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Evaluation of the subchronic toxicity of kefir by oral administration in Wistar rats

Damiana Diniz Rosa[1*], Maria do Carmo Gouveia Peluzio[1], Tania Pérez Bueno[2], Ernesto Vega Cañizares[2], Lilian Sánchez Miranda[2], Betty Mancebo Dorbignyi[2], Dainé Chong Dubí[2] Ivette Espinosa Castaño[2], Łukasz Marcin Grześkowiak[1] and Célia Lucia de Luces Fortes Ferreira[3]

[1]*Department of Nutrition and Health. Federal University of Viçosa. University Campus. Viçosa. Minas Gerais. Brazil.* [2]*National Center for Animal and Plant Health (CENSA). Apdo. 10. San José de las Lajas. Mayabeque. Cuba.* [3]*Department of Food Technology. Federal University of Viçosa. University Campus. Viçosa. Minas Gerais. Brazil.*

Abstract

Introduction: Kefir is obtained by fermentation of milk with complex microbial populations present in kefir grains. Several health-promoting benefits have been attributed to kefir consumption.

Objective: The objective of this work was to conduct a subchronic toxicity study, offering the rats normal or high-doses of kefir and evaluating growth, hematology and blood chemistry, as well as assessing bacterial translocation and the integrity of the intestinal mucosa of animals.

Methods: Wistar rats were randomly divided into three groups (n = 6/group): control group received 0.7 mL of water, kefir group received 0.7 mL/day of kefir, (normodose), and Hkefir group received 3.5 mL/day of kefir (fivefold higher dose). Feeding was carried out by gavage. The animals were housed in individual cages and maintained under standard conditions for 4 weeks.

Results: The normodose and high-dose of kefir supplementation did not harm the animals since growth, hematology and blood chemistry in rats, as well as the potential pathogenicity in tissues were within normal limits, demonstrating that consumption of normodose and high-dose of kefir are safe. In addition, administration of the normodose of kefir reduced cholesterol levels and improved the intestinal mucosa of the rats.

Conclusion: These results demonstrate that the consumption of kefir is safe. Importantly, while damages are not seen for the high-dose, the normodose consumption is recommended due to the pronounced beneficial effects, as safety is concerned.

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Key words: *Kefir. Toxicity. Safety. Histological analysis. Bacterial translocation.*

EVALUACIÓN DE LA TOXICIDAD SUBCRÓNICA DEL KÉFIR POR ADMINISTRACIÓN ORAL EN RATAS WISTAR

Resumen

Introducción: El kéfir es obtenido por fermentación de la leche con una población microbiana compleja presente en sus granos. Al consumo de kéfir se le atribuyen múltiples efectos beneficiosos sobre la salud.

Objetivo: Evaluar la toxicidad subcrónica del kéfir en ratas Wistar, administrado por vía oral en dosis normal (normodosis) y sobredosis. Se evaluaron además, los parámetros de peso corporal, hematología, química sanguínea, translocación bacteriana e integridad de la mucosa intestinal.

Métodos: Se conformaron tres grupos de seis animales de manera aleatoria: grupo control, recibió 0,7 mL de agua; grupo kéfir recibió 0,7 mL/día de kéfir (normodosis) y grupo Hkéfir recibió 3,5 mL/día de kéfir (dosis cinco veces superior). La administración se llevó a cabo mediante sonda. Los animales se alojaron individualmente, y se mantuvieron bajo las mismas condiciones de manejo y alimentación durante 4 semanas.

Resultados: La administración de kéfir en dosis normal y sobredosis no afectó los parámetros evaluados en los animales, el peso corporal, indicadores hematológicos, de química sanguínea, y la patogenicidad potencial en los tejidos se encontraron dentro de límites normales, lo que demostró que el consumo de kéfir en dosis normal y sobredosis es seguro. Además, se evidenció que la administración de normodosis de kéfir redujo los niveles de colesterol y mejoró la mucosa intestinal de las ratas.

Conclusión: Se demostró que el consumo de kéfir es seguro. Destacar que, la administración de sobredosis no evidenció daños, no obstante, se recomienda el consumo de normodosis, debido a los marcados efectos beneficiosos y de seguridad.

