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Lactic acid bacteria administration from Jember tempeh (Indonesia) as a probiotic candidate in intestinal physiology and histology of mice strain Balb-C

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Abstract. *Azizah SN, Rosida, Hidayah AN, Dwijayanti AR. 2023. Lactic acid bacteria administration from Jember tempeh (Indonesia) as a probiotic candidate in intestinal physiology and histology of mice strain Balb-C. Biodiversitas 24: 6969-6978.* The lactic acid bacteria of tempeh have been studied for their potential as a probiotic with highly beneficial effects on health. This study aimed to investigate the potential of lactic acid bacteria from Jember tempeh on white mice's intestinal physiology and histology. The highest population of lactic acid bacteria isolated from three Jember tempeh types with GYP solid media was found in the Kaliwates tempeh (TK) at 2.74 × 10^8 cells/ml. According to the purification and lactic acid activity, the LAB isolates, encoded as TA.1, TB.1, TK.1, TK.2, and TK.4 are all probiotic candidates. All isolates obtained the optimum growth time at the 15th hour in TA.1 and TB.1 isolates, the 6th hour in TK.1 and TK.2 isolates, and the 8th hour in TK.4 isolate. The administration of LAB probiotics from Jember tempeh on the weight gain of mice presents a sig value of 0.38>0.05, which means an insignificant effect on the weight gain among the group. The solid feces condition of mice was also similar within groups. The administration of LAB probiotics on the intestinal histology of mice presents a sig value of 0.028<0.05, which means a significant effect within groups, showing a similar condition between the consortium administration group and the normal group. Groups TA.1, TB.1, TK.1, TK.4 and control *(Lactobacillus acidophillus)* had the same intestinal histology, namely partial necrosis. The administration of LAB probiotics from Jember tempeh can also reduce the population of *Salmonella* and *Escherichia coli* bacteria more effectively than without the probiotics. Thus, a good treatment group used as a probiotic candidate is a consortium.

Keywords: Intestinal histology, probiotic, the growth curve

INTRODUCTION

Probiotics are living microbes in food ingredients with beneficial effects for health when consumed at an adequate level. Probiotics produce various metabolites, such as lactic acid, acetic acid, hydrogen peroxide, lactoperoxidase, lipopolysaccharides, and bacteriocin, that can inhibit the growth of pathogenic bacteria and provide a therapeutic advantage for health. Probiotics can medicate lactose intolerance symptoms, prevent colon cancer, reduce blood cholesterol levels, and act as an antidiarrhea. Probiotics provide an immunomodulatory effect by assisting the formation of vitamin B, pyridoxin, niacin, folic acid, cyanocobalamin, biotin, and important antioxidants such as vitamin K (Rajoka et al. 2017; Sulistiani et al. 2019; Azizah et al. 2021).

Probiotics are mainly found in native fermented food from Indonesia, such as *bekasam* (Sari et al. 2018), *terasi* (Putra and Resti 2018; Fevria et al. 2023), *tape* and tempeh (Sulistiani et al. 2019; Azizah et al. 2021), and *oncom* (Kurniati et al. 2021). Probiotics, known by the community, are commonly produced as drinks, such as cow milk, buffalo milk, cheese, and yogurt (Karami et al. 2017). In pharmacy, probiotics are vaccine-carrier and drug-delivery agents like health supplements, formed as tablets, capsules, and capsules filled with bacteria.

Our previous research discovered that the Indonesian fermented foods obtained from Tanjung Market, Jember, East Java, namely tempeh and *tape*, contained lactic acid bacteria (LAB), which can be utilized as probiotics. Isolate TaJ.14 (*tape*), isolates TeJ.22, and TeJ.25 (tempeh) survived in gastric acid with pH levels of 2.5 and 3. These isolates were also sustained in bile salt with high potential levels of antidiarrhea against *Bacillus subtilis, Escherichia coli*, and *Shigella dysentriae*. These three isolates also presented higher probiotic activity than commercial probiotics, such as *Lactobacillus casei*, which was the comparative material in the previous study (Azizah et al. 2021). Therefore, further investigation into the potential of probiotics from Jember tempeh is necessary because each spontaneously fermented food has a unique taste in each region due to different microorganism diversity. Thus, the potential of each fermented food needs to be studied further.

According to Nurdini et al. (2015), each unique taste in each fermented food from one area is caused by different product ingredients and yeast types, besides the endophytic microbes in the ingredient or natural microbes in the production place environment. Several studies indicate that LABs are microbes that are dominantly found in tempeh at 10^7-10^8 cfu/g density. Several LAB species from tempeh, namely *Enterococcus faecium, Lactobacillus plantarum, Pediococcus acidilactici, Wisella confusa, Pediococcus pentosaceus*, dan *Lactobacillus fermentum*. *Lactobacillus*

fermentum from tempeh can also be sustained in low pH levels and 0.5% bile salt solution, thus potentially being used as a probiotic. Towoliu et al. (2015) also mentioned that the lactic acid bacteria as probiotics are composed of Gram-positive and Gram-negative bacteria, such as *Carnobacterium divergens, Enterococcus faecalis, E. faecium, Lactobacillus alimentarius, L. casei, Lactobacillus coryneformis, Lactobacillus curvatus, W. confusa, Wisella kandleri, Vagoccocus* sp., *Streptococcus parauberis, Lactococcus plantarum, Lactococcus carnosum, P. pentosaceus.*

Based on the background above, the potential of LAB from tempeh production in Jember, East Java, needs to be studied regarding their diversity, potential, and effect on the intestinal physiology and histology of the animals, as another requirement for probiotics is non-pathogenic bacteria. According to Towoliu et al. (2015) probiotics can regulate homeostasis in the intestinal epithelium tissue. They can regulate protective reactio ns, increase epithelial cell function, and improve the balance of normal microbes in the intestine. Probiotics have shown significant potential as a therapeutic option for a variety of diseases. Probiotics affect gut microbes and their hosts. This is because probiotics can communicate with the host through recognition receptors such as toll-like receptors and protein-like receptors containing nucleotide-binding oligomerization domains, which modulate key signaling pathways. This recognition is very important to produce an antimicrobial response that can minimize tissue damage due to inflammation. So, selecting the right probiotic strain for a specific application is required by exploring the potential of new probiotics (Bermudez-Brito et al. 2012).

