

## Effect of Recombinant *Lactobacillus* Expressing Canine GM-CSF on Immune Function in Dogs

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Many *Lactobacillus* strains have been promoted as good probiotics for the prevention and treatment of diseases. We engineered recombinant *Lactobacillus casei*, producing biologically active canine granulocyte macrophage colony stimulating factor (cGM-CSF), and investigated its possibility as a good probiotic agent for dogs. Expression of the cGM-CSF protein in the recombinant *Lactobacillus* was confirmed by SDS-PAGE and Western blotting methods. For the *in vivo* study, 18 Beagle puppies of 7 weeks of age were divided into three groups; the control group was fed only on a regular diet and the two treatment groups were fed on a diet supplemented with either  $1 \times 10^9$  colony forming units (CFU)/day of *L. casei* or *L. casei* expressing cGM-CSF protein for 7 weeks. Body weight was measured, and fecal and blood samples were collected from the dogs during the experiment for the measurement of hematology, fecal immunoglobulin (Ig)A and IgG, circulating IgA and IgG, and canine corona virus (CCV)-specific IgG. There were no differences in body weights among the groups, but monocyte counts in hematology and serum IgA were higher in the group receiving *L. casei* expressing cGM-CSF than in the other two groups. After the administration of CCV vaccine, CCV-specific IgG in serum increased more in the group supplemented with *L. casei* expressing cGM-CSF than the other two groups. This study shows that a dietary *L. casei* expressing cGM-CSF enhances specific immune functions at both the mucosal and systemic levels in puppies.

**Keywords:** Canine GM-CSF, *Lactobacillus casei*, *in vitro*, *in vivo*

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Granulocyte macrophage colony stimulating factor (GM-CSF) is a cytokine with a unique ability to stimulate differentiation of hematopoietic progenitor cells into dendritic cells, a potent antigen-presenting cell [1, 14]. Its main activity is to increase the production, oxidative metabolism, phagocytic activity, and microbicidal activity of granulocytes (mainly neutrophils and eosinophils) and macrophages from bone marrow progenitor cells [10, 22–24, 27, 31, 32, 34]. Therefore, GM-CSF is an obvious molecule to be used clinically to counteract the side effects of treatments that deplete granulocytes and macrophages not only in human medicine, but also in veterinary medicine.

Because of its differentiation and activation properties on antigen-presenting cells, as well as some other immunomodulatory effects, GM-CSF is an interesting molecule to be used as an adjuvant in vaccination strategies, and several studies were already published in this field [6, 33].

The biologic activity of GM-CSF demonstrated different results in different species. Murine GM-CSF does not stimulate human hematopoietic cells, and human GM-CSF (hGM-CSF) does not stimulate murine hematopoietic cells. Bovine GM-CSF has little stimulatory effect on human and murine cells [20]. However, hGM-CSF stimulates canine hematopoiesis, according to *in vitro* assays. Despite of the *in vitro* assays result, neutropenia was observed in dogs to which recombinant human GM-CSF (rhGM-CSF) was administrated for 30 days. Neutropenia is associated with the development of antibody to rhGM-CSF [13, 28]. Therefore, species-specific GM-CSF was required for therapeutic application in veterinary medicine. Despite increasing demand, application of GM-CSF was difficult in veterinary medicine because of its high cost but low

effectiveness. Therefore, a conveniently applicable agent of GM-CSF that is cost-effective and safe was required.

Oral administration of diverse lactic acid bacteria is cost-effective and simple [26, 29]. *Lactobacillus* strains have a number of properties that make them attractive candidates for oral adjuvant purposes. For centuries, lactobacilli have been used in bioprocessing and preservation of food and feed, and they are considered "safe" organisms. On the other hand, other live carriers (e.g., *Salmonella*, *Escherichia coli*, and *Vaccinia*) cannot be classified as "safe." They are themselves highly immunogenic [26]. In addition, certain strains of *Lactobacillus* can colonize the gut to prevent intestinal infections. It was suggested in previous studies that lactobacilli or products made by these organisms have antagonistic effects against undesirable intestinal infections and certain forms of cancer [12]. Previously, it was shown that certain *Lactobacillus* species have the capacity to evoke a mucosal as well as a systemic immune response against epitopes associated with these organisms after oral administration [11]. In addition, individual *Lactobacillus* strains are clearly distinct in various properties, and therefore, strain selection is very important. In this study, *Lactobacillus casei* was chosen for recombinant protein expression because of its desirable adjuvant properties [7, 18, 26].

