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Research Paper

Oral Immunization with Recombinant *Lactococcus lactis* and Retinoic Acid Boost Immune Response in Mice

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Abstract
The COVID-19 vaccine is one of the most important approaches for preventing SARS-CoV-2 virus transmission and spread. Oral vaccines are a promising option for preventing SARS-CoV-2 infection because it can activate both the mucosal and cellular immune systems. Previous research has shown that administering oral and intranasal vaccines can induce an immune response in mice. The effectiveness of the SARS-CoV-2 oral vaccine was evaluated in this study by combining spike protein with a carrier of food-grade recombinant *Lactococcus lactis* bacteria and a retinoic acid adjuvant. Mice were divided into three groups: a negative control (no treatment), a positive control (*L. lactis* recombinant), and a third group (*L. lactis* recombinant plus 300 μg retinoic acid). The vaccines were given three times, with a three-week interval between each. The serum levels of IgG, IgA, and IgE were determined using the ELISA method at the end of the study. CD4 and CD8 cells were detected using immunofluorescence. While not statistically significant, the results showed that the retinoic acid groups had the highest anti-spike antibody levels of the three groups. In comparison to the control group, CD4 and CD8 cells increased in the spleens of mice given retinoic acid. There was no difference in temperature or IgE levels between vaccinated and non-vaccinated mice, indicating that the vaccine caused no allergic reaction. This study's findings suggest that retinoic acid adjuvant can stimulate the cellular and humoral immune system.

Keywords
Allergy Reaction, Antibody, Lactococcus, Mucosal Immune, Vaccine

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1. INTRODUCTION

COVID-19 (coronavirus disease 2019) is a contagious infectious disease characterized by severe acute respiratory syndrome caused by SARS-CoV-2. COVID-19 causes respiratory infections that can progress to severe pneumonia and death (Chen et al., 2022). COVID-19 transmission occurs through two primary pathways. Direct transmission involves respiratory droplets expelled during sneezing or coughing by an individual infected with the virus. Indirect transmission occurs when a person comes into contact with surfaces or objects contaminated with SARS-CoV-2 and subsequently touches their eyes (Xu et al., 2020).

Vaccination represents the most effective preventive strategy against COVID-19. Since the onset of the COVID-19 pandemic, scientists worldwide have been actively engaged in developing vaccines, with more than 250 candidates currently undergoing preclinical and clinical testing (Panahi et al., 2023). Considering the high mutation rate observed in many coronaviruses, a universal vaccine could be more advantageous than strain-specific vaccines, which may require frequent updates as new variants and subvariants continue to appear, therefore the vaccine development remains important (Bartsch et al., 2024; Parhusip et al., 2022). To date, numerous vaccines have been developed and predominantly administered through the systemic route, typically via injection (Tsang et al., 2021). Mucosal vaccines are considered to be more beneficial because they can stimulate both mucosal and systemic immune responses (Yurina, 2018). The mucosa-associated lymphoid tissue (MALT) induces mucosal tissue immunity, with the gut-associated lymphoid tissue (GALT) and the nasopharyngeal-associated lymphoid tissue (NALT) being the most important sites of immunity induction to activate B cells that produce immunoglobulin A (IgA) (Mudgal et al., 2020).

Previous research on the COVID-19 mucosal vaccine used a recombinant *Lactococcus lactis* (*L. lactis*) carrier carrying the high conserve region (HCR) gene of the SARS-CoV-2 spike

protein, which was given orally and intranasally to BALB/c mice. Both routes can induce a humoral immune response against SARS-CoV-2, as evidenced by increased IgA and IgG levels. However, the oral route resulted in a lower increase in IgA than the intranasal route (Yurina et al., 2023). This is unfortunate because the oral route vaccine has several advantages, including that it does not cause pain from needles, is less expensive, and is safer than the systemic route, which is administered via injection (Hameed et al., 2022).

Microorganism has been extensively studied for its health application, including probiotics (Muharni et al., 2023; Sałański et al., 2022; Saleena et al., 2023). Using the bacterial carrier *L. lactis* for vaccine design has several advantages, including the ability to survive enzymatic reactions and low pH in the gastrointestinal area (Wyszyńska et al., 2015; Kesuma et al., 2025). Furthermore, when used as a mucosal vaccine vector, *L. lactis* is a food grade lactic acid bacterium that is safe, non-commensal, and non-colonizing. *L. lactis* can be engulfed by microfold cells (M cells) from Peyer's patches (PP) and brought to antigen presenting cells (APC), which will then present antigens to form a specific immune response after oral administration (Bahey-El-Din, 2012; Robinson et al., 2004; Nabila et al., 2023). However, a strategy for adding adjuvants to lactic acid bacteria-based vaccine formulas is required to induce a stronger and longer lasting immune response (Vilander and Dean, 2019).

