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Effect of Lactic Acid Filtrate and Bacteriocins of *Lactobacillus Acidophillus* on Phagocytosis Activity of Macrophages Cell againts Enteropathogenic *Escherichia coli* (EPEC)

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Immunity development known as one of effective ways in avoiding infection. Antibacterial agent product isolated from *Lactobacillus acidophillus* has been reported can activate T lymphocyte as part of adaptive immunity. This experimental study aimed at investigation of lactic acid and bacteriocins filtrate from *L. acidophillus* in modulating phagocytosis activity of human macrophages infected by enteropathogenic *Escherichia coli* (EPEC). Each of human macrophages culture was supplemented with lactic acid and bacteriocins filtrate at concentration of 3.125, 6.25, and 12.5 µg mL^{-1} as well as control without filtrate addition and incubated for 24 h. Macrophages culture was then infected with EPEC for 30 minutes and was microscopically observed after being stained by Giemsa. Percentage of phagocytosis activity was gained from active macrophages in 100 observed cells. Macrophages cultures supplemented with bacteriocins filtrate showed augmented phagocytosis activity while cultures supplemented with lactic acid filtrate showed decreased phagocytosis activity. ANOVA analysis showed significant difference in phagocytosis activity of macrophage cultures supplemented with lactic acid (p=0.038) and bacteriocins (p=0.016 and 0.023). Tukey HSD analysis for phagocytosis activity of macrophage cultures supplemented by bacteriocins, each group of treatment showed significant difference againts control. In conclusion, lactic acid from *L. acidophillus* has no effect in modulation of macrophages phagocytosis activity while bacteriocins can improve phagocytic activity. Bacteriocins from *L. acidophillus* then can be suggested to have a role as immunomodulator.

Key words: bacteriocins, enteropathogenic *E. coli* (EPEC), lactic acid, *Lactobacillus acidophillus*, phagocytosis

Peningkatan imunitas merupakan salah satu cara yang efektif dalam menghindari penyakit infeksi. Produk antibakteri yang diisolasi dari *Lactobacillus acidophillus* telah dilaporkan dapat mengaktifkan limfosit T yang berperan dalam imunitas adaptif. Penelitian eksperimental ini bertujuan untuk menguji kemampuan filtrat asam laktat dan bacteriosin dari *L. acidophillus* dalam memodulasi aktivitas fagositosis dari makrofag manusia yang terinfeksi oleh enteropathogenic *Escherichia coli* (EPEC). Setiap kultur makrofag manusia diberi filtrat asam laktat dan bacteriosin dengan berbagai konsentrasi, yaitu 3,125, 6,25, dan 12,5 µg mL^{-1}, serta kontrol tanpa penambahan filtrat dan kemudian diinkubasi selama 24 jam. Kultur makrofag yang telah diinkubasi kemudian diinfeksi oleh EPEC selama 30 menit dan diamati secara mikroskopis setelah diwarnai oleh Giemsa. Persentase aktivitas fagositosis diperoleh dengan menghitung jumlah makrofag yang aktif dalam 100 makrofag. Kultur makrofag yang diberikan filtrat bacteriosin menunjukkan peningkatan aktivitas fagositosis, sementara kultur makrofag yang diberikan filtrat asam laktat menunjukkan penurunan aktivitas fagositosis. Analisis ANOVA menunjukkan perbedaan yang signifikan pada aktivitas fagositosis makrofag yang diberikan filtrat asam laktat (p = 0,038) dan bacteriosin (p = 0,016 dan 0,023). Analisis Tukey HSD untuk aktivitas fagositosis pada kultur makrofag yang diberikan filtrat bakteriosin, masing-masing kelompok perlakuan menunjukkan perbedaan yang signifikan terhadap kelompok kontrol. Dari penelitian ini dapat disimpulkan bahwa asam laktat dari *L.acidophillus* tidak berpengaruh dalam modulasi aktivitas fagositosis makrofag sementara bacteriosin dapat meningkatkan aktivitas fagositosis makrofag. Bacteriosin *L. acidophillus* kemungkinan dapat berperan sebagai imunomodulator.

