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Kefir D'Aqua and Its Probiotic Properties

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1. Introduction

Prebiotics are non-digestible molecules produced by probiotic microorganisms [1]. Probiotic microorganisms are generally bacteria or fungi recognized as safe, with their properties based on the production of organic acids, reduction of biogenic amines, digestion/breakdown of carbohydrates and proteins, immunomodulatory and anti-inflammatory responses, reduction of carcinogenic amines, and production of antimicrobial peptides, among others [2]. These days probiotics are mostly consumed as probiotic yogurts and other probiotic dairy products, dietary supplements, spoonable forms, and probiotic cultured drinks for daily dosage packaging, among others. Prebiotics are also claimed to enhance wellbeing through immunomodulatory and metabolic activities, and act as a natural barrier against pathological processes [1]. These molecules are considered to be a targeted for human and animal production and health, and represents a multimillionaire market of the functional foods. Furthermore, the increasing market of prebiotics counts today with a thousands of patented invention, related to isolation, production, preparation, methods of use, or application of newly health enhancing molecules. The global production and consumption of functional foods is a multi-billion industry, with an estimated market size around US\$ 60 billion in 2008-9, several times greater than the health treatment costs only in USA in that years, in the order of US\$ 832 million (Figure 1). As a comparison, the global market of probiotic products was US\$ 15.9 billion in 2008 and US\$ 19 billion in 2009, with a compound annual growth rate (CAGR) of 11.7 % (2009-2014). Furthermore, the probiotic market predicted by 2014 for Europe and Asia comprises, respectively, US\$ 12.9 billion (11.1 % CAGR), and US\$ 8.7 billion. Japan, a global leader of functional foods, devoted US\$ 4.5 billion to the study and commercialization of prebiotics, with US\$ 1.5 billion verted exclusively for the oligosaccharide commerce in 2009 [3]. The USA have occupied the second position in the last decade, with a commercialization of US\$ 110 million for functional oligosaccharides (35 % inulin, 20 % mannan oligosaccharides, and 10 % fructan), and with a CAGR rate of 20 % The European and the U.S. market for prebiotics is projected to reach nearly US\$ 1.2 billion and US\$225 million, respectively, by the year 2015 [3]. This has reached nearly US\$ 21.6 billion in 2010 and is expected to reach US\$ 31.1 billion in 2015, and at a CAGR of 7.6 % for the 5-year period.

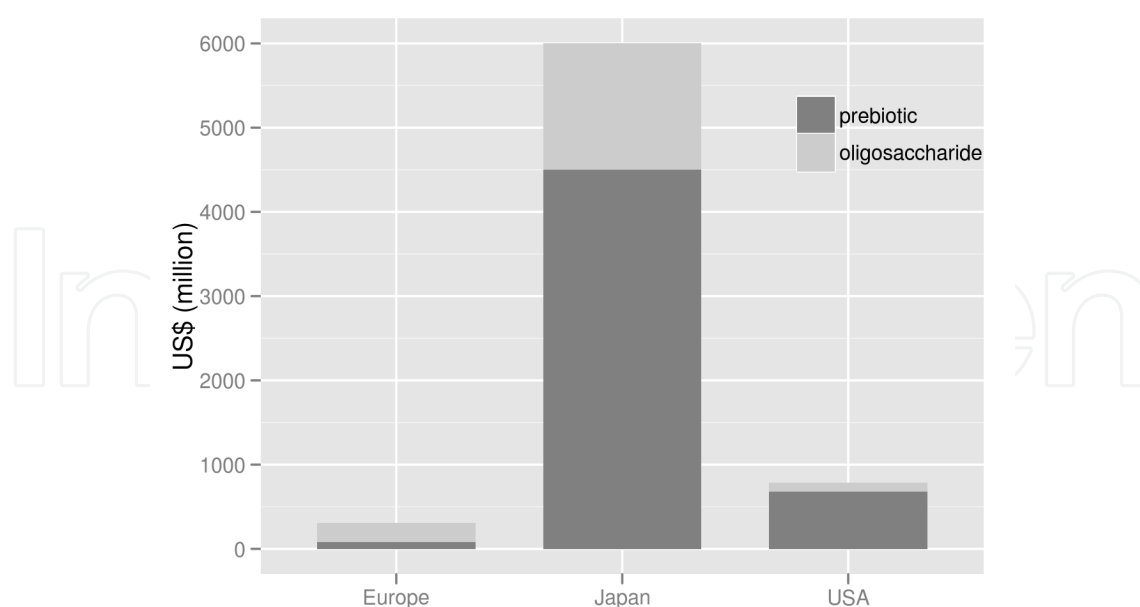


Figure 1. Global market of prebiotics from 2008 to 2010 [3].

2. Studies on water kefir

In general, prebiotics are considered nondigestible but fermentable oligosaccharides, involved on health promotion for the host [4]. Such compounds are known to provide improvements in nutritional status, besides additional health benefits such as protection against carcinogenesis, mutagenesis, prevention of injuries caused by free radicals, control of intestinal flora, gastrointestinal resistance, decrease of blood pressure induced by hypertension, production of β -interferon, cortisol and norepinephrine, increase of phagocytic activity of peritoneal and lung macrophages, increase of IgA cells in these sites, antimicrobial activity, and anti-inflammatory activity, among others [1]. Kefir, an acid-alcoholic fermentation traditionally consumed in Eastern Europe as milky suspensions due its potential health benefits [5], is able to produce peptide and sugar prebiotics (e.g., lactacin, bactericins, KGF, kefiran) [1].

Historically, kefir grains (Figure 2) were considered a gift from Allah among the Muslim people of the northern Caucasian mountains [6]. The word kefir is derived from the Turkish word *keif*, which can be translated to good feeling for the sense experienced after drinking it, or their promoted health claims. Kefir grains were passed from generation to generation among the tribes of Caucasus being considered a source of family wealth [6]. Kefir grains can be also cultivated in a solution of raw sugar and water (e.g., molasses), known as sugary, water or water kefir. Sugary kefir grains are very similar to milk kefir grains in terms of their structure, associated microorganisms and products formed during the fermentation process, albeit without the characteristic cauliflower look of them. Kefir d'aqua, sugary kefir, or water kefir, is generally a home made fermented beverage based on a sucrose solution with or without fruit extracts. Kefir consists of a gelatinous and irregular grains formed by a consortium of yeasts and lactic acid bacteria embedded in a resilient polysaccharide matrix named kefiran [7]. Since 2002 our research group has dedicated to study the properties and beneficial effects of kefir and kefiran extracts [7, 8] and, more recently, an oligosaccharide isolated from water kefir fermentation, and named aqueous kefir carbohydrate (AK) [9].



Figure 2. Sample of water kefir grains after souring a molasses solution.

Different from the milky bacteria-encapsulated polysaccharide kefiran, AK seems to be an oligosaccharide isolated from an aqueous fraction of kefir grains [10].

2.1. Kefir characteristics

2.1.1. Microbial strains

Different sets of yeasts and bacteria in water kefir have been identified from several regions and sources, and with both culture-dependent or molecular methods. Notwithstanding, kefir is able to change their bacterial/yeast ratio, even their microbial strains as a function of time, experimental conditions, temperature, and neighboring microorganism, in the inner grain [11]. A typical consortium appears to consist of mostly lactic acid bacteria plus yeasts promoting alcoholic fermentation, together with some acetic acid bacteria (Table 1), possibly oxidizing the ethanol formed [12]. Despite the great microbial diversity found in kefir samples from different regions, there are common strains prevailing in kefir sources from different countries. The most likely strains found in kefir are *Lactobacillus*, *Leuconostoc*, *Kluyveromyces* and *Acetobacter* genus, although the symbiotic 'organism' had also presented some rare microorganisms, such as *Chryseomonas* and *Kloekera* [13].

2.1.2. Growth

Changes in physical, chemical and microbiological parameters during continuous cultures of water kefir has been studied by several authors since 50's [15]. In our lab grains samples grown in molasses solutions at 50 to 200 g·L⁻¹ in distilled water have been tested for some parameters, as optima temperature and pH of development, ionic strength, some metabolites (glucose and glycerol), growth changes after freezing even at -70 °C, and bacteria/yeast proportions. The results have shown a maximum temperature of growth about 25 °C, and a continuous pH decrease for the suspensions up to 20 h (from pH 6.1 to pH 4.5). While kefir suspensions presented decreasing levels of glucose (7 times), glycerol increased 3 times during cultivation in molasses at physiological conditions for 7 days. The bacteria/yeast quotient of

Bacteria	
<i>Lactobacillus brevis</i>	<i>Lactobacillus hilgardii</i>
<i>Lactobacillus lactis cremoris</i>	<i>Lactobacillus casei</i> subsp. <i>casei</i>
<i>Lactobacillus casei</i> subsp. <i>rhamnosus</i>	<i>Acetobacter aceti</i>
<i>Lactobacillus casei</i> subsp. <i>Pseudoplatantum</i>	<i>Lactobacillus plantarum</i>
<i>Lactobacillus buchneri</i>	<i>Lactobacillus fructivorans</i>
<i>Lactobacillus keranofaciens</i>	<i>Lactobacillus kefir</i>
<i>Lactobacillus collinoides</i>	<i>Lactococcus lactis</i> subsp. <i>lactis</i>
<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>
<i>Leuconostoc mesenteroides</i> subsp. <i>Dextranicum</i>	<i>Enterobacter hormachei</i>
<i>Gluconobacter frateuri</i>	<i>Chryseomonas luteola</i>
Yeasts	
<i>Saccharomyces bayanus</i>	<i>Saccharomyces cerevisiae</i>
<i>Saccharomyces florentinus</i>	<i>Saccharomyces pretoriensis</i>
<i>Zygosaccharomyces florentinus</i>	<i>Candida valida</i>
<i>Hanseniaspora viniae</i>	<i>Hanseniaspora uvaldeensis</i>
<i>Kloeckera apiculata</i>	<i>Candida lambica</i>
<i>Candida colliculosa</i>	<i>Toruspora delbrueckii</i>
<i>Candida inconspicua</i>	<i>Candida magnoliae</i>
<i>Candida famata</i>	<i>Candida kefyr</i>
<i>Kluyveromyces lactis</i>	<i>Kluyveromyces marxianus</i>

Table 1. Some microbial strains found in water kefir samples [13, 14].

water kefir showed a prevalence of lactic acid bacteria in the grains (31±8 % greater), whereas yeasts have been mainly found in the suspensions (63±6 % greater). Surprisingly, water kefir grains have been demonstrated a higher resistance against extreme environment conditions. As an example, the grains were able to growth in KCl up to 5 %, or even at temperatures lower than 4 °C. At household conditions of growth, biomass curves of freezed-stored grains have shown an continuous linear trend up to the 5th month of grains storage, and with a decay rate of 4g/day/month. However, a progressive disruption of the overall metabolism of the self-organized grains have been identified under -70 °C freezing. For testing this highly apparent resistance of kefir grains, we had performed some challenges against antibiotics, irradiation and gas treatments, with water kefir.

