Research Article

Exploring the Protective Efficacy of *Lactobacillus acidophilus* Against Interleukin-1beta (IL-1β) Expression in Mice Induced with Canine Coronavirus Vaccine

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Abstract | The proposed study investigates the protective effects of Lactobacillus acidophilus against the expressions of tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) in the pulmonary section of mice induced with canine coronavirus (CCoV) vaccine. The research utilized male mice (Mus musculus) aged two to three months, divided into three groups: a control group administered with normal saline (D0), a CCoV-induced group (D1), and a group induced with CCoV along with 1 mL/kg body weight of Lactobacillus acidophilus for seven days (D2). The CCoV vaccine was administered subcutaneously at 0.5 mL/kg body weight, while the Lactobacillus acidophilus isolate was given orally for seven days. On the eighth day, all mouse groups were euthanized, and tissue samples were collected for immunohistochemical analysis. The findings revealed that CCoV vaccine induction in the treatment group resulted in duodenal mucosa haemorrhage, necrosis, and severe inflammation. Conversely, in the group treated with Lactobacillus acidophilus, tissue damage was inhibited, demonstrating the protective effect of the probiotic. Lactobacillus acidophilus effectively mitigated lung, liver, and duodenal damage by reducing the expressions of TNF-α and IL-1β in mice.

Keywords | Canine coronavirus, TNF-α, IL-1β, *Lactobacillus acidophilus*

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INTRODUCTION

Since emerging in Wuhan, China in 2019, the coronavirus has become a global pandemic, causing clinical symptoms like fever, chills, wheezing, fatigue, and shortness of breath. Respiratory complications and lung disorders, marked by edema and cellular changes, are evident, but the precise pathogenesis of COVID-19 remains unclear (Xu *et al*., 2020). Recent findings reveal that dysregulated immune responses play a central role, involving lymphopenia, reduced NK cells, CD8+ T-cells, and diminished type I interferon responses, leading to IL-8/CXCL8-induced

neutrophil recruitment and inflammatory thromboses. Alveolar damage may result from direct infection of AT2 cells or indirect effects from local inflammatory responses, causing endothelial activation. This cascade creates an environment conducive to inflammation and coagulation, involving dysfunctional immune cells and fibrin deposition (Lamers and Haagmans, 2022).

Until now, the classification of the main coronavirus (CoVs) groups was distinguished based on their phylogenetic and antigenic analysis and genetic linkage (96% amino acidity), namely Alphacoronavirus (Alpha-CoV) discovered in dogs and cats, Betacoronavirus (Beta-CoV) in bats, and Gammacoronavirus (Gamma-CoV) and Deltacoronavirus (Delta-Cov) in pigs, cattle, and poultry (Rodriguez-Morales *et al*., 2020). SARS-CoV-2, part of the Coronaviridae subfamily, belongs to the beta coronavirus group. Among human-infecting beta coronaviruses, SARS-CoV-2, SARS-CoV (2002–2003 outbreak), and MERS (2012 outbreak) caused severe diseases in human (Platto *et al*., 2021). Companion animals, including cats and dogs, have been found susceptible to various coronaviruses like feline coronavirus (FCoV), canine coronavirus (CCoV), SARS-CoV, and SARS-CoV-2. Studies indicate the potential for transmission of these coronaviruses between humans and animals, posing a potential threat of clinical diseases. There is an urgent need to comprehensively investigate the susceptibility, cross-species transmission, and tissue tropism of animal coronaviruses in close contact with humans to prevent potential transmission (Pandit *et al*., 2023). Meanwhile, the canine coronavirus (CCoV) is a gastrointestinal pathogen from the Alphacoronavirus-1 family that causes fatal diarrhea in puppies if there is a secondary microbial pathogens infection. Infection of CCoV in dogs and puppies causes immunosuppression condition characterized by systemic lowering of the lymphocyte (lymphopenia) together with a significant decrease of the CD4 macrophage in puppies (Buonavoglia *et al*., 2006; Tian *et al*., 2020).

