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Antimicrobial and healing activity of kefir and kefiran extract

Kamila Leite Rodrigues [a], Lucélia Rita Gaudino Caputo [b], Jose Carlos Tavares Carvalho [c], João Evangelista [a], Jose Maurício Schneedorf [c,*]

[a] *Lab. Biologia e Fisiologia de Microrganismos, Unifenas, Alfenas, MG, Brazil*
[b] *Lab. Patologia Experimental, Unifenas*
[c] *Lab. Fitofármacos, Unifenas, Alfenas, MG, Brazil*

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Abstract

Kefir and its insoluble polysaccharide, kefiran, were both tested for antimicrobial and cicatrizing activities against several bacterial species and *Candida albicans* using an agar diffusion method. Comparator antimicrobials were also tested. Cicatrizing experiments were carried out on Wistar rats with induced skin lesions and *Staphylococcus aureus* inoculation, using a topical application of a 70% kefir gel. Both kefir and kefiran showed some activity against all organisms tested; the highest activity was against *Streptococcus pyogenes*. Cicatrizing experiments using 70% kefir gel had a protective effect on skin connective tissue and 7 days treatment enhanced wound healing compared with 5 mg/kg of neomycin–clostebol emulsion.

Keywords: Kefir; Kefiran; Cicatrizing; Antimicrobial

1. Introduction

Nosocomial bacteraemia associated with resistant organisms and postoperative surgical infections is a serious problem [1]. Since antibiotic use became widespread 50 years ago, bacteria have relentlessly developed resistance [2]. Because of this, efforts have been made to develop and study new compounds outside conventional antibiotic therapy [2]. These include new organic compounds [3], peptides isolated from vertebrates [4], honey preparations [5], ozonized oils [6] and probiotic strains [7]. A probiotic may be a single strain or a mixture of different organisms and are claimed to enhance wellbeing through immunomodulatory, metabolic and barrier activities against pathological processes. Kefir is an example of a probiotic mixture of bacteria and yeasts [8].

Kefir is a microbial symbiont mixture that produces jelly-like grains as it grows, that contain both lactic acid bacteria (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Acetobacter* and *Streptococcus* spp.) and yeasts (*Kluyveromyces*, *Torula*, *Candida* and *Saccharomyces* spp.). Both bacteria and yeasts are surrounded by a polysaccharide matrix, named kefiran, a water-soluble branched glucogalactan, which has been reported to have antibacterial, antimycotic and antitumour activity [9]. Kefir is claimed to act against the pathogenic genera *Salmonella*, *Helicobacter*, *Shigella* and *Staphylococcus*, and *Escherichia coli* and to have some anti-inflammatory activities [10]. These two properties of kefir might be of use as an alternative treatment for patients infected with a single or multi-resistant strains of organisms.

This study, therefore, looked at the antimicrobial and cicatrizing activities of kefir and kefiran using agar diffusion experiments and cicatrizing tests on rats.

2. Methods

2.1. Microorganisms used

The microorganisms used were *Staphylococcus aureus* ATCC 6538, *Streptococcus salivarius* ATCC 39562, *Streptococcus pyogenes* ATCC 17568, *Pseudomonas aeruginosa*

* Corresponding author.
E-mail address: fitofarmacos@unifenas.br (J.M. Schneedorf).

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ATCC 27853, *Candida albicans* ATCC 10232, *Salmonella typhimurium* ATCC 14028, *Listeria monocytogenes* ATCC 4957 and *E. coli* ATCC 8739. All strains were kept frozen in liquid nitrogen and cultured on appropriate media following the standard guidelines of NCCLS [11].

2.2. Kefir

Starter grains (5 g) kept at Lab. Fitofarmacos (Unifenas, MG, Brazil) were continuously cultured in 100 g/L of molasses for 15 days prior to experiments. The medium was changed at 24 h intervals and the grains washed with sterile water. Preliminary taxonomic classification of the bacterial isolates was performed on individual colonies by Gram staining, API 20 S system for streptococci, API 20 NE for bacteria and API 20 AUX for yeasts (API Biomerieux, SA, France). Both suspensions and kefir grains contained significant number of *Leuconostoc* spp., *Lactobacillus lactis*, *Acetobacter* spp., *Saccharomyces cerevisae*, *Kluyveromyces marxianus* and *K. lactis* [8,10].

