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Research Note

Inhibition of *Bacillus cereus* in Milk Fermented with Kefir Grains

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ABSTRACT

The effects of kefir-fermented milk were tested against a toxigenic strain of *Bacillus cereus.* The incubation of milk with *B. cereus* spores plus 5% kefir grains prevented spore germination and growth of vegetative forms. In contrast, when 1% kefir grains was used, no effects were observed. The presence of metabolically active kefir grains diminished titers of nonhemolytic enterotoxin A, as assessed by enzyme-linked immunosorbent assay. During fermentation, kefir microorganisms produce extracellular metabolites such as organic acids, which could play a role in the inhibition of spore germination and growth of *B. cereus,* although the effect of other factors cannot be ruled out. Results of the present study show that kefir-fermented milk is able to antagonize key mechanisms involved in the growth of *B. cereus* as well as interfere with the biological activity of this microorganism.

Kefir is a fermented milk drink produced by the action of kefir grains. These grains contain complex microbiota constituted by a symbiotic association of lactic acid bacteria (lactobacilli, lactococci, *Leuconostoc*), acetic acid bacteria, and yeast *(2, 4, 6, 8, 10, 16, 23, 26).* There are several reports about the in vitro inhibitory effect of kefir against enteropathogens *(7, 11, 17, 27, 28).*

Bacillus cereus is a spore-forming, gram-positive, facultative anaerobic bacterium associated with food poisoning in humans. This microorganism is responsible for two distinct types of foodborne illness: (i) diarrheal syndrome, seen occasionally following the consumption of milk, dairy products, meat, sauces, soups, and vegetables; and (ii) emetic (vomit-inducing) syndrome, associated with consumption of cooked meals *(21).* Virulence of *B. cereus* is ascribed to the production of several extracellular factors as well as to bacteria-enterocyte interaction *(12).*

B. cereus spores are heat resistant and survive in extreme conditions (low pH and low water activity) and can be isolated from milk powder *(22, 24).* Concerning the action of lactic acid bacteria on *B. cereus,* Wong and Chen *(25)* reported the death of vegetative forms and the inhibition of spore germination. These effects could be attributed to the high concentration of organic acids produced during fermentation. Røssland et al. *(18)* reported the reduction of *B. cereus* levels in reconstituted powdered milk inoculated with lactococci and lactobacilli, the inhibition being higher with *Lactobacillus casei* than with *Lactobacillus acidophilus (20).*

Kefir-fermented milk is described in the *International Dairy Federation Bulletin (9)* and in the *Código Alimentario Argentino* (Art 576 – Res. Conj. SPyRS y SAGPA no. 33/2006 y no. 563/2006) *(3),* in which production in reconstituted powdered milk is recommended.

Milk fermentation with kefir grains requires incubation times ranging from 24 to 48 h at temperatures between 20 and 30°C. Since concentration of *B. cereus* spores normally present in milk powder range from 0.01 to 1 spore per g (data not shown), it is possible that around 10 spores in 100 ml are present in 10% reconstituted milk powder. Given the high growth rate of this microorganism, high concentrations of vegetative cells could be likely found after a few hours of incubation. In addition, growth-associated toxin production might lead to unsafe products.

The aim of the present work was to determine the effect of kefir grains on germination of spores, survival of vegetative forms, and biological effects of *B. cereus* in milk fermented with kefir grains.

MATERIALS AND METHODS

Preparation of milk samples. In all experiments, reconstituted skim milk (RSM) was prepared in sterile flasks containing 200 ml of sterile water and 10% (wt/vol) of skim milk powder (La Serenisima, Argentina). RSM was inoculated with *B. cereus* spores at concentrations ranging from 10 to 10^6 CFU/g and AGK1 kefir grains *(1)* at 1 or 5 g in 100 ml of milk. Controls with either *B. cereus* or kefir grains were performed. Samples were incubated at 20°C, and aliquots or their supernatants (obtained by centrifugation at 10,000 $\times$ *g* for 15 min) were withdrawn for analysis.

Bacterial strains and growth media. *B. cereus* strain 2 and its growth conditions were described previously *(13, 14).* Spores were obtained by inoculation of an 18-h culture of *B. cereus* in sporulation medium (nutrient broth agar with 1 g/liter of glucose and 10 mg/liter of $MnSO_4$ added). After 24 h of incubation at

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32°C and 7 days at room temperature, bacteria were collected with 10 ml of 0.8% (wt/vol) NaCl solution. Vegetative forms were eliminated by heating at 60°C for 30 min. Spores were maintained at −80°C with glycerol (10%) as a cryoprotectant. Vegetative forms and supernatants containing toxins were obtained from *B. cereus* exponential-phase cultures in brain heart infusion broth plus 0.1% (wt/vol) glucose.

