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Immunomodulatory activity of exopolysaccharides produced by *Leuconostoc mesenteroides* strains isolated from palm (*Borassus flabellifer* L.) sap

A Ma'unatin^{1,2,*}, Harijono¹, E Zubaidah¹ and M Rifa'i³

¹Department of Agricultural Technology, Brawijaya University, Jl. Veteran 6, Malang 65144, East Java, Indonesia

²Department of Chemistry, Maulana Malik Ibrahim State Islamic University Malang, Jl. Gajayana 50 Dinoyo, Malang 65144, East Java, Indonesia

³Department of Biology, Brawijaya University, Jl. Veteran 6, Malang 65144, East Java, Indonesia

*E-mail: a_maunatin@yahoo.com

Abstract. This study was to evaluate the immunomodulatory effects of exopolysaccharides (EPS) produced by *Leuconostoc mesenteroides* strains isolated from palm (*Borassus flabellifer* L.) sap. The EPS used were produced by two strains of *Leuconostoc mesenteroides* (N5 and N7) on different medium which were palm sap (EPS NSN5 and EPS NSN7) and MRS supplemented with sucrose (EPS MSN5 and EPS MSN7). EPS were given to BALB/c mice before infected by lipopolysaccharide (LPS). The results of flow cytometric analysis of spleen lymphocytes showed some EPS were able to increased cytokines production (IL-2, INF- γ and TNF- α) by CD4⁺ cells. IL-2 production decreased with EPS NSN5 and EPS NSN7, INF- γ decreased with EPS MSN7 while TNF- α decreased with EPS NSN7. These results indicated that the EPS produced by two strains of *Leuconostoc mesenteroides* have immunomodulatory activity.

1. Introduction

Lactic acid bacteria (LAB) are widely used to produce several of fermentation products and contribute to improving the texture and viscosity of fermented products because they are able to synthesize exopolysaccharides (EPS) [1]. Exopolysaccharides (EPS) are macromolecules consisting of sugars and sugar derivatives [2], which have wide applications in the pharmaceutical, biomedical and food industries. Dextran is one of the biopolymers produced by *Leuconostoc* species which has many applications in the industry [3]. EPS produced by LAB shows various bioactivity, such as prebiotic, lowering blood cholesterol, antioxidants, anticancer and immunomodulatory [4].

The immune system helps protect the body against several pathogens [5]. Immune system imbalance is one of the causes of inflammation, infection and autoimmune disease, immunomodulators administration is one alternative to prevent this. Immunomodulators are used to stimulate and normalize the activity of the immune system [6]. Nowadays natural immunomodulators receive the most attention because their use is safe. Previous studies have been reported that EPS produced by LAB has potential as an immunomodulatory which can activate macrophages and induce secretion of NO and cytokines (TNF- α , IL-1 β , IL-6 and IL-10) [4], stimulate levels of TNF- α , IL-2



and IL-6 mRNA [7], stimulate IgA production [1], Increased phagocytic activity of macrophages [8] and increase IL-2 and TNF- α production [9].

EPS produced by LAB show the diversity of structure and composition of sugar monomer and its non-carbohydrate substituents, this depends on the species used and the condition of the growth media. Palm (*Borassus flabellifer* L.) sap is abundant availability in Indonesia which contains enough sugar and nutrients making it suitable as an alternative medium for fermentation. The immunomodulatory effect of EPS produced by *Leuconostoc mesenteroides* strains isolated from palm (*Borassus flabellifer* L.) sap have not been reported before, therefore this study was to evaluate the immunomodulatory effects of exopolysaccharides (EPS) produced by *Leuconostoc mesenteroides* strains isolated from palm (*Borassus flabellifer* L.) sap.

2. Materials and methods

2.1. Preparation of LAB

The LAB used in this study were *Leuconostoc mesenteroides* strains which was the result of isolation from palm (*Borassus flabellifer* L.) sap. Each culture stock was transferred to De Man Regosa and Sharpe (MRS) agar (Merck, Germany) and incubated at 30 °C for 48 h.