Kata kunci: asam laktat, bakteriosin, *E.coli* enteropatogenik (EPEC), fagositosis, *Lactobacillus acidophillus*

Probiotic administration in coping of various disease caused by pathogenic bacteria that resistant to antibiotic nowadays has become a trend (Mileti *et al*. 2009). Probiotics are living microorganism which if given in adequate manner will produce many advantages for the health of the host (Kaboosi 2011). Probiotic bacteria are generally used and can be isolated from *Lactobacillus* groups (Romeo *et al*. 2010).

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Lactobacillus acidophillus is one of probiotic bacteria from *Lactobacillus* group. Bacteriocins is a peptide synthesized by bacteria, including probiotic bacteria. Antibacterial agent namely lactic acid and bacteriocins from *L. acidophillus* are known to have inhibitory effect to pathogenic bacterial growth and have an effect to improve immune system through lymphocyte activation (Kim *et al*. 2009; Kaboosi 2011; Khazaie *et al*. 2012). This inhibitory effect by antibacterial agent involves its activity in lowering pH circumstance that influence the viability of pathogenic bacteria. In addition, the filtrate has been reported as an immunomodulator (Bourhis *et al*.2009). Immunomodulator is a substance or drug that recover the function of defect or hampered immune system, while immunostimulator is one of immunomodulator which improve the function of immune system (Borchers *et al.* 2009; Patil *et al*. 2011; Khazaie *et al*. 2012).

Macrophages are cells of innate immune system that can be found in most of many organs. Macrophages play a role as phagocyte as well as an antigen precenting cell (APC) that initiate adaptive immune response by activating T lymphocyte. Macrophages can be activated by Toll-like receptor (TLR) signals after recognize many kinds of pathogen associated molecular patterns (PAMPs) such as component of pathogenic bacteria and virus, fungi, and many others (Taylor *et al*. 2010; Fabriek *et al*. 2011).

Infectious disease is one of global basic problem need to be overcomed comprehensively. Beside antibiotic treatment, natural substance administration which improve immune system must be considered as a part of controlling and eradication of infectious disease (Sivick *et al*. 2010; Fengyi *et al*. 2011). Infectious disease caused by bacteria commonly occured in gastrointestinal tract. *Escherichia coli* is one of causative agent in human gastrointestinal tract infections. Enteropatoghenic *E. coli* (EPEC) is an important causative agent of children diarhhea in developing countries. EPEC infections mostly observed as diarrhea with manifestations life fever, blood, and convulsions (Rodrı´guez-Ban˜o *et al*. 2010).

In this work, we conducted investigations about lactic acid filtrate and bacteriosins effect as immunostimulator on macrophages phagosytosis activity especially againts EPEC. Adminstration of lactic acid filtrate and bacteriosins from *L. acidophillus* is expected to have a role in avoiding and handling EPEC infections in the future.

MATERIALS AND METHODS

Bacteria Strain. The cultures used were *Lactobacillus acidophillus* CPS1 from the isolation of whole milk of Lembang, and Enteropathogenic *Escherichia coli* (EPEC) bacteria culture, from the collection of Microbiology Laboratorium, Medical Technology Department, Sekolah Tinggi Ilmu Kesehatan Jenderal Achmad Yani. *L. acidophillus* and EPEC was grown in the Man Rogosa Sharpe (MRS) agar (OXOID CM0361 B) supplemented by 0.5% $CaCO_3$ and McConkey Agar (MCA) (OXOID CM0007) media respectively at a temperature of 37 °C for 24 h.

Production of Lactic Acid Filtrate of *L. acidophillus*. Bacterial filtrate was obtained by centrifugation of *L.acidophillus* culture that had been active in the Man Rogosa Sharpe (MRS) broth at 6000 rpm at 4 °C for 15 min to separate the cells from the filtrate. Filtrate supernatant was taken and put into a sterile tube. Filtrate was then exposed to UV light at a distance of 40 cm for 40 min (Moghaddam *et al*. 2006; Fauziah *et al*. 2013), this treatment can distinguish bacteriosin from lactic acid filtrate according to uv sensitive characterisitic of bacteriocins. This filtrate was qualitatively confirmed by Uffelman's method (Salkowski 2009) and diluted with sterile aquadest to gain lactic acid filtrate stock equal to 1000 µg mL^{-1} (v/v). Every stock then diluted for the second time so we got the concentration of each microtube consecutively 25, 12.5, and 6.25 µg mL^{-1}.

