Original

Microbial profile of a kefir sample preparations – grains in natura and lyophilized and fermented suspension

Perfil microbiológico de preparações de uma amosta de quefir – grãos in natura*, liofilizado e suspensão fermentada*

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Abstract

Probiotics are supplementary foods developed by microbial strains that improve animal health beyond basic nutrition. Probiotics are consumed orally, regardless of being considered as normal inhabitants of the intestines, able to survive in enzimatic and biliary secretions. Kefir is a probiotic originated from the old continent, fermented by several bacteria and yeasts, encapsulated in a polyssacharide matrix, and resembles jelly grains. Kefir is also presented as its sourish product both in sugary or milky suspensions containing vitamins, aminoacids, peptides, carbohydrates, ethanol, and volatile compounds. Kefir is known to have a diverse microbial content depending on the country and fermentative substrates, which cause distinct probiotic effects. In this sense, the purpose of this work was to isolate, identify, and quantify the microbial content of a native sugary kefir sample (fermented suspension and lyophilized natural grains). Serial dilutions were plated on Rogosa agar (AR) and De Man, Rogosa and Sharpe (MRS), for *Lactobacillus*; Brain Heart Infusion (BHI), for total bacteria; Sabouraud-Dextrose-Agar (SDA), for yeasts and filamentous fungi; Thioglycolate Agar (TA), for *Streptococcus*, *Acetobacteria* and *Leuconostoc*; and Coconut Water Agar (CWA), and CWA supplemented with yeast extract (CWAY), for various genera. Genera and species for all strains were identified through biochemical reactions and specific API systems. The microbial profile of kefir was different from other sources of grains despite the presence of similar microorganisms and others which have not been reported yet. The data obtained with the CWA and CWAE media suggest that both substrates are alternative and salutary media for culture of kefir strains.
Keywords: *aqueous fermentation; kefir; microorganisms; probiotics.*

Resumo

Probióticos são suplementos alimentares desenvolvidos por cepas microbianas. O Kefir é um probiótico constituído por bactérias láticas e leveduras, encapsuladas em uma matriz polissacarídica (grãos). É um produto de fermentação contendo vitaminas, aminoácidos, peptídeos, carboidratos, etanol e compostos voláteis. Possui composição microbiológica variada, dependendo da origem e substrato de fermentação, influenciando na variabilidade de seus efeitos. Este trabalho objetivou a identificação do perfil microbiano de uma amostra de Kefir cultivada em solução aquosa de açúcar mascavo. Diluições seriadas de quefir (caldo fermentado, grãos in natura e liofilizado) foram plaqueadas em Ágar Rogosa (AR), Ágar De Man, Rogosa & Sharpe (MRS), para *Lactobacillus*; Ágar BHI, para contagem de bactérias totais; Sabouraud–Glicose, para leveduras e fungos filamentosos; CaldoTioglicolato, para *Streptococcus, Acetobacter* e *Leuconostoc*; Ágar Água de Coco (AAC) e AAC suplementado com extrato de levedura (AACE), para vários gêneros. Gêneros e espécies foram identificados por reações bioquímicas e sistemas API específicos. O perfil microbiano do Kefir analisado foi distinto daqueles de outras origens encontradas para os grãos, embora tenham sido encontrados microrganismos similares, além de outros ainda não reportados na literatura. Os dados obtidos com AAC e ACCE sugerem que ambos os substratos podem ser alternativas para meio de cultivo.
Palavras-chave: *fermentação aquosa; quefir; microrganismos; probióticos.*

1 Introduction

Functional foods present substances with distinct biological functions, called bioactive components, capable of modulating the organism physiology and ensuring health maintenance (DÂMASO, 2001). The pre and probiotic foods are themselves functional and are rich in micronutrients characterizing a wide range of possibilities for their development (ASHWELL, 2002) They are related to very different physiological areas, other than those of the regular functional foods.

