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Bacteria and yeast microbiota in milk kefir grains from different Italian regions

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ABSTRACT

Kefir grains are a unique symbiotic association of different microrganisms, mainly lactic acid bacteria, yeasts and occasionally acetic acid bacteria, cohabiting in a natural polysaccharide and a protein matrix. The microbial composition of kefir grains can be considered as extremely variable since it is strongly influenced by the geographical origin of the grains and by the sub-culturing method used. The aim of this study was to elucidate the bacteria and yeast species occurring in milk kefir grains collected in some Italian regions by combining the results of scanning electron microscopy analysis, viable counts on selective culture media, PCR-DGGE and pyrosequencing. The main bacterial species found was *Lactobacillus kefiranofaciens* while *Dekkera anomala* was the predominant yeast. The presence of sub-dominant species ascribed to *Streptococcus thermophilus*, *Lactococcus lactis* and *Acetobacter* genera was also highlighted. In addition, *Lc. lactis*, *Enterococcus* sp., *Bacillus* sp., *Acetobacter fabarum*, *Acetobacter lovaniensis* and *Acetobacter orientalis* were identified as part of the cultivable community. This work further confirms both the importance of combining culture-independent and culture-dependent approaches to study microbial diversity in food and how the combination of multiple 16S rRNA gene targets strengthens taxonomic identification using sequence-based identification approaches.

1. Introduction

Kefir grains are a unique symbiotic association of different microbial genera and species, mainly lactic acid bacteria (LAB), yeasts and occasionally acetic acid bacteria (AAB), cohabiting in a natural polysaccharide (called kefiran) and a protein matrix (Farnworth, 2005; Dobson et al., 2011; Guzel-Seydim et al., 2011; Leite et al., 2013a,b; Pogačić et al., 2013). The grains are elastic, slimy, varying from white to light yellow in colour, and with an irregular and lobed-shaped cauliflower-like structure of different size, generally ranging between 1 and 3 cm in length (Farnworth, 2005; Kabak and Dobson, 2011; Leite et al., 2013a). Kefir grains have traditionally been used in the Caucasus from time immemorial as starter cultures for the production of the kefir (Kabak and Dobson, 2011). Indeed, kefir is obtained through a lactic/alcoholic fermentation of milk and it is characterised by a distinctive flavour and viscosity due to a mixture of lactic and acetic acid, ethanol, CO_2, acetaldehyde, acetoin, diacetyl, exopolisaccharides (EPS) and other bioactive compounds produced via microbial fermentation, such as peptides, amino acids, bacteriocins, folic acid, calcium and vitamins B1, B12, and K (Kabak and Dobson, 2011; Guzel-Seydim et al., 2011; Leite et al., 2013a). Today the popularity and the availability of kefir (and consequently of kefir grains) is increasing throughout the world due to the number of well-known health benefits and the longevity related to its daily consumption (Farnworth, 2005; Kabak and Dobson, 2011; Guzel-Seydim et al., 2011; Gao et al., 2012; Leite et al., 2012, 2013a,b; Diosma et al., 2014; Pogačić et al., 2013).

The microbial composition of kefir grains can be regarded as extremely variable and is sometimes not well-defined since it is

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strongly influenced by the geographical origin of the grains, the climatic conditions, the method (temperature and time of incubation, agitation, ratio of grain to milk, etc.) and type of milk used to carry out the periodical sub-culturing (Witthuhn et al., 2005; Filipčev et al., 2007; Dobson et al., 2011; Leite et al., 2013b; Marsh et al., 2013; Altay et al., 2013). Accordingly, the possible modification of the dynamic equilibrium of such a microbiota can be responsible for important changes in the physicochemical, rheological, sensory and nutritional properties of kefir beverages.

Since the late 1990s all the above considerations have stimulated an ever-increasing part of the scientific community to investigate bacterial and yeast diversity occurring in kefir grains collected worldwide: Ireland (Garbers et al., 2004; Dobson et al., 2011), Taiwan (Chen et al., 2008), Tibet, China (Jianzhong et al., 2009; Gao et al., 2012, 2013), Belgium (Ninane et al., 2007), Portugal (Pintado et al., 1996), Turkey (Kesmen and Kacmaz, 2011; Kök Taş et al., 2012), South Africa (Witthuhn et al., 2004), Brazil (Miguel et al., 2010; Magalhães et al., 2011; Leite et al., 2012, 2013b), Canada and the USA (Miguel et al., 2010). Furthermore, some studies have highlighted the importance of combining both culture-dependent and culture-independent methods (i.e. PCR-DGGE and high-throughput DNA sequencing) in order to have a more complete picture of the microbiota thriving in kefir grain ecosystems (Chen et al., 2008; Miguel et al., 2010; Kesmen and Kacmaz, 2011; Dobson et al., 2011; Leite et al., 2012; Marsh et al., 2013; Pogačić et al., 2013; Gao et al., 2013).

To the authors' knowledge, there are no reports on the microbial composition of kefir grains from Italy. Therefore, the aim of this study was to elucidate the bacteria and yeast species occurring in milk kefir grains collected in some Italian regions by combining the results of scanning electron microscopy (SEM) analysis, viable counts on selective culture media, PCR-DGGE and pyrosequencing.

