjang (Kim et al., 2009), kefir (Miguel, Cardoso, Lago, & Schwan, 2010), cauim (Ramos et al., 2010) and caxiri (Santos, Almeida, Melo, & Schwan, 2012).

Studies of a Brazilian beverage produced from cereals such as rice, cassava, peanuts, and cotton seed demonstrated that both bacteria and yeast were present during fermentation. Recently, Santos, Almeida, Melo, & Schwan (2012) reported the presence of *Bacillus subtilis*, *B. pumilus*, *Lysinibacillus fusiformis*, *Lactobacillus fermentum*, *Pediococcus acidilactici*, *Enterobacter* sp., *Saccharomyces cerevisiae*, *Rhodotorula mucilaginosa, Pichia guilliermondii* and *P. membranifaciens* during caxiri fermentation. In cotton and rice fermentations (Ramos, Almeida, Freire, & Schwan, 2011), *L. vermiforme, L. paracasei, L. plantarum, Bacillus subtilis* and *B. cereus* and the yeasts *Candida parapsilosis, C. orthopsilosis* and *Rhodotorula mucilaginosa* were identified. Almeida, Rachid, & Schwan (2007) and Schwan, Almeida, Souza-Dias, & Jerpersen (2007) identified *L. plantarum, L. pentosus, Enterobacter cloacae, B. cereus, B. circulans, B. pumilus* and the yeasts *Saccharomyces cerevisiae, C. parapsilosis, C. intermedia and Pichia guilliermondii*, among other microorganisms, in the beverage cauim.

Microbial activity produces many different chemical compounds, such as the organic acids commonly found in fermented products, as a result of hydrolysis and biochemical metabolism. The quantitative determination of organic acids such as lactic acid, acetic acid, malic acid and propionic acid in fermented foods is important for technical, nutritional, sensorial and microbial reasons (Shukla et al., 2010).

The beverage 'calugi', which is made from different substrates such as rice, cassava and corn, is traditionally produced and consumed by Javaé Brazilian Indians. This non- alcoholic beverage is consumed by adults and children. However, to the best of our knowledge, the physicochemical parameters, volatile composition and microbiota during the fermentation of 'calugi' have not been determined. Thus, the aims of this work were to identify the microorganisms involved in the fermentation of 'calugi' by PCR-DGGE and identify the metabolites present in the fermentation process by gas chromatography-flame ionization detection (GC-FID) and high-performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Beverage preparation and sampling

A 'calugi' of corn and rice was prepared by the local Amerindian Javaé, who live next to the Formoso do Araguaia mountain (Tocantins, Brazil). To prepare the porridge (Fig. 1), approximately 700 g of dried corn (*Zea mays*), 3 kg of rice and 100 g of sweet potato (*Ipomoea batatas* L.) were used. The corn was soaked in water for 30 min before maceration in a rustic wooden pestle. The resulting corn flour was mixed with 3 L of water and sieved to remove the peel. The corn and rice were mixed with7 L of water and cooked for 2 hours; the mixture was stirred every 10 minutes. The sweet potato was peeled and washed before use. Approximately 40 min after the end of cooking (when the porridge

had cooled), the inoculum (mastication juice of the sweet potato) was added. Then, the mixture was homogenized and allowed to ferment at ambient temperature (approximately 30 ºC).

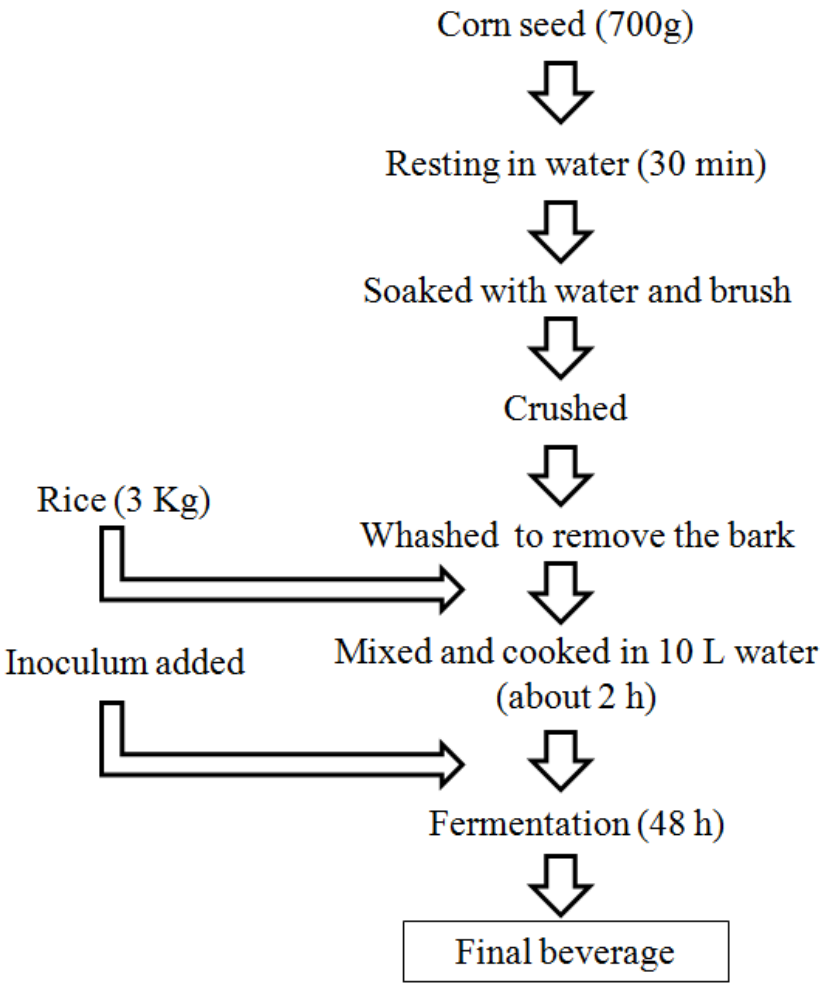

Figure 1 Flow diagram for the manufacture of the beverage from corn and rice

2.2. Sampling

Twenty milliliters of cooked and fermenting substrate was collected at 12 h intervals and added to a sterile bottle containing 180 mL of saline peptone solution (0.1% peptone, 0.5% NaCl, and 0.03% $Na_2H_2PO_4$). The samples were maintained at 4 - 6 ºC until analysis (approximately 4 h).

