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Microbiota of kefir grains

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Summary

Kefir grains represent the unique microbial community consisting of bacteria, yeasts, and sometimes filamentous moulds creating complex symbiotic community. The complexity of their physical and microbial structures is the reason that kefir grains are still not unequivocally elucidated. Microbiota of kefir grains has been studied using many microbiological and molecular approaches. The development of metagenomics, based on the identification without cultivation, is opening new possibilities for identification of previously non-isolated and non-identified microbial species from kefir grains. According to recent studies, there are over 50 microbial species associated with kefir grains. The aim of this review is to summarize the microbiota structure of kefir grains. Moreover, because of technological and microbiological significance of kefir grains, the paper provides an insight into microbiological and molecular methods applied to study microbial biodiversity of kefir grains.

Key words: kefir, kefir grains, molecular methods, microbiota

Introduction

Kefir is a specific dairy product from the group of fermented milks where lactose hydrolysis during fermentation occurs with the simultaneous action of bacteria and yeasts contained in kefir grains. Although lactic acid is a main metabolite, due to yeast activity kefir also contains significant quantities of CO_2 and variable alcohol quantity. Because of associative growth of various microbial species in kefir, during fermentation other organic compounds are formed, like bioactive peptides, exopolysaccharides, bacteriocins which are presumed to have a probiotic effect on human health (Kosikowski and Mistry, 1999; Stepaniak and Fetliński, 2003; Lopitz-Otsoa et al., 2006; Hong et al., 2010).

The microbial population of kefir grains consists of numerous species of lactic acid bacteria, acetic acid bacteria, yeasts and filamentous moulds which develop a complex symbiotic relationship within a microbial community (Marshall et al., 1984; Farnworth, 2005). Also, the presence of certain microbial species within kefir grain is determined by the area of origin (Angulo et al., 1993; Lin et al., 1999). Scientific researchers have, among others, tried to explain the interior and exterior physical structure of kefir grain which represents the unique microbial ecosystem. However, due to numerous species and phenomenon of their associations, microbiota of kefir grains has still not been completely elucidated (Leite et al., 2012; Wang et al., 2012). In investigation of kefir microbiota composition various microbiological and molecular methods of isolate identification have been used, as well as new metagenomic molecular approaches based on the identification of microbial population without the cultivation of microorganisms on a nutrient medium (Unsal, 2008; Leite et al., 2012; Gao et al., 2013). The investigation of the unique eco system typical for kefir grains has a multiple scientific purpose. Apart from the description, these microbial species isolations can be used for composition of

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starter cultures. The purpose of the kefir grain investigation is based also on the isolate isolation with potentially different probiotic and biochemical characteristics (Hertzler and Clanci 2003; Santos et al., 2003; Liu et al., 2005; Farnworth, 2005; Lopitz-Otsoa et al., 2006; Powel, 2006; Ferreira et al., 2010; Hong et al., 2010; Magalhães et al., 2011; Dimitreli and Antoniou, 2011; Purnomo and Muslimin, 2012).

Based on the scientific researches carried out in past several last years, the purpose of this paper was to give a review of research results of kefir grain microbial population. Also, because of the significance of kefir grains in technological and microbiological sense, the paper presents more details associated with microbiological and molecular experimental approaches used in investigation of microbial biodiversity of kefir grains.

Kefir

Kefir is traditional fermented milk product which has been produced and consumed for thousand years in the areas from Eastern Europe to Mongolia. It is believed that the name kefir derives from the mountain areas of Caucas or Caucasia where, according to the legend, the aboriginals got it directly from the prophet Mohammed (Gaware et al., 2011). The name kefir most likely derives from the Turkish word kefy or keif meaning happiness, satisfaction (Kurman et al., 1992). Apart from the name kefir, the following names are used for the same product: kepyr, kephir, kefer, kiaphur, knapson, kepi and kiipi (Rattray and O'Connell, 2011).

Industrial kefir is mostly produced in Russia and other countries of the ex Soviet Union, then in Poland, Sweden, Hungary, Norway, Finland, Germany, The Czech Republic, Denmark and Switzerland. Kefir is also produced in Greece, Austria and Brazil (Saloff-Coste, 1996). Since it is considered as an ethnic product, the popularity of kefir increased in the USA and Japan lately. According to the available data in Croatia, kefir is produced in Croatia in relatively small quantities by only few dairy, exclusively by addition of a commercial culture.

