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REVIEW ARTICLE

Kefir micro-organisms: their role in grain assembly and health properties of fermented milk

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Abstract

Kefir is a homemade viscous and slightly effervescent beverage obtained by milk fermentation with kefir grains, which are built up by a complex community of lactic acid and acetic acid bacteria and yeasts confined in a matrix of proteins and polysaccharides. The present review summarizes the role of kefir micro-organisms in grain assembly and in the beneficial properties attributed to kefir. The use of both culture-dependent and independent methods has made possible to determine the micro-organisms that constitute this ecosystem. Kefir consumption has been associated with a wide range of functional and probiotic properties that could be attributed to the micro-organisms present in kefir and/or to the metabolites synthetized by them during milk fermentation. In this context, the role of micro-organisms in kefir health promoting properties is discussed with particular attention to the contribution of yeast as well as bioactive metabolites such as lactic and acetic acid, exopolysaccharides and bioactive peptides. Even though many advances on the knowledge of this ancient fermented milk have been made, further studies are necessary to elucidate the complex nature of the kefir ecosystem.

Kefir: an ancient fermented milk containing a complex microbiota

Kefir is a homemade, viscous and slightly effervescent fermented milk with an acidic flavour (Garrote *et al.* 2001). Kefir differs from other fermented products because of the particular characteristic of its starter: the kefir grains. They are discrete structures composed of protein and polysaccharide where a complex microbiota is confined. They can be described as gelatinous white or lightly yellow irregular masses with an elastic consistency and size varying from 0·3 to 3·5 cm diameter. Kefir grains contain approximately 83% water, 4 ± 5% of proteins and 9 ± 10% of a polysaccharide called kefiran (Abraham and de Antoni 1999). Lactic acid bacteria (LAB) are the major population in kefir grains accompanied by acetic acid bacteria (AAB) and yeasts (Dong *et al.* 2018). The complex microbiota is an example of a symbiotic community where LAB (10^8–10^9 CFU per gram of grain), yeasts (10^7–10^8 CFU per gram of grain) and AAB (10^5–10^6 CFU per gram of grain) share their bioproducts as energy sources and microbial growth factors (Garrote *et al.* 2010; Nielsen *et al.* 2014; Plessas *et al.* 2016; Tamang *et al.* 2016). Figure 1 shows a SEM micrograph of Argentine kefir grains as well as the main genera described in kefir.

Lactic acid bacteria helps to the preservation of the product through production of lactic acid, acetic acid and antimicrobial compounds (Garrote *et al.* 2000; John and Deeseenthum 2015) and also to organoleptic properties by producing volatile compounds (e.g. acetaldehyde), exopolysaccharides (Rimada and Abraham 2003) or free amino acids (Guzel-Seydim *et al.* 2011; Dertli and Çon 2017). Yeasts produce alcohol and carbon dioxide in the milk that contribute to mouthfeel and taste of kefir (Rosa *et al.* 2017).

The microbial composition of kefir is subjected to variations (Londero *et al.* 2012; Rosa *et al.* 2017). It is