2.3. Isolation and counting

Bacteria and yeasts were enumerated on five different culture media, namely MRS (De Man Rogosa Sharpe, Merck, Darmstadt, Germany), GYC [50 g/L glucose (Merck, Darmstadt, Germany), 10 g/L yeast extract (Merck, Darmstadt, Germany), 30 g/L calcium carbonate (Merck, Darmstadt, Germany), pH 5.6], Nutrient agar medium, VRBG agar (violet red bile with glucose) (Oxoid, Hampshire, England) and YPD agar [10 g/L yeast extract (Merck, Darmstadt, Germany), 10 g/L peptone (Himedia, Mumbai, India), 20 g/L glucose (Merck, Darmstadt, Germany), 20 g/L agar (Merck, Darmstadt, Germany)]. MRS agar containing 0.1% cysteine-HCl was used to enumerate LAB under anaerobic conditions. AAB were enumerated on GYC agar containing 0.1% cycloheximide to inhibit yeast growth and 50 mg/L penicillin (Sigma, St. Louis, USA) to inhibit LAB growth. Nutrient agar medium (Merck, Darmstadt, Germany) was used as a general medium for the growth of mesophilic bacteria. The VRBG medium was used for enterobacteria. For yeast enumeration, YPD agar containing 100 mg/L chloramphenicol (Sigma, St. Louis, USA) and 50 mg/L chlortetracycline (Sigma, St. Louis, USA) to inhibit bacterial growth was used. After spreading, the MRS plates were incubated in acrylic anaerobic jars at 30 °C for 3–4 days. YPD and Nutrient agar plates were maintained at 30 ºC for 2-5 days. VRBG plates were maintained at 36 °C for 3-4 days. GYC plates were maintained at 25 °C for 5–8 days. After the incubation period, the colony-forming units (CFUs) were enumerated.

2.3. PCR analysis

The total DNA was extracted from samples acquired at different times during the fermentation process using the protocol DNA Purification from Tissues [(QIAamp DNA Mini Kit (Qiagen, Hilden, Germany)] in accordance with the manufacturer's instructions. The final samples were stored at −20 °C until further use.

The DNA from the bacterial community was amplified with the primers 338fgc (5' -CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG 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, Forney, Daae, & Torsvik, 1997). A fragment of the D1-region of the 26S rRNA gene was amplified with the eukaryotic universal primers NL1GC (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA TAT CAA TAA GCG GAG GAA AAG-3') (the GC clamp is underlined) and LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3'), which amplified a fragment of approximately 250 bp (Cocolin, Bisson, & Mills, 2000). All reactions were performed in a 25 μL volume containing 0.625 U Taq DNA polymerase (Promega, Milan, Italy), 2.5 mL 10X buffer, 0.1 mM dNTP, 0.2 mM each primer, 1.5 mM $MgCl_2$ and 1 mL of extracted DNA. The amplification was performed according to Ramos et al., (2010). Aliquots (2 mL) of the amplified products were analyzed by electrophoresis on 1% agarose gels before the DGGE analysis.

2.4. PCR-DGGE analysis

The PCR products were separated in polyacrylamide gels [8% (w/v) acrylamide:bisacrylamide (37.5:1)] in 1× TAE buffer with a DCode system apparatus (BioRad Universal Dcode Mutation Detection System, Richmond, CA,USA); solutions containing 35–70% denaturant [100% denaturant

corresponds to 7 M urea and 40% (v/v) formamide] were used for the bacterial community, and solutions containing 30-60% denaturant were used for the yeast community. The gels were run at 60 ºC for 6 h at a constant voltage of 120 V. After electrophoresis, the gels were stained with SYBR-Green I solution (Molecular Probes, Eugene, UK) (1:10.000 v/v) for 30 min, and the images were visualized and photographed with a transluminator (LPix®).

Various DGGE bands were excised from the acrylamide gels. The DNA fragments were purified with a QIAEX II gel extraction kit (Qiagen, Chatsworth, CA, USA) and re-amplified with the primer 338fgc and 518r for bacteria and NL1 and LS2 for yeast. The PCR products were purified and sequenced by Macrogen Inc. (Seoul, South Korea), and the obtained sequences were compared with those available in the GenBank database with the BLAST algorithm (National Center for Biotechnology Information, Maryland, USA).

2.5. Chemical analysis of the fermented 'calugi' beverage

The samples were characterized by pH, total soluble solids (º Brix), protein, fat (AOAC, 2000), total sugars (Dische, 1962) and starch content (Areas & Lajolo, 1987).