Various technologies are used in the production of kefir, but they can be basically described as a traditional or industrial manufacturing process. The traditional way of manufacture is a direct inoculation of kefir grains into the milk, or the milk is inoculated by a technical culture prepared from the kefir grains. Unlike this, the term industrial processes in kefir manufacture, means the use of commercial, mostly DVS cultures (Wszolek et al., 2006). Commercial cultures contain isolates of various lactic acid bacteria and/or yeasts species isolated from kefir grains. In comparison with kefir manufactured from kefir grains, kefir manufactured with a pure culture is significantly lacking its authenticity (Otles and Caginidi, 2003; Farnworth, 2005; Garcia Fontán et al., 2006; Wszolek et al., 2006). The loss of authenticity is the most frequently connected to the comparatively small number of various microbial species contained in a pure culture. However, in Poland, kefir is produced by milk inoculation with its own lyophilised culture produced from kefir grains. Using this method "modified" kefir is less sour than the traditional one and is characterised by creamier consistency, but with a significant improvement in the permanent quality, its authenticity has not been significantly changed (Libudzizs and Piatkoiewicz, 1990; Muir et al., 1999).

Regardless of the manufacturing method and culture type according to Codex Allimentarius standard (Codex Stan 243-2003) a typical microbial population of kefir must contain *Lb. kefiri* as well as species *Leuconostoc*, *Lactococcus* and *Acetobacter* (prepared from kefir grains) and yeasts which ferment lactose (*Kluyveromyces marxianus*) as well as yeasts which do not ferment lactose (*Saccharomyces cerevisiae* and *Saccharomyces exigous*) when kefir grains are used for the culture. According to the same standard, a typical kefir must contain at least 2.8 % proteins, less than 10 % fat, at least 0.6 % lactic acid, while the alcohol percentage is not determined. The total number of specified microorganisms from culture must be at least 10^7 cfu/mL, and the number of yeasts not under 10^4 cfu/mL.

At the end of fermentation, which includes three days of cold ripening, the pH value of a typical kefir is between 4.2-4.7, it contains between 0.8-1.2 % of lactic acid, 0.5-0.7 % of ethanol and approximately 0.20 % of CO2. Apart from these compounds, kefir also contains various aromatic compounds like acetaldehyde, diacetyl and acetoin,

other organic acids like formic, acetic and/or propionic and isoamyl alcohol in traces (Wszolek et al., 2006). Also, many scientific studies confirm that apart from nutritive value kefir also has a strong probiotic effect (Farnworth, 2005; Lopitiz-Otsoa et al., 2006; Rattray and O'Connell, 2011).

Kefir grains

Kefir grains represent a unique ecosystem in nature, formed by a symbiotic relation between bacteria and yeasts. A complex microbial community of kefir grains contains more than 50 various species of bacteria and yeasts and, depending on their origin, several species of filamentous moulds (Angulo et al., 1993; Garrote et al., 2001; Jukić et al., 2001; Stepaniak and Fetliński, 2002; Sarkar et al., 2008; Wang et al., 2008). However, the ration and number of individual microbial species within a kefir grain depends significantly on the origin and method of cultivation (Koroleva et al., 1988; Tamime and Marshall, 1997; Ferreira et al., 2010). Apart from numerous microbial species, a kefir grain is made of a spongy fibrillated structure with reticular laminar matrix and fibrous cluster which, particularly in the grain centre, branches and interconnects with long chains. This complex structure is made of proteins, polysaccharides, various cellular elements and numerous other still undefined components. A water-soluble substance kefiran makes a polysaccharide component with approximately 25 % of dry grain weight. Kefiran, which in its complex exopolysaccharide structure contains D-glucose and D-galactose in 1:1 ratio is responsible for mutual connection of microbial community of a kefir grain (La Rivière et al., 1967; Kander and Kunath, 1983; Marshall et al., 1984; Micheli et al., 1999). Also, it is presumed that kefiran contains microbial community in a kefir grain in symbiosis in a way that microbial population exists according to precisely determined pattern. A peripheral part of a grain almost exclusively contains bacteria, while yeasts dominate in the centre. Areas between the centre and a peripheral part of a kefir grain contain both bacteria and yeasts, but their ratio progressively changes depending on the distance from the grain centre (Bottazzi and Bianchi, 1980; Lin et al., 1999). Thus, homofermentative *Lactobacillus species* which form kefiran like bacteria *Lb. kefiri* and *Lb. kefiranofaciens* are differently placed within a kefir grain. *Lb. kefiranofaciens* can be found in the centre of a kefir grain where growth conditions are anaerobic and where ethanol is present, and *Lb. kefir*i at its peripheral part. Lactobacilli are also located at the peripheral part of a matrix, as well as yeasts which do not form kefiran and which usually cannot pass through a polysaccharide part into its interior (Zhou et al., 2007; Dimitreli et al., 2011). The bacteria *Leuconostoc mesenteroides* and yeast *Kluyveromyces marxianus* are also dominant microbial species of a peripheral layer (Lin et al., 2007). So far, numerous species of lactococci, lactobacilli, streptococci, some type from acetobacter genus, yeasts and moulds have been isolated from a kefir grain (FAO/WHO, 2001).

