KARINA TEIXEIRA MAGALHÃES

PRODUÇÃO DE BEBIDAS FERMENTADAS KEFIR DE SORO DE QUEIJO

LAVRAS – MG

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Tese apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Biotecnologia de Microrganismos, para a obtenção do título de Doutor.

Orientadora
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Co-Orientadores
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Ficha Catalográfica Preparada pela Divisão de Processos Técnicos da Biblioteca Central da UFLA

Magalhães, Karina Teixeira.
Produção de bebidas fermentadas kefir de soro de queijo / Karina Teixeira Magalhães. – Lavras : UFLA, 2010.
133 p. : il.

Tese (Doutorado) – Universidade Federal de Lavras, 2010.
Orientador: Rosane Freitas Schwan.
Bibliografia.

1. Bebida fermentada não alcoólica. 2. Análise microbiológica. 3. Análise química. 4. Bactéria ácido lática. 5. Leveduras. I. Universidade Federal de Lavras. II. Título.

CDD – 663.9

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APROVADA em 21 de Dezembro de 2010

Dra. Lucília Domingues	UMINHO/Portugal
Dr. José António Teixeira	UMINHO/Portugal
Dr. João Batista de Almeida Silva	USP
Dr. Disney Ribeiro Dias	UFLA

Dra. Rosane Freitas Schwan UFLA
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À minha mãe, Gilda,
pelas orações e conselhos.
Ao meu pai, Gumercindo, pela criação.
À minha irmã Kassiana, familiares e amigos, pelo carinho.

DEDICO

Aos orientadores, co-orientadores e professores,
pelos valiosos conhecimentos transmitidos.

OFEREÇO

AGRADECIMENTOS

O trabalho que apresento para apreciação na minha defesa de doutorado foi realizado ao longo de três intensos anos de trabalho, em continuação do trabalho apresentado para a defesa de mestrado em Microbiologia Agrícola. O meu fascínio em conhecer o mundo dos "pequenos organismos" contribuíu para que esta tarefa fosse feita com grande satisfação!

Um doutorado é feito em um longo período de tempo, envolvendo por isso, diferentes cincunstâncias, locais e pessoas que podem contribuir de muitas formas para a sua execução. Estando ciente do apoio, amizade e carinho que algumas pessoas me dispensaram ao longo deste percurso, proporcionando-me um bem-estar imprescindível para a execução desta tarefa, desde já expresso a todos os meus sinceros agradecimentos.

A DEUS, pela vida e pela força para enfrentar todas as dificuldades. Agradeço as inúmeras pessoas que colocastes em meu caminho e que contribuíram imensamente com minha caminhada; agradeço por me dar sabedoria para identificá-las e humildade para receber sua ajuda.

À Universidade Federal de Lavras (UFLA) e ao Programa de pós-graduação em Microbiologia Agrícola do Departamento de Biologia, pela oportunidade concedida para realização do doutorado.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes) e Capes-Grices, pelo apoio financeiro e por me proporcionar tão valiosa experiência do doutorado sanduiche.

À Cooperativa Agrícola Alto Rio Grande Ltda (CAARG), em Lavras, MG, pela grande colaboração na realização do projeto. A Quinta dos Ingleses e Lactogal, em Portugal, pela doação do soro de queijo para execução deste trabalho.

À professora Dra. Rosane Freitas Schwan, pela orientação deste trabalho e por ter confiado em mim e por ter me proporcionado tão valiosas experiências, que levarei por toda a vida. Quero também fazer um agradecimento especial à sua qualidade de ser muito humana. Agradeço pela paciência e dedicação na orientação, pela amizade, pela responsabilidade em transmitir conhecimentos e pelo exemplo de conduta como pesquisadora e professora. Agradeço, principalmente, pela sua presença em minha vida durante o período de mestrado e doutorado. E assim espero que permaneça por todo o meu caminho que seguirei a partir deste momento.

Ao professor Dr. Romildo da Silva, pelos ensinamentos transmitidos, atenção, brincadeiras e, em especial, pela sua alegria.

Ao professor Dr. Eustáquio Souza Dias, pelos ensinamentos transmitidos, pela atenção e dedicação como educador.

À professora Dra. Cristina Ferreira Silva Batista e a Dra. Carla Luiza da Silva Ávila pela amizade e ensinamentos.

Ao professor Dr. Disney Ribeiro Dias, pelos ensinamentos transmitidos, pela atenção, amizade e imensa ajuda na elaboração de artigos.

Ao professor Dr. João Batista de Almeida Silva, que foi contribuidor com a minha grande experiência adquirida no doutorado sanduiche em Portugal.

Ao meu co-orientador, Dr. José António Teixeira por ter me recebido com muito carinho e atenção na Universidade do Minho, para o doutorado sanduiche e por ter me proporcionado tão grandioso aprendizado durante os dez meses que permaneci em seu laboratório.

À minha co-orientadora, Dra. Lucília Domingues, pela sua dedicação e exemplo de docente e pesquisadora, além de seu exemplo de responsabilidade, inteligência e determinação, que foram fundamentais para o desenvolvimento deste trabalho em Portugal.

Ao Dr. José Maria Oliveira, pela imensa ajuda nas análises cromatográficas.

À prof. Dra. Maria das Graças Cardoso e a Dra. Simone Cristina Marques pela participação e colaboração em minha banca de qualificação.

À Engenheira Madalena e a Ana Nicolau, que foram indispensáveis na execução de algumas etapas deste trabalho em Portugal.

Aos pós-doutorandos Dra. Maria Alcina Pereira e Dr. Giuliano Dragone, que participaram diretamente na concretização deste trabalho em todas as etapas.

Aos amigos que conquistei em Portugal, e que foram fundamentais em minha vida durante os dez meses de estadia nesse País. E espero que estes laços sejam eternos, pois para mim, foram a família que tive durante esse período.

Ao amigo Antônio Claret Sales, pela grande ajuda, amizade e incentivo no início desta jornada.

A todos os funcionários do Departamento de Biologia da UFLA, pela amizade. E a Rose pela amizade, dedicação e ajuda sempre.

Ao Gilberto Vinícius de Melo Pereira, agradeço em especial pela imensa ajuda que me proporcionou quando mais precisei. Agradeço pela amizade e carinho sempre. E apesar de as vezes discutirmos, saiba que considero muito sua amizade. Aliás, amigos verdadeiros são para dizer o que precisamos ouvir e não o que queremos ouvir. Sei o quanto foi e é valiosa sua amizade, e espero que ela não se acabe com o término desta estapa em minha vida. Pois grandes amizades aumentam com o tempo, mesmo à distância.

À Mariana, pela amizade e pelo companheirismos tanto no laboratório, quanto nas festinhas. Tenho saudades amiga, das nossas festas.

À Cíntia e ao Euziclei, que sempre estiveram comigo desde o mestrado, e sempre trabalhamos juntos.

A todos os meus amigos, colegas e companheiros de laboratório e mestrado e doutorado. Não citarei nomes, pois não quero cometer o erro de

esquecer alguém. Assim, agradeço a todos, e saibam que cada um tem um lugarzinho em meu coração.

Aos meus amigos Marcele e Assis, pelo carinho.

Aos amigos Sr. José, D. Marta, D. Efigênia, D. Cidinha, Sr. Hélio, D. Olga e Xico pela imensa ajuda. Saibam que são muito importantes para mim.

Aos meus primos Luciane, Denise, Josiane, Janine, Adriano, Lucas Kemps e Júnior, que fizeram parte dessa jornada. Obrigada pela amizade e companheirismo nas horas de lazer.

Em especial, agradeço a meu primo Danton, que participou diretamente desta etapa da minha vida e esteve presente nas horas mais difíceis. Agradeço a atenção e carinho, que foram fundamentais.

Aos familiares, em especial a Malena a Nei e a Tia Nenê, pelas orações e incentivo.

Ao inimigo. Agradeço por ter-me auxiliado a crescer; por me mostrar a cada perseguição e a cada puxão de tapete o valor da fé e da oração. Aprendi a rezar por você, a pedir por seus defeitos e a solicitar da Divindade maior compreensão e tolerância. Você me ensinou a rezar por mim e por você e me aproximou mais de Deus. Agradeço-lhe, meu inimigo, por me ensinar, nos momentos de descrença, o valor das amizades genuínas. Meu inimigo agradeço-lhe por aclarar todos os pontos falhos que tenho, cada defeito, cada erro, cada tropeço dos muitos que cometi, dos pequenos aos patéticos. Cada risada sarcástica que você dava me empurrava para o trabalho incessante em cada ponto que eu poderia melhorar. Você me ensinou o valor da humildade. Agradeço-lhe, meu inimigo, por me ensinar o valor da coragem. Por fim, meu inimigo, agradeço-lhe por ressaltar meus valores publicamente e pelas inúmeras horas gastas no seu dia a dia com minha pessoa. Sou muito feliz por saber que com sua ajuda cheguei até aqui e continuarei no meu caminho.

Finalmente, agradeço aos meus pais, Gumercindo e Gilda e à minha irmã Kassiana, que sempre acreditaram e apoiaram minhas decisões e estiveram presentes em todos os momentos difíceis desta jornada. E ao Puff, pelo carinho.

"Há homens que lutam um dia e são bons; Há outros que lutam um ano e são melhores; Há os que lutam muitos anos e são muito bons; Porém, há os que lutam toda a vida; Esses são os imprescindíveis."

Bertolt Brecht

RESUMO

Preocupações com a valorização do soro de queijo conduziram a um recente interesse na produção de bebidas kefir de soro de queijo. Grãos de kefir oriundos do Brasil (12.5 g) foram inoculados em 250 mL de soro de queijo (CW), soro de queijo desproteinizado (DCW) e leite. Erlenmeyers contendo os grãos de kefir foram estaticamente incubados por 48 h e 72 h a 25 °C. Amostras das bebidas e grãos foram avaliadas. Neste estudo, a comunidade microbiana presente em grãos de kefir e correspondentes bebidas de CW, DCW e leite, foi investigada por eletroforese em gel de gradiente desnaturante (DGGE), seguido de clonagem e sequenciamento. Sucessões microbianas amplificadas se afiliaram para *Kluyveromyces marxianus, Saccharomyces cerevisiae, Kazachatania unispora, Lactobacillus kefiranofaciens* subsp. *Kefirgranum, Lactobacillus kefiranofaciens* subsp. *Kefiranofaciens* e uma bactéria não cultivável também relatada para o gênero *Lactobacillus.* A coloração por fluorescência combinada a microscópio confocal a laser (CSLM) mostrou a distribuição de leveduras em macro-agrupamentos entre a matriz do grão de kefir essencialmente composto de polissacarídeos e bactérias. Nenhuma diferença foi encontrada na estrutura da comunidade microbiana nas bebidas analisadas e grãos de kefir, exibindo uma microbiota altamente estável ao longo das fermentações em diferentes substratos. O consumo de lactose, produção de etanol, bem como a formação de ácidos orgânicos e combinações voláteis foram determinadas durante a fermentação de CW e DCW por grãos de kefir. Estes resultados foram comparados com valores obtidos na produção da tradicional bebida kefir de leite. Os resultados mostraram que os grãos de kefir puderam utilizar lactose de CW e DCW e produziram etanol, ácido láctico e ácido acético. Álcoóis superiores (2-metil-1-butanol, 3-metil-1-butanol, 1-hexanol, 2-metil-1-propanol, e 1-propanol), éster (acetato de etilo) e aldeído (acetaldeído) foram também produzidos em bebidas kefir de CW e DCW e bebidas kefir de leite. Todas as bebidas kefir mostraram boa aceitação por avaliação sensorial. Os grãos de kefir mostraram potencial para serem usados para o desenvolvimento de bebidas kefir de soro de queijo.

Palavras-chave: Soro de queijo. Bebidas kefir. PCR-DGGE. Clonagem. Análise química.

ABSTRACT

Cheese whey valorization concerns have led to a recent interest on the production of cheese whey kefir beverages. Brazilian kefir grains (12.5 g) were inoculated in 250 mL of cheese whey (CW) and deproteinised cheese whey (DCW) and milk. Erlenmeyers containing kefir grains were statically incubated for 48 h and 72 h at 25 °C. Samples of the beverage and grains were analyzed. In this study, the microbial community present in milk kefir, cheese whey and deproteinised cheese whey kefir and correspondent beverages, was investigated by Denaturing gradient gel electrophoresis (DGGE) followed for cloning and sequencing. Amplified microbial sequences were affiliated to *Kluyveromyces marxianus, Saccharomyces cerevisiae, Kazachatania unispora, Lactobacillus kefiranofaciens* subsp. *kefirgranum, Lactobacillus kefiranofaciens* subsp. *Kefiranofaciens* and an uncultured bacterium also related to the genus *Lactobacillus*. Fluorescence staining in combination with Confocal Laser Scanning Microscopy (CSLM) showed the distribution of yeasts in macro-clusters among the grain's matrix essentially composed of polysaccharides and bacteria. No differences were found in the community structure detected in the analyzed beverage and kefir grains, showing that microbiota of kefir grains is highly stable along the fermentations carried out in different substratum. Lactose consumption, ethanol production as well as organic acids and volatile compounds formation were determined during CW and DCW fermentation by kefir grains and compared with values obtained during the production of traditional milk kefir. The results showed that kefir grains were able to utilise lactose from CW and DCW and produce ethanol, lactic acid and acetic acid. Higher alcohols (2-methyl-1-butanol, 3-methyl-1-butanol, 1-hexanol, 2-methyl-1-propanol, and 1-propanol), ester (ethyl acetate) and aldehyde (acetaldehyde) in cheese whey-based kefir and milk kefir beverages were also produced. The kefir beverages showed good acceptance in the sensory analysis. The kefir grains showed potential to be used for developing cheese whey-based beverages.

Keywords: Cheese whey. Kefir beverages. PCR-DGGE. Cloning. Chemical analysis.

SUMÁRIO

PRIMEIRA PARTE - EMBASAMENTO BIBLIOGRÁFICO ABORDANDO OS PRINCIPAIS TEMAS ENVOLVIDOS NO TRABALHO: SORO DE QUEIJO E GRÃOS DE KEFIR

1 INTRODUÇÃO

O soro de queijo, produto secundário da indústria láctea, é reconhecido por apresentar importantes propriedades nutricionais e funcionais, devido ao teor de aminoácidos sulfurados presentes em suas proteínas, caracterizando-as como de alto valor biológico (ALMEIDA et al., 2001). Os teores de aminoácidos essenciais do soro estão de acordo com as exigências da Organização das Nações Unidas para a Alimentação e a Agricultura (FAO) e da Organização Mundial de Saúde (OMS) (MING, 2002). O uso adequado deste tipo de produto ajudaria a indústria a reduzir problemas relativos ao seu descarte (ALMEIDA et al., 2000; ALMEIDA et al., 2001; ZACARCHENCO; ACARCHENCO, 2004; MAGENIS et al., 2006; MAGALHÃES et al., 2010c).

A conversão do soro líquido em bebidas fermentadas, seria uma das mais atrativas opções devido à simplicidade do processo, a possibilidade de uso dos equipamentos já existentes na usina de beneficiamento de leite, além da composição físico-química apresentada. Atualmente, no Brasil, o processo tradicional de produção de bebidas lácteas fermentadas utiliza soro em pó, muitas vezes importado (ALMEIDA et al. 2000; THAMER; PENNA, 2005), enquanto os soros líquidos gerados pelas indústrias Brasileiras, em sua grande maioria, ainda são direcionados ao tratamento de efluentes e alimentação animal.

Mundialmente tem aumentado de maneira notável o consumo de bebidas lácteas fermentadas. No ano de 1995, foi observado aumento de 17 % no

consumo deste tipo de bebida no Brasil (IGLÉCIO, 1995; TAMINE; ROBISON, 2000).

O emprego de soro de queijo na produção de bebida láctea fermentada constitui uma forma racional do aproveitamento do soro em um produto inovador. A produção de bebida produzida por fermentação do soro de queijo através de grãos de kefir poderia ser uma alternativa interessante para utilização do soro. Os grãos de kefir constituem uma associação simbiôntica de leveduras e bactérias ácido láticas, usadas tradicionalmente para a produção de bebida láctea fermentada. Dentre as leveduras encontradas no kefir, representantes dos gêneros *Saccharomyces, Kluyveromyces* e *Kazachatania* foram observados. As bactérias foram do gênero *Lactobacillus* (MAGALHÃES et al., 2010c). La Riviére (1969) encontrou representantes do gênero *Acetobacter* em grãos de kefir.

A fermentação do soro por microrganismos dos grãos de kefir poderia diminuir o conteúdo de lactose no soro de queijo, enquanto produzia ácido, principalmente o lático e outros metabólitos contribuindo com as características organolépticas do produto final (MAGALHÃES et al., 2010c). A fabricação de bebidas por fermentações láticas pode prover perfis sensoriais desejáveis e já foi considerada uma opção para acrescentar valor organoléptico no soro de queijo (THAMER; PENNA, 2005).

Portanto, o objetivo do presente trabalho foi avaliar o uso dos grãos de kefir como cultura iniciadora para produção de bebidas de soro de queijo e soro de queijo desproteinizado, observando o comportamento microbiano durante os processos de fermentação, além de avaliar as mudanças bioquímicas, produção de ácidos orgânicos, formação de combinações voláteis e avaliação sensorial.

2 REVISÃO BIBLIOGRÁFICA

2.1 Produção de soro de queijo

Entre os principais efluentes do setor de laticínios encontra-se o soro de queijo, também denominado por soro de leite, que corresponde ao líquido obtido após precipitação da caseína do leite (ALMEIDA et al., 2001; MAGENIS et al., 2006). O soro de queijo é gerado em grandes quantidades visto que, para cada 10 litros de leite, um quilo de queijo é produzido e 9 litros de soro são obtidos como produto secundário. O soro de queijo constitui um grave problema ambiental devido à sua elevada carga ogânica e difícil biodegradabilidade (1000 litros/dia de soro equivalem ao poder poluente de 600 habitantes/dia). A DQO (demanda química de oxigênio) do soro chega a atingir 60 g/L, impossibilitando a sua incorporação em um qualquer processo tradicional de tratamento de efluentes (ALMEIDA et al., 2001).

Por cada litro de soro são desperdiçados cerca de 50 gramas de lactose e 10 gramas de proteína com elevado valor nutricional e funcional, criando condições para que se considere a valorização do soro com simultânea redução da carga poluente (ALMEIDA et al., 2001).

Com o avanço da tecnologia, a fabricação de queijo passou de um processo tradicional, onde pequenas quantidades de soro produzidas eram despejadas nos campos ou usadas como ração alimentar, para um processo industrial onde litros de soro são produzidos diariamente (ALMEIDA et al., 2000).

De acordo com a Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) a produção brasileira anual de queijo é superior a 495.000 toneladas, gerando cerca de 4,45 milhões de litros de soro de queijo (BRASIL, 2010b). Por outro lado, apesar desta grande produção, o Brasil ainda é um

grande importador de produtos de soro, como por exemplo, o soro em pó. Anualmente é importado em torno de 31,7 mil quilos destes produtos (BRASIL, 2010a).

2.1.1 Composição do soro de queijo

O soro é constituído por cerca de 85 a 90% do volume de leite usado na fabricação de queijo e retém cerca de 55% dos nutrientes do leite (KOSIKOWSKI, 1979; MAGALHÃES et al., 2010b). Entre estes encontram-se cerca de 20% das proteínas do leite, lactose, elementos minerais e vitaminas (ALMEIDA et al., 2000) (Tabela 1).

Tabela 1 Composição média do soro em vitaminas

Vitamina	**µg/g Soro de queijo**
B6	**4,0**
B12	**0,021**
Riboflavina	**23,4**
Niacina	**9,6**
Biotina	**0,37**
Ácido fólico	**0,9**
Ácido pantoténico	**47,3**
Ácido *p*-aminobenzóico	**10,0**

Fonte: (KOSIKOWSKI, 1979; ALMEIDA et al., 2001)

A composição do soro varia de acordo com a sua origem (cabra, vaca, ovelha) e técnica utilizada na fabricação de queijo. Distinguem-se dois principais tipos de soro (Tabela 2), o doce (pH 6-7) e ácido (pH < 5), de acordo com o procedimento utilizado na precipitação da caseína (ALMEIDA et al., 2001).

Tabela 2 Composição do soro de queijo (g/L)

	Soro doce	Soro ácido
Densidade*	**1,239**	**1,0245**
Sólidos	**70,84**	**65,76**
Lactose	**51,81**	**45,25**
Nitrogênio total	**1,448**	**1,223**
Ácido lático	**0,322**	**7,555**
Ácido cítrico	**1,298**	**0,260**
Cinza	**5,252**	**7,333**

• Os valores de densidade estão em Kg/L
Fonte: (KOSIKOWSKI, 1979; ALMEIDA et al., 2001)

Os soros ácidos apresentam maior teor em cinza e ácido lático, e menor teor proteico que os soros doces, sendo limitada a sua utilização na alimentação, precisamente pelo seu paladar acídico e elevado conteúdo salino (KOSIKOWSKI, 1979). Cálcio, fósforo, sódio e potássio constituem cerca de 60% do conteúdo de cinza do soro. A Tabela 3 descreve o conteúdo mineral dos soros ácido e doce.

