Chapter 18

Microbial Interactions in Kefir: A Natural Probiotic Drink

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Kefir is a fermented milk originated in the Caucasian mountains obtained by incubation of milk with kefir grains. These grains contain a relatively stable and specific microbiota immobilized in a matrix of polysaccharides and proteins. Numerous species of lactic acid bacteria (LAB), acetic acid bacteria, and yeasts, held together into the matrix, exist in a symbiotic relationship. Microbial interactions in kefir are very complex due to the composition of kefir grains. In this microbial ecosystem, a delicately balanced population of microorganisms occurs, each interacting with and influencing the other members of the population. In kefir grains, the balanced population of microorganisms determines the synthesis of biologically active metabolites that are essential for the grain growth and the inhibition of external microorganisms like pathogens and food contaminants. Although microbial interactions in kefir grains have not yet been very well characterized, it has been established that these interactions are species- and strain specific. S-layer proteins of Lactobacillus *kefir play an important role in coaggregation between this LAB and* Saccharomyces lipolytica *as well as in the inhibition of* Salmonella *adhesion and invasion to Caco-2/TC7 cells. A better knowledge of microbial interaction will be the basis for understanding the kefir grain ecosystem and its probiotic properties.*

18.1. General Description of Kefir

Kefir is a fermented beverage originated in the Caucasian mountains, which has become popular in many European countries. Kefir is a sour fermented milk, sometimes carbonated, with a low alcohol content. It differs from other milk products because it is not the result of the metabolic activity of a single species but of mixed microbiota confined to a matrix of discrete "kefir grains." They are gelatinous irregular white or light yellow masses with a structure similar to tiny florets of cauliflower varying in size from 0.3- to 3.5-cm diameter. They are composed of proteins and polysaccharides in which the complex microbiota is enclosed. During fermentation, grains increase in size and number, and this is how new biomass is obtained; grains are generally recovered from the fermented milk to be reused.

Despite the wide consumption of kefir, this biological system has not been fully studied, probably due to its complexity. Yeasts, acetic acid bacteria, and lactic acid bacteria (LAB) coexist in a symbiotic association and are responsible for acid–alcoholic fermentation. The activity of the grain depends on the viability of the microbiota. Generally, about 10^8 cfu/g of LAB, 10^6–10^7 cfu/g of yeasts, and 10^5 cfu/g of acetic acid bacteria are present in the kefir grain (Garrote et al. 2001; Witthuhn et al. 2005b). Among LAB, homofermentative and heterofermentative *Lactobacillus*, *Lactococcus*, and *Leuconostoc* are the genera most frequently found. Lactococci, lactobacilli, and yeasts could be mutually stimulated to produce the components of the grain matrix. The distribution of microorganisms within the kefir grain was studied by Bottazzi and Bianchi (1980) using scanning electron microscopy. These authors suggested that the population of

yeasts and lactobacilli were not randomly distributed in the grain. Lactobacilli were located at the periphery of the grain while yeasts were located inside.

The microbiological composition of kefir grains is still controversial. The most common microorganisms isolated from kefir grains are detailed in Table 18.1. Different reports indicate that kefir grain microbiota strongly depends on the grain origin (Ottogalli et al. 1973; Kuo and Lin 1999), on the culture conditions (Molska et al. 1983), and on the storage and elaboration processes (Zourari and Anifantakis 1988; Garrote et al. 1998).

18.2. Kefir Grain Preservation

For propagation of the kefir starter culture, a desirable and adequate proportion of the microorganisms composing the kefir grain is required. A decrease in the yeast content of kefir grains alters the rate of biomass production (Garrote et al. 1997). Kefir grains may be preserved lyophilized, dried, or wet. Some authors recommend storage of wet kefir grains at 4°C or drying at room temperature for 36–48 h. Dried kefir grains retain activity for 12–18 months, whereas wet grains retain activity for only 8–10 days (Kosikowski 1982; Marth and Yousef 1991).

14 **Table 18.1.** Microorganisms found in kefir and kefir grains.