2.1.3. Resistance

As a well-structured gelatinous grains with diverse microbial strains in their composition, it was hypothesize that the bacteria and yeasts present in kefir could be protected inside the polysaccharide matrix, exhibiting a different resistance under physical and chemical stresses than freely strains in solution. Keeping this in mind it has been tested the colony resistance of kefir against three disordering factors: ultraviolet radiation exposure (UV), antibiotic administration, and gas treatment (oxygen and ozone) [16]. After an exponential growth phase the samples were submitted to UV and chemical treatments. Far UV (15 W D₂) was taken daily in tubes containing the grains during 5, 10, 30 and 60 min, up to 9 days. The growth of grains were followed gravimetrically after cutting dried grains into six layers, from the inner core to the outer shell of the grains. Antibiotic treatment was carried out with 1 mL penicillin G (20 µg·L⁻¹), 50 mg nystatin (Fungizon) and 1 mL streptomycin (100 µg·mL⁻¹) dispensed separately in kefir cultures during 12 days at 24 h intervals. Gas treatment was done with continuous ozonization at 1, 5, 10, 30, 60, and 120 min in 0.5 g of kefir starter

grains, following cultivation as described. In all these challenges the grains were able to resist against extreme conditions during cultivation. UV treatment, for example, suggested a relative recovery of growth after the irradiation period (Figure 3). This was revealed comparing the slopes of growth curves obtained before the UV irradiation (1.22 ± 0.15 g/day/g of sample), after 7 days treatment (0.30 ± 0.02 g/day/g of sample) and 15 days treatment (0.56 ± 0.07 g/day/g of sample). With the antibiotic treatment, a decrease in growth rates was observed 72 h after administration in culture media, with bacteria bringing out more biomass to the grain structure than yeasts. In the other hand, the gas treatment resulted an exponential decay for the growth rate up to 41 ± 23 (oxygen) and 25 ± 8 % (ozone) after 7 days after the exposures. Although these disordering factors were able to decrease kefir growth during the challenges, none of them was able to completely disrupt the grain structure or biomass production after exposures. In conclusion, the ancient culture of symbiotic kefir showed a strong resistance against UV, antibiotic and ozone defiances, allowing a retrieval close to the normal growth after the disturbances.

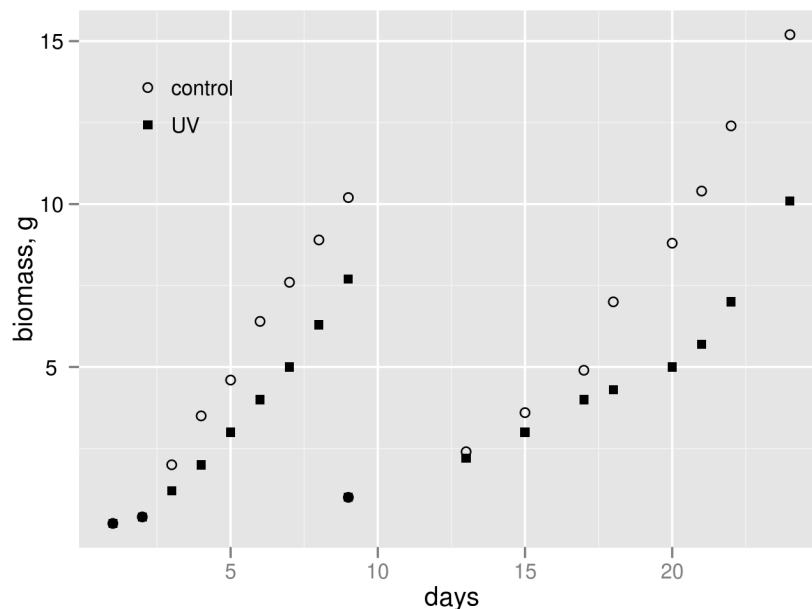


Figure 3. Growth curves of kefir grains submitted to far-UV irradiation up to the 9th day, following normal cultivation with 1 g-starter sample.

2.1.4. Artificial symbiogenesis

The microbial flora present in kefir grains has been studied from a symbiotic community point of view by Linn Margulis since 1995 [17]. Accordingly, it has been stated [18] that separated cultures of microbial kefir grains, either do not grow in milk or have a decreased biochemical activity, which further complicates the study of the microbial population of kefir grains. The mechanism of symbiogenesis of kefir grains from distinct strains of unicellular organisms is unknown, although there are some data about the recover of their structure and probiotic properties from lyophilization, and even so, about the formation of an artificial consortium produced by bits of kefir grains transferred to a yeast extract-sucrose solution [19]. Using a simple approach, we had developed artificial cultures of kefir by trapping their strains in alginate beads [20]. To do so, kefir grains were cultured in $200 \text{ g} \cdot \text{L}^{-1}$ of molasses

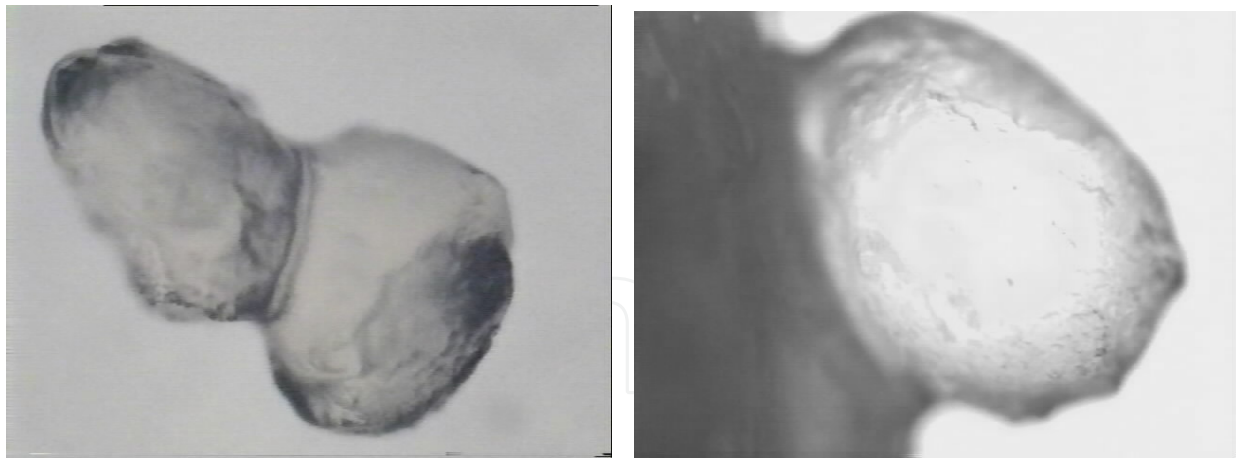
solution for 7 days. Then the supernatant was collected, centrifugated at 7000 rpm during 15 min, resuspended into 5 mL of molasses as above, and filtered to avoid minor grain fragments. For cell immobilization 100 mL of a 4 % sodium alginate solution was mixed with the treated kefir suspension and dropped into 1.5 % of a cold calcium chloride solution. The alginate-kefir beads resulted were then continuously cultivated with molasses replacement at 48 h intervals. Strikingly, novel kefir grains had been arisen from solution after three months of cultivation (Figure 4), resembling the ordinary household grains, as monitored by optical microscopy at low resolution, and with the common budding property exhibited by normal grains (Figure 5).



Figure 4. Fresh alginate-kefir beads (bottom of the image) and the beads cultured with 48-h medium changes for 96 days.

Antimicrobial activity was chosen as a comparison index for native and artificial grains. The assays were carried out introducing 0.1 mL (3×10^8 cells) of *S. aureus*, *S. typhimurium*, *E. coli*, and *C. albicans* in 1.5 mL of kefir suspensions, following incubation for 24 h at 35 °C. After this period 0.1 mL of each tube was swabbed in Petri dishes containing the proper culture media and incubated for 24 and 48 h. By counting the colony unit formers (CUF) for native and artificial grains, the antimicrobial activity of kefir exhibited a similar pattern, with total inhibition for all strains for both kefir types (native and artificial produced). Photomicroscopy showed an increase of grain budding from alginate-kefir beads after the 96th day of incubation, with the novel grains achieving an identical kefir morphology up to 120 days, and presenting a mean diameter of 22 ± 2 mm. These findings indicate a partial maintenance of both structural and probiotic properties of kefir during the grain development unnaturally induced, a high-degree of self-organization for the symbiotic culture. In this goal we also had tested the potential of kefir grains to hold an exogenous strain, trying to incorporate *Saccharomyces cerevisiae* on grain development. The procedure, similar to that described above [21], was conducted by adding different amounts *S. cerevisiae* in the starter cultures before the shaping of alginate-kefir beads.

