https://doi.org/10.48048/tis.2024.8280

Anti-Bacterial Potential of *Multi-strain* Probiotics *Lactiplantibacillus plantarum* (Strain Dad-13 and FNCC-0250) and *Lacticaseibacillus paracasei* GMRMP-001 against *Escherichia coli* FNCC-0091 *In Vitro* and *In Vivo*

Devi Ariska[1], Yunika Mayangsari[1,*], Endang Sutriswati Rahayu[1,2,3], Dian Anggraini Suroto[1] and Wahyu Dwi Saputra[1]

[1]Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia
[2]University Center of Excellence for Integrated Research and Application for Probiotic Industry, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia
[3]Center of Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia

([*]Corresponding author's e-mail: yunika.mayangsari@ugm.ac.id)

Received: 6 April 2024, Revised: 24 June 2024, Accepted: 1 July 2024, Published: 20 October 2024

Abstract

Lactiplantibacillus plantarum Dad-13, *Lactiplantibacillus plantarum* FNCC-0250 and *Lacticaseibacillus paracasei* GMRMP-001 are probiotics strain isolated from Indonesian local and traditional fermented foods. This study aims to evaluate the administration of *multi-strain* probiotics at dose of 10^9 CFU/mL/day for 14 days against the diarrhea-causing bacteria *Escherichia coli* FNCC-0091 at a dose of 10^{11} CFU/mL/day. Thirty rats were randomized and the divided into 5 groups, including control group, skim milk group, *single-strain* group, *multi-strain* group and *E. coli* group. Antibacterial activity was analyzed *in vitro*, feed intake, body weight were analyzed, microbial analysis was carried out on feces and cecum, short chain fatty acids were analyzed and intestinal morphology was measured. The result showed that the *single-strain* and *multi-strain* inhibition zone in *in vitro* test produces a very strong inhibitory potential (> 9 mm). The number of *L. plantarum* and *L. paracasei* increased and the number of *E. coli* decreased significantly in the feces and cecum of Rat indicating the survival rate of the probiotic bacteria in the digestive tract and their ability to fight pathogenic bacteria. The concentration of acetic acid, propionic acid and butyric acid compounds also increased, indicating that the digestive tract is at a low pH so that it can inhibit the growth of pathogenic bacteria in rats. In addition, consumption of *multi-strain* probiotics does not have a negative effect on intestinal morphology. The conclusion is that *multi-strain* probiotics are able to inhibit pathogenic bacteria i*n vivo* and *in vitro*.

Keywords: *Escherichia coli*, Gut histology analysis, *Multi-strain*s, Probiotic, Short chain fatty acid

Introduction

Escherichia coli is a pathogenic bacteria that can cause intestinal infections which called as diarrhea [1]. Based on global data, diarrhea is the second most deadly disease in children under 5 years and every year there are almost 1.7 billion cases of diarrhea in children [2]. To minimize the incidence of diarrhea, it is recommended to consume probiotics. Probiotics are a group of live microorganisms which, if consumed in sufficient quantities, will have health effects on the body [3]. Probiotic bacteria are required to survive in the intestine, stick to the intestinal mucosa, have a potential to balance the intestinal microflora, and have antimicrobial activity in pathogenic bacteria [4], and also being able to play a role in modulating the immune system [5]. Probiotic bacteria come from lactic acid bacteria (LAB) group which is dominated by *Bifidobacterium* and *Lactobacillus* [6].

Lactobacillus is a type of probiotic that is recognized to inhibit intestinal tract pathogens and maintain the balance of intestinal microflora [1]. *Lactiplantibacillus plantarum* Dad-13 and *Lacticaseibacillus paracasei* GMRMP-001 are strains obtained from local fermented Buffalo Milk "Dadih" typical of West Sumatra, Indonesia, while *Lactiplantibacillus plantarum* FNCC-0250 is a strain obtained from the traditional fermented Cassava typical of Yogyakarta, Indonesia [7]. All of these strains have met the requirements as probiotic bacteria and are functional ingredients, one of which is that it can inhibit pathogenic bacteria in the digestion [8].

The *Lactiplantibacillus plantarum* Dad-13 and *Lactiplantibacillus plantarum* FNCC-0250 strains show the ability as probiotic agents by its ability to survive simulated gastric digestion, bile salts and having antagonistic properties against pathogenic bacteria [7]. Meanwhile, the *Lacticaseibacillus paracasei* GMRMP-001 strain is a type of gram-positive bacteria and one of its characteristics is that it is able to inhibit the growth of pathogenic bacteria, this strain has a cell surface hydrophobicity > 50 % and high aggregation [9].

The ability and anti-pathogenic properties can be attributed to *Lactobacillus plantarum* in producing organic acids which contribute to lowering the pH and create unsuitable conditions for the growth of pathogenic bacteria [10]. Studies on *Escherichia coli* infection and administration of the *single-strain* probiotic candidate *Lactiplantibacillus plantarum* Dad-13 have been carried out *in vitro* and *in vivo* and resulted in the conclusion that *Lactiplantibacillus plantarum* Dad-13 can inhibit the growth of *Escherichia coli* bacteria as an agent of diarrhea [8].

