

**IDENTIFICAÇÃO DE MICRORGANISMOS
ISOLADOS DE GRÃOS DE KEFIR DE LEITE
E DE ÁGUA DE DIFERENTES
LOCALIDADES**

MARIA GABRIELA DA CRUZ PEDROZO MIGUEL

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Dissertação apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola para a obtenção do título de “Mestre”.

Orientador:

Profa. Dra. Patrícia Gomes Cardoso

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MINAS GERAIS – BRASIL
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APROVADA em 19 de junho de 2009

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LAVRAS
MINAS GERAIS – BRASIL

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RESUMO

MIGUEL, Maria Gabriela da Cruz Pedrozo. **Identificação de microrganismos isolados de grãos de kefir de leite e de água de diferentes localidades.** 2009. 71 p. Dissertação (Mestrado em Microbiologia Agrícola) - Universidade Federal de Lavras, Lavras, MG.*

O kefir é uma bebida fermentada de leite que teve sua origem nas montanhas do Cáucaso, no Tibet ou na Mongólia, séculos atrás. Além do leite, uma mistura de água e açúcar mascavo na concentração de 3 a 10% também tem sido utilizado como substrato para produção da bebida. Os grãos de kefir são massas gelatinosas irregulares que podem variar de tamanho 3 – 30 mm, possuem uma aparência semelhante a couve-flor e coloração branca amarelada. A estrutura e a composição dos produtos formados durante a fermentação dos grãos do kefir d'água são semelhantes aos grãos cultivados em leite. Neste trabalho objetivou-se identificar os microrganismos presentes nos grãos de kefir de leite e de água de diferentes estados brasileiros, Canadá e Estados Unidos, por técnicas morfológicas, bioquímicas e moleculares. Perfis eletroforéticos de sequências do DNA ribossomal foram analisados por PCR-DGGE. A população bacteriana variou entre 4.95 log₁₀ UFC/g a 10.43 log₁₀ UFC/g nas amostras de Minas Gerais e Estados Unidos, respectivamente, e para levedura de 5.15 log₁₀ UFC/g na amostra do Distrito Federal e 8.77 log₁₀ UFC/g na amostra de São Paulo, quando cultivado em leite. Em água e açúcar mascavo a população nos grãos apresentou valores de 6.04 log₁₀ UFC/g na amostra proveniente de Alagoas a 9.18 log₁₀ UFC/g na amostra da Bahia para bactéria. Para leveduras os valores variaram de 5.92 log₁₀ UFC/g na amostra de Minas Gerais a 8.30 log₁₀ UFC/g na amostra do Rio Janeiro. As leveduras identificadas nos grãos de kefir de leite e água foram pertencentes aos gêneros *Saccharomyces*, *Pichia*, *Dekkera*, *Candida*, *Kluyveromyces*, *Yarrowia*, *Zygosaccharomyces*, *Galactomyces* e *Saccharomycetes* sendo comuns as espécies *S. cerevisiae*, *Pichia fermentans*, *P. membranifaciens*, e *Yarrowia lipolytica*. Nas amostras analisadas, foram identificadas bactérias dos gêneros *Lactobacillus*, *Bacillus*, *Gluconacetobacter* and *Acetobacter*. As espécies *Lac. kefir*, *Lac. paracasei* e *Lac. satsumensis* foram espécies comuns em ambos os grãos. As espécies bacterianas *Gluconacetobacter liquefaciens*, *Acetobacter lovaniensis*, *A. syzygii*, *Bacillus cereus*, *Lactobacillus satsumensis* e as leveduras *Candida parapsilosis*, *C. valdiviana*, *Pichia guillermondii*, *P. cecembensis*, *P. caribbica*, *Zygosaccharomyces mellis* e *Z. fermentati* foram descritas pela primeira vez em grãos de Kefir. Estes resultados mostram uma grande diversidade na população microbiana de grãos de kefir de diferentes origens.

* Comitê Orientador: Dra. Patrícia Gomes Cardoso (Orientadora), Dra. Rosane Freitas Schwan (Co-orientadora).

ABSTRACT

MIGUEL, Maria Gabriela da Cruz Pedrozo. **Identification of microorganisms isolated from kefir grains in milk and water of different localities.** 2009. 71 p. Dissertation (Master in Agricultural Microbiology) - Federal University of Lavras, Lavras, MG.*

The kefir is a fermented milk beverage that had its origin in the mountains of Caucasus, in Tibet or in Mongolia, many centuries ago. Another substrate that is used for production of that drink is water and raw sugar in the concentration from 3 to 10%. Kefir grains resemble small cauliflower florets that vary in size from 3 to 30 mm in diameter, are lobed, irregularly shaped, white to yellow-white in colour and have a slimy but firm texture. The structure, the microbiological constitution and the composition of the products formed during the fermentation of the water Kefir grains are similar to the grains cultivated in milk. The objective of this work was to identify the microorganisms present in the grains of milk kefir and water from different Brazilian states, Canada and United States by traditional and molecular methods. The bacterial population varied between 4.95 log₁₀ UFC/g to 10.43 log₁₀ UFC/g in the samples from Minas Gerais and USA respectively, and for yeast, varied from 5.15 log₁₀ UFC/g in the sample from Distrito Federal to 8.77 log₁₀ UFC/g in the sample from São Paulo, when cultivated in milk. However, for samples cultivated in water, values varied between 6.04 log₁₀ UFC/g in the sample coming of Alagoas to 9.18 log₁₀ UFC/g in the sample from Bahia for bacteria, and for yeasts it varied from 5.92 log₁₀ UFC/g in the Minas Gerais sample to 8.30 log₁₀ UFC/g in that from Rio de Janeiro. The microorganisms present in the kefir grains cultivated in milk or in water are similar, being the species belonging to the *Saccharomyces*, *Pichia*, *Dekkera*, *Candida*, *Kluyveromyces*, *Yarrowia*, *Zygosaccharomyces*, *Galactomyces* and *Saccharomycetes* genera, being common the species *S. cerevisiae*, *Pichia fermentans*, *P. membranifaciens* and *Yarrowia lipolytica*. In the samples the bacteria identified were of the genera *Lactobacillus*, *Bacillus*, *Gluconacetobacter* and *Acetobacter*. The species *Lac. kefir*, *Lac. paracase* and *Lac. satsumensis* species were found in both grains. As to the bacterial species *Gluconacetobacter liquefaciens*, *Acetobacter lovaniensis*, *A. syzygii*, *Bacillus cereus*, *Lac. satsumensis* and the yeasts *Candida parapsilosis*, *C. valdiviana*, *Pichia guillermondii*, *P. cecembensis*, *P. caribbica*, *Zygosaccharomyces mellis* and *Z. fermentati* this study is the first to report their presence in kefir. These results show a great diversity in the microbial population of kefir grains from different sources.

* Guidance Committee: Dra. Patrícia Gomes Cardoso (Adviser), Dra. Rosane Freitas Schwan (Co-Adviser)

1 REFERENCIAL TEÓRICO

1.1 Grãos de Kefir

A fermentação é um dos métodos mais antigos e econômicos para produzir e preservar alimentos (Billings, 1998). Kefir é uma bebida fermentada de leite que teve sua origem nas montanhas do Cáucaso, no Tibet ou Mongólia, séculos atrás. Os caucasianos descobriram que o leite fresco carregado em bolsas de couro poderia ocasionalmente fermentar, resultando em uma bebida efervescente (Irigoyen, et al., 2005). Essa bebida é encontrada em muitos países como Argentina, Taiwan, Portugal, Turquia, França (Farnworth, 2005), Canadá (Mainville et al., 2006), Japão (Cheirsilp et al., 2003), Rússia, Estados Unidos (Ogles & Cagindi, 2003) e Iran (Assadi et al., 2000). No Brasil, a bebida kefir é produzida de forma artesanal e amplamente difundida em todo o país.

A composição bioquímica e microbiológica do kefir demonstra que este é um probiótico (Farnworth, 2005; Yüksekdag et al., 2004; Ogles & Cagindi, 2003). Os probióticos são alimentos funcionais que são usados ao longo dos séculos, e são definidos como microrganismos que fermentam os alimentos, promovendo o equilíbrio na microbiota intestinal (Giacomelli, 2004, Theunissen et al., 2005).

Kefir tem sido usado no tratamento ou controle de várias doenças há muitos anos na Rússia, Estados Unidos e Japão pelos seus aspectos nutricionais e terapêuticos (Ogles & Cagindi, 2003). Algumas propriedades podem ser citadas, como ação antimicrobiana (Rodrigues et al., 2005), antiinflamatória (Rodrigues et al., 2005; Lee et al., 2007), cicatrizante (Rodrigues et al., 2005) e antialérgica (Lee et al., 2007). Na Rússia, o consumo do kefir foi recomendado para reduzir o risco de doenças crônicas. Ele também é indicado à pacientes em tratamento de doenças gastrintestinais, doenças metabólicas, hipertensão, isquemia do coração e alergia (Farnworth & Mainville, 2003).

Os grãos de kefir (Figura 1) são massas gelatinosas irregulares que podem variar de tamanho 3 a 35 mm de diâmetro (Irigoyen et al., 2005), possuem uma aparência semelhante a couve-flor e coloração branca amarelada (Otlés & Cagindi, 2003). Embora o kefir comercial seja tradicionalmente produzido a partir do leite de vaca, ele também pode ser preparado a partir do leite de ovelha, cabra e/ou búfala. Além desses, o leite de soja também tem sido utilizado para produção da bebida (Farnworth, 2005). Outro seria a água e o açúcar mascavo na concentração de 3 a 10%. Esta bebida é conhecida como kefir d'água, sendo principalmente consumida no México (Ulloa et al., 1994). O flavour e aroma do kefir tradicional são os resultados da atividade metabólica simbiótica de várias espécies de bactérias e de leveduras que se encontram naturalmente nos grãos de kefir (Witthuhn et al., 2004; Yüksesdag et al., 2004). *Lactobacillus* compõem a maior parte da população microbiana (65-80%), além de *Lactococcus* e leveduras (Witthuhn et al. 2004). As leveduras são importantes na fermentação do kefir, produzindo etanol e CO₂ (Irigoyen et al., 2005).

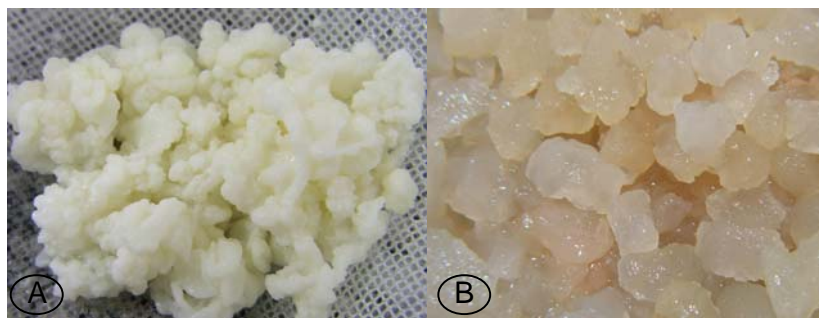


FIGURA 1 Grãos de kefir A) grãos de kefir de leite, B) grãos de kefir de água

A estrutura, a constituição microbiológica e a composição dos produtos formados durante a fermentação dos grãos do Kefir d'água são semelhantes aos grãos cultivados em leite.

O método tradicional de produção da bebida kefir ocorre diretamente pela adição dos grãos de kefir no substrato de preferência. O substrato leite cru, por exemplo, deve ser fervido, resfriado a 25°C e inoculado com 2 a 10% de grãos. Após o período de fermentação, que varia de 18 a 24hs, em temperatura ambiente, os grãos são separados da bebida pronta, por filtração. Eles podem ser secos a temperatura ambiente, mantidos em baixa temperatura e utilizados novamente para inoculação de um novo substrato (Otlés & Cagindi, 2003).

