Identification of Dysbiosis Related Bacteria from New Zealand's White Rabbit Intestinal Treated With *Lactobacillus plantarum* IS-10506 as Probiotics Food Supplementation

Arif S.W. Kusuma[1,*], Rizky Abdulah[2], Melisa I. Barliana[3], Tiana Milanda[1], Febrina A. Saputri[4], Raden M. Febriyanti[1], Sofa D. Alfian[2], Widya N. Insani[2], Dinda Arditta[1], Devinna[2], Inggrid S. Surono[5] and Vesara A. Gatera[2]

[1]*Department of Biological Pharmacy, Faculty of Pharmacy, Universitas Padjadjaran, Indonesia*

[2]*Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Padjadjaran, Indonesia*

[3]*Scientific Consortium of Drug Discovery and Development, Universitas Padjadjaran, Indonesia*

[4]*Department of Analytical Pharmacy and Medicinal Chemistry, Faculty of Pharmacy, Universitas Padjadjaran, Indonesia*

[5]*Department of Food Technology, Faculty of Engineering, Bina Nusantara University, Indonesia*

Abstract: The gastrointestinal microoganisms encompass thousands of bacterial species that constitute a relatively stable ecosystem inside human body. Dysbiosis is an imbalance condition of beneficiary microbacteria cause by newer microorganism. This research aimed to investigate the effect of *L. plantarum* IS-10506 as probiotics supplementation on total bacterial and *Enterobacteriaceae* count; and also to identify the dysbiosis causing bacteria. Total bacterial and *Enterobacteriaceae* number on both control and test group were determined by Total Plate Count method. Identification of dysbiosis related bacteria were determined by fermentation test, gram staining, and Microbact™ 12A kit. The TPC result of total bacterial population on control group was 222.67 x 10^6 cfu/ml, while the probiotics induced group was 210.33 x 10^6 cfu/ml. As for the TPC result for *Enterobacteriaceae* population from control group was 12.00 x 10^6 cfu/ml, *Enterobacteriaceae* population in the probiotics induced group was 11.66 x 10^6 cfu/ml. Four genera related to dysbiosis has been isolated from the intestinal sample and all are rod-shaped Gram negative bacteria. In conclusion, the use of *L. plantarum* IS-10506 as food supplementation has reduced the total bacterial count. Four genera of dysbiosis related bacteria has been identified from the intestinal sample which include *Eschericia, Serratia, Enterobacter,* and *Citrobacter*.

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

INTRODUCTION

The gut microbiota encompass thousands of bacterial species that constitute a relatively stable ecosystem inside human body. These gut microbiota has various roles benefiting the host which include maintenance of normal functions of the intestinal villi, carbohydrates digestion and fermentation, vitamins production, immune responses regulation, and protection from other potential harmful bacteria [1,2].

Dysbiosis is defined as an alteration in microbiota community structure and/or function, capable of driving a detrimental distortion of microbe and host homeostasis that specifically initiates or propagates disease [3-6].

With the constant evolution of microbiota, which could directly affect the host susceptibility, the crucial role of the microbiota in maintaining gastrointestinal system homeostasis is evident [7-9]. A variety of pathologies are associated with changes in the community structure and function of the gut microbiota, suggesting a link between dysbiosis and disease etiology [2,10,11].

Probiotics is defined as living bacteria species which act directly to alter the intestinal microbiota profile. Probiotics are very useful in improving host health conditions by increasing the body's nutritional needs, contributing enzymatically to digestion, inhibition of pathogenic microorganisms, having anti-mutagenic and anti-carcinogenic activity, as well as growth-enhancing factors and enhancing immune responses. Probiotics also play an important role as immunostimulatory and antipathogenic agents. Several in-vivo studies in mice have shown evidence that probiotics may reduce dietary obesity [12,13].

This research aim to investigate the effect of Lacto-bacillu plantarum IS-10506 as probiotics supllemen-

*Address correspondence to this author at the Department of Biology Pharmacy, Faculty of Pharmacy, Universitas Padjadjaran Jl. Raya Bandung-Sumedang Km. 21.Jatinangor, Sumedang, Indonesia; Tel: +62 842 88888 Ext. 3510; Fax: +62 22 7796200; E-mail: arif.satria@unpad.ac.id

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tation on total bacterial and Enterobactericeae count and to identify the dysbiosis causing bacteria isolated from the New Zaeland's white rabbit intestinal system.