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Palabras clave: *Kéfir. Toxicidad. Seguridad. Análisis histológico. Translocación bacteriana.*

Correspondence: Damiana Diniz Rosa.
Departamento de Nutrição e Saúde.
Av. Ph. Rolfs, s/n.
Campus Universitario. Viçosa.
36570-900 Minas Gerais. Brazil.
E-mail: drosa_ufv@yahoo.com.br

Introduction

Fermented dairy products have been consumed by humans for thousands of years. Kefir is a drink obtained by fermenting milk with kefir grains, which contain bacteria and fungi that coexist in a complex symbiotic association[1]. When inoculated into a milk matrix, kefir grains produce an acidified fermented beverage that is self-carbonated, which contains mainly lactic acid and small amounts of alcohol and exopolysaccharides. Furthermore, bioactive peptides[3], antibiotic components[4], and numerous bacteriocins are produced[5].

Microorganisms including probiotics present in fermented milks, are associated with human health benefits. This functional food is considered a probiotic because it contains live microorganisms that confer health benefits when administered in appropriate amounts[6]. The microbial strains present in kefir beverage often belong to species from the genera *Lactobacillus, Bifidobacterium* and *Saccharomyces*, acetic acid bacteria, and several genera of yeasts, whose health benefits have been well characterized[7].

Kefir consumption has been associated with several health-promoting properties, such as antimicrobial[8], anti-inflammatory[9], reduction of cholesterol and triglycerides plasma levels[10] and has also been shown to exert beneficial effect on gut health[11]. Kefir for centuries has been empirically used in many eastern European regions to treat different gastrointestinal diseases. Kefir has gained interest in the scientific community due to its health benefits against numerous diseases and infections[12].

Among probiotic functional foods, kefir stands out because of its low cost; it can be produced at home and can easily be incorporated into the diet. However, little attention has been payed to the safety concern with the use of kefir. The information on the safe levels of kefir intake or the amount that needs to be consumed and the time required to exert beneficial health effects are sparse in the literature. Based on the widespread worldwide kefir consumption, which is increasing daily due to the globalization of food habits, such safety studies are urgently needed.

In general, probiotics have been considered as safe. There are however some theoretical adverse risks regarding the use of beneficial microbes in humans. They include the potential for translocation and negative impact on gastrointestinal physiology and function, including metabolic and physiologic effects. Finally, there is also the potential for antibiotic resistance transfer within the gastrointestinal tract from commensal or probiotic bacteria to other bacteria or pathogens[6,13].

Therefore, the objective of our study was to conduct a subchronic toxicity assay with kefir using rat-animal-model. We offered the animals different doses of kefir for 4 weeks and thereafter evaluated their growth, hematology, and blood chemistry. The potential infectivity and pathogenicity (translocation and mucosal histology) of kefir were also assessed.

Materials and methods

Kefir preparation

Kefir particles (grains of kefir, obtained from a private household in Viçosa, Minas Gerais, Brazil) were washed with distilled water and inoculated in whole cow's milk during incubation at room temperature. The kefir beverage was prepared by inoculation of 5% (wt/wt) kefir grains into pasteurized milk. After incubation at 25-28 °C for 24 hours, the grains were separated from the fermented milk by filtration through a plastic sieve, washed, and kept for next preparation[11]. This process was repeated daily throughout the 4 weeks of the experimental period. Animals received fresh milk and kefir drink every day.

The fresh kefir offered to the animals presented the following physicochemical composition: pH 4.10 ± 0.10; high acidity, 0.461 ± 0.06 g/100 g of lactic acid; lipids 3.30 ± 0.16 g/100 g, proteins 3.00 ± 0.01 g/100 g. The lactic acid bacteria (LAB) were present in kefir at the levels of 2.78×10^7 CFU/mL and yeasts at 2.94×10^8 cell/mL, as determined by selective agar plating.

Animals

Eight-week-old male and female Wistar rats, which were supplied by the Experimental Animal Center, National Center for Animal and Plant Health, Mayabeque, Cuba, were used in this study. They received commercial standard chow (16.0% protein, 56.0% carbohydrate, 2.0% fat, 5.3% cellulose, and 5.0% vitamins and minerals) and tap water *ad libitum* for 1 week to allow adaptation of the animals. They were housed three to four rats per polycarbonate cage with softwood chips as bedding, in a barrier-sustained animal room, air-conditioned at 23-25 °C and 50-60% humidity, on a 12 h light/dark cycle.