1.2 Composição Química dos Grãos de kefir

Os principais produtos formados durante a fermentação para produção da bebida são o ácido láctico, o CO₂ e o álcool (Yüksekdağ et al., 2004; Otlés & Cagindi, 2003). A composição química e os valores nutricionais de 100 g de kefir podem variar dependendo da sua origem e do seu modo de preparo. Geralmente apresentam aproximadamente 65 Kcal de energia, sendo 3,5g de gordura, 3,3 g de proteína, 4g de lactose, 0,12 g de cálcio, 0,10 g de fósforo, 12 g de magnésio, 0,15 g de potássio, 0,05 g de sódio, 1g de ácido láctico, 13 mg de colesterol, 5µg de manganês, 0,05g de triptofano, 0,34g de leucina, 0,21g de isoleucina, 0,17g de treonina, 0,27g de lisina, 0,22g de valina, 0,06 mg de vitamina A, 0,04 mg de vitamina B₁, 0,17 mg de vitamina B₂, 0,05 mg de vitamina B₆, 0,5 mg de vitamina B₁₂, 1 mg de vitamina C, 0,08 mg de vitamina D e 0,11mg de vitamina E (Otlés & Cagindi, 2003).

O exopolissacarídeo obtido do kefir é conhecido como kefiran. É um glucogalactano ramificado solúvel em água, contendo quantidades iguais de D-glucose e D-galactose (Rimada & Abraham, 2001; Frengova et al., 2002; Cheirsilp et al., 2003). O exopolissacarídeo é sintetizado pelos microrganismos

presentes nos grãos de kefir, sendo este liberado no meio. A qualidade e quantidade deste exopolissacarídeo depende de alguns fatores como, linhagens microbianas envolvidas, composição do meio de cultivo incluindo fatores de crescimento e condições de preparo como temperatura de crescimento e tempo de fermentação (Tamime, 2002). Quando a bebida é produzida utilizando leite, o teor de kefiran alcança valores de 218 mgL⁻¹. Já quando o substrato utilizado é o soro de leite, os valores chegam a 247 mg L⁻¹ (Rimada & Abraham, 2003; Rimada & Abraham, 2006). No mundo, há uma grande quantidade de soro resultante da indústria de produtos lácteos. Esse subproduto quando descartado diretamente na natureza pode ocasionar grande prejuízo ambiental. Assim, a utilização do soro para produção de kefir ou para produção de biomassa leveduriforme para panificação seria uma alternativa interessante (Koutinas et al., 2007).

1.3 Microrganismos encontrados em Kefir

Várias bactérias do ácido láctico estão presentes nos grãos de kefir e nos seus produtos, sendo que essas bactérias foram isoladas e identificadas em várias amostras dos grãos e da bebida, utilizando técnicas dependentes de cultivo. As principais espécies de bactérias encontradas em grãos de kefir de leite são *Lactobacillus acidophilus* (Angulo et al. 1993), *Lactobacillus brevis* (Simova et al., 2002), *Lactobacillus paracasei* subsp. *paracasei* (Simova et al., 2002), *Lactobacillus delbrueckii* subsp. (Simova et al., 2002; Witthuhn et al., 2004), *Lactobacillus. helveticus* (Angulo et al., 1993; Simova et al., 2002), *Lactobacillus. kefir* (Angulo et al., 1993; Takizawa et al., 1998; Garrote et al., 2001), *Lactobacillus. plantarum* (Garrote et al., 2001), *Leuconostoc mesenteroides* subsp. (Garrote et al., 2001; Witthuhn et al., 2004), *Lactococcus lactis* subsp. (Garrote et al., 2001; Simova et al., 2002; Witthuhn et al., 2004), *Streptococcus thermophilus* (Simova et al., 2002). Bactérias ácido acéticas

também têm sido identificadas, como *Acetobacter aceti* (Garrtore et al., 2001), *Acentobacter pasteurianus* (Farnworth, 2005). Alguns autores relataram a presença de leveduras como *Zygosaccharomyces* sp.(Witthun et al., 2004); *Candida krusei* e *Candida lambica* (Witthun et al., 2005), *Cryptococcus humicolus* (Witthun et al., 2005), *Saccharomyces cerevisiae* (Garrote et al., 2001). Espécies de *Saccharomyces unisporus*, *Saccharomyces exiguus* e *Saccharomyces humaticus* foram identificadas com base na sequência de genes do rDNA (Latorr-García et al., 2007). Em estudos realizados por Ulloa et al. (1994) com grãos de kefir d'água foram identificadas as leveduras *Candida valida*, *Pichia membranifaciens* e *S. cerevisiae* presentes na bebida fermentada provenientes do México.

Um estudo realizado por Frengova et al. (2002), utilizando grãos de kefir provenientes da Bulgária demonstrou que os *Lactobacillus* isolados destes grãos são os principais responsáveis pela produção de kefiran. Mais de 50% dos isolados de *Lactobacillus bulgaricus* testados foram produtores do exopolissacarídeo. Já *Lactobacillus helveticus* e *Streptococcus thermophilus* apresentaram baixa produção do kefiran, enquanto *Lactobacillus brevis* e *Lactobacillus lactis* não produziram o exopolissacarídeo. Há muitos estudos visando à otimização da produção de kefiran (Rimada & Abraham, 2005; Cheirsilp et al., 2002; Cheirsilp et al., 2003; Rodrigues et al., 2004). O *Lactobacillus kefiranofaciens* é um excelente produtor deste exopolissacarídeo. A associação entre *Lactobacillus kefiranofaciens* e *Saccharomyces cerevisiae* foram testadas por Cheirsilp et al. (2003). Os resultados mostraram que a associação desses microrganismos promoveu aumento na produção do exopolissacarídeo. A levedura *S. cerevisiae* reduz a concentração de ácido láctico, remove o peróxido de hidrogênio, além de produzir compostos que incentivam o crescimento do *Lactobacillus kefiranofaciens*, aumentando assim a produção do exopolissacarídeo.

Kefiran possui atividade antimicrobiana e cicatrizante. Estudos realizados com grãos de kefir provenientes de Alfenas, Minas Gerais, mostraram que o kefir apresentou ação antimicrobiana sobre os microrganismos *Staphylococcus aureus* (ATCC 6538), *Streptococcus salivarius* (ATCC 39562), *Streptococcus pyogenes* (ATCC 17568), *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (ATCC 10232), *Salmonella typhimurium* (ATCC 14028), *Listeria monocytogenes* (ATCC 4957) e *Escherichia coli* (ATCC 8739) (Rodrigues et al., 2005).

1.4 Uso de métodos moleculares para estudo da diversidade microbiana

Muitos estudos foram realizados visando à identificação dos microrganismos presentes na bebida e nos grãos de kefir. Um produto probiótico de confiança requer uma completa identificação das espécies microbianas envolvidas. Assim, são de grande importância a utilização das técnicas tradicionais associadas a técnicas moleculares de identificação das espécies microbianas presentes em diversos produtos fermentados. Na maioria dos estudos, foram utilizadas técnicas tradicionais de identificação que avaliam características fenotípicas e fisiológicas dos microrganismos isolados (Garrote et al., 2001; Schoevers & Britz, 2003; Witthuhn et al., 2004; Yuksekdog et al., 2004; Fontán et al., 2005; Mainville et al., 2006).

Técnicas moleculares oferecem novas oportunidades para determinar e analisar as espécies que compõem as comunidades microbianas (Garbers et al., 2004). Técnicas como ARDRA, RAPD, PCR-DGGE tem sido empregadas na microbiologia de alimentos e tem oferecido melhorias no campo da detecção e identificação microbiana em amostras complexas de alimentos (Cocolin et al., 2004; Ercolini et al., 2004; Theunissen et al., 2004). Golowczyk et al. (2008) utilizaram as técnicas ARDRA e RAPD para caracterizar as espécies de *Lactobacillus* homofermentativo isolados de grãos de kefir e testar suas

capacidades antimicrobianas. Os resultados mostraram que várias linhagens de *Lactobacillus plantarum* inibiram o crescimento de *Salmonella typhimurium*, *S. entérica*, *S. gallinarum* e *E. coli*.

Mainville et al. (2006) utilizaram testes de assimilação em carboidratos e a técnica de RFLP para caracterizar bactérias ácido láctico presentes nos grãos de kefir provenientes de Moscou, na Rússia. Os resultados da identificação polifásica foram semelhantes entre as espécies presentes nos grãos avaliados. Foram encontrados os gêneros *Lactobacillus*, *Lactococcus* e *Leuconostoc*. Poucas espécies de bactérias do ácido láctico foram identificadas, porém, um grande número de linhagens diferentes foram demonstradas pela técnica de RFLP.

RAPD e ARDRA foram utilizados para caracterizar bactérias ácidas lácticas isoladas de grãos de kefir provenientes de La Plata na Argentina (Delfederico et al., 2006). Diferentes linhagens de *Lactobacillus kefir* foram identificadas usando a técnica de ARDRA e sequenciamento. A técnica de RAPD-PCR foi utilizada para posterior comparação com linhagens referencias deste mesmo *Lactobacillus*. Foram observadas nos grãos de kefir de La Plata diferentes linhagens da espécie *L. kefir* (Delfederico et al., 2006).

No início dos anos 90, Muyzer et al. (1993) desenvolveram a técnica eletroforese em gel de gradiente desnaturante (DGGE) com a proposta de caracterizar o perfil dos microrganismos presentes em amostras ambientais. Essa mesma técnica foi posteriormente empregada na área de microbiologia de alimentos (Ercolini, 2004; Cocolin et al., 2000; Cocolin et al., 2007). A técnica de DGGE permite analisar produtos de PCR, de acordo com suas sequências de nucleotídeos e o tamanho dos produtos (Muyzer et al., 1993). Isso possibilita não só a avaliação da diversidade genética das comunidades microbianas bem como, associada ao sequenciamento dos produtos amplificados, a identificação das espécies presentes na comunidade (Monteiro, 2007). Garbers et al., (2004)

utilizaram o DGGE para tipificar e identificar a comunidade microbiana presente em grãos de kefir de leite da África do Sul e Irlanda. Oligonucleotídeos específicos para amplificação de sequências do rDNA de bactérias e leveduras foram usados, e os resultados mostraram que os diferentes tipos de grãos apresentaram uma composição distinta de microrganismos. Somente uma espécie de levedura estava presente em todos os grãos. Análises da sequência da região 18S rDNA de leveduras mostraram que os gêneros predominantes foram *Candida* e *Saccharomyces* e o gênero predominante de bactéria foi *Lactobacillus*. As espécies *Lactobacillus crispatus* e *Lactobacillus gallinarum* não foram isoladas dos grãos de kefir por técnicas tradicionais. Essas espécies foram identificadas somente após o sequenciamento de fragmentos de DNA amplificadas para o DGGE, mostrando o potencial da técnica na identificação de espécies provavelmente não cultiváveis.

A análise da diversidade bacteriana dos grãos de kefir, provenientes de Shanghai, na China, foi realizada por Wang et al. (2006). A técnica de DGGE e sequenciamento da região 16S rDNA foram utilizadas para a identificação. Resultados obtidos pelo meio do sequenciamento da região V3 16S rDNA demonstraram a presença de 4 gêneros sendo estes *Sphingobacterium*, *Lactobacillus*, *Enterobacter* e *Acinetobacter*. O PCR-DGGE e o sequenciamento dos fragmentos são ferramentas importantes na identificação de microrganismos presentes nos grãos de kefir e em produtos probióticos. Em razão das limitações conhecidas pelos métodos de identificação fenotípica, Chen et al. (2008) utilizaram a técnica PCR-DGGE para caracterizar o perfil bacterias ácido lácticas, utilizando os métodos dependente e independente de cultivo de três diferentes grãos de kefir obtidos em Hsinchu, Ilan e Mongólia (Taiwan). *Lactobacillus kefir*, *Lactobacillus kefiranofaciens*, *Leuconostoc mesenteroides*, *Lactococcus lactis* e *Streptococcus thermophilus* foram isoladas destes grãos. Resultados obtidos neste estudo mostraram que a utilização da técnica de PCR

DGGE e sequenciamento dos fragmentos pode ser útil na identificação das linhagens de microrganismos presentes no processo de fermentação.

