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Lactobacillus plantarum FNCC 0137 fermented red *Moringa oleifera* exhibits protective effects in mice challenged with *Salmonella typhi* via TLR3/TLR4 inhibition and down-regulation of proinflammatory cytokines

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ABSTRACT

Background: *Salmonella typhi* is a foodborne pathogenic bacterium that threatens health. S. typhi infection exacerbated the antibiotic resistance problem that needs alternative strategies. *Moringa oleifera* possesses anti-inflammatory and antimicrobial effects. However, there is a lack of information about the pharmacological value of red *M. oleifera*. The fermentation of red *M. oleifera* leaves extract (RMOL) is expected to add to its nutritional value.

Objective: The present study aimed to evaluate non-fermented RMOL (NRMOL) and fermented RMOL (FRMOL) effects on *S. typhi* infection in mice.

Materials and methods: Female *Balb/C* mice were randomly divided into eight groups. The treatment groups were orally administered with NRMOL or FRMOL at doses 14, 42, and 84 mg/kg BW during the 28 days experimental period. Then *S. typhi* was introduced to mice through intraperitoneal injection except in the healthy groups. The NRMOL or FRMOL administration was continued for the next seven days. Cells that expressed $CD11b^{+}$ $TLR3^{+}$, $CD11b^{+}TLR4^{+}$, $CD11b^{+}IL\text{-}6^{+}$, $CD11b^{+}IL\text{-}17^{+}$, $CD11b^{+}TNF\text{-}a^{+}$, and $CD4^{+}CD25^{+}CD62L^{+}$ were assessed by flow cytometry.

Results: Our result suggested that NRMOL and FRMOL extracts significantly reduced ($p < 0.05$) the expression of $CD11b^{+}TLR3^{+}$, $CD11b^{+}TLR4^{+}$, $CD11b^{+}IL\text{-}6^{+}$, $CD11b^{+}IL\text{-}17^{+}$, and $CD11b^{+}TNF\text{-}\alpha^{+}$ subsets. In contrast, NRMOL and FRMOL extracts significantly increased ($p < 0.05$) the expression of $CD4^{+}CD25^{+}CD62L^{+}$ subsets. NRMOL at dose 14 and 42 mg/kg BW was more effective compared to FRMOL in reducing the expression of $CD11b^{+}TLR3^{+}$, $CD11b^{+}TLR4^{+}$, and $CD11b^{+}TNF\text{-}\alpha^{+}$ subsets.

Conclusion: Our findings demonstrated that NRMOL and FRMOL extracts could be promising agents for protection against *S. typhi* infection via modulation of TLR3/TLR4, regulatory T cells, and proinflammatory cytokines.

1. Introduction

Salmonella enterica serovar typhimurium (*Salmonella typhi*) is a Gram-negative bacterium that is an important foodborne pathogen with a worldwide distribution. *S. typhi* has received extensive attention due to its harmful effect on humans and animals,

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including abdominal pain, acute diarrhea, fever, nausea, vomiting, and sometimes lethal septicemia [1,2]. Interestingly, *S. typhi* infection in humans and mice produces many similar symptoms and hence mice are the most widely used animal model for studying *Salmonella* infection [3,4]. *Salmonella* infection was reported to affect around 11–21 million people worldwide, and approximately 128–161 thousand people die annually, with South-East Asia being one of the regions with highest case frequency [5].

As a first-line of defense, body initiates an acute inflammatory response in response to *S. typhi* infection. This response is executed via direct interaction of *S. typhi* with host cells such as macrophages or dendritic cells (DCs), followed by intensifying the inflammatory response, mainly in the liver, spleen, lungs, and intestines [6,7]. During bacterial infection, macrophages expressed Toll-like receptors 4 (TLR4), which primarily recognizes the lipopolysaccharide (LPS) as the major component of the cell wall of *S. typhi*. This recognition subsequently stimulates TLR4 signaling pathways, such as NFκB, to produce proinflammatory cytokines, resulting in increased systemic cytokine production and septic shock in the later stages [8–10].

In the earliest phase of infection, both expressions of TLR3 and TLR4 in macrophages were increased to overproducing proinflammatory cytokines. These circumstances provide a survival advantage during bacterial elimination by producing reactive oxygen and nitrogen species, resulting in increased oxidative stress [11–14]. However, chronic inflammation and oxidative stress can promote tissue damage [15,16]. Therefore, the development of alternative options for the treatment or prevention that will reduce tissue damage caused by *S. typhi* and its virulence are urgently needed since *S. typhi*, and other bacteria become multi-resistant to antibiotics [17].

