

Immunological effects of Kefir produced from Kefir grains versus starter cultures when fed to mice

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ABSTRACT

Background: Natural kefir grains have a unique microbiota. The structure contains lactic acid bacteria (LAB), acetic acid bacteria and yeast in specific ratios in a polysaccharide matrix. Authentic kefir is produced by a traditional method using kefir grains cultured in milk. In contrast, starter cultures are used instead of kefir grains in the industry. The commercial kefir starter cultures used are limited and often very different from the kefir grain microbiota. The resultant commercial “kefir” is just a fermented drink containing some probiotic microorganisms and does not possess the same microbial population or chemical and physical characteristics of authentic kefir. The aim of this project was to determine and compare the effects on the mouse immune system of kefir produced using natural kefir grain versus commercial kefir produced by starter culture.

Methods: Kefir produced with different cultures was fed to Balb/c mice (6-8 weeks, 20-25 grams, male) by gavage for two weeks at 300 µl/day. Intestinal tissues were collected from sacrificed mice at the end of the trial. The control group of mice (CNI group) were fed with phosphate buffered saline (PBS). The experimental treatments were mice fed authentic kefir produced using kefir grains (KGI group) and mice fed kefir produced using starter culture (STI group). Immunoglobulin (Ig) A, Immunoglobulin G, Interleukin (IL)-4, Interleukin-10, Interleukin-12, Toll Like Receptor (TLR)-4 were analyzed immunologically in intestinal fluid samples.

Results: Results indicated that IgA values were 60.87, 72.78 and 55.31 ng/mL; IgG values were 26.59, 38.90 and 29.44 ng/mL; IL-4 values were 84, 40.28 and 53.28 pg/mL; IL-10 values were

110.98, 175.91 and 134.77 pg/mL; IL-12 values were 53.90, 22.93 and 24.75 pg/mL; TLR-4 values were 0.53, 0.43 and 1.37 ng/mL, for the CNI, KGI and STI groups, respectively.

Conclusion: The high probiotic content of grain kefir had the ability to modulate many immunological mechanisms.

Keywords: immune system, *in vivo*, kefir grain, probiotic, starter kefir culture

BACKGROUND

Kefir grains are small, white, gelatinous particles comprised of a primarily polysaccharide matrix in which a wide variety lactic acid bacteria (LAB), acetic acid bacteria and yeast symbiotically co-exist. The water soluble polysaccharide kefiran is found in kefir grains and has antimicrobial, anti-inflammatory and immune modulatory activities. Authentic kefir, produced using kefir grains, is a natural probiotic-containing fermented drink which is composed of organic acids such as lactic acid, formic acid, succinic acid, acetic acid and propionic acid, and aromatic compounds such as acetaldehyde, ethanol, acetone and diacetyl. It also contains vitamins, minerals and essential amino. As authentic kefir contains all the nutrients of milk including protein, fat and carbohydrates, it is a nutritious product. Moreover, digestion of authentic kefir is reported to be easier than milk due to the high enzymatic activity imparted via the kefir microbiota. Formation of fermentation metabolites by the kefir microbiota increases the nutritive value and provides better absorption in the body [1,2,3]. Authentic kefir contains *L. kefiranofaciens* subsp. *kefiranofaciens* [4], *L. kefiri* [5], *L. acidophilus* [4], *L. casei* [9], *L. rhamnosus* [6], *L. plantarum* [7], *L. fermentum* [6], *L. cremoris* [8], *Streptococcus thermophilus* [4], *Bifidobacterium bifidum* [4], *Kluyveromyces marxianus* [9], *Saccharomyces cerevisiae* [10] having the probiotic characteristic.

In industrial kefir production, a kefir starter culture is typically used rather than kefir grains. Kefir starter cultures contain a limited number of bacteria and yeast. *L. kefiranofaciens* subsp. *kefiranofaciens*, *L. kefiranofaciens* subsp. *kefirgranum*, *L. kefiri* and *L. parakefiri* bacteria, which are the characteristic species in kefir grains, are not found in commercial kefir starter cultures.

