Effects of Colostrum Probiotics on ACE-2 Expression and Hematology in Murine Models Immunized Against Canine Coronavirus

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Abstract

In modern society, the human-animal bond has significantly strengthened, with pets evolving into integral family members providing psychological support to their owners. In the current landscape of heightened focus on immune health and the widespread utilization of probiotic advancements globally, this study investigates the potential of colostrum probiotics to influence hematological profiles and ACE-2 expression in a murine model of canine coronavirus (CCV) infection. Twenty-four mice were categorized into four treatment groups: (C) placebo, (T1) CCV induction + isoprinosine, (T2) CCV induction + probiotic-based, and (T3) CCV induction + colostrum-based probiotics. CCV induction was performed via subcutaneous injection of a live-attenuated CCV vaccine at 60 mg/kg body weight for 7 consecutive days. Probiotics were administered via oral gavage for 14 consecutive days. On the 15th day post-treatment, euthanasia was performed, and blood samples were collected to examine hematological profiles and ACE-2 enzymes in the intestinal tissue sections. Data analysis, conducted using ANOVA followed by Duncan's test ($p < 0.05$), revealed a significant improvement in the T3 group, particularly in leukocytes, hemoglobin, lymphocytes, neutrophils, and ACE-2 expression. In conclusion, this study suggests that probiotics, specifically colostrum-based, enhance immune parameters and ACE-2 expression in the intestinal milieu of murine model subjected to CCV immunization.

Keywords: ACE-2, canine coronaviruses, good health and well-being, hematology, probiotic

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INTRODUCTION

In modern society, the human-animal bond has significantly strengthened, with pets evolving into integral family members providing psychological support to their owners (Pachana *et al*., 2011). Among domesticated animals, dogs, historically adapted to high protein and carbohydrate diets aligned with their owners' lifestyle, play a pivotal role in this relationship. The well-being of a dog's digestive system, particularly the intestines, relies on a balanced microbiota facilitating optimal nutrient absorption (Semova *et al*., 2012; Solfaine *et al*., 2024).

The emergence of the canine coronavirus (CCV) significantly impacts the health of dogs, with potentially severe consequences, especially when disrupting the digestive tract, causing diarrhea or, in severe cases, hemorrhage (De Oliveira and Burini, 2009; Hamid *et al*., 2022). Canine coronavirus, a highly contagious viral disease, poses a significant threat to the digestive tract system in dogs, especially affecting younger individuals. Clinical symptoms encompass anorexia, depression, vomiting, and the presentation of watery to slimy diarrhea. Although the disease's indicators are evident in the feces of infected dogs, the virus remains viable for only 48 hours outside the host (Kirana *et al*., 2024).

Accurate diagnosis involves a further examination using the CCV rapid test (Noli and Saridomichelakis, 2014). This contagious ailment transcends age and breed boundaries, with transmission facilitated in crowded and unsanitary environments. The incubation period, spanning 1–4 days from exposure to clinical

signs, precedes a disease onset duration of 2–10 days in most affected dogs (Beaumier *et al*., 2018). The efficacy of probiotics has been extensively investigated in understanding their potential to enhance gut microbiota and nutrient absorption during infection. Numerous studies have identified promising microbiota using techniques such as qPCR, FISH, or 16S rRNA sequencing, suggesting their application to restore equilibrium in the intestinal microflora ecosystem and serve as secondary therapeutic agents (Manichanh *et al*., 2012; Berlina *et al*., 2023). A focal point of intrigue lies in investigating the effectiveness of probiotics against CCV infection, providing significant medical insights to bolster the recovery phase of the disease. This research endeavor aims to scrutinize how probiotics impact the expression of the ACE-2 enzyme in intestinal organs and the hematological profile within a rat model subjected to CCV immunization.

MATERIALS AND METHODS

Ethical Approval

This research received approval from the Animal Ethics and Care Committee at the Faculty of Veterinary Medicine, Universitas Wijaya Kusuma Surabaya, under the reference number 158-KKE An ethical analysis was conducted to ensure the welfare of animals, with a commitment to preventing cruelty and minimizing suffering.

Study Period and Location

This research was performed during June to September 2022 at the animal laboratory, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya.