This paper describes the advanced oral agent of recombinant canine granulocyte macrophage colony stimulating factor (cGM-CSF) through the expression of cGM-CSF protein in *Lactobacillus casei*. The effects of this agent were investigated by *in vitro* and *in vivo* studies.

## MATERIALS AND METHODS

### Construction of cGM-CSF Gene Expressing Plasmid and Insertion into *L. casei*

The gene encoding cGM-CSF (GeneBank Accession No. S49738) without signal sequence and the pHAT-*pgsA* fusion vector (BioLeaders Korea Corporation, Daejeon, Korea) were fused (Fig. 2), and transformed into *E. coli*. Cloned plasmid was extracted and re-transformed into *L. casei*, isolated from human by electroporation, and incubated in deMan, Rogosa, and Sharpe (MRS) medium containing 30 µg/ml of erythromycin.

### Expression of cGM-CSF on *L. casei*

Transformed *L. casei* was cultured in 5 ml of MRS medium containing 30 µg/ml of erythromycin at 30°C, anaerobically. The cells were harvested by centrifugation at 4,200 ×g for 10 min. The cells were resuspended with distilled water and kept at -20°C until use.

Expression of recombinant cGM-CSF protein was analyzed by Western blot. The cellular proteins, adjusted to 80 µg/ml, were loaded onto 12% polyacrylamide gel and separated at 100 V for 2 h. The separated proteins were electronically transferred to nitrocellulose membrane and placed to react with anti-cGM-CSF antibody (R&D Systems, MN, U.S.A.) and biotin-conjugated mouse anti-goat IgG antibody (R&D Systems, MN, U.S.A.) sequentially. Blots were

developed using the Chemiluminescent ECL Detection Kit (Amersham Inc., U.K.) and exposed to Hyperfilm (Amersham Inc., U.K.).

### Fraction of *Lactobacillus*

One ml of overnight cultured recombinant *Lactobacillus* was inoculated to 100 ml of MRS broth containing 30 µg/ml erythromycin. The cells were incubated overnight at 37°C and harvested with a centrifuge at 6,000 ×g 4°C for 10 min. The pellet was washed with pure PBS three times and suspended in 10 ml of PBS, including 2 mM PMSF, and then sonicated three times (each time 1 min) on ice. The lysate was centrifuged at 11,000 ×g, 4°C for 15 min. One ml of supernatant was transferred to an ultracentrifuge tube and ultracentrifuged at 13,720 ×g, 4°C, for 60 min in the vacuum. Finally, 1 ml of the cytoplasm sample in the supernatant was collected. The cell wall and membrane sample in the pellet were resuspended with 500 µl of TE buffer. Each sample was incubated overnight at -20°C and confirmed by SDS-PAGE and Western blot.

### *In Vivo* Assessment of *Lactobacillus casei* Expressing cGM-CSF Protein in Young Dogs

**Animal and material administration.** Unvaccinated 7-week-old Beagle puppies were used in this study. Eighteen dogs were divided into three groups, regardless of sex and body weight; control group (n=6), experimental group 1 with *L. casei* (n=6) administered, and experimental group 2 with *L. casei* expressing the cGM-CSF protein (n=6) administered. All dogs were vaccinated subcutaneously with a killed canine corona virus (CCV) vaccine (Pfizer, U.S.A.) at 12 weeks of age (week-4 trial). During the experiment, the test dogs were cared for according to the Animal Care and Use Guidelines [Institutional Animal Care & Use Committee (IACUC) guideline], and the dogs were each housed in separate cages and fed on commercial dry dog food and water.

The strains of *L. casei* and *L. casei* expressing the cGM-CSF protein were fermented and lysophilized, at a dose of 1×10<sup>9</sup> colony forming units CFU/g, by the Department of Veterinary Bacteriology, College of Veterinary Medicine, Chungbuk National University, Cheongju, Korea. These materials were administered at a dose of 1×10<sup>9</sup> CFU/day orally for 7 weeks, which was the experimental period.