Adjuvants are functional excipients that are added to vaccine formulations to boost the immunogenicity of an antigen. Adjuvants can boost vaccine bioavailability in the mucosa and protect against the body's natural defense systems like pH and mucus, which can reduce interactions with APCs or degrade antigens in vaccines. Retinoic acid is one of the adjuvants that can be used for mucosal vaccination. Retinoic acid is an oxidation product of retinol (vitamin A) that can increase IgA production directly on B cells via immunoglobulin class switching (Correa et al., 2022). Furthermore, when antigens are presented, retinoic acid can increase the expression of $\alpha 4\beta 7$ receptors and the CC chemokine receptor (CCR9) on T cells, resulting in increased transport of T cells to the intestine (Mwanza-Lisulo and Kelly, 2015).

Previous research demonstrated that using retinoic acid as an adjuvant in vaccination using tetanus toxoid intranasally in BALB/c strain mice can significantly increase IgA anti-tetanus toxoid compared to the carrier group (Cirelli et al., 2015). Another study involving 46 Zambian adults using oral thypoid vaccine (Vivotif) was successfully demonstrated a significant increase in IgA specific to Salmonella typhi lipopolysaccharide in the group with the addition of retinoic acid adjuvant compared to no adjuvant (Mwanza-Lisulo and Kelly, 2015). Thus, our study focuses on the addition of retinoic acid as an adjuvant to increase the immune response, which are IgA, IgG, CD4+, and CD8+, in the administration of recombinant *L. lactis*-based COVID-19 mucosal vaccines via the oral route.

To the best of our knowledge, this is the first study to explore the combination of recombinant food-grade *Lactococcus lactis* with retinoic acid as an adjuvant. This innovative approach offers a promising advantage in mucosal vaccine development. The synergy between a safe bacterial carrier and a well-characterized adjuvant may provide a novel, effective, and non-invasive platform for future vaccine formulations, particularly for pathogens entering through mucosal surfaces. We also aim to examine the temperature profile and allergic reaction, demonstrated by the increment of IgE level, during vaccine administration.

2. EXPERIMENTAL SECTION

2.1 Bacterial Culture

Lactococcus lactis strain NZ3900 (MoBiTec GmbH, Goettingen, Germany) with recombinant plasmid pNZ8149-HCR (*L. lactis* HCR) constructed from previous research Yurina et al. (2023) is the main subject of this study. The highly conserved region for the spike protein gene has been inserted in the pNZ8149. The plasmid was transformed to the *L. lactis* NZ3900 using electroporator (2000 V, 25 μF, 200 Ω). The vaccine produced based on our previous methods that has been established (Mubarak et al., 2025). Recombinant *L. lactis* HCR was streak in M17 media (Himedia, India) supplemented with 0.5% lactose for 18-24 hours at 30°C. The overnight culture was refresh to new M17 broth media and incubated at 30°C. After the OD600 reached 0.8, the 40 ng/mL nisin (MoBiTec GmbH, Goettingen, Germany) was added as the inducer, and the culture was incubated for another 18-24 hours at 30°C. Before harvesting, OD600 measurement is performed to determine the number of *L. lactis* colonies formed. Centrifugation was used to harvest the bacteria cells. Then, the pellet cells were washed in PBS and stored in 4°C.

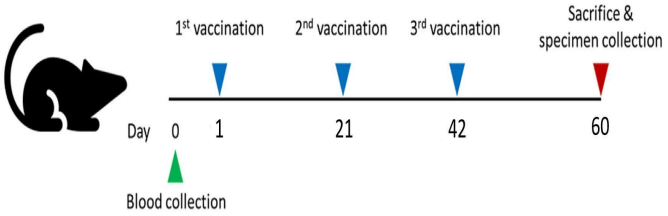

Figure 1. BALB/c Mice Vaccinated on Day -1, -21, and -42. The Mice were Sacrificed on Day-60

2.2 Animal Treatment and Vaccination Protocols

Male BALB/c strain mice weighing 15-20 grams were used in the experiments. This study has received an ethical clearance No.111/KEP/UB/2022 from Universitas Brawijaya Research Ethics Committee. Mice was divided into 3 groups: control negative which did not receive treatment (n=5), the positive control group which received *L. lactis* HCR 5×10^9 CFU without retinoic acid (n=9), and the treatment group which was vaccinated with *L. lactis* HCR 5×10^9 CFU with 300 μg of all-trans retinoic acid (ATRA) (n=9). Mice were vaccinated orally with oral gavage at a dose of 5×10^9 CFU, 3 times with a