Production of Bacteriocins Filtrate of *L. acidophillus*. Bacterial filtrate was obtained by centrifuging *L. acidophillus* bacteria that had been active in the Man Rogosa Sharpe (MRS) broth at 6000 rpm at 4 °C for 15 min to separate the cells from the filtrate. Filtrate supernatant was taken and put into a sterile tube. It was neutralized with NaOH, and the filtrate was sterilized with 0.22 µm Millipore filter (Moghaddam *et al*. 2006; Fauziah *et al*. 2013). This filtrate was qualitatively confirmed by visual zones of inhibition on lawns of L. lactis (Ulrich and Hughes 2001), and was then diluted with sterile aquadest to gain bacteriocins filtrate stock equal to 1000 µg mL^{-1} (v/v). Every stock was then diluted for the second time so we got the concentration of each microtube consecutively 25, 12.5, and 6.25 µg mL^{-1}.

Preparation of Peripheral Blood Mononuclear Cell (PBMC) and Macrophages Culture. PBMC isolated from whole blood of healthy individual

(confirmed by protein electrophoresis analysis as uninfected subject at the time of investigations). PBMC isolation was performed according to Bagiada and Linawati (2009) briefly, leucoytes were separated by centrifugation of whole blood at 1500 rpm for 15 min to form three layers consist of red blood cells at the bottom, buffy coat containing leucocytes, and plasma as the upper layer. The buffy coat was then transferred into falcon tube containing Hank's balanced salt solution (HBSS) (1:1). This mixture was transferred into another falcon tube containing histopaque (1:1) and followed by centrigugation at 1500 rpm for 30 min. Isolated PBMC transferred into new falcon tube and washed by HBSS (1:1). Macrophages culture was performed according to Herawati *et al.* (2013) briefly, *t*wo hundreds microliter of isolated PBMC was dispensed into each well of multidish 24 wells which contains coverslip, and incubated for two hours at 37 ^{o}C and 5% CO_2. Culture supernatants from every well were discarded and washed by HBSS followed by giving 500 µL of complete RPMI (Roswell Park Memorial Institute), incubation was then continued to 7-10 d at 37 ^{o}C and 5% Co_2.

Macrophages Phagocytosis Activity Examination. Macrophages phagocytosis activity examination againts EPEC was performed in duplicate according to Chairul *et al*. (2009) and Herawati *et al*. (2013). Briefly, 500 µL of lactic acid filtrate or bacteriosins from various concentration dispensed into each well of culture containing mature macrophages followed by adding RPMI to get the final indicated concentration of lactic acid filtrate or bacteriosin in each well 12.5, 6.25, and 3.125 µg mL^{-1}, including control without filtrate addition. After incubation for 24 h, culture medium from each well were discarded and washed by HBSS followed by addition of 500 µL of PBS and EPEC suspension into each well including control. The plate was then incubated for 30 min, washed twice by PBS, fixed with absolute methanol for 1 min and stained by Giemsa for 10 min. Every well was then gently washed by tap water and the coverslip was taken from each well and dried before observation under light microspcope using objective lens 100x with immersion oil. Phagocytosis activity measurement was determined by the number of actively phagocytosing macrophages in one hundred macrophage cells. Preparation of EPEC bacteria was started by inoculation of EPEC in MCA, after incubation for 24 h EPEC inoculum was then suspended in 5 mL sterile phosphat buffer saline (PBS), and measured by nephelometer of Mcfarland until its turbidity in proportion to 0.5 Mcfarland (150 x 10^{6}).

