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Potential of multi-strain probiotics extract as an anti-inflammatory agent through inhibition of macrophage migration inhibitory factor activity

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ABSTRACT

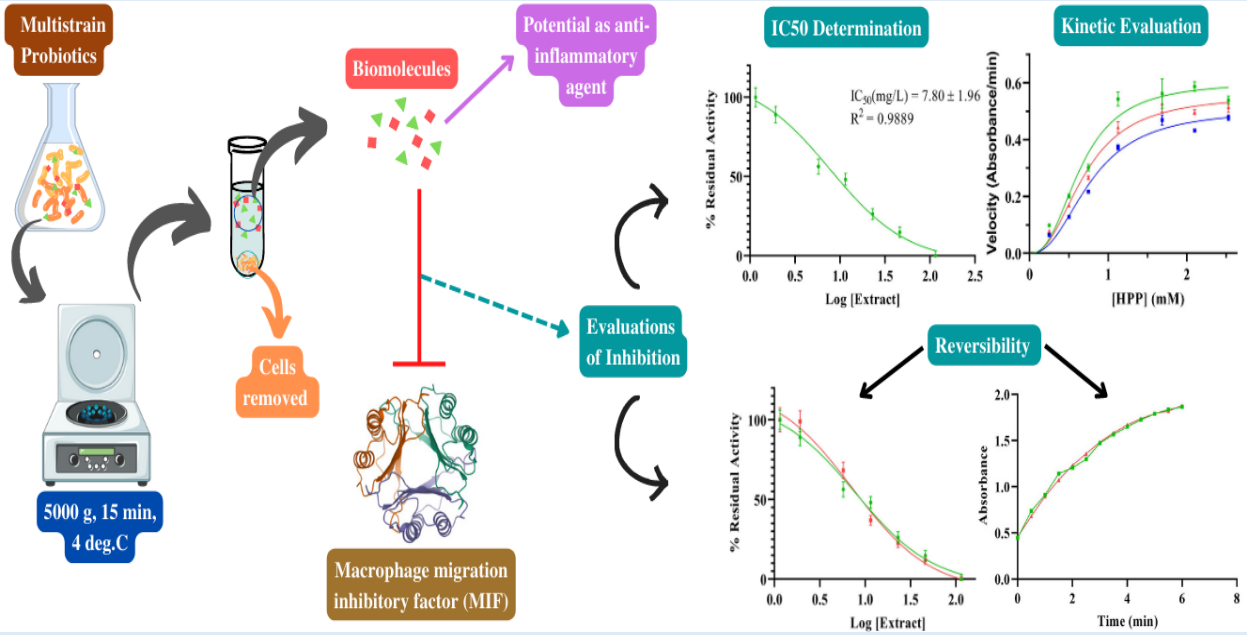
Background: Probiotics are reported to have a role for improving health conditions and reduce the risk of diseases associated with inflammation. However, how they affect inflammation has not been well studied. The inflammation occurs during the progression of chronic diseases could damage the normal function of cells, tissues, and organs. Macrophage migration inhibitory factor (MIF) is recognized as a cytokine playing a key role in the inflammation process. Inhibition of its activity has been used by researchers as an approach for alleviating the inflammation. This study aims to evaluate the potential inhibitory effect of the extract of multi-strain probiotics consisting of *Lactobacillus casei* EMRO 002*, L. casei EMRO 213, L. plantarum* EMRO 009*, L. fermentum* EMRO 211*, L. rhamnosus* EMRO 014*, L. bulgaricus EMRO 212*, and *Rhodopseudomonas palustris* EMRO 201 on MIF tautomerase activity, the reversibility, and the mechanism of inhibition. Hence, the scope of this study is the evaluation of the functional characteristics of multi-strain probiotics extract on MIF activity.

Methods: The multi-strain probiotics were centrifuged and the supernatant was separated from the pellet. The supernatant, i.e. the extract, was mixed with MIF and analyzed for its inhibition effect on MIF tautomerase activity. The reversibility of inhibition was evaluated by preincubation and dilution assays, and the mechanism of inhibition was determined by kinetic evaluation.

Results: The multi-strain probiotics extract inhibited MIF tautomerase activity with an IC_{50} of 7.80 ± 1.96 mg/L. The preincubation and dilution assays showed that the inhibition is reversible, and the kinetic evaluation predicted that the extract components might bind to the enzyme active site and other site(s) of MIF.

