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The Effect of Fermented Kefir as Functional Feed Additive in Post-Weaned Pigs

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Abstract: The control of the immune system of pigs after weaning is important in pig farming because productivity depends on the survival of the post-weaned pigs. Previously, antibiotics would have been administered in the case of infectious diseases to increase the survival rate of post-weaned pigs, but now, the use of antibiotics is strictly restricted in order to prevent other problems such as the occurrence of antibiotic-resistant pathogens. In this study, the effect of fermented kefir as a functional feed additive as a replacement to antibiotics was evaluated in terms of the microbial profile in fecal samples, immunological factors in the blood of pigs, growth performance measured as average daily gain (ADG) and the feed conversion rate (FCR) of post-weaned pigs. In the kefir-treated group, the number of lactic acid bacteria and *Bacillus* spp. in the fecal samples of the pigs increased with the kefir treatments. Interestingly, the number of coliform groups as opportunistic pathogens was reduced in the fecal samples of pigs treated with kefir. We found out that treatment with kefir enhanced the innate immunity of post-weaned pigs though the reduction of IL-6 as a proinflammatory cytokine and an increase in IgG as an immunoglobulin, enhancing immunological defense against pathogens. Finally, after treatment with kefir, we observed that the ADG of post-weaned pigs increased to 135.6% but FCR decreased to 92.2%. Therefore, this study shows that fermented kefir can be used as a functional feed additive and an antibiotic alternative in order to improve both the innate immune system and growth performance of post-weaned pigs.

Keywords: fermented kefir; post-weaned pig; innate immune system; growth performance; functional feed additive

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1. Introduction

In pig farming, it is important that the health of pigs is maintained and supported, especially in the post-weaning period, when they are more susceptible to environmental stresses and infection by pathogenic microorganisms [1]. Pathogenic microorganisms can be lethal to piglets after weaning and treatment with antibiotics is usually increased [2]. However, the use of antibiotics in livestock accelerates the occurrence of antibiotic-resistant pathogens, which is a major concern, like the emergence of antibiotic-resistant microorgansims [3]. Due to these concerns, the use of antibiotics is now strictly prohibited in pig farming for the prevention of infection by pathogenic microorganisms [3]. Therefore, it is now very important to find alternatives that can replace antibiotics.

Kefir is known to be milk fermented using a kefir grain with microbial complexes containing kefiran as the biologically active exopolysaccharide [4]. These microbial complexes in kefir grain possess probiotic characteristics [4]. Many microorganisms isolated

from kefir show high survival against the low pH and bile salts in the gastrointestinal tract, thereby adhering to intestinal mucus. In addition, these microorganisms can produce antagonistic materials like organic acids and antimicrobial peptides to interfere with the adherence of pathogenic bacteria to the intestinal mucus.

Kefir grain has been shown to confer diverse biological activities such as improved digestion and tolerance to lactose, antibacterial effects, hypocholesterolaemic effects, antihypertensive effects, anti-inflammatory effects, antioxidant activity, anti-carcinogenic activity and anti-allergenic activity. It is thought that these diverse activities result from the microbial complexity of the kefir grain's composition [4,5]. In this study, we examined whether fermented kefir could play a role as a functional feed additive for improvement of the growth performance measures average daily gain (ADG) and feed conversion rate (FCR) and the immune condition of pigs through a field trial.

2. Materials and Methods

The experimental protocol (TMCco-2019-01) describing the management and care of animals was reviewed and approved by the Animal Care and Use Committee of Chungnam National University, Daejeon, South Korea.