**Measurement of body weight and hematology.** Body weights of test dogs were measured once a week for 7 weeks (total 8 times; 0, 1, 2, 3, 4, 5, 6, and week 7 of trial). Blood samples were obtained from each group for hematology analysis once a week for 7 weeks (total 8 times; 0, 1, 2, 3, 4, 5, 6, and week 7 of trial). Complete blood cells and differential cells were calculated using a cell counter analyzer MS9-5V (Melet Schloesing, France).

### Measurement of various immunoglobulins by ELISA analysis.

A 0.5 g fecal sample and 1.5 ml of blood sample from each puppy were collected once a week for 7 weeks (total 8 times; 0, 1, 2, 3, 4, 5, 6, and week 7 of trial). Sandwich enzyme-linked immunosorbent assay (ELISA) method was used to quantify the total concentration of IgA and IgG in the feces and serum. A commercially available IgA and IgG antibody ELISA quantitation kit (Bethyl Laboratories, Montgomery, U.S.A.) was used with one alteration. The concentrations of specific IgG to CCV were measured using the indirect ELISA kit mentioned above with one difference. A killed CCV vaccine was used for coating antigen in this study. The microtiter plates were read at optical density (OD) 450 nm by a Model 680 Microplate Reader (Bio-Rad, U.S.A.).

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agg egg atg tgg ctg cag aac ctg ctt ttc ttg ggc act gtg gtc tgc
Arg Arg Met Trp Leu Gln Asn Leu Leu Phe Leu Gly Thr Val Val Cys
1 5 10 15
agc atc tct gca ccc acc cgc tca ccc acc ctt gtc act cgg ccc tct
Ser Ile Ser Ala Pro Thr Arg Ser Pro Thr Leu Val Thr Arg Pro Ser
20 25 30
cag cac gtg gat gcc atc cag gaa gcc ctg agc ctt ttg aac aac agt
Gln His Val Asp Ala Ile Gln Glu Ala Leu Ser Leu Leu Asn Asn Ser
35 40 45
aat gac gtg act gct gtg atg aat aaa gca gta aaa gcg gcc tct gaa
Asn Asp Val Thr Ala Val Met Asn Lys Ala Val Lys Val Val Ser Glu
50 55 60
gtg ttt gac cct gag ggg cca aca tgc ctg gag acc cgc cta cag ctg
Val Phe Asp Pro Glu Gly Pro Thr Cys Leu Glu Thr Arg Leu Gln Leu
65 70 75 80
tac aag gag gcc ctg cag gcc agc ctc acc agc ctc aag aat ccc tta
Tyr Lys Glu Gly Leu Gln Gly Ser Leu Thr Ser Leu Lys Asn Pro Leu
85 90 95
acc atg atg gcc aat cac tat aag cag cac tgt ccc cct acc ccg gaa
Thr Met Met Ala Asn His Tyr Lys Gln His Cys Pro Pro Thr Pro Glu
100 105 110
tct ccc tgt gca acc cag aat att aac ttc aaa agt ttc aaa gag aac
Ser Pro Cys Ala Thr Gln Asn Ile Asn Phe Lys Ser Phe Lys Glu Asn
115 120 125
ctg aag gat ttt ctg ttt aac atc ccc ttt gac tgc tgg aaa cca gtc
Leu Lys Asp Phe Leu Phe Asn Ile Pro Phe Asp Cys Trp Lys Pro Val
130 135 140
aag aag tga
Lys Lys Stop
145
    
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Fig. 1. Canine GM-CSF sequence. There is a 381-base-pair nucleotide sequence encoding canine GM-CSF without signal sequence between the bolded lines.

**Statistical Analysis**

One-way ANOVA was performed to test the overall differences among three groups, and the results were further characterized using Tukey's honestly significant difference test for multiple comparison. All data were analyzed using the SPSS software package (version 12.0, SPSS Inc., Chicago, IL, U.S.A.). A *p* value <0.05 was considered statistically significant.

no remarkable difference by statistical analysis (*p*>0.05). Complete blood count and differential cell count were measured once a week during the test period. There were no difference in the count of white blood cells (WBC), lymphocytes, band neutrophils, segment neutrophils, eosinophils, and basophils among the three groups (*p*>0.05). However, at weeks 4, 5, 6, and 7, the population of monocytes

**RESULTS**

**Generation of Recombinant *L. casei* Expressing cGM-CSF**

The 181-bp nucleotides encoding cGM-CSF without signal sequence were verified (Fig. 1). The expressed *pgsA* (42 kDa)-cGM-CSF (14 kDa) fusion protein (56 kDa) from *L. casei* was detected using anti-cGM-CSF antibody (Fig. 3A). Furthermore, *Lactobacillus* fractionation was performed in order to verify whether the expression of cGM-CSF protein was on the surface or the cytoplasm. At the end, it was found that the expression of the cGM-CSF protein was on the surface of *L. casei* (Fig. 3B).

