Original Research Article

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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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 in 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-6⁺, CD11b⁺IL-17⁺, CD11b⁺TNF-α⁺, 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-6⁺, CD11b⁺IL-17⁺, and CD11b⁺TNF-α⁺ 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-α⁺ 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.

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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,
including abdominal pain, acute diarrhea, fever, nausea, vomiting, and sometimes lethal septicaemia [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 circumstantially 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, fermentation 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 [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 × 108 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 × 107 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 catabolase test. Briefly, one osmium from cultured Salmonella was dipped in object-glass containing H₂O₂ 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 were 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 7D4), 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 phycoerythrin/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-6⁺ (Fig. 2A), shared the same better effect to reduce CD11b⁺IL-17⁺ subsets (Fig. 2C), and NRMOL reduces CD11b⁺TNF-α⁺ subsets more effective FRMOL after S. typhi challenge (Fig. 2E). Based on dot plot analysis, CD11b⁺IL-6⁺, CD11b⁺IL-17⁺, and CD11b⁺TNF-α⁺ subsets were increased in mice challenged with S. typhi. Both NRMOL and FRMOL administration significantly reduced (p < 0.05) the CD11b⁺IL-6⁺, CD11b⁺IL-17⁺, and CD11b⁺TNF-α⁺ 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-6⁺ subsets in mice challenged with S. typhi. Surprisingly, NRMOL at doses 42 and 84 mg/kg BW reduced CD11b⁺IL-6⁻ subdivisions lower than healthy mice groups (Fig. 2B). Interestingly, NRMOL and FRMOL at doses 14 and 42 mg/kg BW reduce CD11b⁺IL-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-α⁻ 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 not entirely effective in combatting 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...
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 overexpression 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 suggests 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 C2=C3 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.
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].
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 effect of 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-6\(^-\) and CD11b\(^+\)TNF-α\(^-\) compared to FRMOL. In addition, NRMOL and FRMOL displayed similar effect in reducing CD11b\(^+\)IL-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 Institute of Health, United States).

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Conflict of interest

None.

Author contributions

MM Rijiani Estri Wuryandari: Conceptualization, Funding acquisition, Methodology, Writing - Original draft preparation. Mochammad Fitri Atho'illah: 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 Rifai: Conceptualization, Resources, Supervision, Writing Review & Editing.

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