Conclusion: The findings show that the extract was able to inhibit MIF tautomerase activity reversibly and its components might bind to the enzyme active site and other site(s) of MIF suggest that the multi-strain probiotics extract has potential to be used as an anti-inflammatory agent for the treatment of chronic diseases associated with inflammation.

Keywords: Multi-strain probiotics, anti-inflammatory, macrophage migration inhibitory factor, reversible inhibition, kinetic evaluation.



INTRODUCTION

Probiotics are composed of live microorganisms which provide health benefit for the host [1]. Bacteria belonging to *Lactobacillus* and *Bifidobacterium* strains, are the most common groups to be used as probiotics [2]. Many foods and beverages are supplemented with these good bacteria to increase their nutrition's value. They are categorized as functional foods due to their beneficial effects which can improve the health status and reduce the risk of diseases. It is reported that consuming probiotics regularly could improve mental health, decrease the level of C-reactive protein and serum insulin, reduce total cholesterol and low-density lipoprotein (LDL), and lower the risk of diarrhea in children and adults [3-6].

Probiotics excrete biomolecules which play important roles in balancing the immune system. Several studies showed that probiotics resolve the imbalance of the cytokines which are produced in the body due to inflammation. Investigations conducted with the oral administration of *Lactobacillus* was reported to induce the production of anti-inflammatory molecules interleukin-10 (IL-10) preventing cells from necrosis [7].

Probiotics were also shown to be able to suppress inflammation in the gut by inhibiting the regulation of TLR expression, TNF-α, and NF-kB signaling [8]. Investigation with animal and human indicated that probiotics have potential beneficial effects for the prevention and treatment of a wide variety of inflammatory diseases such as rheumatoid arthritis, ulcerative colitis, multiple sclerosis, and hepatic encephalopathy [9].

In severe cases of inflammation, excessive production of cytokines creates a condition called cytokine storm, which has detrimental outcomes resulting in organ failure and patient mortality [10]. The lethal effect of cytokine storm can be found in COVID-19, a disease caused by SARS-CoV-2 infection. It is reported that the cytokine storm is the major cause of patients' death in COVID-19 case [11]. In this condition, the level of interleukin-6 (IL-6) increases up to 3-fold higher than that in normal patients [12]. During the infection, a high number of other cytokines is produced to counteract the virus. As a consequence, the permeability of blood vessels is increased which affect the amount of fluid and blood cells entering the alveoli of the lungs – the area of infection. In the end, the condition leads to shortness of breath or dyspnea and failure of the respiratory system. Hence, anti-inflammatory agents are required to treat the condition [10].

Macrophage migration inhibitory factor (MIF) is a key cytokine responsible for the progression of inflammation. It is a homotrimeric protein with a native monomer's size of approximately 12.5 kDa which consists of 115 amino acid residues with two α-helices packed against four β-strands [13]. It was originally discovered as a lymphokine involved in delayed hypersensitivity and various macrophage functions, including phagocytosis, spreading, and tumoricidal activity [14]. Lately, it was re-evaluated and recognized as a pro-inflammatory cytokine that plays a key role in the inflammatory process. Through binding to the CD74 receptor, MIF promotes immune response's cascade by the activation of macrophages and T cells. Hereafter, it triggers the production of multiple cytokines such as TNF, IFN-γ, IL-1β, IL-2, IL-6, IL-8, nitric oxide, COX2, PGE2 [15-16]. It was also reported that MIF inhibits glucocorticoid production which plays an important role for anti-inflammatory effects [17-19]. Hence, inhibiting the MIF activity may alleviate the inflammation.

Besides its role as a cytokine, MIF is also found to possess tautomerase activity which catalyzes the keto-enol tautomerization of D-dopachrome, 4-hydroxyphenylpyruvate (HPP), and thiol-protein oxidoreductase (TPOR) [15,20-21]. One particular important residue for tautomerase activity is the N-terminal proline (Pro-1) which acts as a catalytic base in the tautomerase reaction [21]. Mutation of Pro-1 to glycine substantially reduces the catalytic activity [22].