To increase the effectiveness of probiotics, *multi-strain* probiotics are being researched to increase potency. It is known that *multi-strain* probiotics have better effectiveness than *single-strain*s in their inhibition against pathogenic bacteria [11]. Therefore, this study was conducted to test and evaluate the antibacterial activity of *Lactiplantibacillus plantarum* Dad-13, *Lactiplantibacillus plantarum* FNCC-0250 and *Lacticaseibacillus paracasei* GMRMP-001 *in vitro* and *in vivo*, against *Escherichia coli* FNCC-0091. Several variables were evaluated, including food intake, body weight, gastrointestinal tract morphology, short chain fatty acids (SCFA), analysis of bacteria in feces and cecum and *in vitro* antibacterial activity.

Materials and methods

Reagents and raw material

Rats (age between 8 - 12 weeks, weight 180 - 220 g) were collected from Nutrition Laboratory Center for Food and Nutrition Studies, Gadjah Mada University, Indonesia. Pure cultures of *Lactiplantibacillus plantarum* Dad-13, *Lactiplantibacillus plantarum* FNCC-0250, *Lacticaseibacillus paracasei* GMRMP-001 and *Escherichia coli* FNCC-0091 were obtained from the Food and Nutrition Culture Collection (FNCC) Center for Food and Nutrition Studies, Gadjah Mada University. LAB selection analysis media used de Man Rogosa Sharpe (MRS) agar with a composition of 52.2 g MRS (Merck, Germany), bacteriological agar (Oxoid, UK) and 3 g $CaCO_3$. Meanwhile, the medium for selecting *Latiplantibacillus plantarum* uses *Lactobacillus Plantarum* Selective Medium (LPSM) with a composition of 1 L distilled water, 15 g bacteriological agar (Oxoid, UK), 20 g sorbitol, 10 g bacteriological peptone (Oxoid, UK), 10 g beef extract (Oxoid, UK), 5 g yeast extract (Oxoid, UK), 5 g CH_3COONa, 2 g K_3PO_4, 0.1 g $MgSO_4$, 0.05 g $MnSO_4$, 0.02 g bromocresol purple and 2 mL antibiotics (Ciprofloxacin IV infusion 0.2 %), Tryptone Bile NA) and NaCl 0.85 %. All chemicals and reagents used in experiment were of analytical grade.

Antibacterial activity

Antibacterial activity against *Escherichia coli* FNCC-0091 were tested using agar well diffusion method of Ratna *et al.* [12]. *Lactic Acid Bacteria* (LAB) and pathogenic bacteria were cultured in MRS broth (Merck, Germany) and nutrient broth, respectively. *Escherichia coli* FNCC-0091 at a dose of 10^{11} CFU/mL/day as a pathogenic bacteria were then plated using the pour-plate method and stored in a cool room (4 °C) for 1.5 h. A 7 mm sterile steel cork borer created 4 wells in each petri dish containing NA media. Furthermore, 70 µL of LAB culture at a dose at a dose of 10^9 CFU/mL/day is added to each well and incubated at 37 °C for 24 h. The ability to inhibit pathogen growth was determined by the appearance of a clear zone surrounding the well. The diameter of clear zone minus that of the well in millimeters was used to determine the antibacterial activity of LAB strains.

Bacterial strain production

Lactiplantibacillus plantarum Dad-13*, Lactiplantibacillus plantarum* FNCC-0250 and *Lacticaseibacillus paracasei* GMRMP-001 are strains obtained from the Food and Nutrition Culture Collection, Center for Food and Nutrition Studies, Gadjah Mada University, Yogyakarta. Cell biomass production was carried out by inoculating 0.1 mL of probiotic bacterial isolate into MRS broth media. Next, the strain was inoculated into 1 L MRS broth then incubated at 37 °C for 24 h. After that, the bacterial culture was centrifuged at cold temperatures for 10 min at 3,500 rpm and the resulting pellet was suspended in 20 mL of 10 % skim milk at a concentration of 10^9 CFU/mL. Then, the biomass is enumerated using a colony counter and after reaching the desired dose it is stored in a freezer at a temperature of −20 °C.

Research experimental design

In this scientific research, rats were used as research subjects by following the rules of the Helsinski Declaration. Other requirements in the form of Ethical Clearance issued by the Integrated Research and

Testing Institute, Gadjah Mada University, Yogyakarta, Indonesia were approved as research guidelines with certificate number 00045/04/LPPT/II/2023 dated February 27, 2023. Thirty male rats with the Sprague Dawley strains (8 weeks old, and body weight 200 ± 20 g) was placed in a stainless steel cage with air circulation 12 h/12 h light-dark cycle and air control (22 - 25 °C and 60 % humidity). Rat were fed AIN-93M and given drinking water ad libitum based on the method of Reeves *et al*. [13] with modifications. The composition of AIN-93M rat feed is 62.07 % corn starch, 10 % sucrose, 14 % casein, 4 % soybean oil, 3.5 % mineral mix, 5 % fiber, 0.18 % L-Cystine, 1 % vitamin mix and 0.2 5% choline bitartrate.