2.1. Preparation of Fermented Kefir

Based on the method previously reported, we carried out the preparation of fermented kefir [6]. The kefir grain was inoculated in 4% (w/v) of whole fat milk medium and cultivated at 30 °C for 2 d without agitation. For main fermentation of kefir, the medium used in this study was developed based on De Man, Rogosa and Sharpe (MRS, KisanBio, Seoul, Korea), yeast extract-peptone-dextrose (YPD, KisanBio, Seoul, Korea) and nutrient broth (NB, KisanBio, Seoul, Korea) media. In order to determine the optimum conditions for the main fermentation of kefir, we modified the composition of glucose, whey protein and dipotassium phosphate and the inoculation size and chose the best compositions of each factors to increase number of viable lactic acid bacteria, *Bacillus* spp. and yeast. The main fermentation of kefir was carried out in 300 L of working volume of a 500-L fermenter (JUNGHYUN PLANT, Hwaseong, Korea) at 30 °C for 1 d. For fermentation, sterilized air was supplied at 2 vvm (volume/volume/minute) in the fermenter and mixing rate was kept at 200 rpm through an impeller (JUNGHYUN PLANT, Hwaseong, Korea). After fermentation, the total cells were harvested by continuous centrifugation (HANIL SCIENCE MEDICAL, Daejeon, Korea) at 8000 rpm and the cell pellet was mixed with 20% (w/v) of sterilized skim milk solution. After that, this mixture was lyophilized for 3 d.

2.2. Determination of Vible Colonies in Fermented Kefir

For determination of the number of lactic acid bacteria, *Bacillus* spp. and yeast in the lyophilized kefir, viable colony counting was carried out. Briefly, the sample was diluted by serial dilution to 0.85% with a sterilized saline solution and 100 μL of the diluted sample was spread onto MRS agar for lactic acid bacteria, NB agar for *Bacillus* spp. and YPD agar for yeast. After incubation at 30 °C for 1 d, colonies were counted.

2.3. Experimental Design of Animals

Ninety pigs regardless if sex (4 w of age, 7.66 ± 0.38 kg) were used in this experiment. The weight of each pig at the start and end of this experiment and the amount of feed consumed for 4 weeks was measured. To calculate average daily gain (ADG) and feed conversion rate (FCR), we monitored the weight of each pig and feed consumed for 4 weeks. The groups for this field trial were divided into three groups: the control, experimental I and experimental II group. Each group consisted of 30 pigs. The control group was fed the basic feed only. Experimental I and II groups were fed the basic feed containing 0.1% (w/w) and 0.5% (w/w) of lyophilized kefir of the weight of basic feed,

respectively. The 0.1% and 0.5% of lyophilized kefir contained 1×10^7 and 5×10^7 CFU/mL of lactic acid bacteria, respectively.

2.4. Analysis of Microorganisms in Fecal Sample of Pigs

For analysis of microorganisms (lactic acid bacteria, *Bacillus* spp. yeast and coliform groups) in the fecal samples of pigs, fecal samples were taken from all of pigs at 0, and the second and fourth weeks of the trial. After that, the serial dilution of samples was carried out with sterilized 0.75% NaCl solution and 100 μL of diluted sample was spread onto MRS agar for lactic acid bacteria, NB agar for *Bacillus* spp., OGYE agar for yeast and MacConkey agar for the coliform group. Plates were incubated for 1 d at 30 °C and colonies were then counted.

2.5. Analysis of Porcine TNF-α, IL-6, IgA and IgG in Serum of Pigs

Blood samples were collected from the jugular vein of each pig at the start and end of the trial. Serum was collected using a vacuum tube (Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged at 4000 rpm for 10 min at 4 ℃ before being stored at −80 °C until immunological factors were quantified. Determination of TNF-α and IL-6 in the serum of pigs were carried out using the porcine TNF-α DuoSet ELISA (enzyme-linked immunosorbent assay, R&D Systems, Minneapolis, MN, USA) and porcine IL-6 DuoSet ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions, respectively. Determination of IgA and IgG in the serum of pigs was carried out using the Pig IgA ELISA Quantitation kit (Bethyl Laboratories, INC, Montgomery, TX, USA) and Pig IgG ELISA Quantitation kit (Bethyl Laboratories, INC, Montgomery, TX, USA) according to the manufacturer's instructions, respectively. The concentrations of porcine TNF-α, IL-6, pig IgA and IgG were then calculated from the standard curves.

2.6. Statistical Analysis

Statistical analyses were accomplished using SPSS 22.0 [7]. The obtained data were analyzed using the paired *t*-test for evaluating the association and significance between variables. A p-value < 0.05 was considered significant.