Kefir grains CIDCA AGK1 were characterized as described and originate from CIDCA, Facultad de Ciencias Exactas de la Universidad Nacional de La Plata, Argentina *(1).* They were maintained at −20°C in milk *(7).*

Enumeration of vegetative cells and spores. Endospore and vegetative forms of *B. cereus* in milk and kefir samples were determined by plating serial dilutions on selective nutrient agar containing 0.05 g/liter polymyxin B sulfate (Parafarm, Argentina). Plates were incubated at 32°C for 24 h. Spore content was determined in samples after heat treatment at 80°C for 10 min.

pH and organic acid determination. The pH was measured with a combined glass–calomel microelectrode (Cole Palmer, Chicago, Ill.) in 1-ml aliquots. Organic acids were qualitatively and quantitatively determined by high-performance liquid chromatography as previously reported *(7).*

Detection of nonhemolytic enterotoxin (Nhe). Samples were tested for the presence of NheA by using the Tecra BDE kit (TECRA International Pty., Ltd., Sydney, Australia) according to the instructions of the manufacturers. Absorbance readings were done at 410 nm in a plate reader (Rainbow Reader, SLT Lab Instruments, Salzburg, Austria).

Cell cultures. The enterocyte-like Caco-2 cell line was used *(5).* Caco-2 monolayers of 15 days were used throughout *(13).*

Cytotoxicity assay. Detachment of Caco-2 cells was measured as reported previously *(13).* Each assay was conducted in duplicate over three different passages of Caco-2 cells. Results were expressed as percentage of attached cells.

Statistical analysis. Data were analyzed by the Systat Program version 5.0 (Systat, Inc., San Jose, Calif.) Statistical comparisons were made by means of the Student's *t* test. Significant differences between means were defined at $P < 0.05$.

RESULTS AND DISCUSSION

RSM challenged with *B. cereus* spores showed a decrease in spore concentration and increase of vegetative bacteria, reaching a concentration of 10^7 CFU/ml, which remained constant up to 24 h. No changes were observed in pH (Fig. 1A). RSM challenged with spores and immediately inoculated with 5% kefir grains (Fig. 1B) showed no change in the concentration of vegetative plus sporulated forms during 24 h of incubation. After 48 h, a slight decrease in spore concentration was observed. The germination inhibition could be attributed to the rapid decrease in pH during incubation, which dropped to pH 4.0 after 8 h. In contrast, the same experiment performed with 1% of kefir grains (Fig. 1C) showed diminished spore levels and growth of vegetative forms, correlating with a slow decrease in pH that reach a value of 5.5 after 8 h of incubation.

The organic acid composition of kefir-fermented milk harvested at pH between 4.16 and 4.30 showed concentrations between 4,962 ± 48 and 6,212 ± 333 ppm lactic acid and 728 ± 13 and 949 ± 54 ppm acetic acid, respectively.