2. Material and methods

2.1. Reference strains and culture conditions

Four bacterial reference strains (*Lactobacillus paracasei* NRRL B-4560^T, *Lactobacillus plantarum* DSMZ 2601, *Pediococcus pentosaceus* DSMZ 20336^T, *Lactobacillus parabuchneri* DSMZ 5708) and four yeast reference strains [*Wickerhamomyces anomalus* (synonym *Pichia anomala*) DBVPG 6613, *Starmerella bombicola* DBVPG 3827, *Candida humilis* CBS 6897^T, *Saccharomyces cerevisiae* CBS 1171^T] were used as controls in the PCR-DGGE analyses. These cultures were from: (i) the National Center for Agricultural Utilization Research NRRL (Peoria, USA); (ii) the Deutsche Sammlung von Mikrorganismen und Zellkulturen DSMZ (Braunschweig, Germany); (iii) the Industrial Yeasts Collection DBVPG (University of Perugia, Italy) and (iv) the Centraalbureau voor Schimmelcultures CBS (Utrecht, the Netherlands).

The bacteria were grown at 30 °C for 48 h in anaerobic conditions in MRS agar medium (Oxoid, Basingstoke, United Kingdom). The yeasts were grown at 25 °C for 72 h in aerobic conditions on YPD medium (Oxoid).

2.2. Origin and maintenance of milk kefir grains

Six milk kefir grains of different origin and from five different Italian regions were studied: kefir grains from Trentino Alto Adige (K1), Marche-Recanati (K2), Marche-Ascoli Piceno (K3), and Sicily (K4) were obtained from private households; grains (K5) from Umbria were provided by the University of Perugia, while grains (K6) from Emilia Romagna were supplied by a biotechnology company (Bionova snc, Villanova sull'Arda, Piacenza, Italy). In the laboratory, 100 g of each grain was inoculated (10% w/v) in 1 L of sterile ultra-high temperature (U.H.T.) processed bovine milk and incubated at 20 °C for 24 h. After incubation, filtration through a strainer to remove the coagulated milk and a gentle washing of grains with sterile water were applied; this activation step was repeated three times (Chen et al., 2008; Leite et al., 2012).

2.3. Observation of milk kefir grains using a scanning electron microscope (SEM)

The milk kefir grains were subjected to the treatments proposed by Gao et al. (2012) except for the post-fixation step with osmium tetroxide. The outer surface of the grains was observed using a scanning electron microscope SUPRA™ 40 FESEM (Carl Zeiss SMT, Oberkochen, Germany).

2.4. Enumeration of culturable bacteria and yeasts and bulk cell formation

Ten grams of each kefir grain sample were homogenised in 90 mL of cold sterile 0.1% peptone solution using Stomacher apparatus 400 Circulator (International PBI, Milan, Italy) for 15 min at maximum speed (Kołakowski and Ozimkiewicz, 2012). Serial decimal dilutions were prepared in the same diluent and inoculated in triplicate by surface spreading on specific solid media. The following microorganisms were counted: (i) lactobacilli on MRS agar (Difco, Sparks, MD, USA) at 37 °C under anaerobic conditions (Aquilanti et al., 2012); (ii) mesophilic cocci on M17 agar (Biolife, Milan, Italy) at 25 °C aerobically (Chen et al., 2008); (iii) AAB on three different media incubated at 30 °C under aerobic conditions: (a) Acetic Acid Medium (AAM) agar (D-glucose 1%, ethanol 0.5%, acetic acid 0.3%, peptone 1.5%, yeast extract 0.8%) (Camu et al., 2008), (b) Glucose–Yeast (GY) extract agar (D-glucose 5%, yeast extract 0.5%) (Camu et al., 2008), (c) Gluconobacter Medium (GM) agar (mannitol 2.5%, yeast extract 0.5%, peptone 0.3%) (Gulitz et al., 2011); all the above media were supplemented with 400 mg/L of cycloheximide to inhibit yeast growth (Camu et al., 2008); (iv) yeasts on Dichloran Rose Bengal (DRB) agar chloramphenicol (Difco) at 25 °C aerobically. Microbial enumerations of bacteria and yeasts were carried out after 3 and 10 days, respectively.

The results of the viable counts were expressed as means of colony forming units (cfu) per gram of sample ± standard deviations. The data collected were subjected to one-way analysis of variance (ANOVA) using Statistica StatSoft v.6, and differences were considered non-significant at $P > 0.01$.

After microbial counting, bulk cells were prepared as described by Ercolini et al. (2001). Briefly, colonies were washed off the MRS, M17, AAM, GM, GY, DRB agar media of low (confluent colonies) and high (colonies ranging from 30 to 300) sample dilutions with 2 mL saline solution and glycerol (0.85% NaCl, 50% glycerol); colony washes were stored at −20 °C.

2.5. DNA extraction from milk kefir grains

The microbial DNA was extracted directly from the milk kefir grains using PowerFood™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, USA). In detail, 1 mL of each kefir grain homogenate (dilution 10^{-1}) used for microbial plating was centrifuged to produce a pellet that was processed according to the kit manufacturer's instructions. The DNA quantity and purity were assessed by optical readings at 260, 280 and 234 nm, respectively,