3. Results

3.1. The Optimization of Culture Conditions for Kefir Fermentation

For the main fermentation of kefir, seed culture was carried out in sterilized 10% whole fat milk medium for 2 days at 30 °C without agitation. Thereafter, we optimized the culture conditions for kefir fermentation according to the concentrations of glucose as a carbon source, whey protein as a nitrogen source and dipotassium phosphate as a phosphate source. We optimized the innoculation size of the seed culture. As a result, the number of lactic acid bacteria, *Bacillus* spp. and yeast increased by 123.6%, 115.4% and 113.4%, respectively, when we used 2% of glucose. At 1% of whey protein, the number of lactic acid bacteria, *Bacillus* spp. and yeast increased by 122.7%, 120.2% and 111%, respectively. At 0.02% and 0.1% of dipotassium phosphate, the number of lactic acid bacteria, *Bacillus* spp. and yeast was not reduced, compared to other concentrations of dipotassium phosphate (Figure 1a–c) [6]. The number of lactic acid bacteria, *Bacillus* spp and yeast reached its maximum when 2% seed culture of kefir was inoculated, rather than 10% (Figure 1d) [6]. Based on these results, we finally confirmed the optimum concentration of glucose, whey protein and dipotassium phosphate, and the size of inoculum (Table 1) [6].

