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The effectivity of *jamu* makarens on reducing the number of *E. coli* and improving the small intestine health of Bali local pigs

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Abstract
The study aimed to determine the effectivity of *jamu* makarens in reducing the number of *E. coli* and improving the small intestine health of bali local pigs has been carried out for 12 weeks using 20 heads of male bali local pigs. The experimental design used was a completely randomized design with 4 treatments i.e: P0 (ration without *jamu* makarens), P1 (ration + 2% *jamu* makarens), P2 (ration + 4% *jamu* makarens), and P3 (ration + 6% *jamu* makarens) and 5 replications. The results showed that *jamu* makarens at the level of 2-6% significantly increase lactic acid bacteria, villus height, crypt depth and decrease *E. coli* and pH of the small intestine. Based on the results of the study, it can be concluded that jamu makarens is effective to reducing *E. coli* and improving the small intestine health of bali local pigs with an optimum level of 4%.

Keywords: Bali local pigs, *jamu* makarens, *E. coli*, small intestine health

Introduction
Bali Local pigs are one of the germplasm that needs to be preserved because the population is decreasing and has promising business opportunities. This pig has its own market share because it is more widely used as *babi guling* and religious ceremonial facilities. At first, *babi guling* was an offering in certain ceremonies, but eventually developed into a very famous culinary in Bali and even became a tour package because of its distinctive taste.

According to the Livestock and Animal Health data (2019) [10], the Bali local pig population in Province of Bali has decreased every year. In 2015 the population of Bali lokal pig was 215,321 heads, decreasing to 206,100 heads in 2016; 183,063 heads in 2017; 207,034 heads in 2018 and 155,856 heads in 2019. The main factor that causes the reduction in the population of bali local pigs is because the people prefer to raise purebred pigs that have faster growth. Bali local pigs take 12 months to reach a body weight of 80 kg, while imported purebred pigs only 5-6 months (Budaarsa, 2012) [2].

The growth of livestock is greatly influenced by the health of their digestive tract because a healthy intestine can result in better digestion of feed and absorption of nutrients through its epithelial membrane. In addition, the digestive tract also plays a role in maintaining the immune system because basically the intestine is the first line of defense against microbial pressure from the environment, especially invasive pathogens from the lumen of the digestive tract (Veizaj-Delia and Pirushi, 2012) [19]. To promote growth and maintain the health of the digestive tract of livestock, feed mills usually add Antibiotic Growth Promoters (AGPs) in feed. However, the continued using of AGPs can cause residue in livestock products and antibiotic resistance in people who consume it. Therefore, the use of antibiotics in animal feed began to be prohibited with the issuance of Minister of Agriculture Regulation No. 14 of 2017. With this prohibition, it is necessary to find other alternatives, namely by utilizing herbs, such as *jamu* makarens.

Jamu makarens is a traditional herbal medicine made from ripe maja fruit (*Aegle marmelos* L.), old coconut water, palm sugar and rice washing water that is fermented naturally so that it has potential as a probiotic. Probiotics are living organisms that are able to have beneficial effects on the health of their hosts if consumed in sufficient quantities (FAO / WHO, 2002) [4].

Giving probiotic bacteria will help restore the balance of bacterial populations in the intestine, enrich the intestine with lactobacillus, stimulate the growth of natural bacteria in the body and suppress the population of harmful bacteria (Yuniastuti, 2014) [20]. *Jamu* makarens also contains phytochemical compounds that fuction as antibacterial and antioxidant so that they will be more effective in maintaining the health of the livestock body. Dowarah *et al.* (2017) [3], reported that giving probiotics in preweaning-finisher pig rations can improve intestinal morphology and the number of lactic acid bacteria in feces, reduce the number of *E. coli* and *Clostridia*, and diarrhea scores after weaning. Furthermore, Sukmawati *et al.* (2022) [15] reported that giving *jamu* makarens in broiler rations can reduce pathogenic bacteria (*E. coli*, *Coliform* and *Salmonella*) in the small intestine and increase total lactic acid bacteria, but has not been able to increase growth significantly.

The effectivity of probiotics in the body of livestock is influenced by the dose and how to give it. According to Susinarla (2016) [16], 4% dose in the ration has the best effect on broiler growth and feed convertion ratio (FCR). Furthermore, Simorangkir *et al.* (2021) [13] reported that giving probiotics through feed is most effective compared through the drinking water and force-feeding. Based on this information, this study needs to be conducted to examine the effectivity of *jamu* makarens in reducing the number of *E. coli* and improving the small intestine health of bali local pigs.