Tabela 3 Conteúdo mineral dos soros ácido e doce

Componente[a]	Soro doce	Soro ácido
Ca[b]	**0,88**	**2,40**
P[b]	**1,10**	**1,59**
Na[b]	**1,29**	**1,09**
Mg[b]	**0,18**	**0,22**
K[b]	**1,86**	**1,92**
Zn	**2,10**	**81,0**
Cu	**2,80**	**5,30**
Fe	**9,00**	**13,0**
Pb	**1,15**	**1,68**
Hg	**0,02**	**0,03**
I	**6,80**	**8,60**
Cd	**0,11**	**0,14**
Se	**0,06**	**0,03**
As	**0,77**	**0,59**

[a] ppm, [b] percentagem
Fonte: (KOSIKOWSKI, 1979; MAGENIS et al., 2006)

2.1.2 Processamento e utilização do soro de queijo

Existem relatos da utilização de soro como agente nutritivo e terapêutico desde 460 a.C. (RAMAKRISHNAN, 1991). Na Idade Média, o soro era aplicado como droga farmacêutica em diversas situações, como componente curativo para queimaduras e revitalizador da pele e do cabelo, e as vezes utilizado como alimento para humanos (KOSIKOWSKI, 1979). Nos anos 40 na Europa Central doenças como dispepsia, uremia, artrite, gota, doenças de fígado, anemia e mesmo tuberculose eram tratadas com a ingestão diária de até 1500 g de soro por dia (HOLSINGER et al., 1974).

No entanto, as utilizações para o soro de queijo eram escassas perante a crescente produção de queijo. Tornou-se habitual o descarte do soro produzido para o rio e lago mais próximo. Perante às restrições ambientais, alternativas para a utilização do soro de queijo tais como alimentação animal e/ou como

fertilizante nos terrenos, foram sendo utilizadas (MAGENIS et al., 2006). O soro também era usado como agente terapêutico (revitalizador da pele e cabelo). O primeiro relato de produção industrial de um agente terapêutico do soro foi publicado pela Kraft-Phoenix Cheese em 1938 (RAMAKRISHNAN, 1991).

Para uso industrial do soro, a secagem e concentração térmica estão entre os primeiros métodos aplicados ao processamento industrial e utilização do soro. Estas metodologias proveriam em soro em pó, concentrado de proteína e lactose (Figura 1).

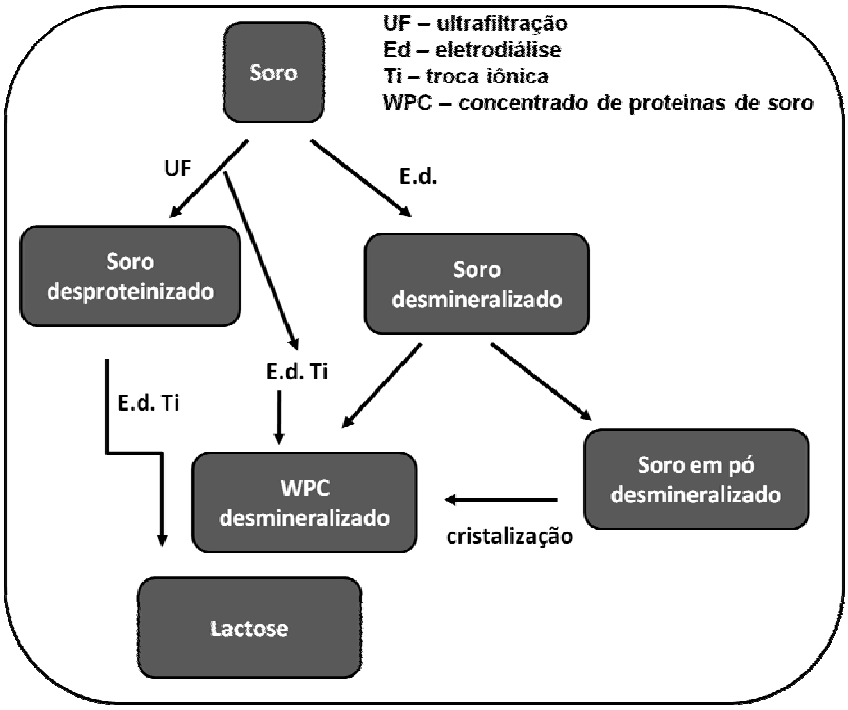


Figura 1 Processamento do soro para obtenção do produto em pó e/ou lactose
Fonte: (RAMAKRISHNAN, 1991; SIVIERI; OLIVEIRA, 2002)

Os produtos obtidos do soro de queijo podem ser divididos em aditivos alimentares (derivados diretamente do soro por simples tratamentos químicos ou físicos) e substratos de fermentação (para a produção de etanol, SCP (*single cell protein*), enzimas, polímeros, bebidas etc).

2.1.2.1 Utilização do soro de queijo como aditivo alimentar

O soro líquido em sua forma natural ou concentrado pode ser utilizado diretamente para alimentação animal ou usado como suplemento de rações. A produção de soro em pó consiste em três operações principais: evaporação, cristalização e secagem. As desvantagens de processar o soro em pó é devido principalmente à elevados custos de produção (SIVIERI; OLIVEIRA, 2002).

Para consumo humano, o soro é utilizado em dois setores da indústria alimentar: (1) laticínios e (2) padarias, sorveterias e/ou lanchonetes, dependendo do processo de tratamento. Por exemplo, nos sorvetes, o soro poderá substituir 25% do leite seco (RAMAKRISHNAN, 1991; SIVIERI; OLIVEIRA, 2002). Existem muitos processos disponíveis para concentração e fracionamento do soro, tais como evaporação, ultrafiltração, osmose inversa, troca iônica, filtração em gel, electrodiálise, etc (MAGENIS et al., 2006).

O concentrado de proteínas de soro (WPC) é o produto obtido em maior quantidade a partir do soro de queijo, apenas ultrapassado pela produção do soro em pó (HORTON, 1996; DOMINGUES et al., 2001). O valor comercial deste produto está diretamente relacionado com o seu teor protéico, podendo servir de aditivo na indústria de panificação (concentrados com 35% de proteína) ou de ingredientes na confecção de alimentos infantis (concentrados com 92% de proteína, denominados isolados de proteína de soro (WPI), (HUFFMAN, 1996), este último com um preço dez vezes superior ao primeiro. O concentrado protéico é obtido tipicamente por ultrafiltração, originando uma fração rica em

lactose, o permeato do soro de queijo. Os WPC e WPI podem apresentar diferentes propriedades funcionais consoante a sua composição em lactose, cinza, gordura e proteína (HUFFMAN, 1996; SIVIERI; OLIVEIRA, 2002). A obtenção do concentrato protéico, por si só, não se apresenta economicamente viável uma vez que apenas 1/6 do volume de soro serve para produção de proteína, restando ainda um considerável volume de permeado de soro de queijo a manusear. O permeado de soro pode ser utilizado para diversos fins, como substrato para fermentação ou para a obtenção de lactose e/ou seus derivados (SIVIERI; OLIVEIRA, 2002; JURASCIK et al., 2006; DRAGONE et al., 2009; MAGALHÃES et al., 2010c, 2010b).

2.1.2.2 Obtenção de lactose e seus derivados a partir do soro de queijo

O processo de obtenção da lactose envolve, essencialmente, a concentração do soro e remoção de gordura, proteína e sais. A molécula de lactose, como outros carboidratos, possui sítios reativos (ligação glicosídica, grupo redutor de glucose, grupos hidroxilo livres, ligações carbono-carbono) que a tornam susceptível de modificações química ou enzimática (SIVIERI; OLIVEIRA, 2002; MAGENIS et al., 2006). Uma variedade de processos com significado comercial envolvendo a modificação química ou enzimática da lactose têm sido investigados (Tabela 4).

Tabela 4 Exemplo de produtos produzidos por modificação química ou enzimática da lactose

Derivado	Processo	Uso potencial	Comentários
Xarope de lactose hidrolisada	**Hidrólise**	**Adoçante alimentar**	**Ácida ou enzimática**
Polímeros	**Polimerização**	**Espuma poliuretano**	
Galactose	**Reação de hidrólise seguida de fermentação da glucose**	**Substituto de sorbitol**	
Lactitol	**Hidrogenação/redução**	**Adoçante não nutritivo**	**tolerado por diabetes, não provoca cáries, GRAS/1993**
Ácido lático	**Fermentação por bactérias do ácido lático e leveduras do gênero *Kluyveromyces* e *Kazachstania***		**Pode se produzido sinteticamente**

Fonte: (YANG; SILVA, 1995; HOLSINGER, 1997; HOFVENDAHL; HAHN-HAGERDAL, 2000; MAGENIS et al., 2006)

Um dos principais derivados da lactose é o lactitol. Este produto tem um poder adoçante, e pode ser utilizado por diabéticos. A hidrólise enzimática apresenta, no entanto, o maior impacto na indústria de laticínios e levou à disponibilade comercial em diversos países de muitos produtos derivados de leite sem lactose (MAGENIS et al., 2006). O xarope resultante da hidrólise da lactose presente no soro resulta em uma mistura de glucose e galactose. Comparado com o poder adoçante da sacarose (100%) este xarope possui poder adoçante de 70% enquanto que a lactose é consideravelmente menos doce (40%) (YANG; SILVA, 1995; HOLSINGER, 1997). Além de poder ser utilizado como adoçante alimentar, pode ainda ser usado como substrato em fermentações.

Quando a lactose é hidrolisada, os monossacáridos, glucose e galactose, aparecem teoricamente em proporções equivalentes (HOFVENDAHL; HAHN-HAGERDAL, 2000; MAGENIS et al., 2006). A hidrólise da lactose pode ocorrer por ação enzimática (Figura 2) da hidrolase β-galactosidase ou por catálise ácida.

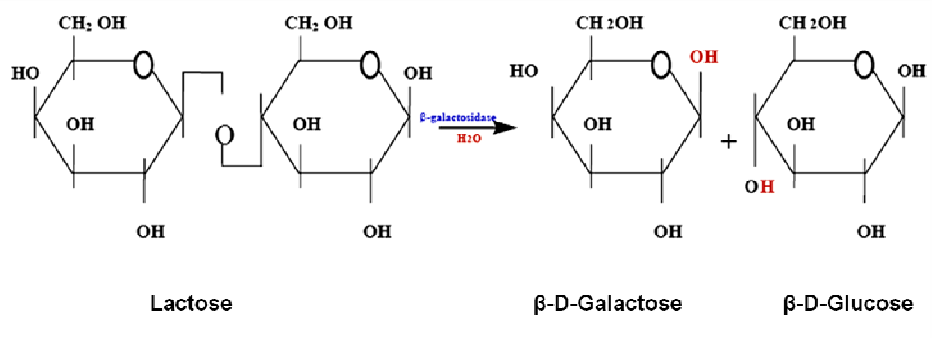


Figura 2 Hidrólise da lactose pela β-galactosidase

Um outro derivado da lactose- é o ácido lático. O ácido lático pode ser obtido por processos fermentativos utilizando bactérias do ácido lático e algumas leveduras do gênero *Kluyveromyces* e *Kazachstania*, ou produzidos sinteticamente através da hidrólise da lactinitrila (HOFVENDAHL; HAHN-HAGERDAL, 2000).

A produção via fermentação possui algumas vantagens. Algumas cepas microbianas podem produzir a forma D- ou L- pura, enquanto a rota química leva sempre à formação de uma mistura racêmica. A fermentação possibilita a utilização de substratos provenientes de fontes renováveis, como a exemplo o soro de queijo. Um processo patenteado para utilização do soro de queijo é o processo contínuo utilizando soro ultrafiltrado como substrato, com recirculação

de células e purificação através de eletrodiálise (HOFVENDAHL; HAHN-HAGERDAL, 2000).

2.1.2.3 O soro de queijo como substrato para fermentações

O soro de queijo utilizado na fabricação das bebidas lácteas pode ser líquido ou em pó. No entanto, os equipamentos para secagem e obtenção do soro em pó não estão disponíveis em laticínios de pequeno e médio porte. Por outro lado, a elaboração de bebidas com soro líquido envolve equipamentos e acessórios comuns, encontrados na maioria dos laticínios. Portanto, a fabricação de bebidas lácteas com soro líquido tornou-se uma opção atrativa no Brasil (SIVIERI; OLIVEIRA, 2002; MAGENIS et al., 2006). A bebida fermentada a partir do soro de queijo, contém compostos voláteis que conferem ao produto características organolépticas desejáveis (MAGALHÃES et al., 2010b). A Figura 3 demonstra os principais compostos voláteis encontrados em soro fermentado.

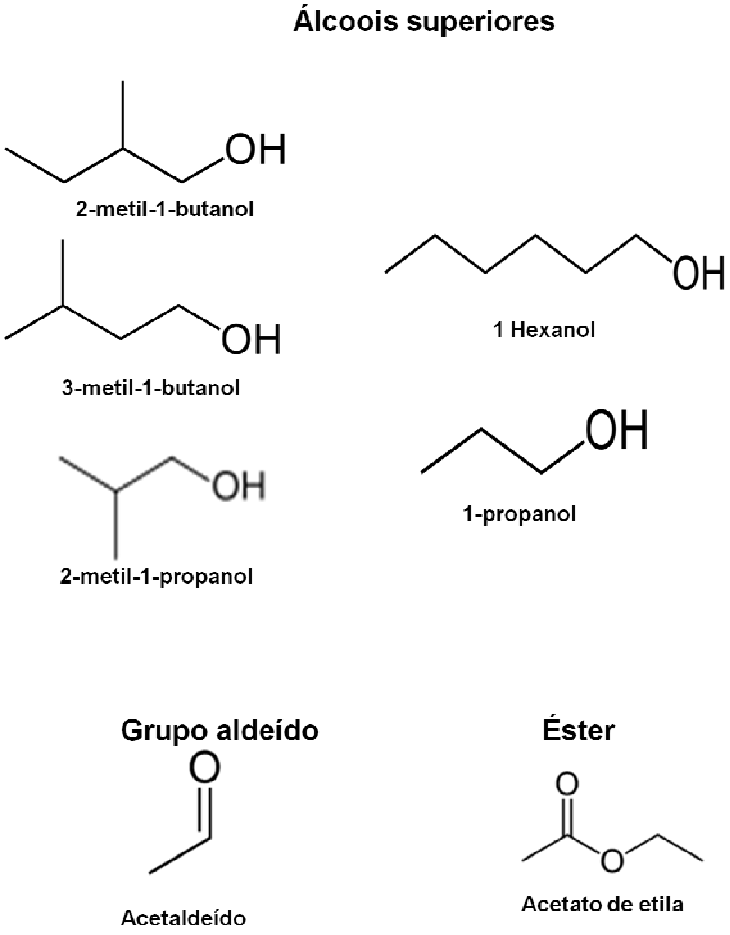


Figura 3 Principais compostos voláteis encontrados em soro de queijo fermentado
Fonte: (MAGALHÃES et al., 2010b)

A principal limitação em utilizar o soro como substrato em fermentações prende-se ao restrito número de microrganismos capazes de utilizar a lactose. Mesmo assim, este é usado em muitos processos industriais. Na Tabela 5 apresentam-se alguns exemplos de produtos obtidos por fermentação do soro

incluindo a produção de álcoois (etanol), biomassa, proteína microbiana, bebidas alcoólicas, ácidos orgânicos (lático, acético e cítrico) e biopolímeros (xantano).

À medida que o recurso à clonagem de genes aumenta, aumentam também as potencialidades na obtenção de protutos por fermentação de soro utilizando microrganismos recombinantes, tendo alguns exemplos sido incluídos na Tabela 5 (FU; TSENG, 1990; GUIMARÃES et al., 1992; COMPAGNO et al., 1993; DOMINGUES et al., 2001; JURASCIK et al., 2006).

Tabela 5 Produtos obtidos por fermentação do soro de queijo

Produtos	**Organismos**	**Referências**
Etanol	***Saccharomyces cerevisiae*** **recombinante**	**DOMINGUES et al., 2001** **JURASCIK et al., 2006**
	Escherichia coli **recombinante**	**GUIMARÃES et al., 1992**
Proteína microbiana	***Kluyveromyces fragilis***	**GHALY et al., 1992**
Biomassa	***Kluyveromyces fragilis***	**GHALY et al., 1992**
Bebidas	***Kluyveromyces marxianus***	**DRAGONE et al., 2009**
Ácido lático	***Lactobacillus bulgaricus***	**MEHAIA; CHERYAN, 1986**
Óleo	***Candida curvata***	**FLOETENMEYER et al., 1985**
B-Galactosidade	***Kluyveromyces marxianus***	**RECH et al., 1999**
Ácido cítrico	***Aspergillus niger***	**EL-SAMRAGY et al., 1996**
Xantano	***Xantomonas campestris*** **recombinante**	**FU; TSENG, 1990**
Glicerol	***Kluyveromyces marxianus***	**RAPIN et al., 1994**
Frutose disfosfato	***Saccharomyces cerevisiae*** **recombinante**	**COMPAGNO et al., 1993**
Proteases	***Bacillus subtilis***	**DIAS et al., 2008**

2.2 Uso de grãos de kefir como cultura iniciadora para fermentação do soro de queijo: microbiologia e características do kefir

Como vimos no tópico anterior (1.1.2.3.), é restrito o número de microrganismos capazes de utilizar a lactose do soro de queijo como substrato. Por esta razão, uma opção de inóculo seria os grãos de kefir (uma cultura simbiôntica de bactérias e leveduras considerada probiótica (GÜZEL-SEYDIM et al., 2005), utilizados tradicionalmente para fermentar o leite, produzindo a bebida láctea kefir.

A bebida kefir de leite contém uma diversidade de bactérias ácido láticas que são grupos fisiologicamente distintos. Elas podem, geralmente, ser descritas como bactérias Gram-positivas, cocos não esporulados ou bastonetes, sendo o ácido lático o maior produto da fermentação dos carboidratos. Tradicionalmente, as bactérias ácido láticas compreendem os gêneros *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Pediococcus* e *Streptococcus* (MACKAY; BALDWIN, 1990). No kefir também é encontrado algumas espécies de leveduras. É conhecido que leveduras têm importante função na preparação de produtos fermentados de leite. Leveduras podem fornecer nutrientes essenciais de crescimento, tais como aminoácidos e vitaminas; elas alteram o pH, secretam etanol e produzem CO_2 (VILJOEN, 2001).

Os grãos de kefir de leite constituem uma associação simbiôntica de leveduras e bactérias ácido láticas, usadas para a produção de uma bebida láctea. Com a transferência diária destes grãos para leite fresco, eles dobram de peso, pela multiplicação, em um período de 7 a 10 dias. Mesmo não tendo assepsia restrita em sua manipulação, geralmente domiciliar, os grãos mantiveram suas características estruturais e de aparência durante várias décadas de propagação (LA RIVIÉRE, 1969; MAGALHÃES et al., 2010a, 2010c).

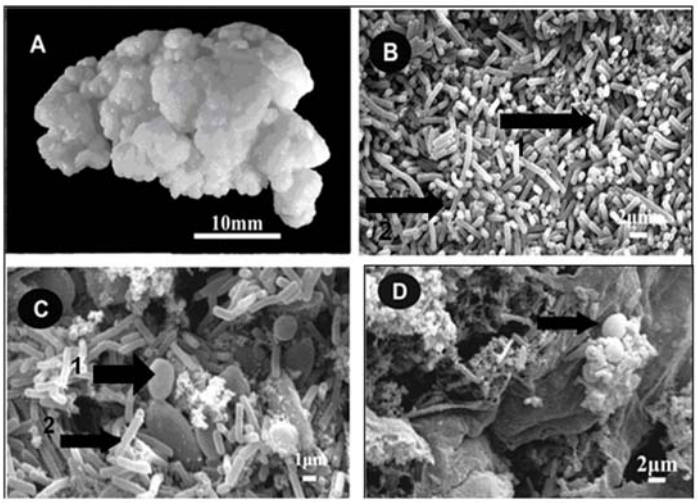
A microbiota da bebida kefir produzida na Argentina foi descrita por constituir bactérias e leveduras. Dentre as leveduras, representantes do gênero *Saccharomyces, Candida* e *Kluyveromyces* foram achadas. As bactérias foram *Lactococcus lactis* subsp. *lactis, Lactobacillus kefir, Lactobacillus parakefir* e *Lactobacillus plantarum*. Representantes do gênero *Acetobacter* também foram observados (GARROTE et al., 2001). E a microbiota da bebida kefir tradicionalmente produzida no Brasil foi descrita como: *Lactobacillus paracasei*, *Lactobacillus parabuchneri*, *Lactobacillus casei*, *Lactobacillus kefiri*, *Lactococcus lactis*, *Acetobacter lovaniensis*, *Kluyveromyces lactis*, *Kazachstania aerobia*, *Saccharomyces cerevisiae* e *Lachancea meyersii* (MAGALHÃES et al., 2010a).

A composição microbiana da bebida kefir é definida dependendo do método de produção, da origem dos grãos e dos métodos de identificação microbiológica (WITTHUHN et al., 2004a, 2004b; MIGUEL et al., 2010). Todos estes fatores contribuem para a variação da população microbiana da bebida. A microbiota dominante do kefir isolada em diferentes localidades encontra-se relacionada na Tabela 6.