This study aimed to isolate the lactic acid bacteria from three different types of tempeh sold at Pasar Tanjung, Jember, East Java, Indonesia. The potential of lactic acid bacterial isolates was selected based on the highest clear zone formation activity on solid GYP media. The LAB probiotic candidates were then applied orally to test animals to determine their effect on the animal physiology based on body weight, fecal condition, and pathogenic microbe contamination in feces. In addition, the effect of LAB isolate administration was also observed on the small intestine histology of the test animals. The future perspective of this research is to obtain strains of lactic acid bacteria as potential probiotics as a starter for typical Indonesian fermented products.

MATERIALS AND METHODS

Location and period

This study was performed from July 2022 to May 2023. This study was carried out in the Laboratory of Microbiology and Pharmacology, Jember Polytechnique of Medicine, and Laboratory of Zoology, University of Jember, Indonesia.

Materials

The tempeh samples originated from three different sub-districts in Jember District, East Java, Indonesia, namely Kaliwates (T.K), Antirogo (T.A), and Baratan (T.B). All tempeh samples were sold in Tanjung Market, the main traditional market in Jember District. The tempeh samples were all cooked and ready to consume after 72 hours of the fermentation process with yeast. The control bacteria, *Lactobacillus acidophilus*, were obtained from the Laboratory of Microbiology, Universitas Jember. The test animals used were male white mice (*Mus musculus*) strain Balb C.

Lactic acid bacterial isolation

The 50 g sample of tempeh was dissolved in 450 mL of NaCl 0.85% (w/v) and minced aseptically by a blender machine. The suspension produced from the following procedure was by its sample with 10^{-1} dilution level. Dilution was performed until the 10^{-7} dilution level with NaCl 0.85%. The 100 µL sample from each 10^{-3} to 10^{-7} dilution level was inoculated on GYP media with spread-plate method. Then, the inoculant was incubated for 48 hours at 37°C. The lactic acid bacteria (LAB) colonies that grew on the media were marked by the clear zone around each colony on GYP agar media. The LAB colony was counted with a colony counter to determine the total population of LAB in three tempeh samples (Azizah et al. 2021).

Purification and observation of potential LAB clear zone activity

The LAB colony was purified by taking and inoculating it with the quadrant streak method on GYP agar media, before being incubated for 48 hours in an incubator at 37°C. The purified sample was presented as a single colony, that produced a clear zone. The clear zone's activity was measured by a vernier caliper. Furthermore, the colony was characterized by its macroscopic and microscopic morphology with the Gram-staining method. The isolate was preserved in a GYP slant media at 4°C for further analysis (Azizah et al. 2021).

Determination of probiotic candidate growth curve

Two loops of five LAB isolates were cultured in 10 ml of GYP broth and incubated on a rotary shaker at 37°C and 120 rpm for 24 h as an inoculum. The 10% LAB inoculum was cultured in 100 ml of GYP broth. Cultures were collected every 1 to 24 h, and their optical density was measured spectrophotometrically at 600 nm (Azizah et al. 2015).

LAB production for test mice strain Balb-C

The 1000 mL of GYP broth was inoculated with 10% of LAB inoculum and incubated on a rotary shaker at 37°C and 120 rpm until the mid-logarithmic phase on each LAB isolate in accordance with the bacterial growth curve result. The same cell cultures were centrifuged at 3820×g for 15 min to obtain a pellet that contained the LAB cell population, while the supernatant was removed. The LAB pellet was washed twice with phosphate buffer (pH 7). Suspension with LAB cell was concentrated at 10^{-8} dilution level with the 7 Mc Farland (2.1×10^8 cel/mL) method using phosphate buffer (pH 7) as a solvent (Azizah et al. 2015).

Test mice strain Balc-C preparation

The test animal remained unfed for the first two weeks as part of the adaptation time to the new environment. The test animals were male mice strain Balb C. Mice strain Balb-C was chosen because these mice were commonly used in research of immunology. Mice were fed with standard pellets and drank regularly, followed by maintaining the cage and replacing the husk once in three days. The animal test was divided into two groups, namely the normal group without LAB probiotics administration and the treatment group with LAB probiotics administration (Andersen and Winter 2019).

LAB probiotics administration on the test mice Balc-C

The adapted mice were divided into eight treatment groups, namely TA.1, TB.1, TK.2, TK.4, LAB consortium of five isolates, LAB control (*L. acidophilus*), and normal treatments. Mice were administered with 0.2 mL of LAB suspension (10^{-8}) orally daily with a feeding tube for 14 days. Observation was performed every day to record the body weight (BW) and feces consistency data. On the 14th day, feces were taken to investigate the pathogenic bacteria in the feces. On the 15th day, mice were euthanized and surged to take their small intestines as histology samples (Nazarudin et al. 2017).

Body weight and feces consistency on the mice Balc-C

Before the treatments were administered, each mouse was weighed and its feces were observed. The body weight was measured with *Ohaus* animal balance. The consistency of the feces was separated into three types, namely liquid, half-solid, and solid (Andersen and Winter 2019).