Material and Methods

Experimental design, animals, cage and rations

The experimental design used was a completely randomized design (CRD) consisting of 4 treatments i.e: P0: ration without *jamu* makarens, P1: ration + 2% *jamu* makarens, P2: ration + 4% *jamu* makarens, and P3: ration + 6% *jamu* makarens. Each of the treatment was repeated 5 times. The livestock used were 20 heads of male bali local pigs that were kept in individual battery cage for 12 weeks. Variables observed include: amount of *E. coli* and lactic acid bacteria, pH, villus height and crypt depth of the small intestine.

Preparation of *jamu* makarens

The main ingredient of jamu makarens is ripe maja fruit (*Aegle marmelos* L.) mixed with palm sugar, old coconut water and rice washing water. Maja fruit is blended first until it is in the form of juice and palm sugar is melted, then all ingredients are put into the barrel to ferment naturally for a month. After a month, *jamu* makarens are filtered and ready to be given to pigs according to the treatment.

Determination the number of *E. coli* and lactic acid bacteria

The amount of *E. coli* and lactic acid bacteria is calculated by the pour method (Fardiaz, 2009) [5]. Faeces samples removed from the small intestine were stirred, then taken 1 g and diluted with 0.1% Bacteriological Pepton water (BPW) solution as much as 45 ml in Erlenmeyer and homogenized to get a dilution of 10^{-1}. Next, a dilution of 10^{-1} is taken and mixed with 9 ml of 0.1% BPW solution in a test tube which is then homogenized so that a dilution of 10^{-2} is obtained. To obtain a dilution of 10^{-3}–10^{-5} is carried out the same way as a dilution of 10^{-2}. Next, 1 ml of the dilution level of 10^{-3}-10^{-5} is put into a sterile petri dish and labeled. After that, sterile MRSA (for BAL) and EMBA (for *E. coli*) media are poured into a petri dish and closed again. Petri dishes are homogenized by moving like the number 8 carefully and left to solidify, then incubated at 37 °C for 24 hours in an incubator in an upside down position. Bacterial colonies that develop over the surface of the medium in order to be counted. After the data is obtained, it is then calculated using the following formula:

$$\text{Number of bacteria (CFU/g)} = \text{number of colonies X} \frac{1}{Dilutions\ factor} \text{x } 10$$

Manufacture of histological preparations of the small intestine

The preparation of histological preparations of the small intestine is carried out according to the method of Kiernan (2001) [8]. The small intestine pieces are fixed by soaking in neutral formalin buffer (NBF) 10% for 24 hours, then sliced (trimming) so that they can be put into boxes for processing in a tissue processor. Next, the intestinal sample was put into 70% alcohol, 80% alcohol, 90% alcohol, 96% alcohol, toluene 1 and toluene 2 for 2 hours each. After that, the intestinal sample was put into liquid paraffin with a temperature of 56° C for 2 hours 2 times. Intestinal samples are then taken with tweezers, followed by blocking using paraffin blocks. Cutting is carried out using microtomes with a thickness of 4-5 µm. A sample of the cut intestine is developed on water in a waterbath, then captured with a glass object. Then dried at room temperature and the preparation is ready to be stained with Hematoxylin Eosin (HE).

Staining is done by soaking the preparation on the glass of the object in xylol I for 5 minutes, followed by xylol II, III for 5 minutes each. Then the preparation is soaked in 100% alcohol I and II for 5 minutes each, next into aquades and then soaked in Harris Hematoxylin for 15 minutes. After that, the preparation is dipped in aquades by lifting and lowering it. The preparation is then dipped in 1% acid alcohol for 7-10 dips, soaked in aquades for 15 minutes, and in eosin for 2 minutes. Next, the preparation was soaked in 96% alcohol I and II for 3 minutes each, 100% alcohol I and II for 3 minutes each, and in xylol IV and V for 5 minutes each. The preparation is dried and mounted using a bundle. Histological preparations are examined under a light microscope connected to a computer and an opticlab camera with an ocular-objective magnification of 10x10 or 10x40. Intestinal histology observed includes villus height and crypts depth.