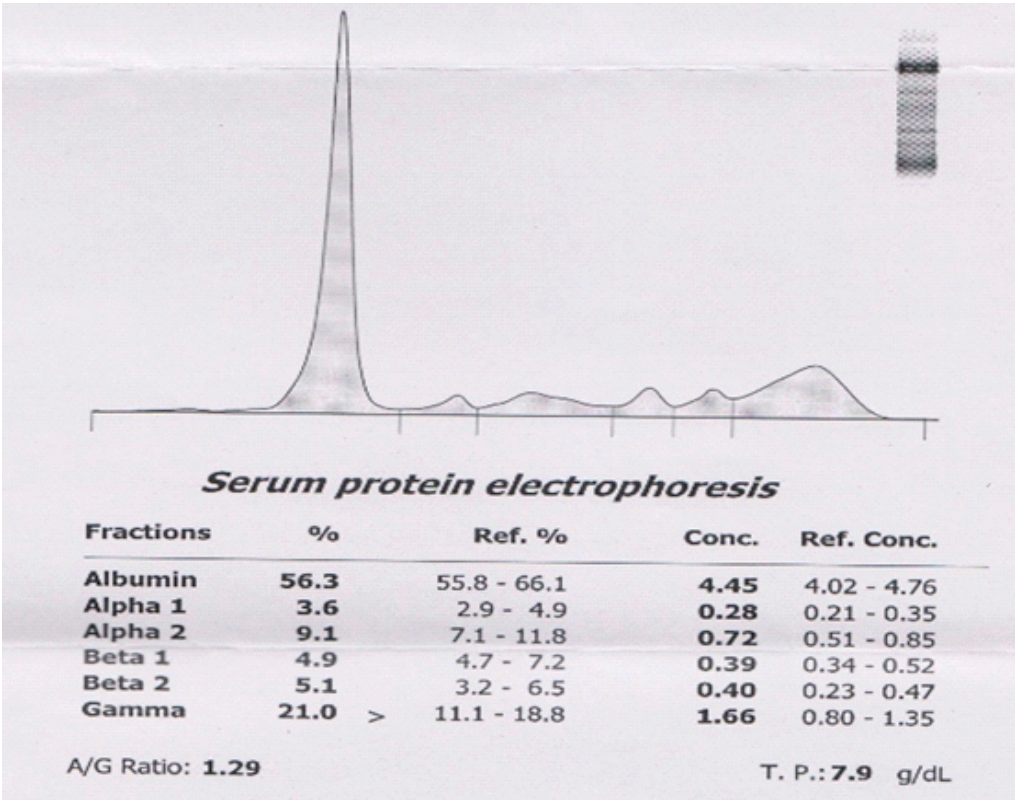
RESULTS

To confirm that the subject of investigations did not encounter any infections, we examine protein electrophoresis of subject blood before used as the source of macrophage culture. The value of albumin, alpha 1 and 2, beta 1 and 2, was at normal range of healthy individual confirmed the subject was not encounter any infections despite gamma globulin level describe increased subject antibody level (Fig 1A). PBMC isolation from blood subject described that centrifugation of whole blood formed three layers consist of red blood cells at the bottom, buffy coat containing leucocytes, and plasma as the upper layer. The isolated PBMC (arrow) used as a source for macrophages culture (Fig 1B). Macrophages maturation from monocyte achieved after 7 d incubation and typical features of the cells was recognized by its attachment characteristic when obeserved on inverted microscope (Fig 1C). Phagocytosis activity of mature macrophages againts EPEC was seen after Giemsa staining and actively phagocytosing macrophages can be recognized by EPEC's present inside the cell when observed on light microscope (Fig 1D).

Measurement of macrophages phagocytosis activity againts EPEC supplemented by lactic acid filtrate of *L. acidophillus* was described. The highest activity level of macrophages occured at concentration 3.25 µg mL^{-1} with 72% active macrophages (Fig 2). Lowest activity occured at concentration 12.5 µg mL^{-1} with 62% active macrophages. ANOVA analysis shows significant difference between control and treatment with $p<0.05$ ($p=0.027$), as control cells activity are better than the treatment. Low phagocytosis activity of treated cells due to more lactic acid supplemented more acid pH circumstance, result in inappropriate optimal condiditon for macrophages phagocytosis. Based on this evidence we suggest that lactic acid as an excretion product of probiotic *L. acidophilus* has no potential role as an immunostimulan.