Tabela 6 Microbiota pertencente ao kefir cultivado em leite, em diferentes locais

Microrganismos	Localidade	Referências
Lactobacillus brevis, Saccharomyces unisporus	**Portugal**	**PINTADO et al., 1996**
Lactobacillus kéfir, Lactococcus lactis* sp. *lactis, Leuconostoc mesenteroides, Lactobacillus parakefir, Kluyveromyces marxianus	**Argentina**	**GARROTE et al., 2001**
Lactococcus lactis, Streptococcus thermophilus	**Turquia**	**YÜKSEKDAG et al., 2004**
Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus delbruekii, Lactobacillus acidophilus	**Espanha**	**SANTOS et al., 2003**
Lactobacillus viridescens, Lactobacillus gasseri, Lactobacillus acidophilus, Candida holmii, Saccharomyces unisporus	**Espanha**	**ANGULO et al., 1993**
Lactobacillus delbruekii* ssp. *delbruekii, Leuconostoc lactis, Lactobacillus curvatus, Zygosaccharomyces sp., Saccharomyces cerevisiae	**África do Sul**	**WITTHUHN et al., 2004c**
Lactobacillus delbruekii* ssp. *delbruekii, Lactococcus lactis* sp. *lactis* 1, *Candida krusei, Candida kefir	**África do Sul**	**WITTHUHN et al., 2004b**
Lactobacillus casei, Saccharomyces cerevisiae	**Rússia**	**PLESSAS et al., 2007**
Lactococcus* sp., *Kluyveromyces marxianus	**Espanha**	**FONTÁN et al., 2006**
Lactobacillus paracasei, Lactobacillus parabuchneri, Lactobacillus casei, Lactobacillus kefiri, Lactococcus lactis, Acetobacter lovaniensis, Kluyveromyces lactis, Kazachstania aerobia, Saccharomyces cerevisiae* e *Lachancea meyersii	**Brasil**	**MAGALHÃES et al., 2010a**
Gluconobacter japonicas, Lactobacillus kefiri, Lactobacillus uvarum, Lactobacillus paracasei, Lactobacillus satsumensis, Lactobacillus plantarum, Lactobacillus paracasei	**Brasil**	**MIGUEL et al., 2010**

Em estudos utilizando a microscopia de luz e a microscopia eletrônica de varredura, com grãos de kefir cultivados em leite, foi observado que a superfície dos grãos de kefir é rugosa. Nas amostras fatiadas, a superfície interna dos grãos também se mostrou rugosa e com porções semelhantes a uma coleção de pequenas crateras. Foram observadas células de leveduras nas superfícies externa e interna dos grãos. Dois tipos de bacilos (curto e longo) foram notados. Os grãos de kefir foram caracterizados como irregulares e variaram de tamanho entre 3 e 35mm. Eles atuam como uma matriz polissacarídica, na quais bactérias ácido láticas e leveduras vivem simbioticamente (GUZEL-SEYDIM et al., 2005; MAGALHÃES et al., 2010a). A Figura 4 demosntra os grãos de kefir visto a olho nu e com auxílio de um microscópio eletrônico de varredura.



A – Grãos de kefir vistos a olho nu. B – Superfície externa dos grãos de kefir, seta 1 – bacilo curto, seta 2 – bacilo longo. C – Superfície externa dos grãos de kefir, seta 1 – levedura, seta 2 – bacilo. D – Superfície interna dos grãos de kefir, seta – levedura.

Figura 4 Estrutura de grãos de kefir
Fonte: (MAGALHÃES et al., 2010a)

2.2.1 Bioquímica do kefir

Além de sua composição microbiológica, o kefir possui uma matriz gelatinosa com teores aproximados de 13% de proteínas e 24% de polissacarídeos e lipídeos (RIMADA; ABRAHAM, 2006). O principal polissacarídeo do kefir é o kefiran, um exopolissacarídeo que contém quantidades aproximadamente iguais de glicose e galactose (CHEIRSILP et al., 2002, 2003; RIMADA; ABRAHAM, 2006).

Descoberto no final da década de 1960, o kefiran assemelha-se a polissacarídeos de glico-galactano, servindo ao kefir como uma matriz de sustentação entre seus diversos componentes (RIMADA; ABRAHAM, 2006). Foi desenvolvido um método para a obtenção de kefiran em laboratório, obtendo o teor de 2g do polissacarídeo por litro da bebida fermentada, a partir da atividade bacteriana presente nos grãos (MICHELI et al., 1999).

Sobre essas investigações sobre os produtores de kefiran há controvérsias, entretanto, Kooiman (1968) reportou que o "*Lactobacillus brevis*, atualmente conhecido como *Lactobacillus kefir*, é responsável pela produção de kefiran." Porém, de acordo com Tada et al. (2007), "o produtor do kefiran nos grãos de kefir é o *Lactobacillus kefiranofaciens."*

A composição química da bebida kefir de leite pode ser variável. Isto depende de fatores tais como o conteúdo de gordura do leite ou a concentração de carboidratos no substrato, a composição microbiológica ou o processo tecnológico de produção (OTLES; CAGINDI, 2003). Os principais produtos formados durante a fermentação são ácido lático, CO_2 e álcool. A composição química da bebida kefir, dada por componente/100g, é apresentada na Tabela 7.

Tabela 7 A composição química da bebida kefir de leite

Composição química do kefir	Valor/100g
Energia	**65kcal**
Gordura	**3,5g**
Proteína	**3,3g**
Lactose	**4,0g**
Água	**87,5g**
Cálcio	**0,12g**
Fósforo	**0,10g**
Magnésio	**12,0g**
Potássio	**0,15g**
Sódio	**0,05g**
Ácido lático	**1,0g**
Colesterol	**13,0mg**
Manganês	**5,0µg**
Triptofano	**0,05g**
Leucina	**0,34g**
Vitamina A	**0,06mg**
Vitamina B	**0,04mg**
Vitamina B2	**0,17mg**
Vitamina B6 e B12	**0,05mg**
Vitamina C	**1mg**
Vitamina D e E	**0,11mg**

Fonte: (OTLES; CAGINDI, 2003)

Os numerosos benefícios das vitaminas B são regulação dos rins, do fígado e do sistema nervoso, além de aumentar a energia e a longevidade do corpo. Triptofano é um dos aminoácidos essenciais no kefir que promovem um

efeito relaxante no sistema nervoso. Cálcio e magnésio são abundantes no kefir, os quais são importantes minerais para a saúde do sistema nervoso. Kefir é também uma boa fonte de fósforo, sendo o segundo mineral mais abundante em nossos corpos e ajuda na utilização de carboidratos, gorduras e proteínas para crescimento, manutenção e energia das células (OTLES; CAGINDI, 2003).

A lactose é um dissacarídeo parcialmente consumido pelos microrganismos presentes na bebida kefir. Estes microrganismos são bactérias láticas e algumas leveduras. Como produto principal do metabolismo da lactose, pontua-se o ácido lático (MAGALHÃES et al., 2010b, 2010a). A gordura presente na bebida fermentada é uma função direta da origem e do tipo do leite utilizado em sua produção, se de rebanho bovino ou caprino. A tipificação, como os baixos níveis de gordura (natural, em pó, desnatado e UHT), também possui função direta na gordura presente na bebida. A escolha da fonte láctica para a fermentação também resulta em propriedades organolépticas distintas para a bebida kefir (OTLES; CAGINDI, 2003).

Amostras de grãos de kefir cultivados em leite e estaticamente, a 4°C, durante 21 dias, mostraram alterações no aroma das suspensões produzidas pelo metabolismo de ácidos voláteis, tais como ácido cítrico, pirúvico, láctico, úrico, acético, propiônico, butírico e hipúrico, em análise cromatográfica de alta eficiência (HPLC), e de etanol, acetaldeído e diacetila, em cromatografia gasosa (CG). Dos compostos testados, ácido lático, cítrico, etanol e acetaldeído apresentaram aumento relevante no 21º dia de cultivo, ao passo que acetoína decaiu em, aproximadamente, 30%. Outros ácidos orgânicos encontrados na bebida foram ácido fórmico, isobutírico, capróico, caprílico e láurico (GUZEL-SEYDIM et al., 2000).

Dióxido de carbono, produzido por algumas bactérias lácticas heterofermentativas e por leveduras, é responsável pela gaseificação típica observada na bebida. Vitaminas do complexo B, como B1, B12, biotina, niacina

(B3) e pirodoxina (B6), além de ácido fólico, vitaminas K, cálcio, manganês e aminoácidos, também constituem parte da suspensão probiótica do kefir (GUZEL-SEYDIM et al., 2000).

O etanol, juntamente com o CO_2 e os compostos aromáticos, é o ingrediente marcante na bebida kefir. Seu teor varia em função do período e da temperatura de fermentação permitida, bem como das condições de produção, se predominantemente aeróbica ou anaeróbica. Não obstante, também pode ocorrer variação na concentração de etanol, dependendo de as amostras serem constituídas por grãos de kefir propriamente ditos, ou amostras liofilizadas, uma apresentação comercial bastante utilizada (HALLÉ et al., 1994).

Existem diferentes faixas de teores alcoólicos encontrados na literatura. Foram encontraram teores variáveis entre 0,03 a 1,8g, a cada 100g da bebida fermentada com os grãos de kefir no leite. O máximo conteúdo de álcool reportado na bebida kefir foi de 38g por litro da bebida, o que equivale a um teor aproximado de 5% de etanol, semelhante ao encontrado na cerveja. Este nível de etanol, porém, só foi alcançado após 7 a 10 dias de fermentação contínua da bebida. O teor alcoólico também varia com a temperatura de fermentação, podendo reduzir-se a quase um terço em 4°C, quando comparado a 30°C (HALLÉ et al., 1994; GUZEL-SEYDIM et al., 2000).

2.2.2 Propriedades terapêuticas do kefir

Um dos primeiros registros literários do emprego terapêutico de kefir data do princípio da década de 1970, quando o pesquisador russo Batinkov sugeriu o emprego da bebida probiótica para o tratamento de úlceras pépticas e duodenais. A partir de então, passaram a surgir diversos trabalhos, em línguas eslavas, sobre a utilização do kefir no tratmento de doenças pancreáticas, pneumonia, bronquite e tuberculose, dentre outras (OTLES; CAGINDI, 2003).

Na atualidade, o kefir tem merecido indicações científicas nos mais variados enfoques, desde a nutrição e a dietoterapia, até mesmo o seu emprego com resultados significativos na oncologia comparada. Vários estudos investigaram os efeitos imunomodulatórios (VINDEROLA et al., 2004), antiinflamatórios (RODRIGUES et al., 2005; LEE et al., 2007), cicatrizantes (RODRIGUES et al., 2004), antialérgicos (LEE et al., 2007), antitumorais (CEVIKBAS et al., 1994; LEBLANC et al., 2006), antimicrobianos (RODRIGUES et al., 2004), antineoplásticos e pró-digestivos (SALOFF-COASTE, 1996) do kefir.

Os microrganismos presentes na bebida kefir processam o leite e tornam os nutrientes mais acessíveis ao organismo. O kefir possibilita que pessoas com problemas de má-absorção da lactose consumam o leite, o qual é modificado e processado pelos microrganismos. O produto também é conhecido por afetar beneficamente o trato intestinal, sendo considerado uma mistura probiótica (OTLES; CAGINDI, 2003; URDANETA et al., 2007). Estudando o metabolismo de indivíduos intolerantes à lactose, foi observado a redução de 30% no teor de lactose, presente em kefir fermentado por 11 dias e foi recomendado o seu consumo por aqueles pacientes (ALM, 1982).

Além da melhoria no metabolismo de carboidratos, para os consumidores, o consumo da bebida kefir também está associado a um aumento na proteólise digestória (VASS et al., 1984).

Em estudo com ratos Wistar, foi verificado melhor digestibilidade de produtos à base de proteínas com dieta suplementada com iogurte e kefir com conseqüente aumento de massa corporal, por grama de proteína consumida pelos animais (VASS et al., 1984). Também foi encontrado correlação direta entre a administração oral de kefir em pacientes com obesidade alimentar e o aumento da proteólise intragástrica (SINTSOVA, 1991). Também foi realizado um estudo com 20 fêmeas de ratos Wistar recebendo a alimentação suplementada com

kefir. Os resultados mostraram que a dieta pode beneficiar a digestão de proteínas, devido ao aumento da atividade intestinal. Além disso, a dieta apresentou relação direta com a redução do índice glicêmico dos organismos examinados (URDANETA et al., 2007).

O primeiro estudo *in vitro* sobre as propriedades antimicrobianas de diferentes linhagens de *Lactobacillus* spp. isoladas do kefir foi realizado em 2003. Foi verificado que as melhores propriedades probióticas foram observadas em *Lactobacillus acidophillus* CYC 10051 e *Lactobacillus kefiranofaciens* CYC 10058 (SANTOS et al., 2003).

Efeitos imunomodulatórios também estão associados ao consumo de kefir. Foi determinado em estudos capacidade imunomodulatória de kefir da resposta imune na mucosa intestinal de ratos e para averiguar a importância da dosagem e viabilidade celular nesta resposta (VINDEROLA et al., 2004). Também foi observado que kefir possui várias substâncias, não especificadas, que podem exercer efeitos benéficos no sistema imune e prevenir certos tipos de câncer. Os autores citam como exemplo a resposta imune em tumores localizados em glândulas mamárias (LEBLANK et al., 2006).

Foi demonstrado, *in vivo,* efeitos antiinflamatórios e antialérgicos em ratos asmáticos. E a atividade cicatrizante de kefir foi verificada usando-se uma pomada à base de kefir, em ratos albinos com ferida dorsal infectada por *Staphylococcus aureus* (LEE et al., 2007). A cicatrização foi mais bem observada nos animais tratados com a formulação com kefir (70%), em relação ao grupo controle tratado com pomada comercial àa base de neomicina-clostebol (RODRIGUES et al., 2004).

2.2.3 Uso de métodos moleculares para identificação microbiana em grãos de kefir: monitoramento microbiano durante o processo de fermentação

Durante muitos anos as técnicas de identificação e classificação de microrganimos mais empregadas foram baseadas em características morfológicas e fisiológicas, denominadas técnicas tradicionais. Entretanto, os procedimentos taxonômicos convencionais para classificar bactérias e leveduras são ainda muito lentos, exigem muito trabalho e não são absolutamente conclusivos.

Várias metodologias têm sido utilizadas para a identificação de microrganimos com base em características morfológicas da colônia, das células ou através da identificação em função das condições de cultivo. Apesar de que informações referentes à capacidade de assimilação de fontes de C e N, em diferentes substratos e caracterização bioquímica e fisiológica são valiosas, características fenotípicas podem ser influenciadas pela linhagem e pelas condições de cultivo (MAGALHÃES et al., 2010a). Algumas limitações podem ser citadas para explicar a incorreta classificação de espécies deste gênero, como a instabilidade na morfologia das colônias das linhagens de uma mesma espécie, os poucos resultados que se obtêm a partir da fisiologia (uma ou duas características) para classificação e a possibilidade de mutação de um único gene alterando as características fisiológicas da linhagem (RAINIERI et al., 2003).

Com o advento da biologia molecular aplicada ao estudo do meio ambiente, vários tipos de marcadores foram desenvolvidos contribuindo significativamente para o avanço do conhecimento sobre a biodiversidade deste grupo. Os métodos moleculares receberam grande impulso com o desenvolvimento da técnica conhecida como reação da polimerase em cadeia (PCR). Nessa técnica descrita por (WOESE, 1987), pequenos e específicos segmentos do genoma de microrganismos podem ser amplificados utilizando-se

primers (seqüências iniciadoras) complementares a seqüências localizadas em regiões específicas do genoma. O que ocorre é a extensão a partir dos *primers*, pela ação de uma DNA polimerase termoestável e a *Taq* DNA polimerase, isolada originalmente do microrganismo *Thermus aquaticus*. O DNA é desnaturado e o ciclo repetido várias vezes, o que permite a amplificação exponencial daquela seqüência específica (WOESE, 1987).

As técnicas moleculares que utilizam à técnica de PCR são cada vez mais utilizadas para identificação de espécies. Dentre as técnicas desenvolvidas a amplificação de regiões do rDNA seguida de seqüênciamento e análise da homologia com seqüências já depositadas em Banco de Dados têm sido utilizada com freqüência para a identificação de espécies. A escolha da região a ser seqüenciada é vital para a análise entre os organismos, sendo normalmente utilizado o rDNA, entre outras razões, por estar presente e ser homólogo em todos os organismos vivos, e por possuir partes muito conservadas intercaladas por outras variáveis (WOESE, 1987).

Os ácidos desoxirribonucléicos (DNA) são considerados os biopolímeros mais adequados para estudos de diversidade. Seus genes, os rRNAs, são universalmente distribuídos entre os diferentes grupos de seres vivos, sendo a molécula com o maior grau de conservação existente. Sua variabilidade pode apresentar-se em maior ou menor extensão em diferentes regiões da molécula (LANE et al., 1985). Organismos procariotos possuem moléculas de rRNA de tamanho 70S (5S, 23S e 16S). A grande maioria dos estudos filogenéticos tem-se centrado no rRNA 16S e existe um vasto conhecimento de seqüências comparativas da subunidade menor do rRNA, em contrapartida, pequeno número de seqüências da subunidade rRNA 23S é conhecido. Informações sobre o espaço intergênico rRNA 16S-23S e a distribuição dos genes de tRNA codificados nessa região são igualmente raras (MENDOZA et al., 1998). A seqüência conservada da região espaçadora 16S-

23S é indicadora direta e estável da divergência evolucionária de cepas de *Staphylococcus* aureus (GÜRTLER; RUDNER 1995). Os genes do rRNA em bactérias são organizados em operons, dentro dos quais os genes que codificam para os RNA 16S, 23S e 5S são freqüentemente separados pela região espaçadora não codificante do DNA. A utilização de sonda, mistura de RNA 16S e 23S, resulta na hibridização apenas com fragmentos no *fingerprint* cromossomal que contêm partes dos genes correspondentes. Por outro lado, a utilização de sondas fragmentos clonados dos genes de rRNA pode resultar na hibridização com os genes correspondentes e a seqüência espaçadora. Então, padrões de hibridização diferentes podem ser obtidos, dependendo da sonda utilizada (SAUNDERS et al., 1990). A maioria das bactérias contém entre 2 a 11 cópias do gene rRNA por célula. As regiões espaçadoras entre os genes rRNA 16S e 23S codificam vários tRNAs e têm várias seqüências repetitivas em regiões não codificantes do grupo de genes (SAUNDERS et al., 1990). Embora os genes do tRNA sejam altamente conservados, o comprimento dos espaçadores intergênicos dos tRNA variam consideravelmente, o que é utilizado para identificação de bactérias.

Organismos eucariotos possuem rRNA 80S (5S, 5.8S, 28S e 18S) divididas em subunidades maior e menor. A região gênica do rDNA em leveduras possui as seguintes estruturas na disposição 5'-3': a região espaçadora externa (ETS), o gene 18S, a região espaçadora interna (ITS1), o gene 5.8S, uma segunda região espaçadora interna (ITS2) e o gene 26S. Este último gene apresenta as seqüências menos conservadas, em relação aos genes 18S e 5.8S, sendo a região de escolha para estudos de filogenia de espécies e grupos taxonômicos mais relacionados. A região D1/D2 do 26S rDNA tem sido utilizada para diferenciar quase todas espécies estudadas (KURTZMAN; ROBNETT, 1998). Porém, o sequenciamento desta região não é capaz de diferenciar todas as espécies de leveduras basidiomicotas, sendo necessário o

sequenciamento conjunto da região ITS (SCORZETTI et al., 2002). Diversos trabalhos de identificação da microbiota pertencente a grãos de Kefir têm sido desenvolvidos a partir das seqüências conservadas de leveduras e bactérias (CHEN et al. 2008, MAGALHÃES et al, 2010c, 2010c; MIGUEL et al., 2010).

Métodos independente de cultivo têm sido utilizados para a investigação da microbiota em alimentos fermentados (ERCOLINI, 2004, MAGALHÃES et al., 2010c; MIGUEL et al., 2010). Técnicas independentes de cultivo têm provado ser uma ótima ferramenta para estudo da complexa microbiota em alimentos (JIANZHONG et al. 2009; MAGALHÃES et al., 2010c; MIGUEL et al., 2010). Dentre os diferentes métodos independentes de cultivo, eletroforese em gel com gradiente desnaturante (PCR-DGGE) é utilizada na microbiologia de alimentos para avaliar a diversidade microbiana (ERCOLINI, 2004). Assim, PCR-DGGE tem sido utilizada para estudo da microbiota em grãos de kefir (JIANZHONG et al. 2009; MAGALHÃES et al., 2010c; MIGUEL et al., 2010).

Na técnica de PCR-DGGE utilizam-se géis de poliacrilamida contendo gradiente linear desnaturante, geralmente ureia e formamida, nos quais fragmentos de DNA com o mesmo tamanho, porém, com sequências de bases nucleotídicas distintas, apresentam padrão de desnaturação diferente. Essa separação baseia-se em um princípio físico simples de que a mobilidade eletroforética do DNA em um gel de poliacrilamida seja sensível à estrutura secundária da molécula com respeito a sua conformação, que pode ser helicoidal, parcialmente desnaturada ou fita simples. As moléculas parcialmente desnaturadas, compostas por partes dupla hélice e partes em fita simples, movimentam-se mais lentamente no gel do que moléculas em fita dupla ou simples (ERCOLINI, 2004).

A diversidade da microbiota em grãos de kefir provenientes do Tibete foi investigada através da técnica PCR-DGGE combinada com a análise da seqüência 16S rDNA (bactérias) e 26S rDNA (levedura). Os microrganimos

dominates isolados através desta técnica foram: *Pseudomonas sp., Leuconostoc mesenteroides, Lactobacillus helveticus, Lb.kefiranofaciens, Lactococcus lactis, La.kefiri, Lb.casei, Kazachstania unispora, Kluyveromyces marxianus* e *Saccharomyces cerevisiae* (JIANZHONG et al., 2009).

PCR-DGGE, mostrou eficiente na análise da diversidade da microbiota em grãos de kefir Brasileiros (MAGALHÃES et al., 2010c; MIGUEL et al., 2010). Os microrganismos dominantes isolados destes grãos foram bactérias do gênero *Lactobacillus* (MAGALHÃES et al., 2010c; MIGUEL et al., 2010) e leveduras do gênero *Saccharomyces* e *Kluyveromyces* (MAGALHÃES et al., 2010c). Esta técnica pode monitorar a presença microbiológica de microrganismos desejáveis e indesejáveis, ao longo de um processo de fermentação, fornecendo informações básicas para o desenvolvimento de culturas iniciadoras (JIANZHONG et al. 2009).

3 CONSIDERAÇÕES FINAIS E PERSPECTIVAS FUTURAS

Para uma visão mais esclarecedora dos trabalhos realizados durante o período de doutorado, sumarizam abaixo as principais contribuições de cada uma das fases, fazendo-se a articulação de todo o trabalho ao final.