Statistical analysis

All data were analyzed using one-way ANOVA to determine the differences among treatments. If differences were found ($p<0.05$), then further analysis was performed with Duncan's multiple range test (Steel and Torrie, 1993) [14]. Data on microbial tests (*E. coli* and lactic acid bacteria) are transformed first in the form of Log X before analysis, so that the data is normally distributed.

Result

The analysis of variance showed that the administration of *jamu* makarens in the ration had a significant effect ($p<0.05$) on the amount of *E. coli*, total lactic acid bacteria, pH, villus height and crypt depth in the small intestine of Bali local pigs (Table 1). The highest amount of *E. coli* was found in the small intestine of pigs that did not consume *jamu* makarens (P0), which was 2.17 x 10^4 CFU / g. The amount of *E. coli* in the small intestine of pigs that consumed *jamu* makarens (P1,

P2 and P3 treatment) was significantly lower (p<0.05) respectively by 78.02%; 97.20% and 97.85% compared to P0, but between P2 and P3 treatment did not show a significant difference (p>0.05).

Table 1: The effect of *jamu* makarens in the rations on the number of *E. coli*, lactic acid bacteria, pH, villus height and crypt depth of the small intestine of bali local pigs

Variable	Treatment[1)]				SEM[3)]
	P0	P1	P2	P3	
E. coli (CFU/gr)	2.17 x 10^{4c}	4.77 x 10^{3b}	6.07 x 10^{2a}	4.67 x 10^{2a}	0.23
Total lactic acid bacteria (CFU/gr)	4.73 x 10^{6a}	5.00 x 10^{6a}	5.20 x 10^{6a}	3.8 x 10^{7b}	0.05
Small intestine pH	7.63^{b}	7.61^{b}	7.46^{ab}	$6.95^{a2)}$	0.24
Villus height (µm)	548.40^{a}	609.50^{ab}	695.99^{b}	645.96^{ab}	51.13
Crypt depth (µm)	289.41^{a}	450.68^{b}	561.03^{c}	546.31^{c}	24.23

Note
Treatment
P0: Ration without *jamu* makarens
P1: Ration + 2% *jamu* makarens
P2: Ration + 4% *jamu* makarens
P3: Ration + 6% *jamu* makarens
Values with different superscripts on the same line indicate a significantly different (p<0.05)
SEM: Standar Error of The Treatment Means

The administration of *jamu* makarens in the ration at the level of 2% (P1) and 4% (P2) did not show a significant effect (P>0.05) on the increase of total lactic acid bacteria in the small intestine, but in the administration of 6% (P3) the results increased significantly (p<0.05) by 87.60% compared to the control (P0). The increase in the number of lactic acid bacteria was followed by a decrease in pH values by 0.26% in the P1 treatment and 2.23% in the P2 treatment, but statistically not significanly difference (P>0.05). The pH value decreased significantly (p<0.05) in the P3 treatment by 8.91% compared to the control (P0).

The reduced amounts of *E. coli* in the small intestine positively affect on the villus height and crypt depth. The height of small intestine villus in P0 treatment (control) was 548.40 µm, while in P1, P2 and P3 treatments was higher than the control respectively by 10.02%, 21.21% and 15.10%, but statistically in P1 and P3 were not significantly different (P>0.05) compare to the control. The crypts depth in the P0 treatment was 289.41µm, while in the P1, P2, and P3 treatments it was significantly higher than the control respectively by 35.78%, 48.41% and 47.02%. Crypt depth between P2 and P3 treatments showed no significantly difference (P>0.05).

Discussion

The administration of *jamu* makarens in the rations at the level of 2-6% in generally effective for maintaining the health of the digestive tract of the pigs with the optimum level of 4%. It was reflected in the reduced amount of *E. coli* and the increased of the villus height and crypt depth in the small intestine. *E. coli* is a species of bacteria that has a natural habitat in the digestive tract of animals and humans that has been widely reported to cause potential damage to the small intestinal mucosa and diarrhea (Savkovic *et al*., 2005) [11]. Many researchers report that the presence of *E. coli* in the digestive tract can be suppressed by administering probiotics lactic acid bacteria.