The highest activity level of macrophages supplemented by bacteriocins occured at concentration 6,25µg mL^{-1} with 93% active macrophages (Fig 3). In addition the lowest activity occured at control cells with 71% active macrophages. ANOVA analysis shows significant difference between control and treatment with $p<0.05$ ($p=0.014$). Tukey HSD test on Table 1

A



Serum protein electrophoresis

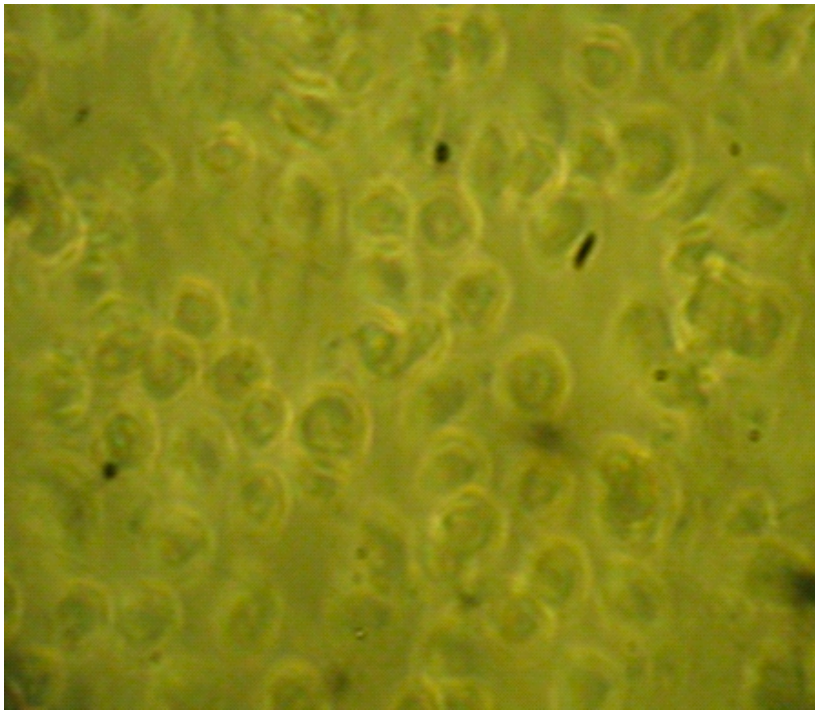
Fractions	%		Ref. %	Conc.	Ref. Conc.
Albumin	**56.3**		55.8 - 66.1	**4.45**	4.02 - 4.76
Alpha 1	**3.6**		2.9 - 4.9	**0.28**	0.21 - 0.35
Alpha 2	**9.1**		7.1 - 11.8	**0.72**	0.51 - 0.85
Beta 1	**4.9**		4.7 - 7.2	**0.39**	0.34 - 0.52
Beta 2	**5.1**		3.2 - 6.5	**0.40**	0.23 - 0.47
Gamma	**21.0**	>	11.1 - 18.8	**1.66**	0.80 - 1.35