Test mice strain Balc-C feces pollution test

A gram of feces was diluted in 9 ml of NaCl 0.85% and homogenized as a 10^{-1} diluted sample. The 100 µL suspension was cultured in SSA and EMBA media, then incubated for 24 hours at 37°C. The colony was observed, following the specification of the bacteria. Bacteria that proliferated on SSA media were *Salmonella* sp. and *Shigella* sp. *Salmonella* is marked by the black color formed around the colony, while *Shigella* is marked by the pink color (Dekker and Karen 2015). Bacteria that proliferated on EMBA media were *E. coli* with intense green metallic sheen color and dark or black color around the colony (Wally 2022).

Intestinal histology of test mice strain Balc-C

The intestinal tissue was fixated in formalin 10% for 2 hours, dehydrated with alcohol 70%, 80%, 95%, and 96%, then added with prusion in each sample for 2 hours, cleared with xylol and impregnated with liquid paraffin (58-60°C) for 2 hours. Embedding was then performed, before tissue-cutting with 4-5 µm thick microtom. Furthermore, object glass was prepared and coated with polylisin as a glue. The tissue was spread or floated on a heater tank filled with warm water, then caught with an object glass. After the tissue was glued to the object glass, deparaffinization was performed on the tissue, and the tissue was stained with hyosin to determine the histopathological condition in the mouse intestine (Sanz et al. 2007)

The intestinal histology of mice was assessed with a scoring method. The scoring assessment is categorized as follows:

Score1 : Good (no degraded villi)
Score2 : Half good/damaged (degraded villi)
Score3 : Damaged (necrotic villi)

Data analysis

The data were presented with tabulations and figures and analyzed with the descriptive-qualitative method for LAB isolation product, total LAB populations, lactic acid bacterial activity, feces consistency, and total pathogenic bacteria in feces. The body weight data were analyzed with a one-way ANOVA. The intestinal histology of mice was analyzed using the Kruskal-Wallis non-parametric test.

RESULTS AND DISCUSSION

The lactic acid bacteria (LAB) have been successfully isolated from three types of tempeh sold at Tanjung Market, Jember, East Java. Tanjung Market became the sampling location as the only main-class traditional market in Jember District, that provides all the needs and requirements of urban and rural communities. There are quite a lot of types of tempeh sold in this market, yet in this study, 3 types of tempeh were selected, which already have trademarks and a better taste than other types of tempeh in the market (Figure 1). The three types of tempeh also have a dense texture and are widely purchased by the public.

Figure 1. Three tempeh samples from Tanjung Market, Jember, East Java, Indonesia: A. Antirogo (T.A); B. Kaliwates (T.K); and C. Baratan (T.B)

According to Nurdini et al. (2015), each tempeh is fermented in a different production location or environment so that the result can represent three tempeh types. For example, a close-up picture may be better, providing the reader with a representation that will result in different tempeh tastes due to the diversity of natural microbes with other species in the environment. These microbes play a role during spontaneous fermentation, as the tools, materials, and environment used are in non-sterile conditions. So, the natural microbes will contribute and grow in the raw materials and fermentation process.

The isolation of lactic acid bacteria (LAB) using GYP media is presented in Figure 2. The LAB isolates in tempeh samples are characterized by the presence of a clear zone around the colonies on solid GYP media. According to Yulinery and Nurhidayat (2013), the clear zone around LAB colonies shows the action of $CaCO_3$ (calcium carbonate), which causes the alkaline condition in GYP and neutralizes the acid production excreted by LAB isolates. Therefore, the addition of $CaCO_3$ to the GYP growth medium is intended as an initial selection stage in the isolation and purification of LAB.

The number of lactic acid bacteria populations that grew in the three tempeh samples is presented in Table 1. The highest LAB population was found in tempeh from Kaliwates (TK). According to Azizah et al. (2021), LAB isolates from Kaliwates tempeh with different isolate codes (TeJ.18, TeJ.22, TeJ.24, TeJ.24) have good probiotic activity to inhibit *E. coli* and *B. subtilis* with strong category, can grow at pH 2.5 and 3, and are resistant to bile salts. Efriwati et al. (2013) also reported that the amount of LAB in tempeh at the end of soaking was 10^6 -10^8 cfu/g. Nurdini et al. (2015) reported that during the fermentation process, LAB gradually grew until 72 hours. The presence of LAB was high with 7-8 log cfu/g, which indicates that these bacteria play an important role during the tempeh fermentation process. Different tempeh production methods and environments also influenced the presence of LAB types. The LAB that dominate the tempeh fermentation stage include *L. plantarum, P. pentosaceus, W. confusa,* and *L. delbruecki.*

According to Barus et al. (2020), LAB contributes to increasing the safety of tempeh through the pH reduction mechanism, producing organic acids and metabolites that inhibit other microbes. During the soaking process, LAB will produce lactic acid as the main fermentation product and other organic acids, which can lower the pH level of the soaking water. This natural acidification can reduce the pH of soybeans, which can control the bacterial populations. Acidification can also provide a good environment for the germination of mold spores and reduce the lag phase time of *Rhizopus oligosporus* mold. The GOS (galacto-oligosaccharide) content in tempeh has a functional value as a prebiotic. Tempeh is produced from soybeans and several other ingredients that support the fermentation process. Tempeh has a function as a probiotic, because tempeh is produced through fermentation, so the living microbes can form a protective layer in the intestines and protect the digestion process from pathogenic bacteria. Therefore, lactic acid bacteria play important roles from the soaking process until the fermentation process is complete.