Species	Reference
Lactobacillus kefir	Kandler and Kunath (1983); Marshall et al. (1984); Angulo et al. (1993); Pintado et al. (1996); Takizawa et al. (1998); Garrote et al. (2001)
Lactobacillus kefiranofaciens	Fujisawa et al. (1988); Toba et al. (1991); Mukai et al. (1992); Takizawa et al. (1998)
Lactobacillus kefirgranum	Takizawa et al. (1994, 1998)
Lactobacillus parakefir	Takizawa et al. (1994); Garrote et al. (2001)
Lactobacillus plantarum	Serot et al. (1990); Garrote et al. (2001); Hertzler and Clancy (2003)
Lactobacillus brevis	Ottogalli et al. (1973); Rosi and Rossi (1978); Marshall et al. (1984); Angulo et al. (1993)
Lactobacillus acidophilus	Ottogalli et al. (1973); Angulo et al. (1993); Marshall (1993)
Lactobacillus viridescens	Molska et al. (1983); Angulo et al. (1993)
Lactobacillus gasseri	
Lactobacillus fermentum Lactobacillus casei	
Lactobacillus helveticus	Kuo and Lin (1999)
Lactococcus lactis subsp. *lactis*	Ottogalli et al. (1973); Angulo et al. (1993); Marshall (1993); Pintado et al. (1996); Garrote et al. (2001)
Leuconostoc mesenteroides	Rosi and Rossi (1978); Angulo et al. (1993); Marshall (1993); Kuo and Lin (1999); Garrote et al. (2001)
Acetobacter aceti	Rosi and Rossi (1978); Angulo et al. (1993)
15 *Candida kefir*	Zourari and Anifantakis (1980); Engel et al. (1986); Angulo et al. (1993); Marshall (1993); Wyder (2001)
Kluyveromyces lactis	Engel et al. (1986); Angulo et al. (1993); Wyder (2001)
Kluyveromyces marxianus	Rohm et al. (1992); Kuo and Lin (1999); Wyder (2001); Garrote et al. (2001)
Saccharomyces cerevisiae	Rosi (1978); Rohm et al. (1992); Angulo et al. (1993); Marshall (1993); Wyder (2001); Garrote et al. (2001)
Saccharomyces delbrueckii	Rosi (1978); Engel et al. (1986); Pintado et al. (1996)
Saccharomyces unisporus	Engel et al. (1986); Angulo et al. (1993); Wyder (2001)
Torulaspora delbrueckii	Angulo et al. (1993); Wyder (2001)
Candida friedricchii	
Pichia fermentum	Rohm et al. (1992); Angulo et al. (1993); Kuo and Lin (1999); Wyder (2001)
Torulopsis holmii	Wyder (2001)
Zygosaccharomyces florentinus	
Issatchenkia occidentalis	
Yarrownia lipolytica	

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Difficulties have been found in maintaining satisfactory quality in grains to produce a beverage with the appropriate and acceptable viscosity. Garrote et al. (1997), comparing two methods for preservation of kefir grains to be employed as starter, evaluated kefir grain metabolic activity when grown in milk after storage and concluded that freezing was the better method for kefir grain preservation. This storage condition maintains the grain activity required for milk fermentation. Since grains stored at −20 or −80°C showed a greater increase in grain weight after successive sub-culturing, a storage temperature of −20°C was suggested for household kefir production (Garrote et al. 1997; Witthuhn et al. 2005a).

18.3. Methods to Study Kefir Grain Microflora

Conventional methods usually employed in microbiology for identifying and classifying bacteria are mainly based on the analysis of morphological and biochemical properties and are sometimes insufficient for some bacterial groups. Garrote et al. (2001) evaluated the microbiological and chemical composition of four Argentinean kefir grains. The grains microbiota comprised lactobacilli, lactococci, acetic acid bacteria, and yeasts; however, significant differences regarding species were observed. *Lactococcus lactis* subsp. *lactis*, *Lactobacillus kefir*, *Lactococcus plantarum*, *Acetobacter*, and *Saccharomyces* were present in all types of kefir grains, while *Leuconostoc mesenteroides*, *L. lactis* subsp. *lactis* biovar *diacetylactis*, *Lactococcus parakefir*, and *Kluyveromyces marxianus* were grain-specific. These isolates were characterized by traditional microbiological methods including cellular morphology, gas production, and sugar fermentation patterns and growth at different temperatures. Also, the analysis of whole cell protein by SDS-PAGE allowed heterofermentative lactobacilli isolated from kefir grains to accurately identified.

Delfederico et al. (2005) confirmed by molecular methods the identity of 17 heterofermentative lactobacilli isolates obtained from Argentinean kefir grains. Results of amplified ribosomal DNA restriction analysis from the closely related reference strains studied established the value of this technique for species differentiation of the *Lactobacillus* genus. The data obtained from the analysis of spacer region confirmed that sequencing of this genome region constitutes a reliable tool for the identification of *Lact. kefir* members. In addition, random amplified polymorphic DNA polymerase chain reaction (PCR) patterns allowed the differentiation of isolates (Delfederico et al. 2005).