Probiotic intervention in rat was carried out after the rat has been adapted for 1 week, while during the adaptation period the rat were given standard AIN-93M feed and drinking water ad libitum. Rats were randomly divided into 5 groups with a good number of rats per group. The first group (P.1) was the group given standard AIN-93M feed for 14 days. The second group (P.2) was the group given standard AIN-93M feed and 1 mL of 10 % skim milk solution for 14 days. The third group (P.3) was the group given standard feed AIN-93M and 1 mL of 10^9 CFU/mL *Lactiplantibacillus plantarum* Dad-13 in 10 % skim milk solution for 14 days. The fourth group (P.4) was the group given standard feed AIN-93M and 1 mL of 10^9 CFU/mL *multi-strain* (*Lactiplantibacillus plantarum* Dad-13, *Lactiplantibacillus plantarum* FNCC-0250 and *Lacticaseibacillus paracasei* GMRMP-001) in 10 % skim milk solution for 14 days. The fifth group (P.5) was the group given standard feed AIN-93M and 1 mL of 10^{11} CFU/mL *Escherichia coli* FNCC-0091 in 10 % skim milk solution for 3 days.

Rats activity during the study was observed per day, in the form of food consumption and body weight. All rat were euthanized with chloroform on day 17 and feces and cecum samples were taken for microbial and short chain fatty acids analysis. Then, the rat intestines were collected aseptically in a refrigerator box using formalin as a preservative for further analysis.

Gut histological analysis

Quantitative analysis of the morphology of the digestive tract (ileum, cecum and colon) was identified using an Eclipse E100 microscope (Nikon, Tokyo, Japan). Hematoxylin and Eosin (H&E) staining was used to view tissue based on the method of Zhou *et al*. [14] with modifications. Tissue samples were soaked in 10 % neutral buffered formalin solution for 48 h. After that, the tissue samples were cut to a thickness of 0.3 - 0.5 mm and then placed in a paraffin block. Then, tissue blocks were cut to a length of 3 - 4 µm. Then, the tissue is stained with Hematoxylin & Eosin (H&E), followed by reading the tissue preparation using a microscope.

Bacterial analyses of the feces and the cecum

Feces and cecum samples were prepared aseptically, then weighed 1 g each, then homogenized and serially diluted with 9 mL of 0.9 % sodium chloride solution. A total of 0.1 mL of liquid samples with various dilutions were poured into MRS Agar (Merck, Germany), *Lactiplantibacillus plantarum* Selective Media (LPSM), Tryptone Bile-X (TBX Agar) and LLV Agar using the spread plate method. Then, it was incubated at a temperature of 37 °C for 48 h. Colonies were enumerated using a Colony Counter (Reichert, New York, USA) based on the method of Bujalance *at al*. [15] with modifications.

Short chain fatty acids analysis

Short Chain Fatty Acid (SCFA) analysis was carried out on cecum samples taken after surgery and after consuming probiotics with an intervention period of 14 days, and injection *Escherichia coli* FNCC-0091 with an intervention period of 3 days. A total of 1 g of cecum sample was added with 1 mL of distilled water (1:3) and homogenized using a vortex for 5 min, after that it was sonicated for 30 min using an ultrasonic cleaner, then centrifuged at a speed of 1,000 rpm for 10 min. The supernatant obtained from centrifugation was then analyzed using a gas chromatography (GC) instrument (Shimadzu, GC 2010 plus series), while the injector specifications were 240 °C, RTX-Wax column, with a column length of 30 m, then a column temperature of 145 °C, followed by column diameter 0.25 μm and column speed of 0.8 min using helium carrier gas. The temperature of the Flame Ionization Detector (FID) is 240 °C.

Statistical analysis

The data obtained is expressed as an average number and standard deviation. Statistical analysis was continued with 1 way ANOVA, Kruskal Wallis and Mann Whitney tests to detect differences in *p*-values from $p < 0.05$ whose significance was expressed using SPSS Statistics 22 Software (IBM Corp., Armonk, NY, USA).

Results and discussion

Antibacterial activity analysis

Antibacterial activity analysis is needed to see the inhibitory potential of probiotic bacteria against pathogenic bacteria. **Table 1** show the results of antibacterial activity testing with the diameter of the inhibition clear zone of several isolates, namely *Lactiplantibacillus plantarum* Dad-13, *Lactiplantibacillus plantarum* FNCC-0250, *Lacticaseibacillus paracasei* GMRMP-001 and *multi-strain* tested on pathogenic bacteria, namely *Escherichia coli* FNCC-0091. The inhibition zone for each isolate ranged from 26.84 to 36.13 mm against *Escherichia coli* FNCC-0091.