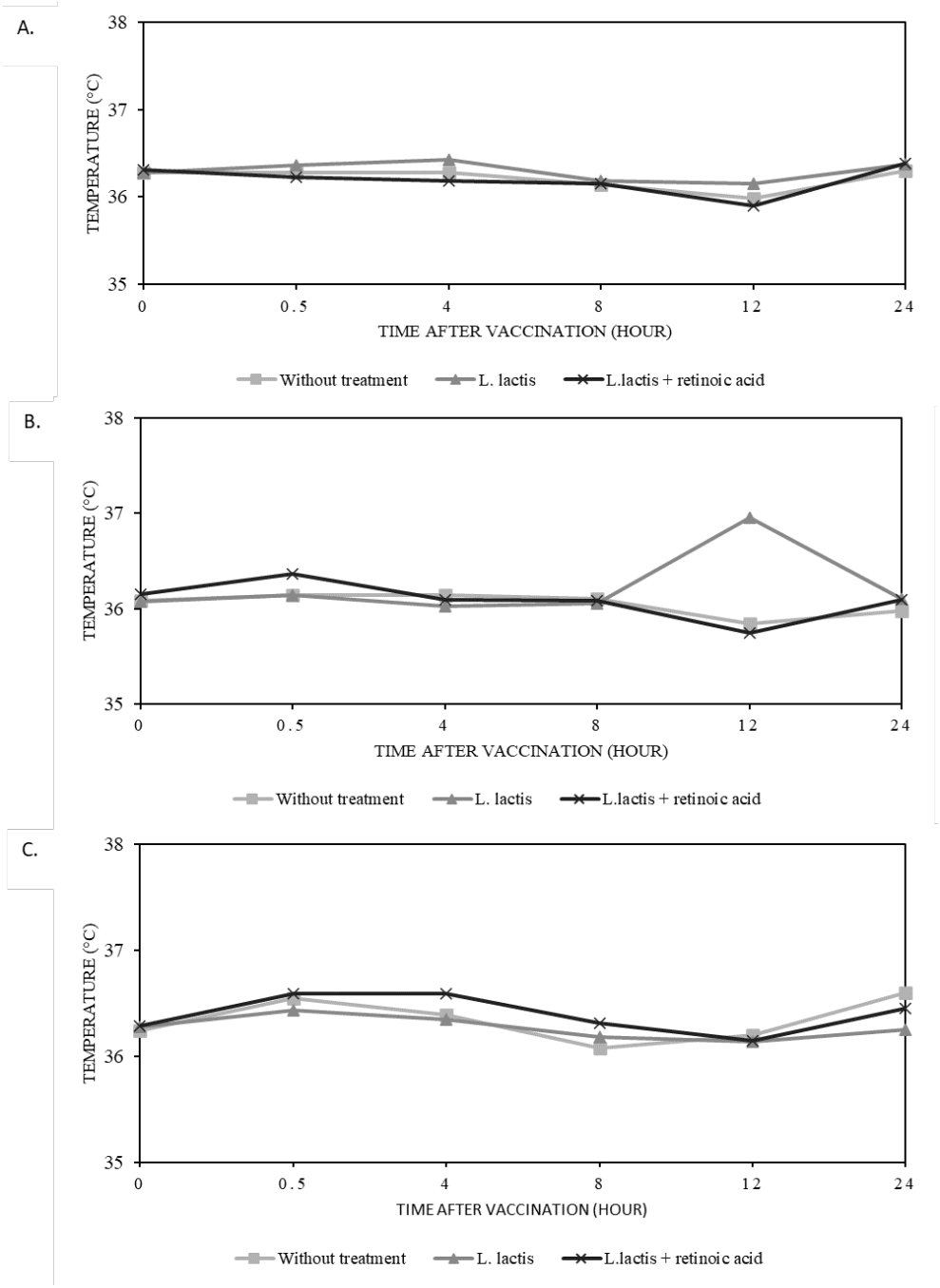

Figure 2. The Graph Showed the Temperature Average of Each Group 24 Hours After Dose 1 (A), Dose 2 (B), and Dose 3 (C). The Temperature Dropped at 8 and 12 Hours After Treatment, Then Rose Again to Approach Temperature Before Treatment. Each Group Showed a Normal Temperature Range (35.5-37.5°C). The Data are Presented as Mean ± Standard Deviation. There is No Significant Difference Among Groups (p>0.05)

3-week interval (day 1, -21, and -42). One hour before vaccination, ATRA was dissolved in canola oil and administered orally via gavage Figure 1 because ATRA reached maximum plasma concentration 60-180 min after being orally administered. Blood samples were taken on days 0, -42, and -60 for antibody measurement. Surface body temperatures were taken 30 minutes after treatment and every 4 hours for the next 24 hours. Furthermore, daily surface body temperature measurements were taken for 14 days. On the 60th day, the mice were sacrificed, and the spleen and small intestine were collected.