**Enhanced Abilities on Production of Monocytes and IgA, and CCV Vaccination in Dogs After Oral Administration of Recombinant *L. casei* Expressing cGM-CSF**

The rate of body weight increase in the control group, experimental group 1, and experimental group 2 showed

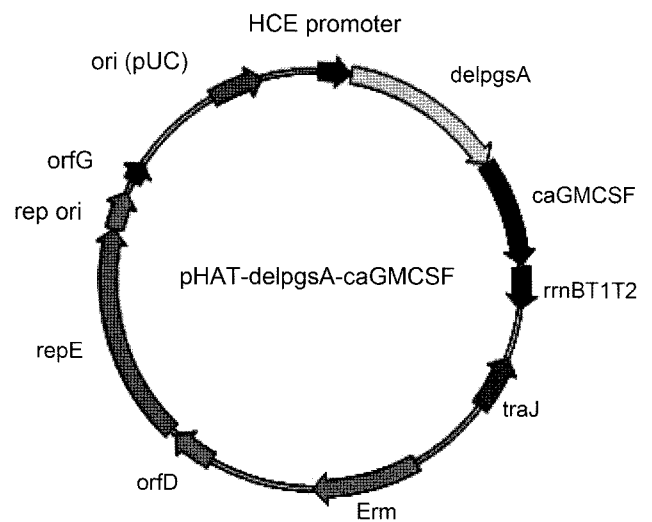
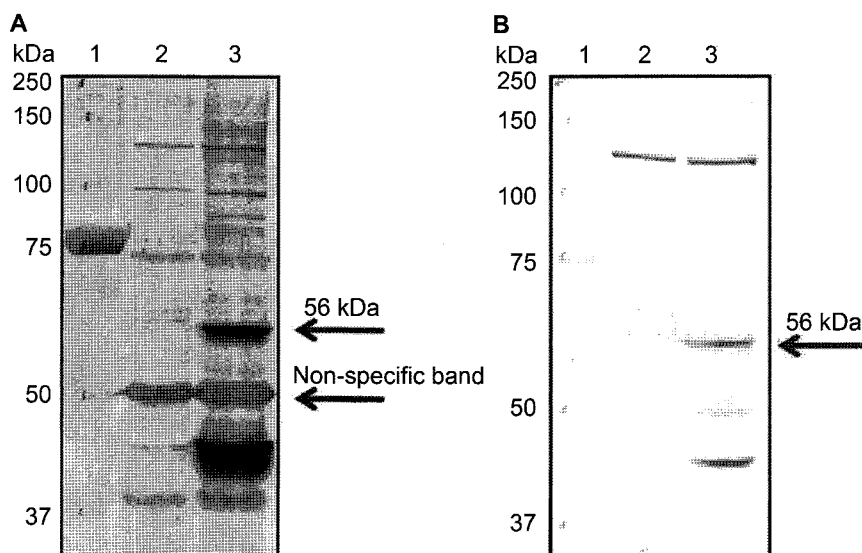


Fig. 2. Diagrammatic representation of the vector containing canine GM-CSF.

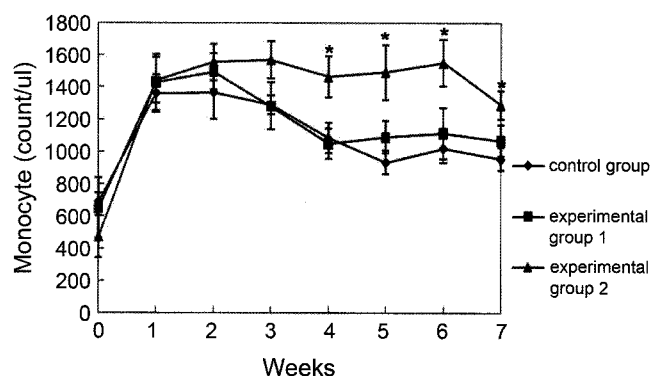


**Fig. 3.** Western blotting analysis.

**A.** Expressed *pgsA*-canine GM-CSF fusion protein from *L. casei*, a band of approximately 56 kDa was detected. Lane 1: size marker; lane 2: wild-type *L. casei*; lane 3: recombinant *L. casei*. **B.** Cytoplasm and cell wall of recombinant *L. casei* from the fractionation method. A band of approximately 56 kDa was detected in the cell wall lane. Lane 1: size marker; lane 2: cytoplasm; lane 3: cell wall.