Table 1 Antibacterial activity test (mm).

Isolates	***Escherichia coli* FNCC-0091**
Lactiplantibacillus plantarum Dad-13	26.84 ± 2.48^{a}
Lactiplantibacillus plantarum FNCC-0250	35.02 ± 3.80^{b}
Lacticaseibacillus paracasei GMRMP-001	36.13 ± 2.87^{b}
Multi-strain (*Lactiplantibacillus plantarum* Dad-13, *Lactiplantibacillus plantarum* FNCC-0250, *Lacticaseibacillus paracasei* GMRMP-001)	27.00 ± 2.41^{a}

Note: No significant differences were detected between the groups ($p < 0.05$).

LAB that are considered probiotic candidates need to be beneficial to the host and possess the ability to inhibit the growth of pathogenic bacteria. LAB can produce large amounts of antimicrobial metabolites,

including bacteriocin, diacetyl, lactic acid and hydrogen peroxide, prohibiting the growth of different pathogenic bacteria [16]. The presence of clear zone formation can confirm the antimicrobial activity against pathogenic bacteria [12]. In the analysis of antimicrobial activity against *Escherichia coli* FNCC-0091, the results of measuring the diameter of the inhibition zone showed large results, the isolate that produced the largest diameter of the inhibition zone was *Lacticaseibacillus paracasei* GMRMP-001, namely 36.13 mm (**Table 1**).

According to Ferdouse *et al*. [17] the antimicrobial activity was grouped into 3 categories, namely no inhibition (negative), moderate (9 mm) and strong (> 9 mm). Based on that criterion, it can be stated that most of the isolates probiotics have moderate inhibition against *Escherichia coli* FNCC-0091. All isolates showed a very high zone of inhibition against *Escherichia coli* FNCC-0091. The highest zone of inhibition was found in the isolate *Lacticaseibacillus paracasei* GMRMP-001 with an inhibition zone of 36.13 mm.

Populations of lactic acid bacteria, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei* and *Escherichia coli* in feces and cecum of rats

Force feeding a 10^9 dose of *single-strain* and *multi-strain* probiotics did not affect the food intake or body weight of any rat group. However, force feeding a 10^9 dose of *single-strain* and *multi-strain* affected the LAB population. Consumption of probiotics for 14 days in rat can increase and maintain the population of LAB in the feces and cecum of rat, this is proven by the quantitative data produced (**Table 2**). This finding is in line with Rahayu *et al.* [18] where consumption of *Lactiplantibacillus plantarum* Dad-13 resulted in up to 10^8 CFU/mL of LAB in digestion. The amount of LAB in the digestion and feces of rat did not differ much. Based on the results of this research, it can be concluded that probiotics are able to survive in the digestive system.

Table 2 The populations of LAB, *L. plantarum*, *L. paracasei* and *E. coli* in the feces and cecum of rats.

Group	Total LAB (log CFU/mL)			
	Feces		Cecum	
	14 Days	17 Days	14 Days	17 Days
P.1	6.56	5.94	NA	6.64
P.2	6.42	6.11	NA	6.21
P.3	6.71	4.73	NA	5.99
P.4	6.45	5.25	NA	5.59
P.5	6.23	5.16	NA	6.09
Group	***L. plantarum* (log CFU/mL)**			
	Feces		**Cecum**	
	Mean	**Min**	**Mean**	**Min**
P.1	6.95	6.83	NA	7.06
P.2	6.96	6.72	NA	5.90
P.3	6.72	6.86	NA	6.78
P.4	6.05	5.25	NA	6.89
P.5	6.89	6.86	NA	7.00

Group	***L. paracasei* (log CFU/mL)**			
	Feces		**Cecum**	
	Mean	**Min**	**Mean**	**Min**
P.1	6.56	6.45	NA	6.80
P.2	8.02	6.52	NA	6.58
P.3	7.07	6.20	NA	6.96
P.4	6.89	6.20	NA	6.43
P.5	6.44	6.35	NA	6.43
Group	***E. coli* (log CFU/mL)**			
	Feces		**Cecum**	
	Mean	**Min**	**Mean**	**Min**
P.1	7.32	7.00	NA	8.00
P.2	6.38	6.67	NA	6.51
P.3	7.26	6.45	NA	6.77
P.4	6.76	5.77	NA	6.64
P.5	9.00	6.84	NA	6.51

Note: P.1 = control group; P.2 = skim milk group; P.3 = *single-strain* group; P.4 = *multi-strain* group; P.5 = *E. coli* group.