Study Design

A total of 24 mice were used in this study and divided into four treatment groups: (C) placebo, (T1) CCV induction + isoprinosin, (T2) CCV induction + probiotic-based, and (T3) CCV induction + colostrum-based probiotics. The CCV induction was achieved through subcutaneous injections of the active CCV vaccine at a dosage of 60 mg/kg body weight over a 7-day period. Concurrently, probiotic therapy was administered orally for 14 days. On the fifteenth day following the treatment, euthanasia was performed across all groups, and blood samples were drawn for the examination of hematological profiles and the assessment of ACE-2 expression in the intestinal tissue sections.

Hematological Evaluation and Immunohistochemical (IHC) Staining

Three milliliters of blood were collected into plain containers and a Vacutainer (BD®, USA) to assess the hematological profile. After allowing clotting for 10 to 20 minutes, all plain tubes were centrifuged at 4000 rpm for 15 minutes using a Hettich EBA 200® centrifuge machine (Germany). The supernatant's biochemical content was analyzed using the Hitachi 902® clinical chemistry analyzer (Roche Diagnostics, USA) (Purnama *et al*., 2021).

The intestinal organ was immersed in 15% formaldehyde for 48 hours before undergoing IHC staining. Alcohol, with concentrations of 70%, 80%, and 96%, served as a dehydrating agent. The cleaning process involved the use of xylol, and the production of paraffin blocks continued at a temperature of 60 °C. The intestine was sliced with a microtome, placed in a water solution, covered with paraffin blocks, and then positioned on a glass slide. Immunohistochemistry staining for one hour at 27°C involved a primary anti-ACE-2 murine antibody. For ACE-2, the mixture was administered in amounts of 10 µL. Subsequently, the material was rinsed three times every five minutes in PBS with a pH of 7.4 (Hamid *et al*., 2018).

Data Analysis

Hematological data were expressed as means with standard deviations (SD), and statistical analyses were conducted employing one-way analysis of variance (ANOVA) alongside the post hoc Bonferroni multiple comparisons test. Meanwhile, the Kruskal-Wallis and Mann-Whitney tests were employed to assess the ACE-2 score. Significance levels were established at $p < 0.05$. The statistical analysis was performed

using SPSS v.25 software (IBM, Armonk, NY, USA).

RESULTS AND DISCUSSION

The results of ACE-2 expression, assessed through immunohistochemical staining, revealed a significant decrease ($p < 0.05$) in the T3 treatment group compared to both the T1 and T2 groups (Table 1). ACE-2 expression was indicated by brown chromogenic staining, prominently observed in intestinal epithelial cells within the mucosa and muscular layers (Figure 1).

Simultaneously, an evaluation of the hematological profile unveiled noteworthy improvements ($p < 0.05$) in leukocytes, hemoglobin, lymphocytes, and neutrophils. Specifically, significant fluctuations ($p < 0.05$) were noted in the leukocyte, hemoglobin, and neutrophil variables in the T3 treatment group compared to both the T1 and T2 groups. Regarding lymphocyte levels, a notable increase ($p < 0.05$) was reported in the T3 treatment group compared to the T1 group (Table 2). These findings underscore an enhancement in ACE-2 expression and the hematological profile following the administration of probiotics with a colostrum basis at a dosage of 5 mg/kg body weight of colostrum.

Table 1. The ACE-2 expression scores differed significantly among treatment groups, indicating distinct levels of ACE-2 expression in each group

Groups	R1	R2	R3	R4	R5	Mean rank ± SE
C	9	8	8	8	9	18.00 ± 0.245^{a}
T1	4	4	5	5	4	7.10 ± 0.245^{c}
T2	6	7	7	6.5	6	13.00 ± 0.224^{b}
T3	4.5	1.5	4	3	3	3.90 ± 0.515^{d}