A/G Ratio: **1.29**

T. P.: **7.9** g/dL

B

C



D

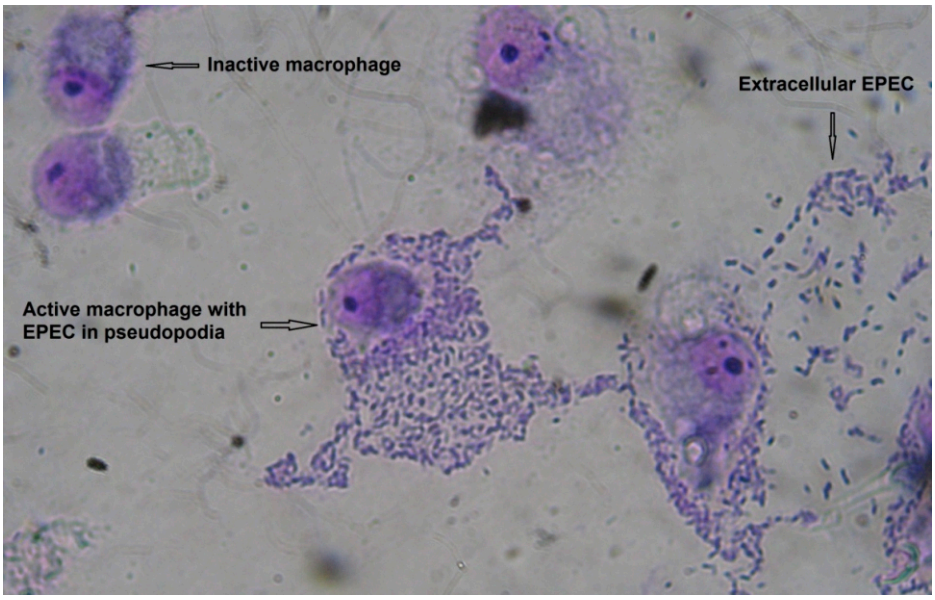


Fig 1(A) Serum protein electrophoresis from whole blood of healthy individual before isolation of PBMC. Albumin, alpha and beta globulin was at normal level despite gamma globulin level increased. This figure indicates that the subject did not encounter any infections. (B) Isolation of peripheral blood mononuclear cell (PBMC) from whole blood of the subject. Blood divided into three layers, red blood cell at the bottom, buffy coat in the middle and plasma at the upper layer. Macrophages was grown from PBMC area in buffy coat (arrow). (C) Macrophages maturation from monocyte. Mature macrophages recognized from their attach features on the coverslip while being observed on inverted microscope. (D) Phagocytosis activity of macrophages againts EPEC after being supplemented with 12,5 ug/ml of bacteriosin of *L. acidophillus* while being observed on light microscope 1000x. Active macrophage can be distinguished by its EPEC engulfing-pseudopodia.

shows significant difference between control and treated cells with concentration of bacteriocins 6.25 and 12.5 $\mu g\, mL^{-1}$.

DISCUSSION

Delphine *et al.* (2009) reported that *L. crispatus* can modulate gen expression of TLR-2 dan TLR-4.TLR-4 has been known as receptor on phagocytic cells recognizing lippopolysachharides (LPS) on the wall of Gram negative bacteria include EPEC. Because *L. acidophilus*is one of probiotic bacteria, increased phagocytosis activity of macrophage culture supplemented by bacteriosin from *L. acidophilus* suggested due to up regulation of TLR-4 gen expression. TLR-4 signal transduction initiated through LPS binding by LPS-binding protein (LBP). The bound LPS to LBP continue to be bound by *cluster of differentiation* 14 (CD14). This LPS-CD14 complex will be recognized by TLR-4 through MD2 (*modulation* - 2). MD2 required for triggering signal induction of TLR-4. TLR-4 signal transduction can activate My88 (myeloid differentiation primary response gene 88) dependent pathway and dan MyD88-independent pathway. These activations will lead to activate *nuclear factor kappa-B* (NF-kB) which mediate gene expression of pro-inflammatory cytokine interleukin-1 (IL-1) and expression of interferon-type gene (Lu *et al*. 2008).

Karlsson (2012) also reported that probiotic bacteria can modify both innate and adaptive immune response dependent on strain-type of probiotic bacteria. *L.rhamnosus* GR-1 is one of probiotic can activate human macrophage NF-kB. NF-kB known as transcription factor play important role in initial immune response againts pathogen. Cytokine production as response to antigen determined by NF-kB translocation into nucleus. The role of probiotic bacteria on improving NF-kB activation made it a basis for improvement of pro-inflammatory cytokine production (Tumor Necrosis Factor/TNF) that also has been reported.