Moringa oleifera Lam. (Family: Moringaceae) originated from the Himalayas and was distributed almost worldwide in tropical and subtropical countries. *M. oleifera* is commonly known as horseradish tree, drumstick tree, or kelor (Indonesian). *M. oleifera* is also considered the magic tree or tree of life due to its abundant macro-, micro-, and phytonutrients [18,19]. Accumulating evidence showed that *M. oleifera* leaves extract (MOL) possessed antioxidant [20], antidiarrheal [21], anti-inflammatory [22], and antimicrobial activities [23] due to its richness in flavonoids, flavanol glycosides, glucosinolate, isothiocyanate, phenolic acid, terpene, alkaloid, and sterol contents [24,25] However, the extract from MOL is not palatable [26].

It has been long recognized that utilization by microorganisms has been commonly used strategy to improve the functional properties of the plant [27,28]. The fermentation of MOL generate a sweet aroma that may increase its appeal and palatability [29]. Besides, MOL fermented by *Lactobacillus plantarum* alters its taste, pH, and viscosity [30]. Interestingly, fermention of MOL with *L. plantarum* reduces its phytate and raffinose content and enhances its peptic digestibility and radical scavenging activity [26,31]. Further, milk fermented with *L. plantarum* demonstrated beneficial effects against *S. typhi* infection [32].

Although many studies have reported MOL's function and its product as an anti-inflammatory agent, there is still a lack of information about red MOL as anti-inflammation in the context of improving specific host immune function related to *S. typhi* infection. Red *M. oleifera* is not very popular as green *M. oleifera*. In contrast, the red *M. oleifera* is frequently used by local tribes in Southeast Sulawesi, Indonesia, as traditional medicine for curing various diseases than the green *M. oleifera* [33]. Herein, we provide evidence that red *M. oleifera* could restore naïve regulatory T cells and TLR3/TLR4 expression that affect proinflammatory cytokines, i.e., interleukin (IL)-6, IL-17, and TNF-α in macrophages of mice infected with *S. typhi*. Red Moringa may have anti-inflammatory activity through modulation of host-immune response in mice challenged with *S. typhi*.

2. Material and methods

2.1. Plant material and identification

The fresh portions of red *M. oleifera* leaves (leaves, seeds, flowers, and roots) were obtained from Sampang, Madura, East Java, Indonesia, during July 2017. The plant specimens then were authenticated and deposited at Purwodadi Botanic Garden, Indonesian Institute of Sciences, Pasuruan Indonesia, with the voucher specimen numbers 1051/IPH.06/HM/VIII/2017.

2.2. Moringa oleifera preparation

Red *M. oleifera* leaves were washed three times using distilled water. Then the leaves were air-dried for 72 h, followed by drying in an oven at 40 °C for 3 h. Dried leaves of red *M. oleifera* were grounded to obtain a powder. The leaves powder (200 g) then macerated with 2 L 70% ethanol for three consecutive days, continuously shaking at 125 rpm for 1 hour per day. The red *M. oleifera* leaves extract (RMOL) was filtered using Whatman No. 1 paper and then concentrated using a rotary evaporator (IKA RV10).

2.3. Moringa oleifera fermentation

L. plantarum FNCC 0137 was received from the Food and Nutrition Study Center, Gadjah Mada University. *L. plantarum* FNCC 0137 was prepared using the MRS broth medium and incubated at 37 °C for 72 hours followed by centrifugation for 20 min at 4 °C. MOL extract at room temperature was fermented with 1×10^8 CFU/g *L. plantarum* FNCC 0137, followed by incubation at 37 °C for 120 h [31]. The fermentation product of RMOL (FRMOL) was supplemented with 10% sucrose and 0.5% NaCl prior to freeze-drying [34].

2.4. Experimental animal

Female *Balb/C* mice were obtained from the Institute of Biosciences, Brawijaya University. Female *Balb/C* mice with 25–30 g weight and six weeks old were housed at Animal Facility, Faculty of Agricultural, Brawijaya University. Mice were allowed to consume food and water *ad libitum*. Mice were acclimatized for seven days at 25–26 °C, RH 60%, and with a 12 h light/dark cycle.