It has been reported that authentic kefir has positive effects on health such as anti-carcinogenic and anti-mutagenic effects [11,12], effects on immune system [13], anti-allergic effects [14], cholesterol-lowering effects [15], blood sugar regulating effects [16], antimicrobial and antifungal effects [17], lactose intolerance-reducing effects [18], effects on blood sugar [19], effects on the digestive system [20], effects against renal failure [21] and positive effects on dental health [22]. Milk kefir and soya milk kefir made from kefir grains as well as kefiran inhibit tumor growth in 180 tumor cells sarcoma-inoculated mice when orally consumed [23]. In a study on breast cancer involving the immune cells associated with milk glands, tumor cells were injected into Balb/c mice that were then fed with milk fermented by the lactic acid bacterial strain *Lactobacillus helveticus* R389. Results indicated an increase in IL-10 and a decrease in IL-6 in mice fed with *L. helveticus* R389 compared to the control group [24].

In studies on mice fed *L. acidophilus*, *L. casei*, *L. kefiranofaciens* and *L. kefiri* or with authentic kefir, increases in IgA [25,26] and IgG [27] were observed when compared to control mice.

Moreover, similar studies indicated increases in the levels of IL-4, IFN (interferon) and IL-10 [27-30] and a significant decrease in the levels of IL-6, IL-8 [31], IL-23, IFN- γ [26].

The aim of the present study was to compare the effects of authentic kefir produced using kefir grains with high natural probiotic content versus kefir produced using starter culture on the immune system.

METHODS

Kefir Production

Kefir grains were obtained from Danem Co. (Suleyman Demirel University, Technopark, Isparta, Turkey). Kefir starter cultures were obtained from CHR Hansen (Istanbul, Turkey). Fresh, whole milk (3.25% fat, 8.1% milk solids not fat) was pasteurized (72°C for 15 minutes) and then cooled to 25°C. Milk was inoculated at a rate of 2% kefir grain for authentic kefir and 2% starter culture for commercial kefir. The inoculated milk was incubated at 25°C and the fermentation was stopped when the pH reached 4.6. Kefir grains were removed by aseptically sieving the product. Both authentic and commercial kefir samples were stored at 4°C. Kefir samples were prepared every day for gavaging mice. The microbial content of each kefir sample was measured.

Animal Maintenance and Experimental Feeding Procedures

Male BALB/c mice (6-8 week-old) weighing 20-25 g were obtained from Akdeniz University (Antalya, Turkey). The mice were kept in a room with a 12 h light dark cycle. The temperature was kept at 24 °C, and the rats were given ad libitum access to food and water. BALB/c mice were divided into three groups of ten as control group (CKI), kefir grain group (GKI) and starter culture group (SKI). The control group (CNI) were gavaged with 300 μ l/d PBS (phosphate buffered saline) daily for two weeks.

Immunological Analyses

Animals were fed in a way that all groups were sacrificed on the same day. Small Intestinal tissue samples of were collected by dissection, and subsequently washed with sterile PBS using Vinderola [27]. Intestinal tissues from the control group were labeled CNI, from the group fed with kefir produced from kefir grains were labeled as KGI, and from the group fed with kefir produced from starter cultures were labeled STI. All intestinal tissue samples were processed in a tissue disruptor (Janke and Kunkel Ultraturrax T-25, Germany). Samples were centrifuged at 10.000 xg for 10 minutes at 4°C, and the resulting supernatant was removed and stored at -80°C until later thawed for analysis.

Immunological analyses were performed using BioTek Synergy HT (Winooski, VT) according to the procedures of Elabscience Mouse IgA, IgG, IL-4, IL-10, IL12 and TLR-4 Enzyme Linked ImmunoSorbent Assay (ELISA) kits (Elabscience Biotechnology Co., Houston, TX).