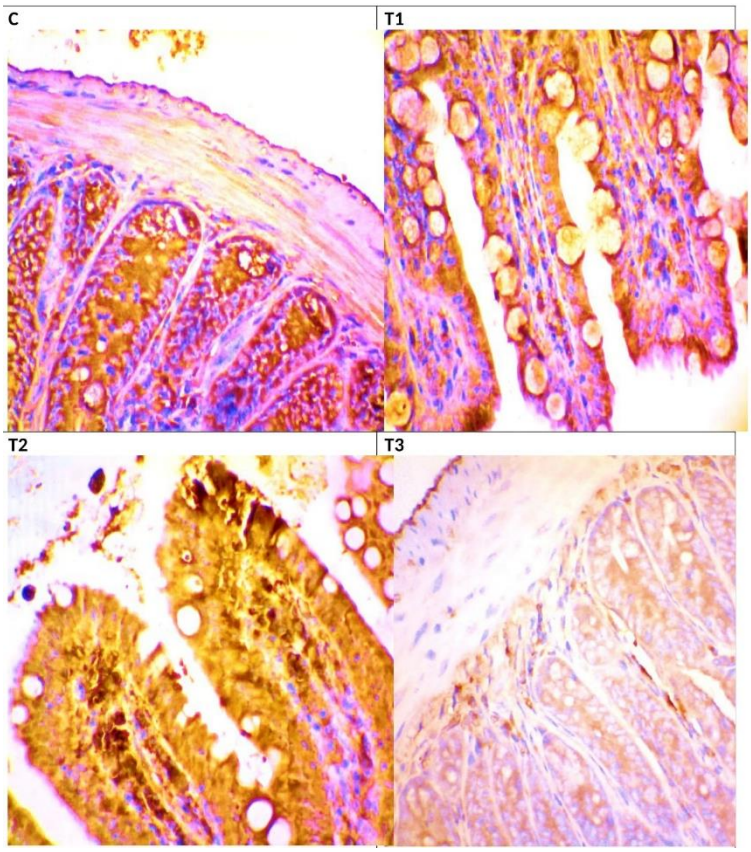

Figure 1. The brown coloration representing ACE-2 expression was prominently observed in intestinal epithelial cells within the mucosa and muscular layers.

Table 2. Hematology evaluation in all treatment groups

Variables	C	T1	T2	T3
Leukocyte (10^3/mm^3)	7.65 ± 0.62^{a}	8.03 ± 0.51^{ab}	8.33 ± 0.45^{b}	7.88 ± 0.54^{a}
Erythrocyte (10^6/mm^3)	8.03 ± 0.46	8.70 ± 0.35	9.15 ± 0.49	8.40 ± 2.12
Hemoglobin (g/dL)	11.84± 0.72^{a}	9.11 ± 1.61^{b}	9.40 ± 0.64^{b}	11.54 ± 1.69^{ab}
Hematocrit (%)	30.15 ± 1.32	24.73 ± 4.12	35.58 ± 2.75	26.45 ± 10.64
Thrombocyte (10^3/mm^3)	641.5 ± 161.24	545.0 ± 158.13	546.0 ± 19.47	513.8 ± 185.54
Lymphocyte (%)	51.23 ± 1.26^{a}	57.73 ± 1.40^{b}	52.07 ± 2.27^{ab}	53.47 ± 2.01^{ab}
Monocyte (%)	5.14 ± 0.51	5.00 ± 0.72	4.64 ± 1.24	4.36 ± 1.23
Eosinophil (%)	2.12 ± 0.50	2.63 ± 1.50	2.12 ± 0.50	2.12 ± 0.50
Basophil (%)	0.55 ± 0.50	1.50 ± 0.00	1.50 ± 0.00	1.25 ± 0.50
Neutrophil (%)	24.50 ± 2.50^{a}	22.5 ± 1.00^{ab}	29.50 ± 2.50^{b}	24.50 ± 2.50^{ab}

The values are presented as mean ± standard deviation (SD), and different superscripts ($^{a, b}$) in the same row signify statistically significant differences ($p < 0.05$).

In addition to the term probiotic, the concepts of prebiotic and synbiotic also contribute significantly to improving the digestive system. Prebiotics are food components that selectively stimulate, encompass short-chain carbohydrates and oligosaccharides like fructooligosaccharides, galactooligosaccharides, and inulin (Gibson, 2004; Al-Sheraji *et al*., 2013; Faiqoh *et al*., 2023).