Lactic acid bacteria are members of gram-positive and no-spore-forming bacteria that produce lactic acid, which is generally non-pathogenic bacteria. Recently, a group of lactic acid bacteria is familiar as probiotics. Several strains belonging to these bacteria are *Pediococcus* spp., *Bifidobacterium* spp., *Lactobacillus* spp., and *Streptococcus* spp. However, the most widely used probiotics are groups of *Lactobacillus* spp., such as *Lactobacillus casei*, *Lactobacillus acidophilus*, and *Lactobacillus rhamnosus*, and *Bifidobacterium* spp., such as *Bifidobacterium bifidum*, *Bifidobacterium longum*, and *Bifidobacterium breve*. Probiotics are beneficial living microorganisms that modulate specific functions and are active with specific molecular pathways in organisms. Probiotics, as a nourishment supplementation, cover several living microbes that benefit animals as their host by increasing the stability of microflora in the gastrointestinal tract. *Lactobacillus acidophilus* is a bacterium with unique activities of exerting both pathophysiological and cellular effects, regulating T-cell activity, inhibiting virus infection in rats, and promoting concentrations of interferon-γ (IFN-γ) (Eguchi *et al*., 2019).

Lactobacillus acidophilus, which can upregulate antiviral replication via specific receptor TLR (Toll-like receptor) signal, can be active in bile acids and maintain the balance of the intestinal milieu and fat accumulation. The lactic acid bacteria group can produce a vital enzyme to digest lactose and stimulate proteolytic-cellulolytic enzyme activity, increasing micro-nutrition absorption. As widely known, probiotics can protect food from microorganism contamination; moreover, some probiotic microorganisms can prevent fatal diarrhea and inhibit Salmonella infection in birds. Furthermore, feeding *Lactobacillus* sp. increases interferon signaling and suppresses apoptotic and inflammatory pathways of SARS-CoV-2 induced in mice (Borchers *et al*., 2009; Cutting, 2011).

Immune responses are divided into innate and adaptive immunities, with specific roles in host defense response. The former has a fast response, temporary defense, and short-term action against pathogens. This immunity includes leukocyte cells and specific receptors to combat bacterial and fungal infections. The latter is a cellular response by lymphocyte cells and their receptors. Both innate and adaptive immunities work synergistically in the host immune system by recognizing infection and memorizing antigen presentation. The critical action of innate cells is producing inflammatory substances, such as cytokines and chemokines, to activate the adaptive systems of B and T lymphocyte cells against pathogen infections and avoid over-immune response (Chon and Choi, 2010; Tellez *et al*., 2012).

Therefore, the objective of this study is to examine the protective role of *Lactobacillus acidophilus* against tumor necrosis factor alpha (TNF-α) and interleukin-1beta (IL-1β) expressions of pulmonary tissues on the canine coronavirus (CCoV) vaccine-induced mice.

MATERIALS AND METHODS

This study was conducted at the Experimental Animal Laboratory in the Faculty of Veterinary Medicine, Universitas Airlangga. The ethical clearance was obtained from Animal Ethics and Care Committee of the Faculty of Veterinary Medicine, Universitas Airlangga, under the reference number 15.KE.017.04.2021. An ethical assessment was carried out to safeguard the welfare of the animals and prevent any form of suffering or cruelty. The study employed mice (*Mus musculus*) meeting inclusion criteria of 2-3 months in age and weighing 30-40 grams. The mice were provided with mice pellets, had access to

distilled water ad libitum, and underwent a one-week rearing period for adaptation. In the treatment groups, mice were administered canine coronavirus (CCoV) vaccines at a dose of 60 mg/kg BW (Vanguard Plus 5®, Zoetis).

Eighteen mice (*Mus musculus*) were divided into three treatment groups. The groups included a control group (D0) receiving distilled water, a CCoV vaccine-induced group (D1), and a CCoV vaccine- and probiotics-induced group (D2). Groups D1 and D2 received a single intraperitoneal dose of 60 mg/kg BW of the CCoV vaccine, while group D2 also received oral probiotics for seven days.