The tautomerase activity of MIF has been used as an approach by researchers discovering multiple classes of MIF inhibitors [19-20]. The enzyme active site of MIF is situated near to the CD74 receptor binding, containing highly conserved amino acids [20]. Interaction between MIF and CD74 is known to be essential for pro-inflammatory activity of MIF [23]. Several studies showed that compounds which bind at or near the enzyme active site are known to affect MIF interaction with the CD74 [13]. Hence, compounds with the ability to inhibit MIF tautomerase activity could be useful for disrupting MIF and CD74 interaction that might result in the reduction of MIF pro-inflammatory activity. In this study, we evaluated the potential inhibitory effect of the extract of multi-strain probiotics comprising of *Lactobacillus* spp. and *R. palustris* on MIF activity. Furthermore, we also conducted the reversibility and kinetic evaluation to investigate the mechanism of MIF inhibition by the extract.

METHODS

Multi-strain Probiotics Extract: The multi-strain probiotics were obtained from AMRO Institute in Surabaya, Indonesia. They consist of *L. casei, L. plantarum, L. fermentum, L. bulgaricus, L. rhamnosus,* and *R. palustris* in a fermented medium (0.20% b/v of citric acid, 1.13% v/v of honey, 1.07% v/v of *Aloe vera* juice, 3.07% v/v mollasses, and 86.87% v/v water) with a pH of 4 in the beginning and 3.2 in the end. The fermentation is conducted under anaerobic condition at 26 ^{o}C for ± 45 days. The number of microorganisms present in the initial probiotics before centrifugation was at least 2 x 10^6 CFU/mL. The extract was obtained by separating the supernatant from the pellet by centrifugation (Sorvall Biofuge Stratos, Thermo Scientific, USA) at 5000 g, 4 ^{o}C for 15 min. The pellet was removed and the supernatant, i.e. the extract, was used for the assays.

Solute Concentration of Extract: The concentration of solutes in the extract was measured as follows: 100 mL of extract was taken and dried at 100 °C in an oven (Memmert, Germany) until constant weight. The solute concentration was calculated in g/L.

IC_{50} Determination of Inhibition: MIF tautomerase activity was measured using recombinant His-tagged MIF from *Escherichia coli* BL21 (DE3), which was purified with Ni-NTA resin (BioRad, USA). The assay was done following the procedure of Kok *et.al.* with few modifications [21].

Stock solution of 10 mM 4-hydroxy phenylpyruvate (4-HPP, Sigma Aldrich, Switzerland) was made in 50 mM ammonium acetate buffer at pH 6.0 and incubated overnight at room temperature to allow equilibration between keto and enol form. Then, it was diluted to 1 mM with the same acetate buffer and used as the substrate for tautomerase activity assay. MIF was diluted in 0.4 M boric acid at pH 6.2 to give suitable concentration for the assay, i.e. 16 nM.

For IC_{50} determination, 90 µL of diluted MIF (16 nM) was put into UV microplate 96 wells (Corning VWR, USA) and mixed with 10 µL of extract solution to give final extract concentrations of 0 mg/L; 1.15 mg/L, 1.92 mg/L, 5.75 mg/L, 11.50 mg/L, 23.00 mg/L, 46.00 mg/L, and 115.00 mg/L. Each solution was then mixed with 100 µL of 1 mM 4-HPP (to give a final concentration of 0.5 mM 4-HPP) and rapidly measured with a microplate reader (FLUOstar Omega, Labtech, Germany). The absorbance at 306 nm was plotted against time, at 37 °C, 15-25 cycles, mode: kinetic, pre-shaking: 3 sec, shaking speed: 300 rpm. For negative control, 90 µL of boric acid buffer was added with 10 µL of distilled water, mixed with 100 µL of 1 mM 4-HPP, and measured as described before.

Reversibility of Inhibition: Reversibility evaluation was conducted by preincubation and dilution assays to examine whether the inhibition occurs reversibly or not.

Preincubation assay was done by incubating MIF with the extract for 2 min (regular IC_{50} assay) and 30 min incubation prior to the addition of substrate, 4-HPP. The % residual activity vs. log [extract] curves and IC_{50}s were compared.

Dilution assay was done by mixing the concentrated MIF (the final concentration of 1600 nM, 100-fold of its regular concentration) without and with the concentrated extract (final concentration of 78 mg/L, 10-fold of its IC_{50}). Subsequently, each mixture was diluted 100-fold with boric acid and 4-HPP in ammonium acetate buffer to meet the regular buffer condition and measured as described before. The absorbance was plotted against time and both curves were compared.