2.2. Production of EPS by *Leuconostoc mesenteroides* strains

The EPS was produced by two strains were *Leuconostoc mesenteroides* N5 and *Leuconostoc mesenteroides* N7 on two media which were MRS broth (Pronadisa, Spain) supplemented with 10% (w / v) sucrose (Pronadisa, Spain) and Palm (*Borassus flabellifer* L.) sap. 10 mL of inoculum *Leuconostoc mesenteroides* strains with optical density (OD = 600 nm) 0.5 which were equivalent to 10⁹ cfu/mL were centrifuged, cells were separated and washed with 0.85% NaCl then inoculated into 100 mL of fermentation media according to the treatment and incubated at 30 °C, 100 rpm for 24 h.

2.3. Extraction of EPS

The fermentation media was centrifuged at 6000 rpm at 4 °C for 15 min. EPS was precipitated by addition of two volumes of cold ethanol (95%) and kept at 4°C for 24 h. EPS was dissolved with distilled water and dialyzed using 14 kDa membrane (sigma) with deionized water for 24 h with two times water change. Each EPS was dried with freeze drying for 18 h and was used for further analysis. This study used 4 types of EPS were EPS from *Leuconostoc mesenteroides* N5 on palm sap (EPS NSN5), *Leuconostoc mesenteroides* N7 on palm sap (EPS NSN7), *Leuconostoc mesenteroides* N5 on MRS-sucrose (EPS MSN5) and *Leuconostoc mesenteroides* N7 on MRS-sucrose (EPS MSN7).

2.4. Calculation of total sugar

Total sugar was determined by the phenol sulfate method using glucose as standard [10]. 0.01 g of EPS was dissolved in 250 mL of distilled water and taken 2 mL. Added 1 mL of 5% phenol and 5 mL of 96% sulfuric acid (v/v) then heated in a boiling bath for 30 minutes. The absorbance was measured at 490 nm. The total sugar of EPS was calculated based on standard curve.

2.5. Animal experiment

Animals used were female mice (*Mus musculus*) strain BALB/c obtained from LPPT-UGM in 6 weeks old, with 18-22 g of body weight. Mice were acclimatized for 7 days and divided into 6 experimental groups (1) EPS (dextran) control, (2) negative (normal) control, (3) NSN5 EPS, (4) NSN7 EPS, (5) MSN5 EPS and (6)) EPS MSN7. Each group consisted of 4 mice. Concentrations of EPS and dextran were 300 μ g/mL. The administration of EPS and dextran was carried out for 20 days and on the 21th day, the mice were injected with LPS of *Salmonella typhimurium* (10 μ g/mL) via intraperitoneal. After 5 days the mice were sacrificed by dislocation of the neck. Spleen organs were taken, destroyed and suspended with phosphate buffered saline (PBS). Homogenates were centrifuged at 2500 rpm at 4 °C for 5 minutes, Pellet resuspended with Phosphate buffered saline (PBS). The homogenates obtained

were taken and PBS was added. Centrifuged at 2500 rpm with a temperature of 4 °C for 5 min and the pellet was taken.

2.6. Cytokines assay

The ability of CD4⁺ T cells in producing pro-inflammatory of cytokines (IL-2, IFN- γ and TNF- α) were analyzed using flow cytometry. Cells isolated from the spleen were incubated with anti-CD4⁺, IL-2, IFN- γ and TNF- α anti-mouse. After incubation, the sample was added with PBS and transferred to cuvette flow cytometry. The results obtained were processed with BD Cell Quest ProTM.

2.7. Statistical analysis

The data obtained were analyzed by one-way ANOVA with Tukey's test using SPSS 23.0 for windows.