The anti-inflammatory activity of this modified grains, as revealed by paw edema assays in rats, showed even higher than native grains (Figure 6). This artificial process of strain internalization for kefir grains suggests a plausible strategy for incorporate some bacteria with specified purposes, e.g., *Lactobacillus acidophilus* for lowering blood cholesterol. In this way, previous studies [6] have demonstrated decreased levels on serum total cholesterol of rats



(a) Binary division of grains obtained from the symbiogenesis produced from alginate-kefir beads cultivated alginate-kefir beads (x15)
(b) A small kefir grain sprouting from the main body of symbiogenesis produced from alginate-kefir beads cultivated alginate-kefir beads (x15)

Figure 5. Symbiogenesis of kefir grains anchored to calcium alginate beads and treated with molasses for 3 months. (a) grain division, and (b) grain sprouting [20].

fed with a high-cholesterol diet supplemented with fermented milk produced by modified kefir grains. This modified kefir was obtained from a mixture of 10 types of *Lactobacillus* and *S. cerevisiae*. In the other hand, the addition of yeast cells of *S. cerevisiae* from a co-culture of *L. kefiranofaciens* and *C. kefir*, or *T. delbrueckii*, did not showed any enhanced effect on kefiran production [22]. Notwithstanding, when yeast extracts were added to *L. kefiranofaciens* cultures, the authors reported an increase in kefiran production, and suggested the role of yeast extracts as mimicking the actions of yeast cells on *L. kefiranofaciens* in the grains as a typically natural co-culture system.

This property of inherent modulation of kefir strains has been also reported with native grains, whenever they were stored for long periods, or even during their cultivation [23]. In this aim, we have evaluated the bacteriocinin activity of kefir from an adaptative potential of growth against some pathogenic strains [24]. To accomplish this, kefir samples were challenged with *Staphylococcus aureus* or *Escherichia coli*, by pipetting 1 mL of 2×10^9 cells/mL of the strains into 70 mL of kefir culture at each 48 h-medium change ($50 \text{ g} \cdot \text{L}^{-1}$ molasses) for 20 days. Kefir grains was then separated, dried and weighted before the medium changes. Then, 0.1 mL of the supernatant was withdrawn from fermented kefir and seeded on EMB agar (*E. coli*) or manitol agar (*S. aureus*, following incubation at 35.5°C for 48 h. The same aliquot was also used for disc diffusion antimicrobial assays. Following, 0.3 mL of inoculated kefir was centrifuged, filtered with 0.22 mm Millipore filter, and pipetted into BHI media containing 3.3 mL of each single inoculated bacteria (unitary Mc Farland's scale). The incubation was done at 35.5°C up to 12 h, and the bacterial growth was monitored spectrophotometrically at 600 nm. After the incubation period, the grains exhibited major morphological changes on their structure for those groups treated with the inoculations. Surprisingly, the filtered kefir sample *S. aureus*-stimulated incubated for 20 days was able to suppress the growth of the same *S. aureus* strains (Figure 7). This finding suggest an epigenetic or adaptative potential for bacteriocinins secretion by kefir to resist to *S. aureus*, as the soured suspension was changed at 48 h-intervals, avoiding the presence of antibiotic molecules previously produced by the symbiotic.

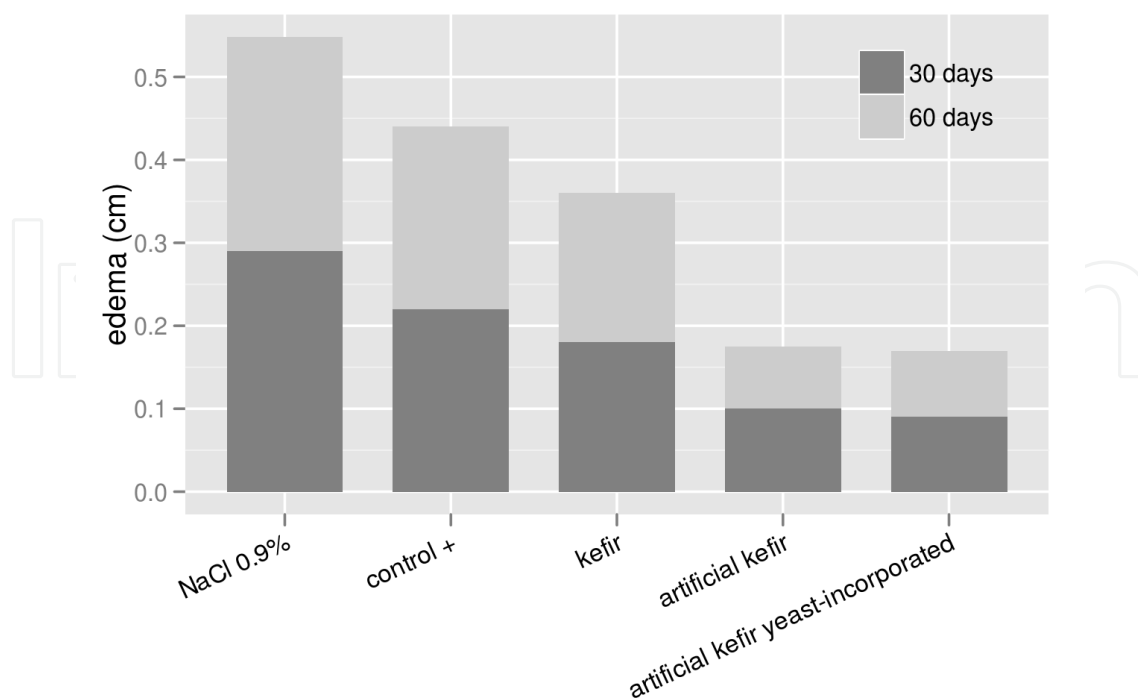


Figure 6. Inhibition of rat paw edema carrageenan-induced (1 mg/paw, 0.1 mL) by kefir suspensions obtained from cultivation of native kefir grains, and those produced by symbiogenesis with or without *S. cerevisiae* incorporation. The assays were carried out for 30 and 60 days after obtained the modified grains. Positive control - 10 mg·kg⁻¹ indomethacin [20].

2.2. Kefir properties

2.2.1. Suspension, grains and kefiran

2.2.1.1. Aqueous kefiran (AK)

There are several studies pertaining to the claimed health properties of the kefir consortium, but mainly with milky preparations. Accordingly, milky kefir is known to present a large antibacterium spectrum, gastrointestinal improvement and proliferation of normal lactic intestinal flora and bacterial colonization, anti-carcinogenic, wound healing and β -galactosidase activities, immuno-stimulatory, anti-diabetes [25], anti-oxidative [26], anti-lipidemic [27], and anti-allergenic effects, among others [28]. In the same way, although there are a lot of data reported about an exopolysaccharide with prebiotic properties isolated from kefir grains, the literature concerns only on the purified molecule from lacteous sources. In this goal our research group had been studied physical-chemical and prebiotic properties of a variation of the milky kefir, an oligosaccharide named aqueous kefiran (AK), and fractionated from molasses solution [29]. Isolated AK solutions prepared at 0.1 % had presented a mean yield, intrinsic viscosity, relative density, and electrical conductivity of, respectively, 1.1 g·kg⁻¹ of dried grains, 0.297±0.03 dL·g⁻¹, 1.044 g·mL⁻¹, and 2.46 μ S·cm⁻¹. Infra-Red spectroscopy (IR) of AK presented strong bands at 3600-3100 (ν O-H) and 10⁷ cm⁻¹ (ν C-O), suggesting a polyhydroxylated nature of the sample. Minor bands were shown at 2950-2880 (ν C-H), 1470 and 1390 cm⁻¹ (δ_x C-H), revealing an aliphatic characteristic of the compound. The composition of monosaccharide residues of AK, as determined from

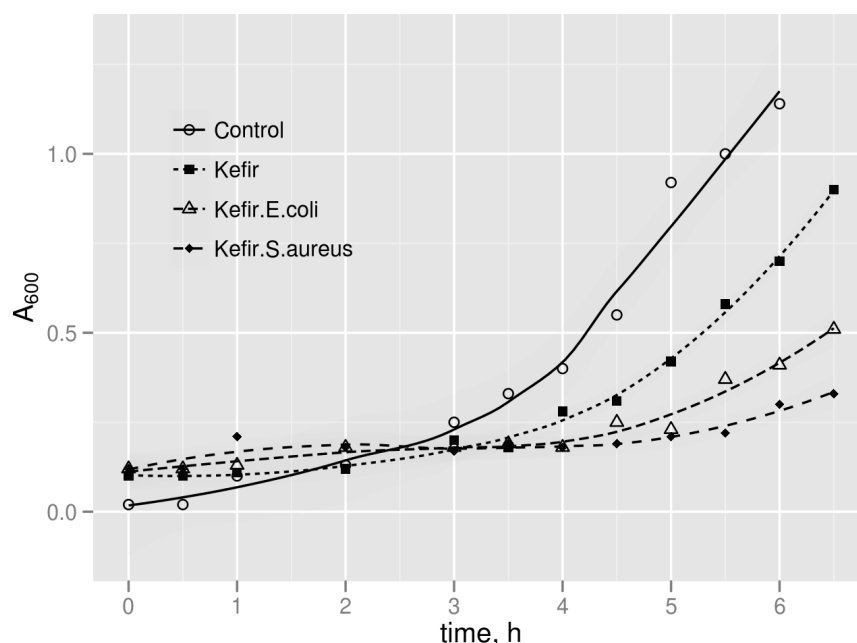


Figure 7. Changes in *S. aureus* growth in the presence of kefir suspensions stimulated for 20 days with *S. aureus* or *E. coli* [24].

thin-layer chromatography and GC, presented mean values of glucose (40 %), ramnose (24 %), galactose (10 %), and arabinose (26 %). From HPLC measurements, the molecular weight of AK was determined as 3534 Da, then suggesting a ten-monomer oligosaccharide structure for the prebiotic. Water kefir is rarely reported in the related literature as well patent depository banks [30]. Nevertheless, both kefir and kefiran, major milk-based, have been used to obtain technically and commercially feasible biotechnological products, as starter cultures by casein immobilization in cheese production [31], food-grade additive of milk gels for fermented products [10], industrial scale-up of alcoholic fermentation of whey [32], for batch alcoholic fermentation [34], for exploiting waste residues from the citrus industry [33], and for development of multipurpose edible films [35], among others.