MATERIALS AND METHODS

Ethical Clearance

To establish a research which used animal model, ethical clearance letter was needed as a scientific and ethical permit. The ethical clearance application was submitted into the Ethical Committee of Medical Faculty, Universitas Padjadjaran.

Animal Preparation

The animal model used in this research are male New Zealand's white rabbits, 2-3 months old, healthy condition, and has normal activity. Body weight ranges from 1-2 kg.

Acclimatization of the rabbits was conducted for a 1 week period. This step was performed to ensure that there were no sick rabbit included into the test. These rabbits were then fed with 200 grams of sterilized animal food pellets daily, morning and afternoon, and enough drinking water.

Each test rabbit was provided with single cage in order to separate the rabbit from one another and to reduce its stress level. Body weight and temperature were monitored daily. Selection criteria of animal model used in this study is based on constant body weight and temperature during one week of acclimatization.

After one week of acclimatization process, only rabbits with constant body weight and temperature were selected as animal model. These rabbit were then divided into two groups which was control and test group, with nine rabbit inside each group.

Probiotics Supplementation

Each rabbit of the test group was given *L. plantarum* IS-10506 for 14 consecutive days, once a day in the morning before meal time. The dosage used was 600 mg of dried probiotics powder, which was dilluted in 10 ml of sterilized aquadest. As comparison, each rabbit of the control group was only given sterilized aquadest without any probiotics. Both probiotics and aquadest solution were delivered by using sterilized feeding tubes.

Small Intestine Collection

At day 15, rabbits from each group were sacrificed. Blood samples were collected for another purpose, while small intestine samples were collected for bacterial count and identification. Small intestine samples that have been obtained, were immediately stored in Phosphate-Buffered-Saline (PBS) and kept in -20^0C refrigerator.

Total Bacterial Count

Total bacterial count were performed by swabbing insides part of intestinal samples using sterilized cotton bud. Samples from cotton bud were then grown into 10 mL of *Mueller-Hinton Broth* medium for 18 hours in 37^0C of incubation temperature. Samples were then dilluted by using sterilized broth medium in other test tube until it reached concentration of 1×10^6 cfu/mL.

20 µL of samples were then poured into the surface of agar medium, spread evenly, and then incubated for another 18 hours. Two types of agar were used, *Mueller-Hinton Agar* was used for total bacterial count, while *MacConkey Agar* was used for *Enterobacteriaceae* bacterial count.

Both agar plates were examined, and the number of colonies was determined after 18 hours of incubation at 37 ^{0}C.

Bacterial Identification

Bacterial identification were conducted by using a series of biochemical/fermentation test and Microbact™ 12A bacterial identification kit.

Data Interpretation

Data obtained from the bacterial identification process were then analyzed by using GIDEON™ software for bacterial identification to determine the genera of each single bacterial colony that has been successfully isolated.

Correlation of total bacterial count from both plates with the effect of probiotic supplementation were analyzed by using statistical analysis

RESULTS AND DISCUSSION

Ethical clearance for this research was granted by the Ethics Committee of Medical Faculty, Universitas Padjadjaran, by reviewing our research proposal and objectives with number 416/UN6.C1.3.2/KEPK/PN/2016.

A total of 18 rabbit's intestinal samples were obtained, which was 9 samples from test group and

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

another 9 samples from control group. From those groups, 29 single colonies of bacteria were successfully isolated. Each single colony was cultured on a *Mueller-Hinton Agar* (MHA) medium to be morphologically observed. Based on its morphological similarities, 29 colonies of bacteria were classified into four main groups. The result of the bacterial morphology identification is explained on Table **1**.

After morphology identification were performed, gram-staining procedure were conducted towards each group to identify bacterial shape. Gram staining differentiates bacteria by the chemical and physical properties of their cell walls by detecting peptidoglycan, which is present in the cell wall of Gram-positive bacteria. Gram-negative cells also contain peptidoglycan, but a very small layer of it that is dissolved when the alcohol is added. This is why the cell loses its initial colour from the primary stain. Gram-positive bacteria retain the crystal violet dye, and thus are stained violet, while the Gram-negative bacteria do not; after washing, a counterstain is added (commonly safranin or fuchsine) that will stain these Gram-negative bacteria a pink color. Both Gram-positive bacteria and Gram-negative bacteria pick up the counterstain. The counterstain, however, is unseen on Gram-positive bacteria because of the darker crystal violet stain. The Gram stain is almost always the first step in the preliminary identification of a bacterial organism. While Gram staining is a valuable diagnostic tool in both clinical and research settings, not all bacteria can be definitively classified by this technique (Table **2**).