The probiotics used in the study contained *Lactobacillus acidophilus* at a concentration of 7.5 x 10^8 CFU/gram. Lactobacillus acidophilus isolate with a concentration of 10^8 CFU/ml was dissolved in a growth medium composed of 100 g nutrient broth (NB) mixed with 500 g molasses, and water was added to reach a total volume of 1 liter. The media-isolate mixture was heated to 100°C for 15 minutes, then fermented for 12 hours and aerated for 14 days.

On the eighth day, all mice were euthanized through cervical dislocation, and liver, lung, and duodenum samples were collected for tissue extraction and immunostaining. Duodenal and lung slices were incubated in 10% neutral buffered formalin, embedded in paraffin, then sectioned to approximately 3-5 microns thickness using a microtome. The sections were subsequently stained using immunohistochemistry and hematoxylin-eosin (HE).

The appearance of TNF-α and interleukin-1β was recognized by indirect immunoperoxidase monoclonal antibodies (mAB), secondary anti-peroxidase antibodies coating, and diaminobenzidine substrate from an immunohistochemistry kit (Bioss, USA) labeled with streptavidin-biotin (LSAB) and horseradish peroxidase (HRP) detection system (ScyTek, USA).

Lung and liver material sections were inspected in the region holding alteration, infiltration cells, and necrotic faction under an Olympus light microscope with 400x magnifications. The proportion of tissue area with alteration was scored 0 (no change or regular), 1 (1-30% necrotic cells), 2 (31-50% necrotic cells), and 3 (51-100% necrotic cells). The analysis of TNF-α and IL-1β expressions included either a definite reaction (brownish mass) or an adverse reaction (no color of brown aggregate), the intensity, and the issue of immunoreaction cells. The immunopositive cells of the material area were scored based on percentage: 0 (no alteration or the cells were expected), 1 (1-30% definite cells), 2 (31-50% definite cells), and 3 (51-100% definite cells) (Muniroh *et al*., 2022).

Data analysis

Data from histomorphology and immunohistochemistry were examined under the light microscope with 400x magnifications. Their scores were then expressed as mean values with a confidence interval of 95% (95% CI) by SPSS 21, with a statistical significance level of $p \leq 0.05$ using the non-parametric Kruskal Wallis and Mann-Whitney test.

RESULTS AND DISCUSSION

The lung histopathology scores in the D0 group, which received normal saline, were significantly lower ($p \leq 0.05$) compared to the necrosis and infiltration cell scores observed in the D1 and D2 groups. Additionally, there was a significant difference ($p \leq 0.05$) in the scores of necrosis and infiltration cells between the treatment groups D1 and D2. These findings suggest that the D0 group displayed minimal tissue alterations in contrast to the D1 and D2 groups.

Meanwhile, the liver histopathology scores for the D0 were quite different ($p \leq 0.05$) from the necrosis and infiltration cells scores of the D1 and D2 groups. The TNF-α and IL-1β expressions increased in the CCoV-induced treatment groups D1 and D2 but decreased in the probiotic group D2. The appearance of inflammatory cytokines, acting as TNF-α and IL-1β, was identified by the presence of brown aggregates on duodenal immunohistochemistry stains (Table 1), compared to the D1 group, the infiltration and necrotic cell scores and the TNF-α and IL-1β expressions significantly dropped in the treatment group (D2) ($p \leq 0.05$) (Figure 1).

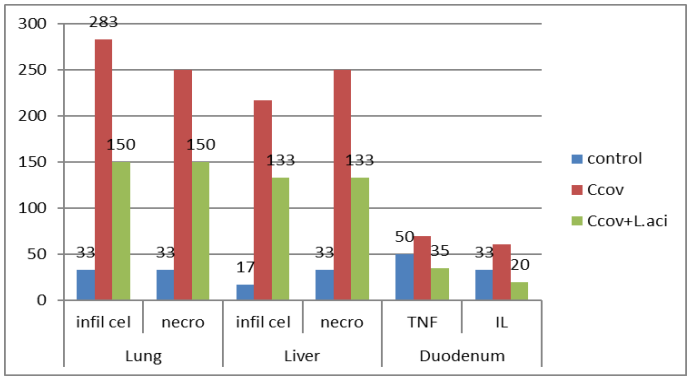

Figure 1: Histomorphological scores for cell infiltration, necrosis, TNF-α, and Interleukin-1beta in different groups.

