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Immunomodulatory effects of *Lactobacillus* biogenic administration in dogs

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Abstract

***Lactobacillus* biogenics were administered to four dogs for 56 days. The regulatory T cell (Treg) count was found to be increased in all four dogs on 56 days. On day 112, 56 days after completion of *Lactobacillus* probiotic administration, the percentage of Tregs was found to be decreased in three dogs. Plasma IgA levels tended to increase after stopping *Lactobacillus* administration. Metagenomic analysis of bacterial flora found in dog feces revealed that *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Fusobacteria* were predominantly found in all 4 dogs. Analysis of fecal microbiomes of species level on day 56 showed that 99 % members of genus *Prevotella* belonged to species *copri*. This study demonstrates some of the immunomodulatory effects of *Lactobacillus* probiotics on dogs.**

Key Words: *Lactobacillus* probiotics, Regulatory T cells

Regulatory T cells (Tregs) play a vital role in the suppression of excessive immune responses, such as in the case of inflammatory bowel disease, in which Tregs have been shown to suppress the immune response and to protect against immune related mucosal injury and in rheumatoid arthritis by suppressing deleterious autoreactive activities[12,16,21,24)]. Tregs are a subset of the CD4+ T cells that express the transcription factor Foxp3 and potently suppress many immune responses[22)]. Tregs play an indispensable role in suppressing excessive immune responses that are deleterious

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to the host and in maintaining immunological unresponsiveness to self-antigens. Tregs are produced in the thymus as a functionally mature subpopulation of T cells and their development can also be induced from naive T cells in the periphery[20]. The study of Tregs is currently focused on human and veterinary research, due to numerous reports about the upregulation of Tregs playing an important role in the suppression of an abnormal immune response, alleviating the symptoms of autoimmune diseases, and treating allergic diseases[1].

The mucosal microbiota is known to affect the immune system; however, the strains of intestinal bacteria that influence the qualitative and quantitative production of Tregs are not known. *Lactobacillus* biogenics include beneficial bioactive substances produced by *Lactobacilli*.[23] *Lactobacillus* biogenics have the potential to affect the intestinal microbiota by activating intestinal immune function through regulatory and immunostimulatory effects[2,3,5,7,19].

In this study, we examined the immunomodulatory effect of *Lactobacillus* biogenics by analyzing Treg levels in the blood of dogs continuously administered biogenics. As a parameter of the mucosal immune status or systemic allergic status, blood levels of immunoglobulin A (IgA) or immunoglobulin E (IgE) in plasma were assessed. In order to analyze the fecal microbiome, 16S ribosomal RNA metagenome analysis was performed.

Four dogs that had been blood donors or owned by the hospital. (weighing 1.8, 4.8, 18.0, 33.0 kg, three castrated males, and one spayed female) were used in this study. They were all kept indoors and were fed a comprehensive nutritional diet. Their blood albumin levels were normal, and they showed no chronic gastrointestinal symptoms such as diarrhea or vomiting. They did not suffer from inflammatory bowel disease (IBD) or allergic diseases such as allergic dermatitis. The dogs were regularly examined medically and dewormed to serve as blood donor dogs. Table 1 shows an overview of the characteristics of dogs used in this study. All dogs were fed the same food containing *Lactobacillus* fermented metabolites (SOPHIA Co., Ltd, Tokyo, Japan) (1 g/day) for the duration of the study. This supplement is made from 16 different types (25 strains) of dead *Lactobacilli*. The day we began administration of the *Lactobacillus* fermented metabolites was designated as day 0. We collected blood and stool samples on day 0 and then at 14-day intervals: on days 14, 28, 42, and 56. Blood sampling sites on the dogs were always disinfected before blood collection. Blood sampling from dogs in this study was performed at the same time with a health check on days 14, 28, 42, 56, and 112. We halted the regimen of administration of biogenics on day 56 and sampled blood and stools for an additional 56 days, that is, for a total of 112 days in the study. This research was approved by Animal Care and Use Committee of Tokyo University Agriculture and Technology (approval number 30-10).