After day 15 of fermentation, kefir was extracted for kefiran production for use in the antimicrobial and cicatrizing experiments. A kefir gel containing 70% grains freshly dried at 60 °C and homogenised with a lanette-based commercially available cream, was used in the rat experiments. The polysaccharide matrix (kefiran) also used was isolated from kefir grains using the method described by Micheli et al. [9]. Briefly, the stirred grains were washed with boiling distilled water for 1 h (one part grains to 100 parts water). The mixture was then cooled and centrifuged at 16000 g for 15 min. The procedure was repeated with the sediment. The polysaccharide dissolved in the combined supernatants was precipitated by the addition of an equal volume of cold ethanol at 4 °C overnight. The precipitate was redissolved in hot water (1:100) for 1 h at 70 °C with stirring and the precipitation procedure was repeated twice. The precipitate was finally dissolved in 100 mL distilled water, dialysed against distilled water until the conductivity reached 1.5 μS/cm and lyophilised (Datamed TS-600 conductivity meter, Brazil).

2.3. Antimicrobial activity

Antibiotic activity of kefir and kefiran extract were evaluated using the disk diffusion method as described by the National Committee for Clinical Laboratory Standards [11]. Cell suspensions of 3×10^8 CFU/mL (using McFarland tubidity standard solutions) were prepared from organisms grown in BHI medium (Difco Lab, Detroit, MI, USA) at 35.5 °C for 24 h. Disks were applied to the agar surface previously inoculated with 0.1 mL organism suspension.

Antibiotics, kefir and purified kefiran extracts were pipetted onto 5 mm diameter paper disks. Amounts used were 0.1 mL of 24 h-fermented kefir (1.2 mg/mL), 5, 20 and 50 μg from a stock solution of 0.5 mg/mL purified kefiran; ceftriaxone (BioChimico Ltda, Rio de Janeiro, Brazil), 100 μg/Ml; ampicillin (Ariston Ind. Quim., São Paulo, Brazil), 10 μg/mL; azithromycin (Prodotti Labs, São Paulo, Brazil), 10 μg/mL; oxacillin (Royton Quim. Farm. Ltda, São Paulo, Brazil), 10 μg/mL and ketoconazole (Hipolabor, São Paulo, Brazil), 10 μg/mL. Inoculated plates were incubated at 35.5 °C for 24 h and the inhibition zones were measured using an analytical pakimeter (Vernier, Beaverton, OR). Experiments were done in triplicates.

2.4. Susceptibility studies

Antimicrobial susceptibility was tested and interpreted using the guidelines for reference broth microdilution method as described by the NCCLS [11]. The MIC was defined as the lowest antimicrobial concentration able to completely inhibit bacterial growth up to 24 h. MIC parameters were determined in triplicates using 0.1 mL of bacterial suspensions (3×10^8 CFU/mL) in tubes containing 10 mL of BHI solution and the same amounts of kefir and kefiran as described above. Tubes were mixed using a Vortex for 60 s and incubated at 35.5 °C for 24 h. MBC values were obtained based on the results for MIC values. Plates containing 25 mL of BHI agar medium were inoculated with 0.1 mL of the tubes showing no growth and incubated for 24 and 48 h at 35.5 °C. Controls were performed using the antimicrobial agents listed above.

2.5. Animals

Male Wistar rats weighting 150–200 g were housed under controlled conditions of light, room temperature and humidity. Procedures and animal health were as defined by the Ethical Commission for Animal Procedures at the University of Alfenas, Brazil. The animals were separated into five groups ($n = 5$) and kept in polyethylene boxes. Food and water were supplied ad libitum.