Experimental Design

Eighteen animals were randomly divided into three groups. Each group consisted of three male (body weight: 210.0 ± 0.35 g) and three female (body weight: 180 ± 0.50 g) rats. The animals were placed in individual cages under controlled conditions. The experimental design is included (fig. 1):

– Control group (Control): standard diet plus oral administration of distilled water at a dose of 0.7 ml/animal/day by gavage;
– Normal dose of kefir group (kefir): standard diet plus oral administration of kefir at a dose of 0.7 ml/animal/day (9.8×10^6 CFU/mL or 4.29×10^7 CFU/ kg body weight/day) by gavage;
– High dose of kefir group (Hkefir): standard diet plus oral administration of kefir at the dose of 3.5 ml/animal/day (4.9×10^7 CFU/mL or 2.1×10^8
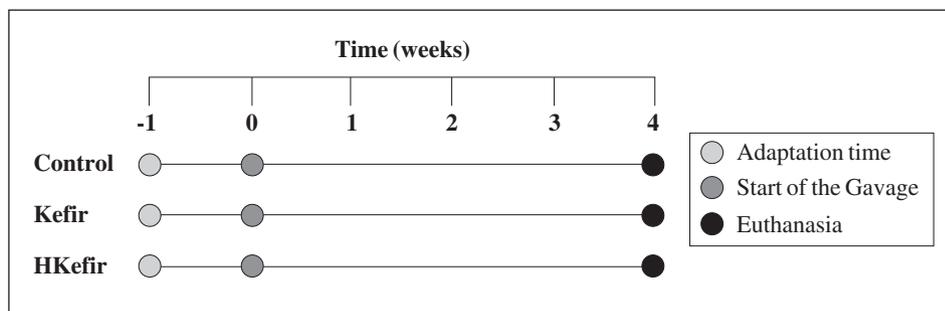
Fig. 1.—Experimental design of the study.

CFU/ kg body weight/day) by gavage. This group received a fivefold higher dose of kefir.

Body weight was measured weekly. At the end of week 4, all of the animals were anesthetized with ethyl ether according to the Guidelines for Ethical Conduct in the Care and Use of Nonhuman Animals in Research Ethical[14]. At the end of the experiment, the organs (spleen, liver, and mesenteric lymph nodes) were collected and weighed under aseptic conditions and inserted into a sterile falcon tube for further analysis of bacterial translocation. Blood was collected by cardiac puncture for hematologic and biochemical analysis. For histological evaluation, samples of the liver, small intestine, cecum and colon were collected and fixed in 10% buffered formalin.

General health status

Throughout the experimental period, changes in behavior and activity, treatment-related illness or death, and differences in hair luster between the treatments and control groups were monitored.

Internal organ indices

Liver, heart, kidney and spleen were collected and weighted immediately after euthanasia. The organ index values were derived from the ratio between weights of the internal organs (mg) of each animal over its final body weight (g).

Hematology and blood biochemistry

The blood was collected by cardiac puncture from anesthetized animals. Blood samples were centrifuged at $700 \times g$ for 10 minutes to obtain serum and thereafter were frozen at -20 °C. Total cholesterol and triacylglycerol were determined using commercial diagnostic test kits (Bioclin®, Diagnostics®, Belo Horizonte, Brazil). The blood samples were collected and transferred to lead-free polyethylene tubes containing EDTA. A cell counter was applied for hematocrit, by the microhematocrit method using heparinized capillary tubes; total leukocyte counts using a Neubauer chamber and Giemsa staining blood smears for differential cell counting by optical microscopy.

Bacterial translocation

Liver, spleen and mesenteric lymph nodes were collected and weighted under strict aseptic conditions to avoid any cross-contamination. The tissues were separately plated in three media. Blood agar based medium (Oxoid, Unipath Ltd., Basingstoke, Hampshire, UK) was prepared according to the manufacturer's instructions. The plates were incubated at 37° C aerobically for 24 h to evaluate the presence of bacteria and yeasts. Sabouraud Maltose Agar (BioCen, Havana, Cuba), plates were incubated at 30 °C aerobically for 48 h to assess the presence of yeasts. The MRS agar (Himedia®, Mumbai, India) plates were incubated at 37° C anaerobically for 48 h to control the presence of bacterial colonies.

