CLÁUDIA PUERARI

DIVERSIDADE MICROBIANA E CARACTERIZAÇÃO FÍSICO QUÍMICA DE BEBIDAS PRODUZIDAS PELOS INDÍGENAS UMUTINA - BRASIL: CHICHA DE ARROZ E DE BACABA

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 obtenção do título de Doutor.

Orientador
Prof.ª Dr.ª Rosane Freitas Schwan

Co-orientadores
Prof.ª Dr.ª Carmen Lucia da Silva - UFMT
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APROVADA em 29 de julho de 2014.

Dr. Disney Ribeiro Dias	UFLA
Dr. Euziclei Gonzaga de Almeida	UFMT
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LAVRAS – MG

2014

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Ao meu irmão, Leandro, pelos sábios conselhos nos momentos mais difíceis.
DEDICO

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“Never underestimate the power of the microbe”

Jackson W. Foster

RESUMO

A chicha é uma bebida tradicional produzida a partir de diversos substratos, como o arroz, abóbora, milho, polvilho de mandioca e bacaba, pelos indígenas Umutina, no Brasil. Sua preparação pode incluir uma etapa de fermentação, mas a bebida também pode ser consumida não fermentada. A fermentação da chicha ocorre de forma espontânea, pela ação dos microrganismos naturalmente presentes na matéria prima e no ambiente em que a bebida é produzida. A chicha de arroz fermentada foi analisada para determinar os microrganismos envolvidos nos diferentes tempos de fermentação e a dinâmica microbiana foi avaliada utilizando combinação de métodos moleculares dependentes e independentes de cultivo. A composição nutricional da bebida e as modificações bioquímicas envolvidas na fermentação foram investigadas. Foi encontrada uma comunidade bacteriana complexa, que muda em estrutura e composição durante o processo fermentativo, como sendo responsável pela fermentação. As bactérias presentes na fermentação pertencem aos gêneros *Lactobacillus*, *Bacillus*, *Leuconostoc*, *Enterococcus*, *Streptomyces*, *Enterobacter*, *Acinetobacter*, *Escherichia*, *Cronobacter* e *Klebsiella*. Análises independentes de cultivo mostraram a presença de espécies não cultiváveis, *Bifidobacterium*, *Propionibacterium* e fungos. O valor de pH decresceu de 5,2 (tempo 0) para 3,9 após 36 h de fermentação em função da produção de ácido lático e ácido cítrico. Não foi detectada a produção de etanol. A chicha de bacaba foi preparada para ser consumida sem fermentação. No entanto, a microbiota presente na superfície da bacaba promove fermentação espontânea no período pós-colheita, entre a coleta e o preparo da bebida (período de aproximadamente 30h). Foram detectadas bactérias do ácido lático (BAL) nas análises dependentes de cultivo, sendo a microbiota composta por *Enterococcus hormaechei*, *Pantoea dispersa* e *Leuconostoc lactis*. As leveduras presentes

foram *Pichia caribbica* e *Pichia guilliermondii*. *Pichia caribbica* também foi detectada na análise independente de cultivo, além de *Propionibacterium avidum*, *Acetobacter* sp., e bactérias não cultiváveis. A fermentação pós-colheita altera as características sensoriais da bebida de bacaba devido à produção de metabólitos pelos microrganismos, tendo sido identificados ácido succínico (2,69g L^{-1}), ácido acético (0,9g L^{-1}) e ácido cítrico (0,49g L^{-1}). A chicha de bacaba pode contribuir com quantidade substancial de gordura (2,2±0,0g 100 mL-1) e de carboidratos (10,87±0,26g 100 mL^{-1}) para a dieta, apresentando valor energético de 76,7 Kcal 100 mL^{-1}.

Palavras chave: Fermentação. Bebida indígena. Comunidade microbiana. PCR-DGGE.

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ABSTRACT

Chicha is a traditional beverage produced using a several substrates as rice, pumpkim, cassava and bacaba by indigenous Umutina people in Brazil. This beverage can be produced with or without fermentation. The fermentation occurs spontaneously, caused by microorganisms naturally found in raw materials and environmental during the beverage production. The rice beverage was analysed to investigate the microbial community dynamic at different fermentation times by culture-based and culture-independent approaches. The nutritional composition and biochemical changes was analysed. A complex bacterial community that changed in structure and composition during the fermentative process assisted the fermentation. The dominant bacteria presented during the fermentation was *Lactobacillus*, *Bacillus*, *Leuconostoc*, *Enterococcus*, *Streptomyces*, *Enterobacter*, *Acinetobacter*, *Escherichia*, *Cronobacter* and *Klebsiella*. DGGE shows species not cultivable, *Bifidobacterium*, *Propionibacterium* and fungus. The pH value decrease from 5.2 (time zero) to 3.9 after 36 h of fermentation since lactic and citric acid was produced by microorganisms. Ethanol was not detected. Bacaba chicha was prepared without fermentation. The microbiote presents at the fruit surface starts a spontaneous postharvest fermentation, at the period between the fruit collection and the beverage production (approximately 30 h). Lactic acid bacteria LAB were detected by plating, been composed by *Enterococcus hormaechei*, *Pantoea dispersa* and *Leuconostoc lactis*. The dominant yeast were *Pichia caribbica* and *Pichia guilliermondii*. *Pichia caribbica*, *Propionibacterium avidum*, and *Acetobacter* spp. were detected using PCR-DGGE. The postharvest fermentation changes the sensorial characteristics in bacaba beverage since microorganism produces metabolites as succinic acid (2.69g L^{-1}), acetic acid (0.9g L^{-1}) and citric acid (0.49g L^{-1}). The bacaba chicha

can contributes with a substantial amount of fat (2.2 ±0.0 g 100 mL^{-1}) and carbohydrates (10.87±0.26 g 100 mL^{-1}) to the diet, showing an energetic value 76.7 Kcal 100 mL^{-1}.

Keywords: Fermentation. Indigenous beverage. Microbial community. PCR-DGGE.

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SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

A fermentação de alimentos e bebidas é utilizada com o intuito de preservar os alimentos, modificar as características sensoriais e obter benefícios nutricionais. Povos indígenas têm aplicado e aperfeiçoado, através de gerações, o conhecimento empírico utilizado nas fermentações rudimentares de variados substratos. Diversos estudos têm sido direcionados à caracterização de processos e da diversidade microbiana de alimentos e bebidas fermentados indígenas, principalmente na Ásia, África e em alguns países da América (Brasil, Bolívia, Chile, México, Colômbia, entre outros).

No Brasil, foram estudados o caxiri, elaborado a partir de mandioca e batata doce, o cauim, que pode ser produzido a partir de diversos substratos, como mandioca, milho, arroz, amendoim, semente de algodão, abóbora e bacaba, o calugi de milho e arroz e a yakupa, que é preparada com puba (produto de mandioca brava fermentada e seca). A associação entre bactérias e leveduras é frequentemente encontrada.

A etnia Umutina produz bebidas fermentadas e não fermentadas, denominadas chicha, utilizando como substratos arroz, abóbora, milho, polvilho de mandioca e bacaba. Estas bebidas são desconhecidas quanto aos métodos de produção, composição química e perfil microbiológico. Estudos sobre a biodiversidade e a função de determinados nichos ecológicos utilizando métodos microbiológicos dependentes e independentes de cultivo são importantes para conhecer o seu potencial metabólico.

A forma de preparo dos alimentos por uma dada comunidade é única e constitui sua cultura alimentar. Simboliza seu patrimônio cultural e aspectos de

etnicidade, ou seja, a afiliação com uma etnia, povo ou grupo cultural. O estudo da diversidade microbiana de alimentos e bebidas fermentados indígenas está intimamente associado ao entendimento do seu uso, significado e representação cultural.

O objetivo do trabalho foi investigar a diversidade microbiana e os produtos do seu metabolismo, a composição nutricional, as características físico-químicas e o modo de preparo das bebidas chicha de arroz e chicha de bacaba, contribuindo para o conhecimento da biodiversidade e para a preservação e proteção do conhecimento tradicional Umutina.

2 REFERENCIAL TEÓRICO

A produção e consumo de alimentos e bebidas fermentados é comum em muitas culturas, as quais descobriram o valor de alimentos fermentados como um método de preservação barato. Estes produtos provavelmente surgiram como resultado de tentativas e erro, ou seja, quando eram produzidas substâncias que conferiam características agradáveis, melhorando ou modificando o *flavor*, odor, sabor e textura e aumentando o tempo de armazenamento, sem causar doenças, continuavam a ser produzidas e que hoje constituem patrimônio cultural e tradicional (RUIZ; RODARTE, 2003).

A fermentação pode ser conceituada como o processo de modificações bioquímicas desejáveis, que consiste na conversão, mediada por microrganismos e suas enzimas, de açúcares em produtos como álcoois, ácidos orgânicos, dióxido de carbono e compostos flavorizantes. Estas modificações controlam o crescimento microbiano, preservando os alimentos e melhorando suas características sensoriais de sabor, aroma e textura, além de melhorar a qualidade nutricional dos alimentos (CAPLICE; FITZGERALD, 1999;

HANCIOGLU; KARAPINAR, 1997; NOUT; MOTARJEMI, 1997; NOUT, 2009).

A biopreservação de alimentos fermentados ocorre devido à inibição do crescimento e sobrevivência de microrganismos deteriorantes e patogênicos quando o pH é inferior a 4,0 e inibição da produção de toxinas por diversas bactérias patogênicas (NOUT; MOTARJEMI, 1997; TAMANG et al., 2009). Esta inibição depende do nível inicial de contaminação, do organismo em questão, do grau de acidificação (quantidade de ácidos não dissociados produzidos), da capacidade tamponante do alimento e do cuidado higiênico sanitário no preparo (MARSHALL; MEJIA, 2012; NOUT; MOTARJEMI, 1997). Os mecanismos antimicrobianos de biopreservação incluem a produção de ácidos orgânicos, peróxido de hidrogênio, dióxido de carbono, diacetil e amplo espectro de bacteriocinas (CAPLICE; FITZGERALD, 1999).

Dentre os benefícios nutricionais atribuídos aos alimentos fermentados estão o decréscimo no nível de carboidratos e de poli e oligossacarídeos não digeríveis (responsáveis pela distensão abdominal e flatulência) e o bioenriquecimento nutricional, pela síntese de aminoácidos, produção de antioxidantes, vitaminas do complexo B e de ácidos graxos ômega 3 poli-insaturados. São reconhecidos ainda os benefícios terapêuticos (redução do teor de lactose) e efeitos imunológicos (LEBLANC et al., 2013; NOUT; MOTARJEMI, 1997; TAMANG et al., 2009).

O consumo de alimentos fermentados introduz microrganismos no organismo humano, os quais podem ter propriedades que permitem sua classificação como probióticos. A produção de alimentos fermentados derivados do leite com propriedades probióticas é comum (ÖZER; KIRMACI, 2010; SILVA et al., 2014). Estudos demonstram a aplicação potencial de probióticos em substratos à base de cereais (RATHORE; SALMERÓN; PANDIELLA, 2012) e de soja (MATIAS et al., 2014). O trabalho de Lee et al. (2014) faz

referência ao potencial do uso de *Lactobacillus* sp., probióticos isolados de kimchi (vegetais fermentados), como cultura *starter* na produção de iogurte.

Algumas matérias primas utilizadas na fermentação, como cereais, tubérculos e legumes, contêm fatores antinutricionais e componentes tóxicos como fitatos, taninos, glicosídeos cianogênicos, oxalatos, saponinas, lectina e inibidores enzimáticos como a alfa-amilase, tripsina e quimiotripsia, que podem diminuir o valor nutricional do alimento (REDDY; PIERSON, 1994). A fermentação promove o aumento da biodisponibilidade de minerais, pois ao reduzir o valor do pH, cria condições para a degradação enzimática de fitatos, presentes em cereais, que de outro modo formariam complexos com cátions polivalentes como o ferro, zinco, cálcio, magnésio e com proteínas (NOUT; MOTARJEMI, 1997).

De acordo com as matérias primas locais disponíveis, grande diversidade de substratos, como hortícolas, leite e carne são fermentados de forma rudimentar, com ou sem a adição de cultura *starter*, sendo possível obter produtos marcadamente diferenciados, com qualidades sensoriais atrativas e seguros do ponto de vista microbiológico (VAN HIJUM; VAUGHAN; VOGEL, 2013).

As fermentações podem ser classificadas de diferentes maneiras, de acordo com a matéria prima utilizada, em categorias, em classes ou de acordo com os produtos majoritários da fermentação. Neste último caso, são descritas quatro tipos principais de fermentação: a alcoólica, ácido-lática, ácido acética e alcalina (STEINKRAUS, 1997).

A fermentação alcoólica resulta na produção de etanol, gás carbônico e compostos aromáticos (ésteres, álcoois superiores), sendo as leveduras os microrganismos predominantes. A produção de vinhos, cervejas e destilados são exemplos deste tipo de fermentação (WAITES et al., 2001).

A fermentação ácido-lática é realizada principalmente por bactérias do ácido lático (BAL), sendo predominantes em derivados de leite fermentados e em cereais fermentados. As BAL produzem ácido lático a partir de hexoses, obtendo energia via fosforilação do substrato. O metabolismo pode ser homofermentativo, quando a glicose é metabolizada majoritariamente a ácido lático via Embden-Meyerhof-Parnas, produzindo duas moléculas de lactato por mol de glicose ou heterofermentativo, pela via da fosfoquetolase, quando ocorre a oxidação parcial da glicose com produção de lactato, dióxido de carbono e etanol via hexose monofosfato (CAPLICE; FITZGERALD, 1999).

A fermentação acética é conduzida principalmente por *Acetobacter*, que produz ácido acético. Na presença de oxigênio, álcool é convertido em ácido acético. Quando uma solução natural de açúcares fermentescíveis é exposta à atividade de microbiota indígena ocorre a fermentação alcoólica por leveduras, seguido pela oxidação do etanol à ácido acético pela ação de bactérias acéticas. Outros produtos minoritários incluem acetaldeído, etil acetato e acetoína (WAITES et al., 2001).

As fermentações alcalinas ocorrem na fermentação de peixes e sementes, popularmente usados como condimento. Os microrganismos essencialmente envolvidos neste tipo de fermentação são *Bacillus subtilis* e outros bacilos, que hidrolisam proteínas a peptídeos e aminoácidos, liberando amônia, que eleva o valor de pH acima de 8,0 (STEINKRAUS, 1997).

As matérias primas tradicionalmente fermentadas incluem derivados de açúcares e amido para obtenção de bebidas alcoólicas (cervejas, vinhos, destilados); leite, para obter queijos, iogurte, leites fermentados e kefir; peixes e produtos cárneos, como molho de peixe e linguiças fermentadas e produtos de origem vegetal (plantas), como pães preparados com cereais e produtos de arroz fermentado, além de frutas fermentadas, vegetais e raízes, incluindo azeitonas, chucrute, molho de soja, tofu, mandioca, cacau e café (WAITES et al., 2001).

Produtos fermentados à base de cereais são populares em regiões tropicais e no continente Africano. A microbiota natural é utilizada para fermentar grãos como milho, milhete, cevada, trigo, aveia, centeio, sorgo ou arroz. Os grãos são frequentemente aquecidos, triturados e algumas vezes filtrados. A população microbiana responsável pela fermentação destes produtos não é bem caracterizada (MARSH et al., 2014).

2.1 Alimentos e bebidas indígenas

As fermentações indígenas caracterizam-se por serem rudimentares, ocorrendo de forma espontânea devido à ação de microrganismos naturalmente presentes nas matérias-primas ou ambiente, com ecologia não controlada. Em algumas preparações, a fermentação pode ser promovida pela adição de cultura *starter*, a qual pode ser uma porção do alimento ou bebida anteriormente fermentada ou matérias primas insalivadas (STEINKRAUS, 1996).