Utilizando a técnica tradicional e de PCR-DGGE, Wang et al. (2008) identificaram leveduras presentes nos grãos de kefir de Taipei, em Taiwan. A associação da identificação tradicional com a identificação molecular por PCR-DGGE e sequenciamento mostraram a presença de três espécies de leveduras nos grãos de kefir de leite, sendo elas *Kluyveromyces marxianus*, *Saccharomyces turicensis* e *Pichia fermentans*.

Nos últimos anos, vem ocorrendo a valorização dos produtos naturais, e um crescente consumo tem sido observado em varias regiões do Brasil. Tendo em vista que os grãos de kefir estão sendo consumidos somente por apresentar valores benéficos e por possuir uma microbiota suscetível a alterações como condições de cultivo, temperatura e outras, conduziu-se este trabalho com a proposta de isolar e identificar a microbiota presente nos grãos de kefir provenientes de diferentes regiões do Brasil, Canadá e Estados Unidos, utilizando técnicas dependentes e independentes de cultivo.

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CHAPTER 2

Microbial diversity of milk kefir grains using culture dependent and independent methods

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1 RESUMO

Kefir é uma bebida fermentada de leite produzida pela adição grãos, consistindo em bactérias ácidas lácticas, bactérias ácidas acéticas e leveduras. O objetivo deste trabalho foi identificar os microrganismos presentes nos grãos de kefir de leite provenientes de diferentes Estados Brasileiros, Canadá e Estados Unidos da América utilizando técnicas dependente e independente cultivo. Bactérias e leveduras foram identificados usando testes bioquímicos e seqüenciamento do 16S rDNA para bactérias e da região ITS de leveduras. Foi realizada análise de PCR-DGGE das amostras obtidas. Um total de 394 isolados foi obtido de todas as amostras sendo que 68.53% dos isolados correspondem a bactérias e 31.48% a leveduras. A população de bactéria variou entre 4.95 log₁₀ CFU/g na amostra de Minas Gerais e 10.43 log₁₀ na amostra dos Estados Unidos de América. Para leveduras a população variou de 5.15 log₁₀ nas amostras de Distrito Federal a 8.77 log₁₀ CFU/g na amostra obtida de São Paulo. Foram isolados bactérias dos gêneros *Lactobacillus* e *Acetobacter* e leveduras dos gêneros *Saccharomyces*, *Zygosaccharomyces*, *Pichia*, *Dekkera*, *Kluyveromyces*, *Yarrowia*, *Candida* e *Galactomyces* utilizando técnicas dependente de cultivo. Utilizando técnicas independentes de cultivo foram identificadas bactérias não cultiváveis, *Gluconobacter japonicus* e *Lactobacillus uvarum*. As espécies *Lactobacillus satsumensis*, *Acetobacter syzygi*, *Candida parapsilosis*, *Pichia guilliermondii*, *Zygosaccharomyces mellis* e *Candida valdiviana* foram descritas pela primeira vez em grãos de Kefir.

Palavras chave: grãos de kefir, bactéria, leveduras, DGGE

2 ABSTRACT

Kefir is a fermented beverage from milk that is produced by adding grains, consisting of lactic acid bacteria, acetic acid bacteria and yeasts. The aim of this research was to identify the microorganisms present in the milk kefir grains from different Brazilian states, Canada and USA by culture dependent and independent methods. The bacteria and yeast were identified using biochemical tests and partial sequence analysis of the 16S rDNA and ITS gene from isolates, and analysis of PCR-DGGE and partial sequence analysis of the 16S rDNA and 18S rDNA of bands excised. The bacteria population varied between 4.95 log₁₀ CFU/g in the sample from Minas Gerais and 10.43 log₁₀ in the sample from United States of America, and for yeast the population varied from 5.15 log₁₀ in the samples from Distrito Federal to 8.77 log₁₀ CFU/g in the sample obtained in São Paulo. A total of 394 isolates were obtained from all samples showing that 68.53% of the isolates corresponded to bacteria and 31.48% to yeast. The bacteria isolates were identified as *Lactobacillus* and *Acetobacter* genera and the yeast isolates were identified in the genera *Saccharomyces*, *Zygosaccharomyces*, *Pichia*, *Dekkera*, *Kluyveromyces*, *Yarrowia*, *Candida* and *Galactomyces* for the culture dependent and culture independent methods was possible to identify uncultured bacterium, *Gluconobacter japonicus* and *Lactobacillus uvarum* species. The *Lactobacillus satsumensis*, *Acetobacter syzygii* bacterial species and the yeast *Candida parapsilosis*, *Pichia guillermondii*, *Zygosaccharomyces mellis* and *Candida valdiviana* have not previously been reported as members of the kefir grain population.

Key words: kefir grains, bacteria, yeast, DGGE

3 INTRODUCTION

Traditionally kefir grains have been used in many countries, especially in Eastern Europe, as the natural starter in the production of the unique self-carbonated dairy beverage known as kefir (Gorsek & Tramsek, 2008). Kefir can be considered a probiotic resource, because it enjoys a variety of health claims (immunomodulatory, anti-neoplastic and pro-digestive effects) in addition to its nutritional value (Yang et al., 2008). Kefir differs from other fermented milks in its starter, which exists in the form of grains (Simova et al., 2002). Kefir grains resemble small cauliflower florets that vary in size from 3 to 30 mm in diameter, are lobed, irregularly shaped, white to yellow-white in colour and have a slimy but firm texture (La Rivière et al. 1967, Garrote et al., 2001).

The properties of kefir are the result of the metabolic activity exerted on the milk substrate by its microbial consortium. That kefir constitutes a complex microbial ecosystem where species, which play a determinant role in the development of the desirable characteristics of the product, may coexist with others whose presence is accidental or even detrimental (Latorre-Garcia et al., 2006). The microorganisms present in kefir grains generally are lactic acid bacteria (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Acentobacter* and *Streptococcus* spp.) and yeasts (*Kluyveromyces*, *Torula*, *Candida* and *Saccharomyces* spp.). Both bacteria and yeasts are surrounded by a polysaccharide matrix, called kefiran, which is a water-soluble branched glucogalactan (Lee et al., 2007).

The direct monitoring of microbial populations in food systems is an important issue that food microbiologists have to pay attention (Cocolin et al., 2007). The microorganisms present in the grains have in the past were identified using selective growth media, and morphological and biochemical

characteristics, however the identification of microbe species is complicated by the fact that certain organisms are not able to grow on synthetic growth media (Garbers et al., 2004). The combination of a conventional isolation strategy with culture dependent identification usually makes microbial analysis of probiotic products relatively time consuming, and results may be influenced by poor viability or low densities of the target organism. For this reason culture independent analysis has been promoted as an alternative and or complementary approach for quality control measurements of probiotic products (Masco et al., 2005).

Polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) is based on amplification of ribosomal DNA and electrophoresis of the PCR product in a polyacrylamide gel containing an increasing gradient of denaturant (Muyzer et al., 1993; Sigler et al., 2004). Recently, DGGE analysis is recognized as one of the most suitable and widely applied techniques to study complex microbial communities originated from food samples or other environments, such as doenjang (Kim et al., 2009), sliced vacuum-packed cooked ham (Hu et al., 2009), rice vinegar (Haruta et al., 2006) and kefir grains (Chen et al., 2008; Wang et al., 2008, Jianzhong et al., 2009).

A method for the mass cultivation of the kefir grains was developed (Shoevers & Britz, 2003), which may result in the commercialization of the kefir grains, rather than the kefir beverage. However, before the grains can be commercialized it is important to know the microbial exact content of the grains, as well as the stability of the microbial population in the grains after mass cultivation.

The aim of this study was to identify the microorganisms present in milk kefir grains obtained from different Brazilian states, and from the countries of Canada and United States of America (USA) by culture dependent and independent methods.

4 MATERIAL AND METHODS

4.1 Kefir grains sampling

Milk kefir grains from Brazilian States of Santa Catarina (Palhoça), São Paulo (Santos), Minas Gerais (Juiz de Fora), Alagoas (Macéio), Rio de Janeiro (Rio de Janeiro), Distrito Federal (Brasília), Rio Grande do Sul (Nova Hartz), Espírito Santo (Aracruz) and Paraná (Curitiba) were obtained from families that traditionally consumed kefir. These were received as handmade, mixed in milk powder. Lyophilized grains were sent from Canada (Bowmanville) and United States of America (Fayette). A sample of kefir grains was removed aseptically for PCR-DGGE.

4.2 Isolation, purification and maintenance of microorganisms

For each sample, 1g of the kefir grains was collected and added to a sterilized tube containing 9 mL of sterile peptone water diluent (0,1% peptone, 0,5% NaCl) and homogenized for 60s. A volume of 0.1 mL of appropriate dilutions was spread plated in duplicate on the following medium for isolation and enumeration of bacteria and yeast. Some samples were necessary for direct plating of grains. Decimal dilutions of the suspended samples were used for microbial enumerations and isolation for bacteria by MRS medium (De Man Rogosa Sharpe, Merck) (Silva et al., 2008) with the addition of 4 mL/L of nystatin (Sigma, St. Louis, USA) to inhibit yeasts growth. The plates were incubated at 28°C and 35°C for 48 hours. Yeasts were isolated on YW agar medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, 2% agar), with the addition of 100 mg/L of chloramphenicol (Sigma, St. Louis, USA) to inhibit the bacterial growth. The YW plates were incubated at 28°C for 120 hours. On plates displaying 30 – 300 colonies, the number of isolates

corresponding to the square root of each morphological yeast and bacterial type observed on YW and MRS plates were picked and recultivated in specific medium for further purification. The purified isolates were freezer stored at -80°C in YW (for yeast) and MRS (for bacteria) containing 20% (v/v) of glycerol.

4.3 Identification by culture dependent methods

Gram-positive bacteria were subdivided into spore-formers and non-spore-formers by inducing spore liberation (80°C for 10 min.). Subsequent identification used motility, catalase and biochemical tests as recommended in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and The Prokaryotes (Hammes et al., 1991). The genera *Lactobacillus* were identified by physiological characteristics – heterofermentative, homofermentative or obligately heterofermentative, by their ability to produce gas (CO₂). The identification of the isolated strains were confirmed by 16S rDNA sequencing. A fragment of approximately 1500 bp of the 16S rDNA was amplified by forward primer 27f (5'AGAGTTTGATCCTGGCTCAG 3') and reverse primer 1512r (5'ACGGCTACCTTGTACGACT 3') (Devereux et al., 2004).

All the yeast isolates were characterized based on their morphology, spore formation, assimilation and fermentation of different carbon sources, according to Barnett et al. (2000). The yeast isolates were identified using the internal transcribed spacer (ITS) ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White et al.,1990).

The sequencing of portions of the 16S for bacteria and ITS region for yeast were accomplished by Central Laboratory of Molecular Biology - LCBM/UFLA (Lavras, MG). Sequence similarity identities were performed using the BLAST database from GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>).

4.4 Identification by culture independent methods

One kefir grain of each sample was placed into sterilized water and homogenized. One milliliter of each sample was transferred into a plastic tube and the DNA was extracted using a NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). The DNA extraction was performed according to the manufacturer's instructions. The genomic DNA was resuspended in sterilized deionized water and stored at -20°C.

