Supplementation of probiotic enterococcus faecium IS-27526 decreasing the population of enterobacteriaceae

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Supplementation of probiotic enterococcus faecium IS-27526 decreasing the population of enterobacteriaceae

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Abstract

Gastrointestinal microbiota composed of trillions of bacteria species bear important role in maintaining stable ecosystem inside the body. In gut, Enterobacteriaceae is present in very low densities below 10^8 cfu/g. Elevated number of enterobacteriaceae in gut can promote gastrointenstinal infection. Probiotics has been used for a long time in treating gastrointestinal related diseases. This reaserch aims to evaluate the effect of Enterococcus faecium IS-27526 supplementation as a novel probiotic from Indonesia on gut microbiota. Species of bacteria that were successfully identified using both morphology and physiology identification test on bacteria resulted in four major genera which are Citrobacter, Enterobacter, Serratia and Escherichia.

Total bacterial and Enterobacteriaceae number were calculated between test and control group. Total bacteria population from Total Plate Count result was 222.67 x 10^6 cfu/ml in control group and 210.33 x 10^6 cfu/ml in probiotics induced group. TPC result for Enterobacteriaceae population from probiotics induced group was 11.33 x 10^6 cfu/ml and 12.00 x 10^6 cfu/ml for the control group. The use of Enterococcus faecium IS-17526 has significantly reduced the total bacterial count as well as enterobacteriaceae population in gut.

Keywords: Dysbiosis, Probiotics, Identification, Total Plate Count, Rabbit Intestine.

Introduction

The gut microbiota composed of a large microbial community consist of over trillions bacterial species that formed a complex ecosystem inside human body. Most gut microbes have various roles in supporting host such as protection against enteropathogens, contributes in normal immune functions, and extracts nutrients and carbohydrates from diets. Alteration in normal microbes ecosystem of the host has been associated with development of gastrointestinal diseases and conditions[1-4].

Enterobacteriaceae is the largest and most diverse group of medically important gram-negative bacilli and cause a variety of human infections including gastrointestinal infections[5]. In the normal gut, *Enterobacteriaceae* is present in very low densities below 10^8 cfu/g[6]. It was found that elevated number of *Enterobacteriaceae* densities in gut will induce gut dysbiosis and bile infection[7].

Enterobacteriaceae can be differentiated with traditional detection methods that rely on *Enterobacteriaceae* ability to ferment glucose resulting in acid, gas, or both gas and acid production[8]. *Enterobacteriaceae* is facultative aerobe, so it has both respiratory and fermentative metabolism. Fermentation products of *Enterobacteriaceae* include formate, lactate, succinate, ethanol, H_2 and CO_2. Most *Enterobacteriaceae* are able to reduce nitrate into nitrite[9].

Probiotics has been defined as "living microorganisms that when administered in adequate amounts confer a significant health benefit on the host"[10]. Probiotics are well known for their ability in promoting good health by reducing the number of harmful bacteria in the body as well as contributing in digestion, enchancing immune responses and inhibiting the growth of pathogenic microorganisms.

Recently, probiotics have been used for clinical purpose to treat diabetes, hypertension, inflammation, colon cancer, immune function and infection, Crohn's disease and many other diseases[11,12].

Enterococcus faecium IS-27526 is a novel probiotic from dadih, a traditional fermented buffalo milk from Sumatra, Indonesia. This probiotic strain has the ability to adhere to the surface of intestinal mucosa, and form aggregates with pathogenic bacteria such as *B. Vulgatus, C. Histolyticum, S. Aureus, Enterobacter sakazakii* dan *E.coli*. Aggregate formation between probiotics and bacteria can trigger growth or colonization of beneficial microorganisms in the gut preventing disease. *In vivo* studies of *Enterococcus faecium* IS-27526 show that these probiotics are resistant under acidic conditions, bile tolerance, hydrophobicity on the surface and are competitive against pathogens[13,14].

This research aims to study the decreasing amount of total bacterial and *Enterobacteriaceae* count with

supplementation of probiotics *Enterococcus faecium* IS-27526 from the New Zealand's white rabbit intestinal system.