The histology of lung tissues with diffuse alveolar cell damages, deposition of hyaline tissues, septa alveolar hemorrhages, leukocyte cell infiltration, and proliferation in alveolar septa areas found in the D1 group. Meanwhile, in the group with probiotics, the damage was decreased by neutrophil cell infiltration and plasma fibrin accumulation, as seen in the D2 group. Histomorphology of liver tissue revealed glycogen accumulation in hepatocyte cells, hemorrhage with dilated sinusoids, lymphocyte cell infiltration in the centrilobular area in the D1 group, and sinusoidal congestion and dilatation in the centrilobular area of the liver tissues in the D2 group (Figure 2).

Table 1: Comparison of varying histomorphological scores of lung, liver, and duodenum tissues among different groups.

Group	Lung (HE)		Liver (HE)		Duodenum (IHC)	
	Infiltration cell	Necrotic cell	Infiltration cell	Necrotic cell	TNF-α	IL-1β
D0 (n=6)	0.33 ± 0.52[a]	0.33 ± 0.52[a]	0,.17 ± 0.41[a]	0.33 ± 0.52[a]	0.50 ± 0.55[a]	0.33 ± 0.52[a]
D1(n=6)	2.83 ± 0.75[c]	2.50 ± 0.55[c]	2.17 ± 0.41[c]	2.50 ± 0.55[c]	7.00 ± 1.55[c]	6.17 ± 1.60[c]
D2 (n=6)	1.50 ± 0.55[b]	1.50 ± 0.55[b]	1.33 ± 0.52[b]	1.33 ± 0.52[b]	3.50 ± 1.38[b]	2.00 ± 0.63[b]

The superscription in the same column shows a significant difference ($p \leq 0.05$). Abbreviations; HE= hematoxylin-eosin, IHC= immunohistochemistry, IL-1β= interleukin-1beta, TNF-α= tumor necrosis factor-alpha, n= several animals, D0 = control group, D1= Canine coronavirus-induced group, D2= Canine coronavirus-induced +*L acidophilus* group.

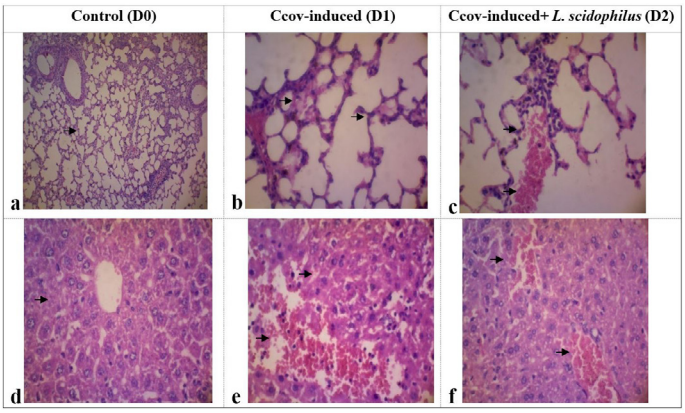

Figure 2: The histological examination of the control group (a, d) demonstrates typical cell structure in the lung tissues and liver. In the D1 group (b), lung tissues exhibit histomorphological feature such as diffuse alveolar cell damages, hyaline tissues deposition, septa alveolar hemorrhages, leukocyte cells infiltration, and alveolar septa proliferation. The D2 group (c) shows neutrophil cell infiltration and plasma fibrin accumulation in lung tissues. In the liver tissues of D1 group (d) shows an accumulation of glycogen in hepatocyte cells, hemorrhage with dilated sinusoids, infiltration of lymphocyte cells. The D2 group (e) present sinusoidal congestion and dilatation (HE, 400x, changes are indicated by arrows).