The primers set, 518r (5' ATTACCGCGGCTGCTGG 3') and 338f (5' CGCCCGCCGCGCGCGGGCGGGGCGGGGCGGGGGCACGGGGGGACTCCT ACGGGAGGCAGCAG 3'), were used for the amplification of the V3 region of the partial ribosomal 16S DNA (Cocolin et al., 2001) and the primers YM951r (5' TTGGCAAATGCTTTTCGC 3') and NS3 (5' CGCCCGCCGCGCGCGGGCGGGGCGGGGCGGGGGCACGGGGGGGCAAGT CTGGTGCCAGCAGCC 3'), used for partial ribosomal 18S DNA amplification for yeast (Haruta et al., 2006). PCR was performed in a total reaction volume of 50 µL containing 0.2µM of each primer, 0.5U of Go *Taq* Flexi DNA polymerase (Promega®), 5µL of 10X PCR reaction buffer with 20mM MgCl₂, 0.125 mM dNTPs mix and 40ng of the DNA template. Amplification was achieved in 0.2 mL tubes by using a model Thermo PCYL220 thermal cycler (Thermo Fisher Scientific Inc., Waltham, USA) with the following parameters for bacteria: initial denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 30s, annealing at 54°C for 60s, extension at 72°C for 1min and final extension at 72°C for 5minutes, and with the following parameters for yeasts: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1min and final extension at 72°C for 7 min.

Amplicons were separated using DGGE (BioRad Universal Dcode Mutation Detection System, USA). PCR samples were directly applied into a gel with 8%

of polyacrylamide in 0.5 x TAE with gradient between 15 and 55% for the bacteria and 12 and 50% for yeast. The gradient was created by polyacrylamide, containing 0 – 100% denaturant (7M urea and 40% of formamide). Electrophoresis was performed at a constant voltage of 70 V for 6h for the bacteria fragments and 200 V for 4h for the yeast fragments and a constant temperature of 60°C. After electrophoresis, they were stained for 30 minutes in a SYBR Green solution and analyzed under UV transillumination. The gel images were photographed using the Loccus Biotechnology – Lpix Image.

Different DGGE bands were excised from the acrylamide gels. The DNA fragments were purified using QIAEX II gel extraction kit (Qiagen, Chatsworth, CA, USA) and then re-amplified by the primer 518r for bacteria and YM951r for yeast and submitted to sequencing at the Central Laboratory of Molecular Biology of UFLA, LCBM/UFLA (Lavras MG). The sequence identities were determined by nucleotide BLAST database from GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>). The reproducibility of DGGE fingerprints was confirmed by repeating the PCR amplification reactions on the DNA isolated from the different kefir grains several times, as well as on DNA isolated from the different grains in the same sample several times.

5 RESULTS AND DISCUSSION

5.1 Microbial population in kefir grains

The eleven types of kefir grains studied here showed similar macroscopic features having a white color and irregular shape. Kefir grains from São Paulo, Minas Gerais, Distrito Federal, Rio Grande do Sul, Espírito Santo, Paraná, Canada and USA presented a more heterogeneous size distribution between 10mm to 25 mm, while Santa Catarina, Alagoas, Rio de Janeiro grains were more homogeneous distribution of size being < 10mm. This varying in size can be the result of different grains sources, original culture conditions, storage and elaboration processes. The Kefir grains obtained were processed immediately to prevent the grains from changing their specific microbial population.

Differential enumeration was observed between the grains samples obtained. The enumeration values (CFU/g) of the isolated viable bacteria and yeast are given in TABLE 1. The bacteria population MRS medium was between $10.43 \log_{10}$ CFU/g at 35°C and $10.18 \log_{10}$ CFU/g at 28°C in the sample from United States of America and $4.95 \log_{10}$ CFU/g at 35°C and $5.59 \log_{10}$ CFU/g at 28°C in the sample from Minas Gerais. Many plating procedures are only partially selective and exclude part of the microbial community. In this work we used MRS because our previous studies show that these media allow the growth of most bacteria present in kefir grains. In general, lactic acid bacteria are more numerous (10^8 - 10^9) than yeast (10^5 - 10^6) and acetic acid bacteria (10^5 - 10^6) in kefir grains, although fermentation conditions can affect this pattern (Koroleva, 1988; Garrote et al., 2001).

The yeast count also varied between samples analyzed. The minimum value was of $5.15 \log_{10}$ CFU/g in the samples from Distrito Federal and the maximum $8.77 \log_{10}$ CFU/g in the samples from São Paulo. The yeast present in Kefir grains was higher than the bacteria population in the samples from Brazilian States of São Paulo, Minas Gerais, Rio de Janeiro, Rio Grande do Sul and

Parana. We have not been able to find data in the scientific literature on the total bacteria and yeast number (CFU/g) without the cultivation of grain.

No bacteria colony was detected on plates for Rio Grande do Sul and no yeast colony was detected on plates for Alagoas and Espírito Santo samples. The microorganisms present in these grains could have been damaged or inactive during transport and/or some species present could be uncultivable in the culture medium used. It is possible that the environmental stress conditions applied during transport of the grains may have impacted the population structure leading to changes in the microbial numbers.

5.2 Identification of isolates by culture dependent methods

A total of 394 isolates were obtained from all samples showing that 68.53% of the isolates corresponded to bacteria and 31.48% to yeast. Primary differentiation and grouping of the strains from the same genus was performed using biochemical methods. For characterization of species within the same genus, sequencing of partial 16S rDNA fragments for bacteria and ITS region for yeasts were used. The identified isolates are listed in TABLE 2. The identified genera of bacteria included *Lactobacillus* and *Acetobacter*, and the genera of yeasts included *Zygosaccharomyces*, *Saccharomyces*, *Pichia*, *Kluyveromyces*, *Dekkera*, *Yarrowia*, *Candida* and *Galactomyces*. The genus *Lactobacillus*, and yeast genera *Zygosaccharomyces*, *Candida* and *Saccharomyces* were also identified kefir grains from Pretoria, South Africa by culture dependent methods (Witthuhn et al., 2004).

It has been reported that genera of bacteria found in the kefir grains included in four genera: *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Acetobacter* (Garrote et al., 2001; Irigoyen et al., 2005). A wide variety of Gram-positive and Gram-negative bacteria are often found in kefir grains (Otlés & Cagindi, 2003). All the bacteria isolated were Gram-positive, except

Acetobacter syzygii which is a Gram-negative isolate from kefir grains from Santa Catarina. The present study showed that both lactic acid and acetic acid bacteria were present in kefir grains. *Lactobacillus kefir*, *Lac. paracasei*, *Lac. helveticus*, *Lac. plantarum*, *Lac. satsumensis* and *Acetobacter syzygii* (TABLE 2) were the main bacteria species found. *Lac. kefir* was the predominating bacteria in almost all kefir grains in this study with the exception of the sample from Rio Grande do Sul, Paraná, Canada and USA. Studie have investigated the composition of the microorganisms present in Taiwanese kefir grains and reported that *Lactobacillus* was the most frequent microorganism and *Lac. kefir* seemed to be the most detected bacteria (Chen et al., 2008).

All the bacteria isolated from grains have previously been reported as members of the kefir grain population, with the exception of *Lactobacillus satsumensis* which was isolated from grains obtained from USA and *Acetobacter syzygii* isolated from grains from Santa Catarina State. *Lactobacillus satsumensis* were isolated from shochu mashes a traditional Japanese distilled spirit made from fermented rice, sweet potato, barley and other starchy materials (Endo & Okada, 2005). The fermented mashes contain 15–18% alcohol and are kept at acidic pH (3.0 – 4.0) as a result of the production of citric acid by the mould during fermentation. *Lactobacillus satsumensis* clustered in the *Lactobacillus casei* – *Pediococcus* group and was closely related to *Lactobacillus nagelii* and *Lactobacillus mali* on the basis of 16S rDNA gene sequence similarity. The isolate was considered to represent a novel species, for which the name *Lactobacillus satsumensis* was proposed (Endo & Okada, 2005). The *Acetobacter syzygii* bacteria belongs to the class of acid acetic bacteria and only *Acetobacter pasteurianus*, *Acetobacter aceti* species have already been reported as present in the kefir grains (Angulo et al., 1993). This is the first report of *Acetobacter syzygii* species in kefir grains. This species was isolated

from flowers, fruits and vinegar fermentation collected in Indonesia (Lisdiyanti et al., 2001; Nielsen et al., 2006).

Lactobacilli were present in all grains indicating the importance of this group for the elaboration of the beverage. Simova et al (2002) identified *Lactobacillus* and yeast species in kefir grains from Bulgaria, indicating that *Lactococcus lactis* was dominant specie representing 58%~70% of the total microbial. Liu et al. (2006) isolated two strains of *Lactobacillus lactis* from Tibetan kefir grains.

Previous studies showed that a vast variety of different species of *Lactobacillus* have been isolated and identified in kefir grains from around the world, such as *Lac. kefir*, *Lac. kefiranofacies*, *Lac. kefigranum*, *Lac. parakefir*, *Lac. acidophilus*, *Lac. brevis*, *Lac. casei*, *Lac. fermentum*, *Lac. delbrueckii*, *Lac. plantarum* and *Lac. helveticus* (Garrote et al., 2001, Navhurs et al., 2003, Irigoyen et al., 2005 Chen et al., 2008). Other species of bacteria also have been identified, such as *Acetobacter aceti*, *Lactococcus latis*, *Streptococcus thermophilus*, *Enterococcus durans*, and *Leuconostoc mesenteroides* (Lopitzotsoa et al., 2006). The bacteria of kefir grains from Argentina included *Lactococcus lactis* subsp. *lactis*, *Lactobacillus kefir*, homofermentative *Lactobacillus* and microorganisms belonging to the genera *Acetobacter* (Garrote et al., 2001). *Lactobacillus lactis* subsp. *lactis*, *Lactobacillus lactis* subsp. *diacetystis*, *Lactobacillus plantarum* and *Lactobacillus parakefir* were also identified in these kefir grains (Garrote et al., 2001).

The isolate of yeasts that were found in this study were *Saccharomyces cerevisiae*, *Zygosaccharomyces mellis*, *Dekkera anomala*, *Pichia fermentans*, *P. membranifaciens*, *P. anomala*, *P. guilliermondii*, *Kluyveromyces marxianus*, *Yarrowia lipolytica*, *Candida parapsilosis*, *C. valdiviana* and *Galactomyces geotrichum* (TABLE 2). The genus *Pichia* was found in kefir grains from São Paulo, Distrito Federal, Rio Grande do Sul and Paraná. Some samples were

identified in only one specie in the culture dependent method being *Yarrowia lipolytica* in grains from Rio de Janeiro, *Pichia anomalia* in grains from Rio Grande do Sul and *Saccharomyces cerevisiae* in grains from Canada. The kefir specific yeasts play a key role in the formation of flavour and aroma (Glaeser et al., 1986). The species present in kefir grains were reported as *Dekkera anomala*, *Brettanomyces anomalus*, *Kluyveromyces marxianus*, *K. bulgaricus*, *K. lodderae*, *K. fragilis*, *K. lactis*, *Torulasporea delbrueckii*, *Candida friedrichii*, *C. humilis*, *C. inconscua*, *C. krusei*, *C. lipolytica*, *C. holmii*, *C. kefir*, *C. pseudotropicalis*, *Saccharomyces cerevisiae*, *S. unisporus*, *S. kefir*, *S. unisporum*, *S. torulopsis*, *P. fermentans*, *Torula kefir*, *Issatchenkia orientallis*, *Debaryomyces occidentallis*, *Debaryomyces hansenii*, *Zygosaccharomyces rouxii*, *Galactomyces geotrichum* and *Yarrowia lipolytica* (Lopitz-otsoa et al., 2006, Păucean & Socaciu, 2008 Lee et al., 2007; Garrote et al., 2001). Some of the yeast isolated from these grains has previously been reported as members of the kefir grain population, with the exception of *Candida valdiviana*, *Candida parapsilosis*, *Pichia guilliermondii* and *Zygosaccharomyces mellis*. The species *Pichia anomala* identified in the kefir grains from Rio Grande do Sul and Distrito Federal is found present in cassava-fermenting (Oyewole, 2001; Lacerda et al., 2005) and also offer alternatives to chemical fungicides for postharvest protection of fruits and vegetables (Melin et al., 2007). This strain reduces the growth of *Penicillium roqueforti* both in vitro and in high-moisture cereal grains in a test-tube version of a malfunctioning storage system (Passoth et al. 2006). Other strains of *P. anomala* have been reported to prevent growth of grey mould, *Botrytis cinerea*, on stored apples (Jijakli and Lepoivre 1998) and on grapevines (Masih et al. 2000). *P. anomala*, *P. guilliermondii*, *C. parapsilosis* among others, were obtained from Hamei samples collected from household rice wine preparations in tribal villages of Manipur (Jeyaram et al., 2008). *P. membranifaciens* found in the milk kefir grains from São Paulo also was

identified in water kefir grains (Ulloa et al., 1994). The specie *Kluyveromyces marxianus* can ferment lactose producing alcohol and formation of the typical yeast flavor (Simova et al., 2002).

