The Effect of *Pediococcus pentosaceus* on Stool Frequency, TNF-α Level, Gut Microflora Balance in Diarrhea-induced Mice

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ABSTRACT

Background: *Enteropathogenic Escherichia coli (EPEC) are pathogenic microorganisms causing inflammation and imbalanced gut microflora that may result in diarrhea. Pediococcus pentosaceus (P. pentosaceus) isolated from "dadih" (milk curd) are used as probiotics containing lactic acid bacteria (LAB), which are useful to improve the balance of intestinal microflora and inhibit the growth of pathogenic microorganisms. This study was aimed to recognize the effect of P. pentosaceus supplementation on stool frequency, tumor necrosis factor-α (TNF-α) and gut microflora balance in experimental mice with EPEC-induced diarrhea.*

Method*: The study was conducted in 60 white mice (Mus muscullus) at Biomedical Laboratory, Biotechnology/ Production and Animal Husbandry Technology Institute, University of Andalas, Padang in April 2012. The frequency of stool, TNF-α level and microflora balance of the mice were measured before and after the EPEC-induced diarrhea and following the administration of antibiotics. Statistical analysis was performed using ANOVA and Duncan test.*

Results: *The highest mean stool frequency was found in positive control group, i.e. 55 times, which was reduced significantly after 12-hour P. pentosaceus supplementation in a dose of 2 x 10^8 cfu/g into 18 times. The mean TNF-α level in positive control group was 128.17 pg/mL that lowered significantly to 48.0 pg/mL. The highest mean total number of LAB was 97.0 x 10^7 cfu/g, which was significantly different from positive control group of 7 x 10^7 cfu/g.*

Conclusion: *P. pentosaceus supplementation in a dose of 2 x 10^8 cfu/g may reduce the stool frequency, lower TNF-α and improve the gut microflora balance following 12-hour supplementation in diarrhea-induced mice.*

Keywords: *Pediococcus pentosaceus, TNF-α, diarrhea, EPEC, gut microflora*

ABSTRAK

Latar belakang: *Enteropathogenic Escherichia coli (EPEC) merupakan kuman patogen yang dapat menyebabkan inflamasi dan ketidakseimbangan mikroflora usus sehingga menyebabkan diare. Pediococcus pentosaceus (P. pentosaceus) pada isolasi "dadih" sebagai probiotik mengandung bakteri asam laktat (BAL) yang bermanfaat dalam memperbaiki keseimbangan mikroflora intestinal dan menghambat pertumbuhan mikroba patogen. Penelitian ini bertujuan untuk mengetahui pengaruh dosis dan lamanya pemberian P. pentosaceus terhadap frekuensi buang air besar (BAB), kadar total necrosis factor- α (TNF-α) dan keseimbangan mikroflora usus mencit diare yang diinduksi EPEC.*

Metode*: Penelitian dilakukan pada 60 mencit putih (Mus muscullus) di Laboratorium Biomedik, Bioteknologi/ Produksi dan Teknologi Peternakan, Universitas Andalas Padang pada bulan April 2012. Dilakukan pengukuran frekuensi BAB, pemeriksaan kadar TNF-α, keseimbangan mikroflora usus mencit sebelum dan setelah pemberian EPEC, serta setelah pemberian probiotik. Analisis statistik dilakukan dengan uji ANOVA dan Duncan.*

***Hasil**: Rata-rata frekuensi BAB terbanyak pada kelompok kontrol positif sebanyak 55 kali dan berkurang sangat nyata setelah 12 jam pemberian P. pentosaceus dengan dosis 2 x 10^8 cfu/g menjadi 18 kali. Rata-rata kadar TNF-α pada kontrol positif sebesar 128,17 pg/mL, menurun sangat nyata setelah 12 jam pemberian P. pentosaceus menjadi 48,0 pg/mL. Rerata kadar total kuman BAL tertinggi pada pemberian P. pentosaceus dosis 2 x 10^8 cfu/g yaitu 97,0 x 10^7 cfu/g dan berbeda sangat nyata dengan kelompok kontrol positif yaitu 7 x 10^7 cfu/g.*

***Simpulan**: Pemberian P. pentosaceus dengan dosis 2 x 10^8 cfu/g setelah 12 jam dapat menurunkan frekuensi BAB, menghambat kadar TNF-α serta memperbaiki keseimbangan mikroflora usus pada mencit diare.*