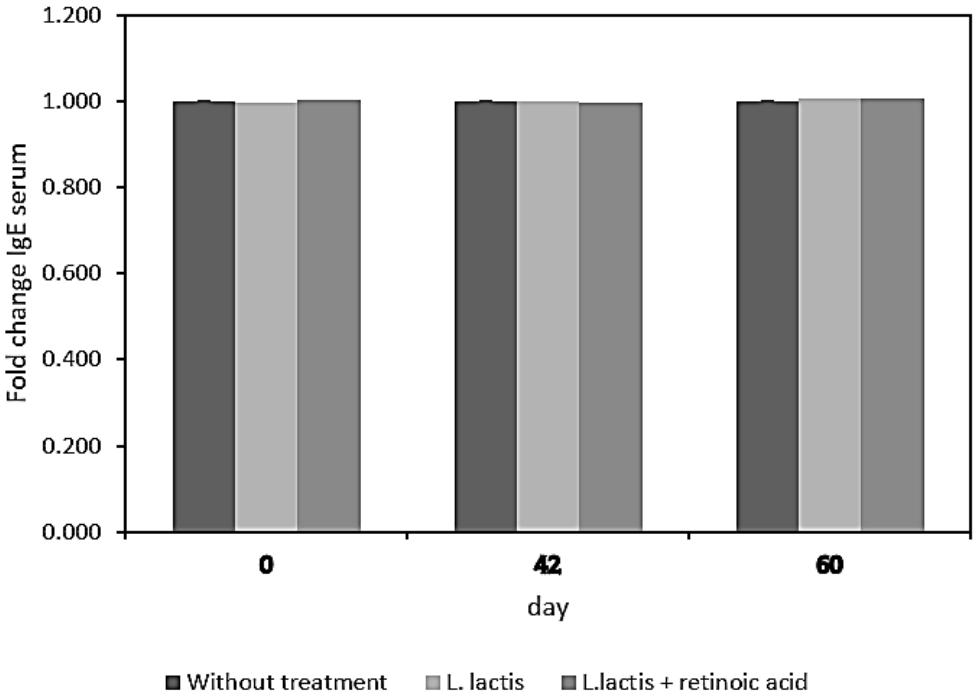

Figure 3. Vaccination Did Not Induce IgE Production After 60 Days of Treatments. All of the Groups Show Similar IgE Level. Fold Change Was Calculated by Dividing the Treatment Groups Antibody Level by the Control Group Antibody Level. There Is No Significant Difference Among Groups (p>0.05)

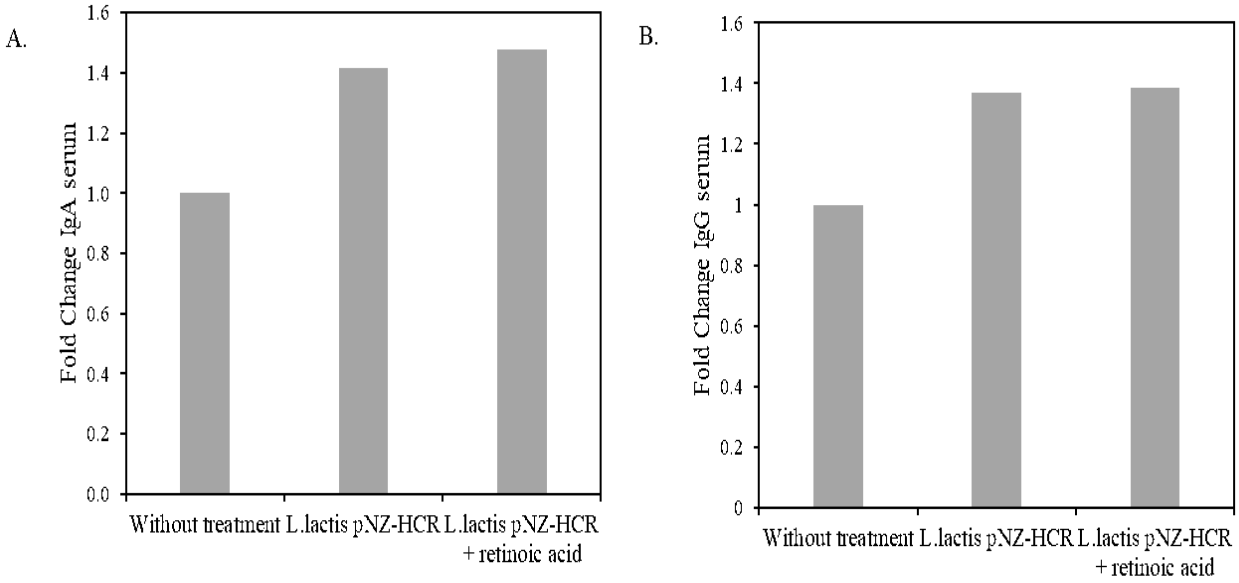

Figure 4. Fold Change in IgA (A) dan IgG (B) Production. The Treatment Groups Showed Higher Antibody Level Production, Both in Aduvant and Non-Aduvant Groups. There Is No Significant Difference Among Groups (p>0.05)