Probiotics are alimentary supplements elaborated with live microorganisms which, when ingested in determined amounts, benefit the host promoting an intestinal microbiota balance. To be considered probiotic, the microorganism must be a usual gastrointestinal inhabitant, survive in its passage through the stomach, and keep its viability and metabolic activity in the intestine (LIMA et al., 2003)

Kefir, considered a probiotic, consists of grains and a supernatant. It is a mix of microorganisms, polysaccharides, aminated molecules, vitamins, acids, ethanol, and volatile substances. Its microbiological composition is very changeable, depending on its origin. The structure of the grains is similar

Recebido para publicação em 25/11/2008
Aceito para publicação em 18/7/2009 (003957)
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to small cauliflower branches, with a diameter of 0.3-3.5 cm, and is composed of about 890-900 $g.kg^{-1}$ of water, 2 $g.kg^{-1}$ of lipids, 30 $g.kg^{-1}$ of proteins, 60 $g.kg^{-1}$ of sugars, and 7 $g.kg^{-1}$ of ashes (ZOURARI; ANIFANTAKIS, 1988; KOROLEVA, 1988; ÂNGULO; LOPEZ; LEMA, 1993; PINTADO; LOPES; FERNANDES, 1996; REA et al., 1996). Many studies have been carried out on the distribution and constitution of the kefir microbiota (PIDOUX et al., 1990; ÂNGULO; LOPEZ; LEMA, 1993; OHARA et al., 1997; WATABE et al., 1998; MITSUE; TACHIBANA; FUJI, 1999; GARROTE; ABRAHAM; ANTONINI, 2001; MARQUINA, 2002; SIMOVA et al., 2002). Bottazzi and Bianchi (1980) suggested that the populations found in the kefir grains are not randomly distributed in the grains; *Lactobacillus*, for example, is found in the grain periphery. Assadi, Pourahmad and Moazami (2000) studied the types of kefir proliferation and concluded that kefir growth is better when its grain is used as a starting colony for its culture. The study results also showed the different sensorial characteristics in accordance to its culture (GARROTE; ABRAHAM; ANTONINI, 1997; KUO; LIN, 1999; LIN; CHEN; LIU, 1999; WSZOLEK et al., 2001).

Kefir culture can be carried out in sugary and leavened, watery, or milky environment. Zubillaga et al. (2001) described kefir grains as a symbiotic association between lactic bacteria, acetic bacteria, and yeasts among other microorganisms. The total composition of the grains has not been completely elucidated (MARSHALL; COLE, 1985; ÂNGULO; LOPEZ; LEMA, 1993).

For over a thousand years of consumption, kefir microorganisms have not been shown to be pathogenic, and kefir suspensions are capable to suppress the growth of some pathogens as *Salmonella* and *Shigella* (KOROLEVA, 1988; ANSELMO; VITORA; LAUSADA, 2001).

The purpose of this investigation was to isolate, quantify, and characterize the microorganisms present in the kefir cultivated at the Unifenas Biology and Physiology Laboratory of Microorganisms, Alfenas, Minas Gerais, Brazil.

2 Material and methods

2.1 Local

This study was developed at the Unifenas Biology and Physiology Laboratory of Microorganisms (BPLM), Alfenas - MG State, Brazil. The kefir cultivated in filtered sugary water (total kefir) was analyzed, and so were the grains, the suspension and the lyophilized total kefir, separately.

2.2 Origin of the kefir grains

The kefir used in these experiments was kindly granted by Prof. Dr. José Maurício Schneedorf Ferreira da Silva, Phytopharmacy Laboratory– Unifenas. It was cultured and microbiologically analyzed at the Unifenas LBFM.

2.3 Methods

Kefir production

Five grams of kefir grains were cultivated in a broth prepared with distilled water and 5% brown sugar. This suspension was transferred into bottles and incubated at 25 °C during 15 days. After reactivation of the microbiota of the grains, they were washed in distilled water and subcultivated in the same broth every 24 hours. With regard to subcultivation, after serial decimal dilutions, analyses of the fermented broth (suspension) and in natura and lyophilized grains.