Basically, some bacteria have similar morphological properties, so further testing method was necessary in order to identify unknown bacterial strain. A series of biochemical tests were performed for identification purposes, which include motility test, Triple Sugar Iron agar (TSIA) test, urease test, Methyl-Red (MR) and Voges Proskauer (VP) test, citrate test, indol test, catalase test. Carbohydrates fermentation test by using various source of sugar which include maltose, saccharose, lactose, glucose, and mannose were also conducted. Based on this identification, each bacterial strain would respond differently for each test given, depending on its ability to break carbohydrates chain or other specific properties. The results of the biochemical-based test for identification is explained on Table **3**.

The Microbact™ Gram-negative system is a standardised micro-substrate system designed to simulate conventional biochemical substrates used for the identification of Enterobacteriaceae and common miscellaneous Gram-negative bacilli (MGNB). Organism identification is based on pH change and substrate utilisations. The 12A strip may be used alone for identification of oxidase-negative, nitrate-positive glucose fermenters comprising 15 genera and may be useful for screening pathogenic Enterobacteriaceae from enteric and urine specimens or identification of other common isolates. The results of bacterial identification by using Microbact™ 12A strip is explained on Table **4**.

The identification data obtained were then analyzed by using web-databased software, namely GIDEON™

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	-	+	+	+
Anytime	-	-	-	-
H_2S	-	-	-	-
Glukosa	+	+	-	+
Manitol	-	+	-	+
Xylosa	+	+	-	+
ONPG	+	+	+	+
Indol	-	-	-	-
Urease	-	-	-	-
VP	-	-	-	-
Sitrat	+	+	+	-
TDA	+	+	+	+

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

(Global Infectious Disease Epidemiology Network). GIDEON™ is a web-based program (https://www.gideononline.com/) for decision support and informatics in the fields of Infectious Diseases and Geographic Medicine. As of 2005, more than 300 generic infectious diseases occur haphazardly in time and space and are challenged by over 250 drugs and vaccines. 1,500 species of pathogenic bacteria, viruses, parasites and fungi have been described. 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 *Eschericia* with 99% probability.

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 on Table **5**. Deviation standard were provided on Table **6**. Independent Samples T-Test results were provided on

Table 5: Colony Counting Result

Test Animal Group	Average Total Microbiota Number (cfu/ml)	Average Total Enterobacteriaceae Number (cfu/ml)
Test Group	210,33 x 10^6	11,67 x 10^6
Control Group	222,67 x 10^6	12,00 x 10^6

Table 6: Standard Deviation Counting

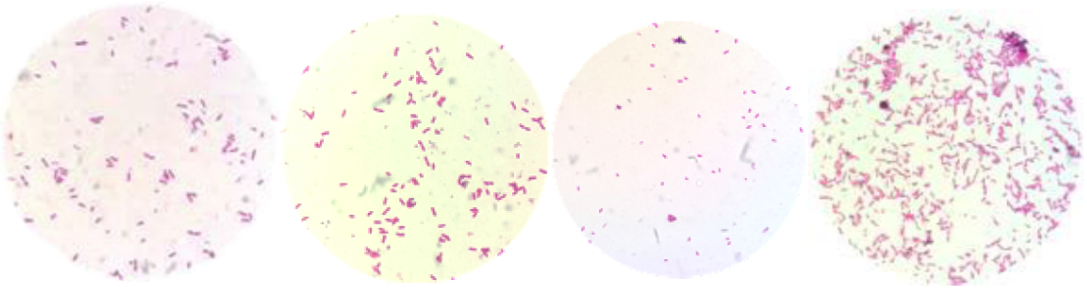
Test Animal Group	Mean	Std. Deviation	Std. Error Mean
Test Group	210,3333	1,15470	0,66667
Control Group	222,6667	3,21455	1,85592

Table 7: 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 8: 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**)

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

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 *L. plantarum* IS-10506 as food supplementation could significantly reduced the total microbiota number. On the other hand, the administration of *L. plantarum* IS-10506 as food supplementation didn't have any significant effect on total *Enterobacter* count, since the value obtained was above 0.05, which was 0.643.

In conclusion, these results suggest that the use of *L. plantarum* IS-10506 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. We also identified four genera of dysbiosis related bacteria from the rabbit small intestinal samples, which include *Eschericia*, *Serratia*, *Enterobacter*, and *Citrobacter*.

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