Statistical Analyses

All statistical analyses were performed using the IBM SPSS v. 23.0 (Statistical Packages for Social Sciences) computer software. One-way ANOVA test was used in parametric conditions and

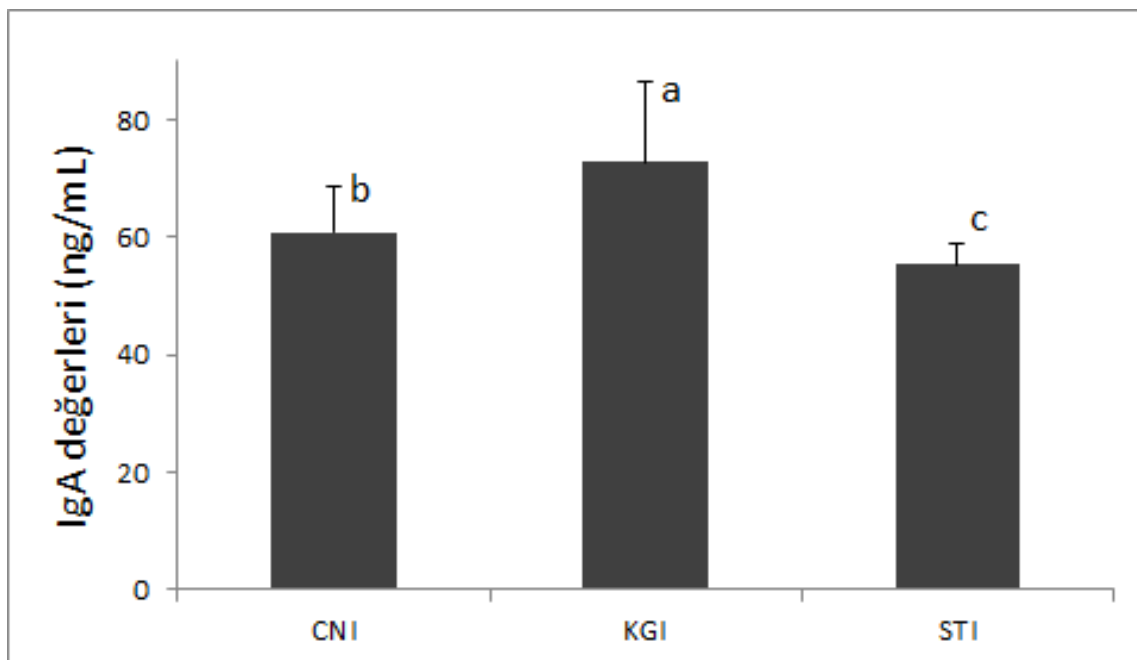
Kruskal Wallis test was used in non-parametric conditions in the difference analysis between groups. Values were considered significantly different when $p < 0.05$.

RESULTS

Microbiological contents of kefir samples

The *Lactobacillus* spp. contents of kefir produced using natural kefir grains was 10.54 log cfu/mL for the kefir produced using kefir grains and 8.40 log cfu/mL for the kefir produced using starter culture. The mean total yeast content of kefir produced from natural kefir grain was 5.69 log cfu/mL whereas it as 2.5 log cfu/mL in starter culture kefir. *L. acidophilus* content of the kefir produced using kefir grain and using starter culture were 9.55, and 8.65 log cfu/mL, respectively. The *Bifidobacterium* spp. content of the kefir produced using kefir grain and using starter culture were 7.83 and 0.12 log cfu/mL, respectively.