However, as traditional and molecular methodologies are tedious, expensive, and time-consuming, the application of other techniques has been considered in the last years. Fourier transformed infrared (FT-IR) spectra of intact bacteria are highly specific patterns that may be unique for individual strains. FT-IR spectroscopy is easy to implement, allows analysis of small quantities of biomass, and requires no specific consumables or reagents (Helm et al. 1991; Naumann 2000; Maquelin et al. 2002). Bosch et al. (2006) developed an approach based on FT-IR spectroscopy in combination with multivariate statistical analysis for rapid differentiation of lactobacilli isolated from kefir grains.

Immunological methods have been used extensively to identify bacteria from a variety of ecosystems. The differential enumeration of *Lact. kefir* or *Lact. parakefir* in kefir in viable counts remains very difficult since the colony morphology of these species is similar to other heterofermentative lactobacilli commonly present in kefir. Serological techniques combined with enzyme-linked-immunosorbent assays (ELISA) have been used to quantify and distinguish physiologically closely related strains in mixed cultures (Ricke and Schaefer 1990; Durant et al.1997; Abraham et al. 2005). Garrote et al. (2005) developed an immunochemical assay employing a specific antiserum against *Lact. kefir* S-layer protein to detect and quantify this microorganism in kefir.

Kefir microorganisms that have been isolated using selective growth media and were biochemically and morphologically characterized cannot produce *de novo* kefir grains, indicating that other bacteria are present in this complex microbial

consortia. Molecular techniques offer new opportunities for determining and analyzing the structure and species composition of microbiological communities. Garbers et al. (2004) showed that denaturing gradient gel electrophoresis (DGGE) fingerprinting can be successfully used to typify the microbial consortium present in kefir grains, as well as to distinguish kefir grains cultured using different methods, or kefir grains that have different origins. The grains can be compared with respect to both eubacterial and yeast species present. Wang et al. (2006) demonstrated that PCR-based DGGE and sequence analysis of 16S rDNA proved to be a valuable culture-independent approach for the rapid and specific identification of the microbial species present in micro-ecosystems and probiotic products. The analysis of the obtained bands allowed finding of sequences similar to *Sphingobacterium* sp., *Lactobacillus* sp., *Enterobacter* sp., and *Acinetobacter* sp.

Chen et al. (2008) showed that bacteria that were not isolated by culture-dependent methods were revealed by DGGE. On the contrary, several LAB strains that were previously identified by culture-dependent methods were not detected by PCR-DGGE. The diversity of LAB strains identified by PCR-DGGE was lower than observed by using initial enrichment stage on nutritive media as the cell counts of certain LAB species were lower than the detection limit of PCR-DGGE. Low sensitivities for the detection of the V2–V3 region in a complex environment (10^7–10^8 cfu/g) by DGGE were reported (Fasoli et al. 2003). This detection limitation is a consequence of high quantities of competitor templates of bacteria present in high concentrations. Moreover, various cell proteins and aged culture may interact with the genomic DNA, thereby affecting primer annealing to the template or the activity of the DNA polymerase (de Barros Lopes et al. 1996; Beh et al. 2006).

18.4. Microbial Interactions

Microbial interactions in mixed cultures occur via multiple mechanisms; they may be direct through physical contact or via signaling molecules (Sieuwerts et al. 2008). The effects of such interactions may either be positive, neutral, or negative and can be divided into five main classes: amensalism, competition, commensalism, parasitism, and mutualism. Amensalism is an interspecies interaction in which one organism adversely affects other organism without being affected itself. It frequently occurs in food fermentations since the major end-products of primary metabolism such as carboxylic acids and alcohols are effective growth inhibitors of indigenous microbiota and spoilage organisms. In the second class of interactions, competition, microorganisms compete for energy sources and nutrients during fermentation. In commensalism, one organism benefits from the interaction while the other strain is not affected. Parasitism occurs when one species benefits at the expense of another. Finally, during mutualism or symbiosis, both participating microorganisms benefit from the interaction. Many food fermentations rely on bacterial interactions; probably the best example of symbiosis is the yogurt consortium, where *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* stimulate in growth and acid production in mixed cultures with respect to single-strain cultures (Sieuwerts et al. 2008).

Which is the real interaction of microorganisms in kefir grains and what is the interaction of kefir microorganisms with exogenous bacteria? The interrelationships of bacteria and yeasts inside kefir grains may have a significant influence on the activities of each strain. The stimulation of growth and lactic acid production by bacteria was observed in the mixed culture; LAB growth was stimulated by growth factors such as vitamins and amino acids produced by yeasts (Simova et al. 2006). Also, bacterial end-products such as lactic acid can be used by yeasts as an energy source. This interaction can be considered symbiotic.