2.6. Induction of wounds

Rats were anaesthetised using 45 mg/kg of sodium thiopental given by the intraperitoneal route. A 6 mm punched wound was made on a shaved dorsal area; this was inoculated with 0.1 mL of *S. aureus* (3×10^8 CFU/mL). After 24 h, the wounds were treated topically with either 0.9% NaCl (negative control group), 5 mg/kg of neomycin–clostebol emulsion (Shering Co., CA, USA, positive control group), or kefir gel (test group) for 7 days as described by Speroni et al. [12].

The decrease in wound diameters during the healing process was measured with an analytical pakimeter. After 7 days, the animals were sacrificed by spinal chord injury. Paraffin-embedded sections were prepared from tissue samples from the wound. The sections were 6 mm thick, cut perpendicular to the skin surface and included the whole thickness of the skin. Serial sections were stained with haematoxylin–eosin.

Any pus present at the site lesion was cultured on BHI agar. After isolation of organisms and coagulase and deoxyribonuclease activity tests (Merck, USA) on these, typical colonies

were transferred to mannitol salt agar (MSA) supplemented with phenol red (Difco Lab., Detroit, MI, USA).

2.7. Statistical analysis

All values were expressed as mean ± S.D. Antimicrobial activity data from diffusion experiments were evaluated using the least squares method adjusted to the data. Animal group comparisons used one-way ANOVA followed by Tukey–Kramer test.

3. Results

3.1. Susceptibility tests

Inhibition ratios of kefiran against the pathogenic strains were determined from minimum least squares applied to diameter values at 5, 20 and 50 μg. The results showed *S. pyogenes* to be the most sensitive microorganism to kefiran, followed by *S. aureus* and *S. salivarius*. *S. typhimurium*, *C. albicans* and *L. monocytogenes* were less sensitive to kefiran and *P. aeruginosa* and *E. coli* the least sensitive. Minimal MIC and MBC values for kefiran against all strains tested ranged from 462 (MIC) to 494 mg/L (MBC) showing only a small increase in concentration to achieve a killing effect. There was a rapid decrease in numbers of organisms surviving concentrations between 450 and 500 mg/L.

Table 1 shows inhibition zone diameters for kefir suspension (0.1 mL culture grown for 24 h), kefiran (50 μg/mL) and the antimicrobial agents used. Results showed activity of both natural compounds against all strains tested (Table 1). The 10 μg/mL oxacillin disks produced zone sizes generally similar to those of the kefir suspension but slightly smaller than those of kefiran at 50 μg/mL. The mean values for kefiran and kefir inhibition zones were 26.3 ± 2.1 and 28.0 ± 2.0 mm, respectively.

3.2. Cicatrizing activity

The antimicrobial and cicatrizing activity of 70% kefir gel in the rat model is presented in Fig. 1. Both the positive control (5 mg/kg neomycin–clostebol emulsion) and kefir gel resulted in a faster reduction of the wound diameter than the negative control (0.9% NaCl). At day 7 of the experiment, the kefir gel-treated wounds were smaller than the clostebol–neomycin emulsion-treated wounds ($P < 0.001$, Tukey–Kramer) (Fig. 1).

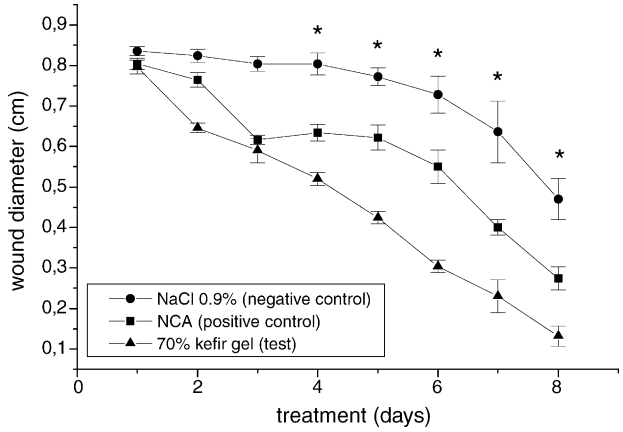

Fig. 1. Cicatrizing activity in skin lesions of animals inoculated with 3×10^8 CFU/mL of *S. aureus*. Data represent untreated animals (●), animals treated with 5 mg/kg of neomycin–clostebol association, NCA (■), and animals treated with 70% kefir gel (▲).