Lyophilized kefir

Five hundred grams of kefir grains were dehydrated at 37 °C for 24 hours, frozen in dry ice, and lyophilized at the Farming Research Company of Minas Gerais (EPAMIG), located at Federal University of Lavras – UFLA, MG State, Brazil.

Microbiological analysis kefir and lyophilized kefir

The microbial quantification process of the lyophilized kefir, total kefir, kefir grains, and supernatant was carried out through decimal dilutions, plated on the following culture media: Rogosa Agar (RA) and De Man, Rogosa, and Sharpe (MRS), specific for *Lactobacillus*; Brain Heart Infusion (BHI) Agar, used for global counting of bacteria; Sabouraud-Dextrose Agar (SDA), for yeasts and filamentous fungi; Thioglycolate Agar (TA), for *Streptococcus, Acetobacter* and *Leuconostoc*; Coconut Water Agar (CWA) and Coconut Water Agar + Yeast Extract (CWAY), for the growth of various genera. With the exception of CWA and CWAY, which were prepared at the BPLM, all others came from MERCK (GERMANY) (INTERNATIONAL..., 2000).

The incubation temperature was 35.5 °C for bacteria and 25 °C for fungi. The incubation time varied in face of the appearance of Colony Forming Units (CFU) macroscopically visible on the surface of the media and/or turbidity in the broths.

Conventional biochemical tests were used for the identification of genera and species, besides specific API galleries (BioMérieux, France). All the procedures were carried out in triplicates.

Statistic analysis

The data for microorganisms global counting were obtained through arithmetic mean and standard deviation of the mean, besides the frequency tables. The comparison between the groups was affected by the non-parametric analysis of Man-Whitney, and the values of $p < 0.05$ were accepted as significant. The analyses were done with the aid of the statistical package XLSTAT version 5.0 (CARR, 2004).

3 Results and discussion

The values found in the samples of grains and suspension of kefir cultivated in AR were respectively 8.5×10^5 and 4.5×10^4 $CFU.g^{-1}$. However, the analyzed lyophilized kefir samples presented no microbial growth in this medium. As it is selective for *Lactobacillus*, these results probably indicate that these microorganisms became unviable either because of the lyophilization procedure or the loss of biosynthetic capacity of some important enzymes for growth in this medium.

This hypothesis is supported by the fact that Lactobacillus strains were found in kefir suspension formulation (Table 1),

and the literature reports several isolated strains of kefir samples. From kefir grains, Marshall, Cole and Farrow (1984) isolated *Lactobacillus desidiosus*, which can ferment L-arabinose and gluconate specifically, while Marquina et al. (2002) detected *Lactococcus brevis* and *Lactobacillus paracasei*. Takizawa et al. (1998) found 120 strains of *Lactobacillus* in kefir grains and separated them into four groups: *Lactobacillus kefirgranum*, representing 49% of strains; *Lactobacillus kefiranofaciens, Lactobacillus kefir,* and *Lactobacillus parakefir*. These data indicate that the *Lactobacillus* genus predominates in other kefir isolates, which was evidenced in this study in the analyses of the suspension's grains and supernatant.

Wszolek et al. (2001) compared the characteristics of kefir cultivated in Scotland and in Poland, both in bovine, caprine, and ovine milk substrata, evaluating the microbiological characteristics and the sensorial properties. The grains were preserved both in saline solution and by lyophilization. The microbiological quality was considered good, predominating bacteria of the lactic acid and yeasts. The type of starter colony and the period of storage affected the sensorial properties, mainly viscosity. Cow and sheep milk were the most viscous. In the six samples of kefir from Iraq, after fermentation of the grains, the global counting of bacteria was of 10^9 CFU.g^{-1} for *Lactococcus*, 10^8 CFU.g^{-1} for *Leuconostoc,* 5×10^5 CFU.g^{-1} for *Lactobacillus*, 10^5 CFU.g^{-1} for acetic acid producers and 10^5 CFU.g^{-1} for yeasts (REA et al., 1996). Some researchers reported the presence of homofermenting and heterofermenting *Lactobacillus, Lactococcus, Leuconostoc*, and *Acetobacter* (ÂNGULO; LOPEZ; LEMA, 1993).