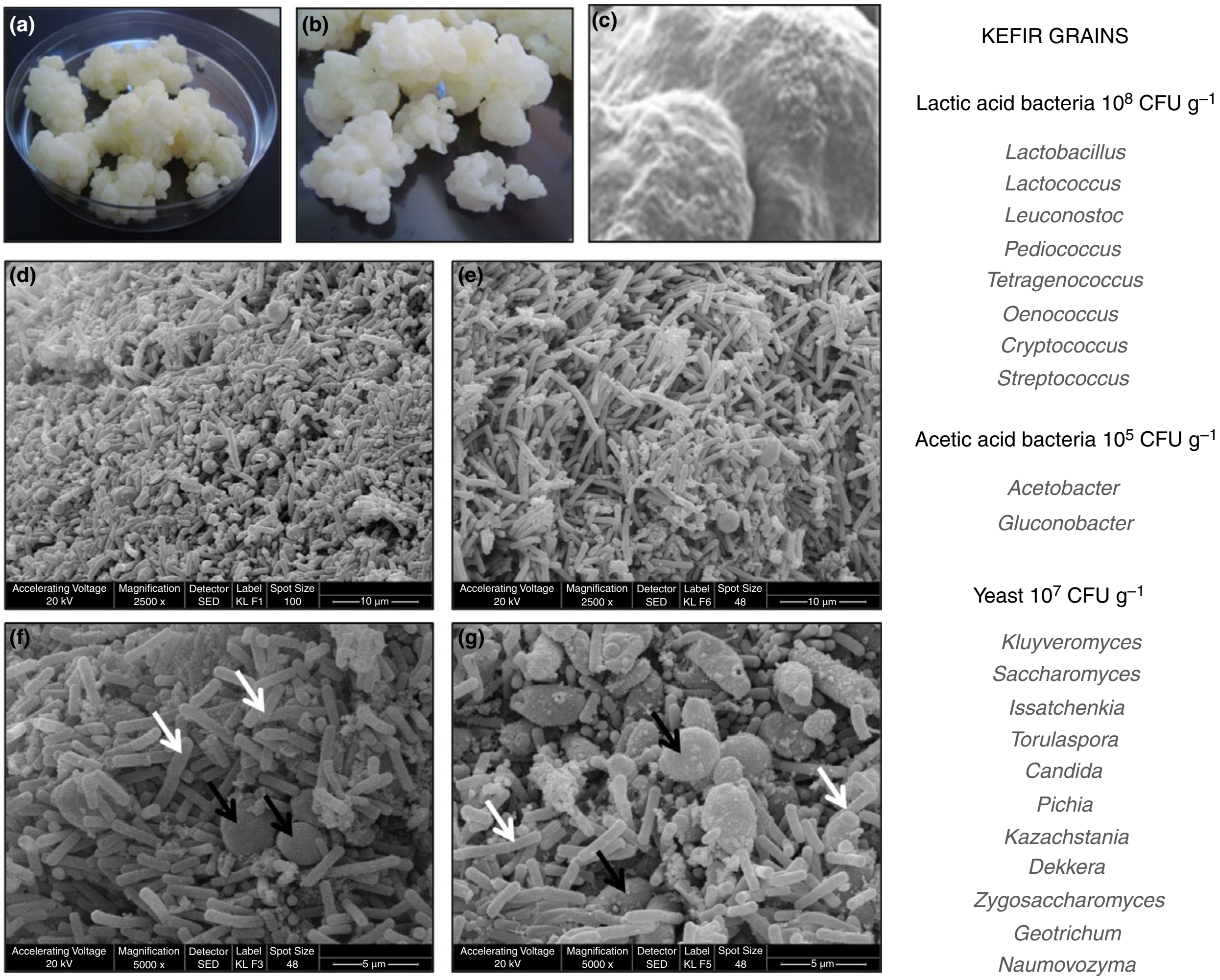


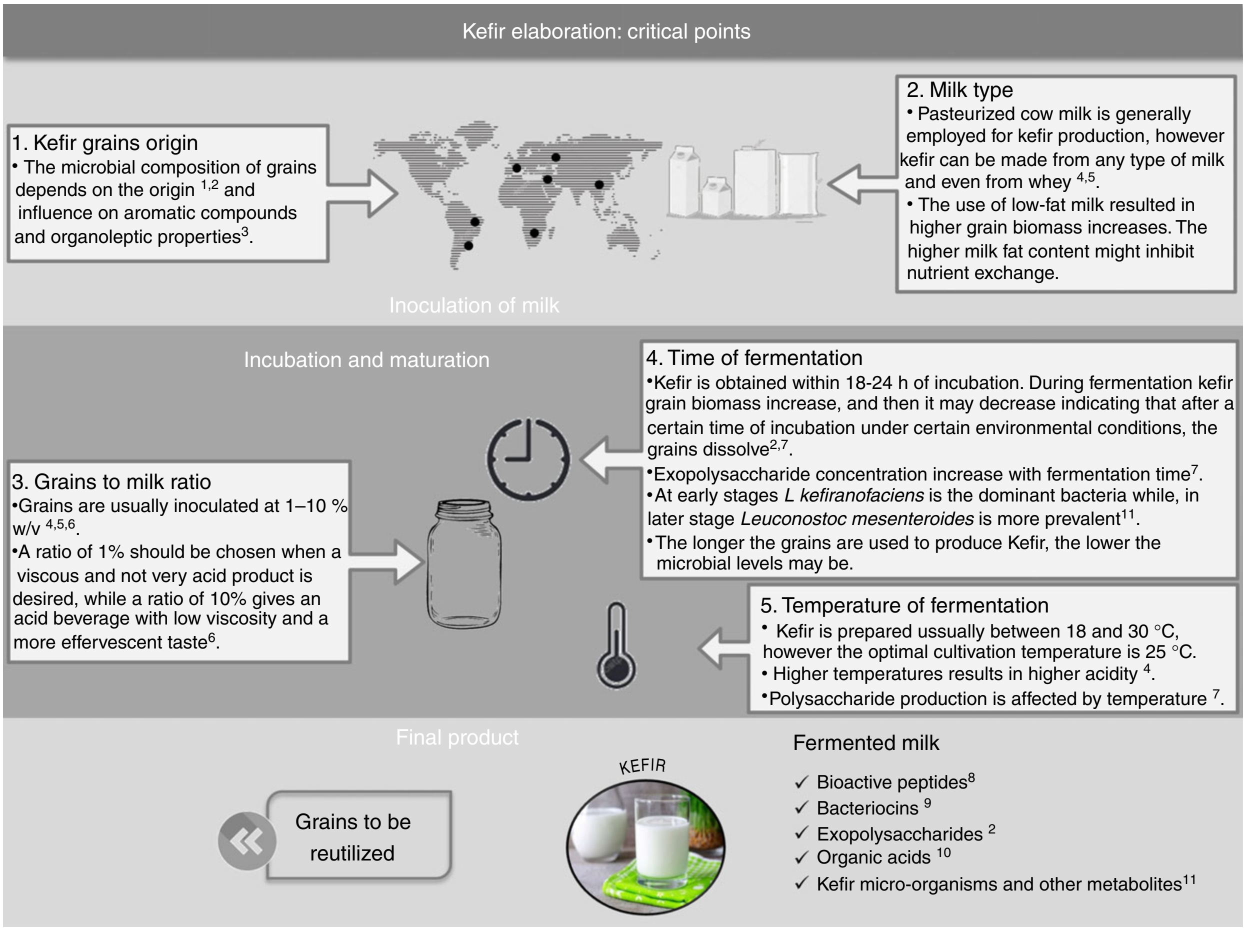
Figure 1 Macroscopic (a, b) and microscopic aspect (c–g) of kefir grains from Argentina and list of the main bacteria and yeasts genera described in kefir grains from different sources. The presence of bacteria (white arrows) and yeasts (black arrows) is indicated in microphotographs f and g.

documented that these variations may be due to factors such as the origin and storage of the kefir grains, the type of milk used as well as the processing conditions of the product, especially the grain/milk ratio and the fermentation temperature (Garrote *et al.* 1998; Nielsen *et al.* 2014).

To obtain kefir, the grains are inoculated in the milk in a certain proportion and when bacteria and yeasts of the kefir grain find the suitable conditions (nutrients, temperature), the fermentation process begins resulting in an increase in the number of micro-organism and the production of different metabolites. At the end of this process, kefir grains that have increased their mass can be recovered from the fermented milk (separated by filtration) and used immediately in a new fermentation (subculture) or stored in suitable conditions to be used as starters. In Fig. 2, a group of variables that must be taken into account during kefir elaboration are recognized. These are considered as ‘critical points’ because they will define the characteristics of the final product with typical chemical, microbiological, organoleptic, nutritional and functional properties.

Several health promoting properties ascribed to kefir consumption were widely reviewed (Nielsen *et al.* 2014; Prado *et al.* 2015; Bourrie *et al.* 2016; Kesenkaş *et al.* 2017; Rosa *et al.* 2017). Kefir benefits can be attributed to the complex microbiota but also to the metabolites produced by them during fermentation process.

The present review describes the methods employed to characterize kefir micro-organisms and their role in grain assembly and health promoting properties attributed to kefir. The role of the nonbacterial fraction as well as the contribution of yeast to health benefits was discussed.



1. Kefir grains origin
- The microbial composition of grains depends on the origin [1,2] and influence on aromatic compounds and organoleptic properties[3].

2. Milk type
- Pasteurized cow milk is generally employed for kefir production, however kefir can be made from any type of milk and even from whey [4,5].
- The use of low-fat milk resulted in higher grain biomass increases. The higher milk fat content might inhibit nutrient exchange.

3. Grains to milk ratio
- Grains are usually inoculated at 1–10 % w/v [4,5,6].
- A ratio of 1% should be chosen when a viscous and not very acid product is desired, while a ratio of 10% gives an acid beverage with low viscosity and a more effervescent taste[6].