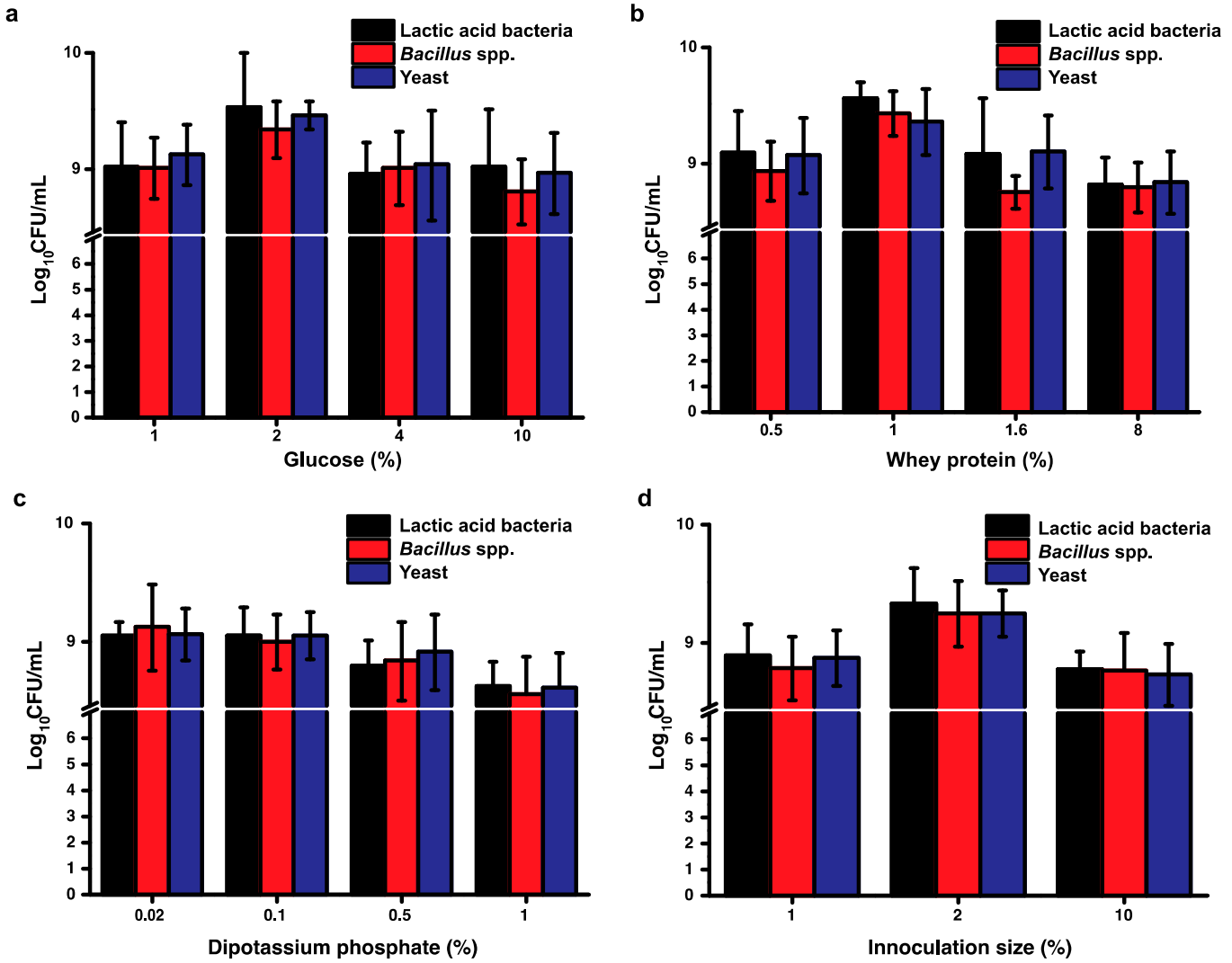

Figure 1. Proportion of lactic acid bacteria, *Bacillus* spp. and yeast in kefir according to culture conditions. (**a**) The proportion of lactic acid bacteria, *Bacillus* ssp. and yeast in kefir by glucose content. (**b**) The proportion of lactic acid bacteria, *Bacillus* spp. and yeast in kefir by whey protein content. (**c**) The proportion of lactic acid bacteria, *Bacillus* spp. and yeast in kefir by dipotassium phosphate content. (**d**) The proportion of lactic acid bacteria, *Bacillus* ssp. and yeast in kefir by inoculation size.

Table 1. The composition of optimized culture medium for kefir fermentation.

Component	Composition (%, w/v)
Glucose	2
Whey protein	1
Dipotassium phosphate	0.02
Yeast extract	2
Ammonium sulfate	0.1
$MgSO_4$	0.01
$MnSO_4$	0.05
Inoculation size	2

3.2. The Effect of Fermented Kefir on Microflora in the Fecal Samples of Post-Weaned Pigs

To examine whether treatment with kefir can alter microorganisms in the gut of pigs, we investigated the number of lactic acid bacteria, *Bacillus* spp., yeast and coliform groups

in the fecal samples before and after two and four weeks of treatment with kefir. An increase in the number of lactic acid bacteria in the fecal sample was observed at the second week in all groups supplied with kefir and kept on it by end of this field test, compared to the control group (Figure 2a). In addition, the number of lactic acid bacteria in the fecal sample of the control group decreased by the end of this field test. This result indicates that direct supplementation of lactic acid bacteria can lead to lactic acid bacteria-enriched microflora. The number of *Bacillus* spp. in the fecal sample also increased in all groups supplied with kefir, similar to the increase in the number of lactic acid bacteria (Figure 2b). However, the kefir supplement did not affect the amount of yeast in the fecal sample (Figure 2c). Interestingly, the kefir supplement reduced the number of coliform groups in the fecal sample at end of this field test with statistical significance (Figure 2d). This result shows the possibility of kefir as antibiotic alternative, because the supplementation of kefir directly inhibited the growth of coliform groups. Therefore, these results indicate that supplementation with kefir was able to directly make the microflora conditions beneficial to the health of post-weaned pigs.