Angulo, Lopez and Lema (1993) studied kefir samples of dairy products from eight domiciliary sources in the Galicia region, northwest of Spain, and found the following bacteria: *Lactobacillus brevis*, *Lactobacillus viridescens, Lactobacillus kefir, Lactobacillus fermentus, Lactobacillus casei* ssp. *rhamnosus, Lactobacillus casei* ssp. *tolerans, Lactobacillus casei* ssp. *pseudoplantarum, Lactobacillus acidophilus, Lactobacillus gasseri, Lactococcus lactis* ssp. *lactis, Leuconostoc* ssp., *Streptococcus salivarius* ssp. *thermophilus.* Among the yeasts, they identified *Torulaspora delbrueckii, and Saccharomyces cerevisae, Saccharomyces unisporus, Candida kefyr, Candida friedrichii, Kluyveromyces lactis,* and *Pichia fermentans*. The authors reported that the important differences in grain composition from the eight sources might be attributed to the different origins of the samples, among other factors. Such interfering factors seem to be mainly geographic and also due to the substratum used for grain proliferation. Wszolek et al. (2001), on the other hand, disagree and affirm that only the substratum differentiates the composition of the fermented milk (kefir) after analyzing the ovine, caprine, and bovine lacteal beverage from Poland and Scotland. These authors recognize, however, that differences in analyses performed different laboratories and in different countries might explain the differences in kefir composition. In this paper, qualitative, and quantitative differences were also evidenced.

Table 1. Characterization of facultative bacteria present in kefir grains, suspension, and lyophilized cultivated in Coconut Water Agar with Yeast Extract, at 35,5 °C.

Identification	Sample	Inoculum
Leuconostoc ssp.	Suspension	Surface
Lactobacillus lactis cremoris	Suspension	Surface
Chryseomonas luteola	Lyophilized	Depth
	Lyophilized	Depth
	Grain	Surface
	Suspension	Surface
	Lyophilized	Surface
Acetobacter sp.	Suspension	Surface

Tables 1 and 2 show some microbial strains found in this study in the various kefir presentations (suspension, grain, and lyophilized).

Similarly, Ângulo, Lopez and Lema (1993) and Ohara et al. (1997) isolated strains of *Leuconostoc* spp. from kefir grains. Garrote, Abraham and Antonini (1997, 2001) isolated strains of *Lactobacillus lactis* from l*actis* subspecies. *Acetobacter* strains were isolated by Garrote, Abraham and Antonini (2001) and Mitsue, Tachibana and Fuji (1999).

Unusual microorganisms were found in this study, such as *Lactobacillus cremoris lactis*, *Chryseomonas luteola, Candida coiliculosa*, *Candida magnoliae, Kloekera* sp., and *Candida famata*. This demonstrates the great microbial diversity in kefir samples from different sources. The present study is the first one to demonstrate the presence of such organisms in kefir.

The literature reports the isolation of unknown microorganisms of kefir, identifying each one of their functions: *Lactobacillus casei* and *Lactobacillus paracasei,* are highly involved with the production of lactate, and *Lactobacillus hilgardü* participates in the production of arabinose and polysaccharide from saccharose. Sugar fermentates were used in these assays (PIDOUX et al., 1990; MAINVILLE et al., 2001). In the present paper, brown sugar was used as substratum for kefir growth, but none of the previously reported microorganisms was isolated. Likewise, brown sugar microbiological analysis showed none of the previously reported microorganisms for identification and characterization (data not shown). However,

Table 2. Characteristics of yeasts in grain, suspension, and lyophilized kefir samples, cultivated in Coconut Water Agar with Yeast Extract and Coconut Water Agar at 35.5 °C.

