Kefir and ayran – traditional fermented products of Russia

M V Kaledina, V P Popenko, N P Shevchenko and S A Chuev
Belgorod State Agricultural University named after V. Gorin, 1, Vavilova st., Mayskiy, Belgorodsky district, Belgorod region, 308503, Russia
E-mail: Kaledina_MV@bsaa.edu.ru

Abstract. The objective of this study was to determine antibacterial activity two traditional fermented milk products from Russia: kefir and ayran. Freshly prepared kefir and ayran was diluted 1/10 with nutrient broth and inoculated with pathogens (log10 5.5-6.5 initial) Escherichia coli EPEC 8621, VTEC 12900VT, Staphylococcus aureus 8632, Clostridia difficile 13832 and Salmonella Typhimurium LT2 and incubated for 24 h at 37°C. Growth of all pathogens was suppressed by kefir and ayran with decrease in viable count below the initial value being seen in the cases of kefir and ayran vs Staphylococcus aureus and kefir vs Salmonella typhimurium. In addition, the influence of novel, potential prebiotics, lactitol and chitozan succinate, added to fermented products on antibacterial effect was studied. We concluded that both additives had no additional effect on the antibacterial activity of the fermented products.

1. Introduction
Traditional fermented milk products, which often involve yeast-lactic fermentation, have been consumed in Russia for centuries. Two widely consumed milk products derived from mixed microbial fermentation in Russia are kefir and ayran. Kefir is a cultured-milk beverage, which many centuries ago originated in the Caucasus Mountains. It is slightly carbonated and contains small amounts of alcohol [1]. Kefir differs from other traditional fermented milks in that it is made only from kefir grains. A variety of different species of organisms have been isolated and identified in kefir grains. The basic microflora contains lactococci, homofermentative and heterofermentative lactobacilli, yeasts and acetic acid bacteria. Among the yeasts isolated from Kefir grains are Candida kefir, Saccharomyces cerevisiae, Saccharomyces delbrueckii, Candida holmii, Saccharomyces unisporus and Saccharomyces lipolytica. The Lactobacillus species present include Lact. caucasicus, Lact. brevis, Lact. kefir, Lact. casei, Lact. plantarum, Lact. acidophilus, Lact. kefiranofaciens, Lact. cellobiosus, Lact. bulgaricus, Lact. helveticus ssp. jugurti, and Lact. lactis ssp. lactis. Lactococci identified include Lactococcus lactis ssp. lactis, Lactococcus lactis ssp. biovar diacetylactis, Lactococcus lactis ssp. cremoris, Lactococcus filant, Streptococcus thermophilus, and Streptococcus durans. Leuconostoc spp identified include Leuconostoc dextranicum, Leuconostoc mesenteroides, and Leuconostoc kefir [2, 3]. The grain is formed via a symbiotic relationship among the complex microflora and held together by a matrix of exopolysaccharides.

Many health benefits have been attributed to kefir, including the enhancement of the immune system and improved digestive health, particularly with regard to lactose digestion. Kefir is claimed to have antitumour, antibacterial and antifungal properties [4].

Ayar is a fermented milk product produced from whole or skim milk (cow, sheep, goat) by the co-culturing of certain lactic acid bacteria and yeasts and has a sour milk taste and aroma. Usually, the
ayran microflora consists of mesophilic and thermophilic lactic acid bacteria and yeast. It is traditionally produced using inoculation from previous ayran culture. This means that over many centuries the ayran microflora has developed stability to environmental factors, including to bacteriophage. The majority of ethnographic researchers consider ayran a dairy product from the Caucasus region. Although, ayran has traditional therapeutic uses there are no studies of health benefits of the fermented product. By comparison with conventional European commercial yoghurt products, which usually contain two microbial starter cultures Lactobacillus delbrueckii var bulgaricus and Streptococcus thermophilus, the more complex microflora of kefir and ayran may yield more bioactive moieties and exert a wide range of health-promoting properties [6].

In the present study we have investigated the antimicrobial activities of kefir and ayran towards a range of Gram-positive and Gram-negative pathogens to define their spectrum of activity [5]. In addition, we have explored the possibility of augmenting bioactivity of the traditional fermented milks by addition of two ingredients that would be expected to influence the microflora of kefir and ayran, namely lactitol and chitosan oligosaccharides.

Lactitol is a disaccharide sugar alcohol (polyol) derived from natural milk sugar lactose [7]. As lactitol is fermented in the large intestine it has beneficial effects on the colonic microflora [8, 9]. Lactitol functions as a prebiotic bifidobacteria along with decreasing in pH of the colon resulting in significant reduction of potential pathogens in the colon [10]. As lactitol contributes almost half the calories of sucrose, has a low glycemic index and does not induce an increase in blood sugar or insulin levels it is considered safe for consumption for people with diabetes. Due to its stability, solubility, and similar taste to sucrose it is used in variety of low fat and low energy foods such as chocolates, sugar free candies preparation, chewing gums, baked goods and sugar substitutes.