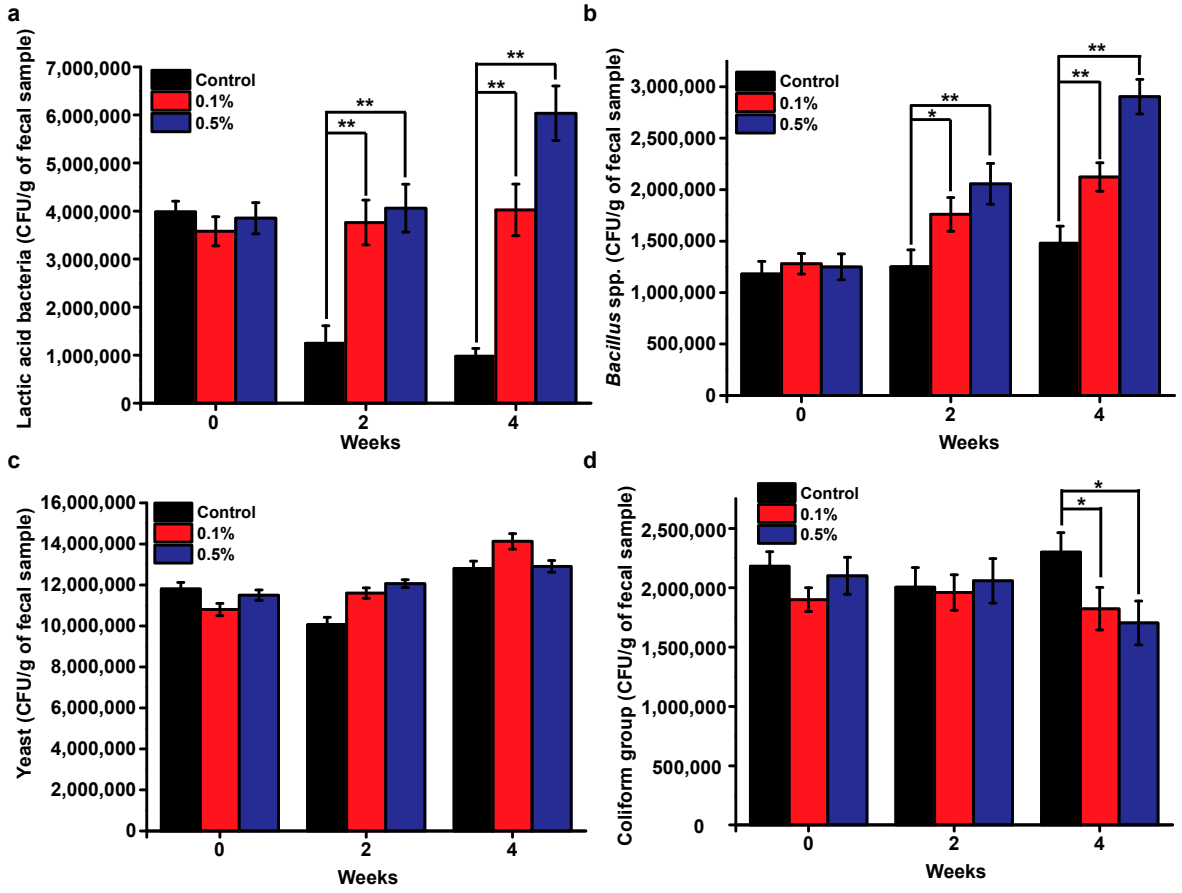

Figure 2. Monitoring microorganisms in the fecal sample of pigs fed fermented kefir. (**a**) Change in the number of lactic acid bacteria. (**b**) Change in the number of *Bacillus* spp. (**c**) Change in the amount of yeast. (**d**) Change in the number of coliform groups. Fecal samples were collected before feeding of kefir, and on the second and fourth weeks. *$p < 0.05$ and **$p < 0.01$, determined using a paired *t*-test.

3.3. The Effect of Fermented Kefir on Innate Immunity of Post-Weaned Pigs

It is known that post-weaned pigs are more susceptible to infection by pathogenic microorganisms, compared to adult pigs [2]. One of the strategies to avoid the use of antibiotics and minimize the damage by infection of pathogenic microorganisms in pig farming is the enhancement of the innate immunity system of these pigs. The levels of TNF-α and IgA in the blood serum were not changed after supplementation with kefir (Figure 3a

and 3c). However, the supplementation of 0.5% kefir dramatically reduced the level of IL-6 as proinflammatory cytokine after the end of this field test (Figure 3b). The level of IgG as immunoglobulin, participating in the defense system against pathogens, was increased in all groups treated with kefir (Figure 3d). This result shows that supplementation of kefir can contribute to the enhancement of the innate immunity of post-weaned pigs.