2.6. Substrates and metabolites

Before GC-FID analysis, volatile compounds were extracted according to Duarte et al., (2010). The extract containing the volatile compounds was analyzed in a Shimadzu GC Model 17A equipped with a flame ionization detector (FID) and a capillary column of silica DB Wax (30m x 0.25mmi.d. x 0.25µm) (J & W Scientific, Folsom, Calif., USA). The temperature program began with 5 min at 50 ºC, followed by a gradient of 50 ºC to 190 ºC at 3 ºC/min; the temperature was then maintained at 190 ºC for 10 min. The injector and detector temperature were kept at 230 ºC and 240 ºC, respectively. The

carrier gas (N_2) was used at a flow rate of 1.2 mL/min. Injections of 1 µL were made in the split mode (1:10). Volatile compounds were identified by comparing the retention times of the compounds in the samples with the retention times of standard compounds injected under the same conditions. Volatile compounds were semi-quantified with 4-nonanol at a final concentration of 312 µg/L as an internal standard. All samples were examined in duplicate.

Carbohydrate (glucose, sucrose, maltose and fructose), organic acid (acetic acid, lactic acid, malic acid, citric acid, propionic acid and succinic acid) and alcohol (ethanol and glycerol) analyses were performed with a Shimadzu liquid chromatography system (Shimadzu Corp., Japan) equipped with a dual detection system consisting of a UV-Vis detector (SPD 10A*i*) and a refractive index detector (RID-10A*i*). A Shimadzu ion exclusion column Shim-pack SCR-101 H (7.9 mm x 30 cm) was used at an operating temperature of 30 ºC for ethanol and glycerol and 50 ºC for acids. Perchloric acid (100 mM) was used as the eluent at a flow rate of 0.6 mL/min. Acids were detected via UV absorbance (210 nm), while alcohols were detected via RID. Carbohydrates were analyzed on a Supelcosil LC-NH_2 column (4.6 mm x 25 cm) operating at 30 ºC with acetonitrile:water (75:25) as the mobile phase at a flow rate of 1 mL/min. The sugars were detected via RID. Individual compounds were identified by comparing their retention times with the retention times of certified standards, and concentrations were determined according to external calibration methodology. All samples were analyzed in duplicate.

2.7. *Statistical analysis*

Principal component analyses were performed with the software XLSTAT 7.5.2 (Addinsoft, New York, N.Y., USA) for group data from microbial counts and metabolites produced during corn and rice ‘calugi’ fermentation.

3. Results and discussion

3.1 Microbial counts

The populations of LAB, AAB, enterobacteria and yeasts increased slightly during 'calugi' fermentation (Fig. 2). After 48 h of fermentation, the counts of these four groups were 4.69 log CFU/mL (LAB), 4.37 log CFU/mL (yeast), 3.29 log CFU/mL (enterobacteria) and 3.14 log CFU/mL (AAB). LAB, AAB and yeast have been reported to be the most common and important groups of microorganisms in the fermentation of indigenous cereal-based foods produced with sorghum, wheat, maize and rice (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb. 2003). These groups of microorganisms are responsible for the production of lactic acid, acetic acid and alcohol.

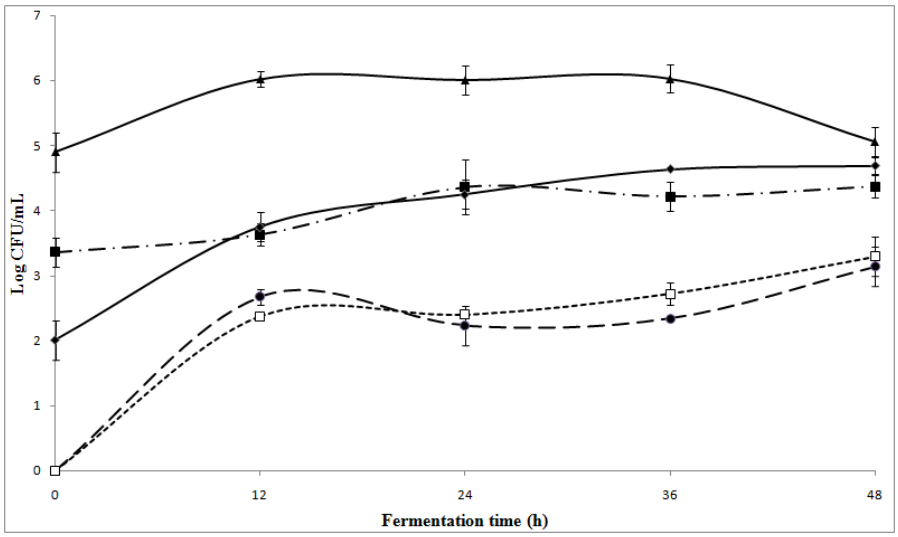

Figure 2 Numbers (log CFU/ml) of total mesophilic bacteria (▲), acetic acid bacteria (●), lactic acid bacteria (♦), *Enterobacter* (□) and yeast (■) during the 'calugi' fermentation

The population of mesophilic bacteria ranged from 4.9 log CFU/mL to 5.06 log CFU/mL. Although mesophilic bacteria are commonly found in different cereal-based fermented foods and beverages as well as roots (Almeida, Rachid, & Schwan, 2007; Foma, Destain, Mobinzo, Kayisu, & Thonart, 2012; Gadaga, Mutukumiraa, Narvhus, & Feresuc, 1999; Ramos, Almeida, Freire, & Schwan, 2011), the population found in 'calugi' was lower than that reported by Ramos et al., (2010) for a cauim beverage produced with peanut and rice. This difference could be due to the inoculum source and the substrate used for the beverage preparation.