4. Time of fermentation
- Kefir is obtained within 18-24 h of incubation. During fermentation kefir grain biomass increase, and then it may decrease indicating that after a certain time of incubation under certain environmental conditions, the grains dissolve[2,7].
- Exopolysaccharide concentration increase with fermentation time[7].
- At early stages *L kefiranofaciens* is the dominant bacteria while, in later stage *Leuconostoc mesenteroides* is more prevalent[11].
- The longer the grains are used to produce Kefir, the lower the microbial levels may be.

5. Temperature of fermentation
- Kefir is prepared ussually between 18 and 30 °C, however the optimal cultivation temperature is 25 °C.
- Higher temperatures results in higher acidity [4].
- Polysaccharide production is affected by temperature [7].

Fermented milk
- ✓ Bioactive peptides[8]
- ✓ Bacteriocins [9]
- ✓ Exopolysaccharides [2]
- ✓ Organic acids [10]
- ✓ Kefir micro-organisms and other metabolites[11]

References: 1. Garofalo et al. 2005. 2. Garrote et al. 2010. 3. Dertli and Çon, 2017. 4. Londero et al. 2012. 5. Nielsen et al. 2014. 6. Garrote et al. 1998. 7. Rimada and Abraham, 2001.8. Ebner et al. 2015. 9. john and Deeseenthum 2015. 10. Garrote et al. 2000. 11. Walsh et al 2016.

Figure 2 Critical points in kefir production that defines the organoleptic and functional characteristics of the fermented product.

Methods for the isolation and identification of kefir micro-organisms

Kefir is a natural reservoir of safe and potentially beneficial healthy strains. Culture-dependent and independent techniques were employed to establish the complex microbial populations that are present in kefir grains or kefir. The strong association that exists between the micro-organisms makes their identification and study a difficult task since some micro-organisms may only grow when they coexist in a symbiotic association (Dobson *et al.* 2011). Methods employed to study microbiota of kefir from different origins and the micro-organisms found in them are listed in Table 1.

The application of culture-dependent methods allowed to isolate and identify a wide collection of LAB and yeasts whose technological and probiotic properties have been studied (Garrote *et al.* 2001; Hamet *et al.* 2013; Prado *et al.* 2015; Kesenkaş *et al.* 2017). Accurate identification of these micro-organisms need the use of traditional phenotypic tests accompanied with molecular techniques (Vandamme *et al.* 1996). Phenotypic characteristics include morphology, mobility, sugar fermentation, Gram staining and spores formation, among others assays. Whole cell protein profiles or methods that involve the analysis of the whole bacteria compounds such as FT-IR were useful tools for discrimination of *Lactobacillus* isolated from kefir (Bosch *et al.* 2006; Hamet *et al.* 2013) as well as molecular techniques such as RAPD accompanying traditional phenotypic test (Golowczyc *et al.* 2008). Sequence-based identification using phenylalanyl-tRNA synthase gene (*pheS*) and Rep-PCR fingerprinting with the (GTG) five primers resulted a rapid and consistent tool for typing of *Lactobacillus* isolated from kefir (Hamet *et al.* 2013). Phenotypic analysis based on the 16S rRNA gene sequence was also employed for taxonomical

Table 1 Microbial composition of kefir grains and beverages from different origin analyzed using different methodologies

Origin	Micro-organisms	Methods employed to study kefir microbiota	References
Argentine kefir grains	*L. kefiri*, *L. parakefiri*, *L. paracasei*, *L. kefiranofaciens* ssp. *kefiranofaciens*, *L. kefiranofaciens* ssp. *kefirgranum*, *L. plantarum*, *Lc. lactis* ssp. *lactis*, *Lc. lactis* ssp. *lactis* biovar *diacetylactis*, *Leu. mesenteroides*, *Acetobacter* sp., *K. marxianus*, *Sac. cerevisiae*, *Sac. unisporus*	Identification of isolates by biochemical test, whole cell protein pattern, FTIR, RAPD-PCR, Rep-PCR fingerprinting (GTG) 5, phenylalanyl-tRNA synthase (*pheS*) gene sequencing, ITS region polymorphism. PCR amplification of 16S and 26S rDNA sequences-DGGE and identification of DGGE bands	Garrote *et al.* (2001) Golowczyc *et al.* (2008) Londero *et al.* (2012) Hamet *et al.* (2013) Diosma *et al.* (2014)
Belgium kefir grains and their products	*L. kefiri*, *L. kefiranofaciens*, *Lc. lactis* ssp. *cremoris*, *Leu. mesenteroides*, *Glu. frateurii*, *Ac.orientalis*, *Ac. lovaniensis, Naumovozyma* sp., *K. marxianus, Kazachastania kefir*	Metagenetic analysis targeting the 16S and 26S ribosomal DNA fragments by pyrosequencing	Korsak *et al.* (2015)
Brazilian kefir grains and beverage	*L. kefiranofaciens*, *L. parakefiri*, *L. kefiri*, *L. amylovorus*, *L. buchneri*, *L. crispatus*, *L. paracasei*, *L. helveticus*, *L. uvarum*, *Lc. lactis*, *Leu. mesenteroides*, *Glu. japonicus*, *Ac. syzygii*, *Sac. cerevisiae*	Identification of isolated micro-organism by phenotypic and genotypic methods. PCR amplification of 16S and 26S rDNA sequences-DGGE and pyrosequencing	Miguel *et al.* (2010) Magalhães *et al.* (2011a) Leite *et al.* (2012) Zanirati *et al.* (2015)
Irish kefir grains and beverage	*L. kefiranofaciens*, *L. kefiri*, *L. helveticus*, *L. parabuchneri*, *L. acidophilus*, *L. parakefiri*, *Leucoconstoc* sp.	16S compositional sequencing analysis.	Dobson *et al.* (2011)
Italian kefir grains	*L. kefiranofaciens*, *Lc. lactis*, *St. thermophilus*, *Enterococcus* sp., *Bacillus* sp., *Ac. fabarum, Ac. lovaniensis*, *Ac. orientalis*, *Dekkera anomala*	PCR-DGGE of kefir grains and identification of DGGE bands Analysis of bacterial and yeast diversity by rRNA gene pyrosequencing	Garofalo *et al.* (2015)
South African kefir grains	*L. plantarum*, *L. delbrueckii* ssp. *delbrueckii*, *L. brevis*, *L. delbrueckii* ssp. *lactis*, *L. curvatus*, *L. fermentum*, *Lc. lactis* ssp. *lactis*, *Leu. mesenteroides* ssp. *cremoris*, *Leu. mesenteroides* ssp. *mesenteroides/dextranicum*, *C. lipolytica*, *C. lambica*, *C. krusei*, *C. kefyr*, *C. holmii*, *Sac. cerevisiae*, *Zygosaccharomyces* sp., *Cryptococcus humicolus*, *Geotrichum candidum*	Isolation in selective growth media and identification by using morphological and biochemical characteristics PCR-DGGE of kefir grains and identification of DGGE bands	Witthuhn *et al.* (2004, 2005) Garbers *et al.* (2004)
Taiwanese kefir grains	*L. kefiranofaciens*, *L. kefiri*, *Lc. lactis*, *Leu. mesenteroides*	PCR-DGGE of isolates and DNA sequencing techniques PCR-DGGE of kefir grains and identification of bands	Chen *et al.* (2008)
Tibetan kefir grains	*L. kefiranofacien*, *L. kefiri*, *L. casei*, *L. paracasei*, *L. helveticus*, *Lc. lactis*, *Leu. mesenteroides*, *St. thermophilus*, *K. marxianus*, *Sac. cerevisiae*, *Kazachstania exigua*, *Kazachstania unispora*	DGGE of partially amplified 16S rRNA or 26S rRNA followed by sequencing of the bands Isolation of micro-organisms and typing by 16S rDNA and 26S rDNA-D1/D2 gene sequencing technology, (GTG)5-Rep-PCR genomic fingerprinting	Zhou *et al.* (2009) Gao and Zhang (2018)