Pure cultures of kefir bacteria and yeasts either do not grow in milk or have a low biochemical activity. Yeasts in kefir provide an environment for the growth of kefir bacteria, producing metabolites that contribute to the flavor and mouthfeel of kefir (Clementi et al. 1989; Simova et al. 2002; Farnworth 2005; Lopitz-Otsoa et al. 2006). Considering the

complexity of the kefir microbiota, all the interactions previously described may coexist to maintain an adequate balance among microorganisms into the grains.

18.4.1. Microbial Interactions and the Biosynthesis of Grain Components

Kefir grains are clusters of microorganisms held together by a matrix of protein and polysaccharides (Bottazzi et al. 1994; Abraham and De Antoni 1999). Kefir grain matrix is synthesized by the complex microbial population included in kefir grains, which is considered an example of a symbiotic community (Witthuhn et al. 2005b). The main marker for evaluating the symbiotic relationship is the increase in biomass during fermentation. Kefir grains may grow in milk (Garrote et al. 1998, 2001), deproteinized whey (Rimada and Abraham 2001), or soy milk (Abraham and De Antoni 1999; Liu and Lin 2000). To increase the biomass of kefir grains, the synthesis of proteins and polysaccharides is necessary. In this aspect, kefir grain can be compared to a complex biofilm where bacteria and yeasts communication occur through several steps. First, the bacterium approaches to a surface so closely that motility is slowed down. Then, the bacterium may form a transient association with the surface and/or other microbes previously attached to it. Once this association has become stable, the microcolonies formed by bacteria are involved in a three-dimensional biofilm development. Occasionally, the biofilm-associated bacteria detach from the biofilm matrix (Watnik and Kolter 2000). During milk or whey fermentation with kefir grains, different events may be observed. Grains increase their weight as a consequence of the growth of microorganisms and the biosynthesis of grain components, and each type of microorganism grows freely in the growth media. Thus, kefir microbiota associated to grain could be considered a biofilm.

The knowledge about grain proteins, as well as the role of the microorganisms present in grains on the synthesis of the matrix components, is limited. Bassete and Acosta (1988) reported that proteins come from the growth medium (milk). In contrast, Abraham and De Antoni (1999) demonstrated that protein is produced by the kefir microbiota by bacteria and yeasts associated to the grains. Whole protein profile of kefir grain grown in milk and in soy milk during a long period presented the same pattern in SDS-PAGE, indicating that grain protein does not depend on the growth media (Abraham and De Antoni 1999). Among grain proteins, two kinds of components were distinguished: those easily extractable by dissolving the grain in water and those soluble only after urea/mercaptoethanol treatment. The tightly associated protein may be the one necessary for grain formation. Grain biomass production depends on the time of fermentation (Rimada and Abraham 2001). During whey fermentation, kefir grain biomass increases up to 96 h, indicating that after a certain time of incubation or under certain environmental conditions, grains dissolve partially, releasing their components to the media. Several authors reported that LAB release cytoplasmic hydrolases after certain incubation periods, which could degrade polysaccharides (Gancel and Novel 1994; Pham et al. 2000). Exopolysaccharides are synthesized by kefir microorganisms and are released into the media, reaching values of 218 and 247 mg/l of kefiran in milk and whey, respectively, as reported by Rimada and Abraham (2001, 2003). It was observed that kefir grains produced similar amounts of polysaccharides in deproteinized whey or in milk (Rimada and Abraham 2003), similar to those obtained with other LAB grown in synthetic media (Cerning 1995; Mozzi et al. 2006).

18.4.2. Microbial Interactions and Production of Biological Active Metabolites in Kefir

Kefir has a long tradition on offering health benefits, especially in Eastern Europe (Zourari and Anifantakis 1988). Several compounds in kefir may have bioactive properties: microorganisms themselves (dead or alive), metabolites produced by microorganisms during fermentation (polysaccharides, bacteriocines), or breakdown-products from the food matrix (peptides); all these compounds may be responsible for the beneficial effects (Farnworth 2005).