The LAB were detected throughout the fermentation process. At the beginning of the fermentation, the LAB population was approximately 2.01 log CFU/mL; after 24 h of fermentation, the LAB population reached 4.25 log CFU/mL and remained at this level until the end of the process (Fig 2). According to McDonald, Fleming, & Hassan (1990), LAB are acid-tolerant and often dominate the fermentation of vegetables and cereals, mainly due to their ability to transport and metabolize different carbohydrates. The LAB behavior observed in this work is in good agreement with results reported by Ramos, Almeida, Freire, & Schwan (2011) for cauim produced with cotton seed and rice or with peanut and rice (Ramos et al., 2010). According to these authors, a symbiotic association occurs between the coexisting mesophilic bacteria, LAB and yeast during fermentation to produce the studied beverages.

The yeast population exhibited moderate variations throughout the fermentation process, with values ranging between 3.36 and 4.36 log CFU/mL (Fig. 2). The growth of yeasts in fermented foods is favored by the acidification of the environment by bacteria, and the growth of bacteria is stimulated by the presence of yeasts, which can provide growth factors such as vitamins and soluble nitrogen compounds (Nout & Sarkar, 1999).

Enterobacteria were not detected at 0 h of fermentation (Fig. 2). At 12 h to 48 h of fermentation, the enterobacterial population ranged from 2.37 log CFU/mL to 3.29 log CFU/mL. Similar results were obtained by Almeida, Rachid, & Schwan (2007) and Madoroba et al., (2011) in their studies of fermented cereal-based foods and beverages. The trend in enterobacterial counts was parallel to that observed for the AAB population throughout the time course of the fermentation (Fig. 2). Moderate variations in the AAB population were observed, which ranged from 2.67 CFU/mL (12 h fermentation) to 3.14 CFU/mL (48 h fermentation). In their studies of ogi, a fermented food produced in Nigeria, Oyarekua, (2011) have also reported a low count of enterobacteria and other gram-negative bacteria, which indicates that their growth may have been inhibited by the presence of lactic acid, resulting in a decrease in the population and in the pH value.

3.2 DGGE analysis of the bacterial and yeast communities

The results of the culture-independent methods (PCR-DDGE) demonstrated that the bacterial population was dominant over the yeast population throughout the fermentation process. In most of cereal-based products, fermentation does not occur spontaneously and requires a mixed culture of yeasts, fungi and bacteria. These groups of microorganisms can act simultaneously in a cooperative manner, and thus, the dominance varies during fermentation (Blandino, Al-Aseeri, Pandiella, Catero, & Webb, 2003). The predominance of bacteria in this study may be due to the secretion of compounds such as organics acids, which may inhibit the growth of some bacteria and yeasts in the fermenting medium. The succession of bacteria and yeasts is determined by the sensitivities of the microorganisms to the very acidic conditions that develop during the fermentation process (Ampe, Sirvent, & Zakhia, 2001). Figures 3 and 4 shows the results of the DGGE analysis of the

bacterial and yeast communities, respectively. Changes in the bacterial community were evident after 12 h of fermentation (Fig. 3). Sequencing of the bands indicated the presence of *Lactobacillus plantarum* (98% similarity), *Streptococcus salivarius* (99%), *Streptococcus parasanguis* (100%), *Weissella confusa* (98%), *Enterobacter cloacae* (100%), *Bacillus cereus* (98%) and *Bacillus sp*. (97%) (Fig. 3) and the yeasts *Saccharomyces cerevisiae* (99%), *Pichia fermentans* (100%) and *Candida* sp. (98%) (Fig. 4).

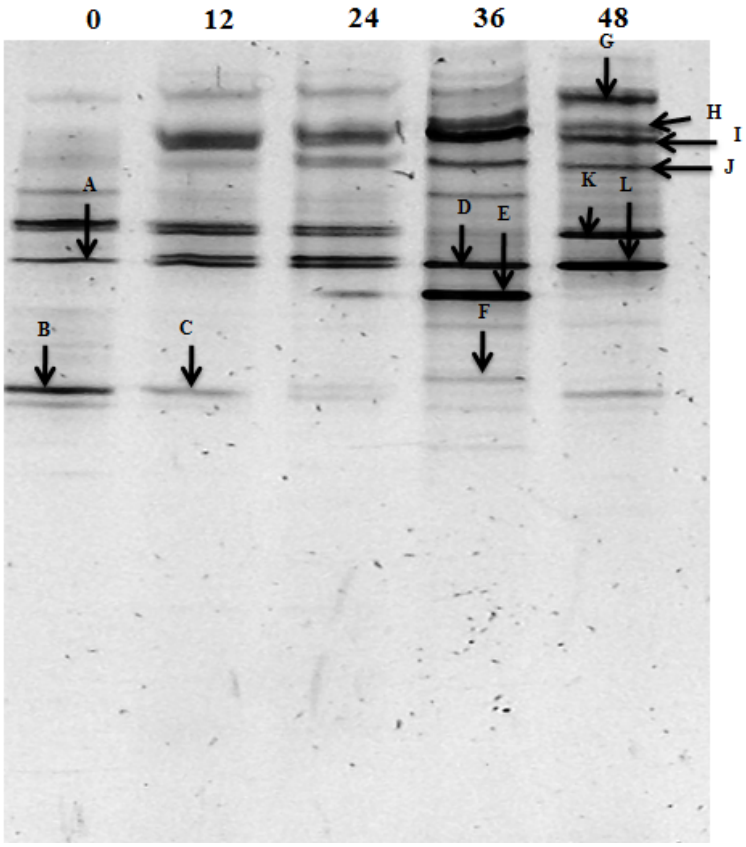

Figure 3 Denaturing gradient gel electrophoresis profiles of the V3 regions of the bacterial 16S rRNA gene amplified from the 'calugi' beverage throughout the 48 h of fermentation. A = *Streptococcus salivarius* (GU425986.1), B = *Uncultured bacteria* (AB241206.1), C = *Uncultured bacteria* (FN780500.1), D = *Streptococcus salivarius* (CP002888.1), E = *Enterobacter cloacae* (HM438951.1), F = *Bacillus cereus* (HQ333011.1), G = *Lactobacillus plantarum* (EF 426261.1), H = *Uncultured bacteria* (FN775581.1), I = *Streptococcus parasanguis* (AM157421.1), J = *Weissella confusa* (HM032145.1), K = *Bacillus* sp. (AY176766) and L = *Streptococcus salivarius* (CP002888.1)