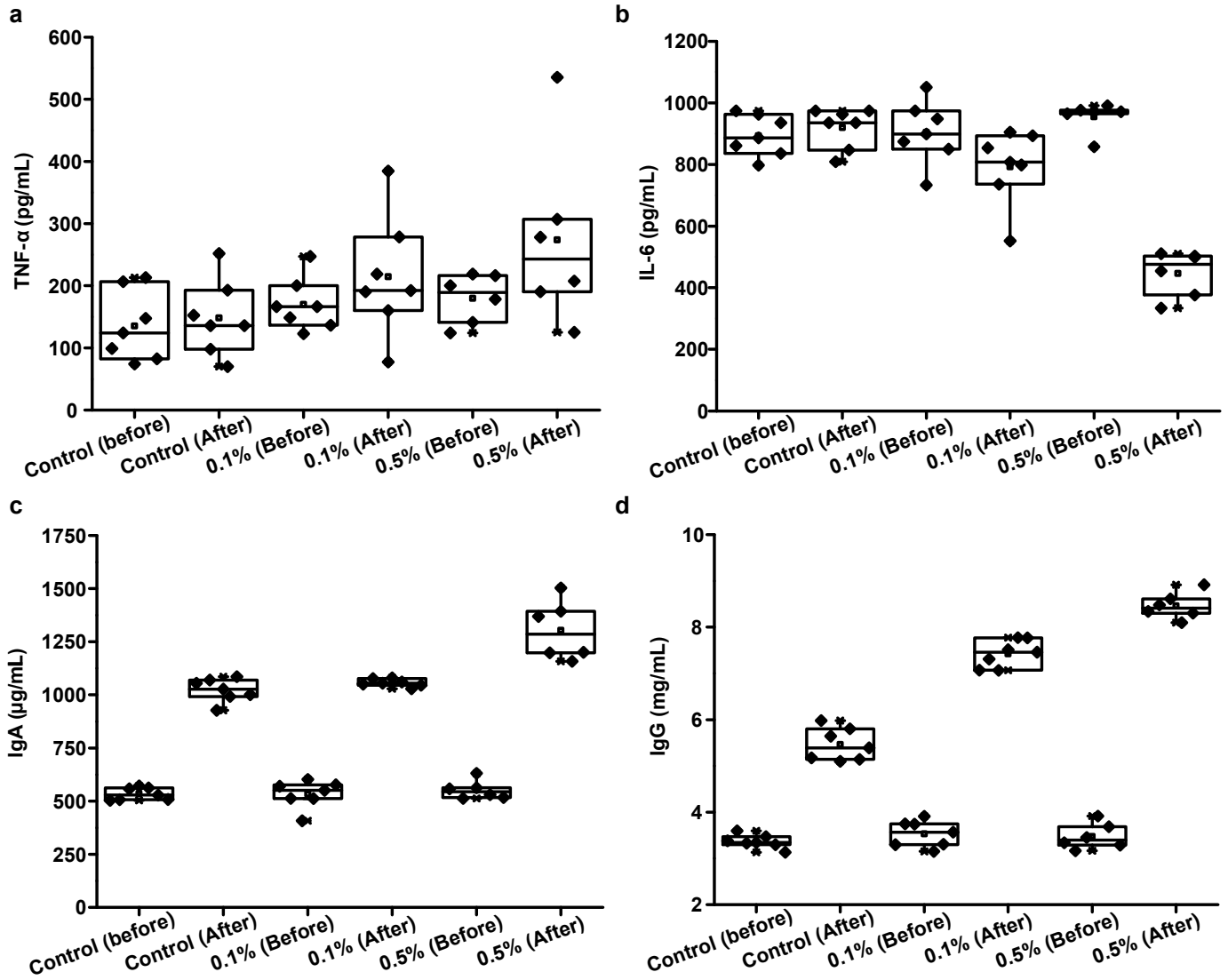

Figure 3. Monitoring cytokines and antibodies participating in innate immunity in the blood of the post-weaned pigs fed fermented kefir. (**a**) The concentration of TNF-α. (**b**) The concentration of IL-6, (**c**) The concentration of IgA. (**d**) The concentration of IgG. Each group consisted of 30 pigs. Blood was collected before and on the fourth week.