Segue abaixo as contribuições de maior relevância.

a) O soro de queijo e o soro de queijo desproteinizado foram utilizados como substratos para a fermentação de grãos de kefir como cultura iniciadora contribuindo com a redução da poluição ambiental, devido ao descarte do soro no meio ambiente. Foram utilizadas técnicas moleculares (PCR-DGGE, clonagem, seqüenciamento) e fluorescência CLMS, além de técnicas para análises químicas (HPLC, GC-FID), que foram determinantes na qualidade deste estudo; além de poderem vir a ser utilizadas em outros propósitos no decorrer de futuros trabalhos científicos;
b) Bebidas kefir com prováveis valores nutricionais foram produzidas. Isto poderá futuramente auxiliar na nutrição diária requerida em uma dieta humana, podendo contribuir diretamente com a saúde da população.

Da articulação dos resultados obtidos no trabalho e de uma forma geral podem retirar-se algumas conclusões partindo dos objetivos principais do projeto, os quais foram todos alcançados. Os estudos abriram perspectivas para a introdução de novos produtos com possíveis valores nutricionais, no mercado. A tecnologia proposta é significante a nível ambiental, devido ao fato de um resíduo industrial muito poluente poder ser empregado para elaborar produtos de valor nutricional, inclusive o uso de grãos de kefir (considerado probiótico)

como alternativo. Além disso, o uso de kefir em forma granular proveria a possibilidade para eliminar o uso de separadores centrífugos que são tradicionalmente usados em indústrias, na produção de bebidas fermentadas, para separação do inóculo. A aplicabilidade desta tecnologia de produção reduziria custos.

Os trabalhos realizados e expostos neste contexto sugerem futuras investigações que constituiriam uma sequência natural dos trabalhos apresentados.

a) Estudo de propriedades terapêuticas - Seria conveniente, para futura aplicação industrial das bebidas kefir de soro de queijo a definição científica de suas propriedades nutricionais. Com isso estaria proporcionando ao mercado uma transformação de ideias em produtos tecnologicamente novos que poderiam proporcionar melhorias à saúde da comunidade;
b) Sugere-se que os grãos de kefir, bem como os microrganismos isolados pertencentes a estas culturas, além do produto final fermentado, sejam testados quanto a possíveis aplicabilidades científicas, ditadas para o kefir: efeitos imunomodulatórios (VINDEROLA et al., 2004), antiinflamatórios (RODRIGUES et al., 2005; LEE et al., 2007), cicatrizantes (RODRIGUES et al., 2004), antialérgicos (LEE et al., 2007), antitumorais (FURUKAWA et al., 1990; CEVIKBAS et al., 1994; LEBLANC et al., 2006), antimicrobianos (SANTOS et al., 2003; RODRIGUES et al., 2004), antineoplásticos e pró-digestivos (SALOFF-COASTE, 1996).

Perante os resultados descritos neste estudo sugere-se ainda a produção de bebidas fermentadas kefir utilizando diferentes substratos.

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SEGUNDA PARTE - ARTIGOS NA ÍNTEGRA

ARTIGO 1

Normas do periódico *Bioresource Technology*

Artigo publicado no periódico indexado:
***Bioresource Technology* 101 (2010) 8843–8850**
doi:10.1016/j.biortech.2010.06.083

Production of fermented cheese whey-based beverage using kefir grains as starter culture: Evaluation of morphological and microbial variations

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RESUMO

Preocupações com a valorização do soro de queijo conduziram a um recente interesse na produção de bebidas kefir de soro de queijo. Neste estudo, a estrutura e microbiota de grãos de kefir de origem Brasileira e bebidas kefir de leite, soro de queijo e soro de queijo desproteinizado foram caracterizados usando técnicas de microscobias e técnicas moleculares. O objetivo deste estudo foi avaliar a estabilidade das bebidas kefir e a possível presença de bactérias probióticas nestas bebidas. Coloração por fluorescência junto a microscopia confocal a laser mostraram a distribuição de grupos de leveduras entre a matriz dos grãos de kefir essencialmente compostos de polissacarídeo (kefiran) e bactérias. Eletroforese em gel de gradiente desnaturante exibiu comunidades microbianas incluindo leveduras afiliadas para *Kluyveromyces marxianus, Saccharomyces cerevisiae* e *Kazachatania unispora,* e comunidades microbianas incluindo bactérias afiliadas para *Lactobacillus kefiranofaciens* subsp. *kefirgranum, Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* e uma bactéria não cultivável também afiliada para o gênero *Lactobacillus.* A estrutura fixa e microbiota dominante, incluindo bactérias probióticas, foram detectadas nos grãos e bebidas kefir analizadas. Estes resultados são determinantes para uma futura implementação de bebidas de kefir de soro de queijo.

Palavras-chave: Kefir. Soro de queijo. Leite. PCR-DGGE. CSLM.

ABSTRACT

Whey valorization concerns have led to recent interest on the production of whey beverage simulating kefir. In this study, the structure and microbiota of Brazilian kefir grains and beverages obtained from milk and whole/deproteinised whey was characterized using microscopy and molecular techniques. The aim was to evaluate its stability and possible shift of probiotic bacteria to the beverages. Fluorescence staining in combination with Confocal Laser Scanning Microscopy showed distribution of yeasts in macro-clusters among the grain's matrix essentially composed of polysaccharides (kefiran) and bacteria. Denaturing gradient gel electrophoresis displayed communities included yeast affiliated to *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *Kazachatania unispora*, bacteria affiliated to *Lactobacillus kefiranofaciens* subsp. *Kefirgranum*, *Lactobacillus kefiranofaciens* subsp. *Kefiranofaciens* and an uncultured bacterium also related to the genus *Lactobacillus.* A steady structure and dominant microbiota, including probiotic bacteria, was detected in the analyzed kefir beverages and grains. This robustness is determinant for future implementation of whey-based kefir beverages.

Keywords: Kefir. Cheese whey. Milk. PCR-DGGE. CSLM.

1 Introduction

Cheese whey is the liquid remaining after the precipitation and removal of milk casein during cheese-making. This byproduct represents approximately 85–90% of the milk volume and retains 55% of milk nutrients. Among the most abundant of these nutrients are lactose (4.5–5.0% w/v), soluble proteins (0.6–0.8% w/v), lipids, and mineral salts (Dragone et al., 2009 and references there in). Cheese whey represents an important environmental problem because of the high volumes produced and its high organic matter content, exhibiting a COD of 60,000–80,000 ppm. Worldwide production of whey is estimated to be in the order of 160 million tonnes per year, showing a 1–2% annual growth rate (Smithers, 2008). The pressure of antipollution regulations together with whey nutritional value challenges the dairy industry to face whey surplus as a resource and not only as a waste problem (Guimarães et al., 2010).

Several methods have been proposed for whey valorization (Guimarães et al., 2010; Koutinas et al., 2009 and references there in). Besides potable ethanol production by lactose converting microorganisms (reviewed by Guimarães et al. (2010)) and genetically-engineered Saccharomyces cerevisiae cells (Domingues et al., 2001; Guimarães et al., 2008; Domingues et al., 2010), the production of alcoholic beverages from whey has also been pointed as an alternative (Holsinger and Posati, 1974), including distilled beverages (Dragone et al., 2009) and kefir-like whey beverages (Paraskevopoulou et al., 2003).

Kefir is made by inoculating milk with kefir grains. These grains are irregular granules that vary in size from 3 to 35 mm in diameter (Güzel-Seydim et al., 2005) contain lactic acid bacteria (*Lactobacillus, Lactococcus, Leuconostoc*), acetic acid bacteria and yeast mixture coupled together with casein and complex sugars by a matrix of polysaccharides denominated kefiran (Güzel-Seydim et al., 2005). Yeasts are important in kefir fermentation because

of the production of ethanol and carbon dioxide. Kefir grains usually contain lactose-fermenting yeasts (*Kluyveromyces lactis, Kluyveromyces marxianus* and *Torula kefir*), as well as non lactose-fermenting yeasts (*S. cerevisiae*) (Farnworth, 2005). This mixed culture of kefir yeast, which ferments lactose, seems to have the potential for beverage production using cheese whey.

Cheese whey utilization by kefir grains has been studied for potable alcohol production (Koutinas et al., 2009) indicating the ability of this biocatalyst to produce high yields in alcoholic fermentations. In addition, the production of kefir-like whey beverages using a cheese whey–milk mixture as substrate has also been reported (Paraskevopoulou et al., 2003). Reports on single cell protein production (using kefir yeasts; Koutinas et al., 2005) and more recently, on starter culture production from whey for use in cheese ripening (Koutinas et al., 2009) can also been found. All these studies show promising perspectives for kefir grains application in whey valorization strategies. Nevertheless, one important aspect has to be clarified for fully application of kefir grains to whey fermentations. Namely, if the microbiota present in the grains change when using whey instead of the traditional milk as substrate. Another relevant issue is whether the kefir probiotic bacteria are present in the beverages. Therefore, the motivation of the present work was to elucidate the stability, organization and identification of the dominant microbiota present in Brazilian kefir grains and correspondent beverages.

2 Methods

2.1 Milk and whey-based fermentation media

Three different substrates with a lactose concentration of 46 g/L were used as fermentation media: pasteurized full cows' milk (M), cheese whey (CW)

and deproteinised cheese whey (DPW). Cheese whey powder, obtained from a regional dairy industry (Quinta dos Ingleses, Caíde de Rei, Portugal), was dissolved in sterile distilled water to the desired lactose concentration. Deproteinised cheese whey was made by autoclaving at 115 °C for 10 min the cheese whey solution, followed by aseptic centrifugation (2220g for 20 min) to remove fines and cream.

2.2 Milk kefir and cheese whey kefir production

Brazilian kefir grains were used in the present study. Inoculum was grown in pasteurized whole milk during 7 days. The substrate was changed daily. Later the grains (12.5 g) were washed with sterile distilled water and inoculated in 250 mL of each substrate. Erlenmeyers containing kefir grains were statically incubated for 48 h and 72 h at 25 °C. Samples of the beverage were aseptically taken in begin and end of the fermentation. Determination of total reducing sugars was used to assess the depletion of substrate. Replicates were used in each fermentation. Lactose and ethanol were further quantified by high-performance liquid chromatography (HPLC), using Jasco chromatograph equipped with the refractive index (RI) detector (Jasco 830-RI).

2.3 Fluorescence staining and CLSM examination of kefir grains

Samples of the grains used as inoculum and collected after fermentation of milk, cheese whey and deproteinised cheese whey were washed in phosphate buffered saline (PBS) and fixated in 3% formaldehyde (v/v in PBS) for 24 h at 4 °C. The grains were washed again in PBS and stored in a solution of 50% ethanol and PBS. To visualize the internal surface, fixed grains were embedded for cryosectioning according to Batstone et al. (2004). The grains in blocks were

sectioned into 10 lm thick slices using a cryostat CM 1900 (Leica, Germany) with the knife temperature of - 20 °C and cabinet temperature of – 18°C. Intact grains and sections were stained with SYTO 9 (20 ng/µl, Molecular Probes, Spain) to visualize cellular nucleic acids, followed by Calcofluor white (25 lM, Sigma, Spain) to stain chitin in cell walls of fungi, and finally Concanavalin A (ConA) conjugated with Alexa Fluor 594 (1 mg/ml, Molecular Probes, Spain) to stain alpha-linked sugar in polysaccharides. The structure of both external (intact grains) and internal (sections) surface of the grains was examined in Confocal Laser Scanning Microscopy (CLSM) (FluoView 1000, Olympus, Germany).
The collection wavelengths of all stains were listed in Table 1.

Table 1 Stains used in the proposed staining scheme

Dye	Excitation (nm)	Emission (nm)	Targets
Syto 9	470	510–540	Cellular nucleic acids
Calcofluor White	405	Maximum 500	Cellulose and chitin in cell walls of fungi
ConA – Alexa 594	590	Maximum 617	Alpha-linked sugar in polysaccharides

2.4 DNA extraction and PCR-DGGE analysis

Kefir grains and fermented product, collected at the end of fermentations, were frozen at the time of sampling and stored at -20 °C. Samples of the grains used as inoculum were also collected. Approximately 1.5 ml of each liquid sample (i.e. beverage) was centrifuged at 13000 rpm for 5 min for five times. Pellets were resuspended in 400 µL of sterile demineralised water. Each sample (grains and beverage) was transferred into a plastic tube and was subjected to DNA extraction using a NucleoSpin Tissue kit (Macherey–Nagel,

Düren, Germany), according to the manufacturer's instructions. The extracted DNA was stored at -20 °C. Genomic DNA was used as template for PCR amplification of bacterial or fungal ribosomal target regions, for denaturing gradient gel electrophoresis (DGGE) analyses. Two primers sets were used for the analysis of each microbial community. Table 2 presents information about the primers and conditions of PCR and DGGE. All PCRs were performed in mix (50 μL) containing: 0.625 U Taq DNA polymerase (Invitrogen, Barcelona, Spain), 2.5 μL buffer 10 X, 0.1 mM dNTP, 0.2 lM of each primer, 1.5 mM $MgCl_2$ and 1 μL of extracted DNA. Aliquots (2 μL) of the amplification products were analyzed by electrophoresis on 1% agarose gels and ethidium bromide staining. The size of the products was estimated using a 100-bp DNA ladder (MBI Fermentas, Vilnius, Lithuania). The PCR products were analyzed by DGGE using a Bio-Rad DCode Universal Mutation Detection System (Bio-Rad, Richmond, CA, USA). Samples were applied to 8% (w/v) polyacrylamide gels in 0.5 X TAE. Optimal separation was achieved with a 30–55% urea-formamide denaturing gradient for bacteria community and 12–60% for the yeast community (100% correspondent to 7 M urea and 40% [v/v] formamide). Gels were run according to the conditions displayed in Table 2. DGGE gels were stained with AgNO3 as described by Sanguinetti et al. (1994) and scanned in an Epson Perfection V750 PRO (Epson, USA).

Table 2 DGGE-PCR primers used to detect yeasts and bacteria in grains and kefir beverage of milk, cheese whey and deproteinised cheese whey

Primer	Sequence (5' – 3')	Community	Target	PCR conditions	DGGE conditions	References
968fGC	AAC GCG AAG AAC CTT AC GC clamp connected to the 5' end of 968f	Bacteria	V6-V8 region of the 16S rRNA gene	condition 1	16h at 85 V at 60°C.	a
1401r	CGG TGT GTA CAA GAC CC					
ITS1fGC	TCC GTA GGT GAA CCT GCG G GC clamp connected to the 5' end of ITS1gc	Yeast	ITS region of the rDNA	condition 1	16h at 85 V at 60°C.	b
ITS2r	GCT GCG TTC TTC ATC GAT GC					
338fGC	GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG GC clamp connected to the 5' end of 338fgc	Bacteria	V3 region of the 16S rRNA gene	condition 1	8h at 85 V at 60°C.	c
518r	ATT ACC GCG GCT GCT GG					
NS3fGC	GCA AGT CTG GTG CCA GCA GCC GC clamp connected to the 5' end of NS3gc	Yeast	18S region of the rDNA	condition 2	16h at 85 V at 60°C.	d
YM951r	TTG GCA AAT GCT TTC GC					

GC clamp – CGC CCG CCG CGC GCG GCG GGC GGG GCG GG
f – forward primer; r – reverse primer.
Condition 1 – Denatured for 5 min at 95 °C. Thirty cycles: denaturing at 92 °C for 60 s, annealing at 55 °C for 60 s and extension at 72 °C for 60 s. Final extension for 10 min at 72 °C.
Condition 2– 35 cycles instead of 30.
a Randazzo et al. (2002); b White et al. (1990); c Ovreas et al. (1997); d Haruta et al. (2006).

2.5 Cloning and sequencing

Bacterial 16S rRNA genes were amplified from genomic DNA with the primer pair 27f (50-AGAGTTTGATCCTGGCTCAG-30) and 1492r (50-CGGCTACCTTGTTACGAC-30). For amplification of fungal ITS region, the primers ITS1 (50-TCCGTAGGTGAACCTGCGG-30) and ITS4 (50-TCCTCCGCTTATTGATATGC-30) were used. PCR was performed according to the method described by Wang et al. (2006) (bacteria) and Naumova et al. (2004) (yeast). The amplification products were visualized by electrophoresis in 0.5% agarose gel at 60–65 V in 0.5X TAE for 1 h. The purification was made using the Kit QIAquick PCR Purification (QIAGEN). The purified products were ligated into the pGEM®-T vector using the vector pGEM®-T vector system I (PROMEGA) and subsequently transformed in competent cells of *Escherichia coli* (JM109) according to the manufacturer's instructions. Fifty white colonies (positive recombinants) were collected for each transformation and screened by PCR-DGGE using the primers 968fGC/1401r (bacteria) and ITS1GC/ITS2 (yeast). Clones whose DGGE mobility corresponded to bands in the community profile of kefir grains and beverage were selected for sequencing. Different clones exhibiting the same DGGE mobility were included as replicates for sequencing. Inserts from the selected clones were amplified using pGEM®-T vector-targeting primers SP6 (50-CAT ACG ATT TAG GTG ACA CTA TAG-30) and T7 (50-TAA TAC GAC TCA CTA TAG GGA GA-30). Sequencing reactions were performed at BIOPREMIER (Lisboa, Portugal) using the same primer pair.

2.6 Phylogenetic analysis

The sequence information was imported into the BioEdit v7.0.9 software package (Hall, 1999) for assembly and the consensus sequences obtained were manually checked and corrected when necessary. Sequence similarity searches were performed in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) using the blast database.

3 Results and discussion

3.1 Kefir fermentation chemical analysis

Table 3 summarizes the main chemical characterization results of kefir beverages fermentation. Lactose was consumed and ethanol was produced during the fermentation. At 48 h the lactose concentration in the milk fermentation was residual while in the whey fermentations a lactose concentration of 15–20 g/L was observed. This likely reflects an adaptation period of the microbial community to the whole and deproteinised cheese whey as kefir grains were preserved in milk. Despite the higher lactose consumption during milk fermentation, the concentrations of ethanol did not show significant differences to those obtained during the cheese whey and deproteinised cheese whey fermentation.

Table 3 Lactose and ethanol concentration in the performed fermentations

Time (h)	**Milk**		**Cheese whey**		**Deproteinised cheese whey**	
Fermentation (48h)	Lactose (g/L)	Ethanol (g/L)	Lactose (g/L)	Ethanol (g/L)	Lactose (g/L)	Ethanol (g/L)
0h	46.06 ± 0.18	n.d.	45.70 ± 0.711	n.d.	46.06 ± 0.18	n.d.
48h	1.26 ± 0.02	8.65 ± 1.65	14.17 ± 2.16	8.3 0 ± 1.22	19.63 ± 0.36	7.81 ± 0.34
Fermentation (72h)	Lactose (g/L)	Ethanol (g/L)	Lactose (g/L)	Ethanol (g/L)	Lactose (g/L)	Ethanol (g/L)
0h	47.12 ± 0.00	n.d.	47.14 ± 0.00	n.d.	47.14 ± 0.00	n.d.
72h	n.d.	12.26 ± 1.42	n.d.	11.72 ± 0.77	n.d.	11.86 ± 0.00

Data are average values of duplicate ± standard deviation.
n.d. – not detected

As total consumption of lactose was not achieved in 48 h of whey fermentation, a second set of fermentations was performed under the same conditions. Total lactose consumption was attained within 72 h fermentation. During this time, ethanol concentration increased up to ~12 g/L and stabilized after lactose was totally consumed. No significant differences were found in the consumption of lactose and ethanol produced when using milk or whey as substrates.

3.2 Structure of kefir grains as revealed by fluorescence staining and CLSM imaging

Micro-scale examination of the structure of kefir grains was performed by fluorescently probing the distribution of cells (bacteria and yeast) and polysaccharides using a triple staining scheme, followed by CLSM examination (results in Supplementary Fig. S1). No significant difference was observed between the structure of kefir grains collected after fermentation of milk, cheese whey and deproteinised cheese whey. The microbial biomass visualized with the Fluor chrome SYTY9 (green), covered great portion of the external surface and was localized both within and between the ConA (red) stained regions, i.e. the polysaccharide matrix. This polysaccharide matrix, called kefiran, is produced by lactic acid bacteria and usually associated to the therapeutic properties of kefir (Tada et al., 2007). Kefiran has frequently been claimed to be effective against a variety of complaints and diseases. Several studies have investigated the antitumor activity, antibacterial and antifungal activities (Otles and Cagindi, 2003; Silva et al., 2009). Recently, the potential of kefiran to modulate key steps in the virulence of *Bacillus cereus* in the context of intestinal infections has been reported (Medrano et al., 2009). *Lactobacillus kefiranofaciens* and several other unidentified species of *Lactobacillus* have been pointed by several authors as the

major producers of the kefiran polymer in kefir grains (Tada et al., 2007). Otles and Cagindi (2003) found that kefiran producing encapsulated *L. kefiranofaciens* are located all over the grain and increased in the center, while some species of *Lactobacilllus* populated only a small region at the surface layer.

Staining with Calcofluor white (blue) was used to highlight yeast cells in the microbial biomass. Blue stained regions were found as smaller portions randomly distributed among the grain's surface (Fig. S1). A similar distribution pattern was observed in the internal surface of the grains, with macro-clusters of yeasts distributed within the grain's matrix, essentially composed of polysaccharides and bacteria. Cells stained in red were also observed, likely due to ConA binding to mannose proteins on yeast surfaces. Altogether, CLSM inspection of the grains revealed the maintenance of the structure and relative proportion of microbiota and polysaccharides in the different fermentation conditions. Interestingly, the structure of the grains was found to develop likewise when using cheese whey (whole and deproteinised) and milk as substrate. Therefore, this suggests that the main characteristics of kefir are maintained when using whole and deproteinised cheese whey instead of milk. To deeper evaluate the stability and composition of relevant microbial groups; the microbiota present in the different fermentations was further analyzed using a molecular approach.