The LAB are required to mediate the fermentation process in most fermented foods; species of the genera *Leuconostoc*, *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Micrococcus* are the most common in the fermentation of food and beverages produced using cereals such as sorghum, wheat, maize and rice (Blandino, Al-Aseeri, Pandiella, Catero, & Webb, 2003). They also frequently predominate in fermented foods due to their ability to tolerate low pH; species of this group produce a variety of antimicrobial substances and create conditions that are unfavorable for the growth of pathogens and toxigenic and spoilage organisms.

S. salivarius was the predominant species throughout the fermentation of the 'calugi' beverage, indicating that this bacterium is most likely derived from the chewed sweet potato inoculum. This species is a prominent member of the oral microbiota of humans and fermented foods such as yogurt and cheese (Talon, Walter, Viallon, & Berdague, 2002; Delorme, 2008) and is closely related to *Streptococcus thermophilus* (Bentley, Leigh, & Collins, 1991).

Saccharomyces cerevisiae was the only yeast detected in the 'calugi' beverage in samples T0 and T12 (Fig.4). The yeast population changed after 24 h of fermentation, at which time *S. cerevisiae, Pichia fermentans* and *Candida* sp. were detected. After 36 and 48 h of fermentation, *Pichia fermentans* and *S. cerevisiae* were detected, respectively (Fig. 4). The *S. cerevisiae* has also been reported by others authors in fermented non-alcoholic caium beverages produced by Brazilian Amerindians (Ramos et al., 2010; Ramos, Almeida, Freire, & Schwan, 2011) and is considered the most common yeast in cereal-based beverages (Blandino, Al-Aseeri, Pandiella, Catero, & Webb, 2003). The main role of *S. cerevisiae* in the fermentation of foods and beverages is the conversion of carbohydrates to alcohols and other aroma components such as esters, organic acids and carbonyl compounds, which are responsible for the

aroma that characterizes the final product (Torner, Martinez-Anaya, Antuña, & Benedito de Barber, 1992).

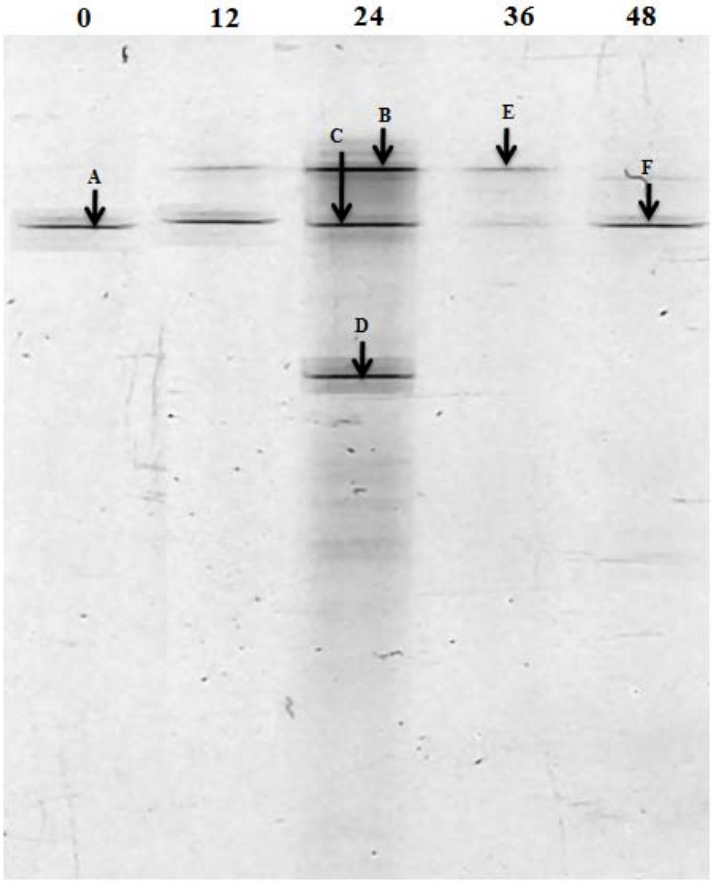

Figure 4 Denaturing gradient gel electrophoresis profiles of the NS3 regions of the yeast 18S rRNA gene amplified from the 'calugi' beverage throughout the 48 h of fermentation. A = *Saccharomyces cerevisiae* (EU649673.1), B = *Pichia fermentans* (FJ770542.1), C = *Saccharomyces cerevisiae* (GM981616), D = *Candida* sp. (GI190714325), E = *Pichia fermentans* (DQ665310.1) and F = *Saccharomyces cerevisiae* (EU649673.1)

3.2 Beverage analysis

3.2.1 Chemical characterization

The pH value decreased from 6.0 (T0) to 3.5 after 48 h of fermentation. The production of organics 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, Al-Aseeri, Pandiella, Catero, & Webb, 2003). The total soluble solids exhibited a slight decrease from 11 ºBrix (0 h fermentation) to 10 ºBrix (48 h fermentation).