2.5. Non-fermented and fermented MOL administration

After one-week acclimatization, forty female *Balb/C* mice were randomly divided into eight groups (n = 5):

Group I = Healthy mice without *S. typhi* injection (HM)
Group II = *S. typhi* only without additional administration (*S. typhi*)
Group III = NRMOL 14 mg/kg BW + *S. typhi* (NRMOL – 14)
Group IV = NRMOL 42 mg/kg BW + *S. typhi* (NRMOL – 42)
Group V = NRMOL 84 mg/kg BW + *S. typhi* (NRMOL – 84)
Group VI = FRMOL 14 mg/kg BW + *S. typhi* (FRMOL – 14)
Group VII = FRMOL 42 mg/kg BW + *S. typhi* (FRMOL – 42)
Group VIII = FRMOL 84 mg/kg BW + *S. typhi* (FRMOL – 84)

Both NRMOL and FRMOL were given orally for 28 consecutive days. On the 29th day, mice except for healthy mice groups were intraperitoneally infected with 1×10^7 CFU/ml *S. typhi* for the next seven days.

2.6. S. typhi confirmation

After 24 h, *S. typhi* infection was confirmed through collected blood via vena caudalis. Briefly, 50 μL blood was collected and

450 µL of 0.9% sterile NaCl was added to it. The mixture was then planted in Luria broth medium and incubated for 24 h at 37 °C with 120 rpm. Followed by isolation from Luria Broth and inoculation in Salmonella selective medium, Xylose-Lysine-Deoxycholate (XLD). The colony of Salmonella is characterized by a colony that formed dark nuclei with a transparent colony. Further confirmation for Salmonella was carried out using the catalase test. Briefly, one ose from cultureed Salmonella was dipped in object-glass containig H_2O_2. formation of bubble was confirmed presence of Salmonella [35,36].

2.7. Splenocyte isolation

Mice were abstained from food overnight with free access to water before being sacrificed through cervical dislocation. Spleen was collected, washed, and crushed to obtain a single-cell suspension. The homogenate was then centrifuged at 2500 rpm for 5 min at 10 °C. The supernatant was discarded, and the pellet obtained was added with 1 mL PBS. Homogenates was aliquoted into 1.5 mL tubes and centrifuged to remove supernatant. The pellet was then stained with appropriate antibodies for flow cytometry analysis.

2.8. Flow cytometry staining

The antibodies used for extra- and intracellular staining were purchased from BioLegend (San Diego, CA, USA) and prepared following routine laboratory procedures [37]. Regulatory T cells (Tregs) were marked by the combination of fluorescein isothiocyanate (FITC) anti-mouse CD4 (clone GK1.5), phycoerythrin (PE) anti-mouse CD25 (clone 3C7), and PE-Cy5 anti-mouse CD62L (clone MEL-14) as cell-surface antibodies for 30 min at 4 °C in the dark. Macrophages were identified by cell-surface antibodies FITC anti-mouse/human CD11b (clone M1/70) for 30 min at 4 °C in the dark, followed by a wash step and added cytofix/cytoperm for 20 min at 4 °C. The supernatant was discarded after centrifugation, then the pellet obtained was stained with PE anti-mouse TLR3 (clone 11F8), PE-Cy7 anti-mouse TLR4 (clone SA15-21), PE anti-mouse IL-6 (clone MP5-20F3), PE-Cy7 anti-mouse IL-17A (clone TC11-18H10.1), and PE anti-mouse TNF-α (clone MP6-XT22). Cells were resuspended in PBS, and a total of 10,000 cell events were acquired using FACS CaliburTM at a low or medium rate. The cells' population was then according to the stained used for further analysis. Cell subsets analysis was performed using FlowJo v10 for Windows (FlowJo LLC, Ashland, OR), following the previously validated protocol [38].

2.9. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine statistically significant differences between groups (*p-value* < 0.05). Tukey HSD test was used for multiple comparisons and post hoc analysis. Analyses were performed using GraphPad Prism 8 (GraphPad Software Inc, La Jolla, CA).

3. Result

3.1. NRMOL restores $CD11b^+$ $TLR3^+$ and $CD11b^+TLR4^+$ subsets better than FRMOL in mice challenged with S. typhi

In the present study, we try to assess the NRMOL and FRMOL efficacy on the expression of $CD11b^+TLR3^+$ and $CD11b^+TLR4^+$ subsets (Fig. 1A–D). Our result suggest that NRMOL is more effective than FRMOL to reduce $CD11b^+TLR3^+$ and $CD11b^+TLR4^+$ subsets after *S. typhi* challenge. Based on dot plot analysis, $CD11b^+TLR3^+$ subsets (Fig. 1A) and $CD11b^+TLR4^+$ subsets (Fig. 1C) were increased in mice challenged with *S. typhi*. Both NRMOL and FRMOL administration significantly reduced ($p < 0.05$) the $CD11b^+TLR3^+$ subsets (Fig. 1B) and $CD11b^+TLR4^+$ subsets (Fig. 1D) compared to mice challenged with *S. typhi* only. Interestingly, NRMOL at doses 14 and 42 mg/kg BW more effective than FRMOL to reduce $CD11b^+TLR3^+$ and $CD11b^+TLR4^+$ subsets in mice challenged with *S. typhi*.