Material and Methods

a. Ethical Clearance: In order to propose a research involving animal subjects, ethical clearance should be sought prior before the work can be undertaken. Ethical clearance application was filed into the Ethical Committee of Medical Faculty, Universitas Padjadjaran.

b. Animal Preparation: Male New Zealand's white rabbit were used as animal subjects of the research with conditions as follow, aged between 2-3 months old, healthy condition with normal activity and weight between 1-2 kg.

Acclimatization was conducted in 1 week period. This step was performed in order to stabilize animal subjects in new enviroment and ensure animal welfare during the test. The animal subjects were fed with 200 grams of sterilized food pellets twice a day, morning and afternoon with access to drinking water.

Each test rabbit was placed in separated single cage to reduce its stress level. Rabbit's body weight and temperature were also monitored daily. After the acclimatization process finished, rabbits with constant body weight and temperature were sorted as animal subjects. The rabbits were divided into two groups, control and test group with nine rabbit inside each group.

c. Probiotics supplementation: *E. faecium* IS-27526 was given to each rabbit of the test group for 14 consecutive days. 80 mg of dried probiotics powder were diluted in 10 mL sterilized aquadest and administered once a day in the morning before meal time. In contrary, control group was given sterilized aquadest without probiotics. Both probiotics and aquadest solution were administered through sterilized feeding tubes.

D. Small intestine collection: Rabbits were sacrificed on the 15th day of the test. Small intestine samples were collected for bacterial count and identification. Samples obtained were immediately transferred into Phosphate-Buffered-Saline (PBS) and kept at -20^{o}C in refrigerator.

e. Total Bacterial Count: Spread plate technique were performed for total bacterial count. Bacteria were obtained by swabbing the inside part of intestine using sterilized cotton bud and then inoculated into 10 mL of *Mueller-Hinton Broth* medium for 18 hours at 37 ^{0}C of incubation temperature. Samples were diluted until 1 x 10^{6} cfu/ml concentration.

20 μl of samples from each dilution were spread evenly on the surface of agar medium and then incubated for another 18 hours. *Mueller-Hinton Agar* was used for total bacterial count while *MacConkey Agar* was used for *Enterobacteriaceae* bacterial count. Colony counts were performed after 18 hours of incubation at 37 ^{0}C.

f. Bacterial Identification: Bacterial identification was conducted by using series of biochemical/fermentation test, gram staining, protease test, catalase test and Microbact™ 12A bacterial identification kit.

g. Data interpretation: GIDEON™ software for bacterial identification was used to analyze the data obtained from Microbact™ 12A bacterial identification kit. Identification was performed to determine the genera of each bacterial colony that had been successfully isolated. Correlation of total bacterial count from both plates with the effect of probiotics supplementation was analyzed by using statistical analysis.

Results and Discussion

Ethical clearance was granted by the Ethics Committee of Medical Faculty, Universitas Padjadjaran with proposal number 416/UN6.C1.3.2/KEPK/PN/2016.

Each intestinal sample obtained from the rabbits was grown on media plate and 29 single colony of bacteria were successfully isolated. Every single colony was then transffered into Mueller-Hinton Agar (MHA) medium and observed. The colony's morphological properties were observed and classified into four main groups. Morphology identification of bacteria is shown in table 1.

Gram-staining procedure was then performed to identify the gram type of bacteria and bacterial shape. Gram staining is always performed for the first step in preliminary identification of bacterial organism. Gram-positive bacteria will generate violet stain on the colony while gram-negative bacteria generate pink stain. Each bacteria groups tested show clear pink stain with rod-shaped colony indicating all the bacteria obtained were gram negative as shown in figure 1 and table 2.

Biochemical tests were conducted to differentiate bacteria groups according to bacteria phsiology properties which include motility test, Triple Sugar Iron agar (TSIA) test, urease test, Methyl-Red (MR) and Voges Proskauer (VP) test, citrate test, indol test, and catalase test. Carbohydrates fermentation test was also tested using various source of sugar include maltose, saccharose, lactose, glucose, and mannose. Each bacteria strain was expected to respond spesifically for each test given, depending on its ability to break carbohydrates chain and other spesific properties. Results of biochemical-based test are shown in table 3.