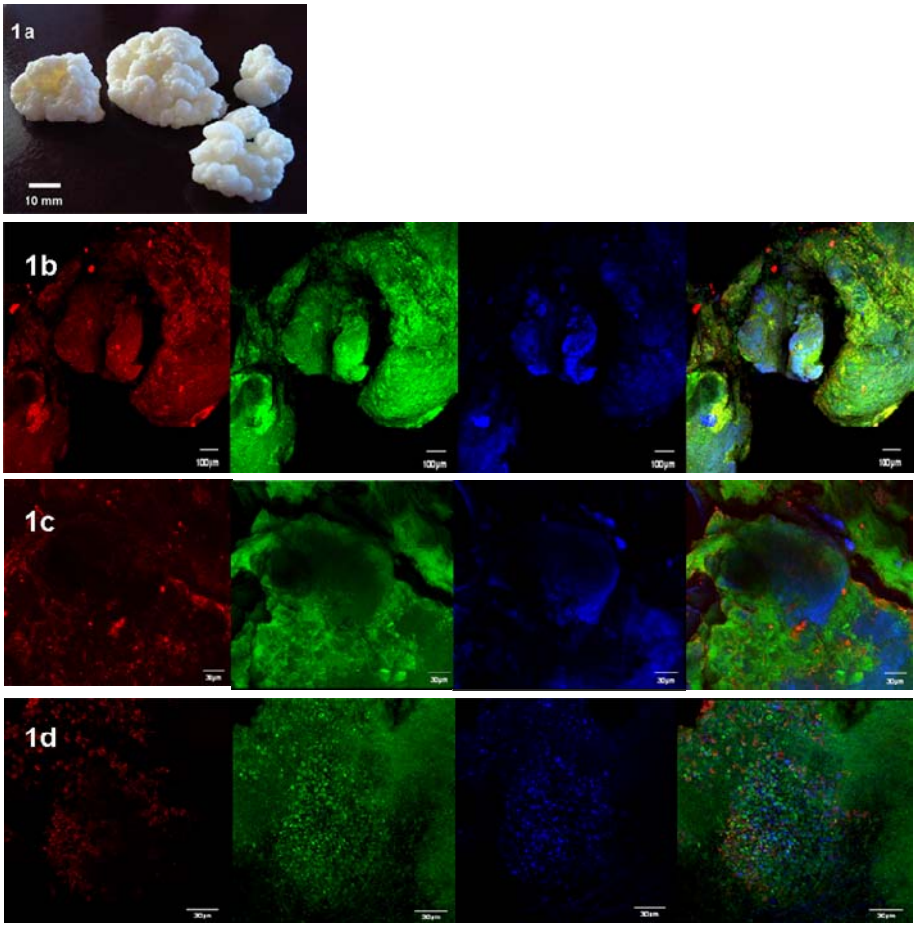

Fig. S1 – Grain kefir under naked-eye examination (1a). Overall view of the distribution of cellular nucleic acids (SYTO 9, green), α-polysaccharides (Con A-Alexa 594, red) and chitin of yeast cell walls (Calcofluor white, blue) obtained by CLSM fluorescence inspection of the outer (1b) and inner (1c and 1d) grain surface. Red: Polysaccharides, Green: DNA (bacteria and yeast), Blue: cellular wall of yeasts

3.3 Evaluation of different primers to assess bacterial and fungal communities in beverage and kefir grains

Although many studies have clearly demonstrated the broad applicability of PCR-DGGE to discriminate among target bacteria, the displayed community profiles can be highly dependent on the PCR primers used (Jianzhong et al., 2009). It has been shown that targeting different rDNA regions may, sometimes, lead to different results in terms of microbial composition. PCR bias (Kanagawa, 2003), co-migration of DNA from different species in the same band (Sekiguchi et al., 2001) and formation of multiple bands in amplification of genes from single genomes (Nübel et al., 1996), may provide incorrect information about dominance and diversity of certain ribotypes in the community. In this study, four of the mostly used primers for PCR-DGGE, were selected to profile microbial communities in fermented products and kefir grains: two primer sets targeting different regions of bacterial 16S rDNA, namely 968fGC/1401r (V6–V8 region) and 338fGC/518r (V3 region), and the primer pairs ITS1/ITS2 and NS3/YM951 targeting fungal ITS (internal transcribed spacer) and 18S rDNA regions, respectively. All the analyzed primer pairs gave satisfactory amplification of the samples. For yeast community, both ITS and 18S rDNA PCR-DGGE analyses yielded the same microbial DGGE profile. Three predominant bands were observed in both gels (Fig. 1).

For bacteria, however, a different profile was generated by the two primer pairs tested. The primer pair 968fGC/1401r, targeting the 16S rDNA V6–V8 regions, yield patterns with two main bands (high intensity) in the microbial profile (Fig. 2a), whereas the pair 338fGC/518r generated profiles with five bands (high intensity), but of similar dominance in the profile (Fig. 2b). Other authors tested the feasibility of different primers pairs for molecular detection of microbial communities. Ercolini et al. (2001) used the primer pair 338fGC/518r

to differentiate and identify lactic acid bacteria (LAB) isolated from food. The analysis of the amplified variable V3 region of the 16S rDNA allowed to differentiate within species of the genera *Enterococcus*, *Lactococcus, Lactobacillus, Pediococcus*, and *Leuconostoc.* However, cases of comigration were also observed, which made it impossible to achieve an unequivocal identification of some species. In another study, the presence of *Leuconostoc* in Stilton cheese could only be detected when targeting the V4–V5 region of the 16S rDNA and not when the V3 region was analyzed (Ercolini et al., 2003). Randazzo et al. (2002) used the 16S rDNA V6–V8 regions to examine the evolution of bacterial community during manufacturing of Ragusano cheese.

This PCR-DGGE analysis was able to successfully identify and differentiate between species of *Lactococcus, Leuconostoc, Streptococcus, Lactobacillus* and *Macrococcus.* Van Beek and Priest (2002) monitored LBA communities during fermentation of Malt whisky by PCR-DGGE of V3 and RT-PCR-DGGE of V6–V8 regions of 16S rDNA. These authors optimized the separation of lactobacilli in DGGE by adopting the V6–V8 region as a target, giving better resolution of several species due to higher heterogeneity in sequences of species from *Lactobacillus.* In a recent study, Magalhães et al. (2010) could not differentiate some species of Lactobacillus by PCR-DGGE migration of fragments of the 16S rDNA V3 region.

Additionally, some individual *Lactobacillus* spp. were found to correspond to more than one band in the DGGE profile, probably due to target sequence heterogeneity among multiple copies of the 16S rDNAs. Multiple bands were also observed in pure culture amplicons produced with the V3 primer pair, but not with the V6–V8, in DGGE profiles of other bacteria species, such as, E. coli, Stenotrophomonas maltophilia and Burkholderia cepacia (Araújo and Schneider, 2008).

Altogether, the results obtained in this work using different pairs of primers, show a stable DGGE profile either in kefir grains or correspondent beverage, under different fermentation conditions (time and/or substrate) suggesting the presence of a robust dominant microbial consortium. This has high industrial relevance in terms of preservation of the properties of the produced beverages.

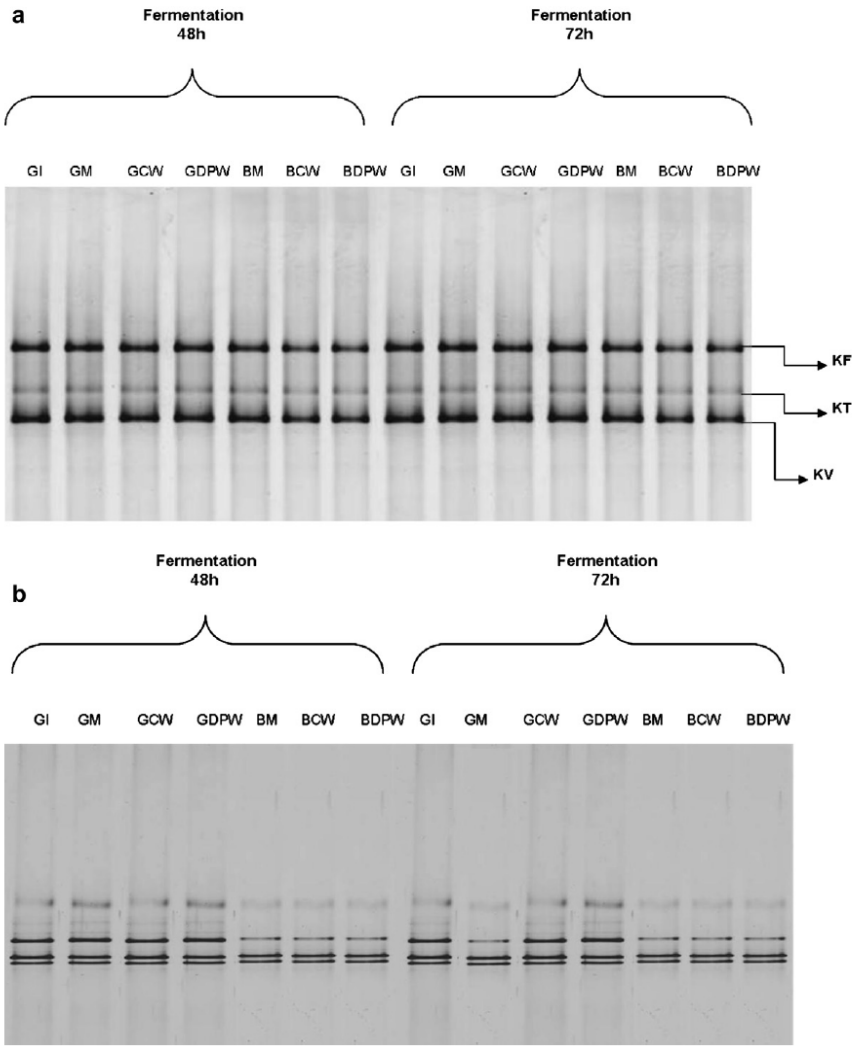

Fig. 1 DGGE profiles of fungal ITS (a) and 18S (b) rDNA fragments amplified from kefir beverages (milk, cheese whey, deproteinised cheese whey) and grains samples. GI = inoculo, GM = grain (fermentation of milk), GCW = grain (fermentation of cheese whey), GDPW = grain (fermentation of deproteinised cheese whey) BM = beverage (fermentation of milk), BCW = beverage (fermentation of cheese whey), DPW = beverage (fermentation of deproteinised cheese whey)

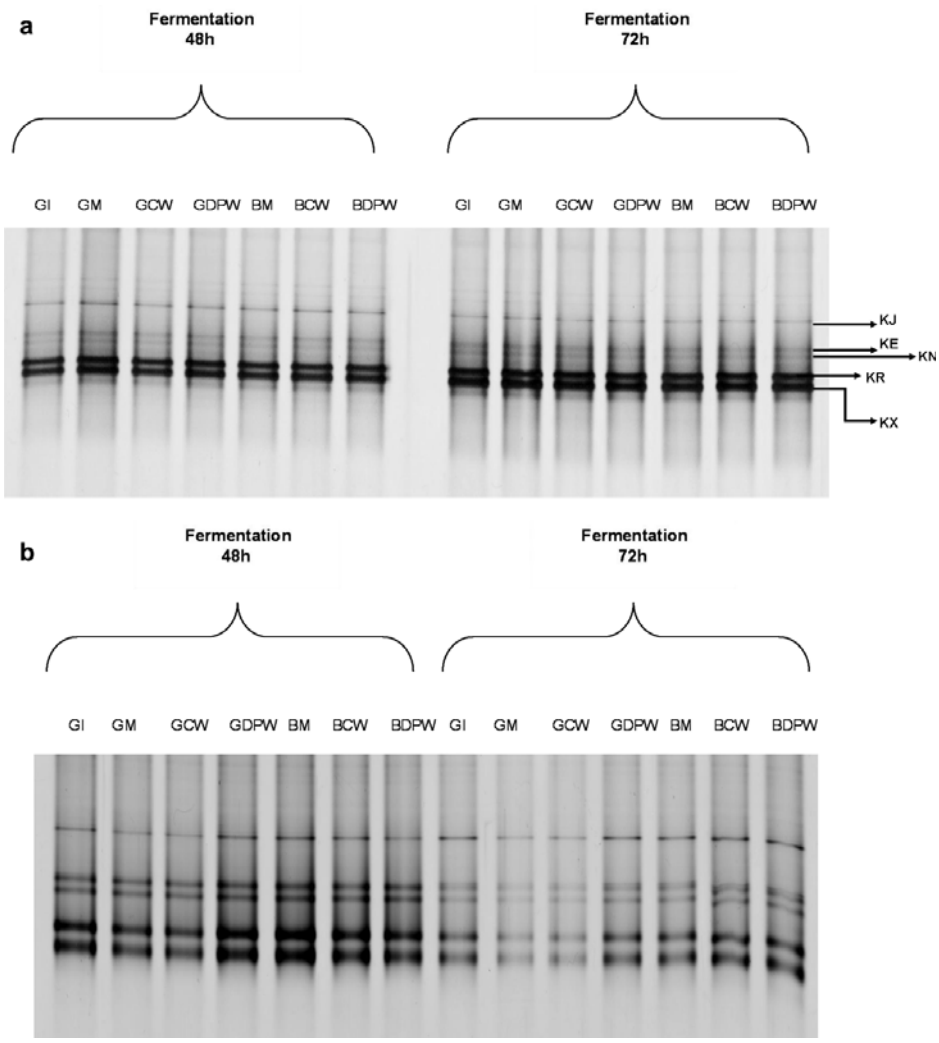

Fig. 2 DGGE profiles of bacterial 16S rDNA V6–V8 regions (a) and V3 region (b) amplified from kefir beverages (milk, cheese whey, deproteinised cheese whey) and grains samples. GI = inoculo, GM = grain (fermentation of milk), GCW = grain (fermentation of cheese whey), GDPW = grain (fermentation of deproteinised cheese whey) BM = beverage (fermentation of milk), BCW = beverage (fermentation of cheese whey), BDPW = beverage (fermentation of deproteinised cheese whey)

3.4 Culture-independent analysis of bacterial and yeast communities

Traditionally, many plating procedures are only partially selective and exclude parts of the microbial community. Thus, in this study the composition of microbiota in kefir grains was evaluated using PCR-DGGE analysis. In addition, the microbial community presents in the fermented beverages obtained from milk, cheese whey and deproteinised cheese whey was also assessed. Representative DGGE fingerprints are shown in Figs. 1 and 2. No differences in community structure were found in all the fermented beverages and kefir grains, suggesting the involvement of the same group of microorganisms in the different fermentations performed. As the ecological conditions remained unchanged, a stable microbiota without changes in species composition could be detected. Furthermore, kefir beverages constitutes an environment characterized by a relatively high pH, produced by LAB – the largest group of bacteria belonging to the kefir microbiota – inhibiting the growth of other groups of microorganisms due to the antimicrobial activity of kefiran. Therefore, only few strains are highly competitive under the prevailing ecological conditions and may persist for decades in continuously propagated fermentative processes (Cheirsilp et al., 2003). Interestingly, a recent study has reported antimicrobial activity of the broth fermented with kefir grains towards common pathogens such as *Candida albicans, Salmonella typhi, Shigella sonnei, Staphylococcus aureus* and *E. coli* (Silva et al., 2009).

To determine the composition of microbiota in grains and kefir beverages (milk, cheese whey and deproteinised cheese whey), nearly full-length bacterial 16S rRNA gene and fungal ITS rDNA fragments were amplified and used to construct clone libraries. Clones containing inserts corresponding to prominent bands in the DGGE profiles were sequenced and the obtained

sequences further compared to sequences deposited in the GenBank database using the NCBI BLAST search program. Table 4 summarizes the obtained similarity search results. Bacterial clones KJ and KR were closest related to *Lactobacillus kefiranofaciens* subsp. k*efirgranum* (98%) and *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* (99%), respectively, whereas KX was affiliated to a yet uncultured bacterium also affiliated to *Lactobacillus.* Bacterial clones KE and KN were not found. They were not recovered for sequencing and may represent other bacterial ribotypes, however with lower PCR amplification efficiency. KE and KN are represented by bands of low intensity in DGGE gel (Fig. 2). Yeast clones, KF, KT and kV were closest related to *K. marxianus* (99%), S. cerevisiae (98%) and *Kazachatania unispora* (99%), respectively. Jianzhong et al. (2009) identified similar species when investigating the microbiota of Tibetan kefir grains by culture independent methods. DGGE of partially amplified 16S rRNA for bacteria and 26S rRNA for yeasts, followed by sequencing of the most intense bands, showed that the dominant microorganisms were *Pseudomonas* sp., *Leuconostoc mesenteroides*, *Lactobacillus helveticus*, *L. kefiranofaciens*, *Lactococcus lactis*, *Lactobacillus kefiri*, *Lactobacillus casei, K. unispora*, *K. marxianus* and *S. cerevisiae*. The bacterial and yeast communities present in three kinds of Tibetan kefir grains, obtained from different regions, showed 78–84% and 80– 92% similarity, respectively. The microorganisms associated with sugary kefir beverage were investigated by Magalhães et al. (2010) using a combination of culture-dependent and independent methods. Bacteria and yeasts were identified via phenotypic and genotypic methods. The bacterial community DNA was amplified with primers 338fGC and 518r spanning the V3 region of the 16S rRNA gene. The yeast community DNA was amplified using the primers NS3 and YM951r. The authors identified similar species when investigating the microbiota of sugary Brazilian kefir beverage. *Lactobacillus paracasei* was the major bacterial isolate identified, followed by

Acetobacter lovaniensis, Lactobacillus parabuchneri, Lactobacillus kefir and *L. lactis. S. cerevisiae* and *K. lactis* were the most common yeast species isolated.

Our data show the presence of *Lactobacillus* in the kefir grains and correspondent fermented beverages. In addition, *L. kefiranofaciens* identified in this study is considered one of the main producers of kefiran polymer (Tada et al., 2007). Previous studies reported a variety of different species of Lactobacillus that have been isolated and identified in milk kefir grains from around the world (Jianzhong et al., 2009). *Lactobacillus* species are important producers of lactic acid. They are probiotics, good at improving the intestinal environment (Jianzhong et al., 2009). The presence of this group in the studied beverages confers a probiotic label to the kefir drinks highlighting its industrial relevance. Based on the DGGE profiles of yeast, a closest relative of the lactose-fermenting yeast *K. marxianus*, was found in this study togetherwith organisms affiliated to non-lactose-fermenting yeast, i.e. *S. cerevisiae* and *K. unispora.* Magalhães et al. (2010) identified similar yeasts species when investigating the microbiota of sugary Brazilian kefir by culture independent and dependent methods.

The yeast flora of sugary kefir was dominated by lactose-negative strains. Among them, *S. cerevisiae* predominated, followed by *Kazachstania aerobia* and *Lachancea meyersii*. *K. marxianus*-related yeast present in this study was, likely, using lactose as carbon source and producing ethanol and carbon dioxide endowing kefir good flavor (Magalhães et al., 2010 and references there in). *S. cerevisiae*-like yeast was detected in this study. The presence of these organisms contributes to the enhancement of organoleptic quality of the kefir beverage, promoting a strong and typically yeasty aroma as well as its refreshing, pungent taste (Magalhães et al., 2010). This yeast also reduces the concentration of lactic acid, removes the hydrogen peroxide and produces compounds that stimulate the growth of other bacteria, thus increasing

the production of kefiran (Cheirsilp et al., 2003). *K. unispora*-like yeast was also detected in this study. Magalhães et al. (2010) affirm that the presence of *Kazachstania* genus yeasts in kefir could be connected with the assimilation of some acids produced by lactic acid bacteria.

In this study, differentiation of the DGGE displayed bacterial and yeast species was possible by using the chosen target rDNA regions. Furthermore, two *Lactobacillus* related sequences were differentiated at the subspecies' level, i.e. *Lactobacillus kefiranofaciens* subsp. *kefirgranum* and *Lactobacillus kefiranofaciens* subsp. *Kefiranofaciens* by targeting the 16SrDNA V6–V8 regions. According to the DGGE profile, members of this specie, considered one of the main producers of kefiran polymer (Tada et al., 2007), were dominant in bacterial community. Compared to other reports, the DGGE displayed dominant bacterial community obtained in this study exhibited much lower diversity at the genus level. Some weaker bands observed on the generated DGGE may represent other bacterial ribotypes, present in lower numbers, or with lower PCR amplification efficiency. Clones with inserts yielding PCR-DGGE fragments corresponding to those faint bands were not found in the screened clone library, hindering further phylogenetic assignment. In spite of specific differences in the microbiota of kefir grains obtained from different origins, the co-existence of a symbiotic association between lactic acid bacteria and yeasts, included in a polysaccharide–protein matrix, enabling lactic-alcoholic fermentation forms the core that characterizes the concept of kefir (Farnworth, 2005). An important probiotic group of bacteria, i.e. *Lactobacillus* spp., is constantly found. Being so, the probiotic properties from whey-based Brazilian kefir beverages found in this study is likely extensible to other kefir beverages.