Identification	Medium	Sample
Saccharomyces cerevisae	CWAYE[1]	Suspension
	CWAYE	Grain
	CWA[2]	Grain
Candida colliculosa	CWA	Suspension
Toruspola delbruechii	CWA	Suspension
Candida inconspicua	CWAYE	Grain
	CWA	Grain
	CWAYE	Lyophilized
Candida magnoliae	CWAYE	Suspension
Kloekera sp.	CWAYE	Lyophilized
	CWA	Grain
Candida famata	CWAYE	Lyophilized
Kluyveromices lactis	CWAYE	Suspension
Kluyveromices marxianus	CWAYE	Suspension
Candida quefir	CWAYE	Suspension

[1]CWAYE = Coconut Water Agar with Yeast Extract; [2]CWA = Coconut Water Agar.

Leroi and Courcoux (1996) also isolated less common strains from kefir kept in sugary water: *Lactobacillus hilgardü* and *Saccharomyces florentinus*. These data supports the thesis that different culture media and regions can present remarkable differences in the kefir microbiota. Table 2 presents the yeasts found in suspension, grains, and lyophilized kefir.

Tables 1 and 2 show that bacteria and yeasts were grown in Coconut Water Agar + Yeast Extract, while only yeasts grew in Coconut Water Agar, in which no bacterium was identified. CWA probably lacks some factors for bacterial growth, which otherwise may be present in yeast extracts.

In the microbial composition of kefir, popularly cultivated in residences of Formosa, Japan, *Lactobacillus helvetícus, Leuconostoc mesenteroides, Kluyveromyces marxianus,* and *Pichia fermentans* were found (LIN; CHEN; LIU, 1999). Among them, *Lactobacillus helvetícus* stands out as the bacterium with the highest growth rate and *Kluyveromyces marxianus* as the yeast with the highest production of lactic acid and ethanol. We also observed the yeast *Kluyveromyces marxianus* in kefir broth cultivated in Coconut Water Agar + Yeast Extract, besides the bacterium *Leuconostoc* sp. The present results did not include an investigation of sub-products. However, it is possible that the isolated microorganisms produce similar compounds, once the fermentate's odor and flavor are similar to the sourish-ethanolic sensorial properties.

The global counting of facultative bacteria in CWA and CWAYE indicated that the method and type of preparation did not affect cell counting of grain and suspension samples, and that there was no significant difference between the plating method, the culture media and growth in grain and suspension (Mann Whitney, $p < 0.05$), except for the lyophilized kefir group, which had low counting. These data confirm the previously found data (Table 3).

Loddi (2001) emphasizes that the probiotics can contain bacteria which are entirely known and quantified or bacterial cultures that has not been defined yet. Some bacteria, such as *Enterocccus, Bacteróides, Eubacterium, Lactobacillus, and Bifidobacterium* are present in defined cultures. When bacteria with probiotic capability are isolated from their conventional habitat and subcultivated and/or lyophilized, some of their properties are lost (FRANCO; OLIVEIRA; CARVALHO, 2006). The lyophilization process could probably interfere with the global counting of the kefir microorganisms due to drying and later rehydration. Since kefir is easily cultivated at home, and seldom industrialized (RETORI, 2008), the purpose of this study was to obtain lyophilized kefir and promote its large-scale production. In face of the results obtained, however, such as the low microorganism counting, this seems to be improbable.

Table 3. Global counting (average) of facultative bacteria in Coconut Water Agar (CWA) and Coconut Water Agar + Yeast Extract (CWAYE) in samples of grain, suspension, and lyophilized kefir.