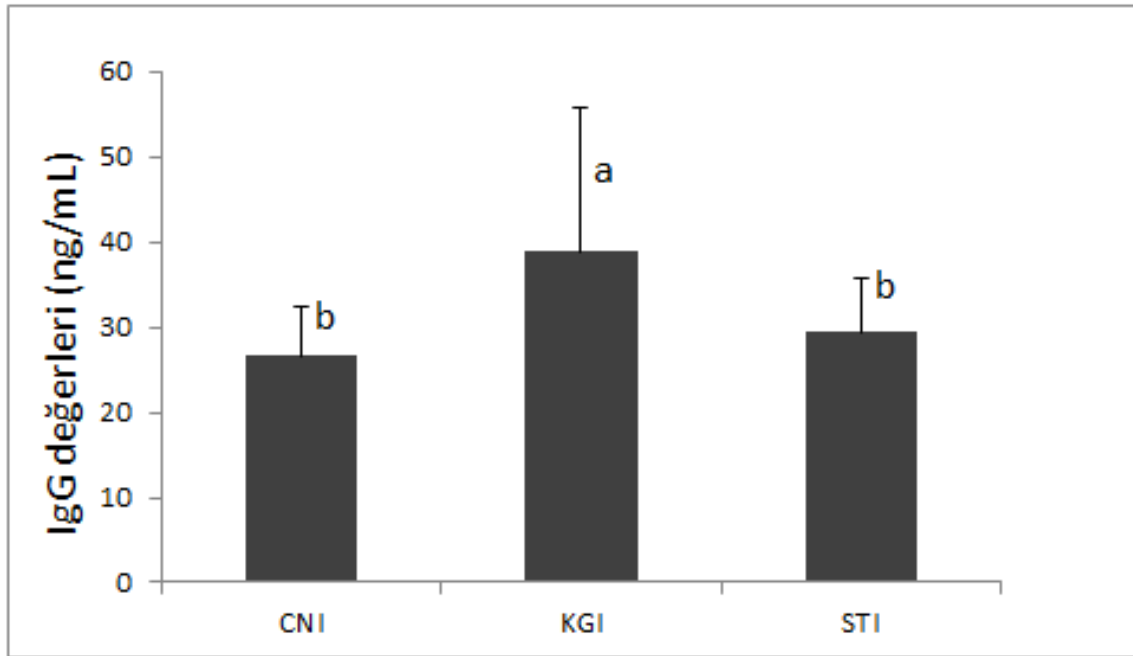
Results indicated the IgA values were statistically higher in the KGI group than in the CNI and STI group ($P < 0.05$). The IgA values in CNI, KGI and STI samples were 60.87, 72.78 and 55.31 ng/mL, respectively (Figure 1).



^{a,b,c} Different letters indicate results were statistically different ($P < 0.05$)

Figure 1. IgA values of CNI, KGI and STI groups

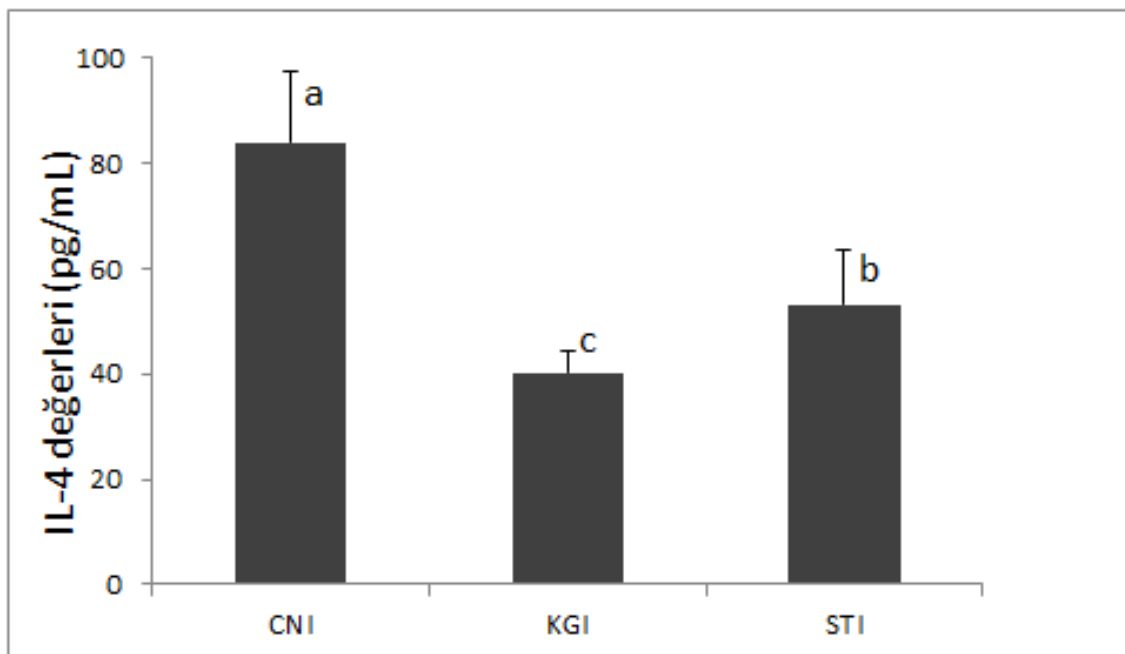
Results indicated the IgG values in the KGI group were higher than those in the STI and CNI groups ($P < 0.05$). In the CNI, KGI and STI samples, IgG values were 26.59, 38.90 and 29.44 ng/mL, respectively (Figure 2). For the IgG results, it was determined that there was a significant difference in all samples ($P < 0.05$).