Chitosan succinate (CS) is a product of enzymatic hydrolysis of chitosan in the presence of succinic acid; it contains ion bound succinic acid (30-50 %). The product is reported to have fungicidal, wound healing and anti-inflammatory properties [11, 12].

2. Material and methods
Materials: Lactitol was obtained from Danisco (UK) and chitosan succinate was obtained from Oligopharm, (Moscow, Russia). All microbiological media were purchased from Oxoid Ltd. (Basingstoke, UK), and antibiotics from Sigma Chemical Company Ltd (Poole, UK).
Kefir used in our study was made from kefir grains (Belgorod Milk Company, Belgorod, Russia) using a protocol defined in a Russian State specification (GOST R 31454-2012). Briefly, kefir starter was prepared by inoculating kefir grains into pasteurized skimmed milk in the proportion of 5% (w/v). Then milk was fermented for 18-20 h at 20-21°C and ripened for 12 h at 4-6°C. The kefir grains were removed by filtration after the fermentation process. The resulting fermented milk was used as starter for making kefir. Skimmed milk was pasteurized at 90-92°C for 2-3 min and cooled down to 22-25°C before the addition of the starter culture. After incubation for 8-12 h, the milk was cooled to 8-12°C for ripening for 8-10 h.

The ayran samples were made using traditional methods for domestic production in Russia. The starter of ayran was obtained from Microbiology Laboratory, Belgorod State Agrarian University. Commercial skimmed milk was pasteurized at 90-92°C for 2-3 min. When the milk had cooled to 40-45°C the starter cultures of ayran from prepared stored stock sample was added. The milk became sour in 4-5 h due to the growth of thermophilic bacteria, after which the temperature was lowered to 20-22°C for 3-4 h to allow growth of mesophiles. The product was subsequently cooled to 6-8°C for 10-12 h to permit growth of yeast.

Additionally kefir and ayran samples were prepared with either lactitol or chitosan succinate. They were added before pasteurization by using the following concentrations: 70 g/liter of lactitol and 0.25g/liter of CS. After cooling in the samples were inoculated with starter cultures 3 % v/v. The choice of concentration of the media supplements was based on preliminary studies that showed that they had minimal influence on the fermentation process and on sensory attributes of the fermented products.
The microorganisms used in this study comprised *Escherichia coli* EPEC 8621, VTEC 12900VT, *Staphylococcus aureus* 8632, *Clostridia difficile* 13832 and *Salmonella Typhimurium* LT2, which were maintained as stock cultures in Microbiological Laboratory, Belgorod State Agrarian University. *Salmonella Typhimurium* and *Clostridia difficile* were grown in Wilkins-Chalgren broth (WC), *Staphylococcus aureus* and *Escherichia coli* strains were grown in Tryptone Soya broth (TS) at 37 °C for 18 hours.

The inhibitory activity of fermentation products against the above Gram-positive and Gram-negative bacteria was tested by co-culture of the pathogenic microorganisms with fermentation product and spread-plating of the samples to enumerate the pathogenic microorganisms. 1 ml fermentation product and 0.1 ml of the overnight pathogen cultures to yield initial pathogen concentrations of log10 5.5-6.0 were added to 9 ml of the appropriate media (WC or TS broth as above). Inoculated tubes were prepared in triplicate. The control included 1 ml sterile water instead ferment product. The samples were then incubated at 37 °C for 24 hours. The samples were serially diluted with peptone water and the viable populations were determined by plating of the diluted samples on appropriate agar in 0, 5, 10 and 24 hours. The plates were incubated at 37 °C for 24-48 hours. At the end of the incubation period, the colonies were counted and their numbers were determined.

The pH of the co-cultivated samples was measured at the beginning and at the end of experiment, using pH indicator paper. At the end of the fermentation period the pH was confirmed using a pH meter.

### 3. Results

At the end of fermentation period used in the preparation of kefir and ayran the pH of the milk fell to 4.60 and 4.46, respectively. Addition of lactitol had little or no effect on the final pH (kefir 4.74; ayran 4.40). Kefir and ayran fermented in the presence of CS had pH 4.87 and 4.60, respectively.

At the beginning of the incubations with the pathogens the experiment pH of the broth with test samples was approximately 6.50 and control sample – 6.8. In all cases, the variation of pH at the end of experiment was 4.50-5.00 for the test sample and 5.00-5.50 for the control sample.