(*Continued*)

Table 1 (*Continued*)

Origin	Micro-organisms	Methods employed to study kefir microbiota	References
Turkish kefir grains	*L. kefiri*, *L. kefiranofaciens*, *L. casei*, *L. paracasei*, *L. parakefiri*, *L. plantarum*, *L. acidophilus*, *L. amylovorus*, *L. brevis*, *L. buchneri*, *L. crispatus*, *L. delbrueckii*, *L.diolivorans*, *L. gallinarum*, *L. gasseri*, *L. helveticus*, *L. johnsonii*, *L. otakiensis*, *L. parabuchneri*, *L. reuteri*, *L. rhamnosus*, *L. rossiae*, *L. sakei*, *L. salivarius*, *L. sunkii*, *Lc. garvieae*, *Lc. lactis*, *Leu. mesenteroides, O. oeni*, *Pediococcus* sp., *Tetragenococcus halophilus*	16S RNA pyrosequencing Whole genome shotgun pyrosequencing	Nalbantoglu *et al.* (2014)
Russian kefir grains	*L. casei*, *L. paracasei*, *L. kefiri*, *L. kefiranofaciens* ssp. *kefirgranum*, *Lc. lactis* ssp. *cremoris/lactis*, *Leu. pseudomesenteroides*, *Sac. cerevisiae*, *Kazachstania unispora*	Classical microbiological analysis and DGGE-PCR method	Kotova *et al.* (2016)

L.: Lactobacillus, *Lc.: Lactococcus*, *St.:Streptococcus*, *Ac.:Acetobacter*, *Glu.: Gluconobacter*, *O.: Oenococcus*, *Sac.*: *Saccharomyces*, *K.: Kluyveromyces*, *C.: Candida.*, Leu.: Leuconostoc.

purpose. However, discrimination of *Lactobacillus kefiranofaciens* at subspecies level was not possible with this approach since genotypic analyses on representative strains from both taxa demonstrated that *L. kefiranofaciens* susbsp *kefiranofaciens* and *L. kefiranofaciens* ssp. *kefirgranum* share 100% 16S rDNA sequence similarity (Vancanneyt *et al.* 2004). However, FTIR analysis as well as whole protein profile allows differentiation of *L. kefiranofaciens* even at subspecies level (Bosch *et al.* 2006; Hamet *et al.* 2013).

Culture-based analyses are limited to species with the ability to grow on the specific medium used. Thus, culture-independent techniques have the potential to provide an in-depth analysis based on the isolation of DNA from dead and living micro-organism (Porcellato *et al.* 2015). Sequence dependent electrophoresis-based fingerprinting methods, such as denaturing gradient gel electrophoresis (DGGE), allow pattern-based visualization of the predominant bacterial groups including those that do not grow and is a first approach for comparing kefir microbiota. Garbers *et al.* (2004) demonstrated that DGGE is a successful method to typify kefir grains' microbial consortium and compared grains of different origins and culture conditions. It has also been described that through DGGE it is possible to detect LAB present in kefir that are not recovered by techniques dependent on culture (Chen *et al.* 2008; Zhou *et al.* 2009). Besides, several LAB that had been previously identified by cultivation were not detected by PCR-DGGE in the same kefir grain (Chen *et al.* 2008; Miguel *et al.* 2010; Leite *et al.* 2012; Londero *et al.* 2012; Hamet *et al.* 2013; Garofalo *et al.* 2015). DGGE analysis followed by sequencing and identification of DGGE bands has some limitation such as detection level and taxonomic resolution. The differential amplification of competitor templates from micro-organisms that are present in high concentration could disadvantage the detection of species that are in low concentration. These results indicate that combining culture-dependent and independent methods allow having a more accurate insight of the microbiota of kefir grain and its fermented milk.