The starch content (4.6% at 0 h of fermentation) decreased during the fermentation period to 4.21% at 48 h of fermentation (Table 1). This reduction in the starch content may be due to its use as a carbon source by the microorganisms present in the beverage, such as *Bacillus*, *Enterobacter*, *Lactobacillus* and *Candida* (Mugula, Narvhus, & Sørhaug, 2003) The fat and protein content decreased to 8.16% and 7.53%, respectively, after 48 h of 'calugi' fermentation (Table 1). The slight reduction in the fat content in this study is likely due to the lipolytic activity of the bacteria and/or yeast genera that are present during the 'calugi' fermentation process, such as *Saccharomyces*, *Candida* and *Bacillus* (Arpigny & Jaeger, 1999). The slight decrease in soluble protein levels can be correlated with the presence of proteolytic organisms such as *L. plantarum*, *Bacillus*, *E. cloacae* and *S. cerevisiae*. *L. plantarum*, which have been isolated from cassava fermentation and can secrete proteases, thereby contributing to the hydrolysis of protein (Holzapfel et al., 1997). After 48 h of fermentation, the total sugar content increased (5.93 g/100g) (Table 1), most likely due to starch degradation (Mugula, Narvhus, & Sørhaug, 2003).

Table 1 Physico-chemical characterization of 'calugi'

	Fermentation time (h)				
Characteristics	0	12	24	36	48
Fat	9.44 ± 0.09	8.74 ± 0.14	9.06 ± 0.58	8.98 ± 0.69	8.16 ± 0.18
Total sugars (g/100g)	5.76 ± 0.36	4.64 ± 0.00	4.44 ± 0.00	4.2 ± 0.00	5.93 ± 0.09
Protein (%)	1.58 ± 0.05	1.34 ± 0.06	1.51 ± 0.06	1.63 ± 0.03	1.53 ± 0.06
Starch (%)	12.6 ± 0.01	6.3 ± 0.05	5.4 ± 0.00	6.3 ± 0.06	1.8 ± 0.01

± Standard deviation

3.2.2 Chromatographic analysis

The results of the HPLC analysis are presented in Table 1. The carbohydrates identified included glucose, fructose, sucrose and maltose. A decrease in all carbohydrates was observed after 12 h of fermentation. At T0, the sample concentrations of glucose, fructose, sucrose and maltose were 1.35 g/L, 0.50 g/L, 1.70 g/L and 58.77 g/L, respectively. Sucrose was not detected after 12 h of fermentation, most likely due to hydrolysis, releasing glucose and fructose, catalyzed by enzymes from microorganisms such as *S. cerevisiae* and *Bacillus* (Uzunova, Vassileva, Ivanova, Spasova, & Tonkova, 2002). Glucose can originate not only from sucrose but also from the conversion of maltose by the action of amylolytic enzymes of *Bacillus* ssp. and *Lactobacillus plantarum* (Almeida, Rachid, & Schwan, 2007; Panda, Parmanick, & Ray 2006). The maltose concentration increased at the end of the fermentation process (88.02 g/L). In our study, species of the genera *Bacillus*, *Lactobacillus* and *Saccharomyces*, which are capable of hydrolyzing starch, were identified. Maltose is continuously produced from the hydrolysis of starch by the enzymatic action of amylases commonly produced by some microorganisms (Almeida, Rachid, & Schwan, 2007).

The alcohols identified by HPLC were ethanol and glycerol (Table 2). The ethanol concentration was 0.19 g/L (after 12 h of fermentation) and

increased to 0.31 g/L at 48 h of fermentation. This compound can result from the heterofermentative and/or alcoholic fermentation of sugars by LAB and yeasts, which were present throughout the 'calugi' fermentation. However, the low ethanol content could be due to the predominance of LAB and the lower yeast population (Fig. 2). In addition to ethanol, glycerol is one of the main products of fermentation by yeasts (Swiegers, Bartowsky, Henschke, & Pretorius, 2005). The glycerol content of the 'calugi' beverage was 0.05 g/L after 12 h of fermentation and 1.70 g/L at the end of the fermentative process (48 h). This increase in the glycerol content coincided with the increase in the yeast population (Fig. 2).

Acetic acid, lactic acid, citric acid, malic acid, succinic acid and propionic acid were measured in the 'calugi' and during the fermentation process (Table 2). Organic acids occur in fermented products as a result of hydrolysis, biochemical metabolism and microbial activity. The quantitative determination of organic acids in fermented foods is important for nutritional, sensorial and microbial reasons (Blandino, Aseeri, Pandiella, Cantero, & Webb, 2003).The concentrations of acetic acid, citric acid and succinic acid decreased after 12 h of fermentation to 0.19 g/L (acetic acid) and 0.08 g/L (succinic acid) at 48 h of fermentation. Citric acid could not be detected after 12 h of fermentation and may have been metabolized to lactic acid and acetic acid. Citric acid can be metabolized by several genera of LAB, resulting in the production of acetic acid, lactic acid and diacetyl (Bartowsky et al., 2004). The acetic acid concentration ranged from 0.55 g/L (0 h) to 0.19 g/L (48 h). This variation can be attributed to the acetic acid bacteria that were identified during the 'calugi' fermentation, as shown in Fig. 2. The production of succinic acid was most likely due to the presence of heterofermentative LAB, which are able to grow during the fermentation of the beverage and produce succinic acid by fermentation of sugars (Swiegers, Bartowsky, Henschke, & Pretorius, 2005).