In the absence of kefir or ayran, numbers of *E.coli* EPEC and VTEC increased by about 3 logs over the 24 hours incubation period, usually reaching a plateau by 5 hours (Fig.1A, 1B, 2A, 2B). The addition of kefir to the incubation medium not only reduced the rate of growth of the EPEC and VTEC *E. coli* strains, but also the final population: a plateau was reached at 5 hours, and was approximately 2 logs lower than the control (Fig. 1A and 1B). Supplementation of the kefir fermentation mixture with lactitol did not increase the inhibitory effect towards the *E. coli* strains and chitosan succinate-supplemented kefir was actually less inhibitory than kefir alone. The samples of *E.coli* EPEC and VTEC co-cultured with ayran also exhibited a slower rate of growth and a lower final population (by approximately 2 logs) than the control and over 18-24 hours the population decreased (Fig. 2A and 2B). Addition of lactitol or CS to ayran did not alter its inhibitory activity towards *E.coli*.

Kefir and particularly ayran showed potent inhibitory effects towards *Salmonella Typhimurium*, actually decreasing the bacterial numbers over 24 hours (Fig. 1C and 2C). In the case of ayran, the decrease in final population of salmonella was about 3 logs in comparison with the starting number. Addition of lactitol or CS to the kefir or ayran had little or no effect on the inhibition of the pathogen.

Similar results were obtained from the tests on kefir and ayran against *Staphylococcus aureus* – in both cases the pathogen population showed a marked decrease of over 2 logs during the 24 h incubation compared with a 3 log increase in the control over the same period (Fig. 1E and 2E). Supplementation with lactitol or CS had no further inhibitory effect and in the case of kefir + CS, the inhibition was reduced (Fig. 2 E).

Kefir and ayran both slowed the rate of growth of *Clostridia difficile* and over the period 18-24 hours clostridial numbers decreased in the presence of both fermented milks (Fig. 1D and 2D). Addition of lactitol to the fermentations decreased the growth of *C. difficile* further, whereas addition of CS had less effect than lactitol in the case of ayran cultures (Fig. 2D) and when added to the kefir culture, virtually abolished the inhibition (Fig. 1D).
**Figure 1.** Growth of *E.coli* EPEC (A), *E.coli* VTEC (B), *Salmonella Typhimurium* (C), *Clostridia difficile* (D), *Staphylococcus aureus* (E) in nutrition broth in the presence of kefir (□), in the presence of kefir with lactitol (Δ) and with CS (○), in the presence of sterile water (◊)
Figure 2. Growth of *E. coli* EPEC (A), *E. coli* VTEC (B), *Salmonella Typhimurium* (C), *Clostridia difficile* (D), *Staphylococcus aureus* (E) in nutrition broth in the presence of ayran (□), in the presence of ayran with lactitol (Δ) and with CS (○), in the presence of sterile water (◊)

4. Conclusion

It has been reported by several investigators that lactobacilli exhibit antimicrobial activity. The antimicrobial effect of lactic acid bacteria could be explained producing wide range of antibacterial compounds including sugar catabolites such as organic acid (e.g. lactic acid and acetic acid), oxygen catabolites such as hydrogen peroxide, proteinaceous compounds such as bacteriocins. Antimicrobial
activity has been reported for several of the species isolated from Kefir and has been attributed to both organix acids and bacteriocins.

In the present investigation, the mechanisms of antimicrobial effects were not explored. Since kefir and ayran has a pH of 4.30-4.70 after fermentation, the inhibitory activity could be due to the production of acids by the lactic acid bacteria.

In the present study, the best antimicrobial effect of kefir was seen against Staphylococcus aureus and Salmonella typhimurium. This confirms the results of other researchers who reported antibacterial effects of Kefir against wide variety of Gram-positive and Gram-negative bacteria. The fermented milk ayran made according to the traditional technology of the North Caucasus region also showed significant antibacterial activity. Among the tested enteric bacteria the most rapid decrease in growth was recorded for Staphylococcus aureus. The results obtained from the tests on kefir against Salmonella and Escherichia coli stains were better than results of ayran on the same stains. The results of both fermented products obtained from the tests on other bacteria were similar.

In our study it was apparent that addition of lactitol to the fermentation mixtures of kefir or ayran did not significantly increase the antibacterial activity apart from that against C. difficile. Chitosan succinate in some cases actually decreased the antimicrobial activity of kefir and ayran, possibly because CS is able to show inhibitory action against lactic acid bacteria.

The data presented here provide evidence that traditional fermented milks from Russia can inhibit the growth and in some cases reduced the numbers of Gram-positive and Gram-negative, pathogenic bacteria in vitro cultures. This study suggests that kefir and ayran have the potential to reduce the risk of enteric infection although it will be necessary to conduct in vivo studies to confirm such activity.

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