Histological analysis

Immediately after euthanasia, liver, ileum, caecum and proximal colon samples were excised and rinsed with ice-cold physiological saline for histological evaluation. Tissue samples of approximately 0.5 cm in length were excised from the ileum (2 cm proximal to the caecum), caecum (middle portion), and colon (2 cm distal to the caecum), and fixed in 10% buffered formalin[15]. After dehydration in an increasing gradient of ethanol, tissue was embedded in paraffin and stained with routine histological hematoxylin and eosin for the histological analysis. Ten images per animal (60 images for each group) were captured and morphometric digital analysis for determining villous height, villous width and crypt depth in ileum tissue were performed according to the procedure described by Rosa et al. (2010)[16]. Crypt depth, mucosa thickness of the caecum and proximal colon samples were measured as described by Kabeir et al. (2008)[17]. The measurements were taken using the Image Pro-Plus® software system, version 4.5 (Media Cybernetics). In liver, hepatic parenchyma was classified as one of the following: hepatocyte cytoplasm or nucleus, hepatic sinusoids, degenerative hepatocytes, central vein, portal space, fatty deposition, and inflammatory infiltrate, according to Predes et al. (2009)[18].

Ethical aspects

This project was approved by the Commission of Ethics in Animal Experimentation of the National Center for Animal and Plant Health, Mayabeque, Cuba.

Statistical Analysis

Results are presented as mean values with their standard deviation (SD). Statistical significance of the difference between groups was assessed by one-way ANOVA followed by Tukey's post hoc multiple comparison test and chi-square analysis using GraphPad Prism (GraphPad Software Inc., San Diego, CA); for statistical analysis $p < 0.05$ was considered statistically significant.

Results

General health status and growth of the animals

During the experimental period, there was no noticeable change in activity, behavior or hair luster in any of the experimental groups. No diarrhea or other treatment-related sickness or death was recorded. At the end of the experimental period, all animals were alive and healthy. The consumption of the different doses of kefir by the animals did not affect weight gain of the animals during the experimental period (fig. 2).

Table I shows the internal organ indices of the different animals groups. The indices of the liver, heart, kidney and spleen revealed no significant differences in the ratio of organ weight/live weight between the groups fed normal dose or high dose of kefir or the control group at the different time points ($p > 0.05$).

Hematology / blood biochemistry

The effects of supplementation with different doses of kefir for 4 weeks on hematological and biochemical parameters were investigated in this study (table II). After 4 weeks of treatment, kefir group showed a reduction in total cholesterol plasma levels when compared to control and HKefir groups ($p = 0.017$). In this period, the levels of triacylglycerol, hematocrit, total leukocytes, and leukocytes fractions remained unchanged ($p > 0.05$).
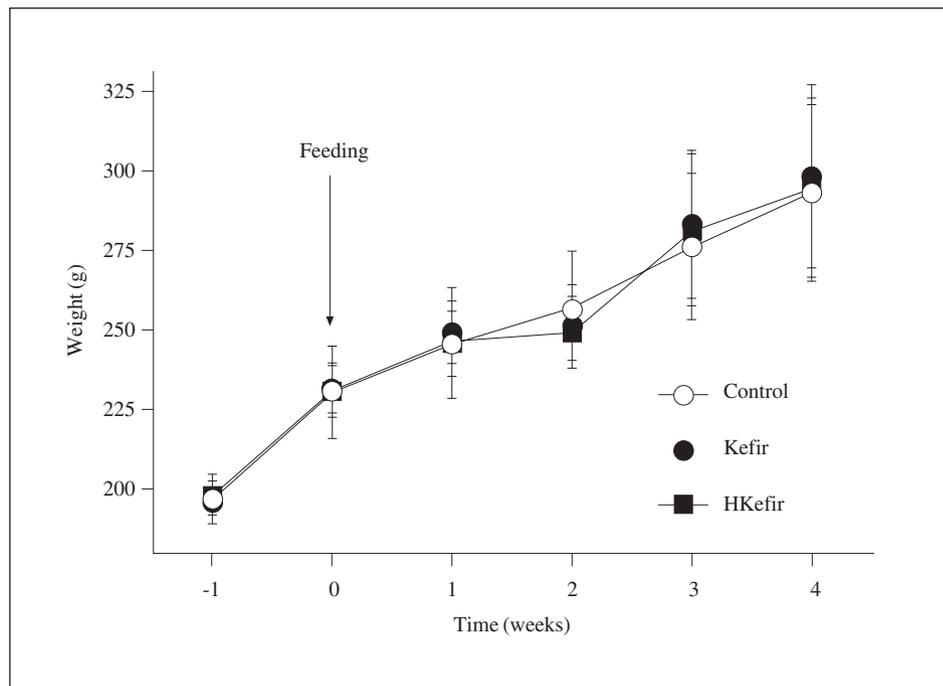

