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International Dairy Journal 15 (2005) 383–389
INTERNATIONAL DAIRY JOURNAL
www.elsevier.com/locate/idairyj

Characterisation of the microbial population at different stages of Kefir production and Kefir grain mass cultivation

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Received 3 November 2003; accepted 28 July 2004

Abstract

Kefir is a fermented milk that is produced by adding Kefir grains, consisting of bacteria and yeasts, to milk. The aim of this study was to determine the microbial population at different stages of traditional Kefir production and Kefir grain mass cultivation. Seven different selective growth media, morphological and biochemical characteristics were used for the isolation and identification of the microbes. The microbial numbers during Kefir production varied between 4.6×10^3 and 2.6×10^8. A *Zygosaccharomyces* sp. was isolated from traditional Kefir grains and after the culturing conditions applied during the mass cultivation *Candida lambica* and *C. krusei* were present. Although these two species are present in other fermented milks, this study is the first to report their presence in Kefir. Species of *Leuconostoc*, *Lactococcus*, *Lactobacillus* and *Cryptococcus* were isolated from traditional grains. *Lactobacillus plantarum* was present in the mass cultivated grains, but not in the traditional Kefir grains.
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Keywords: Kefir grains; Mass cultivation; Lactic acid bacteria; Yeasts

1. Introduction

Kefir is a fermented milk beverage that originated in Eastern Europe. The starter culture used to produce this beverage is an irregularly shaped, gelatinous white/yellow grain (Guzel-Seydim, Seydim, & Greene, 2000). These Kefir grains have a varying and complex microbial composition that includes species of yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB), and mycelial fungi.

Kefir is produced by the diverse spectrum of microbial species present. Lactobacilli are present as the largest portion (65–80%) of the microbial population (Wouters, Ayad, Hugenholtz, & Smit, 2002), with lactococci and yeasts making up the remaining portion of the microbes present in the Kefir grain. The population composition may differ if the grains have different origins, or if the grains are cultured using different methods and substrates. A symbiotic relationship exists between the microbes present in the Kefir grains and it has been shown that there are specific species that always occur in the grains. In contrast, other microbes may either be present depending on the origin of the grains, as well as method of culturing and substrates added (Pintado et al., 1996).

Some LAB that have been isolated from Kefir include: *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus helveticus*, *Lactobacillus kefiri*, *Lactobacillus parakefiri*, *Lactococcus lactis* and *Leuconostoc mesenteroides* (Assadi, Pourahmad, & Moazami, 2000; Cogan et al., 1997; Fujisawa, Adachi, Toba, Arihara, & Mitsuoko, 1988; Kandler & Kunath, 1983; Micheli, Uccelletti, Palleschi, & Crescenzi, 1999; Pintado et al., 1996; Takizawa et al., 1998; Witthuhn, Schoeman, & Britz, 2004). Yeasts isolated from Kefir grains include

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doi:10.1016/j.idairyj.2004.07.016

Kluyveromyces marxianus, *Torula kefir*, *Saccharomyces exiguus* and *Candida lambica* (Assadi et al., 2000; Garrote, Abraham, & De Antoni, 1997; Kwak, Park, & Kim, 1996; Pintado et al., 1996; Witthuhn et al., 2004; Wyder, Keile, & Teuber, 1999; Wyder & Puhan, 1997). *Acetobacter aceti* and *A. rasens*, as well as the mycelial fungus, *Geotrichum candidum* have also been isolated from Kefir grains (Pintado et al., 1996).

A method for the mass cultivation of the Kefir grains was recently developed (Schoevers & Britz, 2003), which may result in the commercialisation of the Kefir grains, rather than the Kefir beverage. However, before the grains can be commercialised it is important to know the exact microbial content of the grains, as well as the stability of the microbial population in the grains after mass cultivation. The aim of this study was to identify the microbial species present at different stages of traditional Kefir production and grain mass cultivation, using selective growth media and morphological and biochemical characteristics.

2. Materials and methods

2.1. Grain activation and traditional Kefir production

The Kefir grains used in this study were obtained from the Department of Food Science, University of Stellenbosch, South Africa. Frozen Kefir grains were activated by adding 18 g of the grains to 500 mL of pasteurised (80 °C for 20 min) full cream milk, followed by incubation at 25 °C. After 24 h, the grains were sieved out and placed in 500 mL of fresh pasteurised full cream milk. This step was repeated for three consecutive days.