Kefir grains obtained from eleven different Brazilian states, Canada and USA were composed of different microorganisms, confirming that the origin and history of kefir grains probably strongly influenced the microbial composition.

5.3 DGGE analyses of bacteria

The independent molecular culture approaches have shown to be a powerful tool in investigation of microbial diversity in food samples (Ercolini et al., 2004). PCR-DGGE generated rDNA gene fragments has been shown to be a useful tool for studying community structure at the species level (Muyzer et al., 1993).

The PCR fragments were resolved on a DGGE gel and DNA banding patterns were obtained for each of eleven different grain types using the Eubacterial specific primers (FIGURE 1). Some bands showed lower intensity and could be related to the presence of low numbers of specific targets in the samples. DNA template number can affect the amplification in complex template mixtures (Candler et al., 1997). Profile results revealed differences between samples from Distrito Federal, Rio Grande do Sul, Espirito Santo, Parana, Canada and USA (FIGURE 1) and the 16S rDNA sequencing culture dependent method also showed a diverse bacterial community (TABLE 2). Individual bands observed in the DGGE profiles were excised from acrylamide gel, re-amplified to sequencing and identified as *Gluconobacter japonicus* (a), uncultured bacterium (b), uncultured bacterium (c), not identified (d), *Lactobacillus uvarum* (e), *Lactobacillus paracasei* (f), Uncultured bacterium (g), *Lactobacillus satsumensis* (h), *Lactobacillus platarum* (i), not identified (j).

uncultured bacterium (**k**) and *Lactobacillus paracasei* (**I**). This sequencing exhibited 100% identity with sequences in the GenBank databases except band **I** whose similarity was 95%.

The clear band **a**, which corresponds to *Gluconobacter japonicus* appeared in the sample from Santa Catarina, and band **b** which corresponds to unculturable bacterium, appeared in sample from Alagoas. Band **i** which corresponds to *Lactobacillus plantarum* appeared in the grain from Alagoas and band **e** which corresponds to *Lactobacillus uvarum* appeared in the grain from Santa Catarina. Meanwhile, band **k** which corresponds to unculturable bacterium present some of the samples with the exception of grains Santa Catarina, Minas Gerais and Rio de Janeiro. Bands in the same position in the gel suggested that the same Eubacteria species are present in all these kefir grain samples. However, several authors report cases of co-migration of amplicons from different species in DGGE gel because many bacteria species are closely related and the 16S rDNA fragment analysed may not contain major differences allowing separation by DGGE (Ercolini et al., 2001, 2003; Meroth et al., 2003). Band **f** appeared in the grain from Minas Gerais, Distrito Federal and Espirito Santo and band **I** appeared in the grain from Distrito Federal, Rio Grande do Sul, Parana, Canada and USA. These amplicons migrated very closely and corresponded to *Lactobacillus paracasei*. These multiple banding patterns may be due to sequence heterogeneity between multiple copies of the 16S rDNA these strain (Nübel et al., 1999). The identity of these bands could be confirmed by comparing their relative position of migration with the PCR products from the *Lactobacillus paracasei* isolate or a reference strain.

The DGGE results obtained were compared to sequencing of partial 16S rDNA fragments for bacteria isolates. The result of DGGE profile of *Lactobacillus paracasei* isolated from the grain from Minas Gerais and *Lactobacillus plantarum* isolated from the grain from Parana and Canada

respectively corresponded to sequencing of partial 16S rDNA fragments for bacteria isolated on the plate. On the other hand, culture dependent procedure showed that *Acetobacter syzygii*, *Lactobacillus kefir*, *Lactobacillus helveticus* and *Lactobacillus parakefir* were detected in the samples and not detected in culture independent procedure. This apparent contradiction between culture dependent and independent methods could have explained the difficulty to obtain good DNA extraction and PCR reaction of the grain samples. Initial template DNA ratio and template competition may affect the detection of microorganisms present in low abundance in a microbial complex (Muyzer et al., 1993; Murray et al., 1996; Zhang et al., 2005). The *Gluconobacter japonicus* and *Lactobacillus uvarum* species identified from São Paulo and Santa Catarina grains, respectively, were not detected in the culture dependent method. The possible explanation might be that only easily culturable microorganism can be detected and the species that are in sublethal or injured states were lost. DGGE analysis does not provide information on the viability of the microorganisms (Rantsiou et al., 2005). The *Gluconobacter japonicus* is fastidious acetic acid bacteria recently identified as novel species of the genus *Gluconobacter* (Malinas et al. 2009). This species has not previously been reported a member of the kefir grain population. *Lactobacillus uvarum* proposed as a new species was identified in the winemaking process, being Gram-positive, motile, non-spore forming (Mañes-Lázaro et al., 2008).

The results obtained in this study showed that a combined method of cultivation with PCR-DGGE and subsequent DNA sequencing could successfully identify bacteria species from different Kefir grains samples. These preliminary results suggest that DGGE analysis is clearly a suitable tool for a rapid and cost effective screening for fastidious microorganisms or those that could not be cultivated.

5.4 DGGE analyses of yeast

The DGGE gel showing the communities of yeasts profile of the eleven different grain type is represented in FIGURE 2. Some bands showed lower intensity and could be related to the presence of low numbers of specific ribosomal 18S DNA amplification targets for yeast in the samples. Band **1** appeared in all of the samples with the exception of Santa Catarina, Alagoas and Parana grains. Band **9** appeared in all of the samples with the exception of Santa Catarina, Alagoas, Rio de Janeiro and Parana grains. This band was especially intense in the samples from São Paulo, Minas Gerais and Distrito Federal. This difference in intensity can be resulting of concentration of the extracted DNA or difference in the amount of specific microorganisms in the samples. Band **13** appeared in the grain from Minas Gerais. Bands **8**, **11** and **14** were found in the grain from Espírito Santo. Band **7** appeared in the grain from Distrito Federal and Espírito Santo. In the Alagoas sample it was not possible to identify yeast by the culture dependent methods and by the culture independent method it was possible to identify two bands. Since in the Espírito Santo sample no colonies were observed when culture dependent method was used, but in culture independent methods, this sample presented several bands. This can be explained by the fact that these yeasts are not cultivable, unviable to grow in plates or fastidious microorganisms.

The bands were excised from the acrylamide gel, re-amplified and the eluted DNA fragments were used for sequencing with the primer YM951r.

6 CONCLUSIONS

The results obtained in this study clearly demonstrated that the microbial species composition of Kefir grains from different Brazilian states, Canada and USA varies and may be influenced by factors such as the method of Kefir production, the origin of the grains and the method of microbial identification, as well as their transportation to our laboratory.

Some bacteria species were identified only by culture dependent methods such as *Lactobacillus satsumensis*, *Lactobacillus kefir*, *Lactobacillus helveticus* and *Acetobacter syzygii*. By culture independent methods it was possible to identify uncultured bacterium, *Gluconobacter japonicus* and *Lactobacillus uvarum* species, that have not been identified by culture dependent methods. From the Espirito Santo sample, it was not possible to isolate any yeast by the culture dependent method, although it presented the highest number of bands in the DGGE gel.

The *Lactobacillus satsumensis*, *Acetobacter syzygii* bacterial species and the yeasts *Candida parapsilosis*, *Pichia guillermondii*, *Zygosaccharomyces mellis* and *Candida valdiviana* have not been previously reported as members of the kefir grain population and were identified by culture dependent methods.

TABLE 1 Microbial population of milk kefir grains from different origins (log₁₀ cfu/ml)

Grains Origin	Aerobica 28°C	Aerobica 35°C	Yeasts
AL	7.99	8	ND
CA	8.96	9.04	7.34
DF	8.30	8.34	5.15
ES	8.11	7.50	ND
MG	5.59	4.95	7.11
PR	8.40	8.08	8.46
RJ	6.48	6.46	8.08
RS	ND	ND	6.11
SC	8.08	8.08	7.54
SP	5.98	5.86	8.77
USA	10.18	10.43	8.45

ND - not determined; MRS - medium for bacterial growth; YW - medium for yeast growth

SC - Santa Catarina; SP - São Paulo; MG - Minas Gerais; AL - Alagoas; RJ - Rio de Janeiro; DF - Distrito Federal; RS - Rio Grande do Sul; ES - Espírito Santo; PR - Paraná; CA - Canadá and USA - Estados Unidos da América

TABLE 2 Species of bacteria and yeast present in the milk kefir grains by culture dependent methods

Samples		Isolates Identified	CFU/g
AL	bacteria	<i>Lactobacillus kefir</i>	1.3 x 10 ⁶
		<i>Lactobacillus helveticus</i>	1.4 x 10 ⁶
	yeast	ND	
CA	bacteria	<i>Lactobacillus plantarum</i>	6 x 10 ⁶
		<i>Lactobacillus kefir</i>	1.6 x 10 ⁷
	yeast	<i>Saccharomyces cerevisiae</i>	1.5 x 10 ⁵
DF	bacteria	<i>Lactobacillus kefir</i>	3.4 x 10 ⁶
	yeast	<i>Candida valdiviana</i>	2 x 10 ³
		<i>Pichia anomala</i>	3 x 10 ³
		<i>Candida parapsilosis</i>	1 x 10 ³
ES	bacteria	<i>Lactobacillus helveticus</i>	1.4 x 10 ⁵
		<i>Lactobacillus kefir</i>	1 x 10 ⁵
	yeast	ND	
MG	bacteria	<i>Lactobacillus kefir</i>	1.3 x 10 ⁴
		<i>Lactobacillus paracasei</i>	7 x 10 ³
	yeast	<i>Kluyveromyces marxianus</i>	7 x 10 ⁴
		<i>Dekkera anomala</i>	8 x 10 ⁴
PR	bacteria	<i>Lactobacillus kefir</i>	2 x 10 ⁵
		<i>Lactobacillus parakefir</i>	2.4 x 10 ⁶
		<i>Lactobacillus plantarum</i>	2.6 x 10 ⁶
		<i>Kluyveromyces marxianus</i>	1 x 10 ⁶
	yeast	<i>Pichia guilliermondii</i>	1 x 10 ⁶
RJ	bacteria	<i>Lactobacillus kefir</i>	2 x 10 ⁵
	yeast	<i>Yarrowia lipolytica</i>	1.7 x 10 ⁶
RS	bacteria	ND	
	yeast	<i>Pichia anomala</i>	1.5 x 10 ⁴
SC	bacteria	<i>Lactobacillus kefir</i>	1.3 x 10 ⁶

TABLE 2 Cont.

Continua...