2.3 IgG, IgA and IgE Level Measurements

Serum was separated from blood by centrifugation at 2200 RCF for 15 minutes. The ELISA kit was used to measure IgA, IgG, and IgE antibody levels in serum. The Mouse Anti-SARS-CoV-2 Spike Protein Antibody IgG ELISA kit and the Mouse Anti-SARS-CoV-2 Spike Protein IgA Antibody ELISA kit (Solarbio Science & Technology, Beijing, China) were used to measure serum IgG and IgA levels. Standards were prepared

with serial concentrations, and samples were diluted in the ratio (1:10) with sample diluent. Before adding 100 μL of diluted sample to each well, the microplate was rinsed three times. Microplates were incubated at room temperature (25±2°C) for 120 minutes before being rinsed four times with a wash buffer. Then, at room temperature (25±2°C), 100 μL of HRP-conjugate antibody was added and incubated. Then 100 μL of HRP-conjugate antibody was added and incubated for 120 minutes at room temperature (25±2°C). Each well was washed four more times with a wash buffer. Each well received 100 μL of tetramethyl benzidine (TMB) substrate and was incubated in the dark at room temperature (25±2°C). By adding 50 μL of stop solution to each well, the reaction was stopped. The absorbance was measured with an ELISA reader at 450 nm.

Total IgE was determined using a mouse IgE sandwich ELISA kit (Bioassay Technology Laboratory, Shanghai, China) in accordance with the manufacturer's instructions. One hundred μL of HRP-conjugate antibody was added and incubated for 120 minutes at room temperature (25±2°C). Each well was washed four times with a wash buffer. Each well received 100 μL of tetramethyl benzidine (TMB) substrate and was incubated in the dark at room temperature (25±2°C). By adding 50 μL of stop solution to each well, the reaction was stopped. The absorbance was measured with an ELISA reader at 450 nm.

2.4 CD4 and CD8 T cells measurements

The immunofluorescence method was used to assess the number of CD4+ and CD8+ T cells in the spleen and intestine. Mouse spleens and intestines were paraffin-coated and stained with antibodies against CD4 conjugated to fluorescein isothiocyanate (SC-13573 FITC) (Santa Cruz Biotechnology Inc, Dallas, Texas, USA) and CD8 conjugated to phycoerythrin (SC-177 PE) (Santa Cruz Biotechnology Inc, Dallas, Texas, USA). For three visual fields, observations were made at a magnification of 200×. The ImageJ software was used to calculate the intensity of CD4 and CD8 T cells.

2.5 Data Analysis

The data is presented in the form of an average and standard deviation. The Shapiro-Wilk and Levene tests were used to determine the normality and homogeneity of the data. The data were statistically analyzed using One-Way ANOVA to determine significant differences between groups (p<0.05), with the Kruskall-Wallis test as an alternative method of analysis. If the results show a significant difference, the Tukey's test for One-Way ANOVA and the Mann Whitney test for the Kruskall-Wallis test is used to perform a post-hoc test. IBM SPSS 25 was used for this analysis.

3. RESULTS AND DISCUSSION

3.1 Vaccination Did Not Cause Fevers or Allergic Reactions

Temperature measurements in the first 24 hours showed that all the mice have normal body temperature (35.5-37.5°C) Figure 2. Observation for 14 days after vaccination showed all