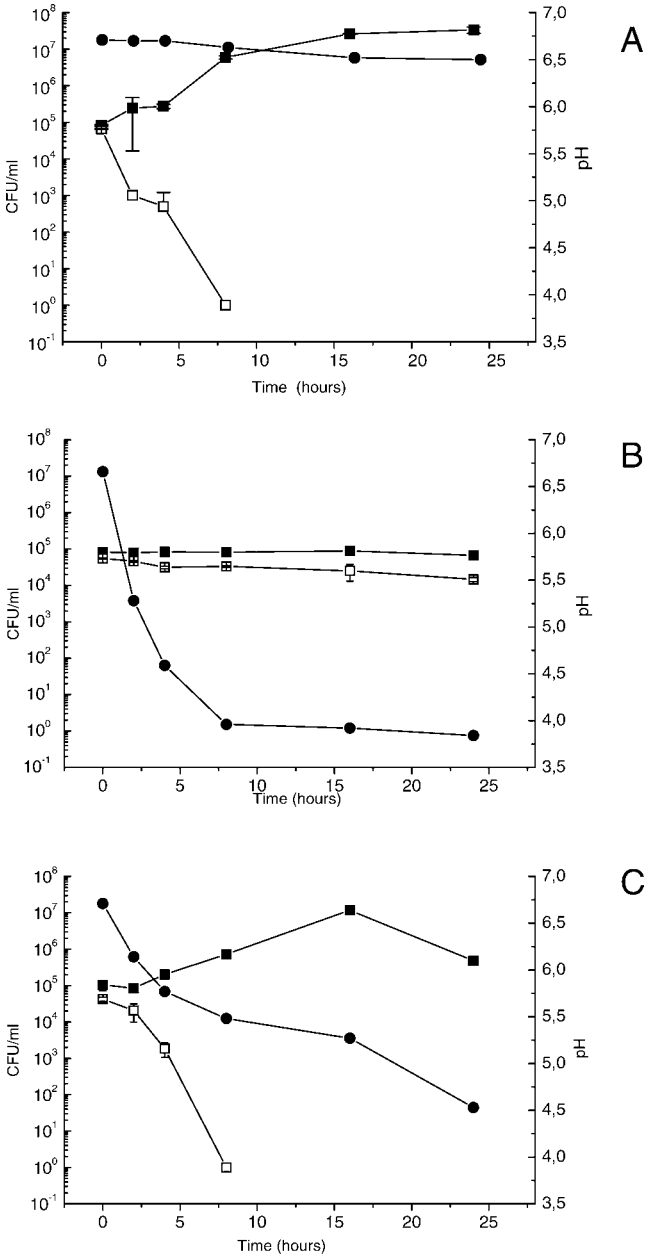

FIGURE 1. *Enumeration of spores (□) or vegetative cells (■) of* B. cereus *in reconstituted skim milk (RSM) (A), or RSM fermented co-incubated with spores and kefir grains at a grain-to-milk ratio of 5% (B) or 1% (C). All samples were inoculated with 10^5 spores per ml of* B. cereus. *The pH of each culture was also determined (●).*

Supernatant of kefir harvested at pH 4.0 produced a decrease of 4 log of vegetative *B. cereus.* No changes in spore concentrations were observed (Fig. 2A). These results are in agreement with those reported by other authors *(19, 20, 25),* showing that the growth of *B. cereus* is inhibited at pH below 5.0 and that vegetative forms are killed at these pH values. The fast drop in pH due to the production of high concentrations of organic acids prevents spore germination. However, the effect of other secreted products in addition to organic acids, such as bacteriocins *(15),* cannot

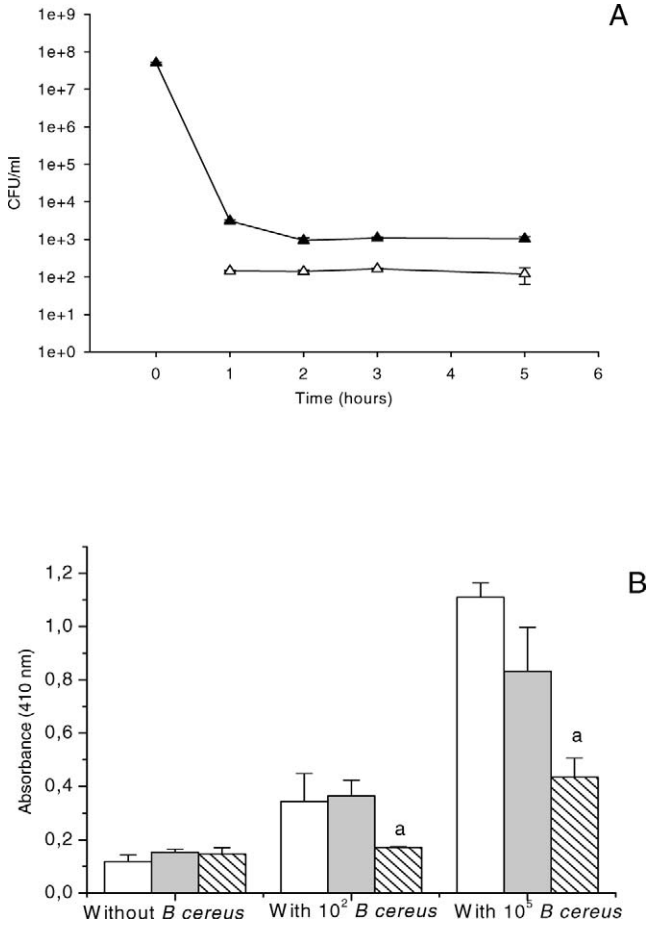

FIGURE 2. *(A) Enumeration of spores (Δ) or vegetative cells (▲) of* B. cereus *as a function of time in the presence of kefir-fermented milk elaborated with 5% kefir grains. (B) Toxin production after 24 h of incubation of samples challenged with* B. cereus *spores at different inoculation rates (10^2 and 10^5 spores per ml). RSM (□) or RSM plus 1% (▒) or 5% (▨) kefir grains. Values represent A_{410} in an enzyme-linked immunosorbent assay for NheA. Values below 0.2 are considered negative. a indicates significant differences were defined at* $P < 0.05$.