Polysaccharides. Kefiran, the polysaccharide present in kefir grains, is a water-soluble branched glucogalactan, containing equal amounts of D-glucose and D-galactose, produced by kefir grains or microorganisms isolated from them (Kooiman 1968; Mukai et al. 1988; Micheli et al. 1999). Kefiran has interesting technological applications such as improvement of viscosity and viscoelastic properties of acid milk gels (Rimada and Abraham 2006) as well as formation of films and gels at low temperatures with interesting viscoelastic properties (Mukai et al. 1991; Piermaria et al. 2008). Kefiran films plasticized with glycerol have good mechanical and water vapor properties (Piermaria et al. 2009). Also, several health-promoting properties of kefiran such as immunomodulation (Vinderola et al. 2006), epithelium protection against toxigenic factors from *Bacillus cereus* (Medrano et al. 2008), or antitumoral activity (Murofushi et al. 1983) have been reported for this exopolysaccharide.

The production of kefiran was ascribed to several lactobacillus species that were isolated from kefir grains such as *Lactobacillus* sp. KPB-167B, *Lactobacillus kefirgranum*, *Lact. parakefir*, *Lact. kefir*, or *Lactobacillus kefiranofaciens* (Toba et al. 1987; Fujisawa et al. 1988; Mukai et al. 1988; Yokoi et al. 1990; Micheli et al. 1999; Taniguchi and Tanaka 2004). Recently, a new strain of *Lact. kefiranofaciens* producing up to 1 g/l of kefiran was isolated from Tibet kefir grains (Wang et al. 2008).

Kefiran production by individual strains in different growth media containing wine, alcohol, or whey (Yokoi et al. 1990), or sago starch (Yeesang et al. 2008) was studied to improve kefiran production. A mathematical model was proposed to determine optimal pH profile for the maximum kefiran production in batch cultures, and it was found that the maximum production was obtained at pH 5.0 during the exponential growth phase (Cheirsilp et al. 2001). Kefiran production by a mixed culture of *Lact. kefiranofaciens* and yeasts was studied on a lactose-containing medium. During co-culture of this LAB strain and *Saccharomyces cerevisiae*, it was shown that lactose was converted into kefiran, lactic acid, and galactose by *Lact. kefiranofaciens* (Cheirsilp et al. 2003a). The consumption of lactic acid by *S. cerevisiae* prevented the accumulation of this compound and therefore the inhibition of *Lact. kefiranofaciens* by this acid (Tada et al. 2007). This fact directly enhances cell growth and kefiran production rates. This co-culture approach has also been described for improving nisin production by a *L. lactis* strain (Shimizu et al. 1999). In addition, kefiran production in a mixed culture under aerobic conditions was higher than that under anaerobic atmosphere (Cheirsilp et al. 2003b). This may be ascribed to the fact that yeasts can grow and produce more growth factors necessary for LAB under aerobic conditions (Lopitz-Otsoa et al. 2006). In addition, the physical contact with *S. cerevisiae* enhanced capsular kefiran production.

In general, optimization of the culture conditions to stimulate production of useful substances by cooperative actions between two microorganisms becomes difficult. Some culture parameters to be considered are pH, temperature, composition of the aeration gas, starter concentration, incubation time, medium nutrient composition, and inoculum percentage of each microorganism (Taniguchi et al. 2001). The development of a mathematical model for kefiran production in a mixed culture constitutes an important tool for defining culture conditions. The model developed by Cheirsilp et al. (2007) considers the impact of *S. cerevisiae* on cell growth, kefiran formation, and substrate assimilation by *Lact. kefiranofaciens*. The construction of mixed culture models for kefiran fermentation allows predicting of the effects of *S. cerevisiae* and environmental factors on growth and kefiran production in a mixed culture.

The production of useful compounds using a single microorganism under culture conditions established by mimicking the actions of yeast cells on *Lact. kefiranofaciens* in kefir grain is an alternative method for optimizing metabolite production. Addition of yeast extract and ethanol, aeration of gas containing CO_2, and their combinations promotes kefiran production by *Lact. kefiranofaciens* in a single culture (Taniguchi et al. 2001).

Bacteriocins. During homemade manufacture, kefir grains are not treated aseptically; in spite of

this, no contamination with undesirable microorganisms has been reported. The high organic acid concentration and the presence of other antimicrobial substances could explain the absence of pathogens in kefir grains manipulated in regular kitchens for thousands of years. Among the antimicrobial substances involved, bacteriocins may be present. Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins (Jack et al. 1995; Ross et al. 2002; for an extensive revision on bacteriocins, see Chapter 5).

A number of lactococci exhibiting antimicrobial activities were isolated from kefir grains in Ireland. The inhibitory substance produced by one of these strains exhibited a broad spectrum of inhibition, similar to that of nisin (Morgan et al. 2000). Also, *Lactobacillus plantarum* ST8KF isolated from kefir produces a 3.5-kDa bacteriocin (bacST8KF) that is active against *Lactobacillus casei*, *Lact. salivarius*, *Lact. curvatus*, and *Listeria innocua* (Powell et al. 2007).