A microbiota da maioria dos alimentos indígenas fermentados é desconhecida e complexa, caracterizada pela associação entre bactérias do ácido lático, bacilos, leveduras e fungos. Esta complexidade estabelece a ecologia de alimentos fermentados como fronteira de descobertas (NOUT; MOTARJEMI, 1997; SCOTT; SULLIVAN, 2008; VAN HIJUM; VAUGHAN; VOGEL, 2013).

A cultura alimentar étnica de muitos povos indígenas tem sido perdida devido às mudanças climáticas, economia global, rápido processo de urbanização e "desenvolvimento" e pelo aumento da disponibilidade de alimentos prontos para o consumo no comércio (TAMANG; KAILASAPATHY, 2010). Para que haja a manutenção das práticas tradicionais e artesanais é essencial que seja realizada a descrição do processo de produção, dos microrganismos presentes nas fermentações rudimentares e o estudo de suas interações (VAN HIJUM; VAUGHAN; VOGEL, 2013). Uma revisão

abordando alimentos e bebidas fermentados indígenas foi publicada por Steinkraus (1996). O autor classifica as fermentações em categorias, tais como: fermentações vegetais proteicas (substitutos da carne), molhos e pastas fermentados, fermentações ácido láticas, fermentações alcoólicas, fermentações acéticas, fermentações alcalinas e pães levedados.

Dentre os substitutos da carne, é citado o tempe, originado na Indonésia e produzido de modo tradicional a partir de soja fermentada por fungos presentes naturalmente ou adicionados como cultura *starter*, com predominância de *Rhizopus* sp., sendo a microbiota variável de acordo com a fonte analisada. Há variação na matéria-prima utilizada e nas técnicas de preparo em função do país que o produz, sendo os principais a Indonésia, Malásia, Holanda, Canadá e Índia.

Os vegetais fermentados são elaborados pela fermentação ácido lática, dentre eles o chucrute, picles e kimchi coreano, elaborado com diversos vegetais; pães e panquecas, como os indianos idli, dosa, khokla e khaman; mingaus ácidos elaborados à base de cereais, como o ogi nigeriano, uji (Quênia), koko (Gana), togwa (Tanzânia), kamu (Nigéria), fubá (Brasil), chika (Peru), entre outros. Exemplos de bebidas ácido láticas são o mahewu, uma bebida não alcoólica obtida por fermentação espontânea de milho, produzida na África do Sul, o gari nigeriano e o pozol mexicano. Algumas fermentações ácido láticas, como os leites fermentados, o leite acidófilo, koumiss e kefir russos, são abordados.

A fermentação acética é caracterizada por diversos tipos de vinagres, enquanto vinhos de mel e pulque mexicano são exemplos de fermentações alcoólicas que utilizam carboidratos como substrato (STEINKRAUS, 1996).

Dentre as bebidas insalivadas, nas quais a saliva é o agente amilolítico, Steinkraus (1996) detalha o processo de obtenção da chicha de milho, uma bebida encontrada em diversos países da América do Sul, como Equador, Brasil,

Peru, Bolívia, Colômbia e Argentina. Cita ainda o caxiri (Kaschiri), feito de mandioca, por indígenas do Brasil.

A insalivação consiste no ato das índias mascarem o substrato, geralmente amiláceos, como a mandioca ou o milho, para em seguida inocular o mosto com leveduras e bactérias úteis, e estes transformam o substrato em cervejas insalivadas. Estes fermentados devem ter surgido a partir do hábito das mulheres nativas em oferecer alimentos mastigados às suas crianças. As bebidas não alcoólicas obtidas pela técnica de sacarificação pela amilase da saliva humana provavelmente foram as precursoras das cervejas do grupo do cauim brasileiro, da chicha andina e das cervejas de cereais maltados (LIMA, 1975).

Os trabalhos pioneiros sobre diversidade microbiana de alimentos e bebidas fermentados no Brasil, utilizando métodos tradicionais e moleculares, caracterizaram o cauim da etnia Tapirapé, elaborado com diferentes substratos como mandioca com arroz (ALMEIDA; RACHID; SCHWAN, 2007), mandioca com arroz (SCHWAN et al., 2007), arroz com amendoim (RAMOS et al., 2010); o caxiri de mandioca (SANTOS et al., 2012); cauim de arroz com semente de algodão (RAMOS et al., 2011), o calugi de arroz com milho (MIGUEL et al., 2012) e yakupa de mandioca (FREIRE et al., 2014).

Por se tratar de uma linha de pesquisa emergente, é necessário ampliar estes conhecimentos para outros alimentos e bebidas fermentados produzidos por indígenas brasileiros. A chicha, encontrada em diversos países da América Latina, como Equador, Brasil, Peru, Bolívia, Colômbia e Argentina, varia em termos de substrato utilizado e de diversidade microbiana.

Alguns alimentos e bebidas fermentados indígenas à base de cereais analisados quanto à diversidade microbiana estão sumarizados no quadro 01.

Quadro 01 Exemplos de alimentos e bebidas indígenas fermentados à base de cereais

Produto	Substratos	Classe	Microrganismos	País	Referência
Ogi	Milho, sorgo	Mingau	*Saccharomyces cerevisiae; Candida krusei;C. tropicalis; Geotrichum candidum; G. fermentans; Rhodotorula graminis; Lactobacillus delbrueckii* subsp. *bulgaricus; Lactobacillus helveticus; L. fermentum;L. plantarum; L. pantheris; L. vaccinostercus; L.bifermentans; L. nantensis; Clostridium*	Oeste africano	Omemu et al. (2007) Oguntoyinbo et al. (2011)
Chicha	Milho	Bebida alcoólica	*S. cerevisiae*	Andes, Peru	Vallejo et al. (2013)
Fura	Milhete	Bolo	*Pediococcus acidilactici; Weisella confusa; Lactobacillus fermentum; L. reuteri; L. salivarius; L. paraplantarum; L. fermentum*	Oeste africano	Owusu-Kwarteng et al. (2012)
Fen-daqu	Sorgo	Licor	*Bacillus licheniformis; Saccharomycopsis fibuligera; Wickerhamomyces anomalus; Pichia kudriavzevii; Aspergillus; Mucor; Rhizopus; Rhizomucor; Penicillium*	China	Zheing et al. (2012)

Continua...

Sobia	Trigo	Bebida	*Lactobacillus cellobiosus; L. buchneri; L. plantarum; L. brevis; L. delbrueckii. Leuconostoc lactis; Pediococcus pentosaceus; Saccharomyces cerevisiae; Candida tropicalis; C. ciferrii; C. guilliermondii; C. lipolytica; Kloeckera japônica; Rhodotorula rubra*	Arábia Saudita	Gassem (2002)
Champús	Milho	Bebida	*Saccharomyces cerevisiae; Issatchenkia orientalis; Pichia fermentans; Pichia kluyveri var. kluyveri; Zygosaccharomyces fermentati, Torulospora delbruekii; Galactomyces geotrichum; Hanseniaspora sp.*	Colômbia	Osorio-Cadavid et al. (2008)
Boza	Arroz, milho, milhete, trigo	Bebida	*Leuconostoc; Lactobacillus; Streptococcus; Pediococcus; Micrococcus; Bacillus; Saccharomyces; Candida; Zygosaccharomyces; Geotrichum; Torulopsis*	Bulgária, Turquia,	Akpinar-Bayizit et al, (2010); Altay et al (2013); Botes et al. (2007); Gotcheva et al. (2000); Hancioglu, Karapinar (1997)
Cauim	Arroz e mandioca	Bebida	*Lactobacillus; Bacillus; Corynebacterium; Paenibacillus; Saccharomyces;Candida; Pichia; Debaryomyces*.	Brasil	Ameida et al. (2007); Schwan et al. (2007)

Continua...

Cauim	Arroz e amendoim	Bebida	*Lactobacillus fermentum; L. plantarum; L. paracasei; Corynebacterium; Bacillus; Pichia; Saccharomyces; Candida; Kluyveromyces*	Brasil	Ramos et al. (2010)
Caxiri	Mandioca e batata doce	Bebida	*Bacillus; Lactobacillus; Enterobacter; Pediococcus; Saccharomyces cerevisiae; Rhodotorula mucilaginosa; Pichia membranifaciens; P. guilliermondii; Cryptococcus luteolus*	Brasil	Santos et al. (2012)
Cauim de arroz e semente de algodão	Arroz e semente de algodão	Bebida	*Lactobacillus plantarum; L. vermiforme;L. paracasei; Candida parapsilosis; C. orthopsilosis; Clavispora lusitaniae; Rhodotorula mucilaginosa.*	Brasil	Ramos et al. (2011)
Calugi	Milho e arroz	Bebida	*Lactobacillus plantarum; Streptococcus salivarius; Streptococcus parasanguis; Weissella confuse; Enterobacter cloacae; Bacillus cereus; Bacillus sp.; Saccharomyces cerevisiae; Pichia fermentans; Candida sp.*	Brasil	Miguel et al. (2012)
Yakupa	Mandioca	Bebida	*Lactobacillus fermentum; L. plantarum; Weissela cibaria; W. confuse; Saccharomyces cerevisiae; Pichia kudriavzevii*	Brasil	Freire et al. (2013)

2.2 Povo indígena Umutina

No Brasil a população indígena é composta por 305 etnias, com uma população de 896,9 mil, dos quais 57,7% vivem em terras indígenas e 42,3% fora das terras indígenas (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2012).

O povo indígena Umutina pertence à família linguística Bororo e conta com população de 445 pessoas, que vivem na terra indígena Umutina, com área de 28.120 hectares, nos municípios de Barra do Bugres e Alto Paraguai, entre os rios Paraguai e Bugres, em Mato Grosso (INSTITUTO SOCIOAMBIENTAL, 2009). Tradicionalmente, os Umutina eram indígenas da mata, coletores, pescadores e caçadores. Com o contato com não índios passam a cultivar roças familiares como fonte de subsistência (ARRUDA, 2003; INSTITUTO SOCIOAMBIENTAL, 2009). A base alimentar era o milho, consumido assado ou cozido e com o qual preparavam pratos como mingaus, caldos, beijus, pães e chicha. Cultivam milho, arroz, cará, batata doce, mandioca, feijão-fava e feijão miúdo, abóbora, pimenta, algodão, urucum, banana e melancia (ARAÚJO; LIMA, 1995; INSTITUTO SOCIOAMBIENTAL, 2009).

Os Umutina produzem diversos tipos de chicha, dentre elas chicha de milho, de arroz, abóbora, polvilho de mandioca e de bacaba. A chicha de arroz é uma bebida utilizada no cotidiano, festas e rituais. Quando é consumida sem fermentação, é denominada *Katazeru*, e a bebida de arroz fermentada, *Ulumuty*. A chicha de bacaba (*Oenocarpus bacaba*), preparada com uma fruta de palmeira nativa, típica da região amazônica e de cerrado do Brasil, pode ser consumida fermentada ou não. A bebida recebe outras denominações, como “vinho de bacaba” ou “leite de bacaba”. Não há registro sobre a forma de preparo e são desconhecidos os aspectos da diversidade e dinâmica microbiana destas bebidas.

Lima (1975) cita um trabalho que trata da etimologia Náhuatl, da expressão chicha (ou chichia), que significa “azedar-se ou tornar-se amargo”. Ainda em Náhuatl o verbo chicha significa “cuspir”, correspondendo à operação fundamental na elaboração da bebida.

Segundo Steinkraus (1997), a chicha é uma bebida alcoólica elaborada de milho, efervescente, consumida pelos indígenas Andinos há séculos, na qual a saliva serve como fonte de amilase para converter o amido em açúcares fermentescíveis. No entanto, o termo chicha também pode ser empregado para bebidas não fermentadas ou não alcoólicas. Além de ser produzida nos Andes, a chicha está presente em algumas regiões do Equador, Brasil, Peru, Bolívia, Colômbia e Argentina. Dentre os substratos utilizados na elaboração da Chicha, Steinkraus (1997) cita, além de variedades de milho, a mandioca, batata doce e quinoa (pseudo-cereal).

2.3 Métodos moleculares para estudo da diversidade microbiana

Os inúmeros fatores que influenciam a microbiota nos alimentos fermentados, como a temperatura, a composição da matéria-prima, a limitação de nutrientes, a pressão e a biodiversidade, com seus efeitos sinérgicos ou antagônicos, fazem com que o estudo desta ecologia seja complexo, pois estes fatores determinam a sucessão ecológica e a dinâmica populacional (RUIZ; RODARTE, 2003).

Na última década, a aplicação de tecnologias moleculares para caracterizar a comunidade microbiana em complemento aos estudos dependentes de cultivo tem facilitado o monitoramento dos ecossistemas fermentativos e a caracterização de espécies microbianas nestes processos (VAN HIJUM; VAUGHAN; VOGEL, 2013).

Como as fermentações indígenas geralmente incluem poucas espécies, se comparado com outros ecossistemas microbianos como o intestino humano ou o solo, o sequenciamento e a genômica comparativa de isolados da fermentação é uma abordagem poderosa para descobrir como o potencial metabólico afeta a fermentação (VAN HIJUM; VAUGHAN; VOGEL, 2013).

A microbiologia investiga os microrganismos utilizando técnicas de isolamento e cultivo com posterior identificação fenotípica. Mais de 90% dos microrganismos presentes em ambientes naturais não podem ser cultivados utilizando as técnicas tradicionais, seja pelo desconhecimento das condições para seu cultivo ou devido à perda da capacidade de reprodução pelos microrganismos, que se apresentam em estado viável (mantém sua atividade metabólica) mas não cultivável, o que limita a compreensão da diversidade microbiana. Mesmo em matrizes alimentares relativamente simples, como em alimentos fermentados, onde os microrganismos cultiváveis predominam, pelo menos 25 a 50% da comunidade microbiana ativa não pode ser cultivada *in vitro* (PONTES et al., 2007; RUIZ; RODARTE, 2003).

O uso associado de métodos tradicionais com métodos moleculares independentes de cultivo tem promovido o conhecimento da diversidade microbiana uma vez que, comparados aos métodos tradicionais, os métodos moleculares são geralmente mais rápidos, mais específicos, mais sensíveis e mais acurados (COCOLIN et al., 2001; JUSTÉ; THOMMA; LIEVENS, 2008; RUIZ; RODARTE, 2003).

Na literatura há inúmeros trabalhos utilizando técnicas moleculares, como o DGGE, para investigar a diversidade de microrganismos em alimentos e bebidas como o vinho (COCOLIN et al., 2002; URSO et al., 2008), sourdough (VUYST et al., 2014; VERA; RIGOBELLO; DEMARIGNY, 2009; ZHANG et al., 2011), queijo (FONTANA et al., 2010; JANY; BARBIER, 2008), café (VILELA et al., 2010), cacau (PEREIRA et al., 2013; NIELSEN et al., 2007;

PEREIRA et al., 2012), kefir (MAGALHÃES et al., 2010; MIGUEL et al., 2010) e cauim (RAMOS et al., 2010).

Os ácidos ribonucleicos (rRNA) são considerados os biopolímeros mais adequados para estudo de diversidade. Os genes rDNA, que codificam o RNA ribossomal (rRNA), são os marcadores moleculares mais frequentemente utilizados por serem distribuídos universalmente, apresentarem propriedades evolucionárias e filogenéticas, alto potencial discriminatório e disponibilidade de bases de dados, como o GenBank (JUSTÉ; THOMMA; LIEVENS, 2008; RUIZ; RODARTE, 2003).