Fig. 2.—Effect of kefir consumption on the weight change during the experimental period. There were no significant differences in weekly weight gain among control group and the groups fed with normal dose and high dose kefir (p > 0.05; Tukey's post hoc ANOVA statistical analysis).

Tabla I
Effect on internal organ (liver, heart, kidney and spleen) indices of Wistar rats fed with normal dose and high dose of kefir

Organs	Control	Kefir	HKefir	p*
Liver	31.66 ± 7.36	29.84 ± 3.02	29.70 ± 3.02	0.560
Heart	3.44 ± 0.55	3.62 ± 0.44	3.62 ± 0.44	0.635
Kidney	7.32 ± 0.93	7.32 ± 0.68	7.32 ± 0.68	0.957
Spleen	2.43 ± 0.29	2.34 ± 0.29	2.31 ± 0.27	0.628

*There were no significant differences in the organ indices (means ± SD, n = 6) between the control group and the groups fed with normal dose and high dose of kefir (p > 0.05; Tukey's post hoc ANOVA statistical analysis).

Parameter	Control	Kefir	HKefir	p
Total cholesterol (mmol/L)	1.82 ± 0.07[a]	1.47 ± 0.14[b]	2.01 ± 0.20[a]	0.017
Triacylglycerol (mmol/L)	0.86 ± 0.09	0.93 ± 0.12	0.74 ± 0.06	0.437
Hematocrit (L/L)	37.00 ± 1.58	39.00 ± 4.18	36.50 ± 1.09	0.602
Total leukocytes ($\times 10^9$/L)	10.18 ± 2.54	8.51 ± 2.44	8.23 ± 2.05	0.494
Neutrophils ($\times 10^9$/L)	1.75 ± 0.44	1.87 ± 0.65	1.37 ± 0.35	0.639
Lymphocytes ($\times 10^9$/L)	8.30 ± 2.07	6.47 ± 1.95	6.69 ± 1.32	0.368
Monocytes ($\times 10^9$/L)	0.07 ± 0.03	0.02 ± 0.01	0.04 ± 0.02	0.524
Eosinophils ($\times 10^9$/L)	0.14 ± 0.07	0.13 ± 0.03	0.13 ± 0.05	0.971

Values are expressed as means ± SD, n = 6. [a,b]Mean values within a row with unlike superscript letters were significantly different ($p < 0.05$; Tukey's post hoc ANOVA statistical analysis).

Bacterial translocation

The incidence of bacterial translocation in rats which received orally different doses of kefir is assessment in liver, spleen and mesenteric lymph nodes. For this analysis, positive animal translocation was defined as an animal that had at least one tissue sample containing one or more viable bacterial cells. None of the animals were positive for the translocation ($p > 0.05$, data not shown).

Histological measurements

Macroscopic evaluation revealed that the animals did not show significant alterations in the liver, spleen, heart, kidney, ileum, cecum, and colon. The morphological analysis did not reveal any histopathological alterations in the liver, as well as in lipid deposition among animals from different treatment groups (data not shown).

Histological evaluations were performed with the three parts of the intestine: ileum, cecum and colon. Four weeks of kefir administration resulted in modulation of the distal small intestine (fig. 3). During this period, the kefir group showed greater villous height and villous width when compared to the control and Hkefir groups ($p < 0.0001$; figs. 3A and 3B). On the other hand, kefir group showed lower crypt depth ($p < 0.0001$; fig. 3C).

The influence of kefir supplementation on the cecum and colon of animals are shown in figure 4. After 4 weeks, kefir group had the highest crypt depth ($p < 0.0001$; fig. 4A). The animals treated with kefir (kefir and Hkefir groups) had higher mucosal thickness in cecum when compared to the control group ($p < 0.0001$; fig. 4B). In the initial portion of the colon, the animals that consumed normal dose of kefir showed lower crypt depth ($p < 0.0001$; fig. 4C) and colon mucosal thickness ($p = 0.0018$; fig. 4D).