		<i>Lactobacillus paracasei</i>	1.4 x 10 ⁶
	yeast	<i>Saccharomyces cerevisiae</i>	8 x 10 ⁵
		<i>Zygosaccharomyces mellis</i>	2 x 10 ⁵
SP	bacteria	<i>Lactobacillus kefir</i>	8 x 10 ³
		<i>Acetobacter syzygii</i>	5 x 10 ³
		<i>Lactobacillus paracasei</i>	9 x 10 ³
	yeast	<i>Pichia fermentans</i>	4 x 10 ⁶
		<i>Pichia membranifaciens</i>	6 x 10 ⁶
USA	bacteria	<i>Lactobacillus satsumensis</i>	2.6 x 10 ⁸
	yeast	<i>Galactomyces geotrichum</i>	9 x 10 ⁵
		<i>Saccharomyces cerevisiae</i>	7 x 10 ⁵

ND not detected; CFU/g – microbial species count; AL - Alagoas, CA - Canada, DF - Distrito Federal, ES - Espirito Santo, MG - Minas Gerais, PR - Parana, RJ - Rio de Janeiro, RS - Rio Grande do Sul, SC - Santa Catarina, SP - São Paulo, USA - Estados Unidos da América

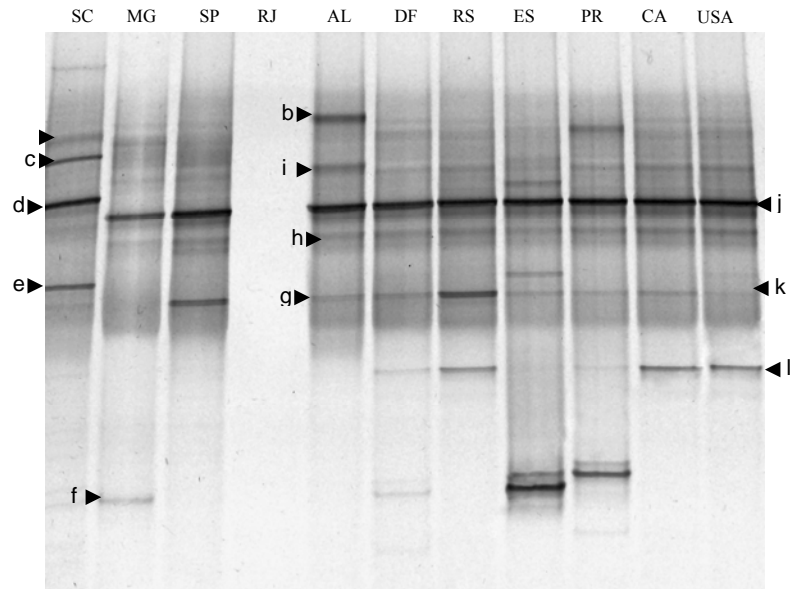


FIGURE 1 DGGE profile of 11 kefir grain bacteria samples with denaturing gradient from 15% to 55%. a – *Gluconobacter japonicus* AB 489253.1; b – uncultured bacterium FJ 838427.1; c – uncultured bacterium AM 921620.1; d – non identified; e – *Lactobacillus uvarum* AY 681128.1; f - *Lactobacillus paracasei* FJ 861111.1; g – uncultured bacterium EF 014703.1; h - *Lactobacillus satsumensis* AB289300.1; i – *Lactobacillus plantarum* EF 426261.1; j – non identified; k – uncultured bacterium EF 014703.1 and l – *Lactobacillus paracasei* FJ 861111.1

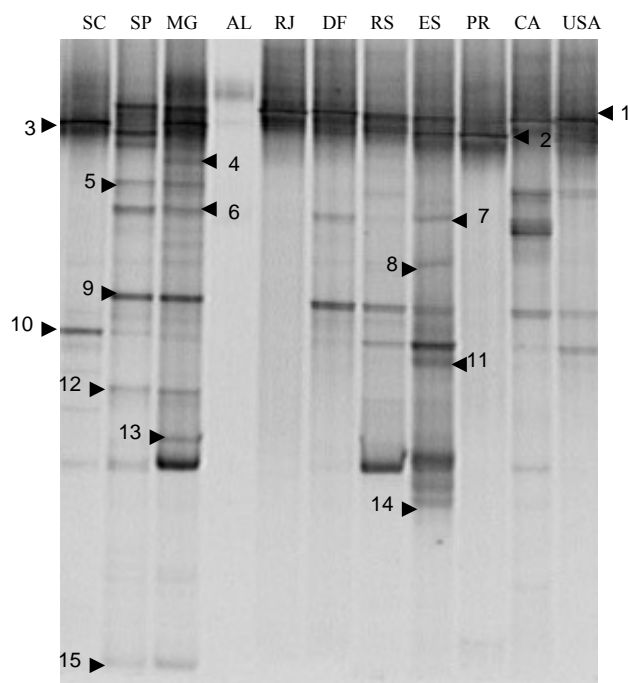


FIGURE 2 DGGE profile of 11 kefir grains yeast samples with denaturing gradient from 15% to 55%. Lane 1 – Santa Catarina; 2 – São Paulo; 3 - Minas Gerais; 4 – Alagoas; 5 – Rio de Janeiro; 6 – Distrito Federal; 7 – Rio Grande do Sul; 8 – Espírito Santo; 9 – Paraná; 10 - Bowmanville (Canada) and 11 - Fayette (USA).

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CHAPTER 3

Diversity of microorganisms present in water kefir grains from different Brazilian States

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1 RESUMO

Kefir é uma bebida fermentada de leite que originou na Europa Oriental. Um substrato também usado para produção desta bebida conhecida como Kefir d'água é açúcar mascavo na concentração de 3 a 10%. O objetivo deste trabalho foi isolar e identificar as espécies microbianas presentes nos grãos de kefir d'água provenientes de 8 Estados Brasileiros utilizando técnicas dependentes e independentes cultivo. Análises de PCR-DGGE foram realizadas com as amostras dos grãos. Um total de 201 bactérias e 85 leveduras foram identificados por técnicas tradicionais e pelo seqüenciamento do 16S rDNA para bactérias e da região ITS para leveduras. A população de bactérias variou de 6.04 log₁₀ CFU/g na amostra de Alagoas a 9.18 log₁₀ CFU/g na amostra da Bahia. A população de leveduras variou de 5.92 log₁₀ CFU/g na amostra de Minas Gerais a 8.30 log₁₀ CFU/g na amostra de Rio Janeiro. Os gêneros *Lactobacillus*, *Gluconacetobacter*, *Acetobacter*, *Bacillus*, *Pichia*, *Candida*, *Yarrowia*, *Saccharomyces* e *Zygosaccharomyces* foram identificados nas amostras por métodos dependente e independente de cultivo. As bactérias *Bacillus cereus*, *Acetobacter lovaniensis* e *Gluconacetobacter liquefaciens* e as leveduras *Pichia cecembensis*, *Pichia caribbica* e *Zygosaccharomyces fermentati* foram descritas pela primeira vez em grãos de Kefir d'água.

Palavras chave: grãos de kefir d'água, bactéria, leveduras, DGGE

2 ABSTRACT

Kefir is a fermented milk beverage that originated in Eastern Europe. The substrate also used for production of that beverage is water and raw sugar in the concentration of 3 to 10%. This beverage is known as water Kefir being mainly consumed in Mexico and Brazil. The aim of this study was to isolate and identify the microbial species present in water kefir grains from different States of Brazil, using culture dependent and independent methods. The samples of the grains were removed for PCR-DGGE analyses and decimal dilutions were prepared for microbiological characterization. A total of 286 isolates (201 bacteria and 85 yeasts) were isolated and identified by means of phenotypic tests, PCR based methods and 16S rDNA gene sequencing. The results demonstrated that enumeration values for bacteria varied between 6.04 log₁₀ CFU/g in the sample from Alagoas to 9.18 log₁₀ CFU/g in the sample from Bahia, and for yeast varied between 5.92 log₁₀ CFU/g in the sample from Minas Gerais to 8.30 log₁₀ CFU/g in the sample from Rio Janeiro. The bacterial genera that were identified include *Lactobacillus*, *Gluconacetobacter*, *Acetobacter* and *Bacillus* and the yeast *Pichia*, *Candida*, *Yarrowia*, *Saccharomyces* and *Zygosaccharomyces*. In this study the *Bacillus cereus*, *Acetobacter lovaniensis*, *Gluconacetobacter liquefaciens* bacterial species and the yeast *Pichia cecembensis*, *Pichia caribbica* and *Zygosaccharomyces fermentati* have not been reported as microorganisms present in kefir grains.

Key Words: water kefir grains, bacteria, yeast, DGGE

3 INTRODUCTION

Kefir is a fermented milk beverage that originated in Eastern Europe and enjoys worldwide consumption (Guzel-Seydim et al., 2005). In addition to milk the kefir grains can also be inoculated in water plus 3 to 10% raw sugar. The kefir grains cultivated in water with raw sugar are known as tibico or tibi (Taboada et al., 1987; Rubio et al., 1993). These tibi grains are very similar to the milk kefir grains, in relation to their structure, microbiological constitution and composition of the products formed during the fermentation of those grains (Ulloa et al., 1994). The tibico grains appear as jellied masses, more or less compact, of whitish or yellowish, translucent color, in an irregular way and of a size that varies from a few millimeters to one or two centimeters (Taboada et al., 1987). The grains have been used to produce a refreshing beverage of low alcoholic and acetic content, when the fermentation duration is short, but when the process is prolonged, this tibico beverage becomes an alcoholic beverage and later a tibico vinegar (Rubio et al., 1993).

The yeast species *Pichia membranaefaciens*, *Candida valida*, *Candida lambica*, *Brettanomyces claussenii*, *Cryptococcus albidus*, *Rhodotorula rubra* and *Saccharomyces cerevisiae* were found in tibico grains from various localities of Mexico (Taboada et al., 1987; Rubio et al., 1993; Ulloa et al., 1994). The species of bacteria described were *Enterobacter aerogenes*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus polymyxa*, *Bacillus pumilus*, *Acetobacter aceti* and bacteria belonging the genus *Lactobacillus* (Taboada et al., 1987; Rubio et al., 1993). Yeasts and lactic acid bacteria coexist in a symbiotic association and are responsible for lactic-alcoholic fermentation (Dimitrellou et al., 2007).

The kefir beverage has been consumed and being considerably probiotic (Garrote et al., 1998; Golowczyc et al., 2008). Various health benefits are related

to the regular consumption of viable probiotic microorganisms and these include the improvement of lactose tolerance, the reduction of cholesterol levels and the control of intestinal infections (Theunissen et al., 2004).

Studies reported the chemical and microbiological composition of kefir grains obtained from La Plata in the Argentina (Garrote et al., 2001). These studies, the biochemical tests and whole-cell protein pattern profiles were employed to characterize and identify the microorganisms present only in milk kefir grains. However, as these methodologies are tedious, expensive and time-consuming the application of other technique should be considered (Bosch et al., 2006). Molecular techniques offer new opportunities for determining and analyzing the structure and species composition of microbiological communities (Garbes et al., 2004). The literature describing the application of PCR-DGGE in microbiology is extremely wide, this fingerprinting technique is in fact, very versatile and has been successfully used in many fields of microbial ecology (Muyzer et al., 1993, 1998). PCR-DGGE is usually employed to assess the structure of microbial communities in food samples or other environments without cultivation, and to determine the community dynamics in response to environmental variations (Ercolini et al., 2004).

The aim of this study was to isolate and identify the microbial species present in water kefir grains from different States of Brazil, using culture dependent and independent methods.

4 MATERIAL AND METHODS

4.1 Kefir grains sampling

Water kefir grains were obtained from families that traditionally consumed the beverage from São Paulo (Santos), Goiás (Caiaponia), Minas Gerais (Governador Valadares), Alagoas (Maceió), Rio de Janeiro (Rio de Janeiro), Distrito Federal (Brasília), Espírito Santo (Aracruz) and Bahia (Vitória da Conquista). The samples of the kefir grains were sent in plastic bags, involved in brown sugar. A sample was aseptically removed for PCR-DGGE analysis.

4.2 Isolation, purification and maintenance of microorganisms

Each 1g sample of the kefir grain was collected and added to a sterilized tube containing 9 mL of sterile peptone water diluent (0.1% peptone, 0.5% NaCl) and homogenized for 60s. A volume of 0.1 mL of appropriate dilutions was spread plated in duplicate on the following medium for isolation and enumeration of bacteria and yeast. For some samples the direct plating of grains was necessary. Decimal dilutions of the suspended samples were used for microbial enumerations and isolation of bacteria by MRS medium (De Man Rogosa Sharpe, Merck) (Silva et al., 2008) with the addition of 4 mL/L of nystatin (Sigma, St. Louis, USA) to inhibit the growth of yeasts. Mesophilic and psychotropic aerobic bacteria on plates were incubated aerobically at 28°C and 35°C for 48 hours. Yeasts were isolated on YW agar medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, 2% agar), with the addition of 100 mg/L of chloramphenicol (Sigma, St. Louis, USA) to inhibit the bacterial growth. The YW plates were incubated at 28°C for 120 hours. On plates displaying 30 – 300 colonies, a number of isolates corresponding to the square root of each morphological yeast and bacterial type observed on YW and

MRS plates were selected and recultivated in specific medium for further purification. The purified isolates were freezer stored at -80°C in YW (for yeast) and MRS (for bacteria) containing 20% (v/v) of glycerol.