Table 2 Concentrations of organic compounds in the fermented ‘calugi’ beverage, as determined by HPLC and GC

	Fermentation time (h)				
Compounds	T0	T12	T24	T36	T48
HPLC analyses (g/L)					
Glucose	1.49 ± 0.4	1.19 ± 0.3	0.84 ± 0.0	0.54 ± 0.0	0.34 ± 0.0
Fructose	0.50 ± 0.1	0.68 ± 0.2	0.36 ± 0.0	0.21 ± 0.0	0.13 ± 0.0
Sucrose	1.70 ± 0.3	nd	nd	nd	nd
Maltose	48.64 ± 24.4	49.03 ± 30.4	30.01 ± 3.1	70.48 ± 7.9	69.07 ± 15.7
Glycerol	0.05 ± 0.0	1.05 ± 0.1	1.45 ± 0.3	1.56 ± 0.1	1.70 ± 0.0
Methanol	nd	nd	nd	nd	0.10 ± 0.0
Ethanol	nd	0.19 ± 0.0	0.27 ± 0.0	0.33 ± 0.0	0.31 ± 0.0
Acetic acid	0.28 ± 0.0	0.01 ± 0.0	0.03 ± 0.0	0.04 ± 0.0	0.08 ± 0.0
Lactic acid	0.24 ± 0.0	2.76 ± 0.1	4.40 ± 0.5	4.56 ± 0.7	4.56 ± 0.2
Citric acid	0.08 ± 0.0	nd	nd	nd	nd
Malic acid	0.09 ± 0.0	0.03 ± 0.0	0.02 ± 0.0	0.02 ± 0.0	0.13 ± 0.0
Succinic acid	0.56 ± 0.0	0.08 ± 0.0	0.06 ± 0.0	0.03 ± 0.0	0.03 ± 0.0
Propionic acid	0.08 ± 0.0	0.07 ± 0.0	0.07 ± 0.0	0.06 ± 0.0	0.07 ± 0.0
GC analyses (µg/L)					
Acetaldehyde	69.46 ± 8.5	53.71 ± 9.7	42.62 ± 9.8	36.32 ± 5.9	457.02 ± 63.7
1,1-Diethoxyethane	14.43 ± 0.2	21.03 ± 0.9	973.96 ± 8.2	62.36 ± 5	17.49 ± 4.2
Ethyl acetate	6.14 ± 1.6	nd	14.79 ± 10.6	nd	24.82 ± 2.8

Table 2, continuation

Isobutyl acetate	42.96 ± 3.9	30.27 ± 7.7	31.53 ± 1.8	37.49 ± 15.8	54.03 ± 9.5
Furfural	nd	nd	nd	187.75 ± 5.12	70.64 ± 0.2
Furfuryl alcohol	nd	87.80 ± 3.93	29.71 ± 9.0	755.82 ± 6.6	705.26 ± 46.0
b-Citronellol	nd	nd	39.66 ± 7.34	165.64 ± 0.3	nd
Hexanoic acid	nd	nd	nd	nd	30.52 ± 0.9
Guaiacol	nd	nd	nd	25.10 ± 7.0	Nd
2-Ethyl caproic acid	nd	nd	69.15 ± 4.4	39.05 ± 5.2	nd
Heptanoic acid	nd	nd	81.27 ± 5.2	77.88 ± 1.7	67.65 ± 4.78
Diethyl malate	nd	nd	33.91 ± 0.6	190.91 ± 2.8	nd
Octanoic acid	nd	88.07 ± 22.0	58.07 ± 11.1	nd	nd
Nonanoic acid	nd	52.04 ± 6.0	22.55 ± 10.3	61.85 ± 7.7	nd
Decanoic acid	113.88 ± 5.1	134.48 ± 1.0	nd	nd	nd

nd – not detected; ± Standard deviation

The other acids identified in the ‘calugi’ by HPLC were malic acid, propionic acid and lactic acid. The highest concentration of malic acid measured in the ‘calugi’ beverage was 0.12 g/L (48 h of fermentation). According to Duarte, Dias, Pereira, Gervásio, & Schwan (2009), high levels of malic acid negatively influence the sensory quality of the beverage. Propionic acid concentrations remained constant throughout the fermentation process (0.07 g/L). The lactic acid concentration increased after 12 h of fermentation, reaching a concentration of 2.00 g/L at the end of the process. The increase in lactic acid occurred when there was an increase in the population of lactic acid bacteria (Table 2 and Fig 2). The LAB may produce several organic acids, including lactic acid, acetic acid and propionic acid, which are responsible for specific tastes in fermented products. 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, Han, & Zhou, 2011).

Fifteen volatile compounds were identified and quantified with GC-FID. These compounds included aldehydes, acids, acetates, terpenes, alcohols and esters (Table 2). The aldehydes identified and quantified were acetaldehyde and furfural. The highest concentration of acetaldehyde was 457.02 µg/L (48 h of fermentation). According to Blandino, Al-Aseeri, Pandiella, Cantero, & Webb (2003), acetaldehyde is commonly formed during the fermentation process in cereal-based (corn, rice, sorghum and others) beverages and food. The highest concentration of furfural, 187.75 µg/L, was detected after 36 h of fermentation. Furfural is virtually ubiquitous in food and is formed from the acid hydrolysis or heating of polysaccharides that contain pentose and hexose fragments (Lake et al., 2001). This compound is common in foods including cocoa, coffee, alcoholic beverages, fruits, vegetables and bread (Adams et al., 1997).

The acids were the largest group of compounds identified in ‘calugi’ and included six compounds: hexanoic acid, 2-ethyl caproic acid, heptanoic acid,

octanoic acid, nonanoic acid and decanoic acid. Different acids were detected at different fermentation times, octanoic acid and decanoic acid were the most abundant acids during the process, with concentrations of 88.07 µg/L and 134.48 µg/L, respectively, at 12 h of fermentation (Table 2).