A *Lactococcus* strain isolated from kefir grains produces a bacteriocin, designated lacticin 3147, which displays the advantage of acidifying milk at sufficient rates to allow commercial manufacture of Cheddar cheese. Lacticin is an effective inhibitor of many Gram (+) food pathogens and spoilage microorganisms; thus, starters made with lacticin-producing strains may provide a useful means of controlling the proliferation of undesirable microorganisms during cheese making (Ryan et al. 1996).

18.4.3. Microbial Interactions and Surface Properties

Adhesion of microorganisms to the matrix and co-aggregation between them could have an important role in the maintenance of the number and species balance in kefir grains over time. The microorganisms attached into the grain probably have advantages over free-living microorganisms with respect to survival in stress conditions such as low pH, low nutrient concentration, and suboptimal temperatures (Garrote et al. 1998; Witthuhn et al. 2005b). Bacterial interactions are mediated by polymeric substances that are present on the outside of the cell wall. Diverse classes of surface constituents have been implicated in bacterial interactions, such as surface exopolysaccharides, surface proteins, lipopolysaccharides, lipoteichoic acids, lectins, S-layers, and fimbriae. The presence of these surface constituents depend on the bacterial genus and strain and the growth and environmental conditions (Navarre and Schneewind 1999).

In bacterial association, ionic or Coulombic interactions, hydrogen bonding, hydrophobic effects, or microbial surface macromolecules such as (glyco) proteins and polysaccharides could be involved. The heterofermentative lactobacilli *Lact. kefir* and *Lact. parakefir* possess S-layer proteins, a macromolecular paracrystalline array of proteins that completely covers bacterial cell surface (Garrote et al. 2004). Yeast surfaces have three major cell wall components, namely glucans, mannans, and chitin (Chaffin et al. 1998; Millsap et al. 1998). Co-aggregation is a process by which genetically distinct microorganisms become attached one to another via specific molecules. Cumulative evidence suggests that such adhesion influences the development of complex multispecies biofilms (Nikolaev and Plakunov 2007). A strong surface interaction between *Lact. kefir* and *Saccharomyces lipolytica* isolated from kefir grains was described. Inhibition of co-aggregation after heating of bacteria and the decrease in the presence of different sugars indicate that the surface molecules involved are thermolabile, suggesting that proteins act as mediators in the aggregation process mediated by a lectin-like activity (Golowczyc et al. 2009).

18.4.4. Microbial Interactions and Probiotic Properties of Kefir

Several health-promoting properties are associated with kefir consumption; in this regard, kefir can be considered a probiotic product. It has been used empirically for the treatment of gastrointestinal and metabolic disorders, atherosclerosis, allergy, and tuberculosis (Saloff-Coste 1996; Lopitz-Otsoa et al. 2006). Several studies demonstrated antitumor activity of kefir (Hosono et al. 1990), stimulation of the immune system (Saloff-Coste 1996; Farnworth

2005), and both antibacterial (Zacconi et al. 1995; Ryan et al. 1996; Garrote et al. 2000) and antifungal activity (Saloff-Coste 1996).

The beneficial action of kefir can be partially attributed to the inhibition of pathogenic microorganisms by metabolic products such as organic acids produced by the kefir microbiota (Garrote et al. 2000). Recent studies demonstrated stimulation of the immune system by kefir (Thoreux and Schmucker 2001; Vinderola et al. 2005) and the role of surface molecules in the protection against enteropathogens (Golowczyc et al. 2008).

The term competitive exclusion was used for the first time by Greenberg (1969) to describe the exclusion of *Salmonella* Typhimurium by normal gut microbiota. Colonization resistance is an analogous 7 term that was introduced by van der Waaij (1971) in studies of the intestinal populations in mice. Microbial interactions and the mechanisms by which indigenous intestinal microorganisms inhibit colonization by invading pathogens are not fully understood. However, *in vitro* and *in vivo* studies suggest that one or more bacterial species may inhibit proliferation or reduce the number of other bacterial types by the following mechanisms (Rolfe 1991): (1) creation of a restrictive physiological environment, (2) production of antibiotic-like substances, (3) competition for bacterial receptor sites, and (4) depletion of or competition for essential substrates. In the development of restrictive environments, the production of organic acids by probiotic microorganisms plays a central role. It is known that lactic acid and volatile short-chain-fatty acids, including acetic, propionic, and butyric acids, inhibit enteropathogens in their non-dissociated state (Helander et al. 1997; Presser et al. 1997).