3. Results and discussion

3.1. Production of EPS

This study showed the EPS was produced by *Leuconostoc mesenteroides* strains in MRS-sucrose have higher level of purity than in palm sap media. The highest total sugar of EPS was 94.37% produced by *Leuconostoc mesenteroides* N5 in MRS-sucrose media (Table 1). The resulting of EPS is influenced by media composition, growth conditions and species of LAB species, as well as the EPS extraction method used. Dextran can be produced by *Leuconostoc mesenteroides* in high quantities [11]. Dextran is an exopolysaccharide composed of D-glucopyranose with the main bonds α - (1,6) and branching bonds α - (1,2), α - (1,3), α - (1,4) produced by strains *Leuconostoc* [1].

Table 1. Total sugar of Exopolysaccharide in different medium.

Media of Fermentation	Total sugar (%)	
	<i>Leuconostoc mesenteroides</i> N5	<i>Leuconostoc mesenteroides</i> N7
Palm sap	58.67 (EPS NSN5)	66.48 (EPS NSN7)
MRS-sucrose	94.37 (EPS MSN5)	90.30 (EPS MSN7)

3.2. Effect of EPS on cytokines production

In this study, we compared the immunomodulatory activity of EPS produced by *Leuconostoc mesenteroides* strains in different media. Determination of immunomodulatory activity of all EPS (EPS NSN5, EPS NSN7, EPS MSN5 and EPS MSN7) were performed by giving EPS to mice and continued stimulation with LPS, then measuring the ability of CD4⁺ to produce pro-inflammatory cytokines (IL-2, IFN- γ and TNF- α) in the lymph using flow cytometry. Lymph contains various immune cells including T and B cells, DC, and macrophages and regulates the immune system to protect the body against bacterial, viral, and fungal infections [12]. Lipopolysaccharide (LPS) stimulates immune cells to release pro and anti-inflammatory cytokines and induce the synthesis of several related enzymes [13].

Previous studies have shown that EPS produced by LAB can modulate the production of various cytokines. Oral administration of EPS for 20 days before being infected with LPS showed significant effect ($P < 0.05$) on IL-2 production by CD4 cells⁺. EPS NSN5 and EPS NSN7 decreased IL-2 while EPS MSN5 and EPS MSN7 increased IL-2 when compared to normal controls (Figure 1). Increased of IL-2 secretion can stimulate T-cell proliferation and IFN- γ production thereby increasing the immune response to cancer and pathogen infections [14]. IL-2 is a cytokine for the survival and proliferation of T cells.

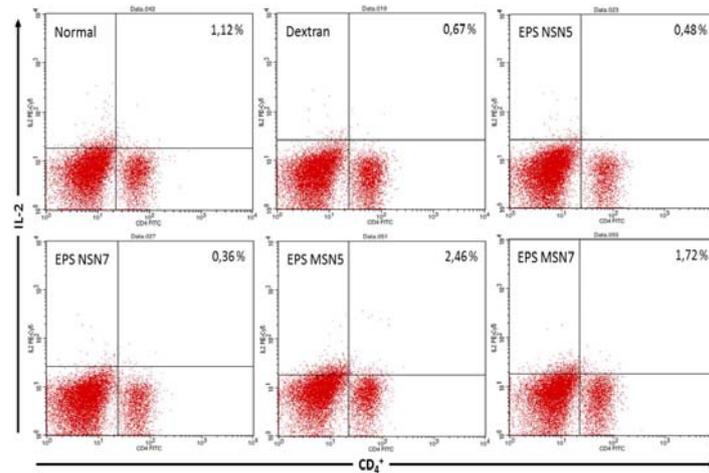


Figure 1. Level of CD4⁺ cells expression in producing IL-2.