Organismos procariotos possuem rRNA de tamanho 70S (50S = 5S e 23S rRNA – subunidades maiores e 30S = 16S rRNA – subunidade menor). O gene 16S, com aproximadamente 1500 nucleotídeos, gera informações importantes para inferências filogenéticas, sendo preferido ao gene 23S devido à maior facilidade de sequenciamento. Em eucariotos o rRNA apresenta tamanho 80S e consiste de uma série repetitiva de três regiões gênicas (18S, 5,8S e 28S) e de regiões espaçadoras intergênicas (ITS e IGS). Entre os vários pares de *primers* existentes para a identificação de leveduras, destaca-se o ITS1–ITS4 (AUSUBEL et al., 2003; MOAT; FOSTER; SPECTOR, 2002). As comunidades microbianas podem ser caracterizadas em função da diversidade, identidade e quantidade, no entanto não há uma técnica molecular única que proporcione uma análise de todas estas funções (JUSTÉ; THOMMA; LIEVENS, 2008).

A Reação em Cadeia de Polimerase (PCR) é uma metodologia baseada na replicação *in vitro.* Consiste em uma reação de polimerização em cadeia na qual se obtém o enriquecimento de um fragmento específico de DNA por meio de sua duplicação em modo exponencial. Utilizam *primers* universais de resolução de várias taxonomias para gerar um *mix* de *amplicons* que podem ser analisados por outras técnicas moleculares. Assim, um gene presente no genoma como cópia única pode ser amplificado a partir de DNA genômico complexo,

podendo ser posteriormente visualizado como uma banda discreta, constituída por moléculas de DNA, por meio de eletroforese em gel de agarose corado com azul de bromocresol (JUSTÉ; THOMMA; LIEVENS, 2008).

A técnica envolve três etapas básicas, que são repetidas por várias vezes, em ciclos: a desnaturação térmica do DNA molde; o anelamento de oligonucleotídeos sintéticos, que funcionam como iniciadores (*primer*) da reação de polimerização, a cada uma das fitas do DNA molde; e, por último, a polimerização das novas fitas de DNA a partir de cada um dos *primers*, utilizando cada um dos quatro desoxirribonucleotídeos trifosfatos (dNTP - dATP; dCTP; dGTP e dTTP) como substrato da reação de polimerização pela Taq polimerase. A Taq DNA polimerase é uma enzima termoestável que permite especificidade e sensibilidade da reação de PCR, que pode ser executada em condições de alta adstringência, assegurando que os iniciadores estejam anelados estavelmente somente às suas sequências perfeitamente homólogas (AUSUBEL et al., 2003; JUSTÉ; THOMMA; LIEVENS, 2008).

Os *primers* de PCR são pequenos fragmentos de DNA de fita simples (15 a 30 nucleotídeos) que são complementares às sequências de DNA que flanqueiam a região alvo de interesse. O objetivo dos *primers* em PCR é fornecer um grupo 3'-OH livre ao qual a enzima Taq polimerase possa adicionar dNTPs. Anelam-se às fitas opostas da sequência-alvo e são orientados de tal forma que a síntese de DNA ocorra através da região compreendida entre estes. Tem-se a duplicação desse segmento de DNA a cada ciclo de reação. Uma vez que os produtos recém-sintetizados também são complementares e capazes de se ligar aos iniciadores, após desnaturação térmica, cada ciclo sucessivo essencialmente dobra a quantidade de DNA sintetizado no ciclo anterior. O resultado é o acúmulo exponencial do fragmento específico de DNA aproximadamente a 2^n, onde n é igual ao número de ciclos realizados. Os produtos de amplificação do PCR podem ser analisados através da eletroforese

em gel com gradiente desnaturante (DGGE) (JUSTÉ; THOMMA; LIEVENS, 2008; RUIZ; RODARTE, 2003).

Como a PCR pode ser utilizada em uma grande diversidade de aplicações, cada situação requer a otimização de um protocolo específico no que diz respeito à concentração da enzima Taq, dos dNTP, de Mg^{+2}, dos iniciadores e da temperatura de anelamento, bem como a composição do tampão da enzima e o número de ciclos a ser usado (AUSUBEL et al., 2003).

Dentre as principais limitações da técnica de PCR podem ser citadas a lise parcial dos microrganismos presentes em uma comunidade durante a extração de ácidos nucléicos, a falta de recuperação de ácidos nucléicos intactos e a necessidade de purificar os ácidos nucléicos para eliminar substâncias que possam inibir a reação de PCR ou a ação de enzimas de restrição (AUSUBEL et al., 2003; JUSTÉ; THOMMA; LIEVENS, 2008).

A reação de PCR pode gerar erros devido a amplificações preferenciais (reassociação de DNA) e formação de moléculas heteroduplex (associação de cadeias simples de moléculas diferentes de DNA) durante a amplificação, dificultando a interpretação de padrões de eletroforese em gel com gradiente desnaturante (DGGE) (JUSTÉ; THOMMA; LIEVENS, 2008; RUIZ; RODARTE, 2003).

O exame da diversidade microbiana utilizando DGGE baseia-se na eletroforese de pequenos fragmentos de DNA (200–700pb) amplificados pela reação de PCR, em um gel de acrilamida com um alto gradiente de desnaturação. A técnica utiliza substâncias químicas como a formamida e a uréia para separar fragmentos de DNA de tamanhos similares, mas com sequências que podem ser separadas por suas propriedades de migrar no gel, o que depende do tamanho dos fragmentos, do conteúdo de Guanina e Citosina (GC) e da sequência de nucleotídeos (JUSTÉ; THOMMA; LIEVENS, 2008).

Na amplificação do DNA através de PCR, um dos iniciadores apresenta uma região rica em G+C (grampo G-C), que visa impedir a total desnaturação da dupla fita do DNA durante a eletroforese. O gradiente desnaturante (uréia e formamida) do DGGE rompe as pontes de hidrogênio entre os nucleotídeos, com fusão parcial em fita simples. A separação das fitas estende-se por todo o comprimento do fragmento, com exceção de um grampo rico em GC (sequência 40-45 de bases ricas em GC), que é ligado ao *primer forward*. Este grampo é altamente estável e mantém os fios parcialmente juntos, reduzindo a velocidade de migração no gel e ocorrendo a separação de outros fragmentos que apresentam composição nucleotídica diferente (AUSUBEL et al., 2003; JUSTÉ; THOMMA; LIEVENS, 2008). Assim, teoricamente, cada banda no gel representa uma espécie ou um grupo de espécies de microrganismos, e a imagem final do gel corresponderá a um padrão referente à comunidade microbiana da amostra estudada.

Pode ser utilizado para comparar mudanças estruturais em comunidades microbianas e dinâmicas populacionais. Apresenta facilidade na interpretação dos resultados e as bandas individuais podem ser recortadas do gel para identificação por sequenciamento, na dependência de que os fragmentos do produto da PCR tenham tamanho suficiente para conter a informação para a classificação taxonômica precisa (JUSTÉ; THOMMA; LIEVENS, 2008). Dentre as desvantagens podem ser citadas a ocorrência de co-migração de diferentes fragmentos com sequências diferentes e que apresentam mobilidade eletroforética idêntica, resultando em falta de reprodutibilidade, e a baixa sensibilidade, ocorrendo perda de bandas de representantes menos abundantes na comunidade microbiana. A co-migração pode ser minimizada pela utilização de um padrão interno, que facilita a normalização de amostras. Outra limitação da técnica é o número máximo de bandas de DNA que podem ser separadas (JUSTÉ; THOMMA; LIEVENS, 2008). Para identificar os microrganismos, o

sequenciamento e pesquisa em bases de dados para sequências homólogas significantes permite rápida identificação de membros da comunidade em nível taxonômico (JUSTÉ; THOMMA; LIEVENS, 2008).

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1 – Physicochemical and microbiological characterization of chicha, a rice-based fermented beverage produced by Umutina Brazilian Amerindians

Artigo aceito para publicação na revista Food Microbiology

Cláudia Puerari

Abstract

Chicha is a traditional, fermented rice beverage produced by the indigenous Umutina people in Brazil. Culture-dependent and independent approaches were used to investigate the microbial community dynamic. The bacterial population ranged from 0.1 to 6.83 log mL^{-1}. Lactic acid bacteria (LAB) and *Bacillus* dominated throughout the fermentation process. Representative colonies were grouped by Repetitive Extragenic Palindromic and Polymerase Chain Reaction (Rep-PCR) and by biochemical features. 16S rRNA partial gene sequences analysis showed that bacteria were represented by the genera of *Lactobacillus, Bacillus, Leuconostoc, Enterococcus, Streptomyces, Enterobacter, Acinetobacter, Escherichia, Cronobacter,* and *Klebsiella*. As shown by Polimerase and Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis uncultivable *Bifidobacterium*, and *Propionibacterium* were found throughout fermetation. Uncultured fungi composed the fungal PCR-DGGE profile. The pH values decreased from 5.2 (time 0) to 3.9 at 36 h of fermentation. Ethanol was not found. The lactic acid concentration increased rapidly throughout fermentation until it reached a high final value (1.4 g L^{-1}) and the average glycerol content in the beverage was 0.425 g L^{-1}. Our results showed that chicha fermentation might be described by the following phenomena: (i) increasing bacterial population, with LAB as the largest group detected; (ii)

increasing concentrations of lactic and citric acids; and (iii) the final product is characterized by a high content of acids and the absence of ethanol, therefore characterizing rice chicha an acidic and nonalcoholic beverage. This study represents the first attempt to examine the microbial population involved in the nonalcoholic fermentation of chicha, which is produced from rice by Amerindians in Brazil. This study is important for promoting the appreciation of and safeguarding this Brazilian indigenous beverage as an immaterial cultural heritage.

Keywords: Nonalcoholic beverage, rice-based beverage, fermented beverage, indigenous beverage

1. Introduction

Generation after generation, indigenous people have improved empirical knowledge about spontaneous fermentation, using different substrates and techniques. In Brazil country, several Amerindian tribes (e.g. Araweté, Kayapó, Karajá, Javaé, Umutina, and Tapirapé) produce foods and beverages, using small-scale fermentation, with nutritional value, and medical and religious importance. Several substrates, such as cottonseed, rice, cassava, corn, peanut and fruit, including pumpkin and bacaba, are used as raw materials to make

fermented beverages (ALMEIDA; RACHID; SCHWAN, 2007; FREIRE et al., 2013a; MIGUEL et al., 2012; RAMOS et al., 2010a, 2011; SANTOS et al., 2012b; SCHWAN et al., 2007b).

Chicha is an umbrella Spanish term for any indigenously brewed alcoholic beverage in the Americas, and there are a wide variety of plants that can be used to brew chicha. Alcoholic chicha is made most often of maize in the Andes, either masticating maize flour or by allowing the maize to germinate and then grinding it into flour (Jennings, 2005).

Among Amerindian people, those of the Umutina produce a rice-based beverage named "chicha", replacing the original corn chicha due to the greater availability of rice. The traditional process Umutina, which was passed down from generation to generation was used. The beverage is consumed daily, and in rituals and celebrations by people of all ages. The nonfermented beverage is called *Katazeru*, and the fermented one is called *Ulumuty*. In the *Ulumuty* preparation, the rice (*Oryza sativa*) undergoes spontaneous and uncontrolled fermentation initiated by indigenous rice microbiota, as well as those associated with utensils used during beverage preparation, hands, and local environments.

Cereals have a significant role in human nutrition worldwide. They are among the most important sources of dietary proteins, carbohydrates, vitamins, minerals, and fiber for people throughout the world (BLANDINO et al., 2003; BRANDT, 2014). Environmental and endogenous determinants, and

technological parameters used to obtain fermented cereal foods and beverages affect the native microbiota in cereal grains. Raw materials affect, among other factors, the degree of fermentability (fermentable carbohydrates, nitrogen sources, and growth factors), viscosity, and dry matter content (ALTAY et al., 2013; VUYST et al., 2002).

In spontaneous cereal-based fermentations is usually verified an association between Lactic acid bacteria (LAB) and yeast, since yeast growth is favored by the acidification (BLANDINO et al., 2003; NOUT; SARKAR, 1999). The bacterial species are belonging to the genera *Bacillus*, *Enterobacter*, *Lactobacillus*, and other LAB; the yeasts species are from the genera *Candida*, *Saccharomyces*, *Rhodotorula*, and *Pichia* (ALMEIDA; RACHID; SCHWAN, 2007; FREIRE et al., 2013a; KOSTINEK et al., 2005; MIGUEL et al., 2012; RAMOS et al., 2011; SANTOS et al., 2012b; SCHWAN et al., 2007b).

There is no information about the microbial community, physicochemical parameters, biochemical characterization during chicha spontaneous fermentation. During a process of spontaneous fermentation undesirable microorganisms and pathogens can develop, so the chemical and microbiological evaluation is necessary. This is the first study to identify microbial diversity in chicha spontaneous fermentation and to document the processing techniques and the biochemical changes during fermentation. Such information will support the selection of starter cultures and the future

production, in laboratories and on an industrial scale, of this traditional beverage of Brazilian Amerindians. This study is important for promoting the appreciation of and safeguarding this Brazilian indigenous beverage as an immaterial cultural heritage.

2. Material and methods

2.1. Chicha production and sample collection

Umutina Amerindian lives in the Indigenous Umutina Land, covering an area of 28,120 hectares, located 15.0738º S and 57.1741º W, between the Paraguay and Bugres rivers, in the city of Barra do Bugres, Mato Grosso State, Brazil (Fig. 1) (ISA, 2009).

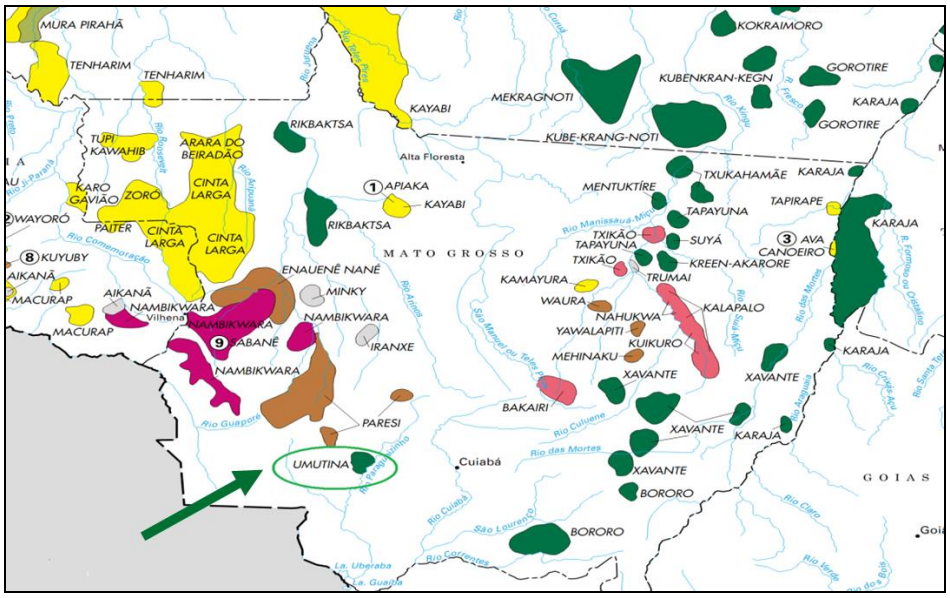

Fig. 1. Umutina Brazilian Amerindians land location.

The Umutina people prepare fermented chicha in the traditional indigenous way. Whole rice grains are cleaned, sun-dried, and soaked under cold water for a few hours before to being finely milled, using a wood pestle, to obtain the rice flour. The beverage is prepared stirring rice flour in boiling water. Separately, some rice flour is toasted, chewed, and used as an inoculum during baking. Next, sugar and more water are added to the so-called pudding, and the beverage is fermented for about 36 h at an ambient temperature (ranging from 30 º to 35 ºC). Traditional protocol was adopted by the Umutina people to make the fermented chicha (Fig. 2).