Most of the studies about the antimicrobial activity of kefir were conducted in *in vitro* experiments. Garrote et al. (2001) demonstrated that kefir obtained with different grains reached a pH of between 3.5 and 4.0 and inhibited the growth of *Escherichia coli*. Thus, milk fermented with grain CIDCA AGK2 was able to halt bacterial growth for at least 25 h. The acid concentration varied between 1.30 and –2.30 g/100 ml in the case of lactic acid, and between 0.13 and 0.29 g/100 ml in the case of acetic acid. Supernatants of kefir abolished *E. coli* growth in broth. However, yogurt supernatants produced an extension of its lag period. Mixtures of lactic and acetic acids at the concentrations present in kefir also increased the lag time. This study suggests that the inhibitory power of kefir can be attributed to the non-dissociated lactic and acetic acids and other compounds not yet identified (Garrote et al. 2000). On the other hand, Gulmez and Guven (2003) compared the microbiological safety of yogurt and kefir in different combinations by using three food-borne pathogenic strains, *E. coli* O157:H7, *Listeria monocytogenes*, and *Yersinia enterocolitica*, as indicators. They concluded that a combination of yogurt and kefir starter may improve the microbiological safety of the end-product.

The antimicrobial activity of sugar broth fermented with kefir grains against *Candida albicans*, *E. coli*, *Staphylococcus aureus*, *Salmonella typhi*, and *Shigella sonnei* was described by Silva et al. (2009). Ulusoy et al. (2007) qualitatively studied the *in vitro* antimicrobial activity of kefir against *Staph. aureus*, *B. cereus*, *Salmonella enteritidis*, *L. monocytogenes*, and *E. coli*, this activity being stable during storage. The effects of kefir were tested against a toxigenic strain of *B. cereus*. The incubation of milk artificially contaminated with *B. cereus* spores plus 5% kefir grains prevented spore germination and growth of vegetative forms. In addition, the presence of metabolically active kefir grains diminished titers of nonhemolytic enterotoxin A, as assessed by ELISA (Kakisu et al. 2007).

Kourkoutas et al. (2006) evaluated a freeze-dried kefir co-culture as starter for Feta-type cheese production, and they could not detect *Staphylococcus* in the cheese. *Staphylococcus* count was significantly lower in unsalted kefir cheese, not only compared with rennet cheese but more important compared with similar cheeses that had been salt-treated. The supernatants of 11 isolates of *Lact. plantarum* from kefir grains produced strong growth inhibition of *Salmonella enterica* serovar Thipymurium and *E. coli*. However, *Salmonella gallinarum, Salmonella enterica* and *Sh. sonnei* were inhibited by some of the strains tested (Gollowczyc et al. 2007). Although some strains of 8

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lactococci isolated from kefir produce bacteriocins as mentioned in this chapter, more research is needed to understand the high inhibitory power of kefir supernatants.

Among the criteria suggested for selection of probiotics, the ability to adhere to the gastrointestinal mucosa and colonization has been proposed (Ouwehand et al. 1999). This property is strain-specific and is related to the structure and molecular composition of the probiotic cell wall. These characteristics also determine the ability to interact with intestinal mucus, other microorganisms of the enteric microbiota, pathogens orally ingested, and their toxins. Adhesion to mucosal surfaces by probiotics probably protect against pathogens through competition for binding sites and nutrients (Ouwehand et al. 2002; Collado et al. 2005) or immune modulation (Salminen et al. 1998). In spite of the lack of definitive proofs, some studies have indicated a relationship between *in vitro* adhesion and *in vivo* colonization (Collado et al. 2007).

Enterohemorrhagic *E. coli* (EHEC) is a food-borne pathogen that causes hemorrhagic colitis and the hemolytic uremic syndrome. Colonization of the human gut mucosa that leads to the development of the histopathological attachment; effacement lesions; and production of Shiga toxins are critical virulent traits of EHEC. It seems that adhesive type IV pili (EHP) are adherence factors (Rendón et al. 2007) that participate in intestinal colonization (Xicohtencati-Cortes et al. 2007). The effect of probiotics against adhesion of EHEC to intestinal epithelial monolayer was studied *in vitro* with some lactobacilli strains isolated from kefir. Hugo et al. (2008) studied the effect of kefir lactobacilli on the biological activity of EHEC and found that strain *Lact. plantarum* CIDCA 83114, viable or dead, prevented detachment of Hep-2 cells. However, other lactobacilli failed to protect eukaryotic cells. Then, the protective effect was not ascribed to pathogen exclusion and lactobacilli could antagonize virulence mechanisms of EHEC either by modification of the microenvironment or by interfering with the signaling cascades triggered by the pathogen (Hugo et al. 2008).