Some of the species like *Lb. kefiri* or *Lb. kefiranofaciens* were named according to kefir. Regarding the ratio of microbial species presence, depending on the origin, a kefir grain contains approximately 10^9 lactococci, between 10^7-10^8 *Leuconostoc* species, 10^7-10^8 thermophile lactobacilli, 10^4-10^5 yeasts and 10^4-10^5 acetic acid bacteria, and among filamentous moulds *Geotrichum candidum* (Kurman et al., 1992). However, it should be emphasised that neither the structure of microbial population nor a single kefir grain is unequivocally determined.

The size of kefir grain is between 0.2-3 cm. They are of irregular form looking like a cauliflower. They are slimy, but of firm consistence. By repeated inoculation into milk, kefir grains increase their mass by approximately 25 % and have a characteristic scent. The colour of kefir grains is ivory or pale-yellowish (Wszolek et al., 2006; Gaware et al., 2011).

Before their next use, kefir grains are conserved by a conventional drying method at the temperature of 33 °C or by drying in a vacuum. In favourable and stable conserving conditions, grains remain stable for several years without losing its activity (Wszolek et al., 2006). Re-activation of kefir grains is obtained by their repeated incubation in pasteurised or reconstituted milk (Sarkar et al., 2008). During incubation, dried grains regain soft structure, first by slow and then by faster growth and the new kefir grains are formed.

Biodiversity of microbial species

Due to complex microbial composition of kefir grains, the isolation and identification of individual species have been methodologically demanding and

complex. Therefore, it is not surprising that the microbial composition of a kefir grain has been differently interpreted in the literature. Apart from different origin of kefir grains, the choice of methods for microbial identification used in numerous studies is definitely one of the significant factors of mentioned diversity.

Cultivation and isolation of bacteria and yeasts

Various microbiological and molecular experimental approaches have been used for investigation of microbial population of kefir grains composition. The most represented and still most accepted experimental approach is a classical cultivation of microorganisms on more or less selective nutrient media (Jukić et al., 2001; Irigoyen et al., 2005; Wang et al., 2008; Chen et al., 2008; Chen et al., 2009) and molecular identification of isolate. The identification without cultivation and isolation of isolates (metagenomic identification) has been used in the last few years in investigation of kefir grains microbial population (Leite et al., 2012; Gao et al., 2013). It is based on the amplification of microbial DNA (certain gene or variable region) isolated directly from a sample (Juste et al., 2008; Ndoye et al., 2011).

The most frequent media used for the classical cultivation of microbial species are standard commercial media for the cultivation of *lactobacillus* (MRS agar, LAW agar, Rogosa agar, LamVab), lactococci (M17 agar), *Leuconostoc* species (MSE agar) and yeasts (Sabouraud agar, potato dextrose agar) (Simova et al., 2006; Irigoyen et al., 2005; García-Fontán et al., 2006., Wang et al., 2008). Also, non-selective nutrient medium PCA (Plate count agar) is most frequently used for determination of aerobic mesophilic bacteria total number (García-Fontán et al., 2006, Wang, et al., 2012).

Isolate purification is a standard procedure which has to be carried out in order to be sure that a microorganism isolated from one colony represents only one isolate - one bacterial species, which very often is not the case after the first cultivation procedure. Therefore, one, two or three subcultivations have to be carried out on the same nutrient media under the same conditions (temperature, with or without the presence of oxygen). Also, after each cultivation, isolated colonies have to be examined under the microscope in order to determine if it is a pure isolate or several morphotypes and if another subcultivation should be carried out in order to obtain one "pure" isolate (Caprette, 2005), which is used later for the isolation of genomic DNA for further molecular identification.