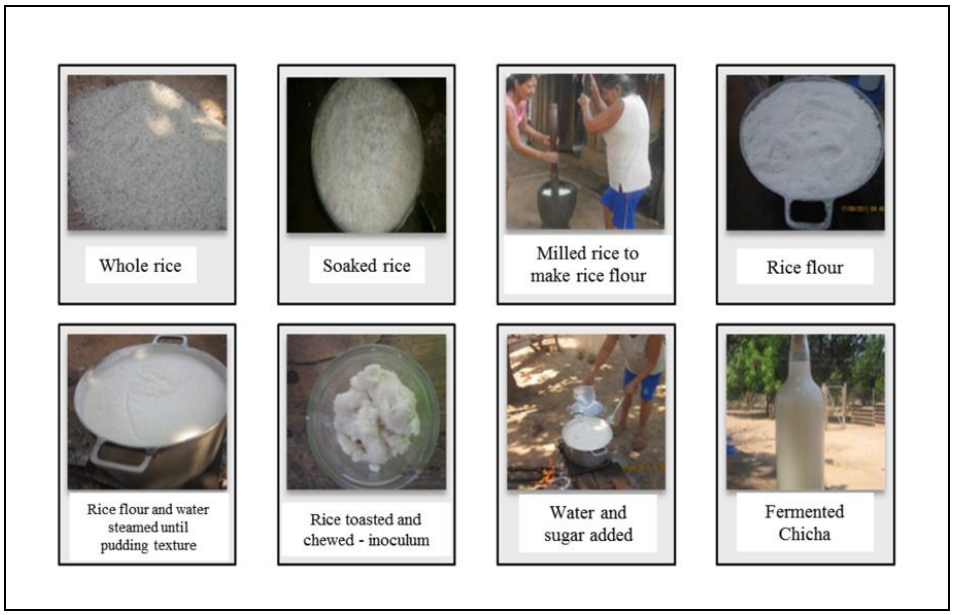

Fig. 2. Steps in the preparation of the rice chicha beverage.

Samples of chicha were taken in duplicate before starting fermentation (T0) and at six-hour intervals during 36 h of fermentation. Sequential tenfold dilutions in 0.1% peptone (0.1% peptone–Himedia, Mumbai, India; 0.5% NaCl–Merck, Darmstadt, Germany) were prepared to quantify the microbial groups. Undiluted samples (50 mL) were taken in duplicate for pH and solids soluble determination, high-pressure liquid chromatography (HPLC) analysis, DNA extraction, and centesimal composition. The samples were frozen until analysis.

2.2. Culture-dependent microbiological analysis

2.2.1. Enumeration and isolation of microorganisms

After mixing the sample in a Stomacher®, tenfold dilutions in saline peptone water (10^{-2}–10^{-6}) were prepared and spread plated in duplicate on nutrient agar (Merck) for aerobic mesophilic bacterial count; the Man Rogosa Sharpe (MRS) agar (Merck) containing 0.1% nystatin (Sigma) for LAB; violet red bile glucose (VRBG) agar (Oxoid) for Gram-negative bacteria; yeast extract peptone glucose (YEPG) agar (1.0% yeast extract–Merck, 2.0% peptone–Merck, 2.0% glucose–Merck, 2.0% agar–Merck), pH 3.5 for yeast count. The plates were incubated aerobically at 28 °C for 48 h and 5 days for bacteria and yeasts, respectively.

Based on macroscopic observations, the square root of total colonies was randomly picked. The isolates were morphologically identified, purified, and preserved in YEPG broth with 20% glycerol at -20 °C for further identification.

2.2.2.Phenotypic and genotypic characterization of isolates

The purified isolates were examined by cell morphology and by performing Gram stain, catalase, oxidase, motility, and sporulation tests were performed as recommended in Bergey's Manual of Determinative Bacteriology (HOLT et al., 1994) and The Prokaryotes (HAMMES; HERTEL, 2003). They were grouped according the features and submitted to other biochemical test.

The Gram-negative strains were identified using Bac tray kits I, II (oxidase negative), and III (oxidase positive) (Laborclin), according the manufacturer's instructions. The Bac tray software for *Enterobacteriaceae* identification (Laborclin) was used to interpret the results.

Gram-positive bacteria were divided in spore-formers and non-spore-formers. Gram-positive, non-spore-forming, catalase-negative and oxidase-negative rods and cocci were presumptively classified as LAB and thus were classified as obligately homofermentative, facultatively heterofermentative, and obligatory heterofermentative by their ability to produce CO_2 from glucose. Biochemical characterization was performed by measuring urease activity, as well as with the gelatinase test, the Triple Sugar and Iron (TSI) test, and the Voges-Proskauer (VP) test. Each isolate was also tested for the ability to ferment carbohydrates. Gram-positive, rods, and spore-forming bacteria were classified as *Bacillus* and grouped using biochemical tests.

2.2.3.Rep-PCR analysis

Following the preliminary phenotypic characterization, molecular biology-based grouping of the isolates was performed using Repetitive Extragenic Palindromic (Rep) PCR. The bacterial DNA from pure cultures was extracted using a 20 μL aliquot of ultra-pure water added to the pellet. The suspension was submitted to the following thermocycling program: 90 ºC / 15 min and submitted to Rep-PCR. The Rep-PCR reaction was carried according to Miguel et al. (2012).

The Rep-PCR profiles were normalized, and a cluster analysis was performed using BioNumerics® v.6.6 software (Applied Maths). The dendrogram was calculated on the basis of Dice's coefficient of similarity with the Unweighted Pair Group Method with the Arithmetic averages clustering algorithm (UPGMA). Based on the phenotypic and genotypic grouping, representative isolates were selected and subjected to 16S rRNA partial gene sequencing using universal primers 27f and 1512r. Sequences were manually corrected and aligned using Vector NTI Suite 10 (Informax, Bethesda, US) and compared to the GenBank database using the BLAST algorithm (National Center for Biotechnology Information, Maryland, US).

2.3. Culture-independent analysis

2.3.1.PCR-DGGE analysis

The culture-independent PCR-DGGE analysis of the V3 region of the 16S rRNA gene was used to monitor the microbial populations from the beginning to the end of spontaneous chicha fermentation. Samples of each beverage (2 mL) were centrifuged at 17.500 x g for 5 min, three times. Pellets were resuspended in 400 µl of sterile demineralized water. Each sample was transferred into a plastic tube and subjected to DNA extraction using the QIAamp DNA stool kit (Qiagen, Valencia, California, US), according to the manufacturer's instructions. DNA extracts were re-suspended in sterilized water and stored at -20 ºC until further analysis.

For DGGE analyses, genomic DNA was used as the template for PCR amplification of the bacterial or fungal ribosomal target regions. Two primer sets were used for the analysis of the microbial community. Bacterial community DNA was amplified with primers 338f / 518r and 27f / 1512r spanning the V3 region of the16S rRNA gene (ØVREÅS et al., 1997). Fungal community DNA was amplified using the primers NS3 and YM951r spanning the 18S region of the rDNA, and the primers ITS1f / ITS4r spanning the ITS region of the rDNA (HARUTA et al., 2006). The PCR mix (25 µl) contained a 12.5 µl Top Taq

Master mix kit (1.25 u of Taq DNA Polimerase: 1X PCR buffer; 1.5 mM $MgCl_2$, 200 mM dNTP) (Qiagen), 1.5 µl of each primer, and 3.0 µl of extracted DNA. Table 1 presents information about the primers and PCR-DGGE conditions. Aliquots (2.0 µl) of the amplification products were analyzed by electrophoresis on 0.8% agarose gels before they were applied to DGGE. The PCR products were analyzed by DGGE using a Bio-Rad DCode Universal Mutation Detection System (Bio-Rad, Richmond, California, US).

Samples were applied to 8% (w/v) polyacrylamide gels in 0.5X TAE. Optimal separation was achieved with a 35–70% urea-formamide denaturing gradient for the bacterial community and a 30–60% gradient for the yeast community, where 100% is defined as 7 M urea and 40% (v/v) formamide. Gel runs were carried out for 5 h at 160 V at 60 ºC, and then gels were stained with SYBR Green I (Molecular Probes, Eugene, OR, US) (1:10.000 v/v) for 30 min. Gels were visualized via UV transillumination (Loccus Biotechnology, LTB 20x20 HE, LPix®, Cotia, São Paulo, Brazil). Individual bands in the DGGE profiles were excised and reamplified to provide a template for sequencing.

Table 1 DGGE-PCR primers used to detect the microorganisms in chicha.

Primer	Sequence (5`-3`)	Community	Target	PCR conditions	References
338fGC	GCA CGC GGG GAC TCC TAC GGG AGG CAG CAG	Bacteria	V3 region of the 16S rRNA gene	1	a
518r	ATT ACC GCG GCT GCT GG				
27fGC	AGA GTT TGA TCC TGG CTC AG	Bacteria	16S rRNA gene	2	
1512r	ACG GCT ACC TTG TTA CGA CT				
NS3fGC	GCA AGT CTG GTG CCA GCA GCC	Yeast	18S region of the rDNA	3	b
YM951r	TTG GCA AAT GCT TTC GC				
ITS1fGC	TCC GTA GGT GAA CCT GCG G	Yeast	ITS region	4	
ITS4r	TCC TCC GCT TAT TGA TAT GC				

GC clamp CGC CCG CCG CGC GCG GCG GGC GGG GCG GG; f forward primer, r reverse primer

PCR Condition 1 – denatured for 5 min at 95 ºC; 30 cycles; denaturing at 92 ºC / 1 min; annealing at 55 ºC / 1 min and extension at 72 ºC / 1 min; final extension at 72 ºC / 10 min.

PCR Condition 2 – denatured for 5 min at 94 ºC; 30 cycles; denaturing at 94 ºC / 40 s; annealing at 52 ºC / 1 min and extension at 72 ºC / 1 min; final extension at 72 ºC / 7 min.

PCR Condition 3 – denatured for 5 min at 95 ºC; 35 cycles; denaturing at 95 ºC / 1 min; annealing at 50 ºC / 1 min and extension at 72 ºC / 1 min;
final extension at 72 ºC / 7 min.

PCR Condition 4 – denatured for 5 min at 95 ºC; 30 cycles; denaturing at 95 ºC / 30 s.; annealing at 52 ºC / 30 s and extension at 72 ºC / 1 min;
final extension at 72 ºC / 10 min.

a – Øvreås et al., 1997

b – Haruta et al., 2006

The conditions for reamplification were equal as those described for DGGE analysis, using the same primer without the GC clamp. The new PCR products were purified using the QIAEX® III purification kit (Qiagen, Chatsworth, CA, US), according to the manufacturer's protocol. The PCR products were sequenced by UNESP (Jaboticabal–SP). The sequences were then compared to the GenBank database using the BLAST algorithm (National Center for Biotechnology Information - NCBI, Maryland, US).

2.3.2. Physicochemical, metabolites, and nutritional composition

The pH value and total soluble solids were measured during sampling according methodology proposed by the Association of Official Analytical Chemists International (AOAC - ASSOCIATION OF OFFICIAL ANALYTICAL CHEMIST, 2000). The pH was measured at room temperature using a digital pH meter (Micronal, B474 model). Soluble solids were determined using a digital refractometer (ATAGO PR–1000) and the results expressed in degrees Brix (ºB).

Moisture, dry matter, fat, and ash content were determined according to the methodology proposed by (AOAC, 2000) in different fermentation times. Total nitrogen content was determined according the Kjeldahl method, and crude protein content was calculated using the conversion factor 5.95 for rice

and rice flour (GREENFIELD; SOUTHGATE, 1992). The concentration of carbohydrates was determined by the difference: 100 – (% moisture + % protein + % fat + % ash content), and energy value was calculated using the Atwater method (WISKER; FELDHEIM, 1990).

The concentrations of alcohols (ethanol, glycerol, and methanol), organic acid (oxalic, citric, tartaric, malic, succinic, propionic, butyric, acetic, and lactic acid), and carbohydrates (glucose, sucrose, fructose, maltose, and raffinose) were determined by HPLC according to Miguel et al. (2012).

3. Results

3.1. Microbial identification by culture-dependent/independent methods

The Fig. 3 shows the microbial population during fermentation identified by the culture-dependent method. Total aerobic mesophilic bacteria are close to the LAB counts. LAB are present in the substrate from the beginning (2.49 Log CFU mL^{-1}) and increase over the following hours, reaching the maximum population after 6 h (5.11 Log CFU mL^{-1}). After this time, the LAB population decreases and remains almost stable until the end of fermentation (4.38 Log CFU mL^{-1}).

Gram-negative bacteria are in low number at the beginning, reaching the maximum at 24 h, decreasing after 30 h, being replaced by LAB. Some Gram-negative bacteria were classified as belonging to the *Enterobacteriaceae* family using Bac tray kits, and the results were confirmed after sequencing.

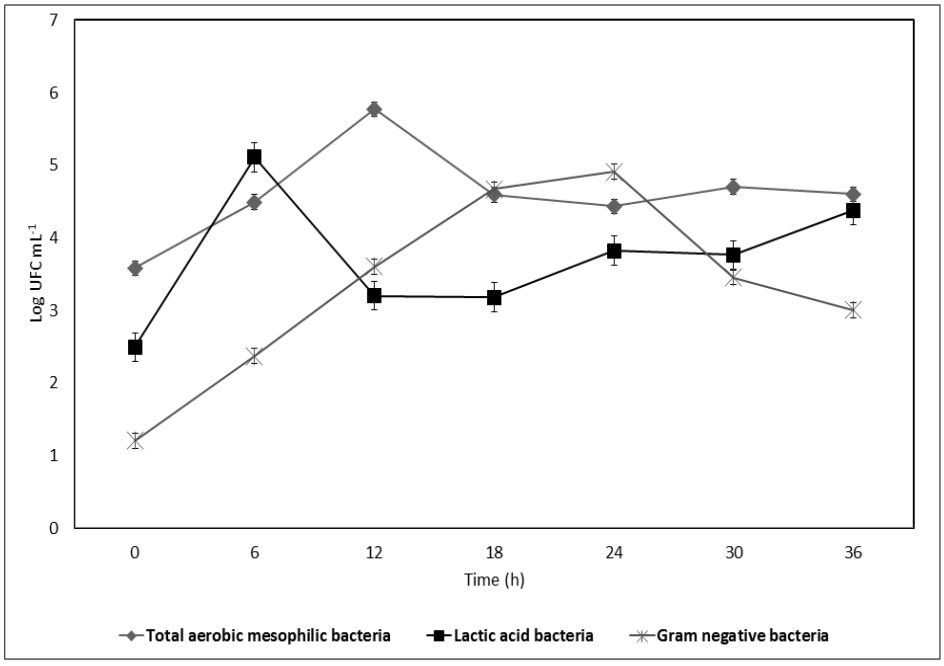

Fig. 3. Distribution of bacteria isolated at different times during chicha beverage fermentation.

The 357 isolates submitted to phenotypic characterization belong to Gram positive (61.5%) and Gram negative (38.5%) groups. Gram-positive, non-sporulating, catalase negative rods were presumptively classified as *Lactobacillus* sp. Gram-positive rods, catalase, and motility-positive and spore-

forming bacteria were classified as *Bacillus* sp. Posteriorly, the bacterial isolates were biochemically classified and grouped by Rep-PCR using BioNumerics (Fig. 4). Considering the patterns obtained, 43 isolates were submitted to DNA-sequencing analysis.