***Kata kunci:** Pediococcus pentosaceus, TNF-α, diare, EPEC, mikroflora usus*

INTRODUCTION

As one of contagious diseases, diarrhea is the third leading cause of death following tuberculosis (TB) and pneumonia.[1] The most common causes of diarrhea are viruses and bacteria including the enteropathogenic *Escherichia coli* (EPEC). Attachment of EPEC to the intestinal mucosa may lead to alteration of cell structure, which subsequently invades into the intestinal epithelial cells and when being consumed in a dose of 10^8 cfu/mL, EPEC may cause diarrhea.[2,3]

Tumor necrosis factor-α (TNF-α) are the main cytokines in acute inflammatory response against negative gram bacteria and other microorganisms.[4] High TNF-α serum level may damage the tight junction in the enterocytes of intestinal mucosa.[5] The cumulative effect of intestinal atrophy and damaged tight junction causes increased membrane permeability which results in disrupted intestinal absorption leading to diarrhea. Changes in normal human gut microbiota may cause various intestinal disorders and diseases; however, in normal condition the microbiota may prevent overgrowth of pathogenic bacteria in gastrointestinal tract.[5,6] The balance of intestinal microflora will be achieved when beneficial microorganism may suppress the bad bacteria by causing the pathogenic microorganisms petered out.[7] Probiotics are living bacteria given as food supplement that may bring beneficial effect to human health by enhancing the balance of intestinal microflora.[8-10] Probiotics may inhibit the production of pro-inflammatory cytokines.[11] Pena et al, demonstrated that in lipopolysaccharide (LPS)-induced mice following *Lactobacillus rhamnosus GG* (LGG) supplementation, there is an inhibition of TNF-α production, which will affect tissue damage and cell apoptosis.[12]

Milk curd or "dadih" is a traditional dish of Western Sumatera containing lactic acid bacteria (LAB), which are useful to enhance human gastrointestinal tract function by inhibiting the growth of pathogenic microorganisms; while, the side product, nisin, are natural antibiotics to neutralize pathogenic gastrointestinal bacteria.[13,14]

One of probiotic microorganism or the lactic acid bacteria that have been successfully isolated from the "milk curd" is *Pediococcus pentosaceus (P. pentosaceus)*.[15] Purwati found that *P. pentosaceus,* the LAB, could produce bacteriocins that can kill pathogenic microorganisms such as *Lactobacillus monocytogenesis*, *Bifidobacterium subtillis, Streptococcus aureus, Escheria coli (E. coli)* and *Salmonella thypii*.[15] The aim of this study was to identify the effect of beneficial dosing and treatment period of *P. pentosaceus* supplementation on the frequency of stool, TNF-α level and the balance of intestinal microflora in mice with EPEC-induced diarrhea.

METHOD

An experimental study with complete random design using the factor pattern of 5 x 4, which was repeated for two times, was conducted in April 2012 at Biomedical Laboratory, Biotechnology/Production and Animal Husbandry Technology Institute, University of Andalas, Padang. The sample were male white mice (*Mus musculus*) obtained from the study and development unit of experimental animal pharmaceutical laboratory in University of Andalas, Padang. There were 60 mice used at eight weeks of age and weighing 25-30 g.

The experimental animals were randomly categorized into two factors. The first factor was the dose, which included a negative control group receiving only standard pellet feed and plain water (C-); other three positive control groups with pellet feed containing EPEC in a dose of 10^8 cfu/g (C+), isolates of *P. pentosaceus* obtained from the "milk curd" at dose of 2 x 10^8 cfu/g, 2 x 10^9 cfu/g, and

2 x 10^{10} cfu/g, respectively. The second factor was the duration of treatment, i.e. 0 hour, 12 hours, 24 hours and 36 hours.

In the first week, the mice were acclimated or went through adaptation process. During this period, 60 mice were given standard pellet feed and water ad libitum. When the adaptation period had been completed, we selected the mice weighing approximately 27 g randomly and categorized them according to the abovementioned groups. There were three mice in each repeated unit. Before providing the EPEC, all mice in the negative control group were measured for their frequency of stools, TNF-α level and the balance of their gut microflora at 0 hour. Afterward, similar measurements were performed for the positive control group that had been adjusted with their duration of treatment.