Kinetic Evaluation of Inhibition: Kinetic evaluation gives the prediction on how the extract components interact with MIF, whether at the enzyme active site or at other site(s) (allosteric), or at both sites (mixed). To this end,

the extract solution was prepared to give a final concentration of 2.88 mg/L and 5.75 mg/L; the substrate solution was prepared to give a final concentration of 0.25 mM; 0.50 mM; 0.75 mM; 1.13 mM; 1.69 mM; 2.10 mM; and 2.53 mM. Then, the velocity of enzyme reaction was measured at increasing substrate concentration in the presence of the extract at concentrations of 0 mg/L, 2.88 mg/L and 5.75 mg/L.

Analysis of data: All measurements were conducted in 3 replicates. For IC_{50} determination and preincubation assays, analysis was done by firstly taking the slope of linear parts of the absorbance vs. time graph and processed further with sigmoidal curve fitting function of GraphPad Prism 8.0 software.

For dilution assay, the analysis was done by plotting the absorbance vs. time, and for kinetic evaluation the analysis was done by plotting the velocity of reaction (absorbance/min) vs. concentration of substrate using enzyme kinetics-sigmoidal curve function of GraphPad Prism 8.0 software, yielding the V_{max}, Hill slope and K_{half}.

RESULTS

Solute Concentration of Extract: A certain volume of multi-strain probiotics was centrifuged to separate the supernatant, i.e. the extract, from pellet. The extract was then dried in an oven until a constant weight. The solute concentration of extract was found to be 23 g/L.

Inhibition of MIF Tautomerase Activity by Extract: The extract was serial diluted to several concentrations and evaluated for its inhibition on MIF tautomerase activity. The assay is based on the detection of the enzymatic enol product of 4-HPP in complex with boric acid. The result showed that the extract inhibits MIF tautomerase activity with an IC_{50} of 7.80 ± 1.96 mg/L (R^2 = 0.989) (Figure 1).