Samples presented a diverse spectrum of species and genera of microorganisms. Gram-positive bacteria dominated the microbiota, including the four genera: *Bacillus* spp., *Enterococcus* spp., *Leuconostoc* spp., and *Lactobacillus* spp. Gram-positive microbiota were composed of homofermentative LAB, belonging to the genus *Lactobacillus* (52.3%), followed by *Enterococcus* (40.9%), and *Leuconostoc* (6.8%), that showed a heterofermentative metabolism. *L. casei* was the most common bacteria species detected, present mainly at the first 18 h of fermentation.

Gram-negative bacteria belonging to the Enterobacteriaceae family were found at all fermentation times, but the cell number was reduced after 30 hours because of the low pH value. Some of them are classified in the coliform group (*Escherichia*, *Enterobacter*, and *Klebsiella*).

The Fig. 5 shows the microbial population from different fermentation times as identified by plating. The bands chosen for sequencing from the PCR-DGGE fingerprints of total bacterial DNA showed a profile different from that of the representative species isolated in plating, including uncultured species as *Bifidobacterium*, and *Propionibacterium*, observed in all fermentation times.

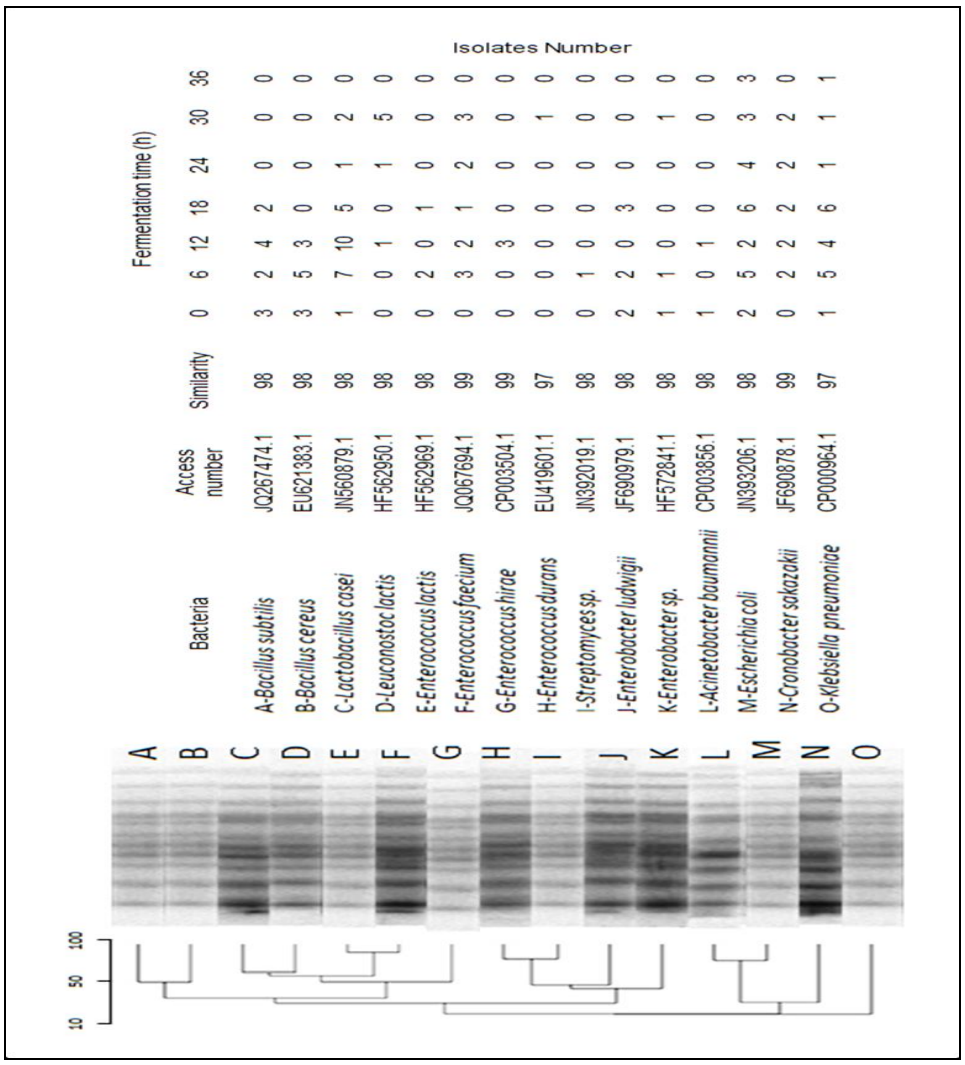

Fig. 4. Dendrogram showing the similarities among band profiles generated by a Rep-PCR analysis of bacteria's strains isolated at different times during the fermentation of chicha.

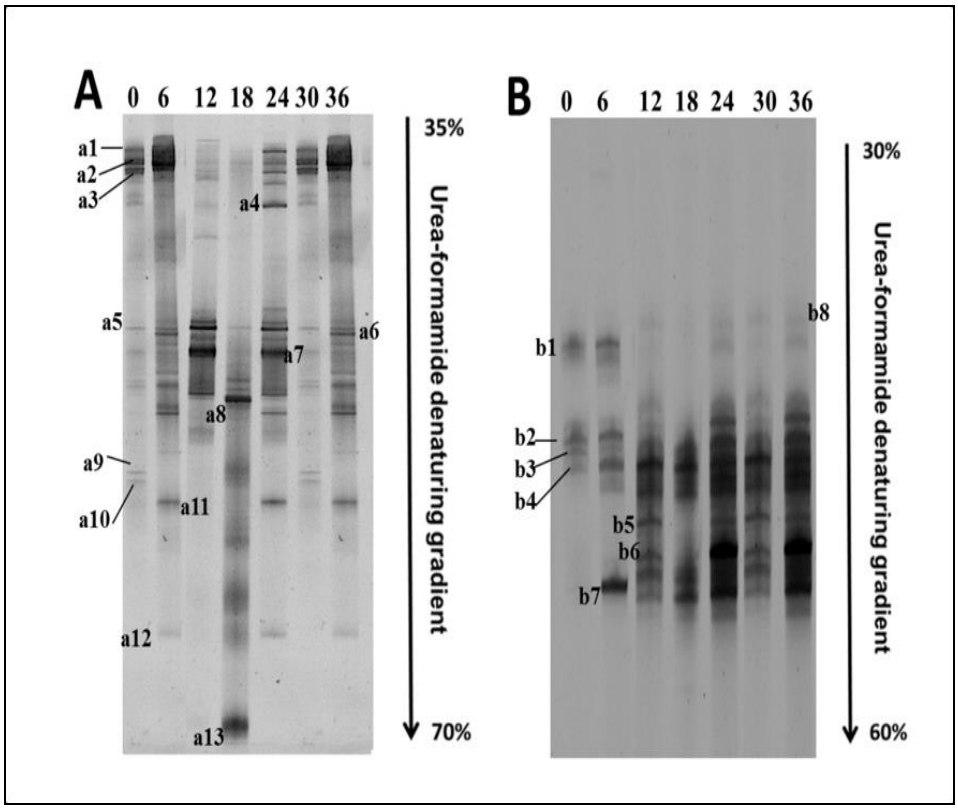

Fig. 5. PCR-DGGE profiles of the bacteria (A) and profiles of the fungal communities (B) in rice chicha during 36 h of fermentation.

(A) The closest relatives of the fragments sequenced, based on a search of GenBank (>99% similarity), were bands a1 – *Propionibacterium* sp.; a2 – uncultured bacterium; a3 – uncultured *Bifidobacterium* sp.; a4 – *Leuconostoc lactis*; a5 – *Lactobacillus casei*; a6 – *Enterobacter* sp.; a7 – *Escherichia coli*; a8 – *Enterococcus lactis*; a9 – *Streptomyces* sp.; a10 – *Bacillus subtilis*; a11 – *Enterococus durans*; a13 – *Klebsiella pneumonia*; a12 – not detected. (B) The closest relatives of the fragments sequenced, based on a search of GenBank (>97% similarity), were bands: b1, b2, b7 – Fungal sp.; b3, b4, b5, b6 – uncultured fungi; b8 – not detected.

The bacteria species *Bacillus cereus, Enterococcus faecium, Enterococcus hirae, Enterobacter ludwigii, Acinetobacter baumannii*, and *Cronobacter sakozakii* were not detected by PCR-DGGE. It should be pointed out that the cell number of these species fell under the detection limit of PCR-DGGE (10^3 CFU / mL) (COCOLIN et al., 2001). The PCR-DGGE profile from total fungal DNA was composed mainly of uncultured fungi. A better microbial community description was reached when using culture-dependent and culture-independent methods together.

3.2. Chicha beverage chemical composition

The macronutrient composition and metabolites during spontaneous chicha fermentation were analyzed (Table 2, Fig. 6 and 7). The pH value decreased from 5.2 (time 0) to 3.9 at 36 h of fermentation (Fig. 6). The production of organic acids in fermented food commonly reduces the pH to values below 4.0. The total soluble solids exhibited a slight decrease from 8.3 ° (0 h fermentation) to 7.2 °Brix (36 h fermentation) (Table 2).

Fermented chicha presented a mean energy value of 34.45 Kcal 100 mL^{-1}, a dry matter content of 8.99 g 100 g^{-1}, a fat content of 0.10 g 100 g^{-1}, protein content 0.42 g 100 g^{-1}, and carbohydrate content 8.47 g 100 g^{-1}. The fat and protein content at the end of fermentation is the same

in nonfermented beverage (Table 2). The final dry matter content of chicha (8.99g 100 mL^{-1}) is similar to Uji content of 6–10 g 100 mL^{-1} (NOUT, 2009). The soluble protein content of chicha ranges from 0.42 to 0.52%.

Table 2 Nutritional composition and physicochemical changes at different fermentation times of rice chicha.

Stage (h)	Moisture	Dry matter	Ash	Fat	Crude Protein*	Carbohydrates	ºB
	g 100 mL^{-1} fresh weight						
0	91.85±0.02	8.16±0.02	0.029±0.0	0.01±0.0	0.42±0.01	7.72±0.01	8.3±0.3
6	91.43±0.10	8.57±0.10	0.028±0.0	0.01±0.0	0.42±0.01	8.14±0.09	7.8±0.1
12	91.24±0.08	8.26±0.62	0.036±0.0	0.01±0.0	0.39±0.01	8.36±0.10	7.8±0.2
18	91.43±0.03	8.57±0.03	0.036±0.0	0.01±0.0	0.45±0.02	8.11±0.05	7.8±0.2
24	91.3±0.010	8.70±0.01	0.034±0.0	0.01±0.0	0.52±0.02	8.17±0.01	7.7±0.1
30	91.49±0.04	8.52±0.04	0.032±0.0	0.01±0.0	0.45±0.02	8.04±0.01	7.4±0.1
36	91.02±0.29	8.99±0.30	0.039±0.0	0.01±0.0	0.42±0.02	8.47±0.27	7.2±0.2

Values are means ±SD for duplicate analysis
*(N x 5.95)

The HPLC results are presented in Fig. 6 and 7. The initial concentration of lactic acid was 0.8 g L^{-1}. Additionally, the lactic acid concentration increased rapidly throughout fermentation until it reached a high final value (1.4 g L^{-1}) (Fig. 6). The concentration of citric acid gradually increased after 30 h of fermentation (0.5 g L^{-1}). Other acids (succinic, malic, tartaric, oxalic, propionic, and butyric) were not detected by HPLC.

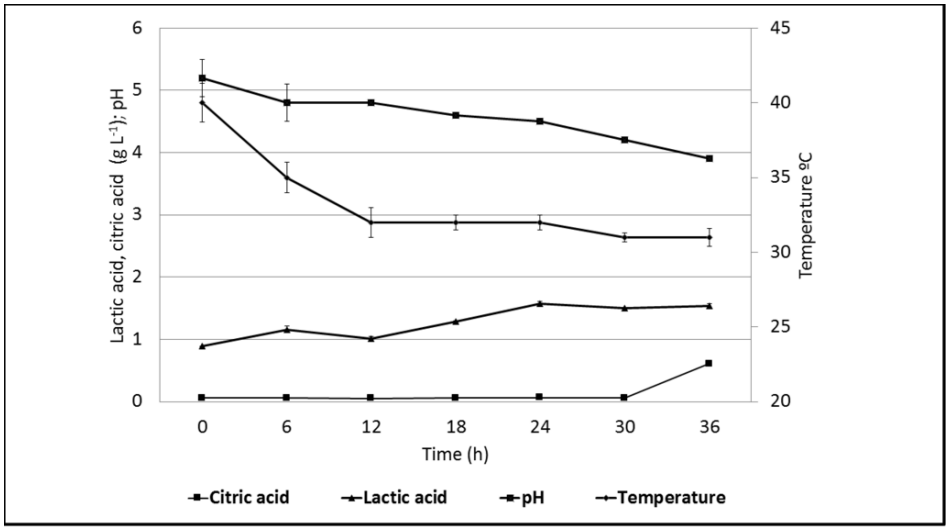

Fig. 6. Trends of pH, organic acids, and temperature during chicha fermentation.

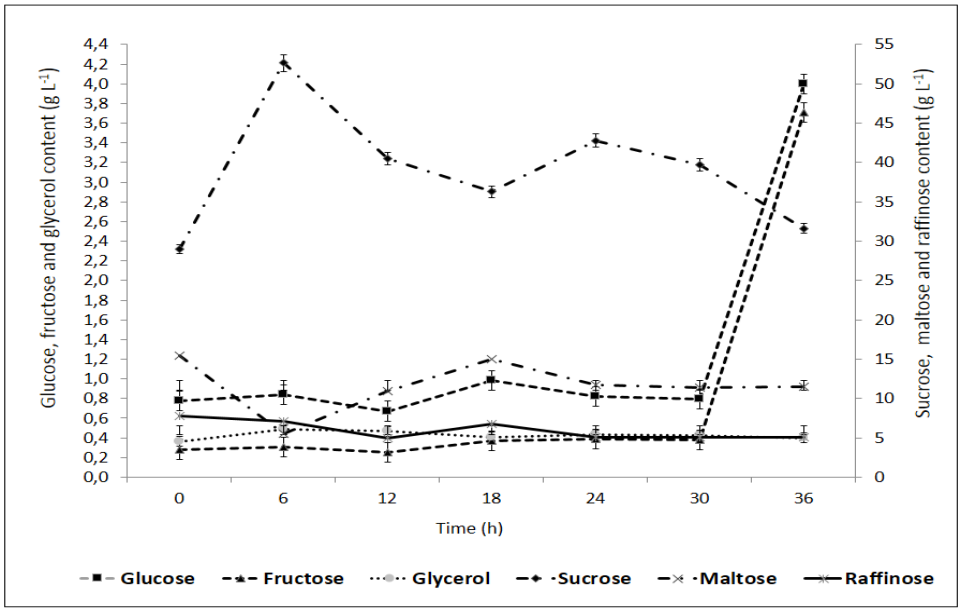

Fig. 7. Sugars and glycerol dynamic during chicha fermentation.

The readily available substrates in rice chicha are, mainly, sucrose, maltose, glucose, and fructose (Fig. 7). Glucose and fructose, products of sucrose hydrolysis, and maltose, product of starch hydrolysis, increased through fermentation. Raffinose, a galacto-oligosaccharide naturally present in rice, appears in high concentration at the beginning and decreases during fermentation. The average glycerol content in the beverage was 0.425 g L^{-1}.

4. Discussion

The study shows the physicochemical and microbiological characterization of chicha, a rice (cereal)-based fermented beverage produced by Umutina Brazilian Amerindians. As observed in other cereal-based fermentations, the process of soaking the rice in water followed by fermentation enables the LAB population to dominate the microbiota. LAB is stimulated by the availability of fermentable sugars generated by endogenous grain amylases and by the action of the *Bacillus* amylases (NOUT; MOTARJEMI, 1997). According to (MCDONALD; FLEMING; HASSAN, 1990), LAB are acid-tolerant and often dominate the fermentation of vegetables and cereals, mainly because of their ability to transport and metabolize different carbohydrates. The LAB behavior observed in this work is in good agreement with results reported

by Ramos et al. (2011) for cauim produced with cottonseed and rice, or with peanuts and rice (RAMOS et al., 2010).