3.2. NRMOL and FRMOL declines proinflammatory cytokines in mice challenged with S. typhi

Proinflammatory cytokines are the main end-product of inflammatory processes. The present study data suggest that NRMOL is more effective than FRMOL to reduce $CD11b^+IL\text{-}6^+$ (Fig. 2A), shared the same better effect to reduce $CD11b^+IL\text{-}17^+$ subsets (Fig. 2C), and NRMOL reduces $CD11b^+TNF\text{-}\alpha^+$ subsets more effective FRMOL after *S. typhi* challenge (Fig. 2E). Based on dot plot analysis, $CD11b^+IL\text{-}6^+$, $CD11b^+IL\text{-}17^+$, and $CD11b^+TNF\text{-}\alpha^+$ subsets were increased in mice challenged with *S. typhi*. Both NRMOL and FRMOL administration significantly reduced ($p < 0.05$) the $CD11b^+IL\text{-}6^+$, $CD11b^+IL\text{-}17^+$, and $CD11b^+TNF\text{-}\alpha^+$ subsets compared to mice challenged with *S. typhi* only. Interestingly, NRMOL at doses 14 mg/kg BW is more effective than FRMOL to reduce $CD11b^+IL\text{-}6^+$ subsets in mice challenged with *S. typhi*. Surprisingly, NRMOL at doses 42 and 84 mg/kg BW reduced $CD11b^+IL\text{-}6^+$ subsets lower than healthy mice groups (Fig. 2B). Interestingly, NRMOL and FRMOL at doses 14 and 42 mg/kg BW reduce $CD11b^+IL\text{-}17^+$ subsets in mice challenged with *S. typhi* towards near healthy mice groups (Fig. 2D). NRMOL at doses 14 and 42 mg/kg BW is more effective than FRMOL to reduce $CD11b^+$ $TNF\text{-}\alpha^+$ subsets in mice challenged with *S. typhi* (Fig. 2F).

3.3. NRMOL and FRMOL restores naïve regulatory T cells in mice challenged with S. typhi

NRMOL and FRMOL had similar efficacy to improve $CD4^+CD25^+CD62L^+$ subsets after *S. typhi* challenge (Fig. 3A–B). Based on dot plot analysis, $CD4^+CD25^+CD62L^+$ subsets were decreased in mice challenged with *S. typhi* (Fig. 3A). Both NRMOL and FRMOL administration significantly increased the $CD4^+CD25^+CD62L^+$ subsets ($p < 0.05$) compared to mice challenged with *S. typhi* only (Fig. 3B). Interestingly, NRMOL and FRMOL restored $CD4^+CD25^+CD62L^+$ subsets in mice challenged with *S. typhi* similar to healthy mice groups, except NRMOL dose 14 mg/kg BW.

4. Discussion

Salmonella constitutes a considerable health burden by causing acute gastroenteritis, contributing almost half of morbidity and mortality attributable to typhoid fever in low- and middle-income countries. Antibiotics are commonly used to treat Salmonellosis in humans, however because of antibiotic resistance antibiotics are not entirely effective in combatting *Salmonella* [39]. Developing new products from natural plants can be an effective strategy however it is still challenging for many researchers for past few decades. Fermentation is a useful method for improving food products' biological properties, promoting the beneficial effects on health. Numerous evidences reported that fermentation using probiotics can be an alternative option to combat *Salmonella* [32,40].

Chronic *Salmonella* infection occurs when the host fails to completely clear bacteria from the body. An impaired immune response or gut microbiota disruption may be associated with the host's inability to clear *Salmonella* [41]. Our result demonstrated