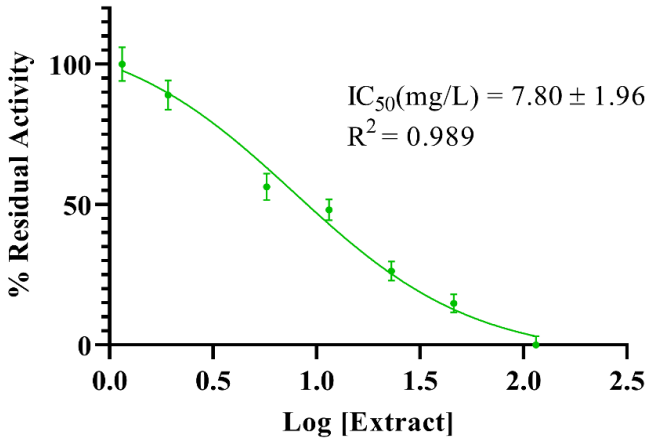
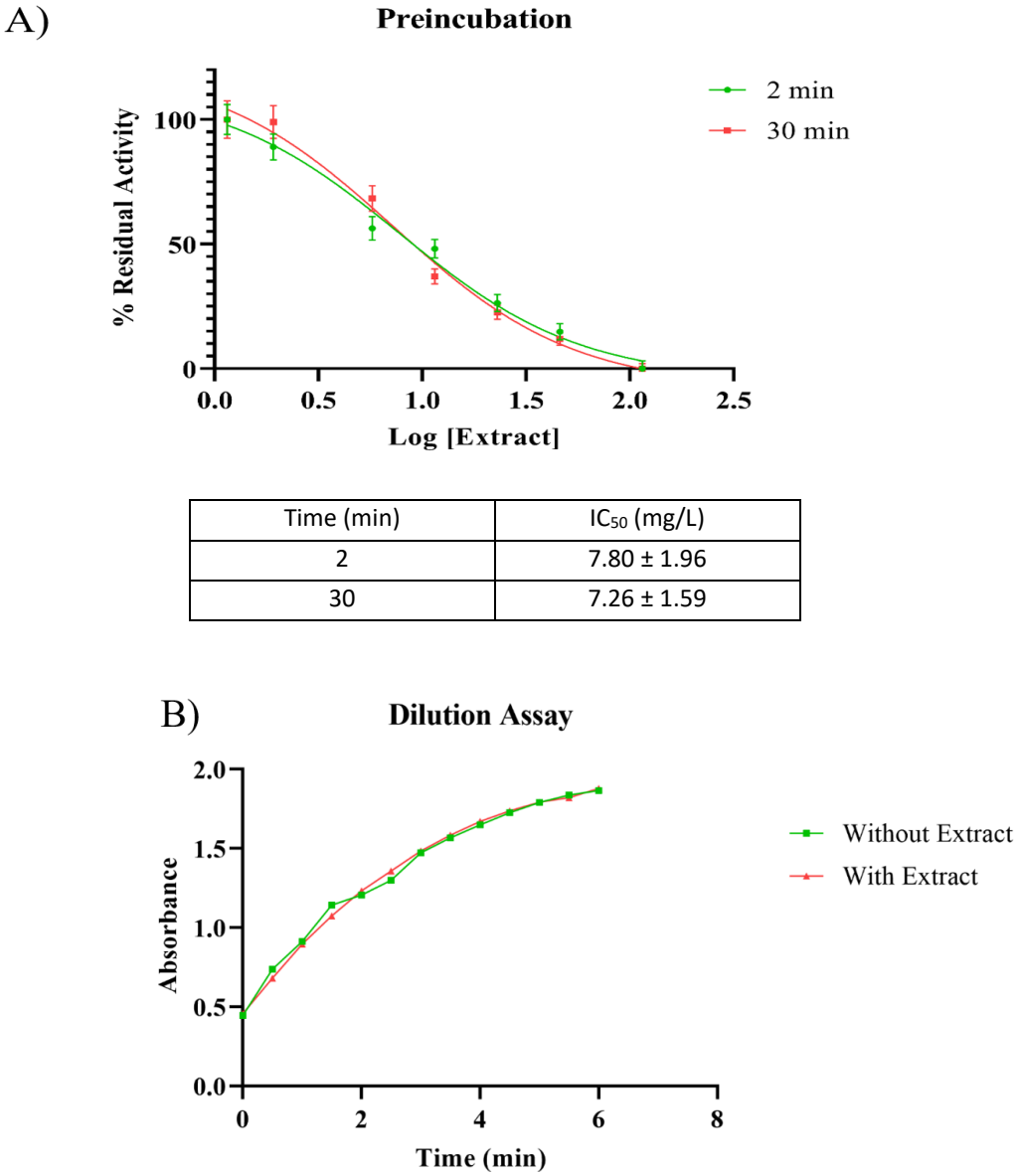


Figure 1. Inhibition of MIF tautomerase activity by multi-strain probiotics extract.

Reversibility of Inhibition: To evaluate the reversibility of the inhibition on MIF tautomerase activity by the extract, we performed preincubation and dilution assays.

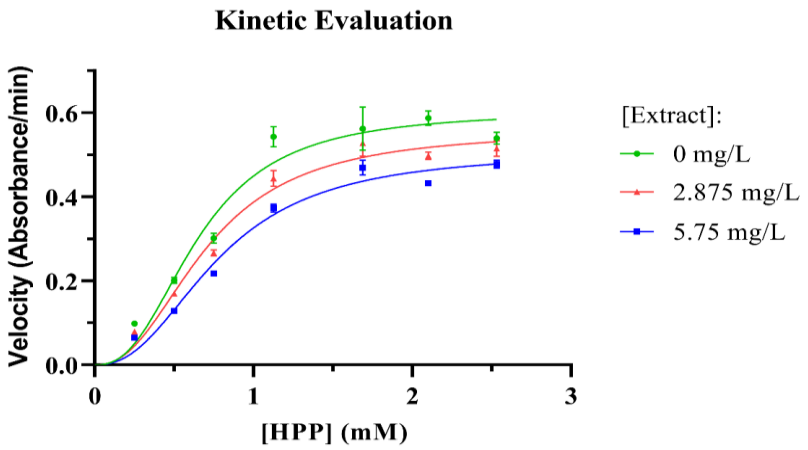
In preincubation assay, the extract was mixed with MIF for 2 min (regular IC_{50} assay) and 30 min incubation before reaction with the substrate. The result showed that both sigmoidal curves are similar and there is no apparent difference between both IC_{50}s, i.e. 7.80 ± 1.96 mg/L (for 2 min preincubation) and 7.26 ± 1.59 mg/L (for 30 min preincubation) (Figure 2A). In dilution assay, 100-fold concentrated MIF was mixed without and with 10-fold of IC_{50} concentrated extract and each mixture was