The results of LAB isolation from mixed isolates were then carried out to purify the isolates and measure the diameter of the clear zone in a single colony. The purification results showed that there were 11 LAB isolates from 3 types of Jember tempeh with different clear zone formations around their colonies. Five LAB isolates produced the highest clear zones (Figure 3 and Table 2). According to Azizah et al. (2021), apart from lactic acid secreted into GYP agar media, LAB also produces other important metabolite compounds, such as hydrogen peroxide and bacteriocins. These metabolites cause probiotics to have therapeutic benefits for lactose intolerance treatment, colon cancer prevention, blood cholesterol level reduction, diarrhea treatment, and immunomodulatory enhancement.

Table 1. The lactic acid bacteria (LAB) population in three types of Jember tempeh

Name	Total (cells/g)
Kaliwates Tempeh (TK)	2.74×10^8
Antirogo Tempeh (TA)	1.8×10^7
Baratan Tempeh (TB)	2.3×10^6

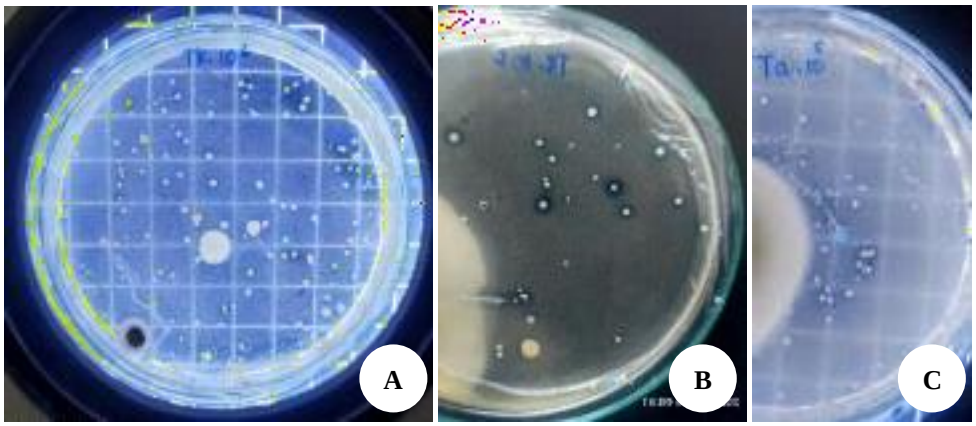

Figure 2. The LAB colonies have been isolated from Jember tempeh after 48 hours of incubation on GYP media. A. Antirogo (T.A); B. Kaliwates (T.K); and C. Baratan (T.B)

After screening the potential LAB isolates, five isolates were highly-qualified, namely TA.1 (Antirogo tempeh), TB.1 (Baratan tempeh), TK.1, TK.2, and TK.4 (Kaliwates tempeh), due to high clear zone activity on GYP agar media (Table 2). These five isolates also had s higher activity than the control LAB isolate, namely *L. acidophilus*. Therefore, the five LAB isolates were further investigated to measure the bacterial growth curve and to determine the optimum time for LAB probiotic cells harvest and LAB inoculum as a probiotic by testing it in vitro using mice as the test animals.

In Table 3, the five LAB isolates had the same colony and cell morphology. The colony is circular in shape, with convex elevation, entire margin, smooth surface, opaque texture, and the same milky white color as *L. acidophilus*. The Gram staining test showed that the six BAL isolates had rod-shaped cells and were characterized as Gram-positive and negative bacteria (Figure 2). According to Mahulette et al. (2016), LAB isolated from Inasua obtained 13 types of Gram-positive bacteria and 10 types of Gram-negative bacteria. LAB from tempeh generally includes Gram-positive and rod-shaped bacteria such as *Lactobacillus heterofermentative* and *Streptococcus non enterococci* (Pisol et al. 2015), *P. pentosaceus* and *L. plantarum* (Sulistiani et al. 2019), *L. fermentum*, *Lactobacillus dellbrueckii*, *Lactobacillus agilis*, and *Weissella confusa* (Barus et al. 2020), *E. faecium* and *Lactiplantibacillus plantarum* (Fallo and Sine 2022). According to Mahulette et al. (2016), rod-shaped LAB generally produces higher levels of lactic acid than coccus-shaped LAB. This opinion is in line with the results of the present study in Figure 4, which shows that five LAB isolates can reduce the pH of liquid GYP media from 7 to 3.

Table 2. Clear zone diameter of LAB isolates from Jember tempeh on GYP media after 48 h of incubation

Code	Clear zone (mm)
TA.1	6.32±1.18
TA.2	0.11±0.11
TB.1	5.53±0.77
TB.2	0.03±0.06
TB.4	0.71±1.14
TB.5	1.42±0.81
TB.6	0.25±0.44
TB.7	2.57±1.68
TK.1	5.31 ±1.18
TK.2	5.62±1.50
TK.4	8.51±2.72
Lactobacillus acidophilus (control)	3.42 ±1.86

Table 3. Characterization of LAB isolates from Jember tempeh

Code	LAB isolate observation						
	Colony morphology					Cells	
	Shape	Elevation	Margin	Surface	Texture	Shape	Gram
TA.1	Circular	Convex	Entire	Smooth	Opaque	Bacil	Positive
TB.1	Circular	Convex	Entire	Smooth	Opaque	Bacil	Negative
TK.1	Circular	Convex	Entire	Smooth	Opaque	Bacil	Negative
TK.2	Circular	Convex	Entire	Smooth	Opaque	Bacil	Negative
TK.4	Circular	Convex	Entire	Smooth	Opaque	Bacil	Positive
Lactobacillus acidophilus (control)	Circular	Convex	Entire	Smooth	Opaque	Bacil	Positive