Jamu Makarens is a fermented herbal medicine that contains of lactic acid bacteria and phytochemical compounds, so that it has potential as a probiotic and anti-bacteria. The results of this study showed that the administration of *jamu* makarens in the ration as much as 2-6% significantly reduce the amount of *E. coli* in the small intestine by 78.02% - 97.85%. This is because of *E. coli* unable to compete with lactic acid bacteria which are increasing in number. Lactic acid bacteria are a group of bacteria capable of converting carbohydrates (glucose) into lactic acid which can lower the pH of the environment to 3 to 4.5. This condition is not suitable for the environment of pathogenic bacteria (*E. coli)* that live at the pH range of 6-8. In addition, lactic acid bacteria also excrete compounds that can inhibit pathogenic microorganisms such as hydrogen peroxide (H_2O_2), diacetyl, CO_2, acetaldehyde, amino acids, and bacteriocins. Bacteriocins can damage membrane permeability and eliminate Proton Motive Force (PMF) thereby inhibiting energy production and protein or nucleic acid production (Syukur, 2017) [17]. The ability of *jamu* makarens to suppress the amount of *E. coli* is strengthened by the presence of phytochemical compounds that are antibacterial such as saponins, tannins and flavonoids. The results of this study are in accordance with Sukmawati *et al.* (2022) [15], that giving *jamu* makarens in the ration by 4-6% can reduce the number of pathogenic bacteria (*E. coli*, *Coliform* and *Salmonella*) in the small intestine of broiler chickens. A comparison graph of the number of *E. coli* in small intestine of pigs fed *jamu* makarens is shown in Figure 1.

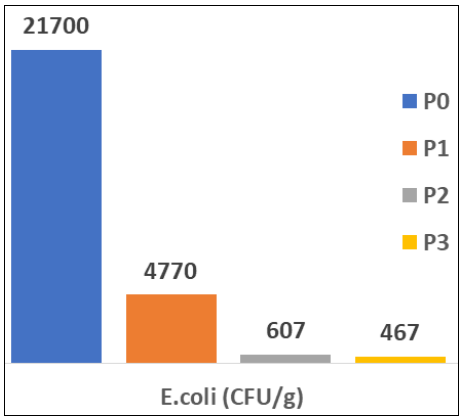

Fig 1: The number of *E. coli* in small intestinal of local bali pigs fed jamu makarens at the level of 0% (P0), 2% (P1), 4% (P2) and 6% (P3)

The decrease in the number of *E. coli* is inversely proportional to the number of lactic acid bacteria. The total lactic acid bacteria in the results of this study tended to

increase with increasing levels of *jamu* makarens given, but at the level of 2% and 4% did not show a significant difference (P>0.05). A significant increase occurred at the level of 6% which was as much as 87.60% compare to the control. This shows that the population of lactic acid bacteria in *jamu* makarens is still less, so the concentration needs to be higher. However, the viability of lactic acid bacteria in *jamu* makarens is quite good as seen from it population that increases in the small intestine. Based on the results of laboratory tests, jamu makarens contains lactic acid bacteria as much as 6.3 x 10^6 CFU/ml while in the small intestine as much as 3.8 x 10^7 CFU/g. This reflects that *jamu* makarens has a good enough potential to be used as a source of probiotics judging from its viability in the digestive tract. This opinion is supported by Shah (2007) [12], who states that to realize health benefits, probiotic bacteria must be viable and must be available in high concentrations, typically 10^6 CFU/g products.

An increase in the number of lactic acid bacteria in the small intestine was followed by a decrease in the pH value with the lowest value at 6% treatment (P3). Giving *jamu* makarens at the levels of 2% (P1), 4% (P2) and 6% (P3) caused a decrease in pH values by 0.26%, 2.23% and 8.91% respectively compared to the control (P0), but at the levels of 2% and 4% did not show a significant difference (p>0.05). Overall, the pH value of the small intestine in this study ranged from 6.95-7.63 (Table 1). Laura *et al*. (2021) [9], reported that the pH of the small intestine results of her study ranged from 6.7-7.5 in the duodenum and 7.6-8.0 in the ileum. These values were comparable to landrace pig gut data sampled under post mortem conditions with pH values of 6.3 – 7.9. Thus, the pH value of the small intestine in the results of this study is still relatively normal. The decrease in pH value is influenced by organic acids produced by lactic acid bacteria, such as lactic acid and acetic acid. According to Syukur (2017) [17], lactic acid bacteria are of two types, namely homofermentative (only produce lactic acid as a carbohydratic fermentation product) and heterofermentative (produce several types of fermentation products, including: lactic acid 40-50%, ethanol, acetic acid and CO_2).