2.3. On biological surfaces

2.3.1. Biomimetic membranes

Albeit kefir has presented diverse prebiotic activities, no direct mechanism of its action on cell membranes have been understood yet. Aiming to help this, the influence of AK on biomimetic membranes composed of 1- α -phosphatidylcholine/cholesterol supported bilayer lipid membrane was studied by voltammetry and electrochemical impedance spectroscopy (EIS) [4]. Our findings suggest that kefir could induce molecular pores at supported bilayer lipid membrane (s-BLM) surfaces up to 5 min at $11.4 \mu\text{mol}\cdot\text{L}^{-1}$, and with a 34 Å of initial radius. The suggested mechanism (Figure 8) seems to involve some hydrogen bonding between the carbohydrate and the phosphate head group of the phospholipid with a carpet-like model of interaction, and is related to the prebiotic concentration. This results can contribute to disclose direct molecular interactions between prebiotic oligosaccharides

and cell surfaces, both related to the biological activity of the prebiotic compound in several experimental models. In this way the prebiotic activity of AK could also be related to some metabolic pathways, as enzyme-kinetic or transport systems. Thinking on it, we have evaluated the plausible action of AK on mitochondrial suspensions, as a model of a whole and independent metabolic system.

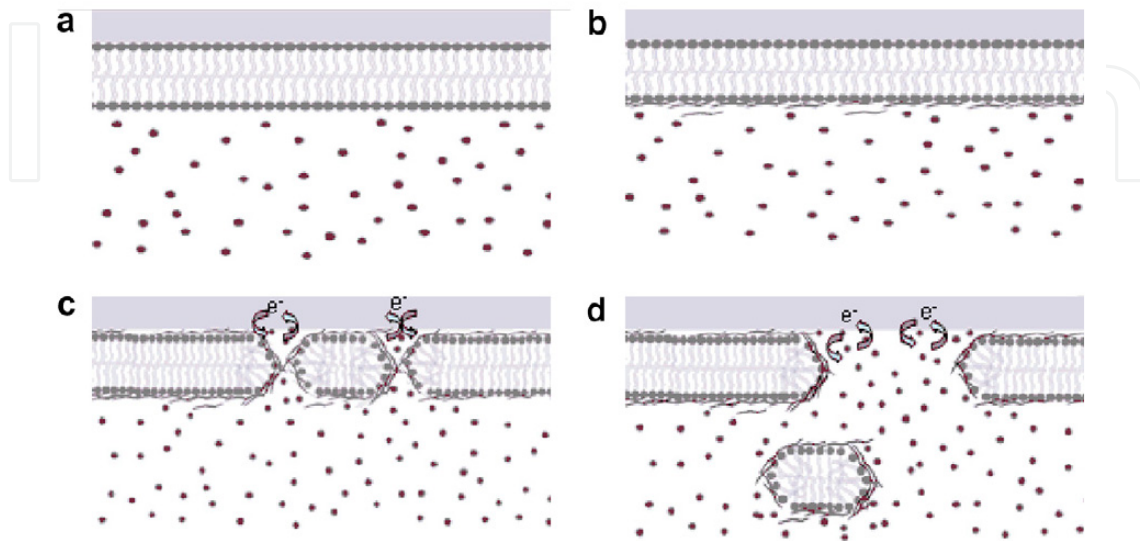


Figure 8. Carpet-like mechanism proposed for water kefiran-membrane interaction. Oligosaccharide molecules line up on the membrane surface (a) until a critical concentration is reached (b) and a detergent-like effect takes place (c). At this stage, oligosaccharides from kefiran and membrane components form aggregates that leave the membrane cause disruption (d) [4].

2.3.2. Mitochondria

Cellular mechanisms of action were investigated to verify the potential activity of water kefiran on the respiratory activity of isolated mitochondria [36]. Samples from rat liver ($1200 \text{ mg}\cdot\text{mL}^{-1}$ protein) were preincubated with kefiran in 20 mM phosphate buffer pH 7.3 containing 70 mM sucrose, 1 mM EDTA, and 5 mM MgCl_2 . The oxygen consumption of mitochondria was determined by chronoamperometry at 50 rpm stirring suspensions in 2 mL using a Clark-type electrode Pt-Ag/AgCl connected to a potentiostat, and with -600 mV of applied potential. The system was previously calibrated with a N_2 -saturated solution and baker yeast suspensions. The current signals after successive additions of buffer, mitochondrial samples, 100 mM succinate, 100 μL of kefiran, and 100 mM malonic acid, were obtained during 90 min. After proper digital filtering and signal amplification, the current values obtained were converted to oxygen concentration and flux. The results for organelle suspensions revealed a total inhibition of mitochondrial respiration with 0.2 % kefiran solution. Aiming to assess the prebiotic properties of AK on the mitochondrial respiratory pathways (Complex I and II), mitochondria suspensions ($300 \text{ mg}\cdot\text{mL}^{-1}$ protein) were preincubated with the prebiotic together with different carbon sources (50 mM Glu, 100 mM malate, 50 mM pyruvate, or 100 mM succinate) [37]. After the incubations, it was found a decrease in absorbance values at 340 nm after addition of 2 mM NADH. Furthermore, some changes at 520 nm were also found, after addition of 5 mM potassium ferricyanide in 50 mM KCN solution, using malonic acid (100 mM) and metformin (1 mM) as inhibitory markers

for Complex I and II, respectively. The inhibition of Complex I showed values of 53 ± 4 % for kefir (50 mg·mL⁻¹), whereas the Complex II showed inhibition values of 54 ± 5 % for AK. Moreover, a mitochondrial swelling test also revealed a mean increased value of 13 % for the kefir tested. These results as a whole point to an inhibitory effect for AK on the oxidative phosphorylation chain of mitochondria.

2.4. On microorganisms

Kefir is well known to resist to a large spectrum of pathological strains, and it seems to be recognized as safe, although its culture contamination has been reported as a source of health impairments. [38]. Antibiotic activity of both kefir and purified AK (50 mg·mL⁻¹) has been evaluated [8] using both the disk diffusion method and susceptibility tests against some well known pathogenic bacteria (*S. pyogenes*, *S. salivarius*, *S. aureus*, *P. aeruginosa*, *S. tiphymurium*, *E. coli*, *L. monocytogenes*, and *C. albicans*). The results of the disc diffusion promoted by kefir are present at Figure 9. A rapid decrease in surviving pathogens with 0.45 mg·mL⁻¹ of kefir in the susceptibility tests was also observed, whereas the prebiotic was able to produce inhibition haloes about 26 ± 2 mm, greater than those found for oxacilin, ampicillin, ceftriaxone, and azithromycin, at their usual concentrations. In these assays, *S. pyogenes* and *S. tiphymurium* were the most sensible bacteria challenged with kefir in vitro [39], as both strains had their growth completely abolished into Petri dishes, as revealed by CFU counting after 24 h of selective cultivation. *Listeria monocytogenes* also presents a valuable target for testing kefir, due to its commonly contamination in dairy products (milk and home made cheese), and its strong resistance at higher temperatures and osmolarity, together with the survival of strains at low pH medium. In this way, we evaluated MIC and MBC values for kefir suspension (0.1, 1.0 and 1.5 mL) pipetting the aliquots together with 0.1 mL *L. monocytogenes* (3×10^8 cell/mL), and following incubation at 35.5 °C for 24 h. After inoculation for 24/48 h, it was found a bacteriostatic property of kefir at 24 h with all aliquots, but a bacteriocidal activity at 48 h with 1.5 mL kefir suspension, suggesting a relative protection of kefir and their prebiotic compounds against *Listeria monocytogenes*. In another work, we tried out antimicrobial activity for both water kefir and its grain extract against *Staphylococcus aureus* [40]. Kefir samples were thawed and continuously cultivated in 100 g·L⁻¹ of molasses solutions during 7 days and 24 h of nourish replacement. The grain extract was obtained from 250 g of kefir grains grinded, boiled in distilled water during 1 h and precipitated twice with cold ethanol for 18 h. Antimicrobial activity was carried out against *Staphylococcus aureus* ATCC 6538 through the agar diffusion method using paper discs. Suspensions of 0.1 mL of *S. aureus* were inoculated into 25 mL BHI medium and swabbed in Petri dishes. Paper discs containing 0.1 mL of 5, 20 and 50 mg of kefir extract, 0.1 mL of kefir suspension, 0.9% NaCl (negative control), and ampicillin (10 µg, positive control) were transferred to growth dishes following incubation at 35 °C for 24 h. The antimicrobial activity of kefir extract against *S. aureus* attained similar values for disc haloes with 50 mg/0.1 mL (20 ± 1 and 27 ± 3 mm), and closer to the ampicillin halo (21 ± 0 mm). Although the polysaccharide extracted from kefir grains presented a lower inhibition area for *S. aureus* as compared to ampicillin, the latter drug is known to exhibit some adverse effects such as diarrhoea, sickness, vomit and kidney disorders.