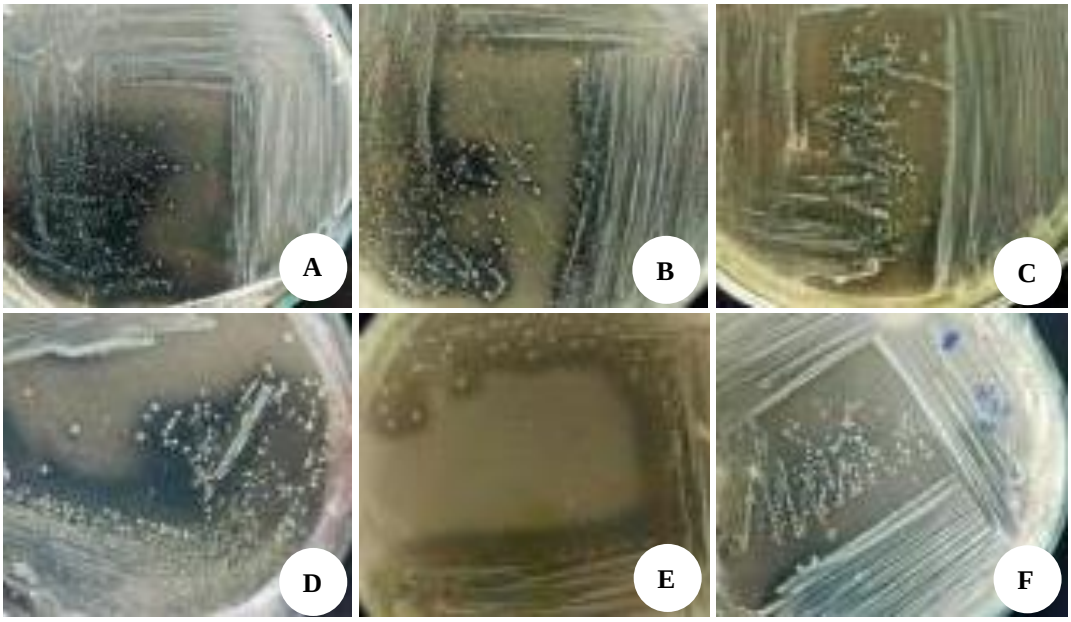

Figure 3. The purification results of LAB and *Lactobacillus acidophilus* isolates on GYP after 48 h of incubation. A TK.1; B. TK.2; C. TK.4; D. TA.1; E. TB.1; F. La

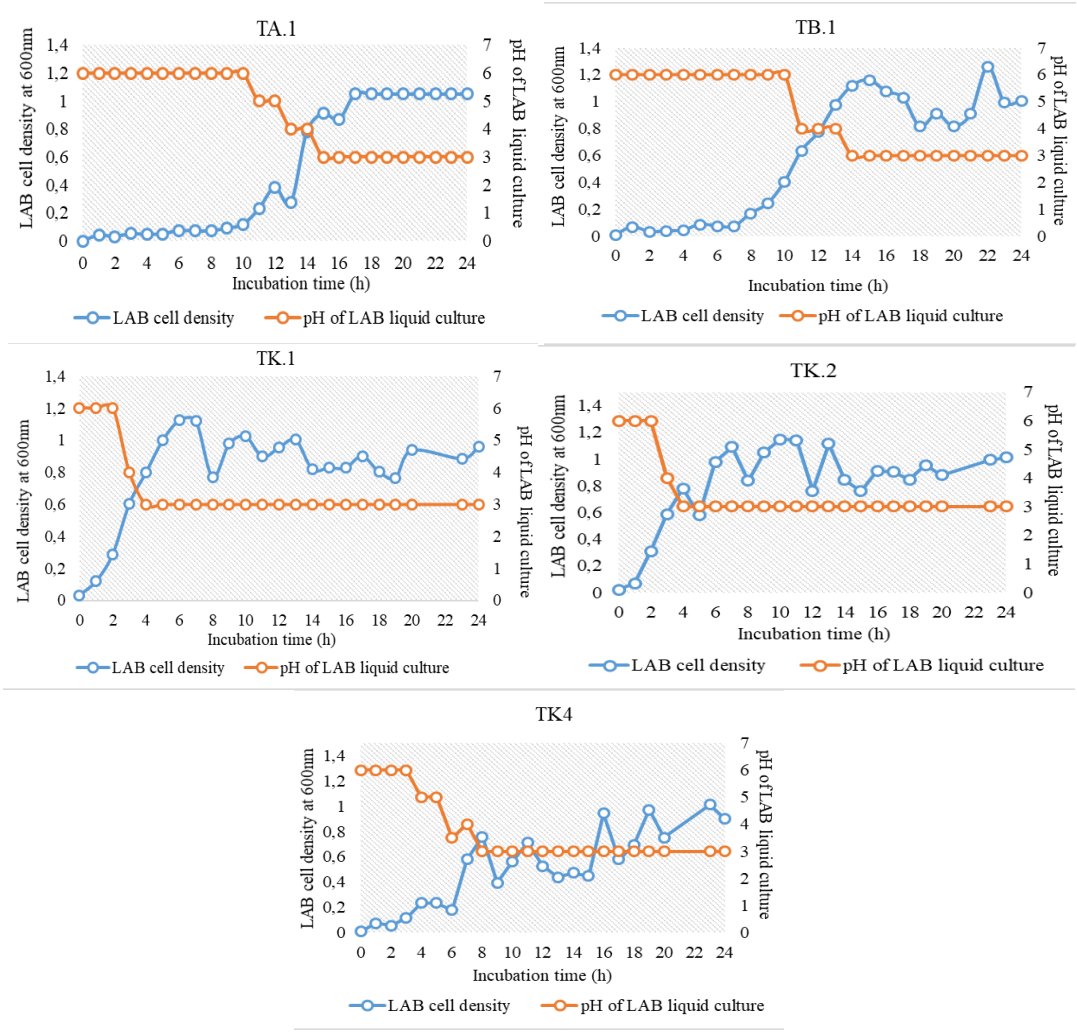

Figure 4. The growth curve of lactic acid bacteria from Jember tempeh on GYP broth for 24 h