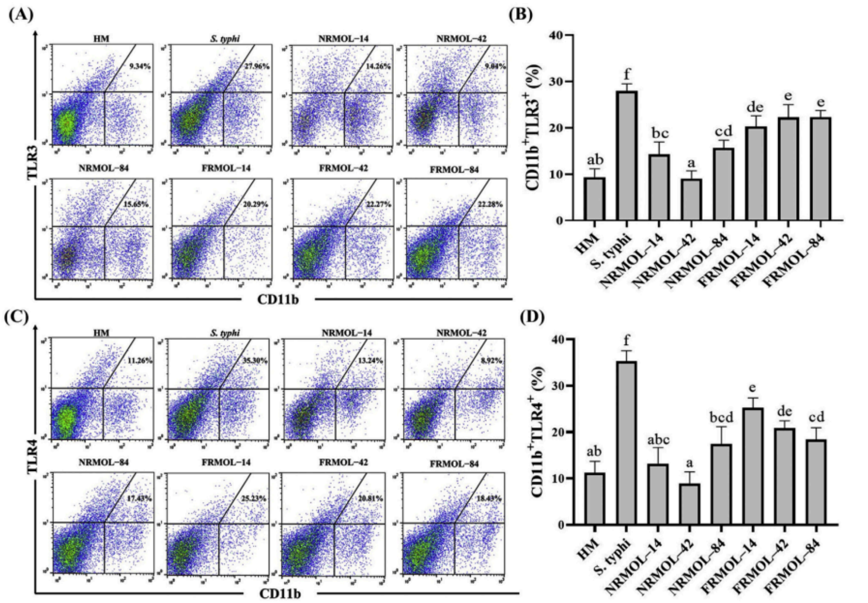

Fig. 1. Effect of NRMOL and FRMOL on CD11b+TLR3+ and CD11b+TLR4+ subsets in mice challenged with *S. typhi*. **(A)** The dot plot analysis of CD11b+TLR3+ subsets in spleen. **(B)** NRMOL reduced $CD11b^{+}TLR3^{+}$ subsets more effective FRMOL on mice challenged with *S. typhi*. **(C)** The dot plot analysis of $CD11b^{+}TLR4^{+}$ subsets in spleen. **(D)** NRMOL reduced $CD11b^{+}TLR4^{+}$ subsets more effective FRMOL on mice challenged with *S. typhi*. The different letter considered significantly different between each group ($p < 0.05$) by post hoc test using Tukey's HSD test. HM = Healthy Mice; NRMOL = Non-fermented red *M. oleifera* Leaves Extract; FRMOL = Fermented red *M. oleifera* Leaves Extract; NRMOL-14 = *S. typhi* + NRMOL 14 mg/kg BW; NRMOL-42 = *S. typhi* + NRMOL 42 mg/kg BW; NRMOL-84 = *S. typhi* + NRMOL 84 mg/kg BW; FRMOL-14 = *S. typhi* + FRMOL 14 mg/kg BW; FRMOL-42 = *S. typhi* + FRMOL 42 mg/kg BW; and FRMOL-84 = *S. typhi* + FRMOL 84 mg/kg BW.

that *S. typhi* elicits an immune response through an increase in expression of $CD11b^{+}$ $TLR3^{+}$ and $CD11b^{+}TLR4^{+}$ subsets. At the earliest infection phase by *Salmonella*, macrophages secrete proinflammatory cytokines, such as interferon-β (IFN-β), through TLR3 and TLR4 signaling pathways [13]. TLR3 and TLR4 share the same adaptor molecule, toll/interleukin-1 receptor (TIR) domain-containing adapter-inducing IFN-β (TRIF), which activates NFκB, interferon regulatory factor 3 (IRF3), and MAP kinase leading to the type I IFN transcription [42]. IFN will protect the host through antimicrobial autophagy by macrophages, while, the over-expression of IFN may result in impaired bacterial clearance [43]. *Salmonella* infection would worsen if the host cannot remove Salmonella from the body completely because the Salmonella present in the body will cause chronic inflammation and will also produce toxins that will induce mucosal damage [44]. A previous study reported that presence of *S. typhi* was observed in the caecum at 7-days post-infection. Moreover, the serum concentration of proinflammatory cytokines, including TNF-α and IL-6, was higher compared to control, indicating that *S. typhi* generated an inflammatory response during infection and led to caecum damage [45].