The application of high-throughput sequencing of 16S amplicons was used to investigate kefir microbial ecosystems in order to achieve a more comprehensive understanding. Pyrosequencing analysis of 16S amplicons was applied for the identification of bacteria and ITS region for yeasts discrimination of kefir from different origins including Italy (Garofalo *et al.* 2015), Brazil (Leite *et al.* 2012), Turkey (Nalbantoglu *et al.* 2014; Dertli and Çon 2017), Tibet (Gao *et al.* 2013; Gao and Zhang 2018) and Ireland (Dobson *et al.* 2011; Marsh *et al.* 2013). It is important to point out that in contrast to DGGE, pyrosequencing analysis allowed to identify micro-organisms that are in low concentration (Leite *et al.* 2012; Garofalo *et al.* 2015). Sequencing of 16S amplicons is limited to genus-level identification and depends on amplification condition and primer selection. Otherwise, it may inaccurately assess the abundance of the community members due to high similarity of the corresponding 16S sequences (Marsh *et al.* 2013; Bourrie *et al.* 2016; Walsh *et al.* 2018). The analysis of the V3 region of the 16S rRNA gene contains insufficient differences for the discrimination of closely related species such as *Lactobacillus kefiri*, *L. buchneri*, *L. sunkii* and *L. otakiensis* or for the discrimination of *L. kefiranofaciens* and *L. helveticus* (Hamet *et al.* 2013; Nalbantoglu *et al.* 2014; Garofalo *et al.* 2015). Nevertheless, the analysis of the V7–V8 region by PCR-DGGE allowed discriminating the presence of *L. kefiri* univocally (Garofalo *et al.* 2015). Metagenomic analysis using whole genome sequencing (WGS-whole genome shotgun) provides a culture-independent approach that does not involve cloning or 16S rRNA gene region amplification. Nalbantoglu *et al.* (2014) studied Turkish kefir grain ecosystem by using amplicon sequencing metagenomics and shotgun metagenomics. They concluded that WGS-based approach identifies novel species and the underlying community with higher resolution and better abundance accuracy. Sequencing based approaches have also identified several yeast species that had not previously been associated with kefir, such as *Dekkera anomala* and *I. orientalis* and have even shown that, in some grains, the yeast population is dominated by a mix of these other species (Marsh *et al.* 2013; Garofalo *et al.* 2015; Bourrie *et al.* 2016). Recently, Walsh *et al.* (2018) compared the performance of three high-throughput short-read sequencing platforms, the Illumina MiSeq, NextSeq 500, and Ion Proton, for shotgun metagenomics of six kefir grains. Compositional analysis of kefir showed that the choice of sequencing platform did not affect the results; nevertheless the bioinformatics tools selected had a more evident impact on results than the choice of sequencer. The advance of 'omic science' allowed understanding kefir ecosystem's dynamic and the role of micro-organisms in physicochemical properties of fermented milks. Walsh *et al.* (2016) used amplicon (16S RNA and ITS) and whole-metagenome shotgun sequencing to study population dynamics during kefir fermentation. Additionally, they were able to identify the contribution of individual micro-organisms in the production of certain metabolites, such as flavour compounds, using a combination of metagenomics and metabolomics tools.

With respect to AAB that have been associated with kefir, culture-dependent and independent methods have revealed *Acetobacter* as the dominant genera present in grains (Garrote *et al.* 2010; Walsh *et al.* 2018). Nevertheless, the studies of AAB of kefir are mainly focused on their role in sugary kefir (De Roos and De Vuyst 2018). Results in milk kefir are scarce and more research is needed to know the role of AAB in the dynamic of this ecosystem.

The role of kefir micro-organisms in grain assembly

Kefir grain is considered an example of a symbiotic community where LAB, yeasts and AAB cohabit in a specific equilibrium (Garrote *et al.* 2010). The symbiotic balance between kefir micro-organisms is evidenced by biomass production during fermentation (Garrote *et al.* 1998), since grains weight increment is a consequence of the growth of micro-organisms and the biosynthesis of matrix protein and polysaccharides. A complex crosstalk between bacteria and yeasts is necessary to obtain new grain biomass that, up to the moment, is only achieved by subculturing pre-existent grains (Londero *et al.* 2012).

Whey fermentation with kefir grains allows obtaining biomass from a byproduct of the dairy industry. However, after a certain time of incubation, the grains dissolve indicating that fermentation time must be controlled when biomass production is required. Fermentation temperature over 37°C produces alterations in the appearance and microbiological composition of the grains as well as a partial dissolution. Thus, fermentation temperature is another factor to be considered (Londero *et al.* 2012).

The existing association of micro-organisms has been maintained through centuries even performing the fermentation in noncontrolled conditions (Bourrie *et al.* 2016; Rosa *et al.* 2017). In this context, Londero *et al.* (2012) evidenced similar bacterial DGGE profiles for grains subcultured in milk or whey at different temperatures while yeasts profiles changed depending on the incubation conditions being the most variable micro-organisms in grains. After 20 subcultures in whey, a loss or reduction of certain yeast populations was detected, since bands corresponding to *Saccharomyces unisporus*, *Kluyveromyces marxianus*, *Kazachstania exigua* or *Kazachstania turciensis* and other bands not identified that appears in the original kefir grains were absent in DGGE profiles of grains grown in whey.

The first approach to understand the role of kefir micro-organisms in kefir grain formation was published some decades ago by Marshall *et al.* (1984). They demonstrated the presence of sheet-like structures formed by a carbohydrate component (lately described as kefiran) and an asymmetric distribution of micro-organisms with kefiran-producing lactobacilli intimately associated to carbohydrate compound on smooth side of the sheet while yeast and other lactobacillus were located on the other. Wang *et al.* (2012) described the distribution of micro-organisms on kefir grain biofilm and demonstrated that the outer layer of the grain was more densely colonized. In contrast, Brazilian kefir grain showed the same distribution in both, the inner and outer layers (Magalhães *et al.* 2011a). SEM observation of the outer portion of different Italian kefir grains showed that all the grains differ in microbial distribution and abundance (Garofalo *et al.* 2015). Recent studies of Tibetan kefir grains by SEM demonstrated that the outside surface was covered by short LAB and the inner surface was covered by long AAB (Dong *et al.* 2018) confirming unequal distribution of micro-organism in the grains. Yeasts distribution evaluated with SEM and *in situ* hybridization with specific oligonucleotide probes indicates that *Saccharomyces cerevisiae*, *K. marxianus* and *Yarrowia lipolytica* are the dominant species which are commonly present on the outer surface (Lu *et al.* 2014). Sequencing data confirmed that the microbial diversity of the grain is not uniform with a greater level of diversity associated with the interior of the kefir grain (Dobson *et al.* 2011). Figure 1 shows macroscopic aspect of kefir grains from Argentine and SEM micrograph of them where lactobacilli and yeast can be visualized on the grain surface.

Kefir grains could be considered a biofilm so different steps are required for its formation including the cell–cell interactions and development of a complex extracellular structure that comprise micro-organism in a stable association (Garrote *et al.* 2010; Wang *et al.* 2012). Aggregation properties of *L. kefiri* strains and their ability to coagreggate with *Saccharomyces lipolytica* were mediated by the lectin-like activity of their surface proteins (S-layer) (Garrote *et al.* 2005; Golowczyc *et al.* 2009). S-layer proteins from aggregating and nonaggregating *L. kefiri* strains were all glycosylated; suggesting that aggregation properties of *L. kefiri* is affected by S-layer glycoproteins structure (Mobili *et al.* 2009; Malamud *et al.* 2017). Cell surface properties, auto-aggregation, co-aggregation and biofilm formation ability of four LAB and three yeast isolated from kefir as well as SEM analysis, allowed Wang *et al.* (2012) to propose a hypothesis to explain kefir grain formation. They suggested a first aggregation/co-aggregation step of *L. kefiranofaciens* and *Saccharomyces turicensis*. Then, other micro-organisms (*L. kefiri*, *K. marxianus* HY1 and *Pichia fermentans* HY3) adhere to the surface of these small grains contributing to biofilm increase till three-dimensional microcolony is obtained.