Analysis of variance or ANOVA was used to evaluate the effect of treatment and the interaction on the observed variables. When the treatment had been proven to have any effect, we continued the analysis by using the Duncan's multiple range test. Statistical analysis and data management were carried out using SPSS 19.

RESULTS

The statistical analysis of Duncan's multiple range test showed that there was a very obvious interaction (p < 0.01) between the dose factor and duration of treatment factor on the frequency of stool (the amount of stool) and TNF-α level (Table 1 and 2).

Table 1. The mean value of stool frequency in mice with various treatment

Dose factor (cfu/g)	Duration of treatment (hour)				Total
	0	12	24	36	
C (-)	4.5^{h}	5.5^{h}	5.5^{h}	5.0^{h}	20.5
C (+)	6.0^{h}	54.5^{a}	36.0^{b}	36.5^{b}	133.0
2 x 10^8	5.5^{h}	17.5^{e}	16.5^{e}	16.0^{e}	60.5
2 x 10^9	4.5^{h}	28.5^{d}	25.5^{d}	12.5^{f}	71.0
2 x 10^{10}	4.5^{h}	31.0^{c}	14.5^{e}	11.5^{g}	61.5
Total	**25.0**	**137.0**	**98.0**	**86.5**	**346.5**

Standard deviation (SD): 1.26; C (-): negative control; C (+): positive control; different alphabet shows significantly difference; Duncan's multiple range test

Table 2. The mean value of TNF-α level (pg/mL) in the stool of mice with various treatment

Dose factor (cfu/g)	Duration of treatment (hour)				Total
	0	12	24	36	
C (-)	13.27^{l}	12.31^{l}	12.23^{l}	12.29^{l}	50.10
C (+)	13.33^{l}	128.17^{a}	123.19^{b}	87.05^{c}	351.74
2 x 10^8	12.30^{l}	48.00^{f}	42.10^{g}	25.04^{j}	127.44
2 x 10^9	12.30^{l}	56.91^{d}	37.65^{h}	17.84^{k}	124.70
2 x 10^{10}	11.29^{l}	65.93^{d}	41.81^{g}	39.86^{i}	158.90
Total	**62.49**	**311.32**	**256.98**	**182.08**	**812.88**

SD: 0.46; C (-): negative control; C (+): positive control; different alphabet shows significantly difference (p < 0.01); Duncan's multiple range test

Table 1 shows the mean value of stool frequency after 12-hour EPEC administration in positive control group. The most frequent was 55 times, which indicated 10 fold increase compared to the normal group that only have 5 times.

After the first 12 hours of *Pediococcus* supplementation in a dose of 2 x 10^8 cfu/g, there was an obvious reduction in stool frequency from approximately 18 times to 3 times.

Table 2 demonstrates the very obvious increase (p < 0.01) of TNF-α level following the 12-hour EPEC administration, i.e. mean value of 128.17 pg/mL, which was 10 times compared to the control group without treatment of 12.31 pg/mL. The TNF-α level lowered almost three fold after 12-hour *P. pentosaceus* supplementation in a dose of 2 x 10^8 cfu/g into 48.0 pg/mL.

The total number of LAB colony increased significantly after 12-hours *P. pentosaceus* supplementation in dose of 2 x 10^8, i.e. 97.0 x 10^7 cfu/g that indicate almost 24 fold increase compared to both negative and positive control group. The lowest mean value was found in positive control group after 24 hours of EPEC supplementation, i.e. 1.0 x 10^7 cfu/g (Table 3).

Table 3. The mean value of total LAB colony (x 10^7 cfu/g) in the mice gut with several treatment

Dose factor (cfu/g)	Duration of treatment (hour)				Total
	0	12	24	36	
C (-)	3.5^{h}	4.0^{h}	5.5^{h}	4.5^{h}	7.5
C (+)	4.0^{h}	6.5^{h}	0.5^{i}	5.5^{h}	16.5
2 x 10^8	5.0^{h}	97.0^{a}	43.0^{f}	61.5^{c}	206.5
2 x 10^9	2.5^{h}	84.0^{b}	60.5^{c}	20.5^{g}	167.5
2 x 10^{10}	6.0^{h}	48.0^{e}	53.5^{d}	22.0^{g}	129.5
Total	**21.5**	**239.5**	**163.0**	**114.0**	**537.5**