Synbiotics represent a synergistic combination of probiotics and prebiotics strategically formulated to enhance the survival and colonization of microorganisms within the intestinal tract (Lokapirnasari *et al*., 2022; Chandra *et al*., 2022). Probiotics play a crucial role in regulating inflammation through both direct and indirect mechanisms (Mileti *et al*., 2009). One key indirect mechanism involves probiotics aiding in the restoration of the hyperpermeable epithelial barrier (Abdurrahman *et al*., 2022). This restoration is pivotal, as hyperpermeability can lead to low-grade endotoxemia, allowing the translocation of macromolecules such as Gram-negative bacteria (Utomo *et al*., 2022). Subsequently, bacterial cell wall lipopolysaccharides or endotoxins may enter the circulation, causing sustained low-grade inflammation (Munford, 2008).

ACE-2 initially emerged as a regulator of cardiac function, influencing hypertension and diabetes by converting angiotensin-2 to angiotensin-1 (Banu *et al*., 2020). Studies on the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) established ACE-2 as the receptor targeted by the virus for cellular entry (Khattab *et al*., 2022). Genetic analysis of the novel SARS-CoV-2 indicates an approximate 80% sequence identity with SARS-CoV, utilizing the same ACE-2 receptor. The expression of ACE-2 in various tissues is intricately linked to the susceptibility and severity of viral infections (Wardiana *et al*., 2021; Solfaine *et al*., 2024).

An additional indirect mechanism involves probiotics enhancing the production of short-chain fatty acids, such as butyrate (Marteau *et al*., 2004). Butyrate serves as an inflammation modulator with anti-inflammatory properties in intestinal epithelial cells, macrophages, and leukocytes (Meijer *et al*., 2010). Its multifaceted actions include activating the inhibitory pathway of NF-κB, regulating various cytokines (TNF-α, IL-2, IL-6, IL-10, NO, and IL12), eicosanoids, and chemokines from leukocytes. Butyrate also enhances the ability of these cells to migrate to sites of inflammation and acts as a nutritional source for colonocytes, contributing to the maintenance of the epithelial barrier (Hamid and Fikri, 2021).

Furthermore, probiotics indirectly contribute by increasing the synthesis of antimicrobial peptides (AMP). AMP, a broad-spectrum antimicrobial, serves as the frontline defense in mucosal tissues, especially in the gastrointestinal and respiratory systems (Zapolska-Downar *et al*., 2004). AMP also plays a vital role in the mechanism of mucosal inflammation, introducing

additional local pathways to control acute inflammatory responses and restore homeostasis in inflamed tissues (Lescheid, 2014).

Probiotics can also act directly as ligands for receptors in the innate immune system, particularly Toll-Like Receptors (TLR), with a notable impact on the activation of TLR-2 and TLR-4. These receptors play a crucial role in inflammatory signaling pathways (Dang *et al*., 2022). The binding of probiotics to TLRs can also influence various pathways, including the NF-κB pathway, Mitogen-Activated Protein Kinase (MAPK), phosphoinositide-3-kinase protein kinase B/Akt (PI3K-PKB/Akt), and peroxisome proliferator-activated receptor gamma (PPAR-γ) (Hoarau *et al*., 2006).

Beyond TLR activity, probiotics possess the capability to activate nucleotide-binding oligomerization domain receptors (NLR) by releasing low molecular weight substances that can permeate epithelial cell membranes and interact with NLRs. Another direct mechanism of probiotics involves their influence on the differentiation and function of immune cells associated with the inflammatory response, such as dendritic cells and T cells (Pratama *et al*., 2021). In dendritic cells, probiotics have the potential to impact maturation, defense, and overall function, critical for maintaining tolerance to commensal microbes and antigens from food sources (Pradere *et al*., 2010).

CONCLUSION

In conclusion, the administration of probiotics at a dosage of 5 mg/kg body weight within the colostrum group improved ACE-2 expression in the intestine and modestly enhanced key hematological parameters, including leukocytes, hemoglobin, lymphocytes, and neutrophils. Further study in larger and diverse animal populations is needed to uncover the synergistic effects of probiotics and colostrum. Investigation into long-term effects, optimal dosage, and potential applications in managing CCV-induced conditions is crucial.

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AUTHORS' CONTRIBUTIONS

ISH: designed, conceptualized, supervised, analyzed data and wrote the manuscript, RS and S: performed the experiment, analyzed data and wrote the manuscript. All authors have read and approve the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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