2.5. On animals

Despite the known probiotic and prebiotic effects of kefir and AK, little is reported about their responses in healthy individuals, e.g. a physiological status of animals naturally receiving

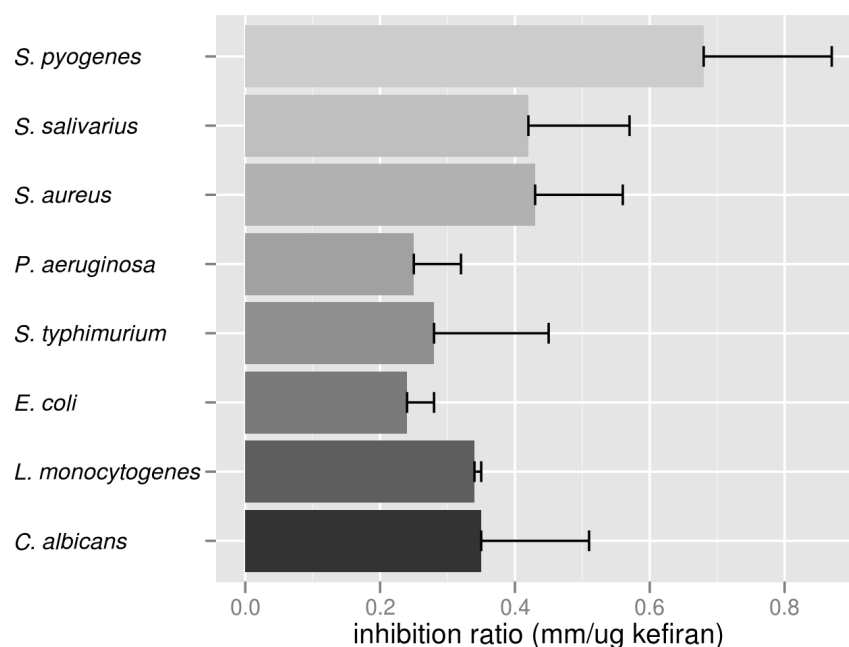


Figure 9. Zone diameters obtained by disc diffusion of haloes produced from the action of water kefiran against some pathogenic strains.

fermented kefir suspensions [41]. Targeting this, it was evaluated the consumption of kefir suspension by Wistar rats ($n=5/\text{group}$) kept in metabolic cages at room temperature, and with water and commercial diet *ad libitum* [42]. After 30 days no mean difference was observed between the animals receiving daily 1 mL of kefir suspension ($50 \text{ g}\cdot\text{L}^{-1}$ 24 h-fermented) by gavage, and the control group (1 mL NaCl 0.9 %). However, the kefir group of male rats excreted more urine ($29\pm14 \%$), consumed more ration ($22\pm6 \%$) and water (18 ± 7), and get more weight ($43\pm16 \%$) than the female group of kefir.

2.5.1. Anti-inflammatory and antimicrobial activity

2.5.1.1. Rodents

Anti-inflammatory responses of sugary kefir and its derivatives are poorly related in the literature. Notwithstanding, kefir may exert a beneficial effect on acute inflammatory responses, additionally improving the immune status of treated animals. In this sense an ED_{50} value of $12.5 \text{ mg}\cdot\text{kg}^{-1}$ was found by rat paw edema, together with inhibitions values about $30\pm4 \%$ and $54\pm8 \%$, for carrageenan (Figure 10) and dextran-induced inflammatory process, respectively ($n=8/\text{group}$). However, no changes in vascular permeability was evidenced in that experiments [29]. When comparing with cyproheptadine, a H_1 -receptor blocker, these results pointed to the antiinflammatory response probably derived from serotonin receptor and arachidonic acid pathways. In another assay, the anti-edematogenic activity of both kefir suspensions and grinded grains were also evaluated with a similar approach through carrageenan, dextran or histamine. Kefir suspensions orally administered 30 min before stimuli were found to be more effective (62 % inhibition) than kefir grains mechanically disintegrated (40 %). The overall data suggest a participation of prostaglandins mediators more than just histamine and serotonin in the anti-inflammatory response as a whole.

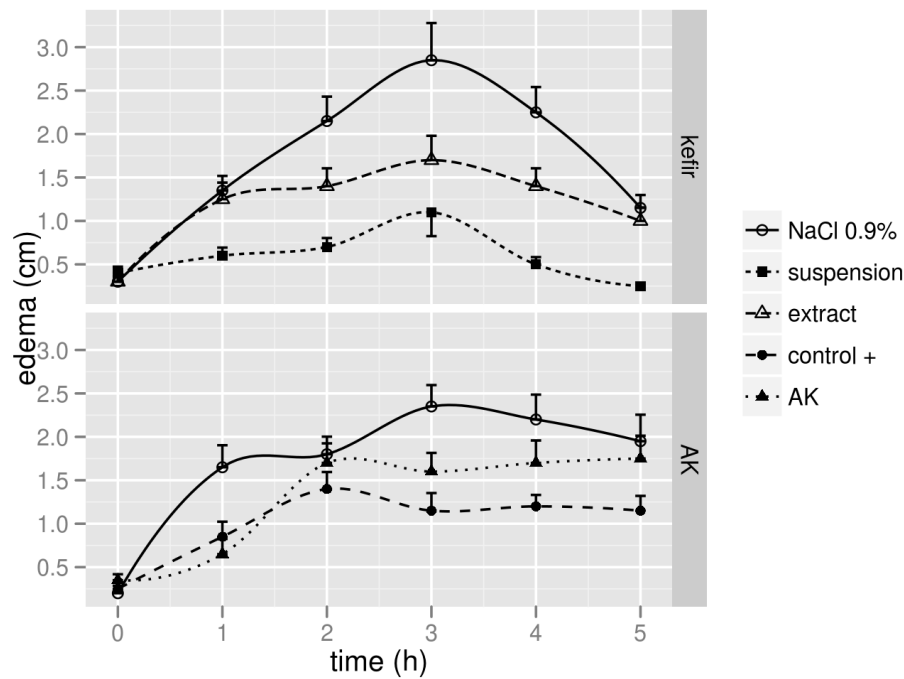


Figure 10. Anti-inflammatory activity of kefir (suspension and extract) and water kefir carbohydrate (AK) on the rat paw edema induced by intraplantar injection of carrageenan ($1 \mu\text{g}\cdot\text{mL}^{-1}$, 1 mL). Positive control - indomethacin, $10 \text{ mg}\cdot\text{kg}^{-1}$ [9, 29].

With the use of an analgesia model of acetic acid-induced writhing reflex in mice [43], both kefir grains and their soured suspensions also exhibited an anti-inflammatory response through abdominal contorsions (28 ± 2 % inhibition, $n=5/\text{group}$), whenever the animals were treated *i.p.* with 0.6 % acetic acid (Figure 11).

Following this findings, cicatrizing activities of both kefir and purified kefiran ($50 \text{ mg}\cdot\text{mL}^{-1}$) were also conducted with rats ($n=5/\text{group}$) [8]. For this test, a 6 mm-punched wound was made on a shaved dorsal area of the animals, following inoculation of *Staphylococcus aureus* at 3×10^8 cels/mL, and treatment of the animals topically with a 70 % kefir gel made with kefiran up to 7 days. The treatment resulted in a faster reduction of the infected-induced wound diameter, as compared with the control group (Figure 12), and even greater than the group treated with a neomycin-clotestebol association at day 7.

The skin samples excised from the animals treated with kefir gel also presented a well developed granulation of the epithelium together with neovascularization areas, suggesting a partial healing in the treated group (Figure 13) [8].

A kefir gel prepared as above was also tested with a prior heat treatment of kefir, aiming do distinguish between probiotic and prebiotic effects of the consortium. In that job, an oitment developed from grinded grains at 70 % was topically used in cicatrizing assays, for testing their microbial resistance against different heat treatments [24]. Cream samples were elaborated with prior treatment of kefir grains by autoclaving (15, 30, and 45 min), or by heating in a water bath at 55°C , for 15 h. The kefir creams were then applied topically to a 8-mm wounded-induced dorsal area of rats ($n=25/\text{group}$), previously inoculated with *P. mirabilis*, following cicatrizing measurements up to 7 days. The positive control group was

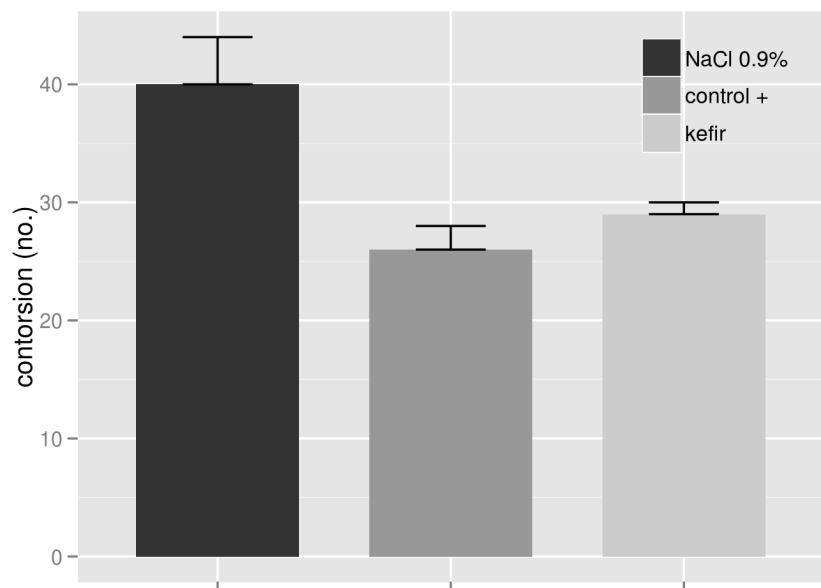


Figure 11. Oral administration of 24h-fermented kefir suspension (1 mL) and indomethacin (10 mg ·kg⁻¹) on the acetic acid-induced writhing reflex in mice, as induced by 0.6% acetic acid [43].