The decrease in the Gram-negative bacteria count observed could be caused by adverse conditions installed after 24 h, when the pH reaches low values (4.5). The pH decrease, because of the rapid production of lactic acid by LAB, inhibits growth and inactivates Enterobacteriaceae, making it difficult for some spoilage organisms in cereals to survive. The inhibitory effect of organic acids lies in the pH-lowering effect as well as the undissociated form of the molecules. The undissociated molecules acts on the bacterial cytoplasmatic membrane, interfering with the maintenance of cell membrane potential, inhibiting active transport, reducing intracellular pH, and inhibiting a variety of metabolic functions (CAPLICE; FITZGERALD, 1999).

LAB metabolism contributes to flavor promotion and beverage preservation. *Enterococcus* is reported as responsible for contributing to flavor development, probably through proteolysis, lipolysis, citrate breakdown, and bio-protection (FOULQUIÉ MORENO et al., 2006; SHORI, 2012).

Generally, LAB and yeasts dominate acidic-fermented foods. Contrary to what other researchers found, in this study a combination of LAB and *Bacillus* spp. (*B. subtilis* and *B. cereus*) dominated this rice fermentation.

Yeasts were not detected. Some authors reported the interaction between LAB and *Bacillus* spp. in other indigenous beverages (ALMEIDA; RACHID;

SCHWAN, 2007; MIGUEL et al., 2012; RAMOS et al., 2010a, 2011; SANTOS et al., 2012b; THORSEN et al., 2011). Enzymes produced by *Bacillus*, degrading starch, proteins, and lipids, play an important role in the fermentation process. They may adapt to the harsh environment of chicha production, leading to accelerated sporulation. Although *B. cereus*, a frequent contaminant in rice-based products, is well known for its toxigenic potential as a food-borne pathogen, the pathogenicity is strain specific, and some varieties of *B. cereus* are used as probiotics (HONG et al., 2008; OGUNTOYINBO; NARBAD, 2012). In addition, *B. subtilis* is being consumed in traditional foods and as a food supplement (HONG et al., 2008). Their ubiquitous distribution makes it inevitable that some members will enter the food chain. This group of bacteria is used as indicator organisms to provide evidence of poor hygiene, inadequate processing, or post-processing contamination of foods (BAYLIS et al., 2011).

Although strains of some species are harmless commensals, such as some strains of *Escherichia coli*, others are important human pathogens. *Enterobacter* has been frequently isolated from different environments, including soil and flies, and was reported in a wide range of foods, including fermented beverages and rice seed (ALMEIDA; RACHID; SCHWAN, 2007; COTTYN et al., 2001; GASSEM, 2002; RAMOS et al., 2010a, 2011; SANTOS et al., 2012b).

The hygienic conditions during chicha production should be taken into account. The presence of these bacterial species underlines the importance of monitoring the hygiene of the fermentation process to ensure that these species do not become dominant and spoil the beverage. One suggested strategy for food safety improvement is the use of well-characterized starter cultures.

The production of organic acids in fermented food commonly reduces the pH to values below 4.0, which ensures the microbiological safety of the product because some pathogens do not survive at this pH (Blandino et al., 2003).

Fermented chicha presented a mean energy value of 34.45Kcal $100mL^{-1}$, a dry matter content of 8.99 g $100\ g^{-1}$, a fat content of 0.01 g $100\ g^{-1}$, protein content 0.42 g $100\ g^{-1}$, and carbohydrate content 8.47 g $100\ g^{-1}$. The fat and protein content at the end of fermentation is the same in nonfermented beverage (Table 2). The food composition can be influenced significantly by the environment, such as soil type, fertilizer, water, or sunlight intensity, and raw material treatment. The final dry matter content of chicha (8.99g / 100 mL) is similar to Uji content of 6–10 g / 100 mL (NOUT, 2009). The soluble protein content of chicha ranges from 0.42 to 0.52%. It was expressively lower than reported in caxiri (1.0%), and corn and rice calugi (1.53%) (Miguel et al., 2012; Santos et al., 2012). Probably, this composition is a consequence of a too-diluted beverage.

The HPLC results are presented in Fig. 6 and 7. The initial concentration of lactic acid (0.8 g L^{-1}) is probably due to LAB growth during the first step of chicha production, when water is added to the rice to obtain flour. Additionally, the lactic acid concentration increased rapidly throughout fermentation until it reached a high final value (1.4 g L^{-1}) because of LAB homofermentative metabolism (Figure 6). The concentration of citric acid gradually increased after 30 h of fermentation (0.5 g L^{-1}).

The readily available substrates in rice chicha are, mainly, sucrose, maltose, glucose, and fructose (Fig. 7). In such fermentations, endogenous grain amylases and *Bacillus* amylases, acting under starch and increasing soluble sugars as glucose and maltose. These sugars serve as sources of energy for LAB. Maltose was the most abundant natural disaccharide through the fermentation. The content decrease after 6 h, probably because of microbial consumption, was followed by a continuous increase until the end of fermentation because of starch hydrolysis by *Bacillus* amylases. Glucose and fructose, products of sucrose and starch hydrolysis, increase through fermentation. The average glycerol content in the beverage was 0.425 g L^{-1}.

Raffinose, a galacto-oligosaccharide naturally present in rice, appears in high concentration at the beginning and decreases during fermentation. This sugar is not hydrolyzed in the human small intestine and is fermented by Gram-negative bacteria in the lower gut, producing gas and causing flatulence. On the

other hand, alpha-galactosidase produced by some *Bifidobacterium* and LAB can hydrolyze raffinose (DINOTO et al., 2006; MEI et al., 2011; MUSSATTO; MANCILHA, 2007; RASTALL, 2010; ZUBAIDAH et al., 2012). Raffinose is a potential prebiotic that is, according to Roberfroid (2007), "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health".

Methanol and ethanol were not identified. Thus, the chicha can be considered a nonalcoholic beverage.

5. Conclusion

This study represents the first attempt to examine the microbial population involved in the nonalcoholic fermentation of chicha, which is produced from rice by Amerindians in Brazil. Lactic acid bacteria and *Bacillus* sp. were the predominant microorganisms in the beverage. This study indicates that the conventional culturing techniques needs to be combined with culture-independent ones to describe to describe optimally the microbiota associated with chicha. Our results indicate that the methods used permit an appropriate characterization of the microbiological and metabolic profile during the spontaneous fermentation of rice to produce chicha. The identification of the

compounds produced during the fermentation processes can facilitate a better understanding of population dynamics and identify the compounds responsible for the flavor and aroma of fermented foods. More studies should be performed to understand and clarify the effects of the dominant microbial species in the fermentation process to develop a controlled process using a selected culture. The study of the chemical composition and the metabolite changes helped in understanding the nutritional importance of the chicha beverage to the Umutina Amerindians. This study is important for promoting the appreciation of and safeguarding this Brazilian indigenous beverage as an immaterial cultural heritage.

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ARTIGO 2 – Bacaba fruit beverage produced by Umutina Brazilian Amerindians: microbiological profile, metabolite composition, and nutritional aspects

Artigo submetido à revista Annals of Microbiology

Cláudia Puerari

Abstract

Bacaba chicha is a beverage prepared by the indigenous Umutina people from the bacaba fruit (*Oenocarpus bacaba*), a purple berry that is rich in fat and carbohydrates, as well as a source of phenolic compounds. In this study, samples of bacaba chicha were collected, and the microbial community was assessed using culture-dependent and -independent techniques. The nutritional composition and metabolite profiles were analyzed, and species belonging to lactic acid bacteria (LAB) and yeasts were detected. The LAB group detected by culture-dependent analysis included *Enterococcus hormaechei*, *Pantoea dispersa*, and *Leuconostoc lactis*. Polymerase chain reaction and denaturing gradient gel electrophoresis (PCR–DGGE) detected additional *Propionibacterium avidum*, *Acetobacter* spp., and uncultured bacteria. *Pichia caribbica* and *Pichia guilliermondii* were detected in a culture-dependent method, and *Pichia caribbica* was confirmed by PCR–DGGE analysis. The pH value of the beverage was 6.2. The nutritional composition was as follows: 16.47±0.73 g 100 mL^{-1} dry matter, 2.2±0.0 g 100 mL^{-1} fat, 3.36±0.44 g 100 mL^{-1} protein, and 10.87±0.26 g 100 mL^{-1} carbohydrate. Spontaneous fermentation occurred during the postharvest period, between fruit collection and beverage production, and the metabolites detected were 2.69 g L^{-1} succinic acid, 0.9 g L^{-1} acetic acid, 0.49 g L^{-1} citric acid, 0.52 g L^{-1} ethanol, and 0.4 g L^{-1} glycerol. This

study is the first step in the immaterial record of this Brazilian indigenous beverage prepared from bacaba fruit.

Keywords: non-alcoholic beverage; indigenous beverage; lactic acid bacteria; yeasts

Introduction

Bacaba (*Oenocarpus bacaba* Mart.), a native palm found in the Brazilian Amazon and Cerrado (Brazilian Savannah) biomes, produces edible purple berries that ripen between December and April. The fruits are collected in the extractive system by indigenous and river communities and are used for feeding as a natural juice or processed into fermented beverages, jelly, and ice cream.

Forest fruits are important sources of food and income for indigenous and rural populations; they are essential for food security, health, and social and economic welfare (Chadare et al. 2010). This type of non-market food, provided by tropical forests, accounts for 47–89% of the gross domestic product (GDP) of poor populations (Sukhdev et al. 2010).

The Umutina indigenous people, who live in the city of Barra do Bugres in the state of Mato Grosso, Brazil, traditionally collect bacaba fruit in the forest

and use this substrate to make a beverage called bacaba chicha. The beverage is also known as bacaba wine or bacaba milk, as the crushed bacaba almonds, prepared with water, produce a beverage that is creamy/light brown in color and has a pleasant taste, like açai (*Euterpe oleracea*) (ISA 2009).

Chicha can be prepared using several cereals and fruits obtained by spontaneous fermentation, or drunk just after preparation (non-fermented) (Vallejo et al. 2013). The Umutina people make chicha using bacaba, pumpkin, rice, corn, sweet corn, and maniva.

The mesocarp of bacaba is rich in fat, carbohydrates, and total dietary fibers (39.3 g 100 g^{-1} fresh weight). It is also a good source of unsaturated fatty acids, with a profile similar to that of olive oil. The total soluble solid of bacaba fruits is 7.89°B and pH 5.3–4.8, with a gross energy value of 606.3±12.8 Kcal 100 g^{-1} fresh weight (Escriche et al. 1999). In addition, bacaba is a promising source of phenolic compounds; it has a high anthocyanin content and significant antioxidant capacity (Abadio Finco et al. 2012).

Considering the nutritional profile and the potentially functional properties of bacaba, its consumption must be stimulated between local communities, as the fruits are widely available and help low-income populations achieve rich and healthful nutrition. Because there is a tendency in these populations to consider industrial preparations superior to local foods, it is

important to demonstrate the value of local produce to ensure an ideal intake of healthy food (Nout and Motarjemi 1997).

The aim of this study was to analyze the bacaba beverage, emphasizing the changes that occur with spontaneous fermentation during the postharvest period and during beverage production and evaluating microbial diversity, metabolites, and nutritional value.

Materials and methods

Bacaba beverage production and sample collection

The bacaba fruits were collected in the indigenous Umutina land, a day before beverage preparation. Figure 1 depicts the production of the bacaba beverage. The first step in the preparation of the beverage entailed selecting the fruit, adding enough warm water to cover the fruit, and letting it stand for half an hour. The water was then changed and the fruit was immediately soaked with wood pestle. The skins were crushed, after which more water was added and a sieve was used to remove the skins and seeds. Sugar cane was added and the substrates were mixed. The beverage can be consumed immediately or after allowing it to ferment for a few hours. The time between harvesting the fruits and preparing the beverage was approximately 30 hours.

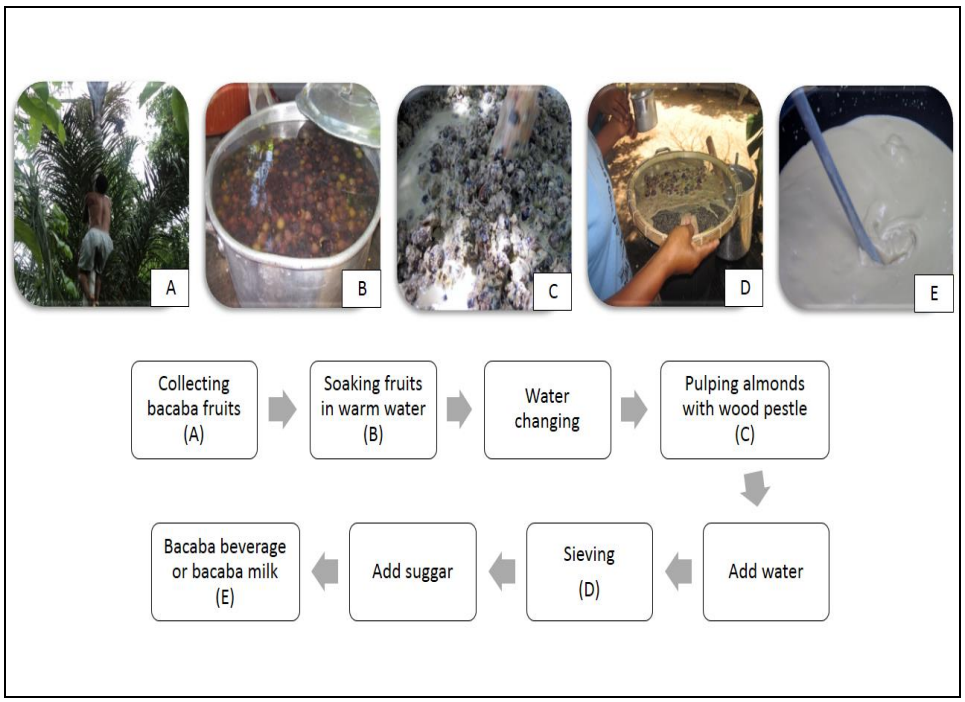

Fig. 1 Photographs and flow diagram representing the bacaba beverage (bacaba chicha) preparation

Samples of the bacaba juice were taken in duplicate. Sequential tenfold dilutions in 0.1% peptone (0.1% peptone; HiMedia, Mumbai, India and 0.5% NaCl; Merck, Darmstadt, Germany) were prepared to quantify the microbial groups. Undiluted samples (50 mL) were taken in duplicate for pH and soluble solid determination, high-pressure liquid chromatography (HPLC) analysis, DNA extraction, and centesimal composition.

Culture-dependent microbiological analyses

Enumeration and isolation of microorganisms

After mixing the sample in a Stomacher® at normal speed for 60 s, tenfold dilutions in saline peptone water (10^{-2}–10^{-6}) were prepared and spread in duplicate on nutrient agar (Merck, Darmstadt, Germany) to perform an aerobic mesophilic bacterial count; on de Man, Rogosa & Sharpe (MRS) agar (Merck, Darmstadt, Germany) containing 0.1% nystatin (Sigma, St. Louis, MO) for LAB; violet red bile glucose (VRBG) agar (Oxoid, Hampshire, England) for Gram-negative bacteria; and yeast extract peptone glucose (YEPG) agar (1.0% yeast extract, 2.0% peptone, 2.0% glucose, 2.0% agar; Merck, Darmstadt, Germany), pH 3.5 for yeast count. The plates were incubated aerobically at 28°C for 48 h and 5 days for bacteria and yeasts, respectively.