In this study, *E. coli* FNCC-0091 was induced at a dose of 10^{11} CFU/mL on day 17. In **Table 2**, it can be seen that before the rat were infected with pathogenic bacteria, the number of *L. plantarum* in the fecal microbiota was relatively higher compared to after infection of pathogenic bacteria. It proves that administration of *single-strain* and *multi-strain* at a dose of 10^9 CFU/mL to rat is able to suppress the growth of *E. coli* at a dose of 10^{11} CFU/mL as evidenced by the persistence of *single-strain* and *multi-strain* in the digestive tract and in the feces of rat. Based on research results, *single-strain* and *multi-strain* as LAB in the digestive tract have an average growth rate of 10^4 to 10^6 CFU/mL. These results are strengthened research by Ikhsani [19] where the average growth of LAB in the digestive tract ranges from 10^4 to 10^5 CFU/mL, indicating that LAB is able to survive in the digestive tract of rat. The results of this research are strengthened by Ratna *et al.* [12] reported that *L. plantarum* and *L. paracasei* have resistance in gastric fluid with a pH of 2, then have resistance in bile salt solutions with a percentage of 0.3 % and have the ability to inhibit pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhi*. The population of LAB and *L. plantarum* in the large intestine can be interpreted as a potential for probiotics to attach to the mucosal surface of the digestive tract. This sticking characteristic can be called adhesion, which is one of the criteria for a strain to be said to be a probiotic bacteria [20].

Short Chain Fatty Acids (SCFA)

Analysis of short chain fatty acid levels is carried out to determine the abundance of acids in digestion. Based on the results of the analysis of short chain fatty acids in the cecum of rats, the concentrations of acetic acid, propionic acid and butyric acid showed results that were not significantly different ($p < 0.05$) (**Table 3**), but these results were in accordance with the concentrations required by the body. One of the

functions of short chain fatty acids in the digestive system is to fight pathogenic bacteria such as *E. coli*. The short chain fatty acids analyzed were acetic acid, propionic acid and butyric acid, these 3 acids have the largest percentage, as much 90 % in the digestive system compared to other acid compositions [21].

Table 3 Level short chain fatty acids in the cecum.

Group	Short chain fatty acid (mg/L)		
	Acetic acid	**Propionic acid**	**Butyric acid**
P.1	3.86 ± 1.84[a]	1.75 ± 0.77[a]	0.71 ± 0.34[a]
P.2	4.15 ± 2.69[a]	2.69 ± 1.96[a]	0.74 ± 0.41[a]
P.3	3.70 ± 1.31[a]	1.74 ± 0.55[a]	0.59 ± 0.18[a]
P.4	5.63 ± 2.10[a]	2.98 ± 1.35[a]	0.77 ± 0.37[a]
P.5	5.90 ± 1.92[a]	2.41 ± 0.47[a]	0.73 ± 0.29[a]

Note: No significant differences were detected between the groups ($p < 0.05$). P.1 = control group; P.2 = skim milk group; P.3 = *single-strain* group; P.4 = *multi-strain* group; P.5 = *E. coli* group.

The results of the SCFA analysis showed that acetic acid had a highest composition, followed by propionic acid and finally acetic acid. The mechanism by which probiotics maintain a healthy colonic environment is that probiotics will increase the number of good bacteria (such as *Bifidobacterium* and *Lactobacillus*) in the colon so that more nutrients are fermented by good bacteria and will increase SCFA. This increase in SCFA puts the colon in an acidic condition because the pH of the colon decreases as SCFA increases. Pathogenic bacteria in the colon that cannot tolerate low pH will experience a decrease in population so that the gut microbiota population and the colonic environment become balanced. These good environmental conditions will help the absorption of nutrients to be more optimal.

Gut histology analysis

Histological examination of the intestine showed inflammation after the induction of *Escherichia coli* FNCC 0091, however there was an influence and effect from consuming probiotics for 14 days, namely it could improve the histological structure of the intestine from the influence of pathogenic bacteria. The picture shows normal intestinal morphology, groups P.3 and P.4 are the groups that were given *single-strain* and *multi-strain* probiotic treatment and induced *Echerichia coli* FNCC-0091, but no erosion was shown on the intestinal mucosa. Meanwhile, group P.5 was a group that only underwent *Escherichia coli* FNCC-0091 induction without probiotic treatment, so it showed erosion of the intestinal mucosa. These results are supported by quantitative morphological analysis (**Table 4**). There was a significant difference in the P.5 group compared to the other groups ($p < 0.05$).

Table 4 Quantitative analysis of the gut histology analysis.