^{a,b,c} Different letters indicate results were statistically different ($P < 0.05$)

Figure 2. IgG values of CNI, KGI and STI groups

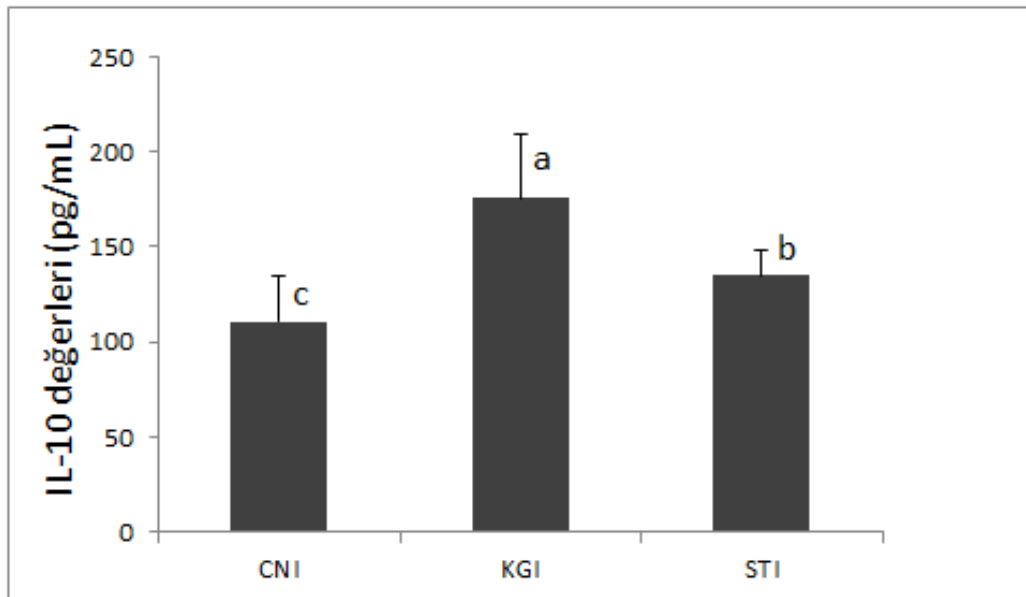
In CNI, KGI and STI samples, IL-4 values were 84.00, 40.28 and 53.28 pg/mL, respectively; there was a significant difference between all samples ($P < 0.05$) (Figure 3). Results indicated authentic kefir prepared from kefir grains lowered IL-4 levels significantly better than starter culture kefir.