The Microbact™ 12A gram-negative kit is a micro-subtrate system designed to simulate conventional biochemical substrates for spesific identification of *Enterobacteriaceae* and common micellaneous gram-negative bacilli (MGNB). Identification is based on pH change and substrate utilisations. Results of bacterial identification by using

Microbact™ 12A strip are explained in table 4. The identification data was obtained and then analyzed with web-databased software, GIDEON™ (Global Infectious Disease Epidemiology Network). Based on this analysis, group 1 was identified as *Citrobacter* with 93% probability, group 2 was identified as *Enterobacter* with 99% probability, group 3 was identified as *Serratia* with 89% probability, and group 4 was identified as *Escherichia* with 99% probability.

Data obtained from GIDEON™ software will be tested further by growing the bacteria group in specific media. The specific media were MacConkey Agar, Eosin Methylene Blue Agar, Skimmed-Milk Agar, Kligler Iron Agar, Xylose-Lysine Deoxycholate Agar, and Brilliant Green Agar. Group 1 suspected *Citrobacter* tested in MacConkey Agar, Eosin Methylene Blue Agar, Skimmed-Milk Agar, Kligler Iron Agar, Xylose-Lysine Deoxycholate Agar, and Brilliant Green Agar. Group 2 suspected *Enterobacter* tested in MacConkey Agar, Eosin Methylene Blue Agar, Skimmed-Milk Agar, Kligler Iron Agar, and Brilliant Green Agar. Group 3 suspected *Serratia* tested in MacConkey Agar and Skimmed-Milk Agar. Group 4 suspected *Escherichia* tested in MacConkey Agar, Eosin Methylene Blue Agar, Skimmed-Milk Agar, Kligler Iron Agar, and Brilliant Green Agar. The colony resulted as shown in table 5.

Citrobacter genus can be identified if the isolates have the following properties: pink colonies on MacConkey Agar at 48 hours, A/AG reaction on TSI and KIA agar with H_2S positive, catalase test positive, nitrate positive, oxidase test positive, non-lactose fermenting, Methyl Red (MR) positive, Voges-Proskauer (VP) negative, Citrate test positive, o-nitrophenyl- β-D-galactoside (ONPG)[15-16].

For Enterobacter: lactose-fermenting, pink colonies on MacConkey Agar, A/A reaction on KIA and TSI with or without gas, indole negative, MR negative, VP positive, citrate positive, oxidase negative, and nitrate positive. For Serratia: late lactose-fermenting, may or may not produce pink colonies on MacConkey Agar at 48 hours, nitrate positive, A/A on TSI and KIA with or without gas, indole negative, MR variable, VP variable, citrate positive, oxidase negative, nitrate positive. For Escherichia: Lactose-fermenting, pink colonies on MacConkey Agar, A/A KIA and TSI with or without gas, indole positive, MR positive, VP negative, citrate negative, oxidase negative, nitrate positive, PYR negative, MUG positive[16].

The average results of the total population of the test group were compared with the control group's total population by using the statistical analysis of the Student's T-Test independent method. The colony counting results obtained can be seen in table 5. Deviation standard were provided in table 6. Independent Samples T-Test results were provided in table 7 and table 8. The results showed that the value Independent Samples T-Test of total microbiota count was below 0.05 so it can be considered that the administration of *E. faecium* IS-27526 as food supplementation could significantly reduce the total microbiota number.

On the other hand, the administration of *E. faecium* IS-27526 as food supplementation did not have any significant effect on total *Enterobacteriaceae* count, since the value obtained was above 0.05 which was 0.643.

The TPC was tested to understand the influence of probiotics supplementation with total microbiota number and total *Enterobacteriaceae* number in gut. Probiotic microorganisms present in intestinal mucosa can prevent pathogens colonization via competitive effects, antimicrobial substances production, and mucosal immunity modulation[17]. The result of TPC test can be seen in table 6.

Table 1
Bacterial Morphology Identification

Bacterial Group	Morphology Characteristics			
	Shape	Color	Texture	Edge Shape
Group 1	Round	Reddish-color	Arised-convex	Intact
Group 2	Round	Reddish-color	Arised-convex	Intact
Group 3	Round	Reddish-color	Arised-convex	Intact
Group 4	Round	Colorless	Flat	Intact

Table 2
Gram-staining Results

Bacterial Group	Shape	Gram's Class
Group 1	Rod-shaped	Gram Negative
Group 2	Rod-shaped	Gram Negative
Group 3	Rod-shaped	Gram Negative
Group 4	Rod-shaped	Gram Negative