in experimental group 2 was significantly greater compared with the other two groups ( $p < 0.05$ ). There was no remarkable difference between the control group and experimental group 1 ( $p > 0.05$ ) (Fig. 4).

The fecal contents were collected, and the concentrations of their IgA and IgG were measured once a week, in all puppies of the three groups. The concentration of IgA in the fecal content tended to be greater in experimental groups 1 and 2 than in the control group at weeks 2, 3, 4, 5, 6, and 7. However, the change was not considered statistically significant ( $p > 0.05$ ). There was no difference



**Fig. 4.** Variation of monocyte population in the blood samples collected from the control group, the experimental group 1 (administration of *L. casei*), and the experimental group 2 (administration of *L. casei* expressing canine GM-CSF protein) during the test period.

Monocyte population in the experimental group 2 was higher from week 2 than those in the other two groups. Each value represents the mean  $\pm$  SEM,  $n = 6$ . \*Experimental group 2 compared with control was significantly different,  $p < 0.05$ .

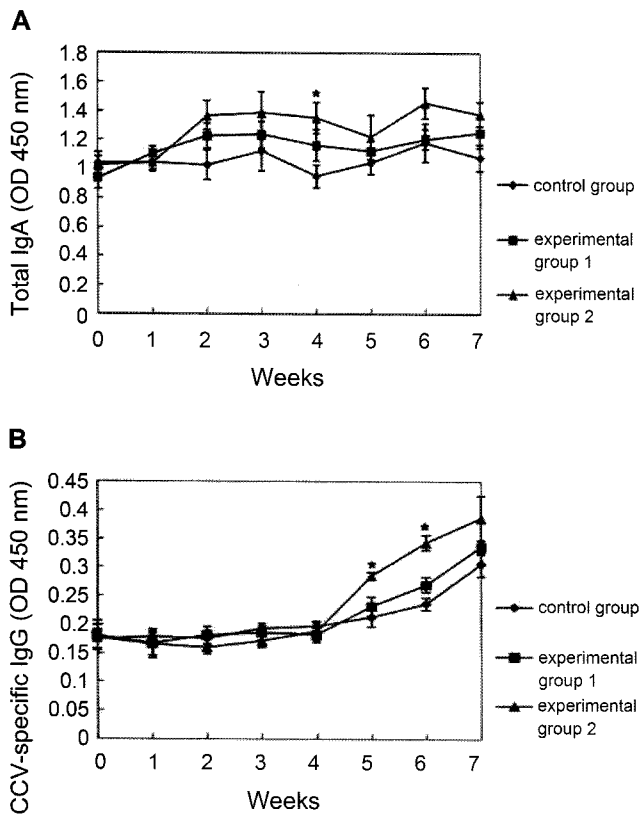
in the concentration of total fecal IgG among the three groups during this test period ( $p > 0.05$ ).

The concentration of total serum IgA tended to be greater in experimental group 2 than that of the control group and experimental group 1 at weeks 2, 3, 4, 5, 6, and 7. The concentration of experimental group 1 tended to be greater than that of the control group at weeks 2, 3, 4, 5, 6, and 7 (Fig. 5A). However, the serum IgA levels in experimental group 2 were significantly higher compared with that of the control group at only week 4 ( $p < 0.05$ ). However, there were no differences in the level of serum IgG among the three groups ( $p > 0.05$ ).

Interestingly, the response to CCV vaccination was stronger in experimental group 2 than both the control group and experimental group 1 (Fig. 5B). After vaccination at week 4 of this trial, the change in experimental group 2 at weeks 5 and 6 was considered statistically significant ( $p < 0.05$ ).