Discussion

Fermented products including functional foods, are consumed by humans widely. They often represent an important dietary component in different geographical regions. Kefir is considered a functional food with probiotic properties that provides health benefits to the host. Indeed, the consumption of kefir has been increasing worldwide. However, there is a dearth of information on the safety of kefir consumption, especially the amount and length of consumption. Our study is the first to assess the *in vivo* safety of kefir supplementation in animal model. We conducted a study to evaluate the effect of two different doses of kefir in a subchronic toxicity assay. General health, organ weight index, hematology and blood biochemistry, and traditional histology were assessed.

After 4 weeks of consumption of different doses of probiotic kefir, the animals did not show differences in bodyweight or internal organ indices. The administration of the normal dose or high dose of kefir did not adversely affect the general health of the animals. In probiotic toxicity studies, behavioral, as well as activity, increasing organs size, especially splenomegaly and hepatomegaly, are the first indicators of undesirable effects. Particularly, the ratio of the spleen weight to body weight is considered to be an indicator of spleen inflammation by enteropathogenic bacteria[19]. However, in our study, we did not detect any toxicity signs related to kefir consumption

The consumption of fermented dairy products including kefir has been proposed as a strategy to reduce levels of circulating cholesterol and to improve lipid profile in humans and animals[20]. In this study, the consumption of kefir at the normal dose was able to reduce the total cholesterol levels. According to Hosono et al.[21], a high count of LAB in kefir ensures binding of cholesterol by up to 33%, probably due to the direct action of microbiota through their metabolic products on total cholesterol. Such beneficial effects of kefir on cholesterol metabolism may be due to the production of short chain fatty acids[22] and by the deconjugation of bile acids[12] by microorganisms. Similarly, significant reductions in cholesterol level in the plasma and liver were observed in hypercholesterolemic rats treated with *Lactobacillus plantarum strains* Lp09 and Lp45[10] and *Lactobacillus acidophilus* LA15, *Lactobacillus plantarum* B23, and *Lactobacillus kefiri* D17[23] isolated from
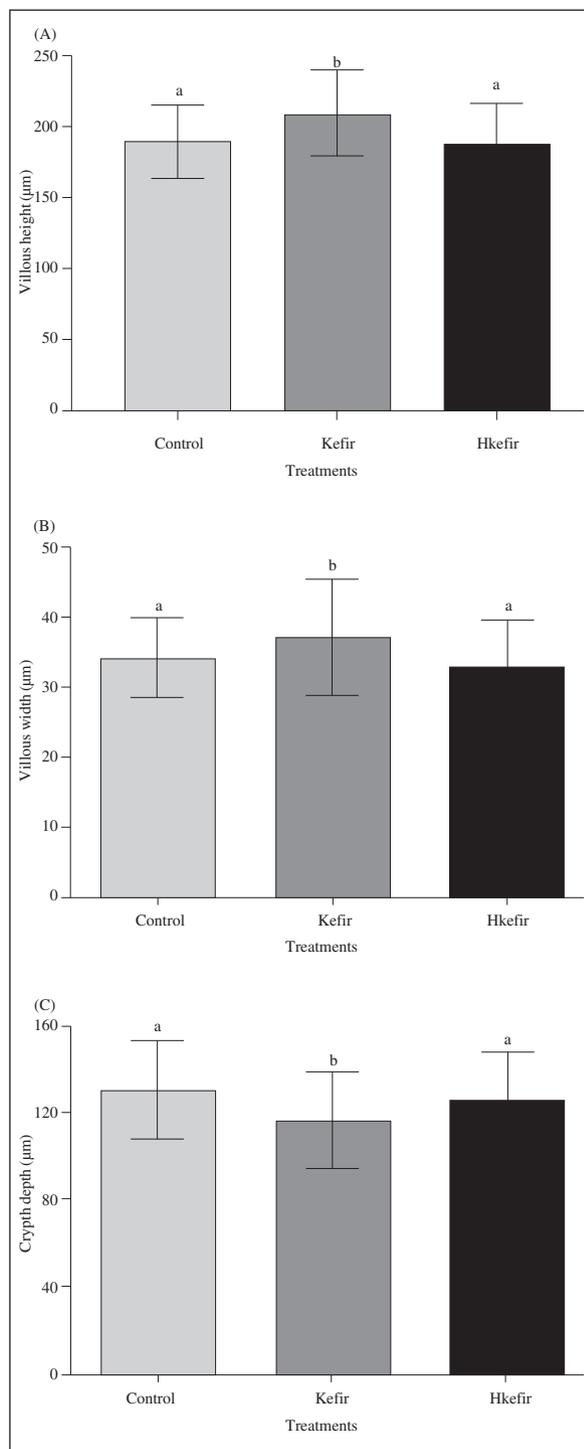
Fig. 3.—Morphometric analysis of small intestine of the animal groups studied. Ileum segments were collected and processed for optical microscopy analysis at the end of the experiment. A) Villous height; B) Villous width; C) Crypt depth; Values are the means ± SD (n = 6). [a,b,c]Differences among treatments were indicated by different letters and were considered statistically significant when p < 0.05; Tukey's post hoc ANOVA statistical analysis.