Table 3
Biochemical Testing Results

Biochemical Properties	Bacterial Group			
	Group 1	Group 2	Group 3	Group 4
Maltose	+	+	+	+
Saccharose	+	+	+	+
Lactose	+	+	-	+
Mannose	+	+	-	+
Glukosa	+	+	+	+
Simmons Citrate	-	+	+	-
TSIA	+	+	-	+
Urea	-	-	-	-
VP	-	-	-	-
MR	+	-	-	+
Motility	+	+	+	+
Indol	-	-	-	-
Catalase	+	+	+	+

Notes: (+) Positive Result; (-) Negative Result

Table 4
Microbact™ 12A Bacterial Identification Results

Biochemical Properties	Bacterial Group			
	Group 1	Group 2	Group 3	Group 4
Lisin	-	+	+	+
Ornitin	-	-	-	-
H_2S	-	-	-	-
Glukosa	+	+	-	+
Manitol	-	+	-	+
Xylosa	+	+	-	+
ONPG	+	+	+	+
Indol	-	-	-	-
Urease	-	-	-	-
VP	-	-	-	-
Sitrat	+	+	+	-
TDA	+	+	+	+

Notes: (+) Positive Result; (-) Negative Result

Table 5
Biochemical Testing Results

Media	Bacterial Group			
	Group 1	Group 2	Group 3	Group 4
MacConkey Agar	Pink to red	Pink to red	Red	Pink to red
Eosin Methylene Blue Agar	Green metallic	Pink	x	Green metallic
Skimmed-Milk Agar	Clear zone	Clear zone	Clear zone	Clear zone
Kligler Iron Agar	Yellow (slant), yellow (butt), produced gas	Yellow (slant), yellow (butt), produced gas	x	Yellow (slant), yellow (butt), produced gas
Xylose-Lysine Deoxycholate Agar	Yellow	x	x	X
Brilliant Green Agar	x	Pinkish white	x	Yellowish green

Notes: (x) Not Tested

Table 6
Colony Counting Result

Test Animal Group	Average Total Microbiota Number (cfu/ml)	Average Total *Enterobacteriaceae* Number (cfu/ml)
Test Group	203,33 x 10^6	11,33 x 10^6
Control Group	222,67 x 10^6	12,00 x 10^6

Table 7
Standard Deviation Counting

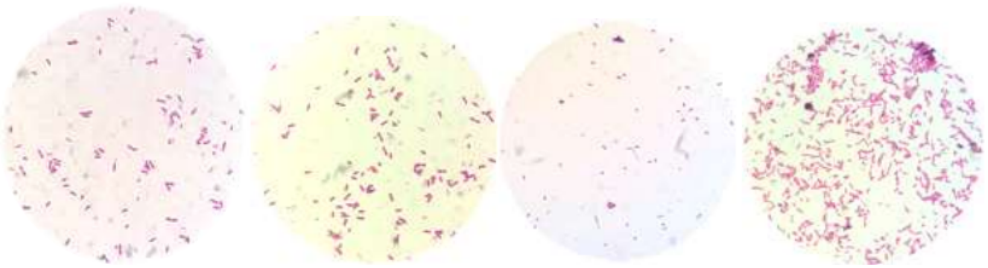
Test Animal Group	Mean	Std. Deviation	Std. Error Mean
Test Group	210,3333	1,15470	0,66667
Control Group	222,6667	3,21455	1,85592

Table 8
Student T-Test Results of Total Microbiota Count

Variances	Sig. (2-tailed)	Mean Difference	Std. Error Difference
Equal variances assumed	0,003	12,33333	1,97203
Equal variances not assumed	0,004	12,33333	1,97203

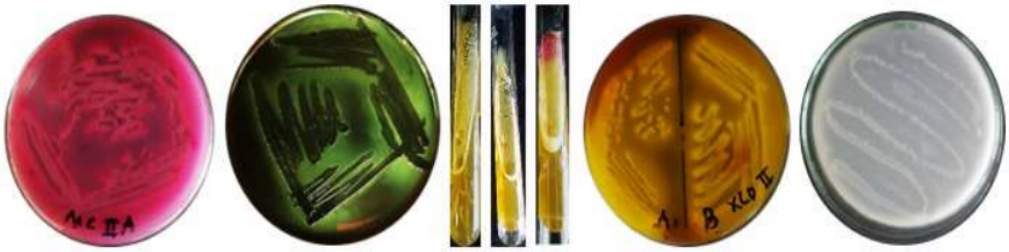
Table 9
Student T-Test Results of Total *Enterobacteriaceae* Count