## DISCUSSION

Methods of making and using the recombinant *Lactobacillus* strains as probiotic composition will be useful in inhibiting enteric bacterial diseases in animals and in maintaining animal health, since *Lactobacillus* in itself, as one of lactic acid bacteria (LAB), beneficially affects the gastrointestinal balance and goes far beyond the conventional nutritional effect [16]. Moreover, GM-CSF may be a particularly effective and attractive factor influencing many immune functions, because GM-CSF has well-documented stimulatory effects on monocyte, macrophage, and antigen-presenting



**Fig. 5.** A. Variation of total IgA in serum samples collected from the control group, the experimental group 1 (administration of *L. casei*), and the experimental group 2 (administration of *L. casei* expressing canine GM-CSF protein) during the test period. The concentration of IgA in the experimental group 2 was higher than those in other two groups. B. Variation of CCV-specific IgG in serum samples from the control group, the experimental group 1, and the experimental group 2 during the test period. After injection of CCV vaccine at week 4, the level of CCV-specific IgG in experimental group 2 was higher than those in other two groups. Each value represents the mean  $\pm$  SEM,  $n=6$ . \*Experimental group 2 compared with control was significantly different,  $p < 0.05$ .

cell functions [1, 14, 21]. In this study, we tried to engineer a set of recombinant cGM-CSF-expressing *Lactobacillus casei* uniquely capable of enhancing specific immune functions at both the mucosal and systemic levels in dogs. For successful cGM-CSF expression in *L. casei*, the signal sequence of the cGM-CSF was deleted and replaced with *pgsA* in the pHAT-*pgsA* and cGM-CSF fusion expression system. The *pgsA* gene is a poly- $\gamma$ -glutamate synthetase complex from *Bacillus subtilis*, which was used as a display motif in this study [2, 3, 19]. The pHAT vector system containing the HCE promoter used in this study is one of the plasmid-based constitutive expression systems. It is a convenient and economical means to mass produce recombinant proteins [25]. Our results of Western blot and fractionation analyses show that the recombinant *L. casei* expresses cGM-CSF protein successfully, and its protein was effectively targeted to the bacterial cell surface, suggesting that *pgsA* acts as a transmembrane anchoring motif (Fig. 3).

Because the pHAT system constitutively produces foreign proteins as cells grow without the need for induction by a chemical compound for the expression of foreign proteins [15, 25], cGM-CSF protein produced in the recombinant *L. casei* could be effective as an oral administration of the recombinant strain. The recombinant *L. casei* expressing cGM-CSF was produced in powder type for easier consumption and longer preservation (it can be preserved in refrigerated state for around one year). Moreover, its effectiveness is satisfactory while the production cost can be maintained at a low level.

Since recently published studies stated that HPV16 E7 expressed in *Lactococcus lactis* using the *Streptococcus pyogenes* M6 protein induced systemic humoral and cellular immunity in a mouse model system [5, 8, 9], *L. casei* expressing cGM-CSF protein was orally administered to dogs in this study on the assumption that its primary target would be the gut-associated lymphoid tissue. Although there were no observations on the various hematologic effects of the proliferative activity of white blood cells, lymphocytes, and granulocytes in peripheral blood, our data showed the stimulatory effect on monocyte proliferation in the experimental group with recombinant *L. casei* expressing cGM-CSF orally administered. The results support that cGM-CSF harboring a hematologic function stimulates the differentiation of hematopoietic progenitors from monocytes. The increase of secretory IgA was observed in the intestine of the experimental group, orally administered with *L. casei* or *L. casei* expressing cGM-CSF, suggesting the possibility of an effective mucosal adjuvant [4, 17, 30, 34]. Interestingly, the response to CCV vaccination was stronger in the experimental group with recombinant *L. casei* expressing cGM-CSF orally administered than both the control group and experimental group orally administered with *L. casei* (Fig. 5B). This improvement of antibody response may enhance the effectiveness of the vaccine in preventing CCV infection.

In conclusion, we demonstrated a successful engineering of the recombinant *L. casei* expressing cGM-CSF, and that the recombinant strain has stimulatory effects on the production of monocytes and IgA, and CCV vaccination in dogs. Our data suggest that the recombinant cGM-CSF expressing *L. casei* could be useful as a good probiotic for enhancing specific immune functions in veterinary medicine. Furthermore, our study could help the development of good probiotic compositions using various cytokine agents, to be applied not only for other animals, but also for humans.

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