Cultivation, purification and isolation of microorganism are very sensitive and important microbiological techniques. The cultivation and/or isolation itself can sometimes represent a much more serious problem problem than the molecular identification which can sometimes be a routine analysis. It has to be emphasised since many autochthonous microbial species are very difficult to cultivate on standard commercial nutrient media. Also, a routine use of standard commercially available media developed in the last thirty years can be suitable for the growth of always the same microbial species regardless of the real number of species in the examined sample (Neviani et al., 2009; Vartoukian et al., 2009), presenting only a partial image of a microbial population which will be cultivated on a nutrient medium.

Since molecular methods based on the isolated DNA and/or RNA are used mostly for the identification of microorganisms, the basics of molecular identification will be described.

Molecular identification

Isolation of DNA from the isolate

The isolation of genomic microbial DNA from the isolate has experienced a significant development from the classic isolation procedure based on the use of phenol-chloroform. Today, commercial kits of renowned manufacturers have been most frequently used in routine DNA isolations and the isolation is carried out according to the manufacturer's protocol. Therefore, only a few general specificities of DNA isolation from the isolate will be mentioned. In order to isolate DNA from the isolate, a colony (previously purified by 2-3 subcultivations) has to be inoculated in a liquid medium, which ensures growth of bacteria in the period from 12-24 hours. The incubation period of isolates for 12-24 hours is usually sufficient to get a necessary cell density for DNA isolation. However, for some isolates, it can be even 48 hours to ensure a sufficient number of cells during incubation, or another inoculation (transplant) of the isolate in a liquid medium is necessary. Namely, the existence of a specific feature of each isolate which

cannot be predicted in advance has to be taken into account. The sample of 1-2 mL is taken from grown cells in a pure culture, for DNA isolation from the liquid medium, or the sample volume is determined by an experienced estimation of medium turbidity or by measuring its optical density. Protocols for DNA isolation differ among themselves to a lesser or greater extent, i.e. there are numerous variations of very similar protocols. These differences are usually in concentration of certain reagents or the composition of certain reagents is a manufacturing secret and the exact composition is not known. However, the initial lysis of the bacterial cell wall with the additional enzyme of lysozyme and proteinase K is common in most protocols for the efficient DNA isolation. After the isolation, DNA concentration (ng/μL) is determined by spectrophotometry method or electrophoretic methods (electrophoresis gel). This procedure is important for determination of the exact DNA microlitres that should be added for certain DNA concentration (ng/μL) in the reaction mixture, in the next step of PCR amplification (Kuchta et al., 2006). DNA concentration in PCR reactions most frequently varies from 20 to 100 ng/μL, which depends on many factors.

However, sometimes much simpler protocols for DNA isolation are implemented, based only on the lysis of a cell wall and the lysed cell is used as a template DNA for PCR reaction (Juste et al., 2008; Ndoye et al., 2011), or the whole colony is used for PCR reaction (colony-PCR) without DNA isolation or previous cell lysis (Unsal, 2008).

Identification of isolates by PCR methods

The identification of isolated microorganisms by methods based on PCR polymerase chain reaction has been applied since mid 1980-ies (Stefan et al., 1988). In that period, many variations of PCR method were developed and introduced by which a certain targeted gene or a variable gene region is amplified in vitro (Bartlett and Stirling, 2003). Primers are added to PCR reaction mixture (artificially synthesised oligonucleotides 5'-3' and 3'-5' direction), isolated DNA (DNA template), enzyme Taq polymerase, deoxyribonucleotides (A,T,C,G), puffer and sterile water. Magnesium which is added can also be an integral part of the puffer or is added separately. PCR reaction mixture is most frequently prepared in volumes of 25 or 50 μL. The very PCR reaction consists of three main cycle steps: denaturation step of a two-strand DNA molecule, primer annealing step and a strand extension step (Kuchta et al., 2006). PCR method is based on the activity of Taq polymerase enzyme, isolated from the bacterium *Thermus aquaticus* which has natural habitats as thermal sources and due to that does not lose the ability of amplifying DNA on temperatures of PCR reactions, generally 60-95 °C (Kuchta et al., 2006).