SD: 0.83; C (-): negative control; C (+): positive control; different alphabet shows significantly difference (p < 0.01); Duncan's multiple range test

The highest mean value of total aerobic microorganism colony in the positive control group after 36 hours of EPEC supplementation was 33.0 x 10^7 cfu/g, which showed 2.4 fold increase compared to the negative control group of 14.0 x 10^7 cfu/g. The mean value of total aerobic microorganism colony was found reduced after 12-hour *P. pentosaceus* supplementation in a dose of 2 x 10^8 cfu/g, which was 5.0 (Table 4).

Based on the result of Duncan's multiple range test, we found a very obvious interaction (p < 0.01) between the dose and duration of treatment regarding the total number of intestinal *E. coli* colony with the highest mean value in positive control group after 12 hours of EPEC administration of 19.0 x 10^7 cfu/g. It indicated three-fold increase compared to the normal value of 7.0 x 10^7 cfu/g. The lowest mean value of total *E. coli* colony was found in the group receiving *P. pentosaceus* supplementation in a dose of 2 x 10^8 cfu/g and obvious decrease was observed ($p < 0.01$) after 12-hours *P. pentosaceus* supplementation with mean value of 3.0 (Table 5).

Table 4. The mean value of total aerobic microorganism colony in the mice gut with several treatment

Dose factor (cfu/g)	Duration of treatment (hour)				Total
	0	12	24	36	
C (-)	12.0^e	12.5^e	11.5^e	14.0^b	50.0
C (+)	12.0^e	7.5^b	32.0^a	32.5^a	84.0
2 x 10^8	10.5^e	4.5^g	5.5^g	3.5^g	24.0
2 x 10^9	8.0^e	14.0^c	9.0^f	5.0^b	36.0
2 x 10^{10}	8.0^e	10.0^d	10.0^d	4.0^g	32.0
Total	**50.5**	**39.5.**	**73.0**	**63.0**	**226.0**

SD: 0.81; C (-): negative control; C (+): positive control; different alphabet shows significantly difference ($p < 0.01$); Duncan's multiple range test

Table 5. The mean value of *Eschericia coli* colony in the mice gut with several treatment

Dose (cfu/g)	Duration of treatment (hour)				Total
	0	12	24	36	
C (-)	6.5^d	7.0^d	7.5^d	6.5^d	27.5
C (+)	5.5^d	19.0^b	21.5^a	11.0^c	57.0
2 x 10^8	7.5^d	2.5^e	1.5^e	3.5^e	15.0
2 x 10^9	5.5^d	5.0^d	2.5^e	2.5^e	15.5
2 x 10^{10}	6.5^d	7.0^d	4.5^e	2.5^e	20.5
Total	**31.50**	**40.5**	**37.5**	**26.0**	**135.5**

SD: 0.62; C (-): negative control; C (+): positive control; different alphabet shows significantly difference ($p < 0.01$); Duncan's multiple range test

DISCUSSION

EPEC are pathogenic microorganisms that can penetrate intestinal mucosa and invade enterocytes, which may result in diarrhea. The diarrhea was observed in this study as the greater stool frequency in mice compared to the negative control group without administration of EPEC. The healthy mice (without EPEC infection) did not experience diarrhea, which could be evaluated by measuring the number of stool in mice. The results of this study is consistent with the study conducted by Liu et al, which demonstrated that the LPS-induced mice (*E. coli* serotype 0128) have experienced diarrhea compared to those without LPS ($p < 0.01$) and the diarrhea was improved after probiotics supplementation.[16]

This study demonstrated that *Pediococcus* are the probiotics that can compete with EPEC in the gastrointestinal tract to reduce stool frequency or even to improve diarrhea into normal condition. *P. pentosaceus* supplementation with different dosing regimen in this study has demonstrated reduced stool frequency compared to the condition following EPEC administration. This study is supported by the results of study conducted by Saavedra.[17] The study found varied doses of probiotics in several other studies that ranged between 10^7 and 10^{10} cfu daily depending on the method being used; however, the most common dose is about 10^8–10^{10} cfu/day.[17]