Intestinal tract	Parameter (µm)	Group				
		P.1	P.2	P.3	P.4	P.5
Ileum	Villous height	1.35 ± 2.11[a]	1.56 ± 2.45[a]	1.53 ± 2.39[a]	2.27 ± 2.62[a]	1.81 ± 2.06[a]
	Epithelial height	0.05 ± 0.08[a]	0.05 ± 0.08[a]	0.05 ± 0.08[a]	0.07 ± 0.08[a]	0.06 ± 0.07[a]
Cecum	Villous height	1.25 ± 1.97[a]	1.57 ± 2.47[a]	1.60 ± 2.47[a]	1.96 ± 2.20[a]	5.27 ± 0.80[ab]
	Epithelial height	0.06 ± 0.09[a]	0.05 ± 0.08[a]	0.04 ± 0.06[a]	0.05 ± 0.06[a]	0.11 ± 0.05[a]
Colon	Villous height	1.12 ± 1.74[a]	1.47 ± 2.28[a]	1.77 ± 2.95[a]	1.67 ± 2.43[a]	2.71 ± 2.18[a]
	Epithelial height	0.04 ± 0.06[a]	0.05 ± 0.07[a]	0.04 ± 0.07[a]	0.06 ± 0.07[a]	0.10 ± 0.07[a]

Note: P.1 = control group; P.2 = skim milk group; P.3 = *single-strain* group; P.4 = *multi-strain* group; P.5 = *E. coli* group.

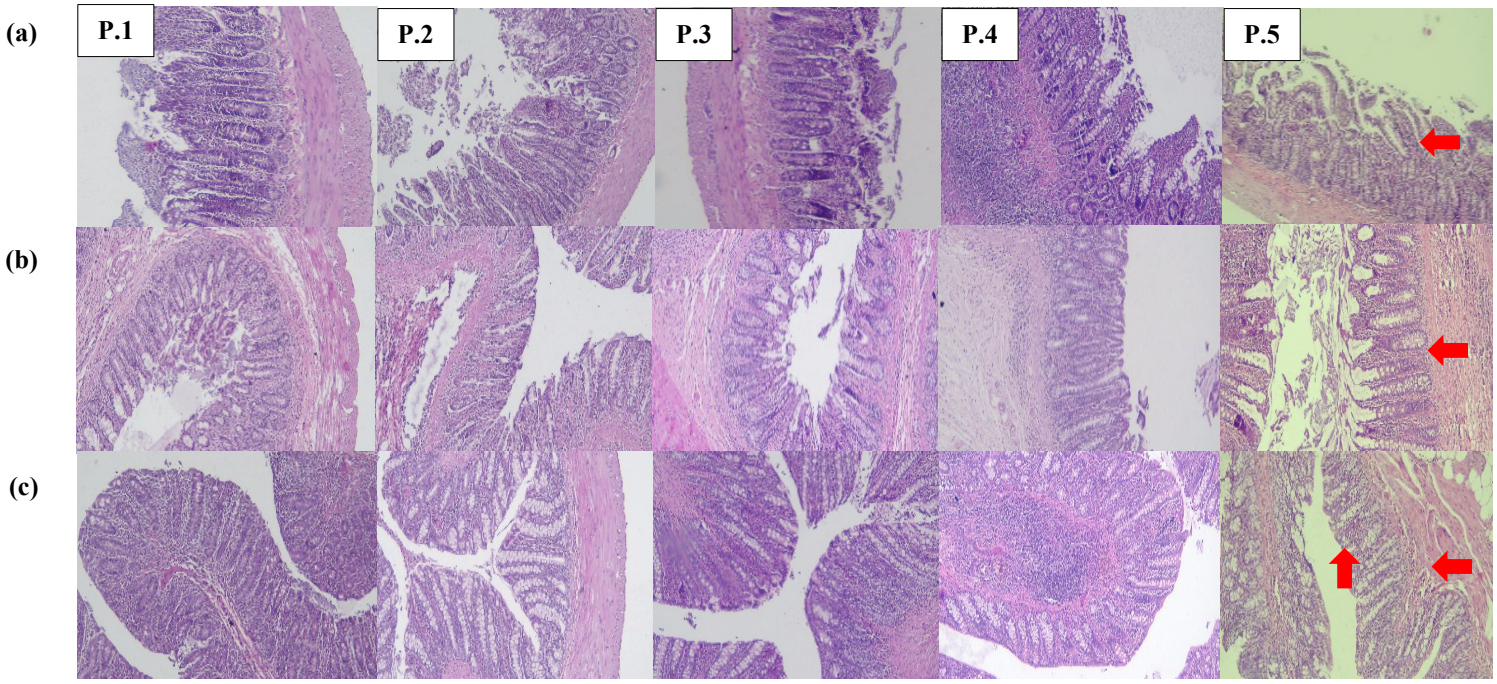

Figure 1 Microscopic appearance of the (a) ileum, (b) cecum and (c) colon. P.1 = control group, P.2 = skim milk group, P.3 = *single-strain* group, P.4 = *multi-strain* group and P.5 = *Escherichia coli* group.