Variances	Sig. (2-tailed)	Mean Difference	Std. Error Difference
Equal variances assumed	0,643	0,33333	0,66667
Equal variances not assumed	0,649	0,33333	0,66667



(a) (b) (c) (d)

Figure 1: Gram-staining Results; (a) Group 1; (b) Group 2; (c) Group 3; and (d) Group 4.



(a) (b) (c) (d) (e)

Figure 2: Group 1 Bacteria on Various Spesific Agar Results; (a) Mac-Conkey; (b) Eosin Metilen Blue; (c) Kliger Iron Agar; (d) XLD; (e) Skimmed-Milk Agar

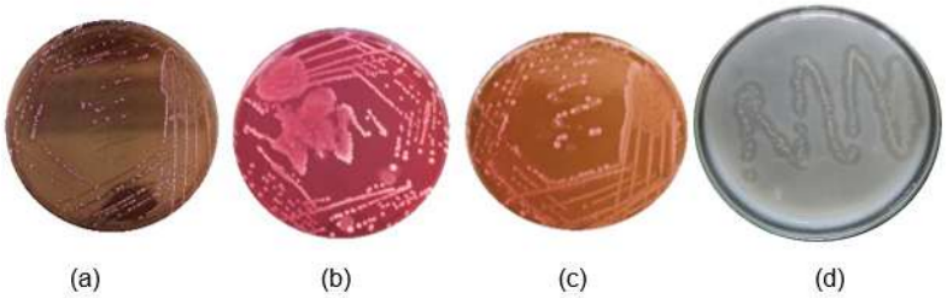


Figure 3: Group 2 Bacteria on Various Spesific Agar Results; (a) Mac-Conkey; (b) Brilliant Green and Oxgall; (c) Eosin Metilen Blue; (d) Skimmed-Milk Agar

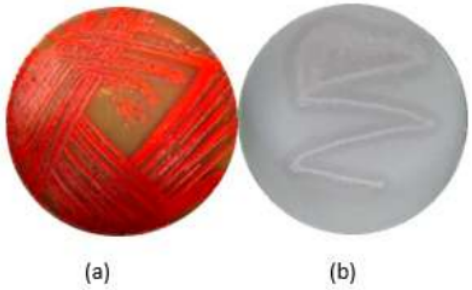


Figure 4: Group 3 Bacteria on Various Spesific Agar Results; (a) Mac-Conkey; (b) Skimmed-Milk Agar

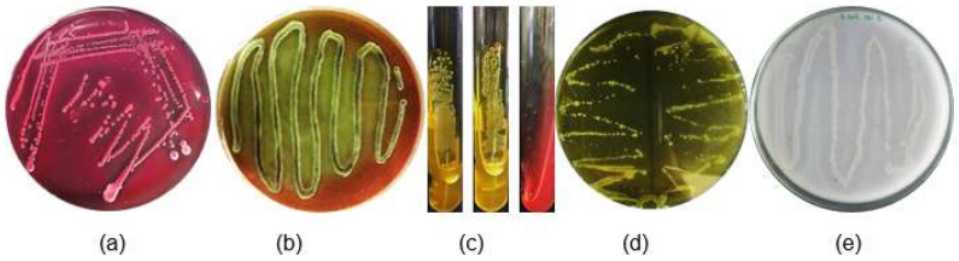


Figure 5: Group 4 Bacteria on Various Spesific Agar Results; (a) Mac-Conkey; (b) Eosin Metilen Blue; (c) Kliger Iron Agar; (d) Brilliant Green Agar; (e) Skimmed-Milk Agar

Conclusion

The use of *E. faecium* IS-27526 as food supplementation in New Zealand's White Rabbit animal model has been proven to significantly reduce the total bacterial count but the effect for *Enterobacteriaceae* bacterial count is insignificant.

Four genera of dysbiosis related bacteria has been identified from the rabbit small intestinal sample which include *Escherichia*, *Serratia*, *Enterobacter*, and *Citrobacter*.

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