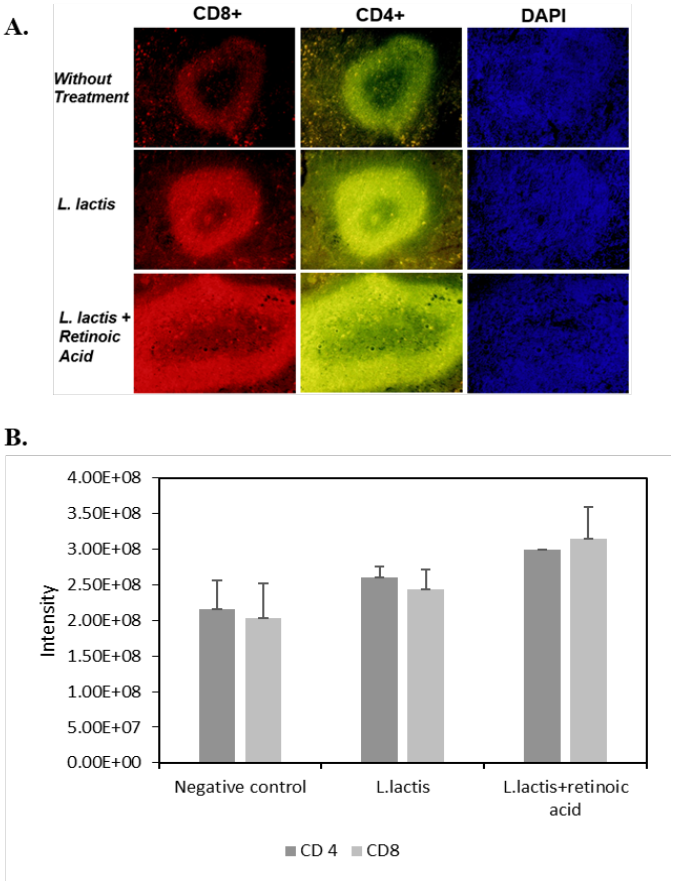

Figure 5. Vaccination Activates CD4 and CD8 T Cells in Mouse Lymph Nodes. Representation of the Immunofluorescence Staining Is Presented (A). Although Not Statistically Significant, the Adjuvant Increased T Cell Response More Potently Than the Control Groups (B). The Data Are Presented as Mean ± Standard Deviation. There Is No Significant Difference Among Groups (p>0.05)

the mice also have normal body temperature (data not shown). This result demonstrated that vaccination did not cause fever. Moreover, there was a slight decrease in temperature when compared to the mice's normal temperature (36.5-38.0°C). Vaccination with and without adjuvant did not significantly elicit IgE production before, during, and after the treatment Figure 3. The results of the study show that there is a slight increase in IgE sensitivity in the group that receives vaccine only and vaccine plus retinoic acid compared to the control group (no treatment), with no statistical significance (p>0.05).

Post-vaccination fever is a frequently observed phenomenon and has been documented as a side effect of COVID-19 vaccine administration. In our study, HCR gene from the S2 spike protein of SARS-CoV-2 exhibits antigenic properties. A non-contact infrared thermometer method was used to measure the surface body temperature profile in the abdominal area of mice in this study. Measuring temperature through the mice's abdomen is preferred over rectal temperature measurements

because it is more comfortable to use, does not harm mice, does not require anaesthesia, and can prevent cross infection (Mei et al., 2018). Observation in the first 24 hours showed that all the mice showed a normal body temperature (35.50-37.50°C) Figure 2. The normal body temperature also occur 14 days after vaccination (data not shown). This result demonstrated that vaccination did not cause fever. Moreover, there was a slight decrease in temperature when compared to the mice's normal temperature (36.50-38.00°C). However, the temperature drop is not classified as hypothermic (Srilata and Jayaram, 2016). This temperature drop can be caused by several factors, including ambient temperature, handling stress, and thermometer condition. Temperature measurement can be affected by environmental temperature because the lower the ambient temperature, the lower the body temperature, which causes thermoregulation to compensate for heat changes (Mei et al., 2018; Srilata and Jayaram, 2016).

Allergic reactions after vaccine administration can occur due to components in the vaccine, such as vaccine carrier, adjuvant, or other ingredients, and can be demonstrated by increasing IgE levels. Our study showed the vaccination with and without retinoic acid did not elicit the IgE level. The use of all-trans retinoic acid (ATRA), which was used as an adjuvant in this study, may have contributed to the decrease in IgE levels.Worm et al. (1998) discovered that ATRA administration can reduce IgE production in B cells by 94±1.8% when compared to the control group, moreover giving ATRA to the OVA-sensitized measuring group can lower total serum IgE levels. IgE levels decrease is possible because ATRA can inhibit cell proliferation mediated by IL-4 and CD40. IL-4 can promote the transition of B cells and induce B cell isotypes to switch to producing IgE antibodies. ATRA can prevent CD40 from binding to the surface of B cells. CD40 can interact with CD40 ligand on T cell surfaces, activating B cells to differentiate, proliferate, and increase their ability to produce antibodies. The CD40-CD40 ligand interaction also activates the cytidine deaminase enzyme, which is involved in the introduction of DNA modification during the Ig class switching process in B cells. Furthermore, ATRA can inhibit the transcription of epsilon germline, which is required for the occurrence of IgE antibody synthesis. ATRA can reduce IgE production by inhibiting IL-4, CD40, and epsilon germline transcription (Worm et al., 1998, 2001).

In this study, using *L. lactis* as a vaccine career may also decrease IgE levels. In Zhang et al study, administration of *L. lactis* NZ3900/p8149 to the mice group with OVA sensitivity resulted in a significant decrease in total serum IgE levels (p<0.01) when compared to the control group (Zhang et al., 2021). *L. lactis* is a type of lactic acid bacteria that has probiotic properties. By preventing inflammation, probiotic bacteria can alter an allergic response (Liu et al., 2017). Th2 cells can be inhibited in their production of IL-4 by *L. lactis*. As previously stated, IL-4 stimulates IgE class switching on B cells, causing them to produce IgE antibodies (Yoshida et al., 2011). An allergic reaction in the body is distinguished by an increase in IgE levels above the normal value, i.e. >1 g/ml. An increase in IgE secretion in the blood can cause an allergic reaction because it can bind to its target receptor, FcRI, on the surface of mast cells and activate them. Mast cells that have been activated can produce and release histamines, lipid mediators, and cytokines, which cause allergic reactions (Abbas et al., 2020; Ünsal et al., 2021). According to the findings of this study, vaccination does not cause an increase in IgE levels or an allergic reaction. This result is inline with other similar study which used *L.lactis* as oral HPV vaccine, the most common adverse effect were mild to moderate nausea and vomitting (Taghinezhad-S et al., 2019).