A study of *L.casei* administration to mice

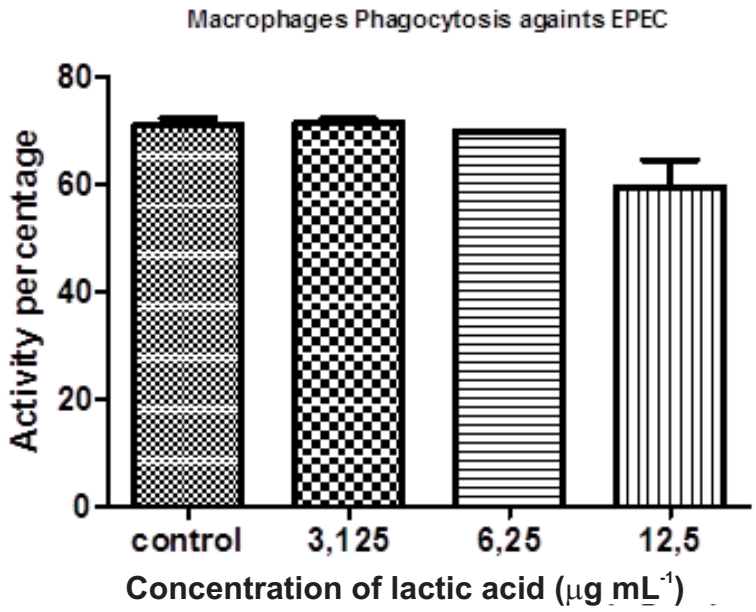


Fig 2 Phagocytosis activity of macrophages againts EPEC from culture supplemented with lactic acid filtrate of *L. acidophillus*. Decrease activity are significant at 12.5 μg mL^{-1} compared to control group with p value = 0.038.

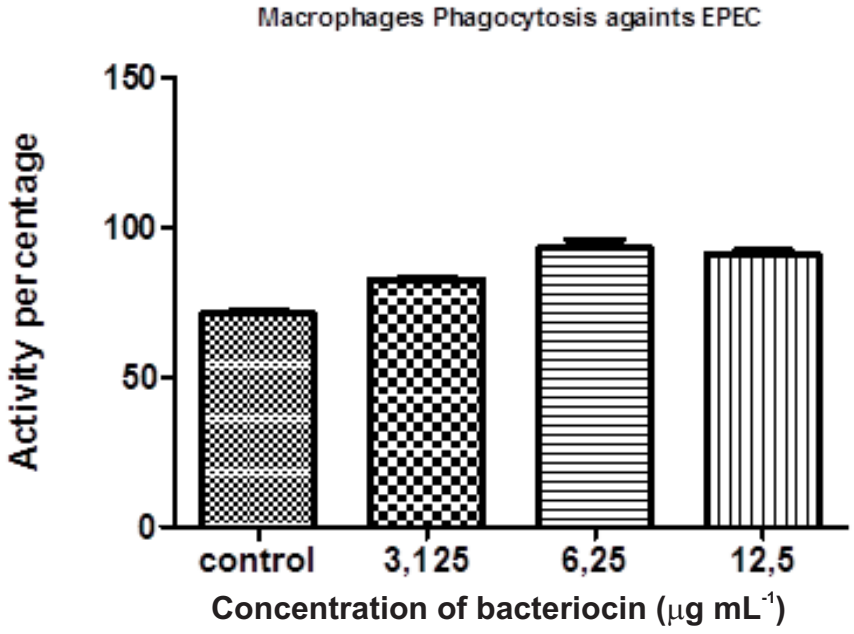


Fig 3 Phagocytosis activity of macrophages againts EPEC from culture supplemented with bacteriosin of *L. acidophillus.* Increase activity are significant at 6.25 and 12.5 μg mL^{-1} compared to control group with p value = 0.016 and 0.023, respectively.

challenged by Salmonella reported that TNF production increased compared to control group with no *L.casei* administration. Nevertheless production of cytokin has not improved for all of pro-inflammatory cytokine, such as IL-6 and IL-8 that showed decrease level as of more investigations about the expression of various cytokine by human macrophage supplemented by bacteriosin from *L.acidophillus* need to be elucidated due to improvement of phagocytosis activity againts EPEC in this study. In addition probiotic bacteria had been reported can modulate adaptive immune response especially limfocyte B function observed from increased antibody level of mice administered by *L.casei* and produce better protective antibody titer againts Salmonella infection. Other investigation reported that *L.rhamnosus* GG administration reduce antibody IgE production by mice so that bacteriosin administration effect on adaptive immune response especially cytokine production need to be revealed through more investigations (Delphine *et al*. 2009; Taylor *et al*. 2010; Karlsson 2012).

Bacteriocins from *L. acidophilus* was able to improve phagocytosis activity of macrophage, while lactic acid had no ability to improve macrophage phagocytosis activity. Bacteriocins from *L. acidophilus* can be suggested play a role as an imunostimulator, therefore require more investigations especially its effect on production of various cytokines.

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