Based on macroscopic observations, the square root of the total colonies was randomly chosen. The isolates were morphologically identified, purified, and preserved in YEPG broth with 20% glycerol at -20°C for further identification.

Phenotypic and genotypic characterization of isolates

The purified isolates were examined by cell morphology, and Gram stain, catalase, oxidase, motility, and sporulation tests were performed as recommended in *Bergey's Manual of Determinative Bacteriology* (Holt et al. 1994) and *The Prokaryotes* (Hammes and Hertel 2003). The isolates were grouped according to their features and subjected to further biochemical testing.

The Gram-negative strains were identified using Bac tray kits I, II (oxidase negative), and III (oxidase positive) (Laborclin, Paraná, Brazil), according to the manufacturer's instructions. The Bac tray software for *Enterobacteriaceae* identification (Laborclin) was used to interpret the results.

Gram-positive bacteria were divided in spore-formers and non-spore-formers. Gram-positive, non-spore-forming, catalase-negative, and oxidase-negative rods and cocci were presumptively classified as LAB. Biochemical characterization was performed by measuring urease activity and conducting gelatinase, triple sugar iron (TSI), and Voges–Proskauer (VP) tests. Each isolate was also tested for its ability to ferment carbohydrates.

REP–PCR analyses

Following the preliminary phenotypic characterization, molecular biology-based grouping of the isolates was performed using repetitive extragenic palindromic (REP)–PCR. The bacterial DNA from pure cultures was extracted using a 20µL aliquot of ultra-pure water added to the pellet. The suspension was then subjected to a 90ºC/15 min thermocycling program and REP–PCR.

The REP–PCR reaction was carried out in a 15µl volume containing a 7.5µl Top Taq Master mix kit (1.25 u of Taq DNA polymerase: 1X PCR buffer; 1.5 mM $MgCl_2$, 200 mM dNTP; Qiagen, Valencia, CA); 2.0 µl of primer GTG5 (5′-GTG GTG GTG GTG GTG-3′, DNA Technologies, Aarhus, Denmark); 0.25 µl of formamide (Merck); 0.25 µl of bovine serum albumin (BSA; New England Biolabs, Beverly, MA); and sterile MilliQ water to adjust the volume. The PCR reaction was performed under the following thermocycling program: 5 min of initial denaturation at 94°C, 30 cycles at 95°C for 30 s, 45°C for 60 s, and 60°C for 5 min, followed by a final elongation step of 60°C for 16 min (Nielsen et al. 2007). The PCR products were separated by 1.5% agarose gel electrophoresis in 1XTAE (300 min, 120 V) using a Bench Top 1 kb DNA ladder as reference (Promega, Milan, Italy). Electrophoresis gels were stained with SYBR Green staining (Invitrogen, Foster City, CA) and documented using a transiluminator (LTB 20x20 HE, LPIX®; Loccus Biotechnology, Cotia, São Paulo, Brazil).

The REP–PCR profiles were normalized, and a cluster analysis was performed using BioNumerics® v.6.6 software (Applied Maths, Belgium). The dendrogram was calculated on the basis of Dice's coefficient of similarity with the unweighted pair group method with the arithmetic averages (UPGMA) clustering algorithm. Based on the phenotypic and genotypic grouping, representative isolates were selected and subjected to 16S rRNA partial gene sequencing using universal primers 27f and 1512r. Sequences were manually corrected and aligned using Vector NTI Suite 10 (Informax, Bethesda, MD) and compared to the GenBank database using the BLAST algorithm (National Center for Biotechnology Information [NCBI], Bethesda, MD).

Culture independent analysis: PCR–DGGE

Total DNA extraction and PCR amplification

Total DNA was extracted from the samples using a QIAamp DNA stool kit (Qiagen) according to the manufacturer's instructions. The DNA from the bacterial community was amplified with primers 338f/518r and 27f/1512r spanning the V3 region of the16S rRNA gene (Øvreås et al 1997) . The DNA from the yeast population was amplified with the eukaryotic universal primers NS3 and YM951r spanning the 18S region of the rDNA and primers

ITS1f/ITS4r spanning the ITS region of the rDNA (Haruta et al. 2006). The PCR mix (25 µl) contained a 12.5µl Top Taq Master mix kit (1.25 u of Taq DNA polymerase: 1X PCR buffer, 1.5 mM $MgCl_2$, 200 mM dNTP; Qiagen), 1.5 µl of each primer, and 3.0 µl of extracted DNA. The DNA extracts were resuspended in sterilized water and stored at -20°C until needed for further analysis.

DGGE analysis

To conduct the DGGE analyses, the PCR products from the microbial community were analyzed using a Bio-Rad DCode universal mutation detection system (Bio-Rad, Richmond, CA). Table 1 presents information regarding the primers and PCR–DGGE conditions.

Aliquots (2.0 µl) of the amplification products were analyzed by electrophoresis on 0.8% agarose gels before they were subjected to DGGE. The samples were applied to 8% (w/v) polyacrylamide gels in 0.5X TAE. Optimal separation was achieved with a 35–70% urea–formamide denaturing gradient for the bacterial community and a 30–60% gradient for the yeast community (100% corresponded to 7 M urea and 40% [v v^{-1}] to formamide). Gel runs were carried out for 5 h at 160 V and 60°C, and the gels were stained with SYBR Green I (Molecular Probes, Eugene, OR) (1:10.000 v/v) for 30 min.

Table 1 DGGE-PCR primers used to detect the microorganisms in chicha.

Primer	Sequence (5`-3`)	Community	Target	PCR conditions	References
338fGC	GCA CGC GGG GAC TCC TAC GGG AGG CAG CAG	Bacteria	V3 region of the 16S rRNA gene	1	a
518r	ATT ACC GCG GCT GCT GG				
27fGC	AGA GTT TGA TCC TGG CTC AG	Bacteria	16S rRNA gene	2	
1512r	ACG GCT ACC TTG TTA CGA CT				
NS3fGC	GCA AGT CTG GTG CCA GCA GCC	Yeast	18S region of the rDNA	3	b
YM951r	TTG GCA AAT GCT TTC GC				
ITS1fGC	TCC GTA GGT GAA CCT GCG G	Yeast	ITS region	4	
ITS4r	TCC TCC GCT TAT TGA TAT GC				

GC clamp CGC CCG CCG CGC GCG GCG GGC GGG GCG GG; f forward primer, r reverse primer

PCR Condition 1 – denatured for 5 min at 95 ºC; 30 cycles; denaturing at 92 ºC / 1 min; annealing at 55 ºC / 1 min and extension at 72 ºC / 1 min; final extension at 72 ºC / 10 min.

PCR Condition 2 – denatured for 5 min at 94 ºC; 30 cycles; denaturing at 94 ºC / 40 s; annealing at 52 ºC / 1 min and extension at 72 ºC / 1 min; final extension at 72 ºC / 7 min.

PCR Condition 3 – denatured for 5 min at 95 ºC; 35 cycles; denaturing at 95 ºC / 1 min; annealing at 50 ºC / 1 min and extension at 72 ºC / 1 min;
final extension at 72 ºC / 7 min.

PCR Condition 4 – denatured for 5 min at 95 ºC; 30 cycles; denaturing at 95 ºC / 30 s.; annealing at 52 ºC / 30 s and extension at 72 ºC / 1 min;
final extension at 72 ºC / 10 min.

a – Øvreås et al., 1997; b – Haruta et al., 2006

The gels were visualized via UV transillumination. Individual bands in the DGGE profiles were excised and amplified to provide a template for sequencing. The conditions for amplification were the same as those described for DGGE analysis, using the same primer without the GC clamp. The new PCR products were purified using a QIAEX® III purification kit (Qiagen, Chatsworth, CA), following the manufacturer's protocol. The PCR products were sequenced by UNESP, Jaboticabal, São Paulo. The sequences were then compared to the GenBank database using the BLAST algorithm (NCBI).

Physicochemical, metabolite, and nutritional composition

The pH was measured during sampling according to the methodology proposed by the Association of Official Analytical Chemists (AOAC) International, at room temperature, using a digital pH meter (Micronal B474 model, São Paulo, Brazil) (AOAC 2000).

Moisture, dry matter, fat, and ash content were determined according to the methodology proposed by AOAC (2000). Total nitrogen content was determined using the Kjeldahl method, and crude protein content was calculated using the conversion factor 6.25. The concentration of carbohydrates was determined as 100 - (% moisture + % protein + % fat + % ash content), and

energy value was calculated using the Atwater method (Wisker and Feldheim 1990).

The concentrations of alcohols (ethanol, glycerol, and methanol), organic acids (oxalic, citric, tartaric, malic, succinic, propionic, butyric, acetic, and lactic), and carbohydrates (glucose, sucrose, fructose, maltose, and raffinose) were determined by HPLC. Analyses were carried out using a chromatograph L(C-10Ai; Shimadzu Corp., Kyoto, Japan) equipped with a dual-detection system consisting of an ultraviolet (UV) detector to analyze acids at UV absorbance (210 nm) and a refractive index detector (RI–10A) to analyze sugars and alcohols. A Shimadzu cation-exchange column (Shim-pack SCR-101H, 7.9 mm × 30 cm) was operated under the following conditions: mobile phase, 100 mM perchloric acid, pH 2.1; flow rate, 0.6 mL min^{-1}; and temperature, 50°C (organic acids) and 30°C (sugars and alcohols). Individual sugars, acids, and alcohols were identified by comparing their retention times with the retention times of certified standards. The quantification of alcohols, sugars, and acids was performed using calibration curves obtained from standard compounds. All samples were examined in triplicate.

Results and Discussion

Microbial identification by culture-dependent and independent methods

The bacaba beverage contained similar mesophilic bacteria, LAB, and a yeast population in quantities of 4.8, 4.9, and 4.8 log CFU/mL^{-1}, respectively. The Gram-negative bacteria population was lower, at 3.3 log CFU/mL^{-1}.

The bacterial and yeast isolates were biochemically classified and grouped by REP–PCR using BioNumerics (Figure 2 and 3). Considering the patterns obtained, 51 bacterial and 26 yeast isolates were submitted to DNA-sequencing analysis. Gram-positive bacteria dominated the microbiota. The sequencing of 42 Gram-positive isolates revealed two genera belonging to the LAB group—*Enterococcus* spp. (n=39) and *Leuconostoc* spp. (n=3). Phylogenetic analysis of the 16S rRNA gene showed that these isolates were closely related to *Enterococcus durans*, the dominant bacteria, *E. hirae*, and *Leuconostoc lactis* (99% similarity). *Enterococcus* spp. are found in human feces and appear in soil, surface water, plants, and vegetables.

This genera is found in dairy product fermentation and fermented sausages and olives (Ong et al. 2012). LAB metabolism contributes to flavor promotion and beverage preservation. *Enterococcus* is reported to be responsible for

contributing to flavor development, probably through proteolysis, lipolysis, and citrate breakdown (Foulquié Moreno et al 2006; Shori 2012).

Representative Gram-negative bacteria isolated from the bacaba beverage were tested and grouped according to morphological and biochemical features. The sequencing of representative isolates by REP–PCR (9) identified four isolates as belonging to the genus *Enterobacter*, with high similarity to *E. hormaechei*, and three isolates were identified as *Pantoea dispersa*.

Enterobacter, a member of the Enterobacteriaceae family, is a ubiquitous bacterium that adapts to a wide variety of environments and can be isolated from different sources and foods, such as infant formula, cereal products, milk powder, environmental samples (Shaker et al. 2007), Chinese liquor (Zheng et al. 2012), grapes (Barata et al. 2012), and sobia, a wheat and malt beverage (Gassem 2002). *E. hormaechei* has been isolated from inyu, a Taiwanese soy sauce (Wei et al. 2013). It is not possible to determine whether the presence of *Enterobacter* in bacaba fruits is due to contamination from the environment or as a constituent of endophytic populations, as reported in strawberries (de Melo Pereira et al. 2012).

The yeast population was composed mainly of *Pichia caribbica* (n=19), and in lower numbers, *Pichia guilliermondii* (n=8). Yeasts are frequently isolated from fruit surfaces, with populations ranging from 2 to 6 log CFU/g^{-1} on apples, grapes, strawberries, and masau fruit (Nyanga et al. 2007).

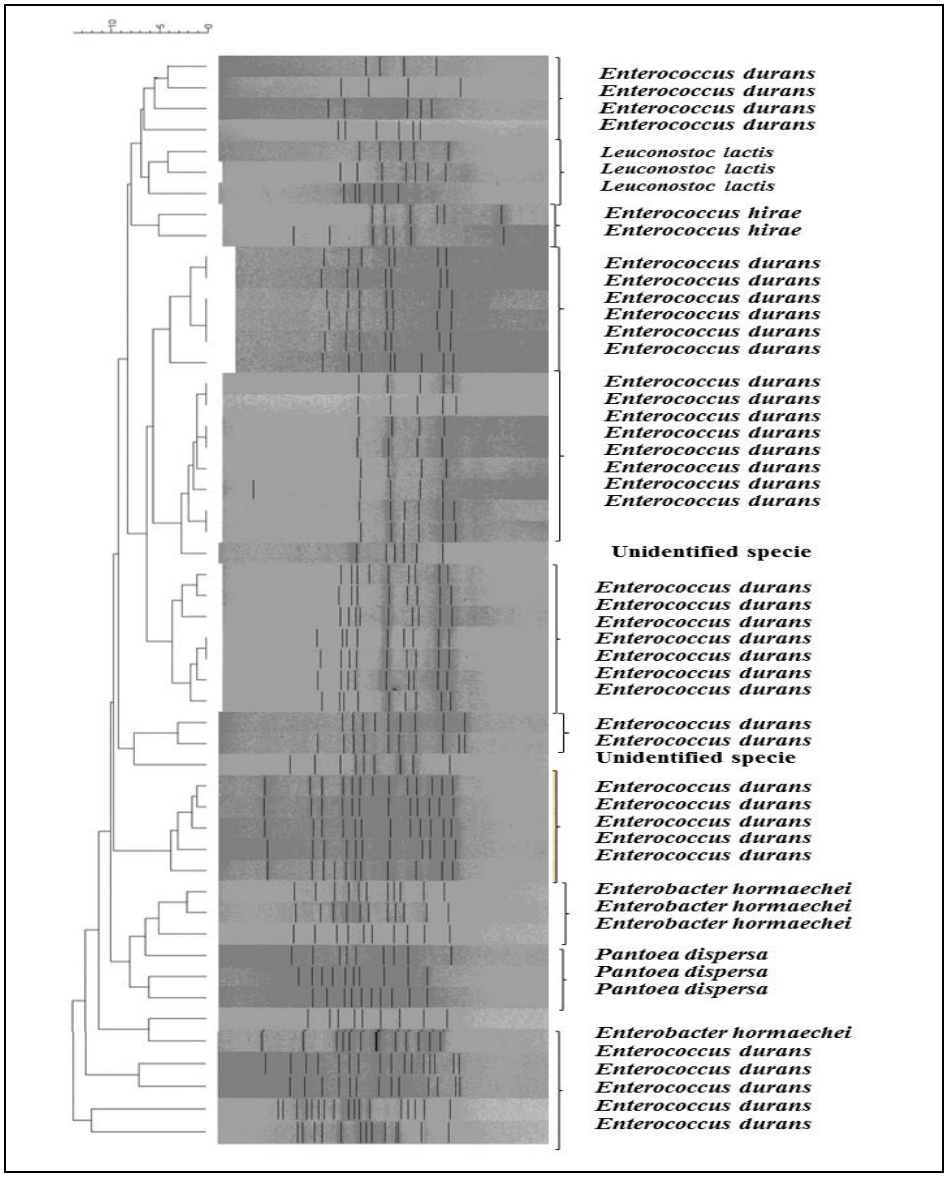

Fig. 2 Dendrogram generated after cluster analysis of Rep-PCR bacterial DNA fingerprints in bacaba beverage