The five LAB isolates, namely TA.1, TB.1, TK.1, TK.2, and TK.4, had their growth measured in the GYP broth to determine the growth pattern and optimal time for the production of probiotic cells and their metabolites. The growth curve of LAB isolates using the spectrophotometer method is presented in Figure 4. The LAB isolates in TA.1 and TB.1 have similar growth patterns compared to other isolates. Isolate TA.1 came from Antirogo Tempeh, Sumbersari sub-district, while isolate TB.1 was from Baratan Tempeh, Patrang sub-district. Meanwhile, TK.1, TK.2, and TK.4 isolates had similar growth patterns compared to isolates TA.1 and TB.1, because isolates TK.1, TK.2, and TK.4 came from the same tempeh product, namely Kaliwates Tempeh, Kaliwates Sub-district.

The TA.1 and TB.1 isolates were still in the adaptation phase at 0-7 hours, as the cell absorbance value was yet to increase and the pH was still 7. The TA.1 isolate began to enter the early log phase at the 11th hour, while TB.1 at the 11th hour, marked by an increased cell absorbance and a decreased pH to 4. The TA.1 and TB.1 isolates showed a mid-logarithmic phase at the 15th hour, marked by a more rapid increase in cell absorbance and a decrease in pH to 3. The TK.1, TK2 and TK4 isolates have faster adaptation character. The TK.1 and TK.2 isolates showed a mid-logarithmic phase at the 6th hour, while TK.4 at the 8th hour, characterized by a faster increase in cell absorbance and a decrease in pH to 3. In this phase, Azizah et al. (2015) stated that the logarithmic number of cells increases rapidly up to a certain limit until it enters the static phase, maximum cell metabolism, and rapid synthesis of cell materials in constant amounts until nutrients are fully used up. This result is in accordance with the previous study results, namely TA.1, TB.1, TK1, TK.2, and TK.4 isolates. When they entered the log phase to the static phase, the pH started to fall from 7 to 3. This shows that lactic acid as a primary metabolite begins to be synthesized and produced during the early log phase and lactic acid production reaches optimum in the late log phase (Figure 4). Therefore, the growth pattern of LAB isolates in GYP growth media presents that the media can supply nutrients for bacterial cell growth well. The growth of bacterial isolates occurred between the 0th hour and continued to increase in the number of cells until the 24th hour.

LAB candidate cell production

Five LAB candidate isolates, namely isolates TA.1, TB.1, TK.1, TK.2, TK.4, and the control, namely *L. acidophilus*, were produced on GYP broth in accordance with the growth curve procedure to gain the number of cells and optima; cell condition on mid logarithmic phase. The LAB suspension in the mid-log phase was harvested and centrifugated to obtain the pellet. The pellet was LAB cells with the highest metabolic activity. The pellet was stabilized in a buffer for further use as probiotics, that were administered orally to the mice (Figure 5). The probiotic population for oral administration had a number of cells of 21×10^8 cells/mL, so each treatment had the same concentration.

The animals used as treatment models for probiotic administration were male white mice strain Balb C. Mice are used as test animals because they have a relatively short life cycle, large number of offspring per birth, easy to handle, similar reproductive characteristics to other mammals, and similar anatomical structure, physiology, and genetics to humans (Fianti 2017; Herrmann et al. 2019). Probiotics were administered every day for 14 days. Observations were performed every day to record the body weight (BB) and feces consistency. After the 14th day, mice were euthanized and surged to take the small intestines for further histological observations on the intestines. The mice's weight after 14 days is presented as an average change in body weight in Table 4.

In Table 4 and Figure 6, the TK.2 treatment group had the highest BW gain, namely 0.288 ± 0.142 g. The results of bodyweight gain were analyzed using the One-way ANOVA with SPSS and showed a significant value of 0.38 (Table 5). The sig value>0.05 indicates that there is no significant difference in weight gain between the treatment groups. According to Utami et al. (2022), consuming probiotics can reduce body weight and is more effective when accompanied by a balanced diet and physical activity. In Table 4, the average BW gain increased in all treatments, as mice did not carry out the physical activity while being administered with probiotics. In addition, the test animal cages were relatively narrow. Mice 4 in TA1 showed a decreased body weight because of external factors such as stress due to treatment. But, the average change in body weight of mice from the day showed an increase in body weight.

The mice's feces consistency was observed every day for 14 days, and the data is presented in Table 6. There are no changes in feces consistency in all treatments from the 1st to 14th day in the single probiotic treatment, consortium treatment, control and normal treatment without probiotic administration of prebiotics. According to Oviani et al. (2015), there was no difference in feces consistency frequency and duration of acute diarrhea in children who received standard therapy compared to standard therapy with the addition of probiotics.

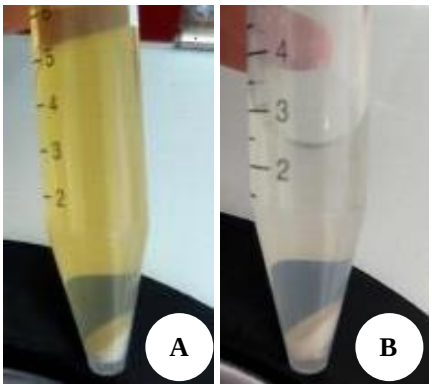

Figure 5. The centrifugated LAB suspension. A. The pellet below the tube was LAB cells; B. Stabilized pellet in a buffer with pH 7 and ready for further administration to mice

Table 5. One-way ANOVA analysis results

Treatment	Sum of squares	df	Mean square	F	Sig
Between groups	0.444	7	0.063	1.123	0.384
Within groups	1.242	22	0.0556		
Total	1.686	29			