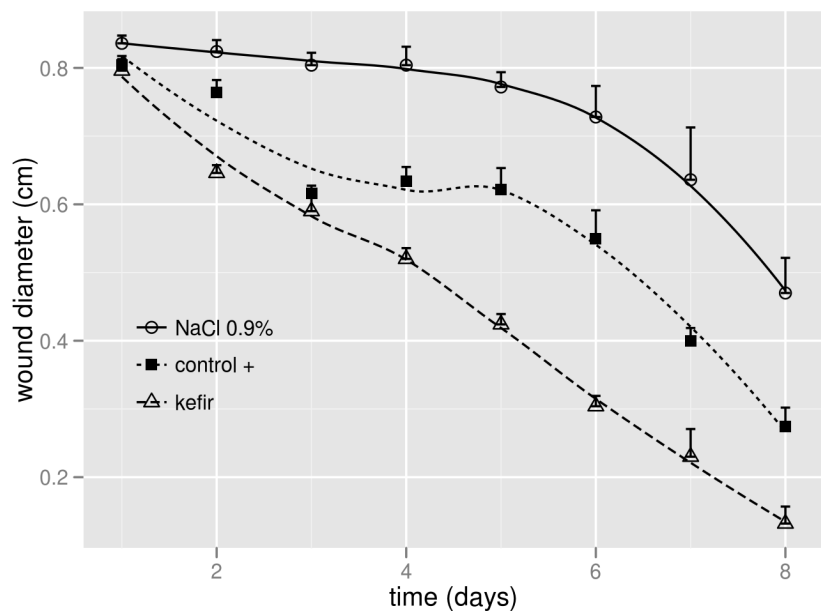


Figure 12. Cicatrizing activity in skin lesions of animals inoculated with 3x10⁸ CFU/mL of *S. aureus*. Data represent untreated animals, animals treated with 5 mg ·kg⁻¹ of neomycin–clostebol association (positive control), and animals treated with 70% kefir gel [8].

treated with a cream made from a chloramphenicol-collagenase association. IL-1 β , TNF- α , and cell blood countings were also determined at the end of the treatments. The main results can be shown at Figure 14. The kefir cream previously treated at 55 °C for 18 h exhibited a similar decrease in dorsal lesion areas as the positive group (chloramphenicol-collagenase association), and even that observed with the untreated kefir group at the 5th and 7th days.

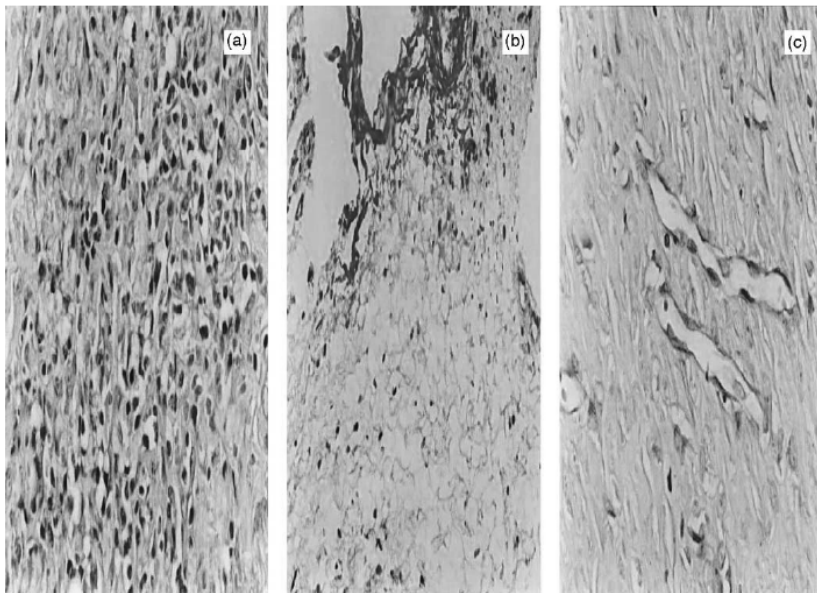


Figure 13. Morphological changes of the skin lesions induced in rats treated with kefir gel 7 days after the abrasions. Haematoxylineosin, 200X. (a) Control rats untreated; (b) rats treated with 5 mg/kg of neomycinclotestebol emulsion; (c) rats treated with 70% kefir gel [8].

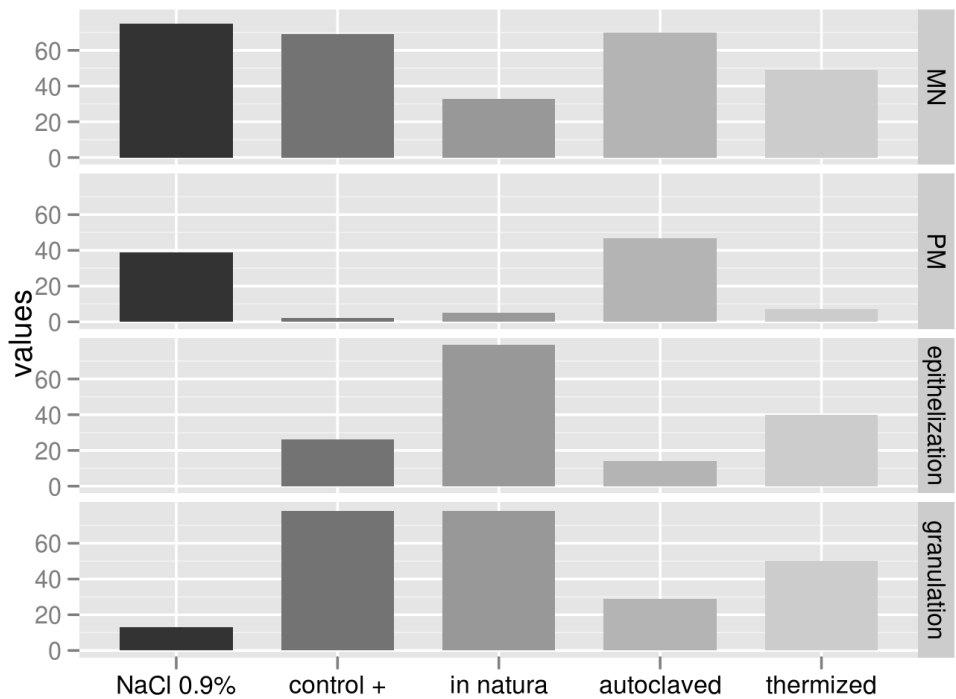


Figure 14. Relative histological findings (MN, PM, epithelization and granulation tissue) from rats infected with *P. mirabilis*, and treated with different preparations of kefir ointments. MN and PM are, respectively, a relative counting for mononuclear and polymorphonuclear cell. The ointments were prepared with native kefir grains, as well with thermized (60 °C, 15 h) and autoclaved grains. Positive control - collagenase-chloramphenicol association [24].

Intriguing, the group treated with autoclaved kefir grains also revealed a meaning decrease of lesion areas, greater than that presented for the negative control group (NaCl 0.9 %).

These findings happened to be so due to a nonproteic molecule taking part in the healing action to the animals, in agreement with the activities of the isolated AK molecule. Furthermore, all tested groups were able to enhance the epithelial tissue proliferation, as compared with the negative control group. In another inflammation model, anti-granuloma assays were also conducted with sugary and milk kefir, together with grinded grains (kefiran extract) and isolated AK. To do this, rats (n=5/group) were challenged with induction of granulomatous tissue by subcutaneously introduction of cotton pellets through abdominal skin incisions, following oral treatment with the agents after 2 h during 7 days [7] (Figure 15).

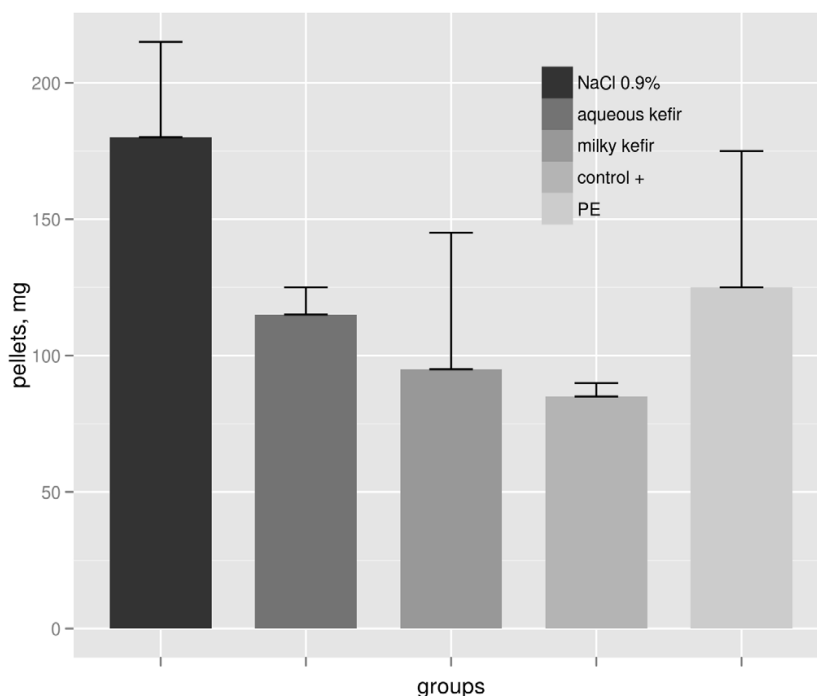


Figure 15. Effect of administration of kefir suspensions in soured milk and molasses ($50 \text{ g} \cdot \text{L}^{-1}$), or aqueous polysaccharide extract (PE, 0.1 %, 1 mL), during 6 days, on the formation of granulomatous tissue in rats. Positive control - dexamethasone ($0.2 \text{ mg} \cdot \text{kg}^{-1}$) [7].

Both aqueous and milky kefir suspensions ($50 \text{ g} \cdot \text{L}^{-1}$) showed similar inhibition values (41 ± 3 and 44 ± 6 %, respectively), whereas the isolated kefiran from molasses suspension lead to a smaller inhibition (34 ± 2 %). As kefir grains is known to stimulate innate immune responses against pathogens [8], we had evaluated the immune activity of neutrophils from rats treated with water kefir suspension [44]. Then cytokine $\text{TNF-}\alpha$ levels, cell recruiting, cellular metabolism, neutrophils oxygen uptake, H_2O_2 production, and myeloperoxidase screening, were tested in animals treated with kefir by gavage. (Figure 16). Wistar rats receiving kefir suspension *p.o.* during 7 days revealed meaning differences as compared as those receiving NaCl 0.9 %. In that animals there were a decrease of 30 ± 3 % in neutrophil recruiting from collected peritoneal cells, 32 ± 3 % in peroxyde production stimulated by forbol ester, and 26 ± 1 % in the myeloperoxidase activity. Then, the orally administered suspensions of water kefir was able to decrease general neutrophil activity in treated animals, probably following antioxidative pathways of the metabolism (Figure 17).