The optimization of certain steps of PCR reaction (temperature, cycle repeating) and concentration of certain reagents (primers, DNA, enzyme, deoxyribonucleotides) are the most frequent problems which can occur during an experiment. Also, potential problems could be contamination of primers or any other reagent or inactivity of Taq enzyme polymerase. It is sometimes difficult to establish the causes of failure of an experiment and with some isolates they can never be established. The aim of PCR reaction is to amplify a targeted gene or gene region important for the identification of microorganisms. The most frequent target of amplification in bacteria is 16S rRNA gene or one of variable regions (V1-V9) of 16S rRNA gene (Cardenas and Tiedje, 2008), for whose amplification universal or genus specific primers are used. With yeasts, the most frequent target of amplification is D1 region of 26S rRNA gene (Cocolin et al., 2002; Wang et al., 2008). In cases when there are many isolates, for certain isolates in order to get a valid result, either the conditions of PCR reaction or primers should be changed, since the applied protocol does not have to be equally efficient for all the isolates.

In further steps PCR product is purified and then mostly separately digested with the enzyme combinations (2, 3 or 4 enzymes). Every enzyme is specific for the digestion of the amplified PCR product. Specific profiles for the exact species are obtained by the combination of various restriction enzymes (Mancini et al., 2012). Products obtained by PCR reaction and enzyme digestion vary in the number of base pairs and are separated by electrophoresis in agarose or polyacrylamide gel in order to obtain specific profiles (Lushai et al., 1999; Kuchta et al., 2006; Copola et al., 2008). The identification of obtained profiles can be carried out in two ways. The first one is the comparison of obtained profiles with profiles of reference strains and the

other can be carried out either independently from the first one or as a supplement to the first one, is a sequencing of 16S rRNA gene from the representative profiles (Copola et al., 2008; Mancini et al., 2012). The obtained sequences are compared to some of available databases on the internet as BLAST. For some species the precise identification by comparison of the obtained profiles is possible by the use of only one restriction enzyme, but in some species which are genetically very close, 3 or 4 enzymes have to be applied for the successful identification of an isolate, since profiles of genetically very close microbial species, obtained by the use of one or two enzymes, can in some cases be identical, which prevents the unequiocal identification of species.

One of the possible procedures of molecular identification of isolates, independent of the enzyme digestion and sequencing of 16S rRNA gene is usage of species-specific primers for proving the presence of a specified species. Such a molecular identification is not used frequently because many species-specific primers have to be used, i.e. as many as the number of expected species. However, this approach can be a final confirmation for the identification of species or subspecies which cannot be identified by other methods and it can be used for the final confirmation of the identification (Temmerman et al., 2004). Also, if the results of 16S rRNA gene sequencing does not provide unequivocal identification, than it leaves the possibility that the result might be two or three genetically close species. The final confirmation of the identification can be carried out with species-specific primers (Temmerman et al., 2004) or DNA-DNA hybridisation (Goris et al., 2007).

Metagenomic identification

The cultivation of microbial population gives a partial insight into the structure of microbial population of complex communities because many species are either not cultivable or cultivation and isolation are doubtful (Giraffa and Neviani, 2001; Copola et al., 2008; Leite et al., 2012). Metagenomic identification, without the cultivation and isolation of microorganisms, represents a wide spectrum of structure investigation possibilities and dynamics of microbial population of any microbial system (Huson et al., 2009). By such a molecular approach it is possible to isolate the total microbial DNA (or RNA) from kefir or kefir grain, for which commercial kits of various manufacturers are used and the targeted region of 16S rRNA gene in bacteria or 26S rRNA gene in yeasts (which are the most frequent, but not the only targets of the amplification) can be amplified by PCR reaction in order to get the insight in the structure of microbial community (Ünsal, 2008; Zhou et al., 2009; Cruz et al., 2010; Gao et al., 2013). For investigation of kefir microbial population by the identification without cultivation, the most frequently used methods are PCR-DGGE (Denaturing Gradient Gel Electrophoresis) and in the last few years pyrosequencing (Wang et al., 2008; Ninane et al. 2007; Chen et al., 2008; Miguel et al., 2010; Leite et al., 2012). Also, the method of cloning the amplified DNA (isolated directly from the kefir grain) was used in *E. colli* and sequencing of V1 and V2 region of 16S rRNA gene (Veronique et al., 2007).