Table 4 Identification of representative bacterial and yeast clones by sequencing of portions of the 16S rRNA and ITS, respectively

Clone	Species	GenBank accession n°	% Similarity	*E* value
KJ	*Lactobacillus kefiranofaciens* subsp. *kefirgranum*	AB372208.1/FJ749467.1	98	$8e^{-}$ 87
KR	*Lactobacillus kefiranofaciens* subsp. *kefiranofaciens*	AJ575260.1/AJ575259.1	99	0.0
KX	Uncultured bacterium clone IMAU 311/Uncultured *Lactobacillus* sp. Clone 2c	GQ267936.1/EF593063.1	97	$1e^{-}$ 24
KF	*Kluyveromyces marxianus*	AF543841.1/EU019227.1	99	0.0
KT	*Saccharomyces cerevisiae*	AM262831.1/AM262824.1	98	0.0
KV	*Kazachatania unispora*	D89896.1/EU789404.1	99	0.0

4 Conclusions

The present study revealed a consistent grain structure and kefir microbiota when replacing milk with whole/deproteinised cheese whey as fermentation substrate. The dominant microbiota, as revealed by PCR-DGGE, was composed by yeast ffiliated to *K. marxianus, S. cerevisiae, K. unispora*, and bacteria affiliated to the *Lactobacillus* genus. Interestingly, this dominant bacterial community was also found in the fermented beverages, conferring probiotic label to kefir beverages. In addition, the observed microbiota stability is determinant for the implementation of this type of kefir beverages and whey valorization. These results open up perspectives for this innovative application of kefir grains.

Acknowledgements

The authors acknowledge Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), CAPES-GRICES.

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ARTIGO 2

Normas do periódico *Food Chemistry*

Artigo publicado no periódico indexado:
***Food Chemistry* 126 (2010) 249–253**
doi:10.1016/j.foodchem.2010.11.012

Comparative study of the biochemical changes and volatile compounds during the production of novel whey-based kefir beverages and traditional milk kefir

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RESUMO

Soro de queijo (CW) e soro de queijo deproteinizado (DCW) foram investigados para o uso como substrato moderno para a produção de bebidas kefir. Consumo de lactose, produção de ethanol como também ácidos orgânicos e formação de combinações voláteis foram determinados durante a fermentação de CW e DCW por grãos de kefir. Os valores obtidos da fermentação de CW e DCW foram comparados com valores obtidos na fermentação de kefir de leite tradicional. Os resultados mostraram que os grãos de kefir puderam utilizar lactose de CW e DCW e produziram quantidades semelhantes de ethanol (7.8-8.3 g/L), ácido láctico (5.0 g/L) e ácido acético (0.7 g/L) quando comparados para valores obtidos durante a fermentação de leite. Além disso, a concentração de álcoóis superiores (2-metil-1-butanol, 3-metil-1-butanol, 1-hexanol, 2-metil-1-propanol, e 1-propanol), éster (acetato de etila) e aldeído (acetaldeído) foram semelhantes em bebidas kefir de soro de queijo e bebidas kefir de leite. Portanto, soro de queijo e soro de queijo deproteinizado podem servir como substratos para a produção de bebidas kefir semelhantes a tradicional bebida kefir de leite.

Palavras-chave: Bebidas. Soro de queijo. Kefir. Lactose. Leite.

ABSTRACT

Cheese whey (CW) and deproteinised cheese whey (DCW) were investigated for their suitability as novel substrates for the production of kefir-like beverages. Lactose consumption, ethanol production as well as organic acids and volatile compounds formation were determined during CW and DCW fermentation by kefir grains and compared with values obtained during the production of traditional milk kefir. The results showed that kefir grains were able to utilise lactose from CW and DCW and produce similar amounts of ethanol (7.8-8.3 g/L), lactic acid (5.0 g/L) and acetic acid (0.7 g/L) to those obtained during milk fermentation. In addition, the concentration of higher alcohols (2-methyl-1-butanol, 3-methyl-1-butanol, 1-hexanol, 2-methyl-1-propanol, and 1-propanol), ester (ethyl acetate) and aldehyde (acetaldehyde) in cheese whey-based kefir and milk kefir beverages were also produced in similar amounts. Therefore, cheese whey and deproteinised cheese whey may serve as substrates for the production of kefir-like beverages similar to milk kefir.

Keywords: Beverages. Cheese whey. Kefir. Lactose. Milk.

1 Introduction

In the past few years there has been an increased interest in the production of fermented dairy beverages containing probiotics due to several health claims that have been associated with their consumption (Özer & Kirmaci, 2010). Probiotics are usually defined as live microorganisms that when ingested in adequate amounts confer a health benefit on the host (Vasiljevic & Shah, 2008). Many of these microorganisms have been identified as lactic acid-producing bacteria and are usually consumed in the forms of fermented milks, yogurt or kefir (Saarela, Mogensen, Fondén, Mättö, & Mattila-Sandholm, 2000; Zajek & Gorek, 2010).

Kefir is a refreshing, naturally carbonated fermented dairy beverage with a slightly acidic taste, yeasty flavor and creamy consistency (Powell, Witthuhn, Todorov, & Dicks, 2007). The traditional production of kefir is initiated by the addition of small (0.3-3.5 cm in diameter), irregularly shaped, yellowish-white kefir grains to fresh milk (Garrote, Abraham, & De Antoni, 1997; Güzel-Seydim, Seydim, Greene, & Bodine, 2000). Kefir grains are mostly composed by proteins and polysaccharides and enclose a complex microflora. Lactic acid bacteria (LAB) and yeasts exist in a complex symbiotic relationship and are responsible for alcoholic and lactic acid fermentation, respectively. Since kefir grains are able to metabolize lactose, they can be used to ferment cheese whey, a lactose-rich waste of negligible cost (Papapostolou, Bosnea, Koutinas, & Kanellaki, 2008).

Cheese whey, the yellow–green liquid remaining after the precipitation and removal of milk casein during cheese making, has been considered as one of the major problems in the dairy industry. It represents an important environmental pollution, exhibiting a biochemical oxygen demand (BOD) equal to maximum allowable limits of 50,000 mg/L and chemical oxygen demand

(COD) equal to maximum allowable limits of 80,000 mg/l (Siso, 1996). Furthermore, deproteinised cheese whey or whey permeate, the liquid fraction obtained through the ultrafiltration or diafiltration of raw cheese whey, account for more than 70% of total whey solids and is mostly responsible for the whey polluting load. Therefore, this liquid generates disposal problems, in terms of volumes produced and polluting load, almost equal to the disposal of raw whey (Guimarães, Teixeira, & Domingues, 2010).

In recent years, considerable efforts have been undertaken to find new ways of using cheese whey and reduce environmental pollution. The lactose content of cheese whey and the presence of other essential nutrients for microbial growth, make this dairy by-product a potential feedstock for the production of valuable compounds through fermentation processes (Panesar, Kennedy, Gandhi, & Bunko, 2007). Besides bio-ethanol fermentation by *Kluyveromyces marxianus* (Sansonetti, Curcio, Calabrò, & Iorio, 2009; Zafar & Owais, 2006), *Candida pseudotropicalis* (Ghaly & El-Taweel, 1995) and genetically modified *Saccharomyces cerevisiae* yeasts (Domingues, Guimarães, & Oliveira, 2010; Domingues, Lima, & Teixeira, 2001; Guimarães, François, Parrou, Teixeira, & Domingues, 2008), the production of alcoholic beverages, including distilled beverages (Dragone, Mussatto, Oliveira, & Teixeira, 2009) and kefir-like whey beverages (Paraskevopoulou, Athanasiadis, Kanellaki, Bekatorou, Blekas, & Kiosseoglou, 2003), has also been considered as an interesting alternative for cheese whey valorisation.

Recently, we characterized the microbiota of kefir grains and beverages obtained from milk and raw/deproteinised cheese whey using microscopy and molecular techniques (Magalhães, Pereira, Nicolau, Dragone, Domingues, Teixeira, et al., 2010). However, scientific information on chemical changes occurring during cheese whey (mainly deproteinised cheese whey) fermentation by kefir grains is still scarce. Therefore, the objective of this work was for the

first time to evaluate the biochemical changes, organic acids production and volatile compounds formation during deproteinised cheese whey (DCW) fermentation by kefir grains, and compare their performance with that obtained during the production of raw cheese whey (CW) kefir beverage and traditional milk kefir.

2 Materials and Methods

2.1 Kefir grains and inoculum preparation

Kefir grains isolated from Brazilian milk kefir beverages were used in the experiments. The inoculum was prepared by cultivating kefir grains in pasteurized whole milk renewed daily during 7 days. After this time, the grains were washed with sterile distilled water and subsequent, the grains (12.5 g) were inoculated in the different fermentation media.

2.2 Media and fermentation conditions

Pasteurized whole cow's milk as well as CW powder solution and DCW powder solution were used as fermentation media for the production of traditional milk kefir and whey-based kefir beverages, respectively. CW powder solution was prepared by dissolving cheese whey powder (Lactogal, Porto/Portugal) in sterile distilled water to the same lactose concentration as in whole milk (46 g/L). DCW powder solution was obtained by autoclaving the CW powder solution at 115 ºC for 10 min, followed by aseptic centrifugation (2220*g* for 20 min) to remove proteins. Kefir grains were cultivated under static conditions in 1-l Erlenmeyer flasks containing 250 ml of medium at 25 ºC for 48 h. The fermentation runs were assessed through periodic sampling in order to

determine lactose consumption, ethanol and organic acids production as well as volatile compounds formation.

2.3 Proteins determination

The protein content of the different samples was assessed at the beginning and at the end of the fermentation process as the nitrogen content based on the Kjeldahl method (AOAC, 1995). The protein content was calculated by multiplying the total nitrogen by 6.38. All protein contents were expressed as g/L.

2.4 HPLC analysis

Lactose and ethanol were quantified by high performance liquid chromatography (HPLC), using a Jasco chromatograph equipped with a refractive index (RI) detector (Jasco 830-RI). Lactic acid and acetic acid were also quantified by high-performance liquid chromatography (HPLC), using a Jasco chromatograph equipped with UV-Vis detector (Jasco 870-UV-visible) and a Chrompack column (300 x 6.5 mm) at 60 °C, using 5 mM sulfuric acid as the eluent, at a flow rate of 0.5 ml/min and a sample volume of 20 µL.

2.5 GC/FID analysis

Higher alcohols (2-methyl-1-butanol, 3-methyl-1-butanol, 1-hexanol, 2-methyl-1-propanol, and 1-propanol), ester (ethyl acetate) and aldehyde (acetaldehyde) in milk kefir and whey-based kefir beverages were determined by extraction with dichloromethane, and subsequent analysis of the extracts by gas chromatography using a Chrompack CP-9000 gas chromatograph equipped with

a Split/Splitless injector and a flame ionization detector. A capillary column (50 m x 0.25 mm i.d., 0.2 μm film thickness; Chrompack) coated with CP-Wax 57 CB was used. The temperature of the injector and detector was set to 250 ºC. The oven temperature was held at 50 ºC for 5 min, then programmed to run from 50 ºC to 220 ºC at 3 ºC/min and then held at 220 ºC for 10 min. Helium was used as the carrier gas at 125 kPa, with a split vent of 15 ml/min. Injections of 1 μL were made in the splitless mode (vent time, 15 s); 4-nonanol (internal standard) was added to the sample to give a final concentration of 122.05 mg/L. The volatile compounds were identified by comparing retention indices with those of standard compounds. Quantification of volatile compounds was performed with the Varian Star Chromatography Workstation software (Version 6.41) and expressed as 4-nonanol equivalents, after determining the detector response factor for each compound.

2.6 Statistical analysis

Each fermentation was carried out in duplicate and mean values are reported. The Tukey's test using Statgraphics Plus for Windows 4.1 software (Statistical Graphics Corp., 1999) was performed to evaluate statistical significance of differences between the beverages and to compare the means among the samples.

3 Results and discussion

3.1 Fermentation performance of kefir grains cultivated in milk, CW and DCW

Fig. 1 shows the time evolution of lactose and ethanol during the fermentation of milk, CW and DCW by kefir grains. It can be observed that most of the lactose present in milk was metabolized within 48 h, resulting in the formation of 8.65 g/L (1.1%) ethanol. Similar results were reported earlier by Papapostolou, Bosnea, Koutinas, & Kanellaki, (2008) during lactose fermentation at 30 ºC by thermally dried kefir cells by conventional drying method at 38 ºC. On the other hand, the use of CW and DCW as substrates for the production of a whey-based beverage resulted in lower lactose consumption than that observed during milk fermentation.

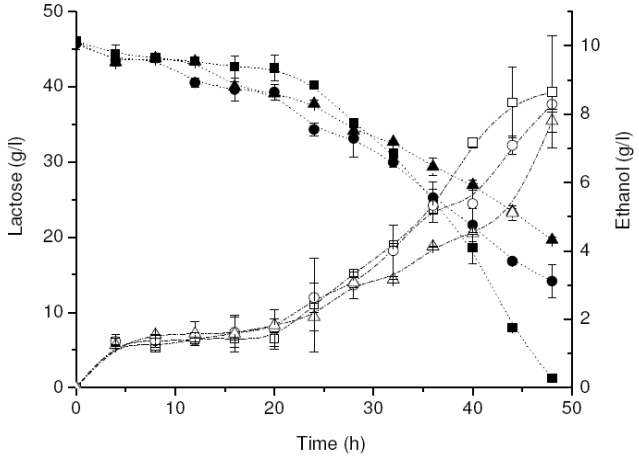

Fig. 1 Lactose consumption (closed symbols) and ethanol production (open symbols) during kefir grains cultivation at 25 ºC using milk (square), cheese whey (circle) and deproteinised cheese whey (triangle) as substrates. Bars represent standard deviation

The higher lactose utilisation during milk fermentation by kefir grains could probably be due to the characteristics of milk that, being richer in nutrients (primarily proteins) than CW and DCW (Table 1), allowed an improved growth for microorganisms. This Table also shows that despite the higher lactose

Table 1 Lactose consumption, ethanol production, ethanol yield factor ($Y_{P/S}$)[a], protein utilisation and increment of kefir grains weight after 48 h of kefir grains cultivation in different fermentation media

Media	Lactose consumption (g/L)	Ethanol production (g/L)	$Y_{P/S}$	Initial protein conc. (g/L)	Final protein conc. (g/L)	Initial kefir grains weight (g)	Final kefir grains weight (g)
Milk	44.8a	8.7a	0.19	34.7a	0	12.5	15.0a
Cheese whey	31.5b	8.3a	0.26	19.1b	0	12.5	14.1b
Deproteinised cheese whey	26.4c	7.8a	0.30	0	0	12.5	14.2b

[a]$Y_{P/S}$ was defined as the ratio between the ethanol concentration (g/l) and lactose consumed (g/l)
Means within the same column with different letters are statistically different at 95% confidence level

consumption during milk fermentation, there was no statistically significant difference ($p<0.05$) among the final ethanol concentrations in the three beverages. A higher lactose utilisation for cell growth could explain the lower ethanol yield obtained at the end of milk fermentation by kefir grains.

The final ethanol concentrations (8.7±1.6 g/L, 8.3±0.2 g/L and 7.8±0.3 g/L for milk kefir, CW-based kefir and DCW-based kefir, respectively) were within the range of ethanol contents (0.5% v/v (3.9 g/L) – 2.4% (18.9 g/L)) reported previously by Papapostolou, Bosnea, Koutinas, & Kanellaki, (2008) for the production of kefir using lactose and raw cheese whey as substrates. Although yeasts such as *Kluyveromyces* sp. are primarily responsible for the conversion of lactose to ethanol during kefir fermentation, some heterofermentative bacteria (e.g. *Lactobacillus kefir*) are also capable of producing ethanol (Güzel-Seydim, Seydim, Greene, & Bodine, 2000). The presence of *K. marxianus* and *Lactobacillus kefiranofaciens* in grains and kefir beverages (milk, CW and DCW) were recently identified by our group using culture-independent methods (PCR-DGGE) (Magalhães et al., 2010).

The mean changes in pH values during cultivation of kefir grains in the three different substrates are depicted in Fig. 2. A sharp decrease in the pH was observed during the first 28 h, from an initial value of about 6.1 to 4.3 at 28 h for all the substrates. Afterwards, the pH decreased slightly, reaching a final value of nearly 4.0. After 48 h of incubation, pH values of the fermented milk kefir and whey-based beverages were not significantly different ($p<0.05$). These pH values were similar to those previously reported for milk kefir (García Fontán, Martínez, Franco, & Carballo, 2006). Athanasiadis, Paraskevopoulou, Blekas, & Kiosseoglou, (2004) suggested an optimal pH of 4.1 for a novel beverage obtained from cheese whey fermentation by kefir granules. According to these authors the flavour of the fermented product was improved at a final pH value of

4.1 due to the higher profile of volatile by-products than for other final pH values.

Production of lactic acid has been linked with lactic acid bacteria metabolism and is of great importance due to its inhibitory effect on both spoilage and pathogenic microorganisms in kefir milk (Magalhães, de M. Pereira, Dias, & Schwan, 2010). As expected, while the pH decreased, the lactic acid concentration increased progressively during milk, CW and DCW fermentations, from a mean value of 0.5 g/L at 0 h to 5.0 g/L at 48 h. This agrees with the finding of Güzel-Seydim, Seydim, Greene, & Bodine, (2000) that kefir has a lower lactic acid content than yogurt (8.8 – 14.6 g/L) probably due to the preferential use of the heterofermentative pathway rather than the homofermentative pathway, with a resultant production of CO_2.

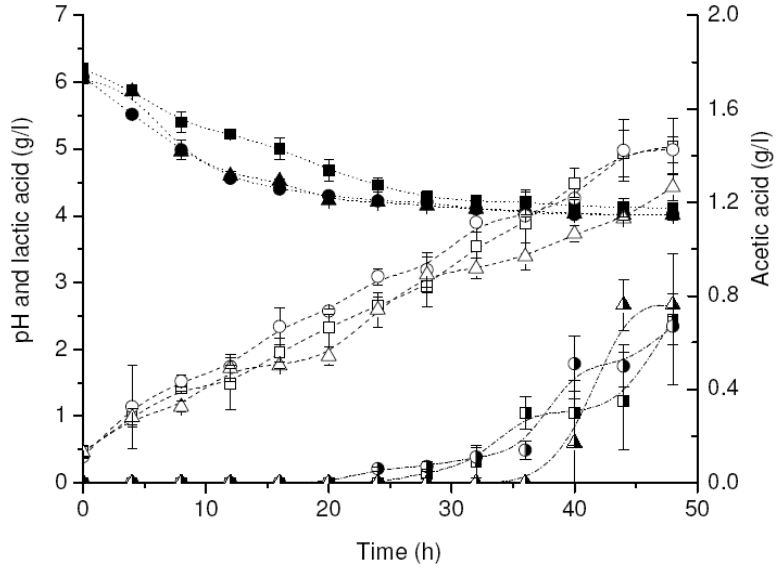

Fig. 2 Time evolution of pH (closed symbols), lactic acid (open symbols) concentration and acetic acid (half-closed symbols) concentration during milk (square), cheese whey (circle) and deproteinised cheese whey (triangle) fermentation by kefir grains. Bars represent standard deviation

The mean concentration of acetic acid was practically zero during the first 24 h of milk, CW and DCW fermentation (Fig. 2), and then increased slightly during the period from 24 to 48 h, reaching a final concentration of 0.7 g/L; this value is similar to those observed by other authors (Rea, Lennartsson, Dillon, Drinan, Reville, Heapes, et al., 1996) during skim milk fermentation by different Irish kefir grains. The presence of acetic acid in the fermented beverages could be attributed to heterofermentative lactic acid and acetic acid cultures present in kefir grains microflora (Magalhães, de M. Pereira, Dias, & Schwan, 2010).

3.1 Volatile by-products identified by GC-FID

Volatile compounds are important contributors to the flavours of beverages, as they determine different desirable sensory characteristics (Arrizon, Calderón, & Sandoval, 2006). Previous studies have shown that the formation of volatile higher alcohols and esters during kefir fermentation is influenced by the composition of the medium (Athanasiadis, Boskou, Kanellaki, & Koutinas, 2001). In our study, a total of 7 flavour-active compounds, including 5 higher alcohols, 1 ester and 1 aldehyde, were identified by gas chromatography coupled with flame ionization detection (GC-FID), and analysed during 48 h of kefir grains cultivation in different media (milk, CW and DCW).

The evolution of each group of volatile compounds during the production of milk kefir and whey-based kefir beverages are illustrated in Fig. 3 and Fig. 4. The higher alcohols identified during milk, CW and DCW fermentations were 2-methyl-1-butanol (active amyl alcohol), 3-methyl-1-butanol (isoamyl alcohol), 1-hexanol (hexyl alcohol), 2-methyl-1-propanol (isobutyl alcohol), and 1-propanol (propyl alcohol) (Fig. 3a, b and c). The levels

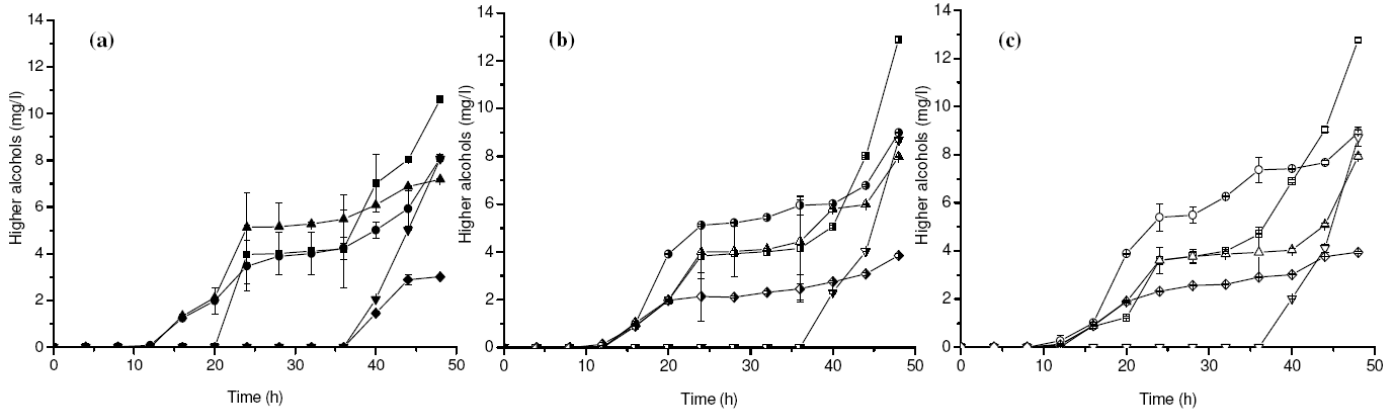

Fig. 3 Formation of higher alcohols - 2-methyl-1-butanol (square), 3-methyl-1-butanol (circle), 1-hexanol (down-triangle), 2-methyl-1-propanol (up-triangle), and 1-propanol (lozenge) - during kefir grains cultivation, using: **(a)** milk, **(b)** cheese whey and **(c)** deproteinised cheese whey as substrates. Bars represent standard deviation

of these alcohols increased from the beginning until the end of the fermentation period for the three different substrates.