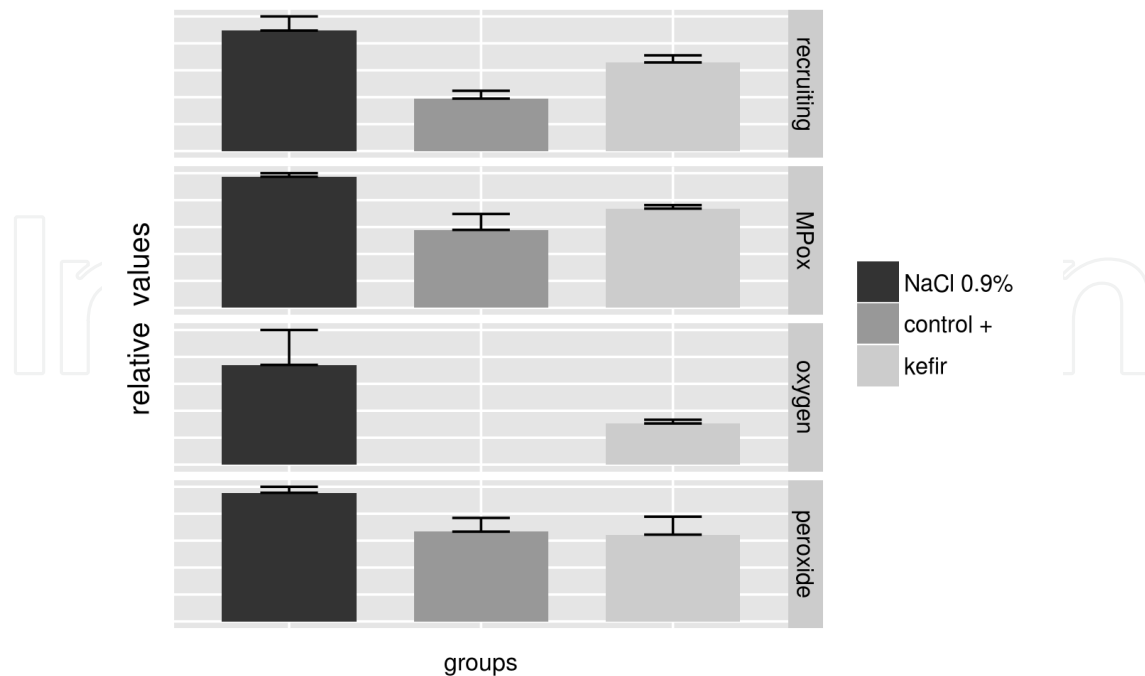


Figure 16. Relative values for neutrophil recruiting, myeloperoxidase index (MPox), oxygen consumption, and H_2O_2 production from peritoneal cells isolated from rats treated *p.o* with water kefir suspensions, and during 7 days after stimuli. H_2O_2 release was stimulated by phorbol 12-myristate 13-acetate (PMA). Positive controls - α -tocopherol (H_2O_2 and MPox assays), dexamethasone (cell recruiting) [44].

2.5.1.2. Intestinal motility

Animal digestibility in rats has been also attempted with kefir samples [45]. In that work it was evaluated changes in intestinal motility induced by a sugary kefir suspension daily administered ($n=6/\text{group}$, Wistar rats) during 15 days. After this period, the animals were kept without food during 24 h and treated with water kefir suspension, water, atropine (negative group), or acetylcholine (positive group). Following, the animals received orally 10 % active charcoal after 30 min. The animals were then submitted to euthanasia after 45 min and the intestinal tracts were exposed from the pylorus to cecum. As a result, kefir suspension was able to enhance intestinal transit up to 65 ± 2 % (Figure 18), closer to the acetylcholine group, and greater than the negative groups. These results indicated an improvement of the peristaltic activity of the intestinal tract of the rats treated with kefir, and evoke its plausible use on treating bowel diseases and gut problems.

2.5.1.3. Dogs

Based on the promising findings obtained with rodents, we had inspect some *in vivo* responses of clinically healthy dogs and rabbits treated orally with kefir suspensions. Dogs presenting balanoposthitis ($n=5/\text{group}$), a common inflammation of the foreskin surfaces of the genital tract of domestic animals, were treated with a 70 % kefir lanette-based ointment, applied daily during 3 days, whereas the positive group was treated with a 0.2 % nitrofurazone solution [46]. After the 25th day, there were more remitted symptoms in the animals treated with kefir cream (62.5 %), as compared as those treated with nitrofurazone solution (37.5 %), a largely compound used in gynaecological infections (Figure 19). Furthermore, the action of

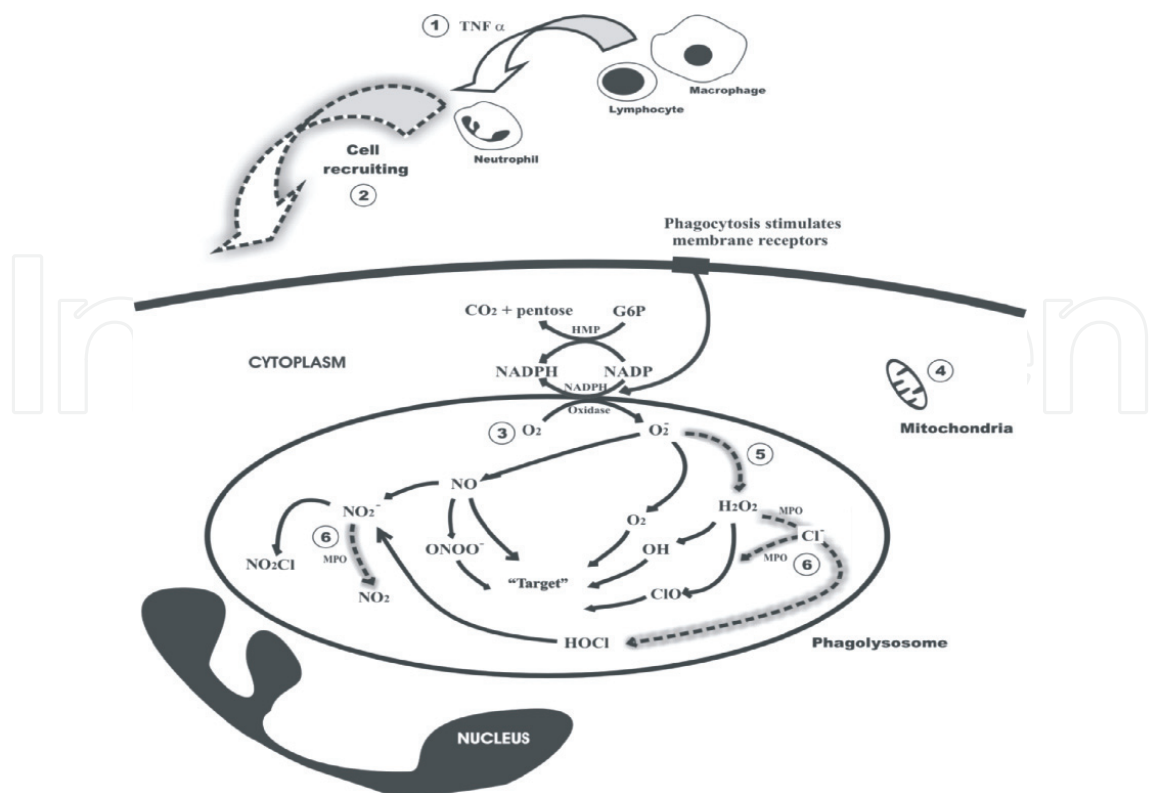


Figure 17. Mapping of cellular and biochemical events evaluated from rat neutrophils treated with water kefir. (Dotted arrows indicates reasonable mechanisms for kefir action). (1) Cellular recruiting; (2) Cellular respirometry; (3) Cellular metabolism; (4) Production of H₂O₂; (5) Identification of the MPO. Hexose monophosphate (HMP); Myeloperoxidase (MPO) [44].

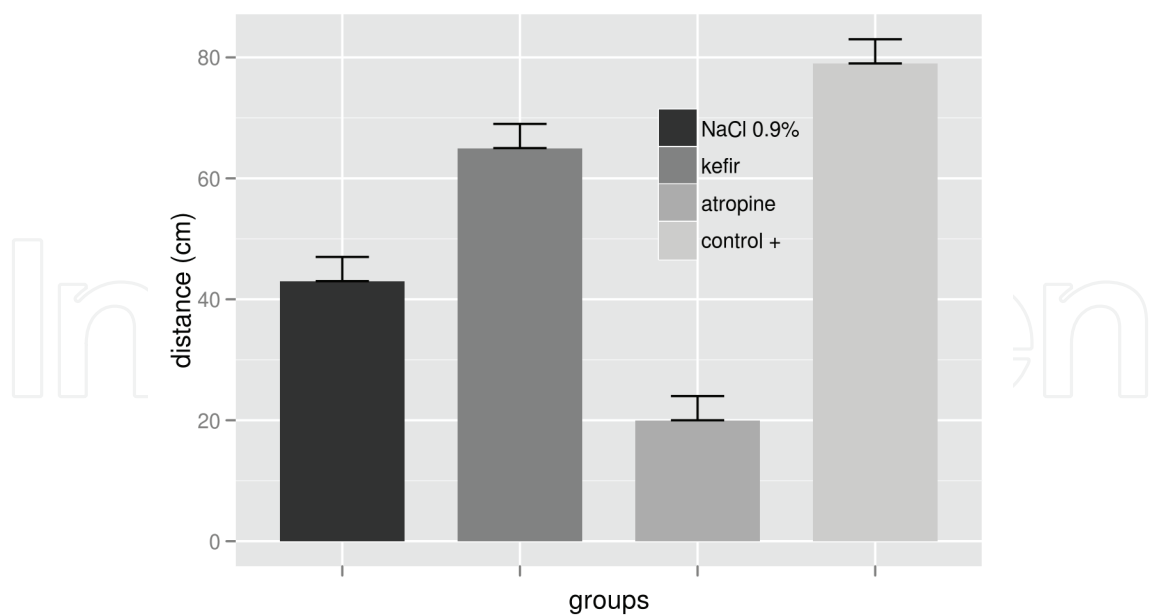


Figure 18. Action of kefir suspension (8.6 g·kg⁻¹), atropine (1 mg·kg⁻¹), acetylcholine (1 mg·kg⁻¹, positive control), and NaCl (0.9 %), orally administered, on the intestinal motility of Wistar rats, as determined by charcoal administration.

the kefir ointment showed more selective for *Staphylococcus* than nitrofurazone, as it was able to decrease 57 % in the frequency of that strains, albeit preserving the naturally-occurring microorganisms of that animals.