Campylobacter jejuni is recognized as the principal cause of human acute bacterial gastroenteritis. This bacterium occurs at high percentage in poultry, which is the primary source of infection (Harris et al. 1986). Competitive exclusion of this bacterium by kefir was studied in chicks. Zacconi et al. (2003) performed *in vivo* studies to verify the competitive exclusion activity of kefir in chicks by assessing the reduction of *Camp. jejuni* colonization of caecum. They found that fresh and frozen kefir could have interesting applications on the control of the diffusion of pathogenic microorganisms in poultry bleedings.

In the competition for bacterial receptor sites, the ability of bacterial to adhere is important in the establishment or maintenance of colonization of mucosal surfaces. The bacterial glycocalyx is thought to mediate bacterial adherence to each other and to the intestinal epithelium. Thus, a layer of protective bacteria *could* block the receptor sites for pathogen attachment. The ability to adhere to epithelial cells *in vitro* is a common property of some lactobacilli strains, this property being strain-specific. *Lact. plantarum* and *Lact. kefir* isolated from kefir grains are able to adhere to Caco-2 cells with different percentage of association (0.97%–10% of adhesion). The ability to associate to Caco-2 cells was not related to hydrophobicity since some highly hydrophobic *Lact. kefir* strains(Golowczyc et al. 2007) and some highly hydrophilic strains of *Lact. plantarum* (Golowczyc et al. 2008) were adherent to Caco-2 (0.97%–5.30% of adhesion) in concordance with previous reports (Reid et al. 1994). In contrast, these two properties, adhesion and hydrofobicity, correlate in the genus *Bifidobacterium* (Perez et al. 1998).

In all isolated strains of *Lact. kefir*, the presence of S-layer was demonstrated. S-layer was constituted by a single polypeptide with an apparent molecular mass of 66–71 kDa. This S-layer conferred to the lactobacilli a high hydrophobicity but could not be associated with the adhesion to Caco-2 cells. In some strains, the presence of S-layer proteins is associated with the ability to autoaggregate and hemagglutinate. However, in other strains with the same surface structure, S-layer

proteins of the same molecular mass and reactivity against monoclonal antibodies, autoaggregation and hemagglutination were not observed, indicating that other surface molecules could be necessary for the expression of these properties (Garrote et al. 2004; Mobili et al. 2009).

To study the protective action of *Lact. kefir* against adhesion and invasion of Caco-2/TC7, several strains isolated from kefir grains and *Salm. enteritidis* were tested (Golowczyc et al. 2007). In contrast to other reports (Lee et al. 2003), no protection against *Salmonella* was observed with lactobacilli adhered to the cells. However, a significant protection was achieved when lactobacilli and *Salmonella* were previously co-incubated. In this case, *Lact. kefir* CIDCA 8321 co-aggregated with *Salmonella* and had the ability to antagonize the invasion of Caco-2/TC7. In contrast, a non-co-aggregating strain (*Lact. kefir* CIDCA 83113) did not produce any protection. These results suggest that the masking of surface structures of *Salmonella* during co-aggregation interfered with the invasion process. In addition, isolated S-layer proteins from *Lact. kefir* had the ability to autoassemble on the surface of *Salmonella*, preventing the invasion of Caco-2/TC7. It could be interpreted that S-layer proteins interact with specific sites on *Salmonella* surface involved in the first step of mucosal infection or could either modify or mask *Salmonella* structures necessary for the invasion of cultured human enterocytes (Golowczyc et al. 2007).

18.5. Conclusions

Microbial interactions in kefir are very complex mainly due to the composition of kefir grains. In this microbial ecosystem a delicately balanced population of microorganisms occurs, each interacting with and influencing the other members of the population. In kefir grains the balanced population of microorganisms determines the synthesis of biologically active metabolites that are essential for grain growth and the inhibition of external microorganisms, like pathogens and food contaminants.

To understand interactions, it is necessary to perform detailed studies on the physiology of the individual predominating microorganisms to establish their requirements with respect to environmental factors such as nutrients, temperature, pH, oxidation-reduction potential, which may be involved in grain growth, and to determine how these factors affect their preservation and probiotic properties. This information altogether will indicate the possible interactions among microorganisms and will be the basis for understanding kefir grain ecosystem. Extensive research remains to be done on microbial interactions in kefir grains to obtain the desired, precise control of these ecological fermentative processes.

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