In the present study, NRMOL and FRMOL inhibited TLR3/TLR4 compared to the increased expression of TLR3/TLR4 detected in the *S. typhi* group. The bioactive compounds from MOL have improved host immunity and inhibited LPS induced inflammatory responses. LPS is well-known as the primary ligand for TLR4 signaling activation [46,47]. Some evidence suggeste that FRMOL could be a potent immunomodulator [35,36,48]. We would like to propose two possible mechanisms through which MOL could downregulate the TLR3 expression. First, the bioactive compounds of MOL influence TLR3/TRIF-dependent pathways. At various degrees, flavonoids can alter TLR pathways. Flavonoids can impact both TLR gene and cell membrane expression, which are both directly related to TLR functionality [49]. Luteolin and quercetin, present in MOL, were demonstrated to suppress the TRIF-dependent pathway. Interestingly, both luteolin and quercetin have a $C_2{-}C_3$ double bond in the carbonyl-containing C-ring, which is important for TANK-binding kinase 1 (TBK1) inhibition [50]. TRIF activates TBK1, phosphorylates IRF3 and induces the IFNs expression [13,42]. Further, luteolin's chemical structure, which has two hydroxyl groups at position 3′ and 4′ in B-ring, was considered an important factor to target TLR3 signaling pathways [50]. Second, the bioactive compounds of MOL may contribute indirectly through tissue repair. Our previous study revealed that FRMOL has a hepatoprotective effect via Nrf2 signaling pathways in mice challenged with *S. typhi*. FRMOL administration also repairs hepatocyte damage through reduced necrotic cells [51]. TLR3 could be activated through extracellular dsRNA released from damaged tissue [52]. The diminished expression of TLR3 by NRMOL or FRMOL may be caused by repairing tissue damage, which reduces the TLR3 main ligand.