The acetates ethylacetate and isobutyl acetate were present at concentrations of 24.82 µg/L (48 h of fermentation) and 54.03 µg/L (48 h of fermentation). Acetates result from the reaction of acetyl-CoA with higher alcohols, which are formed from the degradation of amino acids or carbohydrates (Perestrelo, Fernandes, Albuquerque, Marques, & Camara, 2006). According to Dajanta, Apichartsrangkoon, & Chukeatirote, (2011), ethyl acetate and isobutyl acetate were identified in the spontaneous fermentation of thua nao (a Thai fermented soy product) and are correlated with fruit aromas such as apple, banana and pineapple.

Two terpenes, *b*-citronellol and guaiacol, were found at concentrations of 165.64 µg/L and 25.10 µg/L, respectively, at 36 h of fermentation. The terpenes can be liberated by α-glycosidases from yeasts during the fermentation process (King & Dickinson, 2003) and are considered a positive quality factor because they contribute to the aroma of the beverages (floral nuances) and can be used to differentiate beverages (Calleja & Falqué, 2005).

Furfuryl alcohol was the only alcohol identified in the 'calugi' beverage, with a maximum concentration of 755.82 µg/L after 36 h of fermentation. This alcohol has also been reported in soy sauce (Lee & Kwok, 1987), miso (Ku, Chen, & Chiou, 2000) and Korean doenjang (Park, Gil, & Park, 2003) as important contributors of flavor.

The 1,1-Diethoxyethane was detected throughout the fermentation process, with a concentration of 73.96 µg/L after 24 h of fermentation. Diethyl malate was the only ester identified in this work and was produced after 24 h fermentation with a concentration of 190.91 µg/L after 36 h of fermentation.

This study is the first to report the presence of 1,1-diethoxyethane and diethyl malate in the fermentation of corn and rice.

3.3 Multivariate analyses of count and metabolites

The results obtained for the microbial counts and metabolites produced in the 'calugi' fermentation shown in Fig. 2 and Table 2 were submitted to PCA to identify the compounds that may be directly related to a specific microbial group (Fig. 5). The first (PC 1) and second (PC 2) principal components explain 90.8% and 22.4%, respectively, of the total variance. On the positive side of PC1, the 24 h, 36 h and 48 h time points of the 'calugi' fermentation were correlated with the count in MRS medium, which exhibited the highest population of LAB and lactic acid, showing that these three variables are directly correlated, as expected. On the negative side of PC1 and the positive side of PC 2, the time T0 sample of the 'calugi' fermentation was correlated with the substrate (starch, glucose, sucrose and fructose) subsequently metabolized during the fermentation process.

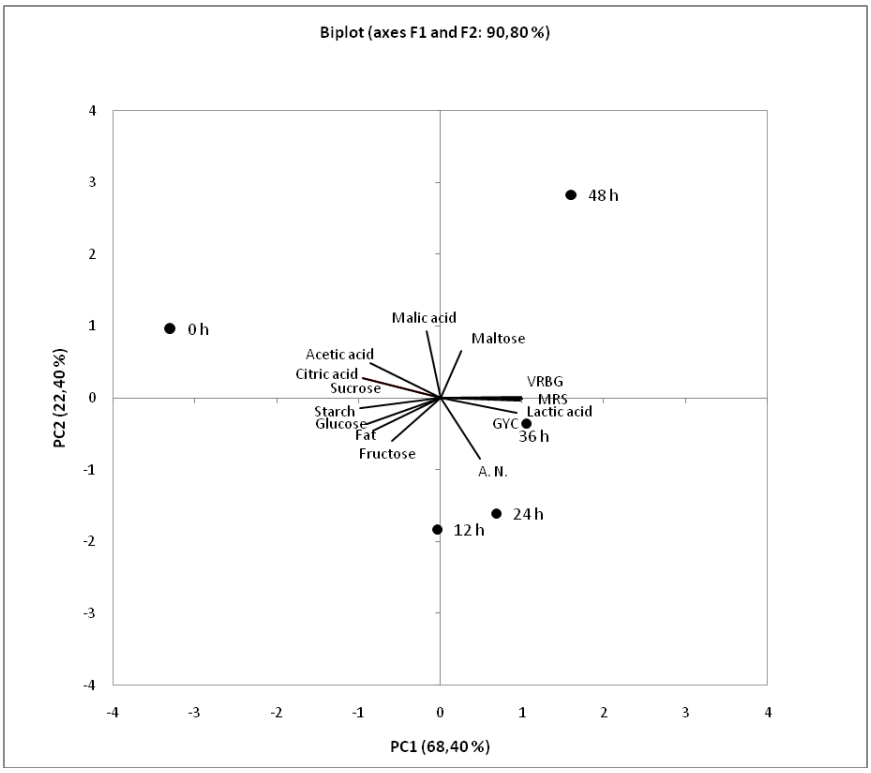

Figure 5 Principal component analysis (PCA) of the count and physical parameters during the fermentation of the corn and rice 'calugi' beverage

4. Conclusions

Lactobacillus and *Saccharomyces* were the predominant microorganisms in the 'calugi' fermentative process. Our results indicated that the methods used permitted an appropriate characterization of the microbiological and metabolic profile during the spontaneous fermentation of corn and rice to produce 'calugi'. The identification of the compounds produced during fermentation processes can facilitate a better understanding of population dynamics and identify the compounds responsible for the flavor and aroma of

fermented foods. This is the first study to report the microbiological and physicochemical characterization of 'calugi' porridge.

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