4.3 Identification by conventional and molecular methods

All the yeast isolates were characterized based on their morphology, spore formation, assimilation and fermentation of different carbon sources, according to Barnett et al. (2000). The yeast isolates were identified using the internal transcribed spacer (ITS) ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White et al., 1990).

Gram-positive bacteria were subdivided into spore-formers and non-spore-formers by inducing spore liberation (80°C for 10 min.). Subsequent identification used was motility, catalase reaction and biochemical tests as recommended in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and The Prokaryotes (Hammes et al., 1991). The genus *Lactobacillus* was identified by physiological characteristics – heterofermentative, homofermentative or obligately heterofermentative, by their ability to produce gas (CO₂). The isolated strains were confirmed by 16S rDNA sequencing. A fragment of approximately 1500 bp of the 16S rDNA was amplified by forward primer 27f (5'AGAGTTTGATCCTGGCTCAG 3') and reverse primer 1512r (5' ACGGCTACCTTGTACGACT 3') (Devereux et al., 2004).

The sequencing of portions of the ITS and 16S region were accomplished and the sequence identities were determined by BLAST program in the GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>).

4.4 PCR-DGGE analysis

One kefir grain of each sample was placed into sterilized water and vortexed. One milliliter of each sample was transferred into a plastic tube and

was subjected to DNA extraction using a NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). The DNA extraction was done according to the manufacturer's instructions. The genomic DNA was resuspended in sterilized water and stored at -20°C.

The primers set, 518r (5' ATTACCGCGGCTGCTGG 3') and 338f (5' CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG 3'), were used for the amplification of the V3 region of the partial ribosomal 16S DNA (Cocolin et al., 2001) and the primers YM951r (5'TTGGCAAATGCTTTCGC3') and NS3 (5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGGCAA GTCTGGTGCCAGCAGCC3'), used for partial ribosomal 18S DNA amplification for yeast (Haruta et al., 2006). PCR was performed in a total reaction volume of 50 µL containing 0.2µM of each primer, 0.5U of Go *Taq* Flexi DNA polymerase (Promega®), 5µL of 10X PCR reaction buffer with 20mM MgCl₂, 0.125 mM dNTPs mix and 40ng of the DNA template. Amplification was achieved in 0.2 mL tubes by using a model Thermo PCYL220 thermal cycler (Thermo Fisher Scientific Inc., Waltham, USA) with the following parameters for bacteria: initial denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 30s, annealing at 54°C for 60s, extension at 72°C for 1min and final extension at 72°C for 7 minutes. For yeasts: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1min and final extension at 72°C for 7 min.

The fragments of amplified PCR were separated using DGGE (BioRad Universal Dcode Mutation Detection System). PCR samples were directly applied into a gel with 8% of polyacrylamide in 0.5 x TAE with gradient between 15 and 55% for the bacteria and 12 and 50% for yeast. The gradient was created by polyacrylamide, containing 0 – 100% denaturant (7M urea and

40% of formamide). Electrophoresis was performed at a constant voltage of 70 V for 6h for the bacterial fragments and 200 V for 4h for the yeast fragments and a constant temperature of 60°C. After electrophoresis, they were stained for 30 minutes in a SYBR Green solution and analyzed under UV illumination and the gel images were photographed.

Different DGGE bands were excised from the acrylamide gels. The DNA fragments were purified using QIAEX II gel extraction kit (Qiagen, Chatsworth, CA, USA) and then re-amplified by the primer 518r for bacteria and YM951r for yeast and submitted to sequencing Central Laboratory of Molecular Biology of UFLA, LCBM/UFLA (Lavras MG). The sequence identities were determined by nucleotide BLAST database from GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>). The reproducibility of DGGE fingerprints was confirmed by repeating the PCR amplification reactions on the DNA isolated from the different kefir grains several times, as well as on DNA isolated from the different grains in the same sample several times.

5 RESULTS AND DISCUSSION

5.1 Microbial enumeration and identification of species associated with water kefir grains

Only a small number of microbiological studies have been performed to examine the microbiota present in water kefir grains. These grains are very similar to the milk kefir grains. Both are also composed of mixed microbial association used as started culture for the preparation of fermented milk products (Ulloa et al., 1994).

The eight different kefir grains showed similar macroscopic characteristics. Kefir grains from São Paulo, Goiás, Rio de Janeiro, Distrito Federal and Espírito Santo presented a distribution of size between 6mm to 9mm, while Minas Gerais, Alagoas and Bahia grains presented size ≤ 5 mm. The milk kefir grains have larger sizes, measuring between 3 to 30mm (La Rivière et al., 1967, Garrote et al., 2001).

The enumeration values (CFU/g) of the isolated viable bacteria and yeast are given in TABLE 1. The yeast counts varied between the minimum value of $5.92 \log_{10}$ CFU/g in the sample from Minas Gerais and the maximum $8.30 \log_{10}$ CFU/g in the sample from Rio de Janeiro. In MRS the average number of CFU/g varied between $6.04 \log_{10}$ at 28°C and $6.36 \log_{10}$ CFU/g at 35°C in the samples from Alagoas and São Paulo respectively and a maximum $9.18 \log_{10}$ CFU/g at 28°C and $9.08 \log_{10}$ CFU/g at 35°C in the sample from Bahia.

A total of 286 isolates were obtained from samples. Among the isolates examined, 201 were found to be bacteria and 85 were yeasts. These bacteria isolated were identified by means of phenotypic tests and PCR based methods and 16S rDNA gene sequencing. Primary differentiation and grouping of the strains from the same genus was performed using biochemical methods. For

separation of species within the same genus sequencing of partial 16S rDNA fragments for bacteria and ITS region for yeasts was used. The bacteria and yeast identified in the kefir grains from eight different Brazilian states are shown in TABLE 2.

The 9 yeast species identified in the water kefir grains in this work by conventional technique were *Saccharomycete* sp., *Pichia cecembensis*, *Yarrowia lipolytica*, *Saccharomyces cerevisiae*, *Pichia membranifaciens*, *Pichia caribbica*, *Pichia fermentans*, *Candida valdiviana* and *Zygosaccharomyces fermentati* (TABLE 2). The species *C. valida*, *P. membranifaciens* and *S. cerevisiae* were also identified in water kefir grains from different locals of Mexico (Ulloa et al., 1994). *S. cerevisiae*, *P. fermentans* and *Yarrowia lipolytica* are species that were described as microorganisms present in the milk kefir grains (Jianzhong et al., 2009; Lopitz-otsoa et al., 2006), whereas *Pichia cecembensis*, *Pichia caribbica* and *Zygosaccharomyces fermentati* have not been reported as microorganisms present in kefir grains.

A new species of the genus *Pichia*, proposed name *Pichia cecembensis* sp. nov., was isolated in papaya fruits (Bhadra et al., 2007). This species was identified from kefir grain sample from São Paulo. The other species found in kefir grain sample from Distrito Federal *Pichia caribbica* has been described as the teleomorphic state of *Candida fermentati* and *Candida guilliermondii* based on molecular data (Vaughan-Martini et al., 2005). The *Zygosaccharomyces fermentati*, identified in Kefir grain from Bahia, were also found in grape musts and orange juice samples obtained in Surat Thani Province (Sukkasem et al., 2007; Romano & Suzzi, 1993).

The 9 bacterial species identified in the kefir grain samples from different Brazilian states were *Lactobacillus casei*, *Lactobacillus sunkii*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei*, *Lactobacillus satsumensis*, *Lactobacillus kefir*, *Bacillus cereus*, *Acetobacter lovaniensis*, *Gluconacetobacter liquefaciens*

(TABLE 2). These species have not been previously reported as members of the water kefir grain population (Rubio et al., 1993). The species belonging *Bacillus* genus such as *B. brevis*, *B. polymyxa*, *B. circulans*, *B. coagulans*, *B. firmus*, *B. macerans* and *B. pumilus* were identified in water kefir grains derived from Mexico (Rubio et al., 1993). The specie *Bacillus cereus* is a saprophytic bacteria commonly found in soil and its presence in dairy products suggest the need of hygienic-sanitary improvements in the whole fluxogram of milk processing (Vidal-Martins et al., 2005). The presence of *Bacillus cereus* in the water Kefir sample from Bahia may be due to contamination at some stage in the preparation or submission of grain. The production of fermented foods under controlled conditions and its safety assurance depend on the knowledge and control of their microbiota. Traditional fermented foods are obtained by natural fermentations in which no inoculum is added and contain microbial complexes. These results show the importance of the identification of the microorganisms present in the grain in the preparation of kefir beverages for human consumption.

Lactobacilli were present in all grain samples in this study, indicating the importance of this group for the production of the beverage (TABLE 2). Previous studies showed that the *Lactobacillus sunkii*, *Lactobacillus rhamnosus*, *Lactobacillus kefir*, *Lactobacillus kefiri*, *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus helveticus* species also have been isolated and identified in the milk kefir samples from around the world (Garrote et al., 2001; Chen et al., 2008; Irigoyen et al., 2005; Narvhus et al., 2003). *Lactobacillus satsumensis*, which was isolated from grains obtained from Bahia and Goiás, was also isolated from shochu mashes a traditional Japanese distilled spirit made from fermented rice, sweet potato, barley and other starchy materials (Endo & Okada, 2005). Fermented mashes contains 15–18% alcohol and are kept at acidic pH (3.0 – 4.0) as a result of the production of citric acid by the mould during fermentation. *Lactobacillus satsumensis* clustered in the *Lactobacillus*

casei – *Pediococcus* group and was closely related to *Lactobacillus nagelii* and *Lactobacillus mali* on the basis of 16S rDNA gene sequence similarity. The isolate was considered to represent a novel species, for which the name *Lactobacillus satsumensis* was proposed (Endo & Okada, 2005).

The acetic acid species *Acetobacter lovaniensis* isolated from the grain from Bahia, and *Gluconacetobacter liquefaciens* isolated from the grain from Bahia and Goiás although present in other fermented beverages, fruits, flowers, honey bees, sugar cane juices, soil, and water, their presence in water kefir was first reported in this study. The species *Acetobacter pasteurianus* consisted of 5 subspecies, the subspecies *Acetobacter pasteurianus* subsp. *lovaniensis* being linked to the fruit and fermented food of Indonesia and Philippines (Lisdiyanti et al., 2000). Yamada et al. (1999) and Seearunruangchai et al. (2003) also demonstrate the presence of *Gluconacetobacter liquefaciencens* linked to fermented food.

5.2 Identification by culture independent methods

Partial ribosomal 18S RNA of yeast were amplified and the resulting PCR products were analyzed by DGGE (FIGURE 1). The fingerprints of the yeast community showed that band **1** appeared in all of the samples. Band **2** appeared in all of the samples with the exception of grains from Distrito Federal. Band **4** appeared in the grain from Distrito Federal. Bands **7** and **8** were found in the grain from Alagoas and bands **13** and **14** appeared in the grain from Bahia. Band **12** appeared in the grain from São Paulo. In the Espírito Santo sample no colonies were observed when the culture dependent method was used, however in cultured independent methods this sample presented two bands. This can be explained by the fact that these yeasts are not cultivable or fastidious microorganisms, or are in low numbers or unviable to grow in plates.

The bands were excised from the acrylamide gel, re-amplified and the eluted DNA fragments were used for sequencing with the primer YM951r.