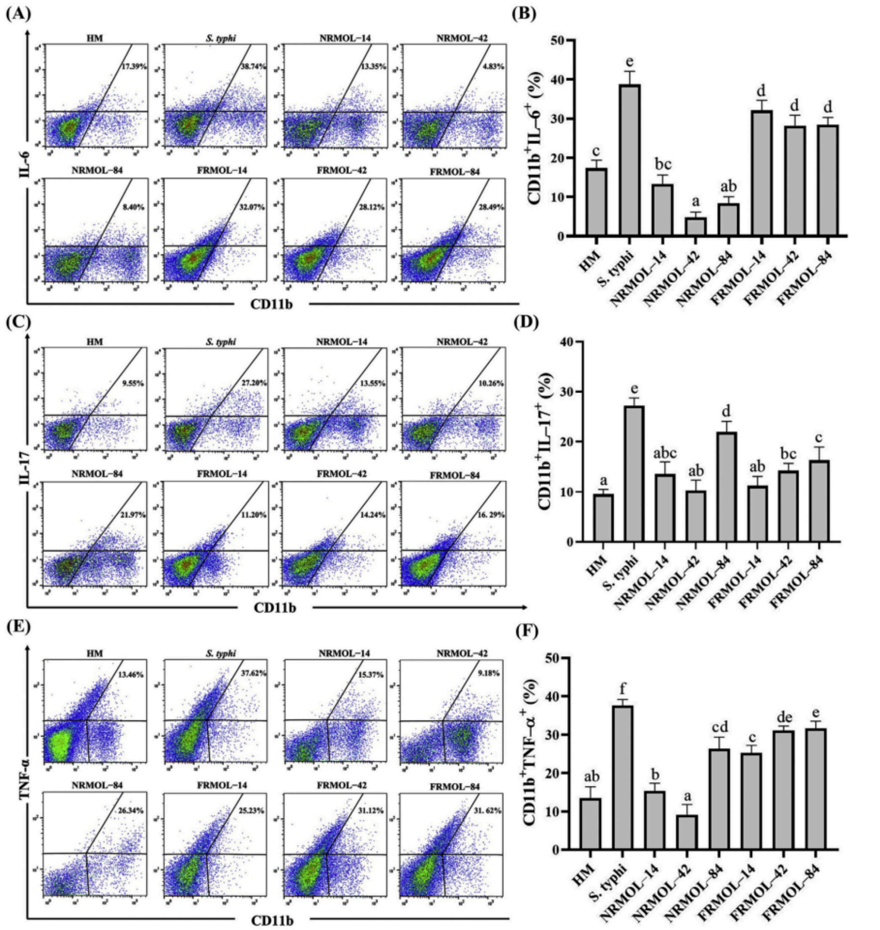

Fig. 2. Effect of non-fermented MOLE and fermented MOLE on proinflammatory generation in mice challenged with *S. typhi*. **(A)** The dot plot analysis of CD11b$^+$IL-6$^+$ subsets in spleen. **(B)** NRMOL reduced CD11b$^+$IL-6$^+$ subsets more effective FRMOL on mice challenged with *S. typhi*. **(C)** The dot plot analysis of CD11b$^+$IL-17$^+$ subsets in spleen. **(D)** NRMOL and FRMOL reduced CD11b$^+$IL-17$^+$ subsets on mice challenged with *S. typhi*. **(E)** The dot plot analysis of CD11b$^+$ TNF-α + subsets in spleen. **(F)** NRMOL reduced CD11b$^+$TNF-α$^+$ subsets more effective FRMOL on mice challenged with *S. typhi*. The different letter considered significantly different between each group ($p < 0.05$) by post hoc test using Tukey's HSD test. HM = Healthy Mice; NRMOL = Non-fermented red *M. oleifera* Leaves Extract; FRMOL = Fermented red *M. oleifera* Leaves Extract; NRMOL-14 = *S. typhi* + NRMOL 14 mg/kg BW; NRMOL-42 = *S. typhi* + NRMOL 42 mg/kg BW; NRMOL-84 = *S. typhi* + NRMOL 84 mg/kg BW; FRMOL-14 = *S. typhi* + FRMOL 14 mg/kg BW; FRMOL-42 = *S. typhi* + FRMOL 42 mg/kg BW; and FRMOL-84 = *S. typhi* + FRMOL 84 mg/kg BW.

The data from the present study demonstrated that NRMOL inhibits proinflammatory cytokines, IL-6, IL-17, and TNF-α secreted by macrophages. These findings are in agreement with the previously published results by our group that inhibition of TLR3 and TLR4 as upstream of signaling pathways would give a beneficial implication. *S. typhi* infection amplifies an inflammatory response and induces macrophages to secrete a huge number of proinflammatory cytokines [9]. TNF-α is essential for generating a systemic inflammatory response that leads to lethal shock [53]. Interestingly, TNF-α collaboration with IL-17 triggers other proinflammatory cytokines production [54]. A recent study reported that red *M. oleifera* contains higher quercetin than green *M. oleifera* [55].