kefir grains. Thus, there is strong evidence supporting the functionality of kefir in the control of cholesterol level.

According to Kabeir and coworkers[17], infectivity and pathogenicity are two important components in safety studies on probiotic bacteria and are expressed as the degree of bacterial translocation. In our study, supplementation of rodents with normal or high doses of kefir did not cause bacterial translocation. Bacterial translocation is defined as the passage of viable bacteria from the gastrointestinal tract through the mucosal epithelium to other tissues. It can occur in cases of physical disruption of the mucosal barrier, thus initiating the first step of infectivity, and in the pathogenesis process of many opportunistic indigenous microbes. If physical disruption of the mucosal barrier occurs, the liver is the first organ to be compromised because of its direct connection through the portal blood. The morphological analyses did not reveal any histopathological alterations in the liver tissue of the study animals.

Some beneficial mechanisms of kefir include competition with pathogenic bacteria for the adhesion sites and strengthening of the physical and immunological barrier function of the intestine. In the present study we performed the histological analyses on the ileum, caecum, and colon. We found that the mucosa, villous and the intestinal crypts were well defined and healthy since the supplementation with different doses of kefir did not result in damage to the intestinal mucosa. Histological evaluation was carried out to corroborate the activity of kefir on the preservation of the structure of the intestinal mucosa in the ileum. Here, the consumption of 0.7 mL/day kefir increased villous weight and width. Our results indicate that a strong hyperplasia process occurred in these groups. This would guarantee the cell turnover rate in order to compensate for the cell loss in the apical region of the villous. In the literature some studies show that in the small intestine, enterocytes generated from stem cells in the crypt base differentiate into absorptive cells and are finally lost from the tips of the villus, resulting in the replacement of lining cells every 2-3 days[16]. On the other hand, the high dose of kefir in our study did not conferred damage to the intestinal mucosa.

Similarly, the normal dose of kefir resulted in better results in the caecum since it increased crypt depth after 4 weeks of consumption. On the other hand, in kefir group we observed a decreased mucosal colon crypt depth and thickness when compared with the other treatments. Our histological analyses of the intestinal mucosa of the animals corroborate the results of the bacterial translocation. The probiotics could prevent the attachment of pathogens and stimulate their removal from the infected intestinal tract. The mechanisms of these beneficial effects are related to the exclusion of pathogenic bacteria by direct antagonism, competition for nutrients, adhesion receptors, and stimulation of host immunity[24].