3.4. The Effect of Fermented Kefir on ADG and FCR as Growth Performance Measures in Post-Weaned Pigs

We also determined the effect of fermented kefir on ADG and FCR, as well as the innate immunity of post-weaned pigs. The ADG in the group supplied with 0.1% kefir was not changed but increased in the group supplied with 0.5% kefir with statistical significance, compared to the control group (Figure 4a). In the case of FCR, 0.1% kefir supplementation had little effect for 4 weeks. However, 0.5% kefir supplementation induced a reduction in FCR, compared to the control group (Figure 4b). Improvement of both ADG and FCR as growth performances by 0.5% kefir supplementation implies that kefir supplementation can contribute to the efficient conversion of nutrient intake to weight gain.

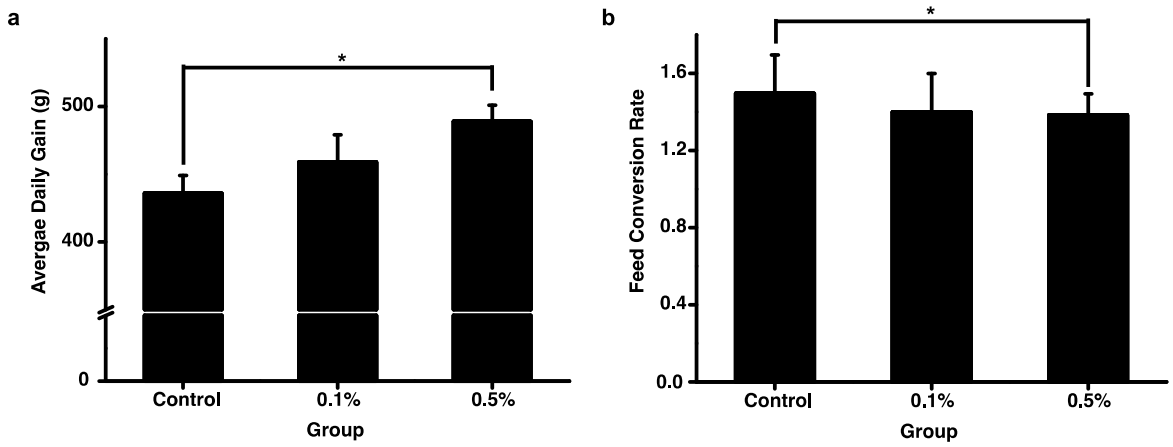

Figure 4. Improvement of average daily gain (ADG) (**a**) and feed conversion rate (FCR) (**b**) in pigs after weaning by supplementation with fermented kefir. Each group consisted of 30 pigs. *$p < 0.05$, determined using a paired *t*-test.

4. Discussion

The use of antibiotics is now restricted for the prevention of microbial infections because of antibiotic resistance; therefore, the need for functional feed additives as antibiotic alternatives in animal farming has increased [8–11]. Indeed, functional feed additives based on probiotic bacteria or natural products have been developed and used as antibiotic alternatives [12–14]. In this research, fermented kefir was evaluated as a functional feed additive for post-weaned pigs, which are relatively susceptible to infection of pathogens.

Firstly, we found that the supplementation with kefir induced an increase in the populations of lactic acid bacteria and *Bacillus* sp. but a reduction in the number of coliform groups. These coliform groups, such as opportunistic pathogens like *Citrobacter* sp., *Enterobacter* sp., *Hafnia* sp., *Klebsiella* sp. and *Escherichia* sp., often damage the intestines of pigs through toxin production, thereby leading to death [14,15]. The use of antibiotics to eliminate these groups is paradoxically one of reasons why antibiotic-resistant bacteria occur [16]. Therefore, it is important to control the number of coliform groups in the intestine to maintain the health and improve the productivity of pigs without the use of antibiotics. Previously, it was known that fermented kefir and kefiran as exopolysaccharide show antimicrobial activity through in vitro and vivo testing [17]. In this study, we showed that the antimicrobial activity of fermented kefir against coliform groups can indeed be realized in weaned pigs through supplementation with kefir.