Table 6. Feces consistency on probiotic treatments

Group	Treatment	Average of feces consistence at the 14th day
1	TA.1	Solid
2	TB.1	Solid
3	TK.1	Solid
4	TK.2	Solid
5	TK.4	Solid
6	*Lactobacillus acidophilus* (control)	Solid
7	Consortium	Solid
8	Normal	Solid

Table 4. Body weight gain of test animals on the 1st-14th day

Group	Treatment	Average body weight gain on the 1st-14th day				Average ± SD
		Mice 1	Mice 2	Mice	Mice	
1	TA1	0.38	0.15	0.23	-0.23	0.133 ± 0.260
2	TB1	-0.08	-0.08	0.69	-0.15	0.170 ± 0.363
3	TK1	0.23	0.08	0.08	-0.23	0.155 ± 0.099
4	TK2	0.38	0.08	0.31	0.38	0.288 ± 0.142
5	TK4	0.23	-0.08	-0.08	-0.38	0.153 ± 0.197
6	*Lactobacillus acidophilus* (control)	0.46	0.08	0.15	0.08	0.193 ± 0.181
7	Consortium	0.23	0.23	0.15	-0.08	0.173 ± 0.07
8	Normal	0	0	0	-0.29	0.145 ± 0.205

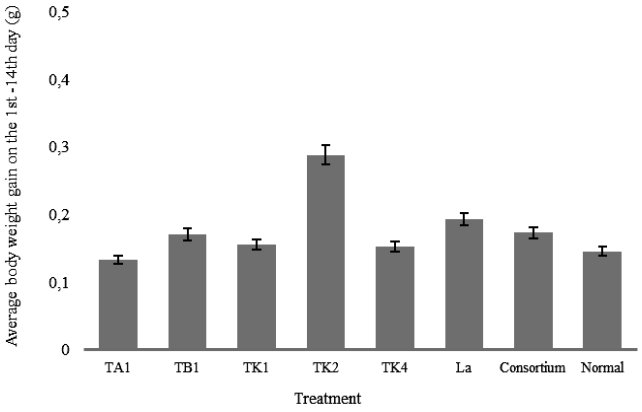

Figure 6. Body weight gain on each treatment

The intestinal histological damage scoring in mice was observed after probiotics administration treatment for 14 days, then counted from the observation results of each field of view. The microscope observation data with a magnification of 400-1000 × is presented in Figure 7, while scoring is presented in Table 7. The lowest scores were found in the normal group (without probiotics administration) and the ABK group (LAB consortium from tempeh). Treatments TA.1, TB.1, TK.1, TK.4 and the control (*Lactobacillus acidophillus*) had a level of intestinal histology damage with a score of 2, namely causing partial necrosis of the intestine. Intestinal histology in normal treatment has a score of 1, which means good or no intestinal necrosis occurs; thus, the best treatment in this study based on the average value of intestinal histology was the consortium group and the normal group.

The observed histology scores were analyzed using the SPSS-Kruskal Wallis method, which showed a significance value of 0.028 and indicated a sig value of <0.05 (Table 8). This value means that there is a significant difference in the intestinal histology among the probiotic treatments. The LAB Consortium has a small score similar to normal. According to Sanz et al. (2007), probiotics function as mucosal defense, protective function, and immune defense in the gastrointestinal tract, such as the epithelial layer, mucus layer, peristalsis and epithelial desquamation, and immunoglobulin A (IgA) secretion, which greatly influences the adhesion of pathogenic microbial, local and systemic immune system modulation. Therefore, the LAB consortium from tempeh can normalize the function of the small intestine without causing damage or necrosis.

Probiotics affect the excretion of mucin genes, that will stimulate the mucus production from the intestinal mucosa as a barrier (García et al. 2019). This probiotic effect was also depicted in the present study that used a probiotic consortium (tempeh LAB mixture) for 14 days. The microscopic image shows a normal small intestinal mucosal architecture with an intact epithelial surface with several cell regenerations. The administration of a fermented probiotic mixture from Jember tempeh in the consortium group showed no damage to the intestinal epithelium of mice. Thus, the score is the same as the normal group.

Tests for fecal contamination in the animals are presented in Table 9. The administration of consortium probiotics could reduce pathogenic bacteria contamination, namely *Salmonella* spand *E. coli*, compared to the normal group without probiotics administration. Treatments with single probiotics, such as those in groups 1 to 6, still suffered from *Salmonella* contamination. According to Sulistiani et al. (2019), probiotics produce substances called bacteriocins, which are actively metabolized proteins that play a role in destroying harmful microorganisms. Probiotics also produce metabolites, such as lactic acid, acetic acid, hydrogen peroxide, lactoperoxidase, lipopolysacharide, to inhibit the growth of pathogenic bacteria, thus having therapeutic benefits in health. According to García et al. (2019), a microbial consortium starter involving lactic acid bacteria will produce various metabolites as the determining factors in the formation of microbiota interaction that occurs positively, negatively, and neutrally. According to Iqraini et al. (2021), the use of a probiotic consortium in arabica coffee fermentation showed that coffee with a fermentation time of 48 hours had the best taste. The GC-MS test results showed that several compounds were detected after fermentation processes, including furans, phenols, propanoic acid, quinic acid, purines, palmitic acid, pyrrole, ascorbic acid, linoleic acid, stearic acid, oleic acid, amines, pyranes, purines, aldehydes, vitamin E, benzadrex, hexene, tocophen.