This study also found increased TNF-α level that may reach 10 fold of normal limit. It suggests that in EPEC-induced mice, there is inflammatory process characterized by increased TNF-α level. The results of this study are consistent with Lapointe's statement that inflammatory responses are associated with EPEC microorganisms that infect intestinal mucosa. It may cause increased proinflammatory cytokines, such as TNF-α, interferon gamma, and IL-1β.[18] In this study, we found that three dose regimens might reduce inflammation as indicated by reduced TNF-α level. This findings are also supported by Galdeano et al who suggest that the recommended dose to bring additional effect for immune response or on the mucosa ranges between 1 x 10^8 and 1 x 10^9 cfu/day, which is also effective when given in 48-72 hours.[19]

This study results are also consistent with the study conducted by Pena and Versalovici, which demonstrated that LGG may specifically inhibited TNF-α against apoptosis or cytotoxic effect.[12] The secretion of TNF-α occurs after 5 hours of LPS supplementation in mice, which was measured using quantitative ELISA. The LGG ability to inhibit LPS that produce TNF-α may also depend on LPS concentration, which will be reduced with increased LPS concentration. Another study that consistent with this study is the study conducted by Kusuma et al showing that supplementation of *Lactobacillus* strain LIS10506 or LIS20506 would inhibit the NFkB activation. Although the mechanism is not clear, but it has significantly reduced TNFR1 expression (as an equivalent of TNF-α activity).[20]

There was also increased total number of LAB microorganisms following probiotics supplementation. Such condition suggests that *P. pentosaceus* of milk curd may overcome various barriers in the gastrointestinal tract such as low pH (in the stomach). Increased total LAB colony may also indicate that *P. pentosaceus* are able to compete with pathogenic microorganisms. This study is consistent with the study conducted by Heriyenni that LAB produces bacteriocin, the extracell of LAB with antimicrobial effect.[21] The WHO also supported this study results stating that the attachment ability of LAB on the bowel is an important factor that must be owned by probiotics.[22] Another study that in agreement with this study is the study conducted by Arief et al. It showed that there was significantly higher total number of LAB attached to caecum mucosa following probiotics supplementation in EPEC-induced mice ($p < 0.01$).[23]

The mean value of total aerobic in this study increased in positive control group (EPEC-induced) and reduced after *Pediococcus* supplementation. The results of this study shows that *Pediococcus* obtained from the milk curd may inhibit *E. coli* population and kill pathogenic microorganism in intestinal mucosa. The findings are supported by Adolffson et al, who suggested that *Lactobacillus sp* originated from the milk curd can overcome invasion of pathogenic bacteria in the intestines.[24] The suggestion is also supported by Surono, who stated that milk curd contains LAB that may exert the function of killing pathogenic bacteria in the intestines.[13]

The results of this study are consistent with the results of study conducted by Arief et al who investigate total number of *E. coli* in caecum mucosa of EPEC-induced mice. They found that by the second week, there was significantly lower total EPEC colony ($p < 0.05$) in EPEC-induced mice that had been supplemented with *L. plantarum* 2C12 and *L. acidophilus* 2B4 probiotics compared to those EPEC-infected mice without supplementation.[23] The results of this study are also corroborated by Asaduzzaman & Sonomoto, who suggests that bacteriocins can inhibit the growth of other microorganism by developing cavity or pore at the sensitive cell membrane or cell wall and lowering the potential or pH gradient which causes cellular damage leading to cell lysis.[25]

The limitation of this study is the short treatment period, i.e. 36 hours; hence, the expected effect has not reached its maximal potency. Further studies should provide longer observation period to investigate the optimal effect of probiotics as has been suggested by Galdeano et al, that probicotics is effective when being used for 48-72 hours.[19] Longer duration of probiotic treatment has also been investigated by Kusuma et al, who evaluated LPS-induced mice with 7-day probiotics treatment which has anti-inflammatory effect.[20]

CONCLUSION

The 12-hour *Pediococcus pentosaceus* supplementation in a dose of 2×10^8 cfu/g may reduce stool frequency, lower TNF-α level and improve the balance of gut microflora in EPEC-induced diarrhea mice.

SUGGESTION

Further studies are needed to evaluate *Pediococcus pentosaceus* supplementation in children with diarrhea.

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