Microscopic images using H&E staining show that *single-strain* and *multi-strain* probiotic intervention at a dose of 10^9 for 14 days in pathogenic bacterial infections causes structural changes and inflammation in the ileum, cecum and colon of mice which can trigger infection, but with the intervention of probiotic bacteria intestinal structure and barrier can be improved (**Figure 1**). Inflammation of the intestine can be characterized by the presence of reddish spots, then characterized by shortened or elongated intestinal villi accompanied by enlarged mucosal granules in the ileum [22]. Apart from that, inflammation can also be characterized by erosion of the intestinal mucosa accompanied by thinning of the outer mucosal layer due to invasion of pathogenic bacteria [23].

Tight junction function plays a very important role in regulating intestinal homeostasis. Transcriptase-polymerase chain reaction showed that 3,5,7,3',4'-pentamethoxyflavone increased transcription of occludin, claudin-3 and claudin-4. 3,5,7,3',4'-pentamethoxyflavone-induced transcription of occludin and claudin-3 is mediated by the transcription factors, KLF5 and EGR1, respectively, while 3,5,7,3',4'-pentamethoxyflavone activates transcription claudin-4 via GATA1 and AP1 [24]. Aberrant expression of claudin-2 is thought to be involved in clinical symptoms, one of which is diarrhea because claudin-2 also functions as a water channel. Claudin-2-mediated influx of Na^+ into the lamina propria activates serum-glucocorticoid salt-sensitive kinase 1 to increase Th1 and Th17 cells [25].

Together with the immune system, the mucosal system in the digestive tract has epithelial cells which can function to prevent or block attacks by pathogenic bacteria [26]. In general, bacteria in the digestive tract in the form of the intestine pass-through epithelial tissue and interact with the body's immune cells under normal conditions, but if the mucosal tissue and epithelial cells in the body are damaged, the interaction of immune cells can be excessive, causing inflammation in the intestinal tract. Based on previous research, it was stated that giving strains of *Lactobacillus rhamnosus* HN001, *Lactobacillus acidophilus* HN017 and *Bifidobacteriumlactis* HN019 to rats did not have a negative effect on intestinal health and integrity.

Conclusions

The inhibitory potential (clear zone) in antibacterial activity shows that *single-strain* and *multi-strain* (*Lactiplantibacillus plantarum* Dad-13, *Lactiplantibacillus plantarum* FNCC-0250 and *Lacticaseibacillus paracasei* GMRMP-001) are able to fight pathogenic bacteria well. In this case it was also shown that consumption of *single-strain* and *multi-strain* as probiotics for 14 days by Sprague-Dawley rats does not have side effects, such as food intake and body weight. The concentration of acetic acid, propionic acid and butyric acid also increased, with this it can be concluded that Short Chain Fatty Acid (SCFA) plays a role in inhibiting pathogenic bacteria. In addition, consumption of *multi-strain* probiotics does not have a negative effect and intestinal morphology. Although this study was limited to animal studies, these findings help assess the potential use of *single-strain*s and *multi-strain*s as probiotics.

Acknowledgements

The research work was funded by PT. Royal Medicalink Pharmalab, Jakarta, Indonesia.

References

[1] Y Hu, Z Mengna, L Zhaoxin, LV Fengxia, Z Haizhen and B Xiaomei. *L. johnsonii*, *L. plantarum*, and *L. rhamnosus* alleviated enterohaemorrhagic *Escherichia coli*-induced diarrhoea in mice by regulating gut microbiota. *Microb. Pathog.* 2021; **154**, 104856.

[2] World Health Organization, Available at: www.who.int/news-room/fact-sheets/detail/diarrhoeal-disease, accessed November 2022.

[3] Food and Agricultural Organization and World Health Organization. *Guidelines for the evolution of probiotics in food.* Food and Agricultural Organization and World Health Organization, Ontario,

Canada, 2002.

[4] S Rodriguez-Sanchez, P Fernandez-Pacheco, S Sesena, C Pintado and ML Palop. Selection of probiotic *Lactobacillus* strains with antimicrobial activity to be used as biocontrol agents in food industry. *LWT* 2021; **143**, 111142.

[5] CM Galdeano, SI Cazorla, JML Dumit, E Vélez and G Perdigón. Beneficial effects of probiotic consumption on the immune system. *Ann. Nutr. Metab.* 2019; **74**, 115-24.

[6] NP Shah, FA Jolene and RR Ramakanth. Populations of *Lactobacillus acidophilus*, *Bifidobacterium spp.* and *Lactobacillus casei* in commercial fermented milk products. *Biosci. Microflora* 2000; **19**, 35-9.

[7] ES Rahayu, Y Agung, Mariyatun, W Linda, U Tyas and W Koichi. Molecular characteristic of indigenous probiotics strains from Indonesia. *Int. J. Probiotics Prebiotics* 2015; **10**, 109-16.

[8] BT Sumaryati, U Tyas and Suparmo. The effect of *Escherichia coli* infection and administration of *Lactobacillus plantarum Dad-13* on the fecal microbiota wistar rats. *agriTECH* 2009; **29**, 165-70.