^{a,b,c} Different letters indicate results were statistically different ($P < 0.05$)

Figure 3. IL-4 values of CNI, KGI and STI groups

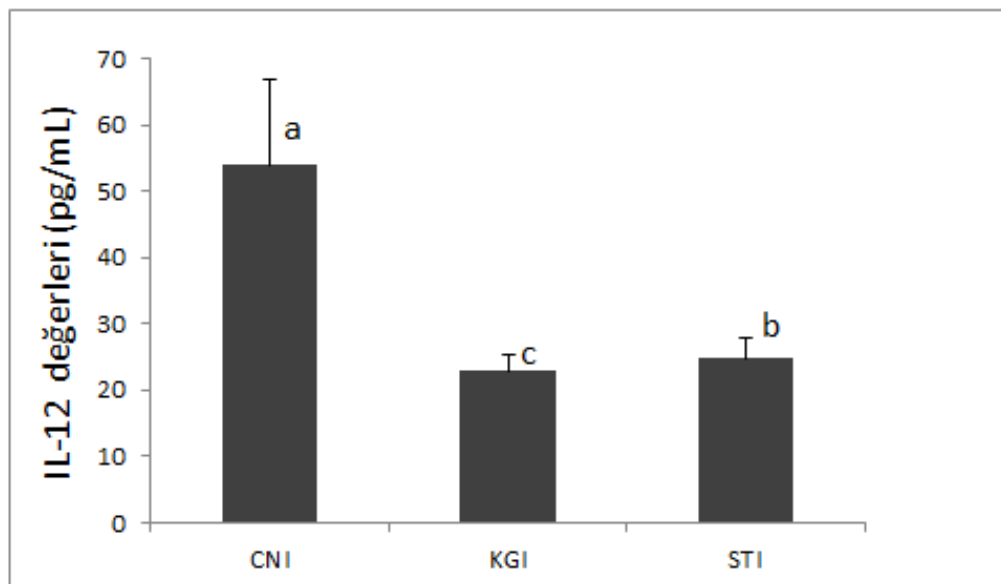
The IL-10 values were significantly higher in the KGI group than those in CNI and STI groups ($P < 0.05$). In CNI, KGI and STI samples, IL-10 values were 110.98; 175.91 and 134.77 pg/mL, respectively (Figure 4).



^{a,b,c} Different letters indicate results were statistically different ($P < 0.05$)

Figure 4. IL-10 values of CNI, KGI and STI groups

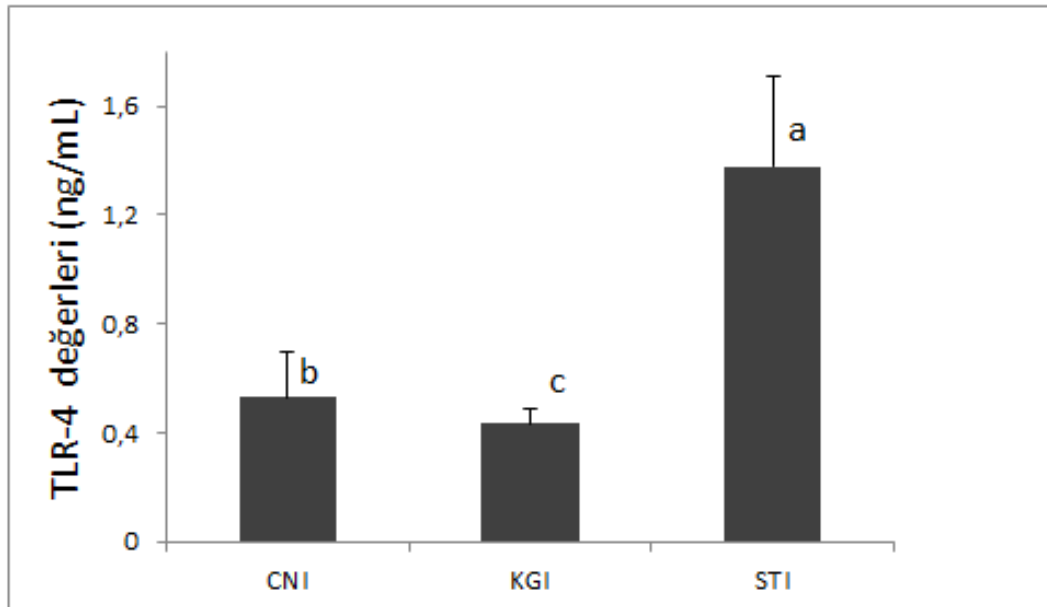
In CNI, KGI and STI samples, IL-12 values were 53.90, 22.93 and 24.75 pg/mL, respectively (Figure 5). All groups were significantly different ($P < 0.05$).