Micro-organisms immersed in kefir grain are responsible for the synthesis of the extracellular components. It has been suggested that milk proteins are attached on grain surface (Prado *et al.* 2015), but no details about structure and composition are available. However, growth of kefir grains in soy milk allowed understanding that proteins are actually produced by kefir micro-organisms since similar SDS-PAGE proteins profiles were observed for grains grown in milk or soy milk (Abraham and de Antoni 1999). The exopolysaccharide produced by the micro-organisms present in kefir grains is called kefiran. Kefiran production was initially ascribed to *L. brevis* (La Rivière *et al.* 1967) and finally to *L. kefiranofaciens* sp. (Fujisawa *et al.* 1988). Recently, it was found that the *L. kefiranofaciens* (Fujisawa *et al.* 1988) and *L. kefirgranum* (Takizawa *et al.* 1994) are phylogenetically identical (DNA 16S with 100% similarity) and were reclassified into two subspecies, *L. kefiranofaciens* ssp. *kefiranofaciens* and ssp. *kefirgranum* (Vancanneyt *et al.* 2004), being only the first subspecies considered responsible for kefiran production (Cheirsilp *et al.* 2018).

Although many approaches were applied to know the population dynamics of kefir, the grains cannot be formed from pure culture and needs pre-existing grains to be produced, indicating that more research is needed to understand kefir micro-organisms' interactions and their role in grain assembly.

Kefir micro-organisms in kefir grain and in kefir fermented milk

The study of kefir ecosystem may involve the knowledge of the ecology of the grain and its maintenance through centuries as well as the population dynamics of the fermented milk. Comparative analysis of microbial composition of kefir and the corresponding kefir grain showed that both microbial communities are different (Dobson *et al.* 2011; Londero *et al.* 2012; Marsh *et al.* 2013; Kotova *et al.* 2016). Marsh *et al.* (2013) studied 23 kefir grains from different countries (Ireland, the United Kingdom, the United States, Spain, France, Italy, Canada and Germany) and the corresponding fermented milk by high-throughput sequencing of 16S genes. They found that kefir grains are dominated by two phyla, Firmicutes, with Lactobacillaceae as the most abundant family, and Proteobacteria. *Lactobacillus* was the dominant genus of kefir grains studied (Marsh *et al.* 2013; Nalbantoglu *et al.* 2014; Garofalo *et al.* 2015; Korsak *et al.* 2015). As an exception, in an Irish kefir grain *Acetobacter* was the dominant bacterial genera (Marsh *et al.* 2013). Other reports also describe the presence of *Bifidobacterium* but they were only identified through culture-independent studies (Dobson *et al.* 2011; Marsh *et al.* 2013). *Lactobacillus kefiranofaciens* is the most dominant species in the bacterial community of kefir grains accompanied by *L. kefiri* and *L. parakefiri* among other species listed in Table 1.

In the fermented milk, Streptococcaceae was the dominant family and the genera that showed higher abundance were *Leuconostoc Lactococcus*, *Lactobacillus*, and

Acetobacter (Marsh *et al.* 2013; Garofalo *et al.* 2015). Furthermore, bacterial population of fermented milk presented lower species diversity than that of the corresponding grains (Marsh *et al.* 2013). Additionally, species that prevails in kefir are modified by fermentation time. According to Walsh *et al.* (2016), *L. kefiranofaciens* is present in the fermented milk at the first stages of fermentation while *Leuconostoc* prevails at late stages of fermentation.

Yeasts population present in the grain or fermented milk also varies. In grains, it was represented by *Saccharomyces* sp., *K. lactis*, *Kazachstania* sp. and *Candida* sp. (Leite *et al.* 2012; Londero *et al.* 2012; Marsh *et al.* 2013). In Irish kefir, *Kluyveromyces* sp. was the predominant genera (Marsh *et al.* 2013; Bourrie *et al.* 2016) and *K. unisporus*, *K. marxianus*, *S. cerevisiae*, *K. meager* or *K. turicensis* were detected in kefir prepared with grains from Argentina (Londero *et al.* 2012) (Table 1).

The analysis of both kefir grain and kefir shows that micro-organisms found at lower abundance in the grain can become dominant in the fermented milk. These findings highlight the need to examine the fermented milk rather than focusing only on the grain population.

Health promoting properties of kefir micro-organisms

The functional and probiotic properties of kefir have been studied by numerous authors and the most relevant findings have been summarized properly (John and Deeseenthum 2015; Prado *et al.* 2015; Bourrie *et al.* 2016; Sharifi *et al.* 2017). Health benefits comprise antimicrobial activity, tumour suppression; wound healing properties, immunomodulation, anti-inflammatory, antiobesity, cholesterol lowering and antioxidant effects, improvement in lactose tolerance, alleviation of fatty liver and enhancement of intestinal bacterial flora.

These beneficial health properties could be ascribed both to the presence of probiotic micro-organisms, as well as to the metabolic products that appear in the fermented milk.

Within the probiotic properties attributed to lactobacilli isolated from kefir can be mentioned the ability of *Lactobacillus plantarum* CIDCA 83114 to prevent the detachment of Hep-2 cells incubated with *Escherichia coli* enterohaemorrhagic (EHEC) to Hep-2 cells (Hugo *et al.* 2008) and antagonize the cytotoxic effects of EHEC Shiga 2 toxin (Kakisu *et al.* 2013). Furthermore *L. kefiri* strains are able to inhibit the adhesion and invasion of *Salmonella enterica* serovar. Typhimurium to Caco-2/TC-7 cells (Golowczyc *et al.* 2007) .