The totally isolated microbial DNA amplified in PCR reaction is detected by PCR-DGGE method on the polyacrylic gel as fragments (of the same size regarding the number of base pairs, but of specific nucleotide sequence for each microbial species) which migrate in a gel to various positions (Muyzer and Smalla, 1998). The identification of DNA fragments is possible either by comparison of a fragment position with the position of the reference strain fragment or with sequencing of fragments cut from various positions in a gel (Muyzer and Smalla, 1998; Copola et al., 2008; Jianzhong et al., 2009). In order to compare fragment positions in a gel, gels are normalised and analysed by bioinformatics programmes.

However, one of the main drawbacks of investigating structures of complex microbial communities is that species which were present in small numbers, most frequently will not be amplified or their DNA will not be isolated at all (Ercolini, 2004). The new method which has been used only recently in microbial population of kefir investigation is pyrosequencing (Dobson et al., 2011; Leite et al., 2012). Pyrosequencing is automated and sophisticated technique based on the synthesis of a single-strained DNA and detection of nucleotide sequences (Magra et al., 2012). The main advantage of this method is that it gives the insight into the structure of minor microbial population present in the investigated microbial system (Quigley et al., 2012).

Table 1. Microbiota of kefir grains

	Microorganism	Reference*
1	*Acetobacter fabarum*	Gao i sur., 2012
2	*Acetobacter lovaniensis*	Unsal, 2008
3	*Acetobacter syzygii*	Unsal, 2008
4	*Acinetobacter*	Gao i sur., 2013
5	*Bifidobacterium spp*	Leite i sur., 2012
6	*Candida inconspicua*	Simova i sur., 2002
7	*Dysgonomonas*	Gao i sur., 2013
8	*Enterococcus faecium*	Unsal, 2008
9	*Geotrichum candidum*	Timara, 2010
10	*Gluconobacter japonicus*	Miguel i sur., 2012
11	*Halococcus spp.*	Leite i sur., 2012
12	*Kazachstania aerobia*	Magalhães i sur., 2011
13	*Kazachstania exigua*	Zhou i sur., 2009
14	*Kazachstania unispora*	Zhou i sur., 2009
15	*Kluyveromyces lactis*	Zhou i sur., 2009
16	*Kluyveromyces marxianus*	Zhou i sur., 2009
17	*Kluyveromyces marxianus* var. *lactis*	Simova i sur., 2002
18	*Lachancea meyersii*	Magalhães i sur., 2011
19	*Lactobacillus amylovorus*	Leite i sur., 2012
20	*Lactobacillus brevis*	Simova i sur., 2002
21	*Lactobacillus buchneri*	Leite i sur., 2012
22	*Lactobacillus casei*	Zhou i sur., 2009
23	*Lactobacillus paracasei*	Magalhães i sur., 2011
24	*Lactobacillus casei* subsp. *pseudoplantarum*	Simova i sur., 2002
25	*Lactobacillus crispatus*	Leite i sur., 2012
26	*Lactobacillus delbrueckii* subsp. *bulgaricus*	Simova i sur., 2002
27	*Lactobacillus helveticus*	Unsal, 2008
28	*Lactobacillus kefiranofaciens*	Unsal, 2008
29	*Lactobacillus kefiranofaciens* subsp. *kefiranofaciens*	Leite i sur., 2012
30	*Lactobacillus kefiranofaciens* subsp. *kefirgranum*	Leite i sur., 2012
31	*Lactobacillus kefiri*	Unsal, 2008
32	*Lactobacillus parabuchneri*	Magalhães i sur., 2011
33	*Lactobacillus parakefiri*	Leite i sur., 2012
34	*Lactobacillus plantarum*	Gao i sur., 2012
35	*Lactobacillus satsumensis*	Miguel i sur., 2012
36	*Lactobacillus uvarum*	Miguel i sur., 2012
37	*Lactococcus lactis* subsp. *cremoris*	Zhou i sur., 2009
38	*Lactococcus lactis* subsp. *lactis*	Unsal, 2008
39	*Leuconostoc lactis*	Gao i sur., 2012
40	*Leuconostoc mesenteroides*	Unsal, 2008
41	*Pelomonas*	Gao i sur., 2013
42	*Pichia fermentans*	Wang i sur., 2008
43	*Pichia guilliermondii*	Gao i sur., 2012
44	*Pichia kudriavzevii*	Gao i sur., 2012
45	*Pseudomonas putida*	Zhou i sur., 2009
46	*Saccharomyces cerevisiae*	Zhou i sur., 2009
47	*Saccharomyces martiniae*	Zhou i sur., 2009
48	*Saccharomyces turicensis*	Wang i sur., 2008
49	*Saccharomyces unisporus*	Zhou i sur., 2009
50	*Shewanella*	Gao i sur., 2013
51	*Streptococcus thermophilus*	Simova i sur., 2002
52	*Weissella*	Gao i sur., 2013