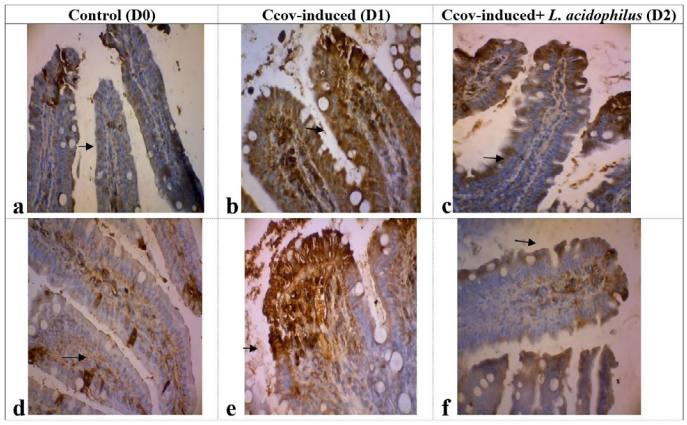

Figure 3: Positive TNF-α expression, shown as brown aggregates in the intestinal mucosa and submucosa duodenal (b), was significantly higher compared to the non-reactive control group D0 (a). in contrast, TNF-α expression in the isntestinal villi showed a moderate decrese (c) with non-specific IL-1β expression in the epithelia and intestinal crypts (d). Strong IL-1β immunoreaction, depicted as brown aggregates in the intestinal villi's epithelial and lamina propria area (e), contrasted with the weak IL-1β immunoreaction in the lamina propria area (immunohistochemistry, hematoxylin counterstained, arrows indicate morphological changes).

The immunohistochemical picture of the duodenal tissue demonstrating a positive reaction with the intensity and wide distribution of expression of the TNF-α, which was shown in brown aggregates in the epithelial and parietal areas, was found in the D1 group. In contrast, the one indicating immunoreactivity of the TNF-α brown aggregates in the epithelium and lamina propria of intestinal villi with moderate intensity was observed in the D2 group. Furthermore, the picture revealing a non-specific reaction to the IL-1β expression in the epithelia and intestinal crypts appeared in the control group. Brown aggregates depicted a strong immunoreaction of the IL-1β in the intestinal villi's epithelial and lamina propria areas in the D1 group. In contrast, a weak immunoreaction of the IL-1β expression was indicated by brown aggregates in the lamina propria area of the intestinal villi in the D2 group (Figure 3).

Antibody-antigen complexes were recognized by the complement system and then attached to the duodenal epithelium as inflammation signals. Meanwhile, the macrophage activation would phagocytose the antigen of the surface duodenal epithelium. TNF-α and IL-1B expression appearance were escalated in the CCoV-induced groups due to the inflammation in duodenal epithelial cells. The cytokines activation would stimulate inflammatory cells as mononuclear, neutrophils, and proteolytic enzymes in tissue destruction. On the other hand, the TNF-α and IL-1β activation stimulated more biological cell signaling, such as inflammation, injury, fibrosis, and cell healing (Lai *et al*., 2020).

The high levels of TNF-α and IL-1β expression indicated inflammation cells in the group induced by the CCoV vaccine. Meanwhile, the immune reactions of TNF-α and IL-1β had a lighter shade of brown, which stated that the probiotics feeding could inhibit the inflammation process

and did not destroy the intestinal tissues, only slight changes in its histological structure. Moreover, probiotic feeding proved to prevent destruction and contained beneficial effects on the gastrointestinal tract. Additionally, feeding probiotic strains of *Lactobacillus acidophilus* lowered cellular changes in the intestinal system.

TNF-alpha and IL-1b play crucial roles in CCoV infection. TLRs may regulate the expression of key inflammatory and immune response biomarkers such as ILs, TNF-α, interferons, and IRFs, contributing to damage control during CCoV infection. In CCoV-infected CRFK cells, significant biomarkers like HSP100, TNF-α, TGIF-1, TGIF-2, mCCL22, iNOS, caspase-1, and caspase-8 were observed in infection-derived EVs at different time points. Caspase-1 and caspase-8 are involved in inducing CRFK cell apoptosis and mediating CoV-induced inflammatory responses through the release of cytokines such as mCCL22, TNF-α, and ILs, along with IRF activation (Pandit *et al*., 2023).