PCR products generated by amplifying V3 region of 16S rDNA gene using primer 338f (GC) and 518r from water kefir samples were analysed by DGGE. The DGGE pattern is shown in FIGURE 2. Individual bands observed in the DGGE profiles were excised from acrylamide gel, re-amplified from sequencing. This sequencing exhibited higher than 97% identity with sequences in the GenBank databases except band **c**, whose similarity was 93%. The bands **a** and **c** were identified as *Lactobacillus helveticus*, **b** as *Lactobacillus sunkii*, **d** and **f** *Lactobacillus kefiranofaciens*, **e** as not identified, **g** as *Lactobacillus parakefiri*, **h** and **i** uncultured bacterium. The *Lactobacillus helveticus* (bands **a** and **c**) and *Lactobacillus kefiranofaciens* (bands **d** and **f**) were found in bands at different positions. These multiple banding patterns may be due to sequence microheterogeneity between multiple copies of the 16S rDNA gene of this strain (Nübel et al., 1999). A single species with multiple rDNA copies can overestimate a community diversity detected by DGGE because the technique could have favored the extraction of this specie.

The *Lactobacillus helveticus* was identified in the sample from Espirito Santo by the culture dependent method, but the analysis of DGGE bands was not observed in the same positions (bands **a** and **c**) in São Paulo and Goiás samples. This apparent contradiction between culture dependent and independent methods could be explained by the difficulty to obtain good DNA extraction and PCR reaction of the grain samples from Espirito Santo. Initial template DNA ratio and template competition may affect the detection of microorganisms present in low abundance in microbial complexes (Muyzer et al., 1993; Murray et al., 1996; Zhang et al., 2005). Also, it is interesting to note that *Lac. helveticus* was not identified by the culture dependent technique in the samples from São Paulo, Goiás and Rio de Janeiro, but was identified in samples analyzed by DGGE. The

possible explanation might be that this microorganism was inviable to grow in the plates of these samples. DGGE analysis does not provide information on the viability of the microorganisms (Rantsiou et al., 2005). The bands **d** and **f** correspond to *Lactobacillus kefiranofaciens* that appeared in samples from São Paulo, Rio de Janeiro and Goiás in DGGE gel. These multiple banding patterns may be also due to sequence heterogeneity between multiple copies of the 16S rDNA of these strains (Nübel et al., 1999). This bacteria was not isolated in plates of the grains sampled, probably for being inviable. In the Rio de Janeiro samples it was not possible to isolated bacteria in plates without diluting the sample. In DGGE analysis of Rio de Janeiro samples bands were observed being identified as *Lactobacillus kefiranofaciens* and *Lactobacillus parakefiri*. In the São Paulo water kefir sample *Lactobacillus kefiranofaciens* and *Lactobacillus helveticus* were identified. These species also were isolated from Taiwanese milk kefir grains (Chen et al., 2008).

Bands **g** which corresponds to *Lactobacillus parakefiri* appeared in all of the samples with the exception of Bahia grains in DGGE gel. This specie was not isolated by culture dependent methods, which may be the result of the fact that this bacteria was damaged in transit or dead. Previous studies showed that *Lactobacillus parakefiri* specie has been reported a member of several milk kefir grains (Garbes et al., 2004; Witthuhn et al., 2004; Garrote et al., 2001).

The *Lactobacillus sunkii* bacterium identified from the São Paulo sample was unique, found by both independent and culture dependent methods. *Lactobacillus sunkii*, *Lactobacillus parakefiri* and bacterium non identified were identified in water kefir grains from Distrito Federal and *Lac. helveticus*, *Lac. kefiranofaciens*, *Lac. parakefiri*, and uncultured bacterium in samples from Goiás by culture independent techniques. These species were not isolated in culture dependent methods.

The specie *Lactobacillus casei*, *Lactobacillus kefir*, *Lactobacillus rhamnosus*, *Lactobacillus satsumensis*, *Gluconacetobacter liquefaciens*, *Acetobacter lovaniensis* and *Bacillus cereus* were identified only by culture dependent methods, not being identified by culture independent methods. This may be due to biases inherent in the PCR technique, which reinforces the idea that PCR based methods cannot account for all organisms from a given sample and a polyphasic approach is needed when a more comprehensive assessment of microbial diversity is sought.

6 CONCLUSIONS

The results obtained in this study show that a combined method of cultivation with PCR-DGGE and subsequent DNA sequencing can identify different microorganisms from water kefir grains from eight Brazilian States.

The *Bacillus cereus*, *Acetobacter lovaniensis*, *Gluconacetobacter liquefaciens* bacterial species and the *Pichia cecembensis*, *Pichia caribbica* and *Zygosaccharomyces fermentati* yeasts have been reported in water kefir grains for the first time, in this work.

The results of this study revealed the presence of some bacteria, such as *Lactobacillus casei*, *Lactobacillus kefir*, *Lactobacillus rhamnosus*, *Lactobacillus satsumensis*, *Gluconacetobacter liquefaciens*, *Acetobacter lovaniensis* and *Bacillus cereus*, only when using 16S rDNA sequencing of the culture in plates.

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TABLE 1 Microbial population of water grain kefir from different origins (log₁₀ cfu/ml)

Grains Origin	Aerobica 28°C	Aerobica 35°C	Yeast
AL	6.04	7.87	7.81
BA	9.18	9.08	6.49
DF	7.65	7.67	7.61
ES	7.23	7.37	NC
GO	8.43	8.28	7.82
MG	7.08	6.98	5.92
RJ	ND	ND	8.30
SP	7.57	6.36	8.04

ND - not determined; MRS - medium for bacterial growth; YW - medium for yeast growth
 SP - São Paulo; MG - Minas Gerais; AL - Alagoas; RJ - Rio de Janeiro; DF - Distrito Federal;
 GO - Goiás; ES - Espírito Santo

TABLE 2 Species of bacteria and yeast present in the water kefir grains by culture dependent methods

Samples		Isolates Identified	CFU/g
AL	bacteria	<i>Lactobacillus rhamnosus</i>	1.6 x 10 ⁶
		<i>Lactobacillus kefir</i>	8 x 10 ⁵
	yeast	<i>Saccharomyces cerevisiae</i>	1 x 10 ⁶
BA	bacteria	<i>Lactobacillus satsumensis</i>	6 x 10 ⁶
		<i>Lactobacillus casei</i>	6 x 10 ⁶
		<i>Gluconobacter liquefaciens</i>	1 x 10 ⁶
		<i>Acetobacter lovaniensis</i>	1 x 10 ⁷
		<i>Bacillus cereus</i>	6 x 10 ⁶
	yeast	<i>Saccharomyces cerevisiae</i>	3 x 10 ⁴
		<i>Zygosaccharomyces fermentati</i>	5 x 10 ⁴
DF	bacteria	<i>Lactobacillus satsumensis</i>	1.5 x 10 ⁶
		<i>Lactobacillus casei</i>	1.5 x 10 ⁶
	yeast	<i>Saccharomyces cerevisiae</i>	5 x 10 ⁵

...Continua...

TABLE 2 Cont.

		<i>Pichia caribbica</i>	2×10^5
		<i>Pichia fermentans</i>	3×10^5
ES	bacteria	<i>Lactobacillus helveticus</i>	1.5×10^6
		<i>Lactobacillus kefir</i>	1.7×10^6
	yeast	ND	
GO	bacteria	<i>Lactobacillus satsumensis</i>	7×10^6
		<i>Lactobacillus paracasei</i>	1×10^7
		<i>Gluconobacter liquefaciens</i>	1.1×10^7
yeast	<i>Pichia membranifaciens</i>	2×10^5	
	<i>Saccharomyces cerevisiae</i>	1×10^6	
MG	bacteria	<i>Lactobacillus kefiri</i>	2×10^5
		<i>Lactobacillus paracasei</i>	7×10^4
	yeast	<i>Yarrowia lipolytica</i>	1.2×10^4
RJ	bacteria	ND	
	yeast	<i>Pichia membranifaciens</i>	8×10^5
		<i>Candida valdiviana</i>	8×10^5
SP	bacteria	<i>Lactobacillus casei</i>	1.3×10^6
		<i>Lactobacillus sunkii</i>	1.7×10^6
yeast	<i>Saccharomycetes</i>	2×10^5	
	<i>Pichia cecembensis</i>	6×10^5	
	<i>Yarrowia lipolytica</i>	4×10^5	

ND not detected; CFU/g – microbial species counts; AL - Alagoas, BA - Bahia, DF - Distrito Federal, ES - Espirito Santo, GO - Goais, MG - Minas Gerais, RJ - Rio de Janeiro, SP - São Paulo

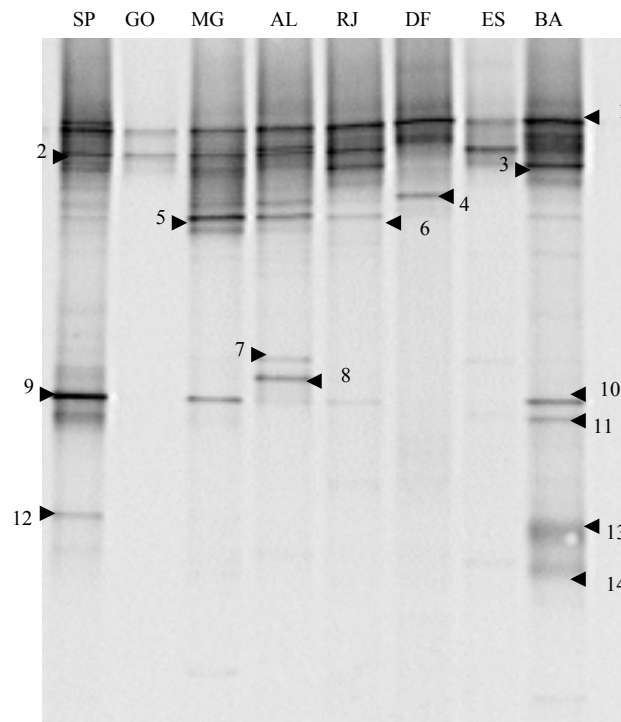


FIGURE 1 DGGE profile of 8 kefir grains yeast samples with denaturing gradient from 12% to 50%. Lane 1 – São Paulo; 2 – Goiás; 3 - Minas Gerais; 4 – Alagoas; 5 – Rio de Janeiro; 6 – Distrito Federal; 7 – Espíritos Santo and 8 – Bahia.

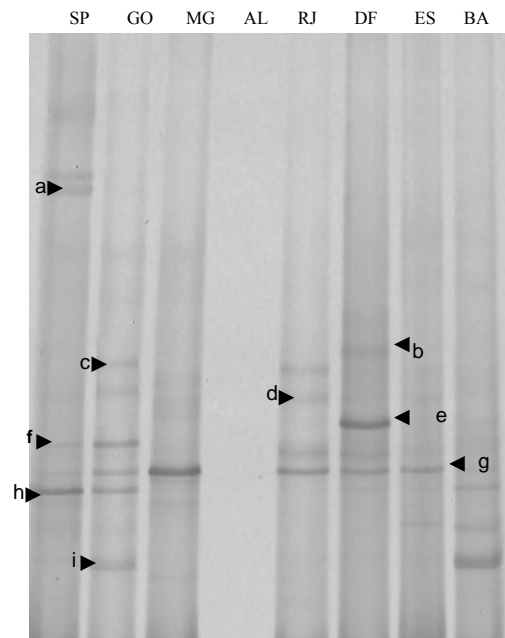


FIGURE 2 DGGE profile of bacteria 8 samples kefir grains with denaturing gradient from 15% to 55%. a – *Lactobacillus helveticus* FJ 861108.1; b – *Lactobacillus sunkii* AB 366385.1; c – *Lactobacillus helveticus* FJ 861108.1; d – *Lactobacillus kefiranofaciens* FJ 845004.1; e – non identified; f - *Lactobacillus kefiranofaciens* FJ 845004.1; g – *Lactobacillus parakefiri* AB 429373.1; h - uncultured bacterium EF 014703.1 and i – uncultured bacterium AM 921620.1.

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