subsequently diluted 100-fold with the boric acid buffer and 4-HPP in ammonium acetate buffer to meet the regular buffer condition. The result showed that after dilution both absorbance curves are practically the same (Figure 2B), suggesting that the activity of MIF mixed with the concentrated extract is recovered after dilution. Both preincubation and dilution assays confirmed that the inhibition of MIF activity by the extract is reversible [21].



Time (min)	IC_{50} (mg/L)
2	7.80 ± 1.96
30	7.26 ± 1.59

Figure 2. A) MIF was mixed with multi-strain probiotics extract for 2 min (regular IC_{50} assay) and 30 min incubation prior to the addition of substrate. It was found that both sigmoidal curves and IC_{50}s are similar. B) Concentrated MIF (100-fold of regular concentration) was incubated without and with concentrated extract (10-fold of IC_{50}). Subsequently, the mixture was diluted 100-fold with the boric acid buffer and 4-HPP in ammonium acetate buffer to meet the regular buffer condition, and the enzyme activity was monitored. Diluting the mixture of concentrated MIF and concentrated extract recovered the enzyme activity.



Parameter	[Extract] (mg/L)		
	0	2.875	5.75
V_{max} (Absorbance/min)	0.60 ± 0.06	0.56 ± 0.04	0.51 ± 0.05
Hill slope	2.50 ± 0.82	2.35 ± 0.56	2.37 ± 0.58
K_{half} (mM)	0.65 ± 0.09	0.70 ± 0.08	0.78 ± 0.10

Figure 3. The velocity of MIF tautomerase reaction was measured at increasing concentrations of substrate, 4-HPP, in the presence of the inhibitor, i.e. the extract, at some concentrations. V_{max}, Hill slope, and K_{half} were determined for each concentration of extract.

Kinetic Evaluation of Inhibition: To complete the study of MIF tautomerase inhibition by the extract, we performed kinetic evaluation. The velocity of MIF tautomerase reaction (absorbance/min) was measured at increasing substrate concentrations in the presence of the inhibitor, i.e. the extract, at some concentrations, and the kinetic parameters were determined (Figure 3).

DISCUSSION

Probiotics are live microorganisms beneficial for human health when administered in proper amounts. In particular, bacteria belonging to *Lactobacillus* and *Bifidobacterium* strains are the most common groups to be used as probiotics. They are reported as advantageous bacteria for prevention of inflammatory bowel diseases, lactose intolerance, intestinal microbial imbalance, and other related diseases [7]. It was well established that probiotic containing *Lactobacillus* modulates the immune system by interaction with *Toll-like Receptor* (TLR) which induces anti-inflammatory cytokine production [8].

In this study, we evaluated the potential inhibitory effect of the extract of multi-strain probiotics consisting of *L. casei, L. plantarum, L. fermentum, L. bulgaricus, L. rhamnosus,* and *R. palustris* on MIF activity.

MIF is a cytokine that plays a key role in the progression of inflammation. The involvement of MIF in immune system is often associated with inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, atherosclerosis, asthma, inflammatory liver disease, and systemic lupus erythematosus [24]. The approach to inhibit MIF by targeting its tautomerase activity has been widely taken as an effort for anti-inflammatory agent discovery. The discovered N-acetyl-p-benzoquinone imine (NAPQI) as a MIF tautomerase inhibitor also showed inhibition on MIF biological activity [25]. Moreover, intensive study on isoxazole-1 (ISO-1) derivatives revealed that the compounds hindering MIF tautomerase activity could interfere with MIF biological functions which are

responsible for inflammation [26]. These studies provided examples that compounds binding to the MIF enzyme active site could have the potential to disrupt its pro-inflammatory functions [20].