Previously, studies on probiotics feeding concluded that it could increase the host animal's immune system by forming an antibody IgA in the gastrointestinal tract. The probiotic microbes' activities produced metabolic enzymes against pathogenic microbes in animals' intestinal systems. TNF-α is an important marker of inflammatory reactions. There have been many study results showing that using probiotics can improve inflammation. The use of probiotics can reduce the level of TNF-α (Nursalim *et al*., 2016). Other analysis results also confirm that Lactobacillus strains can survive in the digestive tract, adhere to the intestinal mucosa, and provide beneficial functions, such as delivering anti-oxidation, inhibiting α-glucosidase activity, lowering cholesterol, and providing anti-inflammatory effects (Oh *et al*., 2018). For example, *L. plantarum* has been shown to increase the production of pro-inflammatory mediators in cells, such as IL-β, IL-6, and TNF-α (Chon and Choi, 2010). Not only *L. plantarum* but other probiotics, such as *Bifidobacterium lactis,* can also improve colitis cases and show anti-inflammatory activity *in vitro* (Philippe *et al*., 2011).

In addition to reducing TNF-α levels, probiotics can regulate cyclooxygenase-2 (COX-2). Specific probiotics are considered an alternative therapy in COX2 regulation and expression in the intestinal epithelium to control inflammatory effects. Pathological pain is triggered by pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α (Oliveira *et al*., 2011). Therefore, it can be suggested that pain can be reduced and possibly prevented if cytokine production is inhibited.

Hart *et al.* (2004) reported that some probiotics could hinder the synthesis of the pro-inflammatory cytokine and regulate the synthesis of anti-inflammatory cytokine IL-10 from dendritic cells. In addition, probiotics can also suppress inflammation by preventing the activation of NFκB signals through IκBα phosphorylation inhibition (Thomas and Versalovic, 2010).

In a previous study, neutrophils were identified as the predominant cell infiltration, constituting 60% of the lung tissue. SARS-CoV-2 infection in the mouse model resulted in noticeable features such as hemorrhage, inflammatory cells, proteinaceous debris, hyaline membrane-like changes, and the formation of thrombi in the lung tissue (Bi *et al*., 2020).

Histomorphology of pulmonary tissues of the control group D0 depicted no alteration and expected findings. In contrast to the D0 group, the induction groups D1 and D2 displayed destructions in the alveolar septa area. The alveolar colors were basophilic and dark acidophilic, indicating more infiltration cell, hemorrhagic, and exudate accumulation. Furthermore, the lungs of the treatment group with *Lactobacillus acidophilus* had slight polymorphonuclear cell and plasma fibrin accumulation on the lumen alveoli, indicating a healing process in the lung parenchyma. Toxic and infectious pathogens, additionally, could develop lung alteration and affect the alveoli structure of the lungs. Thus, alteration in the lumen space, alveolar septa width, and alveolar cells would disrupt the alveolar function. Moreover, the treatment group with probiotics had limited destruction in the alveolar and septa alveolus areas.

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, the current research analysis demonstrates that *Lactobacillus acidophilus* has the potential to inhibit the expressions of TNF-α and IL-1β in the duodenum. Furthermore, the administration of *Lactobacillus acidophilus* in the treatment group is associated with a notable reduction in pulmonary necrosis and inflammation. These findings suggest a promising role for *Lactobacillus acidophilus* in modulating inflammatory responses both in the duodenum and lung.

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NOVELTY STATEMENT

This study introduces a groundbreaking aspect, revealing

that Lactobacillus acidophilus not only inhibits IL-1β and TNF-α expressions in the duodenum but also significantly reduces pulmonary necrosis and inflammation. These unprecedented results underscore the promising and multifaceted anti-inflammatory potential of *Lactobacillus acidophilus*, marking a significant advancement in its therapeutic application against canine coronavirus vaccine-induced responses in mice.

AUTHOR'S CONTRIBUTION

RS and ISH designed and conducted the study. RS collected the samples and analyzed the data. RS wrote the first draft of the manuscript. ISH revised the manuscripts. All authors read and approved the final draft of the manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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