Kefir was then produced by adding the 18 g of grains to 1 L of pasteurised full cream milk at 25 °C. The grains were sieved and transferred to 1 L fresh pasteurised full cream milk every 24 h. This procedure was repeated until the grains were used to isolate the microbial population at specified time intervals.

2.2. Mass cultivation of Kefir grains

The mass production of the Kefir grains was done according to the method developed by Schoevers and Britz (2003). The procedure involved the addition of 2 g $100\,mL^{-1}$ yeast extract (Biolab, supplied by Merck Chemicals (Pty) Ltd. South Africa, 20 Montague Drive, Montague Gardens, Cape Town) and 0.5 g $100\,mL^{-1}$ urea (Biolab) to 400 mL of pasteurised full cream milk. Forty grams of Kefir grains were then added to the pasteurised milk and the containers incubated at 25 °C in a shake waterbath at 130 rpm. The milk mixture was completely replaced every 24 h.

2.3. Microbiological analyses

In each case, 10 g of Kefir grains were homogenised in 90 mL (1:9 dilution) of sterile saline solution (0.85 g $100\,mL^{-1}$ NaCl) in a Stomacher (BagMixer, Interscience, France) for 15 min, or until no grain particles were observed. The concentrations of the viable bacteria and yeasts in the suspensions were obtained by serial plating dilutions in sterile saline solution from 10^{-1} to 10^{-6} on selective media and the results were expressed as colony forming units per gram of Kefir grain (cfu g^{-1}). The different selective media included: de Man, Rogosa and Sharpe Agar (MRS; Biolab) containing 3 g $100\,mL^{-1}$ ethanol (Merck) and 0.5 g $100\,mL^{-1}$ filter-sterilised cycloheximide (Merck Chemicals (Pty) Ltd. South Africa, 20 Montague Drive, Montague Gardens, Cape Town) as described by Pintado et al. (1996) as selective for lactobacilli; potassium carboxymethyl cellulose agar containing 10 mL L^{-1} medium filter-sterilised TTC (Oxoid, supplied by CA Milsch (Pty) Ltd., P.O. Box 943, Krugersdorp, South Africa) (KCA + TTC) as described by Nickels and Leesment (1964) as selective for lactococci; KCA containing 30 μg L^{-1} medium filter-sterilised vancomycin (Fluka, supplied by Sigma-Aldrich S.A. (Pty) Ltd., P.O. Box 10434, Aston Manor, South Africa) (KCA + V) as described by Benkerroum, Misbah, Sandine, and Tandaoui (1993) as selective for leuconostocs; Acetobacter peroxydans medium (APM) as described by the manufacturer (DSMZ, 2001) as selective for acetic acid bacteria; Yeast extract lactate medium containing 0.0002% (v/v) naladixic acid (Oxoid) (YELN) as described by Riedel, Wingfield, and Britz (1994) as selective for the lactate utilisers, such as propionibacteria; Pal propiobac medium (Pal-P) as described by Thièrry and Madec (1995) as selective for propionibacteria; and malt extract agar (MEA) (Biolab) as described by Garrote, Abraham, and De Antoni (1997) and yeast extract chloramphenicol agar (YEC) (Biolab) as described by Rea et al. (1996) as selective for yeasts.

The media that were used for the selection of lactobacilli, lactococci, leuconostocs and propionibacteria were incubated anaerobically using the Anaerocult A system (Merck) for 5–10 d at 30 °C, after which the selected colonies (Harrigan & McCance, 1976) were cultivated on MRS. The plates with the yeasts and AAB were aerobically incubated for 3–5 d at 25 °C. The selected colonies from the MEA and YEC agar that selected for yeasts were cultivated on PDA (Biolab), while colonies from the acetic acid bacteria selective medium (APM) were cultivated on APM. The Harrison disc method (Harrigan & McCance, 1976) was used to determine the prevalent microbes that developed on each dilution. This method was used to select colonies from each plate in a truly random manner for further identification, making it possible to calculate the

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