The volatile higher alcohol identified, 2-methyl-1-butanol, attained the highest concentration at the end of CW and DCW fermentations (12.8-12.9 mg/L) and milk fermentation (10.6 mg/L). This volatile compound is produced during the catabolism of the branched chain amino acid (BCAA) isoleucine, or is synthesized de novo during the biosynthesis of the BCAA (Schoondermark-Stolk, Jansen, Veurink, Verkleij, Verrips, Euverink, et al., 2006). Therefore, the higher concentration of 2-methyl-1-butanol in the whey-based beverages could be related with the higher isoleucine content in CW (0.31-0.69 mg / 100 g powder; (Mavropoulou & Kosikowski, 1973)) in comparison with that found in milk (0.14±0.08 mg / 100 g milk; (Albert, Mándoki, Csapó-Kiss, & Csapó, 2009)). To our knowledge, no previous scientific results are available concerning the presence of 2-methyl-1-butanol in kefir beverages obtained from deproteinised cheese whey (0.12±0.01 mg / 100 g).

Despite the different evolution patterns observed for 1-hexanol and 3-methyl-1-butanol (Fig. 3), both higher alcohols achieved similar concentrations (nearly 9 mg/L) at the end of fermentation, for the different substrates. This alcohols has a positive influence on the aroma of the fermented beverage when it occurs in concentrations up to 20 mg/L. On the contrary, increased concentration of this alcohols, having an volatile description of "coconut-like", "harsh" and "pungent", can contribute negatively to the product aroma (Gómez-Míguez, Cacho, Ferreira, Vicario, & Heredia, 2007; Dragone, Mussatto, Oliveira, & Teixeira, 2009).

Within the group of higher alcohols, 1-propanol, associated with ripe fruit and alcohol aromas, showed the lowest concentration in the different fermented beverages. The final content of this compound in milk kefir (3.0

mg/L) was lower than those found in whey-based kefir beverages (3.9 mg/L). However, these values were well below the odour threshold of 306 mg/L (Peinado, Mauricio, & Moreno, 2006). Similar levels of 1-propanol were also reported in the continuous fermentation of raw cheese whey using delignified cellulosic-supported kefir yeast at 27 ºC (Kourkoutas, Psarianos, Koutinas, Kanellaki, Banat, & Marchant, 2002).

Only one ester characterized by fruity attributes, namely ethyl acetate, was detected during milk, CW and DCW fermentations by kefir grains. The concentration of this volatile compound increased slowly up to 36 h, and then increased markedly until the end of fermentation (Fig. 4a). No statistically significant differences ($p<0.05$) were found in the final concentrations of ethyl acetate (9.7-11.5 mg/L) in the different fermented beverages, using milk, CW and DCW as substrates. Kourkoutas, Psarianos, Koutinas, Kanellaki, Banat, & Marchant, (2002) showed that kefir yeasts immobilized on delignified cellulosic material were capable of producing ethyl acetate from raw cheese whey in a wide range of concentrations (from traces to 95 mg/L). According to these authors, such concentrations are typical of fermented beverages.

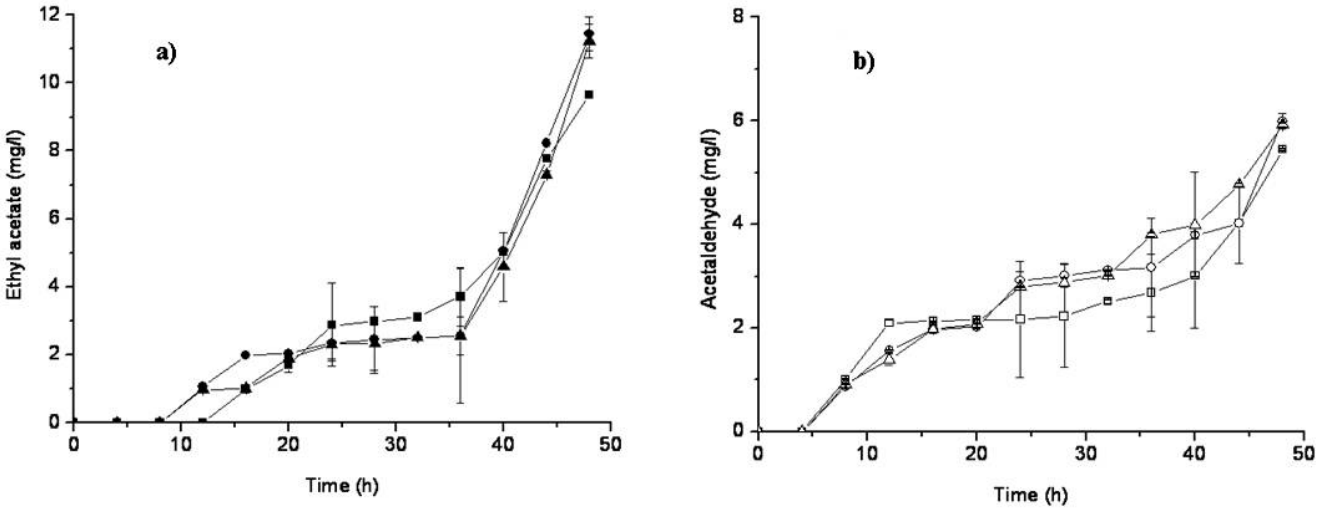

Fig. 4 a) Production of ethyl acetate (closed symbols) and b) acetaldehyde (open symbols) during milk (square), cheese whey (circle) and deproteinised cheese whey (triangle) fermentation by kefir grains. Bars represent standard deviation

Acetaldehyde, which imparts nutty and pungent aromas, was found in milk kefir and whey-based kefir beverages at low concentrations (6.0 mg/L) after 48 h of fermentation (Fig. 4b). These results were consistent with those reported by Ertekin & Güzel-Seydim, (2010) for whole and non-fat milk kefir fermented at 25 ºC during 18±2 days and stored at 4 ºC for 1 day. According to these authors, acetaldehyde is considered the major yogurt-like flavour in fermented milks. Acetaldehyde can be formed by group N streptococci. These microorganisms degrade lactose to galactose and glucose. According to Geroyiannaki et al. (2007) the glucose can be metabolized by the homofermentative Embden-Meyerhof-Parnas pathway to pyruvate, where 2 mol of lactate are formed per glucose molecule. Residual pyruvate, catalyzed by an □-carboxylase, is then converted to diacetyl and acetaldehyde. An aldehyde dehydrogenase may also generate acetaldehyde from acetyl-CoA which is formed from pyruvate by the action of a pyruvate dehydrogenase. Nitrogen metabolism can also result in acetaldehyde formation. Threonine aldolase catalyzes the c1eavage of the amino acid threonine to acetaldehyde and glycine (Zourari, Accolas, & Desmazeaud, 1992).

4 Conclusion

Although a lower lactose utilisation was observed during the production of the cheese-whey based beverages in comparison with that obtained during the traditional cultivation of kefir grains in milk, no significant differences were found among samples at the end of the fermentations when considering final ethanol content, pH, lactic acid and acetic acid concentrations as well as major volatile formation. Therefore, the results of the present study provided evidence indicating that cheese whey and deproteinised cheese whey may serve as substrates for the production of kefir-like beverages similar to milk kefir. The

use of desproteinised cheese whey as substrate in kefir fermentation processes can be considered as a new whey valorisation strategy.

Acknowledgements

The authors acknowledge the financial support from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), CAPES-GRICES and Lactogal for supplying cheese whey powder.

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ARTIGO 3

Normas do periódico *International Journal of Food Science and Technology*

Artigo publicado no periódico indexado:
***International Journal of Food Science and Technology* In Press**
doi:10.1111/j.1365-2621.2011.02570.x

Chemical composition and sensory analysis of cheese whey-based beverages using kefir grains as starter culture

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RESUMO

O objetivo do presente trabalho foi avaliar o uso de grãos de kefir como cultura iniciadora para a produção da tradicional bebida kefir de leite e bebidas kefir de soro de queijo. A fermentação foi conduzida inoculando grãos de kefir em leite (ML), soro de queijo (CW) e soro de queijo desproteinizado (DCW). Os Erlenmeyers contendo os grãos de kefir com diferentes substratos foram incubados estaticamente por 72 h a 25°C. Lactose, etanol, ácido acético, ácido lático, acetaldeído, acetato de etila, álcool isoamil, isobutanol, 1-propanol, álcool isopentil e 1-hexanol foram quantificados por HPLC e GC-FID. Os resultados mostraram que os grãos de kefir puderam utilizar toda a lactose em 60 h de fermentação do ML e 72 h nas fermentações de CW e DCW. Os grãos de kefir produziram quantias semelhantes de etanol (~12 g L^{-1}), ácido lático (~6 g L^{-1}) e ácido acético (~1.5 g L^{-1}), quando se comparou a fermentação do leite com soro de queijo e soro de queijo desproteinizado. Baseado nas características químicas e aceitação por análise sensorial, os grãos de kefir mostraram potencial para serem usados para a produção de bebidas de soro de queijo.

Palavras-chave: Kefir. Bebida de soro de queijo. HPLC. GC-FID. Avaliação sensorial. Bactéria ácido lática. Leveduras.

ABSTRACT

The aim of the present work was to evaluate the use of the kefir grains as a starter culture for tradicional milk kefir beverage and for cheese whey-based beverages production. Fermentation was performed by inoculating kefir grains in milk (ML), cheese whey (CW) and deproteinised cheese whey (DCW). Erlenmeyers containing kefir grains and different substrates were statically incubated for 72 h at 25°C. Lactose, ethanol, lactic acid, acetic acid, acetaldehyde, ethyl acetate, isoamyl alcohol, isobutanol, 1-propanol, isopentyl alcohol and 1-hexanol were identified and quantified by HPLC and GC-FID. The results showed that kefir grains were able to utilise lactose in 60 h from ML and 72 h from CW and DCW and produce similar amounts of ethanol (~12 g L^{-1}), lactic acid (~6 g L^{-1}) and acetic acid (~1.5 g L^{-1}) to those obtained during milk fermentation. Based on the chemical characteristics and acceptance in the sensory analysis, the kefir grains showed potential to be used for developing cheese whey-based beverages.

Keywords: Kefir. Cheese whey beverage;.HPLC. GC-FID. Sensory evaluation. Lactic acid bacteria. Yeasts.

1 Introduction

Cheese whey is the major by-product of the dairy industry and its disposal without expensive sewage treatments represents a major source of environmental pollution. Considerable efforts have been made over the past years to explore new outlets for cheese whey utilization and reduce environmental pollution (Magalhães *et al.,* 2010a). Besides potable ethanol production by lactose converting microorganisms (Guimarães *et al.,* 2010) and the production of distilled beverages (Dragone *et al.,* 2009) and kefir-like cheese whey beverages (Magalhães *et al.,* 2010a) this by-product has also been suggested as an alternative for industrial residue utilization which may reduce environmental pollution.

Traditionally kefir grains have been used in many countries, especially Eastern Europe, as a natural starter in the production of kefir, a unique self-carbonated dairy beverage. Kefir differs from other fermented milks in its starter, which exists in the form of grains (Simova *et al.,* 2002). Kefir grains contain lactic acid bacteria (LAB) including *Lactobacillus, Lactococcus, Leuconostoc* and *Streptococcus* spp. and yeasts (*Kluyveromyces, Torula, Candida* and *Saccharomyces* spp.). Both the bacteria and yeast are surrounded by a polysaccharide matrix, called kefiran, which is a water-soluble branched glucogalactan (Magalhães *et al.,* 2010b).

The production of a functional beverage produced upon whey fermentation by kefir grains could be an interesting alternative for cheese whey utilization. Cheese whey fermentation by kefir microrganisms could decrease the high lactose content in cheese whey, producing mainly lactic acid and other metabolites such as aroma compounds contributing to the flavour and texture and increasing carbohydrate solubility and sweetness of the end product. Manufacture of beverages through lactic fermentations can provide desirable

sensory profiles and have already been considered an option to add value to cheese whey (Pescuma *et al.*, 2008).

Recently, Magalhães *et al.* (2010a) set out to characterize kefir-associated microbiota by using two unrelated techniques, DNA analysis (by denaturing gradient gel electrophoresis (DGGE)) and optical microscopy (combining fluorescence staining with confocal laser microscopy). The composition of microbiota was related to *Lactobacillus kefiranofaciens* subsp. *kefirgranum, Lactobacillus kefiranofaciens* subsp. *kefiranofaciens*, an uncultured bacterium related to the genus *Lactobacillus, Kluyveromyces marxianus*, *Saccharomyces cerevisiae* and *Kazachstania unispora*. No differences were found in the community structure detected in the analyzed beverage and Brazilian kefir grains, showing that microbiota of kefir grains is highly stable along the fermentations carried out in different substratum. However, this characterization was restricted to the microbiota and until recently, we were not aware of any reports concerning the chemical and sensorial characterization of these beverages. Therefore, the aim of the present work was to evaluate the use of the kefir grains as a starter culture for traditional milk kefir beverage and for cheese whey-based beverages production, besides evaluating the biochemical changes, organic acids production and volatile compounds formation during fermentation process.

2 Materials and methods

2.1 Cheese whey-based and milk fermentation media

Three different substrates containing lactose concentration of 46 g L^{-1} were used as fermentation media: pasteurized full cows milk (ML), cheese whey (CW) and deproteinised cheese whey (DCW). Cheese whey powder, obtained

from a regional dairy industry ((Lactogal, Porto/Portugal), was dissolved in sterile distilled water until the desired lactose concentration. Deproteinised cheese whey was made by autoclaving at 115°C for 10 min the cheese whey solution, followed by aseptic centrifugation (2220 xg for 20 min) to remove cream. Confirmation of cheese whey deproteinisation was accomplished for Kjeldahl method.

2.2 Kefir beverages production

Brazilian kefir grains were employed in the present study. The grains (12.5g) were washed with sterile distilled water and inoculated in 250 ml of ML, CW and DCW. The milk is used commonly for kefir beverage, therefore was used to compare the fermentation with cheese whey kefir beverages. Erlenmeyers containing kefir grains were statically incubated for 72 h at 25°C. Samples of the kefir beverages were aseptically taken every 12 h for analysis of organic acids, ethanol, sugars and volatile flavor substances. Determination of total reducing sugars was used to assess the substrate consumption. The pH of the fermented beverages was measured using a Micronal B474 pH meter. Two replicates were done in each fermentation batch.

2.3 Analytical methods

2.3.1 Chemicals

1-Hexanol and ethyl acetate were purchased from Aldrich Chemistry (Munich, Germany). 1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol were purchased from Fluka Analyticals (Seelze, Germany). Ethyl acetate, Acetaldeyde lactose, were purchased from Sigma-Aldrich (Saint

Luis, EUA) and acetic acid, lactic acid, ethanol, methanol were purchased from Merck (Darmstadt, Germany).

2.3.2 Organic acids, sugars and ethanol

Lactose and ethanol were quantified by high-performance liquid chromatography (HPLC), using a Jasco chromatograph equipped with a refractive index (RI) detector (Jasco 830-RI). Lactic acid and acetic acid were also quantified by high-performance liquid chromatography (HPLC), using a Jasco chromatograph equipped with UV-visible detector (Jasco 870-UV-visible). A Chrompack column (300 x 6.5 mm) at 60°C, using 5 mM sulfuric acid as the eluent, at a flow rate of 0.5 ml/min and a sample volume of 20 µl was used.

2.3.3 Volatile flavor substances

In order to identify the volatile compounds, the kefir beverages were analyzed directly without any previous treatment according to Fraile *et al.* (2000). A Chrompack CP-9000 gas chromatograph equipped with a Split/Splitless injector, a flame ionization detector, and a capillary column (50 m x 0.25 mm i.d., 0.2 µm film thickness; Chrompack) coated with CP-Wax 57 CB was used. The temperature of the injector and detector was set to 250ºC. The oven temperature was held at 50ºC for 5 min, then programmed to run from 50ºC to 220ºC at 3ºC min^{-1} and then held at 220ºC for 10 min. Helium was used as the carrier gas at 125 kPa, with a split vent of 15 ml min^{-1}. Injections of 1 µL were made in the splitless mode (vent time, 15 s); 4-nonanol (internal standard) was added to the sample to a final concentration of 122.05 mg L^{-1}. The volatile compounds were identified by comparing the retention times of the samples with those of standard compounds. Quantification of volatile compounds was

performed with Varian Star Chromatography Workstation software (Version 6.41) and expressed as 4-nonanol equivalents, after determining the detector response factor for each compound.

2.4 Sensory evaluation

The final kefir beverages (ML, CW and DCW) were evaluated by 25 untrained tasters, males and females, 25-35 years of age (students of the Centre of Biological Engineering, University of Minho, Campus Gualtar, Braga, Portugal). Randomized, refrigerated (10°C) samples of 10 mL were served (containing 1.0 mg of sucrose) in clear, tulip-shaped glasses with a volume of 50 mL; these were marked with three digit random numbers and covered with Petri dishes. Distilled water was provided for rinsing of the palate during the testing. Tasters were asked to indicate how much they liked or disliked each product on a 9-point hedonic scale (9 = like extremely; 1 = dislike extremely) according to colour, odour, aroma, appearance, taste and overall acceptability characteristics.

2.5 Statistical analysis

Statistical analysis was carried out with Statistica software version 9.0 (StatSoft Inc., Tulsa, OK, USA). Principal component Analysis (PCA) was used to summarize the information in a reduced number of principal components. A one-way ANOVA was performed for chemical parameters, concentration of volatile compounds values to determine significant differences ($P<0.05$) by using the Duncan's multiple range test using the SPSS version 10.0.

3 Results and discussion

3.1 Microbial metabolites

Milk and cheese whey-based kefir beverages were monitored during the 72 h fermentation period by determining the acidity. During the 72 h of incubation, pH values of the fermented milk kefir and whey-based beverages ranged from 6.1 to 3.9, not finding significant differences for all the substrates ($p<0.05$) (*data not shown*). These pH values were similar to those previously reported for kefir beverage (Magalhães *et al.* 2010b). pH is an important factor that can strongly affect the quality of a beverage (Sharma *et al.,* 2009). Furthermore, pH values of the fermentation broth significantly influence the fermentation time of lactose and the levels of volatile compounds, reflecting possible variations in the sensory characteristics of the final product (Athanasiadis *et al.,* 2004).

High performance liquid chromatography (HPLC) was used to analyze organic acids, ethanol e sugars in the produced kefir beverages. The Figs. 1, 2 and 3 shows the concentration of sugars, organic acids and ethanol obtained by ML, CW and DCW fermentation. The production process of organic acids and alcohol was followed by the lactose consumption in kefir beverages (ML, CW and DCW). The total lactose consumption was observed in 60 h in the ML fermentation and 72 h in the CW and DCW fermentation (Figs. 1, 2 and 3). This likely reflects an adaptation period of the microbial community to the whole and deproteinised cheese whey as kefir grains are fermented in milk commonly. Lactose readily degraded to galactose and glucose by Group N streptococci, *Lactobacillus* and by some strains of *Kluyveromyces* (Güzel-Seydim *et al.,* 2000).