In relation to yeasts, strains belonging to species *S. cerevisiae*, *S. unisporus*, *I. occidentalis* and *K. marxianus* were studied by our group, determining their resistance to gastrointestinal conditions both *in vitro* and *in vivo.* Additionally, their capacity to adhere to Caco-2 cells was studied (Diosma *et al.* 2014). The *in vitro* modulation of the epithelial innate immune response was studied, detecting that kefir yeasts modulate the proinflammatory response of flagellin-induced in Caco-2:CCL20 luc cells (Romanin *et al.* 2010). The multiplicity of interaction (relation micro-organisms/epithelial cells) and the incubation time showed to be factors that influence the modulatory effect. Furthermore, the response triggered by other proinflammatory agonists such as IL-1β, TNF-α and LPS was also modulated by the yeasts. Romanin *et al.* (2010) demonstrated that the modulation of gene expression is specific for proinflammatory genes with no alterations in the expression of nonimmunological genes.

The potential use of *K. marxianus* as a probiotic has been suggested in several reports (Maccaferri *et al.* 2012). Romanin *et al.* (2016) deepened the study of the anti-inflammatory capacity of the kefir yeast *K. marxianus* CIDCA 8154 in different models. They demonstrated *in vitro* that the pretreatment of the epithelial cells with yeast reduces the intracellular levels of reactive oxygen species, concluding that the modulation of the intestinal inflammatory response occurs through a mechanism independent of ROS generation. Furthermore, it was demonstrated in a model of *Caenorhabditis elegans* that the yeast was able to protect from oxidative stress. Likewise, mice treated orally with *K. marxianus* CIDCA 8154 presented a less histopathological damage and lower levels of circulating IL-6 in a TNBS-induced colitis model (Romanin *et al.* 2016).

Other authors have also studied the probiotic potential of kefir yeasts. de Lima *et al.* (2017) found that *S. cerevisiae* strains isolated from Brazilian kefir presented interesting *in vitro* probiotic properties. However, Cassanego *et al.* (2017) observed that *S. cerevisiae, Hanseniospora uvarum* and *K. unispora* isolated from other Brazilian kefir were not able to tolerate the passage through the simulated gastrointestinal tract. Xie *et al.* (2012) studied the positive effect of the kefir yeasts on *Lactobacillus* probiotic potentials and Cho *et al.* (2018) recently found that a combination of kefir-derived *Kluyveromyces* KU140723-02 and polyphenol-rich grape seed flour or its extract has an incremented antioxidant activity.

Combination of kefir micro-organisms was also studied in order to obtain blends with improved probiotics properties. It was demonstrated that a combination of two lactobacilli, one lactococcus and two yeasts protected epithelial cells *in vitro* against *Shigella* invasion (Bolla *et al.* 2016). Additionally, this blend exerted a protection against *Clostridium difficile* infection in a mouse model

(Bolla *et al.* 2013). Likewise, Londero *et al.* (2015) showed that the antagonistic properties of a mixed culture of kefir strains against *Salmonella* sp.

Metabolites produced by kefir micro-organism

Since several health promoting properties of kefir were ascribed to its nonmicrobial fraction, it is relevant to gain a better understanding of the metabolites and main changes produced in the milk. Micro-organisms ferment lactose, hydrolyse proteins, produce exopolysaccharides and other metabolites, such us: organic acids, vitamins, ethanol, acetaldehyde, diacetyl, carbon dioxide and bacteriocins.

One activity associated to this fraction was the antimicrobial capacity ascribed mainly to the presence of organic acids sometimes accompanied by other inhibitory compounds such as bacteriocins (Garrote *et al.* 2000; John and Deeseenthum 2015; Iraporda *et al.* 2017). Lactic acid level in kefir varies between 0.078 and 0.255 mol l^{-1} (Garrote *et al.* 2010; Magalhães *et al.* 2011b; Leite *et al.* 2013) and acetic acid concentration range between 0.015 and 0.038 mol l^{-1} depending on the micro-organisms present in the kefir grains as well as to fermentation conditions (Iraporda *et al.* 2014).

The inhibitory activity of nonmicrobial fraction of kefir as well as cell free supernatant of fermented milks with micro-organisms isolated from kefir was demonstrated against several pathogenic bacteria (Garrote *et al.* 2000; Golowczyc *et al.* 2008; Iraporda *et al.* 2017).The inhibitory effect of kefir against *Salmonella* is lost by neutralizing the nonmicrobial fraction even when concentrated five times, indicating that the organic acids in their nondissociated form would be responsible for this effect (Iraporda *et al.* 2017). Otherwise, *in vitro* studies indicate that incubation of *Salm. enterica* serovar. Enteritidis with the neutralized nonmicrobial fraction of kefir did not affect pathogens viability but decrease their invasive capacity to intestinal epithelial cells in culture (Iraporda *et al.* 2017).

Another health benefit attributed to the non-microbial fraction of kefir is its ability to modulate the immune response (Iraporda *et al.* 2014). In this context, de Moreno de LeBlanc *et al.* (2006) demonstrated that the nonmicrobial fraction of kefir delayed breast tumour development inducing an adequately balanced local immune response in the mammary glands. Lactate and other organic acid such as acetate, propionate and butyrate also down regulate pro-inflammatory responses in intestinal epithelial and myeloid cells (Iraporda *et al.* 2014, 2015). The increase in extracellular lactate concentration at the level of the colon could generate a change in the cells metabolism which implies a decrease in the rate of glycolysis that affects the normal activation of myeloid cells against proinflammatory stimuli (Iraporda *et al.* 2015; Brooks 2018).

Intrarectal administration of lactate provides a significant reduction of the intestinal inflammation and the epithelial damage induced by TNBS. On the other hand, when administered in drinking water no protection against acute intestinal inflammation was observed, probably due to the fact that lactate does not reach necessary levels in the colon because it was absorbed and/or consumed by colonic bacteria (Iraporda *et al.* 2016). However, lactate can appear in the gut via the consumption of probiotics and prebiotic containing foods. Probiotic micro-organisms that adhere to epithelial cells can produce lactate in the gut epithelium microenvironment. In this aspect, it is important to point out that some *Lactobacillus paracasei* strains isolated from kefir are able to adhere to Caco-2 cells and mucin with an increase in their adhesion ability after passage through simulated gastrointestinal tract (Bengoa *et al.* 2018b). In the same way, the consumption of prebiotics which are selectively fermented in the colon induces the growth of *Lactobacillus* and *Bifidobacterium* that ferment nondigestible carbohydrates producing mainly lactate. Furthermore, lactate can be used by the gut microbiotia for the production of acetate, propionate and butyrate; short chain fatty acids highly associated to gut's health.

Modulation of intestinal microbiota by kefir administration has been demonstrated in animal trials (Kim *et al.* 2017, 2018). This impact on microbial communities might modify the metabolite profile and is expected to influence immune responses. Recent evidence suggests that products of intestinal microbiota might positively influence inflammatory disease pathogenesis. This modulation may be mediated by kefir micro-organism or the EPS present in the fermented milk.