The results of this study showed that the extract of the multi-strain probiotics can inhibit MIF tautomerase activity, with an IC_{50} of 7.80 ± 1.96 mg/L (Figure 1). The preincubation and dilution assays showed that the inhibition of MIF by the extract is reversible. In preincubation assay, MIF mixed with the extract for 2 min (regular IC_{50} assay) and for 30 min incubation prior to the addition of substrate showed similar sigmoidal curves and IC_{50} values, i.e. 7.80 ± 1.96 mg/L and 7.26 ± 1.59 mg/L, respectively (Figure 2A). Hence, the inhibition of MIF tautomerase activity by the extract is not time dependent. In dilution assay, the tautomerase activity of concentrated MIF mixed with concentrated extract was recovered to its original state after 100-fold dilution, as shown by similar absorbance vs. time curves, suggesting a reversible inhibition (Figure 2B). Most of reversible inhibitors that bind to MIF enzyme active site form hydrogen bonds with Pro-1; Lys-32; Ile-64; and/or Asn-97; and engage in aryl–aryl interactions with Tyr-36; Tyr-95; and/or Phe-113 [20]. In combination with an irreversible inhibitor, MIF will show no activity after dilution and the absorbance vs. time curve of the mixture will be horizontal [21]. The reversibility of the inhibition of MIF tautomerase activity by the extract, confirmed by preincubation and dilution assays, is an expected characteristic for ensuring a safety use of the extract. Irreversible inhibitors, on the other hand, would be accumulated in the body for a long period of time and may cause unintended adverse reactions.

To complete the study, we evaluated the kinetics of MIF inhibition by the extract. In this evaluation, the velocity of enzyme reaction was plotted against increasing substrate concentration in the presence of the inhibitor, i.e. the extract, at some concentrations (Figure 3). The results showed that the velocity of reaction (absorbance/min) of the sigmoidal curves increases with the increasing substrate concentration. Likewise, the K_{half} increases with the increasing extract concentration. Both of the increases, the velocity of reaction and K_{half}, represent a competitive-like type of inhibition. This type of inhibition is indicated by the direct competition between inhibitor and substrate to bind at the active site of an enzyme [27]. However, the increase of velocity of reaction in the presence of the inhibitor, i.e. the extract, did not reach maximum velocity (V_{max}). In this case, the V_{max} decreased with the increasing extract concentration. These indicate that the inhibition of MIF tautomerase activity by the extract is not really competitive. Furthermore, the Hill slope was found larger than 1 suggesting that the inhibition of MIF tautomerase activity by the extract is also of allosteric type, i.e. the binding of extract components also occurs at other(s) site of MIF. This allosteric type of inhibition might lead to the alteration of MIF conformation, causing lack of its pro-inflammatory activity [28]. Taken together, it could be predicted that the inhibition of MIF tautomerase activity by the extract is a combination between competitive and non-competitive one, i.e., the extract components bind not only at enzyme active site, but also at other site(s) of the protein. Such combination could be anticipated because the extract might contain several types of metabolites. Previous study using the symbiosis culture of *L. casei, Lactobacillus farraginis, R. palustris, Saccharomyces cerevisiae, and Candida ethanolica* reported that metabolites produced during fermentation such as 16 alpha-hydroxyestrone, gingerol, embelin, and diallyl sulfide are predicted to possess anti-inflammatory activity [29]. Other studies reported that bacteriocin produced by *L. plantarum* shows activity as an anti-inflammatory agent and bacteria-produced long chain

fatty acid (3-hydroxy octadecaenoic acid) could be implicated in the probiotics anti-inflammatory properties [30-31].

CONCLUSION

Multi-strain probiotics extract from *Lactobacillus* spp. and *R. palustris* was found to be able to inhibit MIF tautomerase activity. Preincubation and dilution assays indicated that the extract inhibits MIF reversibly and kinetic evaluation suggested that the inhibition of MIF activity by the extract is a combination between competitive and non-competitive one, i.e., the extract components bind not only at enzyme active site, but also at other(s) site of the protein. Having such characteristics, it is implicated that the multi-strain probiotics extract could probably reduce the MIF pro-inflammatory activity. Hence, the multi-strain probiotics extract could potentially be used as an anti-inflammatory agent for the treatment of chronic diseases associated with inflammation.

List of Abbreviations: MIF: macrophage migration inhibitory factor, 4-HPP: 4-hydroxy phenylpyruvate, CD74: cluster of differentiation 74, IC: inhibitory concentration.

Authors' Contributions: TK conceptualized and supervised the work, designed the experiments, and critically reviewed the manuscript. DN carried out the experiment, analysis, and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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