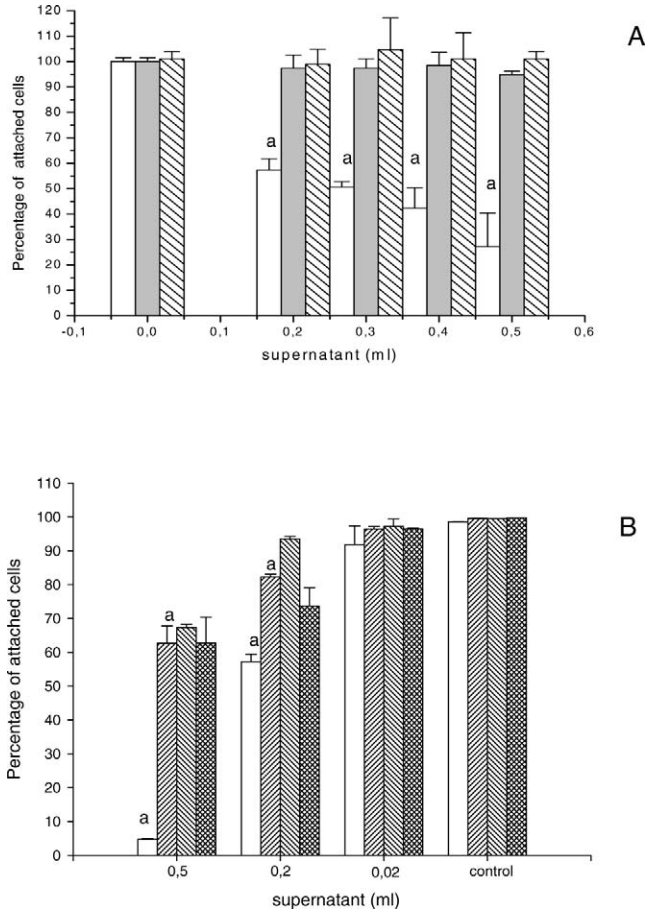

FIGURE 3. *(A) Biological activity in supernatants of RSM (□) or RSM co-incubated with 10^5* B. cereus *spores per ml plus 1% (▒) or 5% (▨) kefir grains. Supernatants were obtained after 24 h of incubation at 20°C, and biological activity was evaluated by detachment of Caco-2 cells. (B) Biological activity of supernatants of brain heart infusion broth incubated with 10^5* B. cereus *spores per ml during 24 h at 20°C (□) and the same supernatant mixed with supernatant of kefir-fermented RSM at pH 4.0 and then neutralized to pH 7.0 with 1 M NaOH (▨), supernatant of artificially acidified RSM with 7,000 ppm lactic acid plus 900 ppm acetic acid and afterward neutralized to pH 7.0 (▨), supernatant of artificially acidified RSM with HCl to pH 4.0 and then neutralized to pH 7.0 (▩). Biological activity was evaluated by detachment of Caco-2 cells. a indicates significant differences were obtained at* $P < 0.05$.

be ruled out. In addition, competition for nutrients could also be relevant for spore germination and growth *(20).*

Concerning toxin production, Figure 2B shows that NheA was detected after 24 h of incubation of RSM added with spore inocula of 10^2 spores per ml or greater. The same result was observed in RSM inoculated with 10^2 spores per ml and fermented with 1% kefir grains. However, no toxin was detected in milk contaminated with 10^2 spores per ml and fermented with 5% kefir grains. In all cases studied, RSM inoculated with 10^5 spores per ml showed toxin production, but samples containing 5% kefir grains showed significantly less toxin than did the others ($P < 0.05$).

To study biological effects of *B. cereus* extracellular factors, cultured human enterocytes (Caco-2) were used. Biological activity on enterocyte-like cells showed a dose-response behavior when different concentrations of *B. cereus* supernatants were tested (Fig. 3A). No detachment of Caco-2 cells was observed with milk or kefir without *B. cereus.* RSM artificially contaminated with 10^5 spores per ml co-incubated with 1 or 5% of grains in RSM had no cytotoxic effect. *B. cereus* enterotoxins present in *B. cereus* RSM supernatants had cytotoxic activity at low dilutions, and this effect was abolished by supernatants of kefir-fermented milk or artificially acidified milk (Fig. 3A and 3B).

We could hypothesize that co-incubation of *B. cereus* with kefir grains antagonizes production of extracellular metabolites, modifies interaction of toxins with their targets, or both. As a result of this antagonism, no cytopathic effects were detected even at inocula much higher than those normally found in milk powder. Even though NheA was detected, biological activity was completely abrogated in cocultures. Results obtained with *B. cereus* supernatants co-incubated with milk supernatants, obtained by addition of organic acids at low and neutral pH, showed a significant

decrease of Caco-2 cells detachment (Fig. 3B). It is worth noting that the same results were obtained with milk supernatant after rennin coagulation (data not shown). Taking into account these results, we could hypothesize that the interaction between *B. cereus* toxins and milk proteins are modified by acid or rennin treatment, thus interfering in the biological activity of *B. cereus* extracellular factors. Our results show that milk fermentation with kefir grains antagonizes *B. cereus* through the organic acids produced during fermentation. These findings contribute to the understanding of beneficial effects of kefir-fermented milk as well as to emphasize the suitability of kefir grains for the preparation of safe milk powder–based foods.

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