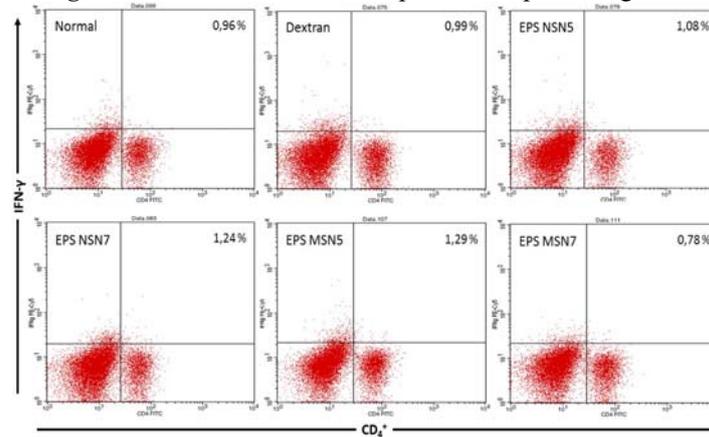


Figure 2. Level of CD4⁺ cells expression in producing IFN-γ.

The administration of EPS showed no significant effect ($P > 0.05$) on the production of IFN- γ by CD4⁺ cells. The EPS (EPS NSN5, EPS NSN7 and MSN5) increased IFN- γ production while EPS MSN7 decreased IFN- γ . The highest IFN- γ production was 1.29% produced by giving EPS MSN5, while the lowest was 0.78% by giving EPS MSN7 (Figure 2). The results showed that most EPS increased IL-2 and IFN- γ production, therefore EPS have potential as immunostimulator by increasing the activity of the Th1 cells immune system.

The administration of EPS showed no significant effect ($P > 0.05$) on the production of TNF- α by CD4 cells⁺. The EPS (EPS NSN5, EPS MSN5 and EPS MSN7) increased TNF- α production. These EPS induced TNF- α suggesting pro-inflammatory immunoregulatory potential. While EPS NSN7 decreased TNF α . The highest TNF- α production was 1.78% produced by administration of MSN5 EPS, while the lowest was 0.16% by dextran administration (Figure 3).

The results of this study support from the previous obtained. EPS from *L. rhamnosus* RW-9595M can increase the production of pro-inflammatory cytokines (TNF α , IL-6 and IL-12) and decrease anti-inflammatory cytokines (IL-10) [15]. EPS from *L. plantarum* JLK0142 is able to increase IL-2 and TNF α production in cyclophosphamide-induced immunosuppressed mice [9]. EPS from *Leuconostoc mesenteroides* NTM048 was able to increase IFN γ production by CD4 cells⁺ [1].

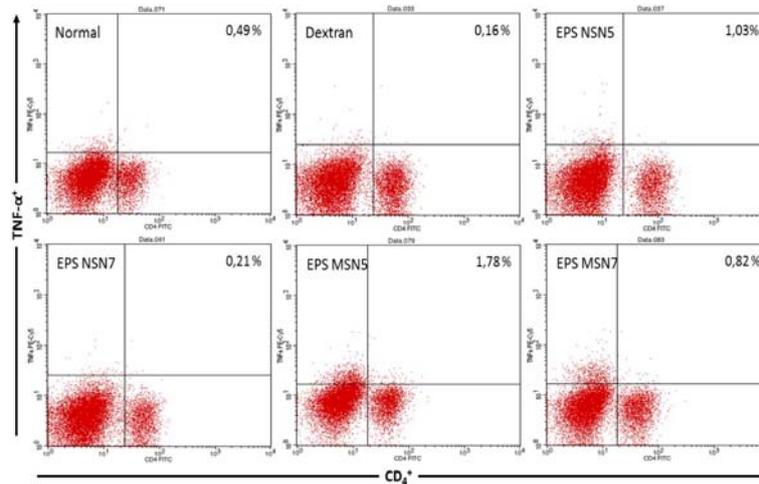


Figure 3. Level of CD4⁺ cells expression in producing TNF- α .

4. Conclusion

In this study, some EPS increased cytokine production (IL-2, TNF- α , and INF- γ) by CD4⁺ cells. On the other hand, production IL-2 decreased with EPS NSN5 and EPS NSN7, INF- γ decreased with EPS MSN7 while TNF- α decreased with EPS NSN7 treatment. EPS were able to different immune responses, these results were indicated that the EPS produced by two strains of *Leuconostoc mesenteroides* isolated from Palm (*Borassus flabellifer* L.) sap have immunomodulatory activity.

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