3.2 Adjuvant Increase Antibody Production

The treatment with *L. lactis* pNZ-HCR increases IgA levels in serum, and the addition of retinoic acid slightly enhances this effect, indicating that both the recombinant bacteria and retinoic acid may promote mucosal immune responses Figure 4A. Similar to IgA, the IgG serum levels increase with the treatment of *L. lactis* pNZ-HCR and slightly more with retinoic acid. IgG is a systemic antibody, thus this result suggests the treatments also stimulate systemic immunity, though the difference between the two treatment groups is modest Figure 4B. These result is suggested similar thred with previous studies, in which the retinoic acic is effectively induced mucosal immunity (Li et al., 2024; Said et al., 2023).

Oral administration of the vaccine can induce the formation of IgA and IgG. IgA plays a crucial role in mucosal immunity, including neutralizing viruses, preventing pathogens from binding to and invading host cells, and blocking viruses from entering the systemic circulation through the mucosa (Zhai et al., 2023). IgG is the most common monomeric antibody, accounting for about 75% in serum, and is important in providing long-term immunity or immunological memory against infectious agents (Alibolandi et al., 2022). In this study, the increased levels of IgA occurring in the group given retinoic acid adjuvants are since retinoic acid can regulate transcription processes involving interaction with the RAR and RXR heterodimer complexes at the integrin chain code $\alpha 4$ (Itg$\alpha 4$) and the CCR9 promoter gene, thereby increasing the formation of the homing receptors of integrin $\alpha 4\beta 7$ and CCR 9 in target cells, T cells and IgA+ B cells. Integrin $\alpha 4\beta 7$ in the surface of the cells will bind to the mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1). On the other hand, CCR9 will bind to CC chemokine ligand 25 (CCL25). As a result of both interactions, IgA+ B cells subsequently migrate to the effector area, the small intestinal proprium. In modulating the immune response of B cells, retinoic acid can enhance the expression of the transmembrane receptor cluster of differentiation 38 (CD38) in B cell so that it can stimulate the process of differentiation and maturing B cell into plasma cells that secrete antibodies (Hao et al., 2021). Retinoic acid is also known to play a role in stimulating the occurrence of IgA and IgG class switch recombination (CSR). This is because retinoic acid can improve the regulation of enzyme activation-induced deami-

nase (AID) expression, which can break down DNA in the α switch region and cause the output of other constant regions of isotypes Cμ, Cδ, Cγ, and Cϵ (Bos et al., 2022; Oliveira et al., 2018). To increase the number of plasma cells that express IgG, retinoic acid regulates the gene expression of the AID enzyme that plays a role in the direct switching from IgM to IgG1 by removing the genes Cμ, Cδ, and Cγ3 so that the variable region can directly merge with the Cγ1 region to form the molecule IgG1 (Valenzuela et al., 2016).

3.3 Adjuvant Increase Expression of CD4+ and CD8+ T Cells

The intensity of CD4+ and CD8+ in spleen in the group with *L. lactis* and *L. lactis* +retinoic acid inclined to increase but not significantly compared to the non-treatment group (p>0.05) Figure 5, while the intestinal CD8 + intensity between the vaccination group and additive addition to the group without treatment was statically significant (p<0.05). The CD4 expression was also increased although not statistically significant Figure 6.

Based on the anatomical location of the lymph, the T cells are located on the white pulp part of the T cell zone (TCZ) or Periarteriolar lymphoid sheath (PALS) (Cesta, 2006; Lewis et al., 2019). The area of the white pulp on the spleen is arranged randomly and irregularly, with varying sizes ranging between 0.5-1 mm (Ali et al., 2023). T cells play a major role in mediating the immune response through the secretion of specific cytokines and memory cells that are able to survive repeated infections. CD4+ T cells play a role in helping to activate macrophages, as well as activating B cells to produce antibodies. In order to carry out its function, CD4+ T cells will secrete certain cytokines after binding to MHC class II. CD8+ T cells, known as cytotoxic lymphocytes, have the ability to directly kill infected cells after binding to MHC class I. Retinoic acid has an activity inducing "T cell gut homing" through increased $\alpha 4\beta 7$ markers integrin and the chemokine receptor CCR9. The administration of retinoic acid before vaccination can increase the accumulation of T cells in the intestinal mucosa. Exposure to retinoic acid will cause T cells to express homing receptors $\alpha 4\beta 7$ and CCR9 so that they can migrate along the intestinal mucous tissue to be deactivated or stimulated by antigens through vaccination (Cirelli et al., 2015).