Using the seven categories of stool consistency described in the fecal scoring chart from Nestle´Purina's guidelines for animal welfare[4], we categorized the scores as follows: 1-2 as constipation, 3-5 as normal stools, and 6-7 as diarrhea. All dogs had hard-to-normal stools, and none of the dogs had soft stools. Thus, stools were found to be stable throughout the study periods in all dogs. Body conditioning scoring (BSC) is categorized on a 5-point scale: 1 is defined as underweight, 2 as slightly underweight, 3 as ideal weight, 4 as slightly overweight, and 5 as overweight[10]. *Lactobacillus* biogenics administration did not appear to have any significant effects on BCS, the total blood cell count, and on the white blood cell count (Supplemental Table 1). We compared the basic information between pre- and post-treatment using the Wilcoxon signed-rank test. Results obtained had $p > 0.01$. During this period, none of the dogs exhibited symptoms such as diarrhea and vomiting.

Table 1. Information of dogs used in this study.

number	Age (year)	sex	breed	BCS	BW(kg)
1	5	S	Chihuahua	4	1.8
2	12	C	Dachshund	3	4.8
3	8	C	Golden Retriever	3	33.0
4	3	C	mix	3	18.0

C: castrated male, S: spayed female.
BCS was determined at day 0.

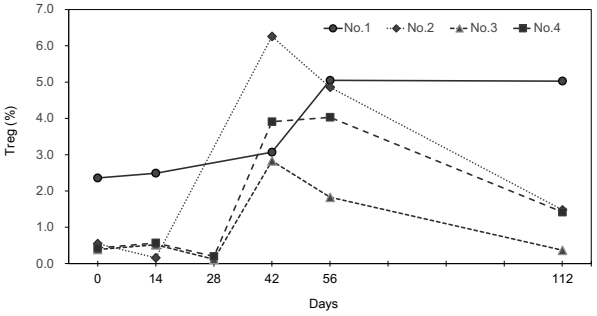

Fig. 1.
Ratio of Tregs to helper T cells. Number of days of *Lactobacillus* administration are shown on the X axis, and the ratio of Tregs to helper T cells is shown on the Y axis.

To elucidate the effects of administration of *Lactobacillus* biogenics on the number of Tregs, PBS (-) was added to blood cells and mononuclear cells (PBMCs) were separated using the Ficoll Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). 5 µL/tube Anti-Fc antibody (Thermo Fisher Scientific, New York, NY, USA) was added to each sample after three washes with the FACS buffer (PBS containing 2 % FCS and 0.1 %NaN_3) and samples were incubated for 20 min at 4 °C. After labeling with the FITC-conjugated anti-CD4 antibody (Thermo Fisher Scientific) and the PE-conjugated anti-CD25 antibody (Thermo Fisher Scientific) for 30 min at 4 °C, the samples were washed three times, and then centrifuged. The Foxp3 fixation/permeabilization working solution was added, and the cell samples were incubated for 60 min at 4 °C and washed three times with the FACS buffer. Samples were stored in the dark at 4 °C. After two washes with the permeabilization buffer at room temperature, pellets were resuspended in the permeabilization buffer. The PE-Cyanine 5-conjugated anti-Foxp3 antibody (Thermo Fisher Scientific) was then added and samples were incubated at room temperature for 30 min. After washing with the permeabilization buffer three times, cells were resuspended in 500-1000 µL FACS buffer and analyzed using the FACSCalibur cytometer[8,15]. This analysis was conducted for 20,000 cell events, and we calculated the ratio of CD25- and FOXP3-double-positive cells (Tregs) to all CD4-positive cells (T helper cells). Fig. 1 shows that the Treg percentage was elevated in four dogs until day 42 compared with that on day 0. In addition, on day 112, 56 days after the last administration of *Lactobacillus* biogenics, the percentage of Tregs decreased in three dogs (No. 2, 3, and 4). A recent report indicated that the percentage of Tregs in dogs that do not suffer from immunological diseases such as IBD and allergic dermatitis, ranges from 1 to 4 %[11]. Therefore, the four dogs used in our study were kept healthy throughout treatment.