^{a,b,c} Different letters indicate results were statistically different ($P < 0.05$)

Figure 5. IL-12 values of CNI, KGI and STI groups

In CNI, KGI and STI samples, TLR-4 values were 0.53, 0.43 and 1.37 ng/mL, respectively (Figure 6). It was determined that there was a significant difference in all samples ($P < 0.05$).



^{a,b,c} Different letters indicate results were statistically different ($P < 0.05$)

Figure 6. TLR-4 values of CNI, KGI and STI groups

DISCUSSION

In a previous study conducted on *L. acidophilus*, *L. reuteri* and *L. casei*, IgA values increased in immunosuppressed mice with administration of these lactic acid bacteria [32]. In a similar study, the number of IgA levels were significantly increased after 7 days of feeding kefir compared to control [27]. With *B. lactis* and *L. acidophilus* consumption, an increase in serum IgA levels was observed compared to the control group [33]. It also was observed that there was an increase in the number of IgA cells in the lamina propria at the end of the 7th day of oral administration of kefir to Balb/c mice [34]. Carasi et al. [26] found an increase in IgA levels in the feces of mice treated with probiotic microorganisms. The research findings of the present study are in agreement with other studies in that probiotics promote IgA production. In the present study, authentic kefir made from kefir grains significantly increased IgA over the control whereas the starter culture kefir caused slightly lower IgA levels compared to both the control and the kefir grain generated product.

In a study on probiotic administration to laboratory rats infected with *Salmonella enteritidis*, it was observed that IgA antibody secretion was significantly increased in the probiotic-fed mice [28]. In the present study, IgA levels significantly increased in KGI samples.

In the previously mentioned study on feeding *L. acidophilus*, *L. reuteri* and *L. casei*, IgG levels increased in immunosuppressed mice when fed the probiotics [32]. In a similar study, after kefir administration, a significant increase in IgG levels was observed on the 5th and 7th day as compared to control mice [27]. In yet another study, healthy human adults who consumed *B. lactis* and *L. acidophilus*, had an increase in serum IgG levels as compared to the control group [33]. In

the present study, IgG levels were significantly higher in mice fed authentic kefir made from kefir grains compared to both the control and IgG levels in mice fed starter culture prepared kefir.

IL-10 promotes the balance between the function of cells that secrete pro-inflammatory cytokines and the function of cells that secrete anti-inflammatory cytokines [36]. In the present study, IL-10 levels significantly increased in KGI samples over both the STI and CNI samples. In a similar study, after kefir administration, IL-4 and IL-10 increased markedly in all periods, while IL-6 and IFN increased on days 2 and 5, respectively [27]. It was determined that with *L. kefiranofaciens* administration, there was an increase in IL-10 level in mice [37]. Generally, probiotics increase the production of intestinal anti-inflammatory cytokines such as interleukin-10 (IL-10) and TGF (Transforming Growth Factor)-beta, while reducing the production of pro-inflammatory cytokines such as IL-8 [38, 39]. While it was observed that there was an increase in IgA levels in the feces of mice treated with probiotic microorganisms, there were significant decreases in the amounts of IL-23, IFN- γ , and IL-6, IL-10 levels with a decrease in the expression of proinflammatory mediators in the lymph node nodules [26]. Again, in a similar study, it was determined that on day 7 of kefir ingestion, IL-10 increased in the intestinal mucosa of the animals, and modulation of intestinal immunity provided the control of the intestinal homeostasis of macrophages [30]. Results of the present study support that authentic kefir produced using kefir grains causes greater IL-10 production than kefir produced from starter cultures.