Such microflora changes to increase the number of beneficial microorganisms can lead to modulation of the production of cytokines and antibodies, controlling the innate and acquired immunity of pigs through interactions between microflora and the intestine, as well as metabolites produced from microorganisms [18]. According to Wang et al., they observed effects of probiotics on levels of inflammatory cytokines in the serum of pigs supplied with *Lactobacillus fermentum* and *Pediococcus acidilactici* [18]. In particular, the level of IL-6 as a pro-inflammatory cytokine was decreased in the probiotic groups. The reduction of IL-6 through treatment with probiotics was also studied by Zhang et al. [19]. They demonstrated that treatment with *L. rhamnosus* GG attenuated the level of IL-6 in the serum of piglets [19]. Other studies have also shown that treatment with probiotic microorganisms induces the expression of IgG and anti-inflammatory cytokines like IL-10 and suppresses the expression of proinflammatory cytokines such as IL-6, IL-8 and TNF-α [20–22]. In agreement with these studies, we found out that the level of IL-6 was reduced and that of IgG was increased in the serum of weaned pigs supplied with kefir, compared to the control group. However, supplementation with kefir did not affect the level of TNF-α and IgA in this study.

Finally, we found out that supplementation with fermented kefir improved both ADG and FCR in post-weaned pigs. Similar to this result, it was previously reported that the supplementation of beneficial microorganisms like probiotics can maintain a healthy intestine and improve conversion rate of feed to energy and nutrients, thereby improving ADG and FCR as growth performances in swine [12–14]. Also,

Therefore, we have demonstrated that fermented kefir can be utilized as a functional feed additive in order to improve ADG and FCR, as well as the immune system in pigs through the alternation of microflora inhabiting in the intestines (Figure 5).

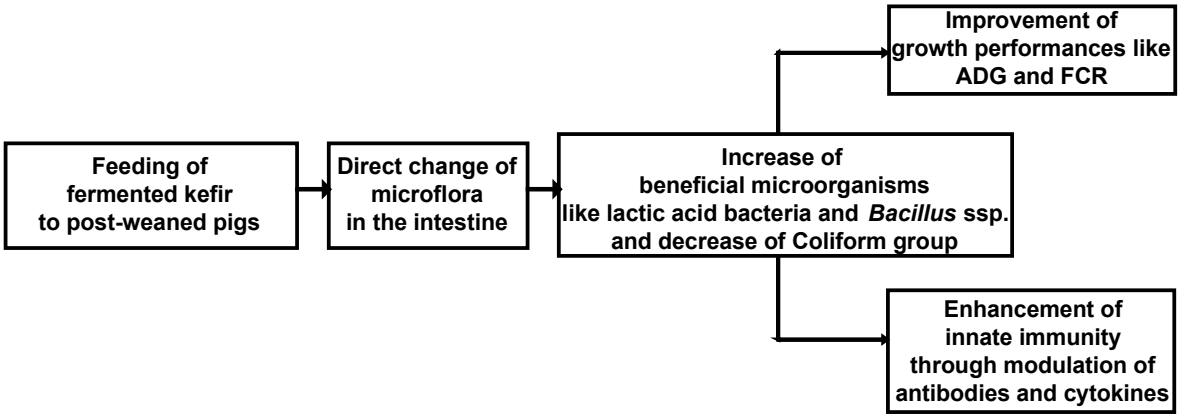

Figure 5. Proposed mode of action of fermented kefir for improving ADG, FCR and innate immunity in post-weaned pigs.

Author Contributions: H.-C.K., H.L., and J.-W.S. designed the research and conducted all experiments. W.C. and D.B.S. carried out the field trial. J.H. and D.J. carried out the fermentation of kefir, preparation of lyophilized kefir, and viable cell counting. H.L. wrote the manuscript. All authors discussed the results and commented on the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by Ethics Committee of Myongji University, Yongin, Korea (TMCco-2019-01, January 10, 2019).

Data Availability Statement: The data presented in this study are available in the article or supplementary material.

Conflicts of Interest: The authors declare no conflict of interest.

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