Next, we measured the concentrations of IgA and IgE in the blood of these dogs using a Dog IgA ELISA Quantitation kit (Bethyl Laboratories, Inc., Montgomery, TX, USA) and Dog IgE ELISA Quantitation kit (Bethyl Laboratories, Inc.). Figs. 2 and 3 show the levels of IgA and IgE, respectively.

The bacterial composition of the stool samples was analyzed using 16S metagenome analysis as described previously[6,18]. Briefly, DNA was extracted from 200 mg of fecal samples using the QIAamp Fast DNA Stool Mini Kit (Qiagen). PCR was performed for the V3-V4 region of 16S rRNA genes using universal primers F341 and R805 according to the high-throughput sequencing protocol (Illumina, San Diego, CA, USA), as previously described[6], and the barcoded amplicons were processed using Premix EX Taq (TaKaRa

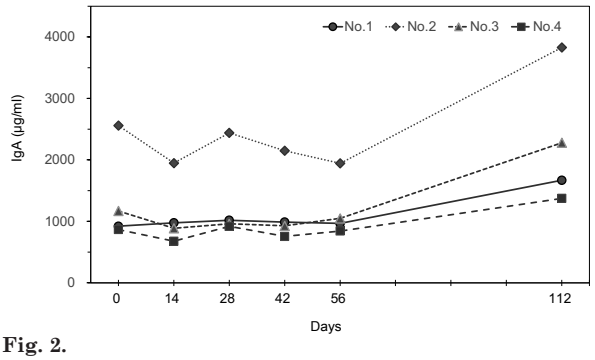

Fig. 2.
Plasma IgA concentration. The X axis shows the number of days of *Lactobacillus* administration and the plasma IgA concentration (μg/ml) is shown on the Y axis.

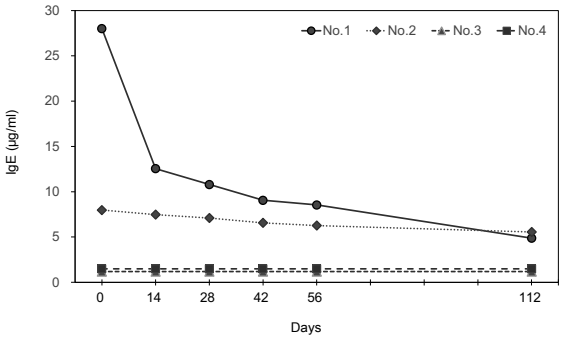

Fig. 3.
Plasma IgE concentration. The X axis shows the number of days of *Lactobacillus* administration and plasma IgE concentration (μg/ml) is shown on the Y axis.