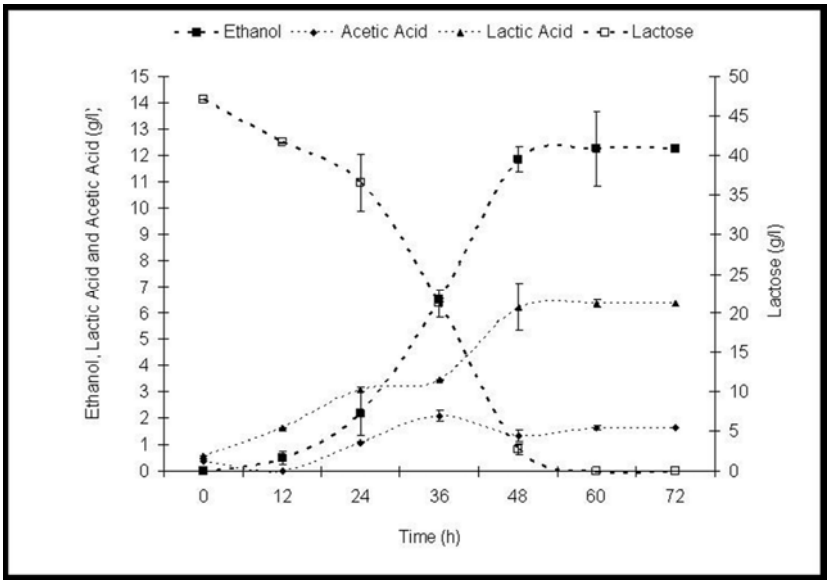


Figure 1 Chemical parameters of the fermentation process of milk kefir beverage

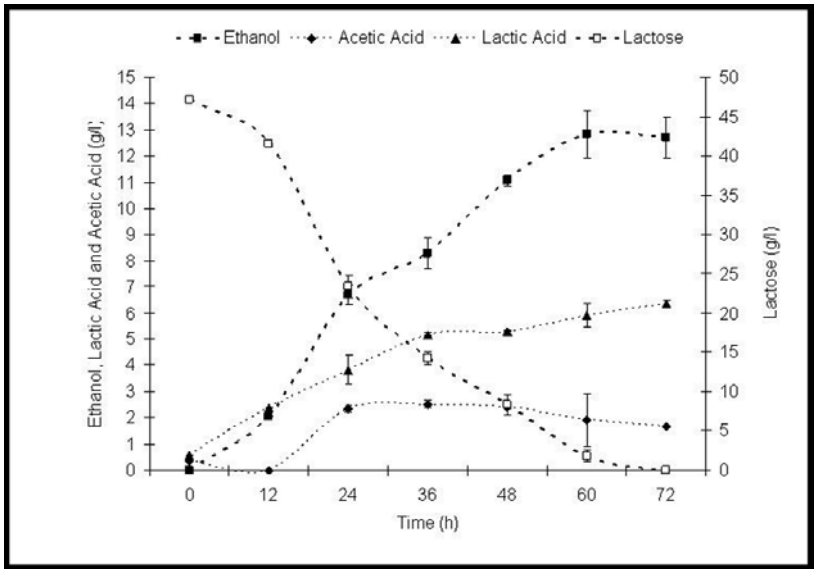


Figure 2 Chemical parameters of the fermentation process of cheese whey kefir beverage

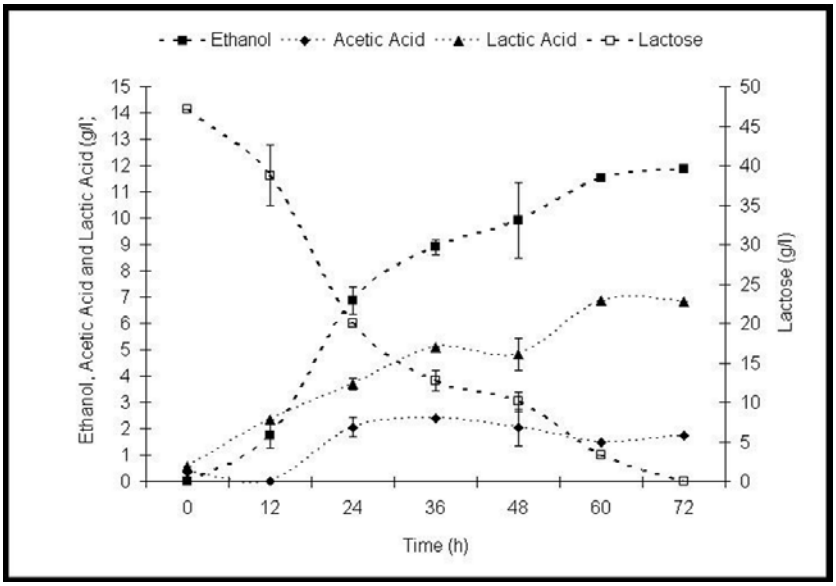


Figure 3 Chemical parameters of the fermentation process of deproteinised cheese whey kefir beverage

In the present work, the lactic acid content increased during the 72 h of fermentation process in kefir beverages, reaching maximum value of 6.35 g L^{-1}, 6.34 g L^{-1} and 6.81 g L^{-1} in ML, CW and DCW kefir beverages, respectively (Figs. 1, 2 and 3). The fermentation of lactose by lactic acid bacteria present in kefir culture can be associated with the increase in lactic acid production through the hydrolysis of sugars released from the glycomacropeptide of casein as well as the glycoproteins associated with the fat globule membrane (Rynne *et al.,* 2007). Acetic acid was also formed during the fermentation process of kefir beverages (ML, CW and DCW), reaching maximum value of ~1.5 g L^{-1} (Figs. 1, 2 and 3). The acetic acid was formed probably by heterolactic bacteria, previously identified in Brazilian kefir beverages (Magalhães *et al.,* 2010b). These results are of great importance since lactic acid and acetic acid provides

pleasant taste and inhibits the development of undesirable or pathogenic microorganisms, due to the substrate acidity increase (Magalhães *et al.,* 2010b.).

Ethanol concentration increased during the kefir fermentation process in all three kefir beverages, reaching maximum concentration of 12.26 g L^{-1}, 12.72 g L^{-1} and 11.86 g L^{-1} in ML, CW and DCW kefir beverages, respectively (Figs. 1, 2 and 3). *Saccharomyces cerevisiae*, previously identified in kefir beverages (Magalhães *et al.,* 2010a), which exhibits strong fermentative metabolism and tolerance to ethanol, is primarily responsible for the alcohol production (Pereira *et al.,* 2010). However, some bacteria from the genus *Lactobacillus* also have the ability to produce ethanol, since they have alcohol-dehydrogenase activity, an enzyme able to convert acetaldehyde to ethanol (Magalhães *et al.,* 2010b). The content of alcohol should be enough to give kefir the flavour of a light alcoholic beverage that is typical of traditional (ancient) kefir of the Caucasus and the yeast aroma ensures the specificity of this type of fermented beverage (Beshkova *et al.,* 2003).

3.2 Aroma-related compounds

Lactic acid bacteria present in kefir grains starter cultures produce a plethora of enzymes that contribute to the formation of volatiles via proteolysis, lipolysis and carbohydrate degradation during ripening. Such enzymes are peptidases which are involved in the transformation of casein into free amino acids which are further degraded to volatile aroma compounds. Other enzymes are esterases and lipases that hydrolyze triglycerides of dairy of fat in free fatty acids (Dragone *et al.,* 2009). GC/FID analysis was employed to determine volatile compounds in kefir beverages (ML, CW and DCW) during 72 h fermentation process. The Table 1 shows the results of the following aroma forming compounds produced in the kefir beverages. Isoamyl alcohol (3-methyl-

1-butanol), isobutanol (2-methyl-1-propanol), 1-propanol, isopentyl alcohol (2-methyl-1-butanol) and 1-hexanol were the alcohols found in the kefir beverages (ML, CW and DCW) (Table 1). The identified ester is represented for ethyl acetate, while among the aldehyde group, acetaldehyde was found in kefir beverages. According to some authors (Apostolopoulou *et al.,* 2005), ethyl esters (mainly ethyl acetate), alcohols with three or more carbon units, and acetaldehyde, are the major agents responsible for the flavour of fermented beverages.

Ethyl acetate has a significant effect on the organoleptic characteristics of fermented beverages. The presence of this ester results in a pleasant aroma with fruity properties, but can turn vinegary at levels above 150 mg L^{-1}, adding spoilage notes to the beverage (Falqué *et al.,* 2001). Thus, the ethyl acetate concentration in kefir beverages (8.18 mg L^{-1}, 8.27 m gL^{-1} and 8.38 mg L^{-1} in ML, CW and DCW, respectively) was found at a level suitable to confer a pleasant flavour. Normally, increased ethyl acetate concentrations are indicative of long term storage of the raw material and probable acetic bacterial spoilage. 1-propanol can also be an indicator of bacterial spoilage. The low final concentration of 1-propanol in kefir beverages (1.97 mg L^{-1} for ML, 2.44 mg L^{-1} for CW and 2.38 mg L^{-1} for DCW) can be compared with levels of other beverages, such as whiskies and cider brandies (Apostolopoulou *et al.,* 2005). The concentration of 2-methyl-1-propanol in kefir beverages (Table 1) can also be well compared with levels in other beverages (Dragone *et al.,* 2005; Apostolopoulou *et al.,* 2005).

Table 1 Concentration of volatiles compounds present in kefir beverages spirit by GC-FID

Kefir beverages fermentation	Compounds volatile						
	Acetaldehyde	Ethyl acetate	1-Propanol	2-Methyl-1-propanol (isobutyl alcohol)	2-Methyl-1-butanol (isopentyl alcohol)	3-Methyl-1-butanol (isoamyl alcohol)	1-Hexanol
	Milk mg/l						
0 h	n.d	n.d	n.d	n.d	n.d	n.d	n.d
12 h	2.16±0.02 acde	n.d	n.d	n.d	n.d	0.08±0.04 bcde	n.d
24 h	2.17±1.12 acde	2.88±1.22 bcde	n.d	5.14±1.56 de	3.97±1.26 de	3.46±1.58 bcde	n.d
36 h	2.68±0.75 acde	3.71±0.86 bcde	n.d	5.49±1.96 de	4.20±1.67 de	4.24±0.47 bcde	n.d
48 h	3.38±0.65 acde	3.77±1.30 bcde	n.d	6.82±0.35 de	4.44±0.90 de	4.40±1.79 bcde	n.d
60 h	5.08±0.82 acde	8.18±1.67 bcde	1.97±0.29 ab	10.89±2.15 de	8.65±1.94 de	5.89±1.73 bcde	0.50±0.02 abc
72 h	5.08±0.82 acde	8.18±1.67 bcde	1.97±0.29 ab	10.89±2.15 de	8.65±1.94 de	5.89±1.73 bcde	0.50±0.02 abc
	Cheese whey mg/l						
0 h	n.d	n.d	n.d	n.d	n.d	n.d	n.d
12 h	1.56±0.01 bcde	1.06±0.08 cde	0.15±0.07 abcd	n.d	n.d	n.d	n.d
24 h	2.92±0.16 bcde	2.35±0.46 cde	2.54±1.22 abcd	3.99±1.10 bcde	3.84±0.14 de	5.62±0.26 de	n.d.
36 h	3.16±0.95 bcde	2.56±1.98 cde	2.46±0.57 abcd	4.43±1.76 bcde	4.15±2.14 de	5,45±0.40 de	n.d.
48 h	3.23±0.98 bcde	2.56±1.84 cde	2.15±0.78 abcd	4.21±0.30 bcde	4.07±0.07 de	5.73±0.05 de	n.d.
60 h	5.97±0.11 bcde	6.42±0.19 cde	2.37±0.13 abcd	8.88±2.03 bcde	8.15±0.95 de	5.75±0.71 de	0.57±0.02 abc
72 h	5.98±0.17 bcde	8.27±0.37 cde	2.44±0.18 abcd	10.51±0.41 bcde	8.88±0.23 de	5.91±0.16 de	0.57±0.06 abc

(Continued overleaf)

Table 1 (Continued)

Kefir beverages fermentation	Compounds volatile						
	Acetaldehyde	Ethyl acetate	1-Propanol	2-Methyl-1-propanol (isobutyl alcohol)	2-Methyl-1-butanol (isopentyl alcohol)	3-Methyl-1-butanol (isoamyl alcohol)	1-Hexanol
	Deproteinised cheese whey mg/l						
0 h	n.d	n.d	n.d	n.d	n.d	n.d	n.d
12 h	1.39±0.11 bcde	0.98±0.03 bcde	0.10±0.01 abcd	n.d.	0.13±0.04 e	0.27±0.24 bcde	n.d.
24 h	2.81±0.01 bcde	2.32±0.50 bcde	2.34±0.93 abcd	3.61±0.55 bce	3.62±0.17 e	5.40±0.57 bcde	n.d.
36 h	2.80±0.04 bcde	2.56±0.57 bcde	2.31±0.04 abcd	3.94±1.07 bce	3.70±0.42 e	5.37±0.52 bcde	n.d.
48 h	3.12±0.82 bcde	2.75±0.35 bcde	2.35±0.5 abcd	4.04±0.07 bce	4.01±0.02 e	5.80±0.15 bcde	n.d.
60 h	5.95±0.08 bcde	6.30±0.36 bcde	2.39±0.16 abcd	8.22±1.10 bce	7.74±0.37 e	5.83±0.74 bcde	0.59±0.01 abc
72 h	5.98±0.61 bcde	8.38±0.25 bcde	2.38±0.25 abcd	10.60±0.28 bce	8.89±0.16 e	5.82±0.02 bcde	0.58±0.04 abc
Odour threshold (mg/l)	25^{n} ~	12.3^{m} §	266^{n} 750^{o} ~	-	7^{m} * Ω	7^{m} * Ω	0.592^{n} * Ω
Descriptors	$Whey^{n}$	$Whey^{n}$	$Whey^{n}$	-	$Cheese^{m}$, $Whey^{n}$	$Cheese^{m}$	$Whey^{n}$

Data are average values of duplicate ± standard deviation. n.d. - not detected.
*Olfactory perception threshold in hydro-alcoholic solution; §Olfactory threshold in model wine; ΩOlfactory perception threshold in water; ~Olfactory difference threshold in beer.
[m]Escudero *et al.* (2004); [n]Dragone *et al.* (2009).
a-e = The averages of the columns with different letter are significantly different (P <0.05).

Amyl alcohols (3-methyl-1-butanol and 2-methyl-1-butanol) are formed during fermentation by deamination and decarboxylation reactions from isoleucine and leucine, respectively (Dragone *et al.,* 2009). Such compounds constitute quantitatively the greater fraction of the alcohols in most fermented beverages (Soufleros *et al.,* 2004). In the kefir beverages produced in our study they were found in the final concentration of 8.80 mg L^{-1} for 2-methyl-1-butanol and 5.80 mg L^{-1} for 3-methyl-1-butanol (Table 1). Increased concentration of amyl alcohols can contribute negatively to the aroma of the beverages (Falqué *et al.,* 2001). The 1-hexanol alcohol was also found in kefir beverages. This alcohol has a positive influence on the aroma of the fermented beverage when it occurs in concentrations up to 20 mg L^{-1}. On the contrary, increased concentration of 1-hexanol, seriously impairs the organoleptic characteristics of the beverage (Falqué *et al.,* 2001). The low 1-hexanol final concentration found in kefir beverages (0.5 mg L^{-1} for ML, 0.57 mg L^{-1} for CW and 0.58 mg L^{-1} for DCW) can be considered to affect positively the flavor of the product. Methanol was not found in kefir beverages, that is benefic since a highly toxic effect has been reported for this compound (maximum legal limit 1000 g hL^{-1} of 100% vol. ethanol – Council Regulation (EEC) No. 1576/89, 1989) (Geroyiannaki *et al.,* 2007). The absence of methanol in kefir beverages is probably due to the lack of pectin in milk and cheese whey.

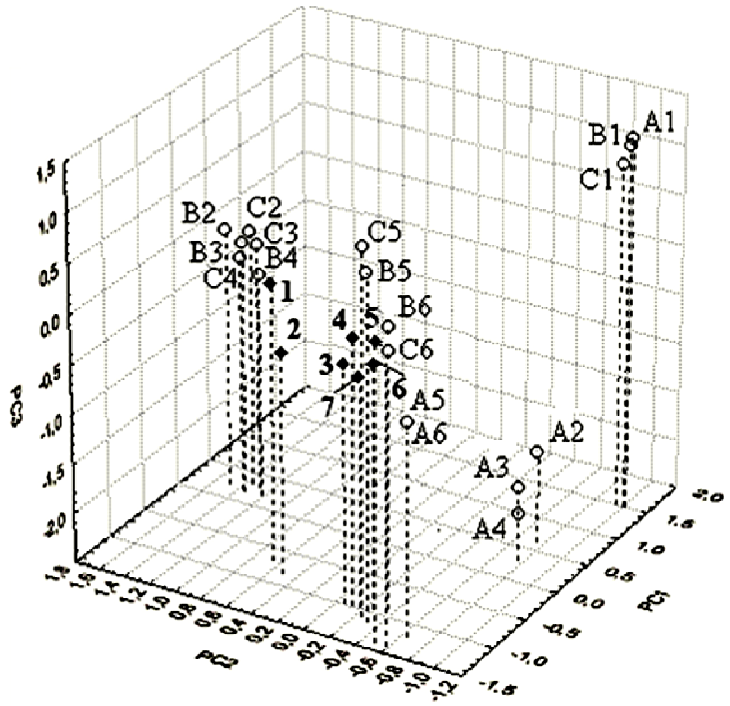
Low molecular mass carbonyl compounds such as aldehydes and ketones are normally found in fermented beverages as by-products of yeasts fermentation, intermediates in the formation of fusel oil and as a result of alcohol oxidation at various stages of beverage production. Nevertheless, their presence is not desirable because some of them are responsible for unpleasant organoleptic properties (Dragone *et al.,* 2009). In the present study, acetaldehyde was the only carbonylated compound identified among the major volatile compounds, but its concentration value (5.08 mg L^{-1} for ML and 5.98 mg L^{-1} for

CW and DCW) was low when compared to other beverages, such as tsipouro or grappa (Apostolopoulou *et al.,* 2005). This low concentration value is interesting, because elevated acetaldehyde concentrations give a pungent irritating odour to the beverage, and can be health hazards (Geroyiannaki *et al.,* 2007).

The results obtained for the volatile compounds (Table 1) were submitted to PCA to obtain a more simplified view of the relationships among the volatile compounds analyzed (Fig. 4). The first principal component accounted for 76.85% of the total variation, while PC2 and PC3 explained 13.29% and 7.86% of the total variation, respectively. A plot of the results shows the formation of four groups (Fig. 4). Two of the groups are located on the first factor (X-axis positive, Y-axis negative and Z-axis positive or negative), and includes A1, B1, C1 and A2, A3, A4 samples. These groups corresponded to the early times of fermentation and were far plotted of the 1-propanol, isoamyl alcohol and isopentyl alcohol point, suggesting that these volatiles compounds made significant contributions to separation between these. The third group is closely related on the second or third part of the axis (X-axis positive, Y-axis positive and Z-axis positive or negative), and includes the samples B2, B3, B4, C2, C3, and C4. These times of fermentation were mainly associated with larger concentrations of isoamyl alcohol and 1-propanol. The following group is also closely related on the second or third part of the axis (X-axis negative, Y-axis negative and Z-axis positive or negative) and includes the samples of end fermentation (A5, A6, B5, B6, C5 and C6). The final concentration of volatiles revealed only little variation among in the samples of beverages produced (ML, CW and DCW), confirming results (Table 1).

Our results indicated a significant contribution of kefir culture in volatile compounds as the composition of organic acids, esters, acetaldehyde and alcohols. The compounds identified in in milk and cheese whey/based kefir

beverages are similar to those present in other beverages, like sake (Teramoto *et al.,* 2002), mouro (Soufleros *et al.,* 2004)., or mescal (León-Rodríguez *et al.,* 2006) produced from *Agave salmiana*, for example. Dragone *et al.* (2009) also found similar compounds in cheese whey alcoholic beverage.



[(1 = 1-Propanol; 2 = isoamyl alcohol; 3 = isopentyl alcohol; 4 = acetaldehyde; 5 = 1-Hexanol; 6 = Ethyl acetate; 7 - isobutyl alcohol.). Samples A, B e C correspond to fermentation of ML, CW and DCW respectively. A1, B1 e C1 12 hours of fermentation; A2, B2 and C2, 24 hours of fermentation; A3, B3 and C3, 36 hours of fermentation; A4, B4 and C4, 48 hours of fermentation; A5, B5 and C5 60 hours of fermentation; A6, B6 and C6 72 hours of fermentation.]

Figure 4 Principal Component Analysis (PCA) 3D plot of concentration of volatiles compounds of kefir beverages

3.3 Sensory analysis

The kefir beverages were subjected to sensory analysis to assess its acceptance. (Table 2). For all attributes assessed the beverages showed good acceptance (at least 5 points or 50% of acceptance). The likeness in sensory analysis found among these three beverages analyzed here might be the result of the similar chemical and volatile compounds compositions of these final products (Figs. 1, 2, 3 and Table 1).

The traditional milk kefir beverage is commonly manufactured in different countries and is known for its organoleptic characteristics assessed. The potential for use of cheese whey as a medium for manufacturing products with a sensory profile similar to that of fermented milk beverages was demonstrated by the results of this study, i.e., there was no evidence that, when using the kefir grains, adversely effect the substrate (cheese whey or milk) on volatile compounds and sensory (Figs. 1, 2, 3 and Table 1).

Assadi *et al.,* (2008) tested the technological potential of various ratios of lactic bacteria, yeasts and acetic acid bacteria isolated from kefir grains starter culture for cheese whey fermentation. The best results were obtained when all the starter cultures were combined, i.e. bacterial mixed yeast fermentation. The potential of starter culture in production of healthy beverage from cheese whey was found and the beverage produced good organoleptic quality and presented taste of artificial butter milk and naturally carbonated. These results confirm the good acceptable of the beverages produced in this study by kefir grains containing mixed bacterial and yeast cultures.

Table 2 Sensory colour, appearance, taste and overall acceptability scores on a 9-point hedonic scale (9 = like extremely; 1 = dislike extremely) of kefir beverages

Kefir beverages	Sensory properties				
	Colour	Aroma	Appearance	Taste	Overall acceptability
Milk	6.02	6.11	5.82	6.17	6.16
Cheese whey	5.78	6.01	5.69	6.08	6.01
Deproteinised cheese whey	5.51	5.92	5.54	5.82	5.89

4 Conclusions

Kefir grains were able to reduce the lactose concentration in cheese whey producing volatile compounds for good quality of the beverages. GC-FID revealed the presence of volatile compounds in the kefir beverages. Most of these compounds are similar to those reported for other fermented beverages, although the concentration values are different. Higher alcohols (mainly isobutyl alcohol and isopentyl alcohol) and ethyl esters (mainly ethyl acetate) were the most dominant compounds present, contributing thus for the greatest proportion of the total aroma. The results of this study indicate that novel beverages of acceptable organoleptic character can be produced by cheese whey-based fermentation by kefir grains.

The proposed technology in this study is environmentally significant due to the fact that a very polluting liquid industrial waste is employed to produce products of nutritional value, including the use of probiotic kefir grains as alternative. The one key point for industrial application of the proposed technology is the promotion of fermentation by kefir of granular biomass which provides the possibility of eliminating the use of centrifugal separators that have a high energy demand and require high industrial investment.

Acknowledgements

The authors acknowledge the financial support from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), CAPES-GRICES and Lactogal for supplying cheese whey powder.

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ANEXO

OS AUTORES AGRADECEM O APOIO RECEBIDO