Table 7. Intestinal histology observation scoring in mice after probiotics administration for 14 days

Group	Treatment	Average of histology score
1	TA.1	2.75±0.43
2	TB.1	2.50±0.87
3	TK.1	1.75±0.43
4	TK.2	2.75±0.43
5	TK.4	2.50±0.87
6	*Lactobacillus acidophilus* (control)	2.75±0.43
7	Consortium	1.25±0.43
8	Normal	1.00±0.00

Table 8. Data analysis results of intestinal histology observation

Kruskal-Wallis H	df	Sig
15.704	7	0.028

Table 9. Fecal contamination in mice on SSA and EMBA media

Group	Treatment	*Salmonella* sp. (cell/g)	*E. coli* (cell/g)
1	TA.1	8.00×10^{1}	0
2	TB.1	1.50×10^{1}	0
3	TK.1	6.70×10^{2}	2.7×10^{1}
4	TK.2	5.00×10^{1}	0
5	TK.4	3.60×10^{2}	8.5×10^{1}
6	*Lactobacillus acidophilus* (control)	1.15×10^{1}	0
7	Consortium	1.00×10^{1}	0
8	Normal	7.80×10^{2}	4×10^{1}

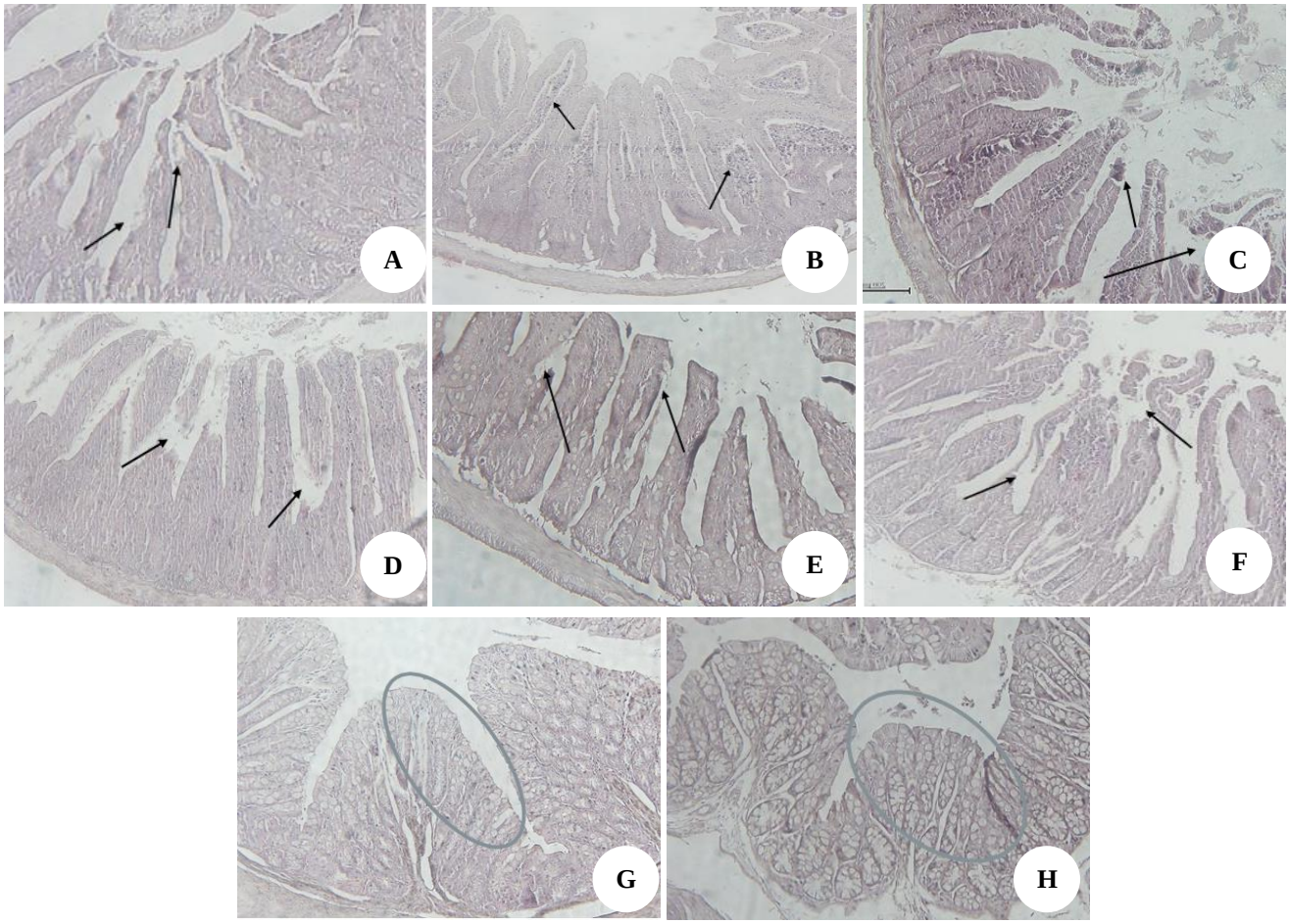

Figure 7. The intestinal histology of mice. A. TA.1 (score 3); B. TK.1 (score 2); C. TK.2 (score 3); D. TK.4 (score 3); E. TB.1 (score 3); F. La (positive control, score 3); G. LAB consortium (score 1); H. Normal (score 1)

Therefore, the consortium treatment in this study is the best probiotic candidate based on of all tests in this study. The consortium treatment was able to normalize the intestinal condition of mice so that it did not cause tissue damage. The probiotic consortium treatment also reduced and prevented the growth of the population of pathogenic bacteria in the feces of mice, namely *Shigella* sp. and *E. coli*; so that the probiotic consortium can be used as a starter in further research in the fields of health, food and industry. The conclusion is supported by previous research that providing a microbial starter in the form of a consortium provides a more beneficial effect regarding the taste of the fermented product and the diversity of chemical compounds desired for health.

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