Our result suggest that the retinoic acid can improve the immune response following vaccination through the increasing CD8 T cells in spleen and small intestine. Retinoic acid has highly lipophilic properties with low water solubility and permeability. This can be the factor that retinoic acid has poor bioavailability when administered via the oral route (Grace and Viswanathan, 2017; Borges et al., 2021). In addition, retinoic acid is rapidly degraded by metabolism by the CYP26 enzyme with a half-life of approximately 1.5 hours. Low ATRA bioavailability may contribute to insignificant increases in CD8+ and CD4+ T cell expression. Our result suggest that the formulation is needed to improve the bioavailability of the retinoic

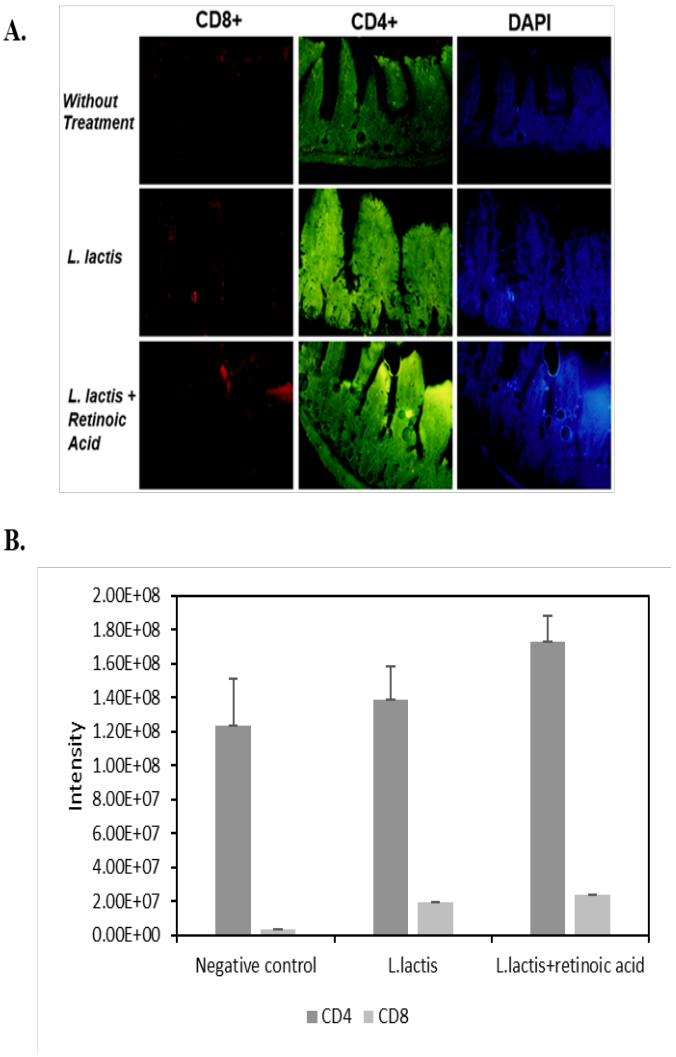

Figure 6. In Mouse Intestine, Vaccination Activates CD4 and CD8 T Cells. A Representation of Immunofluorescence Staining is Shown (A). For CD8 and CD4 T Cells, the Adjuvant Increased T Cell Response More Potently Than the Control Groups (B). The Data are Presented as Mean ± Standard Deviation. Asterisk (*) Show a Significant Difference Between Groups (p<0.05)

acid. The weaknesses of the retinoic acid pharmacokinetic profile can be reduced by developing its delivery systems such as in the form of liposomes, lipid nanoparticles, microspheres, and polymer micelles (Ross and Zolfaghari, 2011).

Since there is a rise in intestinal CD4 and CD8 T cells but no significant rise in antibody titters, there is a possible function for cell-mediated immunity as opposed to humoral immunity. The rise in intestinal CD4 and CD8 T cells may suggest that these immune cells are playing a significant role in defence, particularly in mucosal immunity, even though antibody titters are frequently linked to protection against infections. Coordination of immunological responses, including the activation of B cells,

macrophages, and CD8 T cells, depends on CD4 T cells. To maintain tolerance and fight infections, CD4 T cells can help regulate immune responses in the intestines. Conversely, CD8 T lymphocytes eliminate infected cells directly. Since most infections enter the body through the intestinal environment, the minor increase in these cells raises the possibility that they may be actively involved in reacting to pathogens there.

4. CONCLUSIONS

Our study revealed that the retinoic acid is a potent adjuvant for oral vaccination through the increasing CD4 and CD8 expression in small intestine. Retinoic acid as an adjuvant has been proven to be safe, as the results showed no increase in body temperature or allergic reactions. In addition, the administration of retinoic acid as an adjuvant is tended to increase the serum antibody levels of IgA and IgG . However, the vaccination dose and frequency need to be adjusted to reach higher immune response.

5. ACKNOWLEDGMENT

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