The pus samples all grew *S. aureus* as proven by the tests described previously.

3.3. Histological examination

Histopathology of the lesions is shown in Fig. 1. Specimens from the non-treated group (0.9% NaCl) showed a lack of neovascularization but intercellular infiltration was present (Fig. 2a). Skin from the positive control group, treated with 5 mg/kg neomycin–clostebol emulsion, showed small ulcerative areas and similar histopathology to the negative control (Fig. 2b). Skin from animals treated with 70% kefir gel showed a well developed granulation of the epithelium together with areas of neovascularization, suggesting partial healing (Fig. 2c).

Table 1
Antimicrobial activity of 0.1 mL of 24 h kefir suspension and purified kefiran at 50 μg/mL compared with some antibiotics

Strain	Ketoconazole	Ampicillin[a]	Azithromycin[a]	Ceftriaxone[a]	Oxacillin[a]	Kefiran[b]	Kefir
S. pyogenes	–	16.6	18.8	16.7	19.3	29.0	27.2
S. salivarius	–	17.5	18.8	17.7	19.2	27.1	24.9
S. aureus	–	25.5	23.9	29.8	28.5	28.3	30.0
P. aeruginosa	–	23.6	22.4	21.2	26.7	26.2	30.2
S. typhimurium	–	15.7	17.1	20.5	24.4	26.8	25.6
E. coli	–	19.2	18.3	21.1	23.5	26.0	28.4
L. monocytogenes	–	18.1	19.1	18.1	17.8	234	29.3
C. albicans	20.7	–	–	–	–	23.2	28.0

The results represent the mean zone diameters (in mm) using the agar diffusion method [12]. MIC/MBC values of kefir and kefiran feel within a narrow range of 450–500 mg/L. Means with different letters in the same column are considered statistically different ($P < 0.001$).