The Reduced amounts of *E. coli* in the intestines of pigs have a significant effect on the villus height and crypt depth of the small intestine. The villus is protrusions of the small intestine mucosa that lead to the lumen. The villi are lined by columnar epithelial cells, enterocytes that have microvilli that function to expand the absorption surface of nutrient. The higher the villi, the more optimal the absorption process. At the base between the intestinal villi are digestive glands or crypts that produce digestive enzymes and stem cells that continuously regenerate epithelial cells and goblet cells that have been damaged. In crypts, epithelial stem cells divide and push upward (Luminally), further differentiating into enterocytes or goblet cells. In addition, crypts also contain paneth cells that produce immunoglobulins, lysosomes and bacteriolytic enzymes as natural intestinal defenses from pathogens (Bello and Danmaigoro, 2019) [1].

The administration of *jamu* makarens in the ration at the level of 2% did not show a significant effect (P>0.05) on the increased of villus height, but at the level of 4%, the villus height increased significantly (p<0.05) by 21.21% compared to the control and appeared to decrease at the level of 6%. The same results were also found at the crypt depth, but at the administration of 2% it showed a significant increase (p<0.05), as well as at the level of 4% and the depth of the crypt seemed to decrease at the level of 6% (P3) but not significantly different (P>0.05) from the level of 4% (P2). This reflects that the optimum level of giving *jamu* makarens is at the level of 4%. A comparison graph of villus height and crypts depth of small intestine of pigs fed *jamu* makarens is shown in Figure 2.

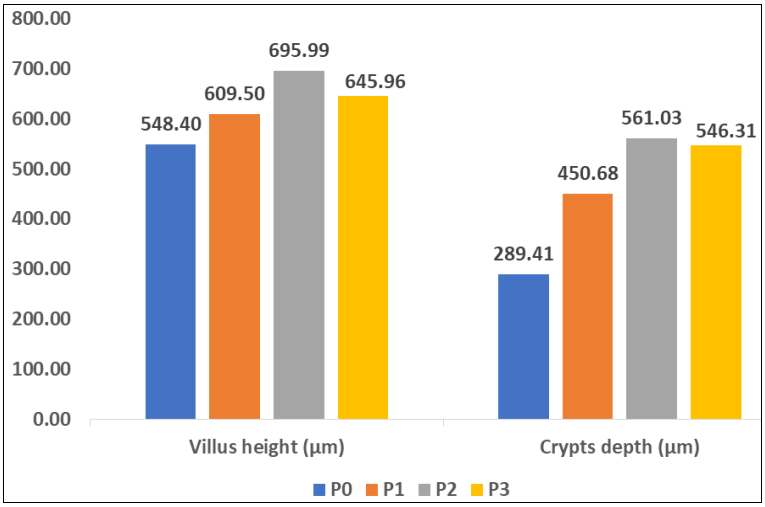

Fig 2: Villus height and crypts depth of local bali pigs fed jamu makarens at the level of 0% (P0), 2% (P1), 4% (P2) and 6% (P3)

The results of this study are in accordance with Harimurti and Rahayu (2009) [7], who reported that probiotic supplementation with single and mixed strains can increase villus height in the duodenum, jejunum, and ileum. But the crypta depth of the jejunum was not significantly different even though the duodenum and ileum were significantly different (p<0.05). Wresdiyati *et al* (2013) [18] reported that indigenous probiotics L. plantarum and L. fermentum can suppress villi damage and increase the thickness of the small intestinal mucosa of rats. The villus height, crypts depth, and their ratio, are important informative indicators on gut health status. A higher villus ensures a greater count of enterocytes, which increases the surface area and promotes several positive effects: higher enzyme production, increased absorptive area, and improved the system of nutrient transport. This is in accordance with Han *et al.* (2013) [6] that crypts are locations of epithelial stem cells that are responsible for proliferation of epithelial cells.

Conclusion

Based on the results of this study, it can be concluded that *jamu* makarens is effective to suppressing the amount of *E. coli* and improving the small intestine health of bali local pigs with an optimum level of 4%.

Suggestion

Based on these conclusions, it is recommended to give *jamu* makarens in the ration of pigs at the level of 4%. Further research needs to be done, namely by giving through drinking water.

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Conflict of interest declaration

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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