It has been reported that the lower IL-12 levels may facilitate the control of inflammation. In the present study, the IL-12 levels of the KGI group were significantly lower than the CNI group. Concurrently, an increase in IL-10 was noted in the KGI group. These results may increase the secretory IgA levels, making the immunological response more effective. The interaction between IL-12 and IFN- γ is necessary for effective immune responses against a large number of pathogens including intracellular bacteria such as mycobacteria. Previous studies using recombinant bovine IL-12 cells cloned in vitro and expressed were found to have IFN- γ -inducing activity [40]. The combination of IL-12 and IL-2 may help to increase IFN- γ expression in the pulmonary tissue, but does not reduce colony burden in the pulmonary tissue or increase the survival rate of the mouse [41]. In addition, IL-10 inhibits IL-12 release from active macrophages and dendritic cells. It was also observed that IL-2, IL-6 and IFN- γ production were significantly increased in probiotic-fed mice, while no significant change was observed in IL-4 levels [28]. Results of a previous study on rats fed *B. breve* and *L. rhamnosus*, indicated a decrease in IL-4 levels [35]. However, IL-2, IL-4, IL-6 and IFN- γ were not measured in the present study.

Toll-like receptor 4 (TLR-4) is found in tissues targeted for insulin activities. Probiotic consumption increases the number of *Bifidobacteria* present in the intestine causes increased expression of adhesion proteins which in turn attenuates intestinal permeability and weakens the activation of TLR-4 by bacterial lipopolysaccharide [42,43]. In the present study, higher *Bifidobacterium* spp. content was provided in authentic kefir produced by kefir grains and therefore, a more effective decrease in activation was observed at the TLR-4 level of the KGI group.

CONCLUSION

Nutritional habits have an important effect on the immune system. Recently studies have increased the understanding of the intestinal microbiota and intestinal health. Concurrently, there is an interaction between the consumption of probiotic microorganisms and the immune response. The probiotic effects of fermented dairy products arise not only from microorganisms but also from metabolites produced during fermentation. This study has determined that authentic kefir produced from kefir grains has a greater ability to modulate immunological responses compared to kefir produced by limited starter cultures as is commonly done for commercial kefir. It was found that secretory IgA and IgG levels were higher in mice fed with authentic kefir grain-generated kefir as compared to control and those fed with starter culture-generated kefir. In addition, the humoral immune system is higher in mice fed with authentic kefir grain-generated kefir. Authentic kefir appears to stimulate a more effective defensive mechanism in blood and in the lumen of the mucosal organs such as the gastrointestinal tract and respiratory tract.

This study has shown that under *in vivo* conditions, the microbiota of authentic kefir produced by natural kefir grain causes enhanced immunomodulator properties as compared to kefir produced using starter culture as is typical in commercial kefir.

List of Abbreviations: *B*, *Bifidobacterium*; CNI, control group; ELISA, Enzyme Linked ImmunoSorbent Assay; IFN, interferon; IG, Immunoglobulin; IL, interleukin; *K*, *Kluyveromyces*; KGI, the kefir produced using kefir grains; *L*, *Lactobacillus*; LAB, lactic acid bacteria; PBS, phosphate buffered saline; *S*, *Saccharomyces*; STI, the kefir produced using starter culture; TGF, Transforming Growth Factor; TLR, Toll Like Receptor.

Author Contributions: ZBGS, TKT and FD designed the research; FD, ZBGS and TKT performed the research; FD and TKT prepared the kefir samples and feed animals. FD, ZBGS and TKT analyzed the data; FD, ZBGS and TKT prepared the manuscript, figures, references.

Competing Interests: There are no conflicts of interest to declare.

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