*The table gives the review of identified kefir grain microorganisms, not mentioning whether the microorganism was identified in that reference for the first time, or if it was only identified in that reference

Microbial species of kefir grains

Numerous microbial species in kefir grains and kefir were identified by different microbiological and molecular techniques. The diversity of identified species of bacteria and yeast confirms the complex microbial structure of that natural microbial system. According to recent scientific sources, the microbial population of the kefir grain includes more than 50 various microorganism species (Table 1). This number will probably be increased with the further development of metagenomic identification, but also with improving classical cultivation, since no single approach is perfect and cannot give the complete insight into the structure of microbial population.

Unsal (2008) isolated and identified by PCR-DGGE method from the kefir grain *Acetobacter syzygii*, *Leuconostoc mesenteroides*, *Enterococcus faecium*, *Lactobacillus kefiri/parabuchneri*, and *Lactococcus lactis s*ubsp. *lactis*, while the following were identified in kefir by metagenomic approach without isolation: *Lactococcus lactis* subsp. *lactis*, *Lactobacillus kefiranofaciens*, *Lactobacillus helveticus*, *Acetobacter lovaniensis*. In this paper the equal number of microorganisms was identified by both approaches. However, Zhou et al. (2009) identified by PCR-DGGE method without isolation 10 bacterial species in the kefir grain: *Lactobacillus kefiranofaciens*, *Lactobacillus helveticus*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus casei*, *Lactobacillus kefiri*, *Leuconostoc mesenteroides*, *Pseudomonas* sp., *Pseudomonas fluorescens*, *Pseudomonas putida* and seven species of yeasts: *Kazachstania unispora*, *Kazachstania exigua*, *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Saccharomyces cerevisiae*, *Saccharomyces martiniae*, *Saccharomyces unisporus*. These 17 microorganisms might have probably been so far the largest number of identified microorganisms in one study. Gao et al. (2012) isolated and identified 11 species of microorganism from Tibetan kefir: *Bacillus subtilis*, *Lactococcus lactis*, *Lactobacillus kefiri*, *Leuconostoc lactis*, *Lactobacillus plantarum*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, *Kazachstania unispora*, *Acetobacter fabarum*, *Pichia guilliermondii*. Simova et al., (2002) isolated and identified from the kefir grain *Lactococcus lactis* subsp. *lactis*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus casei* subsp. *pseudoplantarum*, *Lactobacillus brevis*, *Kluyveromyces marxianus* var. *lactis*, *Saccharomyces cerevisiae*, *Candida inconspicua*, *Candida maris*. Wang et al., (2008) isolated and identified from the kefir grain yeasts *Kluyveromyces marxianus*, *Saccharomyces turicensis*, *Pichia fermentans* and *Saccharomyces unisporus*. Jianzhong et al. (2009) investigated the composition of the Tibetan kefir microbial population by PCR-DGGE method without previous microorganism cultivation. Primers 338F-GC and 518R were used for PCR reaction for bacterial DNA, and the target of amplification was V3 region of 16S rRNA gene, and for DNA of yeasts primers NL1GC and LS2 were used. In the same way, the following bacteria were identified: *Pseudomonas sp.*, *Leuconostoc mesenteroides*, *Lactobacillus helveticus*, *Lactobacillus kefiranofaciens*, *Lactococcus lactis*, *Lactobacillus kefiri*, *Lactobacillus casei*, and yeasts: *Kazachstania unispora*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae* and *Kazachstania exigua* (Jianzhoung et al., 2009). Leite et al., (2012) amplified V3 region of 16S rRNA gene with universal primers F357- GC and R518, to explore microbiota of Brazilian kefir. Specific primers were also used for the identification of lactic acid bacteria: Lac1 and Lac2-GC for the identification of bacteria from genera *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Weissella*, and primers Lac3 for bacteria from genera *Lactococcus*, *Streptococcus*, *Enterococcus*, *Tetragenococcus* and *Vagococcus*. D1 domain of 26S of rRNA yeast gene was amplified by primers NL1-GC and LS2. All GC primers contained 39 bp GC nucleotides in order to prevent total product denaturation (Leite et al., 2012). Investigating the structure of microbial population of Brazilian kefir by pyrosequencing and DGGE method, the potential of both methods in metagenomic identification of microbiota was compared (Leite et al., 2012). Only 5 species of microorganisms were identified by DGGE method: *Lb. kefiranofaciens*, *Lactococcus lactis*, *Lb. kefiri*, *Saccharomyces cerevisiae* and *Kazachstania unispora*, while the same microbe species which were identified by DGGE method were also identified by pyrosequencing, but also representatives of *Bifidobacterium*, *Leuconostoc*, *Streptococcus*, *Acetobacter*, *Pseudomonas*, *Halococcus*, as well as numerous representatives of lactobacilli which were not identified by DGGE method:

Lb. kefiranofaciens subsp. *kefirgranum*, *Lb. kefiranofaciens* subsp. *kefiranofaciens*, *Lb. parakefiri*, *Lb. parabuchneri*, *Lb. amylovorus*, *Lb. crispatus*, *Lb. buchneri*, and one representative of *lactococcus Lc. lactis* subsp. *cremoris* (Leite et al., 2012). It should be emphasised that some of these species were represented with less of 1 % in the total population which emphasises the pyrosquencing potential in investigation of the structure of complex and incompletely investigated microbial communities like kefir (Leite et al., 2012). Also, Gao et al., (2013) identified for the first time in Tibetan kefir grains without cultivation species from genera *Shewanella*, *Acinetobacter*, *Pelomonas*, *Dysgonomonas*, *Weissella* and *Pseudomonas*. Considering the fact that these species were identified for the first time, their role and significance on specific characteristics of the kefir still remains to be elucidated. The mentioned results of the investigation of the structure of kefir grain microbial population prove that the number of identified microbial species is increased by the use of new molecular metagenomic methods in identification and such a trend will be continued. It also indicates a smaller potential of identification based on cultivation of microorganisms on media developed 30 or more years ago, since the fact is that the development of new nutrient media has not been as intensive as the development of metagenomic identification (Huson et al., 2009; Vieites et al., 2010; Quigley et al., 2011; Delmont et al., 2011).

Conclusion

The studies of autochthonous microbial population of kefir grain by the use of contemporary microbiological and molecular methods give new ideas on the complexity of the microbial system of the kefir grain which has so far resulted in more than 50 identified microbial species. The isolation of microorganisms from kefir grains, due to their further technological and probiotic characterisation can potentially result in strains with completely new characteristics. Further development of metagenomics, based on the identification of microbial communities without cultivation, confirm that the microbial culture isolated until now represent only one part of the complex microbial system which influences specific features of kefir. However, the classical cultivation and isolation will still remain irreplaceable for the detailed characterisation of microbial isolates and discovery of new strains.

Mikrobni sastav kefirnih zrna

Sažetak

Bakterije i kvasci, a ponekad i filamentozne plijesni u kefirnim zrnima žive u složenom simbiotskom odnosu koji kefirna zrna čini jedinstvenom mikrobnom zajednicom u prirodi. Složenost i kompleksnost njihove fizičke i mikrobne strukture razlogom su što su kefirna zrna još uvijek mikrobiološki nedovoljno i nepotpuno istražena. U istraživanju mikrobnog sastava kefirnih zrna koriste se različiti mikrobiološki i molekularni pristupi. Razvojem metagenomike, bazirane na identifikaciji bez kultivacije, otvaraju se nove mogućnosti identifikacije do sada još neidentificiranih mikrobnih vrsta sadržanih u kefirnom zrnu. Do sada je identificirano preko 50 vrsta mikroorganizama prisutnih u kefirnom zrnu. U radu su prikazane do danas identificirane mikrobne vrste sadržane u kefirnim zrnima različitog podrijetla. Također, radi tehnološkog i mikrobiološkog značenja koja imaju kefirna zrna sama po sebi, u radu su detaljnije prikazani molekularni eksperimentalni pristupi koji se koriste u istraživanju njihove mikrobne bioraznolikosti.

Ključne riječi: kefir, kefirna zrna, molekularne metode, mikrobne vrste

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