[9] AO Rahmi. 2023, Selection of lactic acid bacteria from Indonesian Dadih originated from Alahan Panjang, West Sumatera, as a probiotic candidate. Master Thesis. Gadjah Mada University, Yogyakarta, Indonesia.

[10] Z Noor, NC Muhammad, I Retno and Sardjono. Screening of *Lactobacillus plantarum* producing lactic acid for mocaf fermentation. *agriTECH* 2017; **37**, 437-42.

[11] W Mastuti and FY Era. Effectiveness of single and *multi-strain* probiotics against *Escherichia coli in vitro*. *J. Sci. Tech*. 2018; **7**, 178-87.

[12] DK Ratna, M Evita, ES Rahayu, MN Cahyanto, R Wikandari and T Utami. Indigenous lactic acid bacteria from halloumi cheese as a probiotic candidate of Indonesian origin. *Int. J. Probiotics Prebiotics* 2021; **16**, 39-44.

[13] PG Reeves, FH Nielsen and GC Fahey. AIN-93 purified diets for laboratory rodents: Final report of the American institute of nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* 1993; **123**, 1939-51.

[14] JS Zhou, Q Shu and KJ Rutherfurd. Safety assessment of potential probiotic lactic acid bacterial strains *Lactobacillus rhamnosus HN001*, *Lactobacillus acidophilus HN017*, and *Bifidobacterium lactis HN019* in BALB/c Rat. *Int. J. Food Microbiol.* 2000; **56**, 87-96.

[15] C Bujalance, M Jiménez-Valera and E Moreno. A selective differential medium for *Lactobacillus plantarum*. *J. Microbiol. Methods* 2006; **66**, 572-5.

[16] Q Zhang, Y Pan, M Wang, L Sun, Y Xi, M Li and Q Zeng. *In vitro* evaluation of probiotic properties of lactic acid bacteria isolated from the vagina of yak (*Bos grunniens*). *PeerJ* 2022; **10**, 15-35.

[17] J Ferdouse, S Paul, T Chowdhury, F Ali, S Islam and TJ Hossain. Probiotic characteristics of *Pediococcus pentosaceus* and *Apilactobacillus kunkeei* strains: The lactic acid bacteria isolated from Bangladeshi natural honey. *Appl. Food Biotechnol.* 2023; **10**, 33-45.

[18] ES Rahayu, HR Ilzamha, A Armita, ZK Rafli, CP Putrika, M Yustinus, U Tyas and W Jaka. Safety assessment of indigenous probiotic strain *Lactobacillus plantarum Dad-13* isolated from dadih using *sprague dawley* rats as a model. *Am. J. Pharmacol. Toxicol.* 2019; **14**, 38-47.

[19] AY Ikhsani. 2016, Safety evaluation of the indigenous probiotic strain *Lactobacillus plantarum Mut 7* using the *sprague dawley* rat model. Master Thesis. Gadjah Mada University, Yogyakarta, Indonesia.

[20] M Kechagia, D Basoulis, S Konstantopoulou, D Dimitriadi, K Gyftopoulou, N Skarmoutsou and EM Fakiri. Health benefits of probiotics: A review. *ISRN Nutr.* 2013; **2013**, 481651.

[21] XF Chen, X Chen and X Tang. Short-chain fatty acid, acylation and cardiovascular diseases. *Clin. Sci.* 2020; **134**, 657-76.

[22] A Takeuchi and H Sprinz. Electron-microscope studies of experimental *Salmonella* infection in the preconditioned guinea pig: II. Response of the intestinal mucosa to the invasion by *Salmonella typhimurium*. *Am. J. Pathol.* 1967; **51**, 137-61.

[23] VO Oyetayo, FC Adetuyi and FA Akinyosoye. Safety and protective effect of *Lactobacillus acidophilus* and *Lactobacillus casei* used as probiotic agent *in vivo*. *Afr. J. Biotechnol.* 2003; **2**, 448-52.

[24] Y Mayangsari, S Natsumi, X Wenxi, M Chinatsu, T Yuki, U Osamu, S Tomohiro, S Yoshiharu, Y Yoshinari and S Takuya. 3,5,7,3',4'-Pentamethoxyflavone enhances the barrier function through transcriptional regulation of the tight junction in human intestinal Caco-2 cells. *J. Agric. Food Chem.* 2021; **69**, 10174-83.

[25] Y Mayangsari, O Mayu, M Chinatsu, T Yuki, U Osamu, S Tomohiro, S Yoshiharu, Y Yoshinari and S Takuya. 5,7-Dimethoxyflavone enhances barrier function by increasing occludin and reducing claudin-2 in human intestinal Caco-2 cells. *J. Funct. Foods* 2021; **85**, 104641.

[26] S Koch and A Nusrat. The life and death of epithelia during inflammation: Lessons learned from the gut. *Ann. Rev. Pathol. Mech. Dis.* 2012; **7**, 35-60.