Bio, Otsu, Japan). PCR conditions were as follows: 94 °C for 3 min; 25 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 oC for 30 s, and a final extension at 72 °C for 10 min. For purification of the PCR products, we used the Agencourt AMPure XP kit (Beckman Coulter, Brea, CA). To attach dual indices and Illumina sequencing adapters, the second round of PCR was performed as follows: 98 °C for 30 s; 8 cycles of 98 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. PCR products were purified, and the amplicons were sequenced using the MiSeq platform (Illumina, San Diego, CA, USA) using the MiSeq Reagent kit v3 (600 cycles) with 300 paired-end reads (Illumina). A total of 26,014,126 reads of 16S rRNA genes were obtained from 23 fecal samples with a range between 399,514 and 1,596,372 reads per sample. A total of 30,000 raw sequence reads per sample were extracted from all the raw reads obtained on MiSeq. Extracted reads were quality trimmed, combined with fastq-join script, and chimera depleted using USEARCH 6.1 using QIIME 1.9.1. Clustering into operational taxonomic units (OTUs) was carried out at 97 % similarity against the Greengenes database 13.8, and taxonomic ranks were assigned to OTUs using QIIME. Canine fecal microbiomes were classified into five phyla (Fig. 4). Although *Fusobacteria* was predominant in stool samples of dog No. 3, *Bacteroidales* was found to be predominant after administration. On the other hand, Fusobacteria was found to be predominant in the No. 1 dog after stopping administration. The microbiome status of dog No. 2 on day 112 and that of dog No. 3 at 0 day was similar. In case of dog No. 4, the percentage of *Bacteroidales* was found to be increased after stopping administration. Furthermore, we analyzed fecal microbiomes of species level and performed a statistical comparison before and after treatment with *Lactobacillus* biogenics to the same individual. We found that 99 % members of *Prevotellacease* belonged to species *copri*, which is an intestinal inflammation-related microbiota. *P. copri* was the only variety with a ratio of 1 % (1.0-44.3%)or more that had a significant difference ($p < 0.01$) based on the Wilcoxon signed-rank test. These results suggest that *Lactobacillus* biogenics administration changed the numbers of *P. copri* in these dogs. Fig. 5 shows that the percentage of the population of *P. copri* decreased in four dogs until day 56 compared with that on day 0.

Results of the present study provide preliminary data with a small data set. This study showed the immunomodulatory effect of *Lactobacillus* biogenics in healthy dogs. A recent report indicated that the percentage of Tregs in dogs that do not suffer from immunological diseases such as IBD and allergic dermatitis, ranges from 1 to 4 %[11]. The percentage of Tregs

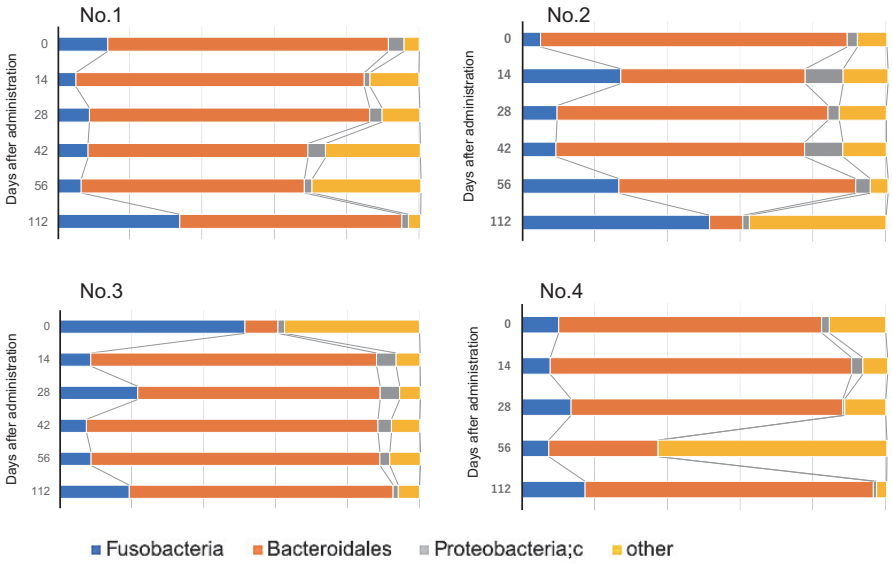

Fig. 4.
Representation of the fecal microbiome population at the phylum level in four dogs. The OTUs obtained using QIIME were classified up to the phylum. The percentage of various phyla found in the fecal microbiome are shown on the X axis and the number of days *Lactobacillus* administration are shown on the Y axis.

in three out of the four dogs used in our study exceeded the ranges in 56 days treatment period.