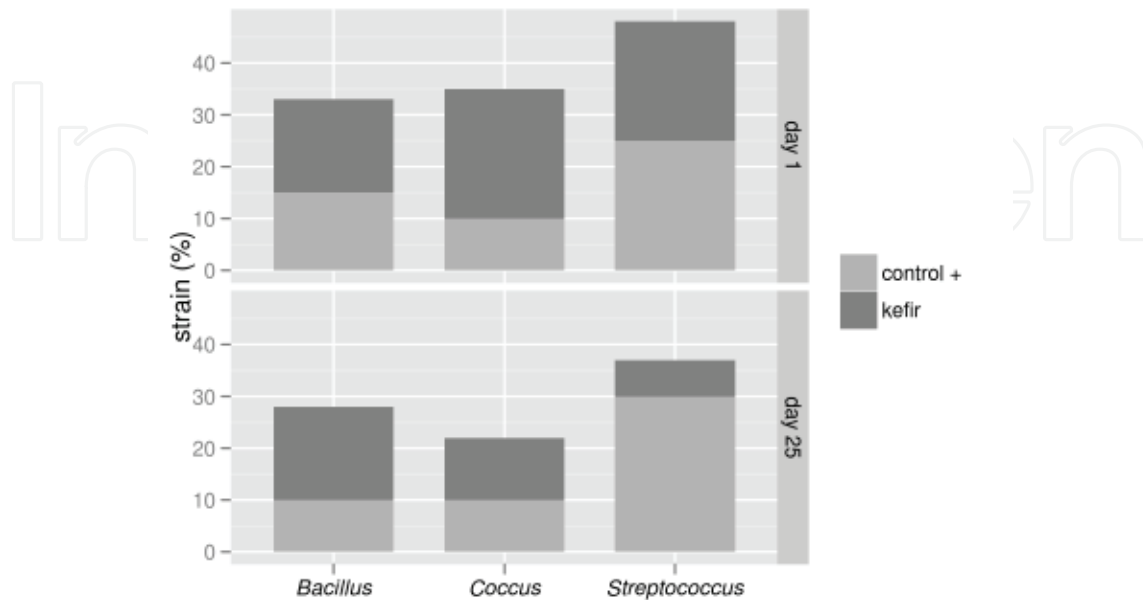


Figure 19. Bacterial counting before and after the treatment of balanoposthitis in dogs with nitrofurazone or kefir gel. Positive control - 0.2% nitrofurazone [46].

2.5.1.4. Lipidemic activity

The intake of soured kefir was tested in the healthy rabbits to identify its plausible effects in serum cholesterol levels. Rabbits (n=10/group) were fed with kefir grains in natura mixed with reconstituted pelletized industrial rations during 30 days, following their growth and serum lipid assessments (total cholesterol, triglycerides, HDL, LDL, and VLDL) [47]. The rabbits who received kefir grains in natura had significantly lesser growth than the control group. Besides, the fraction of total cholesterol and HDL had significant increases, with a mean reduction of the Castelli II index (LDL/HDL ratio) for the kefir group. This datum suggest the increase of total cholesterol as due to the increase of serum HDL, as measured from the rabbit auricular veins. As reported before [27] the total cholesterol levels has been reduced in broiler chicks fed with milk-fermented kefir, in agreement with above findings. In conclusion, these results would suggest that the probiotic can be thought for weight control therapies and prophylactic actions against dyslipidemies.

2.6. On plant

The addition of diverse compounds to plant culture medium has been successfully used for different species in tissue culture techniques. Banana and malt extract, as well as coconut water, e.g., is related to promote the growth of different species of orchids in micropropagation studies [48]. Although the action of kefir in plant physiology is unknown, recent studies demonstrated that kefir was able to induce the synthesis of phytoalexins in soy cotyledons, and also inhibits germination in urediniospores of *Phakopsora pachyrhizi*, a fungus which

cause Asian rust [49]. In this goal, the *in vitro* growth and foliar anatomy of orchids kept in a culture medium with different concentrations of Knudson medium, kefir and sucrose have been evaluated [50]. Biochemical analysis (carotenoids, soluble sugars, chlorophyll, phenolic compounds, and key enzymes of secondary metabolism), foliar anatomy and *in vitro* growth of orchids (*Cattleya walkeriana*) cultivated at different concentrations of Knudson medium, kefir and sucrose, were valued through micropropagation studies. [51].

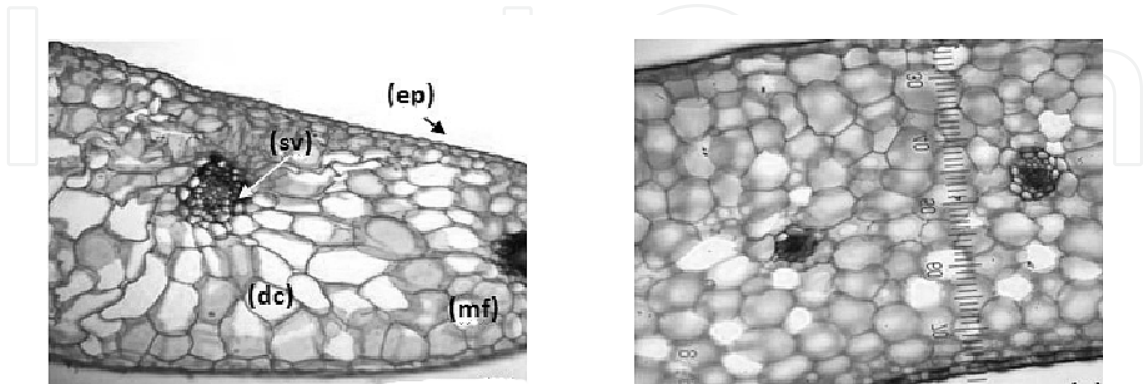


Figure 20. Foliar anatomy of micropropagated orchids (*Cattleya walkeriana*) cultivated *in vitro* with Knudson medium (A), and 25 % Knudson medium and 75 % kefir grains (B). Vascular system (sv), foliar mesophile (mf), epidermis (ep) and cell disorders (dc) [50].

Furthermore, the biochemical data assessed from the micropropagated orchids (Figure 21) evidenced a meaningful increase of the carotene level (up to 24 times greater than control), total phenolic (33 %) and polyphenol oxidase activity (about 3 times greater than control). In this sense, the use of kefir in *in vitro* orchid micropropagation have been promoted more growth, organization and thickness of foliar tissues.

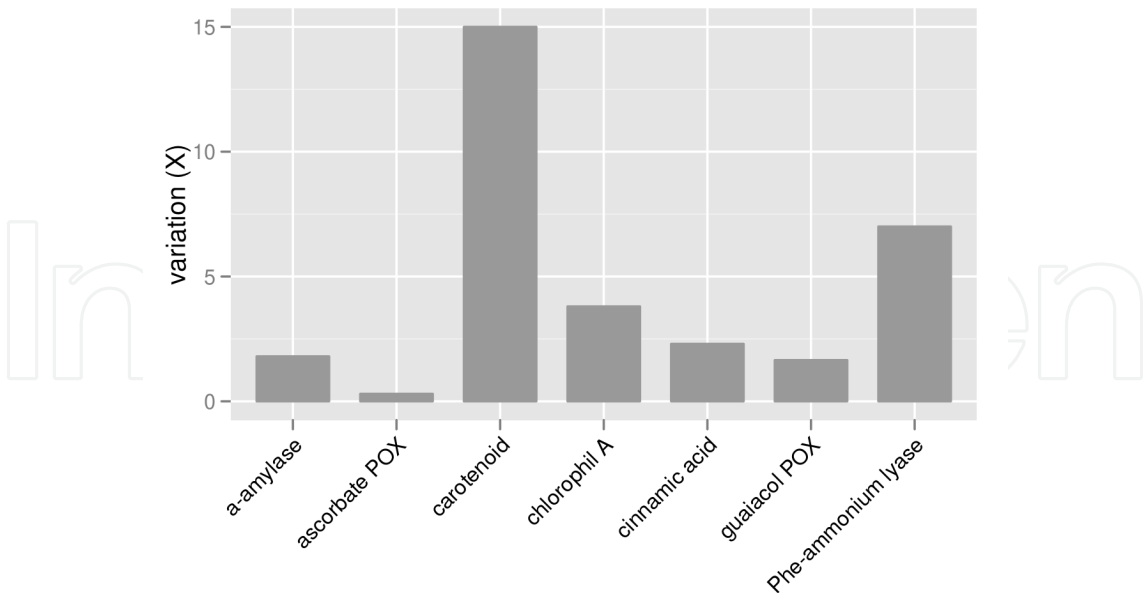


Figure 21. Changes in some compounds and secondary metabolism-key enzymes of micropropagated orchids cultivated with 75 % grinded kefir grains in Knudson medium [51].

The resulted treatment of micropropagated orchids (Figure 20) has been displayed a better organization and larger thickness of the mesophile as observed in culture media at 75 % kefir, when compared with the anatomical development of plants cultivated exclusively in Knudson medium [50].

3. Conclusion

Kefir can be considered an amazing example of coevolution of a microbial consortium. Their grains seems to simulate a multicellular living organism, as they are able to growth, divide, and age. From a survival point of view, kefir is very well adapted to resist to different and even extreme environments, also competing to a large spectrum of microbial strains. As kefir have acquired a strong resistance against several microorganisms, as well to improve the natural immunity of mammals since ancient ages, it is reasonable to think the consortium as a potential naturally-occurring drug able to decrease a large sort of illness afflictions.

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