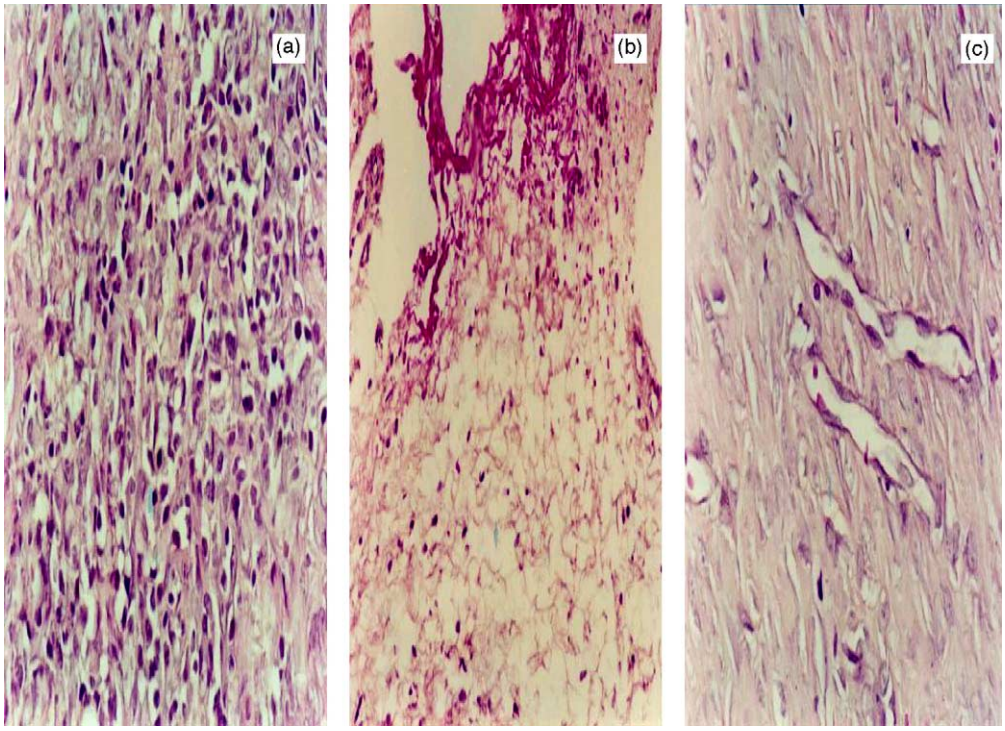

Fig. 2. Morphological changes of the skin lesions induced in animals 7 days after abrasion. Haematoxylin–eosin, 200×. (a) Control rats untreated; (b) rats treated with 5 mg/kg of neomycin–clostebol emulsion; (c) rats treated with 70% kefir gel ($n = 15$, five animals/group).

4. Discussion

The benefits of probiotic microorganisms have been tested in double-blind and placebo-controlled studies of cancer, carcinogen formation, reduction of serum cholesterol, stimulation of immune system and prevention or treatment of human infections [13]. Fermented suspensions of kefir grains are claimed to have clinical effects on diarrhoeal disease, urinary tract infection, salmonella, streptococcal and *Helicobacter pylori* infections [10,14]. Although antimicrobial activity by several isolated strains from kefir grains has been reported [10], antibiotic or cicatrizing properties of the whole kefir grains themselves and their derived products have not been previously described. This work has shown activity by kefir polysaccharide matrix, kefiran and a 70% kefir gel made from ground grains.

Kefiran was able to inhibit the growth of seven bacteria and a yeast. Matijasik and Rojelsj found the MIC of a supernatant from a culture of *Lactobacillus* K7 to be 1495 mg/L for *Clostridium tyrobutyricum* and 1280 mg/L for *C. difficile* and *C. perfringens* [15]. Kumthavee [16] isolated a bacteriocin from *Lactobacillus rhamnosus* that showed antibacterial activity, using an agar diffusion method, at 150 μg/mL. Recently, Padilla et al. have found an antimicrobial peptide isolated from a *Pseudomonas* spp. capable of inhibiting several Gram-positive and -negative bacterial strains [17] with a mean MIC against enterococci of 0.14 mg/L. When compared with the positive controls used, kefir and kefiran had higher MIC values than those previously reported in the literature. This difference could be due to the total yield and purity of the biocide substance produced by the microcosm. In this study kefir suspensions were used after 24-h growth and kefiran extract was obtained by hot water extraction following cold ethanol precipitation. Optimized approaches to kefiran isolation have yielded up to 2 mg/mL, regardless of the grain source [9] or the purified microorganism used [18].

Inhibition zones of kefir suspension (0.1 mL, 24-h growth) and kefiran extract (50 μg mL) were of similar size as those found using other probiotics. Matijasik and Rojelsj [15] showed *Lactobacillus* K7 strain produced inhibition zones of 19 and 22 mm against *C. tyrobutyricum* and *C. perfringens*, respectively. When evaluating the antimicrobial activity of *Bacillus. subtilis* against 21 strains of *H. pylori*, Pinchuk et al. reported inhibition zones of 10–16 mm [19].

Kefir was tested for cicatrizing activity in rats with dorsal injuries infected with *S. aureus*. Cutaneous healing is an important area of dermatology as it is involved in a large number of common conditions; superficial wounds, minor surgery, leg ulcers, scabby lesions or burns. Animals treated with a simple kefir formulation made from dried grains showed better wound healing compared with those treated with the clostebol–neomycin emulsion. Anti-inflammatory and healing activities have been reported for other topical natural products, but only after longer periods of treatment or higher concentrations of the substance being applied. Medeiros et al. [20] treated burns in rats with hyaluronic acid and reduced the cicatrizing process from 38 to 29 days. Protective effects on skin connective tissues have been reported by Speroni et

al. [12] who reported 100 mg of *Echinacea pallida* root to aid healing over 72 h in rats with damaged skins.

There is an urgent need for the development of novel antimicrobial agents against highly resistant pathogenic strains. The data presented in this work suggest that kefir biofilms and their polysaccharide compounds may be good antimicrobial, antiinflammatory and cicatrizing agents for use in a variety of infections.

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