	CWA		CWAYE	
	Depth	Surface (CFU)	Depth	Surface
Lyophilized	1.4×10^2	3×10^2	7.5×10^2	2.5×10^2
Grain	3.3×10^5	2.4×10^4	6.7×10^5	1.2×10^5
Suspention	1.4×10^4	4.6×10^5	1.2×10^4	4.5×10^5

Garrote, Abraham and Antonini (1997) found bacteria, yeasts and filamentous fungi in grains obtained in Argentine domiciliary environments. Four bacteria were isolated: *Lactococcus lactis* ssp. *lactis*; *Lactococcus lactis* ssp. *diacetylactis*, a homofermenting bacillus, probably *Lactobacillus kefiranofaciens* – also isolated by Arihara, Toba and Adachi (1990) – or *Lactobacillus kefirgranum*, and a heterofermenting bacillus that could be *Lactobacillus brevis*, *Lactobacillus kefir* – also isolated by Arihara, Toba and Adachi (1990) – or *Lactobacillus parakefir*. Lactose nonfermenting yeasts were found: *Saccharomyces cerevisae* and *Saccharomyces lipolytic*. The fungus was characterized as *Geotrichum candidum*. Their results indicated the following composition of kefir grains: 0.9% (1.64×10^7 CFU.g^{-1}) of *Lactococci*; 78.3% (1.59×10^9 CFU.g^{-1}) of *Lactobacilli,* and 20.8% ($42.30\ 10^7 \times$ CFU.g^{-1}) of yeasts. The number of bacteria was slightly higher in fermented milk, while the number of yeasts was lower. The authors concluded that the microbial composition of the milk fermented with kefir grain is the same as that of the grains prope, except for *Lactobacillus kefir*, which was not detected after fermentation. Later, the same authors (GARROTE; ABRAHAM; ANTONINI, 2001) isolated *Lactococcus lactis* ssp. *lactis, Lactobacillus kefir, Lactobacillus plantarum, Acetobacter, Saccharomyces, Leuconostoc mesenteroides, Lactococcus lactis* ssp. *lactis biovar diacetylactis, Lactobacillus parakefir,* and *Kluyveromyces marxianus* in Argentine samples. However, qualitative differences were found in the four samples. Our results are very close to those of the above-mentioned authors with regard to isolated bacteria and yeasts.

By virtue of the diversity of microorganisms found in the various samples, in this and other studies, we discuss the use of kefir to improve the digestion of lactose, the functions of the intestines, the physical and mental aptitude, the weight control and weight loss due to the long-lasting use of this fermented beverage, and, still, its use as a disinfectant in agriculture and industry (RETORI, 2008)

4 Conclusion

The total average counting of kefir grain *Lactobacillus* was 8.5×10^5 CFU.g^{-1}, while in kefir suspension it was 4.5×10^4 CFU.g^{-1} and *Lactobacillus lactis cremoris* was isolated.

Microbial analyses revealed the following bacteria and yeasts, respectively: *Leuconostoc* ssp., *Lactobacillus lactis cremoris, Chyseomonas luteola, Acetobacter, Sacharomyces cerevisae, Candida colliculosa, Toruspola delbruechii, Candida inconspicua, Candida magnoliae, Kloekera* sp., *Candida famata, Kluyveromices lactis, and Kluyveromices marxianus, Candida kefir*.

Kefir microbiota is not the same considering all aspects when compared with the available literature data.

With regard to microbial composition, the kefir cultivated at the UNIFENAS Biology and Physiology Laboratory of Microorganisms is similar to those already described in the literature. A few peculiarities, however, have not been described yet. A high number of microorganisms were isolated and identified in relation to the literature data available.

The Coconut Water Agar was shown to be a good medium for microorganism growth, and thus it is considered as an alternate culture medium, mainly when added with yeast extract, which makes it richer for a better growth, besides being less expensive.

Lyophilized kefir presented the lowest bacterial counting, probably due to its smallest contents of water.

Acknowlegments

We are grateful to CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais) for their financial support, and to Prof. Vinicius Vieira Vignoli for language revision.

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