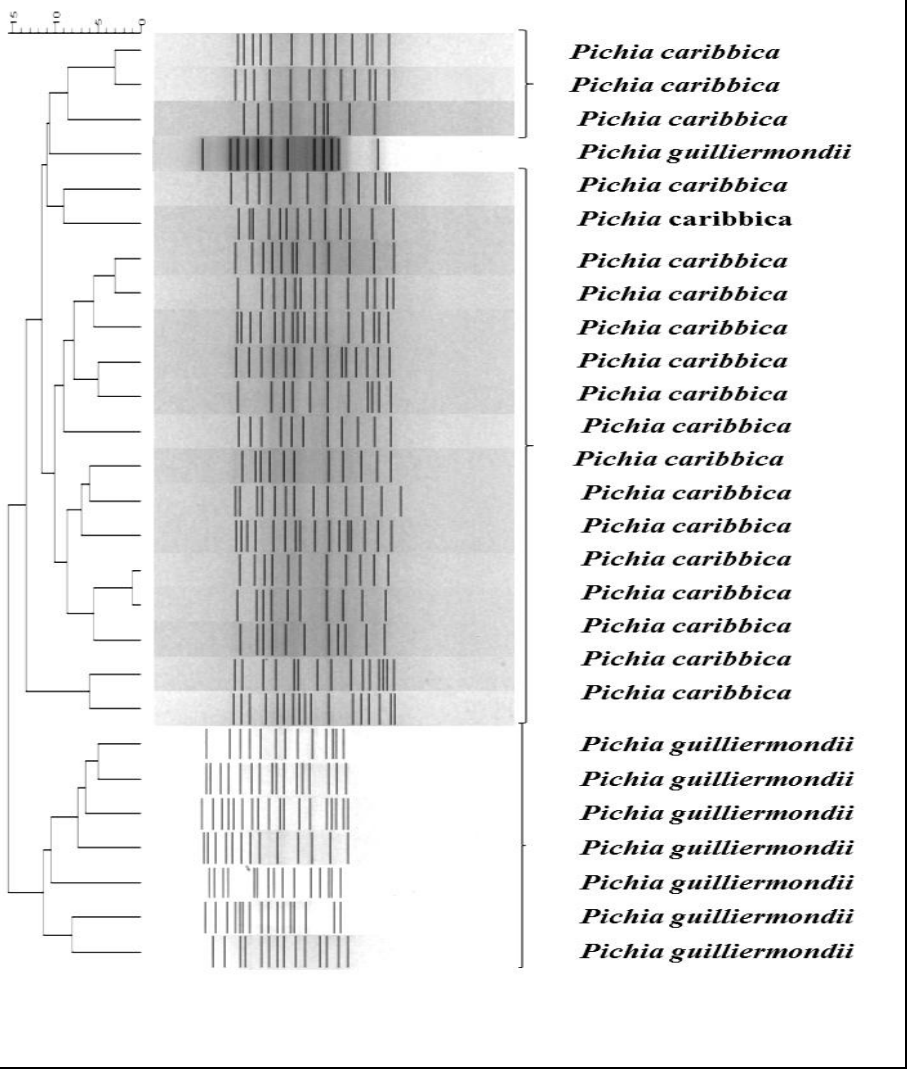

Fig. 3 Dendrogram generated after cluster analysis of Rep-PCR yeast DNA fingerprints in bacaba beverage

Pichia spp. typically form films on liquid media and are known to be important in producing indigenous foods in various parts of the world. *Pichia* is frequently found on grape surfaces, growing during the initial stage of wine fermentation (Chavan et al. 2009; Li et al. 2010; Stringini et al. 2009; Urso et al. 2008) and in rice wine (Lv et al. 2012). *Pichia* spp. are normally present at the beginning of wine fermentation, mainly in damaged ripened fruits, when the sugar is more available (Barata et al. 2012). Chanprasartsuk et al. (2010) reported *Hanseniaspora uvarum* and *P. guilliermondii* as the main species isolated from freshly crushed pineapple juice and natural fermented wine from Thailand and Australia, but *Saccharomyces* yeasts were not found.

The association of yeast and LAB bacteria found in bacaba preparations has also been described in several other fermented foods produced from cassava, rice, peanuts, and fruit (Almeida et al.2007; Caplice and Fitzgerald 1999; Chadare et al. 2010; Freire et al. 2013; Gassem 2002; Miguel et al. 2012; Ramos et al. 2010; Schwan et al. 2007; Wilfrid Padonou et al., 2009). Stringini et al. (2009) verified this association in a West African palm wine, and Nyanga et al. (2007) found an association between LAB and yeast in masau fruit and fermented fruit pulp.

LAB contributes to beverage fermentation by acidification (McDonald et al. 1990), and flavor development is a result of LAB and yeast activity. Yeasts can produce volatile compounds and metabolites that improve the flavor

properties of the final product and enhance LAB growth by the release of nutritive compounds (Tofalo et al. 2012).

Figure 4 shows the DGGE profile obtained after amplification of the V3 region of bacterial 16S rRNA and 18S and ITS region of fungal rDNA, obtained directly from the samples. The bands showed the presence of *Enterococcus* spp. and *Enterobacter* spp., as detected by REP–PCR, and uncultured bacteria. The DGGE assessment revealed the further presence of *Propionibacterium avidum* and *Acetobacter* spp.

PCR–DGGE was not able to detect the *Leuconostoc* species. It should be pointed out that the cell numbers of those species fell below the detection limit of PCR–DGGE (10^3 CFU mL^{-1}) (Cocolin et al. 2001). The PCR–DGGE profile of fungal DNA extracted directly from the samples was composed mainly of *Pichia caribbica* and others uncultured eukaryotes.

Conventional microbiological methods, which require selective enrichments and sub-culturing, are dependent on the ability of a microorganism to grow on the medium under the culturing conditions applied, on the number of isolates, and on the selection methodology. Thus, those methods might fail to detect some microorganisms, excluding members of the microbial community (Jany and Barbier 2008; Magalhães et al. 2010). Culture-independent approaches have the advantage of being able to reveal microorganisms that are difficult or impossible to culture (Quigley et al. 2011).

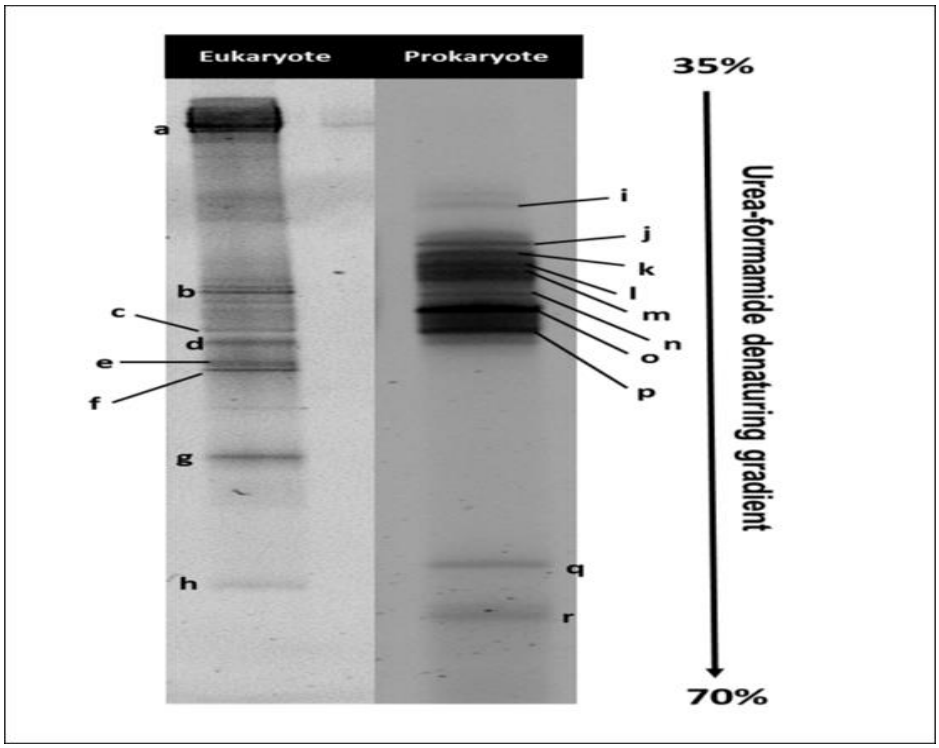

Fig. 4 PCR-DGGE profiles of the Eukariote (A) and Prokariote communities (B) in bacaba beverage.

(A) The closest relatives of the fragments sequenced, based on a search of GenBank (>99% similarity), were bands a – uncultured Eukaryote; b – *Pichia caribbica*; c – uncultured *Malassezia*; d – uncultured Eukaryote; e - not sequenced; f – uncultured Eukaryote; g – not sequenced; h – not sequenced. (B) The closest relatives of the fragments sequenced, based on a search of GenBank (>97% similarity), were bands: i – not sequenced; j – not sequenced; k – *Propionibacterium avidum*; l – *Enterococcus* spp.; m - uncultured bacteria; n – *Enterobacter* spp.; o – *Acetobacter* spp.; p – uncultured bacteria; q – not sequenced; r – uncultured bacteria

Chemical composition of bacaba beverage

The pH value of the beverage was 6.2. The organic acids that were present were probably the result of yeast and bacteria metabolism during the preparation time, when the fruit rested in warm water. Organic acid concentration was low.

From a nutritional point of view, the bacaba fruit mesocarp has a moisture content of 58.0 g 100 g^{-1}, fat content of 30.2, ash content of 1.2, protein content of 4.6, and 606.3 Kcal 100 g^{-1} (Escriche et al. 1999). The bacaba beverage exhibited an energy value of 76.7 Kcal 100 mL^{-1}. The macronutrient composition consisted of 16.47±0.73 g 100 mL^{-1} dry matter content, 2.2±0.0 g 100 mL^{-1} fat content, 3.36±0.44 g 100 mL^{-1} protein content, and 10.87±0.26 g 100 mL^{-1} carbohydrate content. The high carbohydrate content reflects the added sugar. The soluble protein content was considerably higher than that reported in caxiri (1.0%) and corn and rice calugi (1.53%) (Miguel et al. 2012; Santos et al. 2012).

The indigenous Huitoto, Andoque, Yukuna, Muinane, and Miraña tribes that settled in the Amazon region of Colombia consume the bacaba fruit mesocarp in the form of chicha or cahuana, contributing a substantial amount of fat and carbohydrate to their diet (Escriche et al. 1999).

The spontaneous fermentation that occurs in the bacaba beverage during the postharvest period is started by a natural mixed culture of yeasts and LAB that produces metabolites that impact beverage quality. When the fruit rested in warm water, it underwent three types of spontaneous fermentation, confirmed by HPLC results: alcoholic, succinic, and acetic (Figure 5). The fermentative microbiota and the metabolic compounds verified in the bacaba juice occurred because the long postharvest period stimulates spontaneous fermentation. Considering that the first preparation step consists of placing the fruit in warm water for half an hour, the increased water content, the availability of nutrients, and the temperature created ideal conditions for the natural microorganisms present on the fruit surface and environment to grow.

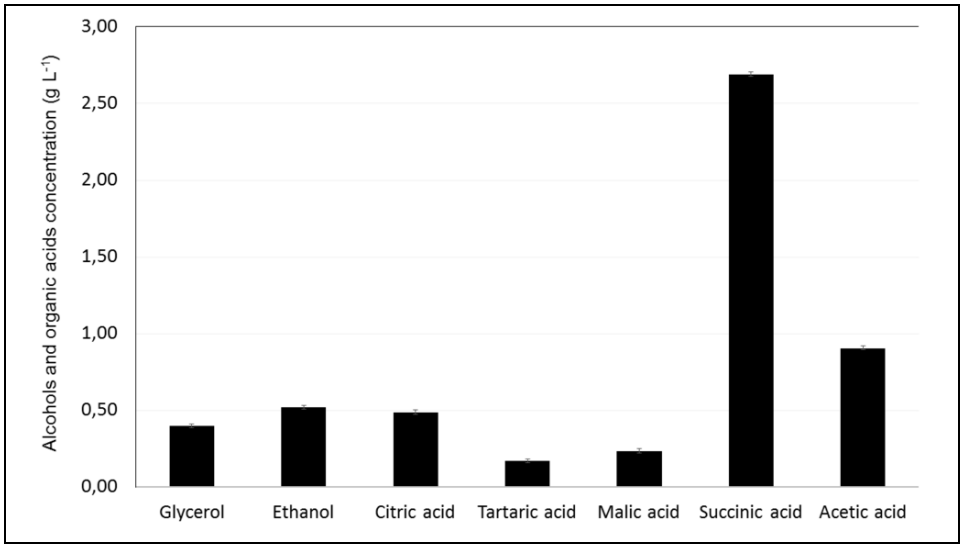

Fig. 5 Organic acids and alcohols production by natural microbiota in bacaba beverage.

A specific postharvest microbiota was established, producing metabolic compounds and modifying the taste and smell of the beverage. These microbial communities vary in composition and diversity according to a range of environmental factors, including pH, sugars, moisture availability, fruit growing conditions, and transport and storage procedures (Aguiar et al. 2013; Leff and Fierer 2013; Nyanga et al. 2007). The spontaneous postharvest fermentation is also favored by intrinsic and extrinsic factors such as sugar content, and the respiratory activity of the fruit, which turn the environment anaerobic and prone to fermentation (Aguiar et al. 2013).

The concentration of succinic acid (2.69 g L^{-1}), acetic acid (0.9 g L^{-1}), and citric acid (0.49 g L^{-1}) was due to the microbiota metabolism (LAB and yeast) during the first step of the bacaba beverage production. Acetic acid in higher concentrations is generally considered an undesirable metabolite in fruit wines (Barata et al. 2012; Duarte et al. 2010). Malic and tartaric acid were present in lower concentrations, but other acids (lactic, oxalic, propionic, and butyric) were not detected by HPLC. The sugars present in the beverage and the respective concentrations (g L^{-1}) were fructose (3.62±0.3), glucose (4.0±0.9), and sucrose (88.4±1.0). The sucrose was added during the preparation of the beverage. Maltose and raffinose were not found.

Ethanol was present at a low level (0.52 g L^{-1}) and the glycerol content was 0.4 g L^{-1}; they are the main products of fermentative yeasts. High

temperatures (19–31°C) increase ethanol production (Zajšek and Goršek 2010). The ambient temperature in the Umutina region during the beverage production ranged from 30° to 35°C. Methanol was not identified. Due to the low ethanol content, the bacaba beverage was classified as a non-alcoholic beverage.

Conclusion

This study represents the first attempt to examine the microbial population involved in the spontaneous fermentation that occurs during the postharvest period in bacaba chicha production. Similar to other fruit wines and juices, the association between LAB and yeast was evident during the postharvest period, prior to fermentation.

The microbiological results indicated that the conventional culturing techniques (plating, REP–PCR, and DNA sequencing) and PCR–DGGE needed to be combined to describe optimally the microbiota associated with bacaba chicha. A better microbial community description could be reached when using culture-dependent and culture-independent methods together.

The study of the chemical composition and the metabolite changes helped in understanding the nutritional importance of the bacaba beverage to the Umutina people and in validating its consumption, as bacaba fruit is widely available and has high nutritional value. This study is important for promoting

the appreciation of and safeguarding this Brazilian indigenous beverage as an immaterial cultural heritage.

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