In the present study, the animals received 0.7 mL of kefir as the normal dose and a dose 5 times higher (3.5 mL) which was considered a high dose. By extrapolating to the human diet and considering an adult man of approximately 70 kg bodyweight, 0.7 mL/day/
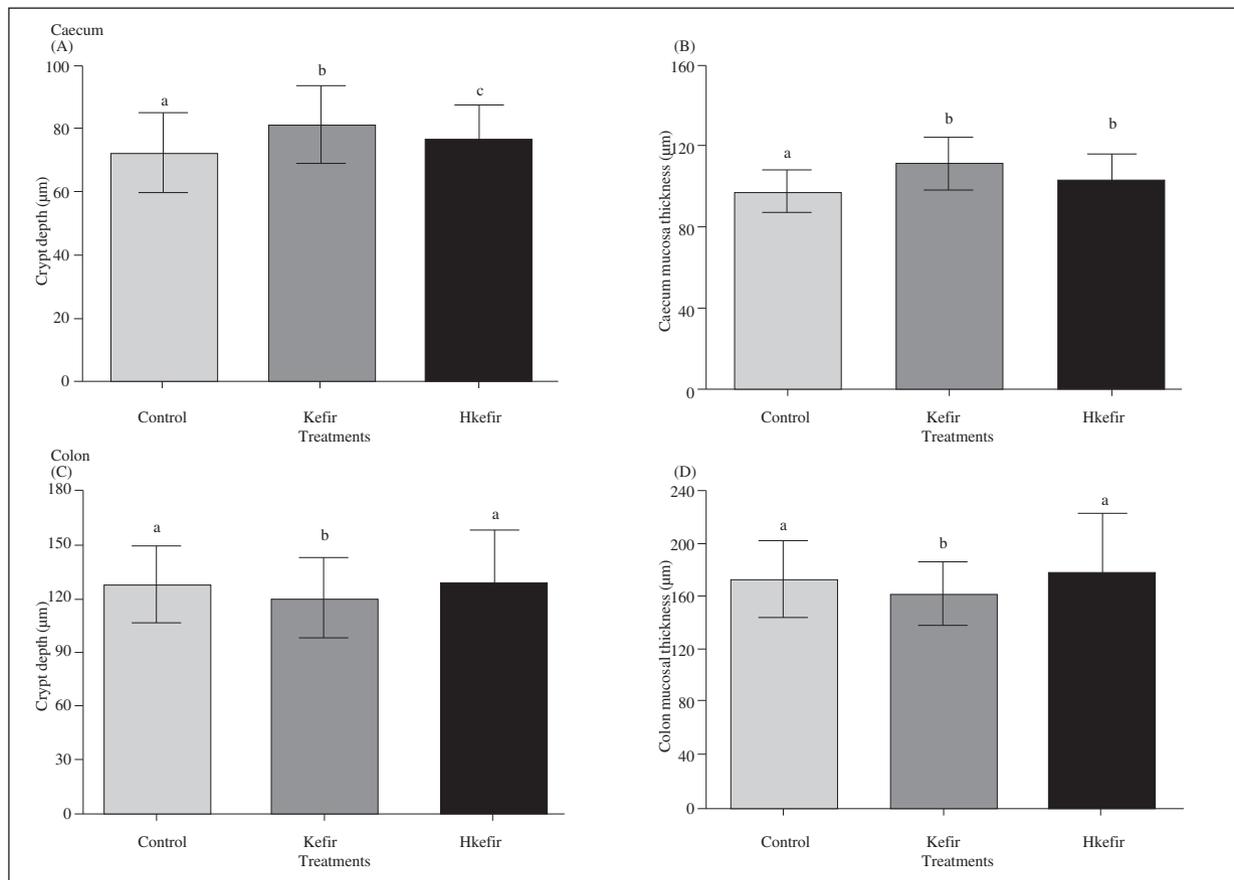
Fig. 4.—Morphometric analysis of large intestine of the animal groups studied. Caecum and proximal colon segments were collected and processed for optical microscopy analysis at the end of the experiment. A) Crypt depth in the caecum; B) caecum mucosal thickness; C) crypt depth in proximal colon; D) colon mucosal thickness. Values are means ± SD (n = 6). ^{a,b,c}Differences among treatments were indicated by different letters and were considered statistically significant when p < 0.05; Tukey's post hoc ANOVA statistical analysis.

animal of this probiotic represents a daily dose of 200 mL of kefir/human/day, a quantity that is easily incorporated into a diet. Likewise, the high-dose kefir for human consumption represents a daily consumption of 1 000 mL of kefir, which is unlikely to be incorporated into the human diet.

Taken together, we conclude that kefir supplementation with normal dose and high dose for 4 weeks in Wistar rats did not demonstrate harmful effects on the animals, as determined by growth, hematology, and blood chemistry in rats, as well as the potential pathogenicity in tissues. These findings clearly demonstrate that consumption of both the normal dose and high dose of kefir are safe. The results emphasize that, although no damages in the mucosa were seen at the high-dose-consumption of kefir, the normal dose is recommended due to the most pronounced beneficial effects, as safety is concerned.

Acknowledgements

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