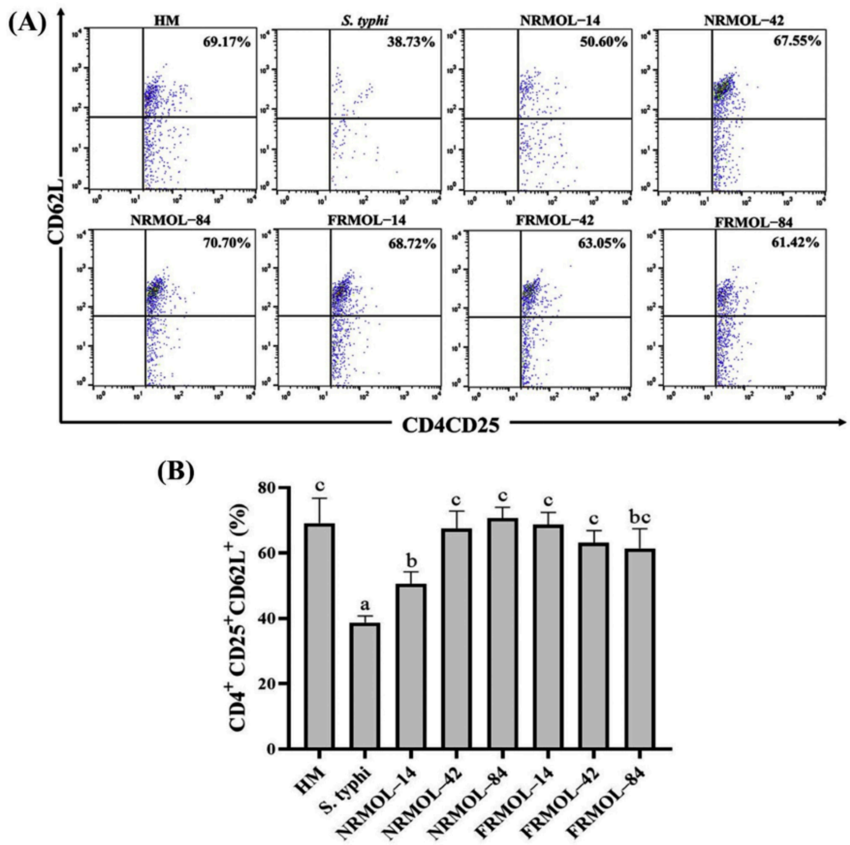

Fig. 3. Effect of NRMOL and FRMOL on $CD4^+CD25^+CD62L^+$ subsets in mice challenged with *S. typhi*. **(A)** The dot plot analysis of $CD4^+CD25^+CD62L^+$ subsets in spleen. **(B)** NRMOL and FRMOL increased $CD4^+CD25^+CD62L^+$ subsets on mice challenged with *S. typhi*. The different letter considered significantly different between each group ($p < 0.05$) by post hoc test using Tukey's HSD test. HM = Healthy Mice; NRMOL = Non-fermented red *M. oleifera* Leaves Extract; FRMOL = Fermented red *M. oleifera* Leaves Extract; NRMOL-14 = *S. typhi* + NRMOL 14 mg/kg BW; NRMOL-42 = *S. typhi* + NRMOL 42 mg/kg BW; NRMOL-84 = *S. typhi* + NRMOL 84 mg/kg BW; FRMOL-14 = *S. typhi* + FRMOL 14 mg/kg BW; FRMOL-42 = *S. typhi* + FRMOL 42 mg/kg BW; and FRMOL-84 = *S. typhi* + FRMOL 84 mg/kg BW.

Quercetin is reported to suppress the lethal shock caused by *S. typhi* infection. The hydroxyl groups of quercetin are important factors for delaying *S. typhi* action [53]. Our previous study also showed that FRMOL increased its total flavonoid content [35]. Our findings are also supported by a study that reported milk fermented with *Lactobacillus* reduced IL-17, TNF-α, and IL-6 in mice challenged with *Salmonella* [32,56]. Acurcio et al., 2017 proposed that the possible mechanism of a protective effect from *L. plantarum* is due to modulation of the host immune system [32]. Further, probiotic use could stimulate the anti-inflammatory cytokine expression, followed by reducing proinflammatory cytokines [57]. The anti-inflammatory properties of MOL have been reported to decline NFκB and proinflammatory cytokines expression in several animal models of inflammation [58,59]. Based on our findings, we assumed that both NRMOL and FRMOL might be useful to also treat other inflammatory diseases through the downregulation of inflammatory cytokines in the TLRs signaling pathway.

Our result also demonstrated that NRMOL and FRMOL restored the naïve Tregs. Tregs are essential to maintain peripheral immune tolerance to self-antigen. Tregs play an important role in controlling immune response during infection [60]. Tregs suppress the effector T cells by various mechanisms, mainly producing IL-10. The balance between Tregs and effector T cells reflects the immune homeostasis [61]. A previous study reported that quercetin and other flavonoids could modulate the aryl hydrocarbon receptor (Ahr), which further influenced Tregs' activation via Foxp3, the main transcription factor for Tregs [62–64]. On the other hand, the activation of heme oxygenase-1 (HO1) via the Nrf2/HO-1 signaling pathway is reported to increase Foxp3 expression. Meanwhile, a previous study reported that FRMOL increased HO-1 expression via the Nrf2/HO-1 signaling pathway. Naïve Tregs ($CD4^+CD25^+CD62L^+$ subsets) could be influenced by nutrients and improve their suppressive function and number [65–67]. Based on our result, we suggest that the bioactive compound in NRMOL and FRMOL may benefit from restoring naïve Tregs, which may implicate Th cell responses during *S. typhi* infection.

5. Conclusion

In summary, our finding showed that NRMOL efficiently reduced $CD11b^+TLR3^+$, $CD11b^+TLR4^+$, $CD11b^+IL\text{-}6^+$ and $CD11b^+TNF\text{-}\alpha^+$ compared to FRMOL. In addition, NRMOL and FRMOL displayed similar effect in reducing $CD11b^+IL\text{-}17^+$ and restoring naïve Tregs. The main goal of anti-inflammatory therapy is inhibition of TLR3/TLR4 and the proinflammatory cytokines followed by the recovery of naïve Tregs. Our study provides evidence that the anti-inflammatory properties of RMOL could become a promising supplement to treat *S. typhi* infection or might be other inflammatory diseases. Future studies are required for exploring, the molecular mechanisms of NRMOL and FRMOL.

Ethical approval

All experimental procedures were approved by the Animal Care and Use Committee of Brawijaya University with approval number: 829-KEP-UB. All the experiments were conducted following the Guide to the Care and Use of Laboratory Animals (National Institutes of Health, United States).

Source of funding

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Conflict of interest

None.

Author contributions

MM Riyaniarti Estri Wuryandari: Conceptualization, Funding acquisition, Methodology, Writing - Original draft preparation. **Mochammad Fitri Athoillah:** Formal analysis, Writing - Original draft preparation, Visualization. **Rizky Dzariyani Laili:** Data curation, Investigation. **Siti Fatmawati:** Data curation, Investigation. **Nashi Widodo:** Supervision, Writing - Review & Editing: **Edi Widjajanto:** Supervision, Writing- Reviewing and Editing **Muhaimin Rifaii:** Conceptualization, Resources, Supervision, Writing Review & Editing.

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