The study revealed that the majority of dogs had an elevated number of Tregs during administration of *Lactobacillus* biogenics. *Lactobacillus* biogenics affect the bacteria responsible for producing short-chain fatty acids, which in turn are involved in the induction of Tregs[12] and thus *Lactobacillus* biogenics were considered to be involved in the reduction of numbers of *P. copri* in canine microbiomes in this study. A previous study reported that *P. copri* is associated with the occurrence of rheumatoid arthritis, and high *P. copri* numbers can significantly increase the susceptibility of mice to colitis induced by glucan sulfate[24]. Other studies have indicated that *P. copri* may help drive chronic inflammation in HIV-infected people[14]. All of these studies showed a strong relationship between *P. copri* and inflammation[24,25]. Our study indicated that short-term administration of *Lactobacillus* biogenics may affect inflammation in dogs by decreasing the population of *P. copri*.

Three out of the four dogs showed a decrease in the number of Tregs 56 days after the end of probiotic administration. These results suggested that the intestinal environment was affected by the cessation of the supply of *Lactobacillus* biogenics. The average plasma IgE concentrations in dogs that do not suffer from immunological disease are reported to be 16.3 µg/ml[13]. Although, the concentration of IgE in dog No. 1 was higher than this range on day 0, IgE concentrations in all dogs, including dog No. 1, were below this range after day 14 (Fig. 3). Thus, three out of the four dogs used in this study showed no apparent changes in plasma IgE concentration, which is related to immediate allergies[9]. We presume that the possible reason is that all the dogs were in good health during the probiotic regimen. A recent study indicated that the range of plasma IgA concentrations in dogs, which do not suffer from immunological disease, ranges from 1000 to 3000 µg/ml[17]. During Lactobacillus biogenics administration from 0 to 56 days in this study, the IgA concentration in the four dogs was within

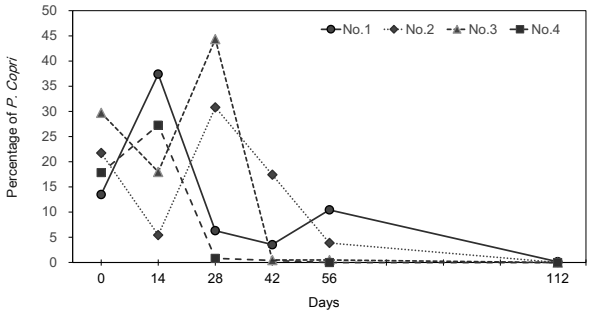

Fig. 5.
The Ratio of *P. copri* to the fecal microbiome of species level. The number of days *Lactobacillus* administration are shown on the X axis and the ratio of percentage of *P. copri* to that of the fecal microbiome of species level on the Y axis.

this range (Fig. 2). The plasma IgA concentration was slightly elevated in all the dogs on day 112, suggesting that IgA production was related to the lack of administration of *Lactobacillus* biogenics.

Treg counts of all dogs used in this study between days 0 and 56 days showed significant difference ($p < 0.01$) as determined using the Wilcoxon signed-rank test. This study reveals the link between *Lactobacillus* biogenics and an increase in Treg concentration. Honda *et al.* observed that mice bred in room temperature and normal humidity environment have a high of Treg counts in their colon, and conversely, mice raised in a sterile environment (germ-free) show a sharp decrease in Treg count[1]. Moreover, it has been discovered that introduction of strains of intestinal bacteria (e.g., bacteria of the *Clostridium* genus) into germ-free mice greatly increases the Treg counts in the intestine. It has also been found that mice infected with *Clostridium* are resistant to intestinal inflammation and are less prone to allergic reactions. Based on these data, it is hypothesized that an improvement in intestinal microbiota could lead to suppression or alleviation of autoimmune diseases through an increase in Treg levels.

This study showed the immunomodulatory effects of *Lactobacillus* probiotics on dogs. For further research, it will be necessary to increase the statistical sample size and to monitor animals with diseases such as inflammatory bowel disease. Lastly, this study covered a short period of only 56 days, future studies however, should ideally involve a longer regimen of probiotic administration.

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Supplemental data

Supplemental data associated with this article can be found, in the online version, at https://doi.org/10.14943/jjvr.69.3.175

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