Kefiran, a water-soluble heteropolysaccharide composed by equal amounts of D-glucose and D-galactose, is the main polysaccharide present in kefir reaching values of about 218 mg l^{-1} (Rimada and Abraham 2003; Zajsek *et al.* 2011). Kefiran has been studied because of its technological properties and several health benefits attributed to its consumption. This polymer is an interesting additive for the food industry since it significantly improves the viscosity and viscoelastic properties of acid milk gels and is capable of forming translucent cryogels and edible films (Abraham *et al.* 2010; Piermaria *et al.* 2015). Kefiran is a nondigestible polysaccharide that can reach the large intestine where it can exert antimicrobial (Rodrigues *et al.* 2005), anti-inflammatory (Vinderola *et al.* 2006) and antiallergenic effects (Kwon *et al.* 2008). Kefiran administration in drinking water increase the number of bifidobacteria population in the colon (Hamet *et al.*

2016) and also produces an increase in the number of mucus-producing cells of the gut (Medrano *et al.* 2011). The biological activity of kefiran could be ascribed to the ability of this polysaccharide to interact with the enterocytes or indirectly mediated by the demonstrated bifidogenic effect. Additionally, this polymer is able to antagonize pathogens virulence factors *in vitro* (Medrano *et al.* 2008) and reduce blood pressure and serum cholesterol levels (Maeda *et al.* 2004).

Many authors have isolated and studied the EPS synthetized by different *L. kefiranofaciens* ssp. *kefiranofaciens* strains from kefir grains in single cultures or in co-culture with yeasts, evidencing the same structure and composition as kefiran (Mitsue *et al.* 1999; Maeda *et al.* 2004; Wang and Bi 2008). Hamet *et al.* (2013) have isolated nine EPS-producing *L. kefiranofaciens* ssp. *kefiranofaciens* strains from different kefir grains observing that the degree of polymerization of the EPS produced in milk was strain dependent. However, none of them produced fractions of a molecular weight higher than 10^5 Da. Jeong *et al.* (2017) demonstrated that *L. kefiranofaciens* DN1 produces a different EPS from kefiran composed of mannose, arabinose, glucose, galactose and rhamnose when it grow in glucose. Otherwise, *L. kefiranofaciens* 1P3 isolated from Brazil kefir grains was able to produce an α-glucan in the presence of sucrose; however, they did not report if the same strains is able to produce EPS from lactose (de Paiva *et al.* 2016).

In addition to *L. kefiranofaciens*, many other EPS-producing LAB species have been isolated from kefir grain (Hamet *et al.* 2015; Jeong *et al.* 2017). Gangoiti *et al.* (2017) studied the structure of the EPS synthetized by *L. plantarum* CIDCA 8327 in milk observing that it corresponded to an α-glucan. It is interesting to note that this strain produces an heteropolysaccharide in a semi-defined medium with glucose and an α-glucan in milk, where lactose is the sugar source indicating that this strain may produce glycan by a different pathway than the one described by homopolysaccharides synthesis.

Lactobacillus paracasei ssp. *paracasei* strains isolated from Argentine kefir grains were able to produce EPS in milk or culture media (Hamet *et al.* 2015; Bengoa *et al.* 2018a). Growth temperature affected EPS production by *L. paracasei* ssp. *paracasei*. These changes were evidenced by the presence of a high molecular weight fraction and an increase in the total amount of produced EPS at lower temperature (Bengoa *et al.* 2018a). The fermented milk obtained with these strains has good rheological (Hamet *et al.* 2015) and health promoting properties such as inhibition of *Salmonella* invasion and modulation of proinflammatory response (Zavala *et al.* 2016; Bengoa *et al.* 2018a). Di *et al.* (2017) studied the EPS produced by *L. plantarum* YW11 isolated from Tibetan kefir evidencing its antioxidant activity. Additionally, it was demonstrated that the consumption of EPS recovers the microbiota diversity and phylotypes in an aging mouse model.

The literature has reported many other benefits of LAB EPS such as antitumour properties, cholesterol lowering capability, antihypertensive activities and epithelium protection from intestinal pathogenic micro-organisms and faecal microbiota modulation (Patten and Laws 2015). Considering this, there is a growing interest in the isolation of new EPS-producing strains that could be included in the food matrix for the development of functional foods with improved technological properties (Torino *et al.* 2015; Zannini *et al.* 2016). In this context, kefir grains are an important source of EPS-producing micro-organisms that synthetized either kefiran or a different EPS in single culture such as the glucan produced by *L plantarum* in milk (Gangoiti *et al.* 2017). Since *Lactobacillus* strains that synthetized different EPS from kefiran have been isolated from kefir grains; it cannot be discarded that the nonbacterial fraction of kefir contains not only kefiran but also small amounts of different EPS that are not determined and may also contribute to kefir health benefit.

Ebner *et al.* (2015) and Dallas *et al.* (2016) described the presence of peptides in kefir samples with biological activity, including antihypertensive, antimicrobial, immunomodulatory, opioid and antioxidative functions. Recent reports demonstrated that the administration of kefir or commercial peptides from kefir reduced weight gain in obese mice (Bourrie *et al.* 2018; Tung *et al.* 2018). Santanna *et al.* (2017) showed that administration of nonmicrobial fraction caused a significant reduction in vascular lipid deposition. Similarly, Brasil *et al.* (2018) evidenced that the nonmicrobial fraction of kefir inhibits angiotensin-converting enzyme and reduces hypertension, attributing this effect to the release of bioactive peptides from milk proteins by kefir micro-organisms.

Conclusion

Kefir has been associated to the healthy status and longevity of consumers over years. However, the scientific bases of the health promoting properties of kefir were demonstrated in the last three decades. The fermented milk is a dynamic product whose properties depend on several factors such as source of milk, growth condition and origin of the grain. The main variations include microbial composition as well as metabolites such as lactic and acetic acid, exopolysaccharides and bioactive peptides. An in-depth comprehension of microbial and chemical composition of kefir is necessary to understand the complex cross talk between kefir micro-organisms that allow the maintenance of this complex ecological system through centuries. Understanding the beneficial role of each kefir micro-organism and the components of the nonmicrobial fraction would allow the design of commercial products containing the best defined blends of micro-organisms and metabolites to obtain tailors made products with specific health benefits.

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Conflict of Interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper**.**

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